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THÈSE EN COTUTELLE PRÉSENTÉE POUR OBTENIR LE GRADE DE

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ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ

ÉCOLE DOCTORALE SCIENCES ET TECHNOLOGIES

Spécialité NEUROSCIENCES

Par Sarah BOU SADER NEHME

**MÉCANISMES CORTICAUX DES INTERACTIONS ENTRE SENSIBILISATION
DOULOUREUSE ET TROUBLE DE L'ATTENTION AVEC OU SANS
HYPERACTIVITÉ (TDAH) DANS UN MODÈLE MURIN**

*CORTICAL MECHANISMS OF COMORBIDITY BETWEEN PAIN SENSITIZATION AND ATTENTION-
DEFICIT/HYPERACTIVITY DISORDER (ADHD) IN A MOUSE MODEL*

Sous la direction de : Pr. Marc LANDRY et Pr. Walid HLEIHEL

Soutenue le 18 décembre 2024

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Résumé

Le trouble du déficit de l'attention avec ou sans hyperactivité (TDAH) et la douleur chronique sont deux syndromes complexes d'origine multifactorielle. Des études confirment une comorbidité entre ces deux pathologies. Cependant, les mécanismes ne sont toujours pas élucidés. Une hyperactivité des neurones du cortex cingulaire antérieur (ACC) et une dérégulation de la voie ACC-insula postérieure (PI) ont été démontrées dans un modèle de souris TDAH. La neuroinflammation serait impliquée dans cette concomitance. Notre hypothèse suggère que la neuroinflammation déclencherait une amplification de l'activité neuronale dans l'ACC, sensibilisant ainsi les voies impliquées dans les symptômes du TDAH et la perception douloureuse. Ce travail de thèse vise donc à identifier les mécanismes inflammatoires à l'origine du TDAH et de la sensibilisation douloureuse qui lui est associée, avec un intérêt porté sur le rôle du récepteur purinergique P2X₄ dans cette comorbidité.

Afin de répondre à cette question, nous avons généré un modèle de souris TDAH par injection unilatérale et intracérébroventriculaire de 6-hydroxydopamine (6-OHDA), à P5. D'une part, des souris sauvages mâles et femelles âgées de deux mois ont été sacrifiées, leurs cerveaux ont été extraits et leurs ACC et PI ont été disséqués. Des tissus fixés ont été utilisés pour des études morphologiques tandis que des tissus frais ont été utilisés pour des études transcriptomique, protéomique et phosphoprotéomique. D'autre part, des souris avec un knock-out total du récepteur P2X₄ ont été soumises à des tests de sensibilisation à la douleur (thermique et mécanique) et d'hyperactivité. Les tissus provenant d'ACC fixés ont été utilisés afin d'étudier les changements morphologiques microgliaux tandis que des tissus provenant d'ACC et de PI fraîchement extraits ont été utilisés pour des analyses transcriptomiques.

En ce qui concerne les marqueurs identifiés chez les souris modèles de TDAH, nos résultats démontrent (i) des changements dans la morphologie des cellules microgliale et astrocytaire, associés à une réactivité cellulaire, dans l'ACC des souris 6-OHDA, (ii) la présence d'un environnement pro-inflammatoire dans l'ACC et la PI des souris 6-OHDA, (iii) des modifications dans l'expression de multiples protéines et l'activité de plusieurs kinases (sérine-thréonine et tyrosine) dans l'ACC et la PI des souris 6-OHDA, associées à des altérations du guidage axonal, de l'apoptose, de la dynamique du cytosquelette, de cascades de signalisation, de neurotrophines et de neurotransmetteurs, et (iv) des variations des interactions neurones-glie, et en particulier de la communication entre les neurones et les astrocytes dans l'ACC des souris 6-OHDA. L'intégration des données a permis d'identifier quatre processus altérés dans l'ACC et la PI des souris mâles et femelles 6-OHDA : l'apoptose, le guidage axonal, la plasticité synaptique (potentialisation à long terme) et la croissance des prolongements neuronaux ; des processus précédemment associés au TDAH et aux douleurs chroniques. De plus, nos résultats suggèrent un effet délétère du récepteur P2X₄ sur l'activité locomotrice des souris et la réactivité microgliale. Il exercerait néanmoins un effet protecteur en limitant l'expression de molécules pro-inflammatoires, probablement sécrétées par des cellules non-microgliales.

En conclusion, nos travaux fournissent des pistes sur les mécanismes inflammatoires qui sous-tendent la comorbidité entre le TDAH et la sensibilisation à la douleur. Ces pistes devront ultérieurement être validées individuellement. Le maintien d'un environnement pro-inflammatoire dans l'ACC et la PI entraîne des changements dans certains processus synaptiques (potentialisation à long-terme, guidage axonal, croissance des composants neuronaux) et apoptotiques. Ces altérations modifieraient la connectivité cellulaire et l'activité neuronale, participant ainsi à la pathogenèse du TDAH et de la douleur chronique.

Mots clés: Mécanismes corticaux, TDAH, sensibilisation douloureuse, neuroinflammation, cortex cingulaire antérieur, insula postérieure

Abstract

Attention deficit/hyperactivity disorder (ADHD) and chronic pain are two complex conditions of multifactorial origins. Clinical and preclinical studies support an association between these two syndromes. However, the mechanisms underlying their comorbidity are not well understood. Previous findings from our team demonstrated a hyperactivity of the neurons of the anterior cingulate cortex (ACC) and a deregulation of the ACC-posterior insula (PI) pathway in ADHD-like conditions. Growing evidence also suggests a role for neuroinflammation in this concomitance. Our hypothesis thus suggests that neuroinflammation triggers an enhanced neuronal activity in the ACC that sensitizes pathways involved in ADHD symptoms and pain perception. Therefore, this Ph.D. work aims to elucidate the inflammatory mechanisms that may underlie ADHD and its associated pain sensitization, with an interest in the role of the purinergic P2X₄ receptor.

To address this question, we generated an ADHD-like mouse model through the unilateral intracerebroventricular injection of 6-hydroxydopamine (6-OHDA) at P5. Two-month-old wild-type male and female mice were sacrificed, their brains were extracted, and their ACC and PI were dissected. Fixed tissues were used to study microglial and astrocytic morphology while fresh tissues were utilized for transcriptomic, proteomic, and phosphoproteomic investigations. Moreover, mice with a total knock-out of the P2X₄ receptor were tested for thermal and mechanical pain sensitization, in addition to hyperactivity. Fixed tissues of the ACC were used to study changes in microglial morphology while fresh tissues of the ACC and PI were utilized for transcriptomic analyses.

Regarding the identification of inflammatory mechanisms in our ADHD-like mouse model, our results report (i) changes in microglial and astrocytic morphology, associated with cellular reactivity, in the ACC of 6-OHDA mice, (ii) the presence of a pro-inflammatory environment in the ACC and PI of 6-OHDA mice, (iii) modifications in protein expression and kinase (serine-threonine and tyrosine) activity in the ACC and PI of 6-OHDA mice, and correlated with impairments in axon guidance, apoptosis, cytoskeleton dynamics, signaling cascades, neurotrophins, and neurotransmitter systems, and (iv) alterations in protein interactions and, therefore, neuronal-astrocytic communication in the ACC of 6-OHDA mice. Finally, data integration identified four processes impaired in the ACC and PI of 6-OHDA males and females: apoptosis, axon guidance, synaptic plasticity (long-term potentiation), and growth of neuronal components. Interestingly, alterations in these processes were not only linked to ADHD and chronic pain conditions but also associated with Eph/ephrin bidirectional signaling cascades. Our findings also indicate a role for the P2X₄ receptor in the worsening of ADHD hyperactivity symptom and the induction of morphological changes in microglial cells that correlate with cellular reactivity. However, it exerts a protective effect by limiting the expression of pro-inflammatory molecules, possibly from non-microglial cells.

In conclusion, our work provides interesting insights into the inflammatory mechanisms that may underpin the comorbidity between ADHD and pain sensitization. A mild and sustained pro-inflammatory environment in the ACC and PI drives changes in synaptic-related (long-term potentiation, axon guidance, outgrowth of neuronal components) and apoptotic processes. These impairments alter cell-cell connectivity and neuronal activity, thus participating in ADHD and chronic pain pathogenesis.

Keywords: Cortical mechanisms, ADHD, pain sensitization, neuroinflammation, anterior cingulate cortex, insular cortex

Aperçu de la Thèse

Le TDAH est un trouble neurodéveloppemental caractérisé par des symptômes d'inattention, d'hyperactivité et d'impulsivité. Il s'agit du trouble infantile le plus prévalent au monde, pouvant persister à l'âge adulte. Environ 8 % des enfants âgés de 3 à 12 ans, 6 % des adolescents âgés de 12 à 18 ans, et 3% des adultes seraient diagnostiqués avec du TDAH. Son étiologie est complexe et multifactorielle. Il est rare qu'une altération génétique ou un facteur environnemental unique soit à l'origine de sa pathophysiologie. Celle-ci repose plutôt sur l'interaction entre plusieurs facteurs de risque génétiques et environnementaux. De plus, le TDAH est associé à des modifications touchant plusieurs structures cérébrales, systèmes de neurotransmetteurs (dopamine, sérotonine, acétylcholine, norépinéphrine) et fonctions exécutives. Les études cliniques confirment une comorbidité entre le TDAH et d'autres maladies psychiatriques et non psychiatriques. Plus intéressant encore, une association entre le TDAH et la douleur chronique a été établie.

Selon l'Association internationale pour l'étude de la douleur (IASP), la douleur est décrite comme étant une « expérience sensorielle et émotionnelle désagréable associée à une lésion tissulaire réelle ou potentielle, ou ressemblant à celle-ci ». La douleur fait partie des mécanismes de défense de l'organisme, avec comme seul objectif le protéger des stimuli nocifs. Trois composantes de la douleur ont été précédemment décrites : une composante sensori-discriminative, une composante émotionnelle, et une composante cognitive. L'aspect sensori-discriminatif englobe les mécanismes neurophysiologiques permettant de renseigner sur l'intensité, la localisation, la durée et la qualité de la douleur. L'aspect émotionnel fait intervenir les émotions et résulte en l'aversion à la douleur. Enfin, la dimension cognitive décrit les pensées et les croyances relatives à la douleur. Elle est influencée par les expériences passées et permet d'interpréter la sensation douloureuse afin de lui donner un sens. Elle englobe également les stratégies d'adaptation mises en œuvre pour réduire et tolérer cette sensation aversive. La douleur est donc une expérience subjective qui peut être conditionnée par différents facteurs biologiques, sociaux et psychologiques.

La douleur est divisée en trois types : la douleur nociceptive, la douleur neuropathique et la douleur inflammatoire. La douleur et la nociception sont des phénomènes distincts. Alors que la douleur renvoie à une expérience complexe et personnelle impliquant plusieurs dimensions, la nociception désigne - et se réduit - à l'activité neuronale qui permet le traitement de l'information suite à des stimuli nocifs. Plusieurs structures cérébrales (« matrice de la douleur ») sont activées par les stimuli nociceptifs et sont impliquées dans les différents aspects de la douleur. Celles-ci incluent les cortex somatosensoriels primaire et secondaire, le cortex préfrontal, le cortex cingulaire antérieur, le cortex insulaire, l'amygdale, le thalamus, le cervelet, l'aire tegmentale ventrale et le noyau accumbens. Les douleurs neuropathique et inflammatoire sont les deux grandes catégories de douleur chronique. La douleur chronique est définie comme une douleur qui dure pendant plus de trois mois, touchant près de 20 % de la population mondiale. Contrairement à la douleur aiguë, elle persiste en l'absence de stimuli nocifs et n'agit donc plus comme mécanisme d'alerte face à ces signaux. La douleur neuropathique résulte d'une lésion nerveuse, tandis que la douleur inflammatoire survient à la suite d'un traumatisme inflammatoire. Ces douleurs chroniques sont associées à des altérations de la perception douloureuse (allodynie et hyperalgésie) ainsi qu'à une sensibilisation accrue à la douleur.

Des études cliniques ont démontré une prévalence élevée de douleurs pathologiques ainsi que des altérations quant à la perception et la sensation douloureuse chez les patients TDAH. Les adultes atteints de ce trouble présentent un seuil de douleur inférieur à celui mesuré chez des individus sains, rétabli par

un traitement au méthylphénidate. Inversement, les patients diagnostiqués avec des douleurs chroniques présentent les symptômes du TDAH. Plus intéressant encore, la douleur a été corrélée à des déficits attentionnel et cognitif; des déficiences qui caractérisent ce trouble neurodéveloppemental.

Le TDAH et la douleur chronique sont deux pathologies complexes interconnectées. Cependant, les mécanismes qui sous-tendent cette comorbidité demeurent peu connus. Des modifications de l'activité neuronale, au niveau des régions corticales impliquées dans la régulation des fonctions cognitives et de la perception douloureuse (ACC, PI), pourraient sous-tendre cette coexistence. Ces altérations pourraient être déclenchées par la neuroinflammation, décrite comme étant la réponse inflammatoire au niveau du système nerveux central caractérisée par (i) l'activation des cellules gliales (astrocytes, microglies), (ii) la libération de médiateurs inflammatoires et (iii) des changements dans l'intégrité de la barrière hémato-encéphalique entraînant l'infiltration de cellules immunitaires périphériques et l'amplification de la réponse immunitaire.

La neuroinflammation participe aux mécanismes de sensibilisation à la douleur ainsi qu'à la pathogenèse du TDAH. Elle pourrait donc agir comme lien entre ces deux pathologies.

Notre hypothèse suggère que la neuroinflammation déclenche l'hyperactivité des neurones de l'ACC qui résulte en la sensibilisation des voies impliquées dans les symptômes du TDAH et la perception de la douleur. Cette thèse de doctorat vise donc à élucider les mécanismes inflammatoires sous-jacents le TDAH et la sensibilisation douloureuse qui lui est associée, à travers :

1. La mise en évidence d'une réactivité microgliale et astrocytaire dans l'ACC de souris 6-OHDA (mâles et femelles)
2. La caractérisation de l'état inflammatoire de l'ACC et de la PI chez les souris 6-OHDA (mâles et femelles)
3. L'étude des changements dans l'expression des protéines dans l'ACC et la PI de souris 6-OHDA (mâles et femelles)
4. La mise en évidence de modifications dans l'activité des kinases dans l'ACC et la PI de souris 6-OHDA (mâles et femelles)
5. L'identification des voies altérées dans l'ACC et la PI de souris 6-OHDA (mâles et femelles)
6. L'étude des modifications touchant la communication astrocytaire-neuronale (interactions protéiques) dans l'ACC de souris 6-OHDA.

Afin de répondre à ces questions, nous avons généré un modèle de souris TDAH par injection intracérébroventriculaire unilatérale de 6-OHDA à P5. Ce modèle de souris est caractérisé, entre autres, par une dégénérescence des neurones dopaminergiques et la présentation des trois symptômes (hyperactivité, impulsivité, inattention) du TDAH. Les souris témoins ont été injectées avec de l'acide ascorbique.

Les souris ont été sacrifiées à l'âge de deux mois, leur cerveau a été extrait et l'ACC et la PI ont été disséqués. Des études morphologique (immunofluorescence), transcriptomique (RT-qPCR), protéomique (spectrométrie de masse, FRET-mFLIM) et phosphoprotéomique (PamGene) ont été réalisées afin d'identifier les changements moléculaires survenant dans l'ACC et la PI de souris 6-OHDA. L'intégration des données a permis de mettre en lumière l'altération de plusieurs cascades de signalisation cellulaire en conditions TDAH.

L'étude de la morphologie des astrocytes et cellules microgliales a indiqué des changements coïncidant

avec une réactivité cellulaire dans l'ACC de souris 6-OHDA. La quantification de cinq paramètres morphologiques a révélé une forme amiboïde pour les cellules microgliales dans l'ACC des femelles 6-OHDA et, au contraire, une structure ramifiée dans l'ACC des mâles 6-OHDA. Une diminution de la complexité morphologique microgliale est généralement associée à une réactivité cellulaire. L'analyse quantitative a également mis en évidence des modifications quant à la forme des astrocytes. Les astrocytes ramifiés étaient nombreux dans l'ACC des mâles 6-OHDA, contrairement à l'ACC des femelles 6-OHDA où ils présentaient une forme moins complexe. Il est intéressant de noter que ces deux types de morphologie ont été précédemment liés à une réactivité astrocytaire. La double immunofluorescence GFAP/vimentine a confirmé la présence d'une astroglie dans l'ACC des femelles 6-OHDA (augmentation de l'intensité de la GFAP et de la vimentine, augmentation du nombre de colocalisations GFAP/vimentine) mais qui serait plus légère dans l'ACC des mâles 6-OHDA (augmentation de l'intensité de la GFAP). En résumé, nos résultats morphologiques indiquent (i) une réactivité microgliale dans l'ACC des femelles 6-OHDA uniquement, et (ii) une astroglie dans l'ACC des femelles et des mâles 6-OHDA.

Les résultats morphologiques ont soulevé de nouvelles questions. Ces cellules réactives sécrètent-elles des molécules pro- ou anti-inflammatoires ? Contribuent-elles à la réponse immunitaire ou, à l'inverse, l'atténuent-elles ? Afin d'élucider le statut inflammatoire de l'ACC et de la PI dans des conditions TDAH, nous avons exploré les changements dans l'expression transcriptomique de nombreux marqueurs inflammatoires. Nos résultats ont démontré une augmentation de l'expression de médiateurs pro-inflammatoires, associée à une diminution des niveaux d'expression de molécules anti-inflammatoires, dans l'ACC et la PI des souris mâles et femelles 6-OHDA. Certaines de ces variations ont été mises en évidence chez les patients atteints de TDAH (augmentation des niveaux d'IL-6, diminution de l'expression du BDNF) et de douleur chronique (contribution du BDNF, de Wnt5a et de MMP9 à la douleur pathologique). Dans les quatre structures cérébrales, des réseaux centrés sur les cytokines pro-inflammatoires (IL-6, TNF- α , IL-1 β) et leur inhibition de la sécrétion de BDNF ont été identifiés.

Nos résultats morphologiques et transcriptomiques pointent vers une réactivité cellulaire et une sécrétion de marqueurs pro-inflammatoires dans l'ACC et la PI des souris 6-OHDA. Nous nous sommes donc intéressés à mieux comprendre les mécanismes intervenant en conditions TDAH. Pour cela, nous avons eu recours à la spectrométrie de masse et la technique PamGene, deux méthodes de criblage à haut débit, permettant de détecter des altérations dans l'expression des protéines et l'activité des kinases, respectivement.

L'analyse par spectrométrie de masse a révélé des changements dans l'expression des protéines, corrélés à des modifications de plusieurs voies: (i) guidage axonal, apoptose et intégrité de la barrière hémato-encéphalique dans l'ACC des femelles 6-OHDA, (ii) guidage axonal, endocytose et adhésion cellulaire dans l'ACC des mâles 6-OHDA, (iii) dynamique du cytosquelette dans la PI des femelles 6-OHDA, et (iv) polymérisation de l'actine et division cellulaire dans la PI des mâles 6-OHDA. Elle a, de même, permis d'identifier (i) un réseau axé sur la dynamique de l'actine et des microtubules dans l'ACC des femelles 6-OHDA, (ii) une interaction entre l'immunité, les ribosomes et l'épissage alternatif dans l'ACC des mâles 6-OHDA, (iii) des interactions entre l'inflammation, la viabilité neuronale et l'intégrité de la barrière hémato-encéphalique dans la PI des femelles 6-OHDA, et (iv) un réseau centré sur l'inflammation dans la PI des mâles 6-OHDA. Ces résultats mettent donc en lumière des variations affectant des processus régulant la réponse immunitaire et la plasticité synaptique, en conditions TDAH.

Nous nous sommes ensuite attardés quant aux conséquences de ces altérations d'expression sur l'activité des protéines. Pour ce faire, nous avons effectué des analyses phosphoprotéomiques et avons confirmé des changements dans l'activité des sérine-thréonine et tyrosine kinases, en conditions TDAH. Une

diminution de l'activité des tyrosine kinases et une augmentation de l'activité des sérine-thréonine kinases ont été observées dans l'ACC des femelles 6-OHDA. Ces variations ont été associées à des altérations touchant la phagocytose, la mort cellulaire, le guidage axonal et la signalisation cellulaire induite par les cytokines. Dans l'ACC des mâles 6-OHDA, une amplification de l'activité des sérine-thréonine et tyrosine kinases a été démontrée. Celle-ci a été corrélée à des modifications visant l'adhésion focale, le guidage axonal, la signalisation des récepteurs sérotoninergiques et d'autres cascades inflammatoires (ErbB, PI3K-Akt). En outre, une augmentation de l'activité des deux familles enzymatiques a été signalée dans la PI des femelles 6-OHDA, induisant des perturbations au niveau de la synaptogénèse, de nombreuses voies de signalisation (MAPK, ErbB, mTOR), neurotrophines et récepteurs sérotoninergiques. Enfin, une suractivation des sérine-thréonine et tyrosine kinases a été observée dans la PI des mâles 6-OHDA et associée à des variations affectant plusieurs cascades de signalisation intracellulaire (MAPK, Ras, ErbB, PI3K-Akt, STAT), les neurotrophines, le guidage axonal et la synaptogénèse. Ces observations suggèrent donc une modification de l'activité des kinases en conditions TDAH, entraînant des altérations dans la modulation de la réponse immunitaire, le guidage axonal, ainsi que les voies de signalisation induites par les neurotrophines et récepteurs sérotoninergiques.

Nos données soulignent le rôle clé joué par les astrocytes en conditions TDAH. Des changements morphologiques associés à une astrogliose ont été observés dans l'ACC des souris 6-OHDA. L'expression des marqueurs pro-inflammatoires, à laquelle les astrocytes contribuent, était amplifiée dans l'ACC et la PI des souris 6-OHDA. Enfin, l'altération de voies auxquelles les astrocytes sont étroitement liés a été suggérée dans l'ACC et la PI des souris 6-OHDA. Par conséquent, nous nous sommes focalisés sur l'étude de la connectivité neuronale-astrocytaire en conditions TDAH. Les résultats de la technique FRET-mFLLIM ont confirmé l'augmentation des interactions entre l'EphA3 (exprimées par les astrocytes) et l'EphA4 (exprimées par les neurones), indiquant une intensification de la communication neuronale-astrocytaire chez les souris 6-OHDA. Bien que les astrocytes participent au maintien de l'homéostasie du système nerveux central, une dérégulation de la communication neuronale-astrocytaire pourrait résulter en (i) une diminution de la quantité de transporteurs glutamatergiques au niveau de la synapse, (ii) une augmentation de la disponibilité glutamatergique et enfin (iii) une excitotoxicité (neurotoxicité).

L'intégration des données transcriptomiques, protéomiques, phosphoprotéomiques et morphologiques a permis d'identifier des voies modifiées dans l'ACC et la PI des souris 6-OHDA telles que l'apoptose, le guidage axonal, la plasticité synaptique (potentialisation à long-terme) et la croissance des composants neuronaux. Nous suggérons que le maintien d'un état pro-inflammatoire entraînerait des changements dans ces processus, se traduisant par des modifications de la connectivité cellulaire et de l'activité neuronale. Ces altérations pourraient être à l'origine de la comorbidité entre le TDAH et la sensibilisation à la douleur qui lui est associée.

Le second objectif de ce travail de thèse visait à étudier le rôle du récepteur purinergique P2X₄ dans la comorbidité entre le TDAH et l'hypersensibilité douloureuse. Le récepteur P2X₄ est un canal ionique activé par des molécules d'ATP permettant le flux de cations (Na⁺, K⁺ et Ca²⁺) à travers les membranes. Au niveau du système nerveux central, ce récepteur est principalement exprimé par les neurones et les cellules microgliales et participe au renforcement des synapses, à la sensibilisation douloureuse, ainsi qu'à la régulation de la neuroinflammation. Il était donc intéressant d'explorer les conséquences d'une délétion totale de ce récepteur sur les symptômes du TDAH et la sensibilisation à la douleur.

Pour ce faire, des souris P2X₄ knock-out et wild-type ont été injectées avec de la 6-OHDA ou de l'acide ascorbique. Des tests comportementaux ont été réalisés pour évaluer l'hyperactivité (Open-field) et la sensibilisation à la douleur thermique et mécanique (test de von Frey et test plantaire). Ensuite, les souris

âgées de deux mois ont été sacrifiées, leurs cerveaux ont été extraits et l'ACC et la PI ont été disséqués. Des études morphologique (immunofluorescence) et transcriptomique (RT-qPCR) ont été menées afin d'analyser les variations affectant la morphologie des cellules microgliales et l'expression des marqueurs inflammatoires.

En conditions TDAH, la délétion du récepteur P2X₄ (i) a supprimé l'hyperactivité locomotrice, (ii) n'a pas eu d'effets sur la sensibilisation à la douleur thermique et mécanique, (iii) a limité les changements morphologiques des cellules microgliales, modulant ainsi la réactivité cellulaire, et (iv) a favorisé un environnement majoritairement pro-inflammatoire dans l'ACC et la PI. Le knock-out du récepteur P2X₄ a également réduit le seuil de la douleur thermique en conditions basales. En conclusion, nos résultats suggèrent que le récepteur P2X₄ aggrave l'hyperactivité locomotrice associé au TDAH et favorise les changements morphologiques microgliaux liés à une réactivité cellulaire. Réciproquement, le récepteur P2X₄ exerce un effet protecteur en restreignant la libération de médiateurs pro-inflammatoires, probablement par des cellules non-microgliales.

En conclusion, nos travaux ont permis d'élucider une partie des mécanismes à l'origine de la comorbidité entre le TDAH et la sensibilisation à la douleur. Nos données suggèrent la présence d'un état pro-inflammatoire dans des régions corticales (ACC et PI) impliquées dans les fonctions cognitives et le traitement de la douleur. Cet environnement inflammatoire est caractérisé par une réactivité microgliale et astrocytaire, une libération de médiateurs pro-inflammatoires, des variations dans l'expression des protéines et des perturbations dans l'activité des kinases. Ces altérations inflammatoires entraînent des changements dans la plasticité synaptique (potentialisation à long-terme), le guidage axonal et l'apoptose ; processus auparavant liés au TDAH et à la douleur pathologique. Par conséquent, la connectivité cellulaire (communication neuronale-astrocytaire, décrite par l'augmentation des interactions EphA4-ephrinA3) et l'activité neuronale (hyperactivité des neurones de l'ACC) se retrouvent modifier, sensibilisant ainsi les voies impliquées dans la perception douloureuse et les symptômes du TDAH.

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Table of Contents

Résumé	2
Abstract	3
Aperçu de la Thèse	4
Acknowledgments	9
Publications and Communications	10
Table of Contents	11
List of Abbreviations	15
List of Tables	18
List of Figures	19
General Introduction	22
Chapter 1 - Literature Review	26
1. Attention-deficit/hyperactivity disorder	27
1.1 Introduction to ADHD	27
1.1.1 History of ADHD	27
1.1.2 Core symptoms and diagnosis	28
1.1.3 Epidemiology	30
1.1.4 Risk factors	30
1.1.5 Comorbidities	32
1.1.6 Treatments	33
1.1.7 Impact of ADHD	34
1.2 Neurobiology of ADHD	34
1.2.1 Macro-anatomical changes	34
1.2.2 Neurotransmitter systems	35
1.2.2.1 Evidence from genetic studies	35
1.2.2.2 Evidence from preclinical and clinical studies	36
1.2.2.3 Evidence from pharmacological studies	37
1.2.3 Neurocognitive profiles	38
1.3 Animal models of ADHD	39
1.3.1 Criteria for animal models validation	39
1.3.2 ADHD-like animal models	41
1.3.3 Neonatal 6-OHDA lesioned mouse	47
2. Pain	50
2.1 Introduction to pain	50
2.2 Nociceptive pain	51

2.2.1	Stages of pain perception	51
2.2.2	Peripheral nociceptive fibers	52
2.2.3	Ascending pathways	53
2.2.4	Cortical structures	53
2.2.5	Descending pathways	53
2.3	Mediators	54
2.4	Chronic pain	56
2.4.1	Generalities	56
2.4.2	Mechanisms of chronic pain	57
2.4.2.1	Peripheral sensitization	57
2.4.2.2	Central sensitization	58
2.4.2.3	Inflammatory pain	59
2.4.2.4	Neuropathic pain	60
2.4.2.5	Implication of the pain matrix and descending pathways	60
3.	Inflammation	61
3.1	Peripheral inflammation	61
3.2	Neuroinflammation	61
3.2.1	Cellular actors	63
3.2.1.1	Microglia	63
3.2.1.2	Astrocytes	65
3.2.2	Molecular actors	68
3.2.3	Deleterious outcomes of neuroinflammation	82
3.2.4	Neuroinflammation and ADHD	83
3.2.5	Neuroinflammation and pain	84
3.2.6	Neuroinflammation as a link between ADHD and pain sensitization	85
4.	Purinergic system	87
4.1	Generalities	87
4.2	Types of receptors	88
4.3	P2X ₄ receptor	89
4.3.1	Generalities	89
4.3.2	Role in pain hypersensitivity	90
4.3.3	Role in inflammation	91
Chapter 2 - Materials and Methods		94
2.1	Animals	95
2.2	P5 surgery	95
2.3	Tissue preparation for immunofluorescence	95

2.4	Iba1/GFAP double immunofluorescence _____	95
2.5	Image acquisition and analysis for Iba1/GFAP double immunofluorescence _____	96
2.6	GFAP/Vimentin double immunofluorescence _____	96
2.7	Image acquisition and analysis for GFAP/Vimentin double immunofluorescence _____	97
2.8	Tissue preparation for RT-qPCR procedures, PamGene assays, mass spectrometry, FACS, and MACS _____	97
2.9	RNA extraction and RT-qPCR _____	97
2.9.1	RNA extraction _____	97
2.9.2	RT-qPCR _____	100
2.10	Protein extraction and mass spectrometry _____	102
2.11	Protein extraction, PamGene STK/PTK assays, and data analysis _____	104
2.11.1	Protein extraction _____	105
2.11.2	STK assay _____	107
2.11.3	PTK assay _____	107
2.11.4	Data analysis _____	108
2.11.5	Data analysis: phosphosite analysis _____	109
2.11.6	Data analysis: kinase analysis _____	109
2.11.7	EnrichR and IPA analysis _____	110
2.12	Förster resonance energy transfer (FRET) _____	110
2.13	Behavioral procedures _____	112
2.13.1	Open-field test _____	112
2.13.2	Von Frey test _____	112
2.13.3	Plantar test _____	112
2.14	Statistical analyses _____	112
2.15	Other experiments _____	113
2.15.1	Magnetic-activated cell sorting (MACS) _____	113
2.15.1.1	Tissue dissociation _____	113
2.15.1.2	Isolation of astrocytes _____	114
2.15.1.3	Isolation of microglia _____	114
2.15.1.4	Isolation of neuronal cells _____	114
2.15.2	Fluorescence-activated cell sorting (FACS) _____	114
2.15.2.1	Trial 1 _____	115
2.15.2.2	Trial 2 _____	116
2.15.2.3	Trial 3 _____	116
2.15.2.4	Trial 4 _____	116
2.15.3	NF- κ B immunofluorescence _____	117

2.15.4 Tryptase immunofluorescence _____	117
Chapter 3 - Results _____	119
3.1 Iba1/GFAP double immunofluorescence staining _____	120
3.1.1 Microglial morphology in the ACC of females _____	120
3.1.2 Astrocytic morphology in the ACC of females _____	121
3.1.3 Microglial morphology in the ACC of males _____	121
3.1.4 Astrocytic morphology in the ACC of males _____	122
3.2 GFAP/Vimentin double immunofluorescence staining _____	123
3.2.1 Astrocyte reactivity in the ACC of females _____	123
3.2.2 Astrocyte reactivity in the ACC of males _____	124
3.3 RT-qPCR _____	125
3.3.1 Inflammatory marker expression in the ACC of females _____	126
3.3.2 Inflammatory marker expression in the ACC of males _____	127
3.3.3 Inflammatory marker expression in the PI of females _____	128
3.3.4 Inflammatory marker expression in the PI of males _____	129
3.4 Mass spectrometry _____	130
3.4.1 Protein screening in the ACC of females _____	130
3.4.2 Protein screening in the ACC of males _____	131
3.4.3 Protein screening in the PI of females _____	133
3.4.4 Protein screening in the PI of males _____	135
3.5 PamGene assay _____	138
3.5.1 Kinase activity in the ACC of females _____	138
3.5.2 Kinase activity in the ACC of males _____	141
3.5.3 Kinase activity in the PI of females _____	149
3.5.4 Kinase activity in the PI of males _____	156
3.6 Study of neuronal-astrocytic interactions _____	163
3.7 Results summary _____	164
3.7.1 Altered pathways in the ACC of 6-OHDA females _____	165
3.7.2 Altered pathways in the ACC of 6-OHDA males _____	166
3.7.3 Altered pathways in the PI of 6-OHDA females _____	167
3.7.4 Altered pathways in the PI of 6-OHDA males _____	169
3.8 Role of the P2X ₄ receptor _____	170
Chapter 4 - Discussion _____	190
Conclusion _____	203
References _____	206

List of Abbreviations

5-BDBD	5-(3-Bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one
5-CSRTT	5-choice serial time task test
5-HT	Serotonin receptor
5-HT2	Serotonin receptor 2
5-HT2A	Serotonin 2A receptor
5-HTT	Serotonin transporter
5-HTTLPR	Serotonin-transporter-linked promoter region
6-OHDA	6-hydroxydopamine
A₁	Adenosine type 1 receptor
A₂	Adenosine type 2 receptor
AC	Adenylyl cyclase
ACC	Anterior cingulate cortex
ADD	Attention-deficit disorder
ADHD	Attention-deficit hyperactivity disorder
ADHD-C	ADHD combined subtype
ADHD-HI	ADHD hyperactive-impulsive subtype
ADHD-I	ADHD inattentive subtype
ADRA2A	Alpha 2A adrenergic receptor
AIM2	Absent in melanoma 2 protein
AMPA-R	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
APE1	Apurinic/apryrimidinic endodeoxyribonuclease 1
Arg1	Arginase 1
ASD	Autism spectrum disorder
ATP	Adenosine triphosphate
BAG2	Bcl-2-associated athanogene
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
cAMP	Cyclic adenosine monophosphate
CASP1	Caspase 1
CCL2	C-C motif chemokine ligand 2
CCL4	C-C motif chemokine ligand 4
CCL5	C-C motif chemokine ligand 5
CCR2	C-C chemokine receptor type 2
CCR5	C-C chemokine receptor type 5
CD	Conduct disorder
CD11b	Integrin alpha M subunit
cGMP	Cyclic guanosine monophosphate
CHA	Convex hull area
CHRNA4	Nicotinic acetylcholine receptor alpha 4 subunit
CHRNA7	Nicotinic acetylcholine receptor alpha 7 subunit
CHT	Choline transporter
CNS	Central nervous system
CNV	Copy number variants
COMT	Catechol-O-methyl-transferase
COX-2	Cyclooxygenase-2
CSF1R	Colony-stimulating factor receptor
CX3CL1	Fractalkine
CX3CR1	Fractalkine receptor
CXCL1	CXC motif chemokine ligand 1
CXCL12	CXC motif chemokine ligand 12

CXCL13	CXC motif chemokine ligand 13
CXCR2	CXC motif chemokine receptor 2
CXCR5	CXC motif chemokine receptor 5
DAG	Diacylglycerol
DAT/DAT1	Dopamine transporter
DHN	Dorsal horn neurons
D1R/DRD1	Dopamine D1 receptor
D2R	Dopamine D2 receptor
DRD4	Dopamine D4 receptor
DRD5	Dopamine D5 receptor
DSM	Diagnostic and statistical manual of mental disorders
DUSP6	Dual specificity phosphatase 6
EAAT3	Excitatory amino acid transporter 3
EP	Prostaglandin E2 receptor type
Eph	Erythropoietin-producing human hepatocellular
ERK	Extracellular signal-regulated kinases
F4/80	EGF-like module-containing mucin-like hormone receptor-like 1
FACS	Fluorescence-activated cell sorting
FLIM	Fluorescence lifetime imaging microscopy
FOX2	Forkhead-Box P2 protein
FRET	Förster resonance energy transfer
GABA	γ -aminobutyric acid
GFAP	Glial fibrillary acidic protein
GPCR	G-protein coupled receptor
GSK3β	Glycogen synthase kinase 3 beta
HTR1B	Serotonin 1B receptor
IASP	International Association for the Study of Pain
Iba1	Ionized calcium-binding adapter molecule 1
IC	Insular cortex
IL	Interleukin
INF-γ	Interferon-gamma
iNOS	Inducible nitric oxide synthase
IRF5	Interferon regulatory factor-5
IRF8	Interferon regulatory factor-8
IP	Prostacyclin receptor
IP₃	Inositol triphosphate
IPA	Ingenuity pathway analysis
JAK	Janus kinases
KCC2	Cl ⁻ -cotransporter K ⁺ -Cl ⁻ exporter 2 channels
LTP	Long-term potentiation
MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinase
mfFLIM	Multiple frequency-domain fluorescence lifetime imaging microscopy
MMP9	Matrix metalloproteinase 9
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor- κ B
NLRP3	NOD-like receptor family pyrin domain containing 3
NLRP6	NOD-like receptor family pyrin domain containing 6
NMDA-R	N-methyl-D-aspartate receptor
NOX2	NADPH oxidase 2
ODD	Oppositional defiant disorder
P2X₃R	P2X purinoceptor 3

P2X₄R	P2X purinoceptor 4
PAG	Periaqueductal gray
PARK2	Parkinson protein two
PBS	Phosphate-buffered saline
PBS-BSA	Phosphate-buffered saline bovine serum albumin
PECAM1/CD31	Platelet endothelial cell adhesion molecule
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PI	Posterior insula
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKB/Akt	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PNS	Peripheral nervous system
PTK	Protein tyrosine kinase
RVM	Rostroventromedial medulla
sCG	Soluble guanylyl cyclase
SEMA6D	Semaphorin 6D
SLC6A2/SLC6A4/NET	Norepinephrine transporter
SNAP-25	Synaptosomal-associated protein 25
SNP	Single nucleotide polymorphism
SOD1	Superoxide dismutase type 1
SORCS3	Sortilin Related VPS10 Domain Containing Receptor 3
STAT	Signal transducer and activator of transcription
STK	Serine-threonine kinase
TAK1	Transforming growth factor-beta-activated kinase 1
TDAH	Trouble du déficit de l'attention avec ou sans hyperactivité
TGF-β	Transforming growth factor-beta
TLR	Toll-like receptor
TMEM119	Transmembrane protein 119
TNF-α	Tumor necrosis factor-alpha
TNFR	Tumor necrosis factor receptor
tPA	Tissue-type plasminogen activator
TRAF6	E3 ubiquitin ligase
TRPV1	Transient receptor potential vanilloid type 1
TSPO	Translocator protein
UKA	Upstream kinase analysis
UTR	Untranslated region
VNTR	Variable number of tandem repeats
Wnt3a	Wnt family member 3A
Wnt5a	Wnt family member 5A

List of Tables

Table 1: ADHD diagnostic criteria.....	29
Table 2: Impaired executive functions in ADHD patients	38
Table 3: Animal models of ADHD	41
Table 4: Pain-associated mediators	54
Table 5: Some markers of reactive astrogliosis.....	67
Table 6: Inflammatory markers in the CNS	69
Table 7-1: Final RNA concentrations following Turbo DNA-free treatment for the ACC.....	98
Table 7-2: Final RNA concentrations following Turbo DNA-free treatment for the PI	99
Table 8: List of genes detected and the forward/reverse primers used.....	101
Table 9-1: Final protein concentrations of ACC samples for mass spectrometry	103
Table 9-2: Final protein concentrations of PI samples for mass spectrometry	103
Table 10-1: Protein concentrations of ACC samples for PamGene assays.....	105
Table 10-2: Protein concentrations of PI samples for PamGene assays	106
Table 11: Criteria used to assess data quality	108
Table 12: QC results for the PamGene STK and PTK assays in the ACC and PI	109
Table 13: Altered signaling pathways identified by EnrichR and IPA in the ACC of 6-OHDA females	140
Table 14: Altered signaling pathways identified by EnrichR and IPA in the ACC of 6-OHDA males	144
Table 15: Altered signaling pathways identified by EnrichR and IPA in the PI of 6-OHDA females.....	151
Table 16: Altered signaling pathways identified by EnrichR and IPA in the PI of 6-OHDA males	158

List of Figures

Figure 1: ADHD overview.	27
Figure 2: Main brain structures altered in ADHD.....	35
Figure 3: Validation criteria for animal models.....	40
Figure 4: Morphological alterations in 6-OHDA mice	48
Figure 5: Spontaneous locomote activity at P24.....	48
Figure 6: Baseline performance of sham and 6-OHDA mice in the 5-CSRTT	49
Figure 7: Hypersensitivity to thermal and mechanical stimuli in 6-OHDA mice in basal and CFA conditions	50
Figure 8: Stages of pain transmission.....	52
Figure 9: Key mediators of central sensitization	58
Figure 10: Effects of PKC, PKA, and ERK pathways activation on central sensitization	59
Figure 11: The neuroinflammatory process.....	62
Figure 12: Core functions of microglial cells	63
Figure 13: Diversity of microglial morphology	64
Figure 14: Astrocyte functions	65
Figure 15: The two main types of astrocytic cells in the CNS	66
Figure 16: Molecular signatures of microglia and astrocyte phenotypes.....	68
Figure 17: The MAPK signaling pathways.....	81
Figure 18: The JAK-STAT signaling pathways.....	82
Figure 19: Neuroinflammation as a link between ADHD and pain sensitization	87
Figure 20: The purinergic receptors, agonists, and downstream effects.....	89
Figure 21: Structure of the P2X ₄ receptor	90
Figure 22: Microglia morphology in the ACC of females.....	120
Figure 23: Astrocyte morphology in the ACC of females	121
Figure 24: Microglia morphology in the ACC of males.....	122
Figure 25: Astrocyte morphology in the ACC of males	123
Figure 26: Astrocytic expression of GFAP and vimentin in the ACC of females.....	124
Figure 27: Astrocytic expression of GFAP and vimentin in the ACC of males	125
Figure 28: Detection of pro-inflammatory and anti-inflammatory mediators in the ACC of 6-OHDA and sham female mice.....	126
Figure 29: Detection of pro-inflammatory and anti-inflammatory mediators in the ACC of 6-OHDA and sham male mice.....	127
Figure 30: Detection of pro-inflammatory and anti-inflammatory mediators in the PI of 6-OHDA and sham female mice.....	128
Figure 31: Detection of pro-inflammatory and anti-inflammatory mediators in the PI of 6-OHDA and sham male mice.....	129
Figure 32: Mass spectrometry analysis in the ACC of female mice	131
Figure 33: Mass spectrometry analysis in the ACC of male mice.....	133
Figure 34: Mass spectrometry analysis in the INS of female mice.....	135
Figure 35: Mass spectrometry analysis in the INS of male mice.....	137
Figure 36: Changes in kinase activity and associated pathways in the ACC of females.....	139
Figure 37: Changes in kinase activity and associated pathways in the ACC of males.....	143
Figure 38: Changes in kinase activity and associated pathways in the PI of females	150

Figure 39: Changes in kinase activity and associated pathways in the PI of males	157
Figure 40: EphA3 and EphA4 interactive states in ADHD-like conditions	164
Figure 41: Schematic summary of the altered markers and their associated pathways in the ACC of 6-OHDA females.....	166
Figure 42: Schematic summary of the altered markers and their associated pathways in the ACC of 6-OHDA males.....	167
Figure 43: Schematic summary of the altered markers and their associated pathways in the PI of 6-OHDA females.	168
Figure 44: Schematic summary of the altered markers and their associated pathways in the PI of 6-OHDA males.....	170

General Introduction

Simone Biles, the greatest and most decorated American gymnast of all time.

Emma Watson, a successful actress and UN Goodwill ambassador.

Michael Phelps, the most decorated Olympian swimmer of all time.

Raven Baxter, a molecular biologist and science communicator.

Agatha Christie, one of the most famous writers in the world.

Nelly Furtado, Justin Timberlake, Channing Tatum, Johny Depp, Will Smith, and other actors, actresses, and singers.

These famous personalities were diagnosed with ADHD.

So, what is ADHD? Is it a “true” mental condition? Are ADHD subjects “deficient” or “disordered”?

No, individuals with ADHD are just different.

These differences make them special.

ADHD is a multifactorial neurodevelopmental disorder characterized by symptoms of inattention, impulsivity, and hyperactivity (American Psychiatric Association, 2013). Its prevalence ranges from 5 to 8% in children and adolescents, making it one of the most common childhood conditions worldwide (Salari et al., 2023). ADHD is comorbid with several psychiatric and non-psychiatric diseases. Patients display impairments in multiple domains of perceptual functioning, particularly pain sensation (Fuermaier et al., 2018).

Chronic pain is a complex and disabling neuropathology affecting around 20% of the worldwide population (Breivik et al., 2006; C. J. Phillips, 2009; Treede et al., 2015). It is characterized by persistent pain, lasting for more than three months, in the absence of noxious stimuli (Bonica, 1991; Merskey & Bogduk, 1986; Treede et al., 2015).

So, how are these two syndromes connected?

Pain disrupts cognitive and attentional functions, features of ADHD (Higgins et al., 2015; D. J. Moore et al., 2019). Conversely, subjects with attentional problems report alterations in pain perception (Fuermaier et al., 2018). Heightened pain sensation, decreased pain tolerance, and sensory processing deficits have been associated with the disorder (Dellapiazza et al., 2021; Panagiotidi et al., 2018; Stickley et al., 2016; Treister et al., 2015). Additionally, ADHD patients are more prone to being diagnosed with chronic pain conditions (Battison et al., 2023; Bou Khalil et al., 2018; Mundal et al., 2024) while ADHD symptoms are commonly observed in patients suffering from chronic pain conditions (Battison et al., 2023; Karaş et al., 2020; Lipsker et al., 2018; Low Kapalu et al., 2018).

So, why are these conditions comorbid?

Several mechanisms could underlie the co-occurrence of ADHD and chronic pain. First, attentional processes can modulate pain perception by acting on brain networks (Kucyi et al., 2013; Villemure & Bushnell, 2002) and descending pathways (Sprenger et al., 2012). Interestingly, the higher the working memory load, the more distracted attention is from pain, and the less painful the cue is (Sprenger et al., 2012; Veldhuijzen et al., 2006). Cognitive distraction therefore acts as an analgesic on pain perception (Lier et al., 2022). Second, attention, cognitive functions, and pain are regulated by common brain structures such as the amygdala, the ACC, the nucleus accumbens, and the insular cortex (IC). Disruptions in these regions could impair attention, executive functioning, pain perception, and impulsivity (Bush et al., 1999; Bushnell et al., 2013; T. Chen et al., 2018; Lopez-Larson et al., 2012; Makary et al., 2020; Neugebauer, 2015; Ossipov et al., 2014; Tajima-Pozo et al., 2018; Yael et al., 2019). Finally, dopaminergic system dysfunction contributes to the pathogenesis of both ADHD and chronic pain thus interlinking these two pathologies (Jarcho et al., 2012; Sagvolden, Johansen, et al., 2005).

So, how is this concomitance triggered?

Previous findings reported a hyperactivity of ACC neurons and a deregulation of the ACC-PI pathway in ADHD-like conditions (Bouchatta et al., 2022). Remarkably, the ACC and the insula are both fundamental regions of the salience network, a prime modulator of attention and behavior (Menon, 2015; Uddin et al., 2019). Not only is the salience network responsible for filtering stimuli, thus guiding attention toward important inputs, but it also appears to participate in pain processing (Borsook et al., 2013; Schimmelpfennig et al., 2023; Seeley, 2019).

The impact of a hyperactive salience network on fibromyalgia pathogenesis has been recently discussed (A. M. Pinto et al., 2023). The authors propose a model of fibromyalgia explained by an aberrant emotional regulation resulting from an imbalance between a hyperactivated threat-handling system and a

downregulated soothing-affiliative network. The authors describe the threat-handling system as a network responsible for detecting harmful stimuli and promoting defensive behaviors, and the soothing-affiliative network as a system engaging in positive affect states (warmth, kindness, etc.). These impairments result in an overactive salience network, which in conjunction with other mechanisms, leads to fibromyalgia occurrence. As per the authors, the alterations are mainly influenced by innate (genetic) and acquired (environmental) risk factors (A. M. Pinto et al., 2023).

We suggest that the comorbidity between ADHD and pain sensitization results from a hyperactivated salience network (mainly the cingulate and insular cortices), triggered by genetic predisposition, environmental influences, and a sustained inflammatory environment. We further hypothesize that this continuous alert mode sensitizes pathways involved in ADHD symptoms and pain processing.

Neuroinflammation, the inflammatory response within the CNS, is thought to partake in chronic pain conditions (Gao & Ji, 2010; Inoue & Tsuda, 2018; T. Li et al., 2019; Miyamoto et al., 2017; H. Ni et al., 2016; Vergne-Salle & Bertin, 2021) and ADHD pathogenesis (Alvarez-Arellano et al., 2020; Corona, 2020; Dunn et al., 2019; Leffa et al., 2018) thus supporting its potential implication in the concomitance of these two pathologies.

Therefore, this Ph.D. work aimed to decipher the inflammatory mechanisms underlying ADHD and its associated pain sensitization. Its main objectives were to:

1. Provide evidence of a sustained pro-inflammatory environment in the ACC and PI in ADHD-like conditions
2. Identify pathways and networks altered in the ACC and PI in ADHD-like conditions
3. Investigate the role of the purinergic P2X₄ receptor in the comorbidity between ADHD and pain hypersensitivity.

To address the first two questions, we have used wild-type mice injected with either 6-OHDA or ascorbic acid and have studied changes in (i) microglial and astrocytic morphology in the ACC of 6-OHDA males and females, (ii) transcriptomic expression of pro- and anti-inflammatory mediators in the ACC and PI of 6-OHDA males and females, (iii) protein expression in the ACC and PI of 6-OHDA males and females, (iv) serine-threonine and tyrosine kinase activity in the ACC and PI of 6-OHDA males and females, and (v) cellular connectivity (neuronal-astrocytic communication) in the ACC of 6-OHDA mice.

To elucidate the role of the P2X₄ receptor on ADHD symptoms and pain sensitization, we have used wild-type and P2X₄ knock-out mice injected with either 6-OHDA or ascorbic acid and have explored modifications in (i) ADHD hyperactivity symptom, (ii) pain sensitization to thermal and mechanical stimuli, (iii) microglial morphology in the ACC of mice, and (iv) transcriptomic expression of inflammatory markers in the ACC and PI of mice.

Chapter 1

Literature Review

1. Attention-deficit/hyperactivity disorder

1.1 Introduction to ADHD

ADHD is a neurodevelopmental disorder outlined by persistent patterns of inattention, impulsivity, and hyperactivity (American Psychiatric Association, 2013).

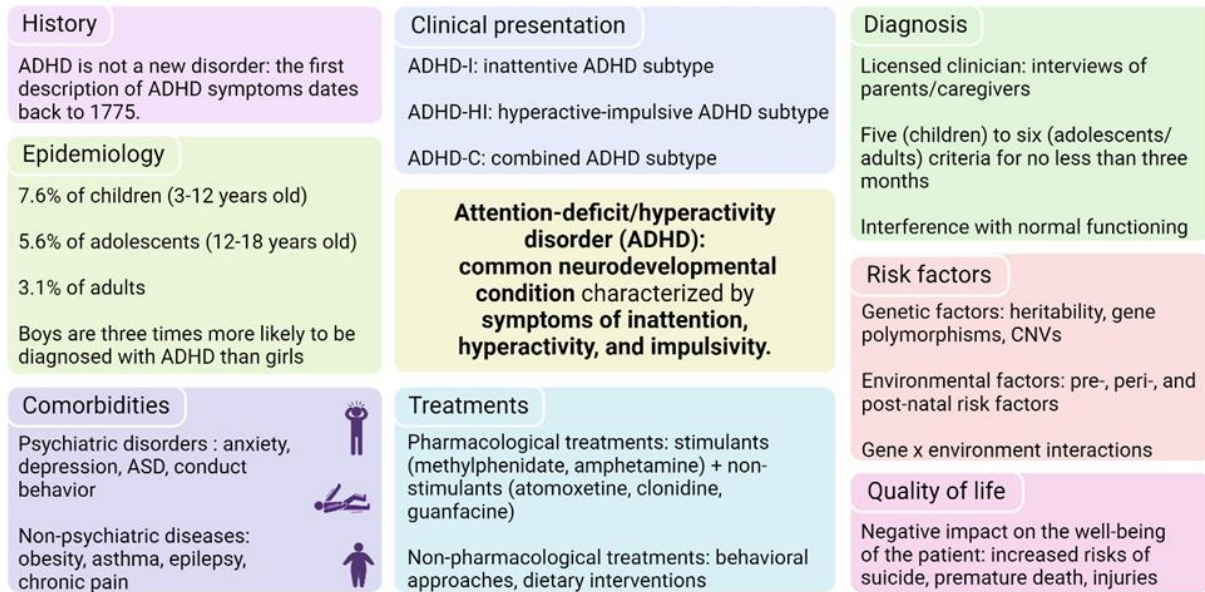


Figure 1: ADHD overview. Created with BioRender.com

1.1.1 History of ADHD

ADHD symptoms have been often mentioned in the literature since the nineteenth century. However, the disorder's history is complex, and its definition has long remained ambiguous (K. W. Lange et al., 2010).

ADHD history started in 1775 when Melchior Adam Weikard, a German physician, was the first to describe symptoms resembling those of the current disorder (Faraone et al., 2021). He was later followed by Sir Alexander Crichton, a Scottish physician, who defined the condition as an "incapacity of attending with a necessary degree of consistency to any one object" and depicted impulsivity as "a degree of anger as borders on insanity" (Crichton, 2008). In 1844, the story "Fidgety Phil" by German physician Heinrich Hoffmann illustrated a boy with excessive activity and symptoms of attentional deficits (Hoffman, 1845; K. W. Lange et al., 2010).

The year 1902 marked the "turning point" of ADHD history with the Goulstonian Lectures of British pediatrician, Sir George Frederic Still. In these lectures, Still described some of his cases as "children with a defect of moral control as a morbid manifestation, without general impairment of intellect and physical disease" (Still, 1902). Five of the twenty children were girls, with symptoms onset before the age of seven, thus reflecting the currently known disproportion and criterion in ADHD diagnosis (Still, 1902).

In 1908, correlations were made between early brain damage and behavioral problems (Tredgold, 1908), and in 1932, the "hyperkinetic disease of infancy" was proposed by Franz Kramer and Hans Pollnow (Kramer & Pollnow, 1932). According to these two German physicians, children diagnosed with this condition presented excessive and urgent motor behavior, educational problems, and difficulty sustaining concentration during complicated tasks (Kramer & Pollnow, 1932). These features appeared during early childhood, between the ages of three and four, and were sometimes implicated in adulthood (Kramer & Pollnow, 1932).

In 1937, Charles Bradley was the first to test the effects of a stimulant drug -benzedrine- on children diagnosed with behavioral problems. To its great surprise, the patients performed better at school, were less hyperactive, were more interested in the tasks, and were more accurate in their work (Bradley, 1937). These children were later confirmed to exhibit signs of hyperactivity, impulsivity, and weak memory – features related to ADHD (Conners, 2000). Benzedrine was then replaced by methylphenidate, a molecule synthesized by Leandro Panizzon in 1944 (Morton & Stock, 2000; Rothenberger & Neumärker, 2005).

Meanwhile, growing evidence started supporting the implication of minimal brain damage in the development of hyperactive behaviors (Ross & Ross, 1976) until 1957, when the work of Laufer suggested that the hyperkinetic syndrome was linked to a brain disturbance rather than damage (Conners, 2000). In 1963, the Oxford International Study Group of Child Neurology changed the name of the disorder from “minimal brain damage” to “minimal brain dysfunction” (MacKeith & Bax, 1963; Ross & Ross, 1976; Rothenberger & Neumärker, 2005). This shift in terminology has caused multiple controversies and has led to the use of more specific terms, such as “hyperactivity”, to label the disorder (Barkley, 2006; Rothenberger & Neumärker, 2005).

In 1968, a novel nomenclature was officially introduced by the second edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-II): Hyperkinetic Reaction of Childhood (American Psychiatric Association, 1968). However, Douglas’ paper in 1972 shed light on the importance of recognizing attentional deficits as a core feature of the disorder (Douglas, 1972), and with the publication of the DSM-III in 1980, the condition was renamed “Attention Deficit Disorder (ADD) (with or without hyperactivity)” by the American Psychiatric Association (American Psychiatric Association, 1980). Hyperactivity was no longer considered a key criterion for the diagnosis, and lists of symptoms were established for hyperactivity, impulsivity, and inattention (American Psychiatric Association, 1980).

Controversy remained as to whether to include hyperactivity as a core symptom of ADHD. Indeed, back in the years, it was still unclear as to whether ADD with hyperactivity and ADD without hyperactivity were two similar or distinct psychiatric conditions (Barkley, 2006). In 1987, and with the revision of the third edition of the DSM (DSM-III-R), the disorder was renamed “Attention-deficit/hyperactivity disorder (ADHD)”. A single list of symptoms regrouped those related to impulsivity, hyperactivity, and inattention (American Psychiatric Association, 1987). In the meantime, findings demonstrated a difference between ADD children with hyperactivity and ADD children without hyperactivity. Some doubts also questioned the central role played by attentional deficits in ADHD. Alterations in the mechanisms underlying motivation and reinforcement, abnormalities in the brain of ADHD patients, and the implication of genetic risk factors were also reported (Barkley, 2006). Moreover, in 1990, ADHD was no longer regarded as a childhood disorder solely but rather a persistent psychiatric condition that could sometimes remain into adulthood (Döpfner et al., 2000).

Finally, in 1994, ADHD was categorized into three subtypes according to specific criteria for each: the predominantly inattentive subtype (symptoms of attentional deficits) (ADHD-I), the predominantly hyperactive-impulsive subtype (symptoms of hyperactivity and impulsivity) (ADHD-HI), and the combined subtype (symptoms of both dimensions) (ADHD-C) (DSM-IV) (American Psychiatric Association, 1994). With the DSM-V publication, minor changes in the diagnostic criteria were undertaken. However, the overall definition of ADHD stayed identical to that of the DSM-IV (American Psychiatric Association, 2013; J. N. Epstein & Loren, 2013).

1.1.2 Core symptoms and diagnosis

ADHD core symptoms include attentional deficits, hyperactivity, and impulsiveness (American Psychiatric

Association, 2013). These features impair the patient’s normal development and decrease his quality of life. Inattention has been associated with academic problems, decreased adaptive functioning, and low self-esteem (Faraone et al., 2021; Willcutt et al., 2012). On the other hand, impulsivity and hyperactivity have been correlated with aggressive and risky driving behaviors (Faraone et al., 2021; Willcutt et al., 2012).

Based on the nature of the symptoms, three clinical presentations of ADHD were defined: ADHD-I, ADHD-HI, and ADHD-C (American Psychiatric Association, 2013). The diagnosis is completed by a licensed clinician and relies on interviews with parents and caregivers. For an individual to be diagnosed with ADHD, at least five (for children) and six (for adolescents and adults) criteria for inattention and hyperactivity/impulsivity (Table 1) should be observed (Substance Abuse and Mental Health Services Administration, 2016), for no less than six months and in different settings (work, school). They should (i) interfere with normal social, occupational, and academic functioning (American Psychiatric Association, 1994), (ii) not be better explained by any other disorder, and (iii) first appear during early to mid-childhood (American Psychiatric Association, 2013; Faraone et al., 2021; World Health Organization, 2018).

Interestingly, new studies speculate an adult-onset for the disorder. More surprisingly, some researchers claim that adult ADHD is not always a continuity of the childhood condition; adult and childhood ADHD may be two distinct mental conditions. Further investigations are needed to refute or confirm these hypotheses (Castellanos, 2015; Faraone & Biederman, 2016).

Table 1: ADHD diagnostic criteria

ADHD core symptom	Diagnostic criteria
Inattention	<ul style="list-style-type: none"> • Failure to give close attention to details. • Makes careless mistakes in schoolwork, at work, or during other activities. • Difficulty to sustain attention in tasks or play activities. • Does not seem to listen when spoken to directly. • Fails to follow instructions and finish duties and tasks at school and the workplace. • Difficulties in organizing tasks and activities. • Avoidance, dislike, or reluctance to engage in tasks that necessitate sustained mental effort. • Loses things necessary for tasks or activities. • Easily distracted by extraneous stimuli (for older adolescents and adults, may include unrelated thoughts). • Forgetful in daily activities.
Hyperactivity	<ul style="list-style-type: none"> • Fidgets, taps hands or feet, squirms in seat. • Leaves seat in situations when remaining seated is expected. • Runs or climbs during inappropriate situations (in adolescents or adults, may be limited to feeling restless). • Unable to play or take part in leisure activities quietly. • “On the go” acting as if “driven by a motor”. • Excessive talks
Impulsivity	<ul style="list-style-type: none"> • Answers before a question has been completed. • Troubles in waiting his/her turn. • Interrupts or intrudes on others.

1.1.3 Epidemiology

ADHD is one of the most common childhood disorders in the world, with persistence into adulthood. Several factors can affect its prevalence such as age, sex, and ethnicity.

ADHD symptoms manifest during early childhood, around the age of four. They are most evident in children and adolescents and tend to decrease, but sometimes remain, into adulthood (Salari et al., 2023). According to a recently published paper that analyzed 61 cross-sectional studies, 7.6% of children aged between 3 and 12 years old and 5.6% of adolescents aged between 12 and 18 years old are diagnosed with the disorder (Salari et al., 2023). The three ADHD subtypes are equally observed in individuals aged below 12 years old. Indeed, the analysis of 24 studies has demonstrated an occurrence of 33.2%, 30.3%, and 31.4% for ADHD-I, ADHD-HI, and ADHD-C presentations, respectively (Salari et al., 2023). However, a discrepancy was detected in patients aged 12 to 18 years old who mainly display the ADHD-I subtype (37.3%) (Salari et al., 2023). In adults, ADHD incidence is equal to 3.1%, with the inattentive dimension being the most common (Ayano et al., 2023).

Studies indicated an increased prevalence of ADHD in boys compared to girls, with boys being three times more likely to develop the disorder (Reale et al., 2017; Rucklidge, 2010; Sadock et al., 2014). ADHD symptoms also seem to differ between the two genders. For example, externalizing symptoms - such as hyperactivity and impulsivity – are more perceived in boys, while girls mainly exhibit internalizing symptoms such as inattention (Rucklidge, 2010; Salari et al., 2023; Weiss et al, 2003).

The disorder's occurrence can diverge across countries. For instance, 8.7% of students aged between 7 and 12 years old have ADHD in Nigeria (Adewuya & Famuyiwa, 2007) while the disorder's incidence is equal to 10.2% in Lebanese adolescents aged between 11 and 17 years old (Maalouf et al., 2016). 6.6% of Spanish children and 7% of Spanish adolescents suffer from ADHD (Catalá-López et al., 2012) while the prevalence varies between 5.74% in Chinese children and 6.72% in Chinese adolescents (T. Wang et al., 2017). Finally, ADHD seems to affect 15.9% of Blacks, 16.6% of Whites, 10.1% of Latinos, and 12.4% of Asian children and adolescents (Cénat et al., 2024).

1.1.4 Risk factors

ADHD etiology is complex and multifactorial. A single genetic alteration or environmental factor rarely triggers the disorder. Instead, its pathophysiology relies on the interactions between genetic predisposition and environmental influences, each exhibiting a small effect (Faraone et al., 2021).

ADHD is a highly transmissible disorder with a heritability ranging from 77 to 88%, according to twin studies (Langner et al., 2013; Lichtenstein et al., 2010). Relatives of ADHD patients are almost five times more likely to be diagnosed with the condition compared to the general population (Biederman, 1992; BIEDERMAN et al., 1990; Faraone et al., 2015).

Mutations in genes involved in neurotransmitter systems (DRD4, DRD5, DAT1, SNAP25, 5HTT, HTR1B) are associated with the disorder (Gizer et al., 2009). The DRD4 gene, encoding for a G protein-coupled receptor (GPCR) belonging to the dopamine D2-like receptor family, has been suggested as a candidate gene for ADHD. The 7-repeat allele of the variable number of tandem repeats (VNTR) polymorphism affecting its exon 3 was found to be more prevalent in ADHD patients compared to controls and was thus considered a "risk" allele (Bhaduri et al., 2006; Gizer et al., 2009; Gornick et al., 2007; LaHoste, Swanson, Wigal, Glabe, Wigal, King N, et al., 1996). The SLC6A3/DAT1 gene encodes for a dopamine transporter that ensures dopamine reuptake from the synaptic cleft to the presynaptic cell. This gene is subject to a VNTR in its 3' untranslated region (UTR) which reduces the transporter's availability and binding potential (Eng

et al., 1971; Heinz et al., 2000; Jacobsen et al., 2000) and was significantly correlated with ADHD (Cook et al., 1995; Gizer et al., 2009; Kopecková et al., 2008; Simsek et al., 2005). Finally, the protein encoded by the SNAP-25 gene plays a key role in axonal growth, synaptic plasticity, and the regulation of neurotransmitter release (Antonucci et al., 2016; Irfan et al., 2019; Osen-Sand et al., 1993). Several mutations affecting this gene were investigated, and the single nucleotide polymorphism (SNP) rs3746544 was linked with ADHD (Gizer et al., 2009; J. W. Kim et al., 2007; Ye et al., 2016). Its homozygous T genotype increased omission errors on a visual continuous performance test (E. Kim et al., 2017).

Other genetic mutations were explored but were not found to participate in ADHD etiology. These modifications encompass a SNP in exon 4 of the COMT gene (Barr et al., 1999; Cheuk & Wong, 2006; Gizer et al., 2009; Hawi et al., 2000), the rs5569 and rs2242447 SNPs of the SLC6A2 gene (Barr et al., 2002; Cho et al., 2008; Gizer et al., 2009; X. Xu et al., 2005), the rs1800544 SNP of the ADRA2A gene (Gizer et al., 2009; B. Wang et al., 2006), and the rs6265 SNP of the brain-derived neurotrophic factor (BDNF) gene (Gizer et al., 2009; J. Lee et al., 2007; Schimmelmann et al., 2007; X. Xu et al., 2007).

Copy number variants (CNVs) are rare (frequency below 1%) genomic duplications and deletions that, according to various studies, contribute to ADHD (Elia et al., 2010; Faraone et al., 2015; Faraone & Larsson, 2019; Williams et al., 2010, 2012). >500 Kb CNVs were detected in 15.6% of ADHD individuals compared to 7.5% of the general population (Williams et al., 2010) while >100kb CNVs were 1.15 times more observed in patients compared to controls (Williams et al., 2012). More importantly, genes affected by CNVs were reported to play key roles in learning, synaptic transmission, neurodevelopment, and behavior (Elia et al., 2010). Rare CNVs affecting the PARK2 gene (Faraone & Larsson, 2019; Jarick et al., 2014) and duplications in the CHRNA7 gene (Williams et al., 2012) were identified in ADHD subjects. Moreover, CNVs spanning chromosomes 7p15.2-15.3 (Lesch et al., 2011) and 16p13.11 (Williams et al., 2010) were shown to increase the disorder's susceptibility. The 7p15.2-15.3 region contains the neuropeptide Y gene, involved in neurogenesis and the modulation of inflammatory responses in the CNS (Decressac & Barker, 2012; Ferreira et al., 2010; Lesch et al., 2011; C. Li et al., 2019). Other CNVs spanning genes that encode glutamatergic receptors were also examined and were related to ADHD cognitive and clinical disabilities (Elia et al., 2010). Genetic linkage has also determined specific chromosomal regions that contribute to ADHD pathogenesis such as 4q13.2, 5q33.3, 8q11.23, 11q22, 17q11.1-q12, and 17p11 (Arcos-Burgos et al., 2004; Gizer et al., 2009; Ogdie et al., 2003).

Several pre-, peri-, and postnatal environmental risk factors take part in ADHD pathophysiology. For instance, maternal smoking and alcohol use (prenatal factors), premature birth and low birth weight (perinatal factors), as well as exposure to environmental toxins such as zinc, lead, pesticides, and biphenyls (postnatal factors) (Curatolo et al., 2010; Faraone et al., 2015). Prenatal exposure to alcohol, smoking, and maternal obesity were demonstrated to increase risks of psychiatric conditions (Andersen et al., 2018; D'Onofrio et al., 2008; Sanchez et al., 2018; Sen & Swaminathan, 2007), including ADHD in offspring (Braun et al., 2006; L. Huang et al., 2018; Jenabi et al., 2019; Kotimaa et al., 2003; Milberger et al., 1996; Sagiv et al., 2013; Sanchez et al., 2018). Prenatal acetaminophen use was correlated with an enhanced likelihood of ADHD in Taiwanese offspring (M.-H. Chen et al., 2019) while mothers exposed to phthalate were more prone to give birth to ADHD infants (Engel et al., 2018). Children with low birth weight (Franz et al., 2018; M. Ni et al., 2023; Pettersson et al., 2015) or with high urinary levels of dialkyl phosphate metabolites of organophosphates (Bouchard et al., 2010) were more likely to be diagnosed with the disorder. Lead-exposed children presented higher odds of developing ADHD symptoms (Braun et al., 2006; Goodlad et al., 2013), especially those exposed to smoking simultaneously (Froehlich et al., 2009).

Gene-environment interactions potentiate the impact of environmental factors on ADHD risk. A variant of 5-HTTLPR (a promoter polymorphism affecting the SLC6A4 gene), combined with stress, predicted ADHD likelihood and was implicated in the hyperactivity and impulsivity symptoms (van der Meer et al., 2014). Maternal smoking was correlated with ADHD-C presentation in genetically predisposed children (Neuman et al., 2007). Smoking-exposed twins carrying the DAT1 440 allele were 2.9 times more at risk of being diagnosed with ADHD compared to twins with no maternal smoking history and DAT1 genetic alterations (Neuman et al., 2007). The odds of ADHD-C subtype diagnosis were also increased in DRD4 seven-repeat allele carriers (Neuman et al., 2007). Finally, men carrying the homozygous genotype for DAT1 10-repeat allele, and who were subject to prenatal smoking, showed enhanced susceptibility to ADHD-HI subtype (K. Becker et al., 2008).

1.1.5 Comorbidities

Clinical studies support the comorbidity between ADHD and health conditions. In the United States, 33% of ADHD children suffer from at least one concomitant disorder (Larson et al., 2011). These include autism spectrum disorder (ASD), learning problems, tic disorders, depression, conduct disorder (CD), anxiety, and oppositional defiant disorder (ODD). ADHD also co-occurs with non-psychiatric problems such as obesity, allergies, asthma, diabetes, psoriasis, epilepsy, chronic pain, and autoimmune diseases (Biederman et al., 1994; Faraone et al., 2021; Gnanavel et al., 2019; Larson et al., 2011).

6% of American ADHD children meet ASD diagnosis criteria (Larson et al., 2011), while 30% of Spanish ASD children present ADHD symptoms (Canals et al., 2024). ADHD youths are four to six times more likely to have chronic tic disorders (Poh et al., 2018), with 1.3% suffering from Tourette's syndrome specifically (Larson et al., 2011). Depression and ADHD co-exist in 13.9% of American children (Larson et al., 2011); ADHD adolescent and young adult females are almost three times more exposed to major depression compared to controls (BIEDERMAN et al., 2008). The comorbidity rate between anxiety and ADHD is estimated to be 17.8% in American youths (Larson et al., 2011) and 38% in the Iranian population (Mohammadi et al., 2021). 46.1% of ADHD patients exhibit learning disabilities (Larson et al., 2011). Finally, oppositional defiant disorder is the most prevalent comorbid condition of ADHD in Iranian (Mohammadi et al., 2021) and Caucasian subjects (Elia et al., 2008).

In adults, the co-existence rate between ADHD and depression, bipolar disorder, anxiety, and personality disorders can reach up to 55%, 35%, 47%, and 34% in the general population, respectively (Q. Chen et al., 2018; Choi et al., 2022).

Alongside its comorbidity with psychiatric disorders, ADHD also coincides with non-psychiatric health problems. 40% of ADHD children and 70% of ADHD adults are overweight (Cortese et al., 2016), 3.9% of ADHD adults are diagnosed with type 2 diabetes mellitus, and 8.51% with hypertension (Q. Chen et al., 2018). ADHD subjects display increased odds of psoriasis (Hegvik et al., 2018) and asthma (Cortese et al., 2018) while enhanced ADHD incidence was found in epileptic patients (Bertelsen et al., 2016; Brikell et al., 2018; Chou et al., 2013). An association between ADHD and pain was also established (Stickley et al., 2016), with 39% of women reporting chronic widespread pain (Asztély et al., 2019).

More importantly, findings demonstrate gender differences regarding ADHD-associated disorders. Older ADHD women tend to report internalizing disorders (anxiety, depression, bulimia, and mood disorders) while externalizing behaviors (substance use disorder, conduct disorder) are more prevalent among younger ADHD boys (Biederman et al., 1994, 2006; Mohammadi et al., 2021; Nussbaum, 2012; Rucklidge, 2010; Solberg et al., 2018; Williamson & Johnston, 2015).

1.1.6 Treatments

ADHD is characterized by alterations in dopamine (del Campo et al., 2011; T. J. Spencer et al., 2005; Volkow, Wang, Newcorn, Fowler, et al., 2007; Volkow, Wang, Newcorn, Telang, et al., 2007; Volkow et al., 2009) and noradrenaline neurotransmitter systems (de Villiers et al., 1995; del Campo et al., 2011; Galgani et al., 2023; Jones & Hess, 2003) which have become the targets of pharmacological interventions aiming to treat the disorder. However, medications are not the only treatment option. Non-pharmacological therapies are also effective in improving ADHD symptoms (JENSEN et al., 2007; Molina et al., 2009).

Pharmacotherapies rely on the use of stimulant (methylphenidate, dextroamphetamine, mixed amphetamine salts) and non-stimulant (atomoxetine, guanfacine) medications (Curatolo et al., 2010). Each molecule has a specific mechanism of action.

Methylphenidate, the most common ADHD treatment, increases dopamine signaling by blocking the transporter responsible for dopamine reuptake, disinhibiting the dopamine D2 receptor (D2R), or amplifying dopamine response duration or tone (Challman & Lipsky, 2000; Curatolo et al., 2010; Volkow et al., 1998). It can also inhibit norepinephrine reuptake (Challman & Lipsky, 2000; Hannestad et al., 2010). Likewise, the use of amphetamine (dextroamphetamine or mixed amphetamine salts) aims to enhance dopamine and norepinephrine activity at the synapses by either intensifying their release into the synaptic gap, abolishing their catabolism or diminishing their reuptake by presynaptic cells (Curatolo et al., 2010; Schrantee et al., 2017). As for non-stimulants, atomoxetine inhibits dopamine and norepinephrine reuptake from the synaptic cleft (Bymaster et al., 2002; Moon et al., 2014) while guanfacine and clonidine selectively stimulate alpha2A adrenergic receptors (Arnsten, 2010; Cinnamon Bidwell et al., 2010; Curatolo et al., 2010; Faraone et al., 2015; Neuchat et al., 2023).

All these pharmacological components reduce ADHD symptoms (Biederman et al., 2003, 2008; Hunt et al., 1995; Jain et al., 2011; Kratochvil et al., 2008; Neuchat et al., 2023; Sallee et al., 2009; Scahill et al., 2001) and are usually well tolerated by the patients. However, some common side effects were noted following their use. These include sleep problems (insomnia), lack of appetite, abdominal pain, vomiting, and headaches (Biederman et al., 2003, 2008; Kratochvil et al., 2008; Sallee et al., 2009; Scahill et al., 2001).

Importantly, symptom severity and the co-occurrence of other disorders are criteria to be considered when prescribing medications. For instance, when ADHD co-exists with more severe conditions (for example, substance use or mood disorders), these disorders should be managed first to ensure the effective treatment of ADHD afterward (C. Binder et al., 2009; Faraone et al., 2015).

Non-pharmacological approaches, such as dietary and behavioral interventions, are alternative ADHD treatments that can be favored when patients (i) poorly respond to or don't have access to medications, (ii) experience serious side effects, or (iii) are considered too young to undergo pharmacotherapies (Faraone et al., 2015; Faraone & Antshel, 2014; Shrestha et al., 2020; Sonuga-Barke et al., 2013).

Behavioral approaches rely on strategies dependent on reinforcement and social learning principles to improve an individual's conduct (Daley et al., 2018; Faraone et al., 2015). They comprise parent training, classroom interventions, cognitive behavioral therapy, and social skills training (Young & Myanathi Amarasinghe, 2010). Parent training is a well-established indirect intervention aiming to optimize parenting skills and ameliorate parent-child relationships (especially during childhood and adolescence). It implements strategies that teach parents and caregivers how to manage the behavior of ADHD children (Faraone et al., 2015; Pelham Jr et al., 1998; Young & Myanathi Amarasinghe, 2010). Even though the results remain controversial, behavioral interventions were shown to exhibit small effect sizes in the

improvement of ADHD symptoms (Faraone et al., 2015; Faraone & Antshel, 2014; Shrestha et al., 2020; Sonuga-Barke et al., 2013). Nevertheless, their efficacy is much higher when combined with medications (Catalá-López et al., 2017; Klein & Abikoff, 1997; Y. Li & Zhang, 2024; Pelham et al., 2005; Rostain & Ramsay, 2006).

Additionally, small reductions in ADHD symptoms were evident following zinc (Bilici et al., 2004; Granero et al., 2021; S. Pinto et al., 2022) and iron (Granero et al., 2021; Konofal et al., 2008; S. Pinto et al., 2022) administration, omega-3 or omega-6 polyunsaturated fatty acids supplementation (J. P.-C. Chang et al., 2018; Döpfner et al., 2021; S. Pinto et al., 2022), and vitamin D supplement combined with methylphenidate (Gan et al., 2019; S. Pinto et al., 2022).

1.1.7 Impact of ADHD

ADHD is associated with severe adverse outcomes that negatively impact the well-being of the patient. Throughout his lifetime, the subject suffers from impairments that not only affect his quality of life (Faraone et al., 2021; Y. Lee et al., 2016) but also compromise the well-being of his family (Dey et al., 2019). These include comorbid health conditions, psychological problems, failure in different settings, risky behaviors, and social disabilities (Faraone et al., 2015, 2021).

Aside from a higher risk of developing comorbid disorders (as detailed previously), ADHD individuals are two to four times more vulnerable to suicide attempts and four to six times more susceptible to suicide deaths (C. Fitzgerald et al., 2019; K.-L. Huang et al., 2018; Septier et al., 2019). They are at higher risk of educational and professional underachievement (class repeat, special education needs, school expulsion, unemployment) (Fleming et al., 2017), in addition to impaired family and peer relationships (Ros & Graziano, 2018; Strine et al., 2006). Individuals with an ADHD history are also more likely to be convicted of a crime and incarcerated (Mohr-Jensen et al., 2019; Young et al., 2015), to perpetrate physical violence, and to be victims of violent behaviors (Christoffersen, 2019; McCauley et al., 2015).

1.2 Neurobiology of ADHD

ADHD is a complex neurodevelopmental disorder associated with alterations in numerous brain structures, neurotransmitter systems, and executive functioning.

1.2.1 Macro-anatomical changes

ADHD is marked by macro-anatomical modifications in various brain regions (Curatolo et al., 2010; Dark et al., 2018; Emond et al., 2009; Krain & Castellanos, 2006) (Figure 2).

Total cerebral and cerebellar volumes were found to be significantly reduced in ADHD patients (Castellanos et al., 1996, 2002; Durston et al., 2004; Mostofsky et al., 2002; Wolosin et al., 2009), with an approximate 4% decrease in intracranial volume of ADHD boys (Durston et al., 2004). The main structures affected include the prefrontal cortex (PFC) (Moreno-Alcázar et al., 2016; Ranta et al., 2009; Sowell et al., 2003), the basal ganglia (Castellanos et al., 1996; Frodl & Skokauskas, 2012), the ACC (Frodl & Skokauskas, 2012; Makris et al., 2010; Moreno-Alcázar et al., 2016), the corpus callosum (M. Yu et al., 2023), the cerebellum (Castellanos et al., 1996; Makris et al., 2015), and the IC (Lopez-Larson et al., 2012). These brain structures are mainly involved in motor control, pain processing, and cognitive functions such as learning, memory, and attention (Bastian, 2011; Bush et al., 2000; Foerde & Shohamy, 2011; Funahashi & Andreau, 2013; Hinkley et al., 2012; Ong et al., 2019; Packard & Knowlton, 2002; Rapoport et al., 2000; Uddin et al., 2017; Xiao & Zhang, 2018).

More specifically, abnormalities in the gray and white matter were demonstrated in ADHD subjects, with

volumetric reductions primarily affecting the PFC (Filipek et al., 1997; Kates et al., 2002; Mostofsky et al., 2002; OVERMEYER et al., 2001). Interestingly, the PFC showed a decreased white matter volume, mainly in the left hemisphere (Mostofsky et al., 2002), and a bilateral volumetric reduction of the gray matter, prominent in the right hemisphere (Filipek et al., 1997; Mostofsky et al., 2002; OVERMEYER et al., 2001). On the contrary, an increase in the grey matter volume was depicted in the posterior temporal lobe and inferior parietal lobule of patients (Sowell et al., 2003). Importantly, reduction in right anterior IC gray matter volume was linked to attention and inhibition problems (Lopez-Larson et al., 2012).

Impairments in cortical thickness and maturation were also reported. A thinning of the cortex (Makris et al., 2007; Shaw et al., 2006) and a three-year delay in brain maturation (Shaw et al., 2007) were evident in individuals with ADHD compared to typically developing controls. Abnormalities in cortical maturation were the most apparent in prefrontal cortices (Shaw et al., 2007). Interestingly, cortical thinning in the right rostral ACC was related to parent-reported ADHD symptoms (Bledsoe et al., 2013). It predicted 37.4% and 39.6% of hyperactivity and inattention symptoms variance in ADHD children, respectively (Bledsoe et al., 2013).

The activation of the aforementioned regions was also impaired during specific tasks. The right inferior frontal cortex, the supplementary motor area, the ACC, and the striato-thalamic areas presented reduced activation during response inhibition tasks (Hart et al., 2013). In attention-demanding tasks, hypoactivation of the right dorsolateral PFC, the posterior basal ganglia, as well as the thalamic and parietal regions was observed (Hart et al., 2013). Neuronal activity within the ventral striatum was also decreased during reward anticipation (Scheres et al., 2007). Remarkably, lower activation of the ventral striatum was associated with increased hyperactivity/impulsivity symptoms (Scheres et al., 2007).

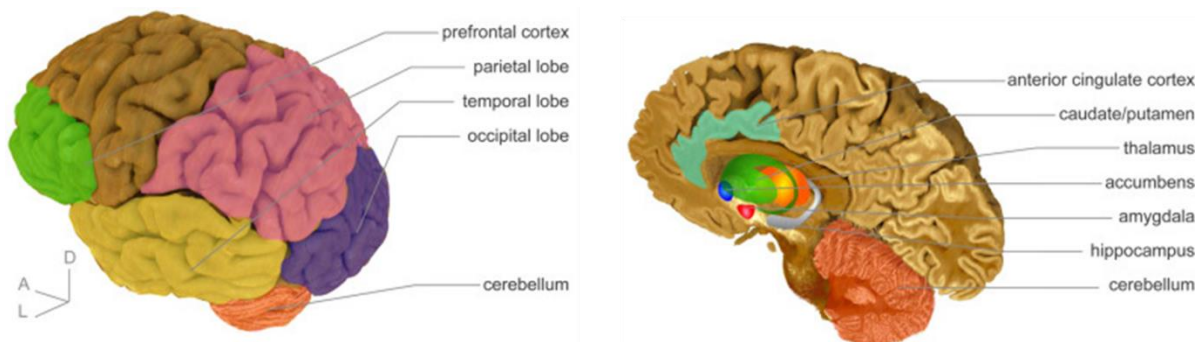


Figure 2: Main brain structures altered in ADHD (Dark et al., 2018)

A anterior, L lateral, D dorsal

1.2.2 Neurotransmitter systems

Imbalances in neurotransmitters (dopamine, serotonin, acetylcholine, norepinephrine), targeted by ADHD pharmacological treatments, are thought to be implicated in the onset of the disorder (Beane & Marrocco, 2004; Liang et al., 2024; Vogt, 2019).

1.2.2.1. Evidence from genetic studies

Genetic risks underlie ADHD pathogenicity, with candidate genes (DRD4, DRD5, DAT1, SNAP25, 5HTT, HTR1B) implicated in neurotransmitter systems (Gizer et al., 2009) (see section “risk factors”). Not only were genetic mutations more prevalent in ADHD patients but they also predicted ADHD features.

Mutations in dopamine receptors and transporters were depicted in ADHD patients. DAT1 9.5-repeat allele was correlated with impulsivity and anger issues (Hasler et al., 2015). Homozygosity in its 10-repeat

allele manifested in smaller caudate volumes in ADHD patients (Durston et al., 2005) and, remarkably, in lower improvements in ADHD symptoms following methylphenidate administration (Froehlich et al., 2011). DRD1 variants conferred susceptibility to ADHD (Bobb et al., 2005; Misener et al., 2004) and attentional deficits (Misener et al., 2004). Morphological changes were reported in DRD4-variant carriers. The 7-repeat allele of the DRD4 gene was significantly linked to decreased frontal gyrification in ADHD patients but not controls (Palaniyappan et al., 2019) while homozygosity in the 4-repeat allele was related to reduced prefrontal gray matter volumes in ADHD siblings (Durston et al., 2005). The 2-allele repeat led to altered connectivity of temporal and frontoparietal lobes (S. Chen et al., 2022) and was positively associated with learning problems (S. Chen et al., 2022).

Associations between increased SLC6A4 promoter methylation and ADHD symptoms were observed. Higher methylation status in the CpG6 and CpG8 sites correlated with hyperactivity and impulsiveness (CpG6 and CpG8 sites) as well as cortical thickness in the right occipitotemporal regions (CpG5, CpG6, CpG7, and CpG8) (S. Park et al., 2015).

Mutations in the nicotinic acetylcholine receptor $\alpha 4$ subunit (CHRNA4) gene were demonstrated. The T allele of the SNP rs6090384 was transmitted in ADHD patients and linked to inattention (Todd et al., 2003; Wallis et al., 2009). Furthermore, the Ile89Val polymorphism (rs1013940) affecting the choline transporter (CHT) gene was two to three times more genotyped in ADHD children and was predictive of ADHD-C subtype diagnosis (English et al., 2009).

Finally, norepinephrine transporter (NET) variants were also found to increase ADHD risk. Homozygosity for the major A allele of SNP rs28386840 and the minor allele of SNP rs3785157 enhanced the odds of ADHD diagnosis while heterozygosity for rs3785157 protected against omission errors in a continuous performance test (Hohmann et al., 2015).

1.2.2.2. Evidence from preclinical and clinical studies

Evidence suggests altered neurotransmitter transmission in ADHD conditions.

Investigations have mainly focused on the dopaminergic system and, despite mixed results, the “ruling” theory proposes dopaminergic deficits in ADHD individuals.

ADHD-like dogs displayed lower dopamine and serotonin serum concentrations compared to controls, correlating with impulsiveness (González-Martínez et al., 2023). Children diagnosed with severe ADHD (Spivak et al., 1999) or with hyperactivity symptoms (Coleman, 1971) demonstrated low blood levels of serotonin. Moreover, deficits in dopamine levels were detected in ADHD adults and were implicated in inattention (Volkow, Wang, Newcorn, Telang, et al., 2007)

An abnormally high accumulation of [^{18}F]DOPA, indicative of increased DOPA decarboxylase activity, was evident in the right midbrain of ADHD children (Ernst et al., 1999). Enhanced enzyme activity was previously associated with low levels of extracellular dopamine or blockade of dopamine receptors (DR - D1R and D2R) (Abercrombie et al., 1990; Hadjiconstantinou et al., 1993; M.-Y. Zhu et al., 1993). These findings are in line with the downregulated expression of D1R observed in the ACC of spontaneously hypertensive rats (SHR) (Satoh et al., 2018), and later replicated in ADHD patients (Yokokura et al., 2021). Importantly, decreased levels of D1R were linked to severe symptoms of hyperactivity (Yokokura et al., 2021).

Dopamine transporter (DAT) dysregulation is also thought to participate in ADHD pathophysiology. DAT density was examined in ADHD subjects; however, inconsistencies in the results were found. Some studies demonstrated increased DAT density (D. D. Dougherty et al., 1999; T. J. Spencer et al., 2007) in ADHD

patients, while others reported decreased density (Volkow et al., 2009; Volkow, Wang, Newcorn, Fowler, et al., 2007) or no differences compared to controls (van Dyck et al., 2002). Enhanced DAT availability was positively correlated with attention problems (Volkow et al., 2009; Volkow, Wang, Newcorn, Telang, et al., 2007).

Disruptions in the noradrenergic and cholinergic transmissions in the PFC resulted in attentional impairments and impulsivity. In rats, norepinephrine depletion led to slower correct response time and reduced accuracy on the 5-choice serial time task test (5-CSRTT) (Milstein et al., 2007). Cholinergic alterations induced deficits in inhibitory control (higher perseverative and anticipatory responses) and accuracy (Dalley, 2004). This is in line with findings supporting the role of these two neurotransmitter systems in attention (Dahl et al., 2020; Klinkenberg et al., 2011).

1.2.2.3. Evidence from pharmacological studies

ADHD pharmacological treatments increase dopamine and norepinephrine extracellular levels and attenuate ADHD symptoms, thus indicating a role for these two systems in the etiology of the disorder.

Methylphenidate exerts its beneficial effects by amplifying catecholamine release in the PFC (Arnsten & Dudley, 2005; Berridge et al., 2006). These enhancing effects were challenged following the administration of idazoxan (α 2-adrenoceptor antagonist) and SCH23390 (D1R antagonist) suggesting that methylphenidate ameliorates behavioral performances through the stimulation of D1 and α 2 receptors (Arnsten & Dudley, 2005; Gamo et al., 2010). This was further confirmed following the reduction of methylphenidate-induced PFC neurons firing in response to SCH23390 administration (Gronier, 2011). At the clinical level, methylphenidate improved ADHD symptoms (MUSTEN et al., 1997; Van der Oord et al., 2008; Wender et al., 2011) and impairments in spatial working memory (KEMPTON et al., 1999; Mehta et al., 2000), spatial short-term memory, planning skills, and set-shifting capacities (KEMPTON et al., 1999).

Both atomoxetine (inhibitor of dopamine and norepinephrine reuptake) and guanfacine (agonist of alpha2A adrenergic receptors) administration alleviated impulsivity in rodents (Milstein et al., 2007; Navarra et al., 2008; Paterson et al., 2012). Atomoxetine further enhanced attentional performances on the 5-CSRTT (Navarra et al., 2008), while guanfacine and dextroamphetamine both reduced ADHD symptoms in adult patients (F. B. Taylor & Russo, 2001).

Impairments in serotonin availability also result in ADHD. Overactivation of serotonin receptor 2 (5-HT₂) worsened impulsivity in rats (Koskinen et al., 2000) while administration of ketanserin, a 5-HT₂ antagonist, decreased premature responses (Koskinen et al., 2000; Passeti et al., 2003). Desipramine, a norepinephrine/serotonin transporter inhibitor alleviated inattentive and hyperactive/impulsive features in children and adolescents with ADHD (T. Spencer et al., 2002). Soficlin, a selective agonist of α 4 β 2 nicotinic acetylcholine receptors, prevented cognitive impairments in monkeys and improved memory in rats (Rueter et al., 2004). In line with these findings, administration of Soficlin 4mg moderated ADHD symptoms in adults (Bain et al., 2013).

ADHD neurobiology is complex and dysregulations in several neurotransmitter systems underlie its pathophysiology. As supported by genetic studies, mutations in genes involved in dopamine, noradrenaline, serotonin, and acetylcholine systems increased the odds of ADHD diagnosis and symptoms. Preclinical and clinical findings investigated changes in the levels of these neurotransmitters, as well as alterations in their transporters and receptors. Despite mixed results, findings demonstrated deficits in dopamine, noradrenaline, serotonin, and acetylcholine concentrations in ADHD individuals, further associated with the disorder's features. Finally, ADHD pharmacological treatments were found to target these abnormalities by acting on the neurotransmitter's transporters and/or receptors.

1.2.3 Neurocognitive profiles

Executive functions represent a family of neurocognitive abilities and processes that enable one to achieve a goal-directed behavior through objectives formulation, action implementation, and obstacles foresight (Perrotta, 2019). They involve diverse neural networks including the PFC, the thalamus, the cerebellum, and the basal ganglia (Funahashi & Andreau, 2013; Middleton & Strick, 2001, 2002; Van der Werf et al., 2003). Dysregulations in several executive functions were reported in ADHD individuals (Mueller et al., 2017; Pievsky & McGrath, 2018; Sergeant, 2005; Willcutt et al., 2005) (Table 2). These encompass but are not limited to, working memory, inhibitory control, temporal information processing, reward, and arousal (Faraone et al., 2015). Response inhibition, vigilance, spatial working memory, and planning are among the most consistently impaired functions in ADHD patients (Willcutt et al., 2005).

Table 2: Impaired executive functions in ADHD patients

Executive function	Results	References
Attention	<ul style="list-style-type: none"> ADHD adults: low scores on tasks of focused and sustained attention 	(SCHOECHLIN & ENGEL, 2005)
	<ul style="list-style-type: none"> Deficits in selective attention (increased reaction times) and divided attention (increased number of omission errors) Performance worsening in tests of alertness, divided attention, and selective attention 	(Tucha et al., 2017)
Inhibitory control (response inhibition and interference control)	<ul style="list-style-type: none"> ADHD children: long reaction time (inhibitory function) 	(Lijffijt et al., 2005)
	<ul style="list-style-type: none"> ADHD adults: impairment in response inhibition Improvement in response inhibition following methylphenidate treatment No differences in interference control 	(Arat Çelik et al., 2022)
Working memory	<ul style="list-style-type: none"> ADHD adolescents and adults: low scores on different working memory tasks 	(Kennedy et al., 2019)
	<ul style="list-style-type: none"> Medication naïve ADHD children: poor performance on a spatial working memory task No differences in the performances between ADHD-medicated patients 	(BARNETT et al., 2001)

	(stimulant treatment) and control participants	
	<ul style="list-style-type: none"> • Beneficial effect of methylphenidate on visual-spatial memory 	(Bedard et al., 2004)
Planning	<ul style="list-style-type: none"> • Deficiencies in motor planning with delay in the initiation of movement and variability in the response velocity during short and long planning conditions 	(Dahan & Reiner, 2017)
Reward system	<ul style="list-style-type: none"> • Choice of immediate rewards over future ones (higher discounting of future rewards) 	(Jackson & MacKillop, 2016; Marx et al., 2021)
Temporal information processing	<ul style="list-style-type: none"> • Poor performance on duration discrimination and reproduction tasks, mainly on the visual modality • No effects of medications 	(Toplak et al., 2006)
Response time variability	<ul style="list-style-type: none"> • Increased variability in reaction time, attenuated by psychostimulants 	(Kofler et al., 2013; Salum et al., 2019; Tamm et al., 2012)
Speech and language	<ul style="list-style-type: none"> • High prevalence of language problems 	(Sciberras et al., 2014)
Arousal	<ul style="list-style-type: none"> • Arousal dysregulation • Mixed results, with most of the findings pointing to hypo-arousal 	(Bellato et al., 2020; Isaac et al., 2024)

1.3 Animal models of ADHD

Animal research has become an essential step in scientific investigation through its contribution to (i) the understanding of biological mechanisms in physiological and pathological conditions, and (ii) the development and validation of new pharmacological compounds.

The scientific field has long been dependent on animal models as they share comparable genetics with Humans (Mouse Genome Sequencing Consortium., 2002), are vulnerable to Human pathologies and infectious agents (Rong & Liu, 2023), enable the establishment of causal relationships through the control of surrounding factors— difficult to implement in clinical trials (Bennett, 2008; N. L. H. Phillips & Roth, 2019), and help in explaining and interpreting behaviors (N. L. H. Phillips & Roth, 2019). More importantly, animal models ensure data translation to Humans (Subramanian, 2022).

Despite the recent advances that have shed light on ADHD neurobiology, some pathophysiological mechanisms remain poorly understood. Consequently, researchers have developed numerous animal models mimicking the disorder to elucidate ADHD etiology and develop new therapeutic strategies.

1.3.1 Criteria for animal models validation

The validation of animal models is critical to certify the reliability, consistency, and reproducibility of the

data obtained. It relies on three main criteria: construct, face, and predictive validity (Figure 3) (Subramanian, 2022).

To perfectly mimic a disorder, the animal model should possess the same fundamental mechanisms underlying the disease's phenotype as those observed in Human patients. Construct validity refers to this homology, and is considered by many the most important validation parameter (Kornetsky, 1989; McKinney, 1969; R. W. Russell, 1964; Sagvolden, Russell, et al., 2005; Sarter & Bruno, 2002; van der Staay et al., 2009). Face validity reflects symptom similitudes between the animal model and the modeled situation (van der Staay et al., 2009; Willner, 1986). Finally, predictive validity denotes the aptitude of an animal model to replicate Human behavior during a certain task (R. W. Russell, 1964). In most studies, it is regarded as the capacity of the model to differentiate between effective and inefficient treatments (D. H. Epstein et al., 2006; Willner, 1986).

Consequently, an ADHD-like animal model should fulfill several criteria to be validated. First, it should present the same neurobiological origins and impairments as the clinical condition. For instance, genetic polymorphisms, alterations in neurotransmitter systems, or impairments in the morphology and activity of brain structures. Second, it should exhibit the three core symptoms of the disorder (hyperactivity, impulsivity, and attentional deficits). Finally, the animal model should forecast the behavioral responses toward the existing ADHD therapeutic strategies (Figure 3) (Regan et al., 2022; Sagvolden, Russell, et al., 2005).

However, it is important to note that few models comply with all three criteria, especially when modeling a complex condition. Therefore, choosing the appropriate animal model can be challenging and should depend on the aim of the research.

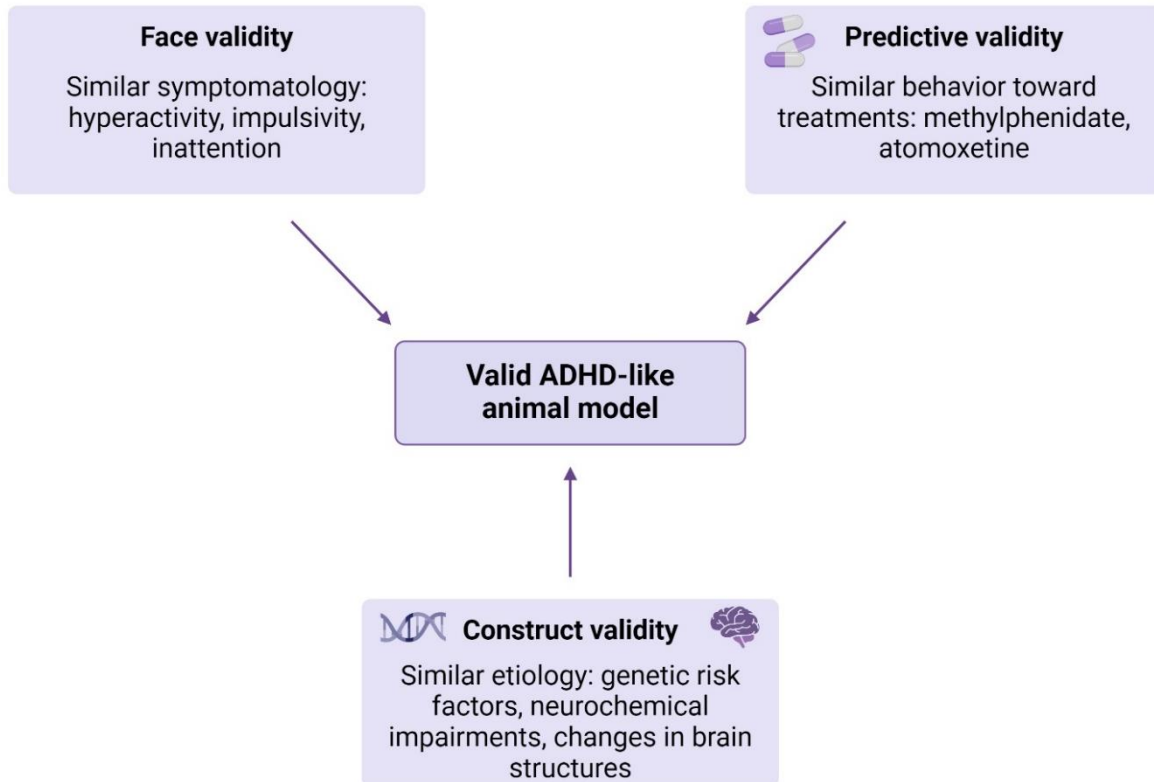


Figure 3: Validation criteria for animal models. Created with BioRender.com

1.3.2 ADHD-like animal models

Several animal models have been developed to mimic ADHD (Fontana et al., 2019; D. Kim et al., 2024; Kostrzewa et al., 2008; Leo & Gainetdinov, 2013; Rahi & Kumar, 2021; Regan et al., 2022; Sagvolden, Russell, et al., 2005). They were induced by genetic mutations, pharmacological lesions, physical trauma, or exposure to environmental toxins (Table 3).

Table 3: Animal models of ADHD

	Animal model	Features	References
Genetic models	Spontaneously hypertensive rat	Structural and neurochemical alterations	(Bendel & Eilam, 1992; De Bruin et al., 2003; Jentsch, 2005; Kantak et al., 2008; Knardahl & Sagvolden, 1979; Myers et al., 1982; V. Russell et al., 1995, 1998; V. A. Russell, 2003; Sagvolden, Metzger, et al., 1992; Sagvolden, 2000, 2006; Tomassoni et al., 2004; Ueno et al., 2002; Wultz et al., 1990; Wultz & Sagvolden, 1992)
		Impulsivity and hyperactivity (development if repeated test), inattention	
		Deficits in memory and spatial learning, preference for immediate rewards	
		Sensitivity to methylphenidate, amphetamine, and guanfacine	
		Drawback: hypertension	
	Wistar-Kyoto hyperactive rat	Hyperactivity	(Drolet et al., 2002; Hendley et al., 1986; Sagvolden, Hendley, et al., 1992)
		Hyperreactivity to novel aversive stimuli	
	Naples high-excitability rat	Inattention	(Gallo et al., 2002; Viggiano et al., 2002)
		Reactivity to novel situations, working memory impairments	
	Hyposexual rat	Hyperactivity, deficits in selective attention	(Kohlert & Bloch, 1993; Kohlert & Block, 1996)
		Controversy over its sensitivity to methylphenidate	
	Poor 5-CSRT rat	Impulsivity, inattention	(Puumala et al., 1996)
		Sensitivity to methylphenidate	
Wig rat	Impulsivity, hyperactivity	(Kamimura et al., 2001; Masuo et al., 2007)	
	Impaired working memory		
	Drawback: panic when exposed to water		

	Letrophilin-3 knock-out rat	Association between ADHD and mutations in letrophilin-3 gene	(Arcos-Burgos et al., 2010; Gomez-Sanchez et al., 2016; X. Huang et al., 2019; Regan et al., 2019, 2021; Sable et al., 2021)
		Impulsivity, hyperactivity in novel environments, inattention	
		Deficits in inhibitory control, working memory, and cognition	
		Sensitivity to amphetamine	
	Letrophilin-3 knock-out mouse	Association between ADHD and mutations in letrophilin-3 gene	(Arcos-Burgos et al., 2010; Gomez-Sanchez et al., 2016; X. Huang et al., 2019; Mortimer et al., 2019; Wallis et al., 2012)
		Impulsivity, hyperactivity	
		Deficits in spatial learning and memory	
	Letrophilin-3 knock-out zebrafish and drosophila	Association between ADHD and mutations in letrophilin-3 gene, decrease in the number of dopaminergic neurons	(Arcos-Burgos et al., 2010; Gomez-Sanchez et al., 2016; X. Huang et al., 2019; M. Lange et al., 2012)
		Impulsivity, hyperactivity	
		Sensitivity to methylphenidate and atomoxetine	
	Mouse with Ala559Val substitution in the DAT gene	Association of the mutation with ADHD	(Davis et al., 2018; Mazei-Robison et al., 2005, 2008; A. Stewart et al., 2022)
		Impulsivity	
SNAP-25 knock-out coloboma mouse	Association of the mutation with ADHD, alterations in dopaminergic and noradrenergic systems	(Bruno & Hess, 2006; Feng et al., 2005; E. Hess et al., 1992; E. J. Hess et al., 1996; Jones et al., 2001; Mill et al., 2004)	
	Impulsivity, hyperactivity		
	Impairment in latent inhibition		
	Sensitivity to amphetamine		
	Drawback: no sensitivity to methylphenidate		
Acallosal mouse	Impulsivity (in a novel environment, but not after repeated testing), hyperactivity (develops over time)	(Magara et al., 2000)	

		Impaired acquisition in conditioned learning tasks	
Dopamine transporter knock-out mouse		Impulsivity, hyperactivity in novel environments	(Gainetdinov et al., 1999; Gainetdinov & Caron, 2000; GAINETDINOV & CARON, 2001; Madras et al., 2005; Trinh et al., 2003; P. Wong et al., 2012)
		Deficits in learning and memory	
		Sensitivity to amphetamine	
		Drawback: increased dopaminergic levels, growth delay, premature death, no sensitivity to methylphenidate	
Dopamine transporter knock-down mouse		Association between DAT gene and ADHD	(Madras et al., 2005; Zhuang et al., 2001)
		Hyperactivity in novel environments	
		Impaired response habituation in novel environments, motivation for a food reward	
		Sensitivity to amphetamine	
Tachykinin-1 (NK1) receptor mouse		Alterations in dopaminergic, serotonergic, and noradrenergic systems, association between tachykinin receptor gene mutations and ADHD	(Fisher et al., 2007; Froger et al., 2001; Herpfer et al., 2005; Yan et al., 2011; Yan, Hunt, et al., 2009; Yan, McQuillin, et al., 2009)
		Impulsivity, minor hyperactivity, inattention	
		Sensitivity to methylphenidate and amphetamine	
DAT-CI (cocaine insensitive) mouse		Association between DAT gene and ADHD, impaired dopaminergic system	(R. Chen et al., 2006; Madras et al., 2005; Napolitano et al., 2010)
		Hyperactivity	
		Sensitivity to methylphenidate and amphetamine	
Alpha-synuclein lacking mouse		Hyperactivity in novel environments	(Senior et al., 2008)

	Steroid sulfatase deletion in mouse	Association between genetic mutations of the steroid sulfatase gene, ADHD, and attentional deficits	(Davies et al., 2009; Stergiakouli et al., 2011; Trent et al., 2012; L.-J. Wang et al., 2017)
		Hyperactivity, inattention	
	TR β PV knock-in mouse	Association between thyroid hormone resistance and ADHD	(Albrecht et al., 2020; Mouri et al., 2014; Siesser et al., 2005, 2006; Weiss et al., 1993)
		Impulsivity, hyperactivity after repeated testing, inattention	
		Impairments in learning and memory	
		Sensitivity to methylphenidate	
	Guanylyl cyclase-C knock-out mouse	Impulsivity, hyperactivity, inattention	(Gong et al., 2011)
		Sensitivity to amphetamine	
	Shati/Nat8l knock-out mouse	Impulsivity, hyperactivity, inattention	(Toriumi et al., 2018)
		Reduced jumping latency in the cliff avoidance test, decreased recognition index in the object-based recognition test	
		Sensitivity to atomoxetine and methylphenidate	
	Mouse with loss of ataxin-1 - transcriptional repressor capicua (ATXN1-CIC) complex	Structural abnormalities	(H.-C. Lu et al., 2017)
Hyperactivity			
Deficits in learning and memory			
Drawback: ASD and intellectual disability comorbidity			
Zebrafish with ablated per1b	Decreased dopaminergic levels	(J. Huang et al., 2015)	
	Inattention, hyperactivity, impulsivity		
	Disruption of the circadian cycle		
Micall2b knock-down zebrafish	Impulsivity, hyperactivity	(L. Yang et al., 2018)	
	Sensitivity to atomoxetine		

Pharmacological models	Neonatal 6-OHDA lesioned rat	Impairments in dopaminergic and noradrenergic systems	(Davids et al., 2002, 2003; D. D. Dougherty et al., 1999; HANNA et al., 1996; LaHoste, Swanson, Wigal, Glabe, Wigal, King, et al., 1996; Luthman et al., 1989; SHAYWITZ et al., 1976; Shaywitz et al., 1976, 1978; K. Zhang, 2001)
		Hyperactivity, inattention	
		Altered spatial discrimination and learning	
		Sensitivity to methylphenidate	
	Neonatal 6-OHDA lesioned mouse	Structural, functional, and neurochemical alterations	(Bouchatta et al., 2018, 2020, 2022)
		Impulsivity, hyperactivity, inattention	
		Anxiety, pain sensitivity, decreased cognitive functions	
		Sensitivity to methylphenidate	
	Perinatal 6-OHDA with adulthood 5,7-DHT lesioned rat	Altered dopaminergic and serotonergic systems	(Kostrzewa et al., 1994)
		Hyperactivity	
Sensitivity to amphetamine			
Rat with neonatal anoxia	Altered monoamine systems	(Dell'Anna et al., 1991; Dell'anna et al., 1993)	
	Transient hyperactivity		
	Impairment in spatial memory		
Cerebellar stunting rat model	Hyperactivity	(Ferguson, 1996; Kostrzewa et al., 1994)	
	Learning deficits		
Rat with nucleus accumbens core lesion	Impulsivity, hyperactivity	(CARDINAL et al., 2004)	
	Learning deficits		
Rat with subthalamic lesion	Impulsivity, inattention	(Baunez & Robbins, 1997)	
Physical trauma models	X-radiation of neonatal rat	Hyperactivity	Czurko et al, 1997 (Czurkó et al., 1997; Diaz-Granados et al., 1992, 1994; Highfield et al., 1998)
		Learning and memory deficits	
		Partial sensitivity to d-amphetamine	
		Drawback: does not comply in terms of construct validity	

	Hypoxia in neonatal rat	Impulsivity, hyperactivity, inattention	(Delcour et al., 2012; Miguel et al., 2015, 2019; PIEŠOVÁ et al., 2020; Row et al., 2002; SPEISER et al., 1983)
Anxiety, learning, and memory deficits			
Partial sensitivity to methylphenidate			
Drawback: does not comply in terms of construct validity			
Environmental models	Rat reared in social isolation	Hyperactivity in novel environments	(Dalley et al., 2002)
		Increased errors of omission and perseveration	
	Rat with prenatal ethanol exposure	Impaired dopamine and noradrenaline systems	(Detering et al., 1980; Dewey Cooper & Kevin Rudeen, 1988; Fahlke & Hansen, 1999; GILBERTSON & BARRON, 2005; Girard et al., 2000; Hausknecht et al., 2005; C. K. Kim et al., 1997; Naseer et al., 2014; Reyes et al., 1989; Shen & Choong, 2006; R. Wang et al., 2020; C. Xu & Shen, 2001)
		Impulsivity, hyperactivity, inattention	
		Deficits in working and spatial memory	
		Sensitivity to amphetamine and methylphenidate	
	Rat exposed to organic pollutants	Impulsivity, hyperactivity	(Eubig et al., 2010; Grandjean et al., 2001; Sable et al., 2009; Sagiv et al., 2021; P. Stewart et al., 2003, 2005)
	Rat exposed to manganese	Hyperactivity	(Kern et al., 2010)
	Rat exposed to organo-phosphate pesticide	Hyperactivity	(C.-H. Chang et al., 2018; Gómez-Giménez et al., 2018; Roberts et al., 2019; Sagiv et al., 2021)
	Rat/mouse with prenatal nicotine exposure	Impulsivity, hyperactivity	(Hoang et al., 2021; Maitre et al., 2021; Nilsen & Tulve, 2020; Paz et al., 2007; Santiago & Huffman, 2014; Sourander et al., 2019; Vaglenova et al., 2004; Xi & Wu, 2021; J. Zhu et al., 2017)
Cognitive deficits, anxiety			
Sensitivity to methylphenidate			

	Rat/mouse/monkeys exposed to lead	Hyperactivity, inattention	(Cory-Slechta, 2003; Eubig et al., 2010; Levin et al., 1987; Levin & Bowman, 1986; Rice & Karpinski, 1988; Stangle et al., 2007)
		Cognitive and memory impairments	
		Sensitivity to amphetamine and methylphenidate	
	Maternally stressed mouse	Hyperactivity	(Ronald et al., 2011; Son et al., 2007)
	Mouse exposed to pyrethroid pesticides	Increased DAT levels	(Pitzer et al., 2019, 2021; Richardson et al., 2015; Wagner-Schuman et al., 2015)
		Impulsivity, hyperactivity, inattention	
		Working memory deficits	
		Sensitivity to methylphenidate	

Few of the above-listed animal models recapitulate all ADHD features. ADHD is, indeed, a complex neurodevelopmental disorder whose modeling can be challenging. Nevertheless, they all remain valid. The choice of the animal model should depend on the objectives of the research project.

The neonatal 6-OHDA lesioned mouse model, previously validated by our team, is the ADHD-like animal model adopted during my Ph.D. thesis.

1.3.3 Neonatal 6-OHDA lesioned mouse

The neonatal 6-OHDA lesioned mouse model is generated by the unilateral intracerebroventricular injection of 6-OHDA at P5 (five days after birth) and is characterized by dopaminergic neuron degeneration (Bouchatta et al., 2018). 6-OHDA is an analog of the dopamine neurotransmitter and a selective catecholaminergic neurotoxin. It is transported by dopaminergic and noradrenergic transporters and leads to the depletion of neuronal cells, once metabolized intracellularly (Wree et al., 2020). Administration of desipramine thirty minutes before surgery protects against noradrenergic cell degeneration (Bouchatta et al., 2018).

The validation process has previously confirmed that this ADHD-like mouse model presents numerous features of the clinical condition.

First, 6-OHDA mice display significant reductions in dopaminergic neurons in the striatum (Figure 4, A1), the substantia nigra (Figure 4, A2), and the ventral tegmental area (Figure 4, A2). Brain weight is decreased by 25% in comparison to sham mice, with no changes in body weight (Figure 4, B1-B2). Cortical thickness is affected in several cortical layers (II-III, V, VI) of the ACC (Figure 4, C1-C2). Alterations in the morphology of layer II-III pyramidal neurons are reported in the dorsal ACC and include reductions in the branching complexity of the medium-sized neurons, dendritic spine size, mean spine density, and area of pyramidal neurons (Bouchatta et al., 2018).

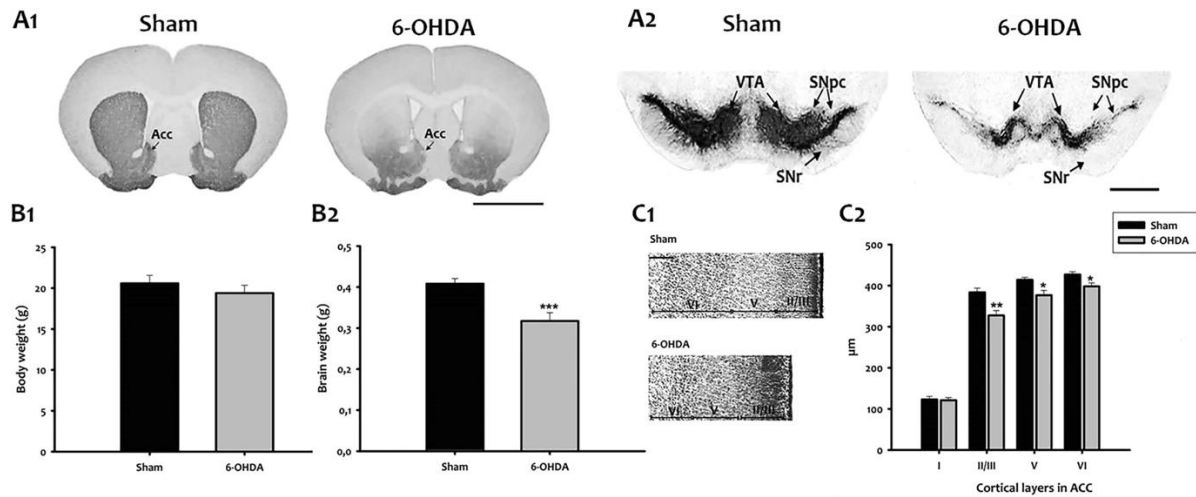


Figure 4: Morphological alterations in 6-OHDA mice (Bouchatta et al., 2018)

Second, 6-OHDA mice exhibit the three core symptoms of ADHD: hyperactivity, impulsivity, and inattention (Bouchatta et al., 2018). Spontaneous locomotor activity was tested at P24 (juvenile mice) and P40 using the open-field test. 6-OHDA mice are more hyperactive than sham mice in both childhood (Figure 5) and adolescence (Bouchatta et al., 2018). Administration of methylphenidate (3.0 and 5.0 mg/Kg) alleviates hyperactivity in P24 6-OHDA mice (Figure 5) (Bouchatta et al., 2018).

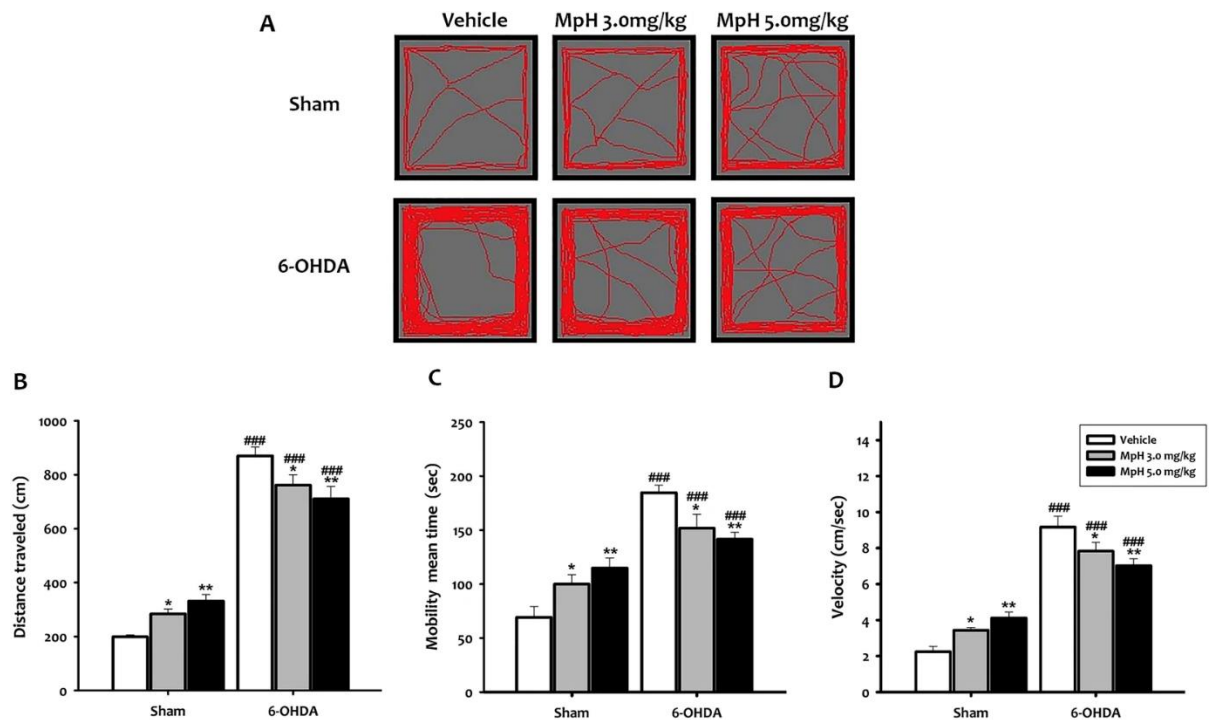


Figure 5: Spontaneous locomotor activity at P24 (Bouchatta et al., 2018)

Inattention and impulsivity were assessed using the 5-CSRTT (adolescence and adulthood) and the cliff avoidance reaction test (childhood) (Bouchatta et al., 2018, 2020). Findings demonstrate impairments in attention and increased impulsivity in 6-OHDA mice as depicted by a decreased accuracy and enhanced number of omission, premature, and perseverative responses (Figure 6) (Bouchatta et al., 2018). Methylphenidate administration (5.0 mg/Kg) ameliorates all parameters (Bouchatta et al., 2018). Moreover, 6-OHDA juvenile mice present abnormal latent inhibition, indicative of poor sustained attention (Bouchatta et al., 2020).

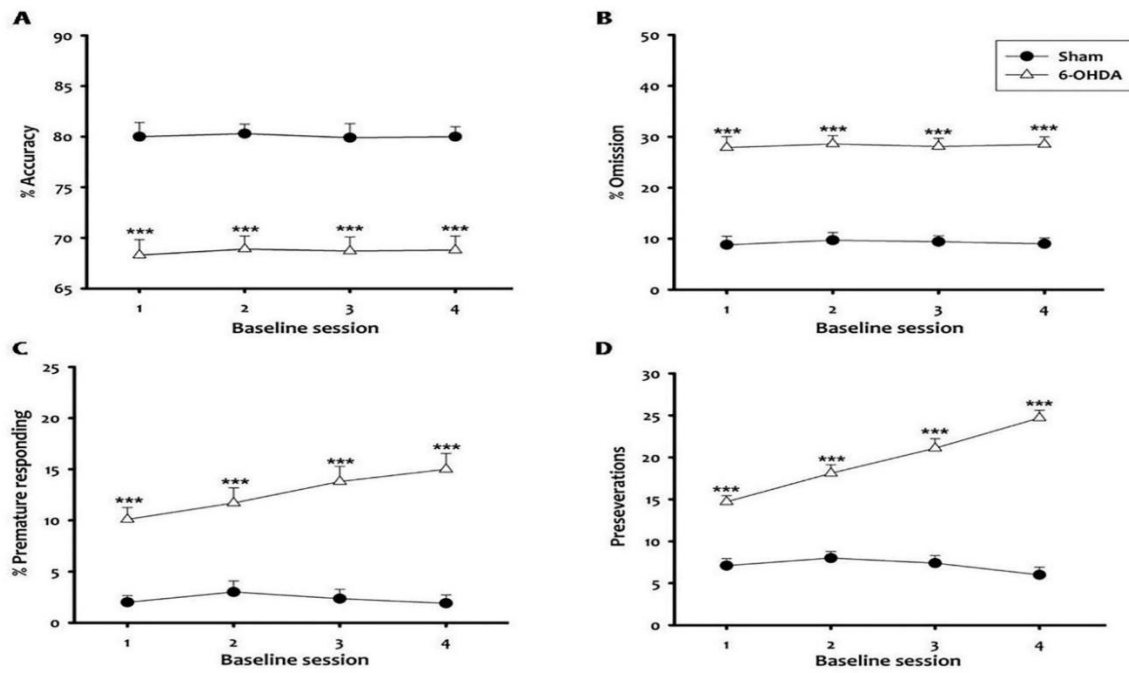


Figure 6: Baseline performance of sham and 6-OHDA mice in the 5-CSRTT (Bouchatta et al., 2018)

Comorbidities are perceived in 6-OHDA mice such as anxiety-like behavior (elevated plus maze test), antisocial and aggressive behaviors (social interactions), as well as deficits in short-term memory (novel object recognition test). Methylphenidate administration improves the animal's social interactions and cognitive impairments but does not affect anxiety (Bouchatta et al., 2018).

Importantly, hypersensitivity to thermal (hot/cold plate test) and mechanical (Von Frey test) stimuli develops in 6-OHDA mice. Both the paw-licking latency (Figure 7, A-B) and the mechanical withdrawal thresholds (Figure 7, C) are shortened in ADHD-like conditions. Methylphenidate administration is ineffective on 6-OHDA-induced pain sensitivity (Bouchatta et al., 2022).

The injection of complete Freund's adjuvant (CFA), inducing a model of persistent peripheral inflammatory pain, further decreases mechanical and thermal thresholds (Figure 7, D1-D3). Remarkably, ADHD-like conditions amplify sensitization to nociceptive stimuli (Figure 7, E1-E3), suggesting a worsening of central sensitization mechanisms (Bouchatta et al., 2022).

In terms of functionality, dorsal horn neuron (DHN) excitability and ACC neuron activity are amplified in basal and nociceptive conditions (Bouchatta et al., 2022). Modulation of the ACC or ACC-PI excitatory pathway modifies DHN activity and mechanical thresholds, supporting the role of these two brain structures in pain processing. More importantly, the effects of this modulation are enhanced in 6-OHDA mice, suggesting impairments of nociceptive mechanisms in ADHD-like conditions (Bouchatta et al., 2022).

Overall, the neonatal 6-OHDA lesioned mouse model recapitulates the main characteristics of the clinical condition: (i) structural and morphological abnormalities, (ii) three core symptoms (attention, hyperactivity, impulsivity), (iii) comorbid disorders (anxiety, pain sensitivity, cognitive deficits), and (iv) functional impairments. It is therefore a reliable model for the study of ADHD.

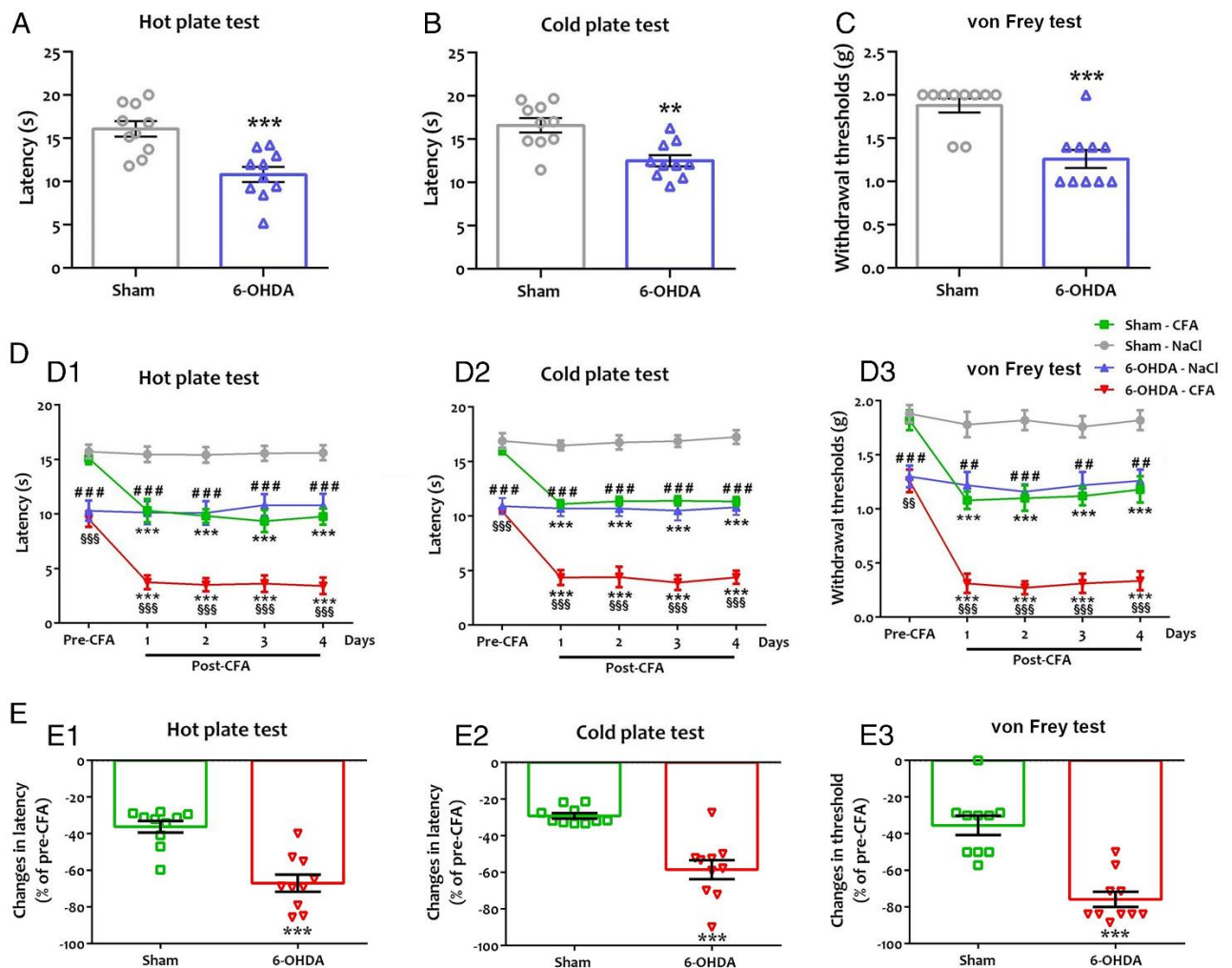


Figure 7: Hypersensitivity to thermal and mechanical stimuli in 6-OHDA mice in basal and CFA conditions (Bouchatta et al., 2022)

2. Pain

2.1 Introduction to pain

According to the International Association for the Study of Pain (IASP), pain can be described as an unpleasant “sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage” (Raja et al., 2020). Pain is part of the body’s defense mechanism as its sole purpose is to protect against harmful stimuli and prevent further damage (Yam et al., 2018). It is a subjective experience that can be affected by different biological, social, and psychological factors (Raja et al., 2020).

Three dimensions of pain were previously described: sensory-discriminative, affective-motivational, and cognitive pain aspects (Melzack & Casey, 1968). The sensory-discriminative dimension relates to the spatiotemporal features (intensity, location, duration) and quality of pain. In contrast, the affective-motivational dimension involves emotion and captures how bad the pain sensation is. The unpleasantness of pain can lead to protective actions, thus implicating motivation as well. It can be accompanied by mood problems such as anxiety and depression. Finally, the cognitive dimension depicts the thoughts and beliefs about pain. It is influenced by past experiences, helps to interpret pain, and provides meaning to it. It also encompasses the coping strategies implemented to reduce and tolerate this aversive sensation (Ciccone & Grzesiak, 1984; McGuire, 1992; Melzack & Casey, 1968; Talbot et al., 2019). Therefore, pain is an

individual experience engaging emotions and cognitive processes, with the ultimate intent to keep the body from harm.

Pain is classified into three main types which are nociceptive, neuropathic, and inflammatory pain (C. J. Woolf et al., 1998; Yam et al., 2018).

2.2 Nociceptive pain

Pain and nociception are distinct phenomena. While pain refers to a complex and personal experience involving several dimensions, nociception designates -and is reduced to- the neuronal activity that enables information processing following noxious stimuli (Raja et al., 2020).

2.2.1 Stages of pain perception

The processing of pain-related information and, therefore, pain perception involves several systems and can be divided into four stages: (i) transduction, (ii) transmission, (iii) modulation, and (iv) perception (Figure 8) (Osterweis et al., 1987; Yam et al., 2018).

Transduction denotes the conversion of noxious stimuli, that have activated peripheral nociceptor fibers, into a transmissible electrochemical signal. Noxious stimuli include mechanical, thermal, and chemical events. Transmission represents the process by which pain-related information is carried out from the site of injury to the brain regions involved in pain perception: (i) the nociceptive signal is transmitted by the axons of the afferent nociceptor fibers from the periphery to the dorsal horn of the spinal cord, (ii) ascending pathways then relay the message to the brain (thalamus, somatosensory cortex, limbic system, brain stem) where it is processed and integrated, and (iii) descending pathways transport the signal from the brain to the organs through the spinal cord. Modulation points out the neural events that participate in the regulation of pain sensation. It takes place during the whole transmission process. Finally, perception is the subjective integration of sensory signals into a clear whole (Figure 8) (Osterweis et al., 1987; Yam et al., 2018).

Remarkably, the intensity of the nociceptive signal and the frequency of neuronal discharge were reported to be positively correlated. The more intense the stimulus is, the higher the discharge frequency is, and the more painful the sensation is (M. Fitzgerald & Lynn, 1977; LaMotte et al., 1983; Osterweis et al., 1987).

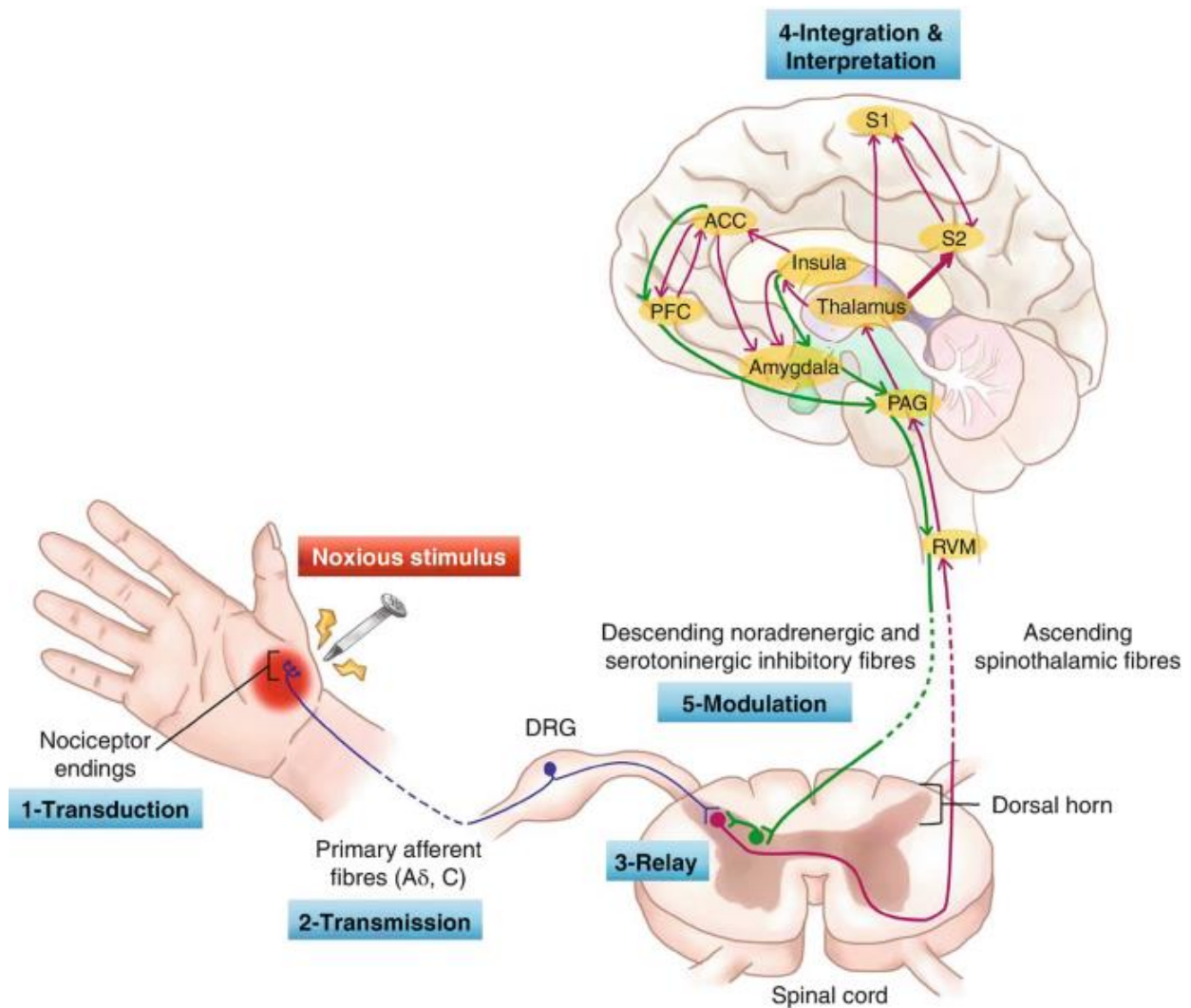


Figure 8: Stages of pain transmission (Y. Chen & Abdi, 2022).

2.2.2 Peripheral nociceptive fibers

Nociception is mediated by the activation of two types of nociceptive primary afferent fibers: the myelinated A δ -fibers and the unmyelinated C-fibers (Figure 8). These nociceptor fibers are silent in homeostatic conditions but are activated in response to noxious stimuli (Yam et al., 2018).

Myelinated A δ -fibers are activated by thermal and mechanical nociceptive signals. They have a diameter of 2 to 5 μ m and a fast conduction velocity of about 30m/s. They are known to respond to transient and pricking pain and to terminate in laminae I and V of the gray matter of the spinal cord. On the other hand, unmyelinated C-fibers respond to thermal, mechanical, and chemical stimuli. They display a small diameter of less than 2 μ m and a slow conduction velocity of 2 μ m/s. Unmyelinated C-fibers are the most abundant, making up 70% of the afferent fibers entering the spinal cord, and terminating in the laminae I and II of its gray matter (Yam et al., 2018).

Other nociceptor fibers were also reported such as (i) myelinated A α with proprioceptive properties, (ii) myelinated A β fibers responding to mechanical stimuli and terminating in laminae III and IV, (iii) myelinated A γ fibers sensitive to mechanical stimuli, and (iv) B fibers that are moderately myelinated with a conduction velocity ranging from 3 to 14m/s (Yam et al., 2018).

Afferent nociceptor fibers convey the signal from the periphery to the spinal cord. In the gray matter of

the dorsal horn, these primary fibers release neurotransmitters (primarily glutamate) from their spinal endings thus activating second-order nerve cells (Figure 8) (Osterweis et al., 1987; Willis, 1985).

2.2.3 Ascending pathways

A subset of second-order pain-transmission cells transfers the noxious information from the spinal cord to specific brain regions. These cells lie in the anterolateral quadrant of the spinal cord and constitute the ascending pathways (Figure 8) (Osterweis et al., 1987).

Two major ascending pathways were described: the spinothalamic tract and the spinoreticular tract. The spinothalamic tract sends sensory signals up to the thalamus (which projects to the somatosensory cortex), thus conveying information related to the stimuli's location and intensity. It is further divided into two pathways: the lateral spinothalamic tract (neospinothalamic tract) responsible for temperature sensation, and the anterior spinothalamic tract (paleospinothalamic tract) involved in non-discriminative touch and pressure sensation (Osterweis et al., 1987; Sengul & Watson, 2012; Yam et al., 2018). The spinoreticular tract reaches the reticular formation of the brainstem. It is mainly implicated in the affective-motivational pain dimension, descending pain modulation, and motor responses to pain (Millan, 1999; Sengul & Watson, 2012).

2.2.4 Cortical structures

Several brain structures are activated by nociceptive stimuli. They are implicated in the different aspects of pain and are referred to as "the pain matrix". This network is composed of the primary and secondary somatosensory cortices, the PFC, the ACC, the IC, the amygdala, the thalamus, the cerebellum, the ventral tegmental area, and the nucleus accumbens (Figure 8) (Apkarian et al., 2005; Bushnell et al., 2013; Ossipov et al., 2014).

The somatosensory cortices mediate the sensory-discriminative dimension of pain (duration and location of pain) while the ACC and IC are responsible for the affective-motivational aspect. The PFC, the amygdala, the nucleus accumbens, and the ventral tegmental area also contribute to the latter dimension (Bushnell et al., 2013; Ossipov et al., 2014).

2.2.5 Descending pathways

Two other regions are critical for pain perception, the periaqueductal gray (PAG) and the rostroventromedial medulla (RVM) (Figure 8).

PAG is responsible for (i) receiving inputs from the ascending nociceptive pathways and relaying this information to the different structures of the pain matrix, and (ii) receiving inputs from the higher brain centers and sending it to the RVM, through their reciprocal connections, to modulate the descending pathways. PAG mainly exerts an inhibitory effect on nociceptive information (Behbehani & Fields, 1979; Ossipov et al., 2014).

The RVM receives inputs from the PAG, the thalamus, the locus coeruleus, and the parabrachial region and carries this information through its descending projections to the spinal cord. It is primarily composed of two serotonergic nuclei, exhibiting pro- or anti-nociceptive effects. This dual impact depends on the subtype of serotonergic receptor (5-HT) that is activated. Following the activation of the descending pathways from the RVM, serotonin is released in the spinal dorsal horns and can bind to different subtypes of 5-HT receptors. Activation of 5-HT_{2A} and 5-HT₃ receptors elicits a facilitatory pain effect, while activation of 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, and 5-HT₇ receptors results in anti-nociception (Ossipov et al., 2014).

Other descending projections to the spinal cord encompass those arising from the locus coeruleus, the

Kölliker-Füße pontine noradrenergic nuclei, and the A5 area in the brain stem. They are constituted of noradrenergic projections that communicate with the PAG and the RVM, and present pro- and anti-nociceptive effects. The release of spinal norepinephrine and its binding to the ADRA2A receptors is anti-nociceptive, while the activation of spinal α 1-adrenergic receptors facilitates pain sensation (Holden et al., 1999; Ossipov et al., 2014; Pertovaara, 2006).

2.3 Mediators

Neurotransmitters, adenosine triphosphate (ATP), growth factors, and other molecules are essential for pain sensation. They can be found in the periphery or the CNS and activate different signaling pathways with pro- or anti-nociceptive effects (Table 4) (Yam et al., 2018).

Table 4: Pain-associated mediators (Yam et al., 2018)

Mediator	Location	Receptor	Affected pathway	Effect on pain	References
Substance P, Neurokinin A, Neurokinin B	CNS and peripheral nervous system (PNS)	Neurokinin type 1, Neurokinin type 2, Neurokinin type 3	Phospholipase C (PLC)/Inositol triphosphate (IP ₃) Diacylglycerol (DAG)/Protein kinase C (PKC)	Excitatory	(Donkin et al., 2007; Gerard et al., 1990; Nagano et al., 2011; Saria, 1999; Takeda et al., 1991; Yip & Chahl, 2001)
Bradykinin	CNS and PNS	Bradykinin receptor 1 and 2	PLC/ IP ₃ DAG/PKC	Excitatory	(Gironacci et al., 2013; Llona et al., 1987; McLean et al., 2000; Walker et al., 1995)
Serotonin	CNS and PNS	5-HT _{2A} receptor, 5-HT ₃ receptor	5-HT _{2A} : PLC/ IP ₃ and DAG/PKC 5-HT ₃ : Na ⁺ /K ⁺	Excitatory	(Aghajanian & Marek, 1999; Bortolozzi et al., 2005; Férézou et al., 2002; Marek et al., 2001; Martin et al., 1998)
Prostaglandin E ₂ , Prostaglandin I ₂	CNS and PNS	Prostaglandin E ₂ receptor type (EP) 1-4, Prostacyclin receptor (IP)	EP ₁ : PLC/ IP ₃ and DAG/PKC IP, EP ₂ , EP ₃ , EP ₄ : Adenylyl cyclase (AC)/cyclic adenosine monophosphate	IP, EP ₁ , EP ₂ , EP ₄ : excitatory EP ₃ : inhibitory	(Claar et al., 2015; Ricciotti & FitzGerald, 2011; Woodward et al., 2011)

			(cAMP)/protein kinase A (PKA)		
Leukotriene B ₄	PNS	Leukotriene B ₄ receptor 1 and 2	AC/cAMP/PKA	Excitatory	(Bäck et al., 2014; J. D. Levine et al., 1986)
Proton	CNS and PNS	Acid-sensing ion channels, Vanilloid receptor for capsaicin	Na ⁺ /K ⁺	Excitatory	(Cui et al., 2006; S. M. Huang et al., 2002; Osmakov et al., 2014; Sluka et al., 2009)
ATP, Adenosine	CNS and PNS	Purine receptor type 3 (P2X ₃), Adenosine type 1 (A ₁) or 2 receptor (A ₂)	P2X ₃ : Na ⁺ /K ⁺ A ₁ , A ₂ : AC/cAMP/PKA	Excitatory A ₁ : inhibitory	(Burnstock, 2000; Elmenhorst et al., 2007; North, 2002; Tawfik et al., 2005)
Nerve growth factor	CNS and PNS	Tropomyosin receptor kinase A	Phosphoinositide 3-kinase (PI3K)/Ras	Excitatory	(Freeman et al., 2004; Riikonen & Vanhala, 1999)
Glutamate	CNS and PNS	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA-R), N-methyl-D-aspartate receptor (NMDA-R)	Na ⁺ /K ⁺ /Mg ²⁺ /Ca ²⁺	Excitatory	(Dingledine et al., 1999; Furukawa et al., 2005; Moriyoshi et al., 1991; Platt, 2007)
Norepinephrine	CNS and PNS	α1, α2, β receptors	α1: PLC/ IP3 and DAG/PKC α2, β: AC/cAMP/PKA	α1, β: excitatory α2: inhibitory	(Rang et al., 2011; Saunders & Limbird, 1999)
Histamine	CNS and PNS	Histamine 1 receptor	PLC/ IP3 DAG/PKC	Excitatory	(Shimamura et al., 2011; West et al., 1990)
Nitric oxide	CNS and PNS	Soluble guanylyl cyclase (sCG)	sCG/cGMP	Excitatory and inhibitory	(Forstermann & Sessa, 2012)
γ-aminobutyric acid (GABA)	CNS and PNS	Ionotropic GABA _A receptors,	GABA _A : Cl ⁻ /K ⁺	Inhibitory	(Hyland & Cryan, 2010;

		Metabotropic GABA _B receptors	GABA _B : AC/cAMP/PKA		Lorenzo et al., 2007)
Opioid peptides	CNS and PNS	μ, δ, and κ- opioid receptors	AC/cAMP/PKA	Inhibitory	(Gramsch et al., 1982; James et al., 1982; Quock et al., 1999)
Cannabinoids	CNS and PNS	Cannabinoid type 1 and 2 receptors	AC/cAMP/PKA	Inhibitory	(Núñez et al., 2004; Pacher & Mechoulam, 2011; Rogers, 2015; Van Sickle et al., 2005)
Glycine	CNS	Glycine receptors	Cl ⁻	Inhibitory	(Baer, 2009; Naas et al., 1991)
Calcitonin gene-related peptide	CNS and PNS	Calcitonin receptor-like receptor	AC/cAMP/PKA	Excitatory	(Arulmani et al., 2004; Brain et al., 1985; L.-J. Chen et al., 2010; McCulloch et al., 1986; Rosenfeld et al., 1983)

2.4 Chronic pain

2.4.1 Generalities

Chronic pain is defined as pain lasting for more than three to six months. Contrary to acute pain, chronic pain persists in the absence of noxious stimuli thus no longer acting as a warning mechanism toward harmful signals (Bonica, 1991; Merskey & Bogduk, 1986; Treede et al., 2015).

Chronic pain is a prevalent condition affecting almost 20% of the worldwide population (Breivik et al., 2006; C. J. Phillips, 2009; Treede et al., 2015). Inequalities among genders are observed as women are more susceptible to developing chronic pain conditions compared to men (Andrews et al., 2018; Brennan, 2020; Larsson et al., 2017; C. J. Phillips, 2009; Souza et al., 2017). For instance, 18% of women in the older population (>60 years old) experience chronic pain compared to 10% of men (C. J. Phillips, 2009).

It is a disabling neuropathology that negatively impacts the well-being of the patient and his daily activities (Brennan, 2020; C. J. Phillips, 2009; Yiengprugsawan & Steptoe, 2018). For instance, chronic pain can result in depression, anxiety, and sleep problems (Cohen et al., 2021). Additionally, patients report insufficient management of their pain (Breivik et al., 2006), with almost 50% citing dissatisfaction (Souza et al., 2017).

It is also a great economic and medical burden. In 2010, loss of productivity and medical bills accounted for costs ranging from US\$560 to US\$635 billion (Institute of Medicine (US) Committee on Advancing Pain Research, 2011). This can be explained by the loss of productivity and the increased absenteeism of chronic pain patients (C. J. Phillips, 2009).

Interestingly, findings suggest an association between ADHD and chronic pain conditions. A high prevalence of chronic pain conditions is observed in ADHD patients and is estimated to be 65.9% among ADHD adolescents. 55.1% are affected by musculoskeletal pain while 31.3% suffer from multi-site pain (Mangerud et al., 2013). Additionally, 31.7% of ADHD patients report chronic abdominal pain or chronic headaches (Asztély et al., 2019). Reciprocally, individuals suffering from chronic pain are frequently diagnosed with ADHD (Battison et al., 2023). For instance, 18% of children with pathological pain conditions have ADHD (Low Kapalu et al., 2018).

2.4.2 Mechanisms of chronic pain

Pathological pain is divided into two main categories: neuropathic pain and inflammatory pain. Neuropathic pain results from nerve injury while inflammatory pain arises following an inflammatory insult. These chronic pain conditions are associated with impairments in pain perception (allodynia and hyperalgesia) and increased pain sensitization (at the periphery or in the CNS).

Allodynia is defined as pain in response to stimuli considered innocuous and non-painful in normal conditions while hyperalgesia is described as an exaggerated pain perception to noxious and painful signals (Merskey & Bogduk, 1986; Vardeh & Naranjo, 2017; Yam et al., 2018). Hyperalgesia can be further divided into two types: primary and secondary. Primary hyperalgesia manifests at the site of injury as a consequence of peripheral sensitization (sensitization of peripheral nerve terminals) while secondary hyperalgesia occurs in the surrounding area of the injury site as a consequence of central sensitization (alterations in the spinal cord and brain) (Vardeh & Naranjo, 2017).

Several functional changes contribute to allodynia and hyperalgesia. These mechanisms mainly involve modifications in the A δ and C-fibers and include (i) recruitment and lowered thresholds of afferent A δ and C-fibers that were initially mechanically insensitive, (ii) amplified responses of A δ and C-fibers to perceptible stimuli, and (iii) increased innervation of the injured site by A δ and C-fibers. Other mechanisms involve the recruitment of myelinated A β -fibers that become sensitive to painful messages, or impairments in pain inhibitory pathways (Vardeh & Naranjo, 2017).

2.4.2.1. *Peripheral sensitization*

Following tissue injury or inflammation, inflammatory mediators are released by immune cells and sensory nerve fibers to (i) activate peripheral nociceptors and (ii) amplify their responsiveness to stimuli (bradykinin, prostaglandins, nerve growth factor, histamine). This decrease in pain thresholds is induced by the stimulation of GPCR or receptor tyrosine kinases that mediate, through the initiation of a signaling cascade, the activation of PKA and PKC. These two proteins are known to phosphorylate and regulate the activity of sensory neuron-specific ion channels such as Na $v_{1.8}$, Na $v_{1.9}$, and TRPV1 (transient receptor potential vanilloid type 1). The prolonged opening of these ion channels leads to an extended depolarization and enhanced response (Vardeh & Naranjo, 2017).

Modifications in ion channel activity can sometimes be accompanied by alterations in the transcription or translation of specific proteins. One example is the upregulation in the expression of the TRPV1 ion channel following the release of the nerve growth factor (Vardeh & Naranjo, 2017).

Peripheral sensitization therefore results from a reduced threshold and an increased excitability of the nociceptive peripheral nerve fibers. It is restricted to the site of injury or inflammation and contributes to primary hyperalgesia. Moreover, peripheral sensitization appears to be involved in thermal (heat) sensitization, but not mechanical hypersensitivity (Latremoliere & Woolf, 2009).

2.4.2.2. Central sensitization

Central sensitization is defined as a state of enhanced responsiveness of nociceptors and results from increased inputs to nociceptive stimuli within the CNS. Factors leading to central sensitization include (i) the recruitment of myelinated A β -fibers (mechanoreceptors initially insensitive to noxious signals), (ii) the shift from high to low pain thresholds, and (iii) the increase in DHN spontaneous activity. It affects non-injured or non-inflamed tissues, giving rise to secondary hyperalgesia (Latremoliere & Woolf, 2009).

At the molecular level, numerous mediators and signaling pathways contribute to central sensitization (Figure 9) (Honore et al., 2000). One of the main mechanisms is the activation of the glutamate receptors (NMDA-R), critical in the initiation and maintenance of central sensitization (Latremoliere & Woolf, 2009).

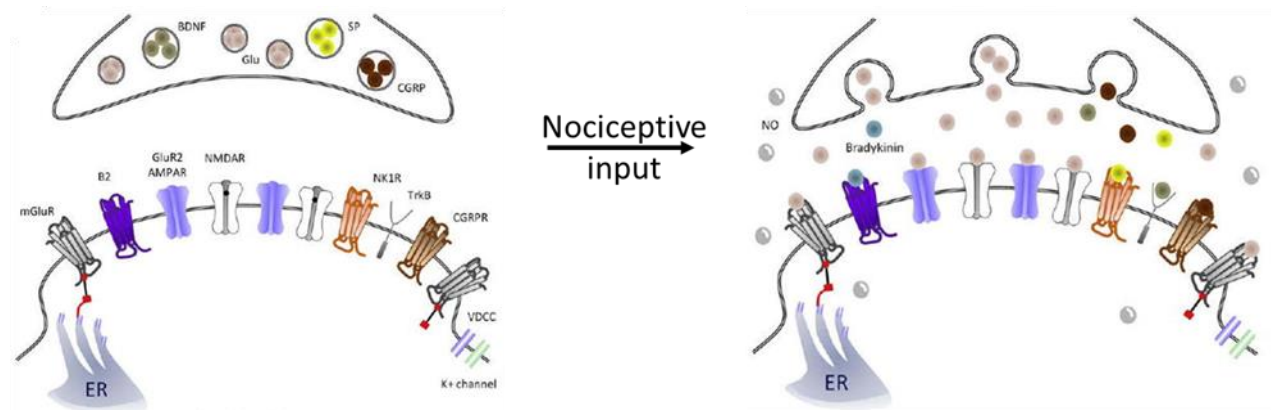


Figure 9: Key mediators of central sensitization (Latremoliere & Woolf, 2009).

In homeostatic conditions, NMDA-Rs are blocked by Mg²⁺ ions (Mayer et al., 1984). This inhibition is lifted by nociceptive stimuli which (i) induce the release of glutamate, calcitonin gene-related peptide, and substance P (ii) lead to membrane depolarization, and (iii) result in the removal of Mg²⁺ ions from the pores of the receptors (Mayer et al., 1984). This is accompanied by the binding of glutamate to NMDA-Rs, the generation of a current, and the entry of Ca²⁺ ions into the neurons. Ca²⁺ ions activate several intracellular pathways that sustain central sensitization. In addition to NMDA-Rs, AMPA-Rs and the metabotropic glutamate receptors type I also engage in central sensitization through PLC/PKC, PI3K, and extracellular signal-regulated kinases (ERK) pathway activation (Latremoliere & Woolf, 2009).

Substance P, co-released with glutamate, participates in central sensitization through its binding to NK1 receptors. It establishes membrane depolarization (Henry, 1976), causes temporal summation of C-fibers evoked synaptic potentials (P. M. Dougherty & Willis, 1991), and activates intracellular pathways. These effects are potentiated by calcitonin gene-related peptide (C. Woolf & Wiesenfeld-Hallin, 1986) which further triggers central sensitization through the activation of PKA and PKC, as well as BDNF release (Buldyrev et al., 2006; Latremoliere & Woolf, 2009; R.-Q. Sun et al., 2003, 2004). BDNF secretion in the spinal cord and its binding to trkB receptors amplifies NMDA-R-mediated C-fiber-induced responses and activates PKC and ERK (Kawasaki et al., 2004; Kerr et al., 1999; Latremoliere & Woolf, 2009; Pezet et al., 2002; Slack et al., 2004, 2005). Finally, bradykinin also contributes to synaptic strengthening through its binding to B2 receptors and the induction of PKC, PKA, and ERK (Kohno et al., 2008; Latremoliere & Woolf, 2009).

ERK, PKA, and PKC pathway activation leads to the recruitment and increased function of glutamatergic receptors (NMDA-R and AMPA-R), in addition to the decrease of K⁺ currents, resulting in enhanced synaptic efficacy and membrane excitability (Figure 10, A-B). ERK pathway also activates several transcription factors driving the expression of genes involved in central sensitization (Figure 10, C) (Galan

et al., 2004; Hu et al., 2006, 2007; Latremoliere & Woolf, 2009; Y. Qin et al., 2005).

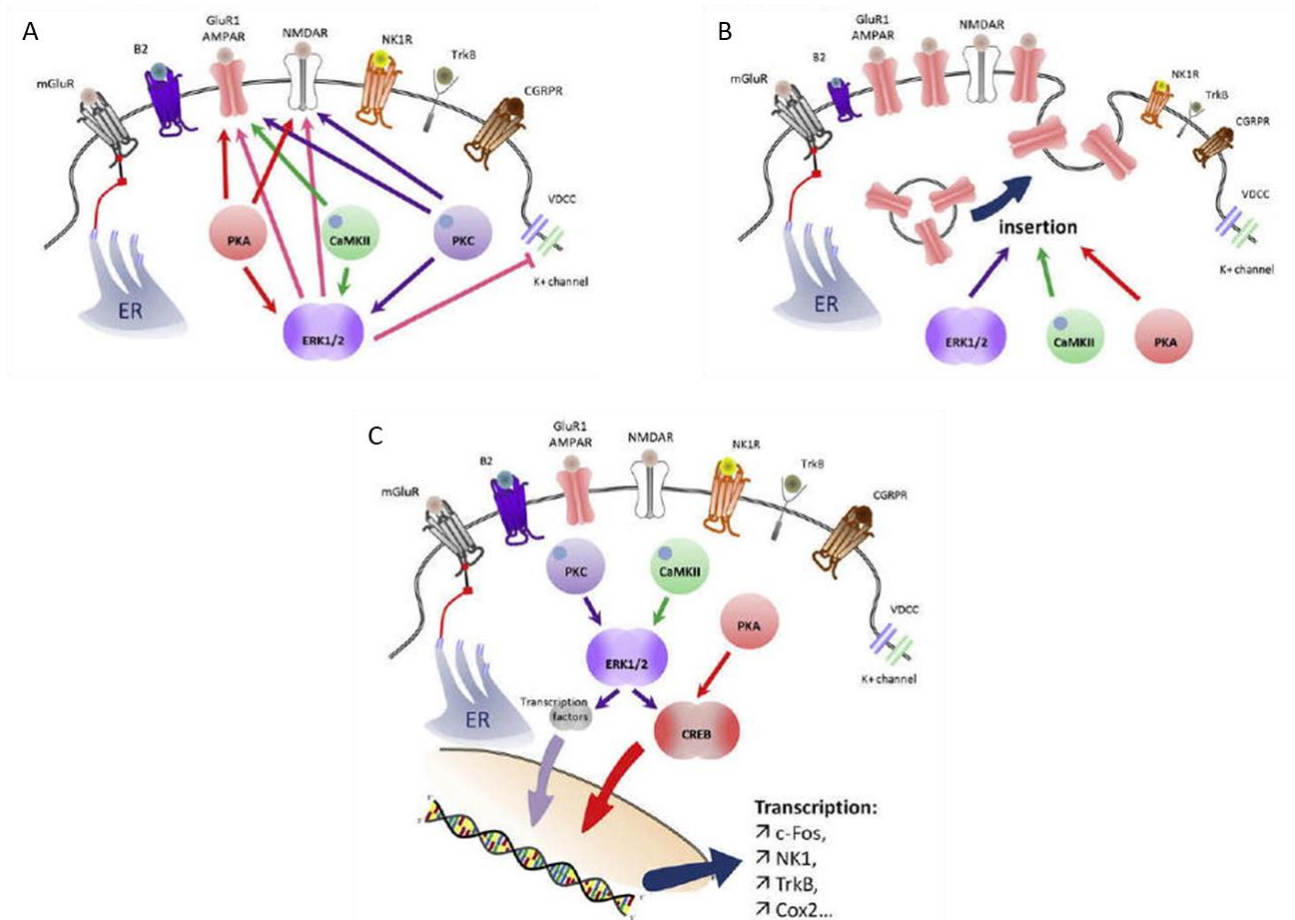


Figure 10: Effects of PKC, PKA, and ERK pathways activation on central sensitization (Latremoliere & Woolf, 2009).

2.4.2.3. Inflammatory pain

Inflammatory pain is a type of chronic pain condition caused by the release of inflammatory mediators that sensitize nociceptors. Mechanisms of central sensitization, along with other unique processes, are responsible for inflammatory pain.

Cyclooxygenase-2 (COX-2) is critically implicated in pain hypersensitivity under inflammatory pain conditions. Its expression in DHN triggers the production of prostaglandin E2 (Samad et al., 2001) which subsequently binds to its receptors EP2 and EP4 and leads to (i) neurotransmitter release from presynaptic cells, (ii) enhancement of NMDA-R and AMPA-R currents, (iii) cation channels activation, and (iv) inhibition of glycinergic transmission (Ahmadi et al., 2002; Baba et al., 2001; Harvey et al., 2004; Latremoliere & Woolf, 2009; F. Müller et al., 2003; Vasko et al., 1994). Another mechanism involves changes in spinal microglial cells which undergo morphological, functional, and transcriptional modifications (Svensson, Hua, et al., 2003; Svensson, Marsala, et al., 2003). In inflammatory pain conditions, p38 mitogen-activated protein kinase (MAPK) is activated (Ji, Gereau, et al., 2009; Svensson, Hua, et al., 2003; Svensson, Marsala, et al., 2003), thus resulting in the synthesis of pro-inflammatory mediators by microglia cells (Ji, Gereau, et al., 2009). Among these inflammatory molecules, interleukin (IL)-1 β and tumor necrosis factor α (TNF- α) activate COX-2 and promote excitatory currents while restricting inhibitory ones (Kawasaki, Zhang, et al., 2008; Latremoliere & Woolf, 2009; Samad et al., 2001).

2.4.2.4. Neuropathic pain

Another type of chronic pain condition is neuropathic pain, induced by nerve damage. Similar to inflammatory pain, neuropathic pain is associated with central sensitization and other unique processes that contribute to pain sensitization.

Following nerve injury, A- and C-fibers generate spontaneous action potentials and undergo changes that initiate and maintain central sensitization. Chemical alterations include the release of new modulators such as substance P and BDNF (Devor, 2009; Fukuoka et al., 2001; Latremoliere & Woolf, 2009; Noguchi et al., 1995; Xiao & Zhang, 2018) or the upregulation of guanosine triphosphate cyclohydrolase 1, an enzyme required for the synthesis of tetrahydrobiopterin (Tegeader et al., 2006). Not only does tetrahydrobiopterin act as a cofactor for nitric oxide synthase, but it also participates in the development of neuropathic and inflammatory pain (Latremoliere & Woolf, 2009; Tegeader et al., 2006). Structural modifications encompass the sprouting of A β fibers from laminae III-IV to laminae I-II and their contact with nociceptive neurons as a consequence of (i) C-fiber terminals degeneration in laminae II and (ii) the increase in axonal growth capacity (Arvidsson et al., 1986; Lekan et al., 1996; Mannion et al., 1996; Shortland et al., 1997; C. J. Woolf et al., 1992, 1995).

Disinhibition was demonstrated to participate in the establishment of neuropathic pain, with the apoptosis of inhibitory interneurons leading to a reduction in GABAergic and glycinergic inhibitory currents (K. A. Moore et al., 2002; Scholz et al., 2005). Loss of GABAergic transmission is attributed to the effects of BDNF on Cl⁻-cotransporter K⁺-Cl⁻ exporter 2 channels (KCC2). The release of BDNF by microglial cells decreases KCC2 expression resulting in diminished or ablated Cl⁻ entry into neurons (Coull et al., 2003, 2005; Latremoliere & Woolf, 2009; V. B. Lu et al., 2009; Miletic & Miletic, 2008).

Finally, the recruitment and activation of peripheral immune cells, microglia, and astrocytes also participate in neuropathic pain states through the release of inflammatory mediators that interact with neurons, thus resulting in central sensitization (Cao & DeLeo, 2008; Latremoliere & Woolf, 2009; Romero-Sandoval et al., 2008; Watkins et al., 2001; Watkins & Maier, 2002).

2.4.2.5. Implication of the pain matrix and descending pathways

Morphological and functional alterations in several regions of the pain matrix and descending pathways underlie pain chronification (S. Yang & Chang, 2019).

Reduced gray matter volume in the IC (Baliki et al., 2012; Schmidt-Wilcke et al., 2008), the nucleus accumbens (Baliki et al., 2012), the ACC (Schmidt-Wilcke et al., 2008), the PFC (Apkarian et al., 2005), and the thalamus (Apkarian et al., 2005) were reported in patients suffering from chronic pain conditions.

Enhanced functional connectivity between PAG and the PFC was detected in subjects with chronic low back pain (R. Yu et al., 2014). Higher functional connectivity between the PFC, the amygdala, and the nucleus accumbens predicted pain chronification in individuals with back pain (Vachon-Preseu et al., 2016). Interestingly, increased functional connectivity between the nucleus accumbens, the PFC, and the basal ganglia contributed to the affective dimension of pain (Baliki et al., 2012) while impairments in the connectivity between PAG and other regions of the pain matrix (IC, amygdala, PFC) were correlated with pain duration and intensity (R. Yu et al., 2014).

Abnormalities in the excitatory synaptic transmission were found in layers II-III of the ACC following peripheral nerve damage (H. Xu et al., 2008) while synaptic potentiation in the amygdala was linked to tactile allodynia in a model of neuropathic pain (R. Ikeda et al., 2007).

Inflammation is a major contributor to pathological pain. Its mechanisms are detailed below.

3. Inflammation

3.1 Peripheral inflammation

Inflammation is the protective response of the immune system toward infection or injury. It is characterized by immune cell activation and signaling molecule release (cytokines, nitric oxide, prostaglandins, etc.) that lead to local and systemic responses. Inflammation can be divided into innate and adaptive, which differ in cellular actors and duration.

At the periphery, the innate immune response is (i) orchestrated by leukocytes and endothelial cells, (ii) effective immediately after the insult, (iii) non-specific, and (iv) usually short-lived. In response to infection or injury, circulating leukocytes are recruited to the site of damage following endothelial cell activation and the expression of cellular adhesion molecules. This extravasation is facilitated by phenotypic changes in the endothelium, resulting in increased permeability to white blood cells (Muller, 2013). Within the tissue, leukocytes act as phagocytes and antigen-presenting cells. They also secrete signaling molecules that attract and mobilize additional effector cells from the periphery (Lyman et al., 2014). On the contrary, the adaptive immune response (i) is highly specialized, (ii) occurs several weeks after the insult, and (iii) involves T and B lymphocyte activation (Lyman et al., 2014).

Inflammation aims to protect the organism from any harm and damaging stimuli. However, an excessive immune response can exert detrimental effects resulting in tissue damage and disease onset. This is mainly caused by the migration of activated immune cells from the initial site of inflammation to other body areas - the local inflammation shifts to a systemic response that can compromise homeostasis.

3.2 Neuroinflammation

Neuroinflammation is defined as the inflammatory response within the CNS. It involves microglia (resident immune cells of the CNS) and astrocyte (family of glial cells) reactivity, inflammatory mediators release (cytokines, reactive oxygen species), and changes in the integrity and permeability of the BBB.

In homeostatic conditions, microglia continuously scan the environment, secrete neurotropic factors, and participate in synaptic remodeling to ensure brain homeostasis. Upon infection or injury, these cells are activated, resulting in morphological, transcriptional, and functional modifications. The release of inflammatory molecules by microglial cells stimulates astrocytes, thus amplifying the immune response to remove the harmful cue.

This inflammatory process is usually time-limited; it disappears with the end of the stimulus. It is beneficial and promotes tissue repair (Sochocka et al., 2017). However, inflammatory responses are not always perfectly regulated. A lack of inflammatory modulation can lead to the overactivation of microglial cells and astrocytes that excessively release inflammatory molecules, leading to (i) the perturbation of BBB integrity, (ii) the recruitment of peripheral immune cells into the CNS that will further secrete pro-inflammatory factors, and (iii) neuronal impairment and degeneration (Figure 11) (Sochocka et al., 2017).

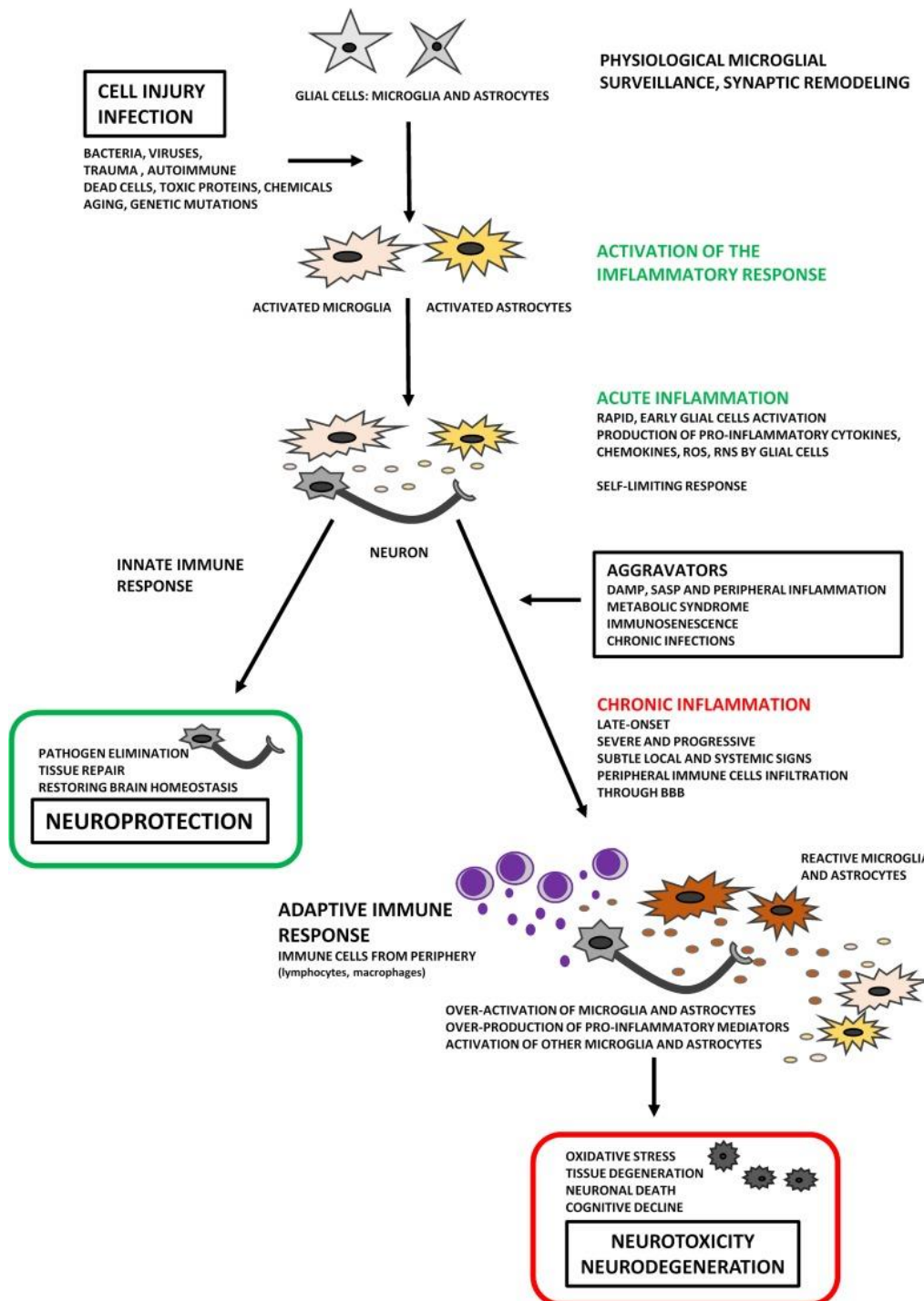


Figure 11: The neuroinflammatory process (Sochocka et al., 2017)

The BBB is the endothelial layer separating the brain from the blood. It protects the brain from detrimental compounds in the bloodstream, drains hazardous molecules from the brain back to the blood, and provides the brain with nutrients required for its good functioning and development (Persidsky et al., 2006). Inflammatory responses can jeopardize the integrity and permeability of the BBB. Studies have demonstrated the harmful effect of some cytokines (TNF- α , IL-6, IL-1 β) on BBB integrity, leading to a higher permeability of the endothelium and leukocyte transmigration into brain tissues (Laflamme et al., 1999; Terrando et al., 2011).

3.2.1 Cellular actors

3.2.1.1. Microglia

Microglia are the resident immune cells of the CNS, deriving from yolk-sac macrophages that have colonized the brain during fetal development. They are widely found in brain tissues and constitute 5 to 12% of the cells (Ginhoux et al., 2010; Lawson et al., 1990). One interesting property of microglia is their capacity for self-renewal, enabling them to repopulate the CNS and persist into adulthood without the involvement of bone marrow-derived cells (Colonna & Butovsky, 2017; Paolicelli et al., 2022).

Microglia are highly dynamic and continuously activated cells that perform a panoply of functions in physiological and pathological conditions (Figure 12). They unceasingly survey the environment and eliminate microbes, antigens, dead cells, protein aggregates, and redundant synapses that may threaten CNS homeostasis. They participate in neuro- and vasculogenesis, synapse remodeling, myelination, and tissue repair but are also essential for BBB integrity and neuronal function. In response to an endangering insult, microglial cells can secrete inflammatory cytokines (Table 6) thus making them fundamental mediators of neuroinflammation (Colonna & Butovsky, 2017; Paolicelli et al., 2022).

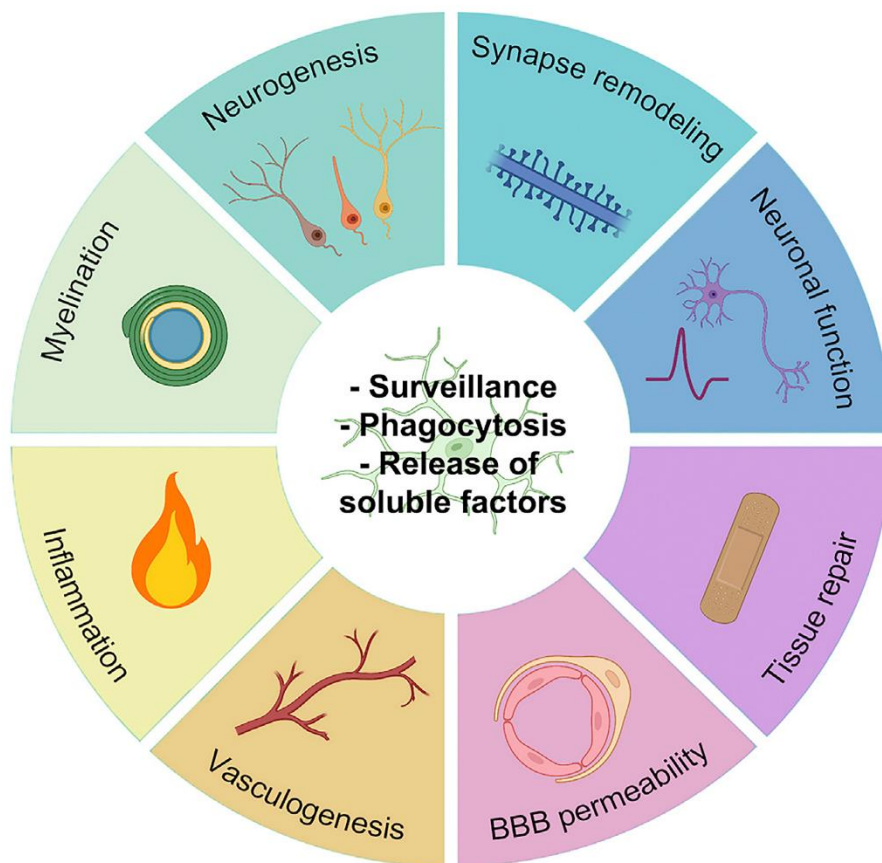


Figure 12: Core functions of microglial cells (Paolicelli et al., 2022)

Microglial cells exhibit various morphologies ranging from ramified to ameboid forms (Figure 13). Under physiological conditions, microglia cells normally present a ramified structure characterized by a small cell soma with long, fine, and numerous processes (Figure 13, A). Upon stimulus detection, microglial cells rearrange their morphology. They retract but thicken their processes and display a more rounded cell body (Figure 13, B) until reaching an ameboid-like form (Figure 13, C).

The “ramified” and “ameboid” microglial shapes have long been associated with “M1” (activated and pro-inflammatory microglial cells) and “M2” (resting and anti-inflammatory microglial cells) phenotypes,

respectively. However, evidence supports the active participation of ramified microglia in the surveillance of the brain and the monitoring of synapses. Ramified microglia can also execute phagocytosis while amoeboid microglia, which have long been considered phagocytes, can lack this ability in certain diseases (Abiega et al., 2016; Colonna & Butovsky, 2017; Paolicelli et al., 2022; Sierra et al., 2010). Consequently, microglia morphology does not clearly define cellular function.

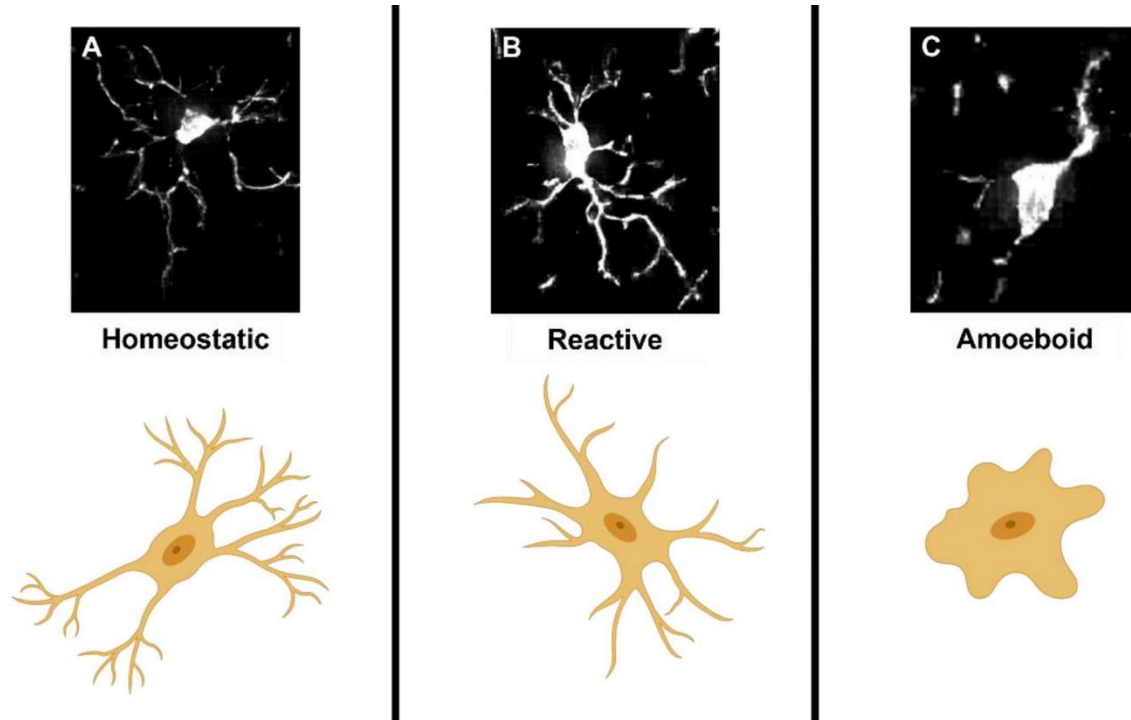


Figure 13: Diversity of microglial morphology (Reddaway et al., 2023)

Several markers help identify microglial cells, in both homeostatic and disease-associated states. These include the cytoplasmic ionized calcium-binding adapter molecule 1 (Iba1), the surface purinergic receptor P2Y₁₂, the transmembrane protein 119 (TMEM119), the colony-stimulating factor receptor (CSF1R), the fractalkine receptor (CX3CR1), the integrin alpha M subunit (CD11b), and the EGF-like module-containing mucin-like hormone receptor-like 1 (F4/80). Microglia express other markers whose expression can change (up- or down-regulation) depending on the functional state of the cells (Paolicelli et al., 2022). For example, inflammatory cytokines (IL-16, IL-18, IL-6, TGF- β , TNF- α , IL-13, IL-4), enzymes (GSK3 β , MMP9), toll-like receptors (TLRs), and transcription factors (IRF5, IRF8, NF- κ B) (Table 6). Additionally, microglial cells express neurotransmitter and neuropeptide receptors that facilitate neuron-glia communications. The activation of these receptors enables microglial cells to monitor neuronal activity, participate in synaptic plasticity and neuronal regeneration, and regulate inflammatory cytokine release (Colonna & Butovsky, 2017).

Microglial cells can be influenced by many factors such as age, gender, local signals, and peripheral cues (Paolicelli et al., 2022). Studies have identified different microglial signatures and distributions according to the temporal stage of development (Hammond et al., 2019; Matcovitch-Natan et al., 2016; Squarzone et al., 2014), brain areas (De Biase et al., 2017), sex (Guneykaya et al., 2018), and maternal microbiota (Thion et al., 2018). A delay in microglial development was observed in males but not females (Hanamsagar et al., 2017), accompanied by differences in transcriptomes and morphologies in response to lipopolysaccharide (LPS) injection (Hanamsagar et al., 2017; Thion et al., 2018).

In concert with astrocytes, they work to ensure CNS normal development, functioning, and protection.

3.2.1.2. Astrocytes

Astrocytes (or astroglia) are the most abundant non-neuronal cell type of the CNS, constituting almost 50% of the cells (Jurga et al., 2021). These star-shaped cells, originating from neural progenitor cells (Villarreal & Vogel, 2021), are tightly linked to neurons and possess an adaptive plasticity enabling the maintenance of CNS homeostasis. At the molecular level, astrocytes partake in ion and proton transport, neurotransmitter catabolism and recycling, and reactive oxygen species neutralization. They participate in embryonic and adult neurogenesis, neuronal guidance, synaptic plasticity, synaptic maintenance, and synaptic elimination. Astrocytes also supply neurons with neurotransmitter precursors and energy substrates, act as chemosensors (pH, Na⁺ concentrations, and energy balance regulation), control the BBB, and contribute to neuroinflammation (Verkhatsky & Nedergaard, 2018) (Figure 14).

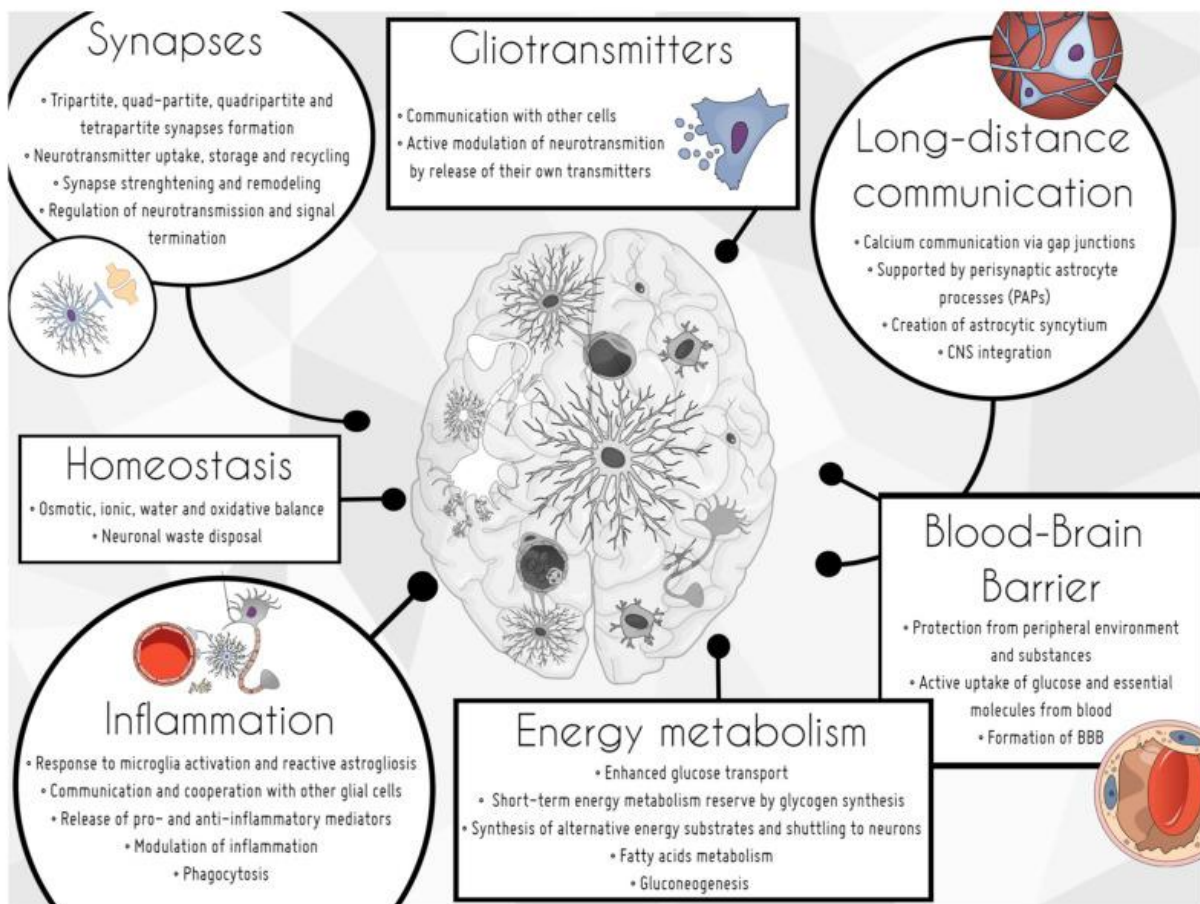


Figure 14: Astrocyte functions (Jurga et al., 2021)

Astrocytes are made of a cell soma from which processes (divided into branches, branchlets, and leaflets) arise, allowing them to communicate with synapses, blood vessels, and other CNS cells. One of the most commonly known structures is the tripartite synapse, consisting of a pre- and postsynaptic neuronal membrane contacted by an astrocytic process. Astrocytes communicate with other cells through gliotransmitter release (for example, ATP, glutamate, and D-serine) and, with each other, through Ca²⁺ signaling (Jurga et al., 2021).

Several astrocytic morphologies have been described, making these cells remarkably heterogeneous. The two main types are the protoplasmic and fibrous astrocytes which reside in the gray and white matter, respectively. Protoplasmic astrocytes are characterized by their numerous ramifications and low glial fibrillary acidic protein (GFAP) expression. On the contrary, fibrous astrocytes are less complex but highly

express the GFAP protein (Figure 15). Other types include the Bergmann and Müller glia, specialized astrocytes in the retina and the cerebellum, respectively (Emsley & Macklis, 2006; Jurga et al., 2021; B. Zhou et al., 2019).

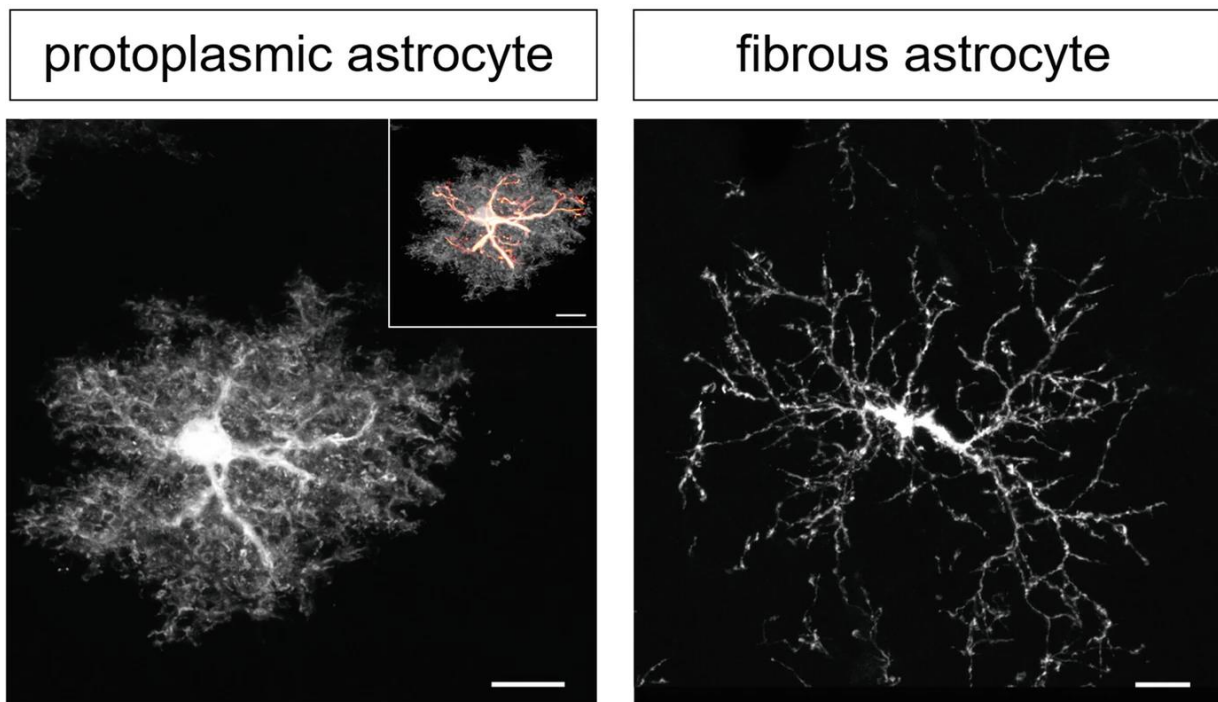


Figure 15: The two main types of astrocytic cells in the CNS (Le Thuc et al., 2021)

In response to injury or infection, astrocytes undergo transcriptional, morphological, and metabolic modifications; a process known as “reactive astrogliosis” or “astrocyte reactivity”. These changes result in the gain of new functions or the loss/upregulation of physiological ones. They can also be associated with irreversible alterations (immune cell recruitment, scar-border formation, astrocytic proliferation) that usually occur when the BBB integrity is compromised. Similar to microglial cells, astrocytes do not polarize into “A1” (neurotoxic and pro-inflammatory state) and “A2” (neuroprotective and anti-inflammatory state) phenotypes. Rather, the overall impact depends on the gene expression profiles and the balance between the detrimental functions acquired and the beneficial ones that were lost (Escartin et al., 2021). Therefore, despite their supportive functions to neurons, astroglia can exhibit cytotoxic functions in certain circumstances.

Numerous markers are up- or down-regulated during reactive astrogliosis (Table 5). One of the most commonly used markers for the study of astrocytes is the GFAP. GFAP is an intermediate filament solely found in astrocytes (Eng et al., 1971) that plays a key role in modulating their motility and shape (Eng et al., 2000). Its expression is usually upregulated (mRNA and protein levels) during reactive astrogliosis (Escartin et al., 2021) making it a good marker for studying astrocytic reactivity. However, evidence suggests that GFAP only labels 15% of the total astrocyte volume (Bushong et al., 2002). Additionally, almost 40% of hippocampal astrocytes do not express it (Walz & Lang, 1998). Consequently, GFAP can’t be considered a universal marker of reactive astrocytes and should always be combined with other molecules.

Reactive astrogliosis is also associated with morphological changes, mainly reported as hypertrophy and extension of astrocytic processes (Escartin et al., 2021). Nevertheless, astrocytes can react differently with a retraction of their branches and a simplification of their shape (Olabarria et al., 2010; D. Sun et al., 2013).

Table 5: Some markers of reactive astrogliosis. Adapted from (Escartin et al., 2021)

	Marker	Type/Function	Changes	References
Cytoskeleton	GFAP	Type III intermediate filament	Upregulated mRNA and protein levels	(Campbell et al., 2020; Carter et al., 2019; Hol & Pekny, 2015)
	Vimentin			(Carter et al., 2019; T. Yamada et al., 1992)
	Nestin	Type VI intermediate filament		(Carter et al., 2019; Moreels et al., 2008)
Metabolism	ALDOC	Glycolytic enzyme	Upregulated protein levels	(J. Levine et al., 2016)
	MAO-B	Catecholamine catabolic enzyme		(Carter et al., 2019)
Secreted proteins	C3	Complement factor	Upregulated mRNA and protein levels	(Liddelow et al., 2017)
	Lcn2	Iron trafficking protein		(Zamanian et al., 2012)
	Serpina3n/ACT	Serine protease inhibitor	Upregulated mRNA levels	
Cell signaling-transcription factors	S100B	Ca ²⁺ binding protein	Upregulated protein levels	(Cerutti & Chadi, 2000; Michetti et al., 2019)
	STAT3	Transcription factor	Phosphorylation and nuclear translocation	(Campbell et al., 2020; Herrmann et al., 2008; Wanner et al., 2013)
Channels-transporters	EAAT1 and EAAT2	Glutamate transporters	Downregulated mRNA and protein levels	(Campbell et al., 2020; R. Jiang et al., 2016)
	KIR4.1	K ⁺ channel		(R. Jiang et al., 2016; X. Tong et al., 2014)

The crosstalk between astrocytes and microglia is necessary for reactive astrogliosis (S.-H. Chen et al., 2015). Microglial-secreted cytokines (IL-1 α , TNF- α , and C1q) promote reactive astrogliosis, resulting in the loss of homeostatic functions (induction and maintenance of synapses, myelin clearance, synaptosome phagocytosis) and gain of neurotoxic properties (neuronal death) (Liddelow et al., 2017). In the hippocampus, the binding of TNF- α to its astrocytic TNF receptor (TNFR) 1 leads to synaptic alterations (increased glutamate release, abnormal excitability of synapses) and cognitive impairments (contextual memory deficits) (Habbas et al., 2015). Reactive astrocytes can, in turn, release inflammatory mediators to amplify the inflammatory response or reduce tissue damage (Table 6). The expressed molecules directly correlate to their functional states (Jurga et al., 2021). They can also act as phagocytes to clear unwanted cell debris (Konishi et al., 2020).

3.2.2 Molecular actors

Cytokines are small cell-signaling proteins and glycoproteins involved in cell communication and inflammation. In normal conditions, cytokines participate in brain development and homeostasis. They can also bind to specific receptors and act as inflammatory mediators. Cytokines are pleiotropic and can exert pro- or anti-inflammatory effects depending on the context, location, and course of the inflammatory response (Figure 16). They are mainly secreted by astrocytes and microglia, on which they also act.

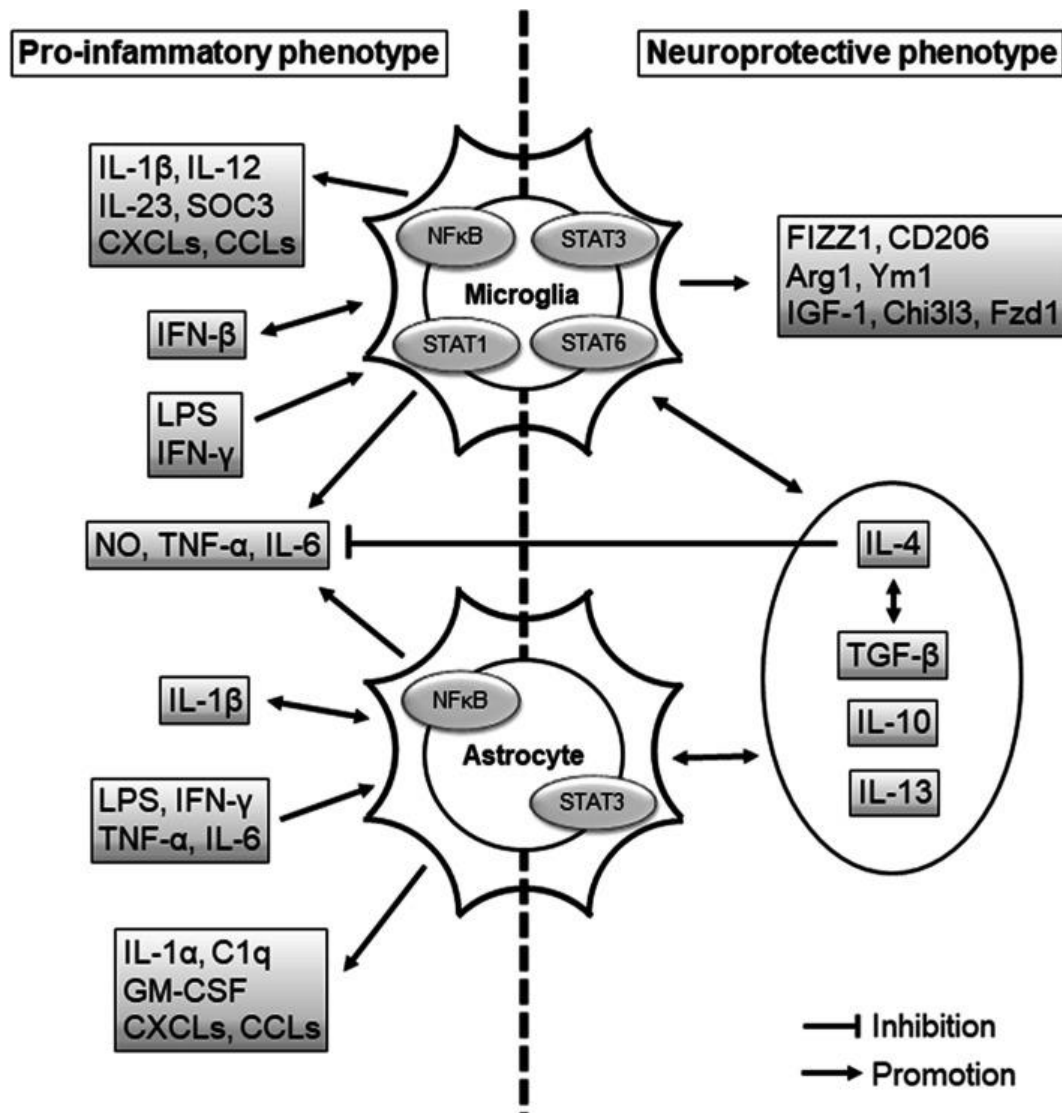


Figure 16: Molecular signatures of microglia and astrocyte phenotypes (Kwon & Koh, 2020)

Their pro-inflammatory properties encompass cellular chemotaxis, further release of inflammatory mediators, activation of pro-inflammatory transcription factors and signaling pathways, in addition to cellular apoptosis (Lyman et al., 2014). On the contrary, their anti-inflammatory effects include the inhibition of pro-inflammatory gene transcription, induction of cell survival, and tissue regeneration.

Importantly, cytokines are not the only contributors to inflammation. Enzymes and specific receptors can also activate signaling pathways and transcription factors that participate in the immune response. The main inflammatory mediators, their properties, and cellular expression are recapitulated in Table 6.

Table 6: Inflammatory markers in the CNS. Adapted from (Bou Sader Nehme et al., 2024) and (Sochocka et al., 2017)

	Markers	Type	Function	Cellular expression	References
Secreted cytokines and receptors	CCL4	Chemokine	<ul style="list-style-type: none"> • Chemotaxis of immune cells (macrophages, T-lymphocytes) • Contributes to neuropathic pain • Pro-inflammatory microglial profile 	Astrocytes	(Saika et al., 2012; Sindhu et al., 2019; Ullah et al., 2020; M. Zhu et al., 2014)
	CCR5	Chemokine receptor	<ul style="list-style-type: none"> • Chemotaxis of microglia • Activation of astrocytes and exacerbation of inflammation • Contributes to neuropathic pain • Pro-inflammatory microglial profile 	Microglia Astrocytes	(Carbonell et al., 2005; Ellwanger et al., 2020; C. Liu et al., 2014; Louboutin & Strayer, 2013; Saika et al., 2012)
	CX3CL1	Chemokine	<ul style="list-style-type: none"> • Involved in neuron-microglia communication • Regulator of microglia activation • Can present anti-inflammatory functions • Pain-mediating chemokine 	Neurons Astrocytes	(Lauro et al., 2015, 2019; Pawelec et al., 2020; R. Silva & Malcangio, 2021; Wilkinson et al., 2015; H. Yoshida et al., 2001)
	CX3CR1	Chemokine receptor	<ul style="list-style-type: none"> • Involved in neuron-microglia communication • Promotes microglia reactivity and inflammatory factors secretion • Pro-inflammatory microglial profile 	Microglia	(M. Lee et al., 2018; Pawelec et al., 2020; Tang et al., 2014)
	CXCL12	Chemokine	<ul style="list-style-type: none"> • Chemotaxis of leukocytes • Induction of NF-κB signaling pathway 	Astrocytes	(Guyon, 2014; Y. Han et al., 2001; Timotijević et al., 2012)

	IL-16	Cytokine	<ul style="list-style-type: none"> • Initiates/sustains the inflammatory response • Modulates T-cell activation • Pro-inflammatory microglial profile 	Microglia	(Guo et al., 2004; Little & Cruikshank, 2008; Mathy et al., 2000)
	IL-18	Cytokine	<ul style="list-style-type: none"> • Induced by NLRP3 inflammasome • Initiates signaling pathways and inflammatory responses in microglia • Exacerbates neuronal cell death (increased Fas-ligand expression) • Pro-inflammatory microglial profile 	Microglia Neurons	(Alboni et al., 2010; Felderhoff-Mueser et al., 2005; L. Song et al., 2017)
	IL-6	Cytokine	<ul style="list-style-type: none"> • Promotes macrophage activation • Role in the acute phase of the inflammatory response • Contributes to nociception and central sensitization • Mainly pro-inflammatory microglia profile (can be anti-inflammatory) 	Astrocytes Microglia Neurons	(Codeluppi et al., 2014; Cunha et al., 1992; Fuster & Walsh, 2014; Garner et al., 2018; Renner et al., 2022; Vazquez et al., 2012; Y.-Q. Zhou et al., 2016)zquez et al., 2012; Y.-Q. Zhou et al., 2016)
	TGF- β	Cytokine	<ul style="list-style-type: none"> • Suppresses the immune response and glia activation • Regulates astrocytic reactivity • Anti-inflammatory microglial profile 	Microglia Astrocytes	(J.-H. Chen et al., 2015; Hisatomi et al., 2002; J. Luo, 2022; Yoshimura et al., 2010)

	TNFR	Cytokine receptor	<ul style="list-style-type: none"> Involved in NF-κB and AP-1 pathway activation Role in central pain sensitization Pro-inflammatory microglial profile 	Astrocytes Microglia Neurons	(Habbas et al., 2015; Probert, 2015; Wajant et al., 2003; L. Zhang et al., 2011)
	TNF- α	Cytokine	<ul style="list-style-type: none"> Role in the acute phase of the inflammatory response Involved in NF-κB and AP-1 pathway activation Involved in nociceptive responses in the CNS Pro-inflammatory microglial profile 	Microglia Astrocytes	(Bhattacharyya et al., 2010; A. Hess et al., 2011; Idriss & Naismith, 2000; Jang et al., 2021; Raffaele et al., 2020)
	CCL2	Chemokine	<ul style="list-style-type: none"> Chemotaxis of microglia, astrocytes, and infiltrating cells Involved in dopaminergic neuron signaling and development Compromises BBB integrity Cytokine production Important for neurogenesis 	Microglia Astrocytes Neurons	(Semple et al., 2010)
	CCR2	Chemokine receptor	<ul style="list-style-type: none"> Upregulated expression during inflammatory responses Cytokine production Cellular chemotaxis Important for neurogenesis 	Microglia Astrocytes Neurons	(Semple et al., 2010)
	CXCL1	Chemokine	<ul style="list-style-type: none"> Neutrophil transmigration and degranulation 	Microglia Astrocytes	(Semple et al., 2010)

			<ul style="list-style-type: none"> • May be involved in synaptic plasticity and release of neurotransmitters 		
	CXCR2	Chemokine receptor	<ul style="list-style-type: none"> • Granulocyte infiltration • Regulator of chemokine levels • May be involved in synaptic plasticity and release of neurotransmitters • Resistance to cell death 	Microglia Astrocytes Neurons	(Roy et al., 2012; Semple et al., 2010)
	CXCL13	Chemokine	<ul style="list-style-type: none"> • Induces the proliferation and recruitment of B cells in the CNS • Involved in the expression of pro-inflammatory cytokines • Contributes to inflammatory and neuropathic pain conditions 	Neurons	(B.-C. Jiang et al., 2016; C. Li et al., 2024; J. Wang et al., 2023)
	CXCR5	Chemokine receptor	<ul style="list-style-type: none"> • Induces the proliferation and recruitment of B cells in the CNS • Involved in the expression of pro-inflammatory cytokines • Contributes to inflammatory and neuropathic pain conditions 	Neurons Astrocytes	(B.-C. Jiang et al., 2016; C. Li et al., 2024; J. Wang et al., 2023)
	IL-10	Cytokine	<ul style="list-style-type: none"> • One of the main anti-inflammatory mediators • Involved in neuronal homeostasis and survival • Inhibits pro-inflammatory 	Microglia Astrocytes	(Burmeister & Marriott, 2018; Milligan & Watkins, 2009; Rubio-Perez & Morillas-Ruiz, 2012)

			<p>cytokine production, receptor expression and activation</p> <ul style="list-style-type: none"> • Anti-nociceptive effects 		
	IL-13	Cytokine	<ul style="list-style-type: none"> • Anti-inflammatory properties • Inhibits pro-inflammatory cytokine and chemokine gene expression 	Microglia Neurons	(Mori et al., 2016; Rubio-Perez & Morillas-Ruiz, 2012)
	IL-1 β	Cytokine	<ul style="list-style-type: none"> • Involved in neuronal degeneration • Role in microglial and astrocytic activation • Induces IL-6 release and iNOS activity in astrocytes • Can induce apoptosis • Role in chronic pain induction and maintenance 	Microglia Astrocytes	(Y. Huang et al., 2011; Mendiola & Cardona, 2018; Milligan & Watkins, 2009; K. Ren & Torres, 2009; Rubio-Perez & Morillas-Ruiz, 2012; Smith et al., 2012)
	IL-2	Cytokine	<ul style="list-style-type: none"> • Involved in astrogliosis and microglial activation • Influences neuronal survival • Modulates neurotransmitter release (acetylcholine, dopamine, noradrenaline) 	Microglia Astrocytes Neurons	(Hanisch & Quirion, 1995; Korneva & Kazakova, 2008)
	IL-4	Cytokine	<ul style="list-style-type: none"> • Anti-inflammatory microglial profile • Induces neurogenesis • Inhibits pro-inflammatory 	Microglia Astrocytes Neurons	(Busch-Dienstfertig & González-Rodríguez, 2013; Latta et al., 2015; Mori et al., 2016;

			<p>cytokine gene expression</p> <ul style="list-style-type: none"> • Anti-nociceptive properties 		Rubio-Perez & Morillas-Ruiz, 2012; Zuiderwijk-Sick et al., 2021)
	INF- γ	Cytokine	<ul style="list-style-type: none"> • Increases TNF activity and nitric oxide • Antiviral activity • Induces antigen-presenting functions in microglia • Astrocytic activator • Can promote neurogenesis and brain repair • Pro-nociceptive properties (hyperalgesia) 	Microglia Neurons	(John et al., 2003; Rubio-Perez & Morillas-Ruiz, 2012; Tan et al., 2021)
Intra-cellular signaling markers	GSK3 β	Serine/ threonine kinase	<ul style="list-style-type: none"> • Promotes inflammatory molecule production through activation of JNK and NF-κB pathways • Altered function is associated with pathological pain • Pro-inflammatory microglial profile 	Microglia Astrocytes	(Abd-Allah et al., 2018; Maixner & Weng, 2013; M.-J. Wang et al., 2010)
	IRF5	Transcription factor	<ul style="list-style-type: none"> • IRF8-IRF5 transcriptional axis induces P2X₄R expression in microglial cells • Involved in neuropathic pain • Pro-inflammatory microglial profile 	Microglia	(Al Mamun et al., 2020; Masuda et al., 2014; Tsuda et al., 2017)
	IRF8	Transcription factor	<ul style="list-style-type: none"> • IRF8-IRF5 transcriptional axis induces P2X₄R expression in microglial cells 	Microglia	(Masuda et al., 2012; Tsuda et al., 2017)

			<ul style="list-style-type: none"> • Contributes to chronic pain • Pro-inflammatory microglial profile 		
	NF-κB	Transcription factor	<ul style="list-style-type: none"> • Induces NLRP3 inflammasome • Role in inflammatory processes in glial cells (cytokines production, iNOS expression) • Pro-inflammatory microglial profile 	Microglia Astrocytes Neurons	(Babkina et al., 2021; T. Liu et al., 2017; Sutterwala et al., 2014)
	NLRP3	Pattern recognition receptor	<ul style="list-style-type: none"> • Results in IL-18 and IL-1β secretion through caspase-1 activity • Pro-inflammatory microglial profile 	Microglia Neurons Astrocytes	(T. Liu et al., 2017; Sutterwala et al., 2014; Voet et al., 2019)
	Wnt3a	Secreted glycoprotein	<ul style="list-style-type: none"> • Involved in synaptic function, formation, and transmission • Involved in axon guidance • Induces microglial activation 	Neurons	(Freese et al., 2010; Hooper et al., 2020; Inestrosa & Varela-Nallar, 2014; Stanganello et al., 2019; Y. Yang & Zhang, 2020)
	Wnt5a	Secreted glycoprotein	<ul style="list-style-type: none"> • Involved in synaptic function and transmission • Involved in axon guidance • Promotes neuronal maturation • Role in pain sensitization 	Microglia Astrocytes Neurons	(Inestrosa & Varela-Nallar, 2014; X. Liu et al., 2022; Simonetti & Kuner, 2020; Yeh et al., 2023)
	TLR-7	Pattern recognition receptor	<ul style="list-style-type: none"> • Promotes pro-inflammatory signals in microglia • Induces neuro-degeneration and apoptosis 	Microglia Astrocytes Neurons	(Butchi et al., 2010; Fiebich et al., 2018; L. Li et al., 2021; Michaelis et al., 2019)

			<ul style="list-style-type: none"> • Upregulates the expression of pro-inflammatory molecules 		
	P2X ₄ R	Purinergic receptor	<ul style="list-style-type: none"> • Involved in pain sensitization • Induces NLRP3 inflammasome activation • Induces cell apoptosis 	Microglia Neurons	(Amadio et al., 2007; He et al., 2022; Long et al., 2020; Molnár et al., 2022; Soto et al., 1996; Stokes et al., 2017; Tsuda et al., 2009)
Astrocytic markers	GFAP	Class-III intermediate filament	<ul style="list-style-type: none"> • Marker of reactive astrogliosis • Involved in pro-inflammatory cytokine release • Increased expression under inflammatory pain 	Astrocytes	(Amalia, 2021; Azzolini et al., 2022; H. Ikeda et al., 2013a)
Oxidative stress markers	Arginase 1	Enzyme	<ul style="list-style-type: none"> • Downregulates nitric oxide production (competes with iNOS) • Anti-inflammatory microglial profile 	Microglia	(Cherry et al., 2014)
	SOD1	Enzyme	<ul style="list-style-type: none"> • Regulates oxidative stress responses • Potential anti-inflammatory effects through its reduction of reactive oxygen species • High SOD1 levels may correlate with postoperative pain reduction 	Neurons Astrocytes Microglia	(Boillée & Cleveland, 2008; Hwang et al., 2020; Jaarsma et al., 2008; KÄRKKÄINEN et al., 2018; Nagai et al., 2007; Shuvaev et al., 2013)
	NOX-2	NADPH oxidase	<ul style="list-style-type: none"> • Increased expression in activated microglia • Reactive oxygen species release 	Microglia Astrocytes Neurons	(Nayernia et al., 2014; Terzi & Suter, 2020)

			<ul style="list-style-type: none"> Involved in microglial chemotaxis Contributes to apoptosis Might be involved in memory and synaptic plasticity Involved in neurogenesis, axonal growth and regeneration 		
	APE1	Endo-nuclease	<ul style="list-style-type: none"> Dual-role enzyme Regulates transcription factor activation and inflammatory cytokine expression Involved in DNA repair Protects neuronal structure and function 	Astrocytes Neurons	(Oliveira et al., 2022)
Other markers	Cathepsin S	Protease	<ul style="list-style-type: none"> Role in antigen presentation Role in microglia-neuron communication Involved in microglia migration towards sites of injury or infection Role in neuropathic pain 	Microglia	(de Mingo et al., 2016; Hao et al., 2007; Lowry & Klegeris, 2018; Nakagawa et al., 1999; Pišlar et al., 2021; R. Silva & Malcangio, 2021; Smyth et al., 2022)
	Iba1	Protein	<ul style="list-style-type: none"> Role in actin-crosslinking and microglial membrane ruffling Induction of microglial phagocytic activity Increased expression is associated with 	Microglia	(Hovens et al., 2014; Ohsawa et al., 2004)

			microglial response		
	CD11b	Surface membrane integrin	<ul style="list-style-type: none"> • Increased expression during microglial activation • Role in pathogen recognition, phagocytosis, and cell survival • Regulates inflammatory pathways such as TLR 	Microglia	(Khan et al., 2018; Latta et al., 2015)
	Pecam1/ CD31	Trans-membrane glycoprotein	<ul style="list-style-type: none"> • Involved in the diapedesis of infiltrating cells • Regulates vascular and BBB integrity 	Endothelial cells of the BBB	(Wimmer et al., 2019)
	TSPO	Outer mitochondrial membrane protein	<ul style="list-style-type: none"> • Involved in the phagocytic ability of microglia • Role in mitochondrial energy metabolism • Role in the formation of reactive oxygen species • Role in neuronal apoptosis, repair, and regeneration 	Microglia Astrocytes	(Cheung et al., 2023; Gui et al., 2020; Nutma et al., 2019)
	tPA	Protease	<ul style="list-style-type: none"> • Involved in neuronal development, plasticity, and protection • Role in axon regeneration • Role in learning and memory • Involved in neuro-inflammation and microglial activation 	Microglia Astrocytes Neurons	(Fredriksson et al., 2016)

			<ul style="list-style-type: none"> • Modulates BBB permeability 		
	MMP9	Enzyme	<ul style="list-style-type: none"> • Contributes to neuro-inflammation • Astroglia migration • Synaptic and axonal plasticity • Involved in the development of neuropathic pain 	Microglia Astrocytes Neurons	(Ji, Xu, et al., 2009; Milward et al., 2007; Reinhard et al., 2015; Rempe et al., 2016)
	BDNF	Neurotrophic factor	<ul style="list-style-type: none"> • Role in neuronal and glial proliferation, differentiation, and survival • Involved in synaptic transmission, plasticity, and formation • Involved in axon growth • Pro-nociceptive properties in the spinal cord 	Microglia Astrocytes Neurons	(Dou et al., 2022; Xiong et al., 2024)

The aforementioned molecular mediators promote the activation of signaling pathways with beneficial or harmful effects on the course of the immune response. Those include PI3K-protein kinase B (PKB/Akt), mammalian target of rapamycin (mTOR), MAPK, as well as Janus kinases/signal transducer and activator of transcription (JAK-STAT) signaling pathways. These pathways are involved in the regulation of cellular processes (cell proliferation, survival, differentiation, and death) and inflammation. They activate the nuclear factor Kappa-B (NF-κB), responsible for inflammatory gene transcription (Shabab et al., 2017; Zarrin et al., 2021).

NF-κB is a family of transcription factors consisting of five monomers: p65 (RelA), RelB, c-Rel, p105/50 (NF-κB1), and p100/20 (NF-κB2). Activated by different stimuli (for example, inflammatory cytokines and oxidative stress), they possess key regulatory roles in the transcription of genes encoding for cytokines, chemokines, regulators of apoptosis and proliferation, factors of the complement cascade, etc. These nuclear factors are thus involved in inflammation, immunity, stress response, and diverse other processes such as cell survival, proliferation, apoptosis, and differentiation. They are generally found in the cytoplasm, in an inactive form, because of their interaction with the IκB complex. Their nuclear translocation and activation depend on the phosphorylation-induced proteasomal degradation of the IκB complex by the IκB kinase (Oeckinghaus & Ghosh, 2009). For instance, the binding of IL-17 to its receptor, upregulated by astrocytes during neuroinflammation, recruits NF-κB activator 1 molecule. This results in the activation of transforming growth factor beta-activated kinase 1 (TAK1) and E3 ubiquitin ligase (TRAF6), stimulation of NF-κB, and consequently, inflammatory gene transcription (Qian et al., 2007). On

the contrary, astrocytic transforming growth factor β (TGF- β) inhibits NF- κ B activation and C-C motif chemokine ligand 5 (CCL5) production, thus limiting neuroinflammation (Cekanaviciute, Dietrich, et al., 2014; Cekanaviciute, Fathali, et al., 2014).

PI3K is a family of lipid kinases (enzymes that phosphorylate other proteins and regulate their activity) that transduce signals elicited by cytokines and growth factors. Signal transduction is performed through the synthesis of phospholipids which activate Akt, a serine-threonine kinase, and other downstream intracellular pathways (P. Liu et al., 2009). The PI3k-Akt pathway modulates inflammation, cell death, and cell activation. It also promotes another serine-threonine kinase, mTOR, whose phosphorylation coordinates microglia activation, NF- κ B activity, and inflammation (Shabab et al., 2017). For example, TNF- α binding to its two receptors, TNFR1 and TNFR2, leads to opposite results. TNFR1 activation causes oxidative stress, neuronal damage, and inflammation (Fischer & Maier, 2015) while TNFR2 prevents cell death through PI3K-Akt pathway stimulation (Fischer et al., 2011). In microglia, the PI3K-Akt pathway is essential for TLR4-induced cell activation, NF- κ B stimulation, and cytokine production (Q.-H. Shao et al., 2024). Activation of the PI3K-Akt pathway by CX3CR1 exerts a dual effect. Findings support its neuroprotective role through the attenuation of microglial activation and cytokine release (Lyons et al., 2009; Zujovic et al., 2000) while others demonstrate its involvement in the synthesis of pro-inflammatory mediators following NF- κ B activation (Castro-Sánchez et al., 2019).

The MAPK family consists of serine-threonine kinases that are successively activated through phosphorylation. Their main functions include the modulation of RNA stability and cytokine translation (Zarrin et al., 2021). These enzymes can be divided into three groups: (i) the MAPK kinase kinase which initiates the cascade following extracellular stimuli, (ii) the MAPK kinase, activated by MAPK kinase kinase, and (iii) MAPK, activated by MAPK kinase, which phosphorylates cytosolic and nuclear effectors leading to modifications in gene expression and protein functions that regulate biological responses (Figure 17) (Morrison, 2012). In microglial cells, activation of three MAPKs (p38 MAPK, ERK1/2, and JNK) contributes to IL-1 β and TNF- α release (Bachstetter et al., 2011; S. H. Kim et al., 2004). More particularly, ERK signaling was reported as an inducer of microglial reactivity and phagocytic activity (M. J. Chen et al., 2021).

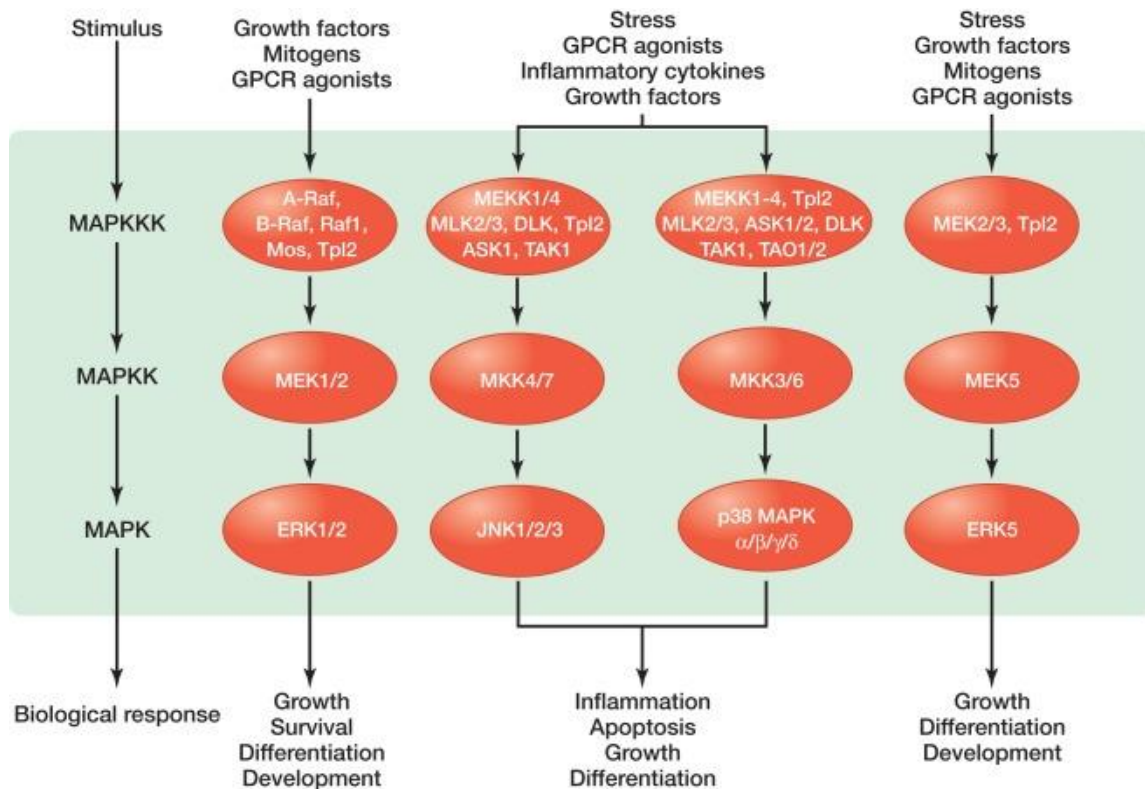


Figure 17: The MAPK signaling pathways (Morrison, 2012)

JAK is a family of tyrosine kinases encompassing JAK1, JAK2, JAK3, and TYK2, a non-receptor tyrosine-protein kinase. These kinases are associated with several cytokine receptors (interleukin, interferon, growth hormone, and erythropoietin receptors). They transduce their signals through the recruitment and phosphorylation of STATs (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6), a family of intracellular transcription factors functionally associated with JAKs. Following the binding of an extracellular cue to the receptor, the associated JAK trans-phosphorylate one another and their downstream substrates (STATs and the receptor itself). The activated STATs translocate into the nucleus, bind to specific enhancer sequences, and regulate target gene transcription (Figure 18) (Harrison, 2012; Zarrin et al., 2021).

Evidence suggests a role for the JAK-STAT signaling pathway, particularly JAK2-STAT3, in reactive astrogliosis. STAT3 is activated by several inflammatory cytokines (IL-6, TNF- α , INF- γ) (R  b   et al., 2013), and both JAK2 and STAT3 were found to be phosphorylated in reactive astrocytes (O'Callaghan et al., 2014; Oliva et al., 2012; Sriram et al., 2004). Notably, their inhibition reduces GFAP expression (Sriram et al., 2004), decreases astrocyte reactivity (Sriram et al., 2004), and disrupts glial scar formation (an astrocytic structure that develops at the site of injury to isolate the region and prevent further damage) (Ceyzeriat et al., 2016; Fawcett, 2009; Herrmann et al., 2008). More interestingly, inhibition of STAT3 in astrocytes also prevents microglial reactivity (Ben Haim et al., 2015; Hristova et al., 2016).

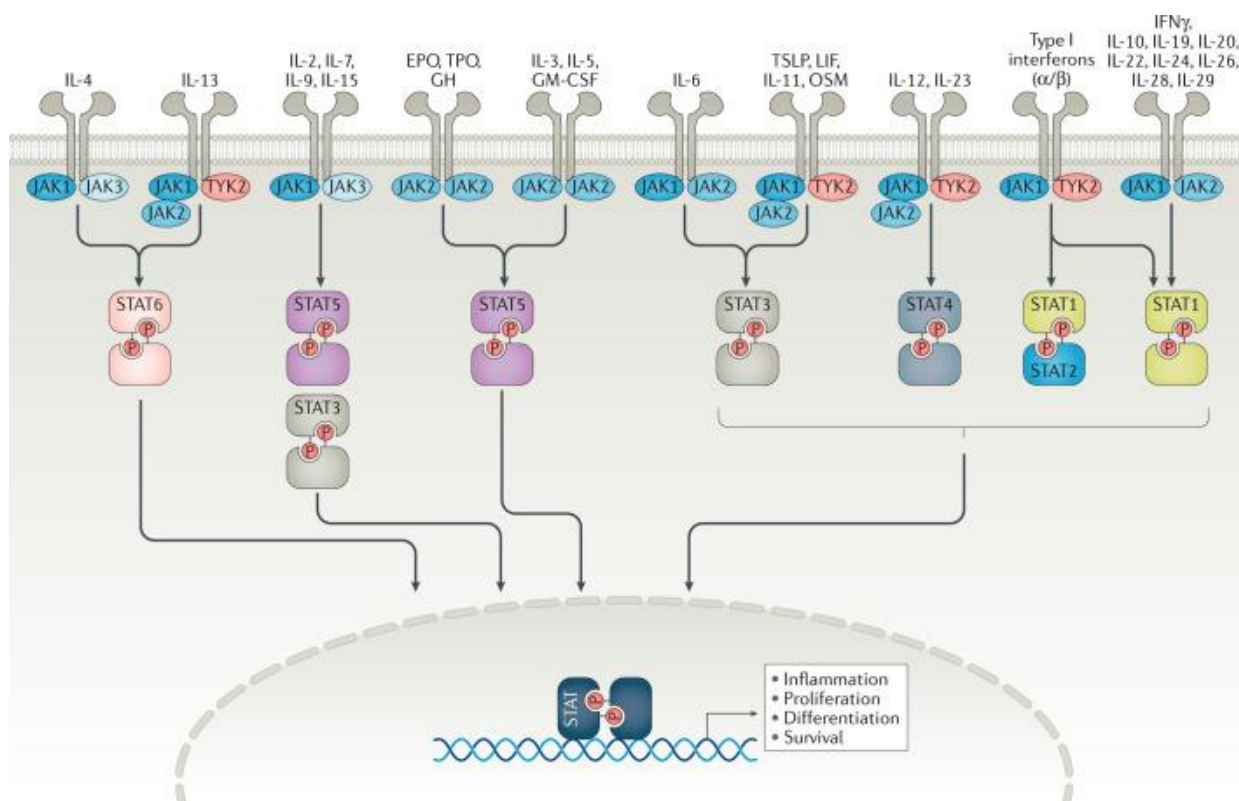


Figure 18: The JAK-STAT signaling pathways (Zarrin et al., 2021)

3.2.3 Deleterious outcomes of neuroinflammation

Neuroinflammation aims to protect the CNS by removing pathogens and promoting tissue repair. However, sustained immune responses and cytokine release result in detrimental outcomes.

Physiological levels of cytokines (IL-1 β , TNF- α) display beneficial effects on synaptic and cognitive functions. However, an excessive production of inflammatory molecules presents harmful consequences such as synaptic dysfunction (loss of synaptic function and deficits in synaptic plasticity), cognitive impairments, neuronal death, and neurogenesis inhibition (Bellingacci et al., 2023; Borsini et al., 2015; Lyman et al., 2014).

In mice with peripheral inflammation, microglial activation paralleled impaired long-term potentiation (LTP) in the hippocampus (Di Filippo et al., 2013). These deficits were further correlated with increased release of IL-1 β and modifications in NMDA-R subunit composition (Di Filippo et al., 2013). Interestingly, IL-1 β seems to exert its detrimental effects through (i) the inhibition of LTP-dependent protein synthesis, (ii) the inhibition of AMPA-R GluA1 subunit expression and phosphorylation, (iii) the impairment of actin cytoskeleton (Machado et al., 2018; Prieto et al., 2019; L. Tong et al., 2018), and (iv) the alteration of BDNF signaling (Golia et al., 2019; L. Tong et al., 2012). In line with these findings, hippocampal injection of IL-1 β led to contextual memory problems and reductions in the total density of CA1 hippocampal dendritic spine (Herrera et al., 2019). Memory impairments, induced by LPS injection, were reversed following IL-1 β knockdown (M. Li et al., 2017). Additionally, elevated levels of TNF- α were associated with increased activation of p38 MAPK and resulted in hippocampal LTP deficits (Butler et al., 2004) while reductions in TNF- α activity rescued LTP suppression (S. Ren et al., 2020).

IL-1 β , IL-8, and TNF- α are among the inflammatory cytokines affecting neurogenesis. IL-1 β was found to reduce the proliferation of hippocampal neural progenitor cells. This was accompanied by a decrease in the expression of the orphan nuclear receptor TLX (Ryan et al., 2013), essential for the maintenance of

neural stem cell self-renewal (Shi et al., 2004). These outcomes were reversed by the inhibition of GSK3 β activity, pointing out the role of this kinase in the modulation of neurogenesis (Green & Nolan, 2012). IL-18 and TNF- α alter neurogenesis by inducing neuronal cell death and inhibiting neuronal differentiation (Y.-P. Liu et al., 2005). One mechanism through which IL-1 β and TNF- α exhibit their deleterious effects is STAT3 pathway activation, which promotes astrocytic differentiation and reduces neuronal one (E. Chen et al., 2013).

Pro-inflammatory mediators contribute to neuronal cell death. Through its binding to the TNFR1 receptor, TNF- α induces excitotoxicity by stimulating microglial cells to secrete glutamate (Takeuchi et al., 2006). Activated astrocytes and microglia also release nitric oxide resulting in neuronal respiration inhibition, glutamate production, and neuronal toxicity (Bal-Price & Brown, 2001).

In conclusion, even though the initiation of an inflammatory response aims to protect the CNS from harmful stimuli, a lack of regulation could be detrimental to numerous physiological processes.

3.2.4 Neuroinflammation and ADHD

Recent findings point out the implication of inflammation in the etiology of neuropsychiatric conditions such as ASD (Matta et al., 2019), schizophrenia (N. Müller et al., 2015; Vallée, 2022), and bipolar disorder (Benedetti et al., 2020). This evidence has led researchers to investigate the potential role of neuroinflammatory mechanisms in ADHD pathophysiology. Increased microglial activation was demonstrated in the dorsolateral and orbitofrontal cortices of ADHD individuals (Yokokura et al., 2021), thus reinforcing the possible contribution of neuroinflammation to ADHD pathogenesis (Alvarez-Arellano et al., 2020; Corona, 2020; Dunn et al., 2019; Leffa et al., 2018).

At the DNA level, mutations in genes encoding for inflammatory cytokines resulted in increased ADHD risk and symptomatology (Anand et al., 2017; Drtilkova et al., 2008; Lasky-Su, Anney, et al., 2008; Lasky-Su, Neale, et al., 2008; Ribasés et al., 2008; Segman et al., 2002; T. F. Smith et al., 2014). For example, the C allele of IL-6 SNP rs1800795 was more frequent in ADHD boys compared to controls (Drtilkova et al., 2008), while two SNP in the IL-16 gene enhanced ADHD-I susceptibility (Lasky-Su, Neale, et al., 2008).

At the protein levels, alterations in the expression of inflammatory mediators were observed in the serum or plasma of ADHD subjects. Importantly, they were associated with ADHD odds and symptoms (Anand et al., 2017; J. P.-C. Chang et al., 2020, 2021; Darwish et al., 2019; Elsadek et al., 2020; Koç et al., 2023; Misiak et al., 2022; Oades et al., 2010; O'Shea et al., 2014). The expression of IL-6 (J. P.-C. Chang et al., 2020; Darwish et al., 2019; Koç et al., 2023; Misiak et al., 2022), IL-2, IL-16, IFN- γ , IL-10, and IL-13 was enhanced in ADHD patients (Oades et al., 2010). ADHD medications reversed this upregulation and returned the expression levels to their basal values (Oades et al., 2010). Additionally, IL-16 and IL-13 were linked to hyperactive/impulsive (errors of commission and motor measures) and attentional deficits (errors of omission), respectively (Oades et al., 2010). On the contrary, low levels of TNF- α (J. P.-C. Chang et al., 2020; Misiak et al., 2022) and BDNF (J. P.-C. Chang et al., 2020) were detected in ADHD individuals. In animal models, IL-2 systemic administration depleted dopaminergic efflux in the nucleus accumbens of animals (Anisman et al., 1996) while IL-1 β , IL-6, and IL-2 peripheral injection intensified serotonin, norepinephrine, and dopamine turnover and activity (Zalcman et al., 1994). Aberrant inflammatory cytokine expression therefore affects many neurotransmitter systems involved in ADHD etiology.

Animal studies have enabled a more in-depth investigation of inflammatory changes under ADHD conditions, leading to further confirmation of the role played by neuroinflammation in the disease etiology. Multiple inflammatory markers (GFAP, Iba1, TNF- α , and neuronal nitric oxide synthase) were overly expressed in the frontal cortex and hippocampus of the SHR model, pointing out microglial and

astrocytic reactivity. This is in line with the results of another study demonstrating increased generation of reactive oxygen species in the hippocampus, striatum, and cortex of the SHR model (Leffa et al., 2017). Neuronal nitric oxide synthase is an enzyme that catalyzes nitric oxide production, and consequently, leads to oxidative stress. Reciprocally, reactive oxygen species can drive microglia and astrocyte activation, thus aggravating neuroinflammation (Corona, 2020). Remarkably, the study of microglial morphology has identified a reactive-like shape (reduced cell area and perimeter) in the ACC and PI of 6-OHDA lesioned mice (Meseguer-Beltrán et al., 2023).

Maternal infections trigger the synthesis of inflammatory molecules that can cross the placenta and the BBB, leading to detrimental consequences (neurodevelopmental and behavioral disorders) on the developing fetus (Massrali et al., 2022; Minakova & Warner, 2018; R. S. Y. Wong, 2022). According to an English cohort study, maternal immune activation accounts for 2.6% of ADHD diagnoses in children (Hall et al., 2022). Mothers suffering from chronic immune disorders (psoriasis, asthma, multiple sclerosis, rheumatoid arthritis) are more susceptible to giving birth to ADHD infants (Instanes et al., 2017; Nielsen et al., 2021). Consistent with those findings, exposure to the immunostimulant poly(I:C) during gestation resulted in attentional shifting impairments and hyperactivity in male mice (Bitanhirwe et al., 2010; Missig et al., 2018). Deficits in cognitive functions (learning and memory) (Giovanolli et al., 2015; Graciarena et al., 2010; X.-W. Li et al., 2016; Ozawa et al., 2006) and neuroanatomical abnormalities (reduced total brain, gray, and white matter volumes) (da Silveira et al., 2017; Short et al., 2010) were also documented. Finally, the vulnerability of neurotransmitter systems to immune maternal activation was established (Dunn et al., 2019). Prenatal exposure to LPS (Depino, 2015; Hsueh et al., 2017) or poly(I:C) (Winter et al., 2009) dramatically attenuated serotonergic levels in the brain and, more particularly, the hippocampus. The dopaminergic system was also affected. Poly(I:C) increased dopamine concentrations in the lateral globus pallidus and the PFC (Winter et al., 2009) while LPS lowered the spontaneous firing rate and activity of dopaminergic neurons (Luchicchi et al., 2016).

ADHD is associated with deficits in dopaminergic transmission. Interestingly, neuroinflammation and dopamine mutually influence each other (Vidal & Pacheco, 2020). Inflammatory cytokines (IL-1 β , TNF- α , INF- γ) control dopamine storage and synthesis, thus regulating dopamine availability (Kitagami et al., 2003; Vidal & Pacheco, 2020). Reciprocally, dopamine shapes the inflammatory response and the activity of immune cells (Dominguez-Meijide et al., 2017). For instance, increased dopaminergic levels attenuate microglial cell activation, their phagocytic activity (Y. Fan et al., 2018), and their associated nitric oxide release (Färber et al., 2005). Moreover, microglial expression and activation of low-affinity dopamine receptors 1 and 2 seem to favor an anti-inflammatory environment (Färber et al., 2005) while stimulation of high-affinity dopamine receptors 3 and 5 promotes inflammation (Pacheco, 2017).

Therefore, these findings argue in favor of a potential implication of neuroinflammation in ADHD pathophysiology.

3.2.5 Neuroinflammation and pain

Chronic pain results from complex neuronal and immune interactions. As previously described, inflammatory mediators are released at the periphery following injury. They sensitize the afferent nervous fibers which, in turn, release neurotransmitters in the spinal cord capable of activating microglial cells. Reactive microglia produce and secrete inflammatory mediators (cytokines, chemokines, neuropeptides) that can modulate the activity of spinal and descending inhibitory neurons. This neuroinflammatory response does not end in the spinal cord, as it can also reach higher brain regions involved in the different dimensions and regulation of pain (Vergne-Salle & Bertin, 2021).

Following injury, the activation of peripheral immune cells promotes the release of inflammatory molecules that bind to their receptors on the primary afferent terminals and initiate pain. Nerve growth factor, secreted by mast cells, induces the phosphorylation and membrane insertion of thermal nociceptor TRPV1, thus participating in peripheral sensitization. Monocytes and macrophages release mediators with pro-inflammatory properties (IL-6, IL-1, and TNF- α) that mediate several nociceptors, leading to thermal and mechanical pain sensitization (Raouf et al., 2018; Vergne-Salle & Bertin, 2021). T cells at the injury site cause pain hypersensitivity, immune cell infiltration, and glial cell activation through IL-17 release (C. F. Kim & Moalem-Taylor, 2011).

The activated cells then migrate to the spinal cord under the chemotaxis effect of attractive cytokines (CCL2, CX3CL1) produced by sensory neurons (Raouf et al., 2018). They further release inflammatory cytokines, whose blockade was shown to attenuate pain-related behaviors in models of arthritis (Boettger et al., 2008; Boettger, Leuchtweis, et al., 2010a, 2010b; Boettger, Weber, et al., 2010; Ebbinghaus et al., 2012). Macrophages also stimulate neuronal calcitonin gene-related peptide secretion which participates in pain maintenance (Massier et al., 2015). Anti-inflammatory molecules mediate the resolution phase. IL-10, generated by macrophages, leads to the attenuation of pain, reduction of immune cell chemotaxis, and decrease of inflammatory cytokine release (Ledeboer et al., 2007; Willemen et al., 2014).

More particularly, microglial reactivity in the spinal cord is a characteristic of pain models. Purinergic and fractalkine signaling (Z. Guan et al., 2016), neuronal colony-stimulating factor-1 (Z. Guan et al., 2016), and neuronal CCL2 (Chun & Kwon, 2019) are some of its mediators. Reactive microglia synthesize cytokines, BDNF, and cathepsin-S that further amplify the inflammatory response, potentiate neuronal excitability, and intensify the associated pain (Inoue & Tsuda, 2018; Vergne-Salle & Bertin, 2021). Microglial cells and neurons also activate astrocytes through the release of mediators such as glutamate, ATP, TNF- α , IL-6, and IL-1 β (T. Li et al., 2019). Astrocytic CCL2 and IL-1 β affect pain sensation by modulating synaptic transmission (increase of excitatory synapse transmission and reduction of the inhibitory one) and activating NMDA-R in DHN (Gao & Ji, 2010).

Immune cells are also activated in higher brain structures, under pain conditions. Following peripheral nerve injury, microgliosis was predominant in regions implicated in pain processing (PFC, thalamus, nucleus accumbens, amygdala, and ventral tegmental area) but absent in brain areas that do not participate in pain perception (A. M. W. Taylor et al., 2017). Additionally, both astrocytes and microglia were activated in the ACC and PAG of neuropathic pain models (Miyamoto et al., 2017; H. Ni et al., 2016). Administration of minocycline (microglial inhibitor) reduced cell reactivity in the ACC and partially decreased mechanical allodynia (Miyamoto et al., 2017). Treatments with astrocytic and microglia inhibitors in the PAG also attenuated pain-related behaviors while blockade of p38 MAPK displayed the same anti-nociceptive effects and was correlated with diminished microglial activation (H. Ni et al., 2016). Neuropathic pain-induced microgliosis and BDNF release were further associated with disrupted dopaminergic transmission within the reward circuitry (A. M. W. Taylor et al., 2015). Of note, the reward system is greatly impaired in ADHD patients.

Consequently, the inflammatory response at the periphery, spinal cord, or different brain structures is tightly linked to pain sensation and modulation.

3.2.6 Neuroinflammation as a link between ADHD and pain sensitization

Clinical and preclinical studies suggest an interaction between ADHD and pathological pain conditions.

Impairments in multiple domains of perceptual functioning were reported in ADHD patients (Fuermaier et al., 2018). More importantly, sensory processing difficulties were positively correlated with ADHD traits

in the general population (Dellapiazza et al., 2021; Panagiotidi et al., 2018). For instance, ADHD adults displayed a lowered threshold in response to cold nociceptive stimuli which was, interestingly, reversed by methylphenidate treatment (Treister et al., 2015). They were also more prone to experiencing pain (Stickley et al., 2016), with the severity of ADHD symptoms being associated with extreme pain sensation (Northover et al., 2015; Stickley et al., 2016). Reciprocally, pain was correlated with altered cognitive functions and attentional deficits in animal models of neuropathic pain (Higgins et al., 2015) and Human patients (D. J. Moore et al., 2019). The two attentional aspects that were predominantly affected by pain involved multiple cues processing and the regulation of attentional deployment (D. J. Moore et al., 2012).

These observations are in line with evidence supporting a higher prevalence of pain conditions in ADHD patients (Battison et al., 2023; Bou Khalil et al., 2018; Mundal et al., 2024). Indeed, 80% of ADHD adults suffer from widespread pain (Stray et al., 2013) while 65.9% of Norwegian ADHD adolescents report chronic pain (Mangerud et al., 2013). Additionally, 39% of women with a main diagnosis of ADHD have chronic widespread pain, which was reduced following the use of stimulants (Asztély et al., 2019). Conversely, ADHD is diagnosed in patients suffering from chronic pain conditions (Battison et al., 2023; Karaş et al., 2020; Lipsker et al., 2018; Low Kapalu et al., 2018). 18% of children with recurrent abdominal pain have comorbid ADHD (Woodbury, 1993) while 44.72% of adults with fibromyalgia suffer from the disorder (van Rensburg et al., 2018).

One explanation for the co-occurrence of ADHD and chronic pain lies in the modulation of pain transmission by attentional processes through their effects on brain networks (PFC, PAG) (Kucyi et al., 2013; Villemure & Bushnell, 2002) and descending pathways (Sprenger et al., 2012). Alterations in neuronal responsiveness to both noxious and nociceptive stimuli were demonstrated in the primary somatosensory cortices and were notably caused by directed attention (Villemure & Bushnell, 2002). Furthermore, a higher working memory load significantly distracted attention from the pain (Veldhuijzen et al., 2006) and reduced neuronal responses in the spinal cord to painful stimuli (Sprenger et al., 2012). The increased functionality between the default mode network and PAG might be one mechanism that diverts attention away from pain (Kucyi et al., 2013).

Several brain regions are involved in both attentional and pain modulation; another argument in favor of a comorbidity between ADHD and pathological pain. The amygdala is a key area that participates in the affective-motivational dimension of pain. The emotional responses to pain account for the hyperactivity of the “nociceptive amygdala” (laterocapsular division of its central nucleus), also resulting in medial PFC deactivation and cognitive deficits (Neugebauer, 2015). Changes in the functional connectivity of the amygdala were associated with emotional lability in ADHD subjects. Indeed, abnormal intrinsic functional connectivity between the amygdala and the PFC was linked to problems in the exteriorization of negative emotions (Hulvershorn et al., 2014). Interestingly, ADHD patients not only display reduced amygdala volumes but also suffer from abnormal amygdala function, correlated with the incapacity of controlling impulsivity (Tajima-Pozo et al., 2018). The ACC is another area of interest with prime roles in attentional and pain processing. Failure to activate the dorsal ACC, and consequently, dysfunction of this structure, was reported in ADHD patients during the Counting Stroop (a functional magnetic resonance imaging task enabling the identification of functional brain modifications during cognitive and attentional tests) (Bush et al., 1999). On the contrary, activation of the ACC potentiates excitatory synaptic transmission in the spinal cord thus contributing to chronic pain (T. Chen et al., 2018). The aversive effects associated with neuropathic pain were attenuated by the optogenetic suppression of ACC hyperactivity (Sellmeijer et al., 2018). Finally, the nucleus accumbens, a component of the reward system, constitutes a signature of ADHD (Yael et al., 2019) and chronic pain conditions (Makary et al., 2020). These findings point to the role

played by the ACC, the amygdala, and the nucleus accumbens at the crossroads of ADHD and chronic pain. Moreover, dysfunction in the dopaminergic system underlies ADHD etiology and pain sensitization. Disorders characterized by deficits in dopaminergic transmission, such as ADHD (Sagvolden, Johansen, et al., 2005), have been associated with pain hypersensitivity while increased dopaminergic transmission was linked to attenuated pain sensitivity (Jarcho et al., 2012).

ADHD and chronic pain are, therefore, two interlinked complex conditions. Several pieces of evidence, detailed previously, support the involvement of neuroinflammation in ADHD (Alvarez-Arellano et al., 2020; Corona, 2020; Dunn et al., 2019; Leffa et al., 2018) and chronic pain (Vergne-Salle & Bertin, 2021). Consequently, neuroinflammation could act as a link between these two pathologies (Figure 19). A sustained neuroinflammatory status could trigger neuronal dysfunction in higher brain regions implicated in ADHD symptoms and pain processing. From a molecular point of view, neuroinflammation could lead to abnormalities in the dopaminergic system; dysfunctions correlated with both pathological pain and ADHD (Kerekes et al., 2021).

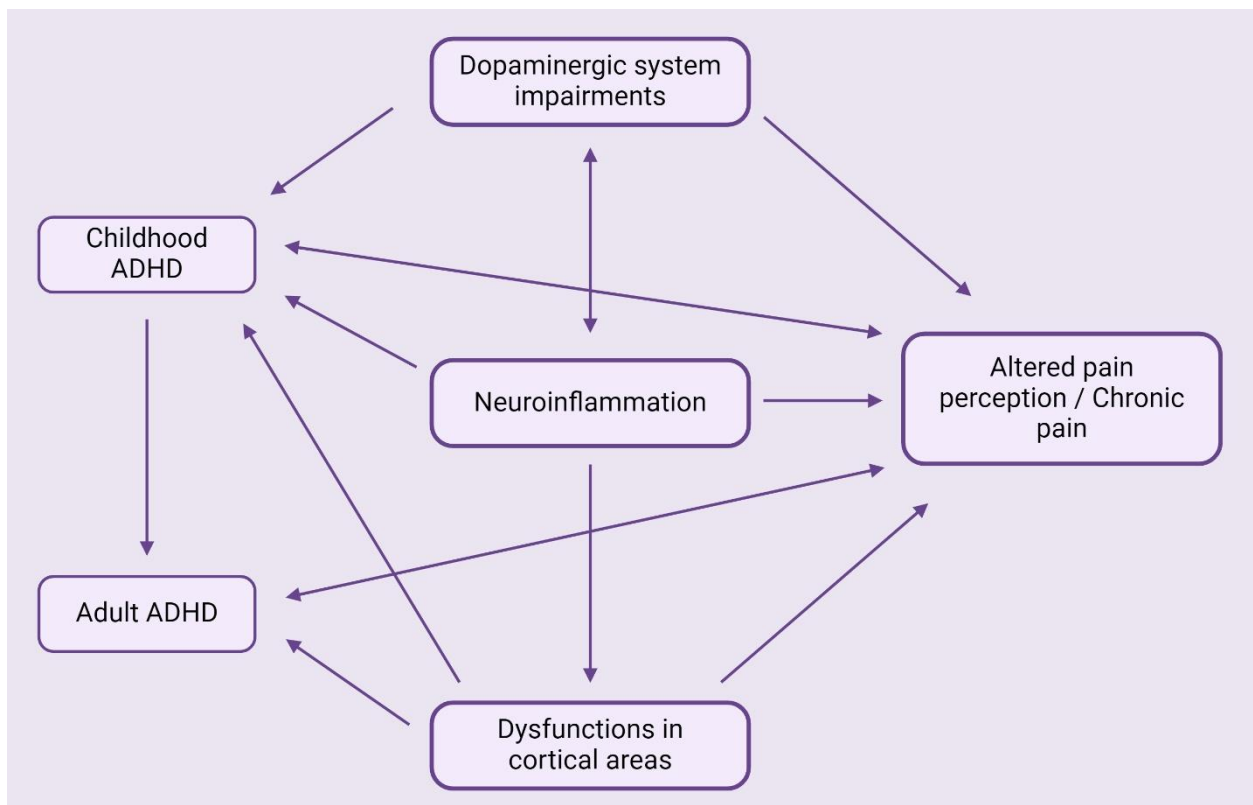


Figure 19: Neuroinflammation as a link between ADHD and pain sensitization. Created with BioRender.com and adapted from (Kerekes et al., 2021)

4. Purinergic system

4.1 Generalities

Purines, pyrimidines, and their derivatives have long been acknowledged as molecules with fundamental roles in nucleotide synthesis and energy homeostasis (Abbracchio & Burnstock, 1998; Z. Huang et al., 2021). However, in the 20th century, findings began supporting the “purinergic transmission” hypothesis, emitted by Geoffrey Burnstock and which suggests that purines can also act as non-cholinergic and non-adrenergic neurotransmitters (Burnstock, 1972; Z. Huang et al., 2021) through the binding to specific receptors, designated as purinergic receptors.

Since then, the purinergic signaling has been implicated in numerous physiological functions (Abbracchio & Burnstock, 1998; Z. Huang et al., 2021) such as cellular growth, proliferation, differentiation, and death (Abbracchio, 1996; Z. Huang et al., 2021; Neary et al., 2008). In the nervous system, it has also been shown to modulate many processes (Mut-Arbona & Sperlágh, 2023) including neuronal-glia communication (Fields & Burnstock, 2006; Stevens & Fields, 2000; Verderio & Matteoli, 2011), synaptic plasticity and transmission (Fields & Burnstock, 2006; Sim et al., 2006; Verderio & Matteoli, 2011; Y. Wang et al., 2004; Wieraszko & Ehrlich, 1994), and astrocytic proliferation (Fields & Burnstock, 2006; Neary et al., 2005).

4.2 Types of receptors

Purinergic receptors are divided into adenosine P1 and ATP P2 receptors (Figure 20) (Abbracchio & Burnstock, 1998; Fredholm et al., 1994; Ralevic & Burnstock, 1998; D. Wang & Zhou, 2023).

The P1 family encompasses four GPCRs activated by adenosine: A_1 , A_{2A} , A_{2B} , and A_3 (Abbracchio & Burnstock, 1998; Fredholm et al., 1994, 2001; D. Wang & Zhou, 2023). These receptors differently affect adenylate cyclase activity and, consequently, cAMP levels (Figure 20) (Fredholm et al., 1994, 2001). For instance, A_1 and A_3 are associated with a G_i protein, whose activation inhibits cAMP synthesis. A_{2A} and A_{2B} , on the contrary, are bound to a G_s protein, whose activation initiates cAMP production (Abbracchio et al., 2009).

As for the P2 type, it is further divided into two sub-classes: the P2X ionotropic receptors ($P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, and $P2X_7$) and the P2Y metabotropic receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) (Burnstock, 2018; Burnstock & King, 1996; Fredholm et al., 1994; D. Wang & Zhou, 2023) (Figure 20).

P2Y receptors possess several ligands, including ADP, UDP, ATP, and UTP (Di Virgilio et al., 2018; Fredholm et al., 2001; Z. Huang et al., 2021; D. Wang & Zhou, 2023). Their downstream effectors are AC and PLC (Figure 20) (Fredholm et al., 1994; Z. Huang et al., 2021). $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ are coupled to a G_q protein, thus activating PLC and Ca^{2+} release from the endoplasmic reticulum. On the other hand, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ are linked to a G_i protein and exert an inhibitory effect on AC, thus decreasing cAMP concentrations. Finally, $P2Y_{11}$ receptors stimulate cAMP and Ca^{2+} signaling following G_q and G_s protein activation (Di Virgilio et al., 2018; Z. Huang et al., 2021).

P2X receptors exist in a homo- or heterotrimeric form (Abbracchio et al., 2009; Z. Huang et al., 2021; Nicke, 1998). They are activated by ATP (Di Virgilio et al., 2018; Z. Huang et al., 2021) and are permeable to Na^+ , K^+ , and Ca^{2+} cations (Figure 20) (Bean, 1992; Di Virgilio et al., 2018; Egan & Khakh, 2004; Fredholm et al., 1994).

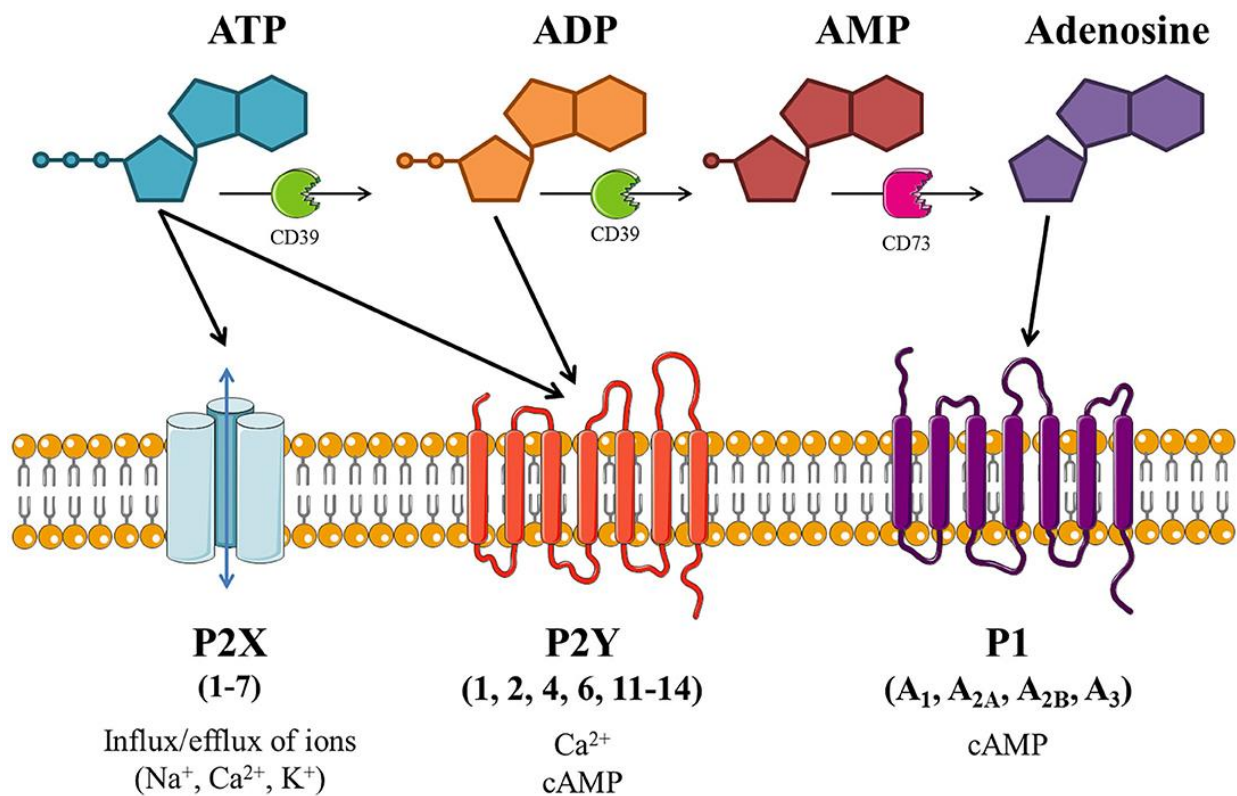


Figure 20: The purinergic receptors, agonists, and downstream effects (Thompson et al., 2021)

The purinergic receptors are characterized by a widespread expression in the human body (Burnstock, 2018; Burnstock & Knight, 2004), with their dysfunctions resulting in severe diseases. For example, P2X₇ dysregulation has been linked with neurodegenerative diseases (Alzheimer's, Parkinson's, Amyotrophic lateral sclerosis), kidney problems (nephritis, hypertension), thrombosis, fibrosis, cardiomyopathy, and many others (Z. Huang et al., 2021). Another example is the P2Y₂ receptor, whose alteration has been associated with gout, tumors, hypertension, and atherosclerosis (Z. Huang et al., 2021).

4.3 P2X₄ receptor

4.3.1 Generalities

The P2X₄R, encoded by the P2X4R gene, is an ATP-gated ion channel whose activation primarily promotes the flux of Na⁺, K⁺, and Ca²⁺ cations across the membranes (Bean, 1992; Di Virgilio et al., 2018; Fredholm et al., 1994; Z. Huang et al., 2021). However, after prolonged exposure to ATP, the receptor can also open non-selective pores and become permeable to larger molecules (Bernier et al., 2012; Kanellopoulos et al., 2021; Khakh et al., 1999; Virginio et al., 1999).

The P2X₄R possesses a trimeric structure, enabling the binding of ATP molecules at three different sites (Burnstock & Knight, 2004; Kanellopoulos et al., 2021). Each subunit is composed of (i) intracellular N- and C-termini, (ii) two transmembrane helices (TM1 and TM2), and (iii) a large extracellular ectodomain that can associate with the ectodomain of another P2X receptor to form an ATP-binding pocket (Kanellopoulos et al., 2021) (Figure 21, A). The subunits of the P2X₄R are characterized by a "dolphin-like" structure with a "tail fluke" anchored in the lipid bilayer but projecting to the cytoplasm and a "body" (dorsal fin, flippers, head regions) protruding into the extracellular region (Hattori & Gouaux, 2012; Kanellopoulos et al., 2021; Kawate et al., 2009; Sophocleous et al., 2022) (Figure 21, B).

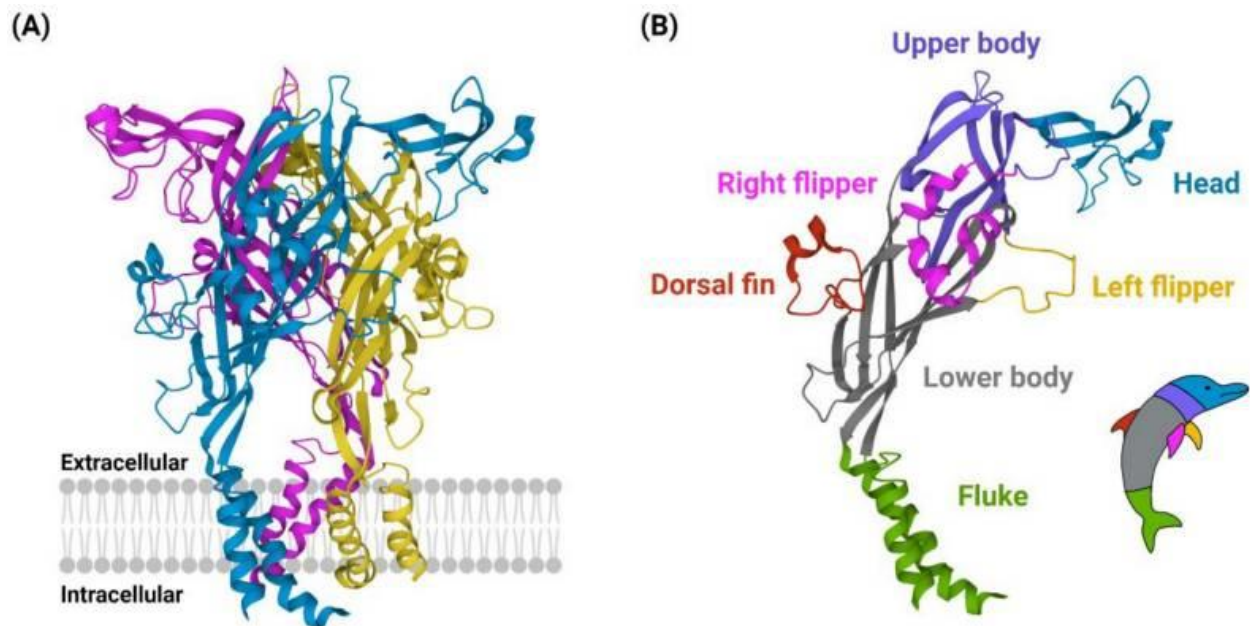


Figure 21: Structure of the P2X₄ receptor (Sophocleous et al., 2022)

Extensive expression and vast distribution of the P2X₄R were identified across mammals (Antonioli et al., 2019; Bo et al., 2003; Sophocleous et al., 2022). The receptor is expressed by various immune cells such as B and T lymphocytes (Sluyter et al., 2001; L. Wang et al., 2004; Woehrle et al., 2010), monocytes (L. Wang et al., 2004), mast cells (Wareham et al., 2009), and macrophages (Coutinho-Silva et al., 2005). Through its expression, P2X₄R participates in numerous processes, including T-cell migration and activation (Ledderose et al., 2018; Manohar et al., 2012), bacterial clearance (Csóka et al., 2018), and mast cell degranulation (K. Yoshida et al., 2017, 2019).

In the CNS, its expression is mainly observed in neurons (Amadio et al., 2007; Soto et al., 1996; Stokes et al., 2017) and microglial cells (Stokes et al., 2017; Tsuda et al., 2003; Ulmann et al., 2008). Notably, P2X₄R knock-out resulted in (i) decreased magnitude of LTP (Sim et al., 2006), (ii) impaired inflammasome signaling following spinal cord injury (de Rivero Vaccari et al., 2012), (iii) attenuated microglial chemotaxis (Ohsawa et al., 2007), (iv) altered BDNF signaling and absence of mechanical hyperalgesia following peripheral nerve injury (Ulmann et al., 2008), and (v) reduced pain sensitivity to tactile allodynia in inflammatory and neuropathic pain models (Tsuda et al., 2009). These results indicate the role of the P2X₄R in synapse strengthening, pain hypersensitivity, and the regulation of neuroinflammation.

The P2X₄R predominantly localizes at the plasma membrane, endosomes, or lysosomes of cells (Robinson & Murrell-Lagnado, 2013; Stokes et al., 2017). In microglia, for example, the lysosomal form is the most prominent (Boumechache et al., 2009; Qureshi et al., 2007; Toyomitsu et al., 2012). Interestingly, the channel's activation seems to be pH-dependent, with the acidic physiological pH (3.5-5.0) neutralizing P2X₄R function in lysosomes. On the contrary, alkalization of the luminal pH induces P2X₄R activation in the presence of ATP molecules (P. Huang et al., 2014; Kanellopoulos et al., 2021; Sophocleous et al., 2022).

Studying the role of the P2X₄R is of interest to us as this receptor has been previously associated with pain hypersensitivity and inflammation.

4.3.2 Role in pain hypersensitivity

P2X₄R was suggested to play key roles in the pathogenesis of pain sensitization and has become a potential target for the treatment of chronic pain conditions.

On one hand, the expression of P2X₄R was upregulated in different models of chronic pain conditions. Examples include models of bone cancer pain (Jin et al., 2014), chronic migraine (Liu et al., 2018; Long et al., 2018, 2020), inflammatory pain (Guo et al., 2005), nerve injury (Tsuda et al., 2003), or diabetes-induced neuropathic pain (Teixeira et al., 2019). Remarkably, P2X₄R expression was associated with hyperexcitability of the DHN in inflammatory pain conditions (Aby et al., 2018).

On the other hand, and more interestingly, the inhibition of P2X₄R expression relieved pain. Knock-out of P2X₄R attenuated pain sensitization to innocuous mechanical stimuli (tactile allodynia) in models of chronic inflammatory and neuropathic pain (Tsuda et al., 2009). The use of antisense oligodeoxynucleotide yielded the same results, blunting tactile allodynia caused by peripheral nerve injury (Tsuda et al., 2003). Antidepressant administration (duloxetine and paroxetine) reversed mechanical allodynia in models of neuropathic pain through the downregulation of P2X₄R-mediated responses (Nagata et al., 2009; Yamashita et al., 2016). 5-BDBD (P2X₄R antagonist) treatment effectively alleviated mechanical and thermal hyperalgesia (Long et al., 2020), in addition to basal hypersensitivity (Long et al., 2018), in a recurrent nitroglycerin-induced chronic migraine model. TNP-ATP (P2X₄R antagonist) limited mechanical allodynia in a rat model of trigeminal allodynia (Liu et al., 2018) while the administration of PSB-15417 (P2X₄R antagonist) further demonstrated the implication of the receptor in the development of diabetes-induced mechanical hyperalgesia (Teixeira et al., 2019). Notably, P2X₄R was mainly expressed in microglial cells (Guo et al., 2005; Liu et al., 2018; Long et al., 2018, 2020; Tsuda et al., 2003), casting light on the importance of microglial P2X₄R in pain sensitization.

Several studies focused on identifying the mechanisms underlying P2X₄R-mediated pain hypersensitivity. In chronic pain conditions, P2X₄R activation was proven to stimulate p38 MAPK and trigger the release of BDNF from microglial or neuronal cells (Lalisse et al., 2018; Long et al., 2020; Trang et al., 2009; Ulmann et al., 2008). Following peripheral nerve injury, BDNF alters anion homeostasis in spinal lamina I neurons via the binding to its TrkB receptor. This results in the switch of GABA response from hyperpolarizing to depolarizing, thus causing net excitation. Interestingly, BDNF also elicits nociceptive hypersensitivity characterized by a decrease in the paw withdrawal threshold to mechanical stimulation (Coull et al., 2005). Consequently, P2X₄R might be involved in pain hypersensitivity through the induction of BDNF release. Additionally, P2X₄R-mediated BDNF-TrkB signaling might also upregulate excitatory amino acid transporter 3 (EAAT3) neuronal expression (Liu et al., 2018), whose blockade lightened pain behaviors (Vincent et al., 2016).

4.3.3 Role in inflammation

P2X₄R contributes to the activation of several inflammatory pathways and, consequently, to neuroinflammation.

After sciatic nerve severing, NLRP3 inflammasome activation and assembly were demonstrated by (i) an increase in the mRNA expression levels of inflammasome components (IL-1 β , CASP1, AIM2, NLRP3, NLRP6) and (ii) the colocalization of NLRP3 with ASC (an indicator of inflammasome assembly). They were initiated exclusively in motoneurons. Interestingly, selective blockade of P2X₄R using 5-BDBD repressed inflammasome priming/activation, enhanced motoneuron survival, and improved functional recovery (Molnár et al., 2022) suggesting a role for this receptor in the initiation of the NLRP3 inflammasome. Likewise, expression of P2X₄R was detected in neurons following spinal cord injury. Knock-out of P2X₄R resulted in altered inflammasome signaling (reduced IL-1 β and CASP1 levels), diminished immune cell infiltration, and improved locomotor recovery (de Rivero Vaccari et al., 2012). Moreover, administration of 5-BDBD attenuated NLRP3, CASP1, and IL-1 β protein levels in a rat model of traumatic brain injury.

P2X₄R inhibition further prevented cell apoptosis and improved neurological functions (He et al., 2022).

However, the NLRP3 inflammasome isn't the only pathway modulated by P2X₄R. Evidence demonstrated a P2X₄R-dependent release of prostaglandin E2 in inflammatory pain conditions (Ulmann et al., 2010). Following carrageenan and CFA injection in the hind paw, tissue-resident macrophages highly expressed P2X₄R, thus stimulating downstream messengers (intracellular Ca²⁺, p38 MAPK), COX-dependent synthesis and release of prostaglandin E2 (Ulmann et al., 2010). Of note, prostaglandin E2 is a mediator of inflammatory responses (Kawahara et al., 2015; Ricciotti & FitzGerald, 2011) thus reaffirming the role of the P2X₄R in inflammatory responses.

P2X₄R was also shown to facilitate P2X₇R-mediated IL-18 and IL-1β release in response to ATP by increasing intracellular Ca²⁺ levels. Pretreatment of dendritic cells with a P2X₄R antagonist or the use of P2X₄R-knock-down cells halted P2X₇R-induced cytokine synthesis. This was further supported by the suppression of cytokine release following chelation of P2X₄R-induced intracellular Ca²⁺ (Sakaki et al., 2013). ATP-evoked intracellular Ca²⁺ signaling was also proven to be induced by P2X₄R in a model of human monocytes and macrophages (Layhadi & Fountain, 2017).

Overall, these findings point to the implication of P2X₄R in chronic pain and inflammatory response, further supporting our interest in studying its role in ADHD and its associated pain sensitization.

With all these data taken together, we hypothesize that neuroinflammation could serve as a bridge between ADHD and chronic pain. We therefore suggest that neuroinflammation triggers ACC hyperactivity and sensitizes pathways involved in ADHD symptoms and pain perception.

This Ph.D. thesis thus aims to elucidate the inflammatory mechanisms underlying ADHD and its associated pain sensitization by:

1. Providing evidence of microglial and astrocytic reactivity in the ACC of 6-OHDA males and females
2. Characterizing the inflammatory status of the ACC and PI of 6-OHDA males and females
3. Studying changes in protein expression in the ACC and PI of 6-OHDA males and females
4. Deciphering alterations in kinase activity in the ACC and PI of 6-OHDA males and females
5. Identifying impairments in specific canonical pathways and networks in the ACC and PI of 6-OHDA males and females
6. Checking for modifications in neuron-astrocyte communication (protein interactions) in the ACC of 6-OHDA mice

To address these questions, we have generated an ADHD-like mouse model through a unilateral intracerebroventricular injection of 6-OHDA at P5. Control mice (shams) were injected with ascorbic acid. Two-month-old mice were sacrificed, their brains were extracted, and the ACC and PI were dissected. Morphological (immunofluorescence staining), transcriptomic (RT-qPCR), proteomic (mass spectrometry, FRET-mfFLIM), and phosphoproteomic (PamGene assay) experiments were performed to decipher variations in the molecular mechanisms occurring in the ACC and PI of 6-OHDA males and females.

Another objective of this Ph.D. work was to investigate the role of the purinergic P2X₄ receptor in ADHD and its associated pain sensitization. To do so, P2X₄ knock-out mice were injected with either 6-OHDA or ascorbic acid. Behavioral tests were conducted to assess hyperactivity (open-field) and sensitization to thermal and mechanical pain (von Frey and Plantar tests). Two-month-old mice were then sacrificed, their brains were extracted, and the ACC and PI were dissected. Morphological (immunofluorescence staining) and transcriptomic (RT-qPCR) studies were carried out to analyze variations in microglia morphology and expression of inflammatory markers.

Chapter 2

Materials and Methods

2.1 Animals

C57BL/6 (Janvier Labs, France) and P2X₄ KO (EOPS facility, Bordeaux Neurocampus, France) pregnant females were housed under a 12h light/dark cycle (lights on at 7 a.m.) at the animal facility of the University of Bordeaux. They were provided with food and water *ad libitum*. The pups were born a few days after the mothers arrived at the animal facility and underwent surgery five days following birth. The study was approved by the ethical committee of the University of Bordeaux and the French Ministry of Higher Education, Research and Innovation (APAFIS#27681 and APAFIS#21135). All efforts were made to minimize animal suffering.

P2X₄ KO mice, generated according to an already-published protocol (Sim et al., 2006), carried a targeted null mutation of the P2X₄ receptor. This mutation was induced by the insertion of a LacZ neomycin cassette in a 10 Kb genomic DNA containing the first two exons of the P2X₄ receptor, between a *Bgl*III site (30 bases upstream of the initiating methionine) and a *Bam*HI site (172 bases downstream of the first exon/intron boundary). This insertion led to the deletion of 337 bases including the first exon, P2X₄-initiating methionine, and 172 bases of the downstream intron.

2.2 P5 surgery

Five days after birth, the pups were injected with either 6-OHDA hydrobromide (Sigma-Aldrich, France) or ascorbic acid (vehicle) following an already-published protocol (Bouchatta et al., 2018). Pups injected with 6-OHDA mimicked the human disease (development of ADHD symptoms) at later stages (Bouchatta et al., 2018), while those injected with ascorbic acid were used as controls.

Thirty minutes before surgery, desipramine hydrochloride (20mg/kg, Sigma-Aldrich, France) was administrated subcutaneously to prevent noradrenergic neuron depletion. The pups were subject to hypothermal anesthesia and received a unilateral intracerebroventricular injection (AP: -2mm, ML: ± 0.6mm, DV: -1.3mm from the bregma) of 6-OHDA (25µg of 6-OHDA dissolved in 3µL of 0.1% of ascorbic acid) or its vehicle. The injection lasted five minutes, with a flow equal to 0.6µL/min. After surgery, the pups were warmed up and returned to their mother. They were provided with enriched food (#SAFED113, Safe-lab, France) to speed up recovery and kept under constant observation. The weaning was performed at P21, and the mice were housed in a cage of two to five to avoid isolation.

2.3 Tissue preparation for immunofluorescence

Two-month-old mice were anesthetized with an intraperitoneal injection of ketamine (100mg/kg, Virbac, France) and xylazine (20mg/kg, Virbac, France) mixture. In the absence of reflexes, they were transcardially perfused with a 0.9% NaCl solution containing 0.01% heparin to clean the brain from the blood, followed by a fixative consisting of 4% paraformaldehyde (PFA) in 0.1M phosphate buffer, pH 7.4. The brains were extracted and post-fixed overnight, at 4°C, in 4% PFA. They were washed three times, ten minutes each, with phosphate-buffered saline (PBS) 0.1M and were cryoprotected in 12.5% sucrose in 0.01M PBS, overnight, at 4°C. The brains were then cut at the cryostat (CM3050S, Leica, Germany) to obtain 25µm sections.

2.4 Iba1/GFAP double immunofluorescence

Iba1 and GFAP are well-established markers for microglial cells and astrocytes, respectively. These proteins, specifically expressed in the two cell types, are widely used to study their morphology through immunofluorescence staining (Eng et al., 1971; Imai et al., 1996; Paolicelli et al., 2022).

The sections were washed three times, ten minutes each, in PBS 0.1M. They were transferred in a blocking

solution consisting of PBS BSA (bovine serum albumin, Sigma-Aldrich, France) 1% with 0.3% Triton X-100, for one hour, at room temperature. The sections were then incubated with the two primary antibodies anti-Iba1 (rabbit, dilution 1/2000, Wako, Japan) and anti-GFAP (chicken, dilution 1/1000, Millipore, France) in a solution of PBS BSA 1%, overnight, at 4°C. The following day, a three-times washing step was performed in PBS 0.1M. The sections were incubated with the secondary antibodies goat anti-chicken Alexa Fluor 488 (dilution 1/500, Invitrogen, France) and goat anti-rabbit Alexa Fluor 568 (dilution 1/500, Invitrogen, France) for two hours at room temperature. They were washed three times, ten minutes each, in PBS 0.1M and were mounted on Superfrost slides (Fisher Scientific, France) using Fluoromount G medium (Invitrogen, France).

The same protocol was followed when comparing wild-type and P2X₄ KO mice. However, the sections were only incubated with the Iba1 antibody.

2.5 Image acquisition and analysis for Iba1/GFAP double immunofluorescence

Images of the ACC were acquired with the x20 oil immersion objective of an SP5 confocal microscope (Leica, Germany). Two optical planes were chosen per picture, and five non-overlapping cells were randomly selected. Not less than 300 and 700 cells were analyzed per group in females (four sham and five 6-OHDA) and males (six sham and six 6-OHDA), respectively. When comparing sham and 6-OHDA wild-type and P2X₄ KO mice (six to seven mice per group), at least 400 cells were assessed per condition. However, gender differences were not considered in this part of the study.

FIJI and FracLac from the ImageJ software were utilized to carry out the analysis (Fernández-Arjona et al., 2017). Five morphological parameters were quantified: fractal dimension, lacunarity, solidity (or density), cell area, and cell perimeter. Fractal dimension has been widely used in the study of microglial cell morphology as a measure of pattern complexity. This parameter enables the identification of microglial shapes ranging from ameboid-like to highly complex branched structures. The higher the fractal dimension, the more complex the pattern and the more ramified the cell. Lacunarity complements fractal dimension in describing cell heterogeneity as it mainly correlates with changes affecting the soma. A higher lacunarity implies a more heterogenous cell image. Cell area represents the total number of pixels found in the filled shape of the microglia image while cell perimeter is assessed based on the outline of the microglial shape. Finally, cell solidity (also known as cell density) is calculated by dividing the area of the cell by its convex hull area (CHA); CHA being the smallest convex polygon fitting the whole cell shape. It describes how compact the cell is: the lower the solidity is, the more ramified the cell is.

2.6 GFAP/Vimentin double immunofluorescence

GFAP and vimentin are both intermediate filaments expressed by astrocytes. The study of these two proteins is important for the characterization of reactive astrogliosis as (i) vimentin is only expressed during development; therefore, an increased expression in astrocytes from two-month-old mice is indicative of reactive astrogliosis, and (ii) GFAP expression is increased when astrocytes are activated (Escartin et al., 2021; S. K. R. Pixley & de Vellis, 1984).

The sections were washed three times, ten minutes each, in PBS 0.1M. They were transferred in a blocking solution consisting of PBS BSA 1% with 0.3% Triton X-100, for one hour, at room temperature. The sections were then incubated with the two primary antibodies anti-vimentin (goat, dilution 1/2000, Sigma-Aldrich, France) and anti-GFAP (chicken, dilution 1/1000, Millipore, France) in a solution of PBS BSA 1%, overnight, at 4°C. The following day, a three-times washing step was performed in PBS 0.1M. The sections were incubated with the secondary antibodies donkey anti-chicken Alexa Fluor 488 (dilution 1/500, Jackson ImmunoResearch, United Kingdom) and rabbit anti-goat Alexa Fluor 568 (dilution 1/500, Invitrogen,

France) for two hours at room temperature. Finally, they were washed three times, ten minutes each, in PBS 0.1M and were mounted on Superfrost slides using Fluoromount G.

2.7 Image acquisition and analysis for GFAP/Vimentin double immunofluorescence

Images of the ACC were acquired with the x63 oil immersion objective of an SP5 confocal microscope. Around three to four non-overlapping two-step z-stacks, with a z-step size equal to 2.014, were taken per picture. Four sham and five 6-OHDA female mice, as well as six sham and 6-OHDA male mice, were assessed. FIJI from ImageJ software and Bioc3D (Dhellemmes et al., 2023) were used to quantify the results. Three parameters were studied: the number of colocalization between GFAP and vimentin, the mean fluorescence intensity of GFAP, and the mean fluorescence intensity of vimentin.

2.8 Tissue preparation for RT-qPCR procedures, PamGene assays, mass spectrometry, FACS, and MACS

Two-month-old mice were anesthetized with an intraperitoneal injection of ketamine (100mg/kg) and xylazine (20mg/kg) mixture. In the absence of reflexes, the mice were sacrificed by decapitation and the brains were extracted. The ACC and PI were rapidly dissected, in sterile conditions, on dry ice (RT-qPCR) or ice (PamGene assay, mass spectrometry, MACS, and FACS). The samples were stored at -80°C to avoid RNA and protein degradation until the day of the experiment.

2.9 RNA extraction and RT-qPCR

RT-qPCR was carried out to identify changes in the RNA expression levels of pro- and anti-inflammatory markers, thus characterizing the inflammatory environment in the ACC and PI of 6-OHDA mice.

Four to ten 6-OHDA female mice, five to nine sham female mice, five to seventeen 6-OHDA male mice, and four to nine sham male mice were utilized for the ACC. Moreover, five to nine 6-OHDA females, four to six sham females, five to eleven 6-OHDA males, and 6 sham males were used for the PI. As for the P2X₄ KO mice, no gender differences were considered; four to eleven mice in the ACC and three to eight mice in the PI were assessed per group.

The RNAs were extracted from the different samples and converted into cDNAs. The cDNAs were then amplified and the expression levels of our genes of interest were measured by qPCR.

2.9.1 RNA extraction

RNAs from the ACC and PI were extracted following the chloroform/isopropanol protocol (CHOMZYNSKI, 1987), as detailed below.

500µL of TRI-reagent (Euromedex, France) were added to each sample to homogenize and lyse the tissue, followed by 100µL of chloroform (Sigma-Aldrich, France) and 12.5µL of glycogen (20mg/mL, Sigma-Aldrich, France) to separate the RNA (aqueous phase) from the DNA/proteins (organic phase). The samples were mixed, incubated for fifteen minutes on ice, and centrifuged for ten minutes at 4°C and 13000rcf. The next steps consisted of the transfer of the aqueous phase onto a new and clean tube, followed by the addition of the same volume of (i) TRI-reagent and its corresponding chloroform volume, (ii) chloroform, and (iii) isopropanol. Between each reagent addition, the samples were mixed, incubated on ice, and centrifuged at 4°C and 13000rcf for (i) ten minutes and (ii) five minutes. After isopropanol addition, the samples were mixed and incubated for ten minutes at room temperature, followed by overnight incubation at -20°C to allow RNA precipitation and pellet formation.

The samples were centrifuged for thirty minutes at 4°C and 13000rcf. The isopropanol was removed and 500µL of ethanol 75% was added to wash the RNA pellet. The samples were centrifuged again for ten minutes at 4°C and 13000rcf, the ethanol was discarded, and the pellet was dehydrated. Once the pellet

became transparent, 20µL of RNase-free water was added.

The samples were incubated for twenty-five minutes at 37°C with Turbo DNA-free (Fisher Scientific, France) to remove DNA residues. A DNase inactivation reagent (Fisher Scientific, France) was then added to the mixture to stop enzyme activity, the samples were incubated for two minutes at room temperature, vortexed, and centrifuged for five minutes at 4°C and 13000rcf. Finally, the supernatant containing the extracted RNA was collected.

RNA quantification was performed by measuring the absorbance at 260nm using a spectrophotometer and its quality was assessed by capillary electrophoresis with the bioanalyzer 2100 (Agilent, France). The results of the RNA quantification for the ACC and PI are shown in Table 7-1 and Table 7-2, respectively.

Table 7-1: Final RNA concentrations following Turbo DNA-free treatment for the ACC

Condition	RNA concentration (ng/µL)
F3559-1 wild-type 6-OHDA	270.596
F3559-3 wild-type 6-OHDA	392.299
F3559-4 wild-type 6-OHDA	267.808
F315-1 wild-type 6-OHDA	460.03
F315-0 wild-type 6-OHDA	413.664
F369 wild-type 6-OHDA	108.661
F4940-0 wild-type 6-OHDA	259.389
F3557-0 wild-type sham	395.238
F2556-1 wild-type sham	383.369
F3312 wild-type sham	95.090
F3313 wild-type sham	209.321
F3591 wild-type sham	142.247
F3592 wild-type sham	139.182
F882 wild-type sham	277.204
F993 wild-type sham	209.38
F994 wild-type sham	199.931
M3594 wild-type 6-OHDA	135.994
M3595 wild-type 6-OHDA	90.394
M584 wild-type 6-OHDA	170.416
M870 wild-type 6-OHDA	202.777
M871 wild-type 6-OHDA	180.667
M872 wild-type 6-OHDA	149.801
M2477 wild-type 6-OHDA	158.033
M2478 wild-type 6-OHDA	269.855
M2479 wild-type 6-OHDA	179.994
M2480 wild-type 6-OHDA	175.179
M2587 wild-type 6-OHDA	233.148
M2589 wild-type 6-OHDA	178.832
M3557-0 wild-type sham	127.603
M3557-1 wild-type sham	138.212
M3557-2 wild-type sham	180.434
M3596 wild-type sham	156
M3597 wild-type sham	143.302
M3598 wild-type sham	222.106

M996 wild-type sham	182.869
M998 wild-type sham	118.368
M997 wild-type sham	92.888
F733-1 P2X4 KO 6-OHDA	519.055
F910 P2X4 KO 6-OHDA	144.978
F739-2 P2X4 KO 6-OHDA	540.601
F738-0 P2X4 KO 6-OHDA	361.372
F955 P2X4 KO Sham	117.479
F995 P2X4 KO Sham	103.666
F957 P2X4 KO Sham	104.763
F956 P2X4 KO Sham	92.256
M731-2 P2X4 KO 6-OHDA	360.188
M737-1 P2X4 KO 6-OHDA	319.048
M736-0 P2X4 KO 6-OHDA	477.514
M735 P2X4 KO 6-OHDA	536.194
M732-3 P2X4 KO 6-OHDA	337.402
M952 P2X4 KO Sham	211.744
M953 P2X4 KO Sham	270.758
M954 P2X4 KO Sham	210.185
M999 P2X4 KO Sham	134.699

Table 7-2: Final RNA concentrations following Turbo DNA-free treatment for the PI

Condition	RNA concentration (ng/ μ L)
F3559-1 wild-type 6-OHDA	483.128
F3559-3 wild-type 6-OHDA	731.044
F3559-4 wild-type 6-OHDA	800.94
F315-1 wild-type 6-OHDA	438.246
F315-0 wild-type 6-OHDA	271.546
F369 wild-type 6-OHDA	155.677
F370 wild-type 6-OHDA	123.511
F3557-0 wild-type sham	159.575
F3557-3 wild-type sham	140.274
F3557-2 wild-type sham	215.902
F2556-1 wild-type sham	149.994
F1292 wild-type sham	192.993
F3313 wild-type sham	164.536
F888 wild-type sham	223.503
F889 wild-type sham	272.329
M3594 wild-type 6-OHDA	125.249
M3595 wild-type 6-OHDA	111.050
M584 wild-type 6-OHDA	128.722
M870 wild-type 6-OHDA	164.304
M871 wild-type 6-OHDA	172.776
M872 wild-type 6-OHDA	125.442
M2477 wild-type 6-OHDA	216.855
M2478 wild-type 6-OHDA	264.73
M2479 wild-type 6-OHDA	249.752

M2480 wild-type 6-OHDA	234.907
M2587 wild-type 6-OHDA	253.926
M2589 wild-type 6-OHDA	200.184
M3557-0 wild-type sham	303.347
M3557-1 wild-type sham	198.209
M3557-2 wild-type sham	421.094
M3590 wild-type sham	103.818
M3597 wild-type sham	116.923
M3558-0 wild-type sham	363.502
M996 wild-type sham	224.656
M997 wild-type sham	321.999
F733-1 P2X4 KO 6-OHDA	500.216
F910 P2X4 KO 6-OHDA	158.233
F739-2 P2X4 KO 6-OHDA	579.611
F738-0 P2X4 KO 6-OHDA	366.163
F955 P2X4 KO Sham	138.7
F995 P2X4 KO Sham	55.823
F957 P2X4 KO Sham	103.695
F956 P2X4 KO Sham	86.150
M731-2 P2X4 KO 6-OHDA	397.102
M737-1 P2X4 KO 6-OHDA	390.9
M736-0 P2X4 KO 6-OHDA	377.548
M735 P2X4 KO 6-OHDA	476.43
M732-3 P2X4 KO 6-OHDA	461.941
M952 P2X4 KO Sham	130.351
M953 P2X4 KO Sham	74.641
M954 P2X4 KO Sham	66.029
M999 P2X4 KO Sham	150.161

2.9.2 RT-qPCR

Following RNA quantification, the samples were normalized according to the lowest concentration. cDNA synthesis consisted of two steps: RNA denaturation and reverse transcription. RNA denaturation was performed using 2µg of RNA, 2µL of random primer (150ng/µL, Fisher Scientific, France), and 1µL of OdT (200ng/µL, Fisher Scientific, France). Water was added in case the final volume did not reach 20µL. The samples were incubated for five minutes at 65°C. Reverse transcription was carried out by adding 20µL of the RT mix to the denatured samples. The RT mix consisted of 8µL of 5x Maxima buffer (Fisher Scientific, France), 2µL of dNTP 10mM Promega, 1µL RiboLock RNase inhibitor (Fisher Scientific, France), 1µL of Maxima RT (Fisher Scientific, France), and 8µL of water. The samples were incubated for ten minutes at 25°C, then for one hour at 50°C, and finally, for fifteen minutes at 70°C. 60µL of water was added to reach a final cDNA concentration of 20ng/µL.

qPCR was performed using a LightCycler® 480 Real-Time PCR System (Roche, France). qPCR reactions were done in duplicate for each sample using 2µL of cDNA at a concentration of 2ng/µL, 3µL of forward and reverse transcript-specific primers at a concentration of 2µM each, and 5µL of LightCycler 480 SYBR Green Master (Roche, France). The PCR program included an initialization step at 95°C for five minutes, followed by 45 cycles of amplification comprising the denaturation of the DNA at 94°C for fifteen seconds and the annealing of DNA strands at 61°C for thirty seconds. A melting curve analysis assessed the specificity of

our qPCR assays.

The list of genes and forward/reverse primers is presented in Table 8. Genes with significantly different expressions in the ACC and/or PI of wild-type and P2X₄ KO 6-OHDA mice, compared to sham mice, are highlighted in bold.

The relative gene expression to the sham group was calculated through the $2^{-\Delta\Delta CT}$ method for each gene of interest (Livak & Schmittgen, 2001). For the determination of the reference gene, the RefFinder method was used (F. Xie et al., 2012). Relative expression analysis was normalized against two reference genes, depending on the tissue: Gapdh (Glyceraldehyde-3-Phosphate Dehydrogenase) and Nono (Non-POU-domain-containing, Octamer binding protein) for the ACC, Gusb (glucuronidase beta) and Ubc (Ubiquitin C) for the PI.

Table 8: List of genes detected and the forward/reverse primers used

Gene	Forward primer	Reverse primer
APE1	GGTTGGCGCCTTGATTACTTT	GACCGGATCTTGCTGTCACAC
Arg1	AACACGGCAGTGGCTTTAACC	GGTTTTCATGTGGCGCATT
BDNF	CCCGTCTGTACTTTACCCTTTGG	TGACTAGGGAAATGGGCTTAACA
Cathepsin S	GGCAAAGATTACTGGCTTGGA	GCCATCCGAATGTATCCTTGAT
CCL2	AGGTGTCCCAAAGAAGCTGT	ATGTCTGGACCCATTCTTC
CCL4	CTCCCGGCAGCTTCACAGA	GCTGGCTTGAGCAAAGACT
CCR2	TGCAACTGCCTCTTTCCTCAT	TTGGCTATTCCATATACACCTTTCC
CCR5	TACTCGGCTTCTAGTCAGGCATT	GTGAGAGGAGGGAGCTTTGTGT
CD11b	CTCATCACTGCTGGCCTATACAA	GCAGCTTCATTATCATGTCCTT
CX3CL1	CATGCTCCACAGAACCAGTTGT	CACCGAAGGAGCCAGGATATAGT
CX3CR1	GTCTGTATGTTTGTGTCGAGGATGA	ACAAAGGGTTGGATATGCCTGT
CXCL1	GGTAGGGCATAATGCCCTTTTA	CGTCTCTGTCCCAGCGA
CXCL12	CAGGCCGTGCACCCTTT	ATGGGCCAGGGTAGCTGTT
CXCL13	TCGAGGAATGAAAAACCTACATGTC	TCCATCTCGCAAACCTCTTGTT
CXCR2	GCTGTCTCCTGAGGAGCTCT	CTCTATCCAATGGATGATTGTGA
CXCR5	GCAAGATAGCAAAGTGGTCCTAGC	AGGAACCTCTGTCTCATTCTCTTA
GFAP	TTTCTCAACCTCCAGATCC	CCGCATCTCCACAGTCTTTA
GSK3β	GCCTTCAGCTTTTGGTAGCAT	TGCCACTACTGTGGTTACCTT
Iba1	CAATTCCTCGATGATCCCAAATA	TTCACCTTGAAGGCTTCAAGTTT
IL-10	AGTTGTGAAGAACTCATGGGTCTT	TGCTGCAGGAATGATCATCAA
IL-13	GAAGAATGGCCTGTTACTCAAG	TGTTCAAGTACAAACCCACCAC
IL-16	GCCATGAAGAACACATTCAAACA	TGGTCCACTCCTTCTAGGCTCT
IL-18	ATTAGCACACATGCGCCTTGT	AAATCATGCAGCCTCGGGTA
IL-1β	GAAGAAGAGCCCATCCTCTG	TCATCTCGGAGCCTGTAGTG
IL-2	ACTAAAGGGCTCTGACAACACATTT	TCAGAAAGTCCACCACAGTTGCT
IL-4	ATCATCGGCATTTTGAACGAG	AGGACGTTTGGCACATCCAT
IL-6	TACTCGGCAAACCTAGTGCGT	ATTTTCTGACCACAGTGAGGAATG
INF-γ	ACAATGAACGCTACACACTGCAT	TGGCAGTAACAGCCAGAAACAG
IRF5	CCTGGGACAGCCACGTAGAC	CAGAACCCTGTAGCCAGCCA
IRF8	CAGCTGTCAATCACTTGAGA	CAAGCAGTATCTACCCCATATT
MMP9	TTGCCCTACTGGAAGGTATTATGT	GAGTGGATAGCTCGGTGGTGT
NF-κB	CAAAGCCCTGACAGTCCATTG	GATTCTCCAGCACCTTTGGATT
NLRP3	ATAGGGTCTGGAGCAAAGGCTT	TCTGGCAGTTTACGTTATCGA

NOX2	TTTCCTGTGTGGCCCTGAA	GGCCGGATTCTGAGTTGGA
P2X4R	TTGGCTGGGTACCTGTTG	CGTGTCTCTGCTCCCATATTCC
Pecam1	CGAGAGTCCTGTGCACGTATTT	TGGATGGCTTGGCCTGAA
Plat (tPA)	AAGATCATGTCTCAACAGCAAAAAA	TCTTGTCCCCAGTGCAAACTTT
SOD1	CCCGCGGATGAAGAGA	CATTGGCCACACCGTCCT
TGF-β	CATGCCAACTTCTGTCTGGGA	GCAAGGACCTTGCTGTACTGTGT
TLR7	GGAAGAGACTCTGCAGGAGCTC	CACTGCCAGAAGTATGGGTGAG
TNFR	AAGAAATGTCCAGGTGGAG	TCTCACTCAGGTAGCGTTGG
TNF-α	GGCACTCCCCAAAAGATG	GCCACAAGCAGGAATGAGAAG
TSPO	TGGCTCTACATAGTCTGGAAAGA	TGTAGAGACCCAAGGGAACCAT
Wnt3a	TCGGATACCTCTTAGTGCTCT	GGGGTCCCACAGCCAAGGA
Wnt5a	CCAGTTCGGCATCGGAGAT	GGCGTTCACCACCCAGCT

2.10 Protein extraction and mass spectrometry

Mass spectrometry experiments were conducted for diverse purposes: (i) to study changes in the expression of pro- and anti-inflammatory mediators at the proteomic level in the ACC and PI of 6-OHDA mice, (ii) to identify signaling pathways that are altered in ADHD conditions, and (iii) to build networks and elucidate the molecular interactions that could result in ADHD symptoms and its comorbid pain sensitization.

The mass experiments were done in collaboration with Dr. Jeffrey GLENNON and his Ph.D. student Mairead SULLIVAN in the facilities of the Conway Institute at the University College Dublin (Ireland).

Protein extraction was performed using the iST sample preparation kit 96x (Preomics, Germany). The procedure, which lasted for two consecutive days, consisted of three main steps: (i) sample lysis at 95°C to extract and denature the proteins, (ii) protein digestion into peptides at 37°C, and (iii) sample purification at room temperature for peptide enrichment.

Seven to eight ACC and PI samples were processed for each condition (6-OHDA males, 6-OHDA females, sham males, and sham females).

50µL of lysis buffer was added to each tube to denature, reduce, and alkylate the proteins. The samples were placed in a heating block and shaken at 1000rpm and 95°C for ten minutes. In the meantime, the resuspension buffer was prepared by adding 210µL of the “resuspend buffer” in the “digest tube” containing a Trypsin/LysC mix. 50µL of this mix was loaded in each sample tube, then placed in a heating block and shaken at 500rpm and 37°C for three hours. This step allowed the digestion and breakdown of the proteins into peptides. Finally, 100µL of the “stop solution” was added to cease enzymatic activity, and the samples were stored at -20°C overnight. The following day, the samples were slightly centrifuged, and a two-times addition of “wash solution 1” and “wash solution 2” was conducted to clean the peptides from any hydrophobic or hydrophilic contaminant, respectively. After each addition, centrifugation was done for three minutes at 2250rcf. Another two-step addition of 100µL of the “elute solution”, followed by centrifugation (2250rcf, three minutes), was performed and the purified peptides were eluted in a collection plate. Finally, the samples were dried in a vacuum evaporator at 45°C for almost two hours, enabling peptide enrichment. They were resuspended in 40µL of “LC-load solution”.

A concentration of 0.2µg/µL was required for the analysis. The final protein concentration of each sample (Table 9-1 and Table 9-2) was measured using a spectrophotometer at a wavelength equal to 215nm. The samples were then sent for analysis to the mass spectrometry platform.

Table 9-1: Final protein concentrations of ACC samples for mass spectrometry

Sample	Protein concentration (µg/µL)
F3602 sham	0.202
F836 sham	0.117
F837 sham	0.281
F888 sham	0.184
F889 sham	0.19
F890 sham	0.189
F891 sham	0.278
F4940/1 6-OHDA	0.329
F4939 1-1 6-OHDA	0.13
F4939 1-0 6-OHDA	0.328
F4939 2-2 6-OHDA	0.195
F4939 1-2 6-OHDA	0.258
F368 6-OHDA	0.185
F370 6-OHDA	0.22
F371 6-OHDA	0.136
M3600 sham	0.231
M3601 sham	0.295
M3599 sham	0.198
M19 sham	0.2
M20 sham	0.278
M879 sham	0.212
M880 sham	0.245
M881 sham	0.263
M2590 6-OHDA	0.299
M2591 6-OHDA	0.285
M560 6-OHDA	0.289
M562 6-OHDA	0.341
M547 6-OHDA	0.238
M548 6-OHDA	0.232
M549 6-OHDA	0.216
M553 6-OHDA	0.153

Table 9-2: Final protein concentrations of PI samples for mass spectrometry

Sample	Protein concentration (µg/µL)
F2500 sham	0.279
F2501 sham	0.269
F835 sham	0.134
F837 sham	0.153
F3591 sham	0.16
F3592 sham	0.137
F3602 sham	0.133
F2547 6-OHDA	0.143
F2548 6-OHDA	0.284
F2549 6-OHDA	0.337
F2658 6-OHDA	0.223

F2659 6-OHDA	0.155
F2665 6-OHDA	0.154
F4939 1-0 6-OHDA	0.21
F4939 1-1 6-OHDA	0.146
M3596 sham	0.306
M3589 sham	0.174
M3598 sham	0.155
M3600 sham	0.223
M3601 sham	0.24
M19 sham	0.168
M20 sham	0.28
M881 sham	0.152
M2590 6-OHDA	0.174
M2591 6-OHDA	0.169
M560 6-OHDA	0.187
M562 6-OHDA	0.259
M547 6-OHDA	0.168
M548 6-OHDA	0.328
M549 6-OHDA	0.279
M553 6-OHDA	0.155

Proteins, with an up- or down-regulated expression in 6-OHDA mice, were identified and displayed in an Excel file. The raw results were analyzed using the Ingenuity Pathway Analysis (IPA) (Qiagen, Germany). For the analysis, three parameters were required: the gene/protein names, the p-values (obtained through the conversion of the log(p-value)), and the student t-test differences (log2fold change). All networks, altered signaling pathways, and upstream regulators were given by the software. Our study analysis was based on the top 10 altered signaling pathways (pathway enrichment analysis) and the most significant network; the network with the highest score and number of interacting molecules (network enrichment analysis).

2.11 Protein extraction, PamGene STK/PTK assays, and data analysis

To explore changes in the functionality of proteins in ADHD-like conditions, we relied on the PamGene assay. In a comparative analysis, this high-throughput screening method deciphers modulations in the activity of serine/threonine and tyrosine kinases.

Serine/threonine kinase (STK) and protein tyrosine kinase (PTK) assays (PamGene, The Netherlands) allow the study of kinase activity based on the phosphorylation of peptide substrates immobilized on the PamChips (PamGene, The Netherlands). The PamChip comprises four similar arrays, each containing 144 (STK) or 196 (PTK) peptide sequences designed based on the literature or through computational predictions. The peptides are formed by 13 amino acids and their phosphorylation sites correlate with one or numerous upstream kinase targets.

In the presence of ATP, kinases in the samples phosphorylate their target peptide sequences. This phosphorylation activity is recorded by a CCD-camera present in the PamStation12 (PamGene, The Netherlands) as a real-time kinetic readout. In the STK assay, a mix of three primary antibodies in the reaction mix binds the phosphorylated Ser/Thr substrates and is detected in a subsequent step with a fluorescently second-labeled antibody. In the PTK assay, the phosphorylation is assessed through a generic fluorescently labeled antibody PY20 that directly binds the phosphorylated tyrosine substrates.

Five to six mice per condition (6-OHDA males, 6-OHDA females, sham males, sham females) were used in each of the STK and PTK assays.

2.11.1 Protein extraction

Samples were ground on ice with a pestle until a homogeneous suspension was obtained. 100µL and 50µL of lysis buffer were added to ACC and PI samples, respectively. The lysis buffer consisted of (for 1mL): 980µL of M-PER mammalian extraction buffer (Fisher Scientific, France), 10µL of Halt phosphatase inhibitor cocktail 100X (Fisher Scientific, France), and 10µL of Halt protease inhibitor cocktail 100X, EDTA free (Fisher Scientific, France). The lysis was forced through pipetting the mixture up and down. Once the solution became clear, the lysates were incubated on ice for fifteen minutes followed by a centrifugation step of fifteen minutes, at 16000rcf and 4°C. The supernatant was collected and transferred to three clean vials. One aliquot was used for protein quantification, and the remaining vials were stored at -80°C until the day of the experiment.

Protein quantification was performed using the Bicinchoninic acid (BCA) protein assay kit (Fisher Scientific, France). In a 96-well plate, 0, 10, 20, 30, and 40µL of 1mg/mL of bovine serum albumin (Sigma-Aldrich, France) solution (triplicate) and 2µL of each sample solution (duplicate) were pipetted onto different wells. Then, 200µL of a mixed solution consisting of 18mL of reagent A (bicinchoninic acid) and 360µL of reagent B (cupric ions) were added. The plate was incubated at 37°C for thirty minutes, and protein concentrations were determined using a spectrophotometer ($\lambda=562\text{nm}$).

Protein concentrations for the ACC and PI samples are indicated in Table 10-1 and Table 10-2, respectively.

Table 10-1: Protein concentrations of ACC samples for PamGene assays

Sample	Protein concentration ($\mu\text{g}/\mu\text{L}$)
F2496 sham	2.3
F2497 sham	3.8
F2498 sham	2.9
F2499 sham	1.4
F2500 sham	1.8
F2501 sham	2.1
F291 sham	1.4
F292 sham	7.6
F22 sham	6.0
F23 sham	3.4
F24 sham	3.0
F25 sham	1.8
F2547 6-OHDA	1.0
F2548 6-OHDA	1.6
F2549 6-OHDA	2.8
F2658 6-OHDA	2.9
F2659 6-OHDA	1.4
F2665 6-OHDA	3.0
F4939 2-0 6-OHDA	2.5
F1349 6-OHDA	4.2
F1350 6-OHDA	1.8
F1803 6-OHDA	2.6
F1358 6-OHDA	3.5

F1359 6-OHDA	2.7
M2494 sham	2.4
M2495 sham	1.7
M2493 sham	2.4
M3311 sham	1.6
M3589 sham	1.7
M3590 sham	1.5
M290 sham	1.5
M295 sham	3.3
M297 sham	3.4
M302 sham	1.1
M303 sham	1.1
M304 sham	1.3
M2661 6-OHDA	3.8
M2662 6-OHDA	0.9
M2663 6-OHDA	3.5
M2664 6-OHDA	2.7
M2660 6-OHDA	2.2
M3593 6-OHDA	2.2
M1343 6-OHDA	2.9
M1344 6-OHDA	2.3
M1345 6-OHDA	1.2
M1346 6-OHDA	4.4
M1347 6-OHDA	1.9
M1353 6-OHDA	3.9

Table 10-2: Protein concentrations of PI samples for PamGene assays

Sample	Protein concentration ($\mu\text{g}/\mu\text{L}$)
F3312 sham	2.1
F2498 sham	2.5
F2497 sham	3.3
F2496 sham	2.2
F2499 sham	0.5
F291 sham	2.1
F4939 2-2 6-OHDA	5.6
F4939 2-0 6-OHDA	6.4
F4940-0 6-OHDA	8.3
F4939 1-2 6-OHDA	1.5
F4939 2-1 6-OHDA	6.5
M2494 sham	2.4
M3589 sham	1.5
M3311 sham	1.3
M2493 sham	0.1
M2495 sham	1.2
M303 sham	3.9
M2663 6-OHDA	0.3
M3593 6-OHDA	0.1

M2662 6-OHDA	1.1
M2661 6-OHDA	1.2
M2664 6-OHDA	0.9
M1346 6-OHDA	2.6
M1347 6-OHDA	6.6

2.11.2 STK assay

The STK assay consists of four main steps: (i) the dilution of lysates, (ii) the blocking cycles, (iii) the incubation with the primary antibody that binds to the phosphorylated substrates, and (iv) the incubation with the secondary antibody, linked to a fluorochrome, and which binds to the primary one.

On the day of the assay (thirty minutes before initiating the run), and following protein quantification, the samples were thawed on ice and centrifuged for five minutes, at 10000rcf and 4°C. The protein amount per array was equalized (to 1µg) by diluting with M-PER solution with inhibitors (2µL protease inhibitor cocktail 100X, 2µL phosphatase inhibitor cocktail 100X, 16µL of M-PER (Fisher Scientific, France)).

Pre-assay general steps: In the meantime, the PamChips were left at room temperature for at least ten minutes, and the buffers were prepared: 3mL of 1X PK-wash buffer (300µL of 10X PK buffer in 2700µL of ultrapure water), 100µL of 4mmol/l ATP solution (4µL of ATP stock solution at 100mmol/l in 96µL of ultrapure water), and 3mL of 1X PBS/0.01% Tween (300µL of 10X PBST in 2700µL of ultrapure water).

The syringe was filled with 3mL of 1X PK-wash buffer and placed in the PamStation. The wash bottle was emptied, the fluid barriers were checked, and the cover glasses were cleaned.

Blocking steps: The protocol was started, the barcodes of the PamChips (article number: 87102) were scanned (710425803, 710425809, 710425808, 710425805, 710425806, 710425807, 710425718, 710425801, 710425802, 710425717, 710425706), the PamChips were loaded in the PamStation, and 30µL of 2% BSA were added to the arrays. During the blocking step, the basic mix containing the primary antibody was prepared (for 30µL): 21µL of water, 4µL of 10X PK buffer, 0.4µL of 100X BSA solution, 0.6µL of the STK antibody mix, 4µL of 4mM ATP.

Incubation with primary antibody mix: Once the blocking step was completed, 30µL of the basic mix and 10µL of the sample solution were mixed and loaded onto the array. The syringe was filled with 1X PBS/0.01% Tween buffer instead of the 1X PK-wash buffer. During the first incubation step, the detection mix containing the fluorescent secondary antibody was prepared (for 30µL): 26.6µL of water, 3µL of 10X Ab buffer, and 0.4µL of STK antibody FITC-labelled.

Incubation with secondary antibody: Finally, 30µL of the detection mix was added to each array. The first images started to appear a few minutes later. Image data were collected at completion of the assay, and sent to PamGene for analysis.

2.11.3 PTK assay

The PTK assay consists of three steps: the dilution of lysates, the blocking cycles, and the incubation with the antibody, linked to a fluorochrome, which directly binds to the phosphorylated substrates.

Lysate dilution steps were similar to the STK assay described above, but the protein amount per array was equalized to 5µg.

Pre-assay general steps: The PamChips were left at room temperature for at least ten minutes, and the buffers were prepared: 3mL of 1X PK-wash buffer (300µL of 10X PK buffer in 2700µL of ultrapure water),

100µL of 4mmol/l ATP solution (4µL of ATP stock solution at 100mmol/l in 96µL of ultrapure water), and DTT solution (dissolution of the solid DTT with the amount of ultrapure water indicated on the bottle). The syringe was filled with 3mL of 1X PK-wash buffer and placed in the PamStation. The wash bottle was emptied, the fluid barriers were checked, and the cover glasses were cleaned.

Blocking steps: The protocol was started, the barcodes of the PamChips (article number: 86412) were scanned (641092323, 641092402, 641092401, 641092319, 641092316, 641092318, 641092315, 641092312, 641092307, 641092304, 641092305), the PamChips were loaded in the station, and 30µL of 2% BSA was added to the arrays. During the blocking step, the basic mix containing the FITC-conjugated PY20 antibody was prepared (for 30µL): 20.6µL of water, 4µL of 10X PK buffer, 0.4µL of 100X BSA solution, 0.4µL of 1M DTT solution, 4µL of 10X PTK additive, 0.6µL of the detection antibody, 4µL of 4mM ATP.

Incubation with primary antibody: Once the blocking step was completed, 30µL of the basic mix and 10µL of the sample solution were mixed and loaded onto the array. The first images started to appear a few minutes later. Image data were collected at completion of the assay, and sent to PamGene for analysis.

2.11.4 Data analysis

Basic processing, phosphosite, and kinase analysis were performed using the BioNavigator software (PamGene, The Netherlands), by Dr. Savithri RANGARAJAN (Scientific Project Leader, PamGene).

Basic processing encompassed image quantification, quality control, and normalizations.

Image analysis and quantification: A series of pictures were acquired during the experiment. For each image, a grid was constructed and several values (quantification types) were calculated. The final “signal” value (Median_SigmBg) referred to the median signal for each image, in which the background was removed. Moreover, each picture was acquired at multiple low and high exposure times to capture high and low-intensity signals, respectively. For each picture, the Median_SigmBg values of the different exposure times were further integrated into a single value called the “S100 signal”.

Quality control: The quality of the data -important to evaluate its reliability- was assessed using PamGene’s QC criteria for (i) signal strength, (ii) number of peptides, and (iii) replicate variation (coefficient of variance) (Table 11). Signal strength is defined as the 99th percentile of the S100 signal. The number of peptides represents how many phosphosites passed the quality control: those that showed an increase of their signal in time on at least 25% of the arrays (PTK assay) or those with a coefficient of variance lower than 50% (STK assay). Replicate variance (or coefficient of variance) indicates the variance between technical or biological replicates.

The QC results for the STK and PTK assays in both the ACC and PI are shown in Table 12.

Table 11: Criteria used to assess data quality

Level	Flag	Signal strength (AU)	QC-passed phosphosites (PTK) of 195	QC-passed phosphosites (STK) of 142	Technical CV	Biological CV
Good	Green	0-4000	> 123	> 90	< 20%	< 30%
Fair	Orange	0-2000	78-123	56-90	20-30%	30-40%
Poor	Red	0-1000	< 78	< 56	> 30%	> 40%

Table 12: QC results for the PamGene STK and PTK assays in the ACC and PI

Assay type	Region	Flag	Signal strength (AU)	QC passed phosphosites	Technical CV	Biological CV
STK	ACC	Green/Good	27.185	100/142	n.a.	5-7%
STK	PI	Green/Good	27.630	103/142	n.a.	11-33%
PTK	ACC	Orange/Fair	29.604	176/195	n.a.	7-50%
PTK	PI	Orange/Fair	26.719	180/195	n.a.	8-40%

Normalizations: The ComBat correction is a batch effect correction technique utilized to remove any technical variation (time, place, and materials) from the data, thus preventing this variation from interfering with the results' accuracy and downstream analysis. It was performed to correct for systematic biases such as the differences that may have arisen between the different runs and the use of different PamChips. The ComBat correction was coupled with an empirical Bayes approach that effectively decreased the noise associated with the correction (C. Chen et al., 2011; Johnson et al., 2007).

2.11.5 Data analysis: Phosphosite analysis

This step aimed to compare individual phosphosites between two conditions (test and baseline) and determine the significance, direction, and size of the effect.

The phosphosites with statistically significant differences between the two conditions (6-OHDA and controls) were identified. To do so, the differential phosphorylation of each phosphosite was compared between the conditions using a two-sided unpaired Student's t-test, with a threshold cut-off at $p < 0.05$.

2.11.6 Data analysis: Kinase analysis

The upstream kinase analysis (UKA) algorithm is a functional class scoring approach that predicts kinase activity between test and control conditions by analyzing changes in its set of peptides. As previously mentioned, each kinase phosphorylates several peptides. The UKA algorithm thus attributes scores to each kinase, indicative of how strongly it correlates with changes in peptide signal. This technique enables (i) the mapping of phosphosite-kinase relationships and (ii) the ranking of the top differential kinases between the conditions.

Four scores are given by the UKA algorithm: kinase statistic, significance score, specificity score, and kinase score (median final score).

The kinase statistic represents the direction of the effect (changes in kinase activity) in the test condition compared to the control one. It represents the median value of peptide statistics (signal-to-noise ratio of peptides between test and control conditions) of the set of peptides phosphorylated by the kinase. When the kinase statistic is below zero, the activity of the kinase is inhibited in the test condition. On the contrary, when the kinase statistic is above zero, the kinase is activated in the test condition.

The significance score is calculated following a permutation test in which samples are randomly permuted. For each permutation, the kinase statistic is calculated and the significance score is assessed based on the differences between the kinase statistic value of the actual sample and that of the permuted ones. The higher the significance score, the more the kinase is differentially active in the test condition compared to the control one.

The specificity score is also assessed through a permutation test during which phosphosites are randomly

permuted. For each permutation, the kinase statistic is calculated and the specificity score is determined based on the differences between the kinase statistic value of the actual sample and that of the permuted ones. The higher the specificity score, the lower the chance that the observed effect is caused by a random set of phosphosites.

The final score combines the significance and specificity scores. Kinase ordering is based on this value.

2.11.7 EnrichR and IPA analysis

The analysis was performed using EnrichR and IPA to match the kinase IDs with functional ontologies. These two software programs were utilized for a more accurate and precise analysis of impaired pathways in ADHD-like conditions.

The uniprot accession numbers of kinases for each comparison, with a median final score above 1.3, were converted into gene names and inserted in EnrichR. EnrichR is an interface containing a large database of gene set libraries that enables pathway enrichment analysis (E. Y. Chen et al., 2013; Kuleshov et al., 2016; Z. Xie et al., 2021).

Peptides with an up- or down-regulated expression in 6-OHDA mice compared to sham, and with a p-value below 0.05, for significance, were identified and displayed in an Excel file. The data was analyzed using IPA. Three parameters were essential for the analysis: the gene/protein names, the p-values, and the log-fold changes. All networks, altered signaling pathways, and upstream regulators were given by the software. Our study analysis was based on the top 10 altered signaling pathways and the most significant network (highest score and number of interacting molecules (QIAGEN Inc., <https://digitalinsights.qiagen.com/IPA>) (Krämer et al., 2014).

Data obtained from the two software was compared, and the statistically significant pathways were identified. Those common to both software were chosen.

2.12 Förster resonance energy transfer (FRET)

Erythropoietin-producing human hepatocellular (Eph) proteins are a large family of tyrosine-kinase receptors, first identified in 1987 and composed of fourteen members in mammals (Hirai et al., 1987). These receptors are activated following the binding of their membrane-integrated ephrin ligands, thus initiating bidirectional signaling pathways that affect both cells (Eph- and ephrin-expressing cells) (Yang et al., 2018).

Ephrin ligands and receptors are involved in axon guidance (repellent cue) and play key regulatory roles in synapse function, formation, and plasticity (Hruska & Dalva, 2012; Yang et al., 2018). For example, EphA4/EphA3 signaling between neurons (EphA4) and astrocytes (EphA3) was found to modify AMPA receptors and spine morphology, and to modulate astrocytic glutamate transporters and synapse plasticity (LTP) in the hippocampus (Carmona et al., 2009; Filosa et al., 2009; Murai et al., 2003; Yang et al., 2018).

Alterations in the functional states of proteins may underlie modifications in cell-cell communication and, consequently, in higher functions. Therefore, to decipher changes in protein interactions under ADHD-like conditions, we relied on the Förster resonance energy transfer method (FRET), which quantifies molecular interactions and conformational changes. The main advantage of using FRET is that the quantification can be performed at a resolution (10nm) exceeding that of proximity ligation (40-100nm) and conventional colocalization (70-100nm) assays (Larijani & Miles, 2022).

FRET is a photophysical process during which energy is non-radiatively transferred from an excited donor

molecule to a nearby acceptor molecule. This event, requiring dipole-dipole interactions, occurs over a distance of 1-10nm and permits the quantification of molecular interactions. Two types of FRET quantifications were previously described: the steady state and the time-resolved. In the former, FRET is determined based on the fluorescence intensity changes of the donor or acceptor. In this case, both chromophores should be equally present (stoichiometry equal to one). In the latter, FRET is measured by assessing the donor's lifetime relative variation in the presence of the acceptor molecule. Lifetime, defined as the average duration during which the fluorophore remains in its excitation state, is an intrinsic property of the chromophore that changes once it undergoes energy transfer. Therefore, one advantage of the time-resolved FRET is that it does not depend on the chromophore concentration (Larijani & Miles, 2022).

Fluorescence lifetime imaging microscopy (FLIM) is a sensitive microscopy approach that exploits the lifetime property of a fluorophore. When a donor transfers its energy to an acceptor, FLIM detects the lifetime change and visualizes modifications undergone near the FRET pairs (Datta et al., 2020).

Two-site amplified time-resolved FRET detected by multiple frequency-domain fluorescence lifetime imaging microscopy (mfFLIM) is a highly specific labeling methodology that uses FRET detected by FLIM. This precise approach provides functional proteomics quantification. Coupled with a new computational tool, functional oncology mapping (FucOmap), it allows to directly map and quantify the functional states of the proteins at a greater spatial resolution (Safrygina et al., 2024). In collaboration with Pr. Banafshé LARIJANI (Centre for Therapeutic Innovation CTI-University of Bath, United Kingdom), we have used this methodology to determine the interactions between neurons and astrocytes under ADHD-like conditions.

Two-month-old mice (one 6-OHDA and one sham) were anesthetized with an intraperitoneal injection of ketamine (100mg/kg) and xylazine (20mg/kg) mixture. In the absence of reflexes, they were transcatheterially perfused with a 0.9% NaCl solution containing 0.01% heparin to clean the brain from the blood, followed by a fixative consisting of 4% PFA in 0.1M phosphate buffer, pH 7.4. The brains were extracted and post-fixed overnight, at 4°C, in 4% PFA. They were washed three times, ten minutes each, with PBS 0.1M and were cryoprotected in 12.5% sucrose in 0.01M PBS, overnight, at 4°C. The brains were then cut at the cryostat to obtain 20µm sections of the ACC. The sections were placed on microscope slides and allowed to dry for a few hours at room temperature. The samples were then sent to Pr. Banafshe LARIJANI.

Primary antibodies directed against EphA3 (rabbit, Bio-Techne, Ireland) and EphA4 (mouse, Fisher Scientific, France) were utilized to quantify the interactions between the two proteins and detect neuron-astrocyte communication.

In brief, the two primary monoclonal antibodies were tagged with Fab fragments conjugated with ATTO488 (donor chromophore, Sigma-Aldrich, United Kingdom) or horseradish peroxidase (HRP, Jackson ImmunoResearch, United Kingdom). Tyramide signal amplification was used to label HRP with Alexa 594, the acceptor molecule (Alexa fluor 594 tyramide Superboost kit, goat anti-rabbit IgG, Fisher Scientific, France). This chromophore-Fab fragments conjugation with the primary antibodies preserved the critical FRET distance (1-10nm). A custom-made semi-automated frequency domain FLIM recorded the donor lifetime, excited by a controlled (40Hz) diode 473nm laser, in the absence and presence of its acceptor. A decrease in the donor lifetime enabled the quantification of protein interactions. After identifying the regions of interest where both molecules were detected, the lifetimes and their standard deviations were automatically calculated and exported to an Excel sheet. FRET efficiency was directly calculated following an equation that involved the donor lifetime in the presence and absence of the acceptor (Safrygina et al., 2024; Sánchez-Magraner et al., 2020).

2.13 Behavioral procedures

The behavioral tests were started at P30 and were conducted during the day, under dim light. The apparatuses were cleaned with a 30% ethanol solution between each trial and animal.

Mice were habituated to the experimenter for four days and to the testing room for thirty minutes before performing the test.

The behavioral tests were conducted by Sandra Sánchez.

2.13.1 Open-field test

The open-field test is a common behavioral procedure that assesses locomotor activity in rodents. Mice were allowed to freely explore and move in an arena (measurements: 40cm x 40cm x 40cm; Ugo Basile, Italy) for ten minutes. Their movements were recorded using a video tracking system (EthoVision XT15, Noldus, The Netherlands). Two parameters were quantified: the distance traveled (cm) and the velocity (cm/s).

Eleven sham and eighteen 6-OHDA wild-type females, eleven sham and nine 6-OHDA wild-type males, ten sham and seven 6-OHDA P2X4 KO females, and eight sham and twelve 6-OHDA P2X4 KO males were used.

2.13.2 Von Frey test

The Von Frey test evaluates nociceptive responses to mechanical stimuli. Its setup (Ugo Basile, Italy) consists of individual cages with a mesh floor to which the mice were habituated before the start of the experiment.

The procedure involved three to five stimulations of the plantar surface of both hind paws, using calibrated von Frey filaments. An interval of thirty seconds between each measurement was set. The filaments were of different grams and enabled the establishment of the withdrawal threshold. The mechanical pain threshold corresponded to the gram of the filament to which a withdrawal of the paw was observed.

Eleven sham and seventeen 6-OHDA wild-type females, ten sham and nine 6-OHDA wild-type males, ten sham and seven 6-OHDA P2X4 KO females, and eight sham and twelve 6-OHDA P2X4 KO males completed the test.

2.13.3 Plantar test

The Plantar test assesses nociceptive responses to thermal stimuli. Its setup (Ugo Basile, Italy) is composed of individual cages with a glass pane floor to which the mice were habituated before the start of the experiment. The procedure consisted of three stimulations of both hind paws by a focused light source: an infrared generator with an intensity of 50 and a cut-off time of fifteen seconds. Consecutive measurements were separated by a two-minute interval. The thermal pain threshold corresponded to the paw withdrawal latency.

Eleven sham and seventeen 6-OHDA wild-type females, ten sham and nine 6-OHDA wild-type males, ten sham and seven 6-OHDA P2X4 KO females, and eight sham and twelve 6-OHDA P2X4 KO males performed the test.

2.14 Statistical analyses

All statistical analyses were done using GraphPad Prism V9 software (GraphPad, United States). Gaussian distribution was evaluated using the Shapiro-Wilk test. Non-parametric one- or two-tailed unpaired Mann-Whitney U tests were performed if data did not reach normality. Otherwise, one- or two-tailed

unpaired t-tests were applied.

Iba1 and GFAP staining results were assessed using non-parametric one-tailed (solidity, Iba1 staining in the ACC of males) or two-tailed unpaired Mann-Whitney U tests. GFAP/vimentin double staining results in the ACC of females were analyzed with a non-parametric two-tailed unpaired Mann-Whitney U test or a two-tailed unpaired t-test. As for the results in the ACC of males, a two-tailed unpaired Mann-Whitney U test was conducted. Three tests were utilized for the analysis of RT-qPCR results in the ACC and PI of females (unpaired one- or two-tailed t-test, non-parametric two-tailed unpaired Mann-Whitney U test) and males (unpaired one- or two-tailed t-test, non-parametric two-tailed unpaired Mann-Whitney U test). For mass spectrometry and PamGene assays' results, a two-tailed unpaired t-test was applied.

In the second part of the study (comparison between wild-type and P2X₄ KO mice), a non-parametric two-tailed unpaired Mann-Whitney U test was used to assess the results of the Iba1 staining. A one-tailed unpaired t-test (ACC) and a two-tailed unpaired t-test (PI) were carried out for RT-qPCR results. For the behavioral data, the analysis was based on a two-tailed unpaired t-test (open-field test, plantar test, amplitude of changes in thermal threshold) or a non-parametric two-tailed unpaired Mann-Whitney U test (Von Frey test, amplitude of changes in mechanical threshold).

Data were expressed as the mean \pm standard error of the mean (SEM), and the probability was set at $p < 0.05$.

2.15 Other experiments

2.15.1 Magnetic-activated cell sorting (MACS)

MACS is a technique developed by Miltenyi Biotec that enables the separation of distinct cellular populations based on their surface antigens. It relies on antibodies linked to magnetic particles that bind to the surface markers of the targeted cells and allow their isolation from the rest of the sample.

This experiment was of great interest to us as one of the objectives of my Ph.D. thesis was to perform RNA-sequencing on isolated astrocytes, microglia, and neurons to study their transcriptome in a specific manner.

The targeted surface antigens were ACSA2 for astrocytes and CD11b for microglia. The isolation of neurons relied on an indirect magnetic labeling system: non-neuronal cells were depleted from the column through their capture by biotin-conjugated monoclonal primary antibodies and a secondary antibody anti-biotin coupled with microbeads.

The adult brain dissociation kit, the anti-ACSA-2 (astrocytes) microbead kit, the CD11b (microglia) microbeads, and the neuron isolation kit were directly purchased from Miltenyi Biotec (France). The reagents used during the experiment were provided with the kits.

2.15.1.1. *Tissue dissociation*

Two 6-OHDA female mice were sacrificed, their brains were extracted, and the ACC and PI were dissected. Two enzymes, along with their buffers, were added to the different tubes: 50 μ L of the enzyme P and 1900 μ L of its buffer Z, as well as 10 μ L of the enzyme A and 20 μ L of its buffer Y. The tubes were placed in the gentleMACS Octo Dissociator with Heaters (Miltenyi Biotec, France), and a specific program was run to dissociate the tissue. They were briefly centrifuged, and the pellet was collected and resuspended in 10mL of cold D-PBS. The samples were centrifuged again (ten minutes, 4°C, 300g), and the supernatant was discarded. 450 μ L of cold debris removal solution, 1550 μ L of D-PBS, and 2mL of overlay solution (D-PBS) were gently loaded. Another ten-minute centrifugation (4°C, 3000g) was performed. Three phases

were formed and the top two were completely discarded. D-PBS was added and the tubes were inverted three times. They were centrifuged (ten minutes, 4°C, 1000g, full acceleration and brake), and the supernatant was removed.

2.15.1.2. *Isolation of astrocytes*

The tubes were centrifuged for ten minutes at 4°C and 300g to discard the supernatant completely. 80µL of buffer, to resuspend the pellet, and 10µL of the FcR blocking reagent were added. The tubes were mixed and incubated for ten minutes in the refrigerator (between 2 and 8°C), then for fifteen minutes with 10µL of anti-ACSA-2 MicroBeads. 1 to 2mL of washing buffer was added to the samples, followed by centrifugation for ten minutes at 300g. The supernatant was removed, and 500µL of buffer was loaded to allow magnetic separation. The samples were applied to the MACS column (Miltenyi Biotec, France), previously rinsed with 500µL of buffer. A three-time washing step was performed with 500µL of buffer to remove all the unlabeled cells, which were collected for the following steps. 1mL of the elution buffer was added and the elute was collected in a clean tube.

2.15.1.3. *Isolation of microglia*

The tubes containing the non-labeled cells (from the previous step) were centrifuged for ten minutes at 300g to remove the supernatant. 90µL of buffer was added to resuspend the pellet. Samples were incubated with 10µL of CD11b MicroBeads, for fifteen minutes, between 2 and 8°C. They were washed with 1 to 2mL of washing buffer, and centrifuged again for ten minutes at 300g. The supernatant was discarded, and 500µL of buffer was added to allow magnetic separation. The MACS column was rinsed with 500µL of buffer, and the samples were applied. A three-time washing step was performed with 500µL of buffer to remove all the unlabeled cells, which were collected for the following steps. 1mL of the elution buffer was loaded and the elute was collected in a clean tube.

2.15.1.4. *Isolation of neuronal cells*

The tubes containing the non-labeled cells (from the previous step) were centrifuged for ten minutes at 300g to remove the supernatant. 80µL of buffer was added to resuspend the pellet. Samples were incubated with 20µL of the non-neuronal cell biotin-antibody cocktail, for five minutes in the refrigerator. They were washed with 1mL of washing buffer and centrifuged again for ten minutes at 300g. The supernatant was discarded. 80µL of buffer and 20µL of anti-biotin beads were added to allow magnetic separation. Tubes were mixed and incubated for ten minutes in the refrigerator. The MACS column was rinsed with 3mL of buffer, and the samples were applied. A two-time washing step was carried out with 1mL of buffer to remove all the unlabeled cells - the neuronal cells of interest.

The results were observed using the counting chamber Malassez. Unfortunately, very few cells were found. Using a single sample to consecutively isolate different cell populations could explain the loss of a consequent number of cells.

2.15.2 Fluorescence-activated cell sorting (FACS)

Since MACS experiments did not yield consistent results in separating cell populations, trials with the FACS method were done. FACS is another technique that isolates a specific cell population from a biological sample. It depends on antibodies linked to fluorochromes, that recognize specific cellular antigens.

These experiments were completed in collaboration with Dr. Jean-Christophe DELPECH.

2.15.2.1. Trial 1

Two naïve female mice, F2314 and F2315, were sacrificed (5mL of PBS 0.1M were injected directly into the heart of the mice to clean the blood vessels), their brains were extracted, and the ACC and PI were dissected.

For this first trial, the targeted surface antigens were CD11b and CD45 for microglia and ACSA2 for astrocytes. As for neurons, the antibody was directed against the intracellular antigen NeuN, thus requiring cell permeabilization. Therefore, samples from the F2314 mice were used to isolate astrocytes and microglia, while samples from the F2315 mice were permeabilized and were utilized to isolate neuronal cells.

The brain structures were introduced into different tubes into which 2mL of Hibernate A (Fisher Scientific, France) were added, enabling neuronal cell preservation. Tissues were homogenized with 2mL glass potters. An additional volume of Hibernate A of 2mL was loaded, and the samples were filtered in FACS tubes (Dutscher, France) to eliminate the remaining debris. Three Percoll gradients (high density, medium density, and low density) were prepared:

High density: 6.852mL of Hibernate A, 1649 μ L of Percoll (Sigma-Aldrich, Cityva, France), 195.6 μ L of NaCl 1M (5.844g of NaCl in 100mL of distilled water, Sigma-Aldrich, France)

Medium density: 7.2mL of Hibernate A, 1301.2 μ L of Percoll, 153 μ L of NaCl 1M

Low density: 7.54mL of Hibernate A, 960.6 μ L of Percoll, 119 μ L of NaCl 1M

1mL of each gradient was added to each sample, starting with the gradient with the highest density.

The tubes were centrifuged (430rcf, 4°C, four minutes, acceleration 5, brake 1), and 2mL from the top of each sample were discarded. The tubes were centrifuged again (550rcf, 4°C, four minutes, brake 9), the supernatants were removed, 5mL of hibernate A was added, and the samples were mixed by inversion. Another centrifugation was performed (five minutes, 4°C, 550rcf), and the supernatant was discarded.

For permeabilization, the ACC and PI of the F2315 mice were resuspended in 500 μ L of Hibernate A. 500 μ L of ethanol (Sigma-Aldrich, France) was slowly added in the middle of the tube. The samples were let on ice for fifteen minutes, centrifuged (five minutes, 550rcf, 4°C), resuspended in 5mL of Hibernate A, centrifuged again (five minutes, 550rcf, 4°C), and finally, resuspended in 500 μ L of PBS BSA 0.2% (0.2g of BSA in 100mL of PBS 0.1M). They were incubated with the clone A60 biotin-conjugated anti-NeuN antibody (dilution 1/1000, Sigma-Aldrich, France) for fifteen minutes on ice. 1mL of PBS BSA 0.2% were loaded, the samples were centrifuged (five minutes, 4°C, 550rcf), the supernatant was discarded, and 300 μ L of PBS BSA 0.2% were introduced along with the secondary antibody streptavidin (dilution 1/1000, Fisher Scientific, France). The incubation lasted for fifteen minutes on ice. The samples were then washed with 5mL of PBS BSA 0.2% and centrifuged again (five minutes, 4°C, 550rcf). The supernatant was removed, and the samples were resuspended in 500 μ L of PBS-BSA 0.2% followed by filtration on FACS tubes.

The ACC and PI extracted from the F2314 mice were not permeabilized and directly incubated with the fluorescent antibodies FITC rat anti-mouse CD11b (dilution 1/300, BD Pharmingen, France), anti-mouse CD45 monoclonal antibody (30-F11) PerCP-Cyanine 5.5 (dilution 1/300, eBiosciences, Fisher Scientific, France), and anti-mouse APC ACSA2 antibody (dilution 1/100, Miltenyi Biotec, France). The incubation with the antibodies lasted for fifteen minutes on ice. The samples were then washed with 5mL of PBS BSA 0.2%, centrifuged (five minutes, 4°C, 550rcf), and filtered on FACS tubes.

Fluorescence minus one (FMO) tubes were prepared following the same protocol. They contained 10 μ L of each sample and all the fluorochromes except the one being measured. These tubes are important controls when interpreting the data as they help identify the fluorescent cells.

The samples were sorted using the cell sorter BD FACSMelody (BD Biosciences, Flow Cytometry Facility, University of Bordeaux).

RNA extraction using the RNeasy plus Mini kit (Qiagen, Germany) and RT-qPCR were conducted to assess the identity of the collected samples. The target genes for microglia were Tmem119, P2RY12, Fcrls, Olfm13, and CX3CR1. Those of astrocytes were GFAP, S100 β , and Aldh1. Those of neurons were Tubb3, Map2, and NeuN. Those of oligodendrocytes were SOX10, Mog, and Mag. The reference genes used were GADPH and 18S. Neuronal cells were isolated; however, microglia were not efficiently sorted as they were also recognized by the anti-ACSA2 antibody.

2.15.2.2. *Trial 2*

In this second trial, a new combination of antibodies was tested: anti-NeuN Alexa fluor 488 conjugated antibody (dilution 1/1000, Millipore, France), PE mouse anti-GFAP antibody (dilution 1/50, BD Pharmingen, France), anti-mouse CD11b PE-Cy7 antibody (dilution 1/300, eBiosciences, France) and Alexa fluor 700 anti-CD45 monoclonal antibody (dilution 1/300, Fisher Scientific, France).

The new anti-NeuN antibody, conjugated to a fluorochrome, helped reduce the number of washes thus limiting cell loss.

One female mouse was sacrificed and the same protocol for sample preparation was followed, as in the first trial.

FMO, “only”, and “unstained” tubes were also prepared following the same protocol. The “unstained” tube contained 10 μ L of the samples (no antibodies were added). Each “only” tube consisted of 10 μ L of the samples and one of the fluorochromes to be analyzed.

Samples were sorted using the cell sorter BD FACSMelody. RNA extraction and RT-qPCR were performed to assess the identity of the collected samples. Neurons were isolated; however, a poor separation between microglia and astrocytes was still observed.

2.15.2.3. *Trial 3*

In this third trial, the same combination of antibodies as in the second trial was tested. However, the dissected sections were smaller, hoping for a better separation of the different cell populations.

One female mouse was sacrificed and the same protocol for sample preparation was used, as in the first trial. The FMO, only, and unstained tubes were also prepared.

Samples were sorted using the cell sorter BD FACSMelody. A better separation of the cellular populations was observed on the cell sorter. RNA extraction and RT-qPCR were effectuated to assess the identity of the collected samples. The results confirmed the isolation of microglial cells and, to a lesser extent, astrocytes. However, and to our surprise, no results were obtained for neurons.

2.15.2.4. *Trial 4*

In this fourth (and final) trial, the dilution of the anti-NeuN was changed from 1/1000 to 1/500. A new anti-CD11b antibody was also used: rat anti-mouse CD11b U450 antibody (dilution 1/300, BD Biosciences, France).

One naïve female mouse F2954 was sacrificed and the same protocol for sample preparation was used,

as in the first trial. The FMO, only, and unstained tubes were prepared following the same protocol.

The samples were sorted using the cell sorter BD FACSMelody. RNA extraction and RT-qPCR were performed to assess the identity of the collected samples. The results of the F2954 mouse indicated an efficient separation of microglia. Few neurons were isolated without being contaminated with oligodendrocytes. However, a poor separation of astrocytes was noticed. More importantly, RNA concentrations were low and unsuitable for RNA sequencing experiments.

2.15.3 NF- κ B immunofluorescence

As previously described, NF- κ B is a family of transcription factors (p65, RelB, c-Rel, NF- κ B1, NF- κ B2) that participates in the transcription of genes involved in inflammation and apoptosis. Their activation and nuclear translocation depend on the degradation of the I κ B complex, which keeps them in a cytoplasmic inactive configuration (Oeckinghaus & Ghosh, 2009). The monomers are found in the form of homo- or heterodimers; the most prevalent dimer is the heterodimer composed of p65 and p50 (Hoffmann & Baltimore, 2006; Maguire et al., 2011). Therefore, to elucidate the activation state of NF- κ B in our 6-OHDA mice, an immunofluorescence staining directed against p65 was run.

The sections were washed three times, ten minutes each, in PBS 0.1M. They were transferred in a blocking solution consisting of PBS BSA 1% with 0.3% Triton X-100, for one hour, at room temperature. The sections were then incubated with the primary antibody anti-p65 NF- κ B (rabbit, dilution 1/200 or 1/500 or 1/1000, Abcam, United Kingdom) in a solution of PBS BSA 1%, overnight, at 4°C. The following day, a three-times washing step was conducted in PBS 0.1M. The sections were incubated with the secondary antibody Alexa fluor 568 goat anti-rabbit (dilution 1/500, Invitrogen, France) for two hours at room temperature. Finally, they were washed three times, ten minutes each, in PBS 0.1M and were mounted on Superfrost slides using Fluoromount G. Unfortunately, no staining was observed. Therefore, an antigen retrieval step was done to improve protein detection.

The sections were incubated in a target retrieval solution (S1699, dilution 1/10, Agilent Dako, France) for twenty minutes at 80°C, and then at room temperature for ten minutes. The same previous immunofluorescence staining protocol was performed, and the primary antibody anti-p65 NF- κ B was diluted at 1/1000. However, the results remained the same and no staining was detected.

The immunofluorescence staining protocol was repeated using another antibody directed against the p65 subunit of NF- κ B (rabbit, dilution 1/1000, Novus Biologicals, France). Unfortunately, no signal was seen.

2.15.4 Tryptase immunofluorescence

Recent evidence points out the role played by mast cells in the etiology of brain inflammation and psychiatric diseases. Indeed, mast cells seem to be involved in the exacerbation of the inflammatory response through diverse mechanisms such as the release of inflammatory markers, their interaction with glial and neuronal cells, or the disruption of the BBB. Moreover, these immune cells appear to take part in the pathogenesis of neurodevelopmental conditions (for example, autism spectrum disorder) or neurodegenerative diseases (for example, multiple sclerosis or Alzheimer's disease)(H. Dong et al., 2014; Y. Song et al., 2020). A recently published review has even suggested the potential implication of mast-cell-mediated neuroinflammation in ADHD (Y. Song et al., 2020).

An immunofluorescence staining of the protein tryptase was done to understand whether mast cells could be involved in ADHD pathophysiology through the amplification of inflammation. Tryptase is an enzyme released during mast cell activation and a powerful modulator of the inflammatory response (Hallgren & Pejler, 2006).

The sections were washed three times, ten minutes each, in PBS 0.1M. They were transferred in a blocking solution consisting of PBS BSA 1% with 0.3% Triton X-100, for one hour, at room temperature. The sections were then incubated with the primary antibody anti-tryptase AA1 (mouse, dilution 1/1000, Abcam, United Kingdom) in a solution of PBS BSA 1%, overnight, at 4°C. The following day, a three-times washing step was done in PBS 0.1M. The sections were incubated with the secondary antibody Alexa fluor 647 plus, goat anti-mouse (dilution 1/500, Invitrogen, France) for two hours at room temperature. Finally, they were washed three times, ten minutes each, in PBS 0.1M and were mounted on Superfrost slides using Fluoromount G. The absence of staining indicated that mast cells may not participate in the inflammatory response observed in 6-OHDA mice.

Chapter 3

Results

3.1 Iba1/GFAP double immunofluorescence staining

Changes in cellular shape accompany cellular reactivity (Escartin et al., 2021; Fernández-Arjona et al., 2017; D. Sun et al., 2013). Importantly, reactive astrocytes and microglial cells have been identified in two ADHD-like animal models, the SHR (P. Zhang et al., 2022) and the 6-OHDA Swiss mouse model (Meseguer-Beltrán et al., 2023). Therefore, an Iba1/GFAP double immunofluorescence staining was performed to investigate microglia and astrocyte morphology, and their possible reactivity, in our 6-OHDA mouse model.

Quantitative assessments of cell area, cell perimeter, and solidity were used to discriminate between ramified and amoeboid-like cellular forms. Fractal dimension and lacunarity were utilized as parameters of branching complexity (Leyh et al., 2021).

3.1.1 Microglial morphology in the ACC of females

Modifications in microglial morphology were observed in the ACC of 6-OHDA females. The micrographs clearly show reduced microglial ramifications and enlarged cell soma in ADHD-like conditions (Figure 22, A). The quantitative assessment is in agreement with these observations. Cell area (2090 ± 37.02 vs. 966.3 ± 22.00 ; $p < 0.0001$), cell perimeter (176.3 ± 1.44 vs. 118.1 ± 1.11 ; $p < 0.0001$), fractal dimension (1.47 ± 0.003 vs. 1.39 ± 0.004 ; $p < 0.0001$), and lacunarity (0.28 ± 0.001 vs. 0.22 ± 0.001 ; $p < 0.0001$) were decreased in 6-OHDA mice compared to shams. On the contrary, solidity (0.19 ± 0.001 vs. 0.23 ± 0.001 ; $p < 0.0001$) was increased in 6-OHDA females compared to controls (Figure 22, B). These results point to the predominance of microglial amoeboid-like morphology in the ACC of 6-OHDA females and suggest cellular reactivity in this brain region.

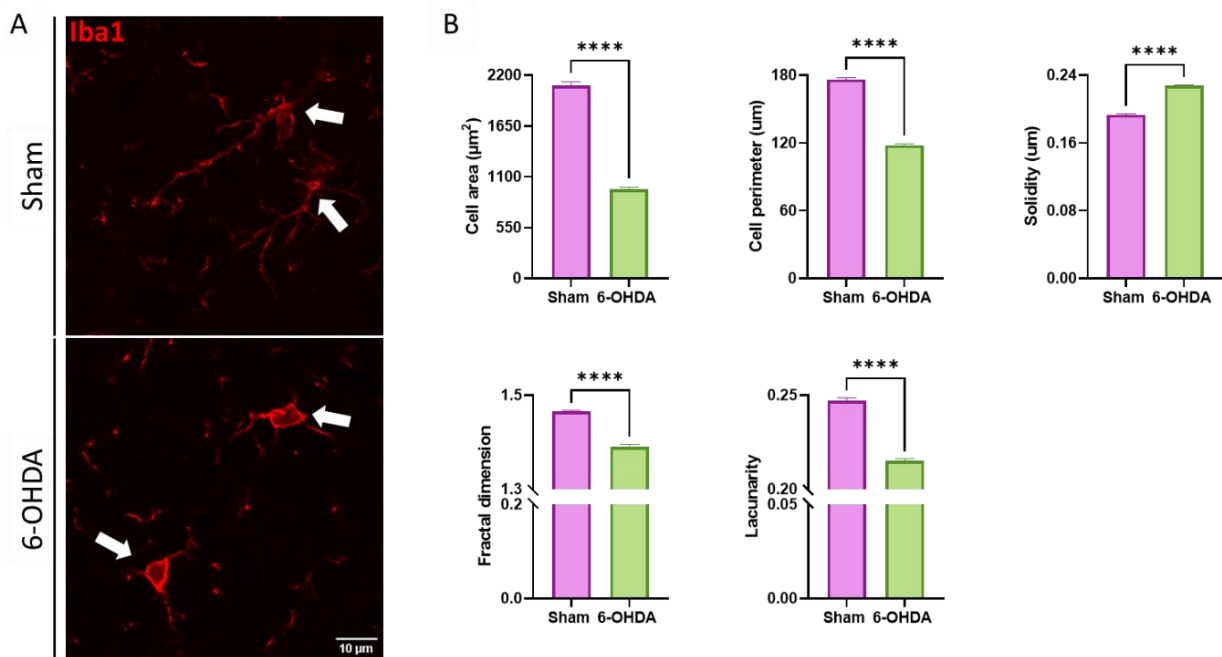


Figure 22: Microglia morphology in the ACC of females. (A) Representative images of microglia in the ACC of sham and 6-OHDA female mice. The arrows indicate microglia with a ramified form and a small cell soma in sham mice, and microglia with a less branched structure but an enlarged cell soma in 6-OHDA mice. (B) Five microglia were quantified per image: cell area (µm²), cell perimeter (µm), solidity (µm), fractal dimension, and lacunarity were assessed. Data are presented as mean ± SEM (n=4-5 mice per group) and were analyzed using a two-tailed Mann-Whitney test (**** $p < 0.0001$).

3.1.2 Astrocytic morphology in the ACC of females

Changes in astrocyte shape were reported in the ACC of 6-OHDA females. The cells appear less ramified in the ACC of 6-OHDA females (Figure 23, A). The quantification of morphological parameters supports these observations. Cell area (1410 ± 27.25 vs. 975.8 ± 21.17 ; $p < 0.0001$), cell perimeter (146.4 ± 1.43 vs. 122.5 ± 1.28 ; $p < 0.0001$), fractal dimension (1.48 ± 0.004 vs. 1.41 ± 0.005 ; $p < 0.0001$), and lacunarity (0.23 ± 0.002 vs. 0.22 ± 0.004 ; $p < 0.0001$) were lessened in 6-OHDA mice compared to shams. In contrast, solidity (0.22 ± 0.001 vs. 0.23 ± 0.001 ; $p < 0.0001$) displayed higher values in 6-OHDA females compared to controls (Figure 23, B). These findings indicate the presence of astrocytes with decreased complexity in the ACC of 6-OHDA females.

Morphological changes of astrocytes under pathological conditions have long remained unclear and controversial. However, several studies point to reduced astrocytic branching in non-homeostatic situations (Olabarria et al., 2010; D. Sun et al., 2013). Consequently, decreased astrocyte branching might correlate with cellular reactivity in the ACC of 6-OHDA females.

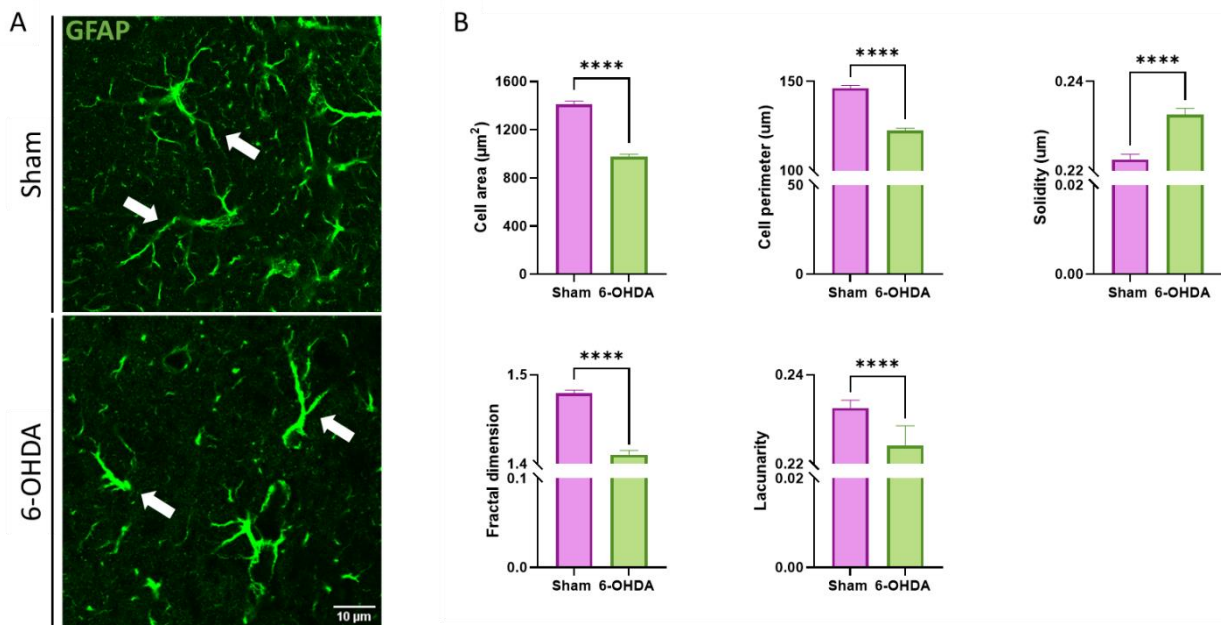


Figure 23: Astrocyte morphology in the ACC of females. (A) Representative images of astrocytes in the ACC of sham and 6-OHDA female mice. The arrows indicate astrocytes with a complex branched shape in sham mice and astrocytes with a less ramified structure in 6-OHDA mice. (B) Five astrocytes were quantified per image: cell area (μm^2), cell perimeter (μm), solidity (μm), fractal dimension, and lacunarity were assessed. Data are presented as mean \pm SEM ($n=4-5$ mice per group) and were analyzed using a two-tailed Mann-Whitney test (**** $p < 0.0001$).

3.1.3 Microglial morphology in the ACC of males

Microglial morphology varied between the ACC of 6-OHDA and sham males. Microglial cells exhibit more numerous and longer processes under ADHD-like conditions (Figure 24, A). Cell area (1472 ± 27.55 vs. 1635 ± 17.62 ; $p < 0.0001$), cell perimeter (149.3 ± 1.38 vs. 157.0 ± 0.85 ; $p < 0.0001$), fractal dimension (1.44 ± 0.003 vs. 1.47 ± 0.002 ; $p < 0.0001$), and lacunarity (0.23 ± 0.001 vs. 0.24 ± 0.001 ; $p < 0.0001$) were amplified in 6-OHDA mice compared to shams. On the contrary, solidity (0.210 ± 0.001 vs. 0.207 ± 0.001 ; $p < 0.05$) was diminished in 6-OHDA mice compared to controls (Figure 24, B). These results imply the predominance of microglia with ramified structure in the ACC of 6-OHDA males and argue against microglial reactivity in this brain area.

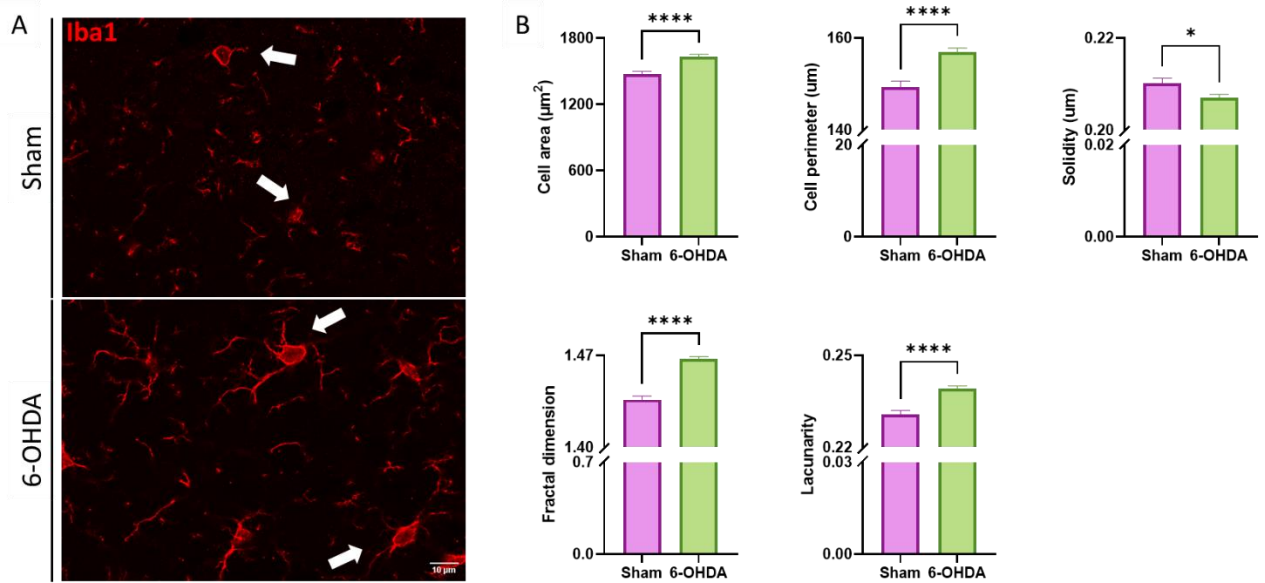


Figure 24: Microglia morphology in the ACC of males. (A) Representative images of microglia in the ACC of sham and 6-OHDA male mice. The arrows indicate microglia with a more ramified form in 6-OHDA mice and microglia with a simpler structure in sham mice. (B) Five microglia were quantified per image: cell area (μm^2), cell perimeter (μm), solidity (μm), fractal dimension, and lacunarity were assessed. Data are presented as mean \pm SEM ($n=6$ mice per group) and were analyzed using a two-tailed Mann-Whitney test (**** $p < 0.0001$) and a one-tailed Mann-Whitney test (solidity parameter; * $p < 0.05$).

3.1.4 Astrocytic morphology in the ACC of males

Variations in astrocyte form were seen in the ACC of 6-OHDA males. The cells display a more complex shape with increased ramifications, hypertrophy, and overlapping domains (Figure 25, A). Cell area (1205 ± 20.89 vs. 1305 ± 15.82 ; $p < 0.0001$), cell perimeter (135.8 ± 1.14 vs. 140.6 ± 0.85 ; $p < 0.0001$), fractal dimension (1.45 ± 0.003 vs. 1.47 ± 0.002 ; $p < 0.0001$), and lacunarity (0.22 ± 0.001 vs. 0.23 ± 0.001 ; $p < 0.0001$) had higher values in 6-OHDA mice compared to shams. No significant changes were identified for the solidity parameter (0.23 ± 0.001 vs. 0.23 ± 0.001 ; $p = \text{not significant}$) (Figure 25, B). These findings indicate the presence of ramified astrocytes in the ACC of 6-OHDA males thus pointing to cellular reactivity.

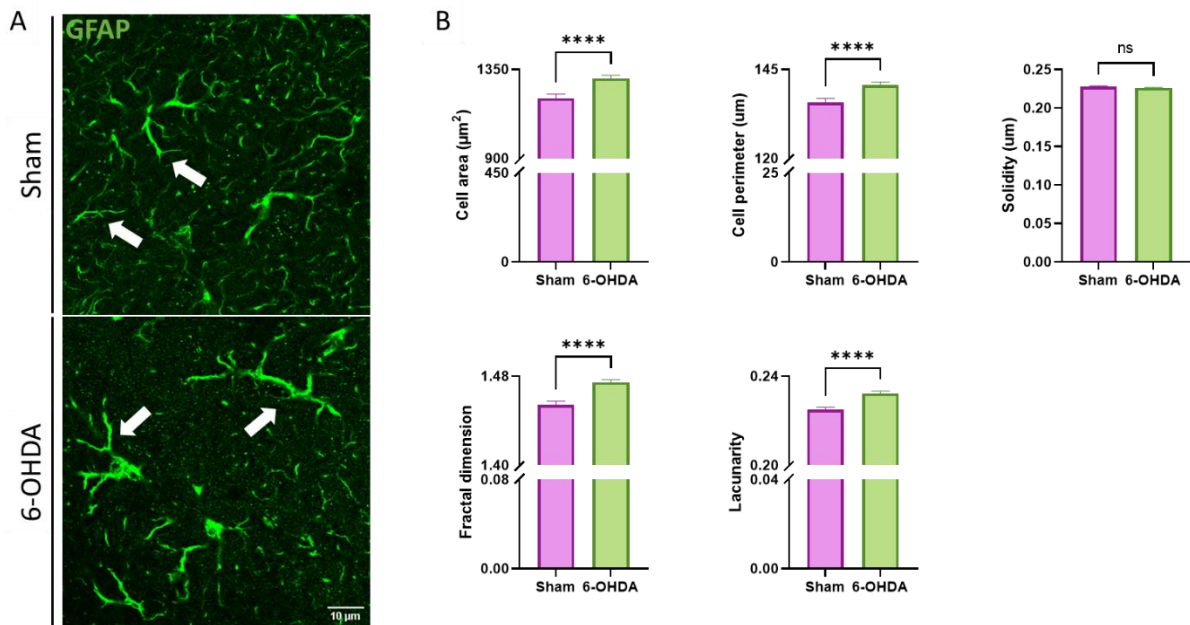


Figure 25: Astrocyte morphology in the ACC of males. (A) Representative images of astrocytes in the ACC of sham and 6-OHDA male mice. The arrows indicate weakly ramified astrocytes in sham mice and astrocytes with numerous branches and processes in 6-OHDA mice. (B) Five astrocytes were quantified per image: cell area (μm^2), cell perimeter (μm), solidity (μm), fractal dimension, and lacunarity were assessed. Data are presented as mean \pm SEM ($n=6$ mice per group) and were analyzed using a two-tailed Mann-Whitney test (**** $p < 0.0001$; ns=not significant).

Remarkably, the morphological changes observed in the ACC of 6-OHDA males are opposite to those demonstrated in the ACC of females. Microglial cells seem to be reactive in the ACC of 6-OHDA females only. Astrocytes display contradictory morphological modifications in the ACC of both genders; however, both variations are indicators of reactive astrogliosis. We therefore performed a double GFAP/Vimentin immunofluorescence staining to confirm astrocyte reactivity in the ACC of 6-OHDA males and females.

3.2 GFAP/Vimentin double immunofluorescence staining

Vimentin is another constituent of the astrocytic cytoskeleton, whose expression peaks during early development and decreases with age. However, astrocytes from adolescent or adult brains were reported to express vimentin once activated, pointing out vimentin as a marker of reactive astrogliosis (Carter et al., 2019; S. K. Pixley & de Vellis, 1984; T. Yamada et al., 1992).

3.2.1 Astrocyte reactivity in the ACC of females

Changes in GFAP intensity, vimentin intensity, and the number of colocalizations between the two proteins were found in the ACC of 6-OHDA females compared to shams (Figure 26, A).

GFAP and vimentin were colocalizing more in ADHD-like conditions (42.09 ± 4.19 vs. 63.29 ± 4.27 ; $p < 0.0001$) compared to controls. Additionally, both filaments displayed increased intensity in the ACC of 6-OHDA females as shown by the enhanced percentage of vimentin intensity (35.04 ± 0.38 vs. 38.07 ± 0.43 ; $p < 0.0001$) and GFAP intensity (64.73 ± 1.05 vs. 68.34 ± 0.83 ; $p < 0.05$) (Figure 26, B).

Overall, these results confirm the presence of reactive astrogliosis in the ACC of females as described by heightened GFAP and vimentin expressions, in addition to amplified colocalizations between the two intermediate filaments.

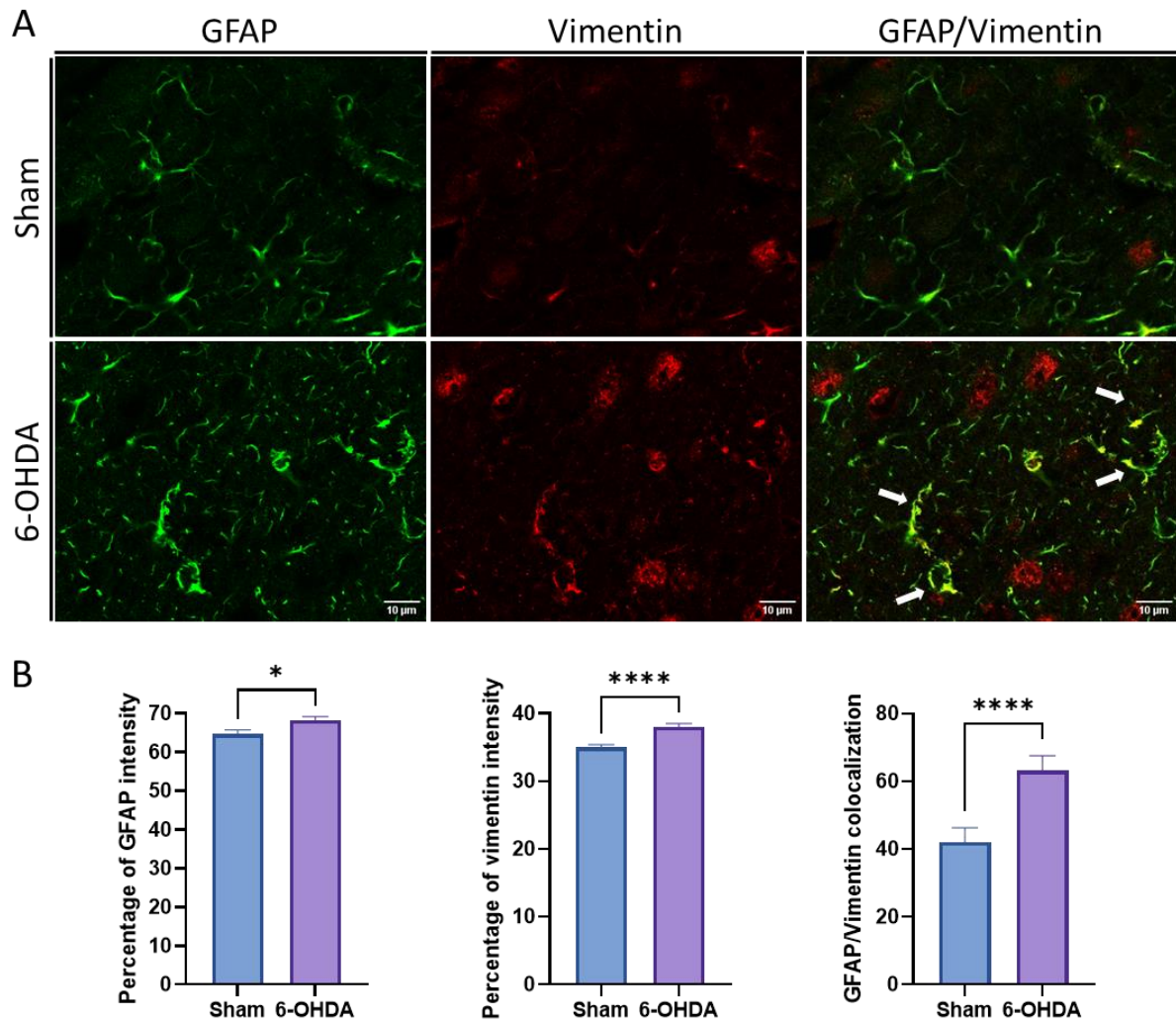


Figure 26: Astrocytic expression of GFAP and vimentin in the ACC of females. (A) Representative images of astrocytes with GFAP and vimentin stainings. Arrows point to astrocytes expressing both intermediate filaments. (B) Astrocytes co-expressing GFAP and vimentin were quantified: number of colocalization, percentage of vimentin intensity, and percentage of GFAP intensity were determined. Data are presented as mean \pm SEM ($n=4-6$ mice per group) and were analyzed using a two-tailed Mann-Whitney test (number of colocalization, percentage of GFAP intensity) and a two-tailed unpaired t -test (percentage of vimentin intensity) (**** $p < 0.0001$; * $p < 0.05$).

3.2.2 Astrocyte reactivity in the ACC of males

Contrary to the ACC of 6-OHDA females, GFAP intensity was the only parameter that varied in the ACC of 6-OHDA males compared to shams (Figure 27, A).

The percentage of GFAP intensity was higher in ADHD-like conditions (60.71 ± 1.50 vs. 70.28 ± 0.93 ; $p < 0.0001$) compared to controls. However, no significant differences were reported for the number of GFAP and vimentin colocalizations (35.05 ± 2.85 vs. 29.12 ± 2.26 ; $p = 0.1168$) and the percentage of vimentin intensity (34.96 ± 0.58 vs. 34.89 ± 0.42 ; $p = 0.6142$) (Figure 27, B).

Despite having no changes in vimentin, our findings point to mild reactive astrogliosis characterized by an increased expression of GFAP in the ACC of 6-OHDA males.

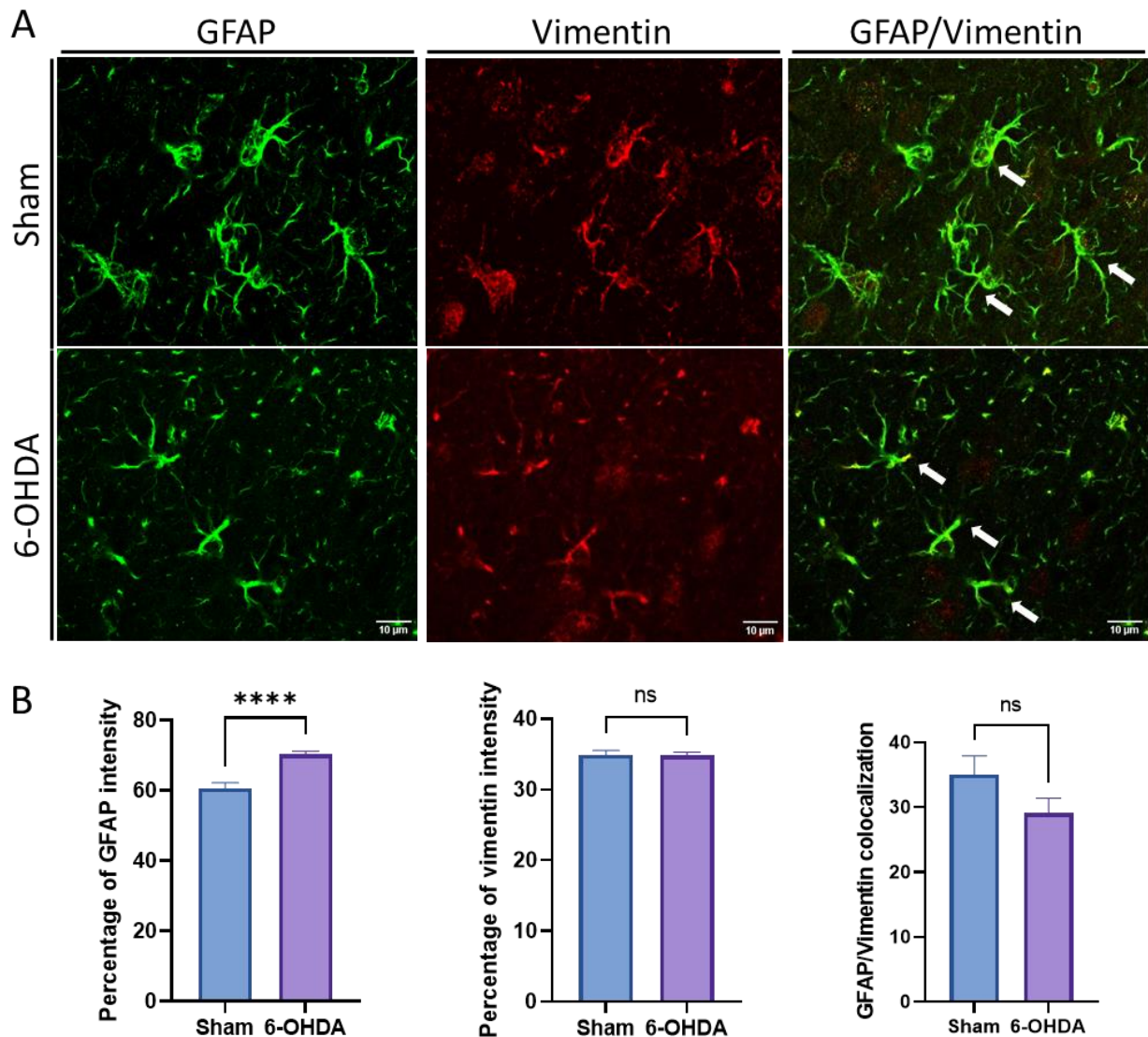


Figure 27: Astrocytic expression of GFAP and vimentin in the ACC of males. (A) Representative images of astrocytes with GFAP and vimentin stainings. Arrows point to astrocytes expressing both intermediate filaments. (B) Astrocytes co-expressing GFAP and vimentin were quantified: number of colocalization, percentage of vimentin intensity, and percentage of GFAP intensity were determined. Data are presented as mean \pm SEM ($n=6$ mice per group) and were analyzed using a two-tailed Mann-Whitney test (**** $p < 0.0001$; ns=not significant).

Our morphological findings point to microglia reactivity in the ACC of 6-OHDA females only and reactive astrogliosis in the ACC of both 6-OHDA males and females. Reactive astrogliosis appears more prominent in the ACC of 6-OHDA females and could be described as mild in the ACC of 6-OHDA males. These results support a more amplified cellular reactivity in the ACC of 6-OHDA females compared to 6-OHDA males.

3.3 RT-qPCR

Modifications in glial morphology were reported in the ACC of 6-OHDA males and females suggesting cellular reactivity and the possible establishment of an inflammatory response.

For a more in-depth study of the inflammatory status of the environment, inflammatory markers were screened using RT-qPCR (Table 8). These comprised cytokines, mediators of neuroinflammation, and markers of microglia and astrocyte reactivity (Table 6).

The experiments were performed in the ACC, the brain region where the morphological changes were

reported, and the PI, which receives inputs from the ACC. Both areas are components of the pain matrix and the salience network.

3.3.1 Inflammatory marker expression in the ACC of females

Pro-inflammatory markers were up-regulated in the ACC of 6-OHDA females compared to shams. These include the canonical cytokine IL-6 (1.08 ± 0.19 vs. 2.08 ± 0.20 ; $p < 0.01$), the transcription factor IRF5 (1.01 ± 0.05 vs. 1.21 ± 0.03 ; $p < 0.01$), the receptor TLR7 (1.03 ± 0.14 vs. 1.32 ± 0.08 ; $p < 0.05$), and the astrocytic marker GFAP (1.30 ± 0.31 vs. 3.13 ± 0.28 ; $p < 0.001$) (Figure 28, A). The expression of anti-inflammatory markers was mitigated. The cytokine TGF- β was less expressed (0.99 ± 0.17 vs. 0.53 ± 0.03 ; $p < 0.05$) while Arg1 presented increased levels (0.81 ± 0.20 vs. 1.34 ± 0.06 ; $p < 0.01$) in ADHD-like conditions (Figure 28, B). The upregulation of arginase 1, an enzyme with protective properties, might indicate its contribution to attenuating the immune response. No significant changes were detected for Iba1 (0.91 ± 0.11 vs. 0.76 ± 0.05 ; $p =$ not significant), TNF- α (1.19 ± 0.31 vs. 1.77 ± 0.21 ; $p =$ not significant) (Figure 28, A), and BDNF (0.53 ± 0.07 vs. 0.39 ± 0.04 ; $p =$ not significant) (Figure 28, B). These results indicate the presence of a predominantly pro-inflammatory environment in the ACC of 6-OHDA females, with some anti-inflammatory molecules counterbalancing the immune response.

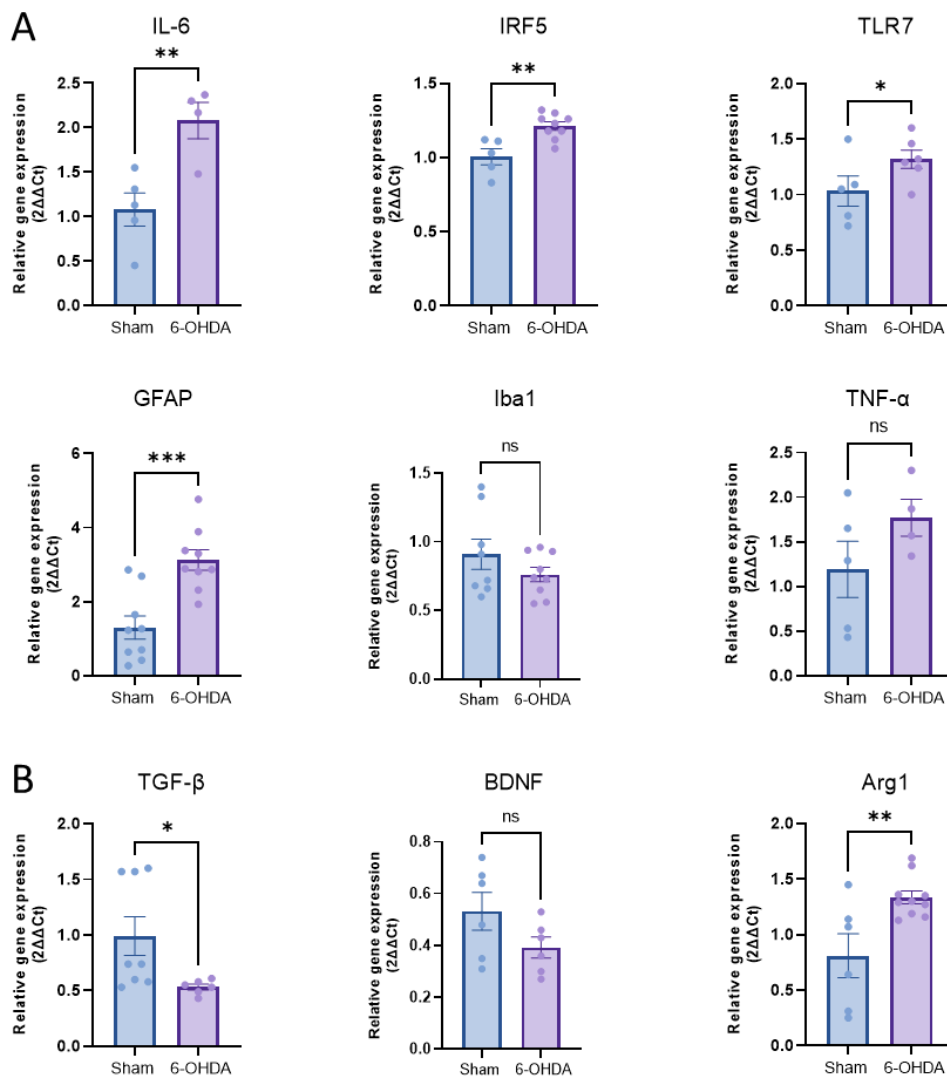


Figure 28: Detection of pro-inflammatory (A) and anti-inflammatory (B) mediators in the ACC of 6-OHDA and sham female mice. Data are expressed as relative gene expression, mean \pm SEM ($n=4-10$ per group). They were analyzed

using a two-tailed unpaired *t*-test (*IL-6*, *IRF5*, *GFAP*, *Iba1*, *TNF- α* , *BDNF*, *Arg1*), a one-tailed unpaired *t*-test (*TLR7*), and a two-tailed Mann-Whitney test (*TGF- β*) (***p* < 0.001; ***p* < 0.01; **p* < 0.05; ns=not significant).

3.3.2 Inflammatory marker expression in the ACC of males

Transcripts of pro-inflammatory markers displayed higher values in the ACC of 6-OHDA males compared to control mice. These encompassed the canonical cytokine *IL-1 β* (0.76 ± 0.08 vs. 1.33 ± 0.11 ; *p* < 0.01), the chemokine *CCL2* (1.01 ± 0.08 vs. 2.09 ± 0.55 ; *p* < 0.05), and the inflammasome *NLRP3* (0.58 ± 0.07 vs. 0.69 ± 0.02 ; *p* < 0.05) (Figure 29, A). In contrast, the anti-inflammatory mediators *IL-13* (1.91 ± 0.45 vs. 0.75 ± 0.08 ; *p* < 0.05) and *BDNF* (0.93 ± 0.11 vs. 0.67 ± 0.06 ; *p* < 0.05) were downregulated (Figure 29, B). No significant changes were detected for *Iba1* (1.09 ± 0.15 vs. 0.95 ± 0.10 ; *p* = not significant) and *GFAP* (1.53 ± 0.39 vs. 2.26 ± 0.45 ; *p* = not significant) (Figure 29, A). These findings suggest that glial cells (mainly astrocytes, as per the morphological findings) act to sustain a pro-inflammatory response in the ACC of 6-OHDA males.

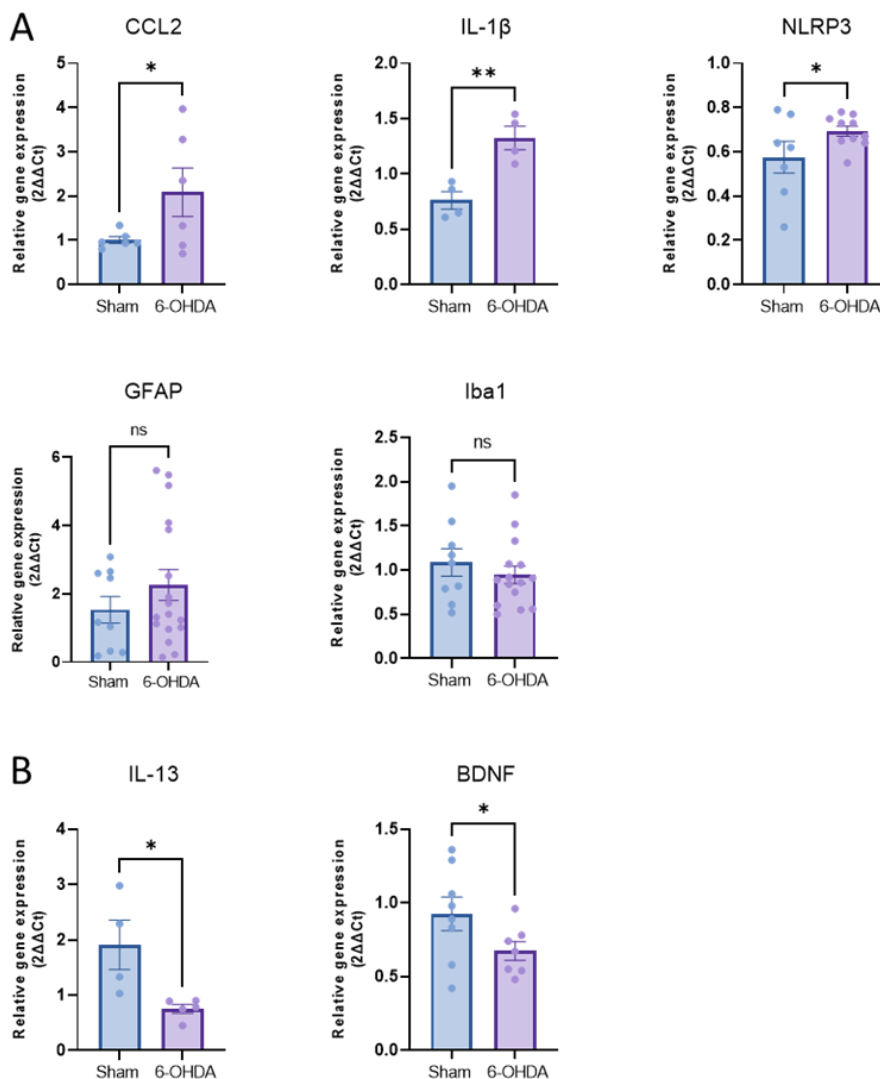


Figure 29: Detection of pro-inflammatory (A) and anti-inflammatory (B) mediators in the ACC of 6-OHDA and sham male mice. Data are expressed as relative gene expression, mean \pm SEM (*n*=4-17 per group). They were analyzed using a two-tailed unpaired *t*-test (*IL-1 β* , *Iba1*, *IL-13*), a one-tailed unpaired *t*-test (*CCL2*, *NLRP3*, *BDNF*), and a two-tailed Mann-Whitney test (*GFAP*) (***p* < 0.01; **p* < 0.05; ns=not significant).

3.3.3 Inflammatory marker expression in the PI of females

Numerous markers with pro-inflammatory roles presented increased mRNA levels in the PI of 6-OHDA females compared to shams. Notably, the canonical cytokine IL-1 β (1.01 ± 0.30 vs. 1.66 ± 0.14 ; $p < 0.05$), the chemokine CCL4 (0.65 ± 0.03 vs. 0.9 ± 0.07 ; $p < 0.05$), the receptor TLR7 (1.00 ± 0.03 vs. 1.26 ± 0.07 ; $p < 0.01$), and the enzymes Wnt5a (1.02 ± 0.09 vs. 1.32 ± 0.11 ; $p < 0.05$) and MMP9 (1.07 ± 0.14 vs. 1.72 ± 0.13 ; $p < 0.01$). tPA (1.00 ± 0.02 vs. 1.29 ± 0.10 ; $p < 0.05$) and GFAP (1.05 ± 0.15 vs. 1.41 ± 0.12 ; $p < 0.05$) were also upregulated (Figure 30, A). Additionally, APE1, an enzyme with mainly protective properties, presented elevated values in ADHD-like conditions (1.00 ± 0.04 vs. 1.21 ± 0.07 ; $p < 0.05$) (Figure 30, B). No significant changes were reported for IL-10 (1.14 ± 0.25 vs. 0.71 ± 0.12 ; $p =$ not significant) and BDNF (1.81 ± 0.21 vs. 1.62 ± 0.13 ; $p =$ not significant) (Figure 30, B). These observations support an established pro-inflammatory response within the PI of 6-OHDA females, with some anti-inflammatory mediators contributing to its regulation.

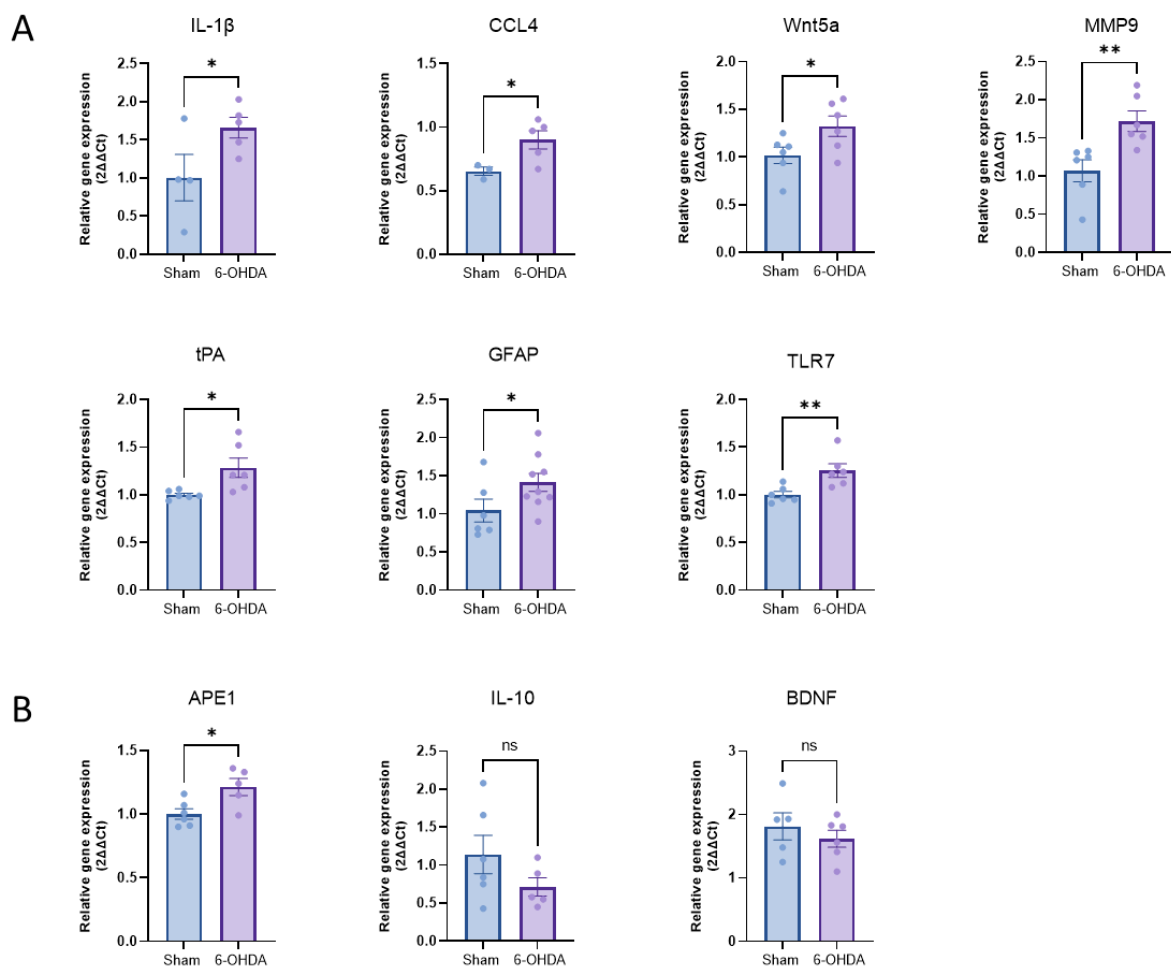


Figure 30: Detection of pro-inflammatory (A) and anti-inflammatory (B) mediators in the PI of 6-OHDA and sham female mice. Data are expressed as relative gene expression, mean \pm SEM ($n=3-9$ per group). They were analyzed using a two-tailed unpaired t-test (CCL4, MMP9, TLR7, tPA, APE1, IL-10, BDNF) and a one-tailed unpaired t-test (IL-1 β , Wnt5a, GFAP) (** $p < 0.01$; * $p < 0.05$; ns=not significant).

3.3.4 Inflammatory marker expression in the PI of males

In agreement with the previous findings, several markers with pro-inflammatory functions displayed increased expression levels in the PI of 6-OHDA males: IRF5 (1.01 ± 0.06 vs. 1.34 ± 0.07 ; $p < 0.01$), Wnt5a (1.02 ± 0.09 vs. 1.30 ± 0.05 ; $p < 0.05$), and GFAP (1.02 ± 0.09 vs. 1.88 ± 0.29 ; $p < 0.05$). No significant differences were observed for the two canonical inflammatory cytokines IL-6 (1.07 ± 0.16 vs. 1.47 ± 0.19 ; $p =$ not significant) and IL-1 β (0.96 ± 0.15 vs. 1.34 ± 0.15 ; $p =$ not significant) (Figure 31, A). The mRNA levels of the anti-inflammatory cytokine IL-10 were also amplified in ADHD-like conditions (1.02 ± 0.09 vs. 1.99 ± 0.47 ; $p < 0.05$) while those of TGF- β were reduced (1.01 ± 0.07 vs. 0.83 ± 0.07 ; $p < 0.05$). No significant difference was reported for BDNF transcript levels (1.06 ± 0.16 vs. 0.91 ± 0.13 ; $p =$ not significant) (Figure 31, B). Taken together, these results point to a predominantly pro-inflammatory environment in the PI of 6-OHDA males.

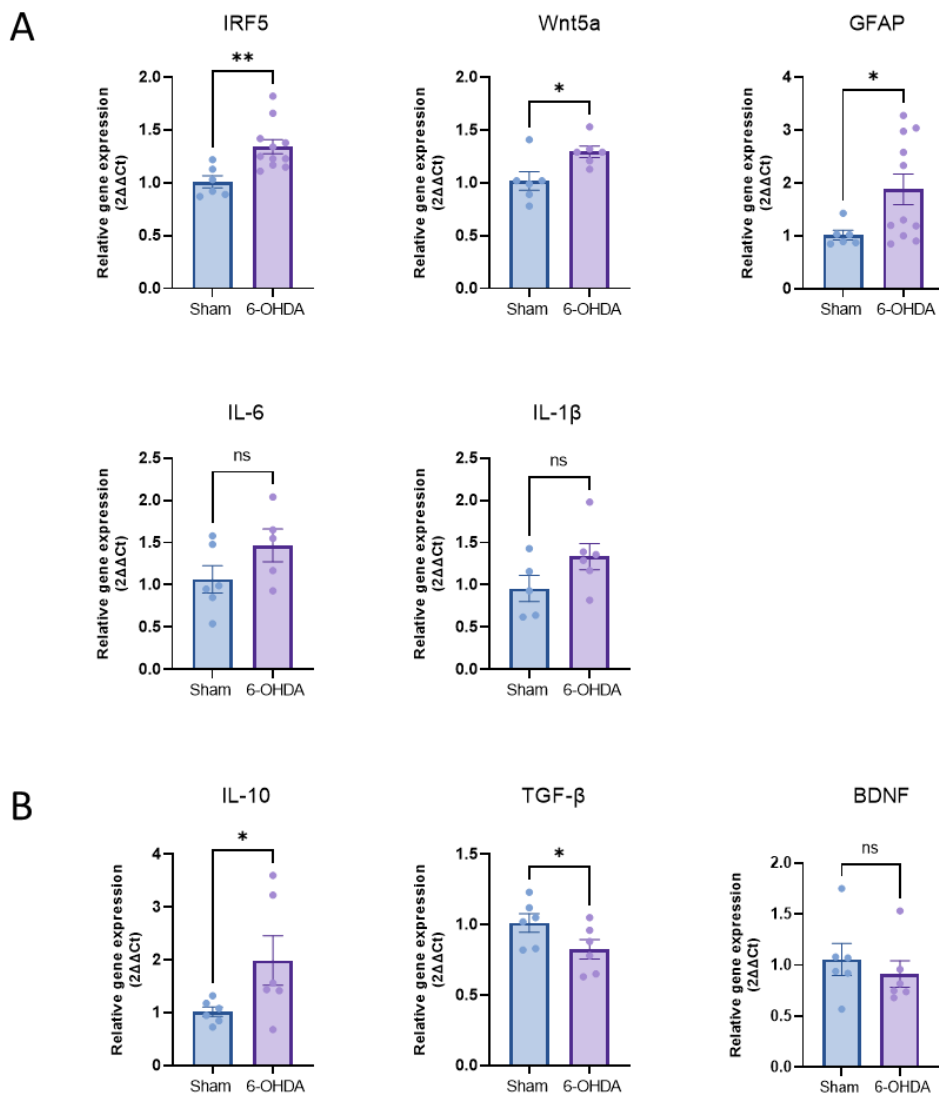


Figure 31: Detection of pro-inflammatory (A) and anti-inflammatory (B) mediators in the PI of 6-OHDA and sham male mice. Data are expressed as relative gene expression, mean \pm SEM ($n=5-11$ per group). They were analyzed using a two-tailed unpaired t -test (IRF5, Wnt5a, GFAP, IL-1 β , IL-6), a one-tailed unpaired t -test (IL-10, TGF- β), and a two-tailed Mann-Whitney test (BDNF) (** $p < 0.01$; * $p < 0.05$; ns=not significant).

Despite identifying different inflammatory mediators with altered transcriptomic expression in ADHD-like conditions, our results point to an overall pro-inflammatory environment in the ACC and PI of 6-OHDA males and females characterized by (i) an increased expression of pro-inflammatory molecules and (ii) a downregulated expression of anti-inflammatory markers.

3.4 Mass spectrometry

The study of inflammatory mediators using RT-qPCR has demonstrated changes in their transcriptomic expression levels. To explore whether these variations were sustained following translation and post-translational modifications, we screened the ACC and PI of both males and females for protein alterations using mass spectrometry. These changes were then analyzed with IPA: pathway (identification of biological pathways overrepresented in a set of genes) and network (identification of the regulatory interactions between the enriched molecules) enrichment analyses were conducted.

3.4.1 Protein screening in the ACC of females

Impairments in protein expression were identified in the ACC of 6-OHDA females compared to controls. According to the volcano plot, numerous proteins displayed either up- or down-regulated levels of expression (Figure 32, A). Overall, downregulated proteins (Figure 32, A, left) were linked to ribosome components, tau protein, and actin interactions while those with an upregulated expression (Figure 32, A, right) correlated with cell-cell adhesion, ATP synthesis, and anti-inflammatory properties.

Pathway enrichment analysis identified cascades involved in the protection of cells against stressful stimuli (KEAP1-NFE2L2, BAG2), apoptosis (FAT10, sirtuin, BAG2), inhibition of proteasomal degradation (FAT10), inflammation (sirtuin), and BBB integrity (ABC-family of transporters). Processes related to axon guidance, synaptic plasticity, and CNS development were also reported (netrin, hedgehog “off” state, sirtuin, BAG2). All these pathways presented a positive z-score (except sirtuin signaling), indicating their possible enhancement. However, the strongest signatures were related to axonal guidance changes, BBB integrity, and apoptosis (Figure 32, B).

Network enrichment analysis highlighted the role played by tau (presented as MAPT) in microtubule and actin dynamics. The represented proteins are mainly involved in actin organization, microtubule development and stability (MAPT, CRMP, IQSEC3, DPYSL2, DPYSL3, alpha-tubulin, MAPRE3), in addition to axon outgrowth (DPYSL2, DPYSL3, L1CAM) (Figure 32, C).

Taken together, these findings support alterations in apoptosis, axon guidance, and cytoskeleton organization. Both the apoptotic and axonal guidance processes seem to be amplified. Neurons could be the target of these apoptotic signals, and axonal guidance changes might constitute compensatory mechanisms for neuronal loss. Impairments in actin and microtubule dynamics could explain microglial and astrocytic morphological modifications, as demonstrated previously.

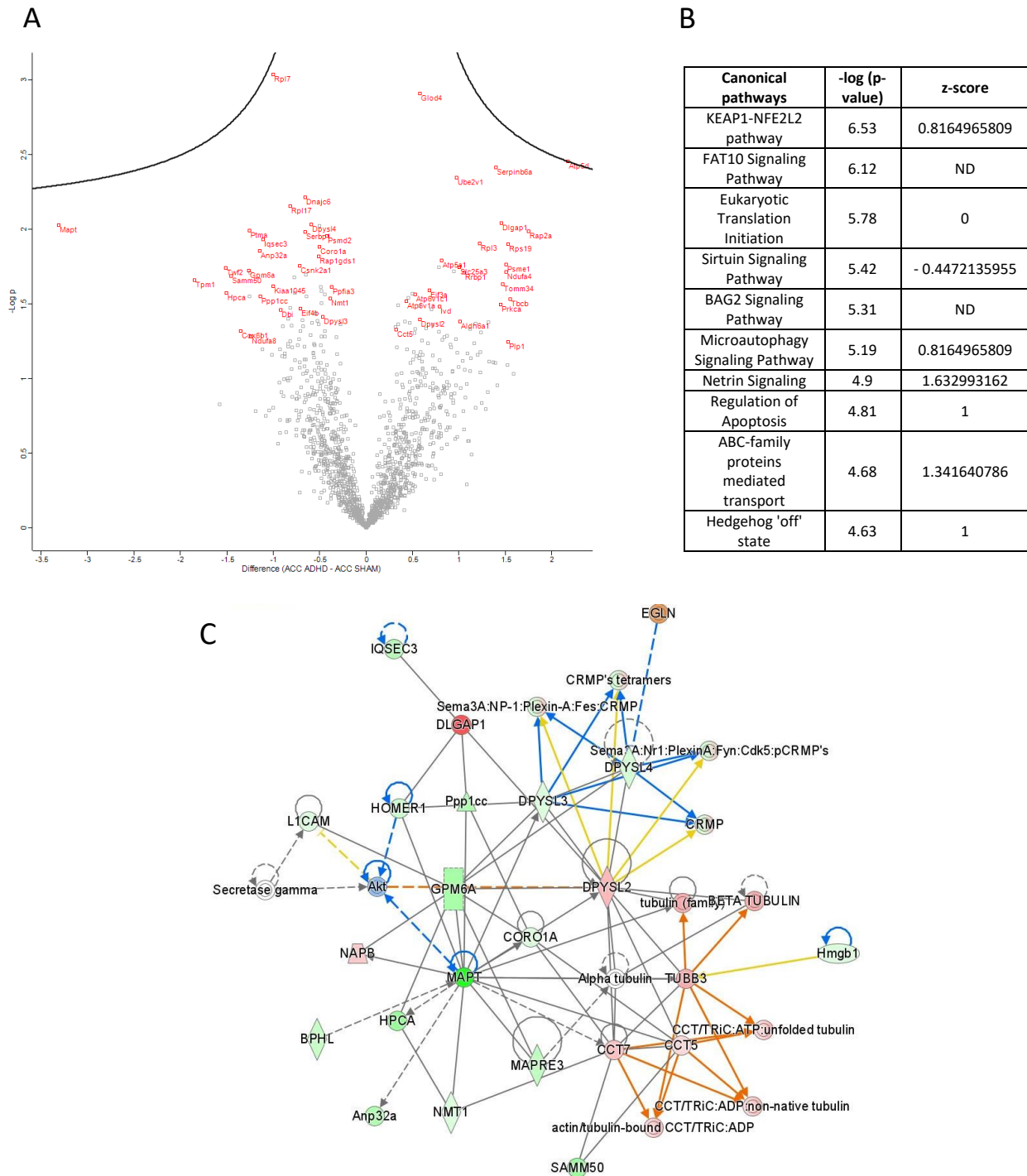


Figure 32: Mass spectrometry analysis in the ACC of female mice. (A) Volcano plot of the differentially expressed proteins (left: downregulation, right: upregulation) between 6-OHDA and sham mice. (B) Top 10 canonical pathways highlighting changes related to axonal guidance, apoptosis, and BBB integrity. (C) Network enrichment analysis centered around actin and microtubule dynamics (blue: downregulation, orange: upregulation). Analysis was performed using IPA software ($n=8$ mice per group), with $p < 0.05$. ND: not determined.

3.4.2 Protein screening in the ACC of males

Several molecules displayed altered expression in the ACC of 6-OHDA males, characterized by either up- or down-regulated protein levels (Figure 33, A). Proteins with decreased expression (Figure 33, A, left) were related to focal adhesion and mitochondrial inner membrane while those with enhanced expression

(Figure 33, A, right) engaged in actin dynamics, synaptogenesis, TNF signaling, and NF- κ B activation.

Based on pathway enrichment analysis, processes with the strongest significance relate to axonal guidance changes (netrin) and synaptic plasticity (PKA signaling). Interestingly, the PKA cascade promotes neuronal development and dopamine synthesis. Both pathways possess protective roles but seem to be downregulated in the ACC of 6-OHDA males (z-score: -1.34164). On the contrary, other upregulated and relevant processes (z-score: 1) include clathrin-mediated endocytosis and cell junction organization. They are complemented by (i) neuroligins and neuroligins, cell-adhesion molecules implicated in synaptic connections, signaling, and function, (ii) PTEN regulation, an inhibitor of the PI3K-Akt pathway, and (iii) the apoptotic execution phase (Figure 33, B).

Network enrichment analysis focused on the interface between immunity (TCR, NF- κ B, caspase, LAMTOR5, COPS6, TPP2, HNRNPM, CD3), ribosomes (NCL, RPS19, RPL7), and alternative splicing (KHDRBS1, HNRNPA1, HNRNPM). Mitochondrial components (HMGCL, MTCH2, CHXH3) were also prominent (Figure 33, C).

These observations endorse impairments in axon guidance and synaptic plasticity whose downregulation negatively affects CNS development and homeostasis. Abnormalities in these two processes can lead to severe neurodevelopmental problems.

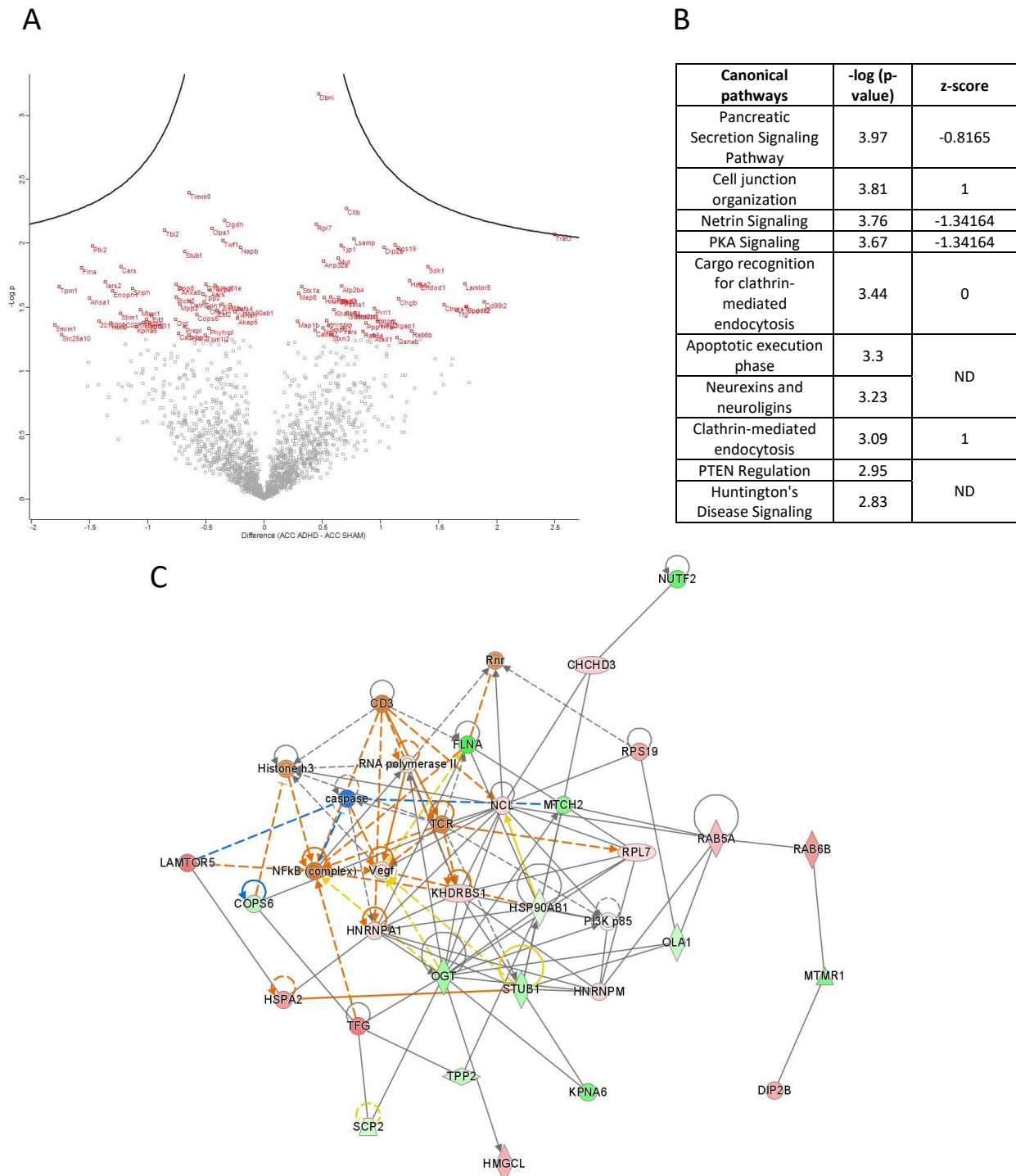


Figure 33: Mass spectrometry analysis in the ACC of male mice. (A) Volcano plot of the differentially expressed proteins (left: downregulation, right: upregulation) between 6-OHDA and sham mice. (B) Top 10 canonical pathways pointing to changes in axonal guidance, endocytosis, and cell adhesion. (C) Network enrichment analysis highlights an interaction between immunity, ribosomes, and alternative splicing (blue: downregulation, orange: upregulation). Analysis was performed using IPA software ($n=8$ mice per group), with $p < 0.05$. ND: not determined.

3.4.3 Protein screening in the PI of females

Several proteins were subject to modifications in the PI of 6-OHDA females. Some were found to be upregulated while others showed a decreased expression (Figure 34, A). Downregulated proteins (Figure 34, A, left) were correlated to ATP synthesis and glutamate receptors while those with an increased

expression (Figure 34, A, right) mainly participated in translation initiation and proteasome activation.

Pathway enrichment analysis was centered around Rho GTPase signaling, involved in actin cytoskeleton modulation. Rho GTPases can activate several of their associated serine-threonine kinases (CIT, ROCK, PAK, PKN) to regulate actin and microtubule dynamics. Complementary canonical pathways were also identified including RHOGDI signaling (inhibitors of GTPase proteins), actin cytoskeleton signaling, and actin nucleation by ARP-WASP complex (initiates the first step of actin polymerization). Importantly, other cascades related to axonal guidance (semaphoring interactions) and the regulation of signaling pathways (ILK signaling) were also detected (Figure 34, B).

Network enrichment analysis accentuated the role of insulin as a central coordinator of the interactions between inflammation (NF- κ B), apoptosis (NF- κ B), neuronal survival (Creb, TARDBP), and BBB integrity (PDGF-BB). The importance of inflammation and cell death was further reinforced as other proteins contributing to these processes were displayed (PI3K, Akt, p38 MAPK, ERK, JNK, ERP29). Synaptic plasticity, synaptic formation (Rac, ERK), and organization of actin cytoskeleton (Ras homolog, DIP2B, ARPC3, SPTBN4, ANK3, FLNa, GRM5) were also pointed out (Figure 34, C).

Overall, abnormalities in inflammatory and apoptotic processes, and cytoskeleton dynamics were underlined in the PI of 6-OHDA females.

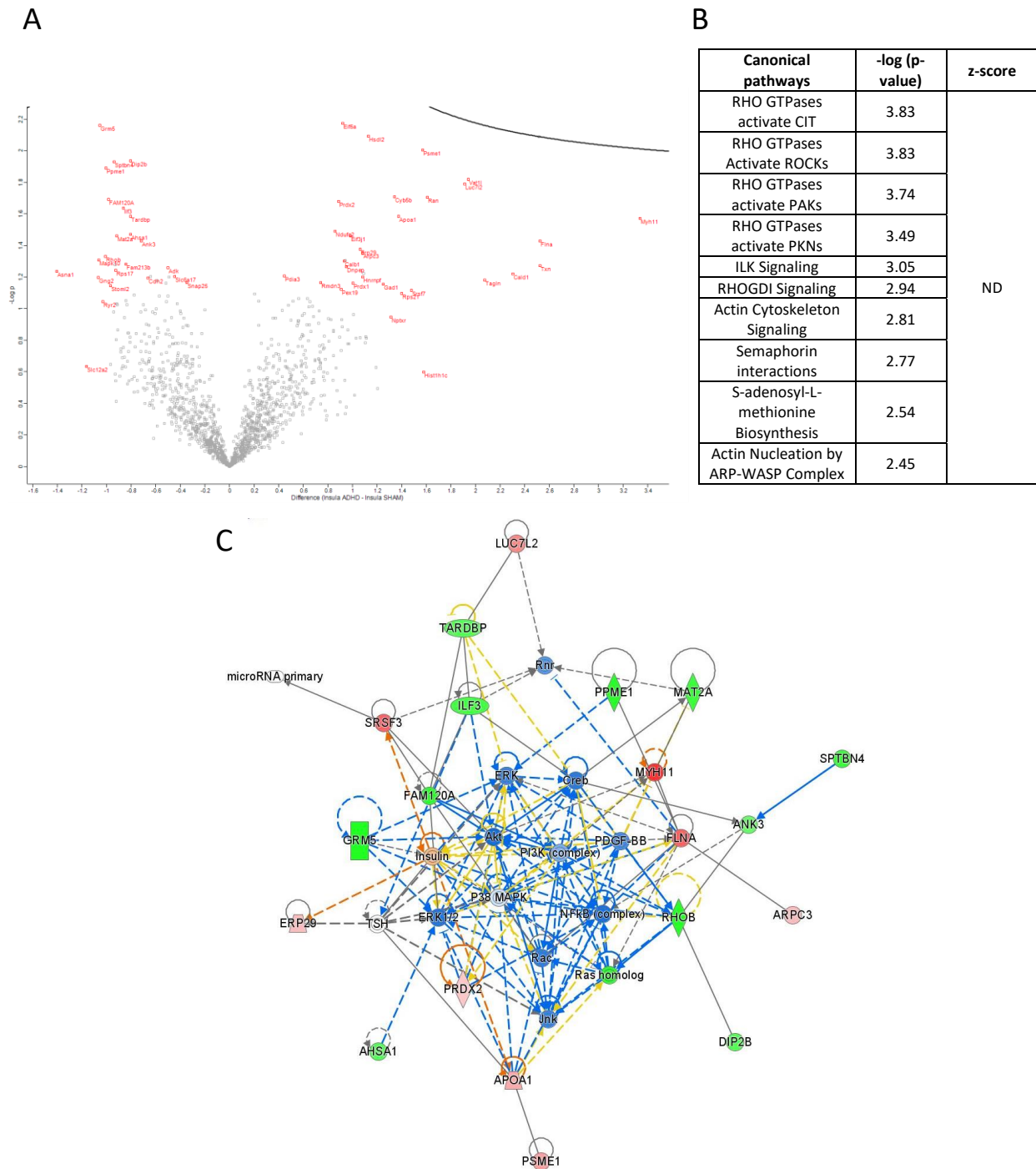


Figure 34: Mass spectrometry analysis in the PI of female mice. (A) Volcano plot of the differentially expressed proteins (left: downregulation, right: upregulation) between 6-OHDA and sham mice. (B) Top 10 canonical pathways pointing to changes in actin cytoskeleton dynamics through Rho GTPase signaling. (C) Network enrichment analysis highlights an interaction between inflammation, neuronal viability, and BBB integrity (blue: downregulation, orange: upregulation). Analysis was performed using IPA software ($n=8$ mice per group), with $p < 0.05$. ND: not determined.

3.4.4 Protein screening in the PI of males

In the PI of 6-OHDA males, a downregulation in the expression of proteins was observed (Figure 35, A, left), with these mainly correlating with lipid metabolism. On the other hand, proteins with increased levels were related to nitric oxide generation and GLUT4 transport. Downregulated proteins were

correlated to ATP synthesis and glutamate receptors (Figure 35, A, right).

Pathway enrichment analysis was centered around mitochondrial respiration (oxidative phosphorylation), cell division (RHOA signaling, signaling by PTK6, mitotic prometaphase), and actin polymerization (RHOA signaling, signaling by PTK6) (Figure 35, B).

Network enrichment analysis mainly pointed towards changes in inflammation and apoptosis, with the involvement of NF- κ B, CD3, SERPINA3, JNK, caspase, CSNK2A2, PDCD6IP, PDCD6, PDCD5, and ERK1/2. Cellular processes, mainly cell cycle control, were also highlighted (KHDRBS1, JNK, CSNK2A2, and ERK1/2) in addition to actin and microtubule dynamics (KIF2A, F-actin, CORO2B, ACTR3, ACTR1A, ACTBL2, UBR4) (Figure 35, C).

Taken together, these observations report alterations in inflammation, apoptosis, cytoskeleton dynamics, and cell cycle control in the PI of 6-OHDA males.

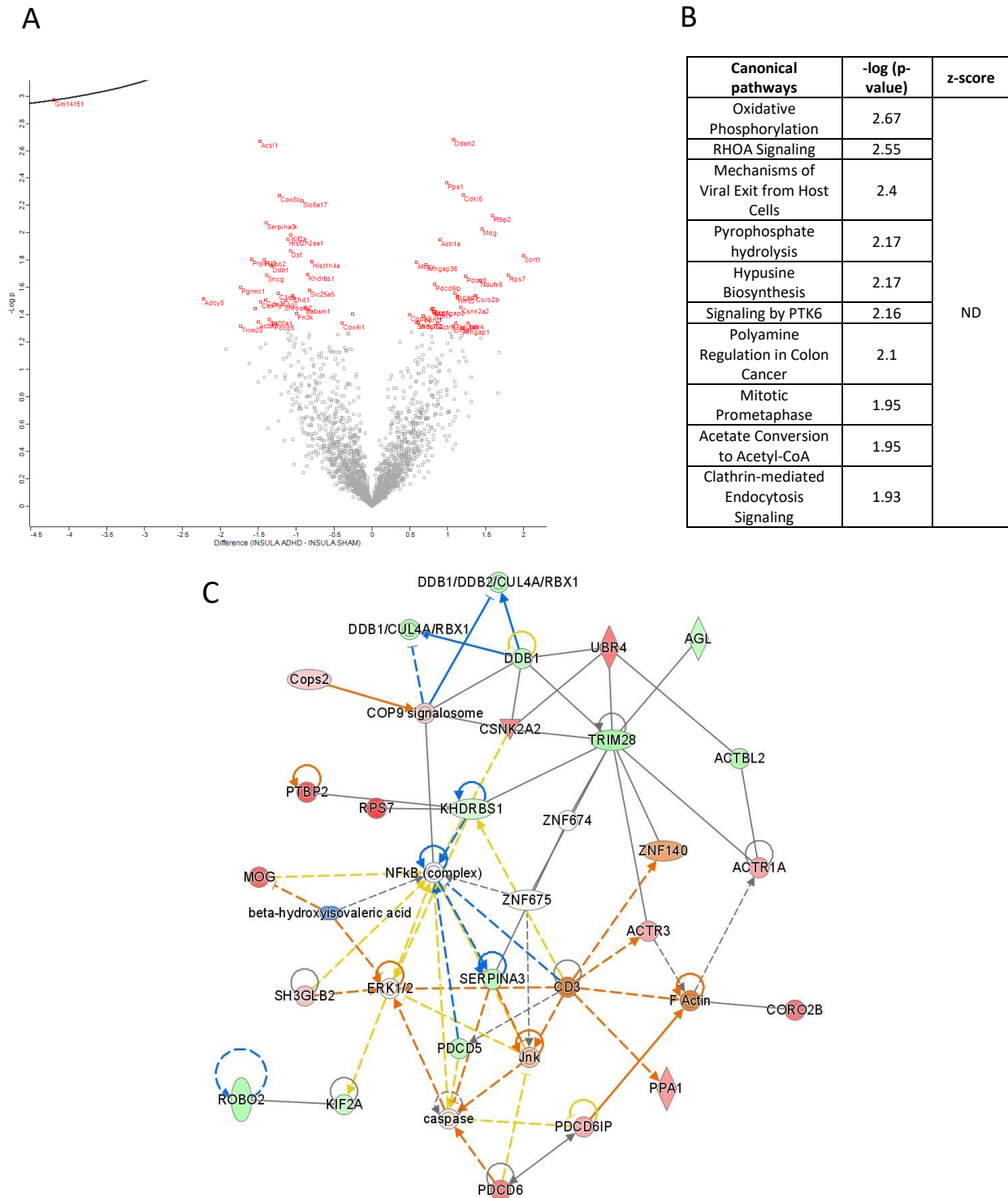


Figure 35: Mass spectrometry analysis in the PI of male mice. (A) Volcano plot of the differentially expressed proteins (left: downregulation, right: upregulation) between 6-OHDA and sham mice. (B) Top 10 canonical pathways pointing to changes in actin polymerization and cell division. (C) Network enrichment analysis focuses on changes in inflammation (blue: downregulation, orange: upregulation). Analysis was performed using IPA software ($n=7-8$ mice per group), with $p < 0.05$. ND: not determined.

Overall, impairments in protein expression were detected in the ACC and PI of 6-OHDA males and females with some proteins being upregulated and others being downregulated. Those modifications were associated with changes in several biological processes including axon guidance, apoptosis, cytoskeleton dynamics, inflammation, and cell cycle.

3.5 PamGene assay

Morphological, transcriptional, and proteomic disruptions were revealed in the ACC and PI of 6-OHDA females and males, pointing to a sustained immune response in ADHD-like conditions.

Importantly, alterations in kinase activity impair downstream pathways that modulate various cellular processes. Abnormalities in signaling cascades also result in aberrant inflammatory responses.

To explore modifications in kinase (serine-threonine and tyrosine) activity and their associated signaling pathways, two phosphoproteomic assays (STK and PTK PamGene assays) and pathway enrichment analysis were performed.

3.5.1 Kinase activity in the ACC of females

Changes in the activity of serine-threonine and tyrosine kinases were evident in the ACC of 6-OHDA females. An overall increase in serine-threonine kinase activity and, on the contrary, a tendency for decreased tyrosine kinase activity, were observed (Figure 36, A-B).

The Kinase top list displays the 20 most significant kinases, their specificity score (effect size), and median kinase statistic (direction) (Figure 36, A). Most serine-threonine enzymes presented a median kinase statistic above 0, suggesting an amplified phosphorylation of their targets (Figure 36, A, left). Interestingly, only a few had a downregulated activity including PKG2 (or PRKG2), IKK α (or CHUK), PKG1 (PRKG1), and GSK3 β . In contrast, all top 20 tyrosine kinases exhibited a median kinase statistic below 0, indicative of a downregulated enzymatic activity in ADHD-like conditions (Figure 36, A, right).

Pathway enrichment analysis identified canonical cascades associated with the altered kinases. Analysis was carried out using two software programs, EnrichR (KEGG 2021 Human gene set library) and IPA, for more rigorous analysis and interpretation of the results. Phagocytosis, axon guidance, and chemokine signaling were among the top 10 pathways determined by EnrichR (Figure 36, C). In agreement with these results, modifications in IL-33 and NTRK1 signaling pathways were depicted in the ACC of 6-OHDA females with IPA (Figure 36, D). Moreover, a comparison of all pathways reported by EnrichR and IPA has selected common processes outlined by both software (Table 13). This more in-depth analysis pointed to modifications in complementary signaling pathways, mainly related to inflammation and glial activity, such as NF- κ B, MAPK, PI3K-Akt, and TLR cascades. Importantly, inflammation is a major contributor to the establishment of chronic pain conditions. Therefore, these pathways point to impairments in immune, neurotrophic, and synaptic plasticity processes that may underlie a sustained inflammatory response, neurodevelopmental deficits, and pain sensitization.

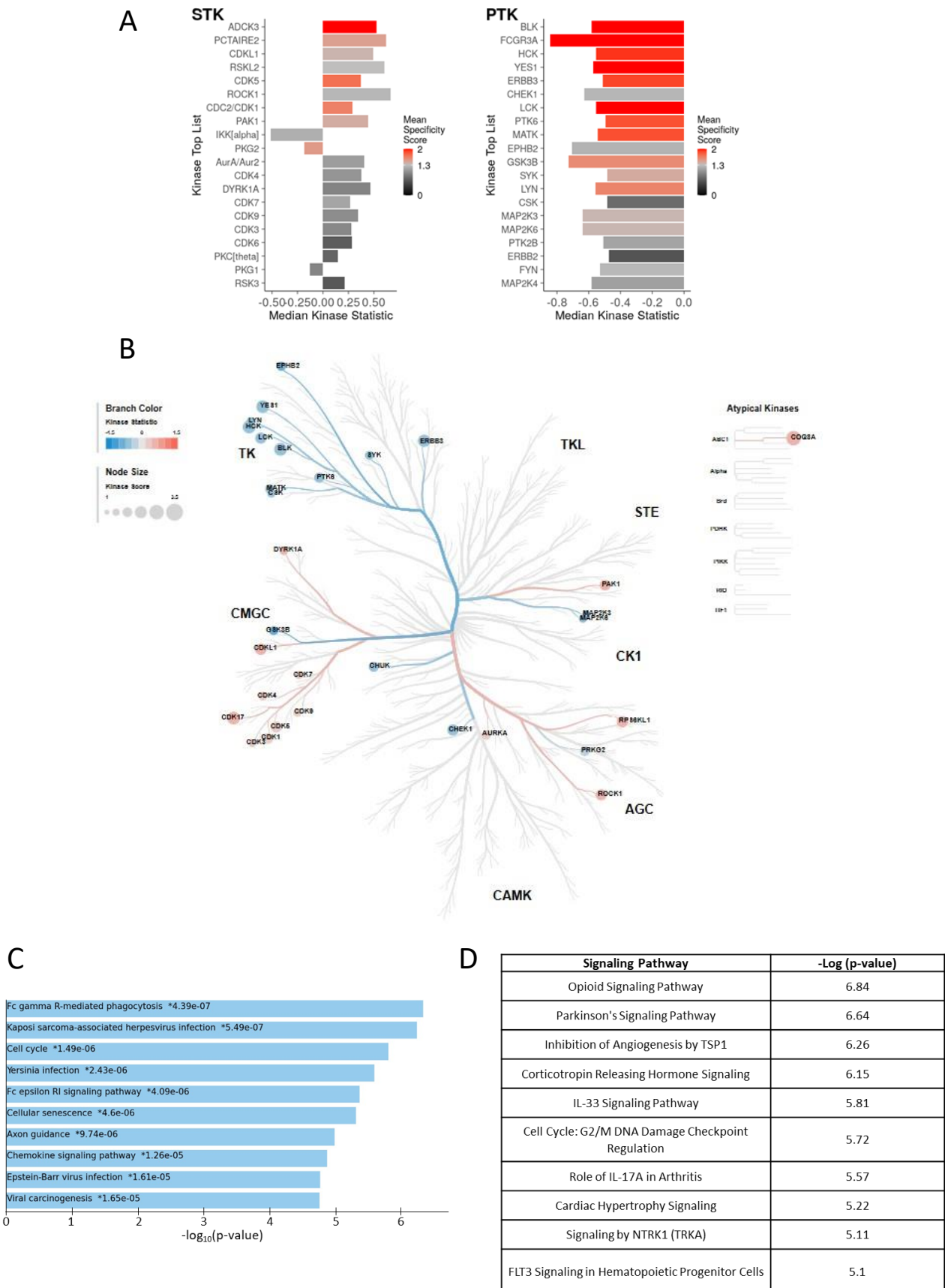


Figure 36: Changes in kinase activity and associated pathways in the ACC of females. (A) Kinase specificity score plot highlighting the top 20 kinases, their effect size, and direction. (B) Kinome phylogenetic tree representing all kinases with a final score above 1.3 (default threshold). Branch color and node size are encoded based on the

kinase statistic (red: upregulation, blue: downregulation) and final scores, respectively. Signals are expressed as log2-fold change. (C) Top 10 canonical pathways from KEGG 2021 Human gene set library displayed based on the $-\log_{10}(p\text{-value})$ with their corresponding p-value ($p < 0.05$). (D) Top 10 canonical pathways from IPA software displayed based on the $-\log(p\text{-value})$ ($p < 0.05$) (6 mice per group).

Table 13: Altered signaling pathways identified by EnrichR and IPA in the ACC of 6-OHDA females

Common Signaling Pathways	EnrichR (KEGG software)	IPA Software
Axon guidance	Axon guidance Adjusted p-value: 1.61×10^{-4}	Netrin-1 signaling p-value: 2.69×10^{-4}
Chemokine signaling	Chemokine signaling pathway Adjusted p-value: 1.83×10^{-4}	IL-33 signaling pathway p-value: 1.55×10^{-6}
		IL-22 signaling pathway p-value: 6.17×10^{-5}
		Chemokine signaling p-value: 7.08×10^{-4}
		IL-7 signaling pathway p-value: 6.61×10^{-4}
		IL-1 signaling p-value: 0.001
		TGF- β signaling p-value: 0.001
	TNF signaling pathway Adjusted p-value: 0.04	IL-13 signaling p-value: 0.001
		IL-27 signaling p-value: 0.002
		IL-6 signaling p-value: 0.002
		IL-4 signaling p-value: 0.003
		IL-10 signaling p-value: 0.003
		IL-17 signaling p-value: 0.004
		IL-15 signaling p-value: 0.03
	p53 pathway	p53 signaling pathway Adjusted p-value: 0.002
TP53 regulates transcription of cell cycle genes p-value: 0.02		
Regulation of TP53 activity through phosphorylation p-value: 0.04		
p53 signaling p-value: 0.05		
ErbB signaling	ErbB signaling pathway	ERBB signaling

	Adjusted p-value: 0.002	p-value: 9.33×10^{-4}
NF- κ B signaling	NF-kappa B signaling pathway Adjusted p-value: 0.003	NLR signaling pathways p-value: 3.39×10^{-4}
		PEDF signaling -log(p-value): 3.12 p-value: 7.59×10^{-4}
MAPK signaling	MAPK signaling pathway Adjusted p-value: 0.006	Signaling by NTRK1 (TRKA) p-value: 7.76×10^{-6}
		p38 MAPK Signaling p-value: 2.51×10^{-5}
		MAPK targets/ Nuclear events mediated by MAP kinases p-value: 1.17×10^{-4}
		MAP kinase activation p-value: 0.02
		CNTF Signaling p-value: 0.03
		ERK5 signaling p-value: 0.04
PI3K-Akt signaling	PI3K-Akt signaling pathway Adjusted p-value: 0.01	Signaling by NTRK1 (TRKA) p-value: 7.76×10^{-6}
		CNTF Signaling p-value: 0.03
Focal adhesion	Focal adhesion Adjusted p-value: 0.02	Paxillin signaling p-value: 0.001
TLR signaling	Toll-like receptor signaling pathway Adjusted p-value: 0.04	Toll-like receptor signaling p-value: 6.61×10^{-4}
Neurotrophin signaling	Neurotrophin signaling pathway Adjusted p-value: 0.046	Signaling by NTRK1 (TRKA) p-value: 7.76×10^{-6}
		CNTF signaling p-value: 0.03
		GDNF family ligand-receptor interactions p-value: 0.04
		Neurotrophin/TRK signaling p-value: 0.04

3.5.2 Kinase activity in the ACC of males

Alterations in the activity of serine-threonine and tyrosine kinases were prominent in the ACC of 6-OHDA males, with an overall increase in their enzymatic activity (Figure 37, A-B). Indeed, all serine-threonine and tyrosine enzymes displayed a median kinase statistic above 0, suggesting an upregulated activity in ADHD-like conditions (Figure 37, A). Remarkably, and in contrast to females, no kinases were downregulated suggesting more amplified kinase activity in the ACC of 6-OHDA males.

Pathway enrichment analysis reported canonical cascades associated with the disrupted kinases. ErbB signaling and focal adhesion were among the top 10 pathways determined by EnrichR (Figure 37, C). Impairments in IL-15 production, serotonin receptor, Ephrin A, and PI3K-Akt signaling pathways were

reported in the ACC of 6-OHDA males with IPA (Figure 37, D). Furthermore, a comparison of all pathways reported by EnrichR and IPA has identified common processes described by both software (Table 14). This more in-depth analysis revealed modifications in inflammatory signaling pathways such as MAPK, PI3K-Akt, JAK-STAT, mTOR, TLR, and chemokine cascades. Several neurotransmitter systems seem to be also affected including dopamine, serotonin, and acetylcholine. Abnormalities in synaptic plasticity and CNS homeostasis (neurotrophins, LTP, LTD, axon guidance) processes were also noted, along with apoptosis, phagocytosis, and the regulation of actin cytoskeleton. Interestingly, both serotonergic and dopaminergic systems were found to be impaired in ADHD, both at the clinical and preclinical levels. Additionally, serotonin participates in the descending modulation of pain. Consequently, and in concordance with the results in the ACC of 6-OHDA females, these cascades underlie changes in neurotransmitter, cellular, inflammatory, and axon guidance processes that may result in a continuous immune response, synaptic plasticity changes, neurodevelopmental problems, and pain hypersensitivity.

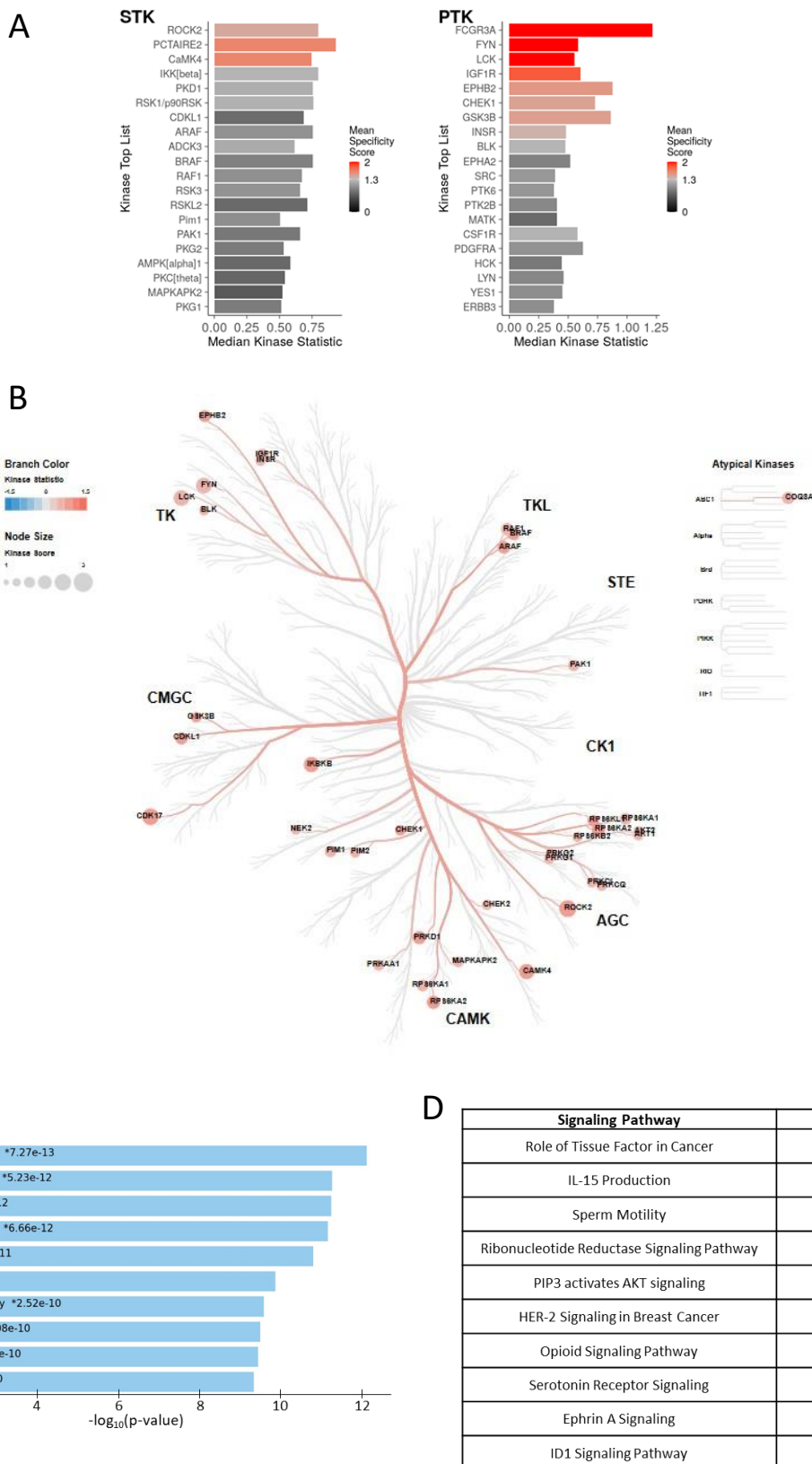


Figure 37: Changes in kinase activity and associated pathways in the ACC of males. (A) Kinase specificity score plot highlighting the top 20 kinases, their effect size, and direction. (B) Kinome phylogenetic tree representing all kinases with a final score above 1.3 (default threshold). Branch color and node size are encoded based on the kinase statistic (red: upregulation, blue: downregulation) and final scores, respectively. Signals are expressed as \log_2 -fold change. (C) Top 10 canonical pathways from KEGG 2021 Human gene set library displayed based on the –

$\log_{10}(p\text{-value})$ with their corresponding p -value ($p < 0.05$). (D) Top 10 canonical pathways from IPA software displayed based on the $-\log(p\text{-value})$ ($p < 0.05$) (6 mice per group).

Table 14: Altered signaling pathways identified by EnrichR and IPA in the ACC of 6-OHDA males

Common Signaling Pathways	EnrichR (KEGG software)	IPA Software
ErbB signaling	ErbB signaling pathway Adjusted p-value: 2.41×10^{-10}	Signaling by ERBB2 p-value: 2.00×10^{-19}
		ERBB signaling p-value: 1×10^{-12}
		Signaling by ERBB4 p-value: 1.20×10^{-5}
		ERB2-ERBB3 signaling p-value: 1.78×10^{-5}
Focal adhesion	Focal adhesion Adjusted p-value: 2.41×10^{-10}	FAK signaling p-value: 1.58×10^{-12}
mTOR signaling	mTOR signaling pathway Adjusted p-value: 8.47×10^{-9}	mTOR signaling p-value: 1.20×10^{-5}
Chemokine signaling	Chemokine signaling pathway Adjusted p-value: 3.34×10^{-8}	IL-15 production p-value: 5.01×10^{-28}
		IL-13 signaling pathway p-value: 3.16×10^{-15}
		IL-33 signaling pathway p-value: 1.58×10^{-14}
		IL-8 signaling p-value: 7.94×10^{-14}
		IL-7 signaling pathway p-value: 2.00×10^{-13}
		IL-22 signaling p-value: 2.00×10^{-11}
		CXCR4 signaling p-value: 2.34×10^{-10}
		IL-6 signaling p-value: 7.76×10^{-10}
		IL-2 signaling p-value: 7.94×10^{-9}
		IL-17 signaling p-value: 1.48×10^{-8}
		IL-3 signaling p-value: 3.47×10^{-8}
		Chemokine signaling p-value: 4.07×10^{-8}
		IL-23 signaling pathway p-value: 8.32×10^{-8}
		IL-10 signaling p-value: 8.32×10^{-8}

		Interleukin-4 and Interleukin-13 signaling p-value: 6.61×10^{-6}
		IL-27 signaling pathway p-value: 1.38×10^{-5}
		IL-4 signaling p-value: 6.61×10^{-5}
	TNF signaling pathway Adjusted p-value: 0.03	IL-1 signaling p-value: 8.32×10^{-5}
		Interleukin-2 family signaling p-value: 1.82×10^{-4}
		IL-15 signaling p-value: 0.002
		TNFR2 signaling p-value: 0.003
		IL-9 signaling p-value: 0.003
		Interleukin-1 family signaling p-value: 0.004
		TNFR1 signaling p-value: 0.007
		Interleukin-1 processing p-value: 0.02
		TGF- β signaling p-value: 0.02
		Interleukin-12 family signaling p-value: 0.046
		Interleukin-7 signaling p-value: 0.049
FoxO signaling	FoxO signaling pathway Adjusted p-value: 4.97×10^{-8}	FOXO-mediated transcription p-value: 1.10×10^{-7}
		FOXO-mediated transcription of cell cycle genes p-value: 7.41×10^{-4}
		FOXO-mediated transcription of cell death genes p-value: 0.04
Axon guidance	Axon guidance Adjusted p-value: 4.20×10^{-7}	Ephrin A signaling p-value: 2.51×10^{-21}
		ROBO SLIT signaling pathway p-value: 2.00×10^{-16}
		Ephrin receptor signaling p-value: 5.01×10^{-11}
		WNT/SHH axonal guidance signaling pathway p-value: 7.94×10^{-11}
		Netrin signaling

		p-value: 3.02×10^{-10}
		EPH-Ephrin signaling p-value: 2.70×10^{-9}
		Semaphorin neuronal repulsive signaling pathway p-value: 6.92×10^{-8}
		Axonal guidance signaling p-value: 3.24×10^{-7}
		Netrin-1 signaling p-value: 2.34×10^{-4}
		Semaphorin interactions p-value: 4.90×10^{-4}
		Semaphorin signaling in neurons p-value: 0.009
		ERK5 signaling p-value: 7.94×10^{-12}
		ERK/MAPK signaling p-value: 2.09×10^{-9}
		RAF/MAP kinase cascade p-value: 1.17×10^{-8}
		MAPK targets/ Nuclear events mediated by MAP kinases p-value: 1.48×10^{-8}
		MAP kinase activation p-value: 2.29×10^{-6}
		Signaling by NTRK2 (TRKB) p-value: 2.88×10^{-5}
		Signaling by NTRK1 (TRKA) p-value: 4.27×10^{-5}
		RAF-independent MAPK1/3 activation p-value: 0.001
		BAG2 signaling pathway p-value: 0.02
		MAPK6/MAPK4 signaling p-value: 0.02
		p38 MAPK signaling p-value: 0.03
		Signaling by NTRK3 (TRKC) -log(p-value): 1.4 p-value: 0.04
		Protein Kinase A signaling p-value: 5.37×10^{-7}
		Dopamine-DARPP32 feedback in cAMP signaling p-value: 5.37×10^{-6}
MAPK signaling	MAPK signaling pathway Adjusted p-value: 6.12×10^{-7}	
cAMP signaling	cAMP signaling pathway Adjusted p-value: 1.19×10^{-6}	

		cAMP-mediated signaling p-value: 2.09×10^{-5}
Phagocytosis	Fc gamma R-mediated phagocytosis Adjusted p-value: 5.60×10^{-6}	Fc gamma receptor (FCGR) dependent phagocytosis p-value: 2.04×10^{-6}
Neurotrophins signaling	Neurotrophin signaling pathway Adjusted p-value: 1.29×10^{-5}	NGF signaling p-value: 10^{-16}
		GDNF family ligand-receptor interactions p-value: 5.89×10^{-10}
		Neurotrophin/TRK signaling p-value: 3.24×10^{-8}
		Signaling by NTRK2 (TRKB) p-value: 2.88×10^{-5}
		Signaling by NTRK1 (TRKA) p-value: 4.27×10^{-5}
		CNTF signaling p-value: 3.47×10^{-4}
		Signaling by NTRK3 (TRKC) p-value: 0.04
AMPK signaling	AMPK signaling pathway Adjusted p-value: 1.31×10^{-5}	AMPK signaling p-value: 5.89×10^{-9}
Actin cytoskeleton	Regulation of actin cytoskeleton Adjusted p-value: 1.39×10^{-5}	ILK signaling p-value: 5.02×10^{-11}
		Paxillin signaling p-value: 2.14×10^{-7}
		Integrin signaling p-value: 4.90×10^{-7}
		RHOA signaling p-value: 0.04
PI3K-Akt signaling	PI3K-Akt signaling pathway Adjusted p-value: 1.71×10^{-5}	PIP3 activates AKT signaling p-value: 3.98×10^{-23}
		PI3K/AKT signaling p-value: 1.58×10^{-12}
		PTEN signaling p-value: 2.51×10^{-12}
		PI3K cascade p-value: 3.72×10^{-6}
		Signaling by NTRK2 (TRKB) p-value: 2.88×10^{-5}
		Signaling by NTRK1 (TRKA) p-value: 4.27×10^{-5}
		PTEN regulation p-value: 4.58×10^{-4}
		Signaling by NTRK3 (TRKC) p-value: 0.04
Synaptic plasticity	Long-term depression	Reelin signaling in neurons

	Adjusted p-value: 1.79×10^{-5}	p-value: 1.58×10^{-19}
		Synaptogenesis signaling pathway p-value: 6.31×10^{-19}
		CREB signaling in neurons p-value: 1.17×10^{-10}
		Synaptic long-term potentiation p-value: 7.41×10^{-7}
		Synaptic long-term depression p-value: 1.10×10^{-4}
	Long-term potentiation Adjusted p-value: 6.28×10^{-4}	NCAM signaling for neurite outgrowth p-value: 4.27×10^{-4}
		L1CAM interactions p-value: 0.003
		Neurexins and neuroligins p-value: 0.008
		Reelin signaling p-value: 0.01
		Synaptic adhesion-like molecules p-value: 0.05
Ras signaling	Ras signaling pathway Adjusted p-value: 1.79×10^{-5}	Signaling by NTRK2 (TRKB) p-value: 2.88×10^{-5}
		Signaling by NTRK1 (TRKA) p-value: 4.27×10^{-5}
		Signaling by NTRK3 (TRKC) p-value: 0.04
JAK-STAT signaling	JAK-STAT signaling pathway Adjusted p-value: 4.08×10^{-5}	STAT3 pathway p-value: 1.12×10^{-9}
		Role of JAK family kinases in IL-6-type cytokine signaling p-value: 4.37×10^{-8}
		JAK/STAT signaling p-value: 4.37×10^{-8}
		Role of JAK1 and JAK3 in γ c cytokine signaling p-value: 2.24×10^{-5}
		Role of JAK1, JAK2 and TYK2 in interferon signaling p-value: 0.002
Role of JAK2 in hormone-like cytokine signaling p-value: 0.01		
Apelin signaling	Apelin signaling pathway Adjusted p-value: 3.29×10^{-4}	Apelin endothelial signaling pathway p-value: 1.26×10^{-12}

Serotonergic system	Serotonergic synapse Adjusted p-value: 0.002	Serotonin receptor signaling p-value: 1.26×10^{-21}
Cholinergic system	Cholinergic synapse Adjusted p-value: 0.002	Acetylcholine receptor signaling pathway p-value: 7.94×10^{-16}
		Adrenergic receptor signaling pathway (Enhanced) p-value: 0.01
Relaxin signaling	Relaxin signaling pathway Adjusted p-value: 0.004	Relaxin signaling p-value: 1.91×10^{-6}
Dopaminergic system	Dopaminergic synapse Adjusted p-value: 0.004	Dopamine-DARPP32 feedback in cAMP signaling p-value: 5.37×10^{-6}
Apoptosis	Apoptosis Adjusted p-value: 0.004	Apoptosis signaling p-value: 1.82×10^{-7}
		Signaling by PTK6 p-value: 2.09×10^{-7}
		Intrinsic pathway for apoptosis p-value: 2.09×10^{-7}
		Sirtuin signaling pathway p-value: 0.005
		Death receptor signaling p-value: 0.02
TLR signaling	Toll-like receptor signaling pathway Adjusted p-value: 0.03	Toll-like receptor signaling p-value: 0.02
		Regulation of TLR by endogenous ligand p-value: 0.048

3.5.3 Kinase activity in the PI of females

Alterations in the activity of serine-threonine and tyrosine kinases were reported in the PI of 6-OHDA females. An overall increase in all kinase activity (serine-threonine and tyrosine) was observed (Figure 38, A-B). All serine-threonine kinases displayed a median kinase statistic above 0, suggesting an upregulated activity (Figure 38, A, left). The same results were found for tyrosine kinases, except for EPHB₄ which exhibited a median kinase statistic below 0, indicative of decreased enzymatic activity in ADHD-like conditions (Figure 38, A, right).

Pathway enrichment analysis described signaling pathways associated with the impaired kinases. MAPK, ErbB, mTOR, and neurotrophin signaling pathways were among the top 10 cascades identified by EnrichR (Figure 38, C). IL-15 production, serotonin receptor, and synaptogenesis signaling were mentioned by IPA (Figure 38, D). Furthermore, a comparison of all pathways cited by EnrichR and IPA has selected common processes outlined by both software (Table 15). This more in-depth analysis highlighted changes related to inflammatory cascades (NF- κ B, Wnt, JAK-STAT, TLR, and PI3K-Akt signaling pathways), neurotransmitter systems (GABA, glutamate, dopamine, acetylcholine, and serotonin), cell death (apoptosis, p53 signaling pathway), and synaptic plasticity (axon guidance, LTP, LTD). Importantly, impairments in these processes can result in deregulated inflammatory response, altered CNS development and functioning, and abnormal pain modulation.

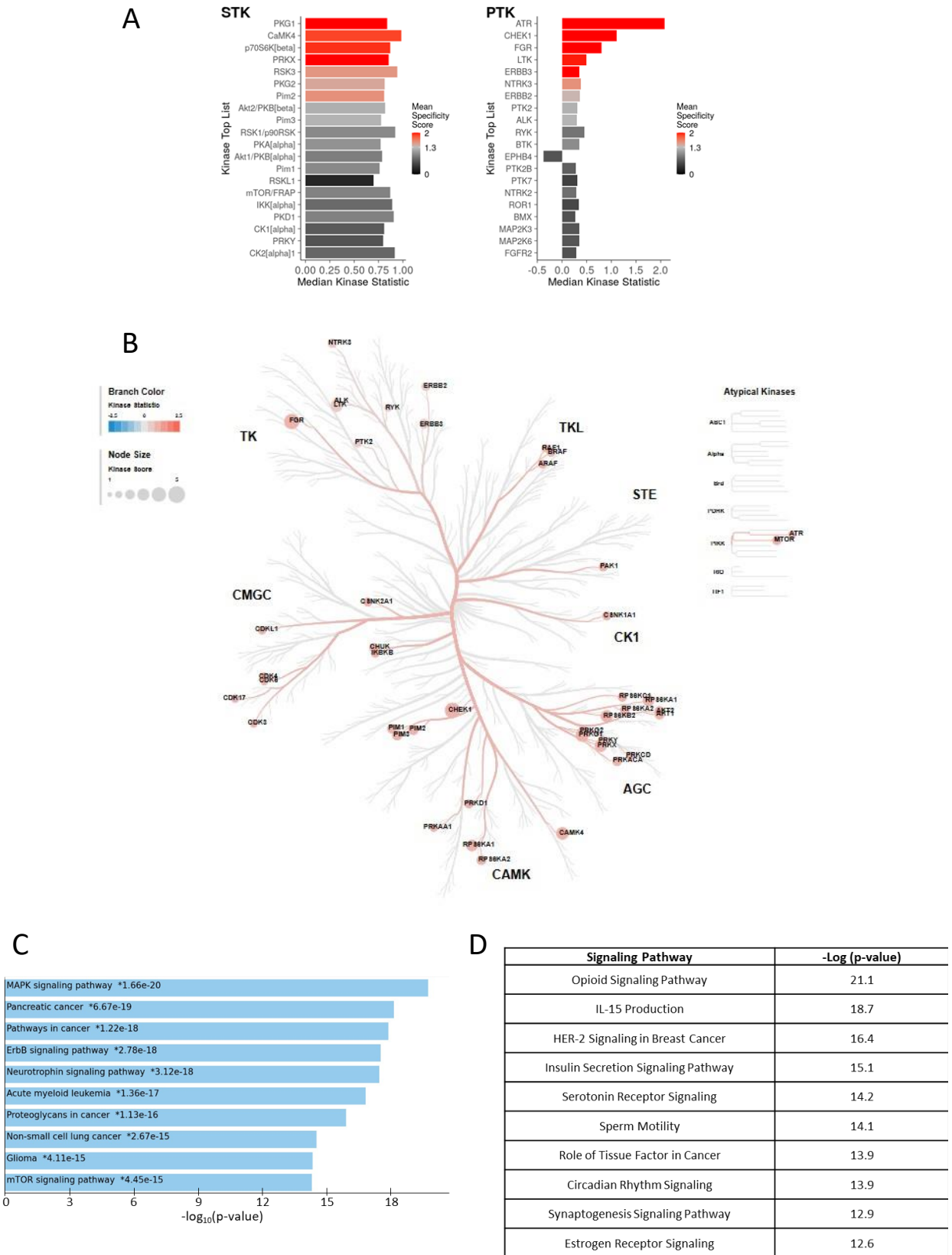


Figure 38: Changes in kinase activity and associated pathways in the PI of females. (A) Kinase specificity score plot highlighting the top 20 kinases, their effect size, and direction. (B) Kinome phylogenetic tree representing all kinases with a final score above 1.3 (default threshold). Branch color and node size are encoded based on the kinase statistic (red: upregulation, blue: downregulation) and final scores, respectively. Signals are expressed as \log_2 -fold change. (C) Top 10 canonical pathways from KEGG 2021 Human gene set library displayed based on the –

$\log_{10}(p\text{-value})$ with their corresponding p -value ($p < 0.05$). (D) Top 10 canonical pathways from IPA software displayed based on the $-\log(p\text{-value})$ ($p < 0.05$) (5 mice per group).

Table 15: Altered signaling pathways identified by EnrichR and IPA in the PI of 6-OHDA females

Common Signaling Pathways	EnrichR (KEGG software)	IPA Software
MAPK signaling	MAPK signaling pathway Adjusted p-value: 2.93x10 ⁻¹⁸	ERK/MAPK signaling p-value: 6.76x10 ⁻⁹
		ERK5 signaling p-value: 5.13x10 ⁻⁸
		Signaling by NTRK1 (TRKA) p-value: 7.08x10 ⁻⁵
		RAF/MAP kinase cascade p-value: 7.41x 10 ⁻⁴
		Signaling by NTRK2 (TRKB) p-value: 0.002
		MAPK targets/ Nuclear events mediated by MAP kinases p-value: 0.004
		p38 MAPK signaling p-value: 0.04
		Signaling by NTRK3 (TRKC) p-value: 0.045
ErbB signaling	ErbB signaling pathway Adjusted p-value: 1.10x10 ⁻¹⁶	Signaling by ERBB2 p-value: 4.68x10 ⁻⁹
		Signaling by ERBB4 p-value: 2.04x10 ⁻⁵
		ERBB signaling p-value: 1.20x10 ⁻⁴
		ERB2-ERBB3 signaling p-value: 7.41x10 ⁻⁴
Neurotrophin signaling	Neurotrophin signaling pathway Adjusted p-value: 1.10x10 ⁻¹⁶	NGF signaling p-value: 9.12x10 ⁻⁷
		Signaling by NTRK1 (TRKA) p-value: 7.08x10 ⁻⁵
		Neurotrophin/TRK signaling p-value: 6.17x10 ⁻⁵
		CNTF signaling p-value: 5.01x10 ⁻⁴
		Signaling by NTRK2 (TRKB) p-value: 0.002
		GDNF family ligand-receptor interactions p-value: 0.02

		Signaling by NTRK3 (TRKC) p-value: 0.045
mTOR signaling	mTOR signaling pathway Adjusted p-value: 7.88x10 ⁻¹⁴	mTOR signaling p-value: 2.95x10 ⁻⁴
PI3K-Akt signaling	PI3K-Akt signaling pathway Adjusted p-value: 1.42x10 ⁻¹³	PIP3 activates AKT signaling p-value: 4.37x10 ⁻⁹
		PTEN signaling p-value: 7.94x10 ⁻⁹
		PI3K/AKT signaling p-value: 7.08x10 ⁻⁸
		Signaling by NTRK1 (TRKA) p-value: 7.08x10 ⁻⁵
		Signaling by NTRK2 (TRKB) p-value: 0.002
		Signaling by NTRK3 (TRKC) p-value: 0.045
FoxO signaling	FoxO signaling pathway Adjusted p-value: 1.08x10 ⁻¹¹	FOXO-mediated transcription of cell cycle genes p-value: 0.045
Chemokine signaling	Chemokine signaling pathway Adjusted p-value: 1.58x10 ⁻¹¹	IL-15 production p-value: 2.00 x10 ⁻¹⁹
		IL-8 signaling p-value: 5.50x10 ⁻⁹
		IL-13 signaling pathway p-value: 7.59x10 ⁻⁷
		IL-33 signaling pathway p-value: 1.12x10 ⁻⁵
		IL-10 signaling p-value: 6.31x10 ⁻⁵
		Chemokine signaling p-value: 7.08x10 ⁻⁵
		CXCR4 signaling p-value: 0.001
		IL-7 signaling pathway p-value: 0.001
		IL-3 signaling p-value: 0.001
	IL-1 signaling p-value: 0.002	
	TNF signaling pathway Adjusted p-value: 1.16x10 ⁻⁶	TNFR2 signaling p-value: 0.003
		IL-9 signaling p-value: 0.004
		IL-27 signaling pathway

		p-value: 0.005
		IL-6 signaling p-value: 0.005
		IL-23 signaling pathway p-value: 0.007
		TNFR1 signaling p-value: 0.009
		IL-4 signaling p-value: 0.02
		Interleukin-1 processing p-value: 0.02
		Interleukin-1 family signaling p-value: 0.047
Synaptic plasticity	Long-term potentiation Adjusted p-value: 4.52x10 ⁻¹¹	Synaptogenesis signaling pathway p-value: 1.26x10 ⁻¹³
		Reelin signaling in neurons p-value: 1.35x10 ⁻¹⁰
		CREB signaling in neurons p-value: 7.59x10 ⁻¹⁰
		Synaptic long-term potentiation p-value: 2.69x10 ⁻⁹
	Long-term depression Adjusted p-value: 3.51x10 ⁻⁸	Synaptic long-term depression p-value: 1.12x10 ⁻⁶
		NCAM signaling for neurite out- growth p-value: 2.29x10 ⁻⁵
		L1CAM interactions p-value: 0.0047
		Reelin signaling pathway p-value: 0.013
Apelin signaling	Apelin signaling pathway Adjusted p-value: 9.71x10 ⁻⁹	Apelin endothelial signaling pathway p-value: 2.34x10 ⁻⁶
Focal adhesion	Focal adhesion Adjusted p-value: 1.45x10 ⁻⁷	FAK signaling p-value: 1.95x10 ⁻⁵
Ras signaling	Ras signaling pathway Adjusted p-value: 3.96x10 ⁻⁷	Signaling by NTRK1 (TRKA) p-value: 7.08x10 ⁻⁵
		Signaling by NTRK2 (TRKB) p-value: 0.002
		Rap1 signalling p-value: 0.04
		Signaling by NTRK3 (TRKC) p-value: 0.045
TLR signaling	Toll-like receptor signaling pathway Adjusted p-value: 7.80x10 ⁻⁷	Toll-like receptor signaling p-value: 0.02

Relaxin signaling	Relaxin signaling pathway Adjusted p-value: 2.48×10^{-6}	Relaxin signaling p-value: 2.14×10^{-7}
p53 signaling	p53 signaling pathway Adjusted p-value: 3.02×10^{-6}	TP53 regulates transcription of cell cycle genes p-value: 0.008
		p53 signaling p-value: 0.03
cAMP signaling	cAMP signaling pathway Adjusted p-value: 3.21×10^{-6}	Protein Kinase A signaling p-value: 2.51×10^{-13}
		cAMP-mediated signaling p-value: 3.63×10^{-6}
		Dopamine-DARPP32 feedback in cAMP signaling p-value: 1.15×10^{-5}
Gap junction	Gap junction Adjusted p-value: 7.27×10^{-6}	Gap junction signaling p-value: 2.88×10^{-5}
Serotonergic system	Serotonergic synapse Adjusted p-value: 2.23×10^{-5}	Serotonin receptor signaling p-value: 6.31×10^{-15}
Cholinergic system	Cholinergic synapse Adjusted p-value: 2.23×10^{-5}	Acetylcholine receptor signaling pathway p-value: 2.75×10^{-9}
AMPK signaling	AMPK signaling pathway Adjusted p-value: 2.89×10^{-5}	AMPK signaling p-value: 1.05×10^{-9}
Dopaminergic system	Dopaminergic synapse Adjusted p-value: 4.38×10^{-5}	Dopamine-DARPP32 feedback in cAMP signaling p-value: 1.15×10^{-5}
		Dopamine receptor signaling p-value: 0.02
Apoptosis	Apoptosis Adjusted p-value: 5.85×10^{-5}	Apoptosis signaling p-value: 9.55×10^{-6}
		MYC-mediated apoptosis signaling p-value: 3.47×10^{-4}
		Sirtuin signaling pathway p-value: 0.001
		BAG2 signaling pathway p-value: 0.002
		Death receptor signaling p-value: 0.03
Calcium signaling	Calcium signaling pathway Adjusted p-value: 6.24×10^{-5}	Calcium signaling p-value: 8.32×10^{-9}
		WNT/Ca ⁺ pathway p-value: 7.76×10^{-4}
		Presynaptic depolarization and calcium channel opening p-value: 0.03
JAK-STAT signaling	JAK-STAT signaling pathway	STAT3 pathway

	Adjusted p-value: 1.05×10^{-4}	p-value: 1.82×10^{-6}
		JAK/STAT signaling p-value: 2.95×10^{-6}
		Role of JAK1, JAK2 and TYK2 in interferon signaling p-value: 0.002
Wnt signaling	Wnt signaling pathway Adjusted p-value: 1.16×10^{-4}	WNT/SHH axonal guidance signaling pathway p-value: 3.24×10^{-6}
		WNT/Ca ⁺ pathway p-value: 7.76×10^{-4}
Axon guidance	Axon guidance Adjusted p-value: 1.72×10^{-4}	ROBO SLIT signaling pathway p-value: 6.31×10^{-11}
		Netrin signaling p-value: 0.000000001
		Ephrin A signaling p-value: 4.07×10^{-9}
		EPH-Ephrin signaling p-value: 6.76×10^{-9}
		Ephrin receptor signaling p-value: 1.29×10^{-6}
		WNT/SHH axonal guidance signaling pathway p-value: 3.24×10^{-6}
		Axonal guidance signaling p-value: 7.08×10^{-5}
		Semaphorin neuronal repulsive signaling pathway p-value: 0.008
		Netrin-1 signaling p-value: 0.008
		Semaphorin signaling in neurons p-value: 0.01
		Semaphorin interactions p-value: 0.01
		Ephrin B signaling p-value: 0.02
NF-kappa B signaling	NF-kappa B signaling pathway Adjusted p-value: 0.003	BEX2 signaling pathway p-value: 2.95×10^{-6}
		NF-κB signaling p-value: 0.005
GABAergic system	GABAergic synapse Adjusted p-value: 0.03	GABAergic receptor signaling pathway (Enhanced) p-value: 3.89×10^{-5}
		GABA receptor signaling p-value: 0.006

Glutamatergic system	Glutamatergic synapse Adjusted p-value: 0.04	Glutaminergic receptor signaling pathway (Enhanced) p-value: 6.31×10^{-11}
		Activation of NMDA receptors and postsynaptic events p-value: 3.31×10^{-6}

3.5.4 Kinase activity in the PI of males

Impairments in the activity of serine-threonine and tyrosine kinases were prevalent in the PI of 6-OHDA males (Figure 39, A-B). All enzymes displayed a median kinase statistic above 0, suggesting an upregulation in their activity (Figure 39, A). Interestingly, none of the detected kinases was downregulated.

Pathway enrichment analysis identified canonical cascades associated with the altered enzymes. Several cascades related to inflammation and CNS development were reported through EnrichR, such as MAPK, Ras, ErbB, PI3K-Akt, T-cell receptor, and neurotrophin signaling pathways (Figure 39, C). Complementary cascades and processes were depicted by IPA including IL-15 production, and synaptogenesis, in addition to Ephrin A, PTEN, STAT3, and ROBO SLIT signaling pathways (Figure 39, D). The comparison of all pathways stated by EnrichR and IPA has identified common processes outlined by both software (Table 16). In addition to the previously mentioned cascades, this more in-depth analysis pointed to modifications in NF- κ B, mTOR, Wnt, and p53 pathways. These changes shed light on the presence and involvement of processes such as inflammation and apoptosis in the PI of 6-OHDA males. Furthermore, dopamine and serotonin are two transmitters that seem to be affected in this brain area. Synaptic plasticity processes (axon guidance, LTP, LTD) were also pointed out. Phagocytosis and actin cytoskeleton suggest changes in the morphology and function of immune cells.

Taken together, these pathways point to immune, neurotrophic, synaptic plasticity, and neurotransmitter abnormalities that may lead to enhanced inflammatory response and cause harmful effects on neurodevelopment and pain modulation.

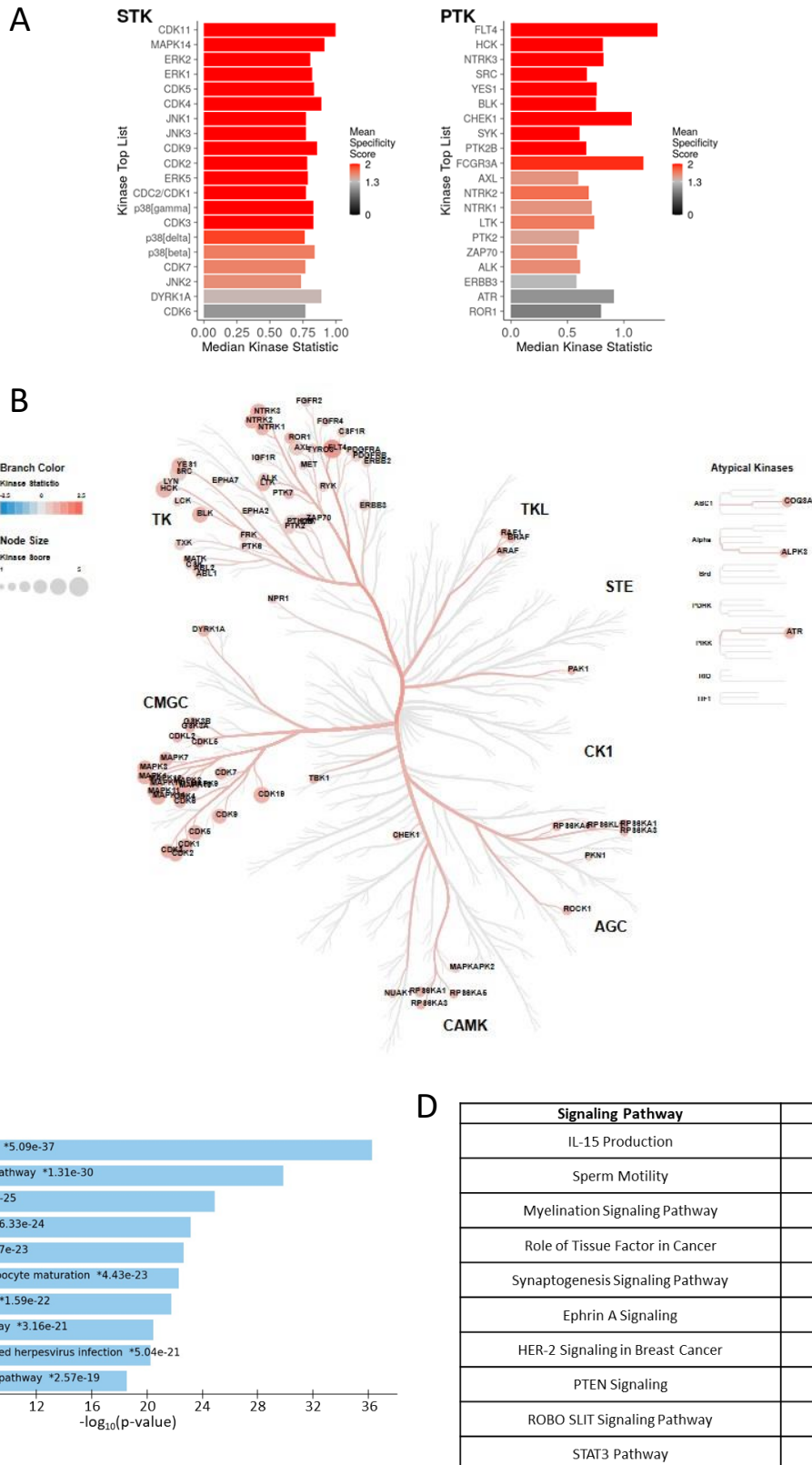


Figure 39: Changes in kinase activity and associated pathways in the PI of males. (A) Kinase specificity score plot highlighting the top 20 kinases, their effect size, and direction. (B) Kinome phylogenetic tree representing all kinases with a final score above 1.3 (default threshold). Branch color and node size are encoded based on the kinase statistic (red: upregulation, blue: downregulation) and final scores, respectively. Signals are expressed as \log_2 -fold change. (C) Top 10 canonical pathways from KEGG 2021 Human gene set library displayed based on the –

$\log_{10}(p\text{-value})$ with their corresponding p -value ($p < 0.05$). (D) Top 10 canonical pathways from IPA software displayed based on the $-\log(p\text{-value})$ ($p < 0.05$) (5 mice per group).

Table 16: Altered signaling pathways identified by EnrichR and IPA in the PI of 6-OHDA males

Common Signaling Pathways	EnrichR (KEGG software)	IPA Software
MAPK signaling	MAPK signaling pathway Adjusted p-value: 8.71x10 ⁻³⁵	ERK/MAPK signaling p-value: 5.01x10 ⁻¹²
		RAF/MAP kinase cascade p-value: 3.99x10 ⁻¹¹
		Signaling by NTRK1 (TRKA) p-value: 1.17x10 ⁻⁹
		ERK5 signaling p-value: 2.88x10 ⁻⁸
		Signaling by NTRK2 (TRKB) p-value: 4.07x10 ⁻⁷
		Signaling by NTRK3 (TRKC) p-value: 9.54x10 ⁻⁶
		RAF-independent MAPK1/3 activation p-value: 0.001
		p38 MAPK signaling p-value: 0.04
Chemokine signaling	Chemokine signaling pathway Adjusted p-value: 6.58x10 ⁻¹²	IL-15 production p-value: 3.98x10 ⁻³⁸
		IL-13 signaling pathway p-value: 5.01x10 ⁻¹⁵
		IL-8 signaling p-value: 1.26x10 ⁻¹³
		IL-2 signaling p-value: 1.74x10 ⁻¹⁰
		IL-7 signaling pathway p-value: 9.12x10 ⁻¹⁰
		IL-3 signaling p-value: 0.000000001
		CXCR4 signaling p-value: 1.91x 10 ⁻⁷
		IL-22 signaling p-value: 3.47x10 ⁻⁷
		Chemokine signaling p-value: 1.74x10 ⁻⁶
		IL-23 signaling pathway p-value: 5.13x10 ⁻⁶
	IL-33 signaling pathway p-value: 6.46x 10 ⁻⁶	
TGF-beta signaling pathway	IL-6 signaling p-value: 1.70x10 ⁻⁵	

	Adjusted p-value: 0.01	Interleukin-2 family signaling p-value: 2.04×10^{-4}
		IL-27 signaling pathway p-value: 2.88×10^{-4}
		IL-15 signaling p-value: 3.09×10^{-4}
		IL-10 signaling p-value: 5.75×10^{-4}
		IL-17 signaling p-value: 0.01
	TNF signaling pathway Adjusted p-value: 1.38×10^{-10}	IL-4 signaling p-value): 0.01
		Interleukin-4 and Interleukin-13 signaling p-value: 0.03
		TGF- β signaling p-value: 0.02
		Interleukin-15 signaling p-value: 0.03
		Interleukin-12 family signaling p-value: 0.047
Neurotrophins	Neurotrophin signaling pathway Adjusted p-value: 1.12×10^{-28}	Neurotrophin/TRK signaling p-value: 1.58×10^{-11}
		NGF signaling p-value: 5.75×10^{-10}
		Signaling by NTRK1 (TRKA) p-value: 1.17×10^{-9}
		CNTF signaling p-value: 2.95×10^{-7}
		Signaling by NTRK2 (TRKB) p-value: 4.07×10^{-7}
		Signaling by NTRK3 (TRKC) p-value: 9.55×10^{-6}
		GDNF family ligand-receptor interactions p-value: 3.80×10^{-5}
Ras signaling	Ras signaling pathway Adjusted p-value: 2.70×10^{-22}	Signaling by NTRK1 (TRKA) p-value): 1.17×10^{-9}
		Protein Kinase A signaling p-value: 4.68×10^{-9}
		Signaling by NTRK2 (TRKB) p-value: 4.07×10^{-7}
		Signaling by NTRK3 (TRKC) p-value: 9.55×10^{-6}
		Rap1 signaling p-value: 7.08×10^{-4}
ErbB signaling	ErbB signaling pathway	Signaling by ERBB2

	Adjusted p-value: 3.87x10 ⁻²¹	p-value: 3.98x10 ⁻¹⁵
		ERB2-ERBB3 signaling p-value: 1.29x10 ⁻⁸
		ERBB signaling p-value: 1.15x10 ⁻⁷
		ERBB4 signaling p-value: 2.45x10 ⁻⁵
PI3K-Akt signaling	PI3K-Akt signaling pathway Adjusted p-value: 6.75x10 ⁻²⁰	PTEN signaling p-value: 1.58x10 ⁻²⁰
		PIP3 activates AKT signaling p-value: 3.16x10 ⁻¹⁹
		PI3K/AKT signaling p-value: 2.00x10 ⁻¹²
		Signaling by NTRK1 (TRKA) p-value: 1.17x10 ⁻⁹
		Signaling by NTRK2 (TRKB) p-value: 4.07x10 ⁻⁷
		PI3K cascade p-value: 4.27x10 ⁻⁶
		Signaling by NTRK3 (TRKC) p-value: 9.55x10 ⁻⁶
		PTEN regulation p-value: 0.006
Focal adhesion	Focal adhesion Adjusted p-value: 5.26x10 ⁻¹⁸	FAK signaling p-value: 2.51x10 ⁻¹³
		Paxillin signaling p-value: 0.03
FoxO signaling	FoxO signaling pathway Adjusted p-value: 2.74x10 ⁻¹⁵	FOXO-mediated transcription p-value: 1.35x10 ⁻⁵
		FOXO-mediated transcription of cell cycle genes p-value: 0.04
Relaxin signaling	Relaxin signaling pathway Adjusted p-value: 2.70x10 ⁻¹¹	Relaxin signaling p-value: 4.07x10 ⁻⁵
Axon guidance	Axon guidance Adjusted p-value: 1.86x10 ⁻¹³	Ephrin A signaling p-value: 6.31x10 ⁻²³
		ROBO SLIT signaling pathway p-value: 7.94x10 ⁻²⁰
		Axonal guidance signaling p-value: 3.98x10 ⁻¹⁵
		Ephrin receptor signaling p-value: 2.51x10 ⁻¹²
		EPH-Ephrin signaling p-value: 7.94x10 ⁻¹¹
		Semaphorin neuronal repulsive signaling pathway

		p-value: 1.17×10^{-10}
		WNT/SHH axonal guidance signaling pathway p-value: 3.24×10^{-9}
		Netrin signaling p-value: 1.05×10^{-8}
		Netrin-1 signaling p-value: 2.57×10^{-4}
		Ephrin B signaling p-value: 7.59×10^{-4}
		Semaphorin interactions p-value: 0.01
		Signaling by ROBO receptors p-value: 0.03
Actin cytoskeleton	Regulation of actin cytoskeleton Adjusted p-value: 2.82×10^{-11}	RHOA signaling p-value: 0.04
Calcium signaling	Calcium signaling pathway Adjusted p-value: 1.15×10^{-9}	Calcium signaling p-value: 0.02
		Presynaptic depolarization and calcium channel opening p-value: 0.03
Gap junctions	Gap junctions Adjusted p-value: 8.57×10^{-9}	Gap junction signaling p-value: 0.03
Dopaminergic system	Dopaminergic synapse Adjusted p-value: 1.09×10^{-8}	Dopamine-DARPP32 feedback in cAMP signaling p-value: 0.01
Synaptic plasticity	Long-term depression Adjusted p-value: 1.37×10^{-8}	Synaptogenesis signaling pathway p-value: 6.31×10^{-25}
		CREB signaling in neurons p-value: 5.01×10^{-19}
		Reelin signaling in neurons p-value: 7.94×10^{-16}
		L1CAM interactions p-value: 2.51×10^{-8}
	Long-term potentiation Adjusted p-value: 2.77×10^{-8}	Synaptic long-term depression p-value: 9.55×10^{-6}
		Synaptic long-term potentiation p-value: 1.91×10^{-5}
		NCAM signaling for neurite outgrowth p-value: 4.68×10^{-4}
		Reelin signaling pathway p-value: 0.01

Phagocytosis	Fc gamma R-mediated phagocytosis Adjusted p-value: 1.69x10 ⁻⁸	Fcgamma receptor (FCGR) dependent phagocytosis p-value: 2.51x 10 ⁻⁶
cAMP signaling	cAMP signaling pathway Adjusted p-value: 5.20x10 ⁻⁸	cAMP-mediated signaling p-value: 1.17x10 ⁻⁷
		Dopamine-DARPP32 feedback in cAMP signaling p-value: 0.01
mTOR signaling	mTOR signaling pathway Adjusted 5.34x10 ⁻⁷	MTOR signaling p-value: 4.47x10 ⁻⁵
p53 signaling	p53 signaling pathway Adjusted p-value: 1.17x10 ⁻⁶	p53 signaling p-value: 4.47x10 ⁻⁶
		Regulation of TP53 activity through association with co-factors p-value: 5.13x10 ⁻⁶
		Regulation of TP53 activity through acetylation p-value: 5.62x10 ⁻⁵
		Regulation of TP53 expression and degradation p-value: 1.23x10 ⁻⁴
		TP53 regulates metabolic genes p-value: 0.001
		TP53 regulates transcription of cell cycle genes p-value: 0.007
Apoptosis	Apoptosis Adjusted p-value: 4.05x10 ⁻⁶	Apoptosis signaling p-value: 5.89x10 ⁻⁶
		Intrinsic pathway for apoptosis p-value: 1.05x10 ⁻⁵
		Apoptotic execution phase p-value: 2.88x10 ⁻⁴
Wnt signaling	Wnt signaling pathway Adjusted p-value: 1.14x10 ⁻⁴	WNT/SHH axonal guidance signaling pathway p-value: 3.24x10 ⁻⁹
		WNT/ β -catenin signaling p-value: 7.08x10 ⁻⁵
Serotonergic system	Serotonergic synapse Adjusted p-value: 1.76x10 ⁻⁴	Serotonin receptor signaling p-value: 5.01x10 ⁻¹⁷
NF-kappa B signaling	NF-kappa B signaling pathway Adjusted p-value: 0.001	NF- κ B signaling p-value: 3.98x10 ⁻¹³
		BEX2 signaling pathway p-value: 5.37x10 ⁻⁸
Apelin	Apelin signaling pathway Adjusted p-value:	Apelin endothelial signaling pathway

	0.03	p-value: 2.63×10^{-5}
JAK-STAT signaling	JAK-STAT signaling pathway Adjusted p-value: 0.04	STAT3 pathway p-value: 2.00×10^{-19}
		JAK/STAT signaling p-value: 5.37×10^{-8}
		Role of JAK1, JAK2 and TYK2 in interferon signaling p-value: 0.002
		Role of JAK2 in Hormone-like cytokine signaling p-value: 0.01
		Role of JAK1 and JAK3 in γ c cytokine signaling p-value: 0.01
		STAT5 activation p-value: 0.02

Our phosphoproteomic experiments indicate an overall upregulation in the activity of serine-threonine and tyrosine kinases in the ACC and PI of 6-OHDA males and the PI of 6-OHDA females. Serine-threonine enzymes presented increased activity in the ACC of 6-OHDA females, unlike tyrosine kinases which were downregulated. Overall, these enzymatic modifications were related to changes in canonical processes such as axon guidance, chemokine and neurotrophin signaling, inflammatory cascades, and serotonergic receptors.

3.6 Study of neuronal-astrocytic interactions

The study of astrocyte morphology has demonstrated cellular reactivity in the ACC of both 6-OHDA males and females. The mRNA levels of GFAP, an intermediate filament only expressed by these cells and upregulated during reactive astrogliosis, were increased in the ACC and PI of 6-OHDA mice thus confirming cellular reactivity. Other inflammatory mediators secreted by astrocytes were also shown to be overexpressed in ADHD-like conditions. Pathway enrichment analysis has identified impairments in numerous processes in which astrocytes play key roles. Taken together, these observations suggest the critical involvement of these glial cells in ADHD-like conditions.

To better comprehend the crosstalk between astrocytes and neurons in ADHD-like conditions, we have used the FRET-mFLIM technique which enables the study of protein interactions whose distances vary from 1 to 10nm. To do so, we have targeted two molecules expressed by each cell type: EphA3, found on astrocytes, and EphA4, expressed by neurons. The FRET efficiency is indicative of protein interactions: the higher the FRET efficiency, the more numerous the interactions.

The areas of coincidence between EphA3 and EphA4 are illustrated in Figure 40. Each pixel within the co-occurrence region represents the median of FRET efficiency. This value ranges from 0% (purple, low or no interactions) to 50% (yellow, high interactive states), as shown in the heatmap. Our results demonstrated an increased coincidence of EphA3 and EphA4 in the ACC of the 6-OHDA mouse (Figure 40, B) compared to controls (Figure 40, A). This is supported by the FRET efficiency values in 6-OHDA mice (25.44-30.31%) (Figure 40, D) which are significantly higher than those observed in the sham animal (0.99-3.69%) (Figure 40, C). Taken together, our findings report increased communication between astrocytes and neurons in ADHD-like conditions.

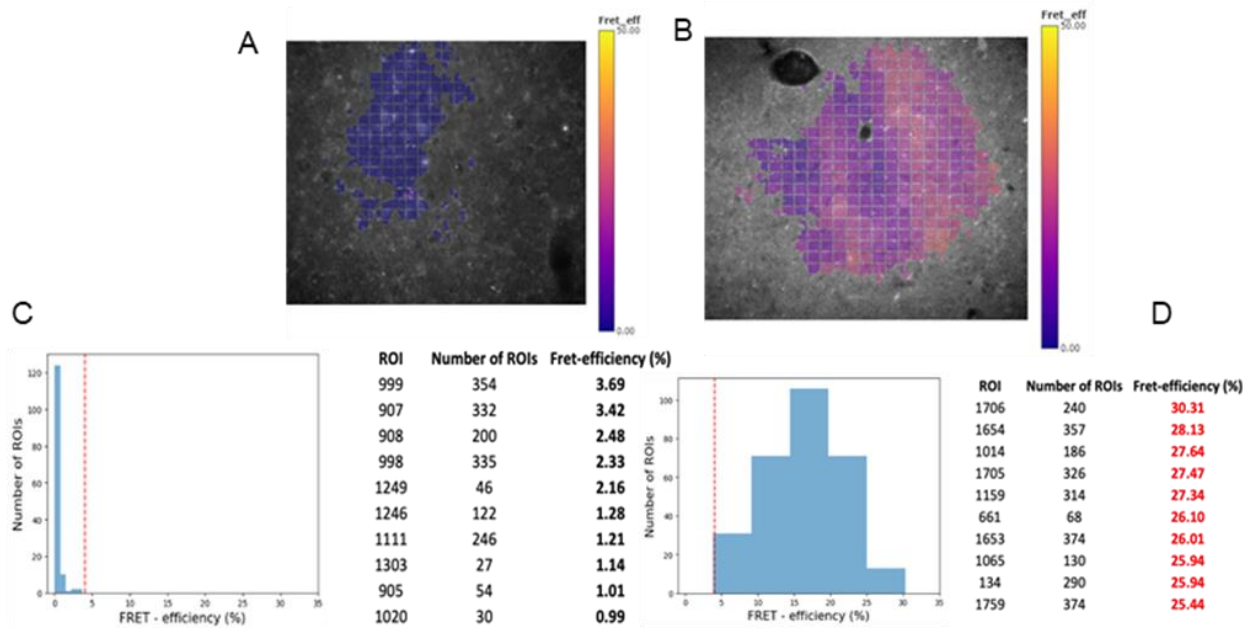


Figure 40: EphA3 and EphA4 interactive states in ADHD-like conditions. Regions of EphA3 and EphA4 co-occurrence in the ACC of sham (A) and 6-OHDA (B) mice. Number of regions of interest (areas of co-occurrence) and their associated FRET efficiency in sham (C) and 6-OHDA (D) mice animals (3 mice per group).

3.7 Results summary

Despite the increased prevalence and disabling consequences of ADHD and chronic pain, the mechanisms underlying their comorbidity are still poorly understood. Growing evidence suggests the role of neuroinflammation in the etiology of these two disorders. Therefore, our work aimed to elucidate the involvement of neuroinflammatory mechanisms in their co-occurrence.

The morphological study of microglia and astrocytes has indicated changes in cellular shape in the ACC of 6-OHDA mice; a remodeling that usually accompanies cellular reactivity. This discovery has led us to investigate possible modifications in the physiological functions of CNS cells. Consequently, we have examined the ACC and PI for potential alterations in inflammatory markers and have demonstrated impairments in their expression levels. These were characterized by an upregulation of pro-inflammatory mediators and a downregulation of molecules with anti-inflammatory properties. Overall, these observations attested to a pro-inflammatory environment in the ACC and PI of 6-OHDA males and females depicted by (i) glial reactivity, (ii) release of pro-inflammatory mediators, and (iii) inhibition of neuroprotective markers.

For a more in-depth understanding of the molecular mechanisms, we have searched for changes in protein expression and activity in the ACC and PI of 6-OHDA mice. High-throughput screening methods (mass spectrometry, phosphoproteomic assays) and pathway enrichment analysis have detected abnormalities in several immune and neuronal processes that could result in neurodevelopmental deficits and chronic pain conditions (pain sensitization).

Interestingly, astrocytes actively participate in the pathways whose alteration was pointed out in the ACC and PI of 6-OHDA mice. Thus, the study of changes in neuronal-astrocytic communication was of interest to us. Our investigations have shed light on modifications in protein interactions and, therefore, neuronal-astrocytic communication in ADHD-like conditions. Our results support increased interactions between these two cell types in 6-OHDA mice. However, even though neuronal-astrocytic crosstalk is necessary for CNS homeostasis and normal functioning, its disruption can have detrimental consequences such as

excitotoxicity, oxidative stress, neurodegeneration, and the development of neurological disorders (Benarroch, 2005).

As our main objective was to identify specific pathways that may underlie the comorbidity between ADHD and its associated pain sensitization, we integrated the data obtained from all different techniques and deduced candidate processes. The results for the ACC and PI of 6-OHDA males and females are shown below.

3.7.1 Altered pathways in the ACC of 6-OHDA females

Reactive astrocytes and microglial cells are predominant in the ACC of 6-OHDA females. This reactivity is accompanied by morphological changes, dictated by a reorganization of their cytoskeleton, and the release of pro-inflammatory mediators.

The activation of surface receptors, such as TLR7 (Michaelis et al., 2019), stimulates the expression of transcription factors in microglial cells. These include IRF5 (Kaur et al., 2018), a pro-inflammatory mediator associated with microglial reactivity (Al Mamun et al., 2020; Z. Fan et al., 2020). Both TLR7 and IRF5 promote the release of cytokines (IL-6 and TNF- α), also produced by reactive astrocytes (Al Mamun et al., 2020; Z. Fan et al., 2020; Kaur et al., 2018; Michaelis et al., 2019). Not only are IL-6 and TNF- α secreted by activated glial cells, but they also act in concert to (i) intensify cellular activation, and (ii) inhibit BDNF release (Calabrese et al., 2014). BDNF is a neurotrophin involved in a wide array of processes such as synaptic plasticity, learning and memory, axonal and neurite growth, and neurogenesis (Bathina & Das, 2015; Benraiss et al., 2001; Cohen-Cory et al., 2010; Cunha, 2010; S. W. Kim & Kim, 2020; K. Yamada et al., 2002; Zigova et al., 1998).

On the contrary, TNF- α stimulates netrin and p53 expression which participate in axonal and neurite growth, axon guidance, and neurogenesis (Di Giovanni et al., 2006; W. S. Lee et al., 2019; Quadrato & Di Giovanni, 2012; X. Shao et al., 2020). p53 is the main positive regulator of cell death (J. Chen, 2016). It acts as an antagonist of NF- κ B (Gudkov et al., 2011; Komarova et al., 2005), which exhibits both pro- and anti-apoptotic functions depending on the cell type and stimulus (Baichwal & Baeuerle, 1997; Mendes et al., 2017). NF- κ B can be negatively regulated by sirtuins, a family of enzymes displaying anti-apoptotic and anti-inflammatory properties (Pan et al., 2022), and can inhibit BAG2 (Bcl-2-associated athanogene), which works to inhibit cell death (L. Qin et al., 2016). Other molecules can modulate apoptotic processes, such as TLR7 and IRF5. TLR7 signaling supports neuronal cell death (Cooks et al., 2014; Michaelis et al., 2019) while IRF5, whose expression is stimulated by p53, was found to intensify apoptosis (Kaur et al., 2018; Yanai et al., 2012).

Finally, microglial and astrocytic reactivity are both regulated by TGF- β . This anti-inflammatory cytokine controls microglial cell maturation, homeostasis, and activation. It also plays a key role in the protection and rescue of dopaminergic neurons by inhibiting microglial reactivity and its mediated neurotoxicity (Spittau et al., 2020). Furthermore, TGF- β is a fundamental modulator of reactive astrogliosis with its effect being highly dependent on the context (J. Luo, 2022).

It is important to be reminded that, at physiological levels, pro-inflammatory cytokines participate in CNS homeostasis and development. For example, IL-6 is involved in neuronal excitability and function (Kummer et al., 2021).

The data is summarized in Figure 41. Based on the scheme, we suggest that apoptosis and axon guidance are the main pathways affected in the ACC of 6-OHDA females. Interestingly, our mass spectrometry results point to alterations in these two processes. An increase in the apoptotic regulatory pathway (z-

score: 1) and a slight reduction in sirtuin signaling (z-score: -0.44721) were reported. As for axon guidance, both the netrin signaling (z-score: 1.632993) and the Hedgehog “off” state (z-score: 1) were enhanced.

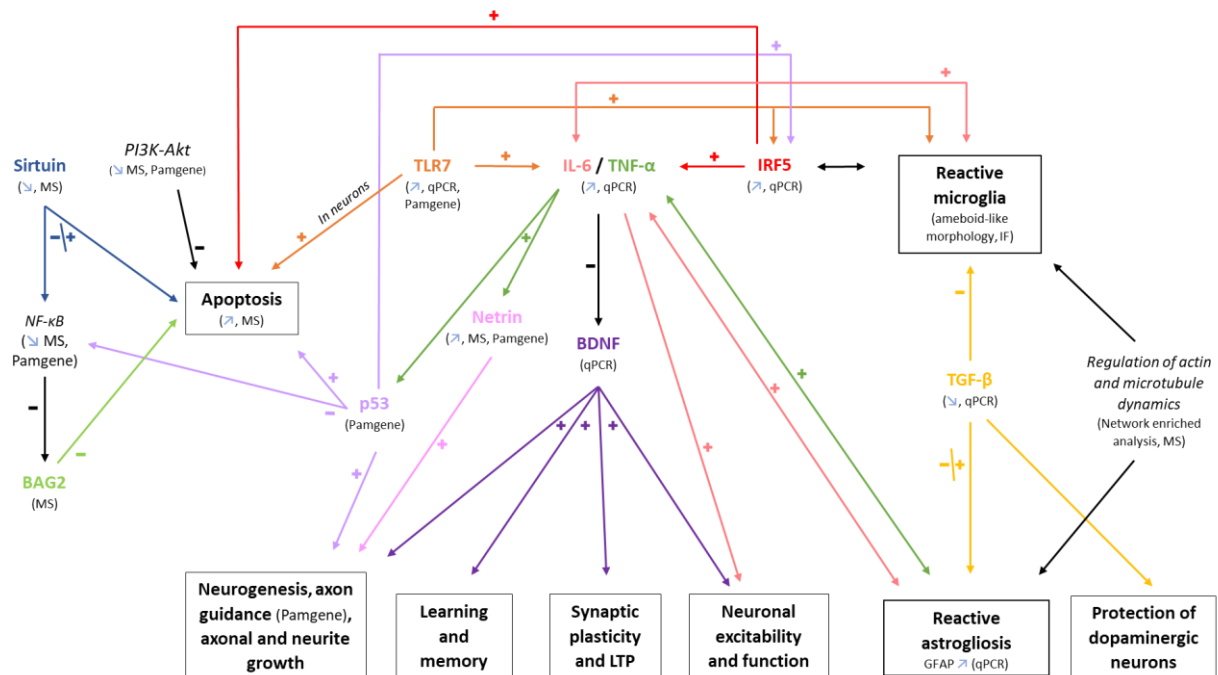


Figure 41: Schematic summary of the altered markers and their associated pathways in the ACC of 6-OHDA females.

3.7.2 Altered pathways in the ACC of 6-OHDA males

Reactive astrogliosis was reported in the ACC of 6-OHDA males and was associated with inflammatory marker release.

IL-1 β is among the secreted molecules by astrocytes and microglial cells, notably following NLRP3 activation (L. Song et al., 2017; Tsai, 2017). Based on its expression levels, this pro-inflammatory interleukin participates in numerous physiological and pathological processes. Homeostatic levels of IL-1 β promote neurite outgrowth through neurotrophin-3 and neurogenin-1 production (S. Y. Park et al., 2018). It enhances mechanisms of synaptic plasticity, such as LTP (Tsai, 2017), and neurotransmitter (serotonin, dopamine, norepinephrine) release (Plata-Salaman, 2002). Moreover, it activates signaling pathways (NF- κ B, Wnt5a) that participate in neuronal proliferation, survival, and differentiation (S. Y. Park et al., 2018; Tsai, 2017). On the other hand, abnormally high levels of IL-1 β stimulate astrocytic and microglial reactivity (Mendiola & Cardona, 2018), induce apoptosis through glutamate release and accumulation (Mendiola & Cardona, 2018; Tsai, 2017), negatively affect learning and memory (Shaftel et al., 2008), inhibit BDNF release (Calabrese et al., 2014; Lima Giacobbo et al., 2019), and promote CCL2 production (Joly-Amado et al., 2020; Semple et al., 2010).

CCL2 is a chemokine secreted by reactive astrocytes. It amplifies astrocytic arborization, causes changes in actin cytoskeleton structure, exacerbates microglial activation, and contributes to astrocytic and microglial chemotaxis. It also acts on the dopaminergic system by directing neurons towards a dopaminergic phenotype, increasing dopaminergic neurons' excitability, and amplifying neurotransmitter release (Bose & Cho, 2013; Joly-Amado et al., 2020; Semple et al., 2010).

In normal conditions, the immune response is attenuated by anti-inflammatory molecules to minimize neuronal loss and damage. IL-13, whose expression was decreased in the ACC of 6-OHDA males, displays

protective properties. These include inhibition of inflammatory molecule release, suppression of microglial reactivity, stimulation of learning and memory, and induction of BDNF synthesis by astrocytes to improve cognitive functions (Brombacher et al., 2017; Miao et al., 2020; Mori et al., 2016).

BDNF is another anti-inflammatory mediator whose downregulation was observed in the ACC of 6-OHDA males. Interestingly, pro-inflammatory cytokines prevent BDNF release (Calabrese et al., 2014). This neurotrophic factor maintains CNS homeostasis and normal functioning. It promotes learning, memory, and signaling cascades (PI3K-Akt, MAPK, and PKC) leading to (i) LTP, synaptic plasticity, and synaptic transmission (Bathina & Das, 2015; Kowiański et al., 2018; Silakarma & Sudewi, 2019; K. Yamada et al., 2002), (ii) inhibition of apoptosis (Lima Giacobbo et al., 2019; Sheikh et al., 2010), (iii) cytoskeleton development, and (iv) neurogenesis, dendritic branching, in addition to dendritic, neurite, and neuronal fibers growth (Bathina & Das, 2015; Benraiss et al., 2001; Cohen-Cory et al., 2010; Cunha, 2010; S. W. Kim & Kim, 2020; Kowiański et al., 2018; Zigova et al., 1998).

The data is summarized in Figure 42. Based on the scheme, we suggest that pathways related to synaptic plasticity (LTP) and the growth of neuronal components are impaired in the ACC of 6-OHDA males. Interestingly, our findings from the PamGene assays point to alterations in these two processes. More importantly, their positive mediators (BDNF, PKA) were found to be downregulated. IL-1 β , another molecular contributor, presented abnormally elevated levels of expression thus suggesting detrimental consequences on these physiological functions.

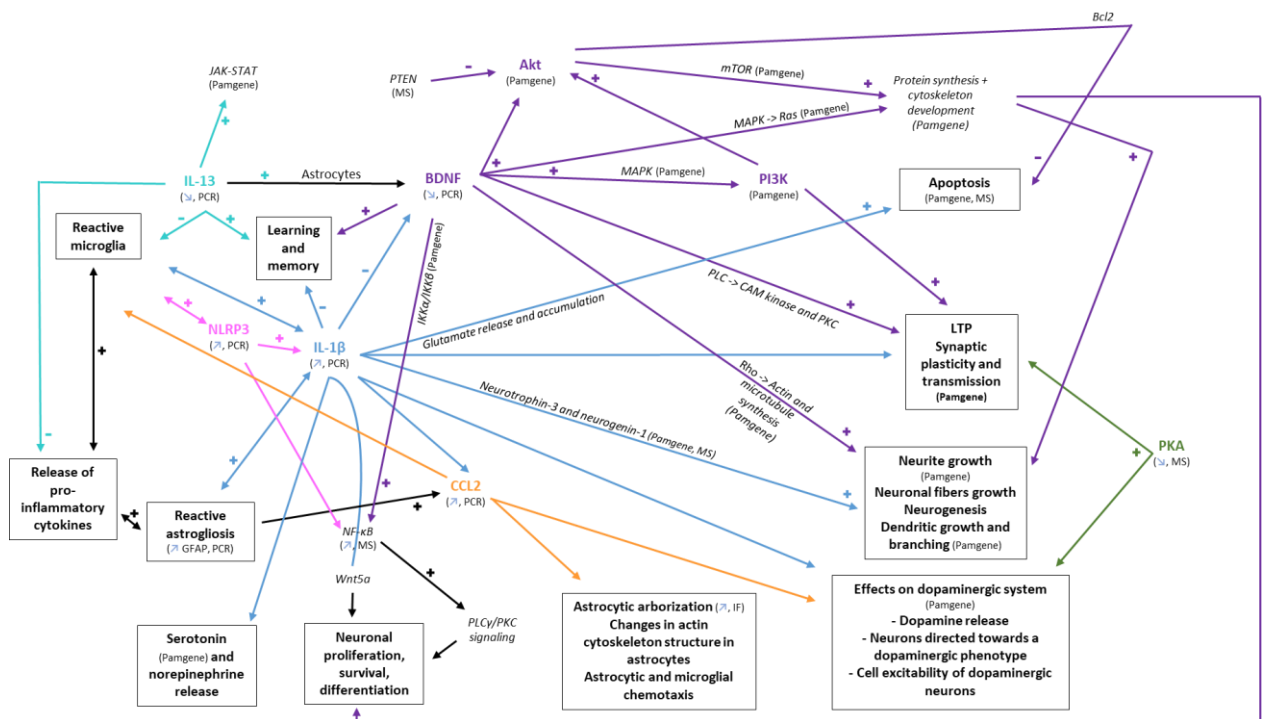


Figure 42: Schematic summary of the altered markers and their associated pathways in the ACC of 6-OHDA males.

3.7.3 Altered pathways in the PI of 6-OHDA females

In the PI of 6-OHDA females, an intensified expression of pro-inflammatory markers hints at glial cell reactivity. Reactive astrocytes and microglia express inflammatory mediators (TLR7, IL-1 β , CCL4, GFAP, MMP9, Wnt5a, NF- κ B, and tPA) to amplify cellular reactivity, enhance inflammatory marker release, and intensify the immune response.

As mentioned previously, IL-1 β possesses a range of physiological and pathological properties. It

reinforces synaptic plasticity and LTP (Tsai, 2017), stimulates the release of neurotransmitters (serotonin, dopamine, and norepinephrine) (Plata-Salaman, 2002), and promotes neuronal processes (S. Y. Park et al., 2018; Tsai, 2017). However, it also inhibits BDNF synthesis (Calabrese et al., 2014; Lima Giacobbo et al., 2019), activates NF- κ B (S. Y. Park et al., 2018), hampers learning and memory (Shaftel et al., 2008), and induces apoptosis through the extensive release of glutamate (Mendiola & Cardona, 2018; Tsai, 2017). TLR7 also enhances neuronal cell death (Cooks et al., 2014; Michaelis et al., 2019), an inducer of IL-1 β production (Michaelis et al., 2019), and p53, its main positive regulator (J. Chen, 2016).

Some activators of the p53 signaling pathway include APE1 and AMPK (Oliveira et al., 2022; Salminen et al., 2011). Together with AMPK, p53 inhibits NF- κ B (Salminen et al., 2011). On the contrary, the APE1 and tPA enzymes can display harmful effects by stimulating this cascade (Lin & Hu, 2014; Oliveira et al., 2022). NF- κ B induces MMP9 and Wnt5a expression which, at normal levels, participate in learning, memory, synaptic plasticity, the establishment of functional connectivity, neurogenesis, axonal and neurite outgrowth (Halleskog et al., 2012; Könnecke & Bechmann, 2013; Mehra et al., 2016; S. Y. Park et al., 2018; Vafadari et al., 2016; Yeh et al., 2023). The implication of BDNF in these physiological functions has been reported (Bathina & Das, 2015; Benraiss et al., 2001; Cohen-Cory et al., 2010; Cunha, 2010; S. W. Kim & Kim, 2020; K. Yamada et al., 2002; Zigova et al., 1998). Apelin and IL-10 exert anti-inflammatory effects by suppressing glial cell reactivity and inflammatory molecule expression (Q. Liu et al., 2022; Lobo-Silva et al., 2016; Porro et al., 2020).

The data is summarized in Figure 43. Abnormalities in synaptic plasticity and apoptosis are evident in the PI of 6-OHDA females. Our PamGene results support alterations in these two processes. Interestingly, TLR7 and IL-1 β are upregulated in this brain area indicating possible irregularities in cell death. MMP9, IL-1 β , and Wnt5a also display increased levels of expression. These molecules mediate synaptic plasticity; modifications in their expression could therefore impair physiological functions.

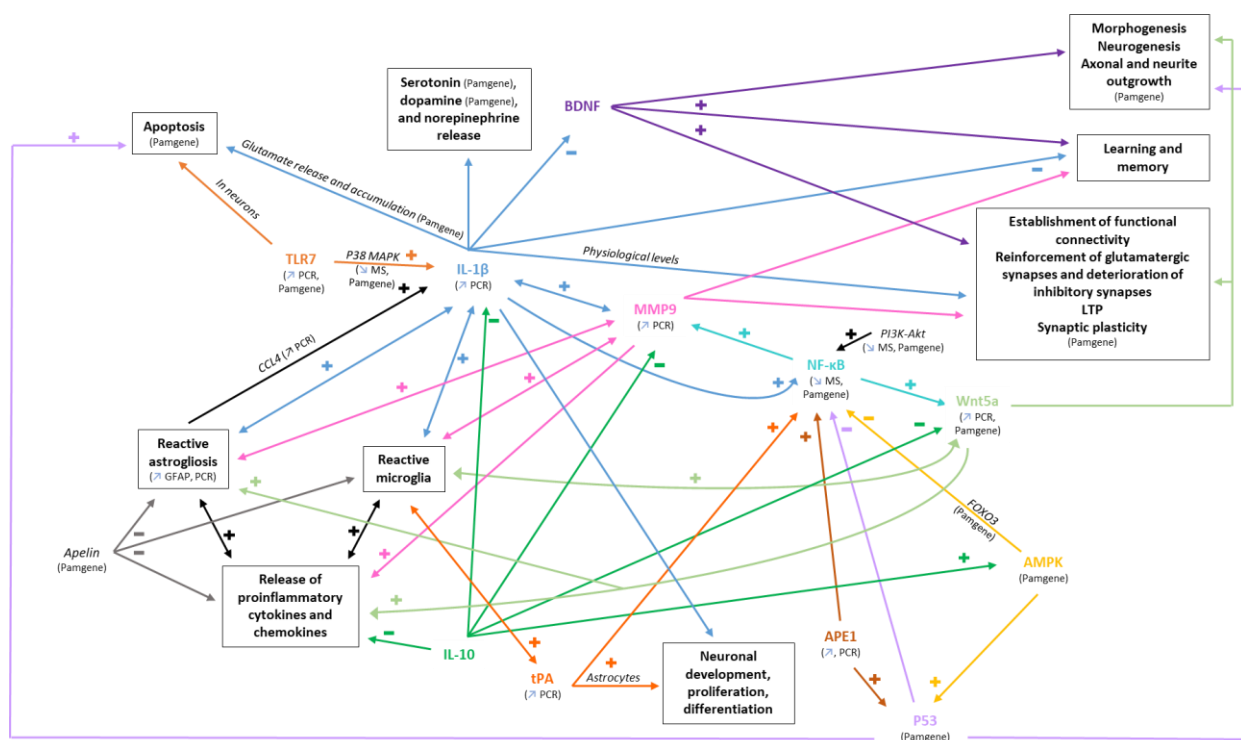


Figure 43: Schematic summary of the altered markers and their associated pathways in the PI of 6-OHDA females.

3.7.4 Altered pathways in the PI of 6-OHDA males

Glial cell reactivity is prominent in the PI of 6-OHDA males, notably due to the increase in Wnt5a, IRF5, and GFAP expressions. Reactive astrocytes and microglia express inflammatory mediators (IL-1 β , IL-6, IRF5, Wnt5a) to amplify the immune response by (i) increasing cellular reactivity, (ii) promoting pro-inflammatory cytokine release, and (iii) inhibiting the production of mediators with neuroprotective effects (such as BDNF) (Lima Giacobbo et al., 2019; Sochocka et al., 2017).

Not only does Wnt5a possess beneficial properties (neurogenesis, morphogenesis) (Halleskog et al., 2012), but it also exerts harmful effects through the induction of glial cell reactivity and inflammatory mediator release (Kumawat & Gosens, 2016; X. Liu et al., 2023).

IRF5, IL-1 β , and p53 signaling promote apoptosis (Kaur et al., 2018; Ozaki & Nakagawara, 2011; Tsai, 2017; Yanai et al., 2012) while the Akt cascade, activated by BDNF, constitutes one of its negative regulators (Sheikh et al., 2010). Nevertheless, physiological activation and expression of IL-1 β , IL-6, and p53 are involved in LTP, synaptic plasticity, neuronal excitability and function, neuronal proliferation and differentiation, neurite and neuronal fiber outgrowth, dendritic growth and branching, morphogenesis, and neurogenesis (Di Giovanni et al., 2006; Kummer et al., 2021; S. Y. Park et al., 2018; X. Shao et al., 2020; Tsai, 2017). p53 also acts as an NF- κ B antagonist and therefore, as a regulator of the inflammatory response (Gudkov et al., 2011; Komarova et al., 2005).

As previously described, BDNF exerts a myriad of functions to sustain CNS homeostasis and normal functioning. It stimulates several signaling pathways (PI3K-Akt, MAPK, PKC, NF- κ B) thus reinforcing learning, memory, synaptic plasticity, cytoskeleton remodeling, outgrowth of neuronal components, and neurogenesis (Kowiański et al., 2018; Lima Giacobbo et al., 2019; K. Yamada et al., 2002). Finally, TGF- β and IL-10, two anti-inflammatory molecules, work to suppress glial cell reactivity and pro-inflammatory cytokines release (Lobo-Silva et al., 2016; J. Luo, 2022; Porro et al., 2020; Spittau et al., 2020).

The data is summarized in Figure 44. Abnormalities in synaptic plasticity, cell death, and neuronal outgrowth are prevalent in the PI of 6-OHDA males. Remarkably, our PamGene assay findings support modifications in these processes. Moreover, IL-1 β , IRF5, wnt5a, and IL-6 are overexpressed in this cortical region suggesting impairments in the apoptotic, neuronal, immune, and synaptic plasticity processes in which they are involved.

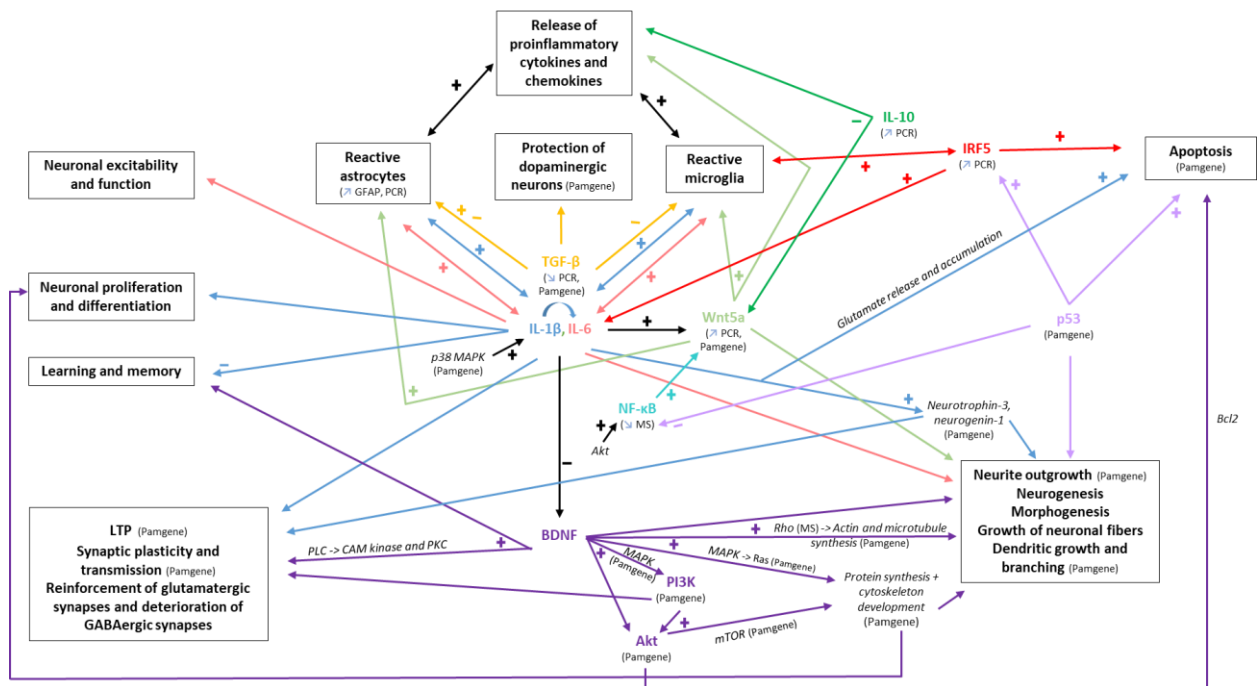


Figure 44: Schematic summary of the altered markers and their associated pathways in the PI of 6-OHDA males.

Our findings indicate a sustained pro-inflammatory environment in the ACC and PI of 6-OHDA male and female mice which, we hypothesize, is triggered by changes in neurotransmitter systems (for instance, the dopaminergic system). Neurodevelopmental problems could modify brain homeostasis and establish a novel environment characterized by glial reactivity. This change in inflammatory status modifies pathways involved in axon guidance, apoptosis, synaptic plasticity, and growth of neuronal components.

3.8 Role of the P2X₄ receptor

The purinergic signaling acts at the crossroads of pain and inflammation. More particularly, the P2X₄R has been implicated in pain sensitization and the activation of several inflammatory signaling pathways, including the NLRP3 inflammasome. This P2X₄R dual function has led us to investigate its role in the comorbidity between ADHD and pain sensitization.

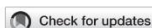
To address this question, transgenic mice carrying a global deletion of the receptor (P2X₄KO) and wild-type mice were injected with either 6-OHDA or ascorbic acid. The effects of the P2X₄R on pain sensitization and ADHD symptoms were tested using behavioral procedures. Locomotor hyperactivity was assessed using the open-field test while pain sensitization toward thermal and mechanical stimuli was measured using the plantar and Von Frey tests, respectively. Moreover, to decipher the impact of the P2X₄R on neuroinflammation in ADHD-like conditions, changes in microglia morphology and alterations in the expression levels of inflammatory mediators were studied in the ACC and PI.

The detailed results can be found in the article attached below. Briefly, our publication demonstrated that P2X₄R deletion (i) prevented locomotor hyperactivity, (ii) does not participate in thermal and mechanical pain sensitization, (iii) limited changes in microglia morphology thus modulating cellular reactivity, and (iv) promoted a predominantly pro-inflammatory environment in the ACC and PI. Interestingly, P2X₄R knockout also reduced the thermal pain threshold in basal conditions.

In conclusion, our findings suggest that the P2X₄R worsens ADHD hyperactivity symptom and promotes morphological changes in microglia that correlate with cellular reactivity. Reciprocally, it exerts a protective effect by reducing the release of pro-inflammatory mediators, possibly from non-microglial

cells. However, it does not participate in pain sensitization to mechanical and thermal stimuli.

Finally, the screening of inflammatory markers in the ACC and PI of 6-OHDA males and females has identified changes in numerous mediators that were also found to be impaired in 6-OHDA wild-type mice compared to shams. These include IL-6, IRF5, GFAP, Arg1, NLRP3, and TGF- β (Chapter 3, RT-qPCR results). This concordance further supports the role of the P2X₄ receptor in regulating inflammatory marker release in ADHD-like conditions.



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P2X4 signalling contributes to hyperactivity but not pain sensitization comorbidity in a mouse model of attention deficit/hyperactivity disorder

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Introduction: Attention deficit/hyperactivity disorder (ADHD) is a common neurodevelopmental disorder characterized by hyperactivity, inattention, and impulsivity that often persist until adulthood. Frequent comorbid disorders accompany ADHD and two thirds of children diagnosed with ADHD also suffer from behavioural disorders and from alteration of sensory processing. We recently characterized the comorbidity between ADHD-like symptoms and pain sensitisation in a pharmacological mouse model of ADHD, and we demonstrated the implication of the anterior cingulate cortex and posterior insula. However, few studies have explored the causal mechanisms underlying the interactions between ADHD and pain. The implication of inflammatory mechanisms has been suggested but the signalling pathways involved have not been explored.

Methods: We investigated the roles of purinergic signalling, at the crossroad of pain and neuroinflammatory pathways, by using a transgenic mouse line that carries a total deletion of the P2X4 receptor.

Results: We demonstrated that P2X4 deletion prevents hyperactivity in the mouse model of ADHD. In contrast, the absence of P2X4 lowered thermal pain thresholds in sham conditions and did not affect pain sensitization in ADHD-like conditions. We further analysed microglia reactivity and the expression of inflammatory markers in wild type and P2X4KO mice. Our results revealed that P2X4 deletion limits microglia reactivity but at the same time exerts proinflammatory effects in the anterior cingulate cortex and posterior insula.

Conclusion: This dual role of P2X4 could be responsible for the differential effects noted on ADHD-like symptoms and pain sensitization and calls for further studies to investigate the therapeutic benefit of targeting the P2X4 receptor in ADHD patients.

KEYWORDS

attention deficit/hyperactivity disorders, pain sensitization, purines, P2X4, anterior cingulate cortex, posterior insula

Introduction

Attention deficit and hyperactivity disorder (ADHD) is classified in the DSM-IV (Diagnostic and Statistical Manual of mental Disorders) (American Psychiatric Association, 2013) as a heterogeneous neurodevelopmental disorder manifested by varying levels of hyperactivity, impulsivity, and inattention in humans. ADHD is the most common neuropsychiatric disorder with a prevalence of 8%–12% in children (Danielson et al., 2018) that remains steady over the last decades (Polanczyk et al., 2014; Song et al., 2021). ADHD exists as three sub-types: Inattentive, hyperactive-impulsive, or combined, and the T.O.V.A test can discriminate between these sub-types (Greenberg et al., 1991). ADHD continues into adulthood in up to 50% of the patients diagnosed during childhood (Faraone et al., 2006), and up to 80% for patients of the combined subtype of ADHD (van Lieshout et al., 2016). Adult symptoms are variable and may include functional impairments (Simon et al., 2009). The Conners' Adult ADHD Rating Scales (CAARS) is used to determine whether an individual may have ADHD and what is the symptoms severity.

In clinics, ADHD patients report impaired perceptual functions (Fuermaier et al., 2018; Panagiotidi et al., 2018; Dellapiazza et al., 2021) and pain sensitivity (Treister et al., 2015). ADHD patients have increased risks of pain disorders and high prevalence of pain among adults with ADHD can reach up to 80% (Lensing et al., 2013; Stray et al., 2013). ADHD severity positively predicts pain sensitivity in human adolescent males, and it is associated to larger physiological response as measured by skin conductance level (Northover et al., 2015). Accordingly, attentional processes have been shown to regulate pain transmission through the modulation of brain networks (Kucyi et al., 2013) and descending pathways (Villemure and Bushnell, 2002; Sprenger et al., 2012). Reciprocally, chronic pain increases impulsivity (Ko et al., 2013) and induces attentional and cognitive deficits in human patients (Moore et al., 2019; Kasahara et al., 2020) and preclinical animal models (Higgins et al., 2015). Among 153 patients with chronic pain, 72.5% had a CAARS and frequently experience symptoms such as hyperactive behavior and other clinical characteristics of ADHD (Kasahara et al., 2020).

The understanding of the mechanisms underpinning ADHD and pain comorbidity requires to investigate animal models of ADHD with good face, construct and predictive validity. Because alterations of dopamine transmission are considered as significant factors for the progression of the disease (Albrecht et al., 2015), a mouse model of ADHD by lesioning dopaminergic fibers with neonatal intracerebroventricular injection of 6-hydroxydopamine (6-OHDA) was developed and validated (Sontag et al., 2010; Bouchatta et al., 2018; 2020). In addition to hyperactivity, inattention and impulsivity, the 6-OHDA mice exhibited a marked decrease of withdrawal thresholds to thermal and mechanical stimuli, suggesting that ADHD-like conditions increase nociceptive sensitivity (Bouchatta et al., 2022; Meseguer-Beltrán et al., 2023; Sifeddine et al., 2023). These previous studies indicated that ADHD and pain sensitization are mutually worsening comorbid disorders. They also pointed to a key role of the anterior cingulate cortex (ACC)

hyperactivity that alters the 'ACC-posterior insula (PI)' circuit, and triggers pain sensitization (Bouchatta et al., 2022).

ADHD patients display a large heterogeneity in symptoms and cognitive performances (Castellanos and Tannock, 2002; Nigg et al., 2005). The exact origin of this heterogeneity remains unknown. However, it has been suggested that inconsistent neurotransmission in neural circuits that engage the prefrontal cortex may account for fluctuations in attentional processing and cognitive performance and hence, for intra-individual variability (Russell et al., 2006). Neurotransmission impairments can be caused by alterations in neurotransmitter release or binding (Posner et al., 2020), neuroglia interactions (Oades et al., 2010; Zhang et al., 2022), or neuroinflammatory processes (Lefa et al., 2018; Corona, 2020).

Purinergic signalling influences numerous physiological processes including neuromodulation, synaptic plasticity, neuroinflammation and neuron-glia communication and is also involved in major CNS disorders. ATP is released by neurons as well as by astrocytes or microglia and activates different P2 receptors and adenosine receptors after extracellular ATP catabolism by ectonucleotidases expressed in various cell types (Rodrigues et al., 2015). In many pathological conditions, the increase of ATP release exacerbates ATP signalling promoting astrogliosis, activation of microglia and inflammatory responses (Rodrigues et al., 2015). Among ATP P2 receptors, P2X receptors are trimeric ATP-gated cation channels made from 7 different subunits (P2X1–P2X7) encoded by 7 genes in mammals. P2X4 is the main subunit in neurons and microglia, and possibly astrocytes (Suurväli et al., 2017). It is the most widely distributed P2X receptor in the brain and is likely to modulate cortical circuits that underpin attentional and cognitive processes as well as pain perception. Since P2X4 is involved in various neurological and psychiatric disorders (Lindberg et al., 2015; Duveau et al., 2020; Montilla et al., 2020), we made the hypothesis that P2X4 could play an important role in ADHD symptoms and comorbid pain by modulating microglia activation and neuronal transmission in the ACC and/or the PI.

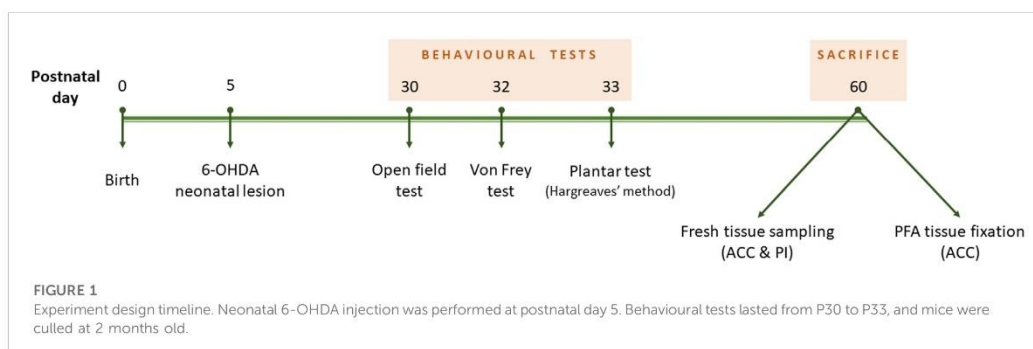
To address this question, we generated an ADHD-like model by neonatal injection of 6-OHDA in a mouse line carrying a global deletion of the P2X4 receptor (P2X4KO) (Sim et al., 2006). To identify possible neural pathways that are influenced by P2X4 signalling in the ADHD model, we explored the expression of neuroinflammatory and microglial activation markers in the ACC and PI of 6-OHDA wild-type (WT) and P2X4KO mice. We further measured hyperactivity, the most common symptom of ADHD, and assessed pain sensitization.

Materials and methods

The time course of the experiments is described in Figure 1.

Animals

C57BL/6 (Janvier Labs, France) and P2X4KO (EOPS facility, Bordeaux Neurocampus) pregnant females and their newborn pups were housed under a 12 h light/dark cycle (lights on at 7 a.m.), provided with water and food *ad libitum*, at the animal facility of the University of Bordeaux. The study was approved by the local ethical



committee and the French Ministry of Higher Education, Research and Innovation (authorization APAFIS#27681 and APAFIS#21135). Procedures were conducted in conformity with the approved institutional protocols. Efforts were made to reduce animal suffering.

6-OHDA neonatal injection (P5 surgery)

At postnatal day 5 (P5), male and female pups were submitted to hypothermal anesthesia and received an intracerebroventricular injection (AP -2 mm, ML ±0.6 mm, DV -1.3 mm from Bregma) of 25 µg 6-OHDA hydrobromide (Sigma-Aldrich, France) dissolved in 3 µL 0.1% ascorbic acid, or vehicle, following a published protocol (Bouchatta et al., 2018). A pretreatment with desipramine hydrochloride (20 mg/kg s.c., Sigma-Aldrich, France) was performed 30 min before surgery to prevent the depletion of noradrenergic neurons. After surgery, the pups were placed back with the mother, kept under constant observation, and provided with enriched food (#SAFED113, Safe-lab, Augy, France) to improve recovery. After weaning, mice were housed in cages of two to five animals to reduce stress due to isolation.

Behavioural procedures

For all the behavioural paradigms, mice were habituated to the researcher for 4 days before starting the procedures and to the testing room 30 min before performing each test. Behavioural procedures started at P30. The tests were conducted during the day, with a dim light. All apparatuses were cleaned using a 30% ethanol solution between trials and animals. All mice performed all the paradigms.

1. **Open Field:** spontaneous locomotor activity was assessed through the open field test. Mice were placed in the center of the arena (measurements: 40 cm × 40 cm × 40 cm; Ugo Basile, Gemonio, Italy) and allowed to freely explore for 10 min. Animals were recorded using a video tracking system (EthoVision XT15, Noldus, Wageningen, Netherlands). Distance traveled (cm) and speed (cm/s) were quantified.

2. **Von Frey test:** nociceptive response to mechanical stimulus was assessed using von Frey setup (Ugo Basile, Gemonio, Italy), as described previously (Bouchatta et al., 2022). During 30 min, mice were habituated to individual cages with a mesh floor. The plantar surface of the hind paws was stimulated by calibrated von Frey filaments of different grams to set the withdrawal threshold. For both hind paws, three to five measurements were registered, with an interval of 30 s between each. The grams of the filament at which the mouse withdrew its paw was considered to be the mechanical pain threshold value.

3. **Plantar test (Hargreaves' method):** nociceptive response to thermal stimulus was assessed using Plantar test setup (Ugo Basile, Gemonio, Italy), as described previously (Bouchatta et al., 2022). During 30 min, mice were habituated to individual cages with a glass pane floor. The plantar surface of the hind paws was stimulated by an infrared (IRed) generator (IRed intensity of 50, cut-off time set at 15 s) to establish the thermal pain threshold. For both hind paws, the latency to paw withdrawal was recorded, and three measurements per paw were performed with an interval of 2 min between each.

Tissue preparation for immunofluorescence

Mice were anesthetized with ketamine (100 mg/kg i.p., Virbac, France) and xylazine (20 mg/kg, i.p., Virbac, France) and were transcardially perfused with 0.9% NaCl containing 0.01% heparin, followed by fixative (4% paraformaldehyde (PFA) in 0.1 M phosphate buffered (PB), pH 7.4). The brains were removed and immersed in 4% PFA overnight at 4°C. After post-fixation, brains were cryoprotected in 12.5% sucrose in 0.01 M phosphate-buffered saline (PBS) at 4°C for 2 days. Coronal sections (25 µm) were obtained using a cryostat (CM3050S, Leica, Heidelberg, Germany).

Immunofluorescence staining

The sections were washed three times, for 10 min each, in PBS 0.1M. They were incubated for 1 h, at room temperature, in a blocking solution consisting of PBS BSA 1% containing 0.3% Triton X-100, and then with anti-Iba1 (rabbit, dilution 1:2000,

Wako, Japan) in a solution of PBS BSA 1%, overnight at 4°C. The sections were rinsed again three times, for 10 min each, in PBS 0.1M. They were incubated with a secondary Alexa fluor 568 goat anti-rabbit antibody (dilution 1:500, Invitrogen, MA, United States), for 2 h at room temperature. A final three-times washing step was performed, and the sections were mounted on Superfrost slides (Fisher Scientific, Illkirch, France) and coverslipped with Fluoromount G mounting medium (Invitrogen).

Image acquisition and analysis

Images of the ACC were acquired using a confocal microscope (Leica TCS SP5), with the 20x oil immersion objective. Two optical planes for each picture were chosen, and five, non-overlapping cells were selected randomly. Six to 7 mice were analyzed per group, with at least 400 cells quantified per group. The analysis was performed using FIJI and FraLac for ImageJ software as previously described (Espinosa-Fernández et al., 2019). Four morphological parameters were assessed: cell perimeter, cell area, fractal dimension, and lacunarity. Cell perimeter was quantified based on the outline of the cell shape, while cell area was measured through the total number of pixels in the filled shape of the cell image. Fractal dimension was used for the interpretation of cell complexity and the characterization of microglial forms. A higher fractal dimension implies a more branched and complex structure. Lacunarity was associated with fractal dimension to describe cell heterogeneity. It is indicative of morphological changes and those of the soma. The lowest lacunarity, the more homogenous the cell image is (Fernández-Arjona et al., 2017; Espinosa-Fernández et al., 2019).

Tissue preparation for RT-qPCR procedure

Mice were anesthetized with ketamine (100 mg/kg i.p., Virbac) and xylazine (20 mg/kg, i.p., Virbac) and then sacrificed by decapitation. The ACC and PI were rapidly dissected under a binocular, under sterile conditions, and frozen in dry ice. The samples were stored at -80°C to avoid RNA degradation.

RNA extraction

RNA of ACC and PI was extracted using the chloroform/isopropanol method. Briefly, 500 µL of the TRI-Reagent (Euromedex, France) was added to each sample to homogenize and lyse the tissue. Chloroform (Sigma-Aldrich, France) was then used to separate the RNA (aqueous phase) from the DNA and proteins (organic phase). The aqueous phase was transferred to a new and clean tube, and isopropanol (Fisher Scientific) was added to precipitate the RNA. Ethanol 75% was utilized to wash the RNA pellet. The samples were then incubated with DNase I (Fisher Scientific) to eliminate DNA residues. Finally, RNA quantification was performed by measuring the absorbance at 260 nm using a spectrophotometer, and its quality was checked by capillary electrophoresis with the bioanalyzer 2,100 (Agilent, CA, United States).

RT-qPCR

cDNA synthesis was performed using 2 µg of RNA, the Maxima Reverse Transcriptase enzyme (Fisher Scientific), and a mix of random and oligo dT primers (Fermentas, MA, United States) at 150 ng/µL and 200 ng/µL, respectively. Transcript-specific primers, 2 µL of cDNA at 2 ng/µL, and LightCycler 480 SY Green Master (Roche, Basel, Switzerland) in a final volume of 10 µL were used for real-time PCR. The list of primers is presented in Supplementary Table S4. The relative gene expression to the vehicle-injected group (sham group) was calculated by using the $2^{-\Delta\Delta CT}$ method for each reaction. Two reference genes were selected for each area of interest: Gusb (glucuronidase beta) and Ubc (Ubiquitin C) for the PI, Gapdh (Glyceraldehyde-3-Phosphate Dehydrogenase) and Nono (Non-POU-domain-containing, Octamer binding protein) for the ACC.

Statistical analyses

The statistical analyses were conducted using GraphPad software (GraphPad Prism V9 software, GraphPad, La Jolla, CA, United States). Gaussian distribution was determined using the Shapiro-Wilk test. For Iba1 staining data, the non-parametric two-tailed Unpaired Mann-Whitney U test for two-sample comparisons was performed, since the data did not meet normality. For RT-qPCR results, a one-tailed (RT-qPCR screening in the ACC data) or two-tailed (RT-qPCR screening in the PI data) Unpaired *t*-test were carried out, since the data reached normality. To analyse behavioural tests data, two-tailed Unpaired *t*-test was applied (Open Field, Plantar test and amplitude of changes in thermal threshold). Since Von Frey test results did not reach normality, the two-tailed Unpaired Mann-Whitney U test for two-sample comparisons was carried out. The amplitude of changes in mechanical threshold was also analysed by Mann-Whitney U test. All data were expressed as the mean ± standard error of the mean (SEM) and statistical tests were performed with probability set at $p < 0.05$. We also indicated the *t*-value (*t*, *t*-test), degree of freedom (df, *t*-test), *U*-value (*U*, Mann-Whitney U test).

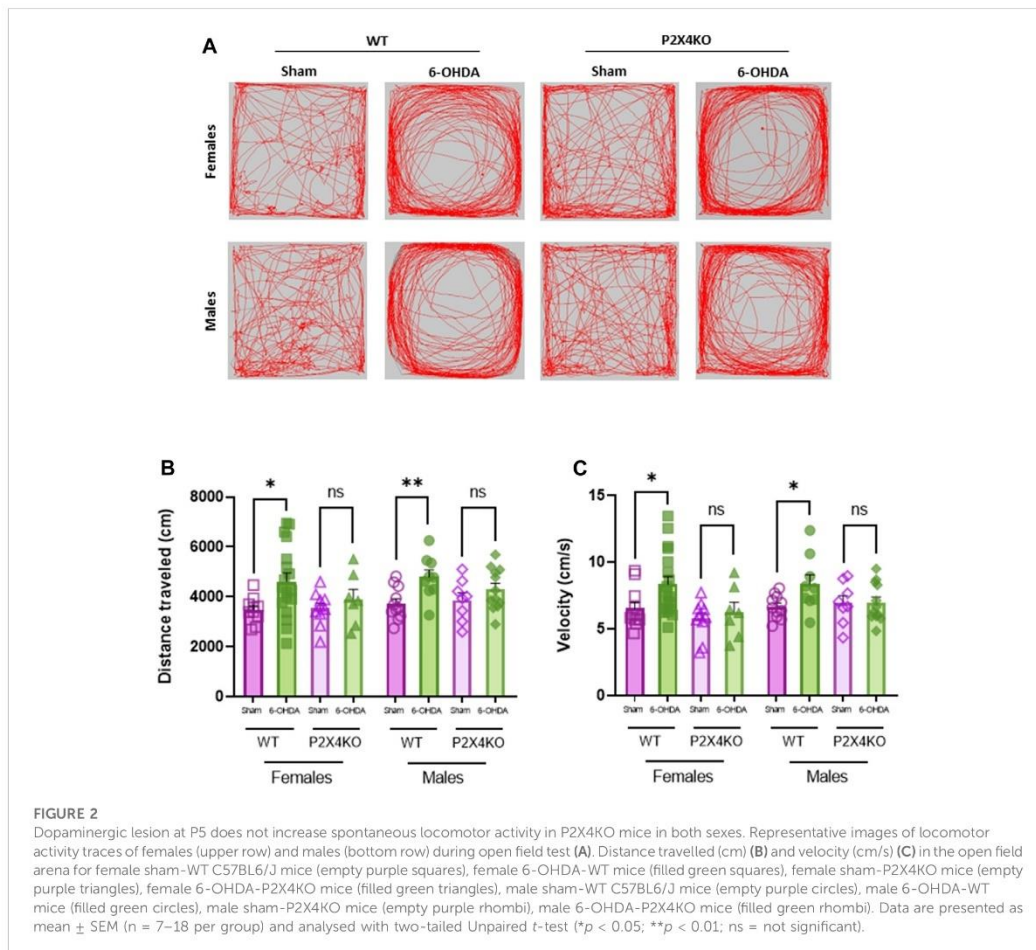
Results

P2X4 receptor deletion prevents locomotor hyperactivity in ADHD-like conditions

We evaluated spontaneous locomotor hyperactivity in WT and P2X4KO mice (females and males) with the open field test (Figure 2). We observed that 6-OHDA-WT animals travelled significantly more distance than sham-WT mice (Figure 2B, Supplementary Table S1A, B, two-tailed Unpaired *t*-test). Furthermore, 6-OHDA-WT group showed higher velocity than sham-WT mice (Figure 2C, Supplementary Table S1A, B).

In contrast, neonatal 6-OHDA lesion did not significantly increase the locomotor activity in P2X4KO female mice nor P2X4KO males compared with sham-P2X4KO females and males (Supplementary Table S1A, B).

We did not observe significant differences in the distance travelled nor the velocity between sham-WT and sham-P2X4KO groups. When comparing 6-OHDA groups, no significant



differences were found either in distance travelled or velocity (Supplementary Table S1A, B).

We did not observe significant differences when comparing females and males of each condition in both mouse lines (WT and P2X4KO) neither in distance travelled nor in velocity (Supplementary Table S1G).

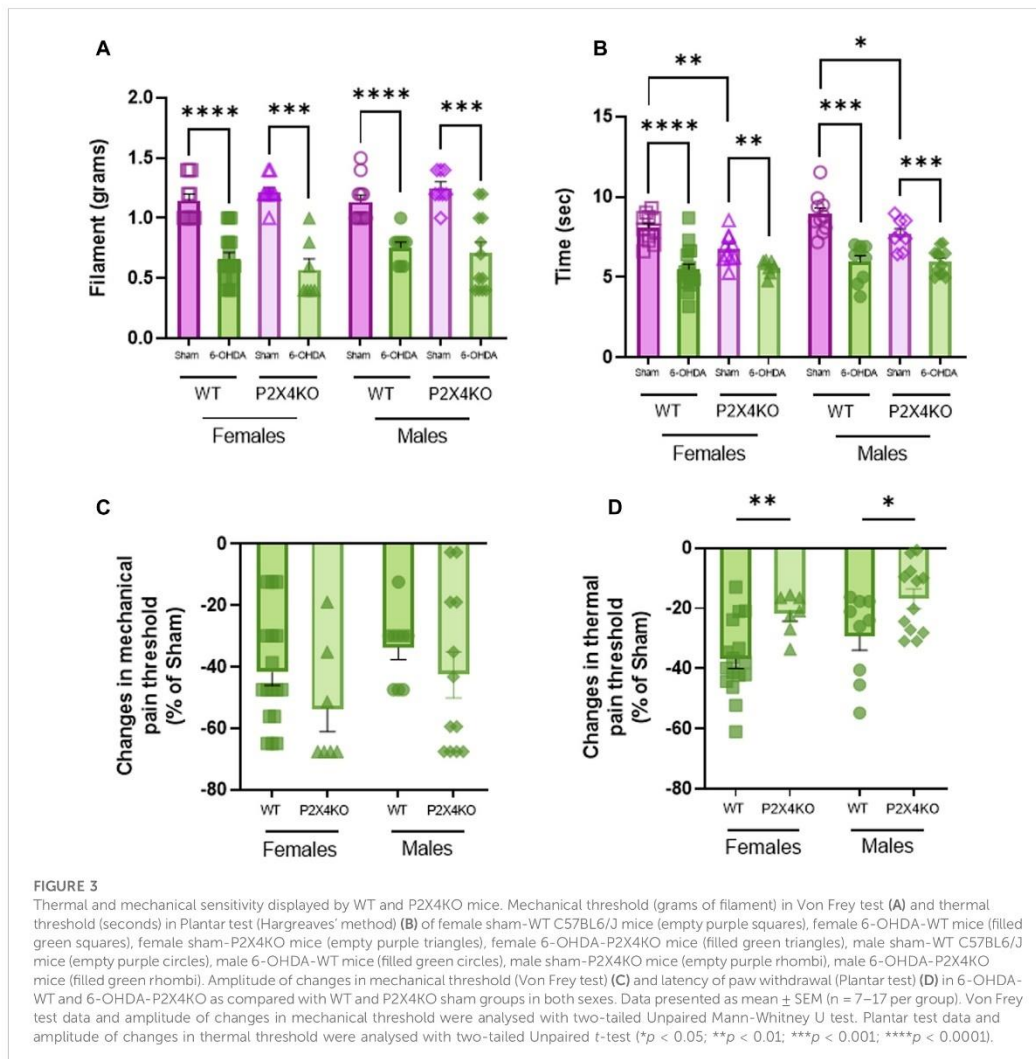
These results indicated that the lack of the P2X4 receptor prevented the development of spontaneous locomotor hyperactivity induced by neonatal 6-OHDA injection.

P2X4 receptor deletion induces sensitization to thermal stimuli in basal but not ADHD-like conditions

Mechanical and thermal pain sensitivity were assessed with Von Frey and Plantar (Hargreaves' method) tests, respectively (Bouchatta et al., 2022).

In the von Frey test, WT mice that received neonatal 6-OHDA injection displayed a significant decrease in the mechanical pain threshold compared with sham animals (Figure 3A, Supplementary Table S1C, D, two-tailed Unpaired Mann-Whitney U test). Moreover, P2X4KO mice injected with 6-OHDA at P5 also showed lower mechanical pain threshold than sham-P2X4KO group (Supplementary Table S1C, D). When comparing WT and P2X4KO groups, we did not find any significant difference in shams (Supplementary Table S1C, D). Similarly, we did not observe significant differences between 6-OHDA groups (Supplementary Table S1C, D).

With the plantar test, we observed in both WT and P2X4KO groups that 6-OHDA females and males were significantly more sensitive to thermal stimulation than sham females and males (Figure 3B, Supplementary Table S1E, F, two-tailed Unpaired *t*-test). Interestingly, P2X4KO sham mice displayed significantly higher thermal pain sensitization than WT sham mice and this effect is irrespective of sex (Supplementary Table S1E, F). No significant



difference was observed when comparing the 6-OHDA groups (Supplementary Table S1E, F).

We did not find significant differences between males and females from each experimental condition neither in mechanical nor thermal pain threshold (Supplementary Table S1G).

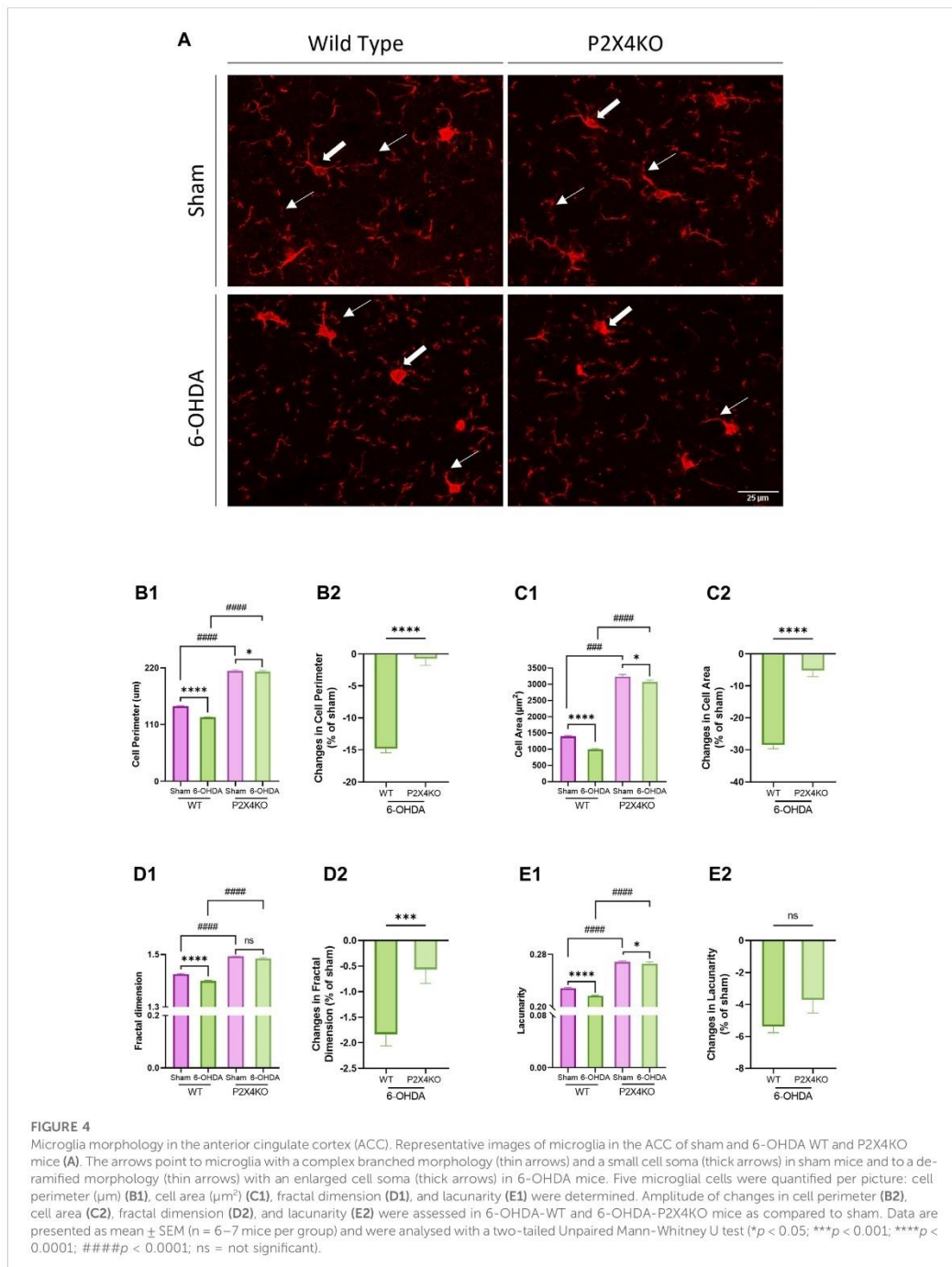
When data was expressed in percentage of changes with respect to sham groups of each mouse line (WT or P2X4KO), we observed that differences in thermal pain threshold between sham and 6-OHDA-P2X4KO mice were significantly lower than between sham and 6-OHDA-WT groups, determined by two-tailed Unpaired *t*-test for females and males (Figure 3D, Supplementary Table S1E, F). Regarding mechanical sensitivity, we did not observe significant differences in the percentage of changes between WT females and

P2X4KO females, nor between WT males and P2X4KO males (Figure 3C, Supplementary Table S1C, D).

Our finding suggested that neonatal dopaminergic lesion induced mechanical and thermal pain sensitization independently of P2X4 receptor suppression, but the lack of this purinergic receptor decreased thermal pain threshold in basal conditions.

The P2X4 receptor modulates microglia reactivity

Microglia activation was previously described in the spontaneously hypertensive rat (Zhang et al., 2022), and the 6-OHDA mouse (Meseguer-Beltrán et al., 2023) models of ADHD. We decided to



verify microglia reactivity in our conditions, and to investigate the consequences of P2X4 deletion on microglia changes. We performed Iba1 staining to analyse morphological modifications in the ACC to compare WT and P2X4KO, and to explore possible differences in microglial reactivity between the two genotypes under ADHD-like vs. sham conditions (Figure 4A). For this purpose, we used quantitative assessments of the cell perimeter and area as indices of the ameboid-like or ramified morphological shape of microglia, and the fractal dimension and lacunarity as indices of the branching complexity (Leyh et al., 2021).

In sham animals, we observed an increase in the baseline homeostatic parameters when P2X4 was deleted compared to WT mice. Cell perimeter, cell area, fractal dimension, and lacunarity exhibited significantly higher values in sham-P2X4KO mice compared to sham-WT mice (Figure 4B1–E1, Supplementary Table S2A, two-tailed Unpaired Mann-Whitney test). Similarly, ADHD-like conditions also resulted in increased cell perimeter, cell area, fractal dimension, and lacunarity in P2X4KO as compared to WT animals (Figure 4B1–E1, Supplementary Table S2B). This suggested that P2X4 receptor deletion influenced microglial morphology toward a more pronounced homeostatic phenotype.

In both genotypes, 6-OHDA neonatal injection caused changes in microglia morphology. Cell perimeter, cell area, fractal dimension, and lacunarity appeared significantly lower in 6-OHDA-WT mice as compared to the sham animals (Figure 4A1, Figure 4B1–E1, Supplementary Table S2C, two-tailed Unpaired Mann-Whitney test). In 6-OHDA-P2X4KO mice, cell perimeter, cell area, and lacunarity displayed decreased values compared to sham-P2X4KO mice, with no significant difference in fractal dimension (Figure 4A1, Figure 4B1–E1, Supplementary Table S2D). Overall, lower values for fractal dimension, lacunarity, cell area and perimeter characterized an ameboid-like microglial morphology, indicating microglia reactivity in 6-OHDA-WT and 6-OHDA-P2X4KO as compared to shams.

Next, we investigated the ability of P2X4 to modulate microglia reactivity in ADHD-like conditions. For this purpose, we assessed the percentage of changes after 6-OHDA neonatal injection for the four morphological parameters in WT and P2X4KO mice using a two-tailed Unpaired Mann-Whitney test. Differences in cell perimeter, cell area, and fractal dimension were significantly higher in 6-OHDA-WT mice compared to 6-OHDA-P2X4KO mice. No significant difference was detected for lacunarity (Figure 4B2–E2, Supplementary Table S2E). This result showed that the absence of the P2X4 receptor limited neonatal 6-OHDA-induced changes in microglia morphology.

P2X4 receptor protects against neuroinflammation

Using RT-qPCR, we studied changes in the expression of several cytokines, intracellular markers of neuro-inflammation and glial activation to determine whether the P2X4 receptor affects the response of ACC and PI to neonatal 6-OHDA injection. All values obtained for the ACC and PI are indicated in Supplementary Table S3, S4, respectively. Among all markers tested (see Material and Methods and Supplementary Table S5), only those for which differences between WT and P2X4KO have

been detected in ADHD-like conditions are presented. These markers and their possible functions are summarized in Table 1.

In the ACC, canonical pro-inflammatory cytokines (IL-6, IL-16, TNF- α) mRNA exhibited decreased amounts in the absence of P2X4. Interestingly, and in agreement with the morphological findings, Iba1 was also expressed at lower levels in 6-OHDA-P2X4KO mice. However, other markers displayed higher levels of expression; most of them being involved in the exacerbation of inflammation (CCL4, CX3CR1, IRF5, and IRF8). Moreover, elevated expression levels of intracellular signalling factors, such as GSK3 β and NLRP3, were also detected (Figure 5A, one-tailed Unpaired *t*-test) and may contributed to the amplification of the immune response through the induction of pro-inflammatory cytokines release. The mRNA level of the astrocyte-specific marker GFAP was also significantly increased in P2X4KO mice (Figure 5A). On the contrary, SOD1 and Arg1, two enzymes with protective roles, displayed lower values (Figure 5B, one-tailed Unpaired *t*-test). This finding indicated the presence of an overall predominantly pro-inflammatory microenvironment in the ACC under ADHD-like conditions in both WT and P2X4KO mice.

In the PI, the transcripts for secreted or intracellular factors intensifying the immune response were mainly upregulated in 6-OHDA-P2X4KO mice (IL-18, IL-16, TNF- α , TNF-R, CX3CR1). IL-6 mRNA, on the contrary, showed a decreased expression level. Intracellular molecules which mainly lead to a pro-inflammatory microenvironment were also overexpressed (GSK3 β , NF- κ B, NLRP3, IRF5, and IRF8), along with some chemokines and their receptors (CX3CL1, CXCL12, and CCR5). Astrocytic expression of GFAP was also enhanced, as well as other molecules involved in antigen presentation such as Cathepsin S (Figure 6A, two-tailed Unpaired *t*-test). Interestingly, higher levels of TGF- β , SOD1, and Arg1 mRNA were observed (Figure 6B, two-tailed Unpaired *t*-test). These latter markers are likely to exert an anti-inflammatory role. Taken together, these data pointed towards a predominantly pro-inflammatory microenvironment in the PI under ADHD-like conditions in both WT and P2X4KO mice.

Discussion

In the present study, we demonstrated that the total deletion of P2X4 prevents neonatal 6-OHDA injection-induced hyperactivity but has only slight effects on pain sensitization that is associated with ADHD-like conditions. Iba1 immunolabeling confirms the activation of microglia in the 6-OHDA mouse model of ADHD. It further indicates that the lack of P2X4 receptor reduces the baseline activation state of microglia and limits, but not suppresses, its reactivity in ADHD-like conditions. Unexpectedly, P2X4 deletion is accompanied by the creation of a proinflammatory environment in the ACC and PI of 6-OHDA adult mice as demonstrated by overexpression of various proinflammatory markers. This situation is further amplified in the ACC by the downregulation of anti-inflammatory molecules.

The 6-OHDA mouse model of ADHD is characterized by alterations of the dopaminergic system that have been extensively considered as a significant factor for the progression of the disease (Albrecht et al., 2015) and may be seen as the primary cause of ADHD pathology (Faraone et al., 2021). Mesocortical dopamine has

TABLE 1 List of markers that exhibit expression changes in 6-OHDA-P2X4KO mice in qRT-PCR experiments. They are sorted according to their functions in inflammation, oxidative stress, or intracellular signalling.

	Markers	Type	Function	Cellular expression	References
Secreted Cytokines and Receptors	CCL4	Chemokine	• Chemoattractant for immune regulatory cells (i.e., macrophages, T-lymphocytes)	Astrocytes	Saika et al., 2012a
			• Contributes to neuropathic pain		Sindhu et al., 2019
			• Pro-inflammatory microglial profile		Ullah et al. (2020)
	CCR5	Chemokine receptor	• Chemotaxis of microglia	Microglia Astrocytes	Carbonell et al., 2005
			• Activation of astrocytes and exacerbation of inflammation		Saika et al., 2012b
			• Contributes to neuropathic pain		Liu et al. (2014)
			• Pro-inflammatory microglial profile		
	CX3CL1	Chemokine	• Neuron-microglia communication	Neurons Can be expressed by astrocytes	Lauro et al., 2015, 2019
			• Regulator of microglia activation		Pawelec et al., 2020
			• Can present neuroprotective functions		Silva and Malcangio, 2021
			• Pain-mediating chemokine		Wilkinson et al., 2015 Yoshida et al. (2001)
	CX3CR1	Chemokine receptor	• Neuron-microglia communication	Microglia	Lee et al., 2018
			• Promote the activation of microglia and stimulate the release of inflammatory factors		Pawelec et al., 2020
			• Pro-inflammatory microglial profile		Tang et al. (2014)
	CXCL12	Chemokine	• Local immune responses in the CNS	Astrocytes Neurons	Guyon, 2014
			• Chemotaxis of leukocytes		Han et al. (2001)
			• Induction of NF- κ B signaling pathway		
	IL-16	Cytokine	• Initiating and/or sustaining an inflammatory response	Microglia	Guo et al., 2004
			• Modulates T-cell activation		Little and Cruikshank, 2008
			• Pro-inflammatory microglial profile		Mathy et al. (2000)
IL-18	Cytokine	• Secretion induced by NLRP3 inflammasome	Microglia Neurons	Alboni et al., 2010	
		• Initiation of signaling pathways and inflammatory responses in microglia		Felderhoff-Mueser et al., 2005	
		• Exacerbation of neuronal cell death through the increase of Fas-ligand expression		Song et al. (2017)	
		• Pro-inflammatory microglial profile			
IL-6	Cytokine	• Promotes alternative activation of macrophages	Microglia	Codeluppi et al., 2014	
		• Inflammatory acute phase response	Astrocytes	Fuster and Walsh, 2014	
		• Contributes to both nociceptor and central sensitization	Can be expressed in neurons	Renner et al., 2022	
		• Pro-inflammatory and anti-inflammatory microglial profile		Zhou et al. (2016)	
TGF- β	Cytokine	• Suppression of the immune response and glial activation	Microglia Astrocytes	Chen et al., 2015	
		• Regulation of astrocyte reactivity		Hisatomi et al., 2002	

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TABLE 1 (Continued) List of markers that exhibit expression changes in 6-OHDA-P2X4KO mice in qRT-PCR experiments. They are sorted according to their functions in inflammation, oxidative stress, or intracellular signalling.

	Markers	Type	Function	Cellular expression	References	
	TNF-R	Cytokine receptor	• Anti-inflammatory microglial profile	Astrocytes Microglia Neurons	Luo, 2022	
			• NF-κB and AP-1 pathway activation		Yoshimura et al. (2010)	
			• Role in central pain sensitization		Probert, 2015	
	TNF-α	Cytokine	• Pro-inflammatory microglial profile	Microglia	Wajant et al., 2003	
			• Inflammatory acute phase response		Zhang et al. (2011)	
			• NF-κB and AP-1 pathways activation		Hess et al., 2011	
			• Nociceptive responses in the central nervous system		Idriss and Naismith, 2000	
				• Pro-inflammatory microglial profile		Jang et al., 2021
						Raffaele et al. (2020)
	Intracellular Signaling Markers	GSK3β	Serine/threonine kinase	• Increases cytokine and chemokine production through activation of JNK and NF-κB pathways	Microglia Astrocytes	Abd-Ellah et al., 2018
• Alterations in GSK3β function are associated with pathological pain				Maixner and Weng, 2013		
• Pro-inflammatory microglial profile				Wang et al. (2010)		
IRF5		Transcription factor	• IRF8-IRF5 transcriptional axis induces P2X4R expression in microglial cells	Microglia	Al Mamun et al., 2020	
			• Involved in neuropathic pain		Masuda et al., 2014	
			• Pro-inflammatory microglial profile		Tsuda et al. (2017)	
IRF8		Transcription factor	• IRF8-IRF5 transcriptional axis induces P2X4R expression in microglial cells	Microglia	Masuda et al., 2012	
			• Contributes to chronic pain		Tsuda et al. (2017)	
			• Pro-inflammatory microglial profile			
NF-κB		Transcription factor	• Induction of NLRP3 inflammasome expression	Microglia Astrocytes Neurons	Babkina et al., 2021	
			• Role in inflammatory processes in glial cells (cytokines production, iNOS expression)		Liu et al., 2017	
			• Pro-inflammatory microglial profile		Sutterwala et al. (2014)	
NLRP3	Pattern recognition receptor	• Induction of IL-18 and IL-1β secretion through caspase-1 activity	Microglia Can be expressed in neurons and astrocytes	Liu et al., 2017		
		• Pro-inflammatory microglial profile		Sutterwala et al., 2014		
				Voet et al. (2019)		
Astrocytic Markers	GFAP	Class-III intermediate filament	• Marker of reactive astrogliosis	Astrocytes	Amalia, 2021	
			• Involved in pro-inflammatory cytokines release		Azzolini et al., 2022	
			• Increased expression under inflammatory pain		Ikeda et al. (2013)	
Oxidative Stress Markers	Arginase 1	Enzyme	• Downregulation of nitric oxide production by competing with iNOS	Microglia	Cherry et al. (2014)	
			• Anti-inflammatory microglial profile			
	SOD1	Enzyme	• Regulates oxidative stress responses	Neurons Astrocytes Microglia	Boillée and Cleveland, 2008	
			• Potential anti-inflammatory effects through its reduction of reactive oxygen species		Hwang et al., 2020	

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TABLE 1 (Continued) List of markers that exhibit expression changes in 6-OHDA-P2X4KO mice in qRT-PCR experiments. They are sorted according to their functions in inflammation, oxidative stress, or intracellular signalling.

	Markers	Type	Function	Cellular expression	References
			<ul style="list-style-type: none"> High SOD1 levels may correlate with postoperative pain reduction 		Jaarsma et al., 2008 KÄRKKÄINEN et al., 2018 Nagai et al. (2007)
Other Markers	Cathepsin S	Protease	<ul style="list-style-type: none"> Role in antigen presentation 	Microglia	Hao et al., 2007
			<ul style="list-style-type: none"> Role in microglia-neuron communication 		Nakagawa et al., 1999
			<ul style="list-style-type: none"> Involved in microglia migration towards sites of injury or infection 		Pišlar et al., 2021
			<ul style="list-style-type: none"> Involved in neuropathic pain 		Silva and Malcangio, 2021
	Iba1	Protein	<ul style="list-style-type: none"> Role in actin-crosslinking which is involved in microglial membrane ruffling 	Microglia	Smyth et al. (2022)
			<ul style="list-style-type: none"> Induction of microglial phagocytic activity 		Hovens et al., 2014
<ul style="list-style-type: none"> Its increased expression is associated with microglial response 			Ohsawa et al. (2004)		

a pivotal role in selective attention, and working memory, which indicates its involvement in the pathophysiology of ADHD (Levy, 2004; Ruocco et al., 2009). Dopaminergic lesions by neonatal intracerebroventricular injection of 6-OHDA in rodents (Sontag et al., 2010) generates ADHD-like models with well-established validity (Avale et al., 2004; Bouchatta et al., 2018). 6-OHDA mice demonstrated good face validity by exhibiting not only the major symptoms of the disease (i.e., hyperactivity, inattention and impulsivity), but also discrete co-existing symptoms (i.e., heightened anxiety, antisocial behaviour and impaired cognitive functions) (Bouchatta et al., 2018; 2020).

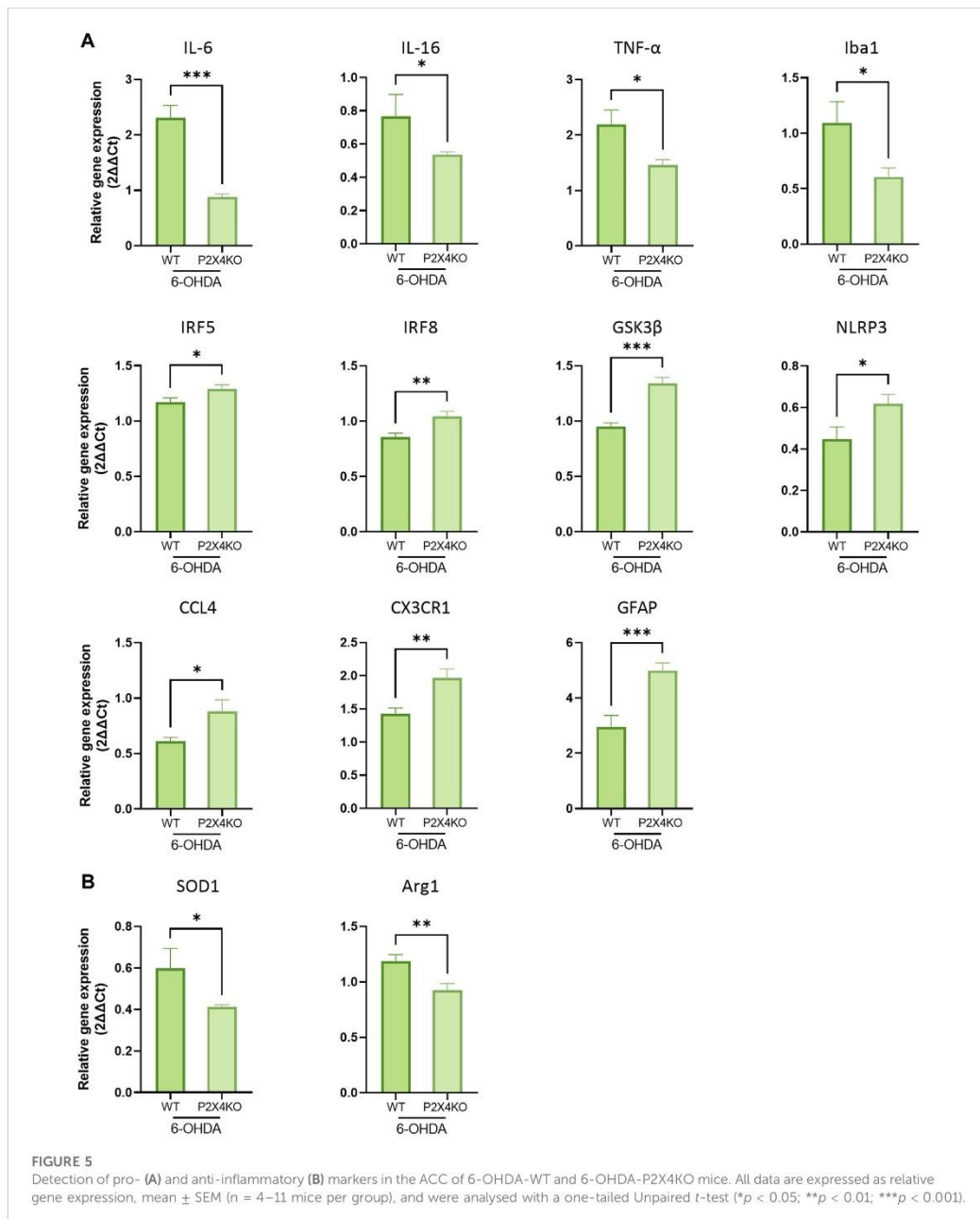
Neuroimaging studies in ADHD patients identified structural and functional abnormalities in brain networks with a key role of the ACC (Faraone, 2015) that is at the crossroad of executive functions and pain processing (Garcia-Larrea and Peyron, 2013; Hart et al., 2013; Sellmeijer et al., 2018). We further identified a key role for the ACC-PI pathways in the interaction between ADHD-like conditions and pain sensitization (Bouchatta et al., 2022). Therefore, we focused the present study primarily on the effects of P2X4 deletion in the ACC and on its consequences in the target PI.

ATP release is triggered by inflammatory conditions and in neurological diseases. Its accumulation in the extracellular space regulates synaptic plasticity and neuron-glia interactions through binding to purinergic receptors, including the P2X4 receptor (Lindberg et al., 2015). The effects of P2X4 deletion on microglia phenotype and secretome are instrumental in our understanding of inflammatory processes underlying neurological diseases. Microglia responds to challenges caused by brain diseases by modifying its morphology, molecular profiles and functions. In contrast to the classical, dualist vision of “good” and “bad” microglia, a recent collective update (Paolicelli et al., 2022) made clear that microglia exist in a broad array of highly dynamic, and multidimensional states. This never-resting microglia can be described as a continuum of configurations according to signals from the external and local

environments. Disruption of microglia functions has been proposed to account for the pathological onset of neurodevelopmental disorders including ADHD (Bordeleau et al., 2019). Moreover, a genome-wide associated study identified causal genes for ADHD in microglia (Fahira et al., 2019). Accordingly, microglia reactivity in ADHD conditions has been already demonstrated in humans (Yokokura et al., 2021) and animal models (Song et al., 2021; Zhang et al., 2022; Sanches et al., 2023). Recent evidence for microglia reactivity has been also provided in the ACC of the 6-OHDA mouse model (Meseguer-Beltrán et al., 2023).

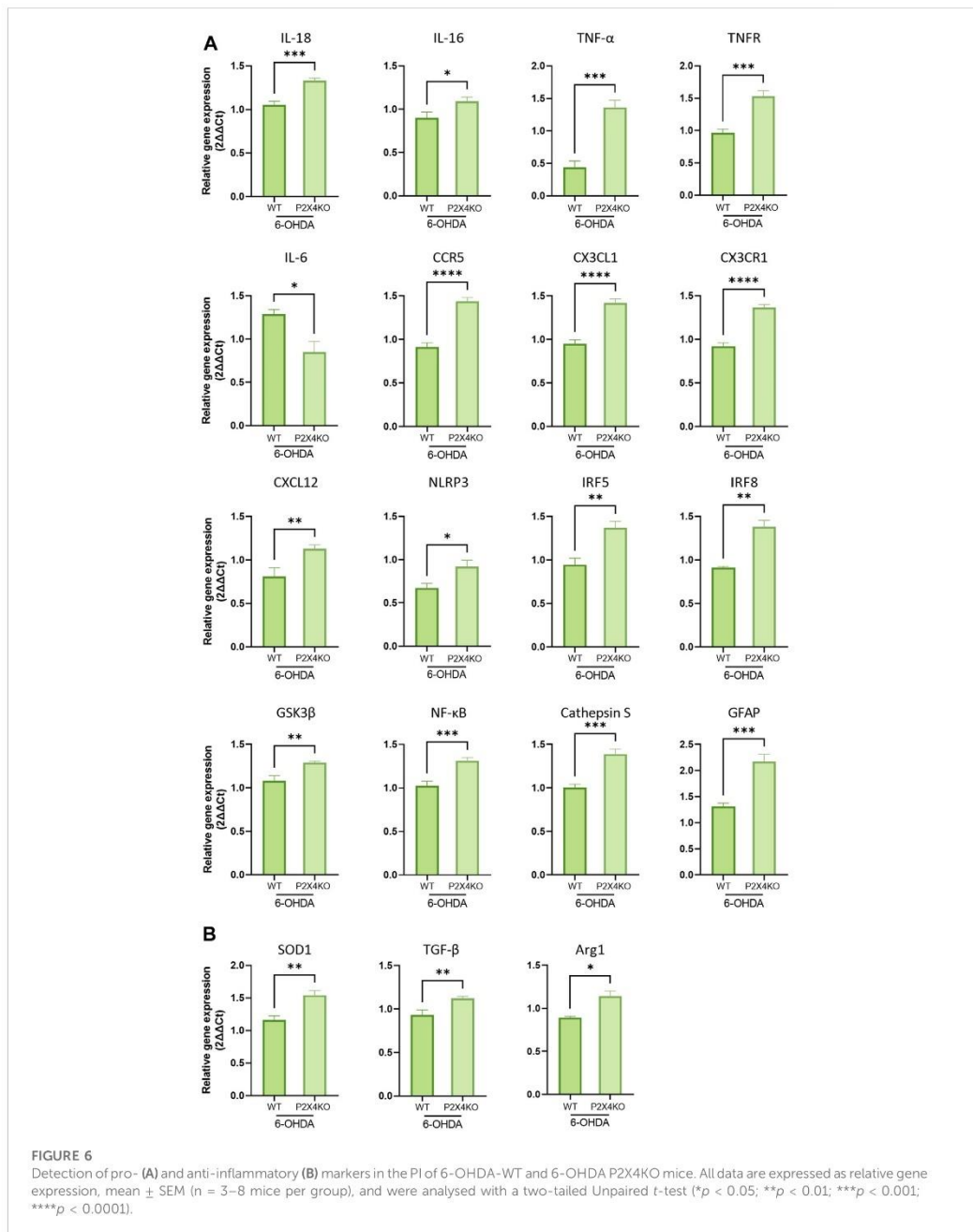
Over the past decades, the role of P2X4 has been extensively studied in microglia that display abundant expression of this receptor (Tsuda et al., 2003; Vázquez-Villoldo et al., 2014; Duveau et al., 2020). P2X4 has been shown to mediate pain hypersensitivity in the spinal cord of animal models of neuropathic (Tsuda et al., 2003; Coull et al., 2005) and inflammatory (Ulmann et al., 2010; Aby et al., 2018) pain. Reactive microglia may also be a causal agent for ADHD-like symptoms in the spontaneously hypertensive rat model (Zhang et al., 2022), although in other models (i.e., traumatic brain injury-induced ADHD-like conditions) microglia inhibition with minocycline did not affect impulsivity and attention deficits (Pechacek et al., 2022). Although P2X4 was proposed as a male-biased microglial mediator of chronic pain (Halievski et al., 2020), recent findings showed that microglial P2X4 is crucial for neuropathic pain, regardless of sex (Gilbert et al., 2023). In agreement, sex comparisons in WT and P2X4KO mice did not reveal any differences between males and females in hyperactivity, impulsivity or sensory thresholds.

Interestingly, the present study demonstrates an unexpected function of P2X4 that may contribute to basal microglia homeostasis. Indeed, its deletion amplifies the features of homeostatic microglia in sham conditions. In 6-OHDA mice, P2X4 suppression attenuates but does not fully prevent microglia



reactivity. In addition, our findings indicate a less reactive microglia phenotype in 6-OHDA-P2X4KO than in 6-OHDA-WT. Beside the morphological analysis, the decreased reactive phenotype of ACC microglia in ADHD-like conditions is also characterized in our study by the decreased expression of i) the microglia-specific marker

Iba1, and ii) the IL16 and TNF- α cytokines that are mostly released by microglial cells (Borst et al., 2021). Limited microglia reactivity in 6-OHDA-P2X4KO mice may explain the lack of hyperactivity (distance travelled) and impulsivity-like (velocity) behaviours induced by ADHD-like conditions in P2X4KO mice.



Despite this reduced microglia reactivity, the qRT-PCR analysis of cytokine expression in ADHD-like conditions suggest that P2X4 deletion favours a predominantly pro-inflammatory environment in the ACC and PI as compared to

WT mice. Table 1 recapitulates these markers sorted according to their possible functions. Microglia pro-inflammatory cytokine receptor (CX3CR1), and transcription factors (IRF5 and IRF8) are upregulated in the ACC and PI of 6-OHDA-P2X4KO mice.

The expression of pro-inflammatory markers of intracellular signalling pathways is also increased in both regions (GSK3 β , NLRP3, NF- κ B). The overexpression of astrocytic markers (GFAP) (Giovannoni and Quintana, 2020) and chemokines (CCL4 in the ACC, CXCL12 in the PI) (Guyon, 2014; Zhu et al., 2014) are indicative of astrogliosis that may also contribute to the pro-inflammatory environment.

The results displayed striking differences in the two brain regions investigated. The pro-inflammatory environment is more pronounced in the PI than in the ACC. As an example, IL18 is overexpressed in the PI while no changes were noticed in the ACC. Interestingly, its release is stimulated by NLRP3 (Amores-Iniesta et al., 2017) that is also upregulated in the PI of 6-OHDA-P2X4KO mice. Modifications of cytokine receptors expression were also detected in the PI (TNFR, CCR5).

Other markers did not show any significant difference in their levels of expression following the deletion of the P2X4 receptor under ADHD-like conditions. Remarkably, some of these molecules were previously shown to be involved in neuropathic pain (MMP9, BDNF) (Kawasaki et al., 2008; Thakkar and Acevedo, 2023) or pain pathogenesis (Wnt5a) (Wang et al., 2020; Liu et al., 2023). These results are in line with the absence of effects of P2X4 deletion on pain sensitization.

Many inflammatory pathways exist in neural cells and some changes in the inflammatory environment may be indirectly triggered by P2X4 deletion. P2X7 is another purinergic receptor known to play prominent roles in inflammatory processes (Di Virgilio et al., 2017) by promoting cytokine production by microglia and astrocytes (Sperlágh and Illes, 2014). P2X7 interacts with P2X4 (Schneider et al., 2017) and P2X4 deletion may thus impact purinergic signalling at large, beyond the sole P2X4 receptor.

The mild pro-inflammatory environment of 6-OHDA-P2X4KO mice is associated with the prevention of hyperactivity/impulsivity and might exert a protective role against ADHD-like symptoms. However, the various pro-inflammatory features described in the 6-OHDA-P2X4KO vs. sham mice are likely to have different, possibly opposite, consequences. Inflammation-induced astrocyte reactivity has been implicated in ADHD-like conditions (Sandau et al., 2012; Zhang et al., 2022; Liu et al., 2023) while in our model it is associated with reduced symptoms. In contrast, a protective role is in agreement with the amplified homeostatic features of microglia observed in P2X4KO mice. In several cases, P2X4 contributes to the development of neurological disorders through well-identified mechanisms, e.g., in hippocampal neurons for Alzheimer's disease (Varma et al., 2009), or in spinal microglia for neuropathic pain (Coull et al., 2005). Conversely, protective roles of P2X4 have been described on motoneurons in amyotrophic lateral sclerosis (Andries et al., 2007) and autoimmune encephalitis (Zabala et al., 2018). In line with our results, the latter study demonstrated an increase in pro-inflammatory gene expression in a P2X4KO mouse model of autoimmune encephalitis as compared to WT mice. In contrast to its effects on hyperactivity and impulsivity, P2X4 deletion does not change mechanical or thermal hypersensitivity in 6-OHDA mice. P2X4 deletion may even induce basal thermal hypersensitivity in sham-P2X4KO mice as compared to WT animals.

The dual effect of P2X4 deletion on ADHD-like symptoms and pain sensitization could result from the dysfunction of distinct cell types. P2X4 is expressed in both neurons and microglia (Bo et al., 2003) and plays different functions in these two cell populations (Duveau et al., 2020). Beneficial impact of both overexpression or deletion of P2X4 described in SOD1 mouse model of amyotrophic lateral sclerosis suggests that P2X4 expressed in motoneurons, and microglia exert opposite effects in ALS pathogenesis (Bertin et al., 2022). In addition, compelling evidence indicates the existence of distinct microglia subpopulations (Stratoulis et al., 2023) that may differ with regard to the expression and/or function of P2X4. The comorbidity of ADHD-like symptoms and pain sensitization may be underpinned by different cell types and/or by the dysfunction of different brain areas.

Our data revealed a complex role of P2X4 likely dependent on a combination of glial and neuronal effects. The lack of P2X4 triggers the development of predominantly pro-inflammatory but distinct environments in the ACC and PI of 6-OHDA mice. In ADHD-like conditions, it decreases microglial reactivity in the ACC and alleviates hyperactivity/impulsivity while only slight alterations of nociceptive behavior are observed. P2X4 deletion has apparent contradictory effects, amplifying the homeostatic characteristics of ACC microglia but promoting the over-expression of pro-inflammatory markers. Importantly, however, these results confirm that microglia activation and inflammation are not equivalent (Paolicelli et al., 2022). In ADHD-like conditions, the lack of P2X4 modulates the microglia phenotype and modifies the "cytokine-enriched secretomes" from microglia and most probably from other cell types as well.

Taken together, our findings suggest that P2X4 facilitates microglia reactivity but at the same time exerts a protective role on the expression of some pro-inflammatory markers, possibly from non-microglia origin. This dual role of P2X4 could be responsible for the differential effects noted on ADHD-like symptoms and pain sensitization.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://github.com/marclandry33/p2x4-adhd>.

Ethics statement

The animal study was approved by the University of Bordeaux local ethical committee and the French Ministry of Higher Education, Research and Innovation (authorization APAFIS#27681 and APAFIS#21135). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SB: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Writing—original draft. SS-S:

Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Writing—original draft. RA: Formal Analysis, Investigation, Writing—review and editing. MT: Investigation, Methodology, Resources, Writing—review and editing. AA: Supervision, Validation, Writing—review and editing. AE: Supervision, Validation, Writing—review and editing. SA: Funding acquisition, Project administration, Resources, Supervision, Validation, Writing—review and editing. WH: Funding acquisition, Project administration, Resources, Supervision, Validation, Writing—review and editing. EB-G: Conceptualization, Data curation, Funding acquisition, Resources, Validation, Writing—review and editing. ML: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing—original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2023.1288994/full#supplementary-material>

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Chapter 4

Discussion

ADHD is one of the most common neurodevelopmental disorders worldwide and is characterized by symptoms of inattention, hyperactivity, and impulsivity (American Psychiatric Association, 2013). ADHD patients display altered sensory processing (Fabio et al., 2024; Fuermaier et al., 2018) and, more particularly, impaired pain perception (Fuermaier et al., 2018; Treister et al., 2015). Chronic pain affects almost 20% of the worldwide population and is caused by modifications in pathways controlling pain processing (Treede et al., 2015). Remarkably, several cortical areas disrupted in ADHD are subject to morphological and functional alterations in pathological pain (Apkarian et al., 2005; Baliki et al., 2012; Schmidt-Wilcke et al., 2008). Additionally, pain was shown to enhance attentional problems and cognitive deficits, hallmarks of ADHD (Higgins et al., 2015; D. J. Moore et al., 2012).

The mechanisms underlying ADHD and chronic pain comorbidity are still poorly understood. It could, however, be explained by neuronal impairments in cortical regions involved in cognitive functions and pain perception (ACC, IC).

The ACC acts as a relay center for neurons coming from the thalamus, the frontal cortex, and the amygdala (Barthas et al., 2015). It is a critical region for cognitive processes (attention, learning, memory, motivation, decision-making, etc.) and emotion but it also participates in pain processing (Apps et al., 2016; Shackman et al., 2011; Zhuo, 2007). Indeed, the ACC is mainly responsible for the affective-motivational dimension of pain (Bushnell et al., 2013; Ossipov et al., 2014). For instance, acute noxious responses were limited by ACC lesions (Johansen et al., 2001; D. E. Lee et al., 1999) while patients with cingulotomies lacked the unpleasantness of pain (Zhuo, 2007). In a neuropathic pain model, repeated stimulations of ACC pyramidal neurons resulted in an anxiodepressive-like behavior (Barthas et al., 2015). Conversely, the development of neuropathic pain-induced anxiodepressive symptoms was associated with ACC hyperactivity, evidenced by enhanced firing rate and bursting activity within this brain area (Sellmeijer et al., 2018). Inhibition of ACC hyperactivity alleviated this anxiodepressive-like behavior, confirming its involvement in the emotional consequences of chronic pain (Sellmeijer et al., 2018). More interestingly, an amplified activity of the basolateral amygdala-ACC circuit induced chronic pain-related depression and was linked to transcriptomic disruptions outlining features of depression, previously depicted in the Human brain (L. J. Becker et al., 2023). These findings further support the role of the ACC in the emotional dimension of pain and encourage an in-depth analysis of the molecular processes occurring in chronic pain conditions and its psychiatric comorbidities. Studies also support the role of the ACC in psychiatric disorders recapitulating impairments in cognitive and emotional functioning. Indeed, ACC hyperactivity was observed in numerous conditions including ADHD, obsessive-compulsive disorder, neuroticism, and anxiodepressive-like behaviors (Bouchatta et al., 2022; Raison, 2015; Sellmeijer et al., 2018).

Like the ACC, the IC partakes in emotion (fear, anxiety, happiness), cognitive functions (learning), and pain perception (Labrakakis, 2023), with numerous findings reporting its recruitment in pain (acute or chronic) conditions (Benison et al., 2011; Greenspan & Winfield, 1992; Isnard et al., 2011). Not only is the IC activated during a nociceptive stimulation but its degree of activation also correlates with the magnitude of the painful stimulus and the perceived intensity of pain (Baliki et al., 2009; Coghill et al., 1999; Labrakakis, 2023). More particularly, activation of the PI was mainly associated with the somatosensory pain dimension in a neuropathic pain model (Barthas et al., 2015), contrasting with the involvement of the ACC in the emotional aspect. PI excitatory pyramidal neurons were found to be sensitized in inflammatory pain conditions, promoting hyperalgesia and reducing pain tolerance (Kadokia et al., 2024). PI activation is also required for the perception of innocuous thermal cues, emphasizing its role in mediating thermoregulation (Vestergaard et al., 2023). Additionally,

impairments in IC volume, connectivity, or activation were reported in numerous disorders such as schizophrenia, ASD, or mood disorders (anxiety, depression) (Avery et al., 2014; Gasquoin, 2014; Mutschler et al., 2019; Paulus & Stein, 2006; Uddin & Menon, 2009; Wylie & Tregellas, 2010), pointing out its relevance in neurodevelopmental and psychiatric conditions.

ACC and IC alterations could be triggered by neuroinflammation, the inflammatory response within the CNS characterized by (i) glial cell activation, (ii) inflammatory mediator release, and (iii) modifications in BBB integrity that result in peripheral immune cell infiltration and immune response amplification (Sochocka et al., 2017). Neuroinflammation is suggested to participate in chronic pain conditions (Gao & Ji, 2010; Inoue & Tsuda, 2018; T. Li et al., 2019; Miyamoto et al., 2017; H. Ni et al., 2016; Vergne-Salle & Bertin, 2021) and ADHD pathogenesis (Alvarez-Arellano et al., 2020; Corona, 2020; Dunn et al., 2019; Leffa et al., 2018), and could therefore act as a link between these two syndromes.

To gain a more in-depth understanding of the inflammatory mechanisms underpinning ADHD and its associated pain sensitization, we have generated an ADHD-like mouse model by unilateral and intracerebroventricular injection of 6-OHDA at P5. We then performed a panoply of experiments (morphological, transcriptomic, proteomic, and phosphoproteomic studies) to elucidate impairments in the molecular processes occurring in the ACC and PI of 6-OHDA mice.

Results summary

The morphological study of astrocytes and microglia has indicated changes associated with cellular reactivity in the ACC of 6-OHDA mice. The quantitative assessment of five morphological parameters revealed an ameboid-like shape for microglial cells in the ACC of 6-OHDA females and, on the contrary, a ramified structure in the ACC of 6-OHDA males. Decreased microglia complexity is usually associated with cellular reactivity (Paolicelli et al., 2022; Reddaway et al., 2023). The quantitative analysis also depicted modifications in astrocytic shape. Ramified astrocytes were prevalent in the ACC of 6-OHDA males, in opposition to the ACC of 6-OHDA females where they displayed a less complex form. Interestingly, both shapes were previously linked to reactive astrogliosis (Escartin et al., 2021; Olabarria et al., 2010; D. Sun et al., 2013). The double GFAP/vimentin immunofluorescence staining results further supported astrocyte reactivity. GFAP intensity, vimentin intensity, and the number of GFAP/vimentin colocalizations were all enhanced in the ACC of 6-OHDA females, concluding on the presence of reactive astrogliosis. However, the results in the ACC of 6-OHDA males were more subtle to analyze as GFAP intensity was the only parameter to differ significantly between 6-OHDA and sham mice. Indeed, no changes in vimentin intensity and the number of colocalizations between the two proteins were detected. These observations suggest mild reactive astrogliosis in the ACC of 6-OHDA males, characterized by an increased GFAP expression. To summarize, our morphological findings point out the presence of possible (i) microglia reactivity in the ACC of 6-OHDA females only, and (ii) reactive astrogliosis in the ACC of both 6-OHDA females and males. This cellular reactivity, which seems more prominent in the ACC of 6-OHDA females, is a component of neuroinflammation. It therefore suggests the presence of an inflammatory response in the ACC of 6-OHDA males and females.

The morphological results led to new questions. Are these reactive cells secreting protective or detrimental molecules? Are they contributing to or attenuating the immune response? To decipher the environmental status of the ACC and PI in ADHD-like conditions, we have searched for changes in the expression of numerous inflammatory markers using RT-qPCR. Our transcriptomic investigations demonstrated an upregulation in the expression of pro-inflammatory mediators, associated with

decreased levels of anti-inflammatory molecules, in the ACC and PI of 6-OHDA males and females. Remarkably, some of these variations were highlighted in ADHD patients (increased levels of IL-6, lower expression of BDNF) (J. P.-C. Chang et al., 2020; Elsadek et al., 2020) and chronic pain conditions (activation of Wnt5a and MMP9 contribute to pathological pain) (Kawasaki, Xu, et al., 2008; X. Liu et al., 2023; J.-F. Wang et al., 2020). In the ACC and PI of 6-OHDA males and females, networks centered around pro-inflammatory cytokines (IL-6, TNF- α , IL-1 β) and their inhibition of BDNF secretion were established. However, it is important to note that despite identifying modifications in numerous inflammatory marker levels in the ACC and PI of 6-OHDA mice, those changes were limited to specific molecules. As some inflammatory mediators did not display alterations in their transcriptomic expressions, our findings suggest a mild pro-inflammatory environment in these brain regions.

A more in-depth study of the role of the purinergic P2X₄R in ADHD and associated pain sensitization highlighted its implication in microglia morphology and inflammatory marker release. Indeed, P2X₄R deletion restrained microglial morphological changes and their release of inflammatory markers in the ACC of 6-OHDA mice (Iba1, IL-6, TNF- α), in line with the alleviation of locomotor activity. However, its suppression favored reactive astrogliosis (GFAP, CCL4, CXCL12) and the expression of pro-inflammatory mediators (GSK3 β , NF- κ B, NLRP3), probably from non-microglial cells, in the ACC and PI of 6-OHDA mice. Reactive astrogliosis has been involved in ADHD occurrence and symptoms (Sandau et al., 2012; P. Zhang et al., 2022) while it was linked to reduced locomotor activity in our 6-OHDA mouse model. Consequently, P2X₄R could exhibit its detrimental effects on hyperactivity through the induction of microglial reactivity while attenuating non-microglial-related inflammatory processes.

With our transcriptomic findings supporting the predominance of a pro-inflammatory environment in the ACC and PI of 6-OHDA mice, a more thorough comprehension of the mechanisms occurring in ADHD-like conditions became of interest to us. Therefore, we used mass spectrometry and phosphoproteomic assays (PamGene technique), two high-throughput screening methods, to detect impairments in protein expression and kinase activity respectively.

Mass spectrometry analysis reported abnormalities in protein expression that correlated with modifications in canonical pathways: (i) axon guidance, apoptosis, and BBB integrity in the ACC of 6-OHDA females, (ii) axon guidance, endocytosis, and cell adhesion in the ACC of 6-OHDA males, (iii) cytoskeleton dynamics in the PI of 6-OHDA females, and (iv) actin polymerization and cell division in the PI of 6-OHDA males. Additionally, network enrichment analysis linked changes in protein expression with (i) a network focused on actin and microtubule dynamics in the ACC of 6-OHDA females, (ii) an interplay between immunity, ribosomes, and alternative splicing in the ACC of 6-OHDA males, (iii) interactions between inflammation, neuronal viability, and BBB integrity in the PI of 6-OHDA females, and (iv) a network centered around inflammation in the PI of 6-OHDA males. These results shed light on variations in immune and synaptic plasticity-related processes in ADHD-like conditions.

We were next concerned with understanding the consequences of protein expression alterations on their functional activity. To do so, we performed two phosphoproteomic assays (STK and PTK PamGene assays) and confirmed an abnormal activity of serine-threonine and tyrosine kinases in ADHD-like conditions. These functional impairments were associated with changes in (i) phagocytosis, cell death, axon guidance, and chemokine signaling in the ACC of 6-OHDA females, (ii) focal adhesion, axon guidance, serotonin receptor signaling, and other inflammatory cascades (ErbB, PI3K-Akt) in the ACC of 6-OHDA males, (iii) numerous signaling cascades (MAPK, ErbB, mTOR), neurotrophins, serotonin receptor, and synaptogenesis in the PI of 6-OHDA females, and (iv) inflammatory signaling cascades

(MAPK, Ras, ErbB, PI3K-Akt, STAT), neurotrophins, T-cell receptor, axon guidance, and synaptogenesis in the PI of 6-OHDA males. Taken together, these observations suggest modified kinase activity in ADHD-like conditions that lead to impairments in the modulation of the immune response, axonal guidance processes, neurotrophin signaling pathways, and serotonergic receptor signaling cascades.

Altogether, our data underline the key role played by astrocytes in ADHD-like conditions with (i) morphological changes associated with reactive astrogliosis in the ACC of 6-OHDA mice, (ii) the expression of pro-inflammatory markers, to which astrocytes contribute, in the ACC and PI of 6-OHDA mice, and (iii) impairments in canonical pathways and processes to which astrocytes are tightly linked in the ACC and PI of 6-OHDA mice. Therefore, we focused on unraveling variations in neuron-astrocyte connectivity in ADHD-like conditions. The FRET-mfFLIM results confirmed enhanced EphA3 (expressed by astrocytes) and EphA4 (expressed by neurons) interactions, pointing to intensified neuron-astrocyte communication in the ACC of 6-OHDA mice.

Transcriptomic, proteomic, phosphoproteomic, and morphological data integration pinpointed specific pathways altered in the ACC and PI of 6-OHDA males and females: apoptosis, axon guidance, synaptic plasticity (LTP), and growth of neuronal components (axons, dendrites, neurites). We thus suggest that a mild and sustained pro-inflammatory state drives changes in these processes, resulting in cell-cell connectivity and neuronal activity modifications. These alterations may underlie the comorbidity between ADHD and pain sensitization.

Methods

Our study relied on a panoply of techniques (immunofluorescence staining, RT-qPCR, mass spectrometry, phosphoproteomic assays, pathway and network enrichment analysis) to elucidate, to the greater extent, the impaired cellular pathways that may underlie the comorbidity between ADHD and pain sensitization.

Immunofluorescence staining is the most established method for the study of cellular morphology. It is a relatively fast and specific technique for protein detection and morphological change visualization. We have chosen to perform Iba1 and GFAP immunostaining as these markers are the most widely used for microglia and astrocyte morphological studies, respectively. Clear changes in cellular structures were identified in 6-OHDA mice compared to shams, hence the non-necessity to complement our investigations by targeting other proteins. However, we acknowledge that using multiple markers could provide us with a better overview of cellular morphology.

RT-qPCR is a sensitive technique used for mRNA quantification. Its protocol is long and time-consuming; however, it allows the detection of low amounts of nucleic acids. As we were blind regarding the mRNA expression of our gene of interest, we relied on this technique to increase the chances of detecting genes expressed at low levels in our samples.

Mass spectrometry and phosphoproteomic assays (PamGene) are two high-throughput screening methods that were performed to investigate changes in protein expression and kinase activity, respectively. Their advantages include wide coverage (screening of all proteins and kinases in the samples), relatively short protocols, and high sensitivity. One additional benefit of using these techniques was the amount of data generated and the relevance of their interpretation to our study as they both enable pathway and network enrichment analysis.

Finally, we have used the KEGG software for a rapid overview of the impaired cellular processes in ADHD-like conditions. However, this analysis was complemented with the IPA software, on which we

mainly focused. The IPA software is user-friendly, and the analysis is fast and compatible with all types of data (proteomic and phosphoproteomic entries). It enables simultaneous pathway and network enrichment analysis (unlike other bioinformatic tools). Not only does it identify the significantly altered pathways but it also provides insights into their activity changes (inhibition, activation).

Impaired pathways in ADHD and chronic pain

The identified cellular processes were not only disrupted in our 6-OHDA mouse model but they were also reported to be impaired in ADHD patients and chronic pain conditions. These observations further support the implication of those signaling pathways in ADHD and its associated pain sensitization. Remarkably, Eph/ephrin bidirectional signaling participates in the above-mentioned canonical pathways by regulating well-identified signaling cascades (J. Yang et al., 2018).

Apoptosis

Subcortical and cortical volumetric reductions were extensively reported in ADHD subjects. Remarkably, the apoptotic pathway was enriched in the affected brain areas, confirming the role of cell death in these volumetric divergences (J. L. Hess et al., 2018). Apoptosis was also closely related to synaptic function and attention. Indeed, it is required for synaptic plasticity (differential regulation of LTP and LTD), learning, memory, and neurite outgrowth (Gilman & Mattson, 2002; Snigdha et al., 2012), and its disruption has been linked to attentional problems (Lo et al., 2015; Valbonesi et al., 2015). Reciprocally, apoptotic processes seem to be influenced by methylphenidate, the most common ADHD treatment. Indeed, methylphenidate administration either reduced or increased the expression of pro- (Bax, caspase-3, cytochrome C) and anti-apoptotic (Bcl-2) molecules age- (young, adult rats) and region-dependently (cerebellum, striatum, hippocampus) (Réus et al., 2014). This indicates the close interaction between apoptosis and ADHD. Moreover, apoptosis participates in the occurrence of chronic pain conditions (Ribeiro et al., 2022). Following peripheral nerve injury, enhanced levels of apoptotic mediators (caspase-3, caspase-8, caspase-12, caspase-7, calpain) were detected in the dorsal root ganglion neurons (Wiberg et al., 2018). The administration of ALCAR, erythropoietin, and other analgesic molecules (isoflurane, paeoniflorin, tetramethylpyrazine) simultaneously suppressed pain-related behaviors and apoptotic activities (Campana & Myers, 2003; Di Cesare Mannelli et al., 2009; Ribeiro et al., 2022), suggesting a link between these two processes. However, peripheral nerve injury interestingly disrupted caspase-3/AMPA-R interactions and prevented LTD in the ACC, resulting in nociceptor behaviors (Y.-J. Wang et al., 2020). Caspase-3 accumulation and LTD restoration relieved mechanical allodynia (Y.-J. Wang et al., 2020), indicating a protective role for caspase-3 in pain hypersensitivity.

Synaptic plasticity and axon guidance

Dysfunctions in synaptic plasticity, axon guidance, and related processes (reduction in hippocampal LTP, low spine density, increased number of immature spines, decreased number in mature spines, impaired working memory) were observed in ADHD animal models and patients (Ugarte et al., 2023). Loci involved in synaptic processes were identified as risk factors for ADHD including FOX2 (synapse formation, learning-related neuronal processes), SORCS3 (neuronal development and plasticity), DUSP6 (control of neurotransmitter homeostasis through synaptic dopaminergic level regulation), and SEMA6D (neural wiring, axon guidance) (Demontis et al., 2019). Even though impairments in their associated proteins were not detected in our 6-OHDA mice, our results point to modifications in their related cellular processes in ADHD-like conditions. Changes in other ADHD-candidate genes (DRD5, SLC6A3, 5-HT1B, GRM1, GRM7) were also not identified in our 6-OHDA mice; however, alterations in

the neurotransmitter systems they are involved in (dopaminergic, serotonergic, glutamatergic) were enriched in ADHD-like conditions (Dark et al., 2018). Nevertheless, BDNF (synaptogenesis, cell death, glial cells), another ADHD-candidate gene (Dark et al., 2018), seems to be drastically affected in 6-OHDA mice.

Dendritic spine plasticity is critical for neuronal circuit development. Dendritic spine density, size, and complexity dictate the number of excitatory inputs a neuron can receive. During childhood, their refinement is required to attain adaptable synaptic circuits and enable the proper functioning of the CNS. Neurodevelopmental disorders are characterized by spine pathology (atypical spines) (Forrest et al., 2018). For instance, decreased spine density and increased spine immature states (thin-type spines) were observed in the prenatal nicotine exposure ADHD mouse model, both restored by methylphenidate treatment (Contreras et al., 2022).

The Eph/ephrin signaling contributes to synapse formation, plasticity, and functioning (Hruska & Dalva, 2012; Murai & Pasquale, 2002; Takasu et al., 2002). In the hippocampus, suppression of EphA4/EphrinA3 signaling resulted in impaired spine shape and organization (Murai et al., 2003). One mechanism by which EphA4 mediates the size and number of dendritic spines is through its tight interactions with GTPase RhoB which lead to actin cytoskeleton remodeling (McKinnell et al., 2004). Moreover, the blockade of EphrinA3 decreased pFAK cascade and reduced dendritic spines on proximal dendrites of Purkinje cells through the inactivation of EphA4 signaling (Heintz et al., 2016). Interestingly, impairments in actin cytoskeleton, Rho GTPase signaling, and pFAK cascade were noted in the ACC and PI of 6-OHDA mice. Furthermore, EphA4 signaling negatively regulates neurite outgrowth and is required for axon guidance (Huot, 2004), two processes found to be altered in ADHD-like conditions. Blockade of EphA4 astrocytic expression promoted neurite extension of the surrounding neurons (Goldshmit et al., 2004) while EphrinA4 activation resulted in the retraction and halt of growth cones (Shamah et al., 2001).

One key characteristic of the CNS is its plasticity and, consequently, its ability to adapt and change synaptic-related processes context-dependently. Synaptic strength can be modified following nerve injury, inflammation, and other detrimental stimuli resulting in synaptic potentiation, depression, or silencing (C. Luo et al., 2014). Mechanisms of synaptic plasticity underlie altered pain processing. These include short- and long-term changes that facilitate (potentiation) or reduce (depression) synaptic transmission (probability of neurotransmitter release). Short-term modifications last from milliseconds to a few minutes while long-term changes persist for at least thirty minutes after the stimulation and can be sustained for hours, days, or months (C. Luo et al., 2014). These processes have been notably demonstrated in regions involved in pain perception such as the ACC (Gemmell & O'Mara, 2002; Shyu & Vogt, 2009), the IC (M.-G. Liu et al., 2013), and the spinal cord (Genzen & McGehee, 2003; Terman et al., 2001; Tsuzuki et al., 2004; H.-M. Zhang et al., 2004). Short-term synaptic changes enable the processing of nociceptive information at all CNS levels and are therefore involved in the perception of acute pain. On the other hand, long-term modifications, particularly LTP, participate in pain-associated hyperalgesia (Sandkühler & Gruber-Schoffnegger, 2012; Zhuo, 2014). Recent evidence also suggests a causal relationship between LTP and working memory. A significant decrease in hippocampal LTP resulted in working memory deficits (Piña et al., 2020), impairments apparent in individuals with ADHD (BARNETT et al., 2001; Kennedy et al., 2019). Importantly, they were reversed by atomoxetine and methylphenidate treatments (Bedard et al., 2004; Piña et al., 2020). These findings endorse the involvement of impaired synaptic processes in ADHD and its associated pain sensitization. Indeed, alterations in synaptic plasticity, and more precisely LTP, have been identified in the ACC and PI of 6-

OHDA males and females and could, therefore, explain pain sensitization previously demonstrated in those mice (Bouchatta et al., 2022).

Evidence suggests a pivotal role for EphrinB2 and EphrinB3 in hippocampal synaptic plasticity processes, such as LTP and LTD. The study notably stated EphA4 as a candidate receptor for both EphrinBs (Grunwald et al., 2004).

Neurotransmission

The ACC and PI are both part of the pain matrix. The ACC is not only critical for the affective-motivational aspect of pain but it also participates in chronic pain and its related anxiety (Labrakakis, 2023; Yao et al., 2023). For instance, nerve injury triggers LTP at some ACC synapses through enhanced AMPA-R number, resulting in intensified pain responses (X.-Y. Li et al., 2010) while blockade of presynaptic LTP in the ACC serves as an anxiolytic in chronic pain conditions (Koga et al., 2015). On the other hand, the PI is known to participate in the sensory-discriminative dimension of pain (Yao et al., 2023). However, findings also demonstrate a correlation between its gray matter volume, connectivity with the postcentral gyrus, and pain thresholds; further highlighting the role of this brain region not only in pain perception but also as a central hub in the determination of pain thresholds (Neumann et al., 2021).

Increased excitatory glutamatergic transmission in these two cortical regions has been linked to synaptic plasticity changes (Zhuo, 2006, 2008, 2014, 2016). Increased glutamatergic transmission is also involved in pathological pain through the upregulation of AMPA-R and NMDA-R expressions and responses (Bliss et al., 2016; T. Chen et al., 2014; S.-B. Liu et al., 2015; Qiu et al., 2013, 2014; H. Xu et al., 2008). Interestingly, the inhibition of insular and cingulate synaptic potentiation was reported to be analgesic (J. Han et al., 2015; X.-Y. Li et al., 2010), suggesting a central role played by insular and cingulate LTP in pain behaviors.

EphA4/EphrinA3 bidirectional signaling was reported to regulate LTP by modulating glutamate transporter expression. The absence of dendritic EphA4 or astrocytic EphrinA3 upregulated glutamate transporter levels, promoted glutamate uptake capacity, and restricted glutamate receptor activation thus leading to an insufficient depolarization of post-synaptic neurons and, consequently, impaired LTP. However, overexpression of astrocytic EphrinA3 or dendritic EphA4 also limited glutamate transporter expression and exacerbated glutamate accumulation, resulting in excitotoxicity (Carmona et al., 2009; Filosa et al., 2009; J. Yang et al., 2014). Remarkably, changes in LTP were dominant in the ACC and PI of 6-OHDA mice.

Pain sensitization

Not only does the Eph/ephrin signaling contribute to synaptic processes, but it is also involved in pain processing (Vasileiou et al., 2013). Inhibition of Ephrin B1 abolished allodynia and thermal hyperalgesia in a lysophosphatidic acid-induced neuropathic pain model (Uchida et al., 2009) while suppression of Ephrin B2 reduced neuropathic pain-associated mechanical allodynia (Kobayashi et al., 2007). Ephrin B1 signaling induces pain behaviors through PI3K-Akt and ERK activation (X.-H. Guan et al., 2010), two signaling pathways enriched in our 6-OHDA mice.

Reactive astrogliosis

EphA4 signaling promotes reactive astrogliosis. Following spinal cord injury, an upregulation in the expression of astrocytic EphA4 was observed near the lesioned area. EphA4 knock-out not only

restricted the astrocytic response to pro-inflammatory cytokines but also limited glial scar formation (Goldshmit et al., 2004).

Implication of the enriched signaling pathways

Interestingly, several signaling pathways enriched in the ACC and PI of 6-OHDA males and females are involved in the above-mentioned altered pathways.

In vitro studies reported the contribution of the Nrg1/ErbB4 pathway in the development of GABAergic neuron dendrites and axons (Fazzari et al., 2010), pointing to the role of this signaling cascade in the outgrowth of neuronal components (pathway identified as disrupted in our ADHD-like mouse model). Interestingly, dendritic arborization of hippocampal neurons relied on ErbB pathway activation and its downstream PI3K kinase (Krivosheya et al., 2008). Additionally, the participation of the ERK/MAPK signaling cascade was demonstrated in neurite outgrowth of both cultured cerebellar (Schmid et al., 2000) and hippocampal neurons (Poplawski et al., 2012).

The PI3K-Akt-mTOR cascade modulates essential biological processes such as cell death and proliferation (L. Wang et al., 2017). More particularly, Akt3 partakes in brain development. Indeed, mice displaying a knockout of Akt3 demonstrated reduced brain size (Easton et al., 2005; Tschopp et al., 2005), impaired features observed in ADHD patients (more details in p34: Macro-anatomical changes).

Overall, deviations in the optimal level of those signaling cascades in the brain could result in impaired functions and could be implicated in the pathophysiology of neurodevelopmental and psychiatric disorders (Iroegbu et al., 2021; Mei & Nave, 2014; L. Wang et al., 2017).

Another type of transmission found to participate in ADHD-like conditions is the P2X₄R signaling. Our findings align with the dual properties of P2X₄R, reported in neurological disorders. P2X₄R participates in the etiology of neurological conditions through well-defined mechanisms (Varma et al., 2009), but protects motoneurons in models of amyotrophic lateral sclerosis (Andries et al., 2007) and autoimmune encephalitis (Zabala et al., 2018). P2X₄R has also been implicated in inflammatory and neuropathic pain, a role particularly dependent on its microglial expression (Guo et al., 2005; C. Liu et al., 2018; Long et al., 2018, 2020; Tsuda et al., 2003). P2X₄R knock-out showed little effect on pain sensitivity in our 6-OHDA mice, in agreement with the absence of expressional changes of pain-related markers (Wnt5a, BDNF, MMP9) (Kawasaki, Xu, et al., 2008; X. Liu et al., 2023; Thakkar & Acevedo, 2023; J.-F. Wang et al., 2020). More interestingly, P2X₄R expression was reported on both microglia and neurons (Bo et al., 2003), displaying cellular-dependent properties (Duveau et al., 2020). For instance, the modulation of neuronal and microglial P2X₄R expression exerted divergent effects in animal models of amyotrophic lateral sclerosis (Bertin et al., 2022). This double impact could therefore explain our findings. Microglial P2X₄R expression may worsen locomotor activity while its neuronal expression may protect against neuroinflammation. Consequently, the differential role of P2X₄R on ADHD symptoms and pain sensitization likely depends on the combination of its neuronal and microglial functions.

Contribution of glial cells

The above-mentioned disrupted pathways were also found to be regulated by astrocytes and microglial cells.

Selective activation of microglial cells was found in the spinal cord, but not in cortical brain structures,

in neuropathic pain conditions (F. Zhang et al., 2008). Minocycline did not affect pre- and post-LTP in the ACC (Q. Song et al., 2015) thus excluding the role of microglial cells in these cingulate processes. Nevertheless, astrocytic activation in the ACC was reported in different chronic pain conditions, raising questions about potential neuronal-astrocytic crosstalk (H. Ikeda et al., 2013b; Kuzumaki et al., 2007; Y. Lu et al., 2011). Astroglia toxin injection suppressed the inflammatory-induced long-term facilitation in the ACC, confirming the effects of cingulate astrocytic activation on synaptic plasticity under pain conditions (H. Ikeda et al., 2013b). These data further support the key role played by astrocytes in ADHD and its associated pain sensitization.

Glial cells and sex differences

More interestingly, and in line with the gender differences that have been highlighted in neurodevelopmental disorders (May et al., 2019), more particularly in ADHD (Babinski, 2024), astrocytes and microglia also display sex differences.

Microglia early inhibition, depletion, or knock-out results in profound neurodevelopmental deficits including locomotor hyperactivity (Nelson & Lenz, 2017; Rosin et al., 2018; VanRyzin et al., 2016). However, these cells remarkably exhibit sex differences regarding their number, morphology, and gene expression in both the developing and adult brains (Breach & Lenz, 2022). Compared to females, microglia were found to be more numerous in the healthy hippocampus and cortex of 3 and 13-week mouse males (Guneykaya et al., 2018) and the cerebral cortex following cortical injury (Acáz-Fonseca et al., 2015). Nonetheless, this enhanced immunoreactivity was attributed to an increased number of microglia with a nonreactive phenotype (ramified structure) near the lesion (Acáz-Fonseca et al., 2015). These results are in line with those of (Schwarz et al., 2012) who reported a decreased detection of round microglial form in several higher structures of P30 male and female rats. In agreement with these observations, microglial morphology significantly differed between the hippocampus of P60 male and female rats. In males, microglial cells exhibited higher process volume and area, increased number of branches, and enhanced number of intersections compared to females (Hanamsagar et al., 2017). In line with our morphological findings, these results point to a more ramified microglial structure in the brains of adolescent and adult males. Additionally, microglial cells were found to display a delay in gene expression, both during development and by early adulthood, in males compared to females. For instance, immune-related genes were upregulated in females compared to males at P60. It is an inflammatory assault (LPS administration) that resulted in the overexpression of those genes in males at E18 and P60, confirming a sex-biased microglial gene expression (Hanamsagar et al., 2017). This could explain the enrichment of immune-associated processes identified by our pathway and network analyses, in the ACC and PI of 6-OHDA males. Any deleterious stimuli could trigger a more amplified expression of inflammatory genes in males than in females.

Likewise, astrocytes exert sex differences in their number, morphology, and properties (Breach & Lenz, 2022). Neonatal astrocytes displayed an increased number of processes (Amateau & McCarthy, 2002) and released more pro-inflammatory cytokines following LPS injection (Santos-Galindo et al., 2011) or TLR4-induced inflammatory response (Loram et al., 2012) in males compared to females. Astrocytes from adult male rats presented increased GFAP immunoreactivity, but no changes in the number of GFAP-expressing cells, compared to females (Chowen et al., 1995). Even though these observations contradict our morphological findings which support a more prominent reactive astrogliosis in the ACC of 6-OHDA females, pronounced differences in cellular morphology were noted in the ACC of 6-OHDA males where astrocytes exhibited a more ramified structure compared to controls. Additionally,

pathways and networks revolving around inflammation were identified in the ACC and PI of 6-OHDA males, pointing to a potential role of astrocytes in the amplification of the immune response. Interestingly, and in line with our transcriptomic findings, astrocytic CCL2 release was detected in the cerebral cortex of males, and not females, following cortical injury (Acaz-Fonseca et al., 2015). CCL2 not only mediates immune cell chemotaxis and cytokine production but further intensifies astrocytic arborization, in agreement with our morphological data (Bose & Cho, 2013; Joly-Amado et al., 2020; Semple et al., 2010).

Overall, these discrepancies could point out differential biological mechanisms underpinning ADHD in boys and girls, with which growing evidence agrees. Moreover, recent data support the key role of the immune system and sex hormones in neurodevelopmental disorders and their associated sex differences (Breach & Lenz, 2022).

Sex hormones drive neurodevelopmental problems and could explain the immune-related sex differences observed during development and adulthood. For instance, prenatal exposure to altered levels of androgen induced changes in brain development and functioning, associated with neurodevelopmental disorders (Lombardo et al., 2020). On the contrary, estrogens have been shown to exert neuroprotective functions with their administration shifting the release of pro-inflammatory mediators to anti-inflammatory molecules, suppressing NF- κ B activation, limiting neuronal injury, and reducing the lesion volume (Gatson et al., 2012; Ghisletti et al., 2005; Giraud et al., 2010; Soldan et al., 2003; Spence et al., 2013). However, estradiol treatment also produced differential effects on microglial cells, sex- and age-dependently. It displayed anti-inflammatory properties on neonatal male microglia while exhibiting a pro-inflammatory impact on neonatal female microglia. More surprisingly, these effects were reversed in adult hippocampal cells with a protective effect on female but not male microglia cells (Loram et al., 2012).

Evidence suggests a crosstalk between the dopaminergic and immune systems. Dopamine exerts immunomodulatory functions that can generate pro- or anti-inflammatory effects, context-dependently. Immune cells, particularly microglia, express dopaminergic receptors. This enables dopamine uptake by microglia, their regulation, and the modulation of their neighboring cells. Microglial functions (chemotaxis, pro-inflammatory cytokine release, phagocytosis) are influenced by dopaminergic signaling. For instance, NF- κ B is suppressed by D1-like receptor activation (anti-inflammatory properties) but is stimulated following D2-like receptor activation (pro-inflammatory effects). In astroglia, dopamine signaling participates in inflammation and neuronal survival. Conversely, the immune system can control the dopaminergic machinery by impacting dopamine receptor expression, metabolism, release, and function (Channer et al., 2023). Interestingly, estrogens also display regulatory effects on the dopaminergic system in females but not males (Bendis et al., 2024). For instance, dopamine release, D2-like receptor binding capacity, and DAT transporter activity were enhanced by estradiol treatment in females only (J. B. Becker, 1990; Yoest et al., 2018).

Estrogens, dopamine, and the immune system mutually influence each other, creating an interplay that ensures brain homeostasis. Disruption in one of these entities (the dopaminergic system, in ADHD conditions) could jeopardize this equilibrium, resulting in an uncontrolled and sustained inflammatory environment. This pro-inflammatory status induces changes in synaptic plasticity and pain modulatory processes, leading to neurodevelopmental deficits and impaired pain perception. This deleterious impact seems amplified in females, probably due to the close interactions between estrogens and dopamine.

Limitations of the work

This Ph.D. work has some limitations. First, our study is based on a predictive analysis of the impaired processes that may underlie the comorbidity between ADHD and pain sensitization. This descriptive investigation should be complemented by an *in vivo* modulation of the identified canonical pathways to confirm their involvement in ADHD symptoms and pain hypersensitivity. Second, changes in proteins encoded by ADHD-candidate genes were not extensively detected in the ACC and PI of 6-OHDA males and females. These differences can raise questions about the relevance of those loci in ADHD or, even, the construct validity of the neonatal 6-OHDA mouse model. They could also be justified by a lack of genetic investigations pinpointing gene polymorphisms in our research study. Third, divergences in glial reactivity were reported between our 6-OHDA mice and existing rodent models (Meseguer-Beltrán et al., 2023). Such variabilities can emerge when working with animal models, highlighting the impact of genetic and environmental factors on biological mechanisms. Fourth, our study misses a transcriptomic high-throughput screening to complement our proteomic and phosphoproteomic data. However, we chose not to perform such an experiment as we were more interested in modifications affecting the structural and functional molecules that govern cellular processes. Fifth, our study did not consider the roles of hormones, despite being involved in inflammation and neurotransmission. It is, therefore, another aspect that could be examined in our project. Finally, as the total deletion of the P2X₄R suppressed its expression in both neurons and microglial cells, the observed effects can't be attributed to a cell-specific expression. Consequently, it would be interesting to repeat the same experiments in mice with targeted receptor ablation to determine the role of neuronal and microglial P2X₄R on ADHD symptoms, pain sensitization, and neuroinflammation.

Perspectives and conclusion

As previously mentioned, one of the main limitations of this Ph.D. work lies in its descriptive analysis of the potentially impaired cellular processes in ADHD-like conditions, with a lack of understanding of the consequences of these alterations on neuronal activity. Additional experiments could complement this work to provide a more in-depth understanding of the functional consequences of neuroinflammation.

Among the techniques that could be used is calcium imaging, which enables the recording of neuronal activity using calcium-dependent fluorescent sensors (Stringer & Pachitariu, 2019). The experiments are currently being performed by Sandra Sánchez. They consist of monitoring ACC pyramidal neuron activity in ADHD-like conditions under nociceptive stimulation or during an attention-demanding task. Briefly, calcium imaging is performed to assess neuronal activity in freely moving mice using microendoscopy. This is achieved following (i) the stereotaxic injection of AAV9-CaMKII α -GCaMP6f-WPRE to selectively express GCaMP6f in ACC excitatory neurons, and (ii) the implantation of GRIN lenses to monitor layer V of ACC neurons. Calcium responses to painful stimulation or attention-demanding tasks are recorded in 6-OHDA and sham mice.

The modulation of Eph/Ephrin bidirectional signaling could also clarify its implication in ADHD and pain sensitization comorbidity. Involved in a wide range of processes impaired in ADHD-like conditions (axon guidance, LTP, neurite outgrowth, reactive astrogliosis), as detailed previously, the EphA4/ephrinA3 signaling could be a noteworthy target. Numerous strategies were developed to specifically interfere with the EphA4 receptor including antibodies (Nb39 and NB53 anti-EphA4 nanobodies) and interfering RNAs (EphA4 overexpression by miR-145 inhibitor, siRNAs targeting

EphA4) (Baudet et al., 2020). The effects of this manipulation on ADHD symptoms, pain sensitization, and LTP should be evaluated using the open-field test (hyperactivity), the 5-CSRTT (attentional deficits and impulsivity), the Von Frey test (pain sensitization to mechanical stimuli), the Plantar test (pain sensitization to thermal stimuli), and the patch-clamp technique (LTP).

In conclusion, our work provides some insights into the mechanisms behind ADHD and its associated pain sensitization. Our data suggest the presence of a sustained, mild, pro-inflammatory state in cortical regions (ACC and PI) involved in cognitive functions and pain processing. This inflammatory environment is characterized by microglial and astrocytic reactivity, pro-inflammatory mediator release, protein expression variations, and kinase activity disruptions. These alterations drive changes in synaptic plasticity (LTP), axonal guidance, and apoptosis; cellular processes previously linked to ADHD and pathological pain. Cell-cell connectivity (neuronal-astrocytic communication, as described by the increase in EphA4-ephrinA3 interactions) and neuronal activity (hyperactivity of ACC neurons) are consequently modified, thus sensitizing pathways involved in pain perception and ADHD symptoms. Our findings also support the role of the P2X₄R in ADHD-like symptoms but not pain sensitization. This purinergic receptor appears to regulate microglia morphological changes associated with cellular reactivity, protect against neuroinflammation, and worsen ADHD-associated hyperactivity.

Conclusion

ADHD and chronic pain are two disabling and prevalent pathologies of multifactorial etiology that often co-exist. However, the mechanisms underlying this comorbidity have not been resolved yet. Interestingly, these two syndromes share impairments in common cortical regions, the ACC and PI, both involved in cognitive functions and pain processing.

Growing evidence suggests a role for neuroinflammation in ADHD pathophysiology and pathological pain conditions. The inflammatory response could therefore act as a bridge between these two disorders. Moreover, previous findings from our team demonstrated a heightened activity of ACC neurons and a deregulation of the ACC-PI pathway in ADHD-like conditions. We, therefore, suggest that neuroinflammation triggers ACC hyperactivity and sensitizes pathways participating in ADHD symptoms and pain processing. This Ph.D. work thus aims to elucidate the inflammatory mechanisms underlying ADHD and its associated pain sensitization.

To address this question, we generated an ADHD-like mouse model, previously validated by our team, by injecting 6-OHDA, at P5, in one of the lateral ventricles. The ACC and PI were extracted, and a panoply of experiments were performed.

We studied the morphology of astrocytes and microglia, two families of glial cells taking part in the inflammatory response within the CNS. Our findings supported microglial reactivity in the ACC of 6-OHDA females only, and reactive astrogliosis in the ACC of both sexes. We then assessed the transcriptomic expression of inflammatory markers in the ACC and PI of 6-OHDA mice to better understand their inflammatory status in ADHD-like conditions. Our RT-qPCR results pointed to a pro-inflammatory environment in both cortical regions, characterized by an upregulated expression of pro-inflammatory mediators and downregulated levels of anti-inflammatory markers. Remarkably, the integration of the identified markers supported a network centered around pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α) and their modulation (inhibition) of BDNF release.

For a more in-depth analysis of the inflammatory processes occurring in the ACC and PI of 6-OHDA males and females, we used two high-throughput screening methods that deciphered changes in protein expressions (mass spectrometry) and kinase activity (PamGene assays) in ADHD-like conditions. Mass spectrometry data supported modifications in protein expression that correlated with impairments in axon guidance, apoptosis, BBB integrity, and cytoskeleton dynamics. Additionally, network enrichment analysis pointed out a prime role for inflammation and cytoskeleton dynamics in these higher cortical structures. The PamGene assays detected variations in serine-threonine and tyrosine kinase activity in the ACC and PI of 6-OHDA mice indicating disruptions in cell death, axon guidance, chemokines, synaptic plasticity, numerous signaling cascades (PI3K-Akt, MAPK, mTOR, ErbB), neurotrophins, and neurotransmitter systems.

As our findings revealed a potential key role of astrocytes in ADHD-like conditions, we examined changes in astrocytic-neuronal connectivity in the ACC of 6-OHDA mice. Using the FRET-mfFLIM technique, we deciphered alterations in EphA3 (expressed by astrocytes) and EphA4 (expressed by neurons) interactions and, consequently, enhanced neuron-astrocyte communication in ADHD-like conditions.

Overall, the integration of our morphological, transcriptomic, proteomic, and phosphoproteomic data identified impairments in four main processes in the ACC and PI of 6-OHDA males and females: apoptosis, axon guidance, synaptic plasticity (LTP), and outgrowth of neuronal components. These modifications are triggered by a mild pro-inflammatory environment that disrupts cell-cell connectivity (most probably, neuronal-astrocytic communication) and neuronal activity (ACC hyperactivity); they

could, therefore, underlie the comorbidity between ADHD and pain sensitization. Interestingly, these canonical pathways were previously associated with ADHD and pathological pain, further supporting our findings. The EphrinA4/EphA3 bidirectional signaling was also reported to be involved in these processes, making it an interesting target to modulate.

The second objective of this Ph.D. thesis aimed to clarify the role of the purinergic P2X₄ receptor in ADHD and its associated pain sensitization. Our observations suggested a role for the P2X₄ receptor in the enhancement of microglial reactivity, protection against neuroinflammation (probably triggered by non-microglial cells), and worsening of ADHD hyperactivity symptom. No effects on thermal and mechanical pain hypersensitivity were noted.

Overall, this Ph.D. work provides insights into the molecular mechanisms that may underlie ADHD and its associated pain sensitization. From changes in glial cell morphology to the increased transcriptomic expression of inflammatory molecules, our results shed light on a pro-inflammatory status maintained in the ACC and PI of 6-OHDA mice. This mild and sustained pro-inflammatory environment impairs protein expression and kinase activity, resulting in altered axonal guidance, apoptotic, and synaptic-related processes. These modifications disrupt neuron-astrocyte connectivity and neuronal activity, thus triggering ADHD and pain sensitization comorbidity.

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