Role of glutamate N-Methyl-D-Aspartate receptor surface trafficking in the firing pattern of midbrain dopaminergic neurons

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Sous la direction de Laurent GROC

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Publications

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Abstract

Midbrain dopaminergic (DA) neurons play several key functions in the brain such as the processing of salient information but are also associated with the emergence of pathologies including Parkinson's disease and drug addiction. Because these processes have in common to modify the firing activity of midbrain DA neurons, it is of crucial importance to understand the mechanisms underlying this activity. Among the various ions channels and receptors involved in the generation of the firing activity of midbrain DA neurons, glutamate N-methyl-D-aspartate receptors (NMDAR) and calcium-dependent potassium SK channels strongly modulate the firing pattern and functionally interact in several neuronal types including DA neurons. However, the mechanisms by which they regulate the firing pattern are poorly understood. Since the functional coupling between NMDAR and SK channels depends on their relative membrane distribution, we hypothesized that the lateral diffusion of NMDAR, which regulates the surface localization of the receptor, could play a role in the firing pattern of midbrain DA neurons through the modulation of SK channel function. We showed first that membrane NMDAR was highly mobile in cultured DA neurons. Alteration of its surface trafficking by a crosslink with NMDAR antibodies profoundly modified the regularity of the firing pattern of DA neurons in midbrain slices, whereas pharmacological blockade of NMDAR did not affect it. Furthermore, a SK channel blocker, which induces a similar change in the firing regularity in control conditions, was less effective when NMDAR surface trafficking was altered. Taken together, these results demonstrate that NMDAR surface dynamics modulate the firing pattern of midbrain DA neurons by regulating SK channel function.

Key words : glutamate NMDA receptors, surface trafficking, dopamine, firing pattern, crosslink, electrophysiology

Résumé

Les neurones dopaminergiques (DA) mésencéphaliques jouent un rôle prépondérant dans de nombreuses fonctions cérébrales telles que la motivation, mais ils sont également impliqués dans l'émergence de pathologies telles que la maladie de Parkinson et l'addiction aux drogues. Ces processus ayant en commun de modifier l'activité de décharge des neurones DA mésencéphaliques, il est d'une importance primordiale de comprendre les mécanismes sous-tendant cette activité. Parmi les différents canaux ioniques et récepteurs impliqués dans la génération de l'activité de décharge des neurones DA, les récepteurs au glutamate de type N-Methyl-D-Aspartate (NMDAR) et les canaux potassiques calcium-dépendants SK régulent fortement le patron de décharge, et interagissent fonctionnellement dans divers types neuronaux incluant les neurones DA. Cependant, les mécanismes mis en jeu dans cette régulation restent méconnus. Le couplage fonctionnel des NMDAR et des canaux SK dépendant notamment de leur distribution membranaire relative, nous avons émis l'hypothèse que la diffusion latérale des NMDAR, processus qui régule la localisation de surface du récepteur, pouvait jouer un rôle dans le patron de décharge des neurones DA *via* la modulation de la fonction des canaux SK. Nous avons tout d'abord montré que les NMDAR membranaires étaient mobiles dans les neurones DA en culture. L'altération de leur trafic de surface par immobilisation avec des anticorps anti-NMDAR modifie profondément la régularité du patron de décharge des neurones DA issus de tranches aigües de mésencéphale, alors que le blocage pharmacologique des NMDAR est sans effet. De plus, j'ai mis en évidence qu'un bloqueur des canaux SK, l'apamine, qui induit un changement similaire de la régularité du patron de décharge en condition contrôle, était moins efficace lorsque la mobilité latérale des NMDAR était altérée. Ainsi, ces résultats démontrent que la dynamique de surface des NMDAR module le patron de décharge des neurones DA en régulant la fonction des canaux SK.

Mots-clés: récepteurs au glutamate de type NMDA, dynamique de surface, dopamine, patron de décharge, crosslink, électrophysiologie

Résumé long

Les neurones dopaminergiques (DA) mésencéphaliques, principalement localisés au sein de la substance noire compacte (SNc) et de l'aire tegmentale ventrale (VTA), jouent un rôle prépondérant dans de nombreuses fonctions physiologiques telles que la recherche de récompense ou la motivation, tandis que des altérations de ce système sont impliquées dans l'émergence de maladies neuropsychiatriques, parmi lesquelles figurent la maladie de Parkinson et la schizophrénie. De façon remarquable, la plupart de ces processus est associée à des modifications de l'activité de décharge des neurones DA mésencéphaliques. Ainsi, la compréhension des mécanismes à l'origine de la décharge des neurones DA revêt une importante capitale. Parmi les canaux ioniques et les récepteurs aux neurotransmetteurs responsables de la génération de cette activité, les récepteurs au glutamate de type N-Methyl-D-Aspartate (NMDAR) et les canaux potassiques calcium-dépendants SK modulent fortement le patron de décharge des neurones DA. En effet, l'activation des NMDAR et le blocage des canaux SK par des approches pharmacologiques favorisent l'apparition de bouffées de potentiels d'action *in vivo* et *in vitro*. De plus, les NMDAR et les canaux SK interagissent de façon fonctionnelle dans divers types neuronaux incluant les neurones DA, et sont situés à proximité l'un de l'autre au sein de la membrane plasmique. Cependant, les mécanismes moléculaires mis en jeu dans cette régulation restent méconnus. Le développement de techniques d'imagerie à haute résolution a mis en évidence que les NMDAR étaient mobiles au sein de la membrane neuronale, et qu'un tel phénomène régulait la localisation des NMDAR à la surface des neurones. Sachant que le couplage fonctionnel entre les NMDAR et les canaux SK dépend notamment de leur distribution membranaire relative, j'ai émis l'hypothèse que la dynamique de surface des NMDAR pouvait jouer un rôle dans l'activité de décharge des neurones DA mésencéphaliques, *via* la modulation de la fonction des canaux SK.

Dans un premier temps, l'objectif était de caractériser la mobilité latérale des NMDAR dans des neurones DA car celle-ci n'a été étudiée que dans des neurones d'hippocampe. Pour cela, j'ai utilisé des techniques d'imagerie de suivi de particules uniques pour suivre la diffusion des NMDAR membranaires en temps réel sur des neurones DA mésencéphaliques en culture issus de souris Tyrosine Hydroxylase (TH)-tdTomato. La culture mésencéphalique n'étant pas uniquement composée de neurones DA, ces souris permettent d'identifier ces neurones par la présence de la protéine fluorescente tdTomato car elles expriment la protéine tdTomato sous le contrôle du promoteur de la TH, qui est l'enzyme de synthèse de la DA. Ainsi, j'ai pu mettre en évidence que les NMDAR étaient hautement mobiles à la surface des neurones DA, dû en partie à la faible proportion de NMDAR immobiles. De plus, j'ai caractérisé la diffusion membranaire des NMDAR dans des neurones DA humains issus de cellules souches. L'analyse des trajectoires des NMDAR sur ces neurones a révélé que

les NMDAR étaient aussi fortement mobiles dans les neurones DA issus de cellules humaines. Contrairement à la situation *in vivo* où les neurones DA sont présents au sein d'un tissu neuronal très dense et sont contactés par de multiples afférences glutamatergiques, les neurones DA en culture sont plus isolés et reçoivent très peu d'afférences excitatrices puisque la culture primaire mésencéphalique est majoritairement composé de neurones DA et *Gamma*-AminoButyric Acid (GABA)-ergiques. Pour étudier la dynamique de surface des NMDAR dans des préparations neuronales plus intégrées et/ou sous l'influence d'afférences glutamatergiques excitatrices, j'ai mis en place plusieurs stratégies. La première consistait à exprimer *in vivo* des récepteurs NMDAR « taggés » dans les neurones DA par électroporation *in utero* afin de suivre la diffusion de ces NMDAR exogènes sur des tranches aigües de mésencéphale grâce à des techniques d'imagerie de suivi de particules uniques. Bien que cette approche développée dans le laboratoire ait permis de suivre la diffusion de récepteurs membranaires dans des neurones hippocampiques, il n'a pas été possible d'adapter l'électroporation *in utero* au mésencéphale en raison de contraintes techniques. Une seconde stratégie envisagée était d'estimer la diffusion de surface des NMDAR grâce à des approches d'électrophysiologie combinées à l'utilisation du bloqueur MK-801 des NMDAR. Cette technique, développée par Tovar et Westbrook dans des cultures hippocampiques, utilise la propriété du MK-801 de ne bloquer que les NMDAR activés en raison de son site de liaison localisé au niveau du pore du récepteur. Ainsi, après le blocage des NMDAR synaptiques par stimulation synaptique en présence de MK-801, les auteurs ont noté qu'une récupération des courants synaptiques dépendants des NMDAR (NMDAR-EPSC) avait lieu lors du rinçage de la drogue. Curieusement, cette récupération était absente lorsque la totalité des NMDAR membranaires (synaptiques et extrasynaptiques) étaient bloqués par l'application conjointe de NMDA et de MK-801. Le MK-801 se liant de façon irréversible aux NMDAR, les auteurs en ont conclu que la récupération observée après le blocage des NMDAR synaptiques provenait du recrutement des NMDAR extrasynaptiques à la synapse. Cette approche permettant d'estimer la population mobile de NMDAR par la récupération des NMDAR-EPSC, j'ai voulu la tester sur des neurones DA de la VTA en tranches aigües. A la différence des neurones hippocampiques en culture où la stimulation synaptique en présence de MK-801 induit la suppression quasi-totale des NMDAR-EPSC en quelques minutes (3-4 minutes), la cinétique de blocage est beaucoup plus lente sur les neurones DA, n'atteignant que 50% d'inhibition après 15-20 minutes de rinçage. Or il est nécessaire d'obtenir une diminution nette et rapide des NMDAR-EPSC pour pouvoir observer la récupération des NMDAR-EPSC. En effet, dans le cas d'un blocage lent des NMDAR-EPSC, il est possible qu'une récupération des courants se fasse en même temps que le blocage, masquant ainsi le phénomène de récupération. Augmenter la concentration de MK-801 ou changer le protocole de stimulation synaptique (double pulse de stimulation au lieu d'un seul) ne modifiant pas la cinétique de blocage des NMDAR-EPSC, cette méthode ne peut pas être utilisée pour estimer la dynamique de surface des NMDAR dans des neurones DA en tranches. En

parallèle de ces approches menées sur des tranches aigües de cerveau, j'ai également mis en place des co-cultures de mésencéphale et de cortex pour recréer un système *in vitro* dans lequel les neurones DA reçoivent des informations excitatrices corticales, et pourvoir suivre la dynamique des NMDAR dans les neurones DA dans ces conditions. Pour cela, des neurones de mésencéphale et de cortex ont été mis en culture dans des chambres microfuidiques permettant de séparer les deux populations neuronales. Ces deux chambres étant reliés par des micro-canaux très fins, seules les projections neuronales peuvent traverser ces micro-canaux pour rejoindre les chambres. Alors que ce système a été développé avec succès avec des cultures embryonnaires de mésencéphale et de cortex, il n'a pas été possible de maintenir viables des cultures postnatales de mésencéphales issus des souris TH-tdTomato, condition nécessaire pour pouvoir identifier les neurones DA en culture et suivre la dynamique des NMDAR de surface.

En parallèle, j'ai testé l'impact de l'activation des NMDAR sur l'activité de décharge des neurones DA dans des tranches aigües de mésencéphale. Pour cela, j'ai enregistré l'activité de décharge des neurones DA en tranches par la technique de patch clamp en configuration cellule-attachée, en réponse à l'application de NMDA. Alors que l'activité basale des neurones DA en tranches est caractérisée par une décharge régulière de potentiels d'action, la présence de NMDA en augmente la fréquence et l'irrégularité. De façon remarquable, la présence de NMDA active les NMDAR mais aussi modifie la dynamique de surface des récepteurs, suggérant ainsi que l'un et/ou l'autre de ces paramètres pouvait être responsable des effets observés sur le patron de décharge. Cependant, le blocage des NMDAR par des agents pharmacologiques tels que le MK-801, ou l'antagoniste compétitif D-APV ne modifie pas l'activité de décharge des neurones. Aux vues de ces données, l'activation basale des NMDAR semble peu impliquée dans le patron de décharge spontané des neurones DA en tranches, ouvrant la possibilité que la dynamique de surface des NMDAR joue un rôle dans l'activité de décharge des neurones DA.

Dans un second temps, j'ai testé cette hypothèse en modulant de façon expérimentale la dynamique de surface des NMDAR par un protocole de *crosslink* précédemment développé dans le laboratoire. Ce protocole, reposant sur l'incubation des neurones avec des anticorps anti-NMDAR dirigés contre un épitope extracellulaire de la sous-unité GluN1 des NMDAR, immobilise les NMDAR membranaires sans en altérer leur fonction ; et a été utilisé avec succès *in vitro* et *in vivo* dans des neurones hippocampiques. De façon similaire, j'ai démontré que ce protocole réduit drastiquement la diffusion des NMDAR dans des neurones DA de souris et dérivés de cellules humaines en culture. De façon remarquable, l'injection *in vivo* d'anticorps anti NMDAR dans le mésencéphale n'altère pas la fonction des NMDAR synaptiques dans les neurones DA par rapport à la condition contrôle où des anticorps non spécifiques sont injectés. De plus, le mésencéphale et en particulier la VTA étant aussi

composée de neurones GABAergiques qui contactent les neurones DA, il est possible que le *crosslink* affecte les neurones GABAergiques, qui en retour, modifient la décharge des neurones DA. J'ai donc vérifié que le *crosslink* ne modifiait pas l'activité des neurones GABAergiques en enregistrant et en comparant les courants spontanés GABAergiques sur des neurones DA issus de rats injectés par des anticorps anti NMDAR ou des anticorps non spécifiques. Aucune différence de cinétique et d'amplitude des courants n'étant noté, cela indique que le protocole de *crosslink* ne modifie pas la transmission GABAergique sur les neurones DA. Une fois ces vérifications faites, j'ai testé l'effet du *crosslink* sur l'activité de décharge des neurones DA en tranches. L'injection d'anticorps anti-NMDAR diminue la fréquence de décharge et augmente l'irrégularité de la décharge de ces neurones par rapport à la condition contrôle. De façon remarquable, il a été reporté dans la littérature qu'une modification de l'activité des canaux potassiques SK calcium-dépendants par l'application du bloqueur apamine, ou l'expression d'un mutant inactif des canaux potassiques SK augmentait de façon similaire l'irrégularité de la décharge spontanée des neurones DA sur des tranches aigües de mésencéphale. Ceci suggère que l'effet induit par le protocole de *crosslink* résulte d'une modification des canaux SK. Ces canaux nécessitant une entrée de calcium pour être activés, ils sont couplés fonctionnellement avec des sources calciques, notamment les NMDAR. Etant donné qu'un tel couplage requiert une proximité entre les canaux SK et leurs sources calciques, l'altération de la dynamique membranaire des NMDAR pourrait affecter le couplage fonctionnel avec les canaux SK, entrainant ainsi une diminution de fonction de ces canaux et une augmentation de l'irrégularité de décharge. Avant de vérifier cette hypothèse, j'ai tout d'abord testé l'effet du blocage pharmacologique des canaux SK par l'apamine sur la décharge des neurones DA. Comme reporté dans la littérature, le blocage de ces canaux induit une irrégularité de la décharge neuronale. Si la modification de la dynamique membranaire des NMDAR par le protocole de *crosslink* diminue la fonction des canaux SK, l'effet de l'apamine devrait être moindre qu'en condition contrôle. Pour cela, j'ai comparé l'effet de l'apamine sur la décharge des neurones DA issus de rats injectés avec le *crosslink* ou des anticorps non spécifiques. Comme prédit, la présence d'apamine induit une augmentation de l'irrégularité de 300% en condition contrôle et de seulement 190% en condition *crosslink*. En accord avec ces résultats, le nombre de neurones répondant à l'apamine est diminué en condition *crosslink,* indiquant ainsi que les canaux SK sont altérés lorsque la dynamique des NMDAR est modifiée. De façon intéressante, le contenu total des sous-unités SK3, l'isoforme prédominant des canaux SK dans les neurones DA, est inchangé en condition contrôle. Ainsi, ces résultats suggèrent que la diminution de l'effet de l'apamine en condition *crosslink* n'est pas due à une internalisation et une dégradation du canal mais à une perte de sa fonction.

Ce projet de thèse avait pour but de tester l'hypothèse innovante selon laquelle la dynamique membranaire des NMDAR pouvait moduler le patron de décharge des neurones DA mésencéphaliques. J'ai ainsi pu démontrer que d'une part, les NMDAR étaient hautement mobiles au sein de la membrane plasmique des neurones DA en culture, et d'autre part, que l'altération de cette mobilité modulait le patron de décharge des neurones DA en tranches par une diminution de la fonction des canaux potassiques calcium-dépendants SK. Ainsi, ces travaux ouvrent un nouveau regard sur les mécanismes de régulation de l'activité neuronale, et suggèrent que la dynamique des récepteurs et/ou canaux joue un rôle à part entière dans cette régulation.

Table of content

Index of figures

INTRODUCTION

I. The dopaminergic system in the brain

1. Role of midbrain DA neurons

The brain is composed of several areas connected to each other to evoke suitable behaviors. Communication between neurons is possible through the release of neurotransmitters and their capture by receptors in specific junction areas called synapses. Among these neurotransmitters, a lot of attention has been focused on dopamine (DA) given its involvement in fundamental physiological functions and neuropsychiatric disorders. The presence of DA was suggested in 1951, when Raab and Gigee found a catecholamine in several brain structures that was neither noradrenaline nor adrenaline (Raab and Gigee, 1951). Few years later, Montagu confirmed by paper chromatography the presence of DA in brains of several species (Montagu, 1957). The first visualization of catecholamines, including dopamine, was allowed by Falck and Hillarp in 1962 with the development of a formaldehyde-based histofluorescence method (Falck et al., 1982). Although this method was successfully used to unravel the catecholamines circuit, it requires special equipment, and thus was replaced by immunohistochemistry against tyrosine hydroxylase (DA-synthesis enzyme). Besides being more accessible for most laboratories, immunohistochemistry enabled to distinguish DA (positive for TH) from noradrenergic and adrenergic systems (positive for TH and dopamine beta-hydroxylase, the synthesis enzyme of the noradrenaline) and thus, helped to map more accurately the DA circuit in the brain.

a) Midbrain DA pathways

The main source of dopamine comes from three neuronal groups located in the midbrain: the retrorubral area (A8), the substantia nigra pars compacta (SNc; A9) and the ventral tegmental area (VTA; A10) (Fallon and Moore, 1978). SNc DA neurons mainly project to the dorsal part of the striatum (caudate nucleus and putamen) forming the nigrostriatal pathway (**Figure 1**) (Ungerstedt, 1971;

Beckstead et al., 1979; Loughlin and Fallon, 1984). By innervating the cortex and the ventral striatum (nucleus accumbens), the VTA is involved in the meso-cortical and the meso-limbic pathways, respectively (**Figure 1**) (Beckstead et al., 1979; Swanson, 1982). In return, midbrain DA neurons receive inputs from the cortex, the basal ganglia and the brainstem (Watabe-Uchida et al., 2012). In particular, SNc DA neurons integrate inputs from somatosensory and motor cortices, the subthalamic nucleus and the globus pallidus (Watabe-Uchida et al., 2012). VTA DA neurons receive glutamatergic inputs from various structures including the prefrontal cortex (Sesack and Pickel, 1992), the bed nucleus of the stria terminalis (Georges and Aston-Jones, 2001), the pedunculopontine tegmental nucleus (Floresco et al., 2003), the laterodorsal tegmental nucleus (Omelchenko and Sesack, 2005) and the dorsal raphe nucleus (Beier et al., 2015). It also receives inhibitory information from the lateral hypothalamus, the ventral pallidum and the rostromedial mesopontine tegmental nucleus (Morales and Margolis, 2017). Within the midbrain and especially the VTA, GABAergic (GABA : gamma-aminobutyric acid) and glutamatergic neurons are also present and modulate the neighboring DA neurons (Johnson and North, 1992; Omelchenko and Sesack, 2009; Dobi et al., 2010). Given the wide heterogeneity of the connections of the midbrain DA neurons, they have been extensively studied to unravel their functions in physiological and pathological conditions.

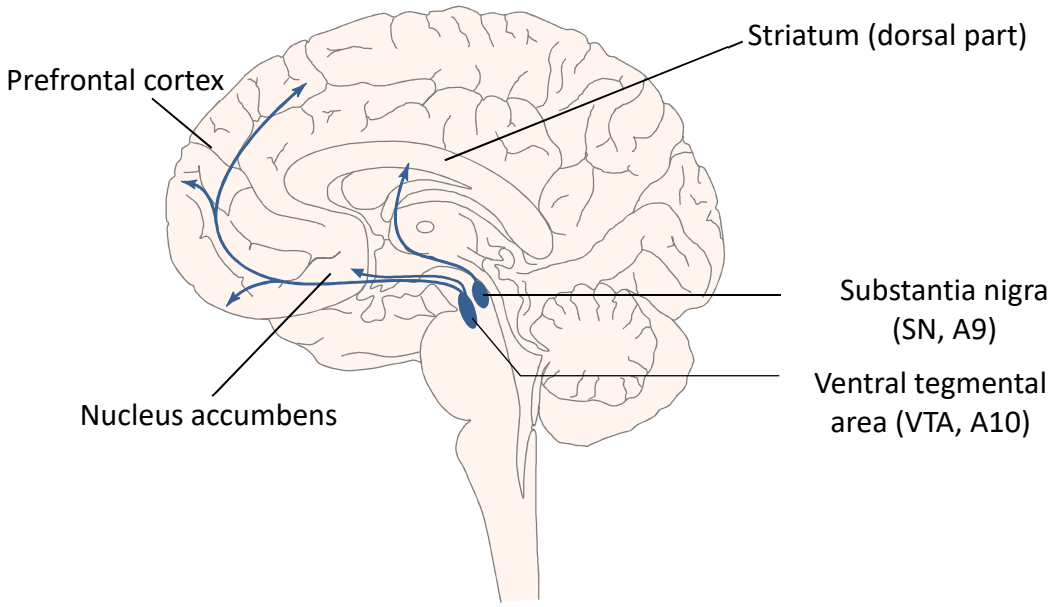


Figure 1. Schematic representation of the principal dopaminergic pathways in an human brain, in a sagittal view. SNc DA neurons mainly project to the dorsal part of the striatum, forming the nigro-striatal pathway. As VTA DA neurons contact the cortex and the ventral part of the striatum (nucleus accumbens), they belong to the meso-cortical and the meso-limbic pathways respectively.

b) Action on DA receptors

DA is synthetized in the cell body and transported into vesicles along the axons. When the vesicles release DA in synaptic clefts, it binds to dopamine receptors (DAR) located in the membrane of post synaptic neurons. Besides its classical release of neurotransmitters by axons, DA neurons also release locally DA from somato-dendritic regions, which act on presynaptic DA auto-receptors (Groves et al., 1975; Jaffe et al., 1998). DAR are G-protein-coupled receptors and have been classified into two families regarding the nature of the G proteins. D1-like family, composed of D1 and D5 receptors, is coupled to Gs protein that activates the adenylate cyclase and thus, stimulates cyclic adenosine monophosphate (AMP) production (Missale et al., 1998; Beaulieu and Gainetdinov, 2011). By contrast, D2, D3 and D4 receptors, which belong to the D2-like family, are coupled to Gi protein. This latter decreases the concentration of cyclic AMP by inhibiting the adenylate cyclase (Missale et al., 1998; Beaulieu and Gainetdinov, 2011). Because Gs and Gi proteins differentially regulate the level of second messengers such as cyclic AMP, different molecular cascades and protein kinases are triggered following the activation of D1 or D2-like family. For example, the production of cyclic AMP induced by the activation of D1-like receptors activates the phosphokinase A and DARPP-32, which in turn modulate the glutamate AMPA and NMDA receptors (Greengard, 2001). DA auto-receptors, which comprised D2R localized in the somato-dendritic and axonal compartments of DA neurons (Sesack et al., 1994), are coupled to Gi proteins and inhibit neurotransmitter release by modulating potassium channels (Congar et al., 2002). Therefore, the activation of DAR triggers a variety of intracellular signaling cascades that modulate the fast synaptic transmission, and enables to exert various brain functions.

c) Role of midbrain DA in physiological and pathological conditions

Role in the control of voluntary movements and Parkinson's disease

One of the main roles of DA is their involvement in the control of voluntary movements and Parkinson's disease (PD) through the nigrostriatal pathway. PD is a degenerative disorder associated with dopaminergic cell loss in the SN, causing symptoms such as bradykinesia (slowness of movement), rigidity and loss of posture (Bernheimer et al., 1973). In the 60's, Carlsson and Sano used chromatography to quantify DA content in various tissues and found that DA was present in the brain (Carlsson et al., 1958) and especially enriched in the striatum (Sano et al., 1959). Because the striatum belongs to the extrapyramidal system, it was first hypothesized that DA could be involved in the control of movements. Relying on this study, Hornykiewicz studied the DA content of PD patients' brains and noticed a decrease in the nigral and striatal DA levels (Hornykiewicz, 2006), suggesting that the cell

loss in SNc could cause the deficit in striatal DA in PD. Since this discovery, following studies have confirmed the role of the nigrostriatal DA pathway in the control of voluntary movements and in PD. For example, PET (position emission tomography) studies in healthy volunteers using a radio ligand binding to DAR revealed that a sequential finger movements elicits the release of DA in the striatum, pointing out the role of this pathway in the control of voluntary movement (Goerendt et al., 2003). In mice trained to press a lever to earn a reward, Jin et al. reported that DA neurons from the nigrostriatal pathway increased their firing before level pressing (Jin and Costa, 2010), indicating that these neurons signal the initiation of a specific learned action sequence and further supporting their involvement in the control of movements. Regarding their implication in PD, there is a well-documented and abundant literature investigating the factors involved in the vulnerability and degeneration of SNc DA neurons (Olanow and Tatton, 1999; González-Hernández, 2010; Surmeier et al., 2010; Dragicevic et al., 2015).

Role in the coding of reward and salient information

Midbrain DA neurons are also implicated in the reward system, which is a group of different neuronal structures responsible for pleasure (positive emotional state) and motivation (voluntary behavior). The pioneering study of Schultz et al. showed that midbrain DA neurons were phasically activated when a natural reward such as fruit juice droplet was given to monkeys (Schultz, 1999). A neutral stimulus could also induce activation of DA neurons if it was previously associated with the delivery of reward (Ljungberg et al., 1992), and confirmed the role of midbrain DA neurons in reward-related events. Interestingly, a novel stimulus non-associated with a reward such as door opening can also elicit activation of these neurons (Ljungberg et al., 1992), suggesting a more general role of midbrain DA neurons in encoding behavioral relevant stimuli. This hypothesis, along which DA neurons would code not only for reward-related events but also for salient information, was further supported by more recent studies. Li et al. showed that a form of long-term synaptic plasticity was facilitated in the hippocampus when the rat was previously exposed to novelty but prevented by direct infusion of DAR antagonists in the hippocampus (Li et al., 2003). This implies that novelty stimulus is sufficient to stimulate DA neurons and its release in the hippocampus. Surprisingly, Brischoux et al. discovered that a subgroup of neurons located in the ventral part of the VTA from anesthetized rats was excited by noxious stimuli (footshocks) (Brischoux et al., 2009). By discovering that DA neurons might also fire in response to unpleasant events, these data raise the following question : how can DA neurons fire and code for both reward delivery and aversive experiences ? To address this question, Lammel et al. took advantage of the recent advancements of optogenetics to selectively activate two DA VTA neuronal subpopulations innervating two different areas (Lammel et al., 2012). They found that, depending on the innervated areas, some DA subpopulations responded to either reward or aversive-related events.

Therefore, midbrain DA neurons form a complex and heterogeneous center, and are essential to sense and respond to behaviorally relevant situations, be they pleasant or unpleasant.

Role in drug abuse and addiction

Functional alterations of midbrain DA neurons have been observed in various pathologies including addiction to drugs of abuse, which is characterized by a loss of control of intake of a particular substance. Acute and chronic administration of cocaine increases extracellular DA level in the striatum of rats (Di Chiara and Imperato, 1988; Pettit and Justice, 1989) and is associated with changes in the firing activity of VTA DA neurons (Einhorn et al., 1988; Henry et al., 1989). On the cellular level, acute administration of cocaine induced a form of plasticity of excitatory glutamatergic synapses specifically on VTA DA neurons in mice (Bellone and Lüscher, 2006; Bellone et al., 2011) and not on neighboring VTA GABAergic neurons (Ungless et al., 2001), highlighting the central role of VTA DA neurons in cocaine-mediated alterations (Koob and Bloom, 1988). Interestingly, other drugs of abuse such as ethanol and opioids also affect midbrain DA neurons. In particular, they modify the extracellular striatal DA concentration (Rada et al., 1991; Wozniak et al., 1991; Weiss et al., 1993), the firing activity of VTA DA neurons (Matthews and German, 1984; Mereu et al., 1984), and induce synaptic plasticity of excitatory synapses on these neurons (Saal et al., 2003). In addition, withdrawal from drug exposure is also characterized by a change in the spontaneous firing activity of midbrain DA neurons (Diana et al., 1998). In view of these data, VTA DA neurons appear as one of the major targets of drugs of abuse and undergo molecular adaptations in response to drug exposure and other aspects of addiction such as withdrawal.

Role in schizophrenia

Besides its role in addiction, Carlsson and colleagues suggested a link between DA and schizophrenia since the 1970's (Carlsson, 1977). Schizophrenia is a mental disorder characterized by hallucinations or thought disorders (positive symptoms), reduction of pleasure and motivation (negative symptoms) and cognitive dysfunctions. Pioneering studies have discovered that various neuroleptics (reserpine, chlorpromazine and haloperidol), used to treat schizophrenic patients (Hollister et al., 1955; Davis, 1965), increased the turnover of catecholamines (Bertler, 1961; Carlsson and Lindqvist, 1963; Andén et al., 1972). The development of radioactive 3H-DA and 3H-Haloperidol binding to DAR demonstrated that the neuroleptics compete with the radioligands for the same binding sites, mainly located in the striatum (Creese et al., 1975). Then, it was inferred that neuroleptics attenuated psychosis by interfering with DAR. On the behavioral level, local application of DAR agonists in the striatum induced alteration in the sensorimotor gating in rats (Wan and Swerdlow, 1993), which is one of the well-documented symptoms of schizophrenic patients (Braff et

al., 1978). Moreover, immunohistochemical studies performed on post mortem schizophrenic patients brains revealed a decrease of dopaminergic innervation in the prefrontal cortex (Akil et al., 1999). Therefore, it was hypothesized that the symptoms of schizophrenia came from a DA imbalance between different brain areas, with an hypodopaminergia in the prefrontal cortex resulting in negative symptoms and an hyperdopaminergia in the striatum responsible for the positive symptoms (Howes and Kapur, 2009). Although the etiology of the pathology is not fully understood and involves other neurotransmitters such as glutamate (Brisch, 2014), DA alterations remain a hallmark of schizophrenia.

In conclusion, by innervating the dorsal and ventral parts of striatum and cortex, DA neurons are essential to multiple physiological functions, such as the coding of salient information or motor control. As a consequence, alterations of this system are associated with the emergence of different pathologies including PD, drug addiction and schizophrenia.

2. Electrical activity of midbrain DA neurons

a) Electrophysiological identification of DA neurons

Given the wide range of behavioral functions involving the midbrain DA system, a lot of efforts have been put to understand the physiology of DA neurons, by electrophysiological approaches conducted in *in vivo* and *in vitro* preparations. However, the presence of other neuronal types in the midbrain made their study challenging. Quantifications based on TH-immunohistochemistry estimated that DA neurons represent between 55% and 75% of the neurons in the VTA (Swanson, 1982; Margolis et al., 2006), and almost 90% in the SNc (Margolis et al., 2006). The great majority of the non-DA neurons were classified as GABAergic, since they were identified by immunohistochemistry against GABA (Van Bockstaele and Pickel, 1995) or GABA-transaminase (the GABA-metabolizing enzyme) (Nagai et al., 1983) and more recently, by *in situ* hybridization for glutamid acid decarboxylase (the GABA-synthesis enzyme) (Nair-Roberts et al., 2008). Using the same technique for the vesicular glutamate transporter VgluT2, several studies reported the presence of a weak proportion (2-3%) of glutamate neurons in the VTA (Yamaguchi et al., 2007; Nair-Roberts et al., 2008). Thus, scientists have established several electrophysiological and pharmacological criteria to identify DA neurons *in vivo* and *in vitro* (**Figure 2**). Compared to GABAergic neurons, they discharge at low frequency (4 Hz) with a broad action potential (AP) (>1ms) *in vivo* (Grace and Bunney, 1983; Steffensen et al., 1998) and *in vitro* (Grace and Onn, 1989). Besides, their firing is inhibited by DA through D2 auto-receptors (Lacey et al., 1987; Cameron et al., 1997). In midbrain slices, the presence of an hyperpolarization-activated

cation current (named Ih) is the main electrophysiology criterion used to identify DA neurons (Johnson and North, 1992; Bonci and Malenka, 1999; Neuhoff et al., 2002; Bellone and Lüscher, 2006). Although the morphology alone is not sufficient to discriminate DA neurons (Margolis et al., 2006), some authors noticed that they exhibit a larger cell body than GABA neurons (Chieng et al., 2011). It is worth noting that a group reported the existence of non DA neurons that were indistinguishable from DA neurons regarding various electrophysiological parameters such as the body size, the presence of an Ih current and the width of the AP; but differed by the spontaneous firing activity (Margolis et al., 2006). These data, in disagreement with others reporting the strong reliability of the presence of an Ih conductance to identify DA neurons (Wanat et al., 2008; Mao et al., 2011), raise the question of the reliability of this criterion alone to discriminate DA neurons. However, some caution should be taken about the limitation of this study. Indeed, Zhang et al. observed that recording DA neurons from TH-EGFP mice for more than 15 minutes dramatically decreased the number of TH immunolabelled neurons to 20 % (Zhang et al., 2010), which may lead to false "negative" DA neurons in the study of Margolis. Taken together, these data provide evidence that the identification of DA neurons by electrophysiology is possible both in *in vivo* and *in vitro* preparation*s*, but it might be preferable to use several criteria to confirm the DA nature.

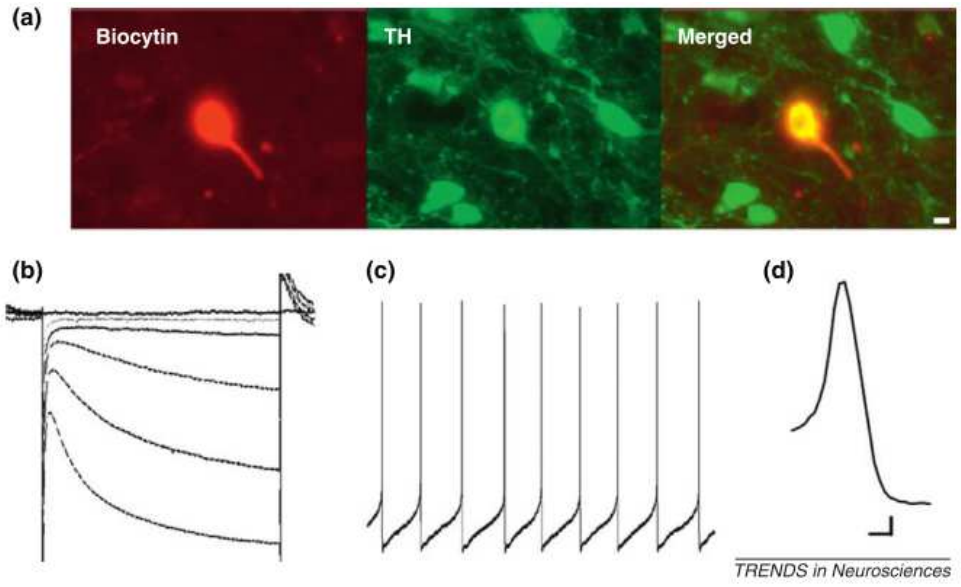


Figure 2. Electrophysiological characteristics of DA neurons in acute midbrain slices (from Ungless and Grace, 2012). a) A recorded neuron with an intracellular solution containing biocytin is positive for TH-immunolabelling, confirming its DA nature. b) Hyperpolarizing voltage steps induce an Ih inward current. c) Typical electrophysiological trace in current clamp of a DA neuron recorded in slices with a slow regular firing pattern. d) Magnified image of a broad action potential, characteristics of DA neurons. Scale bar : (a) 10 µm; (b) 80 ms, 80 pA; (c) 350 ms, 6.25 mV; (d) 1 ms, 10 mV.

b) Discharge patterns exhibited *in vivo* and *in vitro*

In vivo

In vivo extracellular and intracellular recordings in anesthetized and freely moving rats have shown that midbrain DA neurons can be inactive, probably due to strong inhibition mediated by GABAergic neurons, or spontaneously active. When active, they can fire either in a single spike manner or in bursts of action potentials (AP) (**Figure 3**). The single spike pattern (or tonic activity) is characterized by an irregular discharge of single AP at low frequency (4.5 Hz) (Grace and Bunney, 1984a). Bursts, which are encountered in more than 50% of midbrain DA neurons, are composed of trains of AP firing at high frequency (<15Hz), with AP of decreasing amplitude within the bursts (Grace and Bunney, 1984b). To define these latter, Grace and Bunney established the following parameters in the early eighties : a burst starts with a pair of AP with an interspike interval (ISI) ≤ 80ms and ends with an ISI ≥160ms. *In vivo*, burst generation was promoted by the iontophoretic application of glutamate or N-methyl-D-aspartate (NMDA), an agonist of glutamate NMDA receptors (NMDAR) (Overton and Clark, 1992; Christoffersen and Meltzer, 1995), whereas NMDAR antagonists decreased burst firing (Overton and Clark, 1992; Christoffersen and Meltzer, 1995; Connelly and Shepard, 1997). By contrast, application of the glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) blocker did not affect bursting activity, showing a preferential role for NMDAR to modulate this activity. Besides, bursts were prevented by the intracellular injection of calcium chelators (Grace and Bunney, 1984b), pointing out the fundamental role of calcium in the spontaneous burst firing of DA neurons. From these data, it was postulated that excitatory glutamate inputs were responsible for the induction of burst firing in midbrain DA neurons through NMDAR, via a calcium-dependent mechanism.

What could be the relevance for DA neurons to exhibit different activity patterns ? Growing evidence suggests that tonic and phasic firing patterns differentially regulate DA release. The tonic activity of DA neurons would contribute to the steady state of extracellular DA (Keefe et al., 1993; Nissbrandt et al., 1994; Moore et al., 1998; Floresco et al., 2003) and the phasic burst firing would be associated with transient massive DA release (Floresco et al., 2003; Zweifel et al., 2009). By evoking large DA release in targeted areas, burst firing strongly influences animals' behavior, and thus receives a great attention. The pioneering works of Shultz on behaving monkeys, as mentioned earlier, emphasized the role of burst activity of midbrain DA in reward-related events, and more generally in the encoding of salient information (Schultz, 2010). One of the most prominent proofs confirming the link between this activity pattern and some behavioral outcomes was given by Tsai et al., who used optogenetics to spatially and temporally control the activity of midbrain DA neurons (Tsai et al., 2009). Evoking phasic

but not tonic activation in these neurons was sufficient to induce a conditioned place preference in these mice, meaning that mice moved selectively in the compartment associated with phasic activation and in the absence of any reward. On the contrary, reducing burst frequency by inhibiting NMDAR specifically in midbrain DA neurons impaired the acquisition of a conditioned place preference for cocaine (Zweifel et al., 2009). Unlike bursting pattern, tonic activity receives less attention and the physiological relevance of this pattern is still elusive. A recent study using optogenetics found that tonic (5 Hz) but not phasic (50 Hz) stimulation of midbrain DA neurons decreased ethanol self-administration behaviors in mice (Bass et al., 2013), implying that the tonic activity *per itself* modulates some animal behaviors, independently from burst firing.

In vitro

Unlike the *in vivo* situation, DA neurons in midbrain acute slices do not exhibit bursting activity but rather a regular single spike firing called pacemaker activity (Grace and Onn, 1989). One exception comes from the study of Mereu et al., who found that a small proportion of midbrain DA neurons has spontaneous bursting activity in midbrain slices from immature rats (PD 15-21) (Mereu et al., 1997). Because most studies only report the presence of a pacemaker activity (Grace and Onn, 1989; Seutin et al., 1990; Mercuri et al., 1992; Soden et al., 2013), it is thought that the excitatory synaptic inputs contacting DA neurons are severed during the slicing process, which prevents spontaneous burst generation. On the contrary, pacemaker activity does not appear to depend on glutamate inputs and might result from the intrinsic properties of the DA neurons. In line with this, administration of the excitatory amino acid antagonist kynurenate induced a pacemaker-like firing *in vivo* in midbrain DA neurons (Grenhoff et al., 1988). In slices, the pacemaker activity is characterized by a low frequency (1-5 Hz) and a high regularity, assessed by the regular coefficient of variation of the inter-spike intervals (CV-ISI) (Wolfart and Roeper, 2002; Deignan et al., 2012; de Vrind et al., 2016). Since pacemaker activity was initially only encountered in *in vitro* preparations, the relevance of this pattern to the *in vivo* physiology of DA neurons has been questioned. However, few studies noticed the presence of a regular single spike firing *in vivo* in rodent midbrain (Hyland et al., 2002; Herrik et al., 2010) and thus, challenges the view that only *in vitro* DA neurons fire with a pacemaker pattern.

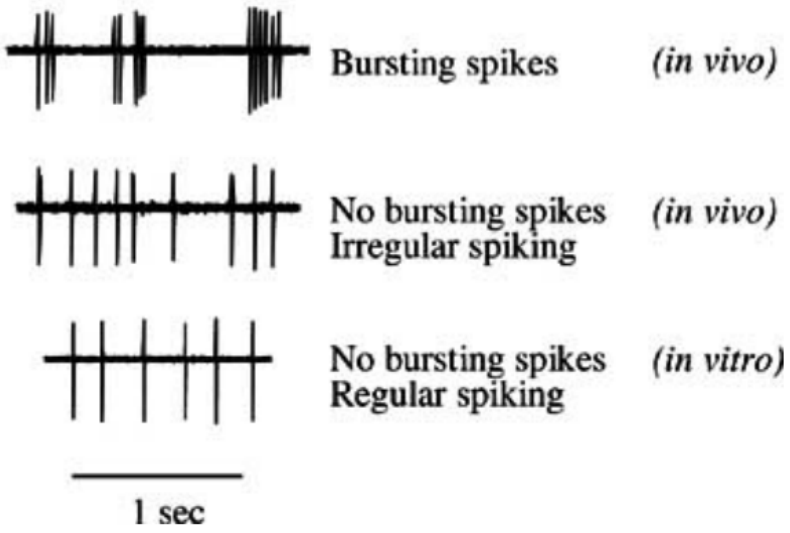


Figure 3. Firing patterns of midbrain DA neurons recorded in *in vivo* and *in vitro* preparations (from Marinelli et al., 2006). *In vivo*, DA neuron can fire in two modes: a bursting pattern with bursts of AP at high frequency, or a slow irregular pattern. *In vitro*, DA neurons fire in a slow regular manner, called pacemaker activity.

Despite the fact that DA neurons do not exhibit spontaneous bursts, these can be induced in acute slices by several pharmacological agents. Depending on the agents used, the induced-bursts display different characteristics that are summed up in **Table 1**. When hyperpolarizing DA neurons, bath application of NMDA evokes bursts of 2 to 10 AP (Johnson and Wu, 2004). Contrary to the *in vivo* situation, NMDA-induced bursts are calcium independent and do not show decreased AP amplitude within bursts. Application of apamin, a blocker of SK2 and to a lesser extent of SK3 channels (Ishii et al., 1997b) has given conflicting results. Whereas Shepard and colleagues could evoke burst activity in 50% of the recorded DA neurons by applying apamin alone (Shepard and Bunney, 1991; Ping and Shepard, 1996), Johnson et al. could not replicate these results unless a continuous depolarizing current was injected in neurons (Johnson and Wu, 2004). These apamin-induced bursts share some similarities with the *in vivo* bursts such as decreasing AP amplitude and calcium-dependence. The combination of NMDA and apamin evokes bursts in 90% of the recorded neurons, appearing as the most efficient way to induce bursts in slices (Seutin et al., 1993). Of note, it is possible to induce bursts with NMDA in combination with BMI which blocks SK channels (Johnson and Seutin, 1997), or with the K-ATP channels blocker NN414 (Schiemann et al., 2012). As nickel inhibits T-type calcium channels responsible for the activation of SK channels in DA neurons, it can also elicit bursts (Wolfart and Roeper, 2002). Finally, iontophoretic application of glutamate also triggers bursts (Deister et al., 2009). Therefore, the modulation of NMDAR and/or potassium channels such as K-ATP and SK channels permits burst generation in DA neurons *in vitro*.

Table 1. Different ways to induce bursts firing in DA neurons *in vitro.*

Burst Induction	Species and age	Recording method	Ca dep.	APs/ burst	AP red.	Burst ind. rate (%)	Reference
Spontaneous	SD rats Adult (200-300g)	In vivo (SNc) Extra and intra	Yes	2,9	Yes	-	Grace and Bunney, 1984
NMDA (10-30µM) + current inj	Male SD rats Adult	In vitro (VTA) Intra	No	2-10	No	-	Johnson et al, 1992
NMDA (20-30µM) + current inj	Male SD rats Adult (150-300g)	In vitro (VTA) Intra	-	9,6	No	33%	Johnson and Wu, 2004
APA (100-200nM) + current inj.	Male SD rats Adult (150-300g)	In vitro (VTA) Intra	Yes	-	Yes	50%	Johnson and Wu, 2004
APA (1µM)	Male SD rats Young (125-175g)	In vitro (SNc) Intra	-	3-12	Yes	30%	Shepard and Bunney, 1991
APA (100nM) + NMDA (20-30µM)	Male SD rats Adult	In vitro Intra	-	3-15	No	90%	Seutin et al, 1993
Nickel (100µM)	C57BL/6J mice 10-14 days	In vitro (SNc) Perforated patch	-	2,3	No	11%	Wolfart and Roeper, 2002
Ni (100µM) + APA (300nM)	C57BL/6J mice 10-14 days	In vitro (SNc) Perforated patch	-	2,6	No	31%	Wolfart and Roeper, 2002
NMDA (10µM) + BMI (30µM)	Male SD rats Adult	In vitro Intra	-	At least 3	No	55%	Johnson and Seutin, 1997
Glutamate (iontophoresis)	SD rats 15-37 days	In vitro (SNc) Whole cell	-	4.9	Yes	-	Deister et al, 2009
NMDA (30µM) + NN414 (10 µM)	WT mouse Adult	In vitro (SNc) Whole cell	-	-	-	80%	Schiemann et al, 2012

As the reference, the characteristics of the spontaneous bursts described *in vivo* by Grace and Bunney is presented in the first line.

Current inj: current injection, intra : intracellular , extra : extracellular, Ca dep. : calcium dependence, APs/burst : number of AP per burst, AP red. : reduction of the AP amplitude within the burst, Burst ind. Rate : burst induction rate, indicating the percentage of neurons exhibiting bursts following the application of the pharmacology agents, apa : apamin.

c) Regulation of the discharge pattern

The firing activity of midbrain DA neurons is modulated by multiple factors. This has been thoroughly reviewed (Marinelli et al., 2006; Marinelli and McCutcheon, 2014). Here, I will mostly discuss two main parameters influencing the neuronal activity of DA neurons, i.e the stage of brain development and the pathological conditions.

Regulation during development

During development, midbrain DA neurons from 2-week-old animals fire in a single spike mode *in vivo* whereas burst activity is encountered in more than 50% of these neurons in adults (Levine et al., 1982; Pitts et al., 1990), suggesting that the discharge pattern of DA neurons is strongly regulated during development. Recordings of spontaneous activity of SNc DA neurons at several postnatal days (PD) in rats showed that most of the changes occur during the first three postnatal weeks. In the first PD, DA neurons fire at low frequency in a random pattern, switch to a regular discharge with doublets within the second postnatal week, then to a more mature pattern with longer bursts after the third postnatal week (Tepper et al., 1990). Wang and Pitts observed that VTA DA neurons followed similar pattern changes (single spike mode, doublets mode, then a mature firing with bursts) during this time window (Wang and Pitts, 1994). Of note, bursts appear as early as PD 14, although their properties are still immature in terms of burst number and duration, and they gradually increase in numbers during development. Some changes in the firing pattern also occur later in development, between adolescence and adulthood. Indeed, VTA DA neurons fire faster in adolescent (PD 37-48) than in adult (PD 82-100) rats *in vivo* (McCutcheon et al., 2012). In particular, the single spike frequency and the number of spikes per burst are higher during adolescence. This change is accompanied by a decreased amplitude and frequency of GABA receptor-mediated events, suggesting that the increased tone of GABA during development may be responsible for the lower firing frequency of these neurons in adulthood. Once the animal reaches adulthood, the basal firing rate and the bursts properties of midbrain DA neurons seem to be relatively stable; as no age-related differences were found between rats aged of 3 and 28 months (Freeman et al., 1989).

The spontaneous firing exhibited in acute slices is also subjected to developmental maturation. Contrary to the mature pacemaker activity encountered in animals above PD 15 (Grace and Onn, 1989; Liss et al., 2001; Soden et al., 2013), Cui et al noticed that midbrain DA neurons from PD 6-12 rats fire in an irregular manner (Cui, 2004). A detailed analysis performed on each age during the three postnatal weeks of rats established that these neurons undergo three major changes in their firing pattern: they are purely bursters between PD 2-3, become irregular between PD 5-11 and switch to a regular pacemaker activity after PD 12 (**Figure 4**) (Dufour et al., 2014a). So, the pattern activity of

midbrain DA neurons is regulated during development, in particular during the first postnatal weeks of life.

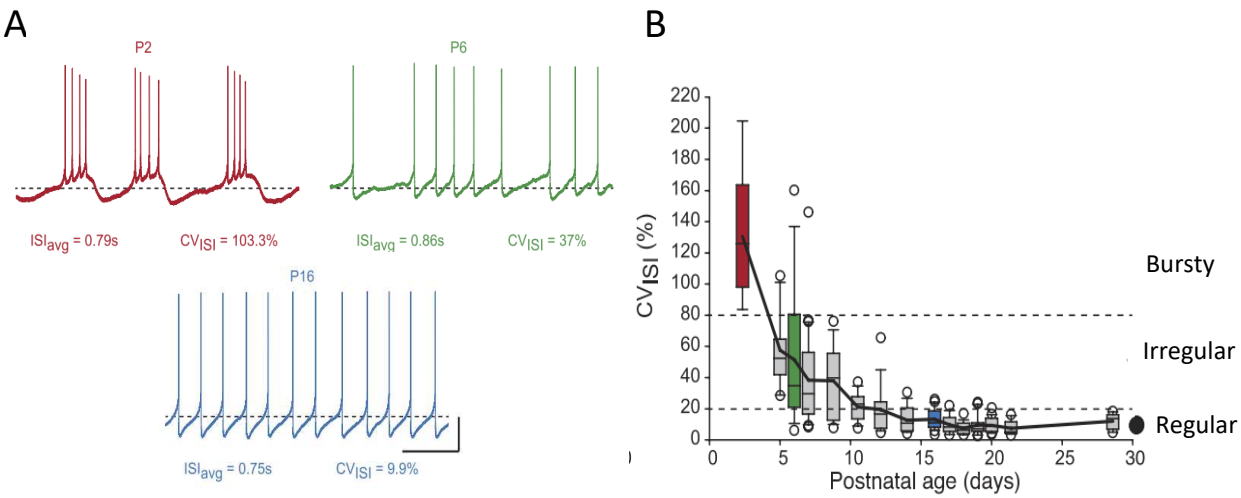


Figure 4. Postnatal development of the firing pattern of DA neurons in SNc acute slices (from Dufour et al., 2014). A) Electrophysiological traces of the spontaneous firing of DA neurons in current clamp from PD 2 (red), PD 6 (green) and PD 16 (blue) rats. At PD 2, DA neurons have a bursting activity, whereas they exhibit an irregular and regular pacemaker activity at 6 PD and 16 PD respectively. B) Plot of the coefficient of variation of the inter-spike intervals (CV-ISI, representing the regularity of the firing) as a function of the postnatal ages of the rats. Burst patterns (characterized by a CV-ISI > 80%) are encountered in PD 2 rats, irregular (80% <CV-ISI< 20%) in PD 5-12 rats and regular (CV-ISI < 20%) after PD 12.

Regulation in response to drugs of abuse and addiction

As mentioned earlier, acute administration of drugs of abuse modifies the firing pattern of VTA DA neurons. *In vivo*, the firing rate was decreased by systemic administration of cocaine (Einhorn et al., 1988; Bunney et al., 2001) whereas the neurons were excited by acute exposure to morphine (Matthews and German, 1984) or ethanol (Mereu et al., 1984; Bunney et al., 2001). These effects seem to be dependent on the state (anesthetized *versus* non anesthetized) of the rat, as cocaine induced an increase in the firing rate in awake rats (Koulchitsky et al., 2012) and ethanol failed to excite DA neurons in anesthetized rats (Mereu et al., 1984). Interestingly, modifications of the firing activity persist in absence of drugs in rats trained to self-administer drugs. Regarding cannabinoids, withdrawal from a chronic treatment with this drug resulted in a decline of midbrain DA neurons activity (Diana et al., 1998). On the contrary, DA neurons had an increase firing rate and modifications of the bursting activity after cocaine self-administration (Marinelli et al., 2003). In fact, the amount of bursts, the number of spikes per burst and the proportion of neurons with a high level of bursts were enhanced after self-administration. The increase in the burst activity was very transient and was only observed the first day of withdrawal whereas the firing rate returned to baseline value after 10-30 days of withdrawal. Therefore, chronic exposure to drugs can induce long-lasting modifications of the activity

that can persist in the absence of the substance. On the behavioral level, the firing activity is also related to individual vulnerability to drugs of abuse, since animals exhibiting higher basal firing rate and bursting activity in VTA DA neurons were more prone to self-administer drugs (Marinelli and White, 2000).

Besides affecting the firing pattern of the neurons, drugs of abuse can also change the number of spontaneous active DA neurons. Indeed, repeated administration of cocaine increased the number of active DA neurons in the VTA (Henry et al., 1989) whereas exposure to ethanol dramatically reduced this number (Xu and Shen, 2001). Therefore, drugs of abuse strongly regulate the DA system, by modulating both the firing pattern and the number of spontaneous active DA neurons.

Regulation in Parkinson disease

Alterations of the firing pattern of SNc DA neurons are a common feature encountered in various animal models of PD. In midbrain organotypic slices treated with 6-OHDA, a neurotoxin that selectively kills DA neurons and models the cell loss observed in PD, the firing activity of DA neurons switched from a pacemaker activity to an irregular pattern with bursting activity (Wang et al., 2014). Consistent with this study, Branch et al. found that the firing properties of *in vitro* SNc DA neurons from MitoPark mice, a genetic model of PD mimicking the progressive loss of these neurons, were modified. After 16 weeks, the pacemaker activity of SNc DA neurons in slices from MitoPark mice was increased and more irregular than the control littermates (Branch et al., 2016). Janezic et al. reported that the firing rate of SNc DA neurons was also enhanced *in vivo* in aged transgenic mice from a different transgenic PD model (Janezic et al., 2013). In addition, the firing pattern was more irregular in SNc DA neurons from slices of PINK 1 mice model of PD, which promoted burst firing *in vivo* (Bishop et al., 2010). Therefore, animal models of PD are associated with an enhancement in the firing rate and in the irregularity of the pattern, favoring bursts generation. Interestingly, a similar increase in the bursting activity was reported *in vivo* in SN DA neurons from PD patients (Schiemann et al., 2012), confirming the importance of DA neuron activity in the etiology of the disease.

Modifications in schizophrenia

Dopaminergic dysfunctions are a hallmark of schizophrenia. In particular, alterations of the firing activity of midbrain DA neurons have been associated with schizophrenia. The psychotomimetics MK-801 and PCP, which induce psychotic symptoms that resemble those expressed in schizophrenia patients (Javitt and Zukin, 1991), enhanced the firing rate and the bursting activity of VTA DA neurons i*n vivo* (French et al., 1993). Inactivation of the delta 1 glutamate receptor (GluD1), whose gene has been linked to schizophrenia (Greenwood et al., 2011), prevented the burst firing of midbrain DA neurons *in vivo* without affecting the overall firing frequency (Benamer et al., 2017). Besides, the

expression of a human mutation of the calcium-dependant potassium SK3 channels, identified in schizophrenic patients, favoured the bursts generation of VTA DA neurons *in vitro* and *in vivo* (Soden et al., 2013). This was accompanied by alterations of the sensory gating, a process known to be disturbed in schizophrenia patients (Swerdlow and Qeyer, 1975). Of note, Valenti et al. observed that developmental rat model of schizophrenia exhibited a higher number of spontaneously active DA neurons *in vivo* in the VTA (Valenti et al., 2011). Remarkably, acute and chronic treatments with antipsychotic drugs used to treat schizophrenia patients such as haloperidol, reduced the DA neuron population activity in these rats. Taken together, these data give credence to the fact that dysfunctions in the DA neurons activity likely contribute to the physiology of schizophrenia.

3. Channels involved in firing activity of midbrain DA neurons

The firing of midbrain DA neurons results from the interplay of a number of ion channels and receptors (**Figure 5**). As mentioned earlier, it is thought that ions channels give the intrinsic membrane properties responsible for the pacemaker activity whereas inhibitory and excitatory synaptic inputs modulate the firing through the activation of ligand-gated receptors. These aspects have been thoroughly reviewed (Liss and Roeper, 2008; Paladini and Roeper, 2014; Dragicevic et al., 2015). Here, I will first describe the ionic conductances contributing to the generation of the firing activity of DA neurons. A particular focus will be put on SK channels, and therefore a brief overview regarding their structure, trafficking, distribution and their role in DA neurons will be given. In the second part, the main receptors modulating the firing pattern of midbrain DA neurons will be presented.

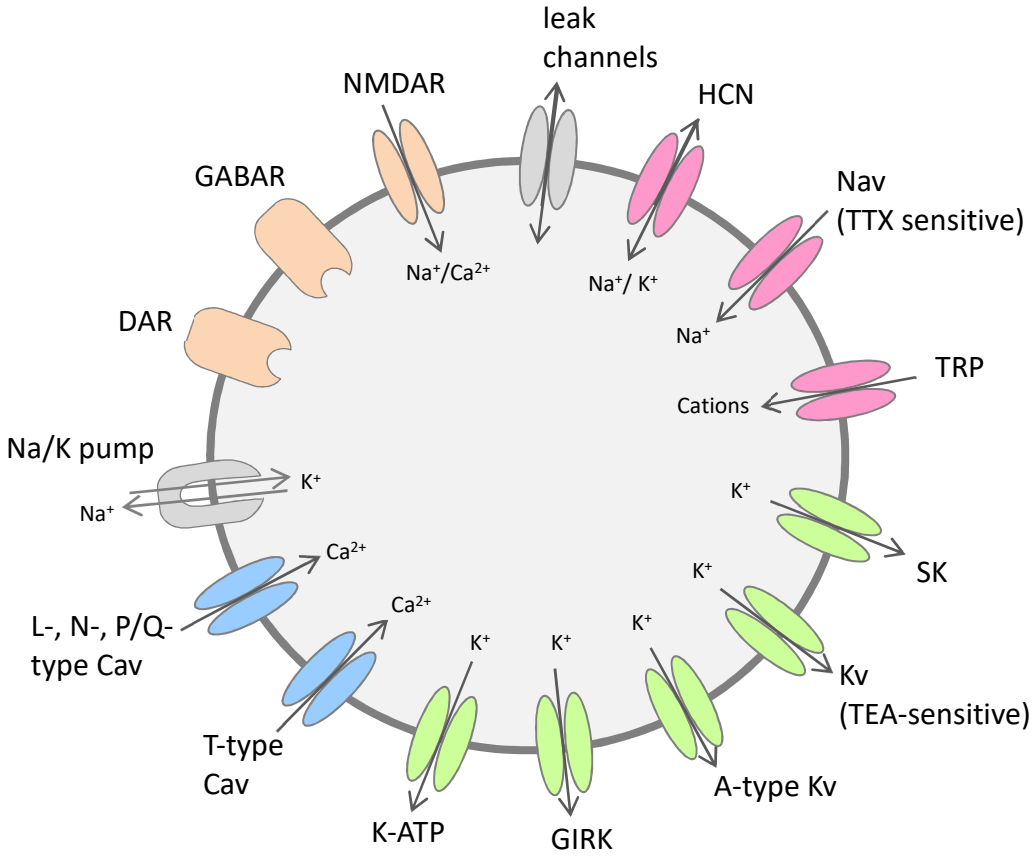


Figure 5. Channels and receptors involved in the generation and modulation of the firing activity of midbrain DA neurons. Non selective cations (HCN and TRP channels) and sodium channels (Nav) are in pink, potassium channels (SK, Kv, A-type Kv, GIRK and K-ATP channels) in green, the calcium channels (L-, P/Q- and N-type) in blue and the modulators (DAR, GABAR, NMDAR) in orange. Leak channels and the Na+/K+ pump are in grey.

a) Ionic conductances involved in the generation of the firing activity

a.1) Overview of SK channels

Structure

The small conductance calcium-activated potassium channels family, so called because of their slow unitary conductance in the order of 5-20 pS (Sah, 1996), can be made of 4 subunits (SK1, SK2, SK3 and SK4) (Köhler et al., 1996; Ishii et al., 1997b), though channels formed by SK4 subunits are often referred as IK (intermediate) channels because of their relatively higher conductance (30-70 pS). Functional channels are composed of four subunits associated to form homo- or hetero-tetramers (MacKinnon, 1991; Ishii et al., 1997b) (**Figure 6**). Each subunit is composed of 6 transmembrane domains, with intracellular N and C terminals (Faber, 2009). The SK subtypes are highly homologous in their structure and mainly differ by their C and N termini. The pore region lies between the 5th and the last transmembrane domain. The calcium sensitivity is conferred by the constitutive binding of the

calmodulin protein with the CAM-binding domain (CAMBD) located in the C terminal of SK channels (Xia et al., 1998). Binding of calcium to calmodulin induces a change in the conformation of SK channels, resulting in the opening of the pore (Schumacher et al., 2001). Interestingly, SK1, SK2 and SK3 homomers exhibit a different pharmacology regarding the SK channel blocker apamin. Indeed, SK2 is the most sensitive, followed by SK3, then SK1 channels. This difference is due to the binding site of apamin, which is located in the pore region and on a serine residue placed in the extracellular region between the 3rd and the 4th transmembrane domain (Ishii et al., 1997a; Nolting et al., 2007). In SK1 channels, the serine is replaced by a threonine residue, lowering the apamin sensitivity. Although they superficially resemble calcium-dependent big conductance potassium channels BK, they differed in some aspects : SK channels are voltage-insensitive and are not blocked by tetraethylammonium (TEA), a known potassium channel blocker (Lancaster and Nicoll, 1987; Köhler et al., 1996).

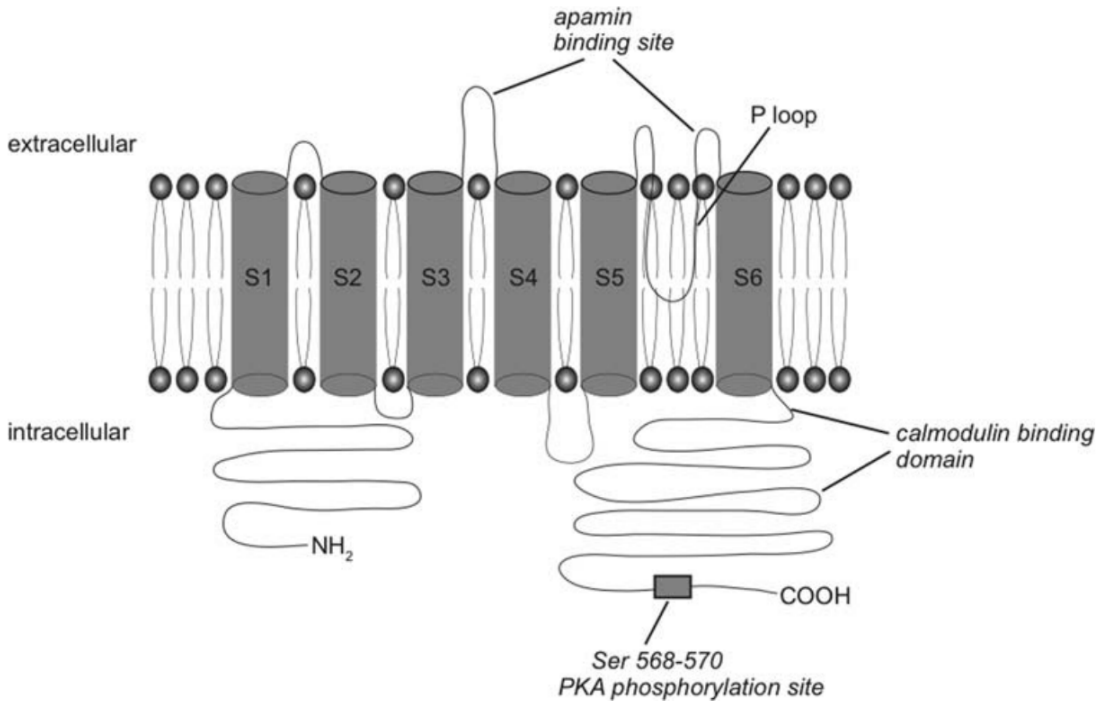


Figure 6. Schematic structure of one SK channel subunit. Each subunit is composed of 6 trans-membrane domains and intracellular C and N terminals. The binding site of apamin is localized between the pore region (between S5 and S6) and a serine residue between S3 and S4. Calmoduline is constitutively bound to the C-terminal of the channel subunit.

Distribution

In the brain. *In situ* hybridization of SK mARN revealed a differential distribution of SK channels, with SK3 being the prevailing form in the midbrain (Tacconi et al., 2001; Sailer et al., 2004). Accordingly, studies focusing more specifically on DA neurons showed that these neurons highly express SK3

channels (Wolfart et al., 2001; Bosch et al., 2002).The presence of SK2 in the midbrain, and in particular in DA neurons is more controversial. Some authors detected its presence in low amount in the midbrain (Stocker and Pedarzani, 2000), whereas others did not (Gymnopoulos et al., 2014). Moreover, Deignan et al. detected SK2 channels in the dendrites of DA neurons with immunogold electron microscopy (Deignan et al., 2012). On the contrary, Wolfart et al. failed to find SK2 mARN by single cell PCR (polymerase chain reaction) in midbrain DA neurons (Wolfart et al., 2001). In addition, the death of midbrain DA neurons induced by administration of the neurotoxin 6-OHDA was correlated with a reduction of SK3 channel expression and left SK2 channels expression unchanged, which also argues against the presence of SK2 subtypes in DA neurons (Mourre et al., 2016). Regarding SK3 channels expression, some regional differences in the distribution exist in the midbrain, with VTA neurons expressing less SK3 channels than SNc neurons (Wolfart et al., 2001). Within the VTA, their expression is heterogeneous since the medial part of the VTA is less enriched than the lateral part (Sarpal et al., 2004). Besides, SK3 channel expression is up-regulated during development in the brain (Gymnopoulos et al., 2014). Indeed, their expression is higher in the midbrain from young rats than from adults (Sarpal et al., 2004). However, the expression of SK channels seems to stabilize in the adulthood, as the SK channel-mediated currents were not different in SNc DA neurons from adult and aged mice (Branch et al., 2014).

In neurons. Recent evidence shows that the neuronal distribution of SK channels plays an important role in the regulation of several biological processes (Faber, 2009). For example, it was shown that the distribution of SK channels differently affected the firing activity in midbrain DA neurons, with somatic (and to a lesser extent, dendritic) SK3 channels contributing to the frequency and the precision of the pacemaker activity, and dendritic SK2 channels contributing only to the latter (Deignan et al., 2012). Moreover, the subcellular localisation of membrane SK channels also impacts their coupling with calcium sources in hippocampal neurons. Dendritic SK channels are tightly coupled with R-type calcium channels, whereas somatic SK channels are weakly coupled with other calcium channels (Jones and Stuart, 2013). Thus, the distribution of SK channels regulate their neuronal function but also their coupling with calcium sources.

Sparse information is available on the distribution of SK channels in DA neurons. Immunolabelling of SK3 channels in cultured midbrain DA neurons showed the strongest staining in the soma and the presence of clusters in dendrites (Herrik et al., 2012). In accordance with these observations, Deignan et al. also found by electron microscopy (EM) that SK3 immunogold particles were localized in the soma and to a lesser extent in both proximal and distal dendrites of midbrain DA neurons (Deignan et al., 2012). They also found dendritic SK2 channels mostly localized in the proximal part of the dendrites.

Of note, SK3 channels were detected into excitatory synapses and in the extrasynaptic compartment in VTA DA neurons (Soden et al., 2013).

Trafficking

Because SK3 is the main isoform found in DA neurons (Stocker and Pedarzani, 2000; Tacconi et al., 2001), I will focus on the trafficking of SK3 channels. A lot of mutants have been created to identify the molecular domains necessary for the correct membrane addressing of SK channels (**Figure 7**). The different SK3 variants, their deletions and/or mutations, their expression pattern and the effects on SK channel-mediated currents are summarized d in the **Table 2**.

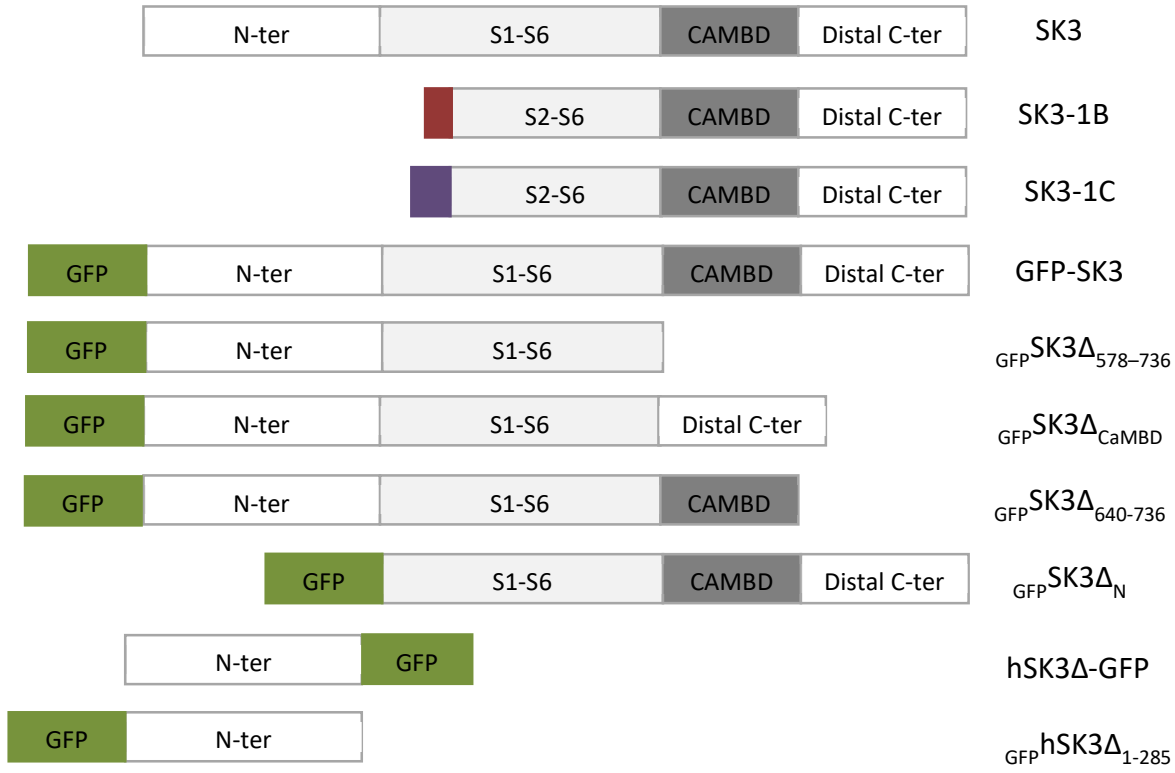


Figure 7. Schema of the structure of the SK3 channel mutants. The native SK3 channels are composed of an N-terminal (N-ter), 6 transmembrane domains (S1-S6), a calmodulin binding domain (CAMBD) and a distal C-terminal (distal C-ter). For SK3 1-B and SK3 1-C, the red and the purple segments represent the exon 1B and the exon 1C respectively. Green GFP segments represent the addition of Green Fluorescent protein (GFP).

The mutant $_{GFP}SK3\Delta_N$, for which the N terminal (N-term) of SK3 was deleted, was mostly found expressed in the intracellular compartment. Similarly, alteration of N-term in the mutants SK3-1B and SK3-1C induced the sequestration of endogenous SK3 in the intracellular space and leads to the abolition of endogenous SK mediated currents. Besides, co-immunoprecipitation experiments done by Roncarati et al. revealed that $_{GFP}SK3\Delta_N$ could interact with wild type (wt) SK3 in HEK cells (Roncarati et

al., 2005). Taken together, these data indicate that the N-term of SK3 is required for its membrane delivery but does not seem necessary for SK oligomerization. Interestingly, SK3-1B and SK3-1C have a dominant negative effect on the endogenous SK channel current. Thus, one can hypothesize that N-term mutants of SK3 associate with wt SK3 subunits and, as the mutant SK3 cannot be delivered in the membrane, sequester wt SK3 intracellularly. Disruption of the entire C terminal (C-term), which contains both CAMBD and the distal part of C-term of SK3, resulted in a total loss of $_{GFP}SK3\Delta_{578–736}$ mutant surface expression and their retention in the endoplasmic reticulum (ER). Deletion of CAMBD caused a similar distribution of $_{GFP}SK3\Delta_{CaMBD}$ mutants in the ER whereas $_{GFP}SK3\Delta_{640-736}$ mutants, for which the distal part of C-term was removed, were also found in the Golgi apparatus. These results imply that the distal part of C-term and CAMBD would control different steps in the SK3 secretory pathway, the CAMBD being required to exit the RE and the distal part of C-term to exit the Golgi apparatus. In addition, both $_{GFP}SK3\Delta_{640-736}$ and $_{GFP}SK3\Delta_{CaMBD}$ mutants were capable of interacting with wt SK3 in co-immunoprecipitation, but not the SK3 mutants with the deletion of the entire C-term (Roncarati et al., 2005), suggesting that several domains in the entire C-term would contribute to SK3 tetramers assembly. Finally, hSK3-Δ mutants and its derivatives ($_{GFP}hSK3\Delta_{1-285}$ and hSK3ΔNLS-GFP), which only contained the N-term of SK3, suppressed SK channels endogenous current, as previously seen with the N-term SK3 mutants. These data strongly suggest that these mutants can assemble with wt SK3 and prevent their membrane delivery. Because hSK3-Δ mutants and their derivatives are devoid of C-term, this hypothesis is in conflict with the results obtained by Roncarati et al. that describe that the C-term domain and not the N-term was required to form SK3 tetramers (Roncarati et al., 2005). However, Frei et al. challenged this finding and revealed interaction between N- and N-term, and between N- and C-term of SK3 in yeasts (Frei et al., 2006). Thus, it might be possible that N-term of hSK3-Δ mutants and their derivatives interact with N- or C-term of wt SK3, and alter their distribution and/or function. To conclude, N-term and C-term are important for the correct membrane delivery of SK3 tetramers but much work is needed to fully understand the mechanisms underlying the assembly and membrane trafficking of these receptors.

Once in the membrane, it is known that glutamate receptors such as AMPAR or NMDAR are anchored in the synapses through the binding to several sets of scaffold proteins, such as the PDZ domain-containing proteins (Kim and Sheng, 2004). Surface SK channels seem to also be anchored in the synapses, through actin-dependent processes (Allison et al., 1998). Indeed, pharmacological disruption of the cytoskeleton prevents the enhancement of synaptic transmission induced by SK channel blockade in amygdala neurons (Faber et al., 2008). In addition, the surface content of various receptors such as AMPAR or NMDAR is regulated by clathrin-mediated endocytosis (Carroll et al., 1999; Roche et al., 2001). SK channels appear to share a similar mechanism of internalization, as

demonstrated by Faber et al. who used blockers of dynamin, an essential component of the clathrin-mediated endocytosis (Faber et al., 2008).

It is interesting to mention that the lateral trafficking of receptors, among which NMDAR, has been identified as a key modulator of their surface distribution (Groc et al., 2006). Although SK channel lateral trafficking has not been investigated yet, several hints favor the idea that SK channels are dynamic in the membrane as well. Given that NMDAR and L-type calcium channels, two calcium sources for SK channels, diffuse in the neuronal membrane (Groc et al., 2004; Biase et al., 2012) and that SK channels need to be located in the vicinity (distance 100-150 nm) of their calcium source (Jones and Stuart, 2013), the lateral mobility of SK channels would allow to regulate their distance from the calcium sources, and also their synaptic and extrasynaptic membrane distribution. Besides, an elegant study using atomic-force microscopy revealed that protein kinase A regulates both somatodendritic distribution and nanoclustering of native SK channels in hippocampal neurons (Abiraman et al., 2016). This reinforces the idea that the SK channel distribution is highly dynamic and modulated by activity.

Table 2. Principal characteristics of the SK3 channels mutants

SK3 variant name	Size of SK3	Mutation / deletion	Used expression system	Effect on SK current	Expression pattern of endogenous/ mutant SK3	Reference
Endogenous human SK3	Human : 736 aa	Exon 1 A	-	-	-	-
SK3-1B	418 aa	Alternative Exon 1B (Lack of the N-term + 1st transmb segment)	PC12 Human Jurkat T lymphocytes	Suppression of endogenous SK3 current Suppression of endogenous SK2 current	Endogenous SK3 : mostly intracellular	Tomita, 2003
SK3-1C	640 aa	Alternative Exon 1C	PC12 HEK -293T	Suppression of endogenous SK3 current Suppression of exogenous SK1, SK2 and IK_{Ca} currents	Endogenous SK3 : mostly intracellular	Kolski-Andreaco, 2004
$_{GFP}$SK3	736 aa (+239 for GFP)	Addition of GFP in the N-term	PC12 HPC culture	Biophysical and pharma-cological properties similar to wt SK3 channels	Colocalization between mutant and endogenous SK3 (plasma mb + intracellular) Mutant SK3 : present in cell body, axon, dendrite	Roncarati,2005 Decimo,2006

$_{GFP}SK3\Delta_{578–736}$	578 aa (+239 for GFP)	Addition of GFP in the N-term Deletion of the entire C-term (CaMBD and C-term distal domains)	PC12, HEK293 HPC culture	-	Mutant SK3 : loss of surface channels colocalization with ER Mutant SK3 : Only somatic, colocalization with ER	Roncarati,2005 Decimo,2006
$_{GFP}SK3\Delta_{640-736}$	640 aa (+239 for GFP)	Addition of GFP in the N-term Deletion of C-term distal domain	PC12, HEK293 HPC culture	-	Mutant SK3 : reduction of 70% of cell surface localization Colocalization with ER and Golgi Mutant SK3 : present in cell body, axon, dendrite	Roncarati,2005 Decimo,2006
$_{GFP}SK3\Delta_{CaMBD}$	677 aa (+239 for GFP)	Addition of GFP in the N-term Deletion of CaMBD	PC12, HEK 293 HPC culture	-	Mutant SK3 : mostly intracellular Mutant SK3: small clusters in soma and proximal dendrites, colocalization with ER	Roncarati,2005 Decimo,2006
$_{GFP}SK3\Delta_{N}$	465 aa (+239 for GFP)	Addition of GFP in the 1st transmb segment Deletion of N-term	PC12, HEK293 HPC culture	-	Mutant SK3 : loss of surface expression Retention in the ER Mutant SK3 : Only somatic Colocalization with ER	Roncarati,2005 Decimo,2006
hSK3Δ-GFP	286 aa (+239 for GFP)	Addition of GFP after the last aa Frame shift mutation in exon 1 → expression of only the first 283 aa (before the 1st transmb segment) of SK3 + 3 aa (Thr, Met, Leu)	In the VTA DA neurons, in vivo	Suppression of endo-genous SK currents	Mutant SK3 : localization in dopamine neuron processes and nucleus	Soden,2013

hSK3ΔNLS-GFP	255 aa (+239 for GFP)	Addition of GFP after the last aa Deletion of the last 31 aa from hSK3-Δ	In the VTA DA neurons, in vivo	Suppression of endogenous SK currents	Mutant SK3 : localization in soma and processes (no more in the nucleus)	Soden,2013
$_{GFP}hSK3\Delta_{1-285}$	285 aa (+239 for GFP)	Addition of GFP in N-term Frame shift mutation in exon 1 → expression of only the first 283 aa (before the 1st transmb segment) of SK3 + 2 aa (Ser+ Asp)	COS-7, Human Jurkat T lymphocytes	Suppression of endogenous SK2 currents	Mutant SK3 : exclusively in the nucleus	Miller,2001

Role of SK channels in DA neurons

When activated by calcium, SK channels induce an efflux of potassium contributing to the after-hyperpolarization (AHP) following an AP. The AHP is composed of 3 phases: a fast (fAHP), a medium (mAHP) and a slow (sAHP) components. Whereas BK channels contribute to the fAHP (Lancaster and Nicoll, 1987), SK channels mediate the mAHP (Sah, 1996) and control the firing activity of many neurons, including DA (Wolfart et al., 2001). First, SK channels contribute to the control of firing frequency. Inhibition of SK channels by apamin induced an increase in the firing rate of DA neurons in midbrain slices (Shepard and Bunney, 1988; Wolfart et al., 2001; Deignan et al., 2012) and *in vivo* (Waroux et al., 2005; Soden et al., 2013; Creed et al., 2016), whereas a positive SK2/SK3 modulator decreased it in midbrain slices (Herrik et al., 2010). It should be noted that some authors did not find a change in the firing rate following apamin application *in vitro* (Soden et al., 2013) and *in vivo* (Ji and Shepard, 2006). However, the most striking effect of SK channels is on the firing pattern of DA neurons. In midbrain slices, apamin alters the precision of pacemaker activity of DA neurons (Wolfart et al., 2001; Deignan et al., 2012; Soden et al., 2013). Therefore, they fire in a more irregular pattern, quantified by an increase in the CV-ISI. Occasionally, apamin alone can trigger the apparition of bursts of AP in some neurons (Shepard and Bunney, 1988, 1991; Ping and Shepard, 1996) but is mostly used in combination with NMDA to induce bursts in slices (Seutin et al., 1993; Johnson and Wu, 2004). It is worth pointing that VTA DA neurons, which express less SK3 channels, exhibited a less regular pattern than SNc DA neurons (Wolfart et al., 2001), and that the increase in the firing regularity of SNc DA neurons observed *in vitro* during the development (Dufour et al., 2014a) was paralleled with a greater membrane expression of SK3 channels (Dufour et al., 2014b). Therefore, all these data strongly support a role of these channels in the control of the pacemaker precision *in vitro*. *In vivo*, pharmacological inhibition of SK channels promoted burst discharge of midbrain DA neurons by increasing both the number of spikes within bursts and the CV-ISI (Waroux et al., 2005; Ji and Shepard, 2006). Using a mutated inactive form of SK3 channels initially identified in patients with schizophrenia, Soden et al found a similar result *in vivo* with an enhanced burst discharge in midbrain DA neurons (Soden et al., 2013). On the contrary, systemic administration of the SK opener 1-EBIO decreased the number of spikes within a burst and the CV-ISI (Ji and Shepard, 2006). Thus, by regulating the regularity of midbrain DA neuron firing, SK channels might control the switch between the tonic and bursting patterns.

Functional coupling to calcium sources

To be activated, SK channels require calcium entry in the cell. In the literature, different sources of calcium have been identified in neurons and include voltage gated calcium channels (N-, P-,Q-, R-, L- and T-type), NMDAR or calcium release from intracellular stores. As mentioned earlier, the

coupling of SK channel with its calcium source, and by extension their function, seems to be quite variable in different types of neurons (Stocker, 2004). In DA neurons, several calcium sources have been characterized so far. In SNc DA neurons, the almost full inhibition of SK channel-mediated AHP by the T-type calcium channel blocker mibefradil highlighted the predominant position of T-type calcium channels as a calcium source for SK channels (Wolfart and Roeper, 2002). However, this result was at odds with the study of De Vrind et al., who found that another more specific T-type calcium channel blocker TTA-P2 was not effective in blocking SK channels-mediated AHP (de Vrind et al., 2016). To explain this discrepancy, the authors pointed out the difference in the age of animals, juvenile mice (PD 10-14) for Wolfart et al. and adults rats (6-8 weeks) for De Vrind et al. Indeed, T-type channels can spontaneously open in DA neurons in young rats (PD 6-12) (Cui, 2004) and this could contribute to the irregular firing of DA neurons observed in young animals (Dufour et al., 2014a). As the firing becomes regular in adults, T-type calcium channels might no longer be involved in the coupling with SK channels, explaining their absence of contribution in the study of de Vrind et al. Interestingly, this raises the question of whether the coupling of SK channels with calcium sources is also regulated during the development in DA neurons. By contrast, both studies of Wolfart. and De Vrind. agreed on the involvement of N-type calcium channels in activating SK channels, although the results of De Vrind. indicate that these channels participate to a greater extent to the coupling with SK channels in mature DA neurons. There is also evidence to suggest the contribution of L-type calcium channels as a source of calcium for SK channels (Nedergaard et al., 1993). Finally, given that the broad voltage-gated calcium channel blocker cobalt did not fully block SK channel-mediated AHP (Wolfart and Roeper, 2002; de Vrind et al., 2016), activation of SK channels may require other calcium sources. In line with this, Seutin et al. found that SK channel-mediated spontaneous hyperpolarisations occurring in midbrain DA neurons from young rats were dependent on calcium release form intracellular stores (Seutin et al., 1998, 2000) and thereby, confirms the presence of other calcium sources used by SK channels.

a.2) Other channels

In addition to SK channels, other conductances are also involved in the generation of the firing activity of midbrain DA neurons.

Leak channels

Contrary to non-pacemaker cells, midbrain DA neurons exhibit few leak potassium channels and more nonselective cation leak and sodium leak channels (Talley et al., 2001; Khaliq and Bean, 1996). Given the low value of the potassium equilibrium potential (-90mV) and the relatively high value

of the sodium equilibrium potential (+40mV), this would explain the relative positive membrane potential (-60mV) of these neurons.

Voltage-gated sodium and non-selective cations channels

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels. HCN channels, formed of four subunits, are non-selective cation cannels. Among the 4 subunits identified (HCN1-4) (Ludwig et al., 1998; Santoro et al., 1998), HCN2, HCN3 and HCN4 are expressed in midbrain DA neurons (Franz et al., 2000; Santoro et al., 2000; Notomi and Shigemoto, 2004). When activated by hyperpolarization, HCN channels open and allow the entry of sodium (and to a lesser extent the exit of potassium) responsible for the "Ih current", which depolarizes the membrane. In current clamp, the presence of the Ih conductance is recognizable by a "sag response", resulting from the depolarization that counters the hyperpolarization. The Ih current is the main criteria to identify DA neurons in slices (Grace and Onn, 1989; Neuhoff et al., 2002; Wanat et al., 2008; Mao et al., 2011). However, its contribution to the firing activity is ambiguous. Mercuri et al. found that blocking Ih current by cesium had no effect on the frequency of pacemaker activity (Mercuri et al., 1995). By contrast, application of ZDZ7288, which is a more specific Ih blocker, inhibits the spontaneous firing of a subset of midbrain DA neurons (Seutin et al., 2001; Puopolo et al., 2007). Accordingly, Neuhoff et al. also noticed that this agent slowed the pacemaker activity of a subpopulation of SNc DA neurons, whereas it has not effect on VTA DA neurons (Neuhoff et al., 2002). Thus, it seems that the effect of Ih channels on DA neuron excitability depends on the population of recorded midbrain DA neurons.

Voltage gated sodium channels. TTX-sensitive voltage-gated sodium channels (Na_V channels) are well-known for their crucial role in the depolarization phase leading to the AP generation in neurons. The Nav family contains 9 members (Nav 1.1-1.9). They are present in pacemaker cells (Raman and Bean, 1999; Taddese and Bean, 2002), including midbrain DA neurons where they drive the pacemaker activity (Khaliq and Bean, 1996). To better understand the role of Na_V channels in these neurons, Tucker et al. used advantage of the dynamic clamp, which consists in inserting virtual conductances generated by computer in a living neuron, in real time (Tucker et al., 2012). They found that the low pacemaker rate could be simulated by a low density of somatic Na_V channels, suggesting that midbrain DA neurons contain a low density of these channels in the soma. Despite their important role in generating pacemaker activity, Na_V channels are not the only contributors to the depolarization during AP generation because some DA neurons still exhibit pacemaker activity with broader and smaller AP in the presence of TTX (Puopolo et al., 2007).

Transient receptor potential (TRP) channels. TRP channels, composed of 28 members divided into three subfamilies (TRPC, TRPV and TRPM), are non-selective cations channels, with some of which permeable to calcium (Bollimuntha et al., 2011). In midbrain slices, activation of the temperature gated TRPV-4 channels increased the firing rate of SNc DA neurons *in vitro* (Guatteo et al., 2005). Besides, application of a TRP channel blocker decreased the pacemaker activity and eliminated the bursts induced by NMDA application in slices (Mrejeru et al., 2011). Together, these data suggest a role for TRP channels in modulating the firing pattern and the frequency of *in vitro* DA neurons. In a worm mutant with degeneration of DA neurons, Nagarajan et al. identified gain-of-function mutations in TRP channels that were responsible for the necrosis of DA neurons through a calcium dependent mechanism (Nagarajan et al., 2014), suggesting that TRP channels might be also involved in the survival of DA neurons.

Potassium channels

Voltage gated potassium channels channels. The presence of voltage-gated potassium channels (K_V) in DA neurons was first suggested by Chiodo and Kapatos in primary dissociated mesencephalic cultures (Chiodo and Kapatos, 1992). By depolarizing the membrane of DA neurons, they discovered an outward potassium current that was non-inactivating during steps duration (500ms), TEA-sensitive and had a delayed onset. In others neuron types, it was shown that these channels are responsible for the repolarization phase during AP generation (Kang et al., 2014). Although these channels were poorly studied specifically in midbrain DA neurons, one can assume that they could play a similar role in midbrain DA neurons. Interestingly, Kv channels seem to play a critical role in the vulnerability of midbrain DA neurons to the neurotoxin 6-OHDA. Application of 6-OHDA, widely used in PD models, enhances both DA neuronal cell death and a Kv channel-mediated current in DA neurons (Redman et al., 2006). The toxic effect of 6-OHDA could be reversed by application of TEA, suggesting an important role of Kv channels in the vulnerability of midbrain DA neuron to neurotoxin 6-OHDA.

A-type potassium channels. Belonging to the Kv channels family, voltage-dependent A-type potassium (A-type Kv) channels are present in midbrain DA neurons, with a predominance of the Kv4.3 channels (Serôdio and Rudy, 1998; Liss et al., 2001). When activated by membrane depolarization, they induce an outward current (named $\mathbf{I_A}$) carried by potassium ions, slowing the subsequent AP generation (Koyama and Appel, 2006). Besides, they quickly inactivate. In SNc DA neurons, it was suggested that A-type Kv channels play a role in tuning pacemaker activity because a strong correlation exists between pacemaker frequency and their number (Liss et al., 2001). Accordingly, Hahn et al. found that the upregulation of A-type potassium channels slowed the pacemaker activity of midbrain

DA neurons (Hahn et al., 2006). By using dynamic clamp to further investigate the role of these channels, the authors showed that the regularity of the firing was also modulated by A-type Kv channels.

G protein-coupled inwardly-rectifying potassium (GIRK) channels. GIRK channels are potassium channels activated by intracellular cascades mediated by G protein-coupled receptors (GPCRs) (Andrade et al., 1986). In midbrain DA neurons, activation of $GABA_B$ or D2 receptors, which are two well-known GPCR coupled to Gi/o proteins (Odagaki and Koyama, 2001; Beaulieu and Gainetdinov, 2011) induces an outward potassium current mediated by GIRK channels, resulting in a late hyperpolarizing potential (Johnson and North, 1992). Notably, Lalive et al. found that GIRK channel-mediated currents were modulated by the firing pattern of midbrain DA neurons, with phasic firing enhancing these currents and tonic current depressing them (Lalive et al., 2014). By inhibiting DA neurons after burst firing or promoting their excitability after tonic firing, GIRK channels may participate in controlling the switch between phasic and tonic firing.

ATP sensitive potassium (K-ATP) channels. K-ATP channels, composed of four Kir6.2 subunits and four regulatory SUR1 subunits (Nichols, 2006), have been detected in DA neurons from midbrain slices (Liss et al., 1999). In K-ATP channel knockout mice, Schiemann et al. observed that the firing pattern in SNc DA neurons was altered *in vivo* (Schiemann et al., 2012). Instead of an irregular discharge of AP with bursts, they fire in a regular manner with no more bursts. In the same study, the authors also showed that co-activation of NMDA and K-ATP was sufficient to induce bursts in DA neurons from midbrain slices, corroborating the fact that K-ATP channels play a role in the firing pattern of midbrain DA neurons.

Calcium channels

Pioneering studies of Grace and Bunney investigated the calcium dependence of the spontaneous activity of midbrain DA neurons i*n vivo*. Injecting calcium or a calcium chelator such as EGTA changed the single spike and the bursting firing patterns respectively (Grace and Bunney, 1984a, 1984b), pointing out the fundamental role of calcium in the spontaneous firing of DA neurons. So far, several voltage gated calcium channels have been identified in midbrain DA neurons and include the high voltage activated L-, N-, P/Q-type (Wolfart and Roeper, 2002; Puopolo et al., 2007; de Vrind et al., 2016; Philippart et al., 2016) and the low voltage activated T-type channels (Cui, 2004). As previously reported, some SNc DA neurons still exhibit pacemaker activity *in vitro* with broader and smaller AP in presence of TTX (Puopolo, 200). This TTX-insensitive activity was calcium dependent and could be slowed down by both L-type and P/Q channel blockers, suggesting their involvement in the generation

of the pacemaker activity in midbrain DA neurons. Besides, most of these calcium channels are involved in the regulation of the firing precision of DA neurons, likely due in part to their functional coupling with SK channels. Indeed, modulation of L-type and N-type channels impacted the firing regularity of midbrain DA neurons (Wolfart and Roeper, 2002; de Vrind et al., 2016; Philippart et al., 2016). The T-type channels, responsible for spontaneous miniature hyperpolarizations in immature DA neurons, contributed to their irregular firing at immature stages (Cui, 2004).

Sodium-potassium ATPase (Na+/K+-ATPase) pumps

The Na+/K+ ATPase is an electrogenic pump, which transports potassium and sodium ions against their electro-chemical gradients to maintain the resting membrane potential of neurons (Vassalle, 1987). In DA neurons, the sodium pump also contributes to modulation of the firing pattern of *in vitro* midbrain DA neurons. The sodium pump blocker ouabain prevented the bursts induced by NMDA application but had no effect in the pacemaker activity of DA neurons in slices (Johnson et al., 1992). Thus, the authors proposed that sodium ions entered the cell through NMDAR following NMDA application and activated the sodium pump, which hyperpolarized the neuron and ended the burst. In accordance with this hypothesis, Shen and Johnson found that the current evoked by the sodium pump was increased in response to higher intracellular sodium concentration (Shen and Johnson, 1998). However, because NMDA-induced and *in vivo* bursts differed in some aspects, the role of the sodium pump in the burst activity remains questionable *in vivo*.

b) Receptors modulating the firing activity

Auto-receptors

In the 70's, Di Chiara et al. (1977) observed that pharmacological destruction of striatum had no impact on the self-inhibition of DA metabolism induced by the DAR agonist apomorphine (Di Chiara et al., 1977). Thus, they postulated the existence of a negative feedback loop mediated by DA auto-receptors present in the somatodendritic compartment of midbrain DA neurons. The presence of such receptors was confirmed by autoradiography, and revealed that both D2R and D3R were present in DA neurons (Filloux and Wamsley, 1988; Morelli et al., 1988; Tepper et al., 1997). The pioneering study of Lacey et al (1897) showed that application of DA induced a potassium channel-mediated hyperpolarization that inhibits the spontaneous firing rate of DA neurons in slices. Since then, several potassium conductances modulated by D2R have been identified. Hahn et al. showed that D2R activation slowed the firing of DA neurons *in vitro* by modulating the A-type Kv channels (Hahn et al., 2006). Besides, GIRK channels are also controlled by D2R (Congar et al., 2002). Therefore, auto-

receptors tune the excitability of midbrain DA neurons by activating potassium conductances, as do many other receptors coupled to Gi/o proteins.

GABA receptors

Recordings of VTA DA neurons from midbrain slices revealed the presence of hyperpolarizing synaptic potentials sensitive to bicuculline, which is an antagonist of the ionotropic $GABA_A$ receptors (Johnson and North, 1992). By RNA analysis and *in situ* hybridizations, Okada et al. demonstrated the presence of transcripts coding several $GABA_A$ receptors subunits in dopaminergic neurons (Okada et al., 2004). The metabotropic $GABA_B$ receptors are also present in rat midbrain DA neurons (Wirtshafter and Sheppard, 2001). *In vivo*, $GABA_A$ and $GABA_B$ receptors modulate both the firing pattern and the firing rate of midbrain DA neurons. Although several authors reported the antagonist effects of $GABA_A$ and $GABA_B$ in regulating both parameters (Engberg et al., 1993; Paladini and Tepper, 1999; Brazhnik et al., 2008), they observed contradictory effects with GABA receptors antagonists and agonists, depending on the methods of drugs administration. Local application of $GABA_A$ and $GABA_B$ receptors antagonists respectively increased and decreased the firing rate *in vivo* in midbrain DA neurons (Paladini and Tepper, 1999; Brazhnik et al., 2008). By contrast, systemic administration of $GABA_B$ receptors antagonists and $GABA_A$ receptors agonists induce a modest increase in the firing frequency (Engberg et al., 1993; Erhardt et al., 2002). Similar discrepancies were noted regarding the firing pattern. Whereas local $GABA_A$ receptor blockade favors the bursting activity, inhibition of $GABA_B$ receptors regularize the firing pattern (Paladini and Tepper, 1999; Brazhnik et al., 2008). As for the firing rate, Erhardt and Engberg found the exact opposite effect with intravenous and systemic administration of drugs (Engberg et al., 1993; Erhardt et al., 2002). However, these studies converge to the idea that both $GABA_A$ and $GABA_B$ receptors differently affected the firing of midbrain DA neurons. By using dynamic clamp experiments on *in vitro* DA neurons, Lobb et al. confirmed the role of $GABA_A$ receptors in the firing pattern by showing that removal of $GABA_A$ conductances favored the burst generation (Lobb and Paladini, 2010).

Glutamate NMDA receptors

The glutamate N-methyl-D-aspartate receptors (NMDAR), which are one of the principal modulators of the firing of midbrain DA neurons, are essential for the bursting pattern. Earlier *in vivo* studies showed that iontophoretic administration of NMDA, an NMDAR agonist, increased the firing rate and the burst activity of midbrain DA neurons by enhancing the burst frequency and the number of spikes per bursts (Overton and Clark, 1992; Christoffersen and Meltzer, 1995). On the contrary, exposure to NMDAR antagonists reduced the burst activity *in vivo* (Overton and Clark, 1992; Christoffersen and Meltzer, 1995) and increased the regularity of the firing (Engberg et al., 1993;

Connelly and Shepard, 1997). These observations were confirmed by *in vitro* experiments on brain slices. Bath application of NMDA increases the frequency of the pacemaker activity of DA neurons in a concentration-dependent manner (Seutin et al., 1990; Mercuri et al., 1992; Wang et al., 1994; Mereu et al., 1997). In addition, bursts could be evoked by NMDA application in midbrain slices under certain circumstances (**Figure 8**) (Johnson et al., 1992; Johnson and Wu, 2004). Indeed, NMDA application alone is not sufficient to induce bursts (Seutin et al., 1990; Mercuri et al., 1992), and it requires the concomitant injection of an hyperpolarizing current in DA neurons to prevent an excessive depolarization and allow burst generation (Johnson and Wu, 2004). Of note, NMDA alone might be sufficient to induce bursts in slices (Mereu et al., 1997). Interestingly, even when an hyperpolarizing current was injected in neurons, application of NMDA only evoked burst in 33% of the recorded neurons (Johnson and Wu, 2004), suggesting that NMDAR activation is necessary but not sufficient to induce bursts in slices. Regarding the effects of NMDAR antagonists, D-APV alone had not effect on the basal pacemaker activity of midbrain DA neurons but reversed the changes in the firing activity induced by NMDA (Seutin et al., 1990; Mercuri et al., 1992; Mereu et al., 1997).

Although these data strongly support the role of NMDAR in the firing pattern of midbrain DA neurons, they do not directly prove that the effect is mediated by NMDAR localized in DA neurons. More recently, Zweifel et al. showed that genetic inactivation of GluN1, the obligatory subunit of the NMDAR, specifically in midbrain DA neurons induced a strong reduction of the *in vivo* burst frequency by 6-fold (Zweifel et al., 2009); and thus, provide the first direct evidence of the role of NMDAR in the burst generation of midbrain DA neurons.

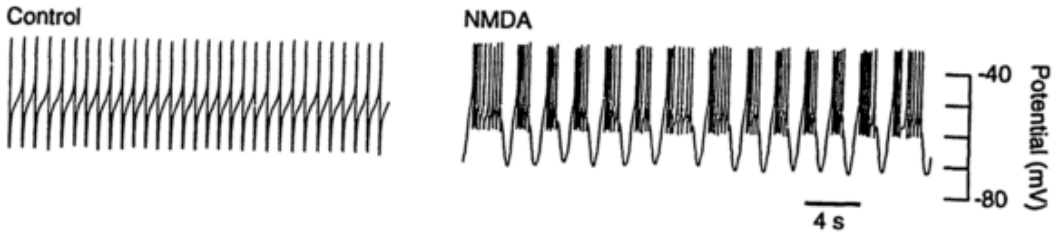


Figure 8. Burst generation in DA neurons from midbrain slices by bath application of NMDA (30µM) (adapted from Johnson et al., 1992). In control conditions, DA neurons fire in a regular pacemaker mode. Following NMDA application and injection of an hyperpolarizing current, rhythmic bursts are elicited in DA neurons.

II. Glutamate NMDA receptors (NMDAR)

Glutamate is the main excitatory neurotransmitter in the brain. Three ionotropic glutamate receptors were pharmacologically identified according to their relative affinity to the following agonists: α-amino-3-hydroxy-5-méthylisoazol-4-propionate (AMPA), N-méthyl-D-aspartate (NMDA) and kainate (Dingledine et al., 1999). Because they differ in various parameters including ion selectivity, conductance and activation-deactivation kinetics, they have different roles in the synaptic transmission:

- AMPA receptors (AMPAR) are responsible for fast synaptic transmission
- Kainate receptors are also involved in synaptic transmission and regulate glutamate release
-Compared to the other ionotropic receptors, NMDA receptors (NMDAR) have a special role in synaptic plasticity due to their unique channel properties. First, as their opening requires the concomitant release of glutamate by the presynaptic neuron and membrane depolarization of the postsynaptic element, they are considered as coincidence detectors. This characteristic is consistent with the Hebbian rule in which the synapse between two neurons should be strengthened if cells are activated at the same time. Second, they have a high permeability for calcium (MacDermott et al., 1986), which is known to have a key role in synaptic plasticity (Bliss and Collingridge, 1993).

In contrast to ionotropic receptors which induce a rapid entry of cations when activated, glutamate can also bind to metabotropic receptors (mGluR). These channels belong to the G-protein-coupled receptors and transmit signals by intracellular cascades (Niswender and Conn, 2010). Given the slow onset of their responses, metabotropic receptors are said to modulate synaptic transmission.

A. On neurons in general

1. Structure and composition of NMDAR

a) NMDAR subunits

NMDAR are one of the three ionotropic glutamate receptors. They form hetero-tetramers composed of two GluN1 and two GluN2 or GluN3 subunits. Each subunit is composed of 4 transmembrane domains (M1-M4), a N terminal domain in the extracellular space (NTD) and an internal C terminal domain (**Figure 9**) (Paoletti et al., 2013). The channel pore is formed by the M2

loops of the four subunits. To be fully activated, NMDAR require the binding of an agonist such as glutamate or NMDA (MacDonald and Wojtowicz, 1980) and a co-agonist, which can be glycine (Kleckner and Dingledine, 1988) or D-Serine (Mothet et al., 2000). These binding sites are located in different subunits of the receptor. The agonist binding site is in the extracellular part of the GluN2 subunit (Monyer et al., 1992; Laube et al., 1997) whereas the co-agonist can bind the extracellular part of the GluN1 or GluN3 subunits (Kuryatov et al., 1994; Yao and Mayer, 2006). On the functional level, NMDAR are permeable to potassium and sodium ions (Mayer and Westbrook, 1987) but also to calcium, for which they have a high permeability (MacDermott et al., 1986). Interestingly, at resting membrane potential, NMDAR are blocked by extracellular magnesium ions. This confers a voltage-dependence because a membrane depolarization is required to remove the magnesium block (Nowak et al., 1984).

The cloning of GluN1 in oocytes showed that expressed homomeric NMDAR, although functional, exhibit low current amplitudes (Moriyoshi et al., 1991; Yamazaki et al., 1992), predicting that natural NMDAR should occurred in heteromeric configuration. It appears that this subunit is obligatory, as functional NMDAR cannot be expressed in the absence of GluN1 subunit in heterologous cells (Monyer et al., 1992; Ciabarra et al., 1995; Chatterton et al., 2002) or in GluN1 knock out mice (Fukaya et al., 2003). In this latter study, inhibiting GluN1 expression retains and aggregates GluN2 subunits in the endoplasmic reticulum, preventing its trafficking to the membrane. For GluN2 and GluN3 subunits, multiple isoforms exist in the brain. GluN2 and GluN3 present 4 (GluN2A-2B-2C-2D) and 2 isoforms (GluN3A-3B) respectively, which confers different characteristics for the NMDAR regarding the activation and deactivation kinetics, the conductance, the calcium permeability, the magnesium sensitivity, the agonist and the antagonist sensitivity (Cull-Candy et al., 2001). For example, GluN2A and GluN2B-containing NMDAR have a higher conductance, sensitivity to magnesium and permeability to calcium than GluN2C or GluN2D-containing NMDAR. Incorporation of GluN3 subunits results in a decrease of ionic conductance, magnesium sensitivity and is accompanied by a drastic diminution of calcium permeability (Ciabarra et al., 1995; Henson et al., 2010). Except GluN1 which is expressed at all embryonic stages from 14 embryonic day (E14) (Laurie and Seeburg, 1994), GluN2 and GluN3 isoforms are differentially distributed and regulated during development in the brain (Monyer et al., 1994). GluN2B and GluN2D subunits are first detected between E14 and E17 in the spinal cord, and in the hypothalamus for GluN2B and the midbrain for GluN2D subunits. During the postnatal development, GluN2B subunit expression spreads and is found in the cortex and the hippocampus whereas GluN2D is mainly expressed in the midbrain, the spinal cord and to a lesser extent in the hippocampus. GluN2A and GluN2C subunits appear at birth, with GluN2A in the hippocampus and cortex and GluN2Csubunit restricted to the cerebellum. In adult rodents, GluN2A subunit is the most

ubiquitous subunit as it is found in almost all brain regions. GluN2B subunit is restricted to the forebrain regions, GluN2C subunit to the cerebellum and GluN2D subunit to thalamic, mesencephalic and brain stem structures (Wenzel et al., 1995). Regarding the less studied GluN3 subunit isoforms, the GluN3A subunit is detected early in the development (E15) and is present in moderate amount in several areas including the cortex and the midbrain during the first postnatal week (Ciabarra et al., 1995; Al-Hallaq et al., 2002). GluN3B subunit expression, detected at birth, seems to be constant during postnatal development and is found mainly in the midbrain, the medulla and the spinal cord in the adults (Matsuda et al., 2002).

b) NMDAR subtypes

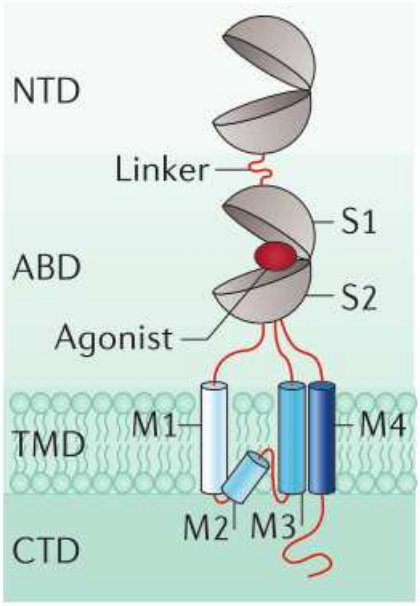


Figure 9. Schematic structure of a GluN subunit (from Paoletti et al., 2013).
Each GluN subunit is composed of an extracellular N terminal domain (NTD), an agonist binding domain (ABD), 4 transmembrane domains (M1-M4) and a C terminal domain (CTD). The pore is formed by the assembly of the M2 transmembrane domains from the four subunits.

As described above, NMDAR are heterotetramers formed by the assembly of the obligatory GluN1 subunits with either GluN2 or GluN3 subunits. The comparison of native and recombinant NMDAR channel characteristics by electrophysiology has enabled to infer the composition of native NMDAR in several brain region (Cull-Candy et al., 2001). For example, when Farrant et al. recorded NMDAR single channel currents in cerebellar granule cells, they noted that these neurons exhibited some atypical NMDAR properties like low conductance (20-30pS) and short opening kinetics (Farrant et al., 1994). These characteristics being strikingly similar to those reported for recombinant GluN1 and GuN2C-containing NMDAR expressed in heterologous systems, it was assumed that NMDAR-containing GluN2C subunits were present in cerebellar granule cells. Moreover, the discovery that several drugs have different affinities for specific subunits has facilitated the identification of the NMDAR composition in the brain. Among these drugs, ifenprodil inhibits recombinant GluN2B-

containing NMDAR currents with a concentration 400 fold lower than GluN2A-containing NMDAR in Xenopus oocytes (Williams, 1993). Given this high selectivity for GluN2B versus GluN2A-containing NMDAR, this drug has been widely used to characterize NMDAR composition in brain structures like the SNc (Brothwell et al., 2008). Up to now, several pharmacological agents are available to target specific NMDAR subunit. They include zinc or DQP 1102, which preferentially block GluN2A and GluN2C/2D-containing NMDAR respectively (Paoletti et al., 1997; Acker et al., 2011).

Based on these tools, it is admitted that most native NMDAR are di-heteromers composed of two GluN1 with two similar GluN2 or GluN3 subunits. Growing evidence also points to the existence of triheteromers in several brain regions. Co-immunoprecipitation experiments have highlighted the presence of tri-heteromers containing GluN1 with both GluN2A and GluN2B subunits in the hippocampus (Al-Hallaq et al., 2007) and since then, other combinations have been proposed, such as GluN1/2A/2D (Dunah et al., 1998) or GluN1/2A/3A (Perez-Otano et al., 2001). Interestingly, electrophysiological and pharmacological approaches also support the existence of tri-heteromers in the brain: GluN1/2B/2D in the midbrain (Brothwell et al., 2008), GluN1/2A/2C in the cerebellum (Chazot et al., 1994) or GluN1/2B/3A in the cortex (Das et al., 1998).

2. NMDAR distribution, function and regulation of its properties

a) Neuronal localization

Synaptic distribution

Subcellular distribution of NMDAR was first described by immunohistochemistry against the GluN1 subunit and revealed that NMDAR are present in synaptic sites and in close apposition to axons (Aoki et al., 1994). Within the synapses, NMDAR accumulate in the post-synaptic density (PSD) (**Figure 10**) (Valtschanoff and Weinberg, 2001; Barrow et al., 2009). The latter, visualized by a dense electron area in electron microscopy, is composed of signaling molecules, cytoskeleton proteins and scaffold molecules such as the MAGUK proteins (Kennedy, 2000), which anchor NMDAR in the synapses (Bard et al., 2010). Because the PSD faces the presynaptic terminals (Valtschanoff and Weinberg, 2001), synaptic NMDAR are activated by glutamate release from synaptic vesicles.

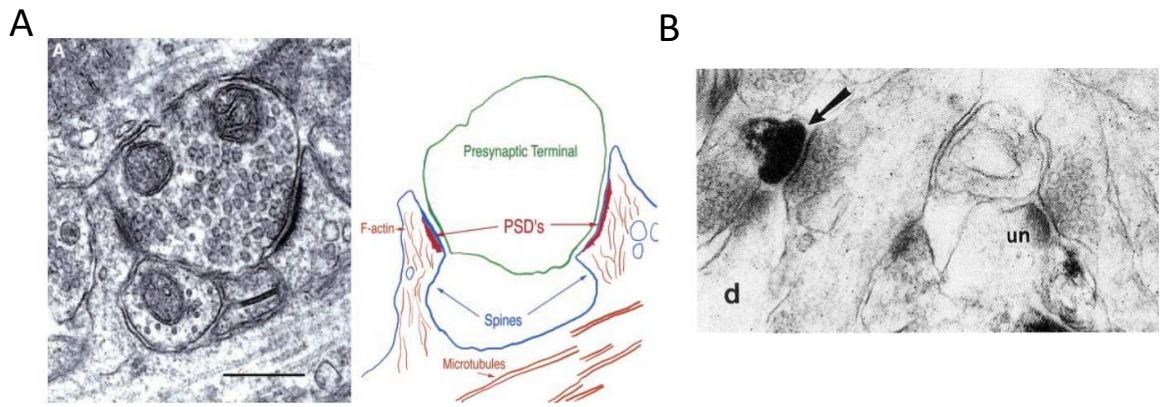


Figure 10. NMDAR are expressed in the excitatory synapses of hippocampal neurons, and are enriched in the PSD (adapted from Petralia et al., 1994; Kennedy, 2000). A) Left, Electron micrograph of the excitatory synapse of a pyramidal neuron. Right, Schematic representation of the major synaptic structures present in A). The presynaptic terminal forms two spines and the PSDs are recognizable by the dense electron areas. B) Electron micrograph of hippocampus immunostained for GluN1 subunits. A dense labelling (arrow) is observed in the PSD of a dendritic spine.

To decipher the composition of synaptic NMDAR, electrophysiological approaches using selective NMDAR subunits blockers were used. The kinetics of NMDAR-mediated EPSC were not altered by the GluN2B subunits blocker ifenprodil in the hippocampus of young (> PD 16) and mature rats (Dalby and Mody, 2003; Bellone and Nicoll, 2007), but was affected by the GluN2A blocker zinc (Bellone and Nicoll, 2007); predicting that synaptic NMDAR preferentially contain GluN2A subunits. In accordance with these studies, electron microscopy experiments performed in the hippocampus and the retina found that GluN2A-containing NMDAR predominate in synapses (Petralia et al., 2005; Zhang and Diamond, 2009). Depending on the developmental stages and the nature of the brain structure, other subunits can also be found in synapses such as GluN2B subunits in hippocampal neurons (Harris and Pettit, 2007), GluN2D in the SNc (Brothwell et al., 2008) and GluN2C in the cerebellum (Farrant et al., 1994).

Extrasynaptic localization

High resolution electron microscopy with immunogold GluN1 also detected NMDAR in extrasynaptic sites along the dendrites (Petralia et al., 2010). By using the open channel blocker MK-801 to "remove" the contribution of synaptic NMDAR, electrophysiological experiments estimated that 75% of the NMDAR are extrasynaptic in hippocampal neurons during the first week *in vitro* (Tovar and Westbrook, 1999). This proportion decreased to 30-50 % by two weeks (Ivanov et al., 2006). Of note, a rather similar proportion of extrasynaptic NMDAR was obtained in hippocampal acute slices from PD 14-P21 rats by Harris and Pettit, who found that around 35% of NMDAR were extrasynaptic (Harris and Pettit, 2007). Intriguingly, extrasynaptic receptors are not uniformly distributed in the

membrane but form clusters adjacent to axon terminals or glia (Petralia et al., 2010). In cortical and hippocampal neurons, GluN2B subunits are present in the extrasynaptic compartment during postnatal development (Stocca and Vicini, 1998; Tovar and Westbrook, 1999; Harris and Pettit, 2007). It has been shown that GluN2B-containing NMDAR can be activated by glutamate spillover following synaptic stimulation of neighboring neurons (Scimemi et al., 2004), suggesting that extrasynaptic NMDAR could be activated by glutamate escaping from the synaptic cleft. In addition, glutamate released from astrocytes preferentially acts on extraysynaptic NMDAR (Fellin et al., 2004). Therefore, extrasynaptic receptors might be activated by the glutamate release evoked by the activation of neighboring neurons or astrocytes. Regarding the contribution of other NMDAR subunits, Brickley et al. identified the presence of extrasynaptic GluN2D-containing NMDAR in isolated membrane patches from cerebellar Golgi neurons (Brickley et al., 2003). Furthermore, GluN3A subunits were also localized outside synapses in hippocampal neurons by conventional and electron microscopy (Pérez-Otaño et al., 2006).

Presynaptic NMDAR

The existence of presynaptic NMDAR was first demonstrated by electron microscopy, and revealed that GluN1 subunits could be found in the presynaptic membrane (Aoki et al., 1994). In addition, electrophysiological approaches further supported the presence of presynaptic NMDAR. The study conducted by Berretta and Jones showed that the application of the NMDAR antagonist D-APV reduced the frequency of spontaneous EPSC in entorhinal cortical neurons, even when postsynaptic NMDAR were blocked by intracellular infusion of MK-801 (Berretta and Jones, 1996). Because this protocol also affected the paired-pulse facilitation, a process known to involve presynaptic changes, it was speculated that presynaptic NMDAR modulated the spontaneous neurotransmitter release. Similar findings were found in the hippocampus (Mameli et al., 2005) and in the visual cortex (Corlew et al., 2007). Of note, the modulation of neurotransmitter release by presynaptic NMDAR seems to be regulated during development, as presynaptic NMDAR facilitated it early in the development in the cortex (<PD 20) and the hippocampus (PD 3-4) but not at later developmental stages (Mameli et al., 2005; Corlew et al., 2007). Besides, growing evidence indicates that presynaptic NMDAR are also involved in synaptic plasticity, in particular in long-term depression (Sjöström et al., 2003; Corlew et al., 2007, 2008).

b) Role of NMDAR

NMDAR have different roles depending on their membrane distribution. Whereas synaptic NMDAR are mostly associated with synaptic plasticity, it is thought that extrasynaptic NMDAR contribute to excitotoxicity and cell death. Furthermore, a new concept has emerged that NMDAR could exert functions independently of the ion flux. These functions would be briefly described in the following section.

Role of NMDAR in synaptic plasticity

In hippocampal neurons and other cell types, glutamatergic synapses can undergo long lasting changes of their synaptic strength, a process called synaptic plasticity. The synaptic strength can be either potentiated (long-term potentiation, LTP) or depressed (long-term depression, LTD). It has been well established that NMDAR play a key role in both plastic processes. Application of various NMDAR blockers such as the competitive antagonist D-APV, the open channel MK-801 and an antagonist of the allosteric glycine site prevented the induction of NMDAR-dependent LTP following tetanic stimulation in hippocampal slices (Collingridge et al., 1983; Coan et al., 1987; Bashir et al., 1990). Activation of NMDAR by a strong synaptic stimulation induces a massive entry of calcium that activates the calmoduline kinase II (Gardoni et al., 2001; Lisman et al., 2012), which in turn contributes to the membrane insertion of AMPAR (Ehlers, 2000) and increases their conductances (Benke et al., 1998). As a consequence, excitatory synaptic transmission is enhanced. Besides being required for the induction of NMDAR-dependent LTP, the receptors are also involved in the expression of LTP. For example, Kullman et al. reported that LTP induced by a pairing protocol or a tetanic stimulation in hippocampal slices was accompanied with a potentiation of NMDAR-mediated synaptic currents (Kullmann et al., 1996).

Following repeated stimulations at low frequency (1-3 Hz), long-term depression of the excitatory transmission could be observed in hippocampal slices, and was prevented by application of D-APV (Dudek and Bear, 1992), confirming that LTD induction requires NMDAR activation. It is thought that moderate entry of calcium through NMDAR activates a protein phosphatase cascade including calcineurine, which downregulates AMPAR synaptic function (Mulkey et al., 1994; Lüscher and Malenka, 2012). Interestingly, Bhouri et al. showed that low frequency stimulation of the Schaffer collateral pathway also evoked a decrease of the NMDAR-mediated currents in hippocampal neurons, that was mGluR dependent (Bhouri et al., 2014). Altogether, these data revealed that NMDAR play a prominent role in the induction and the expression of both LTP and LTD.

Role in excitotoxicity

It was earlier hypothesized that extrasynaptic NMDAR were only a reserve pool of receptors that was mobilized in synapses through lateral diffusion. However, this view was challenged by evidence showing that extrasynaptic NMDAR *per se* play specific roles. Since applying NMDA in the bath will activate all membrane NMDAR and cannot help to discriminate the role of extrasynaptic NMDAR, a specific protocol has been developed to selectively activate extrasynaptic receptors. It consists of NMDA or glutamate bath application after blockade of synaptic NMDAR by synaptic stimulation in presence of MK-801 (Tovar and Westbrook, 1999; Ivanov et al., 2006; Xu et al., 2009). Stimulation of extrasynaptic NMDAR increased the release of the lactate deshydrogenase and reduced the MAP-2 labelling in cortical neurons (Xu et al., 2009). Because these two changes are associated with cell death (Legrand et al., 1992; Brooke et al., 1999), it indicates that extrasynaptic stimulation promotes the neuronal death. Accordingly, activation of extrasynaptic NMDAR was accompanied with a depolarization of the mitochondrial membrane and the swelling of the cell body in cortical culture (Leveille et al., 2008), two hallmarks of excitotoxicity. Finally, extrasynaptic stimulation of NMDAR inactivated CREB pathway and consequently blocked the expression of brain-derived neurotrophic factor (BDNF), which contributes to neuronal survival (Alderson et al., 1990). On the contrary, synaptic NMDAR were mostly associated to a pro-survival function, as they activated the CREB and ERK pathways involved in cell survival (Hardingham et al., 2002; Ivanov et al., 2006; Leveille et al., 2008).

Non-ionotropic function of NMDAR

Intriguingly, it was proposed that NMDAR can have a role independently of the ion influx, so called non-ionotropic functions. One of the first studies proposing a non-ionotropic role for NMDAR was conducted by Alvarez and colleagues in 2007 (Alvarez et al., 2007). They showed that inhibiting NMDAR expression by shRNA GluN1 decreased spine density in hippocampal organotypic slices, whereas pharmacology blockade of NMDAR with D-APV was ineffective. Because D-APV inhibits ion influx, this result suggested that the physical presence of NMDAR but not the ionotropic function was required for spine stability. Since then, other studies have investigated the putative role of the non-ionotropic function of NMDAR. Nabavi et al. found that LTD induced by low frequency stimulation was blocked by the competitive NMDAR blocker D-APV but not by the open channel blocker MK-801 or the competitive antagonist of the co-agonist binding site 7-chlorokynurenate (7CK). Therefore, the authors hypothesized that the binding of the ligand to the glutamate binding site but not the ion channel flow was necessary to induce LTP. Moreover, LTD induction required basal free calcium in the cell but not calcium rise through the NMDAR, and involved p38 MAPK signaling. Of note, some of these results were challenged by Babiec et al., who found that MK-801 effectively blocked LTD induction in young and adult rats (Babiec et al., 2014). However, another study confirmed the involvement of non-

ionotropic functions of NMDAR in LTD (Stein et al., 2015). Indeed, the authors reported that 7CK did not block either the LTD or the shrinkage of spines, a process commonly associated with LTD (Nägerl et al., 2004). The spine shrinkage was abolished with a selective p38 MAK inhibitor and surprisingly, could be evoked by a LTP induction protocol (tetanic stimulation) in the presence of MK-801 to prevent NMDAR ion flow. Thus, these data suggest that the binding of glutamate to NMDAR could induce LTD independently of the ion channel flow, through p38 MAPK signaling. Since then, other studies reported the involvement of non-ionotropic functions of NMDAR in neuroprotection through glycine (Hu et al., 2016; Chen et al., 2017). Therefore, although this concept of non-ionotropic functions of NMDAR is still debated, growing evidence indicates that the signaling through NMDAR is more complex than previously thought. Interestingly, the recent discovery that its binding of NMDA to the binding site induced a conformational change of the NMDAR C-term in the presence of 7CK and MK-801 but not D-APV (Dore et al., 2015), and that this change altered interaction with kinases (Aow et al., 2015), opens the possibility that signaling pathways could be mediated independently of ion flow by receptor conformational changes.

c) Regulation by functional coupling with ion channels: focus on SK channels

NMDAR signaling is subjected to many regulatory process, from phosphorylation (Chen and Roche, 2007) to protein-protein interactions (Dingledine et al., 1999; Fan et al., 2014; Ladépêche et al., 2014). Among the family of membrane proteins, I will here described the modulation of NMDAR signaling by the SK channels. First, electron microscopy experiments detected that NMDAR and SK2/SK3 channels colocalize in the PSD of spines from hippocampal neurons (Lin et al., 2008; Ballesteros-Merino et al., 2014), indicating their close proximity in the neuronal membrane. Using two-photon imaging techniques combined with electrophysiology, Ngo-Anh et al. showed that blocking SK channels increased the excitatory synaptic responses in the hippocampus, by enhancing the amplitudes of NMDAR-mediated synaptic responses and calcium influx (Ngo-Anh et al., 2005). From these data, the authors concluded that synaptic activation evoked calcium entry activating SK channels, which in response, reduced both synaptic responses and calcium influx through NMDAR. Other authors found a similar “shunt” of NMDAR-mediated synaptic responses mediated by SK channel activation in the ventral hippocampus (Babiec et al., 2017), the amygdala (Faber et al., 2005), and the cortex (Faber, 2010). Besides, pharmacological inhibition of SK channel activation promoted LTP induction in the hippocampus (Babiec et al., 2017) and increased its intensity in the amygdala (Faber et al., 2005). On the contrary, genetic overexpression of SK2 channels enhanced the restriction of the excitatory synaptic currents and reduced the induction of LTP in the hippocampus (Hammond, 2006). Therefore, by regulating NMDAR function, SK channels can modulate the synaptic plasticity. Furthermore, NMDAR

also regulate the activity of SK channels. Indeed, enhancing NMDAR activity by reducing magnesium concentration amplify the responses of SK channels to the blocker apamin (Faber et al., 2005; Ngo-Anh et al., 2005; Hammond, 2006), consistent with an increased activation of SK channels. Whereas calcium influx though NMDAR is sufficient to activate SK channels and the effects they mediate in the amygdala (Ngo-Anh et al., 2005) and the hippocampus (Faber et al., 2005), multiple sources of calcium contribute to SK channel activation in the cortex (Faber, 2010). In conclusion, SK channels finely regulate the excitatory responses mediated by NMDAR, which in turn modulates the activation of these channels.

Given the role of NMDAR in synaptic plasticity, one could expect that the modulation mediated by SK channels is accompanied with behavioral outcomes. It seems to be the case, as Hammond et al. demonstrated that transgenic mice overexpressing SK2 channels, which exhibited reduced hippocampal LTP, had impairments of hippocampal-dependent learning and memory in the Morris water maze and a contextual fear conditioning (Hammond, 2006). In line with these findings, blockade of SK channels facilitated the LTP induction in the hippocampus and accelerated spatial and non-spatial learning (Stackman et al., 2002).

3. Surface expression of NMDAR

a) Synthesis of NMDAR in the endoplasmic reticulum

The obligatory GluN1 subunits being expressed in excess compared to the others, it is thought that NMDAR expression highly depends on the amount of GluN2 subunits. Overexpression of GluN2A or GluN2B but not GluN1 subunits increased the number of functional NMDAR in cerebellar and cortical cell cultures (Prybylowski et al., 2002). Once the subunits are translated into proteins, they assemble into tetramers in the endoplasmic reticulum (ER). Monomeric or uncompleted assembled NMDAR are retained in the ER (McIlhinney et al., 1998; Fukaya et al., 2003) and thus, cannot be delivered to cell membrane. The correct assembly into tetramers appears crucial to overcome the ER retention, which serves as a quality control mechanism to guarantee the production of functional NMDAR in the neuronal membrane. Indeed, it is currently hypothesized that the assembly between GluN1 and GluN2 subunits mask retention signals, enabling the exit of the heterotetrameric receptors from the ER (Qiu et al., 2009). Several retention signals have been identified so far. Qiu et al highlighted the presence of a retention signal located in the N-terminal domain of GluN2A, which is silenced by the association with the N-terminal domain of GluN1 subunits (Qiu et al., 2009). Interestingly, this retention signal was not found in the N-terminal domain of GluN2B subunit, indicating that the exit of GluN2A and GluN2B-containing NMDAR from the ER may be differentlialy regulated. In line with this idea, another retention signal located in the M3 transmembrane domains of the GluN1 and GluN2B

subunits has been identified by Horak et al. (Horak et al., 2008). This signal can be masked by the assembly of these M3 domains together with the M4 domain of GluN1. However, other mechanisms seem to regulate the exit of NMDAR from the ER. It has been shown that a retention signal in the C-terminal tail of the GluN1 subunits can be overcome by protein kinase-mediated phosphorylation on specific serine residues (Scott et al., 2003). Moreover, Mu et al pointed out that the export of NMDAR from the ER could be accelerated by modifications of GluN1 mRNA splicing in response to activity blockade, suggesting that the activity also influences the NMDAR secretory pathway (Mu et al., 2003). Besides, GluN2B subunit mutants with a reduced affinity for glutamate exhibited a decreased expression in the Golgi apparatus and the neuronal membrane, implying that the glutamate binding is required for the forward trafficking of NMDAR to the cell surface. Therefore, these data revealed that NMDAR early trafficking is also modulated by the agonist binding (She et al., 2012).

b) Membrane addressing of NMDAR

Once released from the ER, assembled NMDAR are directed to the somatic Golgi apparatus, where they undergo post translational modifications like palmitoylations (Hayashi et al., 2009) and then, reach the membrane by vesicle exocytosis. However, the work of Jeyifous et al. has challenged the idea that NMDAR would only traffic through this classical secretory pathway. Indeed, the authors observed that preventing the formation of intracellular vesicles induced an accumulation of AMPAR but not NMDAR in the somatic Golgi. Instead, an increase of these receptors in the Golgi membranes located in the dendrites was reported, suggesting that unlike AMPAR, NMDAR trafficking could bypass the somatic Golgi and use an atypical secretory pathway through dendritic Golgi membranes (Jeyifous et al., 2009).

After their export form the ER, NMDAR are trafficked toward different cell compartments through intracellular vesicles. These receptors are not exported alone but are associated with protein complexes along their intracellular trafficking toward the plasma membrane (**Figure 11**). Among these proteins, SAP102 and SAP-97, two synaptic scaffold proteins from the PDZ family, seem to play a major role in membrane delivery. After exiting from ER, GluN2B subunits associate with SAP102, enabling the interaction with Sec8 protein which belongs to the exocyst complex (Sans et al., 2003). This complex, composed of 8 proteins, drive vesicles to the plasma membrane and thus, enables the correct delivery of transmembrane receptors. Altering the interaction between Sec8 and the GluN1/GluN2B-SAP102 complex results in a decreased expression of membrane receptors (Sans et al., 2003). In addition, immunohistochemistry combined with immunoprecipitation techniques revealed that SAP97 associates with NMDAR in ER-derived vesicles and is absolutely necessary for the transport of

receptors through the atypical secretory pathway described earlier (Jeyifous et al., 2009). Up to now, the export of GluN2A-containing NMDAR has been less investigated. It was shown that GluN2A and SAP97 colocalized in the ER in hippocampal neurons and that the phosphorylation of SAP-97 in specific positions regulate GluN2A-containing NMDAR membrane insertion (Mauceri et al., 2007), supporting the model that GluN2A-containing NMDAR traffic required the same partners. In conclusion, NMDAR transport requires the association with PDZ-domain proteins in the early steps of intracellular trafficking for their correct membrane delivery.

Once intracellular vesicles containing NMDAR and their partners are formed, they are transported along the microtubule network (**Figure 11**). It is known that motor proteins such as kinesin superfamily proteins (KIFs) are able to carry vesicles and move them along the microtubules (Hirokawa, 1998). Setou et al. provided the first evidence that NMDAR-containing vesicles could be also transported via these proteins. Indeed, the authors showed that GluN2B subunits interact with KIF17 via a protein complex including a PDZ-domain protein called mLin-10 (Setou et al., 2000). The proposed interaction was supported by the combination of live and fixed immunostaining studies on hippocampal neuron cultures showing colocalizations between these 3 partners (Guillaud et al., 2003). This interaction between KIF17 and GluN2B-containing NMDAR appears crucial for the surface distribution of these receptors. Generation of transgenic mice overexpressing KIF17 results in an increased expression of GluN2B subunits (Wong et al., 2002). On the contrary, inhibiting KIF17 by using dominant –negative mutants or antisense oligonucleotides induced a decrease in the GluN2B subunit expression (Guillaud et al., 2003). Moreover, a recent study showed that septin9, a member of the septin protein family involved in microtubule-dependent transport, physically interfered with the binding of mLin10 to KIF17 by competing with mLin10 to bind KIF17 (Bai and Karasmanis, 2016). By doing so, septin9 down-regulated the GluN2B subunit surface expression in hippocampal cultures. This confirms the predominant role of KIF17 in GluN2B-containing NMDAR transport. Another kinesin protein KIF1bα has been shown to interact with several MAGUK proteins like PSD-95 and SAP97 (Mok et al., 2002), and thus, could also be involved in the transport of NMDAR-carrying vesicles along the microtubules.

c) Reaching the synaptic compartment

To incorporate NMDAR at the surface of neurons, the vesicular membrane containing NMDAR needs to fuse with the neuronal membrane. This mechanism is mediated by the SNARE complex. By imaging NMDAR exocytosis with total internal reflection fluorescence (TIRF) that enables to visualize membrane events, Gu and Huganir identified several components of this SNARE complex (Gu and

Huganir, 2016). Knocking down SNAP-25, VAMP-1, and syntaxin4 proteins by interfering RNA reduces surface expression of GluN1 subunits in the synaptic and extrasynaptic compartments, indicating that these proteins regulate the surface content of NMDAR. Intriguingly, the authors noted that most of the exocytotic events occurred in the extrasynaptic compartment. This observation is in line with previous evidence suggesting that NMDAR delivery would preferentially occur outside synapses. Indeed, Guillaud et al did not find any colocalization of KIF17 with GluN2B and PSD-95 or with GluN2B and synaptophysin (a presynaptic marker of glutamatergic synapse) whereas KIF17 and GluN2B clearly colocalize in the dendritic shaft (Guillaud et al., 2003). The absence of KIF17 and GluN2B in synapses clearly revealed that kinesin proteins carrying NMDAR do not enter directly into glutamatergic synapses. Moreover, Prybylowski and colleagues reported that overexpression of GluN2 subunits increased the number of NMDAR clusters but decreased colocalization with the synaptic marker synaptophysin (Prybylowski et al., 2002). From these data, it can be hypothesized that newly formed NMDAR are delivered in the extrasynaptic part of the membrane. As a consequence, other mechanisms are needed for receptors to reach synapses. Super resolution techniques have deciphered that one key regulator of NMDAR distribution is the lateral diffusion of receptors along the membrane (Groc et al., 2009), which enables receptors to enter or exit synapses.

d) Endocytosis

Similarly to AMPAR (Carroll et al., 1999), several studies indicated that NMDAR are endocytosed by a clathrin-mediated mechanism. Roche et al. identified on the C-terminal of GluN2B subunits a consensus internalization motif involved in clathrin-dependent endocytosis (Roche et al., 2001). This motif enables the binding of AP-2 adaptors that link internalized cargo to clathrin. As this motif is in close proximity to the PDZ-binding domain of GluN2B subunit, the authors discovered that PSD-95, a synaptic scaffold protein which binds to the PDZ-binding domain, can inhibit NMDAR internalization and probably stabilize these receptors at the synapses (Roche et al., 2001). As many other biological processes, the internalization of receptors is regulated by several factors. In this same study, the authors observed that the rate of NMDAR endocytosis is decreased as neurons mature. This in accordance with the study of Petralia et al. who found that clathrin-coated pits were commonly encountered at glutamatergic synapses at early postnatal days but rarely in adults (Petralia et al., 2003). Besides being regulated during development, NMDAR internalization is also modulated by its co-agonist. Stimulation of the glycine co-agonist site “primes” NMDAR for clathrin-mediated endocytosis. It also requires activation of the agonist site, as glycine application alone (without NMDA agonist) do not promote NMDAR internalization in hippocampal neurons (Nong et al., 2003).

Interestingly, co-labelling of NMDAR and clathrin-coated pits by immunogold particules with electron microscopy was found in extrasynaptic sites but not within synapses (Petralia et al., 2003). In addition, Blanpied et al. reported that endocytosis occurs in specialized zones located in the vicinity of the postsynaptic density in mature neurons (Blanpied et al., 2002). These data and the others reported in the previous section strongly indicate that NMDAR endocytosis and exocytosis would take place in dedicated areas located outside synapses.

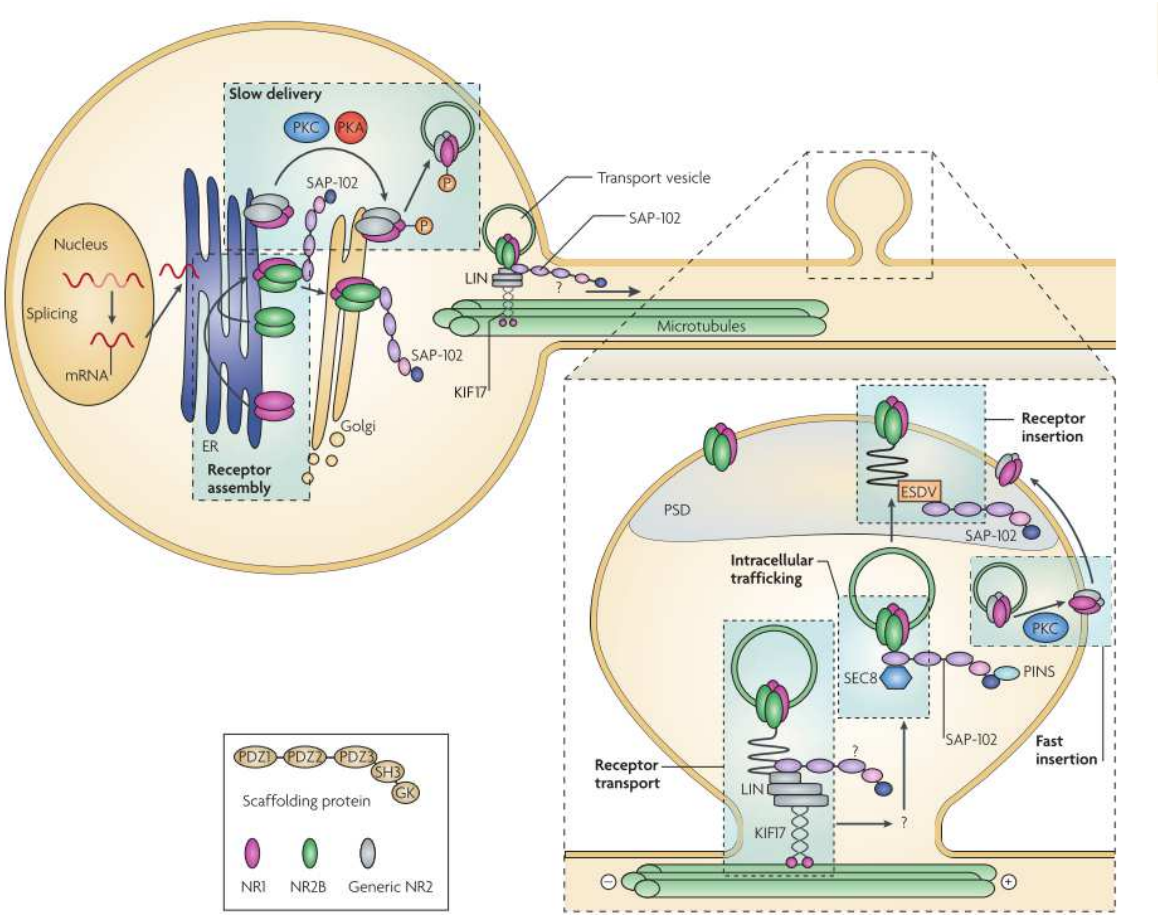


Figure 11. Schema of NMDAR assembly and trafficking to spines (adapted from Lau and Zukin, 2007). NMDAR subunits are translated and assembled into tetramers in the endoplasmic reticulum. Once the tetramer is formed, NMDAR is transported with PDZ-domain proteins in the Golgi apparatus. The vesicles containing NMDAR are carried by kinesin proteins along the microtubule. Finally, newly formed NMDAR are mostly delivered outside synapses, in dedicated places.

4. Role and regulation of NMDAR surface trafficking

The concept of lateral diffusion of receptors started 40 years ago when Axelrod et al. used fluorescence imaging techniques to measure the mobility of surface acetylcholine receptors in muscles (Axelrod et al., 1976b). But it was not until 2002 that the lateral diffusion of NMDAR was demonstrated

in living neurons (Tovar and Westbrook, 2002). Since then, various techniques have been successfully used to characterize NMDAR surface dynamics in neurons (Groc et al., 2009). These techniques can be subdivided in 2 categories: a) ensemble methods measuring the average diffusion of population of receptors and b) single-molecule detection techniques which enable to track individual receptors over time.

a) Measure of NMDAR lateral mobility

a.1) Ensemble methods

Electrophysiology

One of the first hints that NMDAR diffuse within the membrane was provided by electrophysiological approaches combined with the NMDAR blocker MK-801. As MK-801 is an irreversible open channel blocker (Huettner, 1988), it enables to irreversibly tag and block synaptic (opened) NMDAR. After blockade of NMDAR-mediated synaptic responses with MK-801 and subsequent washout, a recovery of responses was observed after several minutes (**Figure 12**) (Tovar and Westbrook, 2002). This recovery being absent when all surface NMDAR were blocked by co-application of NMDA and MK-801, it could not be attributed to the insertion of new NMDAR within the membrane. Instead, the authors suggest that NMDAR move laterally and contribute to the recovery of responses when entering synapses. As far as 65% of synaptic NMDAR were exchanged within 7 minutes, showing that a large portion of NMDAR are mobile in young hippocampal neurons. Using a similar approach, Harris and Pettit did not find any recovery of the NMDAR synaptic currents after synaptic blockade with MK-801 and washout (Harris and Pettit, 2007). Such a discrepancy could be due to differences in the brain preparation (hippocampal culture *versus* hippocampal slice) or the developmental age of the neurons (1-week *versus* 2-3 week old).

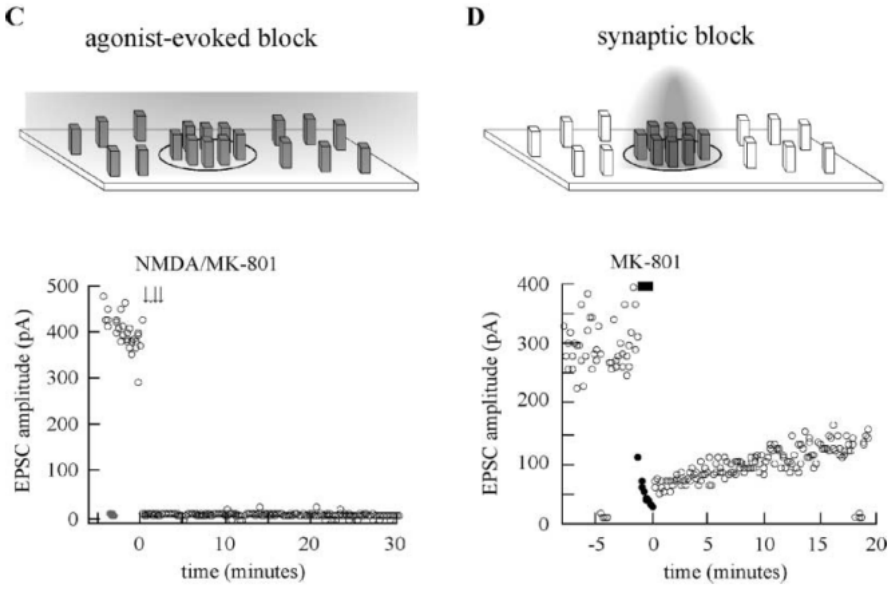


Figure 12. NMDAR-mediated synaptic currents recovered following the blockade of synaptic NMDAR but not total surface receptors (adapted from Tovar and Westbrook, 2002). Left, Following bath application of NMDA and MK-801 to block all membrane NMDAR, no recovery of the NMDAR-EPSC was observed. Right, After synaptic stimulation in the presence of MK-801, recovery of the NMDAR-mediated currents was observed after few minutes of wash. It was hypothesized that the recovery is due to the recruitment of extrasynaptic membrane NMDAR to synapses.

Fluorescence recovery after photobleaching (FRAP)

The FRAP technique was developed by Axelrod and Webb in the 70's (Axelrod et al., 1976a). It consists to bleach (or "switch-off") fluorescent molecules in a specific area and to observe the recovery of the fluorescence in the same area. As the photobleaching is an irreversible process, the recovery is caused by the entry of fluorescent molecules inside the "bleached" area and thus, can provide information about the diffusion of a population of receptors. In fact, two parameters can be extracted from the recovery curves: the coefficient of diffusion of the molecules and the extent of recovery at the end of the experiment, which characterizes the mobile fraction of molecules. The first FRAP experiment on NMDAR was done by labelling the receptors with conotoxin (a NMDAR blocker) bound to tetramethylrhodamine on cortical neuronal cultures (Benke et al., 1993). However, because the neurons were incubated during 1h, a time lapse sufficient to induce NMDAR endocytosis (Roche et al., 2001), it is unlikely that the NMDAR mobility was characterized in a pure population of surface NMDAR, but rather involved the contribution of internalized NMDAR. Taking advantage of the Super Ecliptic Fluorin (SEP), which is a pH-sensitive derivative of GFP that fluoresces at extracellular pH but not in acid intracellular compartments (Ashby et al., 2004), Bard et al. characterized the surface diffusion of NMDAR in hippocampal neurons (Bard et al., 2010). The recovery of fluorescence reaches 50% in dendrites and 20% in synapses, meaning that most synaptic NMDAR (80%) are immobile. Accordingly,

Sharma et al. found an almost similar fraction (70%) of immobile synaptic NMDAR in neuronal cultures (Sharma et al., 2006).

a.2) Single particle techniques

High resolution techniques : Quantum dots

Quantum dots (QD) (10-30 nm) are passive nanoparticules composed of an inorganic semiconductor crystal, coated and functionalized for biological applications (Groc et al., 2007b). Compared to single dyes, QD exhibit several advantages. First, they are brighter, so that they can be excited by a mercury lamp and they are much more photostable, allowing to record longer trajectories of receptors. In addition, they are subjected to blinking, a random alternance between “on” and “off” states of fluorescence, which provides a criterion to identify individual QD (Michalet et al., 2005). Because of their high signal-to-noise ratio, fluorescence signals of a single QD can be fitted with a two-dimensional Gaussian function as it acts as a single point emission. This allows to identify the centroid of the signal with a pointing accuracy of 5-20 nm (Groc et al., 2007b), which is well below the diffraction limited resolution of a typical wide-field fluorescence microscopy (250 nm). Therefore, membrane receptors can be tracked during several seconds by single QD coupled to primary antibodies against an extracellular part of the receptors (**Figure 13**). From the reconstructed trajectories, several parameters can be extracted (Triller and Choquet, 2008):

1) The mean square displacement (MSD) over time, $MSD(t) = \langle r^2 \rangle (t) = 4Dr$, with D the instantaneous diffusion coefficient (**Figure 13**). As it is the mean area explored by receptors over time, MSD reflects the diffusion behaviour of the receptors. In the case of Brownian diffusion, the MSD plot is linear whereas it tends to a plateau when the diffusion is confined.

2) The instantaneous coefficient of diffusion (D) ($\mu m^2/s$), estimated from the linear fit of the first four points of the MSD plot over time, represents the speed of the receptors within the plasma membrane

3) The immobile fraction, estimated by the first point of the cumulative distribution of the diffusion coefficient, characterizes the proportion of immobile receptors.

Although QD display several advantages, they are bulky and bigger than single dyes. Because the total size of the QD-antibody-receptor can reach a size of 30 nm, it may influence the diffusion properties in restricted areas, such as synapses. Accordingly, receptors followed by QD were less localized in synapses than when tracked by single dyes (Groc et al., 2007b). However, receptors coupled to QD still enter the synapses (Dahan, 2003). In addition, the diffusion coefficient of mobile receptors and the immobile fraction were not different between receptors tracked by QD and single

dyes (Groc et al., 2007b). Therefore, QD tracking enables to follow membrane receptors in the synaptic and extrasynaptic compartments, and the recent development of smaller probes should allow a better access to synapses (Chamma et al., 2016).

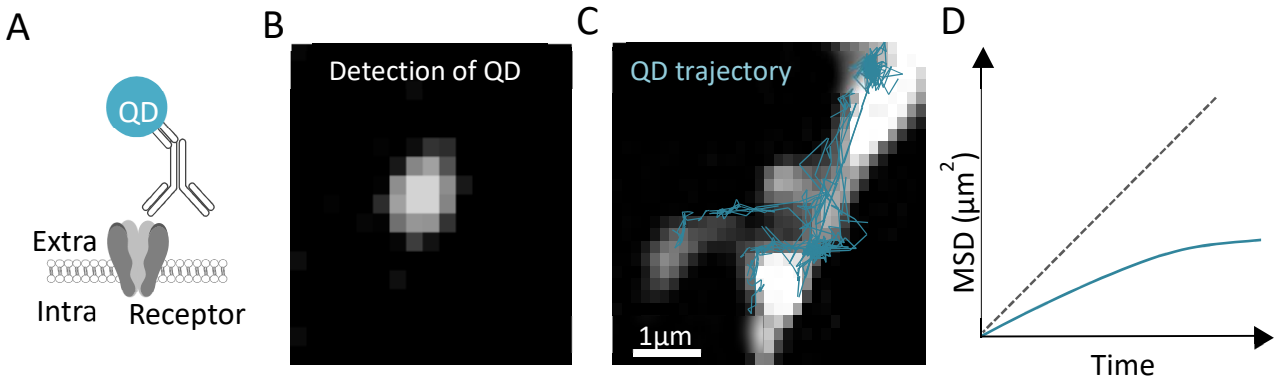


Figure 13. Detection and tracking of single receptors with quantum dots (QD) imaging. A) Schematic representation of a receptor targeted by a QD-primary antibody (Ab) complex in the extracellular compartment. B) Single QD-Ab complexes are detected and tracked by imaging 500 frames at 20 Hz. C) Trajectories of single QD-Ab complexes collected over 500 frames are reconnected with a dedicated multi-dimensional image analysis software. To take into account the blinking of the tracked QD, the software allows to reconnect trajectories constructed during the non-blinking states. D) Plot of mean square displacement (MSD) over time for a receptor trajectory (blue curve). Compared to the behaviour of a freely diffusing molecule (dotted grey line), receptors display a more confined behaviour, due to the alternance of diffusion in the extrasynapic sites and stabilization in the synapses.

Super resolution techniques

To decipher the localization of molecules with a nanometer spatial resolution, several super resolution methods based on image reconstruction such as the photoactivatable localization microscopy (PALM) (Betzig et al., 2006) or the points-accumulation-for-imaging-in-nanoscale-topography (PAINT) (Sharonov and Hochstrasser, 2006) have been developed and give access to the localization of a high number of individual molecules. Using total internal reflection fluorescence (TIRF) that enables to illuminate the cell surface, single particle tracking (spt) of membrane proteins was obtained at high density by sptPALM with a high spatial resolution in living cells (Manley et al., 2008). Besides, an optimized version of PAINT, called uPAINT, was successfully used to study the diffusion of endogenous glutamate AMPA receptors on living neurons (Giannone et al., 2010). By imaging diffusing proteins at high density with a nanometer resolution, sptPALM and uPAINT appear as powerful techniques to better understand the diffusing behaviour of membrane receptors in living neurons.

b) Role of NMDAR surface trafficking

Since the discovery that NMDAR diffuse laterally within the plasma membrane, several studies have investigated their putative functional role. As surface trafficking regulates the membrane distribution of the receptor, it seems that the surface dynamics contributes to several processes involving a change in NMDAR membrane localization.

Role in synaptic maturation during development

It is well established that NMDAR subunits composition change over development. Indeed, *in situ* hybridizations showed that the GluN2B subunit is predominant in the early development in several structures whereas, after the second postnatal week, GluN2A predominates (Monyer et al., 1994). Using electrophysiological approaches, Carmignoto and Vicini observed that the NMDAR-EPSC decay time was longer in cortical neurons from young rats than from adults (Carmignoto and Vicini, 1992), consistent with a developmental change in NMDAR subunit composition occurring in the brain. A similar switch was reported in hippocampal neurons, with NMDAR-EPSC decay time becoming faster with age (Kirson and Yaari, 1996; Bellone and Nicoll, 2007). Besides, NMDAR-mediated synaptic currents were highly sensitive to the GluN2B specific antagonist ifenprodil at early developmental stages in hippocampus slices, and weakly sensitive after 2-3 postnatal weeks (Bellone and Nicoll, 2007). Accordingly, western blot analysis revealed that the GluN2A subunit level was dramatically increased after 2 weeks *in vitro* in hippocampal neurons and in the cortex of PD 15 rats (Sheng et al., 1994; Ferreira et al., 2015). Thus, these data indicate that synaptic GluN2B- are replaced with GluN2A-containing NMDAR during postnatal development in several brain regions. Remarkably, this developmental switch of NMDAR subunit composition is influenced by sensory experience and activity. Visual deprivation by rearing rats in the dark enhanced the sensitivity to ifenprodil while decreasing the NMDAR-mediated EPSC decay kinetics in the cortex, congruous with a decreased GluN2A/GluN2B subunits ratio (Philpot et al., 2001). On the contrary, visual experience decreased the proportion of GluN2B-containing NMDAR, showing that this effect is reversible (Philpot et al., 2001). Blocking NMDAR activity with D-APV prevented the switch from GluN2B to GluB2A subunits (Matta et al., 2011) and therefore, confirms the activity-dependent regulation of the NMDAR composition switch. Interestingly, this process was independent of protein synthesis (Matta et al., 2011), possibly suggesting that a redistribution of surface NMDAR subunits could occur through lateral diffusion. In line with this hypothesis, Groc et al. showed by single particle tracking that the developmental switch of synaptic NMDAR composition occurring during the first two postnatal weeks in culture was correlated with a developmental change of the time spent by the GluN2 subunits within the synapses (Groc et al., 2007a). The residence time (time spend within the synapses) of GluN2B-contaning NMDAR

was drastically decreased by a factor 3 between 8 and 15 days and became inferior to the resident time of GluN2A-containing NMDAR. From these data, it emerges that the developmental switch of NMDAR composition results from the differential stabilization of surface GluN2B and GluN2A subunits within synapses, with a higher stabilization of synaptic GluN2B and GluN2A subunits at 8 and 15 days in culture respectively. What could be the factors impacting the synaptic stabilization of NMDAR? One candidate is the extracellular matrix protein reelin. Using a combination of single particle tracking and electrophysiology in hippocampal culture, Groc et al. demonstrated that reelin contributed to the developmental maturation of excitatory synapses by regulating the lateral diffusion of GluN2B subunits and their time spent in synapses (Groc et al., 2007a). Recent evidence also highlighted that NMDAR co-agonists D-serine and glycine regulate the surface trafficking and the synaptic content of NMDAR subunits. Since D-serine and glycine availability is regulated during the postnatal development (Le Bail et al., 2015), it was proposed that the NMDAR co-agonists modulate the developmental switch of NMDAR subunits (Ferreira et al., 2017).

Role in synaptic plasticity

Although the contribution of NMDAR to synaptic plasticity (LTD and LTP) is well established, the involvement of NMDAR subunits and the relative change in NMDAR signaling following plasticity is less clear. Regarding LTP, the prevailing view was that GluN2A is the predominant subunit required for its expression. Indeed, LTP induced by tetanic stimulation was reduced in the hippocampus of GluN2A knock-out mice (Sakimura et al., 1995) and in mice expressing a C-terminal truncated form of GluN2A subunit (Sprengel et al., 1998). Pharmacological approaches using GluN2A preferential blockers confirmed these findings (Liu et al., 2004; Papouin et al., 2012). Contrary to Liu et al. who found that blocking GluN2B subunit did not impair LTP, evidence for the contribution of GluN2B subunit in LTP was provided by others (Bartlett et al., 2007; von Engelhardt et al., 2008). For instance, blocking GluN2B containing NMDAR by ifenprodil or replacing synaptic GluN2B with GluN2A subunits by overexpression of GluN1/GluN2A subunits significantly decreased the extent of LTP in hippocampal organotypic slices (Barria and Malinow, 2005). In view of these data, the relative contribution of GluN2A and GluN2B subunits is still not fully understood but both subunits seem to be required for LTP. Besides, NMDAR subunit composition is changed following synaptic potentiation. Therefore, it seems that both GluN2A and GluN2B subunits are involved in LTP. Besides, NMDAR undergo changes in the subunit composition following synaptic potentiation. After LTP induction in hippocampal slices from young rats, the NMDAR-EPSC were less affected by the GluN2B blocker ifenprodil and exhibited faster decay kinetics (Bellone and Nicoll, 2007; Matta et al., 2011), which is characteristic of a loss of GluN2B subunits in the synapses and the insertion of GluN2A subunits. This switch was reversible by a depotentiation protocol and was absent after a LTP induction protocol in mature slices (Bellone and

Nicoll, 2007), suggesting that this form of plasticity was specifically expressed in immature synapses, when Glu2B-containing NMDAR predominate in synapses. Other forms of plasticity also involved a change in surface distribution of NMDAR. For instance, synaptic plasticity induced by cocaine exposure drives the insertion of GluN2B- and GluN3A-containing NMDAR in excitatory synapses from VTA DA neurons (Yuan et al., 2013). Moreover, Grosshans et al. could elicit a synaptic potentiation that required insertion of membrane GluN2A subunits In hippocampal slices from adults rats (Grosshans et al., 2002). Remarkably, although both GluN2A and GluN2B subunits likely contribute to LTP, several lines of evidence point to the predominant role of the synaptic distribution of GluN2B subunits in its induction. Transgenic rats overexpressing GluN2B subunits in the cortex and hippocampus exhibited enhanced LTP in the hippocampus (Wang, 2009), suggesting that the increased expression of synaptic GluN2B could promote LTP. Another study tackled directly the role of synaptic GluN2B-contaning NMDAR on LTP by disrupting the interaction between the synaptic anchoring protein PSD-95 and GluN2B subunits with a competing peptide (Gardoni et al., 2009). Treatment of hippocampal slices with this peptide specifically reduced synaptic GluN2B-containing NMDAR, and was accompanied with a reduction of LTP. Thus, these data highlight the changes in the NMDAR signaling following plasticity, although lacking direct evidence for such physical changes in receptors.

To tackle this point, Dupuis et al. used single molecule imaging techniques to track the diffusion of GluN2A and GluN2B subunits following LTP (Dupuis et al., 2014). The authors showed that LTP induction was associated with an increased lateral diffusion of GluN2B subunits while GluN2A trafficking remained unaffected in immature hippocampal neurons. Remarkably, GluN2B subunits were displaced out of the synaptic and perisynaptic compartments. To further assess the role of GluN2B dynamics in synaptic plasticity, NMDAR surface trafficking was immobilized with a cross-link protocol, which consists of incubating neurons with primary GluN1 antibodies targeting the extracellular part of NMDAR and secondary antibodies (Dupuis et al., 2014). This protocol had the advantage of impairing specifically NMDAR surface trafficking without altering their function. Surface NMDAR cross-link prevented LTP induction by altering the redistribution of CAMKII into spines. As GluN2B subunit and CAMKII directly interact (Leonard et al., 1999; Barria and Malinow, 2005), these data support the view that the increased NMDAR surface trafficking during synaptic stimulation contributes to the synaptic translocation of CAMKII into the spines, which promotes LTP. Of interest, a similar approach of surface NMDAR cross-link was used *in vivo* in adults rats (Potier et al., 2015). Direct injection of GluN1 antibodies in the hippocampus blocked LTP and was accompanied by impairments of associative memory. Taken together, these data suggest that, beyond NMDAR activation, the surface trafficking of NMDAR *per se* is involved in LTP and the acquisition of associative memory.

Regarding LTD, fewer studies are available and give conflicted results about the role of NMDAR subunits. Selective blockade of GluN2B-containing NMDAR abolished the induction of LTP in hippocampal slices from young rats (Liu et al., 2004). Similarly, i.p injection of a selective GluN2B subunit antagonist fully blocked hippocampal LTD *in vivo* (Fox et al., 2006). By contrast, overexpression of GluN2B subunits in forebrain structures, which up-regulated the contribution of synaptic GluN2B, failed to affect LTD induced by two different protocols in hippocampal slices (Tang et al., 1999). Bartlett et al. also found that LTD was unaffected by a GluNB2 selective antagonist, but was blocked in presence of a GluN2A selective antagonist (Bartlett et al., 2007). Such discrepancies between studies could result from differences in the preparation (slice or *in vivo*), the age of animals or the induction protocol used. Although no study has investigated the role of NMDAR surface diffusion in LTD, several hints suggest that the mobility of NMDAR could be involved as well. Peng et al. elicited an LTD of the NMDAR-EPSC in hippocampal slices that was unaffected by exocytosis and endocytosis inhibitors (Peng et al., 2010). In addition, this LTD increased the NMDAR-EPSC decay kinetics and the contribution of ifenprodil-sensitive synaptic currents; and was blocked when actin dynamics was altered by pharmacology. Given that the actin cytoskeleton contributes to the synaptic anchoring of NMDAR (Allison et al., 1998), these data support a model in which LTD induces a redistribution of surface GluN2B-containing NMDAR into synapses by lateral diffusion within the membrane.

In conclusion, it is challenging to determine the precise role of NMDAR subunits in LTP and/or LTD. However, these studies shed a new light on the contribution of NMDAR subunits, by showing that their membrane dynamics could be a common mechanism to regulate both LTP and LTD.

c) Modulators of NMDAR surface trafficking

NMDAR lateral mobility is regulated by several proteins. These proteins can be in the extracellular environment, intracellular partners or receptors anchored in the plasma membrane.

Extracellular interactors

Proteins of the extracellular matrix: example of Reelin. Reelin is a glycoprotein from the extracellular matrix. This protein modulates the glutamatergic transmission, as application of reelin enhanced both NMDAR-mediated synaptic transmission and LTP in hippocampal neurons (Qiu et al., 2006). Groc et al. further investigated the molecular mechanisms underlying the modulation of NMDAR by reelin in culture. Inhibiting reelin function prevented the decrease of GluN2B subunits-mediated synaptic currents occurring during development and was correlated with a greater stabilization of GluN2B subunits in synapses. On the reverse, chronic treatment with reelin

dramatically reduced the time spent by GluN2B subunits in immature synapses and suppressed their contribution to the NMDAR-EPSC in immature neurons. Remarkably, the developmental switch of synaptic GluN2B to GluN2A subunits occurring between 8 and 10-12 days in culture was concomitant with an upregulation of reelin within synapses (Groc et al., 2007a). Thus, reelin modulates NMDAR surface trafficking and thereby, likely contributes to the maturation of excitatory synapses in neurons.

NMDAR co-agonists. One of the major hints arguing that NMDAR co-agonists could modulate the receptor surface dynamics comes from the work of Papouin et al., who showed that glycine and D-serine co-agonists differentially regulated the surface trafficking of GluN2A- and GluN2B-contaning NMDAR in the hippocampus (Papouin et al., 2012). GluN2A subunit-mediated currents were slowed down by exogenous application of glycine but unaffected by D-serine. On the reverse, the diffusion of GluN2B subunits was reduced following D-serine but not changed by glycine. By further investigating the mechanisms involved in the modulation of NMDAR trafficking by co-agonists, Ferreira et al. found that application of D-serine reduced the synaptic content of GluN2B subunits by altering the binding of NMDAR with the synaptic anchoring protein PSD-95 (Ferreira et al., 2017). Fluorescence resonance energy transfer (FRET) revealed that this alteration resulted from a conformational change of the C-terminal of NMDAR (Ferreira et al., 2017), which is known to regulate the binding of several intracellular proteins like PSD-95 (Bard et al., 2010). Since the developmental switch of GluN2A and GluN2B subunits occurring during the development in the hippocampus is paralleled by a change in the co-agonist levels (Le Bail et al., 2015; Ferreira et al., 2017), it is proposed that the co-agonist availability may underlie the maturation of excitatory synapses during development by regulating NMDAR membrane dynamics.

Intracellular proteins

MAGUKs (membrane-associated guanylate kinases). MAGUKs are a superfamily of proteins with PDZ-binding domains, which accumulate in the PSD. Among them, PSD-95, PSD-93, SAP-102 and SAP-97 participate to the regulation of NMDAR trafficking. Triple knock down of PSD-95, PSD-93 and SAP-102 reduced the NMDAR-mediated synaptic transmission in hippocampal neurons and was accompanied by a decrease in the number of NMDAR assessed by electron microscopy tomography (Chen et al., 2015), indicating that MAGUK proteins contributed to the stabilization of NMDAR in synapses. Accumulating evidence converge to the view that this stabilization is mediated by the interaction of the MAGUK proteins with the C-terminal of GluN2 subunits. Overexpression of C-terminal mutants of GluN2B subunits increased the total number of NMDAR at the surface without changing NMDAR synaptic current, indicating the incapacity of C-term mutants NMDAR to enter the synapses (Prybylowski et al., 2002). Furthermore, Gardoni et al. found that application of a permeable

peptide mimicking the last amino-acids of the C-term of GluN2B (TAT-2B) disrupted the interaction with PSD-95, SAP-102 and SP-97, and redistributed GluN2B-containing NMDAR outside synapses (Gardoni et al., 2006). Regarding GluN2A subunits, similar interactions with MAGUK proteins seem to regulate the synaptic retention of NMDAR. GluN2A subunits with a truncated C-term were less concentrated in synapses than the full-length wt, and decreased the NMDAR-mediated synaptic currents (Steigerwald et al., 2000). As extrasynaptic NMDAR were not affected by the truncated form of GluN2A, this indicated that the C-term was required to enter synapses. Using combined single particle tracking and co-immunoprecipitations, it was shown that the presence of a divalent peptide mimicking the last amino-acids of the C-terminal of GluN2A subunits increased the surface diffusion of synaptic GluN2A subunits by disturbing the interaction between NMDAR and PSD-95 in hippocampal neurons (Bard et al., 2010). As a consequence, GluN2A subunits were displaced out of synapses; and as the kinetics of the NMDAR-EPSC were slower and more sensitive to the GluN2B blocker ifenprodil in the presence of the biomimetic peptide (Bard et al., 2010), it indicated a compensatory insertion of synaptic GluN2B-containing NMDAR. Therefore, MAGUK proteins modulate the trafficking of NMDAR by contributing to their synaptic anchoring.

CAM kinase II (calcium/calmodulin-dependent protein kinase II). CAMKII is a kinase protein involved in a multitude of biological processes including LTP (Lisman et al., 2012). Following NMDAR activation, the increase of intracellular calcium activates calmodulin, which in turn activates CAMKII and promotes its autophosphorylation (Shen and Meyer, 1999). Once activated, CAMKII is translocated into the PSD (Shen and Meyer, 1999), which favors its binding with NMDAR (Leonard et al., 1999). Besides, the interaction between CAMKII and the C-terminal of GluN2B subunits locked the kinase in an active conformation, enabling to sustain CAMKII activity without calmodulin (Bayer et al., 2001), thereby enabling LTP (Barria and Malinow, 2005). Notably, this effect is specific to GluN2B subunits, as GluN2A subunits weakly interact with CAMKII (Leonard et al., 1999; Barria and Malinow, 2005). Application of CAMKII blockers strongly reduced the surface dynamics of synaptic GluN2B while it did not change the diffusion of synaptic GluN2A subunits in hippocampal culture (Dupuis et al., 2014), consistent with a negligible binding between GluN2A subunits and CAMKII. Moreover, the expression of GluN2B mutants with a dual point mutation that prevented its binding with the kinase (Strack et al., 2000), blocked the increased diffusion of membrane GluN2B subunits induced by chemical LTP (Dupuis et al., 2014). Therefore, NMDAR surface trafficking is regulated by CAMKII activity and by direct interaction with the kinase. In this study, other kinases such as casein kinase II were also shown to affect the lateral mobility of NMDAR in culture (Dupuis et al., 2014).

Transmembrane receptors : example with DAR

At the functional level, it was first shown that NMDAR synaptic transmission was influenced by DA receptors (Harvey and Lacey, 1997). Application of D1/D5 agonists induced either a depression or a potentiation of the NMDAR-EPSC in hippocampal CA1 neurons, depending on GluN2 subunits (Varela et al., 2009). Activation of the D2-family receptors depressed NMDAR-dependent synaptic currents in hippocampal, cortical and striatal neurons (Cepeda et al., 1993; Beazely et al., 2006). In addition to this functional coupling, NMDAR and DAR physically interact, with D1R associating with GluN1 and GluN2A subunits *via* its C-tail (Lee et al., 2002) and D2R with GluN2B subunits (Liu et al., 2006). Acute disruption of D1R-GluN1 interaction by a cell permeable peptide mimicking the last amino-acids of the C-term of D1R altered the clustering of D1R in the vicinity of PSD and displaced the receptors out of the synapses by increasing their lateral diffusion (Ladepeche et al., 2013). This alteration was accompanied with a redistribution of GluN1 subunits in synapses, leading to an enhancement of NMDAR-mediated synaptic currents and LTP in hippocampal neurons. Since similar modifications were observed following pharmacological activation of D1R (Ladepeche et al., 2013), it was proposed that D1R activation disrupted the interaction with NMDAR. Therefore, D1R were redistributed in the extrasynaptic compartment and NMDAR reached synapses, which promoted the NMDAR-mediated synaptic transmission.

d) Altering NMDAR trafficking with NMDAR antibody

Crosslink of NMDAR with commercial antibodies

The first studies using commercial antibodies to modulate receptors surface trafficking were conducted on AMPAR on cultured hippocampal neurons (Groc et al., 2008; Heine et al., 2008) Incubating the cultures with GluA2 antibodies targeting an extracellular epitope in the N-term of AMPAR immobilized (or crosslinked) the receptors in both the synaptic and the extrasynaptic compartments (Heine et al., 2008). However, the amplitude and the kinetics of AMPAR-EPSC were unchanged, allowing to directly investigate the role of AMPAR trafficking *per se* with the crosslink without modifying receptor activity. Few years later, a similar approach was used to study the role of NMDAR trafficking. Incubating cultured hippocampal neurons with GluN1 antibodies targeting an epitope on the N-terminal of NMDAR (X-link GluN1) coupled to secondary antibodies dramatically reduced the surface diffusion of GluN1 (**Figure 14**) but did not change the synaptic content of NMDAR in hippocampal culture (Dupuis et al., 2014). Besides, it did not affect either the calcium influx through NMDAR following NMDA application, or the properties of spontaneous NMDAR-EPSC regarding the frequency, the amplitude and the kinetics. The absence of effect of the X-link GluN1 on synaptic transmission was further demonstrated *in vivo*, where stereotaxic injection of GluN1 antibodies in the

hippocampus did not modify basal synaptic transmission (Potier et al., 2015). Finally, since X-link GluN1 effectively reduced the lateral diffusion of NMDAR in dissociated neurons (Dupuis et al., 2014) and in organotypic slices (Varela et al., 2016) and induced a similar alteration of the synaptic plasticity in cultured neurons and in acute slices (Dupuis et al., 2014), this protocol is a reliable and powerful way to specifically study the impact of NMDAR surface trafficking both *in vitro* and in more integrated preparations.

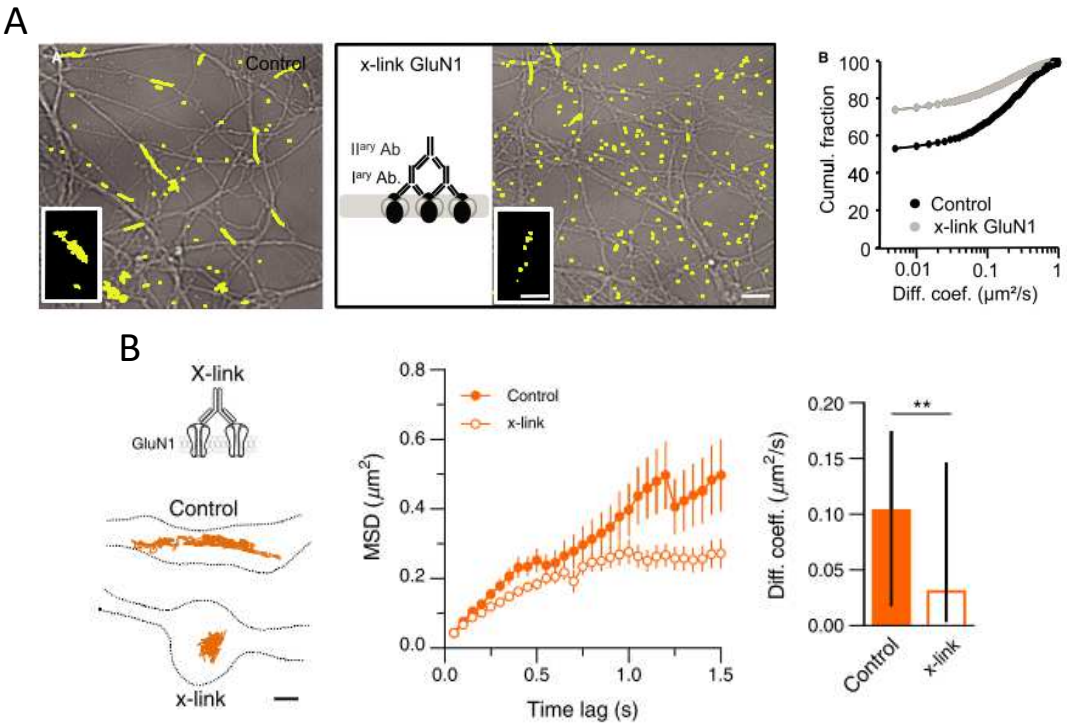


Figure 14. X-link GluN1 immobilizes the diffusion of membrane NMDAR in hippocampal neurons from primary culture and organotypic slices (adapted from Dupuis et al., 2014; Varela et al., 2016). A) Trajectories of single QD-GluN1 (yellow traces) in hippocampal culture before (left) and after X-link GluN1 (middle). Application of GluN1 Ab (primary Ab) and secondary Ab shorten the GluN1 trajectories. Right, The cumulative distribution of GluN1 diffusion coefficient in control and X-link GluN1 conditions showed that GluN1 subunits diffuse less with X-link. B) Left, Schematic representation of X-link GluN1 protocol, consisting of incubating GluN1 primary Ab in hippocampal organotypic slices. Trajectories of single QD-GluN1 complexes in control (middle) and X-link conditions are represented (bottom). Middle, Plot of MSD over time of GluN1 subunits in control and X-link conditions in organotypic slices. The behaviour of GluN1 subunits is more confined in the presence of X-link GluN1. Right, The diffusion coefficient of NMDAR is significantly decreased by X-link GluN1.

Alteration of NMDAR surface trafficking with auto-antibodies against NMDAR

In 2007, a severe form of encephalitis associated with auto-antibodies (Ab) against the GluN1 subunits of NMDAR was reported (Dalmau et al., 2007). Patients with anti NMDAR encephalitis first developed several symptoms consisting of headaches, fever, nausea and after few weeks, psychiatric symptoms appear like anxiety, paranoia and social withdrawal (Dalmau et al., 2011). Expression of a mutated form of GluN1 subunits in HEK cells decreased the reactivity of serum patients, suggesting that NMDAR auto-Ab target an extracellular epitope on GluN1 subunits (Dalmau et al., 2008).

Compelling evidence indicates that these Ab affect the membrane content of NMDAR. Chronic treatment with patients' Ab decreased the number of NMDAR clusters in hippocampal neurons *in vitro* (Dalmau et al., 2008) and *in vivo* (Hughes et al., 2010). In this latter study, the authors reported that this chronic treatment also reduced the synaptic NMDAR clusters *in vitro*, and was associated with a decrease of NMDAR-mediated synaptic currents. In line with these studies, long exposure with patients auto-Ab prevented the induction of LTP in cultured neurons (Mikasova et al., 2012). Therefore, the chronic treatment of NMDAR auto-Ab impaired NMDAR synaptic function by promoting its endocytosis. Although less investigated, acute treatment with patients' Ab seems to involve other mechanisms than endocytosis. Indeed, 2-5h exposure with auto-Ab increased the diffusion of synaptic GluN2A subunits and altered their interaction with the receptor kinase EphB2 (Mikasova et al., 2012). This latter interacts with NMDAR by the extracellular domains in response to the binding of the EphB ligand (Dalva et al., 2000) and favours the clustering of synaptic NMDAR (Henderson et al., 2001). From these data, it emerged that acute exposure to auto-Ab decreased the synaptic retention of GluN2A-containing NMDAR by altering its coupling with the receptor kinase EphB2. In addition to encephalitis, auto-Ab against NMDAR were also associated to schizophrenia, which is a debilitating disorder characterized by psychotic symptoms including delusions and hallucinations (Tsutsui et al., 2012; Pearlman and Najjar, 2014). Noteworthy, the presence of NMDAR auto-Ab in schizophrenia patients is still a matter of debate, and might depend on the detection methods used (Sinmaz et al., 2015). Similarly to encephalitis, acute exposure to auto-Ab from schizophrenic patients increased the surface trafficking of GluN2A subunits and dramatically reduced their synaptic stabilization, impairing the NMDAR-dependent LTP (Jezequel et al., 2017). Since the synaptic content of EphB2 receptors was also impaired in this condition, it favours a scenario in which auto-Ab alter the synaptic stabilization of NMDAR-containing GluN2A subunits by disturbing the synaptic anchoring partner EphB2 receptor. Notably, the acute exposure to auto-Ab did not change the membrane content of synaptic NMDAR, neither the NMDAR-mediated calcium transients (Jezequel et al., 2017), indicating that these effects were not mediated by NMDAR endocytosis or alterations of NMDAR channel activity but rather results from an abnormal NMDAR surface diffusion.

B. On midbrain DA neurons

Since decades, investigating the role of NMDAR in midbrain DA neurons was challenging because of technical limitations. The midbrain and in particular the VTA are composed of several neuronal types such as DA neurons, GABA interneurons and some glutamatergic neurons (Swanson, 1982; Yamaguchi et al., 2007; Nair-Roberts et al., 2008; Dobi et al., 2010; Margolis et al., 2012). This

renders the pharmacological approaches using injection of NMDAR antagonists in the VTA quite hard to interpret, as the effects observed in the presence of NMDAR modulators can be due to a direct effect on NMDAR contained in DA neurons or to a "network" effect caused by action on NMDAR from another neuronal type. The role of NMDAR was also investigated with transgenic GluN1 null mice who had a ubiquitous deletion of GluN1 subunits (Forrest et al., 1994). But using these mice could be problematic because they died several hours after birth. Besides, as the deletion is ubiquitous, it is challenging to draw final conclusions about the role of NMDAR in the studied structure. Fortunately, the generation of conditional knock out mice has enabled to selectively remove NMDAR in midbrain DA neurons (Zweifel et al., 2009) and thus, helped to decipher the involvement of NMDAR in various processes. For this reason, studies using conditional knock out mice will be more developed in this part. However, it should be mentioned that one cannot rule out that some compensatory mechanisms can occur during development and account for some effects observed in these mice.

1. Role of NMDAR in physiological and pathological conditions

Role in synaptic plasticity

NMDAR is responsible for the induction of NMDAR-dependent LTP and LTD, which mostly rely on the incorporation and removal of synaptic AMPAR respectively (Lüscher and Malenka, 2012). Because synaptic plasticity was mostly described in the hippocampus in the CA3-CA1 synapse, one can wonder if similar plasticity (LTP and/or LTD) can occur in midbrain DA neurons. In 1999, two independent teams tested if LTP could also be elicited *in vitro* in DA neurons from VTA (Bonci and Malenka, 1999) and SNc acute slices (Overton et al., 1999). In both groups, strong electrical stimulation induced a potentiation of the responses amplitude that lasted at least 40 minutes and was completely abolished in the presence of NMDAR blockers, two properties similar to NMDAR-dependent LTP encountered in the hippocampus. The presence of NMDAR-dependent LTP was further supported by the fact that mice lacking NMDAR selectively in midbrain DA neurons did not exhibit LTP following high frequency stimulation (Zweifel et al., 2008). Beyond the role of NMDAR in the induction of "classical' LTP, another form of plasticity was identified in midbrain DA neurons and was expressed by an enhancement of NMDAR-mediated synaptic transmission (Harnett et al., 2009). This potentiation was induced by presynaptic stimulation paired with delayed bursts of AP evoked in the postsynaptic neuron and required both NMDAR activation and calcium release from intracellular stores. The induction of LTP of NMDAR did not change the extent of NMDAR-EPSC inhibition by GluN2B and GluN2A blockers (Harnett et al., 2009). Although the authors concluded that this LTD did not require change of synaptic NMDAR composition, one cannot exclude that LTP drives the synaptic insertion of tri-heteromers, which are less sensitive to these blockers than "pure" GluN2A and GluN2B di-heteromers, (Schilström

et al., 2006). In parallel, other groups investigated the presence of NMDAR-dependent LTD in DA neurons. Despite the fact that Jones and Kauer found that LTD could be elicited in midbrain DA neurons, this depression was distinct from hippocampus NMDAR-dependent LTD, because this LTD was NMDAR-independent and rather involved mGluR for its induction (Jones et al., 2000). Of note, pairing burst firing before presynaptic stimulation induced a depression of the NMDAR synaptic transmission (Harnett et al., 2009). Taken together, these data confirm the critical role of NMDAR in the induction and the expression of synaptic plasticity in midbrain DA neurons.

Role in drug-induced modifications and addiction

In the hippocampus, LTP is thought to be one of the mechanisms underlying memory formation, whereas it is thought to be associated with drugs exposure in the midbrain. Indeed, a single exposure to cocaine induced a potentiation of excitatory synapses in midbrain DA neurons that resembles the LTP (Ungless et al., 2001). In particular, cocaine enhanced the excitatory synaptic strength measured by the ratio of AMPAR- over NMDAR-mediated synaptic currents (AMPA/NMDA ratio) in DA neurons, increased AMPAR synaptic currents, and occluded LTP induction in these neurons. Finally, this plasticity was prevented when the NMDAR blocker MK-801 was co-administered (Ungless et al., 2001) or more recently, when GluN1 subunit was genetically inactivated in midbrain DA neurons (Engblom et al., 2008). This indicates that, similarly to hippocampal LTP, induction of cocaine-driven potentiation requires activation of NMDAR. However, western blot analysis failed to reveal changes in AMPAR expression (Ungless et al., 2001), which clearly contrasts with the increased AMPAR expression occurring after LTP induction (Lüscher and Malenka, 2012). Bellone and Lüscher further unraveled the molecular mechanisms underlying this potentiation and found that the increase in AMPA/NMDA ratio was paralleled with a rise in the AMPAR rectification index (Bellone and Lüscher, 2006). As calcium-permeable AMPAR displayed a strong rectifying synaptic response, it was proposed that cocaine-induced potentiation was mediated by a switch to calcium permeable AMPAR at excitatory synapses onto DA neurons. Interestingly, this form of plasticity modulated not only AMPAR synaptic transmission but also NMDAR synaptic transmission. Electrophysiological recordings revealed that injection of cocaine changed NMDAR subunits composition with the insertion of GluN2B and GluN3A subunits at the excitatory synapses (Yuan et al., 2013). Given that GluN3A subunits confer a low conductance to NMDAR (Ciabarra et al., 1995; Henson et al., 2010), these results are consistent with the diminution of NMDAR-mediated synaptic transmission following cocaine administration previously reported (Mameli et al., 2011). By contrast, Schilstrom et al. observed the reverse effect on NMDAR synaptic currents following cocaine exposure (Schilström et al., 2006). A single injection of cocaine or the direct incubation of slices with cocaine enhanced the NMDAR-EPSC in VTA DA neurons, and was correlated with an incorporation of GluN2B subunits in the membrane (Schilström et al., 2006).

Although in disagreement, these studies demonstrate that the role of NMDAR is not limited to the induction of plasticity but that the receptors also participate to the expression of plasticity. To note, *in vivo* administration of other addictive drugs like morphine or nicotine also elicits potentiation of excitatory synapses in midbrain DA neurons (Saal et al., 2003), and enhances the AMPAR rectification index by a redistribution of calcium permeable AMPAR into synapses (Brown et al., 2010). Besides, nicotine-induced potentiation is blocked in presence of NMDAR antagonists, demonstrating the requirement of NMDAR to induce drug-driven synaptic potentiation (Mansvelder and McGehee, 2000). Given the wide range of action mechanisms of these drugs, it appears that NMDAR is a common target necessary to mediate synaptic plasticity driven by drugs of abuse.

On the behavioral level, NMDAR contribute to various aspect of drug addiction. Locomotor sensitization is characterized by an increased locomotor activity of the animals following repeated drug administration, and it persists after drug administration stops. Genetic inactivation of NMDAR in midbrain DA neurons attenuated the locomotor sensitization after 21 days of withdrawal but not 3 days, meaning that NMDAR are involved in the late phase of withdrawal-induced sensitization (Zweifel et al., 2008). Using a similar genetic approach, Engblom et al. discovered that the relapse was also altered in mice lacking NMDAR in DA neurons (Engblom et al., 2008). The relapse, which is the reinstatement of drug-seeking behavior, can be triggered by drug exposition. Therefore, the authors investigated the effect of a single dose of cocaine to reinstate a conditioned place preference, a test modeling the drug seeking behavior. They found that, contrary to control mice that spent more time in the compartment associated with cocaine, mice lacking NMDAR on midbrain DA did not display any preference for this compartment (Engblom et al., 2008), indicating that these mice did not "relapse" following drug re-exposure. In view of these data, NMDAR seem to be particularly involved in the persistence of drug addiction.

Role in burst firing

As already mentioned in the section "I.3. Channels and receptors involved in the firing of midbrain DA neurons", there is a bulk of data highlighting the central role of NMDAR in the generation of burst firing of midbrain DA neurons.

Role in cue-dependent learning and habit

By contributing to burst firing and synaptic plasticity, NMDAR appears as a key modulator of the activity of midbrain DA neurons. Because DA neurons activity is thought to encode reward–related and salient information like aversive stimuli (see section I.1. Role of midbrain DA in physiological and pathological conditions) that drives learning, the view has emerged that NMDAR might be involved in

all these processes as well. In accordance with this hypothesis, injection of NMDAR antagonists in the VTA modified the reward responses induced by electrical self-stimulation in rats (Bergeron and Rompré, 2013) and prevented the development of cue-reward association (Stuber, 2008). Taking advantage of the conditional knock out mice (KO $GluN1_{DAT}$ mice), Zweifel et al. reported that inactivating NMDAR function in midbrain DA neurons impaired burst firing and altered a cue-dependent learning associated with reward (Zweifel et al., 2008, 2009). To assess it, the authors used several tests including t-maze test, which consists of 2 arms with different visual cues, one arm (with vertical stripe cues) associated with accessible food and the other with inaccessible food (horizontal strip). Wild type mice chose more often to go to the arm associated with the accessible reward, reflecting that mice learned which arm was rewarding (Zweifel et al., 2009). By contrast, KO $GluN1_{DAT}$ mice entered in both arms with the same frequency, and thus were unable to correctly perform this task. Intriguingly, some DA-dependent behaviors like working memory or locomotor activity were not altered in these mice (Zweifel et al., 2008, 2009). These findings suggest that NMDAR and subsequent burst firing are necessary for cue-dependent learning associated with reward but do not contribute to other DA-dependent tasks. As tonic activity was not affected in these mice (Zweifel et al., 2009), it was interpreted that tonic DA could be sufficient to evoke some behaviors. Because DA neurons activity was also associated with the coding of aversive stimuli (Brischoux et al., 2009), the KO $GluN1_{DAT}$ mice were also used to test the implication of NMDAR onto DA neurons in this process (Zweifel et al., 2011) The startle responses of mice to a sound was monitored after fear conditioning was established by several presentation of aversive stimuli (foot shock) associated with a neutral stimuli (light pulse). As expected, wild-type mice startled more in presence of the cue (light pulse) previously associated with the foot shock, whereas mice lacking NMDAR in DA neurons startle to the same extent if the neutral cue was present or not. The absence of an increased startle responses suggest a deficit in the learning associated with aversive information. DA signaling being also required for habit learning (Faure et al., 2010), Wang et al. investigated the implication of NMDAR by assessing navigation in water maze in these mice (Wang et al., 2011). Extensive training caused the wild type mice to switch from a cue-based navigation that engages spatial memory to a habit strategy because the pathway in the maze was still the same to reach the searched target and did not require the use of cues anymore. On the contrary, KO $GluN1_{DAT}$ mice were unable to switch to a habit strategy (Wang et al., 2011), demonstrating that NMDAR on DA neurons are necessary for habit learning.

Role in excitotoxicity and Parkinson's disease (PD)

It has long been hypothesized that glutamate, and in particular NMDAR, contribute to excitotoxicity on SNc DA neurons and might be involved in the etiology of PD (Surmeier et al., 2010). Chronic injection of MPTP, a neurotoxin that mimics PD by killing DA neurons, elevated extracellular

level of glutamate in the SN and was accompanied by the appearance of apoptosis and autophagy markers (Meredith et al., 2009). Besides, the anti-parkinsonian drug pramipexole attenuates the DA neuron loss induced by glutamate application in midbrain culture (Izumi et al., 2007). *In vitro* application of NMDAR antagonists prevented the loss of midbrain DA neurons induced by various toxic stimuli such as glutamate application (Sonsalla et al., 1998; Izumi et al., 2007) or a metabolic stress (Zeevalk et al., 1995) and attenuated it in response to both combined (Marey-Semper et al., 1995). Consistently, the presence of the NMDAR blocker memantine, used to treat neurodegenerative diseases including PD, prevented the DA cell loss evoked by NMDA application in SNc slices (Wild et al., 2013). *In vivo*, co-injection of MPP+, a derivative of the MPTP, with NMDAR antagonists such as MK-801 or CPP directly into the SNc provided protection against MPP+ induced toxicity (Turski et al., 1991). However, some studies gave conflicting results about the importance of NMDAR in mediating toxicity induced by glutamate and/or metabolic stress. Several authors found that application of NMDAR antagonists provided a mild protection against the DA neuron loss induced by glutamate (Weller et al., 1993) or glutamate combined with a metabolic stress (Marey-Semper et al., 1995), and no protection for MPP+ induced toxicity (Sawada et al., 1996). In the study of Marey-Semper, the toxicity was also attenuated with AMPAR antagonists (Marey-Semper et al., 1995), suggesting that likely both NMDAR and AMPAR mediated excitotoxicity in midbrain DA neurons. Remarkably, the maturation of NMDAR synaptic transmission was altered in SNc DA neurons during development in a genetic mouse model of PD (Pearlstein et al., 2016). In this mouse, NMDAR-mediated synaptic transmission was severely decreased; in particular the bursts of NMDAR-mediated EPSC occurring in wild type mice during the first two weeks of postnatal development were almost absent. In addition, whereas GluN2D subunits contributed to NMDAR synaptic currents in SNc DA neurons from 1 week-old wild type mice (Pearlstein et al., 2015), it was not the case in age-matched PD model mice (Pearlstein et al., 2016).

In view of these data, the role of NMDAR onto DA neurons in excitotoxicity and PD is unclear and much remains to be worked out to fully understand its contribution in these processes. However, the recent discovery of Pearlstein and collaborators suggests that early alteration of NMDAR might lead to dysfunction of DA system, and thereby could contribute to long-term DA impairments in PD.

2. Influence of NMDAR co-agonists on the DA system

The NMDAR co-agonists D-serine and glycine were first detected by high performance liquid chromatography in the rat midbrain (Hashimoto et al., 1993). Accordingly, the enzyme D-amino acid oxidase (DAO) responsible for the D-serine degradation was also found in the midbrain, and especially in DA neurons from SNc and VTA (Horiike et al., 1994; Verrall et al., 2007; Betts et al., 2014). Injection

of the DAO blocker sodium benzoate in the VTA increased the extracellular levels of DA and its metabolites in the cortex, suggesting a functional role of the co-agonist in the DA system (Betts et al., 2014). Injection of the glycine binding site agonist D-cycloserine in the SNc also enhanced the extracellular level of DA in the dorsal striatum, and attenuated motor deficits induced by antipsychotic injections (Shimizu et al., 2017). Furthermore, DAO knock out mice exhibited a greater number of burst-firing VTA DA neurons *in vivo,* but no changes in the bursts properties and the overall firing rate were observed compared to control mice (Schweimer et al., 2014). Although the authors did not assess the D-serine content in the VTA, the absence of DAO should up-regulate its level and thereby, influence the firing of DA neurons. Because the firing rate of non VTA DA neurons (probably GABAergic neurons) was decreased in DAO knock out mice (Schweimer et al., 2014), the enhancement of burst firing might result from the decreased activity of GABAergic neurons and/or direct effects on the NMDAR-mediated transmission onto DA neurons.

Up to now, the direct influence of the NMDAR co-agonist glycine on DA neurons activity was poorly investigated. Application of glycine transporter (Glyt1) inhibitors enhanced the amplitude of the NMDAR-mediated evoked synaptic currents in DA neurons from SNc acute slices (Schmitz et al., 2013), which is similar to what was found in hippocampal neurons (Bergeron et al., 1998). Besides, Glyt1 inhibitors favoured the DA reinnervation of the striatum following loss of DA neurons by 6-OHDA injection, and restored motor deficits (Schmitz et al., 2013). Such recoveries were not observed in presence of Glyt1 inhibitors in mice line lacking NMDAR in DA neurons, confirming that the effects required the presence of NMDAR in DA neurons. Altogether, it appears that the NMDAR co-agonists influence the DA neuronal activity and the resulting behaviours. However, whether the reported effects are due to the direct modulation of NMDAR onto midbrain DA neurons or involved indirect effects through other neuronal types is still elusive and further investigations are needed to address this question.

3. NMDAR expression and modulation in DA neurons

Since NMDA application modify the firing pattern of midbrain DA neurons *in vivo* and *in vitro* (Seutin et al., 1990; Overton and Clark, 1992; Mereu et al., 1997), it was postulated that active NMDAR were present in these neurons. This was further corroborated by immunohistochemistry and *in situ* hybridization revealing the presence of GluN1 subunits in DA neurons from SNc and VTA (Paquet et al., 1997). Finally, synaptic stimulation evoked EPSC in DA neurons from midbrain acute slices that were partly inhibited by NMDAR antagonists (Mereu et al., 1991), confirming the existence of functional NMDAR in midbrain DA neurons.

a) NMDAR subtypes expression

The NMDAR composition in midbrain DA neurons was inferred from *in situ* hybridizations, immunohistochemistry and electrophysiology approaches. In one week-old rats, Bellone et al. found that the NMDAR-mediated synaptic currents on VTA DA neurons were inhibited by the GluN2B antagonist ifenprodil, indicating the presence of GluN2B subunits in the early development (Bellone et al., 2011). In older rats (3 weeks-old), the GluN2A antagonist zinc produced a robust inhibition of NMDAR-EPSC whereas ifenprodil was poorly effective (**Figure 15**). These data reveal that excitatory synapses onto VTA DA neurons undergo a switch of synaptic GluN2B to GluN2A subunits during the postnatal development, similarly to what was found in the hippocampal synapses (Bellone and Nicoll, 2007). It is worth noting that GluN3A subunits were detected by immunoblots in midbrain from young rats (Al-Hallaq et al., 2002) and in synaptosomes from both cortex and midbrain (Wee et al., 2016). Besides, exposure to cocaine drive the insertion of GluN3A subunits in VTA DA neurons (Yuan et al., 2013; Creed et al., 2016). As cocaine mimics some changes observed during development and is thought to induce the re-juvenilation of synapses with the re-appearance of synaptic GluN2B subunits (Dong and Nestler, 2014), it is possible that midbrain DA neurons contain GluN3A subunits during the postnatal development. Therefore, it seems that synaptic NMDAR on VTA DA neurons are composed of GluN2B and potentially GluN3A subunits early in the development, and are replaced by GluN2A-containining NMDAR after 2-3 postnatal weeks.

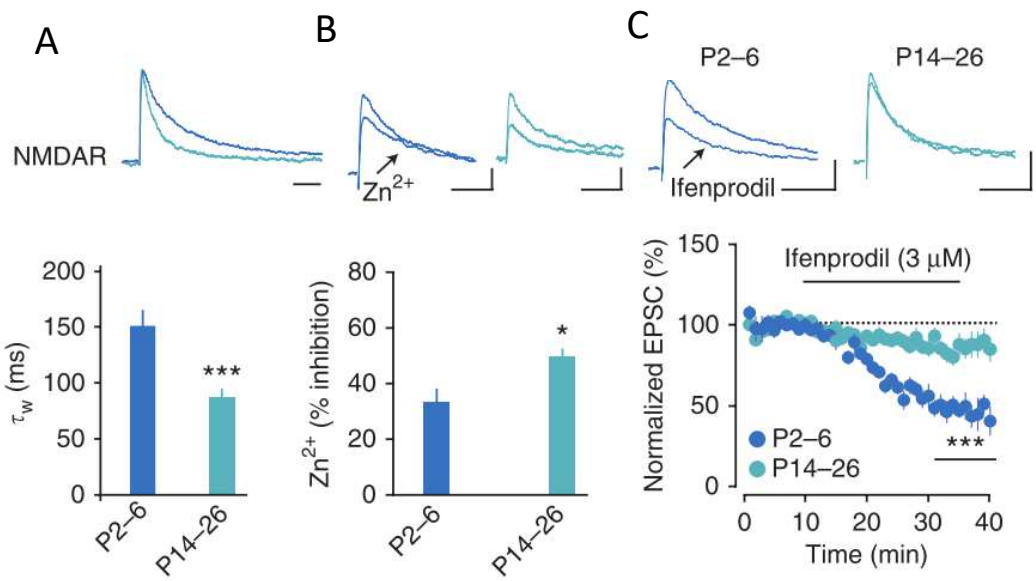


Figure 15. Developmental switch of synaptic GluN2B- by GluN2A-containing NMDAR in DA neurons from VTA acute slices (adapted from Bellone et al., 2011). A) Top, Electrophysiology traces of NMDAR-EPSC in DA neurons from P2-P6 rats (Blue) and P14-P26 rats (light blue). Down, The NMDAR-EPSC decay kinetics is faster in PD 14-26 rats. B) Top, Electrophysiology traces of NMDAR-EPSC in DA neurons from slices of P2-P6 rats (Blue) and PD 14-

26 rats (light blue) before and after the application of the GluN2A blocker zinc. The NMDAR-EPSC is significantly more sensitive to zinc in PD 14-26 rat slices. C) Up, Electrophysiology traces of NMDAR-EPSC in DA neurons from slices of PD 2-6 rats (Blue) and PD 14-26 rats (light blue) before and after the application of the GluN2B blocker ifenprodil. Down, The ifenprodil inhibited to a larger extent the NMDAR-EPSC in slices from PD 2-6 (blue) than PD 14-26 rats (light blue).

In agreement with *in situ* hybridizations showing a preponderance of GluN2D in the midbrain in rats from 1 week-old and a further decline during development (Monyer et al., 1994; Wenzel et al., 1995), Brothwell et al. found that GluN2B and GluN2D subunits were present in SNc DA neurons from P7 rats (Brothwell et al., 2008). During development, the inhibition of NMDAR-EPSC by the GluN2D preferential antagonist UBP141 was unchanged whereas the ifenprodil block was reduced. These data argue against the replacement of GluN2B-containing NMDAR by GluN2D di-heteromers, but rather support the contribution of GluN2B/GluN2D tri-heteromers along development (Brothwell et al., 2008), which was previously suggested by analysis of single NMDAR channel properties (Jones and Gibb, 2005). Pearlstein et al. also highlighted the presence of both GluN2B and GluN2D subunits in SNc DA neurons during early development and further showed that GluN2D subunits did not contribute to NMDAR-EPSC in young adult rats (Pearlstein et al., 2015), consistent with the observed decline of GluN2D mARN in the midbrain during development (Monyer et al., 1994). Strikingly, NMDAR-EPSC of SNc DA neurons were not affected by GluN2A preferential antagonists in juvenile rats (Jones and Gibb, 2005; Brothwell et al., 2008; Suárez et al., 2010). Besides, Salamone et al. reported that DA release evoked by NMDA in synaptosomes from adult rat midbrain was not inhibited by GluN2A antagonists (Salamone et al., 2014). This suggests that NMDAR onto SNc DA neurons are devoid of GluN2A subunits. Taken together, these data indicate that immature SNc DA neurons the synaptic transmission is mediated by GluN2B and GluN2D subunits, probably assembled in diheteromers. During the development, these latter might be partially replaced by GluN2B -GluN2D tri-heteromers.

In conclusion, these data highlight a marked difference in the synaptic NMDAR composition in DA neurons from SNc and VTA, and revealed that, similarly to other brain regions, this composition is developmentally regulated.

b) Functional coupling of NMDAR and ionic channels: focus on SK channels

As observed in the hippocampus (Ngo-Anh et al., 2005; Hammond, 2006; Babiec et al., 2017), the amygdala (Faber et al., 2005) and the cortex (Faber, 2010), SK channels also modulate the synaptic activity of NMDAR in VTA DA neurons. Application of apamin potentiates NMDAR-mediated synaptic responses in DA neurons *in vitro* (Soden et al., 2013) and *in vivo* (Creed et al., 2016). Moreover, the NMDAR-EPSC in DA neurons from VTA slices from mice expressing a dominant negative mutant of SK

channels had a slower decay time than the EPSC from control mice (Soden et al., 2013), consistent with a “shunt” of NMDAR activity by SK channels in midbrain DA neurons. In this latter paper, the authors immunolabelled NMDAR and SK3 channels with gold particles and further demonstrated that these two proteins were in close proximity in the post-synaptic density of midbrain DA neurons (**Figure 16**), which is consistent with the modulation exerted by SK channels on synaptic NMDAR.

Conversely, do NMDAR regulate SK channel activity? Whereas calcium influx through NMDAR activates SK channels in the hippocampus (Ngo-Anh et al., 2005), the amygdala (Faber et al., 2005) and the cortex (Faber, 2010), the situation appears less clear in the midbrain. When Paul et al. investigated the modulation of SK channels by NMDAR activity, they unexpectedly found that bath application of NMDA decreased the SK channels-mediated currents in DA neurons from midbrain slices (Paul et al., 2003), implying that NMDAR negatively modulated SK channel function in these neurons. Nevertheless, even if Creed et al. did not directly investigate the coupling of NMDAR with SK channels, their work suggest that NMDAR activation could promote the activation of SK channels (Creed et al., 2016). Indeed, exposure to cocaine, which drives the insertion of the calcium impermeable GluN3A subunits (Henson et al., 2010; Yuan et al., 2013), significantly reduced the function of SK channels in wild type mice but not in mice knocked down for GluN3A (Creed et al., 2016). These data indirectly suggest that in control conditions, calcium influx through NMDAR activate the SK channels, and that insertion of calcium impermeable GluN3A subunits impairs this coupling. Therefore, it is difficult to draw a clear cut conclusion about the direction of the modulation of SK channels but altogether, these data revealed the presence of a reciprocal coupling between NMDAR and SK channels in midbrain DA neurons, with NMDAR activation regulating SK channels function.

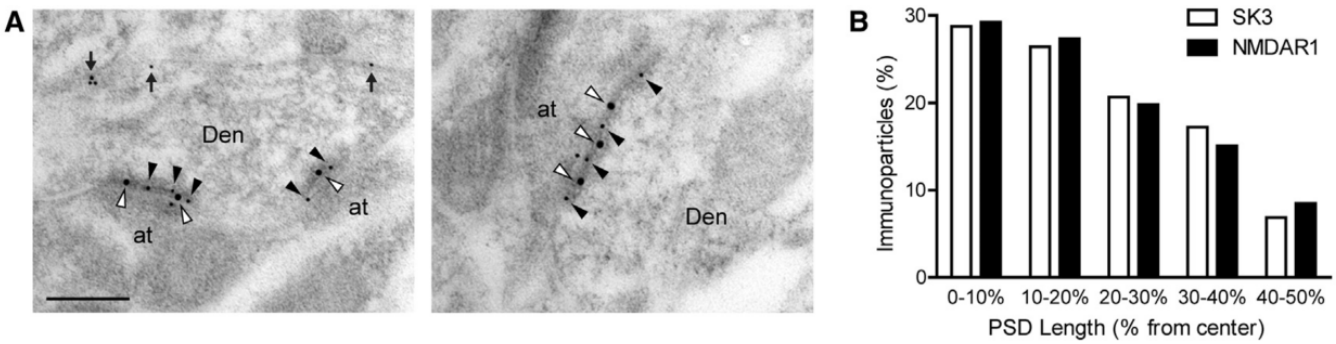


Figure 16. NMDAR and SK3 channels are found in close proximity in the PSD of VTA DA neurons (adapted from Soden et al., 2013). A) Electron microscopy image of a double immunogold labelling of GluN1 subunits and SK3 channels in VTA DA neurons. SK channels (10 nm gold particule) were detected in the extrasynaptic (black arrow) and in the synaptic (black arrowhead) compartments. Within the PSD, NMDAR (white arrowhead) and SK3 channels (black arrowhead) are close to each other. Den : dendrite, at : axon terminal B) Analysis of the relative distribution of GluN1 subunits and SK3 channels along the PSD. NMDAR and SK3 channels follow a similar pattern of distribution within the synapses.

The existence of a functional coupling between these 2 partners was also inferred from their relative action on the firing pattern of midbrain DA neurons in slices. Interestingly, combining the activation of NMDAR and the inhibition of SK channels was more effective to elicit bursts in midbrain slices (Seutin et al., 1993; Prisco et al., 2002) than the modulation of SK channels or NMDAR alone (Shepard and Bunney, 1988; Johnson et al., 1992; Johnson and Wu, 2004), indicating that they both interact to promote the burst generation. Therefore, the coupling of NMDAR and SK channels appears to play a crucial role in the regulation of the firing pattern of midbrain DA neurons.

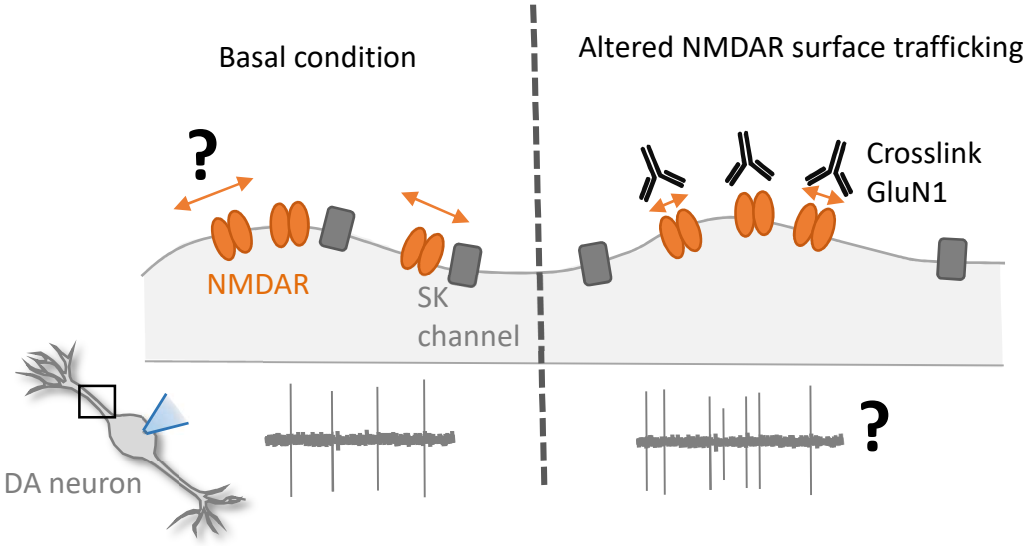
PhD OBJECTIVES

It is well established that NMDAR and other ion channels such as SK channels modulate the firing pattern of midbrain DA neurons. However, the molecular mechanisms by which these two partners control the firing pattern remain elusive. The development of single molecule imaging revealed that NMDAR are mobile within the plasma membrane of hippocampal neurons. This appears as a key mechanism to regulate their membrane distribution. Since the functional interaction between NMDAR and SK channels highly depends on their relative membrane localization, NMDAR membrane trafficking might modulate the firing pattern of midbrain DA neurons by regulating its interplay with SK channels.

In the first part of my thesis, the objective was **to investigate the surface dynamics of NMDAR in midbrain DA neurons**. To do so, single particle tracking with quantum dots was used to track the diffusion of membrane NMDAR in cultured DA neurons from TH-tdTomato mice and in human induced pluripotent stem cells (iPSC)-derived DA neurons. Since cultured DA neurons lack the glutamatergic inputs and the tissue architecture of native preparations, I also wanted to characterize the lateral mobility of NMDAR in a more integrated preparation, i.e rodent midbrain acute slices. Although challenging, our team recently developed a protocol to track surface tagged receptors in hippocampal acute slices by single molecule imaging techniques, which was the subject of a publication (**annexe 1**). However, due to technical limitations described in the **annexe 2**, it was impossible to adapt this technique to the midbrain. Therefore, a second strategy based on electrophysiological approaches combined with MK-801 was considered to estimate the diffusion of membrane NMDAR in midbrain acute slices. Thus, I implemented midbrain acute slices in the laboratory and tested the protocol developed by Tovar and Westbrook to characterize the surface diffusion of receptors. Here again, it seemed that this approach was not appropriate for midbrain DA neurons (**annexe 3**). Finally, as the characterization of the NMDAR surface diffusion was not possible in acute brain slices, I attempted to circumvent the problem of the low glutamatergic tone in cultured midbrain DA neurons by co-culturing DA neurons with cortical neurons. Because both cultures required different culture media, I privileged the development of co-culture in microfluidic chambers. Although the co-culture between embryonic midbrain and cortical neurons was successfully established, the postnatal midbrain culture from TH-

tdTomato mice did not survive in microfluidic devices (**annexe 4**), currently limiting the full characterization of NMDAR surface trafficking in cultured DA neurons under the influence of glutamatergic inputs.

In the second part of the project, I aimed at **testing if NMDAR surface trafficking modulated the firing pattern of midbrain DA neurons.** If yes, the second objective was **to decipher the mechanisms responsible for the modification of the firing pattern.** For this aim, I used a crosslink protocol developed in the team to specifically alter the NMDAR surface diffusion, and studied its impact on the firing pattern of DA neurons. The firing activity was recorded in DA neurons from acute midbrain slices because the spontaneous firing is highly reliable and conserved across laboratories in this preparation.



RESULTS

NMDA receptor trafficking tunes the firing pattern of midbrain dopaminergic neurons

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ABSTRACT

Midbrain dopaminergic (DA) neurons play a central role in major physiological brain functions such as motivation, while their dysfunctions have been associated with neuropsychiatric diseases including schizophrenia and addiction. The activity and firing pattern of midbrain DA neurons are controlled by various ion channels and neurotransmitter receptors, such as the glutamate NMDA receptor (NMDAR) and small conductance calcium-dependent potassium channel (SK). Although intracellular regulatory cascades have been identified, the cellular pathway by which these channels interact and tune the firing pattern of midbrain dopaminergic (DA) neurons remains unclear. As the functional interplay between NMDAR and SK channels likely depends on their surface distribution, we tested the hypothesis that the surface dynamics of NMDAR tunes the firing pattern of midbrain DA neurons by regulating the function of other channels such as SK channels. Using single molecule imaging, we report that NMDAR are highly diffusive at the surface of cultured midbrain DA neurons from rodents and humans. Strikingly, altering *in vivo* the NMDAR membrane dynamics with an artificial crosslink of the receptors, which leaves intact the ionotropic function, strongly modified the firing pattern of midbrain DA neurons. In this condition, the SK channels blocker apamin, which perturbs the DA neuron firing pattern, was poorly effective. As this loss of function was not associated with a reduced membrane content of SK channels, these data fuel a model in which the altered surface dynamics of NMDAR downregulated the function of SK channels. Collectively, these data unveil that the surface dynamics of NMDAR, and not solely its ionotropic function, can tune the firing pattern of midbrain DA neurons through control of the function of SK channels.

INTRODUCTION

The midbrain dopaminergic (DA) system, composed of neurons from the retrorubral area, the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA), is involved in several key physiological functions, like motivation, learning and processing of salient information (Bromberg-Martin et al., 2010; Schultz, 2010). Dysfunctions of this system have been reported in neuropsychiatric diseases such as schizophrenia (Carlsson, 1977) and addiction (Koob and Bloom, 1988). Interestingly, most of these processes are associated with a change in the firing activity of midbrain DA neurons (Einhorn et al., 1988; Ljungberg et al., 1992; French et al., 1993; Brischoux et al., 2009). Therefore, deciphering the mechanisms responsible for the firing activity of midbrain DA neurons is of crucial importance to shed lights on the DA-dependent behaviors and pathological alterations.

In the rodent brain, DA neurons fire in a tonic or phasic mode with bursts of action potentials (Grace and Bunney, 1984a, 1984b), whereas these neurons only exhibit regular pacemaker activity in brain slices (Grace and Onn, 1989; Wolfart et al., 2001; Deignan et al., 2012). It is well-accepted that the firing activity is generated and modulated by the combination of ion channels and neurotransmitter receptors (Liss and Roeper, 2008; Paladini and Roeper, 2014; Dragicevic et al., 2015). Among these, the ionotropic glutamate NMDA receptors (NMDAR) and small conductance calcium-dependent potassium channels SK are of particular interest, since they strongly regulate the firing pattern of midbrain DA neurons *in vitro* (Johnson and North, 1992; Wolfart and Roeper, 2002; Johnson and Wu, 2004) as well as *in vivo* (Overton and Clark, 1992; Waroux et al., 2005; Ji and Shepard, 2006; Herrik et al., 2012) . Besides, NMDAR and SK channels functionally interact in midbrain DA neurons (Paul et al., 2003; Soden et al., 2013) and thus, might work in synergy to tune the firing pattern of these neurons. Consistently, NMDAR and SK channels have been detected in the area of the postsynaptic compartment of glutamate synapses onto midbrain DA neurons (Soden et al., 2013). Thus, the pathways that regulate membrane distribution of these two channels are likely to play an important role in their functional interaction and, consequently, on the firing pattern of midbrain DA neurons.

The membrane and synaptic distributions of NMDAR are processed by exocytosis, endocytosis and lateral diffusion (Bard and Groc, 2011; Paoletti et al., 2013; Lussier et al., 2015). Once they laterally reach the synapse (Tovar and Westbrook, 2002; Groc et al., 2004, 2006; Bard et al., 2010), NMDAR are actively anchored through protein-protein interaction and phosphorylation processes (Lussier et al., 2015). Most of these findings emerged thanks to the single molecule imaging that has now unveiled that neurotransmitter receptors as well as ion channels are highly dynamic at the surface of brain cells (Triller and Choquet, 2008), shedding new and unexpected lights on the molecular regulation of brain cell communication. In forebrain neurons, the surface dynamics of NMDAR is finely tuned by intracellular, transmembrane and extracellular regulators (Groc et al., 2009; Dupuis et al., 2014; Ladépêche et al., 2014; Ferreira et al., 2017), constituting one of the major site for regulation of NMDAR-mediated transmission, synaptic plasticity and behavior (Dupuis et al., 2014; Potier et al., 2015). Our understanding of the receptors and channels surface trafficking onto midbrain DA neurons is in comparison still in its infancy. It has been shown that the DA transporter laterally explores large areas in cultured midbrain neurons (Eriksen et al., 2009), with biophysical properties similar to the ones of transporters in hippocampal cells (Chamma et al., 2013). Thus, it is likely that neurotransmitter receptors and ion channels in midbrain DA neurons are also highly diffusive at the neuronal surface, with specific regulations related to the morphology of the dendritic architecture of midbrain neurons. Here, we hypothesized that NMDAR surface trafficking plays a role in the firing pattern of midbrain DA neurons by regulating SK channel function. Combining single molecule imaging and electrophysiological approaches, we show that NMDAR diffuse at the surface of cultured midbrain DA neurons from mice and human induced pluripotent stem cells (iPSC)-derived neurons, and that the alteration of their surface trafficking *in vivo* modifies the firing pattern of DA neurons in acute midbrain slices. Remarkably, impairing NMDAR trafficking or blocking SK channels similarly increased the irregularity of the firing. This increased induced by SK channel inhibition was less pronounced when NMDAR surface trafficking was impaired, while the membrane content of the channels was not affected. Together, these results revealed that NMDAR surface dynamics *per se* modulates the firing

activity of DA neurons and suggest that this effect is mediated by the modulation of SK channels function.

RESULTS

NMDAR diffuse at the plasma membrane of cultured midbrain DA neurons

Although surface NMDAR diffuse broadly in cultured hippocampal neurons, as well as in cultured and acute hippocampal slices (Bard et al., 2010; Dupuis et al., 2014; Groc et al., 2009; Groc et al., 2004; Groc et al., 2006; Tovar and Westbrook, 2002; Varela et al., 2016), their surface mobility in midbrain DA neurons has not been investigated. To assess whether surface NMDAR are mobile onto DA neurons, we first focused on mice cultured DA neurons. Since DA neurons represent only a weak proportion of neurons in our cultures (<5%, unpublished observations), we used tyrosine hydroxylase (TH)-tdTomato mice obtained by crossing homozygous male TH-Cre mice (Savitt et al., 2005) with homozygous female Cre-reporter tdTomato Ai9 (Madisen et al., 2010) (Fig 1 A1). Indeed, TH is the synthesis enzyme of dopamine, allowing then to image live DA cells in our preparation (identified by tdTomato protein expression). Accordingly, 86% of the TH-tdTomato neurons colocalized with TH immuno-labelling in our cultures (n=3 different cultures, data not shown), demonstrating the high specificity of the tomato protein expression in DA neurons (Fig 1 A2). To study the lateral mobility of endogenous NMDAR on cultured DA neurons, we used the single nanoparticle tracking approach to track single quantum dots (QD) coupled to antibodies against the extracellular N terminal of GluN1, the obligatory NMDAR subunit (Fig 1 B1,B2). Analysis of QD-NMDAR trajectories revealed that the mean square displacement (MSD), which reflects the mean area explored by QD, did not vary linearly with time (Fig 1 C). This non-linearity indicates that NMDAR are not freely diffusing, but rather confined at the surface of DA neurons from mice midbrain culture, as demonstrated for virtually all neurotransmitter receptors (Triller and Choquet, 2008). When compared to the dynamics of NMDAR in cultured hippocampal neurons, the instantaneous diffusion coefficient was rather high (0.127 $\mu m^2/s$, IQR = 0.038-0.206 $\mu m^2/s$, n=750 trajectories) and exhibited a low proportion of immobile receptors (9%) (Fig 1 C). In parallel, we explored the dynamics of membrane NMDAR in human iPSC-derived DA neurons (Fig 1 D1;D3), which are enriched in TH-positive neurons (Fig 1 D2). Accordingly, co-labelling of the nuclear

marker DAPI and the TH enzyme confirmed the high proportion of DA neurons in this culture (40%, data not shown). Tracking of endogenous NMDAR with GluN1 Ab coupled to QD in iPSC-derived DA neurons showed that the receptors were highly diffusive at the membrane of neurons, with limited confinement behavior (Fig 1 E). Notably, the fraction of immobile receptors was very similar between DA neurons from mice culture and human iPSC-derived neurons, indicating that most of surface NMDAR were mobile in mice and human DA neurons. Thus, the NMDAR located at the plasma membrane of midbrain DA neurons are quite dynamic, exploring a large area of the DA neuron dendritic tree.

NMDAR activation alters the receptor surface dynamics and the firing pattern of midbrain DA neurons

The NMDAR membrane dynamics is highly regulated by several modulators, including intracellular, extracellular and transmembrane partners(Groc et al., 2009). In addition, the activation of the receptor by its agonist or co-agonist instantaneously modulates its surface trafficking in hippocampal neurons (Papouin et al., 2012; De Rossi et al., 2016; Ferreira et al., 2017). To test whether NMDAR activation also modulated the receptor dynamics in DA neurons, we investigated the diffusion properties of membrane GluN1-NMDAR in human iPSC-derived DA neurons before and 5 minutes after NMDA application in the recording chamber (Fig 2 A1;A2). Acute NMDA application significantly decreased the instantaneous diffusion coefficient of NMDAR, and subsequently increased the confinement behavior of the receptor (Fig 2 B). Therefore, NMDA application, beyond activating the receptor, efficiently modifies its membrane trafficking in cultured DA neurons.

A remarkable feature of DA neurons is their reliable and robust pacemaker activity in acute midbrain slices (Shepard and Bunney, 1988; Grace and Onn, 1989; Wolfart and Roeper, 2002). Therefore, we performed electrophysiological recordings of DA neurons in acute VTA slices and assessed the impact of NMDA application on the neuronal firing pattern (Fig 2 C1). We privileged the cell-attached voltage clamp configuration for recording spontaneous firing activity to avoid dialysis of the cytoplasm that

might affect the firing pattern. The VTA is composed of a heterologous population of neurons such as GABAergic and DA neurons. In our experiments, these latter were identified by the presence of a hyperpolarization-activated current (Ih) (Johnson and North, 1992; Bonci and Malenka, 1999; Neuhoff et al., 2002) (Fig 2 C1). In addition, we further confirmed the DA phenotype of the recorded neurons by adding neurobiotin in the intracellular patch solution, and assessed the colocalization of the labelled neurons with TH immuno-staining (Fig 2 C2). Cell-attached recordings in voltage clamp showed that DA neurons exhibit a robust pacemaker activity in basal condition characterized by a mean frequency of ~1.7 Hz and a coefficient of variation of the inter-spike intervals (CV-ISI), which estimates the firing regularity (Fig 2 D1;D2), of ~10% (Figure 2 D1;D2). Bath application of NMDA (20 µM) induced a significant increase in both the frequency and CV-ISI of midbrain DA neurons ((Fig 2 D1;D2), indicating that they fired both faster and with a more irregular pattern. Consistent with former studies (Overton and Clark, 1992; Connelly and Shepard, 1997), these observations highlight the role of NMDAR activation in the frequency and regularity of the DA neuronal firing. However, NMDA activation will simultaneously activate the receptor and change its surface dynamic distribution, raising the possibility that the trafficking of NMDAR contributes to the neuronal firing pattern.

The basal surface dynamics, but not the spontaneous activity, of NMDAR tunes the firing pattern of midbrain DA neurons

To elucidate the relative contribution of the activation and membrane dynamics of NMDAR in the midbrain DA neuron firing pattern, we first investigated the impact of NMDAR blockade on this process. In basal conditions, the frequency of spontaneous excitatory post-synaptic events from VTA DA neurons was approximately ~0.14 Hz (data not shown), a value consistent with recordings from young SNc DA neurons (Pearlstein et al., 2015). Application of the NMDAR non-competitive open channel blocker MK-801 (10 µM) change neither the frequency nor the CV-ISI of DA neurons (Fig 3 A;B). As MK-801 only blocks opened receptors, we tested another NMDAR blocker: the competitive inhibitor D-APV (50 µM). Similarly to MK-801, neither the frequency, nor the CV-ISI were altered by D-

APV application (Fig 3 C,D). Thus, these results revealed that the spontaneous activation of NMDAR in VTA DA neurons does not contribute to the neuronal firing pattern.

We next investigated the putative role of NMDAR basal dynamics in this process. To this aim, we used a previously described protocol, i.e GluN1 subunit cross-link (X-link) that alters the lateral dynamics of NMDAR *in vitro*, *ex vivo* or *in vivo*, leaving intact their ionotropic transmission (Mikasova et al., 2012; Ladepeche et al., 2013; Potier et al., 2015). Schematically, the X-link consists of incubating neurons with primary antibodies directed against an extracellular epitope of the GluN1 subunit (Fig 4 A). As the X-link GluN1 was only described in hippocampal neurons, we first tested the reliability of this approach in midbrain DA neurons. We performed these experiments onto DAT-tdTomato neurons as DAT-tdTomato mice exhibit a greater colocalization rate with TH than TH-tdTomato mice (94% in midbrain slices, n= 3 mice, data not shown), and similar median diffusion coefficients of NMDAR onto cultured DA neurons (data not shown). NMDAR surface diffusion in DA neurons was imaged before and 30 minutes after X-link GluN1 (Fig 4 A). As expected, the NMDAR membrane dynamics was greatly reduced following X-link GluN1, with an increased proportion of immobile and confined receptors (Fig 4 B), indicating that X-link GluN1 strongly immobilized the receptors. Similarly, pre-incubating human iPSC-derived DA neurons with X-link GluN1 severely decreased the mobility of membrane NMDAR and confined the receptors (Fig 4C-D). Together, this result confirmed that X-link GluN1 modulates NMDAR surface trafficking in both mice and human DA neurons.

We next investigated the impact of such NMDAR immobilization on the firing activity of DA neurons. To this aim, we injected either IgG against GluN1 (so called, X-link GluN1) or control IgG (goat anti-rabbit) in the VTA of young rats (Fig 5 A1; A2). VTA DA neurons were recorded in acute slices 2-3h after the injection. To assess that neither the stereotaxic injection nor the presence of IgG itself modify the firing of DA neurons, we recorded pacemaker activity in acute slices from both naive rats (no surgery) and rats injected with control IgG, and compared the frequency and CV-ISI. The frequency and CV-ISI were undistinguishable between naive and control IgG rats (Fig 5 B1;B2), indicating that the injection procedure and the presence of IgG did not affect the firing of DA neurons. Although it has been

demonstrated that X-link GluN1 impairs NMDAR surface dynamics in hippocampal neurons without altering their current (Dupuis et al., 2014; Potier et al., 2015), we recorded the NMDAR and AMPAR-mediated currents in X-link or control IgG conditions. The AMPAR/NMDAR ratio was calculated as the ratio between the peak amplitude of evoked AMPAR-mediated excitatory postsynaptic currents (eEPSC) at -60 mV and the peak amplitude of evoked EPSC at +40mv, 50 ms after the onset to isolate the NMDAR-mediated component, in the presence of the $GABA_A$ antagonist bicuculline (20 µM) (Fig 5 C1). The AMPAR/NMDAR ratio from X-link GluN1 and control IgG were not statistically different (Fig 5 C2). In addition, the mean amplitude of the AMPAR- and NMDAR-mediated eEPSCs, as well as the NMDAR decay time, were not altered by GluN1 X-link (Fig 5 C2). The impact of X-link GluN1 on the VTA firing pattern was then tested by recording the spontaneous firing activity of DA neurons in the cell-attached voltage clamp configuration. Strikingly, the X-link GluN1 altered the firing pattern of midbrain DA neurons. The CV-ISI was increased in X-link GluN1 condition, reflecting an increase in the irregularity of the neuronal firing (Fig 5 E1;E2). Of note, the frequency was also decreased in X-link GluN1 condition (Fig 5 E2). Collectively, these data indicate that the basal surface dynamics, and not the spontaneous activity, of NMDAR tunes the firing pattern of midbrain DA neurons recorded in acute brain slices.

GABA neurons represent a significant proportion of VTA neurons (Nagai et al., 1983; Johnson and North, 1992; Nair-Roberts et al., 2008), which project locally on DA neurons and express NMDAR (Steffensen et al., 1998; Bonci and Malenka, 1999; Omelchenko and Sesack, 2009). Therefore, we tackled the possibility that X-link GluN1 altered GABAergic neuron activity and consequently the GABAergic drive onto VTA DA neurons. Spontaneous $GABA_A$ receptor ($GABA_AR$)-mediated IPSC were recorded from DA neurons in X-link GluN1 and control IgG conditions in presence of the AMPAR blocker NBQX (10 µM) (Fig 5 D1). Neither the amplitude, inter-event intervals, rise or decay time were affected by the GluN1 X-link (Fig 5 D2;D3), indicating that the $GABA_AR$-mediated transmission onto DA neurons is not likely to contribute to the firing pattern change observed after reducing NMDAR surface dynamics.

Downregulation of SK channel function in immobilized surface NMDAR condition

Similar change in the irregularity of midbrain DA neuron firing has been described in DA neurons expressing genetically- or pharmacologically (apamin)-inactivated SK channels (Ping and Shepard, 1996; Wolfart et al., 2001; Soden et al., 2013; Creed et al., 2016). This raised the intriguing possibility that the irregular pattern observed when NMDAR were artificially immobilized results from a downregulation of SK channel function. Indeed, SK channel are activated by calcium and functionally coupled to calcium sources such as NMDAR (Faber et al., 2005, 2008; Ngo-Anh et al., 2005) and other channels (Nedergaard et al., 1993; Wolfart et al., 2001; de Vrind et al., 2016). Given that such a coupling requires a close proximity between SK channel and its calcium source (Marrion and Tavalin, 1998; Jones and Stuart, 2013), an altered NMDAR trafficking and distribution might uncouple SK channels from calcium sources and tune the firing pattern of DA neurons. To test the functionality of SK channels, we used the SK channel blocker, apamin. Consistent with the role of SK channels in the firing of DA neurons (Deignan et al., 2012; Creed et al., 2016), apamin (200nM) modified the firing pattern, favoring an irregular pattern while the frequency was unchanged (Fig 6 A1;A2). We then compared the effect of apamin on the firing pattern of DA neurons in acute VTA slices from rats injected with either GluN1 antibodies or control IgG. Similarly to naive rats, SK channels blockade did not affect the firing frequency of neurons in both control IgG and X-link GluN1 conditions (Fig 6 B1). However, apamin was less effective in X-link GluN1 conditions, as the firing irregularity was increased by 300% in control IgG and only by 190% in X-link GluN1 conditions (Fig 6 B2). This suggests that injection of GluN1 antibodies impairs SK channels function. We then calculated the number of apamin responders in rats injected with control IgG or GluN1 antibodies. Neurons were considered as responders if the normalized CV-ISI after apamin was higher than the normalized CV before the drug plus one standard deviation. Consistent with the above data, the number of apamin responders in X-link GluN1 conditions (45%) was lower than in control IgG (75%) or naïve conditions (83%) (Fig 6 C). Altogether, these data demonstrate that blocking SK channels or disturbing NMDAR trafficking similarly promote DA neurons to fire with an irregular pattern in acute slice.

Such a downregulation of the SK channels function can originate from a reduced function or membrane content of the channel. To discriminate between these two possibilities, we estimated the membrane content of GluN1 subunits and SK3 channels, which are the predominant SK channel isoforms in the VTA (Wolfart et al., 2001; Bosch et al., 2002), in X-link GluN1 and control IgG conditions by western blot (Fig 6 D1). We report that GluN1 subunit and SK3 channels membrane fractions from midbrain slices were unchanged between these two conditions (Fig 6 D2). Therefore, it is unlikely that the decreased SK channels function originates from internalization. In conclusion, these data showed that *in vivo* acute modulation of NMDAR membrane trafficking by GluN1 X-link impaired the firing pattern of midbrain DA neurons, likely through a loss of function of SK channels.

DISCUSSION

Here, we investigated the role of NMDAR surface trafficking in the firing pattern of midbrain DA neurons. Using a combination of high-resolution single nanoparticle tracking and electrophysiology approaches, we showed that NMDAR are highly mobile in DA neurons from rodents and humans. Quite surprisingly, an acute alteration of NMDAR trafficking *in vivo* profoundly affects the firing pattern of DA neurons. Since this artificial manipulation did not alter the NMDAR transmission and since the pharmacological blockade of NMDAR did not alter neuronal firing pattern, it emerges that NMDAR surface trafficking *per se* plays a critical role in the regulation of the firing pattern of midbrain DA neurons. Such change in the firing was paralleled to a loss of function (no change in the membrane content) of the SK channels, fueling a model in which the NMDAR surface dynamics and distribution regulate the function of SK channel and thus the firing activity of DA neurons.

Alteration of NMDAR surface trafficking with X-link GluN1. To investigate the role of NMDAR trafficking, we used a crosslink GluN1 protocol previously described in hippocampal neurons (Dupuis et al., 2014; Potier et al., 2015). This manipulation consists in incubating neurons with antibodies directed against the GluN1 subunit to immobilize the receptors without altering their channel properties. We confirmed that this protocol strongly decreased NMDAR membrane dynamics in DA neurons from mice or human-derived iPSC neurons. Furthermore, injecting *in vivo* GluN1 antibodies (X-link GluN1) did not alter the synaptic function of NMDAR and the spontaneous $GABA_AR$-mediated transmission in DA neurons from acute midbrain slices. Therefore, X-link GluN1 enables to study specifically the role of NMDAR surface trafficking in DA neurons and does not involve indirect effects through neighboring GABAergic neurons. This protocol does not appear to change the membrane content of NMDAR, through favored endocytosis for instance. Indeed, western blot experiments showed that the GluN1 subunits membrane fraction was unchanged in X-link GluN1 and control IgG conditions. In addition, the NMDAR-mediated synaptic currents remained unaltered in these

conditions. Thus, consistent with former studies (Dupuis et al., 2014; Potier et al., 2015), our data indicate that X-link GluN1 does not favor NMDAR endocytosis or channel blockade.

Modulation of the firing pattern of DA neurons: unsuspected role of NMDAR basal dynamics. We found here that blocking NMDAR spontaneous activation with two different antagonists, either the open channel blocker MK-801 that mostly blocks synaptic NMDAR or the competitive D-APV that inhibits all membrane receptors, did not change the firing pattern of midbrain DA neurons. These results, consistent with the literature reporting that D-APV application was ineffective in modifying the firing pattern of midbrain DA neurons in acute slices (Seutin et al., 1990; Mereu et al., 1997), imply that the spontaneous activation of NMDAR does not contribute to the pacemaker activity of DA neurons in acute slices. By contrast, we observed that the acute alteration of the basal membrane dynamics of NMDAR affected the firing pattern of DA neurons. Besides, exogenous activation of NMDAR strongly modifies the firing pattern of DA neurons *in vitro* (Johnson et al., 1992; Johnson and Wu, 2004) and *in vivo* (Overton and Clark, 1992; Chergui et al., 1993). Therefore, we propose that NMDAR membrane dynamics modulates the firing pattern of midbrain DA neurons. In basal conditions, the receptor membrane dynamics, but not its ionotropic function, would mainly regulate the firing pattern whereas NMDAR ionotropic function would be required following strong channel activation.

Alteration of SK channels with X-link GluN1. As previously reported in the literature (Wolfart et al., 2001; Waroux et al., 2005; Ji and Shepard, 2006), we found here that SK channels inhibition increased the irregularity of the firing of midbrain DA neurons. Interestingly, a similar modification of the firing pattern was observed when NMDAR trafficking was altered by X-link GluN1, suggesting that both processes share similar mechanisms. In addition, the firing irregularity induced by SK channels blockade was less pronounced when NMDAR surface diffusion was impaired. Interestingly, the membrane content of SK channels was not altered in midbrain slices in this condition. Thus, these results suggest that the alteration of NMDAR surface trafficking affects the SK channels function, but

not its membrane expression. What cellular scenario could explain such an observation? The SK channels need calcium entry through calcium sources, among which NMDAR and voltage gated calcium channels such as T-type, N-type or L-type calcium channels (Nedergaard et al., 1993; Wolfart and Roeper, 2002; Paul et al., 2003; Ngo-Anh et al., 2005; de Vrind et al., 2016). The calcium influx through channels being restricted to micro- or nano-domains (Augustine et al., 2003), SK channels have been found in close proximity to NMDAR (Soden et al., 2013) and voltage gated calcium channels (Marrion and Tavalin, 1998; Jones and Stuart, 2013). Altering NMDAR surface trafficking likely disturbs NMDAR surface distribution, un-coupling SK channels from the receptor. Consequently, the decreased function of SK channels would increase the firing irregularity in midbrain DA neurons. Noteworthy, blocking the spontaneous activity of NMDAR did not alter the firing pattern, indicating that in basal conditions the functional coupling between NMDAR and SK channels does not contribute to the pacemaker activity. Alternatively, altered NMDAR membrane dynamics and distribution might modify the availability of key kinase and phosphatase proteins that regulate SK channels function such as the casein kinase II and the phosphatase 2A (Allen et al., 2007; Maingret et al., 2008). Indeed, both SK channels and NMDAR interact with these proteins (Chan and Sucher, 2001; Chung et al., 2004; Allen et al., 2007). Such a scenario is further supported by the former observation in hippocampal neurons that NMDAR surface dynamics control the spine content of the calmoduline kinase II (Dupuis et al., 2014). Therefore, we unveil a functional interplay between NMDAR surface dynamics and SK channels function, opening a new avenue of research to identify the molecular complex underlying this process.

Relevance of X-link GluN1 to *in vivo* physiology. In this study, we artificially alter NMDAR surface diffusion by injecting GluN1 antibodies. What kind of relevance such alteration may have in functional brains? Auto-antibodies against NMDAR were identified in the serum and the cerebrospinal fluid of patients suffering from a severe form of encephalitis (Dalmau et al., 2007) and psychiatric disorders (Tsutsui et al., 2012). These auto-antibodies recognized an epitope localized in the extracellular part of GluN1 subunits (Dalmau et al., 2008), profoundly altered NMDAR surface trafficking (Mikasova et

al., 2012; Dupuis et al., 2014), and blocked the synaptic long-term potentiation LTP in hippocampal neurons (Dupuis et al., 2014; Jezequel et al, 2017). Therefore, these studies highlight the link between alteration of NMDAR surface dynamics and psychiatric conditions. Whether these human autoantibodies alter the firing pattern of DA neurons, through a dysfunction of the receptor surface dynamics, is an opened and intriguing question.

Changes in the firing pattern of midbrain DA neurons have been associated with the coding of reward and salient information (Schultz, 2010) and are also observed in neuropsychiatric disorders such as in animal models of schizophrenia or in response to drug exposure (Matthews and German, 1984; Mereu et al., 1984). Given the crucial role of DA neurons, a lot of efforts have been put to understand the physiology of these neurons. So far, studies investigating the regulation of the firing pattern mainly focused on testing the role of channels/receptors ionotropic functions (Johnson et al., 1992; Ji and Shepard, 2006; Herrik et al., 2010, 2012). Investigating the surface dynamics of receptors and channels in controlling DA neurons firing is likely to shed new and unsuspected lights.

MATERIELS AND METHODS

All experiments were carried out in accordance with University of Bordeaux guidelines and regulations. The animal procedures were approved by the ethical committee of the University of Bordeaux (Laurent Groc experimentation authorization number 3306009).

Neuronal cultures

Postnatal mice midbrain culture. Tyrosine hydroxylase (TH) – tdTomato and dopamine transporter (DAT)-tdTomato mice were generated by crossing TH-Cre (Savitt et al., 2005) or DAT-Cre mice (Turiault et al., 2007)with Cre-reporter line tdTomato Ai9 (Madisen et al., 2010). Neonatal pups were sacrificed by decapitation. The brains were quickly removed and put on petri dish containing cold Leibovitz's L-15 medium (ThermoFisher). The mesencephalon was dissected and incubated at 37°C for 15 minutes with a papain solution for chemical dissociation. Then, the neurons were mechanically dissociated by going up and down with pipettes, and the cellular suspension was centrifugated at 300 Xg for 5 minutes. The supernatant was removed and 1 mL of DMEM (ThermoFisher) supplemented with fetal bovine serum (10%, ThermoFisher) was added. The viable cells were counted with a Malassey cell and plated at a concentration of 150 000 cell/mL on poly-lysine pre-coated coverslips, in petri dish containing 5 mL of DMEM. Cultures were maintained at 37°c in an incubator (5% C02). Two days later, the medium was replaced by EF12 medium.

Human iPSC-derived DA neurons. The bDopa.4U cell culture was obtained from Ncardia (USA) and prepared according to the bDopa.4U handling guide. Briefly, the cryopreserved culture (2 million cells/vial) was thawed with a Neuro.4U basal medium supplemented with Neuro-Supplement 4. Then, the neurons were seeded (50 000 cells/cm^2) and cultured with bDopa.4U culture media on coverslips coated with poly-L-ornithine (10 µg/ml, diluted in PBS, Sigma) and laminin (10 µg/ml, diluted in PBS, Roche). The medium was changed twice a week by replacing half of the medium with the same amount of fresh medium. Experiments were done on cultures from 9 to 13 days *in vitro* (DIV).

Single particle tracking of NMDAR

Postnatal midbrain cultures (10-12 DIV) or human iPSC-derived DA neurons (11-13 DIV) were incubated 15 minutes with rabbit antibodies (Ab) against GluN1 (1/200 Alomone labs, Jerusalem) at 37°C. After 3 washes with equilibrated EF12 medium, coverslips were incubated 10 minutes with quantum dot (QD) 655 coated with goat Ab fragments anti rabbit (1/10 000, Invitrogen) at 37°C. Non-specific binding was blocked by adding 1% of bovine serum albumin (BSA, Sigma) during antibody and QD incubation. After 3 washes with EF12, coverslips were mounted on a heated chamber for image acquisition. A wild field epifluorescence microscope (Nikon) equipped with a mercury lamp, a Evolve EMCCD camera (Photometrics) and appropriate emission/excitation filters was used to detect QD (QD 655) and TH fluorescence (tdTomato). 500 consecutive frames were recorded at 20 Hz and processed with Metamorph software (Molecular Devices). Recording sessions did not exceed 30 minutes to minimize receptor endocytosis. The instantaneous coefficient of diffusion of GluN1, which reflects the diffusion speed, was calculated for each trajectory, from linear fits of the first four points of the mean square displacement vs. time function using $MSD(t) = \langle r^2 \rangle (t) = 4Dr$.

Stereotaxic injections of antibodies

Antibodies (GluN1 or control immunoglobulins (IgG)) were injected in male and female Sprague Dawley rats between 12 postnatal day (PD 12) and PD 16. After ip injection of buprenorphine (0.05mg/kg), the animals were anesthetized and maintained with isoflurane during the surgery at 5% and 2.5% respectively. Then, the rats were placed on a stereotaxic frame and constantly warm at 37°C with a heating pad during the surgery. After subcutaneous injection of lurocaine (50µL), a bilateral craniotomy was made in the VTA at the following coordinates (AP : -5.60 to 6.10, L : 0.85 and P : -5.60 to -5.80). To inject the antibodies (Ab), glass capillaries were pulled with a horizontal puller. 1,5 µL of solution containing GluN1 Ab or control IgG (diluted at 1/5 with bromophenol blue) were injected per side and the pipette was maintained during at least 3 minutes to avoid liquid reflux. Once the injections

were done, the wound was closed with surgical glue and the animal was allowed to recover during 2-3 h.

Electrophysiology

Slices preparation. Electrophysiological recording were performed on Sprague Dawley rats (male and female), between PD 12 and PD 16. After isoflurane anesthesia, animals were sacrificed by decapitation.The brain was quickly removed and put into cooled artificial cerebro-spinal fluid (ACSF) - Sucrose solution containing (in mM): 250 Sucrose, 2 KCl, 7 $MgCl_2$, 0.5 $CaCl_2$, 1.15 NaH_2PO_4, 11 glucose, and 26 $NaHCO_3$, and bubbled with 95% O_2 and 5% CO_2. Horizontal slices (250 µM) containing the ventral tegmental area (VTA) were prepared in ACSF-Sucrose using a vibratome (Leica Instruments, Germany), and then transferred in an ACSF solution containing (in mM): 126 NaCl, 3.5 KCl, 2 $CaCl_2$, 1.3 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, and 12.1 glucose bubbled with 95% O_2 and 5% CO_2. Slices were first maintained at 34°C for 40 minutes and then at room temperature.

Patch clamp recordings. Slices were submerged and continuously perfused (2.5 mL/min) at 30-31°C. The VTA was determined as the area surrounded by the rostral interstitial nucleus of medial longitudinal fasciculus and the medial terminal nucleus of the accessory optic tract (Atlas Paxinos). Dopaminergic neurons were identified by their regular firing at low frequency (between 1 and 4 Hz, Grace & Onn, 1989) in cell-attached mode and the presence of an Ih current in whole-cell mode (Neuhoff, 2002). The presence of Ih was assessed by -20mV steps from -60 to -140 mV in voltage clamp mode and/or by -50 pA steps from 0 to -200 pA in current clamp to see a sag potential. Recordings were made using an HEKA Patch clamp amplifier (double EPC 10 USB, Harvard BioScience, US). For the cell-attached mode, pulled pipettes (R>10mΩ) containing (in mM) : 142 potassium gluconate, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 EGTA, 2 Na_2ATP; Na_3GTP (pH 7.3-7.4, osmolarity between 290-300mosm) were used. Spontaneous activity of VTA DA neurons in the cell-attached voltage clamp was recorded at the holding potential that gives a holding current of 0 pA (Perkins, 2006). Basal firing activity was recorded

for 10 minutes and when a drug was applied, the firing activity was recorded at least after 5 minutes of drug perfusion. For the whole-cell mode, 5-6 MΩ pipettes were filled with either $CsMeSO_4$- or KCl-based solutions to measure the evoked excitatory postsynaptic currents (eEPSC) and the spontaneous inhibitory postsynaptic currents (sIPSC), respectively. The composition of each solution was (in Mm) : 135 $CsMeSO_4$, 8 NaCl, 10 HEPES, 4 Na_2ATP, Na_3GTP, 5 TEA-Cl, 10 EGTA for $CsMeSO_4$- ; 142 KCl, 10 HEPES, 8 NaCl, 4 Mg_2ATP, 0.3 Na_3GTP, 0.5 EGTA for KCl-. For some immunohistochemistry experiments, neurobiotin (0.1%) was added in the internal solution to label the recorded neurons. To measure AMPAR/NMDAR ratio, AMPAR and NMDAR-mediated synaptic currents were evoked at 0.05Hz with bipolar electrodes placed rostral to the VTA, and recorded with the $GABA_A$ receptor blocker bicuculline (20µM) at a holding potential of -60 and +40mV, respectively. Because both AMPAR and NMDAR are activated at +40 mV, NMDAR-mediated synaptic current amplitude was measured 50 ms after the onset of the mixed EPSC. The sIPSC were recorded at -60 mV in presence of the AMPAR blocker NBQX (10µM) during 10 minutes. For both eEPSC and sIPSC recordings, the access resistance was monitored by a hyperpolarizing step of -5 mV for each sweep and experiments were discarded if the access resistance varied more than 20%.

Drugs. Electrophysiology drugs : NBQX (10 µM), MK-801 (10 µM), Bicuculline methochloride (20 µM), D-APV (50 µM), NMDA (20µM) and Apamin (200 nM) were purchased at Tocris (UK). Neurobiotin (0.1%) was obtained from Vectorlabs (US).

Immunostaining

Cell culture. Mice postnatal midbrain culture and iSPC-derived DA neurons (9-12 DIV) were fixed 10 minutes with paraformaldehyde (PFA) 4%. After PBS washes, cells were incubated 10 minutes with NH_4Cl to eliminate the background noise, permeabilized with 0.1% Triton X-100 in PBS and saturated with bovine serum albumin (BSA) 1%. Coverslips were then incubated 1h with primary antibodies (anti TH, 1/1000, Millipore) followed by 30 minutes with secondary antibodies (donkey anti mouse

Alexa488, 1/1000, Invitrogen) after washes in PBS, at room temperature. Coverslips were mounted on glass slides with Mowiol medium and kept in a black box at 4°C before imaging.

Brain slices. After patching neurons with an intracellular solution containing neurobiotin (0.1%), acute VTA slices were washed with PBS and saturated 2h with a PBS solution supplemented with 2% normal donkey serum and 0.1% Triton. Primary antibodies (mouse anti TH, 1/1000, Millipore) were incubated overnight at 4°C and after PBS washes, slices were incubated 2h with the secondary antibodies (streptavidine A488, 1/1000, ThermoFisher and donkey anti-mouse A568, 1/1000, Invitrogen) at room temperature. After washes, floating slices were mounted on glass slides with Vectashield medium (Vectorlabs).

Western blot experiments

Horizontal midbrain slices (250 µm thick) were homogenized and dissociated in STE (0.32M sucrose, 20mM tris pH8, 2mM EDTA, and protease inhibitor cocktail (1:1000, Calbiochem)). After centrifugation (1,000 g; 10min; 4°C), the supernatant was saved and centrifuged (20,000g; 1h; 4°C). Supernatants were collected and kept at -80°C. Before loading on a gel, the samples were boiled at 95°C for 5 minutes. 30µL of samples were separated by SDS/PAGE (Mini-Protean TGX precast gels 7.5% Stain-Free, Biorad) for 1h at 150V and blotted onto nitrocellulose membrane during 1h at 100V. After blocking 1h in 5% milk in Tris-saline – 0.05% tween 20 (TBST), the membranes were hybridized with an anti-SK3 Ab (3.2µg/mL, Rabbit polyclonal Ab, Alomone) or an anti-GluN1 (0.5µg/mL, Mouse monoclonal Ab, BD Biosciences), diluted in TBST 5% milk, during overnight at 4°C. Corresponding secondary antibodies were used at 1/5000 in TBST 5% milk. Detection was performed using the SuperSignal West Dura Extended Duration Substrate detection System (ThermoFisher) revealed with a ChemiDoc system (Biorad). Quantification of band intensity was performed using Image Lab software (Biorad); GluN1 and SK3 detection were normalized on total protein detection with Stain-Free technology.

Data and statistical analysis

For single particle tracking, the analysis was performed with Metamorph software. The instantaneous coefficients of diffusion were expressed as median +/- interquartile range (IQR) 25%-75%, and compared with unpaired Mann-Whitney tests. Electrophysiology data were analyzed with Clampfit software 10.7.03, except sIPSC that were analyzed with Mini analysis software. Group values are expressed as mean +/- SEM. Data were compared with impaired or paired Student's t tests. Significance levels were defined at *p<0.05, **p< 0.01, ***p<0.001, ****p<0.0001

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Figure 1

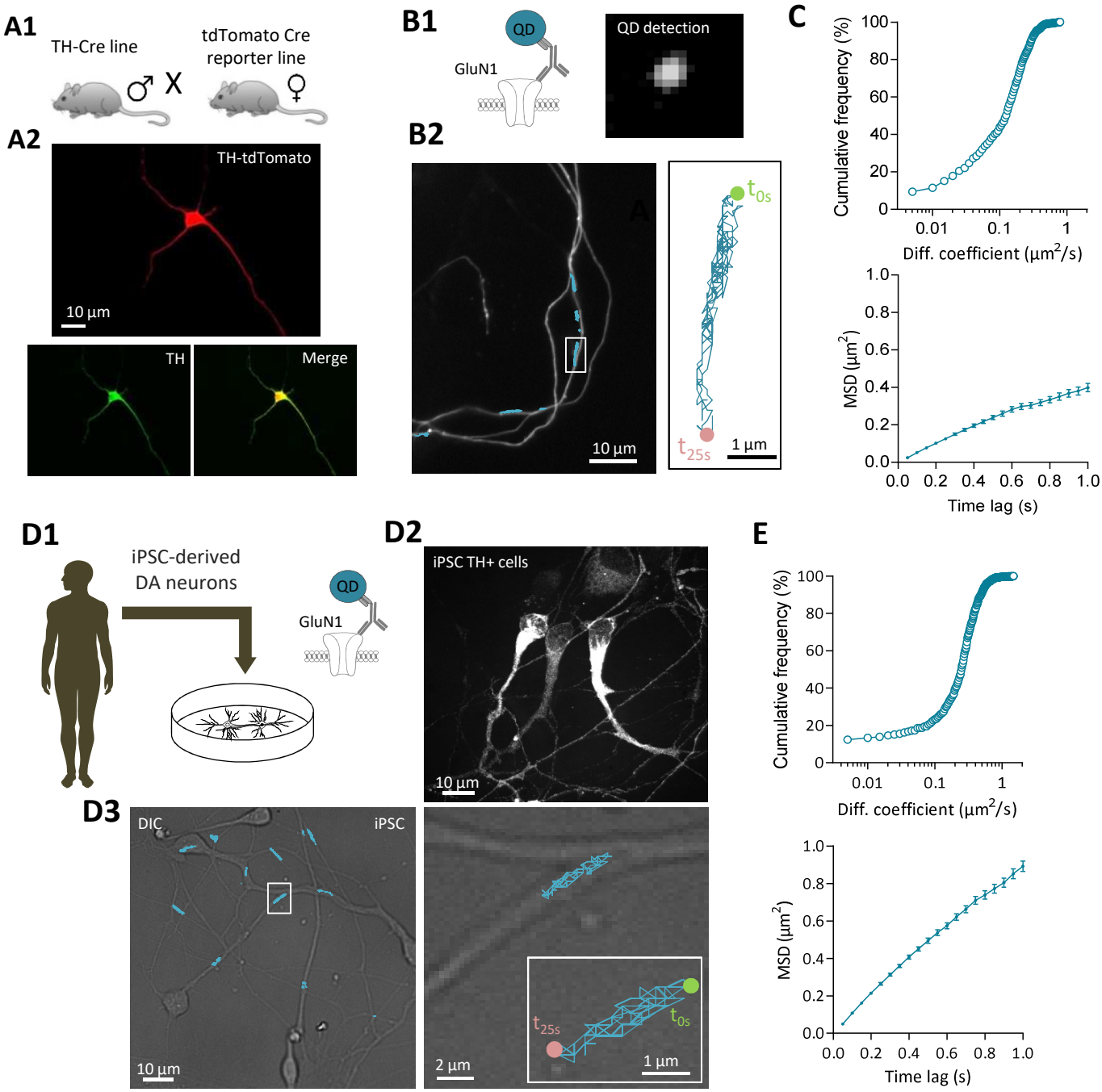
A1
TH-Cre line
tdTomato Cre reporter line
X
A2
TH-tdTomato
10 µm
TH
Merge
B1
QD
GluN1
QD detection
B2
A
10 µm
t_{0s}
t_{25s}
1 µm
C
Cumulative frequency (%)
Diff. coefficient (µm²/s)
MSD (µm²)
Time lag (s)
D1
iPSC-derived DA neurons
QD
GluN1
D2
iPSC TH+ cells
10 µm
D3
DIC
iPSC
10 µm
2 µm
t_{25s}
t_{0s}
1 µm
E
Cumulative frequency (%)
Diff. coefficient (µm²/s)
MSD (µm²)
Time lag (s)

Fig 1. Endogenous NMDAR diffuse at the surface of dopaminergic neurons both from mice midbrain culture and derived from human iPSC.

A1) TH-tdTomato mice were obtained from male TH-Cre mice crossed with female Cre reporter tdTomato Ai9 mice. A2) Example of a fluorescent TH-tdTomato neuron (9 DIV) that is TH immuno-positive, in postnatal mice midbrain culture (86% of tdTomato/TH colocalization, n=3 cultures).

B1) Schematic drawing of a single QD-antibody complex targeting the GuN1 subunit of surface NMDAR (left). QD were detected with an exposure time of 50 ms (right). B2) Representative trajectories (25 s, 20 Hz acquisition, blue traces) of QD-GluN1 complexes on neurites from a TH-tdTomato neuron. Insert, Magnified reconstructed trajectory of a single QD-GluN1 complex.

C) Top, Cumulative distribution of the NMDAR instantaneous diffusion coefficient on TH-tdTomato neurons (median diffusion coefficient = 0.13 $\mu m^2/s$, IQR = 0.04-0.21 $\mu m^2/s$, n=750 trajectories, 5 different cultures). The immobile fraction, indicated by the first point of the curve, is 9%. Down, Plot of the mean square displacement (MSD) over time of QD-GluN1 trajectories.

D1) Schematic drawing of the experimental procedure to track membrane NMDAR on human iPSC-derived DA neurons. D2) Example of TH immuno-positive human iPSC-derived DA neurons. D3) DIC images of cultured human iPSC-derived DA neurons with representative membrane QD-GluN1 trajectories (blue traces). Insert, Magnified reconstructed trajectory of a single QD-GluN1 complex (25 s, 20 Hz acquisition).

E) Top, Cumulative distribution of the NMDAR instantaneous diffusion coefficient on human iPSC-derived DA neurons (median diffusion coefficient = 0.25 $\mu m^2/s$, IQR = 0.12-0.36 $\mu m^2/s$, n=738 trajectories, 1 culture). The immobile fraction is 12.5 %. Down, Plot of the MSD over time of QD-GluN1 trajectories.

Figure 2

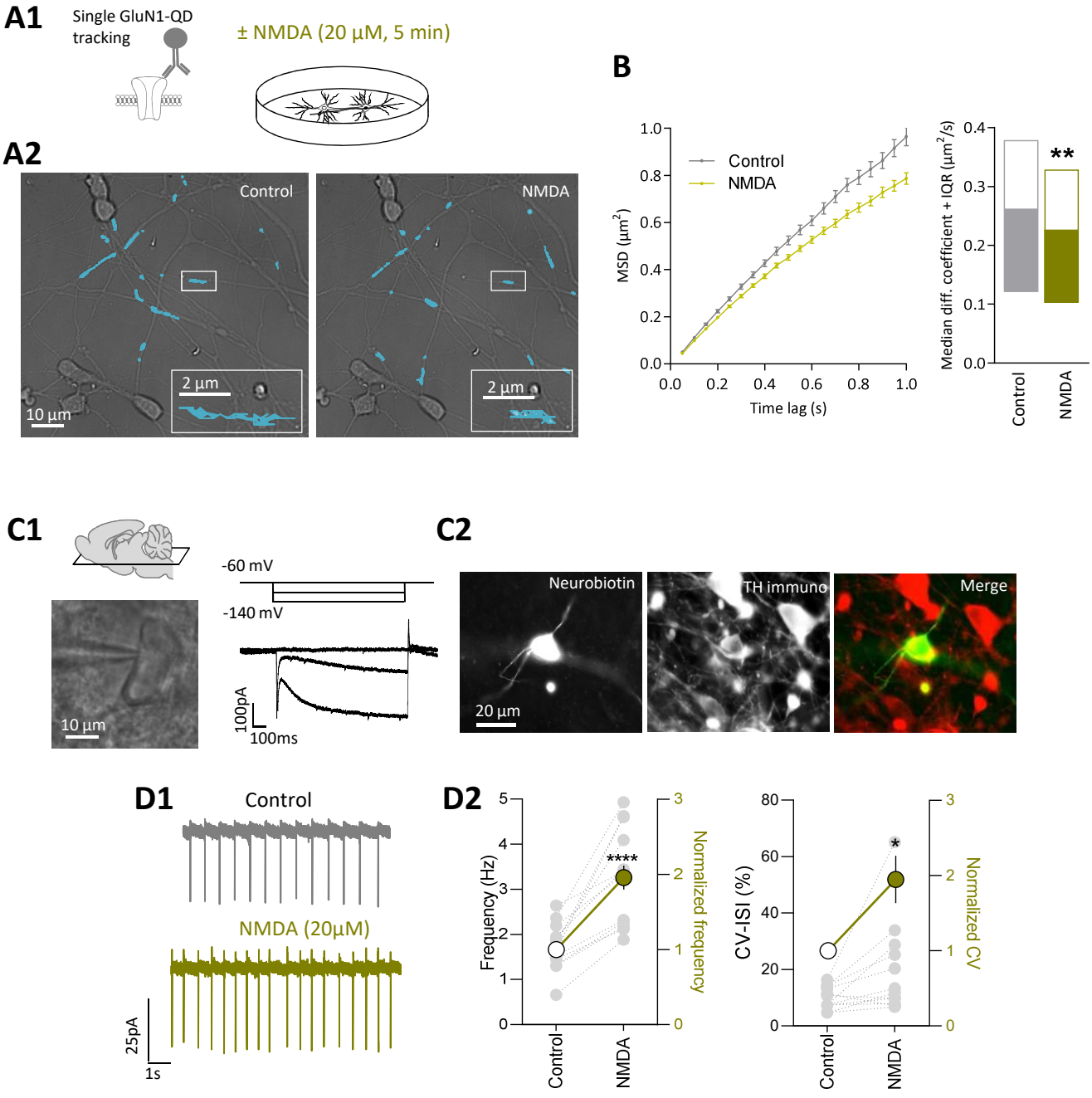
A1
Single GluN1-QD tracking
± NMDA (20 µM, 5 min)
A2
Control
NMDA
2 µm
10 µm
B
Control
NMDA
MSD (µm²)
Time lag (s)
Median diff. coefficient + IQR (µm²/s)
**
C1
-60 mV
-140 mV
100pA
100ms
10 µm
C2
Neurobiotin
TH immuno
Merge
20 µm
D1
Control
NMDA (20µM)
25pA
1s
D2
Frequency (Hz)
Normalized frequency
****
CV-ISI (%)
Normalized CV
*

Fig 2. **NMDAR activation modifies the receptor membrane dynamics on human iPSC-derived DA neurons and the firing pattern of DA neurons in VTA acute slices.**

A1) Schematic drawing of the experimental procedure to track surface NMDAR following pharmacological NMDAR activation. A2) DIC images of human iPSC-derived DA neurons with representative QD-GluN1 trajectories (blue traces) before (left) and after NMDA application (20 µM, right).

B) Left, Plot of the MSD over time of QD-GluN1 trajectories on human iPSC-derived DA neurons before and after NMDA application. Right, Median NMDAR diffusion coefficients before (0.26 $\mu m^2/s$, IQR = 0.12-0.37 $\mu m^2/s$, n=462 trajectories, 1 culture) and after NMDA (0.23 $\mu m^2/s$, IQR = 0.10-0.33 $\mu m^2/s$, n=817 trajectories, 1 culture). Unpaired Mann Whitney test, ** p = 0.0053.

C1) Left, Schematic drawing of the section plane (horizontal) for VTA acute slices (up) and transmission image of a patched neuron (down). Right, Example of an Ih current on a midbrain DA neuron by stepping the membrane potential from -60 to -140 mV (increment of 40 mV). C2) Example of a patched neuron filled with neurobiotin (0.1%) positive for TH immunolabelling.

D1) Representative firing pattern of a midbrain DA neuron recorded in VTA slices in cell-attached voltage clamp mode before (control) and after NMDA application (20 µM, 5 minutes). D2) Left, Firing frequency (left Y axis) and normalized frequency (right Y axis) of midbrain DA neurons before (1.72 ± 0.15 Hz, n=12 neurons) and after NMDA application (3.26 ± 0.32 Hz; mean normalized frequency = 1.96 ± 0.16, n=12). Paired Student's t test, **** p<0.0001. Right, Coefficients of variation of the inter-spike intervals (CV-ISI) (left Y axis) and normalized CV-ISI (right Y axis) before (9.91 ± 1.15, n=12) and after NMDA application (19.99 ± 1.15; normalized CV-ISI = 1.95 ± 0.32, n = 12). Paired Student's t test, * p=0.0121. Each frequency and CV-ISI values after drug application were normalized to the values before the drug. Data are expressed as mean values ± SEM.

Figure 3

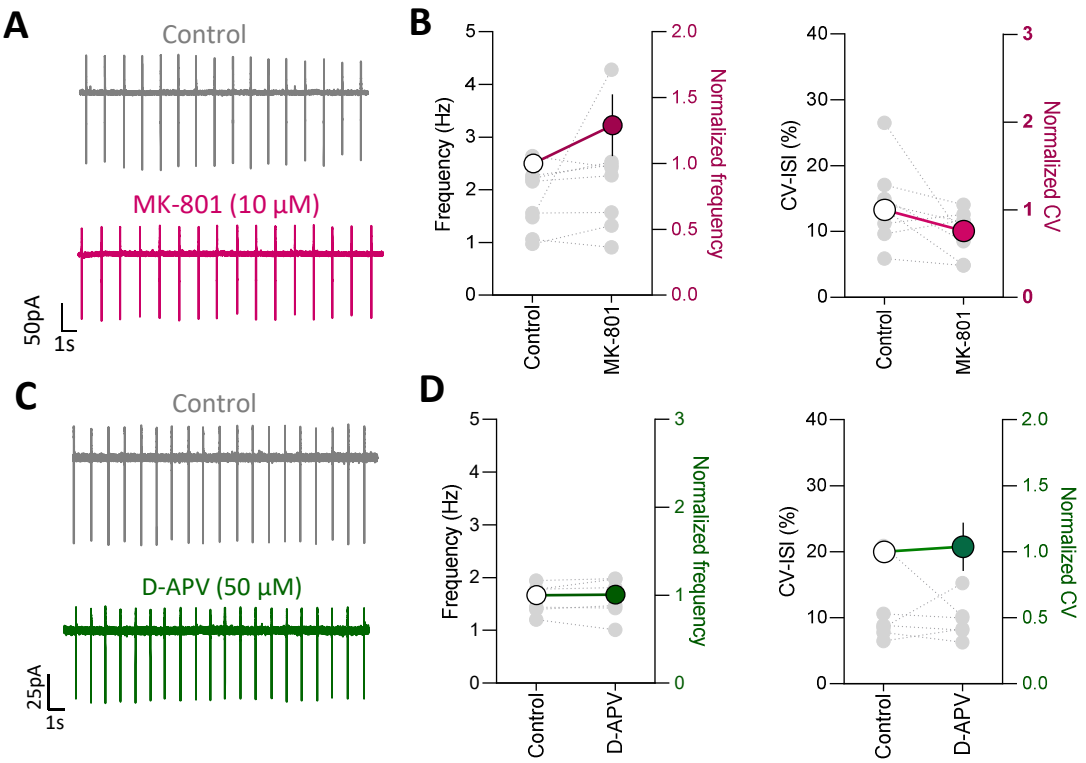
A
Control
MK-801 (10 µM)
50pA
1s
B
Frequency (Hz)
Normalized frequency
Control
MK-801
CV-ISI (%)
Normalized CV
C
Control
D-APV (50 µM)
25pA
1s
D
Frequency (Hz)
Normalized frequency
Control
D-APV
CV-ISI (%)
Normalized CV

Fig 3. Pharmacological blockade of NMDAR does not affect the firing pattern of midbrain DA neurons in VTA slices.

A) Firing pattern of a midbrain DA neuron in VTA slices before (control) and after bath application of the open channel blocker MK-801 (10 µM, 5-10 minutes).

B) Left, Frequency (left Y axis) and normalized frequency (right Y axis) before (1.79 ± 0.22 Hz, n=8 neurons) and after MK-801 application (2.22 ± 0.36 Hz; normalized frequency = 1.29 ± 0.24, n=8). Paired Student's t test, p=0.257. Right, CV-ISI (left Y axis) and normalized CV-ISI (right Y axis) before (14.09 ± 2.15, n=8) and after MK-801 (9.61 ± 1.22; normalized CV-ISI = 0.76 ± 0.11, n=8). Paired Student's t test, p=0.068.

C) Firing pattern of a DA neuron in VTA slices before (control) and after bath application of the competitive antagonist D-APV (50 µM, 5-10 minutes).

D) Left, Frequency and normalized frequency before (1.59 ± 0.12 Hz, n=6 neurons) and after D-APV (1.61 ± 0.16 Hz; normalized frequency = 1.01 ± 0.04, n=6). Paired Student t-test, p = 0.898. Right, CV-ISI and normalized CV-ISI before (10.51 ± 2.14, n=6) and after D-APV application (9.69 ± 1.26; normalized CV-ISI = 1.04 ± 0.18, n=6). Paired Student's t test, p = 0.842. Each frequency and CV-ISI values after drug application were normalized to the values before the drug. Data are expressed as mean values ± SEM.

Figure 4

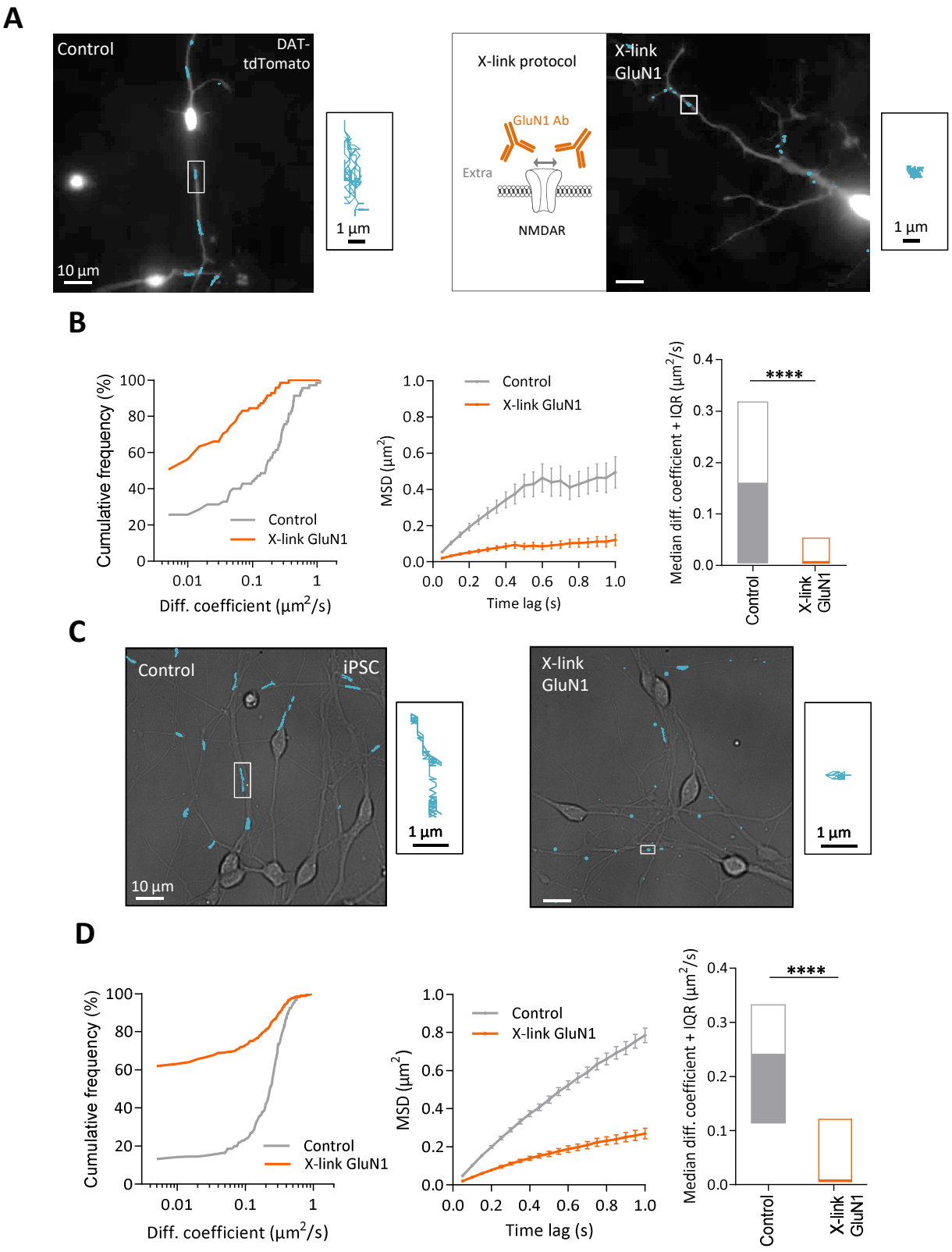
A
Control
DAT-tdTomato
10 µm
1 µm
X-link protocol
GluN1 Ab
Extra
NMDAR
X-link GluN1
1 µm
B
Cumulative frequency (%)
Control
X-link GluN1
Diff. coefficient (µm²/s)
MSD (µm²)
Time lag (s)
Median diff. coefficient + IQR (µm²/s)
****
Control
X-link GluN1
C
Control
iPSC
10 µm
1 µm
X-link GluN1
1 µm
D
Cumulative frequency (%)
Control
X-link GluN1
Diff. coefficient (µm²/s)
MSD (µm²)
Time lag (s)
Median diff. coefficient + IQR (µm²/s)
****
Control
X-link GluN1

Fig 4. X-link GluN1 strongly reduces the NMDAR surface diffusion in DA neurons from mice midbrain culture and derived from human iPSC.

A) Example of fluorescent DAT-tdTomato neurons with representative QD-NMDAR trajectories (blue traces) in control (left) and X-link GluN1 conditions (right). Middle panel, Schematic drawing of the NMDAR X-link with primary GluN1 Ab (1/5, 30 minutes incubation). Inserts, Magnified reconstructed trajectories of single QD-GluN1 complexes in control (left) and X-link GluN1 conditions (right).

B) Left, Cumulative distribution of the NMDAR instantaneous diffusion coefficients in DAT-tdTomato neurons before (n= 70 trajectories, 1 culture) and after X-link GluN1 (n= 71 trajectories, 1 culture). The immobile fractions are 26% and 51% in control and X-link GluN1 conditions, respectively. Middle, Plot of the MSD over time of QD-GluN1 trajectories before and after X-link GluN1. Right, Median NMDAR diffusion coefficients before (0.16 $\mu m^2/s$, IQR = 0.01-0.32 $\mu m^2/s$) and after X-link GluN1 (0.01 $\mu m^2/s$, IQR = 0.0003-0.0507$\mu m^2/s$). Unpaired Mann Whitney test, **** $p < 0.0001$.

C) DIC images of human iPSC-derived DA neurons with representatives QD-GluN1 trajectories (blue traces) in control (left) and X-link GluN1 conditions (1/5, 30 minutes incubation, right). Inserts, Magnified trajectories of single QD-NMDAR complexes in control (left) and X-link GluN1 conditions (right).

D) Cumulative distribution of the NMDAR instantaneous diffusion coefficients in human iPSC-derived DA neurons before (n= 276 trajectories, 1 culture) and after X-link GluN1 (n= 501 trajectories, 1 culture). The immobile fractions are 13% and 62% in control and X-link GluN1 conditions, respectively. Middle, Plot of the MSD over time of QD-GluN1 trajectories before and after X-link GluN1. Right, Median NMDAR diffusion coefficients before (0.23 $\mu m^2/s$, IQR = 0.12-0.33 $\mu m^2/s$) and after X-link GluN1 (0.0004$\mu m^2/s$, IQR = 0.00006-0.0118 $\mu m^2/s$). Unpaired Mann Whitney test, **** $p < 0.0001$.

Figure 5

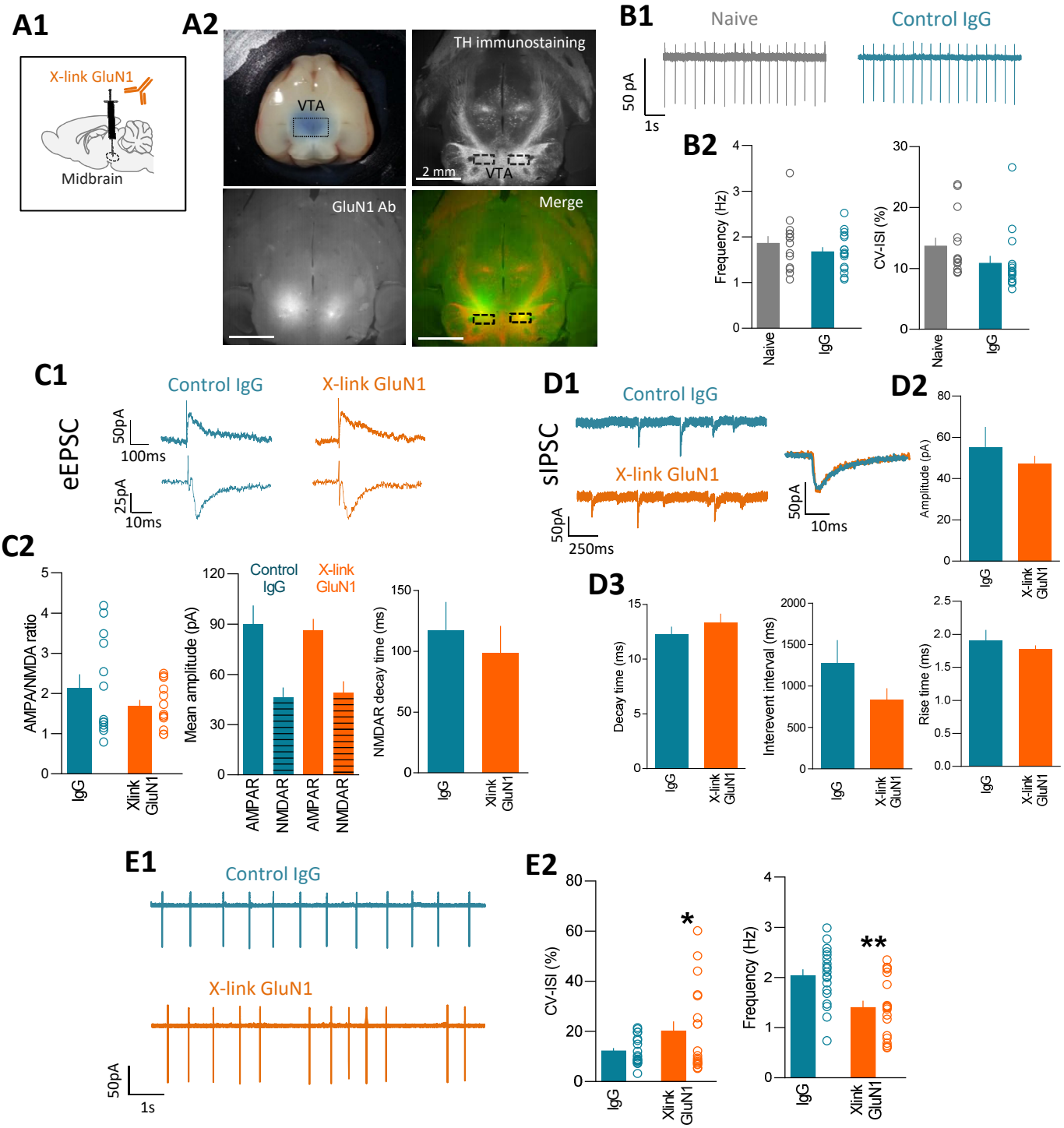
A1
X-link GluN1
Midbrain
A2
VTA
TH immunostaining
2 mm
VTA
GluN1 Ab
Merge
B1
Naive
Control IgG
50 pA
1s
B2
Frequency (Hz)
CV-ISI (%)
Naive
IgG
C1
Control IgG
X-link GluN1
eEPSC
50pA
100ms
25pA
10ms
C2
AMPA/NMDA ratio
IgG
Xlink GluN1
Mean amplitude (pA)
Control IgG
X-link GluN1
AMPAR
NMDAR
AMPAR
NMDAR
NMDAR decay time (ms)
IgG
Xlink GluN1
D1
Control IgG
X-link GluN1
sIPSC
50pA
250ms
50pA
10ms
D2
Amplitude (pA)
IgG
X-link GluN1
D3
Decay time (ms)
Interevent interval (ms)
Rise time (ms)
IgG
X-link GluN1
E1
Control IgG
X-link GluN1
50pA
1s
E2
CV-ISI (%)
*
Frequency (Hz)
**
IgG
Xlink GluN1

Fig 5. Acute alteration of NMDAR membrane dynamics modifies the firing pattern of midbrain DA neurons in VTA slices, without affecting NMDAR- and $GABA_A$ receptor-mediated synaptic currents.
A1) Schematic drawing of the *in vivo* X-link, based on the stereotaxic injection of GluN1 antibodies (Ab) in the midbrain. A2) Horizontal brain section showing the injection site identified with bromophenol blue (top left). Injected GluN1 Ab revealed by immunolabelling are present in the VTA, localized thanks to the TH immunostaining.
B1) Representative firing pattern of DA neurons in VTA slices from a naive and control IgG (goat anti rabbit) injected rats. B2) Firing frequency (left) (1.86 ± 0.16 Hz, n=14 neurons for naïve and 1.67 ± 0.10 Hz, n=16 for control IgG) and CV-ISI (right) (13.68 ± 1.37, n=14 for naive and 10.84 ± 1.23, n=16 for control IgG) of DA neurons from naive and control IgG injected rats. Unpaired Student's t tests, p=0.326 for the frequency, and p=0.132 for the CV-ISI. Data are expressed as mean ± SEM.
C1) AMPA receptors (AMPAR)-mediated evoked excitatory postsynaptic currents (eEPSC, below) and mixed AMPAR/ NMDAR-mediated eEPSC (top) recorded in whole cell configuration at -60 and +40 mV respectively, in the presence of bicuculline (20 µM), in control IgG and X-link GluN1 conditions. The amplitude of NMDAR-eEPSC was obtained 50 ms after the onset of the eEPSC. C2) AMPAR/NMDAR ratio (2.14 ± 0.34, n=13 neurons for control IgG and 1.89 ± 0.18, n=13 for X-link GluN1), mean amplitudes of NMDAR- (46.38 ± 5.85 pA, n=13 for control IgG and 48.91 ± 6.99 pA, n=13 for X-link GluN1, middle) and AMPAR-eEPSC (89.98 ± 11.13 pA, n=13 for control IgG and 86.25 ± 6.86 pA, n=13 for X-link GluN1), and NMDAR decay time (117.00 ± 23.54 ms, n=13 for control IgG and 98.68 ± 22.29 ms, n=13 for X-link GluN1). Unpaired Student's t tests : p=0.513, p=0.784, p=0.777, p=0.579 respectively.
D1) Left, Spontaneous inhibitory postsynaptic currents (siPSC) recorded in DA neurons at -60 mV in presence of NBQX (10 µM) in VTA acute slices from control IgG and X-link GluN1 injected rats. Right, Overlay of single sIPSC in control IgG and X-link GluN1 conditions. D2) Mean amplitudes (56.49 ± 9.56 pA, n=6 neurons for control IgG and 47.38 ± 3.65 pA, n=7 for X-link GluN1). D3). Decay time (12.35 ± 0.67 ms, n=6 for control IgG and 13.31 ± 0.85 ms, n=7 for X-link GluN1), inter-event intervals (1378 ± 282 ms, n=6 for control IgG and 836 ± 138 ms, n=7 for X-link GluN1), and rise time (1.90 ± 0.17 ms, n=6 for control IgG and 1.77 ± 0.07 ms, n=7 for X-link GluN1) of sIPSC. Unpaired Student's t test : p=0.437, p=0.407, p= 0.097, p=0.459, respectively.
E1) Representative firing pattern of DA neurons in VTA slices from control IgG or X-link GluN1 injected rats. E2) Firing frequency (2.03 ± 0.13 Hz, n=19 neurons for control IgG and 1.40 ± 0.14 Hz, n=19 cells for X-link GluN1) and CV-ISI (12.14 ± 1.27, n=19 for control IgG and 20.15 ± 3.89, n=19 for X-link GluN1). Unpaired Student's t tests : **p=0.0018,* p=0.0291, respectively. Data are expressed as mean ± SEM.

Figure 6

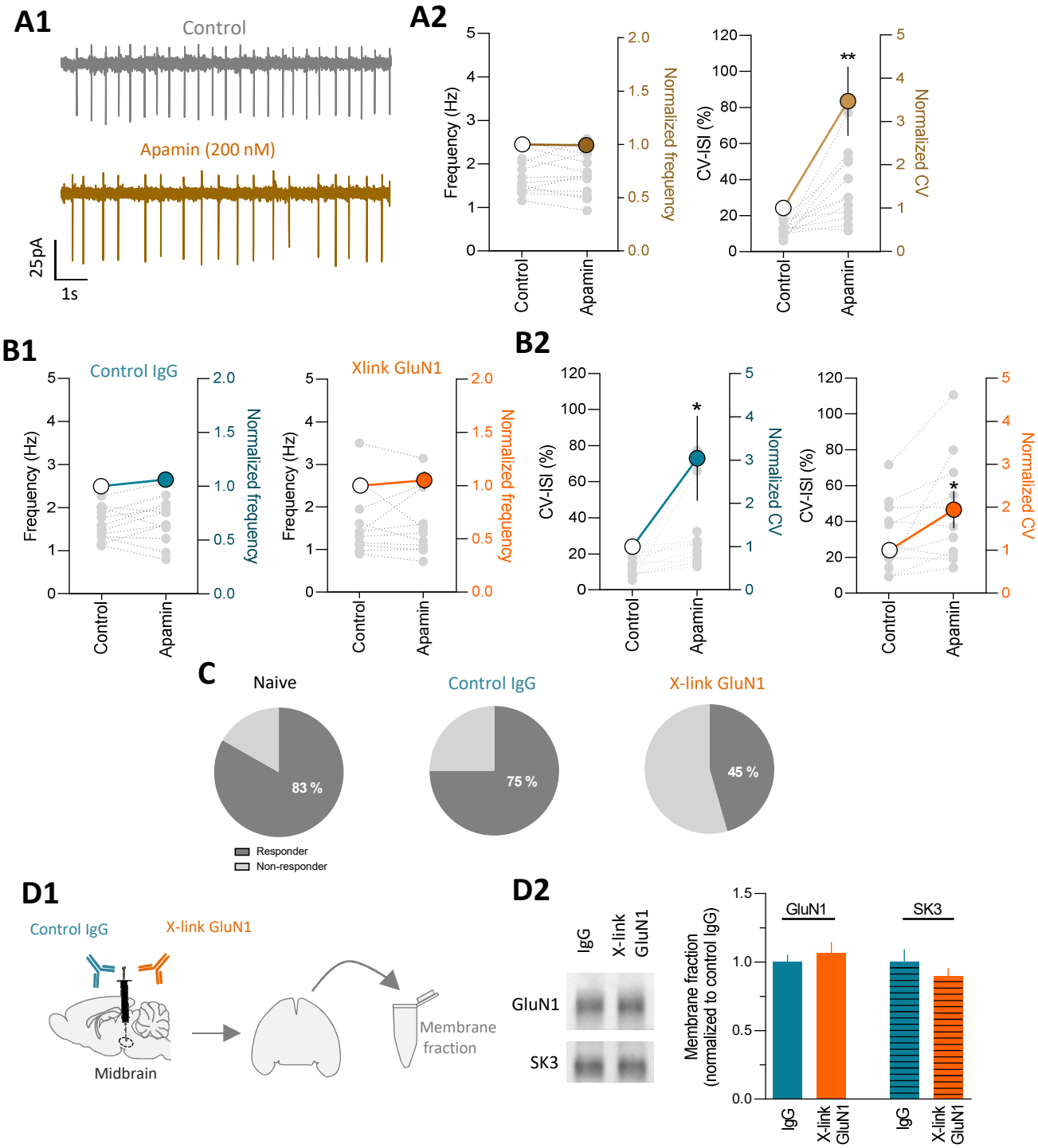
A1
Control
Apamin (200 nM)
25pA
1s
A2
Frequency (Hz)
Normalized frequency
Control
Apamin
CV-ISI (%)
Normalized CV
**
B1
Control IgG
Xlink GluN1
B2
*
C
Naive
Control IgG
X-link GluN1
83 %
75 %
45 %
Responder
Non-responder
D1
Control IgG
X-link GluN1
Midbrain
Membrane fraction
D2
IgG
X-link GluN1
GluN1
SK3
Membrane fraction (normalized to control IgG)

Fig 6. Acute alteration of NMDAR membrane dynamics by X-link GluN1 decreases SK channels modulatory effect but not its membrane content in the midbrain.

A1) Representative firing pattern of a midbrain DA neuron in acute VTA slices from a naive rat before (control) and after the blockade of SK channels by apamin (200 nM, 5-10 minutes). A2) Left, Firing frequency (left Y axis) and normalized frequency (right Y axis) of DA neurons before (1.75 ± 0.13 Hz, n=12 cells) and after apamin application (1.73 ± 0.14 Hz, normalized frequency = 0.99 ± 0.05, n=12). Paired Student's t-test, p=0.894. Right, CV-ISI and normalized CV-ISI before (12.67 ± 1.6, n=12) and after apamin application (29.62 ± 5.96; normalized CV-ISI = 3.47 ± 0.80, n=12). Paired Student's t test, **p=0.0052.

B1) Firing frequency and normalized frequency of DA neurons in control IgG (1.60 ± 0.11 Hz and 1.71 ± 0.15 Hz before and after apamin respectively; normalized frequency after apamin = 1.06 ± 0.07, n=12 neurons) and X-link GuN1 injected rats (1.63 ± 0.23 Hz and 1.61 ± 0.23 Hz before and after apamin, respectively; normalized frequency after apamin = 1.05 ± 0.08, n=11 neurons). Paired Student's t tests: p=0.390 for control IgG and p=0.565 for X-link GluN1 respectively. B2) CV-ISI and normalized CV-ISI before and after apamin in control IgG (CV-ISI = 12.67 ± 1.60 and 29.62 ± 5.96 before and after apamin respectively; normalized frequency after apamin = 3.04 ± 0.98, n=12 neurons) and X-link GluN1 rats (CV-ISI = 31.68 ± 5.99 and 45.93 ± 9.08 before and after apamin respectively; normalized CV-ISI after apamin = 1.94 ± 0.43, n=11 neurons). Paired Student's t tests: *p=0.031 for control IgG and *p=0.027 for X-link GluN1 respectively.

C) Diagram of the proportion of apamin responders and non-responders DA neurons. DA neurons are considered as responders if the value of the CV-ISI after apamin was higher than the CV-ISI before the drug plus the standard deviation.

D1) Schematic drawing of the experimental procedure to obtain midbrain membrane fractions of rats injected with either control IgG or X-link GluN1. Briefly, antibodies were injected in the midbrain and acute midbrain slices were prepared to perform western blotting experiments. D2) Left, western blots of GluN1 and SK channels membrane factions revealed in midbrain slices from control IgG and X-link GluN1 injected rats. Right, Normalized membrane fraction of GluN1 (1.06 ± 0.08, n=6 animals for X-link GluN1) and SK3 channels proteins (0.90 ± 0.06, n=6 animals for X-link GluN1). Unpaired Student's t tests: p=0.530 for GluN1, p=0.380 for SK3. All the values were normalized to the control IgG condition (n=6 animals). Data are expressed as mean ± SEM.

DISCUSSION AND PERSPECTIVES

The aim of the PhD project was to investigate the interplay between NMDAR surface trafficking and the firing pattern of midbrain DA neurons. To address this question, I used a combination of single particle imaging techniques in primary midbrain cultured neurons and iPSC-derived DA neurons, and electrophysiological approaches in rat midbrain acute slices. I showed that both NMDAR and SK channels activity regulate the firing pattern of midbrain DA neurons, whereas blocking the spontaneous activity of NMDAR was ineffective. As NMDAR activation produced both ion fluxes and modulates the membrane diffusion of the receptor, I investigated the specific role of NMDAR surface dynamics in the firing of DA neurons. Altering this dynamics by X-link GluN1 profoundly modified the firing pattern of midbrain DA neurons and reduced the efficacy of SK channel blockers, while the total membrane content of SK channels remained constant. Together, the data collected during my PhD thesis fuel a model in which NMDAR dynamic distribution at the surface of DA neurons tunes the firing pattern of these neurons through an alteration of SK channel function. These different points will be discussed in the following sections.

Modulation of the firing activity of midbrain DA neurons by NMDAR and SK channel activity

SK channel inhibition. A large broad of evidence showed that SK channels regulate the firing precision of midbrain DA neuron *in vitro* (Shepard and Bunney, 1988; Wolfart et al., 2001; Deignan et al., 2012; Herrik et al., 2012), and also the *in vivo* burst generation (Waroux et al., 2005; Ji and Shepard, 2006; Herrik et al., 2010; Soden et al., 2013). Consistent with the literature (Shepard and Bunney, 1988; Wolfart et al., 2001; Deignan et al., 2012; Herrik et al., 2012), I found that the SK channel blocker apamin decreased the regularity of the pacemaker activity in DA neurons from midbrain slices. In addition, several studies reported that SK channels modulate the firing rate of DA neurons, since exposure to SK channel blockers increased the global firing rate both *in vitro* (Shepard and Bunney, 1988; Wolfart et al., 2001; Deignan et al., 2012) and *in vivo* (Waroux et al., 2005; Herrik et al., 2010; Creed et al., 2016). I and others have found that apamin exposure did not affect the firing rate of DA neurons *in vitro* (Seutin et al., 1993; Soden et al., 2013) and *in vivo* (Ji and Shepard, 2006). Shepard and Bunney reported that 20% of the recorded DA neurons did not change their firing rate in response to apamin application (Shepard and Bunney, 1988). Note that Wolfart and collaborators revealed that

the *in vitro* effect of SK channel blockade on the firing rate was highly dependent on the firing frequency of the neurons before the drug (Wolfart et al., 2001). Pharmacological inhibition of SK channels had a small effect on the firing rate, quantified by a relative change of 1.2 in DA neurons with a low firing frequency (<2 Hz), whereas it increased to 1.7 and 2.3 for DA neurons with a firing frequency of 2-4 Hz and 4-6 Hz respectively. In my study and the ones from Soden and collaborators (2013) and Seutin and collaborators (1993) where apamin did not affect the rate, the mean firing frequency was between 1.5 and 1.7 Hz for *in vitro* DA neurons (Seutin et al., 1993; Soden et al., 2013), whereas Deignan and collaborators and Shepard and Bunney, who observed an effect of apamin, reported a firing rate above 2 Hz (Shepard and Bunney, 1988; Deignan et al., 2012). Thus, it is possible that the frequency was too low to detect a significant change in the firing rate induced by apamin. Nevertheless, this relationship between the firing frequency and the effect of SK channels blockade does not fully explain the discrepancies between studies. Although the firing frequency of *in vivo* DA neurons was comprised in the same range of 3-4 Hz in several studies (Waroux et al., 2005; Ji and Shepard, 2006; Herrik et al., 2010; Creed et al., 2016), only Ji and Shepard found that blocking SK channels did not affect the overall firing rate. As different SK channel blockers and different doses were used in these studies, these parameters likely contribute to the discrepancies. Therefore, the effect of SK channels on the firing rate of midbrain DA neurons is less consistent than the effect on the firing precision and likely depends on firing frequency, SK channel blockers and the concentrations used.

NMDAR activation. NMDAR regulate the burst firing and the firing rate of midbrain DA neurons *in vivo* (Overton and Clark, 1992; Christoffersen and Meltzer, 1995; Connelly and Shepard, 1997). *In vitro*, the firing frequency was increased by NMDA application (Seutin et al., 1990; Mercuri et al., 1992; Mereu et al., 1997) but its effect on the firing pattern was less clear. Whereas some authors observed the presence of bursts in DA neurons from midbrain slices in response to NMDA (Mereu, 1997; Johnson, 1992), others failed to evoke bursts (Mercuri, 1992; Seutin, 1990). Similarly, NMDA application did not induce burst firing in midbrain DA neurons in our study. These discrepancies could be explained by differences in brain preparations and experimental procedures. Indeed, injection of a hyperpolarizing current in DA neurons is required to prevent the excessive depolarization induced by NMDA application and enables the bursts generation (Johnson et al., 1992; Seutin et al., 1993; Johnson and Wu, 2004). Unlike most of the studies that only reported the existence of a pacemaker activity in midbrain DA neurons from slices (Grace and Onn, 1989; Seutin et al., 1990; Mercuri et al., 1992; Soden et al., 2013), Mereu et al. showed that *in vitro* DA neurons from immature rats also exhibit irregular and bursting patterns, and reported that NMDA application induced bursts in a different proportion of neurons, depending on their basal firing patterns. The existence of different firing patterns could be

attributed to the different section plane of the midbrain slices (coronal for Mereu *versus* horizontal for the others), and therefore, it is possible that the effect of NMDA application also differs according to the orientation of the brain slices. It is interesting to note that in these studies, the effect of NMDA *in vitro* was mostly qualitative (presence or not of the bursts) and eventually quantified by the number of bursty neurons and the percentage of spikes occurring in bursts (Seutin et al., 1990; Mercuri et al., 1992; Mereu et al., 1997; Johnson and Wu, 2004). My study provides a quantitative analysis of the *in vitro* effect of NMDA application on the firing pattern of midbrain DA neurons by evaluating the CV-ISI. Remarkably, I found that the pharmacological activation of NMDAR decreased the firing precision. Given that the regularity of the firing is correlated with the occurrence of bursts, my results are in accordance with *in vivo* studies demonstrating that NMDAR activation increased the burst firing (Overton and Clark, 1992; Christoffersen and Meltzer, 1995) whereas their inhibition decreased both the bursts activity and the CV-ISI (Christoffersen and Meltzer, 1995; Connelly and Shepard, 1997). We can conclude that activation of NMDAR increases the irregularity of the firing, which likely favours burst generation in midbrain DA neurons.

Blockade of the basal NMDAR activity. In agreement with others (Seutin et al., 1990; Mereu et al., 1997), I report that the basal firing pattern of DA neurons from midbrain slices was not changed following NMDAR antagonist application. Since DA neurons receive excitatory glutamatergic inputs, it is unlikely that the blockade was ineffective because DA neurons were devoid of glutamatergic synapses. However, the frequency of the spontaneous excitatory events was very low (<0.2 Hz) and thus, it is conceivable that the inhibition of these few events is not sufficient to change the firing rate. Also, such spontaneous activity may only partially activate NMDAR that need membrane depolarization for full activation. Given that application of D-APV prevented the effects mediated by SK channels *in vitro* in the hippocampus and the amygdala (Faber et al., 2005; Ngo-Anh et al., 2005), which is consistent with the requirement of NMDAR to active SK channels, the absence of effect of NMDAR blockade on the firing pattern may appear surprising. Of note, the coupling between NMDAR and SK channels is less clear in midbrain DA neurons since activation of NMDAR might decrease or increase SK channel function (Paul et al., 2003; Creed et al., 2016). Still, the concept that NMDAR can modulate the activity of SK channels in midbrain DA neurons is likely of interest.

How is it that D-APV and MK-801 do not change the firing of midbrain DA neurons? Growing evidence indicates that GluN3A subunits are present in midbrain DA neurons. GluN3A subunits are inserted in excitatory synapses from midbrain DA neurons after cocaine exposure (Yuan et al., 2013), inducing a "re-juvenation" of the excitatory synapses with the re-emergence of synaptic GluN2B subunits (Bellone et al., 2011; Dong and Nestler, 2014). This subunit has also been detected by immunoblots in the

midbrain and in synaptosomes from both midbrain and forebrain, with the highest level between PD 10-14 (Al-Hallaq et al., 2002; Wee et al., 2016). Since I recorded DA neurons between PD 12-16, midbrain DA neurons might express GluN3A-containing NMDAR. These receptors being weakly permeable to calcium (Henson et al., 2010), they may poorly contribute to the regulation of SK channels that can be activated by other calcium sources (Nedergaard et al., 1993; Wolfart and Roeper, 2002; de Vrind et al., 2016). Alternatively, the sources of calcium of SK channels can be multiple in midbrain DA neurons. Therefore, if NMDAR are inhibited, other calcium sources identified in midbrain DA neurons such as the voltage gated calcium channels of L-, N-, P/Q or T-type (Nedergaard et al., 1993; Wolfart and Roeper, 2002; Cui, 2004; de Vrind et al., 2016) might be sufficient to compensate the NMDAR blockade and activate SK channels. In accordance with this hypothesis, De Vrind et al. found that blocking only one type of voltage gated calcium channels induced none to moderate decrease of SK channel-mediated AHP and did not reverse the increased irregularity of midbrain DA neurons induced by apamin application, except for the blocker of L-type channels that partially reversed it. Moreover, even if T-type blockers almost abolished the SK channel-mediated AHP and induced bursting activity in SNc DA neurons, the combination with apamin strongly favoured bursts (Wolfart and Roeper, 2002), suggesting that several calcium sources cooperate to maintain SK channel function in midbrain DA neurons.

Modulation of the firing pattern of midbrain DA neurons by NMDAR surface trafficking

Lateral mobility of NMDAR. The surface trafficking of NMDAR was never investigated in DA neurons, so I took advantage of the TH-tdTomato mice to identify live DA cultured neurons and tracked NMDAR at the neuronal surface using single particle tracking approaches. I showed that membrane NMDAR diffused with a mean instantaneous diffusion coefficient of 0.127 $\mu m^2/s$ and an immobile fraction of approximately 9% (fraction of NMDAR diffusing with an instantaneous coefficient <0.005 $\mu m^2/s$). I also characterized for the first time the NMDAR membrane dynamics in human iPSC-derived DA neurons and reported that NMDAR diffused even faster in human cultured neurons but exhibited a similar low fraction of immobile receptors. Of note, it is strikingly different from what is found for surface NMDAR tracking in embryonic hippocampal culture, where the median diffusion coefficient is approximately 0.070 $\mu m^2/s$ and the immobile fraction estimated at 15%. Because NMDAR are stabilized and anchored in glutamatergic synapses (Groc et al., 2004), a low number of glutamatergic synapses in midbrain cultured neurons could explain the high diffusion of NMDAR in these neurons when compared to hippocampal ones. In line with this possibility, several studies reported that rodent postnatal midbrain cultures are mainly composed of GABAergic and DA neurons (Chiodo and Kapatos, 1992; Masuko et al., 1992) and that cultured DA neurons exhibited none to moderate spontaneous

activity (0% for Masuko et al., 1992; , 11% for Rayport et al., 1992; 47% for Chiodo and Kapatos, 1992- but see 68% for Cardozo, 1993). Thus, cultured midbrain DA neurons likely have moderate glutamatergic connections. Furthermore, DA neurons are aspiny, meaning that synapses are mostly localized onto dendritic shafts. As the receptor diffusion is reduced in spine, possibly through the high compartmentalization, a low level of spines will further favor NMDAR diffusion.

Alteration of NMDAR surface trafficking with X-link GluN1. Since membrane NMDAR are highly mobile in cultured DA neurons, the functional role of this process was investigated by using the X-link GluN1 protocol. Primary antibodies against the GluN1 subunit reduce the lateral mobility of NMDAR without affecting their function or the global NMDAR membrane content, allowing to specifically study the effect of the trafficking *per se.* Notably, X-link GluN1 impaired both the frequency and the firing precision of DA neurons. Since the blockade of the spontaneous NMDAR activity was ineffective in changing the firing activity, I propose that, in basal conditions, NMDAR surface trafficking, but not its ionotropic activity, is involved in the pacemaker activity of midbrain DA neurons.

Models of the alteration of SK channels by X-link GluN1. In my thesis, I unveiled that application of the SK channel blocker apamin, which induces irregularity in DA neurons in control conditions, was less effective when NMDAR surface trafficking was altered with X-link GluN1. Therefore, several scenarios can be considered to explain this effect (**Figure 17**). Firstly, a loss of SK channel function could be due to a massive channel endocytosis induced by the NMDAR immobilization (*scenario 1*). However, I did not detect any change in the SK3 channel content in membrane fraction by western blots, arguing against the reduction of SK channel function through internalization and degradation. Given that SK channels are coupled with NMDAR in several neurons including DA neurons (Paul et al., 2003; Faber et al., 2005; Ngo-Anh et al., 2005; Creed et al., 2016), altering NMDAR trafficking might disturb the membrane distribution of the receptor and alter the spatial relationship between the calcium source and SK channels (*scenario 2*). As a consequence, the decreased activity of SK channels would induce a more irregular pattern of midbrain DA neurons. Although tempting, this hypothesis is inconsistent with the results obtained with NMDAR blockers. Indeed, if NMDAR-mediated calcium influx was necessary to activate SK channels, I would expect that blocking NMDAR would impair its functional coupling with SK channels and affect the firing pattern. As NMDAR blockers were ineffective, this scenario seems not the most plausible to explain the alteration of SK channels. A third hypothesis is that the X-link GluN1 disturbs both the distribution of NMDAR and SK channels, uncoupling the channel from other calcium sources (*scenario 3*). This hypothesis relies on the fact that NMDAR and SK channels can interact directly or indirectly through macromolecular complexes. There is no evidence of a direct interaction between NMDAR and SK channels and they are

only observed in close proximity (no colocalization) (Lin et al., 2008; Soden et al., 2013). However, NMDAR and SK channels interact with similar partners including casein kinase II (Bildl et al., 2004; Chung et al., 2004) and calmoduline protein (Xia et al., 1998; Krupp et al., 1999), making possible the existence of large macromolecular complexes. Therefore, the X-link GluN1 might alter the distribution of both NMDAR and SK channels, and uncouple these latter from their calcium sources. Alternatively, alteration of NMDAR surface trafficking might disturb the distribution of protein kinases and phosphatases, modulating the function of SK channels (*scenario 4*). In support of this possibility, NMDAR physically interact with casein kinase 2 (CKII) (Chung et al., 2004) and protein phosphatase 2 (PP2A) (Chan and Sucher, 2001), which can associate with SK channels and regulate the channel properties (Allen et al., 2007; Maingret et al., 2008). Interestingly, altering the surface dynamics of NMDAR, and not its activity, changed the intracellular trafficking and the spine content of CAMKII in hippocampal neurons (Dupuis et al., 2014). Therefore, impairing NMDAR dynamic distribution might affect SK channel properties by disturbing the availability of these proteins. To shed lights on these possibilities, several experiments can be envisioned. First, I plan to confirm the loss of SK channel function by recording and comparing the SK channel-mediated currents in X-link GluN1 and control conditions. Besides, to investigate if X-link GluN1 affects the membrane distribution of SK channels (*scenario 3*), live immunohistochemistry against SK channels is needed to label membrane channels. Because available antibodies against SK3 channels are directed against the intracellular N and C terminals, and thereby require permeabilization, I favour the use of apamin-biotin complexes, which should allow to only label surface channels. In parallel, it would be interesting to perform co-immunoprecipitations to highlight the presence of NMDAR-SK channels macrocomplexes. To investigate whether kinases/phosphatases such as CKII or PP2A are involved in the change of the firing activity of midbrain DA neurons induced by X-link GluN1 (*scenario 4*), a first hint could be to test the contribution of these proteins in the basal activity of SK channels. Indeed, if blockers of CKII or PP2A do not change the spontaneous firing rate in basal conditions, the scenario 4 would be unlikely to explain the effect induced by X-link GluN1.

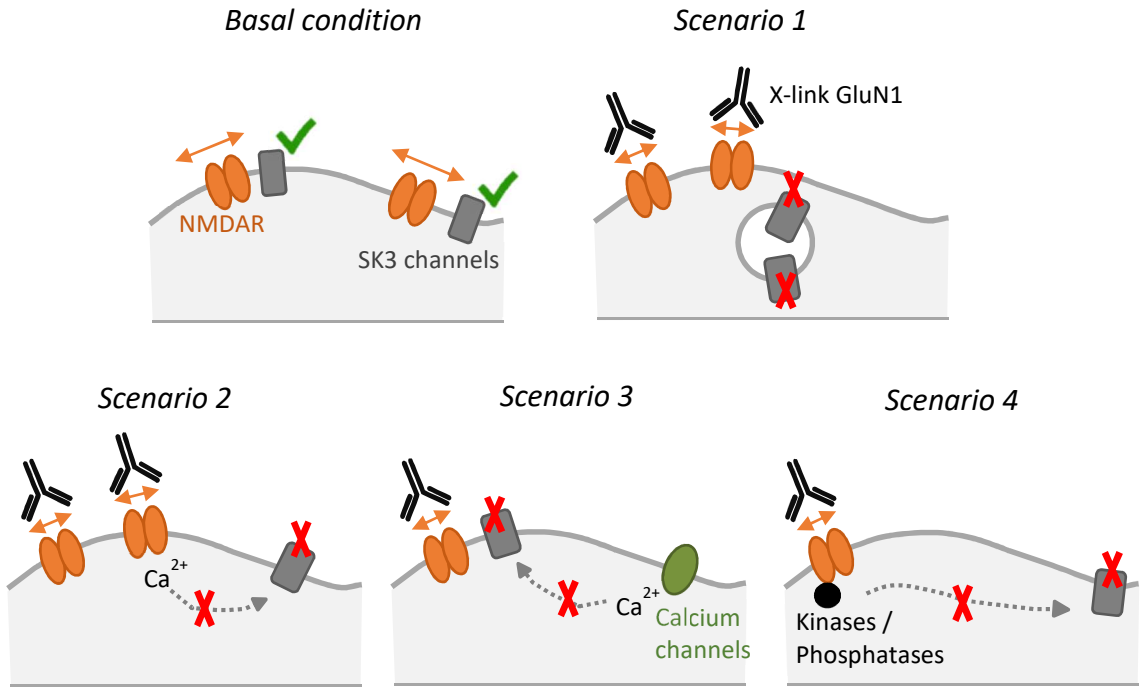

Figure 17. Scenarios to explain the loss of SK channel function by X-link GluN1. In basal conditions, SK channels are functional but lose their function when NMDAR membrane dynamics is altered by X-link GluN1.The different scenarios are described in details in the text.

Implications of NMDAR surface trafficking in physiological and pathological conditions

Role of NMDAR surface trafficking in burst firing. The increased irregularity of the firing pattern observed *in vitro* following NMDAR activation and SK channel inhibition was correlated with an enhancement of the bursting activity in midbrain DA neurons *in vivo* (Seutin et al., 1990; Overton and Clark, 1992; Soden et al., 2013). Given that disrupting NMDAR membrane dynamics also increased the irregularity of the pacemaker activity, NMDAR surface trafficking might regulate the burst firing *in vivo*, and it would be interesting to confirm it by recording *in vivo* the firing of midbrain DA neurons with the X-link GluN1 protocol. The burst generation induced by NMDA application *in vivo* was mostly attributed to NMDAR activation and the resulting calcium entry (Grace and Bunney, 1984), but the fact that NMDA also modified NMDAR surface trafficking in DA neurons opens the possibility that this latter process might contribute to the burst induction as well. Therefore, the presence of NMDA might promote burst activity by modulating both channel activity and its trafficking. Except for artificial application of NMDA, what could be the factors influencing the NMDAR surface trafficking and the burst firing in physiological conditions ? Interestingly, several studies revealed that NMDAR co-agonists D-serine and glycine modulate the lateral mobility of NMDAR in cultured hippocampal neurons (Papouin et al., 2012; Ferreira et al., 2017). Besides, mice lacking D-amino acid oxidase, which degrades D-serine, exhibited a greater number of bursting DA neurons in the VTA (Schweimer et al., 2014). From

these data, the possibility emerges that the availability of NMDAR co-agonists might regulate the NMDAR membrane dynamics, which in turn might control the bursts firing of DA neurons. Moreover, it has been shown in the hippocampus that the availability of the co-agonists is regulated during development, with glycine predominating before the third postnatal week and D-serine after this period (Le Bail et al., 2015; Ferreira et al., 2017). Since appearance of bursts in midbrain DA neurons is also subjected to developmental regulation - they appear only after the third postnatal week *in vivo* (Tepper et al., 1990; Wang and Pitts, 1994) - a change in the co-agonist availability might be involved in the control of the burst appearance. Thereby, it would be interesting to test whether a similar switch of NMDAR co-agonists occurs around the third postnatal week in the midbrain. Up to now, information about the midbrain content of D-serine and glycine and their regulation during development is very scarce (Hashimoto et al., 1993; Nagata et al., 1994) and do not allow to confirm or refute this hypothesis.

Role of NMDAR surface trafficking in pathological conditions. Anti-NMDAR auto-antibodies, including anti-GluN1 antibodies, were detected in several neuropsychiatric disorders such as anti NMDAR encephalitis (Dalmau et al., 2007) and schizophrenia (Tsutsui et al., 2012; Pearlman and Najjar, 2014). Chronic exposure with NMDAR auto-antibodies from schizophrenia and encephalitis patients was associated with synaptic NMDAR endocytosis and thereby, prevented LTP induction (Dalmau et al., 2008; Hughes et al., 2010; Mikasova et al., 2012; Jezequel et al., 2017). Remarkably, acute treatment with auto-antibodies did not affect the membrane content of NMDAR but impaired its surface diffusion (Mikasova et al., 2012; Jezequel et al., 2017). As X-link GluN1 with commercial antibodies also altered NMDAR membrane dynamics without disrupting the receptor synaptic content (Dupuis et al., 2014), it raises the possibility that the changes in firing pattern observed with commercial anti-GluN1 antibodies could also be induced by pathogenic auto-antibodies. It is interesting to note that psychotomimetics drugs such as MK-801 or PCP, which reproduce psychotic symptoms expressed by schizophrenic patients (Javitt and Zukin, 1991), modify the firing rate of VTA DA neurons by increasing both firing rate and bursting activity (French et al., 1993). Besides, mutated inactive forms of the delta 1 glutamate receptor and the SK3 channels, whose genes have been associated with schizophrenia (Greenwood et al., 2011; Askland et al., 2012), profoundly impact burst generation in VTA DA neurons *in vivo* (Soden et al., 2013; Benamer et al., 2017). In light of my results and these data, it would be interesting to test whether NMDAR auto-antibodies from schizophrenia patients affect the firing activity of midbrain DA neurons, because it might partly explain the causes of the DA dysfunction encountered in schizophrenia patients.

Conclusion and perspectives

By revealing that NMDAR membrane dynamics tunes the firing pattern of midbrain DA neurons, my PhD study sheds new and rather unsuspected light on the molecular mechanisms contributing to the regulation of DA neuron activity. Indeed, in addition to the ionotropic function of receptor/channel, their membrane dynamics appears to play a key role in this process, challenging our classical view of the regulation of neuronal activity. The firing pattern appears as a highly dynamic process with diffusive receptors and future investigations are needed to fully understand how the activity and the membrane dynamics of channels and receptors cooperate to regulate the neuronal activity, essential for communication within the brain.

ANNEXES

Annexe 1. Article “Targeting neurotransmitter receptors with nanoparticles *in vivo* allows single-molecule tracking in acute brain slices”.

Annexe 2. Single particle tracking in midbrain acute slices

Annexe 3. Electrophysiological approach with MK-801 in midbrain slices

Annexe 4. Co-culture of midbrain and cortical neurons in microfluidic devices

ANNEXE 2: Investigating NMDAR surface trafficking with single particle tracking in acute midbrain slices

Surface trafficking of NMDAR is regulated by proteins that can be intracellular like kinases and MAGUK proteins, extracellular such as proteins from the extracellular matrix or anchored in the plasma membrane as transmembrane receptors (Groc et al., 2009). Up to now, most of the studies investigating the lateral diffusion of receptors were done in dissociated neuronal culture. In this preparation, neurons are dissociated and cultured with specific media in petri dishes and thus, lack the extracellular environment and the tissue architecture. To circumvent this technical limitation, we developed in the laboratory a new protocol to track the lateral mobility of tagged membrane receptors such as NMDAR or DAR in acute brain slices (**Figure 1**) (Varela et al., 2016a, 2016b). To track NMDAR, the first step was to incorporate DNA constructs coding for GluN1-superecliptic pHluorin (SEP), a pH sensitive derivative of the green fluorescent protein (GFP). We introduced GluN1-SEP DNA construct by *in vivo* electroporation (*in utero* or postnatal electroporation) to express tagged NMDAR and we added GFP to identify electroporated neurons. Then, quantum dot nanoparticles coupled to antibodies against the GFP tag are delivered *in vivo* through intraventricular injections. Once acute brain slices are prepared, tagged NMDAR are tracked by single particle tracking imaging techniques in electroporated neurons. This protocol, initially developed for DAR (Varela et al., 2016a), was successfully used to track NMDAR lateral diffusion in hippocampal acute brain slices and was subjected to a publication (Varela et al., 2016b). In this latter paper, the authors took advantage of this new approach to compare NMDAR surface trafficking in three different hippocampal preparations: in dissociated culture, and in organotypic and acute brain slices. The fractions of immobile and mobile receptors were similar between the preparations but the mobility of NMDAR in acute slices was decreased, probably because of the dense extracellular environment in slices. Therefore, although these different preparations share similarities, it seems that the extracellular environment influences NMDAR membrane dynamics.

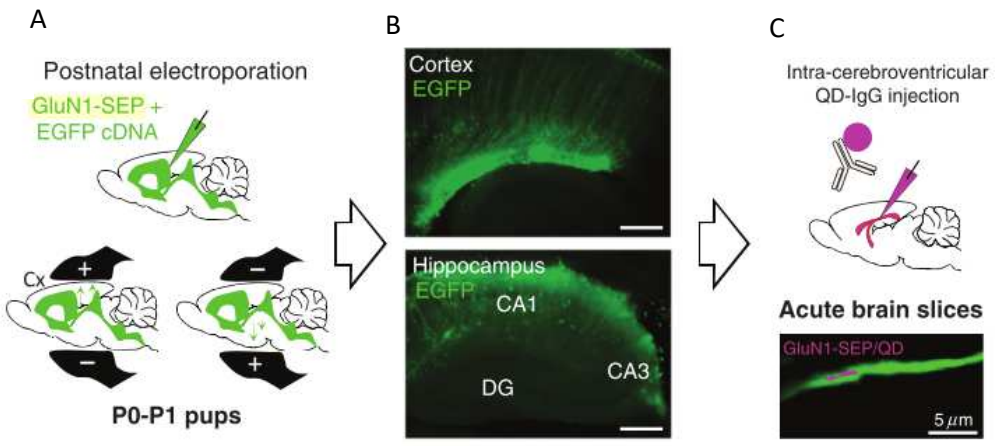

Figure 1. Protocol allowing the tracking of tagged NMDAR in acute brain slices (adapted from Varela et al, 2016). A) *Top,* DNA constructs coding for GluN1-SEP and EGFP are injected in the cerebral ventricles of P0-P1 rat pups to express tagged NMDAR and identify electroporated neurons, respectively. Note that *in utero* electroporation is also possible to express these DNA constructs. *Down,* Then, five electrical pulses (150 V, 50 ms, 1s interpulse interval) are given by bipolar electrodes for each brain hemisphere. B) After few days, electroporated neurons can be observed in the cortex or hippocampus, depending on the targeted structure. C) Three days after electroporation, pups received an intraventricular injection of quantum dots (QD) with anti GFP antibody. 2-3h after injection, brain slices are prepared and imaged with a spinning disk confocal microscope. In the bottom, an example of a QD GluN1-SEP trajectory tracked at the surface of a GFP positive neuron.

During my phD, I investigated NMDAR lateral mobility in mice midbrain primary culture. This latter is characterized by a very low proportion of DA neurons (usually less than 5%, except for co-culture with astrocytic layer for which the yield of DA neurons can reach more than 20%, see Masuko et al., 1992; Rayport et al., 1992) and a high proportion of GABA neurons (Chiodo and Kapatos, 1992; Masuko et al., 1992; Rayport et al., 1992; Gaven et al., 2014; Weinert et al., 2015). This is strikingly different from what is found *in vivo*, where DA neurons represents more than 50% and almost 90% in VTA and SNc respectively, and GABA neurons around 35% in the VTA (Swanson, 1982; Margolis et al., 2006; Nair-Roberts et al., 2008). Therefore, the environment of DA neurons is quite different in culture regarding the neuronal inputs and the tissue architecture and thereby, should impact NMDAR surface trafficking. For this reason, I wanted to characterize the lateral mobility of receptors in a more preserved environment, i.e in midbrain acute slices, using the protocol developed in the laboratory. The first step was to electroporate GluN1-SEP in midbrain DA neurons. *In utero* electroporation is based on the introduction of DNA in neuronal progenitors bordering the cerebral ventricles that will become mature neurons after undergoing maturation and migration to their final localization. DNA is injected in the cerebral lateral ventricles of the rat embryo with a micropipette through the uterine wall and the yolk sac (**Figure 2A**). Bromophenol blue is added to the DNA solution to check the injection site (**Figure 2B**). After DNA injection, electrical pulses are delivered through forceps-type circular

electrodes (**Figure 2C, 2D**). These electrical shocks destabilize the plasma membrane and create pores in the membrane. Then, negatively charged DNA will migrate toward the positive electrode and enter the neurons through the pores.

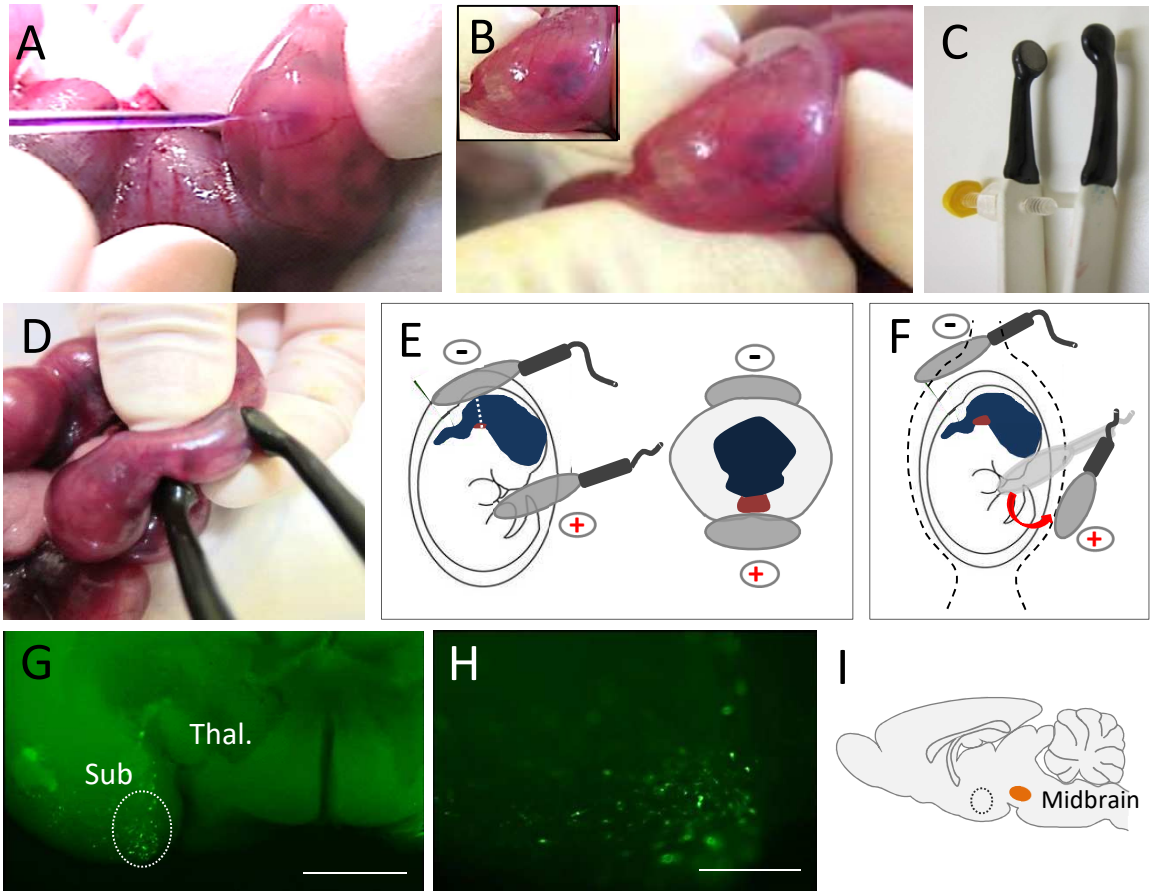

Figure 2. *In utero* electroporation of the midbrain to express tagged NMDAR in DA neurons. A) Solution containing GluN1-SEP, GFP DNA constructs and bromophenol blue is injected in one ventricle of E16 embryos with a glass micropipette. B) The injection is validated if the blue staining spreads in the other ventricle, as observed in the image. C) Bipolar electrodes with a positive (yellow marker) and a negative poles are used. D) Injected embryos are electroporated by given 5 electrical pulses (50 V, 50 ms, 1s interpulse interval). E) Schematic representation of the configuration of the bipolar electrodes on a E16 embryo, in a sagittal (left) and coronal (right) view. The ventricular system is represented in blue and the position of the DA neuron progenitors in orange. To target these progenitors, the positive electrode is placed under the muzzle of the embryo. F) Schematic representation of the configuration of the bipolar electrodes in "real" conditions. Indeed, because of the uterin wall (dotted line) and the albumen yolk (continue line circling the embryo), the positive electrode cannot be placed under the muzzle but rather along the uterine wall. G) Fluorescence image of a coronal brain slice (500 µM) with electroporated GFP neurons (white circle with dotted line) localized in the subiculum. Thal. = Thalamus Bar scale = 2mm H) Magnification of the circled area in Fig 1G. Several electroporated neurons are observed in this area. I) Schematic representation of a rat brain in the sagittal plane. The circled area from fig 1G is represented by the black circle with dotted line. It is worth noting that the electroporated neurons are localized in a more anterior part than the midbrain, resulting from the misplacement of the electrodes due to the uterin wall and the yolk sac.

To successfully electroporate midbrain DA neurons, several parameters had to be considered. First, I chose to electroporate pregnant rats at the embryonic stage 16 (E16) because most of midbrain DA neurons are generated between E13 and E16 (Altman and Bayer, 1980). Another parameter to take into account is the positioning of the bipolar electrodes. As midbrain progenitors are localized in the floor plate of the mesencephalon, the positive electrode has to be positioned under the muzzle to enable DNA to migrate ventrally (**Figure 2E**). However, due to the presence of the uterine wall and the yolk sac, it is very difficult to put the electrode under the muzzle (**Figure 2F**). Consequently, I could not find electroporated neurons in the midbrain. Instead, GFP positive neurons were present in more anterior structures, confirming the misplacement of the bipolar electrode (**Figure 2G-2I**). To circumvent this problem, I wanted to test triple electrodes recently developed by Maschio et al., which enables to target brain structures such as the cerebellum that was not accessible with standard bipolar electrodes (dal Maschio et al., 2012). These triple electrodes are not commercialized and thus, we developed our home-made triple electrodes in the laboratory. Unfortunately, I was not able to reach the midbrain with this configuration and up to now, no study reported successful *in vivo* electroporation of the midbrain. Therefore, despite the recent improvement of *in utero* electroporation, it seems that this technique remains unadaptable to the midbrain.

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ANNEXE 3: Investigating NMDAR surface trafficking on DA neurons by electrophysiology with MK-801

Before the development of high resolution imaging techniques, NMDAR membrane dynamics was first suggested by using pharmacological tools such as the irreversible open channel blocker MK-801. As the binding site of MK-801 is located inside the receptor pore (Huettner, 1988), this pharmacological agent binds selectively to activated NMDAR that opened in response to synaptic glutamate release. Besides, the blockade of NMDAR persists after the drug was washed out (Huettner, 1988), making the MK-801 a powerful tool to investigate the properties of synaptic NMDAR. Tovar and Westbrook took advantage of these characteristics to selectively and irreversibly block synaptic NMDAR, by stimulating synapses (paired-pulse stimulation at 0.125Hz) in the presence of MK-801 (5-20µM) (Tovar and Westbrook, 2002). After blockade of synaptic NMDAR and subsequent washes, the authors observed a gradual recovery of the evoked NMDAR-mediated post-synaptic currents (EPSC) (**Figure 1A**). Surprisingly, this recovery was not observed when synaptic and extrasynaptic NMDAR were blocked by co-application of MK-801 (20 µM) and the agonist NMDA (1mM) (**Figure 1B**), ruling out the possibility that this effect was due to insertion of new NMDAR inside the membrane. It rather suggests that in the case of synaptic block with MK-801 (Fig 1A), membrane extrasynaptic receptors are recruited to synapses, thereby contributing to the recovery of synaptic currents. Since this discovery, the surface trafficking of NMDAR was later confirmed by single particle tracking and other imaging techniques such as FRAP (Groc et al., 2004; Bard et al., 2010). Thus, the use of electrophysiology combined with MK-801 appears as a robust way to explore NMDAR membrane trafficking, especially in brain slices where application of such imaging techniques is still challenging.

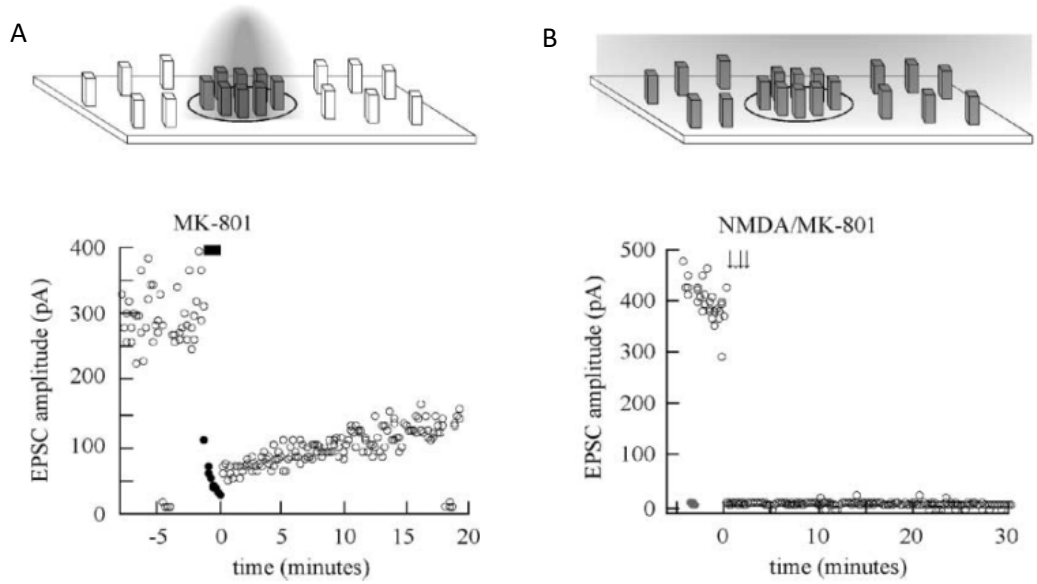

Figure 1. Recovery of evoked NMDAR-mediated post-synaptic current (EPSC) following synaptic block with MK-801 application (adapted from Tovar and Westbrook, 2002. A) Top, Schematic drawing of NMDAR (black rectangles) in the synaptic compartment (delimited by the black circle), which are blocked by synaptic stimulation with MK-801 bath application. Extrasynaptic NMDAR (white rectangles) are not blocked. Down, Application of MK-801 (5-20µM) during synaptic stimulation induces a fast and almost full inhibition (90%) of NMDAR-mediated EPSC. After 15 minutes, the ESPC showed a recovery of more than 25% of the control EPSC amplitude. B) Top, Schematic drawing of the synaptic and extrasynaptic membrane NMDAR following NMDA and MK-801 co-application. Note that this protocol blocked both extrasynaptic and synaptic NMDAR. Down, Four times (indicated by arrows) co-applications of MK-801 (20 µM) and NMDA (1 mM) result in a complete abolition of NMDAR-mediated EPSC. No recovery was observed in this case, even after 30 minutes.

My phD project aimed at investigating the role of NMDAR membrane dynamics in the firing pattern of midbrain DA neurons. As this was done in acute midbrain slices, the idea here was to characterize the lateral mobility of NMDAR in the same preparation with the protocol used by Tovar and Westbrook. To do so, evoked NMDAR mediated EPSC (NMDAR-eEPSC) were recorded in DA neurons from VTA acute slices from young rats (PD 10-P15). To inhibit synaptic NMDAR, MK-801 (10 µM) was bath applied for 5 minutes when stimulating synapses at 0.05Hz with a single pulse protocol. The kinetics of MK-801 blockade was quite slow, reaching 40-50% of inhibition after 15-20 minutes of drug application (**Figure 2A**). In contrast, Tovar and Westbrook reported an almost full inhibition (90% inhibition) after few minutes. To note, the synaptic block should be fast and strong enough to see recovery. Indeed, if the recovery process starts when the synaptic block is still occurring, it would mask the recovery and result in a stable value of current amplitude.

Patching neurons during several minutes (>20 minutes) can induce a significant rundown of the NMDAR-mediated currents probably because of cell dialysis (Rosenmund and Westbrook, 1993; Wu et al., 2007; Wild et al., 2013b). So, it raises the possibility that the gradual inhibition of synaptic currents was due to nonspecific rundown instead of MK-801 action. To test it, I patched neurons during

at least 25 minutes without adding any drug. As no rundown was observed after 25 minutes of patch (**Figure 2B**), it is unlikely that the slow decreased of synaptic current resulted from nonspecific rundown.

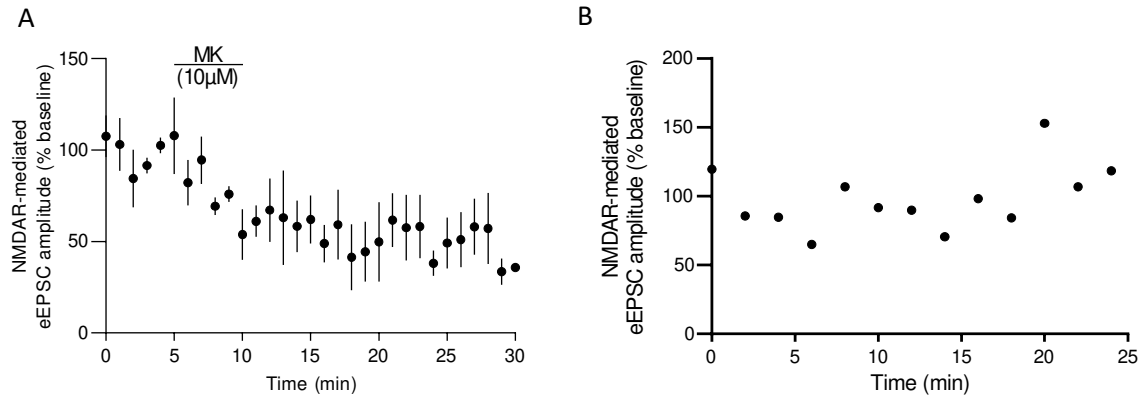

Figure 2. The gradual decrease of NMDAR-eEPSC observed in midbrain DA neurons following MK-801 application is not due to nonspecific rundown of currents. A) MK-801 (10 µM, 5 minutes) application during synaptic stimulation evoked by bipolar electrodes placed rostral to the VTA induces a gradual and progressive decrease of the NMDAR-eEPSC amplitude. After 5 minutes of MK-801, only 40-50% of the EPSC amplitude are inhibited. The EPSC amplitudes are normalized to the mean amplitude of ESPC recorded before the drug (5 minutes). Bars represent SEM. n=5. B) Example of the NMDAR-eEPSC recorded in a midbrain DA neuron in whole-cell patch clamp configuration. Note that the recording is quite stable after 25 minutes, showing that synaptic NMDAR currents do not undergo rundown due to intracellular dialysis.

The slow kinetic of MK-801 could also be due to a problem of drug penetration into slices. To discard this possibility, I recorded NMDAR-eEPSC in the presence of another NMDAR blocker D-APV. D-APV (50µM) is a competitive antagonist that inhibits NMDAR independently of its state (opened or closed). Contrary to MK-801, D-APV decreases current by 78% after 5 minutes (**Figure 3**),indicating that the low access of drug into slices is unlikely to account for the slow kinetic of MK-801.

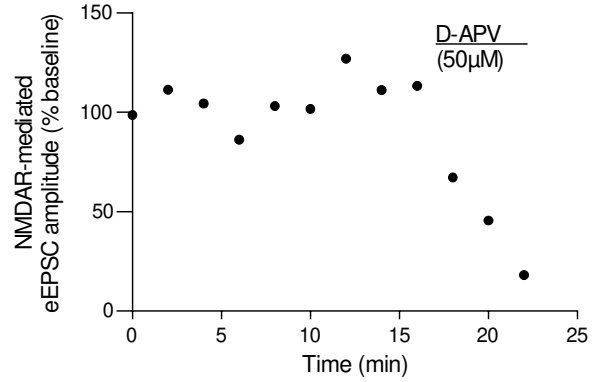

Figure 3. D-APV induces a rapid inhibition of NMDAR-eEPSC in DA neurons from VTA slices. Here is an example of the NMDAR-eEPSC recorded in a DA neuron. Application of the antagonist D-APV (50 µM, 5 minutes) induces a strong and fast decrease of synaptic currents. After 5 minutes, the residual current is about 18%, meaning that the drug easily penetrates brain slices to act on NMDAR.

One of the main differences between my preparation and the one described by Tovar and Westbrook is that they used autaptic primary mouse culture whereas I worked on acute brain slices. Although the authors mentioned that recovery from MK-801 occurred with single or paired-pulse stimulation, it is possible that the single pulse stimulation activated too few synapses and thus, was not sufficient to induce a massive block of synaptic NMDAR in acute slices. Remarkably, Harris and Pettit, who used MK-801 to block synaptic NMDAR in acute hippocampal slices, stimulated synapses with single pulses given at 0.1 Hz (Harris and Pettit, 2007). However, they increased the concentration of MK-801 to 50 µM. Since their protocol induces a rapid and strong block of NMDAR-mediated synaptic current in brain slices (**Figure 4**, black curve), the same conditions were used to optimize the extent of the NMDAR synaptic block. When stimulating synapses with paired pulses given at 0.1 Hz in the presence of a high concentration of MK-801 (50µM), the inhibition of the synaptic currents was still very gradual, reaching 80% of block 10 minutes after MK-801 application (**Figure 5**). To compare, Harris and Pettit obtained the same extent of synaptic block after 5 minutes of MK-801 application. Therefore, these stimulation conditions were sufficient to abolish synaptic currents in hippocampal acute slices, but not in DA neurons from acute VTA slices.

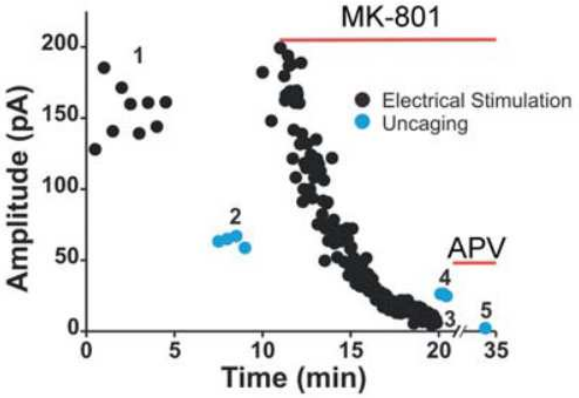

Figure 4. Synaptic stimulation at 0.1 Hz in presence of MK-801 (50 µM) in the bath induced a rapid and robust inhibition of the NMDAR-mediated synaptic currents in acute hippocampal slices (adapted from Harris and Pettit, 2007). After 5 minutes of MK-801, approximatively 75-80% of the NMDAR-mediated EPSC amplitude is blocked.

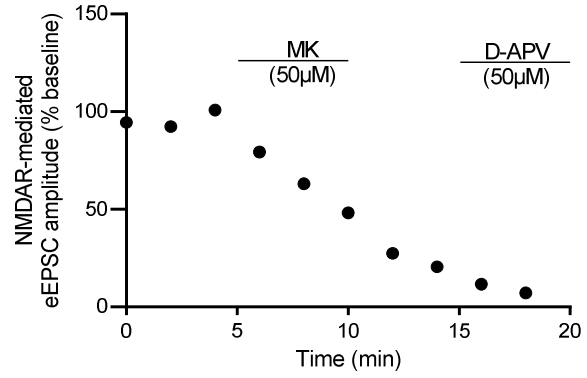

Figure 5. Application of a high concentration of MK-801 (50 µM) with a paired-pulse synaptic stimulation protocol at 0.1 Hz causes a slow inhibition of NMDAR-eEPSC in a DA neuron from acute VTA slices. 5 minutes of MK-801 application reduces the NMDAR-eEPSC by 52%. Addition of D-APV (50µM) further decreases the residual synaptic currents, indicating that the MK-801-induced block was not total after 15 minutes of recording.

Interestingly, Wild et al. found a similar kinetics of the MK-801 blockade in DA neurons from acute SNc slices (Wild et al., 2013a). Synaptic stimulation at 0.1 Hz with a bipolar electrode in the presence of 10 µM of MK-801 very progressively blocked synaptic NMDAR-mediated currents (**Figure 6**, black curve). Unlike hippocampal neurons, 15 minutes were required to inhibit 70% of the synaptic currents in SN DA neurons. Note that I obtained a similar extent of block within 10 minutes in VTA DA neurons, which clearly differs from the almost full blockade observed in hippocampal neurons in less than 5 minutes (**Figure 1A, Figure 4**). Therefore, these data suggest that the slow kinetic of the drug seems to be "specific" of midbrain DA neurons.

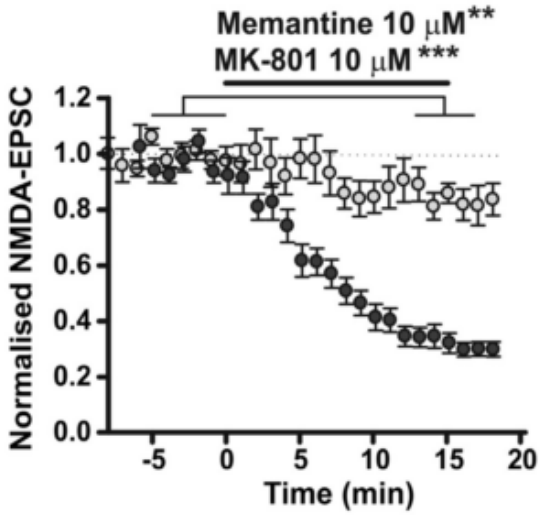

Figure 6. Repetitive synaptic stimulation at 0.1 Hz in presence of MK-801 (10µM) produced a slow reduction of the NMDAR-mediated EPSC in DA neurons from acute SNc slices (adapted from Wild et al. 2013).
After 15 minutes of MK-801 perfusion, NMDAR-eEPSC are diminished by 70% (black curve).

How can we explain such difference of the MK-801 kinetic between hippocampal and midbrain preparations ? One hypothesis could be a difference in the release probability of excitatory glutamatergic synapses in the midbrain compared to the hippocampus. Synapses with a low release probability need more stimulations to trigger release of vesicles than synapses with a high probability. If glutamatergic synapses onto DA neurons have a low release probability, few synapses will be activated by each synaptic stimulation. Consequently, the synapses will be gradually activated and inhibited by MK-801. Although tempting, no data in the literature confirm or invalidate this hypothesis, since no study estimated the release probability of excitatory synapses onto DA neurons. It is believed that the 2 glutamate transporters VGLUT1 and VGLUT2 are associated with low and high release probability respectively (Fremeau et al., 2001). Therefore, the relative distribution of VGLUT1 and VGLUT2 on terminals contacting DA neurons could give a hint on the release probability of these synapses. However, both VGLUT1 and VGLUT2 were detected in glutamatergic terminals onto VTA DA neurons (Omelchenko and Sesack, 2007) and thus, prevent to draw definitive conclusions regarding the release probability of glutamatergic synapses on VTA DA neurons.

Another parameter to take into account is the type of stimulation electrode. The hippocampus is a very well organized structure. Because axons form a single tract, it is possible to use monopolar electrode to obtain a single-fiber stimulation. On the contrary, glutamatergic inputs contacting the VTA are far less organized and come from various directions. Bipolar electrodes are most commonly used in this structure because they enable to stimulate a broader area (Comte, 1982). Therefore, we can hypothesize that each stimulation with bipolar electrodes would recruit a lot of synapses, among which new synapses that are not blocked by MK-801. In this condition, it would require a lot of stimulations to effectively block all the synapses that are potentially activated by bipolar electrodes.

Finally, the slow kinetics observed with MK-801 in my conditions might be caused by a difference in the NMDAR composition between the hippocampal and the midbrain neurons. Whereas hippocampal neurons mostly express GluN2A and GluN2B-containing NMDAR (Groc et al., 2006; Bellone and Nicoll, 2007), several evidence suggest that the GluN3A subunits are present in midbrain DA neurons during postnatal development (Ciabarra et al., 1995; Yuan et al., 2013; Wee et al., 2016). Incorporation of GluN3 subunits modifies the properties of NMDAR, including their sensitivity to various NMDAR blockers. McClymond et al. investigated the efficacy of open channel blockers when GluN3A or GluN3B was co-expressed with GluN1 and GluN2A subunits in Xenopus oocytes, and found that the efficacy of MK-801 block was reduced by GluN3 subunit incorporation (McClymont et al., 2012). Thus, the presence of GluN3A subunits might decrease the sensitivity of MK-801 and slow down drug efficacy. In contrast, D-APV inhibition does not seem to be influenced by GluN3A expression (Yuan et al., 2013), which could explain the relative fast block of NMDAR-mediated EPSC observed with D-APV (**Figure 3**) and not with MK-801. The slow kinetics of MK-801 was also observed in SNc DA neurons. Although GluN3A subunit was detected in the whole midbrain, including the SNC and the VTA (Ciabarra et al., 1995; Wee et al., 2016), no studies reported its presence in DA neurons from the SNc, in contrast to the VTA (Yuan et al., 2013; Creed et al., 2016). Thereby, this explanation is not completely satisfactory to explain the slow kinetics of MK-801.

In conclusion, although the electrophysiological approach combined with MK-801 was a promising tool to investigate NMDAR surface trafficking in midbrain DA neurons from acute slices, this technique cannot be applied in these neurons. Indeed, the very low kinetics of the MK-801 block is not compatible with the observation of the recovery of the synaptic NMDAR-mediated currents, essential to characterize the NMDAR membrane dynamics.

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ANNEXE 4: Establishment of a compartimentalized cortex-midbrain co-culture in microfluidic devices

Primary midbrain cultures are mainly composed of DA and GABA neurons (Silva et al., 1988; Chiodo and Kapatos, 1992; Masuko et al., 1992). Therefore, DA neurons likely received few excitatory glutamate inputs and a massive GABAergic drive. In accordance with this hypothesis, few DA neurons exhibited spontaneous activity in primary culture (0% for Masuko et al., 1992; , 11% for Rayport et al., 1992; 47% for Chiodo and Kapatos, 1992- but see 68% for Cardozo, 1993). It is worth noting that, although some DA neurons have been shown to co-release glutamate *in vitro*, they represent less than 15% of DA neurons in standard midbrain culture (Mendez et al., 2008); supporting the view that midbrain cultures exhibit a low glutamatergic tone. As the NMDAR membrane trafficking is strongly regulated by its agonist NMDA (De Rossi et al., 2016), I wanted to study the receptor dynamics in the presence of a higher glutamatergic drive. To do so, we recreated the projections from glutamatergic neurons to DA neurons by co-culturing these two neuronal populations in a microfluidic device. Since midbrain DA neurons receive a dense glutamatergic innervation from the cortex (Carr and Sesack, 2000; Omelchenko and Sesack, 2007), we decided to co-culture cortical and midbrain neurons. The objective was then to track NMDAR membrane dynamics by single particle imaging techniques in postnatal DA neurons from TH-tdTomato mice co-cultured with cortical neurons in the microfluidic chambers.

In microfluidic devices, each neuronal population is plated in one microfluidic chamber, composed of 2 wells opened in the top and linked by a "covered" bridge (**Figure 1**). The 2 chambers are separated by microgrooves thin enough (10 µm width) to only allow the crossing of neurites but not the cell bodies. Thus, neurons contained in the bridge can send projections to reach the other chamber. By compartmentalizing each neuronal population, this device displays several advantages. First, it enables to culture 2 populations requiring different culture media, which is not possible in classical co-culture on petri dish. It is the case here because cortical neurons grow in neurobasal medium whereas midbrain neurons are cultured with EF 12 medium. The configuration of such devices also allows to selectively modulate one neuronal population. For example, application of drugs such as glutamate in the chamber containing cortical neurons would activate only these neurons without affecting the other chamber.

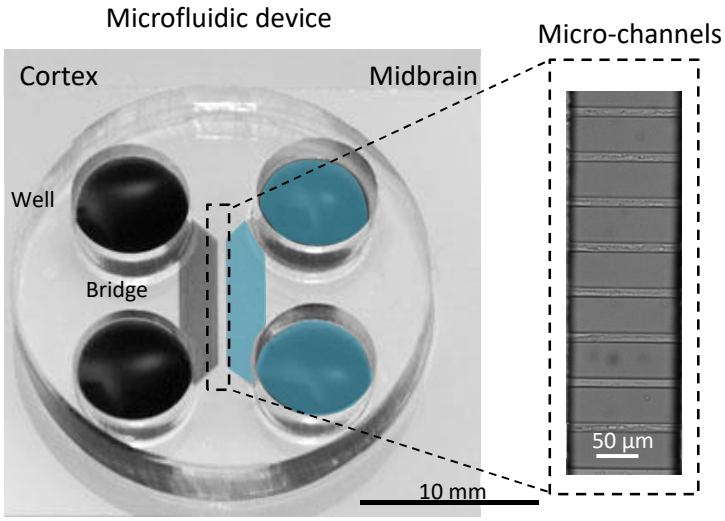

Figure 1. Picture of the microfluidic device developed by Millipore (AXIS 150). It is composed of 2 chambers where 2 neuronal populations (cortical and midbrain culture) are plated. Each chamber is composed of 2 wells and one bridge. Each bridge is connected by micro-channels (150 µm long) thin enough to enable the crossing of neurites but not the cell bodies.

As embryonic cultures (midbrain and cortical from E14 and E18 rats embryos, respectively) were routinely made in the laboratory, I use them first to test the growth and the survival of cultures in the microfluidic chambers, before using the postnatal midbrain culture from TH-tdTomato mice. Given that these cultures derived from embryos at different stages, I plated first the midbrain culture and 1 to 2 days later, the cortical neurons. To find the best conditions for neuronal survival, I tested several cell concentrations in the microfluidic chambers. When plated with a concentration higher than 100 000 cells/chamber, midbrain neurons fasciculate and form big "clusters" of neurons (**Figure 2A**) whereas when lower than 50 000 cells/chamber, the neurons did not survive. Thus, the dilution ranging from 100 to 50 000 cells/chamber was used in the following experiments (**Figure 2B**).

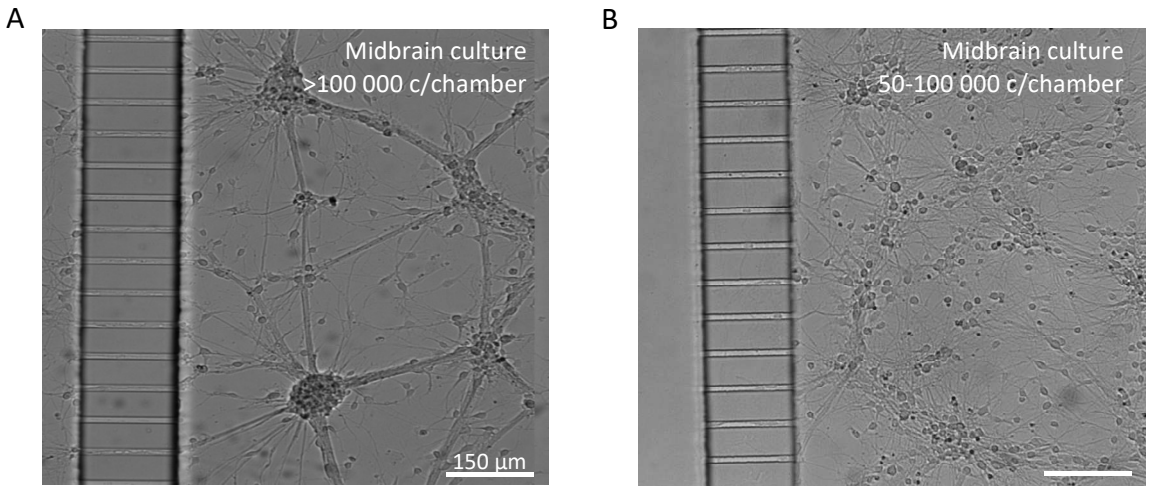

Figure 2. Plating concentrations affects the confluence of midbrain neurons into the microfluidic chambers. A) DIC image of midbrain neurons at 11 days *in vitro* (DIV), when plating at a concentration of more than 100 000 cells/chamber. Note that the neurons fasciculate in the microfluidic chambers. B) DIC image of midbrain neurons at 11 DIV, with the plating concentration comprised between 50 and 100 000 cells/chamber. The neurons are mostly distributed homogenously in the chamber.

After several days *in vitro* (DIV), immunostainings were done in microfluidic chambers to assess if neurons could send projections through the micro-channels. Cortical neurons were labelled for Tau, a microtubule associated proteins enriched in axons, to investigate if cortical neurites, and especially axons, crossed the micro-channels. Midbrain culture was stained with TH antibody to check the presence of DA neurons and the crossing as well. Given the low proportion of DA neurons in our midbrain culture (< 5%), we observed very few DA neurons sending projections through the channels to reach the cortical chamber (**Figure 3**). On the contrary, a lot of cortical neurites, among which axons, crossed the microgrooves to project to the midbrain culture (**Figure 3**). We never observed TH positive neurons in the cortical chamber, confirming the efficient compartmentalization of the co-culture.

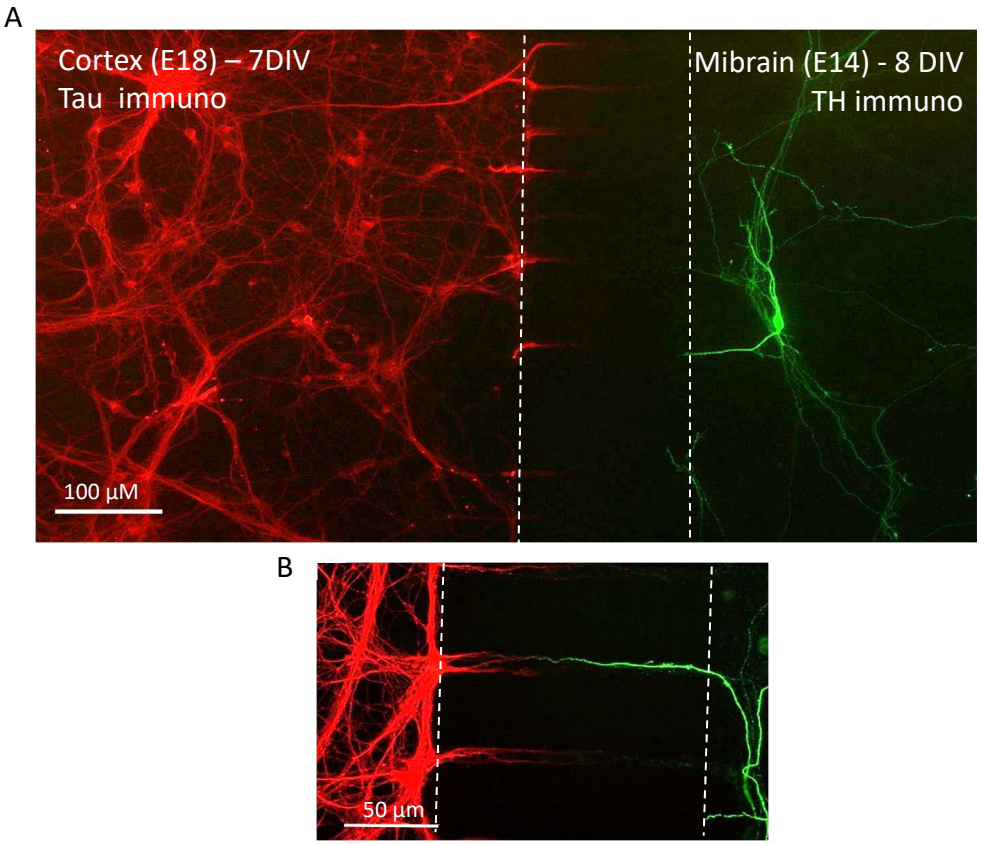

Figure 3. Cortical and midbrain co-culture send projections to each other through the microchannels. A) Fluorescence image of the co-culture cortex – midbrain in the microfluidic chambers. Cortical chamber was labelled with anti Tau antibody (1:1000) and the secondary antibody anti mouse Alexa 568 (A568). Midbrain neurons were labelled with TH antibody (1:1000) coupled to A 488 to identify DA neurons. Because cortical and midbrain culture were not placed at the same time, cortical and midbrain neurons are at different DIV, 7 and 8 DIV respectively. Both cultures send projections through the micro-channels (delimited by the dotted white lines). The high density of Tau labelling suggests that cortical neurons send axons through the micro-channels. B) A higher magnification of another microfluidic device stained with Tau and TH antibodies for cortical and midbrain chambers respectively. Here again, both culture send projections. In the micro-channels.

Because the cortical culture was very dense in the microfluidic chambers, I took advantage of the transfection technique that enables to label few neurons, to observe the global morphology of cortical neurons. After 13 DIV, green fluorescent protein (GFP) transfected cortical neurons were highly ramified and exhibit spines along thin processes (**Figure 4**), as observed in standard primary cortical culture (Li et al., 1998). By contrast, the morphology of DA neurons was very different. They were often fusiform, bipolar and had thicker dendrites, which are the morphological characteristics of DA neurons in classical midbrain culture (Silva et al., 1988; Rayport et al., 1992). So, the relative development of the midbrain and cortical cultures do not seem to be altered by the configuration of the microfluidic device.

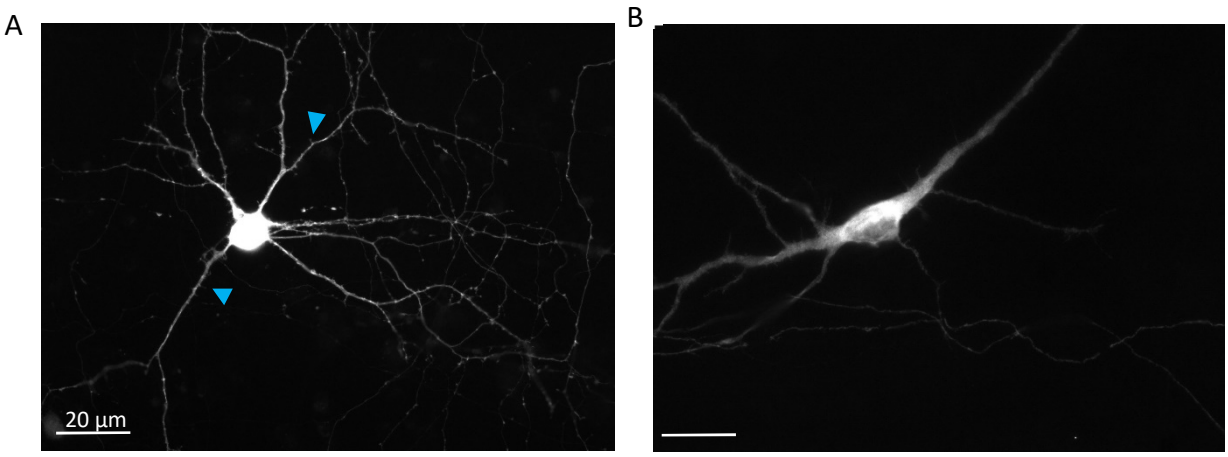

Figure 4. Typical morphology of cortical and midbrain DA neurons in the microfluidic device. A) Fluorescence image of a GFP transfected cortical neuron in the microfluidic chamber. The neuron is highly ramified, with several dendrites coming out the soma. Some protusions (blue arrowheads), presumably spines or filopodia, can be observed along thin dendrites. B) Fluorescence image of a TH positive neuron (TH : 1:1000) stained with A568 in the midbrain chamber. The morphology is quite different from the the cortical neuron. The neuron is fusiform and bipolar with 2 thick primary dendrites coming out the cell body. These morphological features are generally associated to midbrain DA neurons in standard culture.

The objective being to track NMDAR surface diffusion in midbrain DA neurons, the presence of surface NMDAR in DA neurons was also assessed. Since the co-culture became confluent and very dense after 2 weeks *in vitro*, NMDAR immunostainings were performed in 1 week-old cultures. I immunolabelled the GluN2B subunit because it is the predominant NMDAR subunit in immature midbrain DA neurons (Brothwell et al., 2008; Bellone et al., 2011). Even if the staining was very dense as early as 7 DIV, some clusters of GluN2B could be detected in neurites and cell bodies from DA neurons (**Figure 5**), supporting the presence of surface NMDAR in DA neurons maintained in microfluidic chambers.

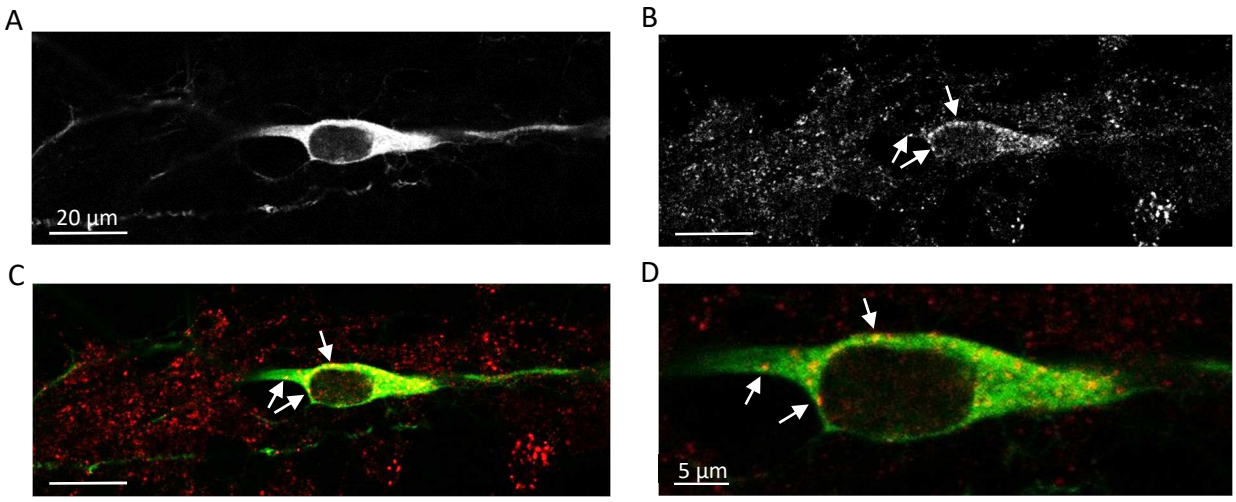

Figure 5. Midbrain DA neurons express surface GluN2B-containing NMDAR in microfluidic chamber. A) Fluorescence image of a TH positive neuron stained with A488, presumed to be DA. B) Fluorescence image of surface GluN2B staining. To label only surface NMDAR, the microfluidic chamber was incubated 15 minutes with anti GluN2B rabbit antibodies at 37°C. Then the cells were permeabilized with Triton and incubated with secondary antibodies anti rabbit A568. Note the presence of GluN2B clusters (white arrows) along the cell body. C) Fluorescence image of the superposition between TH and GluN2B labellings. The GluN2B clusters (white arrows) observed in B) are present in the cell body and proximal dendrites. D) Higher magnification of the cell body in C). The GluN2B clusters are around the nucleus and in proximal dendrites.

After validating that the co-culture developed in microfluidic chambers and that cortical neurons sent projections to the midbrain, the viability of postnatal culture from TH-tdTomato mice was tested. When postnatal midbrain neurons were plated at the range of concentrations used with embryonic cultures, they did not survive. Notably, changing the cell concentration or the coating did not improve the neuronal survival. Therefore, it was not possible maintain postnatal midbrain DA neurons in microfluidic chambers. It should be mentioned that this culture is particularly vulnerable to neuronal death, even in standard culture. Indeed, postnatal cultures are known to have a lot of debris and less neuronal survival than embryonic ones. In addition, DA phenotype seem to be particular vulnerable to death in culture. The number of DA neurons is dramatically reduced 24h after plating (Cardozo, 1993), and they represent less than 5% of the neurons in standard midbrain culture (Silva et al., 1988; Cardozo, 1993; Gaven et al., 2014). The microfluidic device has a configuration that could exacerbate the neuronal loss. There is not direct access to the covered bridge and thus, neurons are plated in one well and are transported in the bridge through the liquid fluid going to the second well. In this way, we cannot control the neuronal concentration in the bridge and this can affect the neuronal survival. The covered bridge could also cause an insufficient exchange between the air and the media and thereby, might alter the oxygen availability. Finally, the volume of the well could be too small to feed the culture.

In conclusion, I successfully implemented embryonic midbrain and cortex co-culture in microfluidic devices. However, the postnatal midbrain culture from TH-tdTomato mice did not survive

in these conditions. Because these cultures are required to label live DA neurons, the investigation of NMDAR membrane dynamics in microfluidic devices is currently limited due to technical limitations.

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