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# Rôles des récepteurs cannabinoïdes de type 1 dans le cortex piriforme antérieur

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THÈSE PRÉSENTÉE  
POUR OBTENIR LE GRADE DE  
**DOCTEUR DE  
L'UNIVERSITÉ DE BORDEAUX**

École doctorale des Sciences de la Vie et de la Santé

Spécialité - Neurosciences

Par Geoffrey TERRAL

**ROLES DES RECEPTEURS CANNABINOÏDES DE TYPE 1  
DANS LE CORTEX PIRIFORME ANTERIEUR**

Sous la direction de : Dr. Federico Massa et Dr. Giovanni Marsicano

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## List of Publications

### Papers published

- Layer-specific potentiation of network GABAergic inhibition in the CA1 area of the hippocampus. 2016 Jun 27;6:28454. *Scientific Reports*.  
Michelangelo Colavita, **Geoffrey Terral**, Clement E. Lemerrier, Filippo Drago, Giovanni Marsicano#, Federico Massa#
- A Cannabinoid Link Between Mitochondria and Memory. 2016 Nov 9;539(7630):555-559. *Nature*.  
Etienne Hebert-Chatelain#, Tiffany Desprez#, Roman Serrat#, Luigi Bellocchio#, Edgar Soria-Gomez, Arnau Busquets-Garcia, Antonio Christian Pagano Zottola, Anna Delamarre, Astrid Cannich, Peggy Vincent, Marjorie Varilh, Laurie M. Robin, **Geoffrey Terral**, M. Dolores García-Fernández, Michelangelo Colavita, Wilfrid Mazier, Filippo Drago, Nagore Puente, Leire Reguero, Izaskun Elezgarai, Jean-William Dupuy, Daniela Cota, Maria-Luz Lopez-Rodriguez, Gabriel Barreda-Gómez, Federico Massa, Pedro Grandes, Giovanni Benard#, Giovanni Marsicano#
- Hippocampal CB1 receptors control incidental associations. 2018 Sep 19;99(6):1247-1259. *Neuron*.  
Arnau Busquets-Garcia, Jose Fernando Oliveira da Cruz#, **Geoffrey Terral**#, Antonio Christian Pagano Zottola#, Edgar Soria-Gomez, Andrea Contini, Hugo Martin, Bastien Redon, Marjorie Varilh, Christina Ioannidou, Filippo Drago, Federico Massa, Xavier Fioramonti, Pierre Trifilieff, Guillaume Ferreira#, Giovanni Marsicano#

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- CB1 receptors dynamically control olfactory processes in the anterior Piriform Cortex *In preparation*  
**Geoffrey Terral**, Gabriel Lepousez, Marjorie Varilh, Arnau Busquets-Garcia, Astrid Cannich, Antoine Nissant, Federico Massa, Edgar Soria-Gomez, Pierre-Marie Lledo, Guillaume Ferreira# & Giovanni Marsicano#.

- Quantitative assessment of hippocampal network dynamics by combining Voltage Sensitive Dye Imaging and Optimal Transportation Theory. *In preparation*  
Michelangelo Colavita, Afaf Bourharguane, Andrea Valenti, **Geoffrey Terral**, Mark W. Sherwood, Clement E. Lemerrier, Fabien Gibergues, Marion Doubeck, Filippo Drago, Giovanni Marsicano, Angelo Iollo, Federico Massa
- Pharmacogenetic stimulation of hippocampal adult-born neurons rehabilitates memory after forgetting. *In preparation*  
Marie Lods, Emilie Pacary, **Geoffrey Terral**, Wilfrid Mazier, Fanny Farrugia, Vanessa Charrier, Federico Massa, Daniela Cota, Djoher Nora Abrous#, Sophie Tronel#
- Post-weaning isolation desensitizes cannabinoid type-1 receptors in mice. *In preparation*

# equal contribution/supervision

## Oral communication

The endocannabinoid system controls olfactory processes in the anterior piriform cortex.  
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## Poster communication

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2017 April. *Doctoral school in Bordeaux-France - Best poster award*  
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2017 August. *Gordon Research Seminar and Conference – Cannabinoid Function in the CNS in Waterville Valley-USA*  
**Geoffrey Terral**, Yohan Wards, Marjorie Varilh, Astrid Cannich, Guillaume Ferreira, Arnau Busquets-Garcia, Edgar Soria-Gomez, Giovanni Marsicano and Federico Massa
- Role of the endocannabinoid system in conditioned odor preference.  
2018 July. *Federation of European Neuroscience Societies 11<sup>th</sup> FENS Forum of Neuroscience in Berlin-Germany*  
**Geoffrey Terral**, Marjorie Varilh, Astrid Cannich, Luigi Bellocchio, Aude Panatier, Federico Massa, Edgar Soria-Gomez, Arnau Busquets-Garcia, Guillaume Ferreira, Giovanni Marsicano.
- The CB1 receptor in the anterior piriform cortex controls olfactory memory.  
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**Geoffrey Terral**, Arnau Busquets-Garcia, Marjorie Varilh, Svein Achicallende, Astrid Cannich, Luigi Bellocchio, Federico Massa, Nagore Puente, Edgar Soria-Gomez, Pedro Grandes, Guillaume Ferreira# & Giovanni Marsicano#.

# Rôles des récepteurs cannabinoïdes de type 1 dans le cortex piriforme antérieur

## Résumé :

Impliquée dans de nombreuses fonctions comportementales, l'olfaction joue un rôle majeur quant à l'orientation de nos actions. Les odeurs communiquent avec le système nerveux central par l'intermédiaire de récepteurs situés dans l'épithélium olfactif du nez qui génèrent des signaux neuronaux, transmis et traités dans de nombreuses régions du cerveau. En particulier, le cortex piriforme antérieur (CPa) est une région olfactive importante impliquée dans la perception et l'intégration des odeurs. Étant donné le rôle du principal récepteur aux cannabinoïdes de type 1 (CB1) dans les fonctions sensorielles et les processus de mémoire, nous avons émis l'hypothèse que ces récepteurs pourraient moduler le traitement des odeurs dans le CPa. Pour ce faire, en combinant des approches anatomiques, électrophysiologiques et pharmacologiques, nous avons d'abord caractérisé la répartition des récepteurs CB1 et évalué leur capacité à réguler les circuits du CPa. Nous avons observé que ces récepteurs sont principalement exprimés dans les interneurons GABAergiques et que leur activation régule la transmission et la plasticité inhibitrice. Puis, nous avons cherché à déterminer le rôle et l'impact des récepteurs CB1 dans le traitement des odeurs dans le CPa. Grâce à une technique d'imagerie calcique *in vivo*, nous avons montré que l'altération de la signalisation des récepteurs CB1 affecte l'activité des neurones du CPa en réponse aux odeurs. En agissant très semblablement sur les circuits inhibiteurs locaux, nous avons mis en évidence que le fonctionnement physiologique des récepteurs CB1 dans le CPa est nécessaire pour le rappel d'une information olfactive apprise dans un contexte appétitif mais pas aversif. De façon générale, ces travaux permettent de mieux comprendre comment les récepteurs CB1 modulent les processus olfactifs dans le CPa.

Mots clés : récepteurs CB1, CPa, odeur, mémoire.



# **Roles of cannabinoid type-1 receptors in the anterior piriform cortex**

## **Abstract:**

Being involved in many behavioral functions, olfaction has powerful influence in guiding our actions. Odors communicate with the central nervous system *via* specialized receptors in the nose olfactory epithelium that generate neuronal signals, which in turn are eventually distributed and processed in many brain regions. In particular, the anterior piriform cortex (aPC) is an important olfactory area involved in perception and integration of odors. Given the extended role of the main cannabinoid type-1 (CB1) receptor in sensory and memory brain functions, we hypothesized that CB1 receptors could modulate odor processing in the aPC. To this aim, using a combination of anatomical, electrophysiological, and pharmacological approaches, we first characterized the distribution of CB1 receptors and their ability to regulate aPC circuits. We found that CB1 receptors are mainly expressed in GABAergic interneurons where their activation regulates inhibitory transmission and plasticity. Then, we evaluated the role and the impact of CB1 receptor modulation on odor-related aPC processing. *In vivo* calcium imaging revealed that odor-evoked aPC activity is affected by alteration of CB1 receptor signaling. Additionally, we demonstrated that physiological aPC-CB1 receptors functioning is necessary for retrieve appetitive but not aversive olfactory memory, likely through modulation of local inhibitory circuits. Overall, this work contribute to a better understanding of how CB1 receptors modulate olfactory processes in the aPC.

Keywords: CB1 receptors, aPC, odor, memory.

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## Long résumé

Les systèmes sensoriels permettent d'adapter nos actions face aux variations environnementales. Parmi les cinq sens, l'olfaction est souvent un sens négligé chez l'Homme mais qui en réalité joue un rôle majeur dans notre quotidien. Par exemple, une simple odeur de viennoiserie en passant devant une boulangerie, est capable de nous ouvrir l'appétit, de stimuler des mémoires ou de modifier plusieurs de nos comportements. Ainsi, et plus précisément, les informations olfactives sont impliquées dans de nombreuses fonctions comportementales telles que la prise alimentaire, l'état émotionnel, les interactions sociales, la détection de danger, et leur mémoire, jouant un rôle très important dans nos décisions quotidiennes. En bref, le système olfactif repose sur la capacité à percevoir et à discriminer les odeurs à partir de molécules odorantes. En arrivant dans la cavité nasale, les molécules odorantes activent les neurones sensoriels de l'épithélium olfactif qui traduisent le message chimique en information électrique. Le signal olfactif est ensuite transféré au bulbe olfactif avant d'être transmis à différentes régions cérébrales dont le cortex piriforme (CP) qui représente la cible principale des arrivées olfactives. Le CP est composé de trois couches cellulaires comprenant des neurones glutamatergiques principalement retrouvés dans la couche II et III et des neurones GABAergiques plus largement distribués au sein du CP. Ce cortex peut être divisé en deux régions anatomiquement et fonctionnellement distinctes, la partie antérieure (CPa) et la partie postérieure (CPp). En particulier, le CPa reçoit de nombreuses informations, à la fois sensorielles provenant du bulbe olfactif et associatives de la part de plusieurs régions cérébrales. Ces caractéristiques font du CPa une région majeure dans le traitement des odeurs et dans la mémoire olfactive.

Contrairement aux autres systèmes sensoriels, les informations olfactives ne sont pas directement relayées par le thalamus. En effet, seulement deux synapses séparent le monde extérieur du CPa ce qui implique que le traitement des odeurs est finement régulé dans cette région. De façon générale dans le cerveau, la régulation de la transmission synaptique est assurée par une variété de neuromodulateurs. Ces effets modulateurs sont notamment observés par l'activation de récepteurs couplés aux

protéines G (RCPGs), dont le récepteur aux cannabinoïdes de type 1 (CB1) est probablement le plus abondant du cerveau. Les récepteurs CB1 régulent de nombreuses fonctions cérébrales comme par exemple la perception sensorielle et la mémoire. Cependant, bien que ces récepteurs soient décrits depuis les années 1990 dans le système olfactif chez le rongeur, leurs fonctions dans les processus olfactifs n'ont commencé à être étudiées qu'au cours des dix dernières années. Plus précisément, plusieurs études ont décrit et déterminé le rôle des récepteurs CB1 dans le premier relais de l'information olfactive, c'est-à-dire dans les neurones sensoriels et le bulbe olfactif. Cependant, aucune étude à ce jour n'a évalué l'importance de ces récepteurs dans le CPa. Etant donné la contribution des récepteurs CB1 dans le contrôle global des fonctions cérébrales et le rôle du CPa dans le traitement des odeurs et dans la mémoire olfactive, **le principal objectif de ma thèse a été d'identifier l'impact de ces récepteurs dans les circuits et les processus olfactifs liés au CPa.**

D'abord, en réalisant une approche anatomique, nous avons caractérisé la présence des récepteurs CB1 dans le CPa. Ces récepteurs sont retrouvés majoritairement dans des fibres GABAergiques localisées dans la couche II du CPa. De plus, les interneurones GABAergiques les produisant sont répartis de façon homogène dans les différentes couches et représentent près de deux-tiers des cellules GABAergiques. Puis, grâce à des expériences électrophysiologiques, nous avons déterminé la fonctionnalité et l'impact des récepteurs CB1 dans le circuit du CPa. L'activation exogène de ces récepteurs diminue la transmission inhibitrice des neurones GABAergiques sur les cellules glutamatergiques de la couche II. Egalement, l'activation endogène des récepteurs CB1 dans les différentes couches est capable d'induire des plasticités inhibitrices à court et long termes. Néanmoins, nous avons observé que la couche I n'était pas capable de produire de plasticité inhibitrice à long terme qui dépend du récepteur CB1, possiblement dû à l'absence d'un type particulier d'interneurones dans cette couche exprimant le neuropeptide cholécystokinine. D'autre part, à travers une approche *in vivo* nous avons évalué comment les récepteurs CB1 étaient capable de réguler les fonctions du CPa en réponse aux odeurs et lors de tâches comportementales. Suite à la présentation d'odeurs, une perturbation de l'activité des récepteurs CB1 affecte la réponse des neurones du CPa. De plus, en agissant vraisemblablement sur les circuits

inhibiteurs locaux, le fonctionnement physiologique des récepteurs CB1 dans le CPa est nécessaire pour le rappel d'une information olfactive apprise dans un contexte appétitif mais pas aversif.

En conclusion, nous avons caractérisé pour la première fois la distribution et le rôle des récepteurs CB1 dans les circuits et les fonctions olfactives liées au CPa. De façon générale, ces résultats permettent de mieux comprendre l'importance de la régulation synaptique induite à travers les récepteurs CB1 dans le système olfactif. Etant donné le rôle des récepteurs CB1 et de l'olfaction dans le développement de pathologies, ces travaux pourront ouvrir de nouvelles perspectives de recherche sur les mécanismes pathophysiologiques liés à l'altération des récepteurs CB1 dans l'olfaction.



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## LIST OF ABBREVIATION

<b>2-AG</b>	2-arachidonoylglycerol
<b>AAV</b>	adeno-associated virus
<b>ACSF</b>	artificial cerebro spinal fluid
<b>AEA</b>	anandamide
<b>AMPA</b>	$\alpha$ -amino-3-hydroxy-5-méthylisoazol-4-propionate
<b>AOB</b>	accessory olfactory bulb
<b>AON</b>	anterior olfactory nucleus
<b>AONpE</b>	anterior olfactory nucleus pars externa
<b>AONpP</b>	anterior olfactory nucleus pars pincipalis
<b>aPC</b>	anterior piriform cortex
<b>APV</b>	(2R)-amino-5-phosphonovaleric acid
<b>ATP</b>	adenosine 5'tri-phosphate
<b>B</b>	bitufted cells
<b>BLA</b>	basolateral amygdala
<b>Ca<sup>2+</sup></b>	calcium
<b>CaMKII</b>	calcium/calmodulin-dependent protein kinase II
<b>cAMP</b>	cyclic adenosine monophosphate
<b>CB</b>	calbindin
<b>CB1</b>	cannabinoid-type 1
<b>CB2</b>	cannabinoid-type 2
<b>CCK</b>	cholecystokinin
<b>CFF</b>	corticofugal fibers
<b>COA</b>	conditioned odor aversion
<b>COP</b>	conditioned odor preference
<b>CR</b>	calretinin
<b>DMSO</b>	dimethyl sulfoxide
<b>dSAs</b>	deep short axon cells
<b>DSE</b>	depolarization-induced suppression of excitation
<b>DSI</b>	depolarization-induced suppression of inhibition
<b>ECS</b>	endocannabinoid system
<b>EPSCs</b>	excitatory post-synaptic currents
<b>eTCs</b>	external tufted cells
<b>FAAH</b>	fatty acid amid hydrolase
<b>FISH</b>	fluorescent <i>in situ</i> hybridization
<b>fMP</b>	fast-spiking multipolar cells
<b>G</b>	neurogliaform cells
<b>GABA</b>	gamma-Aminobutyric acid
<b>GAD</b>	glutamic acid decarboxylase
<b>GCaMP</b>	genetically encoded calcium indicator
<b>GCs</b>	granular cells
<b>GFP</b>	green fluorescent protein
<b>GPCRs</b>	G-protein-coupled receptors
<b>H</b>	horizontal cells

<b>HFS</b>	high frequency stimulation
<b>iLTD</b>	inhibitory long-term depression
<b>IP</b>	intraperitoneal
<b>IPSCs</b>	inhibitory post-synaptic currents
<b>LEC</b>	lateral entorhinal cortex
<b>LiCl</b>	lithium chloride
<b>LOT</b>	lateral olfactory tract
<b>LTD</b>	long-term depression
<b>LTP</b>	long-term plasticity
<b>M/T</b>	mitral tufted
<b>mAChR</b>	metabotropic acetylcholine receptor
<b>MAGL</b>	monoacylglycerol lipase
<b>MAPK</b>	mitogen-activated protein kinase
<b>mGluR</b>	metabotropic glutamate receptor
<b>MOB</b>	main olfactory bulb
<b>mRNA</b>	messenger ribonucleic acid
<b>mTOR</b>	mammalian target of rapamycin
<b>NAPE</b>	N-arachidonoyl phosphatidyl ethanol
<b>NBQX</b>	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline
<b>NMDA</b>	N-methyl-D-aspartate
<b>NPY</b>	neuropeptide Y
<b>OB</b>	olfactory bulb
<b>OE</b>	olfactory epithelium
<b>OR</b>	olfactory receptor
<b>OSNs</b>	olfactory sensory neuron
<b>PC</b>	piriform cortex
<b>PKA</b>	protein kinase A
<b>PTX</b>	picrotoxin
<b>PG</b>	periglomerular
<b>pPC</b>	posterior piriform cortex
<b>PPR</b>	paired-pulse ratio
<b>PV</b>	parvalbumin
<b>Rim</b>	rimonabant
<b>rMP</b>	regular-spiking multipolar cells
<b>SL</b>	semilunar cells
<b>SOM</b>	somatostatin
<b>SP</b>	superficial pyramidal cells
<b>sSAs</b>	superficial short axon cells
<b>TBS</b>	theta burst stimulation
<b>THC</b>	$\Delta^9$ -tetrahydrocannabinol
<b>TTX</b>	tetrodotoxin
<b>TRPV-1</b>	transient receptor potential vanilloid 1
<b>VIP</b>	vasoactive intestinal peptide
<b>VGCC</b>	voltage-gated calcium channel

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# **PART I - GENERAL INTRODUCTION**

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# I.1 THE OLFACTORY SYSTEM

## I.1.1 Why studying olfaction?

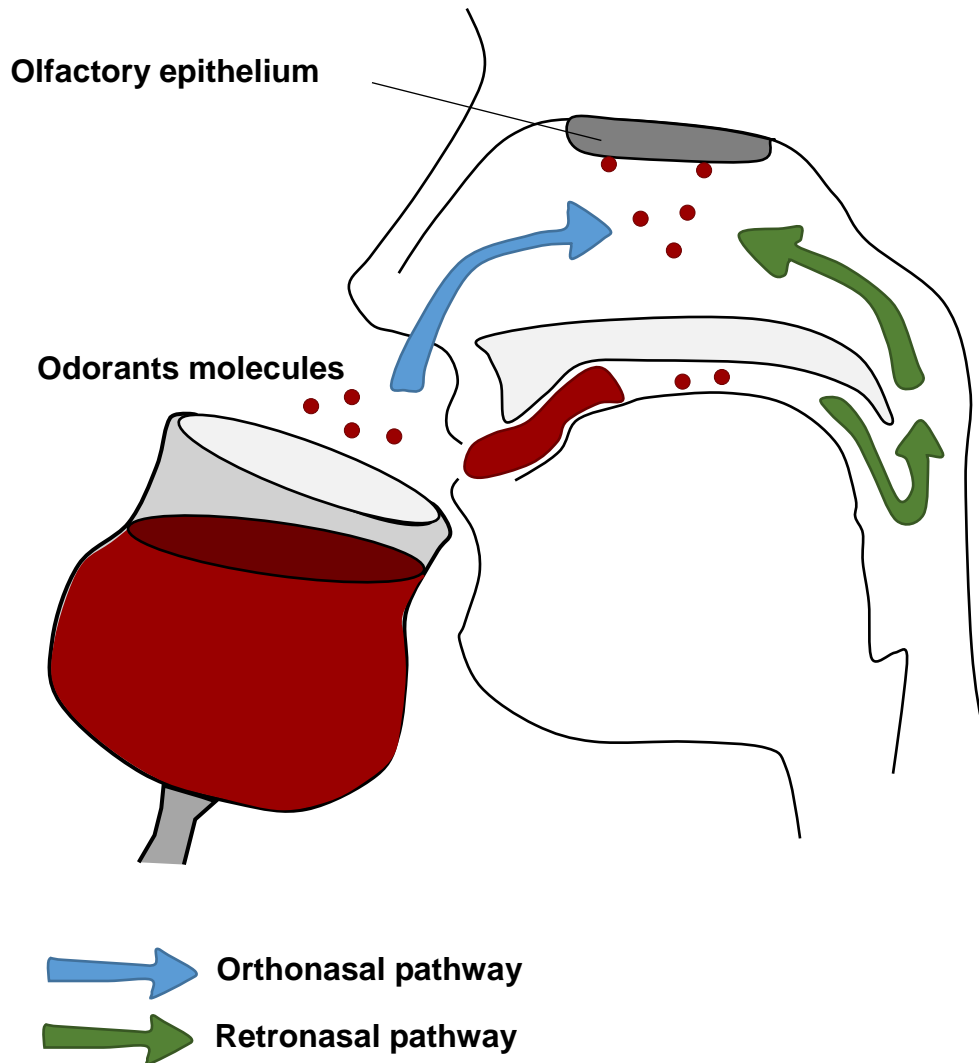
The sensory information provided by the environment is crucial to adapt our actions. Most of the time in daily life, information is perceived through different stimuli and involves several senses. For example, eating food requires identifying it by the shape and the color through the vision, the texture by the touch, the flavor by the taste and the smell, and possibly the audition if it is crunchy. By considering the complexity to process each stimulus with multiple senses, it becomes challenging to understand how sensory information is processed in the brain. Olfaction plays a preponderant role with other sensory modalities such as the taste in feeding behavior (Auvray and Spence, 2008; Prescott, 2012). However, among the five senses, olfaction is often considered as the "ugly duckling" in humans, because it is proposed lower importance for behavioral choices (Gilad et al., 2003; McGann, 2017). However, human sense of smell is more important than what it is thought (De Groot et al., 2017; Gottfried, 2010; McGann, 2017). Odors are powerful stimuli that participate in a plethora of functions including emotional states, food intake, social interactions, warn of dangers and learning and memory processes (Sullivan et al., 2015). Consistently, early alterations of olfactory functions are involved in many neurological, metabolic or neuropsychiatric conditions, such as Alzheimer's disease, obesity or depression (Godoy et al., 2014; Philpott and Boak, 2014). A loss of the sense of smell or anosmia leads to an impairment of the pleasure of eating by affecting the "taste" as described by the subjects (Philpott and Boak, 2014; Rozin, 1982). Moreover, olfaction is a relatively passive sense that depends on respiration (Youngentob et al., 1987) and humans unconsciously take many behavioral decisions based on olfactory cues, (Hoover, 2010; Köster, 2009; Stevenson and Attuquayefio, 2013). For instance, passive perception of fruity odors prior food choice test influences the decision towards fruity desserts (Gaillet-Torrent et al., 2014), indicating that odors are able to drive feeding behaviors (Köster, 2009). Furthermore, the feeding state of individuals modify olfactory perception (O'Doherty et al., 2000; Peger et al., 1972). For all these reasons, understanding the

neurobiological mechanisms of olfaction represents a fundamental question for the human beings.

Unlike audition or vision where the stimuli can be measure regarding a simple physical property (e.g. wave frequency or length), the odorant is defined by complex properties and often based on unconscious perception making it difficult to measure and define (Agapakis and Tolaas, 2012; Stevenson and Attuquayefio, 2013). Odorants are composed of volatile molecules with a large range of physicochemical properties, from molecular weight to pressurization state (Arctander). Moreover, a single odorant is composed most of the time by dozens or even hundreds of volatile molecules with different structural composition such as aldehyde, alcohol, phenols, esters... Thus, how does the olfactory system process volatile molecules in an odor?

### **I.1.2 Arrival of the odor**

To perceive an odor, the volatile molecules have to reach olfactory structures. Through the respiration, these molecules are transported from the environmental air into the nasal cavity. Two distinct paths can lead to olfactory perception: the orthonasal or the retronasal pathways (Pierce and Halpern, 1996). One of the best example (at least for French people) of these two pathways is occurring during wine tasting (**Figure 1**). For the orthonasal pathway, odorant molecules are directly conveyed into the nasal cavity through the nostrils by inhaling with the nose above the glass. The amount of molecules arriving in the cavity is then control by the sniffing pattern (frequency and amplitude of inhalation) (Youngentob et al., 1987). Conversely, the retronasal pathway is used when the food or beverage (e.g. wine) reach the nasal cavity after being in the mouth. The mastication and the expiration release the volatile molecules that provide rich information regarding the ingested foods and beverages. As for the orthonasal pathway, the amount of molecules arriving in the cavity is modulated by the dynamic of the mastication and expiration (Burdach and Doty).



**Figure 1. Representative scheme of the orthonasal and retronasal pathways.**

*Odorants molecules reach the nasal cavity through the nostrils (orthonasally-blue arrow) or the mouth (retronasally-green arrow).*

Although the olfactory system responsible for odor processing is the same between the two pathways, the olfactory perception may slightly differ regarding the route taken by the molecules (Hannum et al., 2018). An explanation of this discrepancy results in the modification that occurs in the retronasal pathway. Indeed, once the molecules reach the mouth, the saliva modify the properties of volatility, the structure and the amount of molecules arriving to the nasal cavity and so the resulting chemical message (Goldberg et al., 2018). Another explanation for this difference is the multisensorial aspect. In the

mouth, both the gustatory and the somatosensory system are involved in the discrimination of the food or the beverage (Bult et al., 2007) which can lead to the activation of different brain structures that in turn trigger different responses (Small et al., 2005). Apart from the mouth, it has been shown that vision can shape the olfactory response. Indeed, colors are able to control odor intensity by increasing orthonasally but decreasing retronasally the odor value (Koza et al., 2005). Finally, the last explanation for the variation in perception between these two pathways is the non-homogenous organization of olfactory receptor neurons in the olfactory epithelium (Ressler et al., 1993). Therefore, the two routes taken by volatile molecules might activate different sensory neurons responsible for different olfactory processes leading to different percepts.

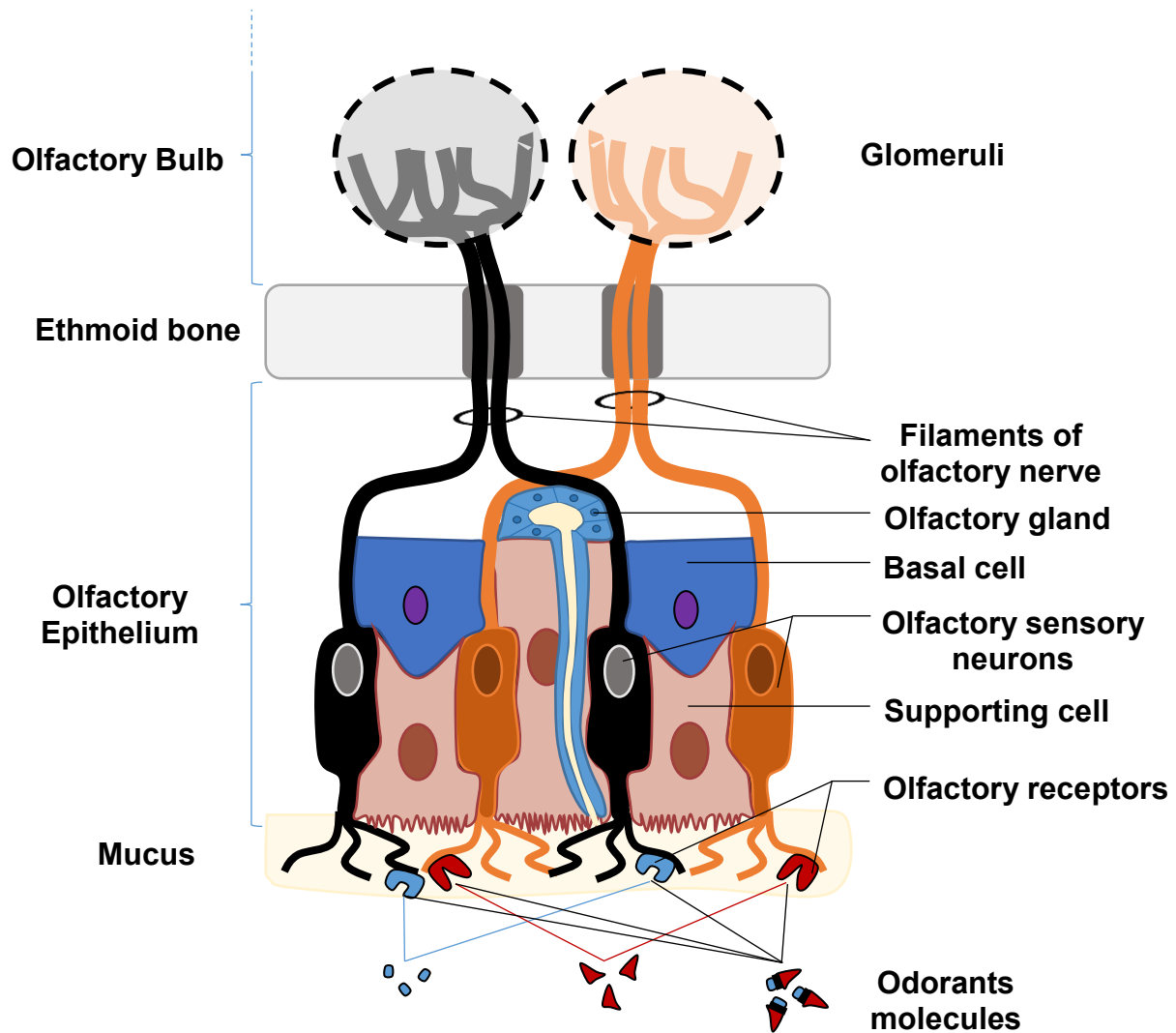
### **I.1.3 Functional architecture of the olfactory system**

#### ***I.1.3.1 Olfactory epithelium***

In the nasal cavity, the volatiles molecules are first detected in the olfactory epithelium (OE), whose convoluted architecture of this structure creates a large surface of detection. The OE is pseudostratified and composed of four types of cells (supporting cells, basal cells, brush cells and olfactory sensory neurons OSNs) protected by a mucus (**Figure 2**). Among these cells, the OSNs are the first protagonist of the olfactory response. Odorants molecules bind olfactory receptors (ORs) located in the ciliary membrane surface of OSNs dendrites. Although ORs are organized in four zones within the OE, each OR shows a widely dispersed distribution within each zone (Ressler et al., 1993). Thus, there is a partial topography of odor detection already in the OE.

The binding with the OR transduces the stimulus provided by an odorant molecule into an electrical signal (Buck and Axel, 1991). This signal is then transmitted onto the main olfactory bulb (MOB) through the olfactory nerve that represents the clustering of OSNs axons. A single OSN expresses only one molecular olfactory receptor, whereas a single OR is able to bind several odorants molecules (**Figure 2**) (Malnic et al., 1999). Given that there are about 1000 OR genes coding for ORs in the mouse genome and

about 400 in the human genome (Glusman et al., 2001; Zhang and Firestein, 2002), the olfactory system is using a combinatorial code allowing perceiving and recognizing a large range of odorants.



**Figure 2. Representative organization of the olfactory epithelium.**

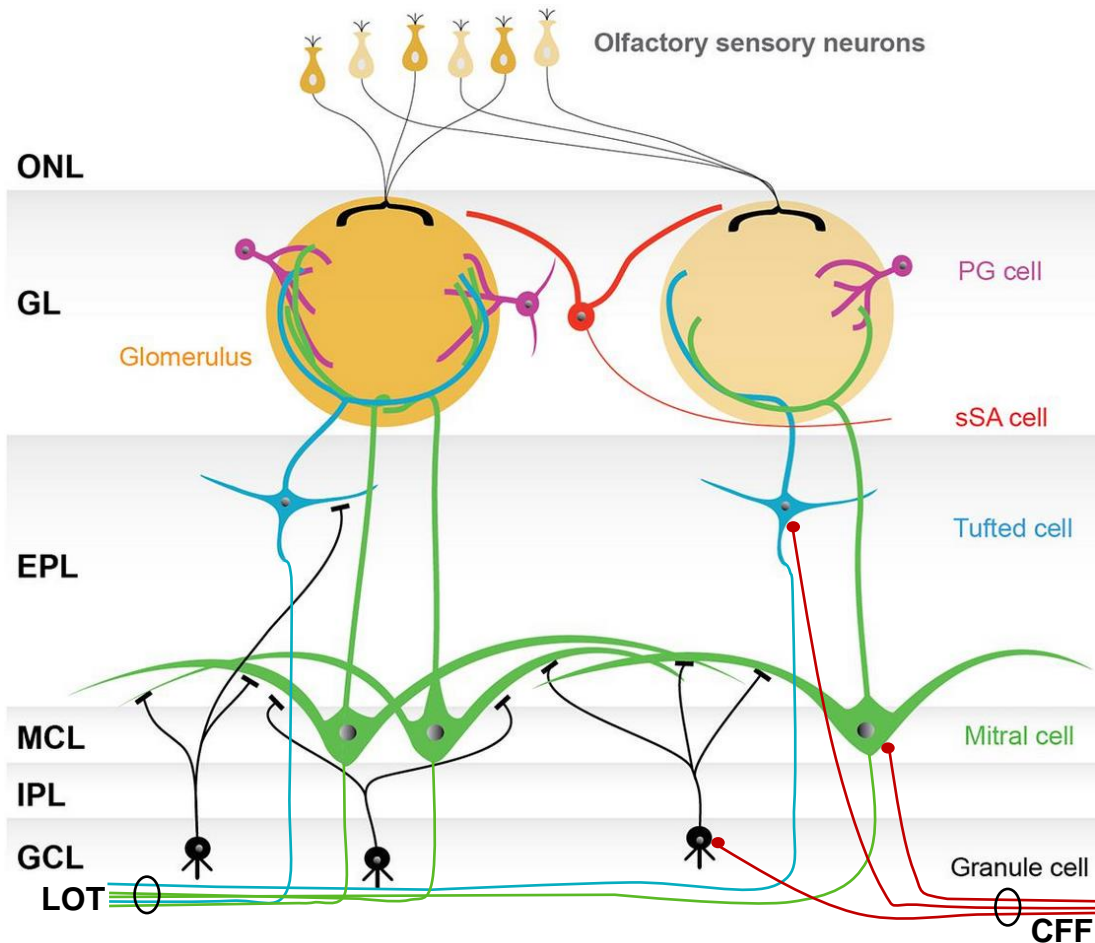
*Odorants molecules bind olfactory receptors on the olfactory sensory neurons. In turn, the signal is transmitted in the olfactory bulb through the olfactory nerve.*

### ***1.1.3.2 Main olfactory bulb***

The olfactory bulb (OB) is composed of the accessory olfactory bulb (AOB) and the MOB. Although the AOB and the MOB may have complementary function, the AOB is thought to play important role in the detection of pheromones (Mucignat-Caretta et al., 2012). Thus, in sake of clarity in the context of this study, only the MOB will be described in the following sections of this Thesis.

The MOB is the first brain relay of the olfactory information. Each OSN expressing only one OR projects to single processing modules in the MOB called glomeruli (**Figure 2**). Thus, each glomerulus receives thousands inputs from the same OSNs and conveying the same OR-mediated signal. However, the same group of OSNs axons can target two or three specific glomeruli, with an invariable position across individuals (Mombaerts et al., 1996). Moreover, given that odorant receptors are expressed in four zones in the OE (see above in Olfactory epithelium section), zonal organization is preserved to some extent in the MOB (Astic and Saucier, 1986). Therefore, these features allow a spatial representation of the odor in a topographic map called chemotopy.

The MOB is composed of six different layers, from external to internal: the olfactory nerve layer, the glomerular layer, the outer plexiform layer, the mitral cell layer, the inner plexiform layer and the granular layer (**Figure 3**) (Pinching and Powell, 1971). OSNs axons coming from the olfactory nerve layer terminate on glomeruli in the glomerular layer. Within the glomeruli, excitatory synaptic connections are established with dendrites of mitral and tufted (M/T) cells, which represent the principal neurons in the MOB. Their bodies are located respectively in the outer plexiform layer and in the mitral cell layer, and their axons assemble form the lateral olfactory tract (LOT), eventually projecting to higher olfactory centers including the anterior olfactory nucleus (AON), the olfactory tubercle (OT), the piriform cortex (PC), the amygdala, the *taenia tecta* and the lateral entorhinal cortex (Haberly and Price, 1977).

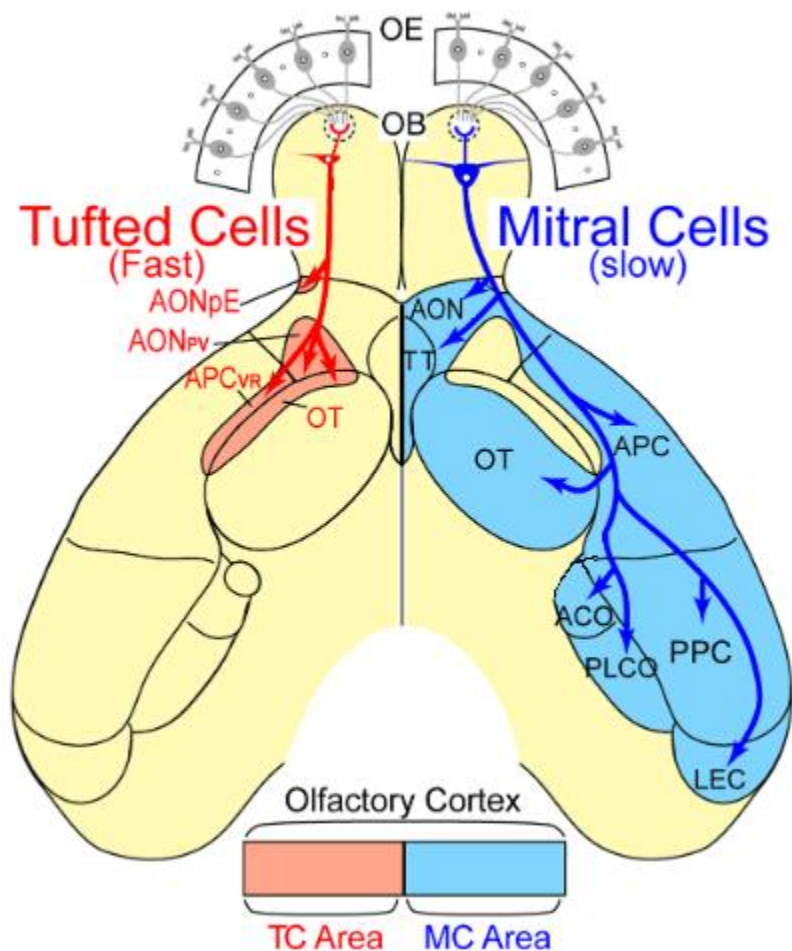


**Figure 3. Representative organization of the olfactory bulb circuitry.**

Olfactory sensory neurons contact mitral and tufted cells within the glomeruli. The output response forming the lateral olfactory tract (LOT) is shaped by the corticofugal fibers (CFF) and several interneurons: periglomerular (PG), superficial short axon (sSA) cells, external tufted cells and granule cells. ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer; MCL, mitral cell layer; IPL, internal plexiform layer; GCL, granule cell layer. Adapted from Nagayama, Homma and Imamura 2014.

Although mitral and tufted axons have similar pathways route, they target different olfactory structures (Igarashi et al., 2012). Whereas mitral cells project to all the above-mentioned areas, tufted cells target olfactory structures relatively close to the olfactory bulb, such as the AON, the anterior PC (aPC) and the OT (**Figure 4**). In addition, these two types of projection neurons differ in their response of odor concentrations, suggesting that both cells transmit distinct odor information (Igarashi et al., 2012).





**Figure 4. Representative connectivity between olfactory structures and mitral and tufted cells.**

*Projections from mitral (MT) and tufted (TC) cells differ regarding olfactory areas. OE, olfactory epithelium; OB, olfactory bulb; AON, anterior olfactory nucleus; pE, pars externa; pV, pars ventroposterior; TT, taenia tecta; OT, olfactory tubercle; APC, anterior piriform cortex; VR, ventrorostral; ACO, anterior cortical amygdaloid nucleus; PLCO, posterolateral cortical amygdaloid nucleus; PPC, posterior piriform cortex; LEC, lateral entorhinal cortex. Adapted from Igarashi et al. 2012.*

The MOB does not function as a simple relay structure but it has integrative roles. Indeed, there are different interneurons that shape the olfactory response in the MOB: the juxtglomerular and the granular cells (GCs) (Nagayama et al., 2014). The juxtglomerular neurons are composed of periglomerular (PG), superficial short axon cells (sSAs) and external tufted cells (eTCs) located in the glomerular layer. The anatomical organization of their synapses together with glial cells restricts the glomeruli to a spherical size of 100 to 200  $\mu\text{m}$  in diameter (Pinching and Powell, 1971). The PG and the sSAs provide mainly GABAergic inhibition in the glomerular layer. In addition to GABA, certain

subpopulations of PG produce the neuromodulator dopamine (Kosaka and Kosaka, 2016). Conversely, eTCs are glutamatergic neurons that activate juxtglomerular neurons and M/T cells. Thus, juxtglomerular neurons participate in the tuning of glomerular output. On the other hand, the GCs are located in the granular layer and are the most numerous cellular population of cells in the OB. Their dendrites establish reciprocal dendro-dendritic inhibitory synapses with M/T cells, thereby regulating the output signal (**Figure 3**). Moreover, olfactory information in the MOB is tightly regulated by feedback projections from cortical areas. These fibers, called corticofugal fibers (CFF), mainly target GC and M/T cells, further refining olfactory responses (Stowbridge, 2009).

### ***1.1.3.3 Higher olfactory structures***

Because several olfactory structures integrate olfactory information, the anatomical organization and connection of these areas will be only briefly described for the AON, OT, amygdala, lateral entorhinal cortex, the neocortex, the thalamus and the hypothalamus. More details will be provided in a separated sub-chapter for the PC, which represents the main subject of the present Thesis.

#### ***1.1.3.3.1 Anterior olfactory nucleus***

Located between the MOB and the aPC, the AON is the more rostral cortical olfactory area and it is the first region receiving inputs from the MOB. In turn, AON neurons project to different brain regions such as back to the MOB, the PC, the OT, the contralateral AON and non-olfactory brain areas (Brunjes et al., 2005). The AON can be divided into two separate structures, *pars externa* (AONpE) and *pars principalis* (AONpP). AONpE is composed of a thin ring of cells encircling the rostral end of the olfactory peduncle. AONpE neurons receive input from ipsilateral MOB and activate contralateral M/T cells, allowing at the same time the discrimination of odor source from the ipsi- and contra-nostril and the maintenance of the topographical organization (odotopic map) of

the odor as in the MOB gomeruli (Grobman et al., 2018; Kikuta et al., 2010). Given that OSNs and M/T cells project their axons to ipsilateral MOB and olfactory areas, respectively, AONpE neurons seem to be mainly involved in the harmonization of the odor responses between the two hemispheres. Located across the entire olfactory peduncle, AONpP is the major part of the AON. It is a two-layered structure with an outer layer (layer I) subdivided into a superficial layer (layer Ia) containing axons from the LOT and a deeper layer (layer Ib) containing dendrites and interneurons. The deepest layer (layer II) is composed of a thick pyramidal layer and diverse interneurons (Kay and Brunjes, 2014). Moreover, the AONpP can be divided into four distinct areas: *pars lateralis*, *pars dorsalis*, *pars medialis* and *pars ventroposterior*. The cellular and input/output variations between these areas suggest that they play distinct roles in olfactory processing (Hamrick et al., 1993). However, the different functions of AONpP subdivisions remain poorly studied.

#### I.1.3.3.2 Olfactory tubercle

The OT is located along the rostral ventral region of the brain, between the PC and below the ventral striatum. The OT receives input from the MOB and is interconnected with the PC and other brain regions involved in sensory, cognitive, endocrine and reward-related center (Wesson and Wilson, 2011). This massive connectivity makes the OT a crucial structure integrating multi-modal information. The OT is a three-layered structure constituted of a molecular layer (layer I), a dense cell layer (layer II) and a multiform layer (layer III). Contrary to classical sensory cortex (such as the auditory cortex or the PC) that show smooth layers, the TO is formed of several hills and clusters of cells called islands of Calleja (Fallon et al., 1978).

#### I.1.3.3.3 Amygdala

Three main groups of nuclei are observed in the amygdala (basolateral, centromedial and cortical (McDonald, 2003), however, olfactory areas project mainly to

the cortical nucleus (Root et al. 2014). Indeed, the MOB sends projections to anatomically distinct areas of this region, suggesting that the odor map from the MOB is conserved in the amygdala (Sosulski et al., 2011). These projections are necessary for innate behaviors such as for aversive and appetitive behaviors (Choi et al. 2011; Root et al. 2014). Moreover, the amygdala receives also PC and OT projections (Haberly and Price 1978) that participate in odor fear conditioning (Otto et al., 2000).

#### I.1.3.3.4 Lateral entorhinal cortex

The lateral entorhinal cortex (LEC) is a cortical area interface between the olfactory system and the hippocampus. It receives both information from the MOB and the PC and projects back to these two structures (Haberly and Price, 1978; Kay et al., 1996; Sosulski et al., 2011). As mentioned, the LEC is highly interconnected with the dentate gyrus and the CA3 area of the hippocampus. The high LEC density of amyloid- $\beta$  in Alzheimer's disease has been suggested to underlie the olfactory dysfunctions associated with the disorder, such as impairment in odor identification (Wilson et al., 2007). Thus, together with its hippocampal connectivity, the LEC appears to play important roles in the modulation of odor-related memory.

#### I.1.3.3.5 Neocortex

Olfactory afferents target two main regions in the neocortex: the orbitofrontal cortex (OFC) and the agranular insula. These two areas receive both projection from the MOB and the PC (Haberly and Price, 1978; Shipley and Geinisman, 1984). The neocortex is involved in decision-making of affective value (agranular insula), subjective pleasantness ratings, reward and motivational system. These structures are multisensorial areas where, for example, olfactory information and taste information converge for the first time, possibly underlying the sensation of flavor (Small et al., 2004).

#### I.1.3.3.6 Thalamus

Unlike other sensory modalities, sensory processing of smell is not directly relayed *via* the thalamus. However, some of the olfactory cortical areas project to the mediodorsal thalamic nucleus which in turn, like in other sensory systems, modulate the sensory information processing (Tham et al., 2009).

#### I.1.3.3.7 Hypothalamus

Different olfactory areas project to the hypothalamus. More precisely, the lateral hypothalamus receives inputs from the AON, PC, OT and the anterior cortical nucleus of the amygdala (Price et al., 1991). Given that the lateral hypothalamus contains orexin neurons involved in promoting feeding behavior and arousal, this olfactory-hypothalamic axis might be important for the control of food intake (Soria-Gomez et al., 2014).

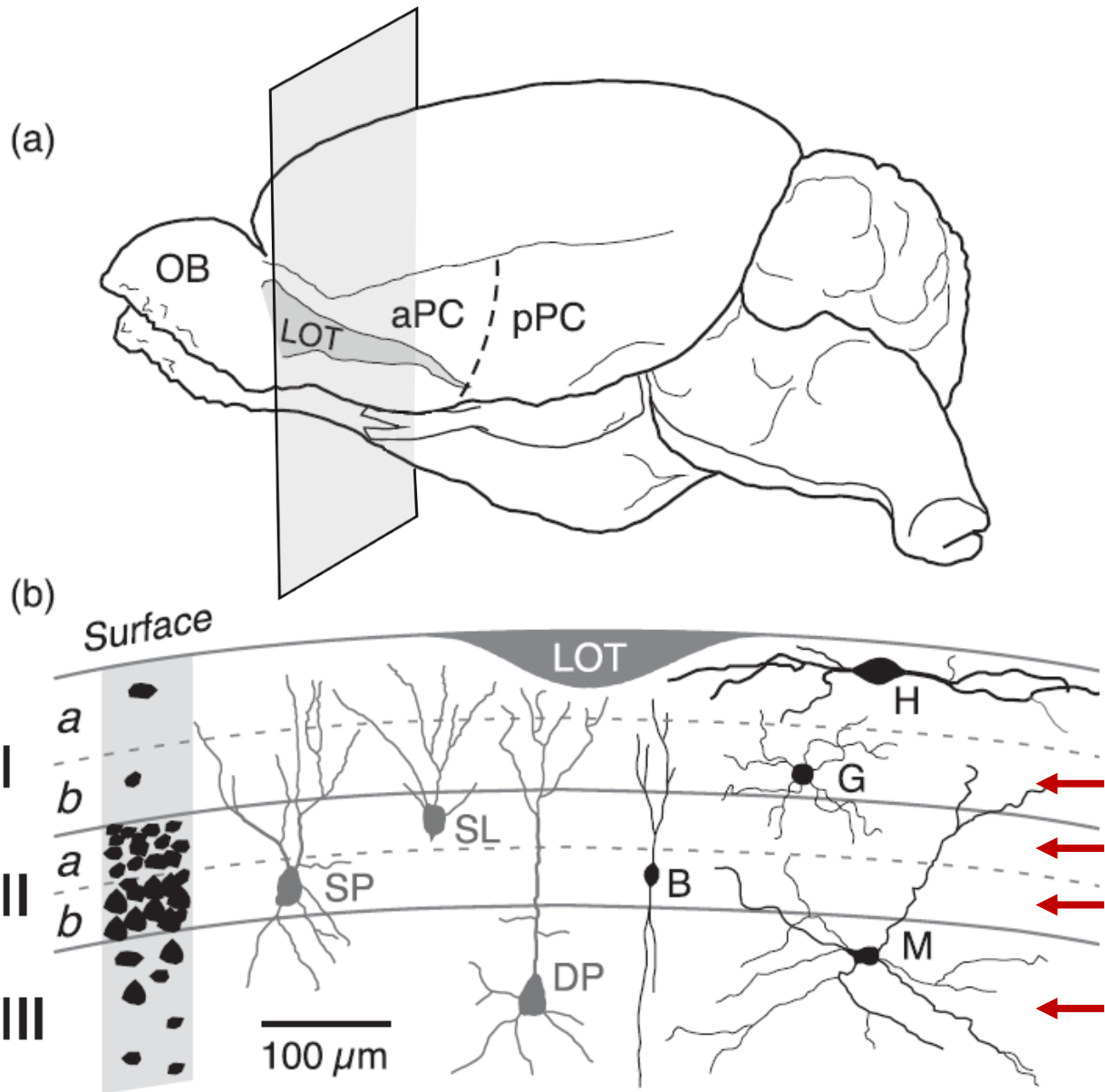
### ***1.1.3.4 The piriform cortex: the main gateway of olfactory processes***

#### I.1.3.4.1 Anatomical characteristics

The PC represents the largest olfactory structure within the brain. It is located in the ventrolateral surface of the brain and is commonly divided into two parts regarding the anteroposterior axis, with the boundary at the caudal end of the LOT (Haberly, 2001), providing an anterior (aPC) and a posterior part (pPC) (**Figure 5A**). The aPC and the pPC show several differences. First, aPC but not pPC contains LOT and, therefore, the main inputs to the aPC come from M/T cells, as well as AON and few associational fibers. Conversely, pPC receives more associational inputs from several brain areas and few axons from mitral but not tufted cells (Bekkers and Suzuki, 2013; Haberly, 2001; Igarashi et al., 2012). Consistent with the associative features prominent in the pPC, the neural

activity in response to odor cues appears highly plastic during learning in the pPC whereas it is more influenced by the sensory attributes of the odor cues in the aPC (Calu et al., 2007). These anatomical differences may explain a functional divergence in response to odors. Indeed, (Litaudon et al., 2003)) demonstrated that odor evoked activity results in a decreasing recruitment of responding cell along the anterior/posterior axis of the PC, together with a difference in synchronization within the respiratory activity. Furthermore, it has been shown that the PC can predict an odor. However, whereas the pPC is capable to discriminate between expectation and actual odor exposure, the aPC responds to the attended odor target for several seconds rather than the current smell, indicating that aPC activity reflects more the sought-out odor than the actual one (Zelano et al., 2011). Moreover, the processing of odor properties differ between aPC and pPC: whereas the aPC encodes odor identity (structural component of an odor, e.g. aldehyde), the pPC encodes odor quality (perceptual character of an odor, e.g. almond-like) allowing the perception of a unified odor (Gottfried et al., 2006; Kadohisa and Wilson, 2006a). Together, these observations suggest that the pPC is mainly involved in higher associative functions rather than being a primary sensory cortex.

Like for other phylogenetically ancient cortices (paleocortex), the PC is laminated with only three main layers. Layer I is the most superficial layer containing sparsely populated neurons, whereas layer II is distinguishable by densely packed principal neurons. Both layer I and layer II can be subdivided into two parts, “a” for the upper and “b” for the lower part. The deep layer, layer III, comprises scattered deep pyramidal neurons (**Figure 5B** ((Neville and Haberly, 2004). Between layer III and the ventral part of the claustrum appears the endopiriform cortex, which, because of the important connections with the PC, is sometimes considered as layer IV (Haberly and Price, 1978). The endopiriform cortex is an epileptogenic structure connecting with other regions of the cerebral cortex by glutamatergic multipolar neurons. Its functions are still unclear but its extensive intrinsic excitatory connections between the PC and other higher brain structure might participate in olfactory memory storage (Behan and Haberly, 1999).



**Figure 5. Schematic representation of the location and anatomy of the piriform cortex.**

**(a)** Ventrolateral illustration of the piriform cortex in the rat brain. **(b)** Schematic laminar structure of the anterior piriform cortex (aPC) according to a coronal section (grey panel in **(a)**). OB, olfactory bulb; LOT, lateral olfactory tract; pPC, posterior piriform cortex; I, II, III, layers. Glutamatergic neurons in grey; SL, semilunar; SP, superficial; DP, deep pyramidal. GABAergic neurons in black; H, horizontal cell; G, neurogliaform cell; B, bitufted; M, multipolar. Red arrows, associational fibers. Adapted from Suzuki and Bekkers, 2007.

#### *1.1.3.4.2 Excitatory circuits*

Although an odotopic map is observed in glomeruli of the MOB, widespread and diffuse odorant information from individual glomeruli is integrated in the PC (Ghosh et al. 2011; Poo and Isaacson 2009; Sosulski et al. 2011; Stettler and Axel 2009). Moreover, single neurons in the PC receive convergent synaptic inputs from multiple glomeruli (Apicella et al., 2010), suggesting that the PC is able to unify odor features, thereby allowing the construction of an odor percept. Indeed, the PC is composed of a complex circuitry implying different inputs, principal cells and inhibitory neurons. Two types of inputs arrive to the PC, the sensory input fibers coming from the MOB target the layer Ia, whereas associational and commissural fibers from PC's neurons and elsewhere appear to be confined to the other layers (Ib, II and III) (Bekkers and Suzuki, 2013). Afferent inputs from the MOB to the PC are influenced by anatomical and intrinsic electrical properties of the receiving cells. There are two subclasses of receiving principal neurons, the semilunar cells (SL) with the soma located mainly in layer IIa with large spines and apical dendrites find in layer Ia and superficial pyramidal cells (SP), with their soma concentrated mainly in layer IIb and with both basal and apical dendrites. These morphological features imply that SL cells receive stronger afferent input from the MOB than SP cells which are more likely to receive associational inputs (Suzuki and Bekkers, 2006, 2011). Furthermore, SL and SP cells show different action potential firing patterns and intrinsic synaptic plasticity. While SP cells display paired-pulse facilitation and fire bursts of action potential following LOT activation, SL cells demonstrate non-facilitating dynamics and fire in a non-bursting manner followed by powerful after-hyperpolarization (Suzuki and Bekkers, 2006, 2011). These distinctive properties provide cell-dependent processing in the PC. In addition, associational inputs increase the complexity of neuronal responses in the PC. There are two kinds of associational connections, intrinsic connections that rely on internal communication within the PC and extrinsic connections linking the PC with other brains regions. The intrinsic connections, also known as autoassociative connections, ensure the communication of pyramidal cells among each other and with interneurons. Autoassociative connections maximize the convergence of signal within PC neurons. (Yang et al., 2017) demonstrated that 78% of the total length and 79% of the total number



of boutons from axon collaterals of single aPC-SP neurons are found in the PC, covering 18% of the total area of the aPC and 4% of the pPC. It has been estimated that each principal neuron receives about 200 afferent inputs from the MOB but more than 2000 recurrent excitatory inputs from other principal neurons within the aPC (Franks et al., 2011). Both SL and SP cells are responsible for recurrent connections. However, different extents are observed between aPC and pPC. While the recurrent connectivity within the aPC is sparse and weak, the pPC has denser and higher intrinsic connection probability (Hagiwara et al., 2012), suggesting once again that pPC is more involved in functional association than the aPC. Conversely, extrinsic connectivity with other olfactory structures ensures the complexity of the odor percept. All the brain areas described above send different projections to the aPC or the pPC. This could be explained in part by the proximity between the olfactory structures. For example, the aPC receives more inputs from the MOB and the AON (Hagiwara et al., 2012; Igarashi et al., 2012) whereas the pPC, which is closer to the amygdala, receives more inputs from the basolateral amygdala (BLA) (Luna and Morozov, 2012).

Taken together, the complex meshwork provided by afferent inputs and by associational fibers may explain the distributed organization of the activation of PC principal neurons, and the lack of evident topographical functional mapping in response to odor.

#### *1.1.3.4.3 Inhibitory circuits*

Balance between excitation and inhibition is tightly regulated in the brain. A dysfunction in this balance is associated with alterations in neuronal global activity that all underpin neuropsychological disorders (Tatti et al., 2017). In the PC, inhibition plays an important role in maintaining both appropriate PC neurons firing and in controlling potential epileptogenic activity in the endopiriform cortex. As in other cortical areas, the PC contains high proportion of glutamate-releasing principal neurons and a much smaller number of GABA-releasing interneurons. PC-interneurons are not a homogenous population and, like in the hippocampus or the neocortex, large diversity in interneuron classes is present.

Based on canonical inhibitory circuits, two types of inhibition are observed in the PC: feedforward and feedback inhibition. According to electrophysiology, laminar location, morphology and molecular markers expression, five classes of interneurons uniformly distributed across all the layers have been pointed out in the PC (Suzuki and Bekkers 2007, 2010a, 2010b, 2012; Young and Sun 2009).

Feedforward inhibitory interneurons are restricted to the layer I, which receives mainly afferent inputs from the LOT (Neville and Haberly, 2004; Suzuki and Bekkers, 2007). Two main classes of interneurons has been reported providing feedforward inhibition, the horizontal (H) and neurogliaform (G) cells. H cells have large and elongate soma and long horizontal dendrites. Their axon is mainly restricted to layer I and they are exclusively found in layer Ia. G cells are located in all the layers but with larger proportion in superficial than in deeper ones. They display small soma with short dendrites and profusely ramifying axons often restricted to the same layer as the soma. Both H and G cells seem to not express any of the common molecular markers characteristic of GABAergic interneurons in other cortical areas (calbindin, CB; calretinin, CR; parvalbumin, PV; cholecystokinin, CCK; neuropeptide Y, NPY; somatostatin, SOM; vasoactive intestinal peptide, VIP; **Figure 5B**).

Feedback inhibitory neurons are restricted to deeper layers (Neville and Haberly, 2004; Suzuki and Bekkers, 2007). In this case, associational fibers (intrinsic or extrinsic) activate interneurons, which in turn inhibit principal neurons. Four classes of interneurons are responsible of feedback inhibition, G cells, bitufted (B) cells, fast-spiking multipolar (fMP) cells, and regular-spiking multipolar (rMP) cells. As mentioned above, G cells are responsible of feedforward inhibition but play also a role in feedback inhibition if located in deeper layers. Indeed, some passive electrical properties differ with the laminar location of G cells. For example, G cells from layer Ia has a lower resting potential ( $V_{rest}$ ,  $-78.1 \pm 0.9$  mV) and higher rheobase ( $R_h$ ,  $317 \pm 28$  pA) than in layer III ( $V_{rest}$ ,  $-71.8 \pm 0.4$  mV;  $R_h$ ,  $188 \pm 14$  pA). B cells have small and bipolar soma mainly located in layer II with long dendrites extended across the three layers. Axon collaterals are mainly found in layer IIb and III forming basket terminations around principal neurons somas. B cells are characterized by the expression of the neuropeptides VIP. fMP cells have their soma

located mainly in layer II and III and have multipolar morphologies with sparsely dendrites located in all layers and strongly ramified axons projecting to layer II. fMP cells fire at high frequency (fast-spiking phenotype) similar to fast-spiking (FS) cells observed in other brain regions. They express either CB or PV or both markers. Finally, as fMP cells, rMP cells have a multipolar shape, but they have profuse dendrites and their axon is not restricted to layer II. rMP cells are mainly found in layer III. They display moderate frequency of firing (regular phenotype) and express mainly SOM or a combination of both SOM and CB markers (**Figure 5**).

Functional difference can be observed between feedforward and feedback inhibition. (Franks et al., 2011) demonstrated that feedback inhibition is stronger than feedforward inhibition in the PC. This could be explained by the ten times higher recurrent intrinsic inputs from principal neurons than afferent inputs from the MOB. These inputs activate both other glutamatergic neurons and GABAergic interneurons. As the axon collaterals are covering a large part of the PC (mainly in layer Ib, II and III; (Yang et al., 2017), they essentially target feedback inhibitory interneurons. However, feedforward and feedback inhibition likely play a synergistic role in global and powerful inhibition to counter the recurrent excitatory circuitry. It has been shown that principal neurons receive unbalanced inputs between excitation and inhibition. Odor-evoked activity induces widespread and nonselective inhibition, whereas excitation is sparse and odor specific, suggesting that GABAergic interneurons receive more odor information than principal neurons (Franks et al., 2011; Poo and Isaacson, 2009).

Inhibitory interneurons classes may be different between the aPC and pPC. Two main studies evaluated the interneurons properties within the PC. Suzuki and Bekkers (2007, 2010a, 2010b, 2012) used several characteristics to classify aPC interneurons (see above), whereas (Young and Sun, 2009) classified the interneurons from the pPC based on firing and morphological properties only. Although different names were given for each interneuron type within these two areas, they appear to have similar morphology and firing patterns. However, differences in inhibition are observed along the aPC rostro-caudal axis, with larger inhibition occurring in caudal parts as compared to the rostral part (Luna and Pettit, 2010). A recent study highlighted that this asymmetric inhibition results in an

opposing inhibition onto principal glutamatergic neurons and interneurons (Large et al., 2018). Principal neurons display stronger inhibition in the caudal part, whereas inhibition of interneurons is higher in the rostral part. The rostral enhancement of inhibition onto interneurons arises from the dense population of somatostatin-expressing interneurons, whose density decreases along the rostro-caudal axis. These studies may underlie spatial variation of odor processing regarding the rostro-caudal inhibitory system.

While PC-interneurons share many electrical features with interneurons found in other cortical regions, such as the hippocampus and neocortex, they do not express exactly the same molecular markers associated with the electrical characteristic. For example, G cells display similar firing phenotypes as G cells in the hippocampus and neocortex but do not express NPY as it is usually the case in the hippocampus (Suzuki and Bekkers, 2010b, 2010a). Although there are some studies discriminating the function of canonical inhibitory circuitry (i.e. feedforward and feedback inhibition) in odor processing, the exact role of the different interneuron classes and the gradient of inhibition within the PC remains unclear.

## **Conclusion on the anatomy of the olfactory system**

Olfactory perception and processing start with an odorant reaching into the nasal cavity through two functionally and anatomical distinct routes: the orthonasal or the retronasal pathways. OR binding transduces odorant molecules into electrical activity in the OSNs that is transmitted to specific glomeruli of the MOB. This signal is then sent to different olfactory structures interconnected with each other. The main olfactory structure receiving MOB inputs and associational afferent inputs from other brain regions is the PC. The PC is composed of different cells types: principal and pyramidal glutamatergic neurons (layer II/III) and several types of interneurons scattered across the three layers. Functional and anatomical differences indicate that the PC can be divided in two parts, the aPC and pPC (for reviews: Gottfried, Winston, and Dolan 2006; Suzuki and Bekkers 2011; Wilson and Sullivan 2011). Mainly aPC functions will be described in the following section.

## **I.2 OLFACTORY CODING IN THE PIRIFORM CORTEX**

### **I.2.1 Olfactory perception**

How olfactory stimuli are transformed into a perceptual representation in the brain is still unclear. Perception requires the process of olfactory information by olfactory systems. First, olfactory perception is a relatively passive phenomenon that depends on alternative phases of stimulation and suppression of odor information regulated by respiration. Respiration patterns (frequency and amplitude) play an important role in the modulation of odor perception (Youngentob et al., 1987). Then, the OE and the MOB shape olfactory perception before being represented in the brain as an odor object mainly by cortical areas. Sensorial attributes of an odor is not only encoded by local neurons from the same structure but also by ensembles of distant neuronal areas (Courtiol and Wilson, 2017; Varela et al., 2001). Thus, perception is supported by highly hierarchically organized functional systems that involve dynamic interactions between brain areas. Considering its connectivity with the external world and association fibers from other brain regions, the PC is considered as an epicenter associative cortex playing key roles in olfactory perception (Courtiol and Wilson, 2017; Gottfried, 2010; Haberly, 2001).

Different approaches are used for the study of perception. Behavioral experiments allow the investigation of odor-induced responses in the whole animal during specific tasks. Conversely, the measure of brain activity by electrophysiological and functional assays provides indications concerning the cellular processes occurring in specific brain regions during olfactory functions. Thus, the combination of behavioral paradigm together with recording neuronal activity allows building relationship between a sensation that can be associated with odor properties and/or behavioral responses, and cellular activity.

### ***1.2.1.1 Odor detection***

Perceived features of a smell depend on three different factors, the intensity, the identity and the affective value (pleasantness or repulsiveness). To be processed, an odorant has to be first detected. The minimal concentration that leads to a percept represents the detection threshold. To study this threshold, single odorant is presented at increasing concentrations. However, detection thresholds are very variable regarding odorants, individuals and genders (Amoore and Hautala, 1983; Dalton et al., 2002). For example in humans, isoamyl acetate (banana-like odor), has an odor threshold in the air around  $10^{-6}\%$ , whereas this value is around 10% for ethane. Moreover, the sensitivity can be modulated by prolonged or several exposition of an odor, which decreases its odor threshold (Dalton et al., 2002; Li et al., 2006; Rabin and Cain, 1986). Once perceived, about 25 to 35% of aPC principal neurons respond to a single odorant (Tantirigama et al., 2017). Odorant stimulation can activate, suppress or induce mixed responses in neurons. Depending on the technique used to study odor-evoked responses, activated and suppressed cells are ranging from 6 to 20% of total aPC principal neurons (Bolding and Franks, 2017; Roland et al., 2017; Tantirigama et al., 2017).

### ***1.2.1.2 Odor identity vs intensity***

As previously mentioned (cf. 1.1.3.3.8), identity and quality features of odors are encoded mainly by the aPC and the pPC, respectively. The identification of an odor corresponds to the ability to name what we smell in humans and to discriminate different odors in animals. For example, isoamyl acetate smells like banana and animals can discriminate it from other odors. Odor identity is coded by piriform neurons firing, which convey reliable odor information observed by specific firing pattern code (Miura et al., 2012; Rennaker et al., 2007). However, the complexity to study odor representations is reflected in the widespread and diffuse odorant information integrated by the PC (Ghosh et al. 2011; Poo and Isaacson 2009; Sosulski et al. 2011; Stettler and Axel 2009). Given

that the responses of piriform neurons are spatially dispersed, the activation or the suppression of neurons allows a control of activity narrowly tuned to odorant. Interestingly, a given neuron responds to multiple odorants, but individual odorants regulate the activity of multiple neurons. This overlapping strategy increase the number of possible responses and representations of odors within the PC. Additionally, aPC pyramidal neurons are necessary to distinguish an odor from background information. Whereas prolonged exposition to an odorant (considered as background) induces sustained activity of MOB M/T cells, its response is reduced in the aPC, favoring the discrimination of a new different odor. Thus, particular tuning properties of aPC neurons allow filtering odor background distracter in order to produce relevant responses for new odors (Kadohisa and Wilson, 2006b; Wilson, 1998, 2003).

Odor intensity is proportional to the concentration of the odorant inhaled. For example, over-ripe bananas release more volatiles molecules that are perceive like a strong smell. Contrary to odor identity, firing-rate of pyramidal neurons weakly depends on odor concentration but instead aPC cells are synchronously more activated at higher concentration. Interestingly, it has been shown that an increased number of aPC neurons are suppressed by high odor concentrations, suggesting that odor intensity is temporally sharpened by inhibition (Bolding and Franks, 2017; Roland et al., 2017). Cumulative evidence suggests that inhibitory interneurons play a major role in odor representation. Feedforward interneurons appear to participate mainly in odor identity, whereas feedback interneurons are recruited to implement odor intensity (Bolding and Franks 2018; Franks et al. 2011; Poo and Isaacson 2009; Stettler and Axel 2009; Zhan and Luo 2010).

As previously mentioned, odor identity is coded by pyramidal neurons' firing-rate. Thus, the change of pyramidal neurons' activity observed other range of odor concentration may degraded the firing that codes its identity. Nevertheless, the aPC is composed of a population of concentration-invariant piriform neurons that encodes odor identity across odor concentrations. This particular feature of the aPC allows maintaining odor representation independently of the odor concentration (Bolding and Franks, 2017; Roland et al., 2017). Recently, Bolding and Franks (2018) demonstrated that recurrent associational fibers from principal neurons are necessary to maintain odor representation.

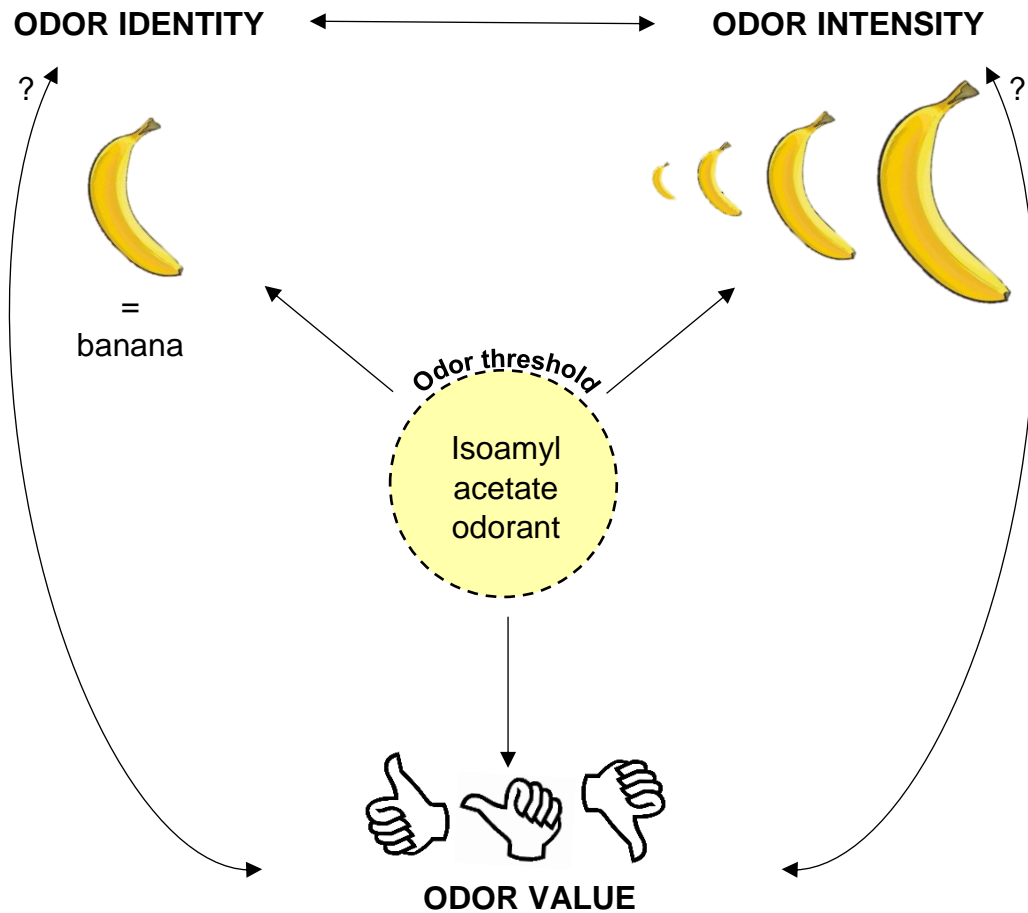


Indeed, they showed that recurrent circuitry elimination induces impairment for odor identity specificity together with more concentration dependence responsiveness. In summary, balance between odor identity and intensity shape odor representation in the aPC.

### ***1.2.1.3 Affective value of odors***

The value of an odor (i.e. its ability to attract or repulse) depends on innate and experienced behaviors. For example, isoamyl acetate is considered as a relatively neutral odor in mammals (Root et al. 2014), but it assumes a positive value after banana ingestion, when this odor is associated with positive properties such as the palatability and nutritious properties of the fruit. Affective values are important features of the olfactory percept. Indeed, most of the time the first reaction about an odorant is given by the hedonic value “I like” or “I don’t like” this smell. Indeed, the olfactory system is highly connected with several structures involved in emotional coding such as the amygdala, OFC, ventral tegmental area and nucleus accumbens (Haberly and Price 1978; Root et al. 2014; Shipley and Geinisman 1984; Wesson and Wilson 2011). Although innate aversive odor responses are mainly coded in the amygdala (Root et al. 2014), most of the odors perceived trigger adaptive response that engage the PC (Choi et al., 2011; Roesch et al., 2007). Activation of only few assemblies of spatially localized neurons in the PC by an odor is capable to elicit both appetitive and aversive behaviors (Choi et al., 2011). The information of odor valence has been proposed to be encoded by firing rate of aPC neurons (Gire et al., 2013). However, more studies are required to investigate how firing rates code for both odor identity and value and to understand the interactions between these features. Moreover, positive correlation exists between the familiarity or the intensity and the hedonic value attributed to the odor (Distel, 1999). For example, an odor judged pleasant is perceived more intense, likely due to an increase of sniffing activity (Youngentob et al., 1987). However, odorants that are pleasant at low concentrations can become repellent at higher concentrations (Yoshida et al., 2012).

In conclusion, the aPC is an important structure that allows building an odor perception through different odor features such as odor identity, intensity and affective value (**Figure 6**). Nevertheless, more work is required to understand the exact relationships between distinct neuronal activities in the aPC to provide specific odor features.



**Figure 6: Representative scheme of different features controlling by the aPC for an odor percept.**

*Once odor threshold is reached, odorant (isoamyl acetate) is identified by its identity (banana object representation), intensity (how strong it smells) and affective value (positive, neutral or negative). Relationship between the three features allow forming an appropriate perception of the odor.*

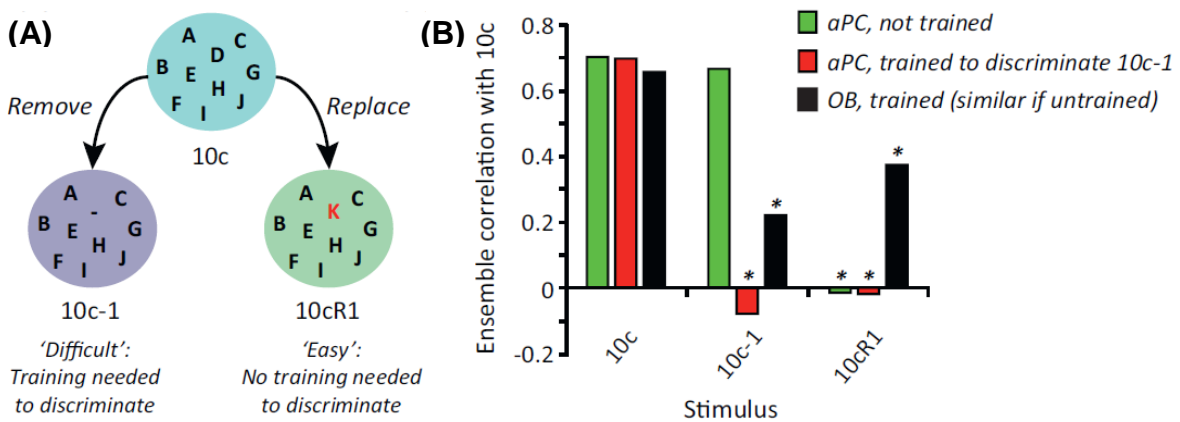
## **I.2.2 Olfactory memory**

In his book “In Search of Lost Time” (Proust, 1913), Marcel Proust describes a childhood memory related to the odor and taste while eating madeleine cakes. Although the association between odor/taste and the madeleine appeared when he was child, his memory remains intact at adulthood. In fact, the remembrance of memories evoked by smells provides more powerful and stronger feelings than memories evoked by verbal or visual information (Willander and Larsson, 2006).

The coding of olfactory memory occurs in several structures in the brain such as the MOB, the OFC, the LEC, the amygdala and the PC (Courtiol and Wilson, 2017; Gottfried, 2010; Suzuki and Bekkers, 2011; Wilson and Sullivan, 2011). Given the functional and structural similarities with the hippocampus and that both structures are considered as auto-associative areas, the PC may lie at the heart of olfactory memory (Haberly, 2001, 1985). Auto-associative areas are composed of recurrent collaterals projections responsible for retroactive control, which enables the formation and storage of memory (Rolls and Treves, 1993). As previously mentioned, the PC expresses a dense network of intrinsic association fibers that allows producing recurrent connections between PC pyramidal neurons (Franks et al., 2011). This particular feature plays a crucial role to evaluate similitude (generalization or pattern completion) and distinguish difference (pattern separation) from partial or overlapping and distinct odors previously experienced. This mechanism allows maintaining perceptual stability when irrelevant variations occur (e.g. fluctuation from the environment, odor  $A = \text{odor } A'$ ) and distinguishing relevant information from distinct odors (e.g. odor  $A \neq \text{odor } B$ ). For example, the same cheese (e.g. camembert) may smell different when it was forgotten in a corner of our fridge for a long period as compared to a fresh one coming from the supermarket. Although the fresh cheese will produce less odorants than the old cheese, through pattern completion we will still be able to recognize it as “camembert odor” but pattern separation will allow discriminating its odor from another type of cheese such as roquefort. Odor pattern separation can occur in both the MOB and aPC. In contrast, pattern completion is narrowly tuned by the aPC (Barnes et al., 2008; Wilson, 2009). Interestingly, depending on the

training, the aPC is able to adapt its responses by switching between pattern separation and pattern completion (**Figure 7**) (Chapuis and Wilson, 2012).

Additionally, as mentioned in the previous section, meaningful olfactory signals are filtered from irrelevant background by aPC neurons (Kadohisa and Wilson, 2006b; Wilson, 1998, 2000a, 2003). Prolonged exposition (tens of seconds) of individual or mixed odorants induces a decrease of aPC pyramidal neurons response, which can extend up to full blockade, while M/T cells activity is maintained. Thus, when an odorant mixture (composed of several odorant components) is presented up for 50s (habituation), new or single odorant components from this mixture are discriminated from background odorants (mixture) by aPC neurons (dishabituation) but not by M/T cells. Nonetheless, when background odorants are presented less than 10s, aPC and M/T neurons do not discriminate between odorants, suggesting that, depending on experience, aPC may adapt its activity in order to distinguish relevant information from the environment (Kadohisa and Wilson, 2006b; Wilson, 1998, 2000a, 2003).



**Figure 7: Pattern completion and separation.**

**(A)** From an initial stimulus composed of 10 odorant component (10c), pattern completion and separation can be evaluated by comparing aPC to OB responses when after presentation of a new odor with one component removed (10c-1) or replaced (10cR1) from the mix. **(B)** Cross-correlation analyses of single-unit ensemble responses in the aPC to the standard 10c mix shows that aPC activity does not display distinction between 10c and 10c-1 in untrained rats (pattern completion), whereas a decorrelation response is observed in trained rats (pattern separation). Thus, based on prior experience, aPC but not OB is able to switch between pattern completion and separation. \*,  $p < 0.05$  compared with 10c. Adapted from Chapuis and Wilson (2012) and modified from Bekkers and Suzuki (2013).

Moreover, odor familiarization (repetitive exposition to odors) influences discriminative responses. Indeed, training to discriminate a rewarded mixture odorant against non-rewarded odorants (single component from the mixture) increases the ability of aPC neurons to discriminate the mixture from its components (Kadohisa and Wilson, 2006a; Wilson, 2000b). In contrast, pPC neurons respond more to both components and mixture, indicating that odor familiarization increases their associative capacity to eventually ensure odor quality (Kadohisa and Wilson 2006a). Consistent with the function of the aPC in odor identity, altogether, these features indicate that this brain region is a key structure for perceptual learning, able to balance perceptual discrimination and perceptual stability (Gottfried et al., 2006; Kadohisa and Wilson, 2006a).

Considering the sparseness and wide distribution of odor-evoked responses in the PC (Ghosh et al. 2011; Poo and Isaacson 2009; Sosulski et al. 2011; Stettler and Axel 2009), representation and discrimination of a given odor may differ among different individuals and across aPC in each hemisphere of a single individual. However, how specific aPC neurons store and retrieve odor information remains unclear. In the aPC, odor discrimination and association are based on different or overlapping coding rate from ensembles of neurons, whose connectivity is experience-dependent (Haberly, 2001; Miura et al., 2012; Rennaker et al., 2007). A recent study proposed that similarities and dissimilarities across odors are supported by random connectivity from several ensembles of piriform neurons. They proposed that this specific feature allows to have a global odor “image” within distinct PC from single or different individuals (Schaffer et al., 2018). The generalization of the odor helps to conserve odor information and to optimize behavioral responses based on similar odorant pattern memory obtained by experience. For example, even if you never smelled roquefort, you will be able to associate its odor with a cheese-like odor. The ability to generalize odors depends on the number of randomly wired neurons that may require the process of all PC cells, whereas odor discrimination may require far fewer neurons (Schaffer et al., 2018). Accordingly, lesions of the aPC impair olfactory discrimination for complex but not simple odorants, suggesting that acquisition of complex odor information requires broad circuitry and their association

across the entire PC, whereas lower numbers of neurons are needed to perceive single odorants (Staubli et al., 1987).

### ***1.2.2.1 Impact of the aPC in the different phases of olfactory memory***

Throughout the present Thesis, the term olfactory memory will be used to refer learning, consolidation and retrieval of olfactory information. In the aPC several modifications occur at different stages of these processes. For example, it has been shown that lesions of the aPC produce a dramatic impairment of olfactory memory acquisition (Staubli et al., 1987). Similar conclusions were obtained by several studies showing the tuning of aPC neuronal activity during olfactory-dependent learning, indicating that aPC neurons are strongly involved in the acquisition phase of olfactory memory (Kadohisa and Wilson, 2006b; Schoenbaum and Eichenbaum, 1995). Moreover, synaptic modifications induced by learning can be observed in the aPC, suggesting that this brain region is able to store at least some features of odor information (Barkai, 2014; Chapuis and Wilson, 2012; Kadohisa and Wilson, 2006a; Quinlan et al., 2004; Roesch et al., 2007). This storage is then reinforced during slow-wave sleep, during which the replay of odor activity patterns produced by the initial learning occurs (Barnes and Wilson, 2014b). Finally, aPC is activated to retrieve odor memory (Gottfried et al., 2004; Kadohisa and Wilson, 2006b; Wilson, 1998, 2000a, 2003). In addition, it has been proposed that transient disruption of synaptic transmission of *Drosophila* mushroom bodies (structure involved in associative learning of olfactory information, similar to the mammal PC) disturbs retrieval, but not acquisition nor storage of memory (Dubnau et al., 2001; McGuire et al., 2001). Altogether, these studies suggest that PC neurons can play different roles during the different memory phases that may depend on various parameters such as the duration of training or the task difficulty.

### ***1.2.2.2 Synaptic plasticity***

Experience-dependent modification of synaptic transmission and efficacy are currently believed to be the cellular mechanisms for the storage of information involved in learning and memory (Ho et al., 2011). Synaptic plasticity is defined by the temporal modification and the ability to adapt the strength of a synapse. Two forms of synaptic plasticity based on their duration are classified: short-term plasticity is the results of synaptic change lasting from a range of tens of milliseconds to few minutes, whereas long-term plasticity lasts from tens of minutes to hours or days. These forms of plasticity can strengthen or weaken the synapse resulting in an enhancement (named potentiation) or a decrease (named depression) of the synaptic efficacy. In the following section, the different forms of synaptic plasticity that occurs in the PC will be reviewed.

#### **1.2.2.2.1 Short-term plasticity**

Afferent and association fibers express different form of short-term modification that can narrowly tuned odor responses. On the one hand, short-term plasticity lasting tens of seconds has been characterized when the aPC is adapting the odor-related response during habituation (Wilson, 1998). This adaptation is mainly due to the activation of group III metabotropic glutamate receptor on LOT afferent inputs leading to a short-term depression of excitatory synapses from M/T cells that project onto aPC neurons (Best and Wilson, 2004). Consistent with the transient adaptation of M/T fibers during odor habituation that last for several seconds ((Best and Wilson, 2004)), train stimulations of afferent inputs produce short-term depression plasticity of postsynaptic potentials at principal neurons whereas the same stimulation protocol at associational fibers is responsible for short-term potentiation response (Hasselmo and Bower, 1990). On the other hand, even shorter (tens of milliseconds) forms of synaptic plasticity can be observed in PC principal neurons. This short-term plasticity occurs when repeated paired stimulation of input fibers are imposed with short inter-stimulus intervals. It has been shown that the two main classes of principal neurons, SL and SP, display different

patterns of this plasticity. In SL neurons, low frequency stimulation (<20 Hz) of LOT or Ib fibers triggers slight facilitation responses whereas stronger stimulations produce short-term depression. For SP neurons, similar results were obtained by stimulating afferent fibers (Suzuki and Bekkers, 2006, 2011). However, stimulations at any frequency induces depression in the synapse between associative fibers and SP cell (Suzuki and Bekkers, 2006, 2011).

Odor discrimination can often occur with a simple sniff, suggesting that short-term odor processing can be modulated by changes in respiration. Indeed, it has been proposed that, together with the timing of short-term plasticity between M/T fibers and principal neurons, transitions from passive breathing to active sniffing shape odor information in the aPC (Oswald and Urban, 2012).

#### 1.2.2.2.2 Long-term plasticity

In the aPC, long-term dependent plasticity research mainly focused on the potentiation effect induced by stimulations in slices. Long-term potentiation (LTP) can occur by stimulating afferent or associative inputs using strong stimulation protocols (e.g. theta burst stimulation, TBS, or high frequency stimulation, HFS). Independently of the stimulating fibers, LTP-mediated plasticity is N-methyl-D-aspartate receptor- (NMDAR) dependent because are blocked by NMDA receptor antagonist (Jung et al., 1990; Kanter and Haberly, 1990). Afferent synapses exhibit relatively weaker LTP as compared to associative inputs that induce stronger and more stable plasticity (Jung et al., 1990; Kanter and Haberly, 1990). However, the ability of afferent fibers to induce LTP differs over development. Indeed, like in other sensory systems, olfactory functions and plasticity are tightly regulated during a specific time window named critical period (Hensch, 2004). During this period (e.g. first two weeks in rodents), LOT synapses show an enhanced sensitivity to LTP induction, whereas association fiber synapses express robust LTP throughout adulthood, indicating that sensory synapses in the aPC are more plastic at early development stage than in adulthood (Best and Wilson, 2003; Franks and Isaacson, 2005; Poo and Isaacson, 2007).



Some studies have shown that olfactory learning induces long-term changes of PC circuits (Barkai, 2014; Cohen et al., 2008; Lebel, 2001). However, these changes are likely not specific to an odor storage mechanism, but they reflect the ability of PC to underlie rule learning rather than to create long-term memory for specific odors. Particularly, learning of an olfactory discrimination task results in modifications in intrinsic neuronal properties and in synapse connectivity (Barkai, 2014). For example, olfactory learning is accompanied by reduced sensitivity to LTP but increased capability to undergo long-term depression (LTD) induction by stimulation of afferent fibers *in vitro*, suggesting that learning-related modifications results in LTP formation *in vivo* that blunts *in vitro* LTP (Lebel, 2001). Moreover, exposing animals to learning olfactory tasks induces long-term enhancement of synaptic connectivity from the OFC to the aPC, indicating that other brain regions play important roles in encoding olfactory information (Cohen et al., 2008).

Although fewer studies evaluated the impact of local inhibitory neurotransmission on memory, it should be noted that olfactory learning induces modification of synaptic inhibition in the PC (Reuveni et al., 2018). In particular, olfactory-discrimination results in a hyperpolarization shift of chloride reversal potential in pyramidal neurons, in an increase of post-synaptic GABA<sub>A</sub> channel conductance and in an enhancement of pre-synaptic GABA<sub>B</sub>-mediated inhibition (Brosh and Barkai, 2009; Kfir et al., 2014; Reuveni et al., 2013).

### ***1.2.2.3 Aversive vs appetitive learning***

Associative memories play a fundamental role in feeding behavior. Indeed, animals must be able to link the sensory characteristics of the food (e.g. smell, taste, appearance, texture) to the consequences of its ingestion. For example, after several conditioning between an odor and a sweet taste (conditioned odor preference, COP; e.g. sucrose), the odor will be considered as appetitive and a positive hedonic value will be associated to it. In contrast, repetitive associations between an odor and a bitter taste (conditioned odor aversion, COA; e.g. quinine) will assign a negative value to the odor, which will be considered as repulsive. As mentioned in the previous section (1.2.1.3), most of the

perceived odors trigger small innate responses, but associative odor features are responsible for adaptive behaviors (Choi et al., 2011; Roesch et al., 2007). These processes are stronger during critical periods at early stages of life (Johanson and Teicher, 1980; Rudy and Cheatele, 1997). Particularly, early odor preference learning induces long-term NMDA-dependent plasticity of LOT to aPC pyramidal neurons synapses, suggesting that aPC is a critical structure for COP during early stages of development (Morrison et al., 2013; Mukherjee et al., 2014). Although few distinct ensembles of spatially localized PC neurons can drive either appetitive or aversive responses at adulthood (Choi et al., 2011), strong evidence suggest that aPC controls mainly COP behaviors (Mediavilla et al., 2016; Roesch et al., 2007), whereas other parts of the olfactory circuits are mainly engaged when odor stimuli are endowed with negative values (Bermudez-Rattoni et al., 1983, 1986; Desgranges et al., 2008; Ferry and Di Scala, 1997; Laviolette and Grace, 2006; Otto et al., 2000; Sevelinges, 2004; Soria-Gómez et al., 2015). For instance, Roesch et al. demonstrated that olfactory cues associated with sucrose activate more aPC neurons than odors associated with quinine. Accordingly, Mediavilla et al. observed a higher c-Fos activity after COP behavior in the aPC as compared to other regions and showed that lesions of this brain region affect COP, but not COA. Therefore, these studies suggested that aPC play an important role in appetitive behaviors (Mediavilla et al., 2016; Roesch et al., 2007). As noted, this behavior might rely on the association of the aPC with multiple brain structures involved in decision making such as the OFC or in the reward system like the nucleus accumbens (Gottfried et al., 2002; Mediavilla et al., 2016; Roesch et al., 2007).

### **1.2.3 Modulatory system**

Neuromodulators play crucial roles in shaping neuronal functions in the brain. Neuromodulatory systems consists either in small pools of neurons such as brainstem and basal forebrain noradrenergic, cholinergic, dopaminergic and serotonergic neurons or small molecules broadly expressed throughout the central nervous system such as peptides (e.g. neuropeptides), gases (e.g. nitric oxide) and lipids (e.g. endocannabinoids) (Avery and Krichmar, 2017; Nadim and Bucher, 2014). The cellular effects of noradrenaline and acetylcholine has been relatively well characterized in the PC (Linster

and Cleland, 2016), however, the impact of neuropeptides, nitric oxide and endocannabinoids remains poorly investigated. Therefore, these systems will not be discussed in this section.

### Noradrenaline

In the PC, noradrenergic inputs modulate association fibers activity and contribute to the cortical adaptation by enhancing signal-to-noise ratio (Hasselmo et al., 1997). Thus, stimulation of noradrenergic fibers from the locus cœruleus induces an odor-evoked increase of PC neurons activity suggesting a better treatment and detection of odor information (Bouret and Sara, 2002). Indeed, together with group III metabotropic glutamate receptor, noradrenaline participates in synaptic depression of LOT synapses (Best and Wilson, 2004). By controlling the odor arousal state, noradrenergic receptors are activated in response to a novel stimulus, allowing the perception of new odors in the environment, through a mechanism known as dishabituation ((Smith et al., 2009).

### Acetylcholine

Similarly, cholinergic inputs modulates PC activity and plasticity. Acetylcholine plays important roles in associative olfactory memory (Hasselmo et al., 1992). Notably, it is involved in synaptic plasticity and facilitates LTP in PC pyramidal neurons (Hasselmo and Barkai, 1995; Patil et al., 1998). As revealed by injection of muscarinic receptor antagonists, endogenous acetylcholine seems to regulate mainly the acquisition phase of olfactory tasks (De Rosa and Hasselmo, 2000; Saar et al., 2001). Interestingly, local administration of this antagonist in the aPC do not affect the spontaneous odor-evoked activity of pyramidal neurons, but it enhances the generalization of odors during olfactory tasks, suggesting that disruption of cholinergic activity in this brain region alters the memory of similar odors (Fletcher and Wilson, 2002; Wilson, 2001).

### Serotonin

Although serotonin receptors are highly present in the aPC (Pazos et al., 1985), few studies investigated their physiological functions. Injection of serotonin receptor antagonists have been shown to disturb the acquisition of an olfactory association (Marchetti et al., 2000). Moreover, a recent study demonstrated that serotonin inhibits the spontaneous neuronal activity of aPC neurons (Lottem et al., 2016). Thus, these studies suggest that serotonergic system modulate olfactory learning and might control olfactory processes.

### Dopamine

Several brain structures project dopaminergic inputs to the PC such as the locus cœruleus and the ventral tegmental area. However, the distribution of these fibers are not homogenous and are mainly segregated in the medial part of pPC (Datiche and Cattarelli, 1996). Therefore, to my knowledge, the specific role of the dopaminergic system in aPC has not been investigated.

## **Conclusion on the olfactory coding in the piriform cortex**

Behavioral responses associated to olfactory processing are based on complex mechanisms that require the transformation of an odorant from a chemical signal into a mental representation. Strikingly, aPC is a key associative structure that allows controlling simultaneously odor perception and olfactory memory. Odor perception depends on characteristics that encode an odor (i.e identity, intensity, value). These basic features can be associated with other sensory characteristics that are responsible for the hedonic value assigned to the odor and for its integration into a memory (likely through long- or short-term synaptic plasticity). Finally, together with various inputs from other brain regions and neuromodulatory systems, aPC narrowly tunes odor information to produce relevant behavior.

## I.3 THE ENDOCANNABINOID SYSTEM IN OLFACTORY PROCESSES

### I.3.1 Characteristics of the endocannabinoid system

#### I.3.1.1 General overview

*Cannabis sativa*, also known as marijuana or cannabis, has been used for thousands of years for its therapeutic and recreational properties. In the late decades, large interest has been aroused in the scientific community about the mechanisms behind these effects (Mechoulam et al., 2014; Russo, 2007). Nowadays, after tobacco, coffee and alcohol, cannabis is the most consumed drug of abuse, with between 119 and 224 million of cannabis users in the worldwide (2012). Technical progresses in the middle of the 20<sup>th</sup> century allowed discovering the main psychoactive components of cannabis,  $\Delta^9$ -tetrahydrocannabinol (THC) (Adams, 1942; Gaoni and Mechoulam, 1964). More than 20 years later, the studies of the biological effects of THC highlighted the identification of the first cannabinoid receptor (CB1) in the brain, providing evidence that cannabis act as a neuromodulatory system (Devane et al., 1988; Herkenham et al., 1990; Matsuda et al., 1990). Subsequently, another receptor was discovered in the periphery, the cannabinoid-type 2 (CB2) receptor (Munro et al., 1993). The characterization of these receptors uncovered the presence of endogenous cannabinoids ligands named endocannabinoids such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG; Devane et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995). Finally, the metabolic mechanisms responsible for synthesis and degradation of endocannabinoids were identified (Di Marzo, 2006; Di Marzo et al., 1994; Marsicano and Kuner, 2008). The identification of cannabinoid receptors (CB1 and CB2), their endogenous ligands (endocannabinoids), and the synthetic and degradative enzymes regulating endocannabinoid levels promoted the concept of the “endocannabinoid system” (ECS), participating in the regulation of physiological processes (Araque et al., 2017; Busquets-Garcia et al., 2018a; Piomelli, 2003).

CB1 and CB2 receptors belong to the superfamily of G-protein-coupled receptors (GPCRs) that consist of seven transmembrane domains with an extracellular N-terminal and an intracellular C-terminal tail (Matsuda et al., 1990; Munro et al., 1993). However, CB1 and CB2 receptors share only 44% of amino acid sequence and present very different patterns of expression and of functions (Pertwee et al., 2010). CB1 receptors is widely expressed in the central nervous system and likely represents the most abundant GPCR in the brain (Herkenham et al., 1991; Howlett, 2002). Given its ubiquitous expression in multiple brain areas, CB1 receptors modulate a variety of functions including learning and memory, mood, stress, anxiety, locomotion, social behaviors, arousal state, food intake, pain and sensory perception (Chaouloff et al., 2011; Corcoran et al., 2015; Lutz et al., 2015; Marsicano and Lafenêtre, 2009; Morena and Campolongo, 2014; Murillo-Rodriguez et al., 2011; Soria-Gómez et al., 2014a; Wei et al., 2017). In contrast, CB2 receptors are primarily found in the immune system at the periphery (Munro et al., 1993). However, increasing evidence indicates that CB2 receptors are present also in the central nervous system (CNS) where their activation can modulate neuronal and glial activity (Kim and Li, 2015; Li and Kim, 2015; Stempel et al., 2016), and CB1 receptors are expressed in peripheral tissues where they participate in metabolic functions (Mazier et al., 2015; Pagotto et al., 2006; Piazza et al., 2017).

In addition, other receptors respond to endocannabinoids such as the transient receptor potential vanilloid 1 (TRPV-1), peroxisome proliferator-activated receptors and some deorphanized GPCRs (Pertwee et al., 2010). For example, TRPV-1 is involved in the transduction of pain sensation and appears to regulate synaptic function when activated by AEA (Chávez et al., 2010; Grueter et al., 2010; Marsch et al., 2007; Puente et al., 2011). However, in sake of clarity and considering the context of this Thesis, the following sections will be focused on the properties of CB1 receptors.

### ***1.3.1.2 Distribution of CB1 receptors***

#### *Distribution in brain structures*

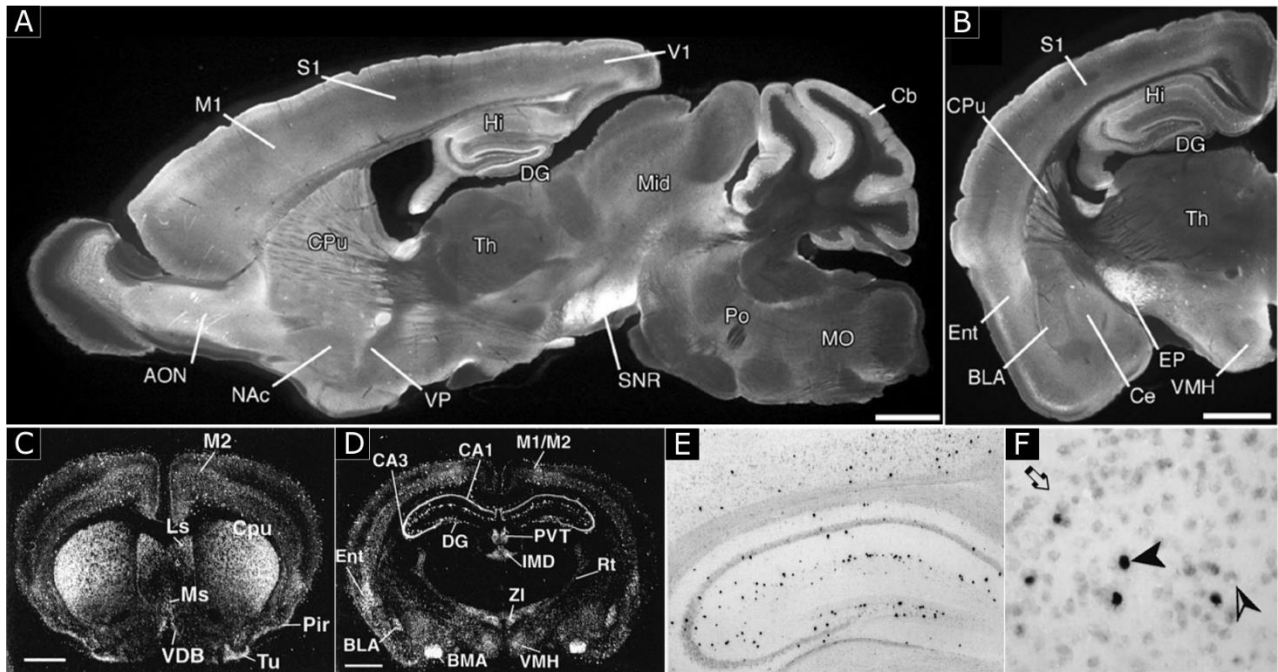
CB1 receptors are mainly reported in the CNS but several studies described their presence in peripheral tissues (Busquets Garcia et al., 2016). Two different approaches are commonly used to study CB1 receptors localization in the brain. Immunohistochemistry or autoradiography provide information on the protein localization, whereas *in situ* hybridization reveals CB1-positive cells containing receptor transcript mRNA. Importantly, given that CB1 receptor protein is mainly expressed at presynaptic terminals (Herkenham et al., 1990, 1991), these approaches have to be distinguished and used as appropriate in order to evaluate either the presence of the receptor (fiber terminals) or the localization of cells producing it (soma), respectively. Indeed, projection neurons have terminals far from their soma. Thus, the localization of the CB1 receptor transcript can differ from the protein expression within a structure. For example, *Substantia Nigra pars reticulata* contains very low CB1 mRNA, but it expresses a large amount of CB1 receptor protein that is localized at terminals of neurons whose cell body is elsewhere in the brain (mainly in dorsal striatum; Chan et al., 1998; Herkenham et al., 1990; Kano et al., 2009). Immunohistochemistry or autoradiography studies revealed high density of CB1 protein in the cerebellum, basal ganglia, hippocampus, cerebral cortex and olfactory system and moderate in the in the amygdala, hypothalamus, thalamus and habenula (Herkenham et al., 1990, 1991; Kano et al., 2009; Pettit et al., 1998). Using *in situ* hybridization, two distinct types of expressing cells can be distinguished by the level of CB1 mRNA expression (Marsicano and Lutz, 1999; Matsuda et al., 1993). High-CB1 expressing cells are considered with round-shaped and very intense staining surrounding or covering the nucleus, whereas low-CB1 expressing cells are defined by discontinuous shape with low staining intensity (Marsicano and Lutz 1999; **Figure 8 E,F**). High-CB1 containing cells are found in the cerebral cortex, hippocampus and amygdala where they are characterized as GABAergic neurons co-expressing the neuropeptide CCK (Marsicano and Lutz, 1999). Instead, low-CB1 expressing cells are present in many more



brain regions including the ones with high-CB1 levels and belong to different cell types. Importantly, this wide range of expression levels makes rather difficult the detection of cells expressing very low amounts of CB1. For instance, astrocytes contain functional CB1 receptors (Navarrete and Araque, 2008, 2010), but their levels are so low that they can be visualized only by very sensitive immunogold electron microscopy (Katona et al., 1999; Rodríguez et al., 2001). Thus, combinations of functional and anatomical assays are required to determine if a cell contains or not CB1 receptors.

### Cell-type and subcellular distribution

CB1 receptors control multiple neurotransmitters release including glutamate, GABA, glycine, acetylcholine, noradrenaline, dopamine and serotonin (Kano et al., 2009). Generally, inhibitory synapses are believed to express higher levels of CB1 than other cells types, suggesting that cannabinoids control different synapses in time- and dose-dependent manners (Kano et al., 2009). Indeed, virtually all high-CB1 expressing cells are GABAergic neurons (Marsicano and Lutz, 1999). Moreover, CB1 receptors have been observed in glial cells such as astrocytes (Han et al., 2012; Navarrete and Araque, 2008; Robin et al., 2018; Rodríguez et al., 2001).



**Figure 8: Distribution of CB1 receptors in the brain of the adult mouse.**

**(A,B)** Immunohistochemistry showing the overall distribution of CB1 receptor. High expression of the protein is found in cerebellum (Cb), basal ganglia, hippocampus (Hi), cerebral cortex and olfactory system and moderate in the amygdala, hypothalamus, thalamus (Th) and habenula. Scale bar, 150 $\mu$ m. **(C-F)** *In situ* hybridization against CB1 mRNA. Cells containing CB1 transcript are broadly found in the whole brain. Scale bar, 1mm. **(E,F)**: CB1 mRNA expression in the hippocampus. Different levels of CB1 mRNA are expressed in the hippocampus, as shown by filled arrowhead, high CB1- and open arrowhead, low-CB1 expressing cells. Open arrow, CB1-negative cell. AON, anterior olfactory nucleus; BLA, basolateral amygdala; CA1 and CA3, cornu ammonis 1 and 3; Ce and BMA, central and basomedial amygdaloid nucleus; DG, dentate gyrus; EP, entopeduncular nucleus; Ent, entorhinal cortex; IMD and RT, intermediodorsal and reticular thalamic nucleus; Ls and Ms, lateral and medial septum; M1, M2 primary, secondary motor cortex; Mid, midbrain; MO, medulla oblongata; NAc, nucleus accumbens; Pir, piriform cortex; Po, pons; S1 and V1, primary somatosensory and visual cortex; SNR, substantia nigra pars reticulata; Tu, olfactory tubercle; VMH, ventromedial hypothalamus; VP, ventral pallidum; ZI, zona incerta. Adapted from Kano et al, 2009 for **A,B**; Mariscano and Lutz, 1999 for **C-F**.

In addition, CB1 receptors are localized in different subcellular compartments. Although the classical protein distribution has been described at the cellular plasma membrane, (endo)cannabinoids are lipid molecules that can diffuse inside cells. Notably, CB1 receptors have been reported in intracellular organelles, such as in endosomes (Katona et al., 1999; Rozenfeld and Devi, 2008) and in mitochondria of neurons (Bénard et al., 2012; Hebert-Chatelain et al., 2016), astrocytes (Gutiérrez-Rodríguez et al., 2018) and muscles (Mendizabal-Zubiaga et al., 2016). The functional implications of these non-canonical localizations of CB1 receptors are being currently dissected (Bénard et al.,

2012; Hebert-Chatelain et al., 2016). Importantly within the context of the present work, the mitochondrial localization of CB1 receptors at presynaptic terminals has been shown to be compatible with the (endo)cannabinoid-mediated regulation of synaptic transmission (Bénard et al., 2012; Hebert-Chatelain et al., 2016). However, in the present work, no experiments were conducted to discriminate the precise subcellular location of the CB1 receptors involved. Therefore, the definition of presynaptic CB1 receptors in the rest of this Thesis will include both classical plasma membrane and intracellular localization.

### ***1.3.1.3 Endocannabinoids, synthesis and degradation***

Endocannabinoids are endogenous ligands of cannabinoid receptors. Despite the recent description of additional compounds acting as endogenous activators or inhibitors of CB1 receptors such as peptides (Pepcans; Bauer et al., 2012) and neurosteroids (pregnenolone; Vallée et al., 2014), the classical and best-characterized endocannabinoids are lipid amides or esters of the long chain fatty acid arachidonic acid. Thus, most endocannabinoid-mediated synaptic modulation appears to be provided by two compounds: arachidonoyl ethanolamide, also known as anandamide (AEA; Devane et al., 1992) and the 2-arachidonoylglycerol (2-AG; Mechoulam et al., 1995; Sugiura et al., 1995). The lipid property of endocannabinoids leads to particular features of their metabolism. Thus, their synthesis and degradation appear to occur in a short period of time, thereby allowing the control of cell activity “on demand” by limiting the temporal window of CB1 receptor activation (Piomelli, 2003). Distinct enzymatic machinery regulate the production and degradation of these molecules.

#### **Endocannabinoid synthesis**

The endocannabinoid lipophilic nature prevents them from the classical storage of signaling molecules in synaptic vesicles. Instead, endocannabinoids are synthesized “on

demand” by the hydrolysis of post-synaptic cell membrane phospholipids (Piomelli, 2003). Synthetic pathways of endocannabinoids are complex and redundant (Fowler et al., 2017; Lu and Mackie, 2016). Here, I will limit to the general description of the best-characterized enzymatic routes. AEA is produced by the cleavage of the N-acyl-phosphatidylethanolamine into the precursor N-arachidonoyl phosphatidyl ethanol (NAPE; Di Marzo et al. 1994). NAPE is then catalyzed by the NAPE-phospholipase D into AEA or undergoes a transformation into phosphoanandamide before being dephosphorylated into AEA (Piomelli, 2003). In contrast, 2-AG is classically synthesized by the phospholipase C from phosphatidylinositol to diacylglycerol and then converted into 2-AG by DAG lipase (Piomelli, 2003).

### *Endocannabinoids degradation*

Once synthesized, endocannabinoids are rapidly degraded. Despite the recent description of alternative pathways, the mechanisms of endocannabinoid degradation are quite well characterized (Lu and Mackie, 2016). Indeed, two specific enzymes degrade AEA and 2-AG, respectively. AEA is degraded by the fatty acid amid hydrolase enzyme (FAAH), which transforms AEA in arachidonic acid and ethanolamine (Cravatt et al., 1996). Instead, 2-AG is hydrolyzed by monoacylglycerol lipase (MAGL) which generates arachidonic acid and glycerol (Farooqui et al., 1989). Later, the degraded-produced compounds can be re-used to generate new endocannabinoids.

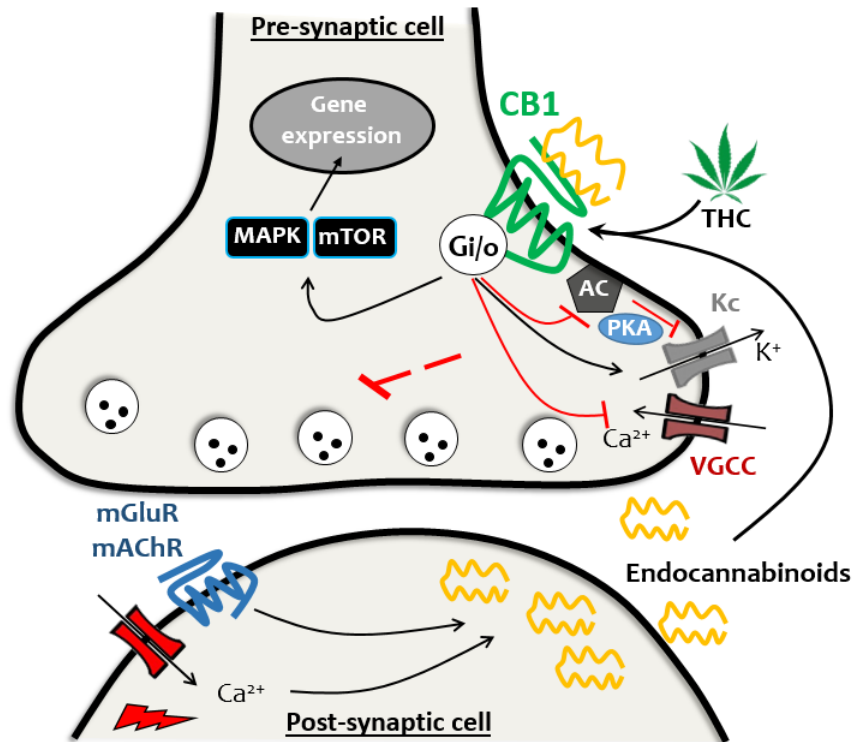
#### ***1.3.1.4 Mechanism of action***

At synaptic level, endocannabinoids are synthesized by post-synaptic intracellular calcium elevations, which can be caused by various stimuli including depolarization (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001), activation of metabotropic acetylcholine (mAChR; Kim et al., 2002) and glutamate

receptors (mGluR; Varma et al., 2001). Once produced, endocannabinoids act at pre-synaptic CB1 receptor, suggesting a retrograde transport. Although different hypothesis are postulated to explain endocannabinoid trafficking with membrane transporters (Di Marzo, 2006), further studies are necessary to investigate the exact retrograde mechanism (Alger, 2012). Classically described, endocannabinoid-CB1 receptor binding decreases neurotransmitter release through Gi/o coupled protein that regulate several intracellular signaling pathways (Castillo et al., 2012; Howlet et al., 1986; Zou and Kumar, 2018). Activation of CB1 receptors inhibits adenylyl cyclase thereby downregulating the production of cyclic adenosine monophosphate (cAMP), which in turn inhibits cAMP-dependent protein kinase A (PKA) involved in the regulation of potassium outward currents (Davis et al., 2003). Moreover, Gi/o stimulation by CB1 receptors induces the activation of inwardly-rectifying potassium channels and the inhibition of presynaptic calcium influx through various forms of voltage-gated calcium channels (Mackie et al., 1995; Piomelli, 2003). The hyperpolarization and with the decrease of calcium availability induced by these events contribute to the reduction of neurotransmitter release from the pre-synaptic terminal (Di Marzo, 2009). Moreover, CB1 receptors are able to modulate additional intracellular pathways including mammalian target of rapamycin (mTOR) and others associated with the mitogen-activated protein kinases (MAPK) pathway (**Figure 9**), such as the extracellular signal-regulated kinase and c-Jun N-terminal kinase (Puighermanal et al., 2012; Turu and Hunyady, 2010). These different signaling pathways regulate several functions including learning and memory, synaptic plasticity and food intake (Alberini, 1999; Puighermanal et al., 2012; Turu and Hunyady, 2010).

Importantly, this mechanism of action is not unique. CB1 receptors can be coupled with different G proteins subunits such as Gs, Gq or Gi/o (Busquets-Garcia et al., 2018a; Turu and Hunyady, 2010). Considering its distribution in various cell types, the existence of different ligands and its ability to activate diverse G proteins, CB1 receptors can induce several signal transduction pathways, suggesting that different effects can be observed depending on the signaling pathway involved. For example, despite the lowest amount of CB1 receptors in hippocampal glutamatergic neurons, G-protein signaling appears more potent in these cells than in GABAergic interneurons (Steindel et al., 2013). Therefore, the signaling pathway of CB1 receptor is not homogeneous, contributing to explain the

several implications of the receptor at different levels (e.g. neurons vs astrocytes; plasma membrane vs mitochondria; brain structure vs another one).



**Figure 9: Mechanisms of endocannabinoid action.**

*Post-synaptic calcium elevation (depolarization or activation by metabotropic glutamate, mGluR or muscarinic acetylcholine receptors, mAChR) induces the synthesis of endocannabinoids that bind pre-synaptic CB1 receptors (CB1). Activation of the protein Gi/o coupled to CB1 receptor modulates neurotransmitter release and gene expression through several intracellular pathways. AC, adenylate cyclase; MAPK, mitogen-activated protein kinases; Kc, potassium channels; mTOR, mammalian target of rapamycin; PKA, protein kinase A; THC,  $\Delta^9$ -tetrahydrocannabinol; VGCC, voltage-gated calcium channels).*

### **1.3.1.5 Modulation of synaptic transmission and plasticity**

CB1 receptor activation can modulate synaptic plasticity, inducing different forms of endocannabinoid-mediated plasticity (Castillo et al., 2012). Neurotransmitter release can be suppressed either transiently or persistently at various synapses in the CNS, presumably contributing to different pathophysiological processes (Araque et al., 2017).

### Endocannabinoid-mediated short-term plasticity

The first evidence of short-term modulation of synapses by endocannabinoids was demonstrated in hippocampal cultures and slices (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). The authors found that the characterized reduction of inhibitory responses after a brief depolarization of post-synaptic hippocampal neurons (Pitler and Alger, 1992) were blocked by CB1 receptor antagonist. Therefore, this plasticity was named depolarization-induced suppression of inhibition (DSI) and was the first demonstration of a retrograde endocannabinoid signaling. In parallel with these studies, a similar phenomenon was observed at excitatory synapses in cerebellar Purkinje cells, and called depolarization-induced suppression of excitation (DSE; Kreitzer and Regehr, 2001). Since these discoveries, other brain regions have shown to express DSI and DSE, including the amygdala, the neocortex, the striatum, the hypothalamus, the ventral tegmental area, the olfactory bulb and others (Castillo et al., 2012; Kano et al., 2009; Pouille and Schoppa, 2018). This form of short-term plasticity seems to be exclusively mediated by 2-AG (Min et al., 2010). However, how calcium elevations can stimulate specifically 2-AG but not AEA synthesis is still unclear (Alger, 2012; Busquets-Garcia et al., 2018a).

A quick depolarization (ranging from hundreds of milliseconds to 10s) induces an elevation of calcium concentration in the post-synaptic cell responsible for the “on demand” production of 2-AG that activates CB1 receptors at the pre-synapse and decrease neurotransmitter release for few seconds to one/two minutes (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001).

As previously mentioned, CB1 receptors are expressed in different neuronal types and in glial cells. Astroglial CB1 receptors have been shown to modulate glutamatergic transmission and plasticity in hippocampal pyramidal neurons (Han et al., 2012; Navarrete and Araque, 2008, 2010; Robin et al., 2018). Transient activation of these receptors coupled with Gq/11 proteins lead to short-term potentiation of excitatory neurons (Navarrete and Araque, 2010). Therefore, short-term depression or potentiation can be induced by activation of CB1 receptors in neurons or in astrocytes, respectively.

Nevertheless, the functional impact of these short-term forms of CB1 receptor-dependent synaptic plasticity on behavior is not clear yet.

### *Endocannabinoid-mediated long-term plasticity*

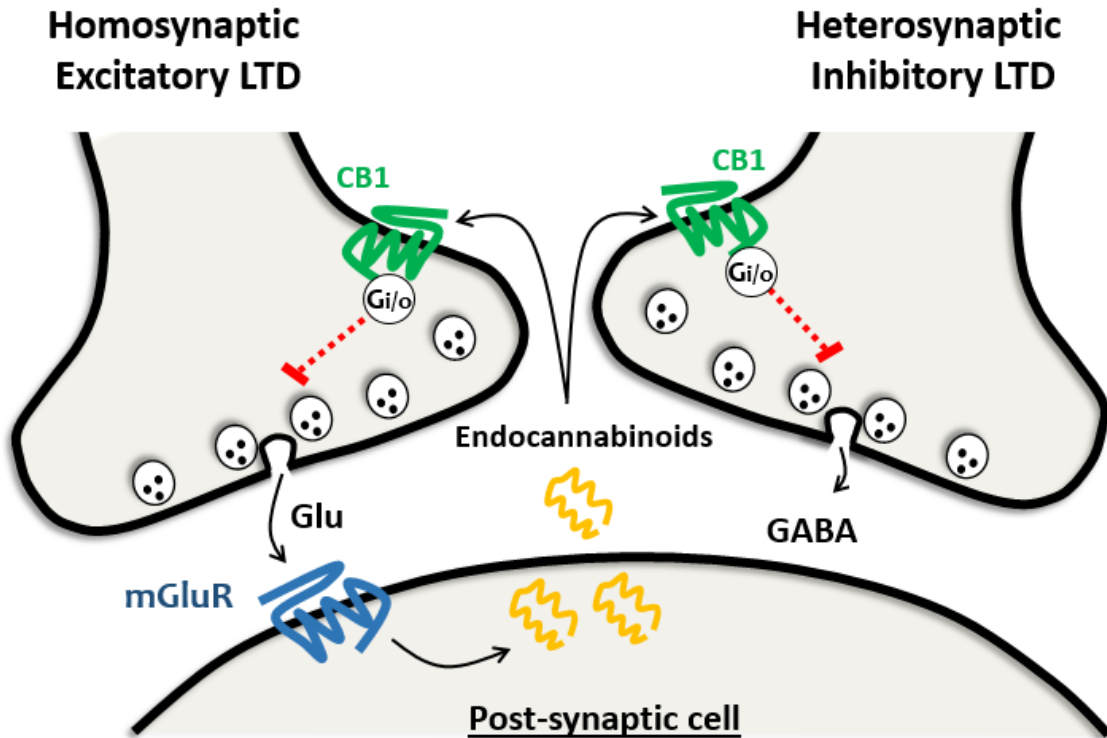
Following the discovery of DSI and DSE, endocannabinoids were shown to mediate long-term form of synaptic plasticity. Like for the endocannabinoid-mediated short-term plasticity, several pieces of evidence led to the discovery of these long-term forms. Previous studies reported that HFS in cortico-striatal glutamatergic fibers induces elevation of calcium in post-synaptic medium-spiny neurons responsible for pre-synaptic LTD of glutamatergic transmission (Calabresi et al., 1992, 1994), suggesting a retrograde mechanism. The first evidence of the endocannabinoid involvement in this form of plasticity was demonstrated by the failure to induce LTD upon application of CB1 receptor antagonists (Gerdeman et al., 2002). Using different stimulation protocols, similar observations were reported in various brain structures such as the nucleus accumbens, the cerebral cortex, the dorsal cochlear nucleus and the cerebellum (Castillo et al., 2012; Kano et al., 2009). Moreover, endocannabinoid-mediated LTD was also characterized at inhibitory synapses from the amygdala, hippocampus and hypothalamus and was named inhibitory long-term depression (Chevalleyre and Castillo, 2003; Crosby et al., 2011; Marsicano et al., 2002). In contrast to short-term plasticity, LTD induction requires long-lasting mobilization of endocannabinoids that activate CB1 receptors for several minutes (Castillo et al., 2012). However, LTD maintenance does not rely on persistent activation of CB1 receptors, but rather on molecular changes, likely at presynaptic level. Indeed, while DSI does not seem to require long-lasting modification at the pre-synapse, iLTD depends on pre-synaptic protein synthesis and intracellular cascades (Younts et al., 2016). More physiologically, it has been shown that iLTD can be induced by evoking theta-burst firing in hippocampal pyramidal neuron (Younts et al., 2013). Interestingly, this type of induction restricts iLTD to only a single active pyramidal neuron in the hippocampus, suggesting that synaptic strength is temporally and spatially modulated by endocannabinoids (Younts et al., 2013). Moreover, iLTD is not induced in silent inhibitory



interneurons indicating that a certain level of spontaneous inhibitory activity is necessary for iLTD induction (Heifets et al., 2008). Conversely, double patch electrophysiological experiments showed that excessive frequency of presynaptic inhibitory neuron firing ( $\geq 20$  Hz) inhibits the functions of CB1 receptors (Foldy, 2006). Despite their molecular mechanisms are not currently known, these observations indicate that CB1 receptors require a “window” of presynaptic activity of the inhibitory interneurons to exert suppression of GABA release.

Depending on the brain structure and type of synapses investigated, LTD induction involves different mechanisms (Kano et al., 2009). Most of the endocannabinoid-mediated LTD require the activation of post-synaptic mGluR receptors (Kano et al., 2009). In case of excitatory neurotransmission LTD, the same synapse undergoing plasticity appears to be the source of the glutamate to induce it (homosynaptic plasticity). In contrast, inhibitory synapses are obviously modulated by the activation of mGluR receptors through glutamatergic release at different synapses, indicating a heterosynaptic mechanism (**Figure 10**; Castillo et al. 2012). For instance, HFS of hippocampal glutamatergic synapses activates post-synaptic mGluR I receptor which in turn lead to the production of 2-AG that decreases neurotransmitter release at inhibitory GABAergic synapses (Chevalleyre and Castillo, 2003).

In addition, astroglial CB1 receptors are involved in long-term plasticity. In the hippocampus, activation of these receptors induces LTP at single synapses (Gómez-Gonzalo et al., 2015) and is required for both *in vivo* LTD induced by exogenous cannabinoids (Han et al., 2012) and *in vivo/in vitro* HFS-induced LTP (Robin et al., 2018). These forms of plasticity are associated with memory functions. For example, injection of CB1 agonists induces an astroglial-dependent hippocampal LTD associated with working memory impairment (Han et al., 2012). In contrast, object recognition memory and hippocampal LTP imply endogenous CB1 receptor activation on astrocytes (Robin et al., 2018). Thus, further experiments are needed to understand how astroglial CB1 receptors modulate different forms of synaptic plasticity.



**Figure 10: Endocannabinoid-mediated LTD in excitatory and inhibitory synapses!.**

*Release of glutamate (Glu) activates post-synaptic metabotropic glutamate receptor (mGluR) which stimulate the synthesis of endocannabinoids. In turn, endocannabinoids can either binds pre-synaptic CB1 receptor (CB1) at the same synapse that produces glutamate (homosynaptic excitatory LTD) or to another GABAergic synapse (heterosynaptic inhibitory LTD). Adapted from Castillo et al, 2012.*

### Non-retrograde endocannabinoid-mediated plasticity

Besides the classical retrograde signaling responsible for pre-synaptic plasticity (DSE/DSI and LTD of inhibitory or excitatory transmission), non-retrograde pathways can induce long-term endocannabinoid-mediated neuronal modifications. Although the presence of post-synaptic CB1 receptors is still debated (Busquets-Garcia et al., 2018a), cumulative evidence suggests that specific neurons can control their own activity *via* autocrine endocannabinoid activation of somatodendritic CB1 receptors. Indeed, multiple trains of evoked action potentials in presence of sodium-channel blocker (tetrodotoxin, TTX) induce long-lasting hyperpolarization in post-synaptic low-threshold-spiking

interneurons of somatosensory cortex, called slow self-inhibition (Bacci et al., 2004). This slow self-inhibition results from the activation of CB1 receptor in CCK- or somatostatin-expressing interneurons (Bacci et al., 2004; Marinelli et al., 2008). Later, this form of plasticity was also characterized in layer 2/3 pyramidal neurons from the somatosensory cortex (Marinelli et al., 2009). Moreover, autocrine endocannabinoid signaling was found in hippocampal pyramidal neurons located close to *stratum radiatum*, where the activation of presumably post-synaptic CB1 receptors was shown to decrease dendritic excitability leading to LTP and spatial memory impairment (Maroso et al., 2016).

### ***1.3.1.6 How to study CB1 receptors contribution?***

A combination of different approaches is needed to understand the temporal, spatial and cell type-specific control of CB1 receptor in specific brain regions. To answer a defined question such as the role of CB1 receptor in synaptic function (through electrophysiology/imaging) or in particular behavior (task related to the brain structure studied), two main approaches are commonly used. In the one hand, pharmacological tools allow the investigation of the temporal and the spatial function of the receptor. For example during a behavioral task, local injection of a drug that disturbs CB1 receptor functions will highlight its relative contribution within the brain region studied at the time of the injection. In the other hand, genetic approaches allow understanding the implication of the receptors within a brain structure and/or a cell type (or subcellular compartments), but they provide less information on the CB1 temporal function.

#### *Pharmacological approaches*

Besides natural ligand components present in *Cannabis sativa* (e.g. THC), numerous synthetic cannabinoids were designed to act on orthosteric or allosteric sites of the CB1 receptor. The best characterized synthetic agonists that present even higher

affinity and potency for CB1 receptor than THC are WIN 55,212-2, HU-210 and CP55940 (Pertwee et al., 2010). Most of the time, these exogenous cannabinoids do not only bind CB1 receptor but can act also at CB2 receptors. For example, HU-210 displays high affinity and potency for both CB1 and CB2 receptor (Pertwee et al., 2010). However, selective CB1 receptor antagonists are used to claim receptor specificity. For example, rimonabant (SR141716A) and AM251 can block agonist-induced activation of CB1 receptors, but they have negligible affinity of CB2 receptors (Pertwee et al., 2010). Moreover, antagonists abolish the activation of endocannabinoids, thus revealing the impact of the ECS. Interestingly, electrophysiological experiments investigated endocannabinoid-mediated plasticity by using both CB1 receptors agonists and antagonists, showing that both types of drugs impede the expression of CB1-dependent synaptic plasticity (Chevalleyre and Castillo, 2003; Kano et al., 2009; Kreitzer and Regehr, 2001; Marinelli et al., 2009; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). Whereas blockade of plasticity by CB1 antagonists is intuitively understandable, the same effect induced by agonists appears almost paradoxical. This paradox can be explained by an occlusion phenomenon. Administration of excess exogenous agonists will bind CB1 receptors, thereby occupying them. Independently of the specific signaling induced by the exogenous agonist, endocannabinoids endogenously mobilized by cell or synaptic stimulation will not find available binding sites on CB1 receptors, thereby impeding the temporal expression of the synaptic plasticity. Thus, physiological endocannabinoid effects at presynaptic or somatodendritic level are hidden by CB1 receptor occlusion by pharmacological agonist applications.

By acting at orthosteric sites, CB1 receptor antagonists block all the signaling pathways associated to the G protein, possibly inducing undesirable side effects (Chorvat, 2013). In contrast, recent allosteric molecules (e.g pregnenolone and derivatives) have been characterized to act as signaling-specific negative modulators. By binding at allosteric sites, might be used to block pathological excessive CB1 receptor activation (e.g. cannabis addiction or cannabis-induced psychoses) without evident side effects (Busquets-Garcia et al., 2017a; Vallée et al., 2014).

## Genetic tools

Using genetic approaches, two classical approaches allow targeting CB1 receptors. First, generation of mutant mouse lines is used to determine the global function of CB1 receptor in the whole body or in different cell types (Marsicano et al., 2002, 2003; Monory et al., 2006). In order to delete the expression of CB1 receptors, mice carrying the CB1 gene (*Cnr1*) flanked by two loxP sites were generated (Marsicano et al., 2002, 2003). This mutant mouse line was then crossed with transgenic mice expressing the Cre recombinase ubiquitously or under the control of specific promoters. The Cre-lox system allows specific recombination in cells containing Cre (Sauer and Henderson, 1988; Sternberg and Hamilton, 1981), leading to conditional deletion in all (using ubiquitous promoters and generating full CB1-KO mice; (Marsicano et al., 2002) or in specific CB1-expressing cells (conditional mutagenesis; Gutiérrez-Rodríguez et al., 2017; Han et al., 2012; Monory et al., 2006). Despite conditional mutagenesis using specific Cre-expressing mouse lines is a powerful tool to dissect cell type-specific functions of genes, the generation of mice expressing Cre recombinase in specific brain structures remains very challenging and the constitutive gene deletion may generate compensatory mechanisms (Morozov, 2008). Thus, using similar recombination approach, the local injection of viruses expressing the Cre recombinase allows refining the contribution of CB1 receptor within a brain region (Zimmer, 2015).

### **I.3.2 Role of the endocannabinoid system in olfaction**

It is known since long time that one of the predominant subjective effects of cannabis intoxication is to altered sensorial perception, including olfactory processes (Tart, 1970). However, although CB1 receptors have been described in the 1990s in many olfactory brain areas of rodents (Herkenham et al., 1990, 1991; Marsicano and Lutz, 1999; Pettit et al., 1998), their modulating odor-related functions started to be studied only during the last ten years. Notably, the involvement of CB1 receptors in specific odor-related processes has been reported in the olfactory sensory neurons (OSN; Breunig et al., 2010;

Czesnik et al., 2007; Hutch et al., 2015), in the main olfactory bulb (MOB; Pouille and Schoppa 2018; Soria-Gómez et al. 2014; Wang, Sun, and Heinbockel 2012) and in the piriform cortex (PC; Ghosh et al. 2018; Hill et al. 2010; Zenko et al. 2011).

The first hypothesis for a physiological involvement of endocannabinoids in olfactory processes came from two observations. 1) Olfactory perception were shown to be changed with the feeding state of individuals (O'Doherty et al., 2000; Payer et al., 1972) and 2) the ECS were proposed to be involved in food intake (Di Marzo and Matias, 2005). Thus, Czesnik et al. (2007) and Breunig et al. (2010) provided the first evidence that cannabinoids were able to modulate olfaction. These studies revealed the presence of CB1 receptors in OSN of *Xenopus laevis* and demonstrated that 2-AG modulates odor-evoked responses. Additionally, they found that the production of 2-AG depends on the hunger state of the animal, responsible for changes in odor sensitivity activity. Although CB1 receptor expression was still observed in OSN of rodents, another study suggested that odor perception was not affected in CB1-KO mice (Hutch et al., 2015). However, despite the species differences, several divergences appear between these studies. First, using CB1-KO mice, Hutch et al. (2015) investigated the involvement of CB1 receptor in odor habituation/discrimination behavior. In contrast, the two first studies evaluated the impact of cannabinoids on odor sensitivity by recorded cellular activity of OSN with calcium imaging and electrophysiology. In addition, the use of total CB1-KO mice lacks brain specificity and might be confounded by compensatory mechanisms (Zimmer, 2015). Thus, the role of CB1 receptor in the OE remains still unclear and need further investigations.

Wang et al. (2012) studied for the first time CB1 receptor functions in MOB glomeruli. Using pharmacological approaches combined with *in vitro* patch-clamp experiments, the authors found that CB1 receptors modulate the firing pattern of periglomerular (PG) and external tufted cells (eTCs). Moreover, they demonstrated that eTCs display spontaneous DSI, suggesting that endocannabinoids are capable to control eTCs activity through CB1 receptor on PG cells. Another study from our laboratory showed that CB1 receptors control granule cells (GCs) activity in the MOB *via* glutamatergic corticofugal fibers (CFF) coming from projecting neurons in anterior cortical

olfactory areas (Soria-Gómez et al., 2014b). Consistent with the idea that cannabinoids in the olfactory system might control feeding state, our laboratory revealed that the hypophagic phenotype observed in mice lacking CB1 receptor in glutamatergic neurons (Glu-CB1-KO) is due to the increased activity of CFF onto GCs. Notably, evidence was provided that endocannabinoid levels increase in the MOB during fasting, which in turn reduces the excitation of GCs. Given that GCs control mitral cell activity, CB1 receptor activation on CFF induces a disinhibition of mitral cells. This effect is followed by a fasting-related enhancement in olfactory sensitivity, which correlates with the amount of food ingested upon refeeding. These results suggest that the endocannabinoid-mediated regulation of olfactory output information control olfactory perception and food intake (Soria-Gómez et al., 2014b). Moreover, CB1 receptors expressed on CFF terminals were recently shown to also regulate another type of MOB neurons, the so-called deep short axon cells (dSAs; Pouille and Schoppa, 2018). Indeed, the authors found that depolarization of dSAs induced transient suppression of excitatory CFF inputs (DSE), dependent on pre-synaptic CB1 receptors. In addition, they observed that dSAs can inhibit GCs thereby suppressing GC to mitral cells inhibition. Interestingly, depending on the CFF strength, Pouille and Schoppa showed that either CB1 receptor can control the synapses from dSAs to GC or directly from GC to mitral cells, suggesting a double dissociation in the control of olfactory bulb output neurons. However, the behavioral consequences of this bidirectional effect remain to be elucidated.

As mentioned in a previous section (I.1.3.3.8), the PC is a brain area capable to generate epileptiform activity (Behan and Haberly, 1999). Considering the importance of CB1 receptor to protect against seizures in other brain structure such as the hippocampus (Marsicano et al., 2003; Monory et al., 2006), the anticonvulsant effects of cannabinoids were assessed in PC slices (Hill et al. 2010). These authors found that CB1 receptor agonists reduce seizures, indicating that CB1 receptor activation is able to control PC activity. Furthermore, another study demonstrated that the ECS in the PC indirectly affects social behavior (Zenko et al., 2011). Although they did not affect social interactions *per se*, local injections of a CB1 receptor antagonist into the posterior PC (pPC) reversed the impairment of social sniffing time induced by activation of dopamine receptors, suggesting that the ECS in the pPC has deleterious effect on social behavior when coupled with

dopamine activation. Finally, a recent study in the pPC proposed that odor-discrimination task training leads to endocannabinoid-mediated modification of inhibitory synapses (Ghosh et al., 2018). They provided evidence that learning of a complex olfactory rule induces activation of CB1 receptors, which in turn enhances GABAergic conductance in post-synaptic pPC pyramidal neurons, indicating a postsynaptic effect. Despite the possible post-synaptic CB1 receptors localization, it is not yet understood how CB1 receptor activation allows controlling GABAergic conductance. Nevertheless, this study suggests that the ECS might play important roles in olfactory memory processed in the PC.



## **Conclusion on the endocannabinoid system in olfactory processes**

Growing evidence revealed that the ECS modulates olfactory processes. However, much less is known about the relative contribution of CB1 receptors during behavior and across different olfactory brain regions (e.g. OE, MOB, PC). Beside the studies of the ECS in primary olfactory structures, it is important to take into account that CB1 receptors are present and modulate associated olfactory areas (i.e amygdala, OFC, hippocampus; (Araque et al., 2017; Kruk-Slomka et al., 2017; Lutz et al., 2015; Wei et al., 2017), suggesting that olfactory processing that involves different brain structures might be regulate by the ECS. Thus, the interconnectivity between olfactory areas together with the tight control of various types of cells (and subcellular location) by the ECS makes the determination of the different roles of CB1 receptors in the olfactory system very challenging.

## **PART II - AIMS OF THE THESIS**

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## AIMS OF THE THESIS

The ability to perceive and retrieve sensory information is crucial for survival and appropriate behavioral responses. The PC is the largest cortical area receiving convergent inputs from the external world and association fibers from other brain regions (Ghosh et al., 2011; Igarashi et al., 2012; Sosulski et al., 2011), thereby playing key roles in odor perception and olfactory memory (Bekkers and Suzuki, 2013; Gottfried, 2010; Haberly, 2001; Wilson and Sullivan, 2011). As pointed out in the introduction, neuromodulatory systems shape olfactory processes, notably by regulating PC functions (Linster and Cleland, 2016). Besides the well characterized regulation of PC circuits by noradrenaline and acetylcholine (Linster and Cleland, 2016), the involvement of other neuromodulators (e.g. neuropeptides, nitric oxide and endocannabinoids) in this brain region remains poorly investigated. One of the most important neuromodulatory systems in the brain is the ECS (Chaouloff et al., 2011; Corcoran et al., 2015; Lutz et al., 2015; Morena and Campolongo, 2014; Murillo-Rodriguez et al., 2011; Soria-Gómez et al., 2014a; Wei et al., 2017). However, the only few studies investigating the contribution of CB1 receptors in the PC were performed in the posterior part (pPC) and the available evidence does not allow determining the exact impact of the ECS in anterior part of PC (aPC) circuits and in related olfactory-guided behaviors (Ghosh et al., 2018; Zenko et al., 2011). Considering the importance of the aPC in processing odor perception and memory functions, and the presence of CB1 receptors in this brain area, it is very likely that the ECS modulates aPC functions.

Therefore, **the general objective of this thesis work is to unravel the role played by CB1 receptors in aPC circuits and functions.** In order to address if and how CB1 receptors modulate aPC processes, we divided this work into two aims.

**Aim 1 – Anatomical characterization of CB1 receptors and their role in aPC-related olfactory function.**

Using a combination of immunohistochemistry, electron microscopy (in collaboration) and *in situ* hybridization, we first dissected the localization and the type of cells expressing CB1 receptors. We then evaluated the functionality of these receptors in aPC circuits with electrophysiology experiments in aPC slices. The ECS regulates learning and memory processes in various brain structures (Kruk-Slomka et al., 2017; Marsicano and Lafenêtre, 2009; Morena and Campolongo, 2014). However, how the ECS modulates olfactory memory is poorly understood. Given the functional and structural importance of aPC in this function (Bekkers and Suzuki, 2013; Haberly, 2001; Wilson and Sullivan, 2011), we developed and study the contribution of CB1 receptors (with viral deletion and pharmacological tools) in a behavioral paradigm known to involve aPC processing (Mediavilla et al., 2016). Finally, we examined the impact of this memory-based behavior on aPC circuitry.

This first aim is part of the submitted manuscript:

**THE CB1 RECEPTORS IN THE ANTERIOR PIRIFORM CORTEX CONTROL MEMORY RETRIEVAL OF ODOR PREFERENCE**

Geoffrey Terral<sup>1,2</sup>, Arnau Busquets-Garcia<sup>1,2</sup>, Marjorie Varilh<sup>1,2</sup>, Svein Achicallende<sup>3,4</sup>, Astrid Cannich<sup>1,2</sup>, Luigi Bellocchio<sup>1,2</sup>, Federico Massa<sup>1,2</sup>, Nagore Puente<sup>3,4</sup>, Edgar Soria-Gomez<sup>1,2,3,4</sup>, Pedro Grandes<sup>3,4</sup>, Guillaume Ferreira<sup>2,5\*</sup> & Giovanni Marsicano<sup>1,2\*</sup>

## **Aim 2 – Impact of CB1 receptor modulation in aPC processes**

Based on the results from the first aim, we wanted to better understand the effects and decipher the mechanisms by which CB1 receptors modulate aPC processes, ranging from the regulation of synaptic plasticity to behavioral functions. First, using *in vitro* patch clamp techniques, we explored the ability of aPC to undergo ECS-dependent forms of synaptic plasticity. Second, we explored the *in vivo* impact of aPC-CB1 receptor activation and blockade (pharmacological approach) on odor-evoked calcium activity (in collaboration) and on olfactory-guided behavior.

This second aim is part of the following manuscript in preparation:

### **CB1 RECEPTORS DYNAMICALLY CONTROL OLFACTORY PROCESSES IN THE ANTERIOR PIRIFORM CORTEX**

Geoffrey Terral<sup>1,2</sup>, Gabriel Lepousez<sup>3,4</sup>, Marjorie Varilh<sup>1,2</sup>, Arnau Busquets-Garcia<sup>1,2</sup>, Astrid Cannich<sup>1,2</sup>, Antoine Nissant<sup>3,4</sup>, Federico Massa<sup>1,2</sup>, Edgar Soria-Gomez<sup>1,2,5,6</sup>, Pierre-Marie Lledo<sup>3,4</sup>, , Guillaume Ferreira<sup>2,7,\*</sup> & Giovanni Marsicano<sup>1,2,\*</sup>

My contribution to these works was to design, perform and analyze immunohistochemistry, *in situ* hybridization, *in vitro* electrophysiology and behavioral experiments as well as writing the manuscripts.



# **PART III - RESULTS**

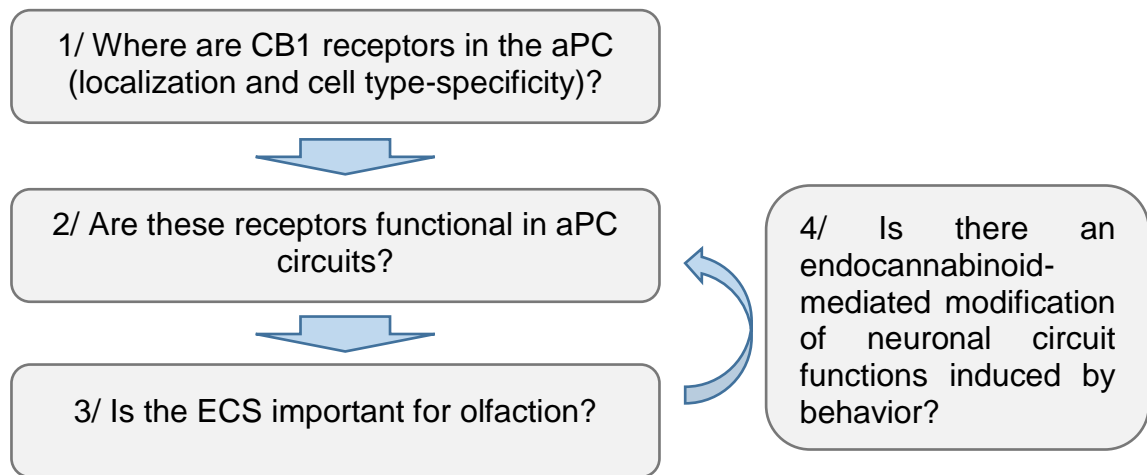
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### III.1 AIM 1 – ANATOMICAL CHARACTERIZATION OF CB1 RECEPTORS AND THEIR ROLE IN APC-RELATED OLFACTORY FUNCTION.

In brief summary, in order to answer the first aim, we asked different questions:



We found that, 1/ CB1 receptors are mainly present in GABAergic neurons and are scattered across the three layers of aPC. 2/ GABAergic aPC-CB1 receptors are functional because they modulate inhibitory transmission. 3/ The ECS in the aPC is necessary for odor memory and in particular for conditioned odor preference retrieval, 4/ odor preference retrieval in turn induces CB1-dependent modifications of inhibitory transmission.



# The CB1 receptors in the anterior piriform cortex control memory retrieval of odor preference

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## **Abstract**

Retrieval of positive or negative odor-related memories is a key determinant of animal behavioral choices. However, how odor memory is formed and retrieved and whether its mechanisms depend on the appetitive or aversive nature of the learning process is still unclear. The anterior piriform cortex (aPC) corresponds to the primary olfactory cortex in the brain and it plays important roles in odor-related processes. Cannabinoid-type 1 (CB1) receptors are present in the aPC, but their potential impact on olfactory memory has never been explored. Here, we used a combination of anatomical, electrophysiological, genetic, pharmacological and behavioral approaches to characterize the physiological functions of aPC CB1 receptors in the regulation of appetitive and aversive olfactory memory. CB1 receptors are mainly found on GABAergic interneurons in the aPC, where they modulate inhibitory transmission. Pharmacological blockade or genetic deletion of CB1 receptors in the aPC specifically impairs the retrieval of conditioned odor preference (COP). Interestingly, expression of conditioned odor aversion (COA) was unaffected by CB1 receptor blockade, indicating that the role of endocannabinoid signaling in the aPC is specific for appetitive memories. COP, but not COA retrieval induces a modulation of inhibitory transmission, which is abolished by blockade of CB1 receptors. Altogether, these data indicate that CB1 receptor-dependent mechanisms physiologically control the retrieval of appetitive odor memory through modulation of inhibitory transmission in the aPC.

## **Significance Statement**

The anterior piriform cortex (aPC) is the primary olfactory cortex but its role in olfactory memory remains unclear. A major modulatory system of memory functions is the endocannabinoid system (ECS) but how it regulates aPC functions has never been explored. Here we find that cannabinoid-type 1 (CB1) receptors, the main ECS receptors in the brain, are mainly expressed by GABAergic neurons in the aPC, where they control inhibitory transmission. Moreover, CB1 receptors in the aPC play a specific role in the retrieval of learned odor preference but not aversion, likely through CB1-dependent modulation of inhibitory transmission. These findings provide a novel role of CB1 receptors in the olfactory system and their impact on memory functions.

## Introduction

Thanks to its involvement in several behavioral functions such as emotional state, food intake, social interactions and learning and memory processes, olfactory information is crucial for the survival of humans and other animals (McGann, 2017; Youngentob et al., 1987a). Indeed, a large part of animal behavior relies on the capacity to perceive odor information and to retrieve its potential meaning based on experiences. Olfactory perception starts with the binding of odorant molecules to olfactory receptors on sensory neurons located in the olfactory epithelium (Buck and Axel, 1991). These neurons project to the olfactory bulb that in turn transmits the signal to other brain regions, of which the major target is the piriform cortex (PC) (Ghosh et al., 2011; Sosulski et al., 2011; Stettler and Axel, 2009b).

Once detected, odors are identified as novel or familiar by comparing their molecular properties with previous odor presentations (Barnes et al., 2008; Chapuis and Wilson, 2012; Gottfried et al., 2006). Whereas the olfactory epithelium and the olfactory bulb are already endowed with some discriminative abilities, the PC and particularly its anterior part (aPC) is amongst the first and most important brain regions where olfactory information is integrated. Indeed, the PC receives inputs both from the olfactory bulb and from many other brain regions making it an ideal structure for olfactory processing and memory (Bekkers and Suzuki, 2013; Haberly, 2001; Igarashi et al., 2012; Wilson and Sullivan, 2011). However, the specific circuits and mechanisms governing how odor information is stored and retrieved in the PC are still unclear.

Cannabinoid type-1 (CB1) receptors together with their endogenous ligands (endocannabinoids) form the so-called endocannabinoid system (ECS, 14), which, in the

brain, is an important modulator of many functions, including learning and memory (Drumond et al., 2017; Kruk-Slomka et al., 2017; Marsicano and Lafenêtre, 2009; Morena and Campolongo, 2014). Retrograde activation of presynaptic CB1 receptors by endogenous ligands is well-known to physiologically control the release of several neurotransmitters in many brain regions (Araque et al., 2017; Castillo et al., 2012; Kano et al., 2009). In the last decades, growing evidence indicate that CB1 receptors are present in different olfactory structures (Gutiérrez-Rodríguez et al., 2017; Herkenham et al., 1991; Marsicano and Lutz, 1999; Pettit et al., 1998), where they can modulate olfactory processes (Breunig et al., 2010; Czesnik et al., 2007; Ghosh et al., 2018; Laviolette and Grace, 2006; Pouille and Schoppa, 2018; Soria-Gómez et al., 2014a, 2014b; Wang et al., 2012; Zenko et al., 2011). For instance, activation of CB1 receptors modulates odor sensitivity in the olfactory epithelium and in the olfactory bulb (Breunig et al., 2010; Czesnik et al., 2007; Pouille and Schoppa, 2018; Soria-Gómez et al., 2014b; Wang et al., 2012). However, little is known about the specific impact of CB1 receptor signaling on odor-dependent learning and memory in olfactory brain structures. Considering that the aPC is a key region for the processing of olfactory memories (Barnes and Wilson, 2014a; Barnes et al., 2008; Bekkers and Suzuki, 2013; Haberly, 2001; Mediavilla et al., 2016; Schoenbaum and Eichenbaum, 1995; Wilson and Sullivan, 2011), we hypothesized that the ECS in this brain region could modulate odor-related memory processes.

In this study, we anatomically and functionally characterized the presence of CB1 receptors in the aPC. Our data show that aPC-CB1 receptors are involved in the control of GABAergic synaptic transmission and they specifically control expression of appetitive, but not aversive, odor memory. Moreover, retrieval-induced changes in local aPC



inhibitory neurotransmission depend on CB1 receptor signaling. Altogether, these results indicate that physiological activation of CB1 receptors in the aPC is necessary for retrieving odor information associated with a positive hedonic value, likely through modulation of local inhibitory circuits.

## Results

### **CB1 receptors are mainly present in GABAergic neurons in the aPC.**

In order to study the role of CB1 receptors in the aPC, we first examined in which cell-types CB1 receptor protein is present. As previously reported (Gutiérrez-Rodríguez et al., 2017; Herkenham et al., 1991; Pettit et al., 1998), CB1 receptors are highly expressed in the aPC of wild-type animals (Fig. 1). More precisely, CB1 receptors are observed in a dense meshwork in layer II, where the so-called aPC principal neurons are localized (Suzuki and Bekkers, 2006, 2011). In order to identify in which specific cell-type CB1 receptors are expressed, immunohistochemistry was conducted in aPC tissues from conditional mutant mice carrying exclusive cell type-specific expression of the receptor (rescue mice; see methods; 22, 41, 42). Similar pattern of expression was observed between the full re-expression of CB1 receptors (CB1-Rescue), the specific re-expression of CB1 receptors in GABAergic neurons (GABA-CB1 Rescue) and WT mice (Fig. 1). In contrast, CB1 receptor expression was barely detectable in mice expressing CB1 receptors only in glutamatergic neurons (Glu-CB1 Rescue) and in mice with a full deletion of the receptor (CB1 Stop mice, Fig. 1). Moreover, as observed in other brain regions such as the hippocampus (Bénard et al., 2012; Gutiérrez-Rodríguez et al., 2017; Katona et al.,

1999), high density of CB1 receptor immunoreactivity was detected by immunogold electron microscopy at presynaptic membranes of GABAergic terminals, whereas glutamatergic synapses contained much lower amounts of gold particles (S1). In addition, few mitochondrial CB1 receptors were observed (S1), similarly as described in the hippocampus (Bénard et al., 2012; Gutiérrez-Rodríguez et al., 2018; Hebert-Chatelain et al., 2016). Altogether, these results indicate that CB1 receptors in the aPC are mainly localized presynaptically at GABAergic synapses. In particular, the high expression of CB1 protein in the pyramidal layer suggests that, similarly to other brain regions (Kano et al., 2009; Katona et al., 2001; Marsicano and Kuner, 2008), the receptor is mainly present on terminals of perisomatic interneurons innervating the somas of pyramidal cells.

As in other brain regions, the localization of the largest part of CB1 receptor protein at presynaptic terminals makes immunohistochemistry unsuitable to identify the precise locations of the cells expressing the receptor. Therefore, to more precisely localize the cell bodies of CB1-expressing neurons in the aPC and to identify their neurochemical nature, we performed double Fluorescent *In Situ* Hybridization (D-FISH) using probes targeting the mRNAs of CB1 and of the marker of GABAergic neurons glutamic acid decarboxylase 65KDa (GAD) (Fig. 2A). Consistent with previous observations (Suzuki and Bekkers, 2010a), the majority of GAD-expressing cells were located in deep layer III (Fig. 2A, B). CB1 mRNA was also highly expressed in layer III, with only scattered positive cells observed in layer I (Fig. 2A,C). Consistently with this partially overlapping distribution, semi-quantitative counting revealed that the majority (63%; Fig. 2A,D) of GAD positive neurons contain also CB1 mRNA, following the distribution of GAD across layers (Fig. 2A,B,E). Similarly to other cortical regions such as the hippocampus (Marsicano and Lutz,

1999), CB1 mRNA was expressed at very different levels across CB1-positive aPC cells. Whereas a majority of cells expressed low-to-moderate amounts of transcript, scattered cells containing very high levels of CB1 mRNA were observed, especially in layers II and III (Fig. 2A). Interestingly, virtually all high CB1-expressing neurons co-express GAD 65 mRNA across the layers (Fig. 2F). In contrast, clear layer differences were observed concerning low CB1-expressing neurons. Whereas they are virtually all GAD-positive in layer I, this proportion is strongly reduced in layers II and III (Fig. 2F). Similarly to the hippocampus, GAD-negative low CB1-expressing cells in layer II and III are glutamatergic pyramidal neurons (data not shown). Altogether, these results show that GABAergic neurons express the large majority of CB1 receptor protein across the three layers, which, however, display distinct distribution patterns of CB1-positive cells.

### **CB1 receptors modulate GABAergic transmission in the aPC.**

The results showed above suggest that CB1 receptor activation could modulate synaptic transmission in the aPC. To test this hypothesis, we first recorded inhibitory transmission onto principal semilunar cells in presence of the CB1 receptor agonist (WIN 55,212-2). Miniature inhibitory post-synaptic currents (mIPSC) frequency, but not the amplitude, was significantly decreased by WIN applications (Vehicle,  $2.1 \pm 0.2$  Hz vs WIN,  $1.80 \pm 0.2$  Hz,  $p=0.017$ ; Fig. 3A-C), suggesting a presynaptic inhibitory effect of the drug. This effect was dependent on CB1 receptors, as it was fully reversed by the application of the CB1 receptor antagonist AM251 ( $2.2 \pm 0.2$  Hz,  $p=0.14$ ; Fig. 3A-C). In contrast, the CB1 receptor agonist did not affect either frequency or amplitude of the miniature excitatory post-synaptic currents (mEPSCs; Vehicle,  $2.95 \pm 0.46$  Hz vs WIN,  $2.92 \pm 0.43$  Hz for frequency,  $p=0.70$ ; Vehicle,  $22.0 \pm 0.8$  pA vs WIN,  $21.5 \pm 0.6$  pA for amplitude,  $p=0.10$ ; Fig. 3D-F).

Similar results were obtained by evoking post-synaptic currents with an electrode placed in layer I of aPC (ePSCs). Thus, WIN reduced the inhibitory (eIPSCs), but not the excitatory (eEPSCs) transmission ( $\Delta$ eIPSCs,  $-62.9 \pm 5.1$  mV,  $p < 0.0001$ ;  $\Delta$ eEPSCs,  $0.38 \pm 4.5$  mV,  $p = 0.93$ ; Fig. 3G,H). Interestingly, this effect was accompanied with a significant rise in the paired-pulse ratio (PPR) of eIPSCs (Vehicle,  $0.65 \pm 0.05$  vs WIN,  $0.98 \pm 0.10$ ,  $p = 0.01$ ), but not of eEPSCs (Vehicle,  $0.88 \pm 0.09$  vs WIN,  $0.84 \pm 0.15$ ,  $p = 0.83$ ; Fig. 3I). These results support the idea that CB1 receptors mainly control GABAergic neurons in the aPC, regulating presynaptic inhibitory transmission onto aPC pyramidal neurons.

### **CB1 receptors in the aPC are necessary for memory retrieval of odor preference.**

According to our findings, CB1 receptors might be involved in odor-related behavioral responses by modulating GABAergic transmission in the aPC. Interestingly, lesion studies recently indicated that aPC is important for conditioned odor preference (COP) in rats (Mediavilla et al., 2016). However, the role of aPC-CB1 receptors in these processes has never been investigated. To address this issue, we first set up a behavioral protocol to assess COP in mice (Fig. 4A). Briefly, mice were exposed to 4 pairings of an odor (C+) with sucrose and simultaneously to a different odor (C-) in water Almond C+ Banana C-. During this conditioning, mice preferred the sucrose-flavored solution, independently of the associated odor (either banana or almond, S2A,B). One day after the last training session, animals were exposed to a choice test between the two odors (C+ versus C-) in the absence of sucrose (Fig. 4A). In these testing conditions, they displayed a reliable and strong preference for the odor previously associated to sucrose (C+) as compared to the other one (C-), revealing the formation of COP (C-,  $0.95 \pm 0.08$  ml vs C+,  $1.53 \pm 0.08$  ml,  $p < 0.001$ ; Fig. 4B), regardless of the odor used as C+ (Banana C-,  $0.9 \pm 0.13$  ml vs Almond

C+,  $1.5 \pm 0.14$  ml,  $p=0.022$ ; Almond C-,  $1.0 \pm 0.10$  ml vs Banana C+,  $1.57 \pm 0.09$  ml,  $p=0.016$ ; Fig. 4C), and without any difference in total liquid consumption (Almond C+ Banana C,  $2.40 \pm 0.24$  ml vs Banana C+ Almond C-,  $2.57 \pm 0.40$  ml; Fig. 4D).

To evaluate the role of the aPC-CB1 receptors in COP, we first locally deleted them. Adeno-associated viruses expressing the Cre recombinase were infused into the aPC of mice carrying the “floxed” CB1 gene (Busquets-Garcia et al., 2018b; Marsicano et al., 2003; Monory et al., 2006) to generate aPC-CB1-KO mice (Fig. S2C, see methods). These mice did not display any preference for the odor previously associated with the sucrose (C-,  $0.99 \pm 0.14$  ml vs C+,  $1.16 \pm 0.15$  ml,  $p=0.89$ ; Fig. 4E and S2B-D). Genetic deletions do not allow determining the temporal dynamics of the involvement of CB1 receptor signaling in different phases of the COP protocol. Thus, we next adopted acute pharmacological approaches to distinguish whether aPC-CB1 receptor activation is necessary for the acquisition or the retrieval of COP. Local injections into the aPC of the CB1 receptor antagonist AM251 prior to each odor-sucrose pairings did not affect COP performance (AM pairings; C-,  $0.60 \pm 0.07$  ml vs C+,  $1.78 \pm 0.08$  ml,  $p<0.0001$ ; Fig. 4E and S2C-E). Conversely, AM251 acutely injected into the aPC prior to the retrieval test abolished COP expression (AM test; C-,  $1.04 \pm 0.08$  ml vs C+,  $1.06 \pm 0.09$  ml,  $p=0.99$ ; Fig. 4E and S2C-E). To test whether the effect of CB1 receptor blockade was still present in over-trained animals and to verify that it did not permanently erase the ability to express COP, all animals injected before retrieval received 4 additional odor-sucrose pairings. Then, animals previously injected with vehicle received AM251 before the second retrieval test [Veh(AM) test], and inversely for the other group [AM(Veh) test]. Veh(AM) test mice displayed clear COP (C-,  $0.54 \pm 0.08$  ml vs C+,  $1.42 \pm 0.09$  ml,  $p<0.001$ ; Fig. 4F and

S2E,F), whereas AM(Veh) test ones were impaired (C-,  $0.97 \pm 0.15$  ml vs C+,  $0.98 \pm 0.15$  ml,  $p=0.91$ ; Fig. 4F and S2E-F). Showing that over-trained animals are still sensitive to CB1 receptor blockade, these results support our previous findings and indicate that CB1 blockade in aPC specifically and transiently impairs COP retrieval without affecting conditioning. The impairment of COP by CB1 receptor blockade could be explained merely by a loss of attraction to sucrose. To control for this possibility, we repeatedly exposed mice to one bottle containing water and another one containing sucrose solution (Fig. S2G). Remarkably, local injections of AM251 in aPC did not impair sucrose preference (Vehicle, C-,  $0.61 \pm 0.11$  ml vs C+,  $1.46 \pm 0.18$  ml,  $p=0.004$ ; AM251, C-,  $0.49 \pm 0.06$  ml vs C+,  $1.70 \pm 0.08$  ml,  $p<0.0001$ ; Fig. 4G and S2H). Overall, these results indicate that endogenous activation of aPC-CB1 receptors is specifically required at the moment of the retrieval of learned, but not spontaneous sucrose preference.

### **CB1 receptors in the aPC are not involved in conditioned odor aversion.**

We next asked whether aPC-CB1 receptors specifically control the retrieval of learned odor preference or are generally involved in the retrieval of both appetitive and aversive memory for odors. We first evaluated whether aPC-CB1 receptors are also necessary for the retrieval of conditioned odor aversion based on association of an odor with gastric malaise induced by lithium chloride (LiCl) injection (conditioned odor aversion - lithium, COA-L) (50, 53–55; Fig. 5A). Briefly, mice were exposed to a conditioning phase consisting of pairings of one odor (C+) with IP injections of LiCl and pairings of the other odor (C-) with IP injections of saline. Finally, AM251 was injected into the aPC before the two-choice test between the C+ and C- odors (Fig. 5A). Notably, mice treated with AM251 did not impair the retrieval of the aversive memory (Vehicle, C-,  $1.28 \pm 0.12$  ml vs C+,  $0.58$

$\pm 0.09$  ml,  $p < 0.001$ ; AM251, C-,  $1.32 \pm 0.09$  ml vs C+,  $0.55 \pm 0.07$  ml,  $p < 0.001$ ; Fig. 5B and S3A). However, COA-L is based on odor-malaise associations whereas COP was based on odor-taste associations. The differential effect of aPC-CB1 receptors blockade on the retrieval of COA-L and COP could therefore be due different types of associations (sensory-gastric versus sensory-sensory), rather than the specific processing of appetitive versus aversive memory. Therefore, we next performed a COA using the aversive taste quinine instead of LiCl (COA-Q), using a very similar procedure to COP, only substituting sucrose by quinine (56, 57; Fig. 5C). Again, mice treated with Vehicle or the CB1 receptor antagonist before the retrieval test displayed the same avoidance towards the odor previously associated with quinine (Vehicle: C-:  $1.64 \pm 0.09$  ml vs C+:  $0.66 \pm 0.09$  ml,  $p < 0.0001$ ; AM251: C-:  $1.34 \pm 0.12$  ml vs C+:  $0.68 \pm 0.08$  ml;  $p < 0.001$ ; Fig. 5D and S3B,C). Altogether, these data indicate that aPC-CB1 is specifically necessary for the retrieval of COP but not COA, implicating that acquired odor choices rely on different mechanisms depending on the appetitive or aversive nature of the unconditioned stimulus.

### **COP retrieval induces pre-synaptic activation of aPC-CB1 receptors at inhibitory synapses.**

We next hypothesized that CB1 receptor-dependent control of GABAergic transmission might be engaged in the aPC during retrieval of COP. To test this idea, we sacrificed animals immediately after COP retrieval test and we measured mIPSCs in aPC slices. Animals undergoing COP retrieval displayed a significant reduction of the frequency of mIPSCs in comparison to a group exposed to the same number of only water-drinking sessions (Water,  $2.47 \pm 0.15$  Hz vs COP,  $1.83 \pm 0.11$  Hz,  $p = 0.003$ ; Fig. 6A,B). We next evaluated if this effect was due to the mere exposure to odor or to sucrose (sucrose-free

or odor-free groups). Thus, mice received the same number of “training” sessions as in COP, but without the presence of sucrose or odor, respectively. These conditions did not induce any reduction of mIPSCs frequency (Sucrose free,  $2.63 \pm 0.22$  Hz; Odor free,  $2.39 \pm 0.14$  Hz vs Water,  $p > 0.8$ ; Fig. 6B). Considering that aPC-CB1 receptors regulate COP but not COA retrieval (Fig. 4 and 5), we evaluated the effect of COA-Q retrieval on mIPSCs frequency and compared it to water exposure. COA-Q retrieval did not affect mIPSCs frequency (COA,  $2.46 \pm 0.115$  Hz vs Water,  $p > 0.9$ ; Fig. 6B). Notably mIPSCs frequencies in all these control groups were significantly different from COP conditions (Sucrose-free,  $p = 0.004$ ; Odor-free,  $p = 0.029$ ; COA-Q,  $p = 0.015$ ; Fig. 6B). Moreover, no difference in mIPSCs amplitude was observed across the groups and COP (Water,  $74.0 \pm 2.4$  pA; Sucrose free,  $76.6 \pm 3.8$  pA; Odor free,  $78.6 \pm 2.7$  pA; COA,  $86.8 \pm 4.6$  pA; COP,  $81.6 \pm 4.2$  pA;  $p = 0.09$ ; Fig. 6C). Altogether, these results underline that COP, but not exposure to water, odor alone, taste alone or COA, specifically reduces pre-synaptic inhibitory transmission onto aPC principal neurons.

Considering that brain local injections are incompatible with successive slicing procedures, we used systemic CB1 receptor blockade to verify that the COP-induced reduction of mIPSCs was acutely due to CB1 receptor activation. Similarly to local applications of AM251, the systemic injection of the CB1 receptor antagonist Rimonabant (1 mg/kg, Rim) blocked the retrieval of COP (Vehicle, C-,  $0.82 \pm 0.12$  ml vs C+,  $1.46 \pm 0.12$  ml;  $p = 0.003$ ; Rim 1mg/kg, C-,  $0.72 \pm 0.08$  ml; C+,  $0.91 \pm 0.08$  ml,  $p = 0.42$ ; Fig. 6D and S4A,B). Animals with systemic injection of vehicle before COP retrieval displayed mIPSCs frequencies similar to untreated mice undergoing COP retrieval and lower than control groups (COP vehicle,  $1.89 \pm 0.07$  Hz vs COP,  $p > 0.9$ ; COP vehicle vs. Control



groups,  $p < 0.05$ ; Fig. 6A,B,E,F). Conversely, systemic administration of Rim before COP retrieval significantly increased mIPSCs frequency as compared to vehicle-treated animals (Vehicle,  $1.89 \pm 0.07$  Hz vs Rim,  $2.58 \pm 0.22$  Hz,  $p = 0.002$ ; Fig. 6F) up to levels undistinguishable from Control untreated mice ( $p > 0.8$ ), with no effect on amplitudes (Vehicle,  $79.5 \pm 3.0$  pA vs Rim,  $83.6 \pm 3.2$  pA,  $p = 0.36$ ; Fig. 6G). These results show that COP retrieval induces a specific CB1-mediated decrease of inhibitory transmission onto aPC principal neurons, indicating a mechanism likely underlying the expression of odor preference memory.

## Discussion

Here we anatomically and functionally characterized the role of endogenous CB1 receptors in the aPC. We found that aPC CB1 receptors appear to be specifically involved in the retrieval of positive, but not of negative, olfactory memories likely through the modulation of aPC inhibitory transmission.

CB1 receptors are present in a high proportion of GABAergic neurons located in the three aPC layers. Moreover, similarly to the hippocampus (Marsicano and Lutz, 1999), cells expressing high levels of CB1 mRNA are exclusively GABAergic. Interestingly, depending on the layer, a portion of low CB1-expressing cells does not co-express GAD mRNA and is presumably glutamatergic neurons (Soria-Gómez et al., 2014b), as also indicated by immunogold electron microscopy experiments. In the main olfactory bulb CB1r present in glutamatergic centrifugal fibers coming from aPC control olfactory perception and food intake (Soria-Gómez et al., 2014b). Although in many brain regions low expression of CB1

receptors in specific cell types is not necessarily linked to lower functional significance (e.g. hippocampal glutamatergic neurons or astrocytes; 48, 52, 58–60) our data suggest that within the aPC there might be a more direct match between the high expression of CB1 receptors on GABAergic interneurons and their behavioral and physiological functions. Indeed, at odds with other brain regions (Domenici, 2006; Kano et al., 2009; Monory et al., 2006, 2015), pharmacological activation of CB1 receptors robustly decreases evoked and miniature inhibitory currents in aPC slices, whereas the same manipulation applied to excitatory transmission has no effect. Thus, though a potential impact of (endo)cannabinoids on glutamatergic synapses within specific excitatory aPC circuits cannot be completely ruled out, our results suggest that the predominant CB1 receptor-mediated effects in this brain region are exerted on local inhibitory neurotransmission. Interestingly, local aPC inhibitory transmission has been shown to be strongly recruited in olfactory-dependent processes (Franks et al., 2011; Poo and Isaacson, 2009; Reuveni et al., 2018; Zhan and Luo, 2010). For instance, *in vivo* odor exposure mainly stimulates activation of GABAergic interneurons in the aPC (Poo and Isaacson, 2009), potentially underlining the importance of specific CB1 receptor signaling functions in this brain region.

In other brain structures, such as the hippocampus or the amygdala, the ECS has been reported to play crucial roles in different phases of learning and memory processes (De Oliveira Alvares et al., 2008; Drumond et al., 2017; Kruk-Slomka et al., 2017; Marsicano and Lafenêtre, 2009). Our data reveal that activation of aPC-CB1 receptors is necessary for COP retrieval but it is dispensable for its acquisition, thereby enhancing the spectrum of ECS involvement in different learning and memory phases.

Our results show that the retrieval of olfactory memories bearing negative values provided by associations of odors with LiCl or quinine (COA) is independent of aPC-CB1 receptor signaling. These intriguing results might be explained by two possibilities: either COA is coded in aPC but it does not involve CB1 receptor signaling, or this function depends on the activity of other brain regions. Our data do not solve this issue. However, olfactory cues associated with sucrose activate more aPC neurons than odors associated with quinine (Roesch et al., 2007) and aPC lesions impair COP but not LiCl-induced COA (Mediavilla et al., 2016). Moreover, CB1 receptor signaling can mediate negative olfactory memories in other brain regions. For instance, Laviolette and Grace (2006; 29) showed that CB1 receptors in the medial prefrontal cortex are required for odor-dependent fear conditioning, and we recently demonstrated that deletion of the *CB1* gene specifically in medial habenular neurons selectively abolishes COA but not COP (Soria-Gómez et al., 2015). Although more experiments will be required to address the exact locations of negative and positive odor memory coding, the present results together with data from the literature support the idea that the aPC is somehow specialized in processing positive acquired values of odors and that CB1 receptors play a key role in this function. Interestingly, it has been shown that the basolateral nucleus of the amygdala (BLA) is essential for COA (Bermudez-Rattoni et al., 1983, 1986; Desgranges et al., 2008; Ferry and Di Scala, 1997). The BLA is more strongly connected with the posterior PC (pPC) than with the aPC, and BLA-pPC interactions are important for aversive conditioning (Hegoburu et al., 2014). This raises the possibility that a double dissociation in the role of aPC and pPC in COP and COA exists. Therefore, a natural follow-up of the present study is to investigate the potential impact of CB1 receptor signaling in the pPC on appetitive and aversive odor memories.

Whereas exposure to odor alone, sucrose alone or COA does not change inhibitory synaptic transmission onto principal neurons, we observed a very clear and robust decrease of mIPSCs frequencies in aPC slices obtained from mice undergoing COP retrieval. Together with the fact that mIPSCs amplitudes were not affected by any of these conditions, these results indicate that COP retrieval is associated with presynaptic reduction of inhibitory transmission. Strikingly, systemic CB1 receptor blockade abolishes at the same time the retrieval of COP and its associated electrophysiological reduction of mIPSCs frequencies. These data suggest that CB1 receptor-dependent physiological presynaptic control of local inhibitory transmission might play a causal role in the retrieval of COP. These processes might be specific of aPC, as a recent study showed that the mechanisms underlying CB1-dependent control of synaptic functions in the pPC might be very different (Ghosh et al., 2018).

In conclusion, this study provided a first characterization of the importance of CB1 receptor signaling in aPC circuitry and related behaviors, thereby contributing to better understand how fine-tuned control of excitatory/inhibitory balance in the aPC regulates olfactory functions and in particular the control of odor information storage and retrieval. Considering that alterations of these functions are associated with many important psychiatric and neurodegenerative conditions (Basavarajappa et al., 2017; Godoy et al., 2014; Philpott and Boak, 2014; Yin et al., 2018), the present data suggest that interference with CB1 receptor signaling might represent a novel conceptual frame to better understand and ultimately tackle these diseases.

## **Experimental Procedures**

Experimental procedures are described in *SI Materials and Methods*. This section discussed the animals used, immunohistochemistry and fluorescent *in situ* hybridization procedures, chemical and drugs preparation, behavioral and surgery procedures, electrophysiological experiments and statistical analysis.

## **Conflict of Interest**

The authors declare no competing financial interests.

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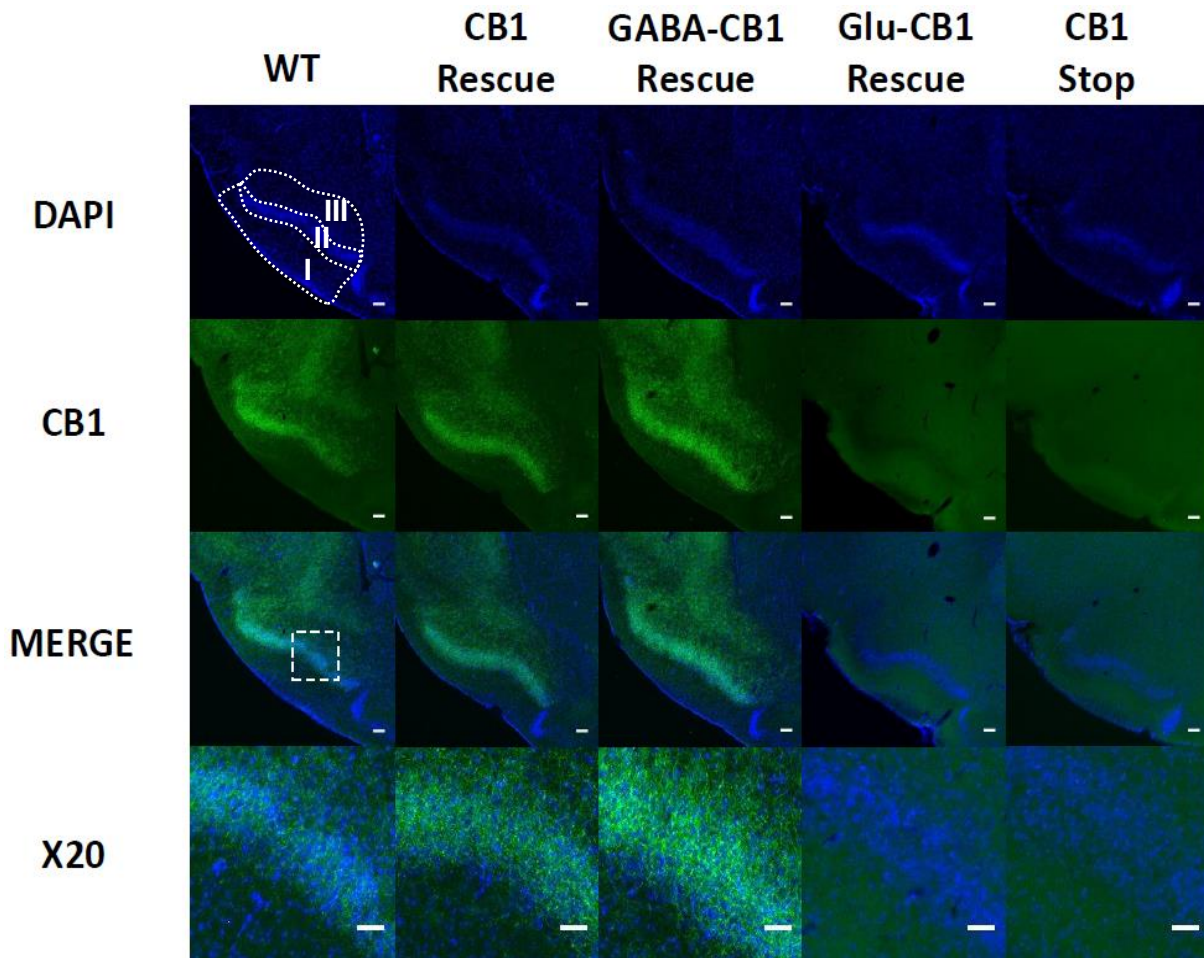
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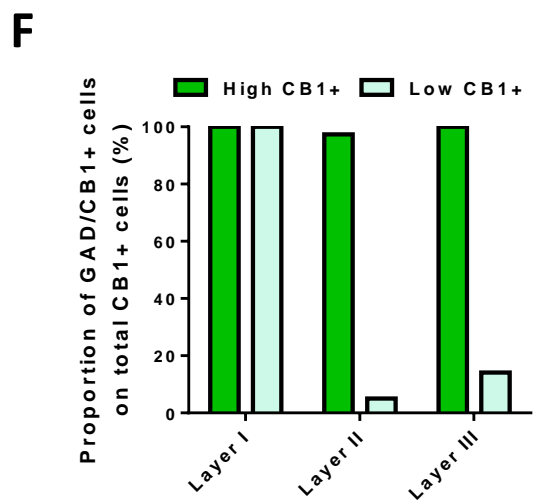
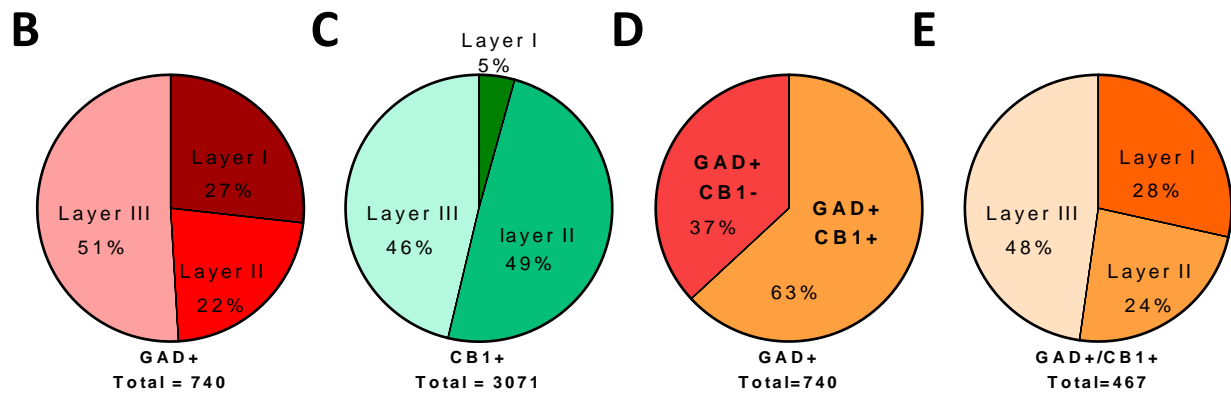
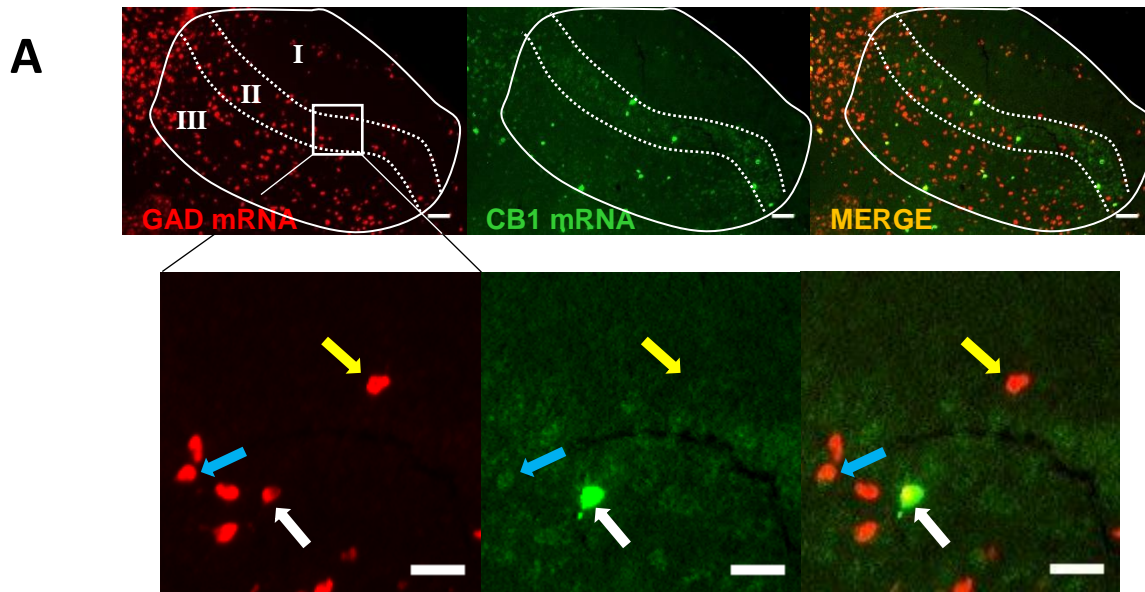
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## Figures



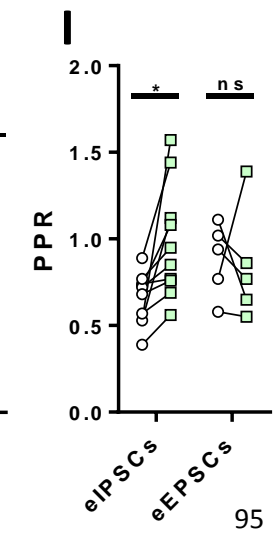
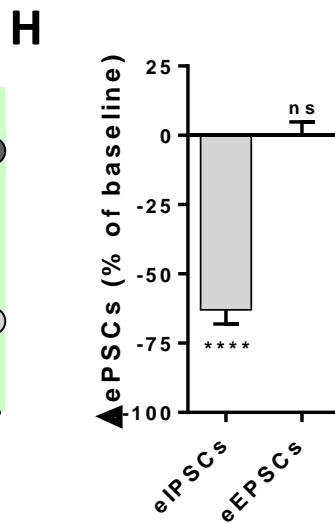
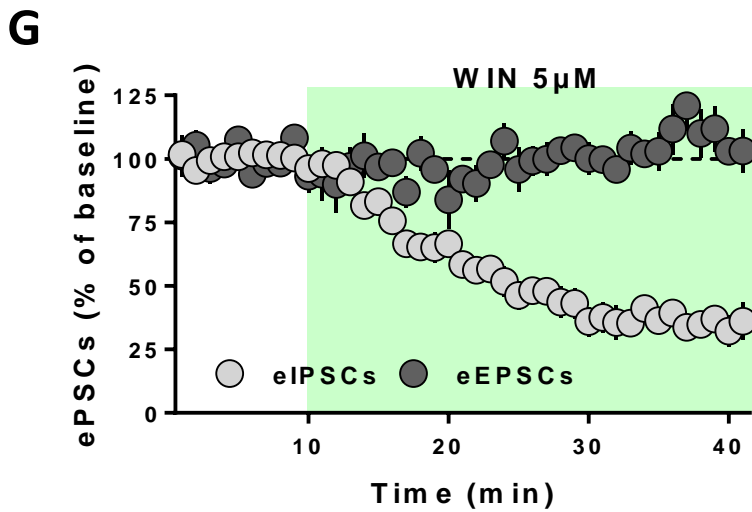
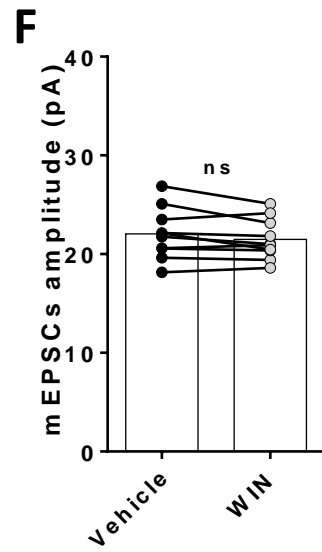
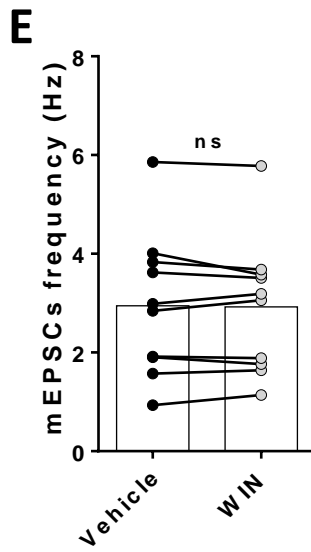
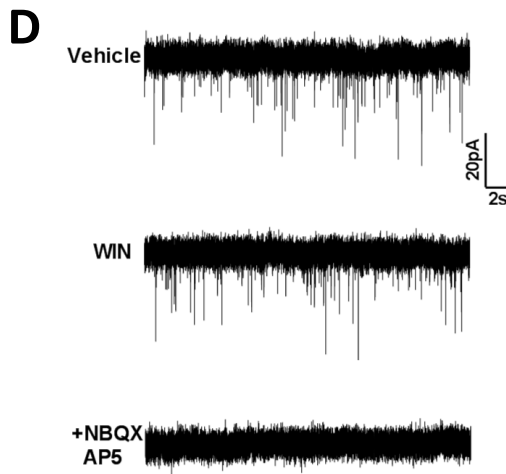
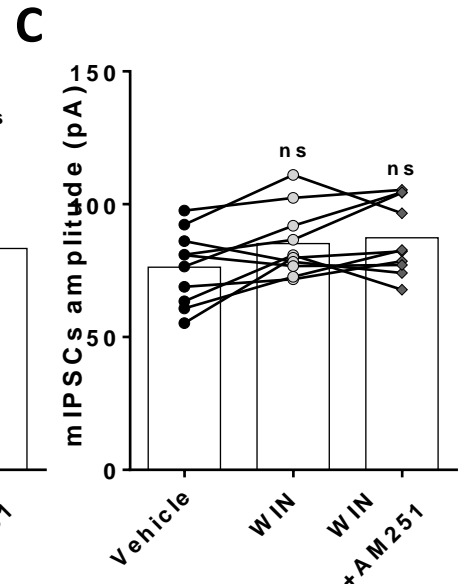
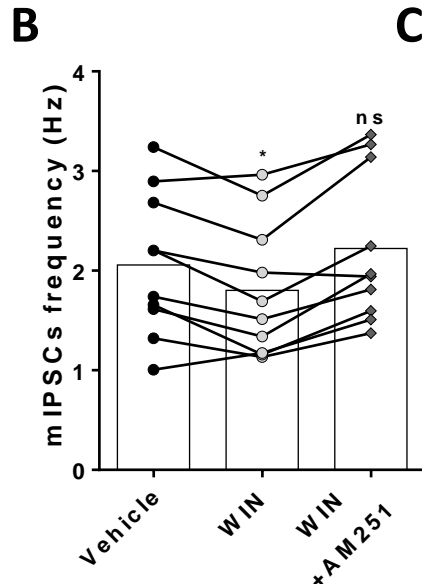
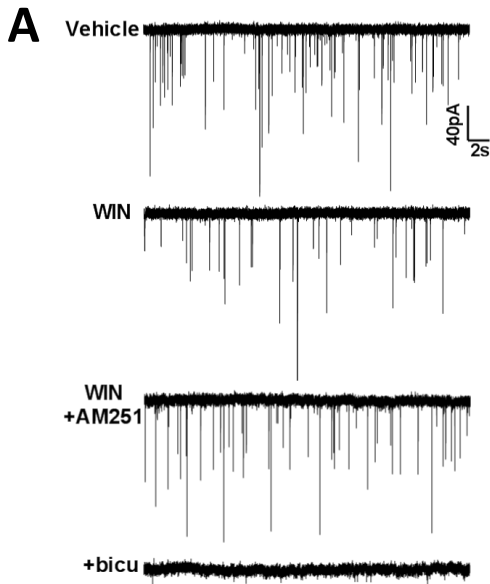
**Figure 1. CB1 receptors are mainly present in GABAergic neurons in the aPC.**

Immunostaining against CB1 receptor. Representative coronal brain sections of the aPC in wild-type (WT), CB1 Rescue, GABA-CB1 Rescue, Glu-CB1 Rescue and CB1 Stop mice. CB1 receptors are highly (and similarly) expressed within the pyramidal layer (layer II) in WT, CB1 Rescue and GABA-CB1 Rescue mice suggesting that aPC-CB1 receptors are mainly present in GABAergic synapses. Note the similar absence of CB1 receptors expression in Glu-CB1 rescue and CB1 Stop mice. Dotted lines represent the different cortical layers (I, II and III). Scale bar, 100 $\mu$ m.



**Figure 2. GABA-CB1 mRNA are scattered across the three layers of the aPC.**

**(A)** Representative images showing double Fluorescent *In Situ* Hybridization against CB1 mRNA (green) and GAD 65 mRNA (red). Lower panels are higher magnifications of the boxed aPC regions in the top panels. Arrows point to GAD positive cells that do not express CB1 (yellow arrow) or are also low and high CB1-expressing cells (blue and white arrows, respectively). Lines represent the different cortical layers (I, II and III). Scale bar, 100µm (top) and 50µm (bottom). **(B)** Representation of the distribution of cells expressing GAD, **(C)** CB1, **(D)** total GAD/CB1 and **(E)** GAD/CB1 in the different layers of the aPC. Note that the distribution of cells co-expressing both GAD and CB1 mRNA follows the distribution of GAD 65 mRNA. **(F)** Numerical evaluation for the double FISH between low and high CB1-expressing cells with GAD positive cells. Note that high CB1-expressing cells are exclusively GABAergic.



### Figure 3. CB1 receptors modulate GABAergic transmission in the aPC.

Effect of the CB1 receptors agonist WIN 55,212-2 on aPC neuronal transmission. **(A)** Representative mIPSCs traces (bicu: GABA-A receptors antagonist bicuculline 10 $\mu$ M). **(B)** WIN (5 $\mu$ M) decreases mIPSCs frequency **(C)** but do not affect amplitude. Note that the CB1 receptors antagonist AM251 (4 $\mu$ M) reverses WIN effect on mIPSCs frequency. One-way ANOVA, repeated measures. \*,  $p < 0.05$ , WIN vs Vehicle; ns, not significant, WIN or WIN+AM251 vs Vehicle (n=10). **(D)** Representative mEPSCs traces (NBQX AP5: AMPA/kainate 10 $\mu$ M and NMDA 50 $\mu$ M receptors blockers). **(E)** Neither the frequency **(F)** nor the amplitude of mEPSCs is affected by WIN. Paired t-test (n=10). **(G)** ePSCs time course, **(H)** percentage reduction and **(I)** paired-pulse ratio (PPR; right) after WIN application. WIN reduces eIPSCs and increases their PPR but had no effect on eEPSCs. One sample t-test and paired-t test. \*\*\*\*,  $p < 0.0001$ , vs baseline; \*,  $p < 0.05$ , WIN vs Vehicle (eIPSCs, n=10; eEPSCs, n=5). Values are represented  $\pm$  SEM.

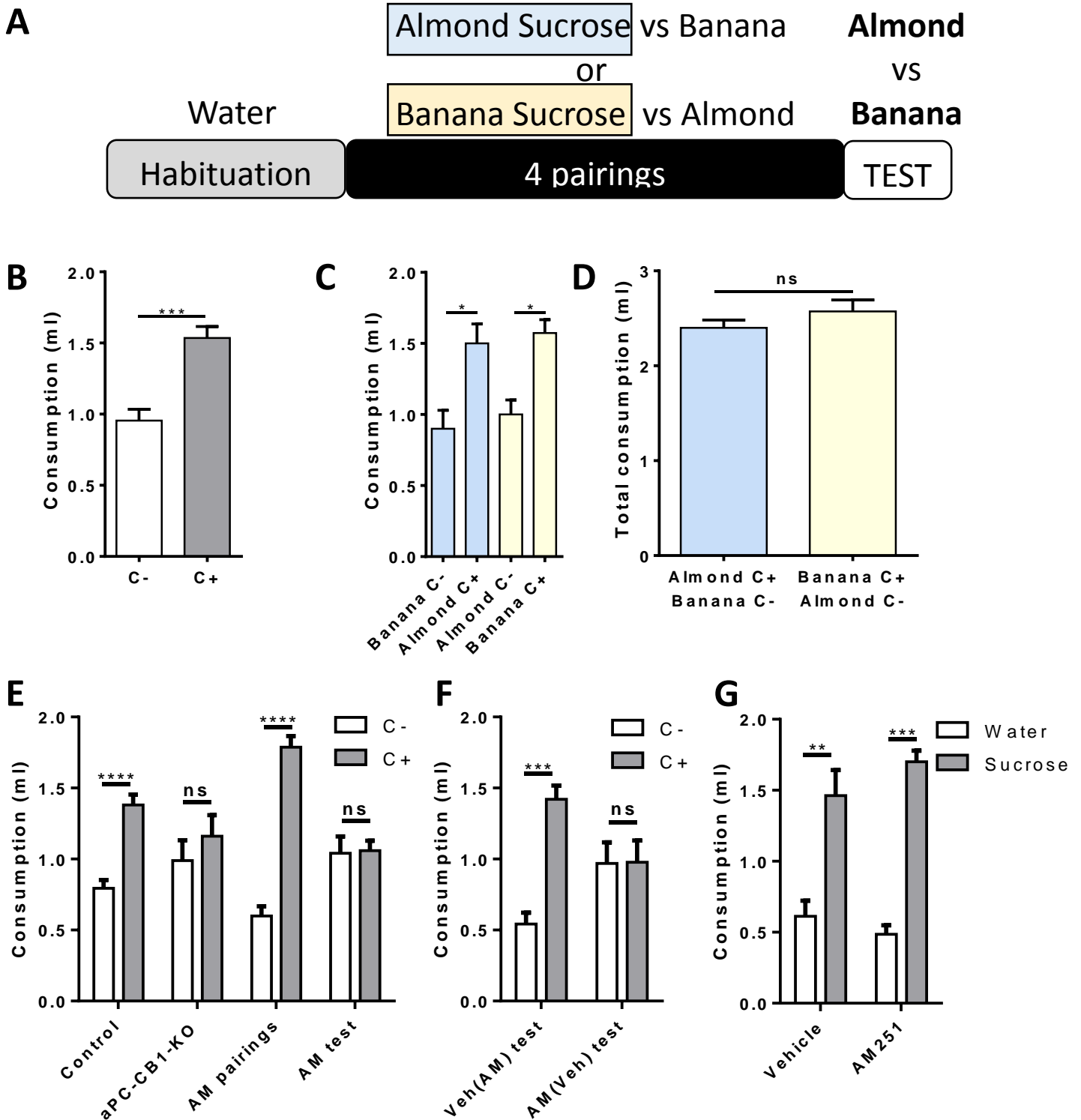
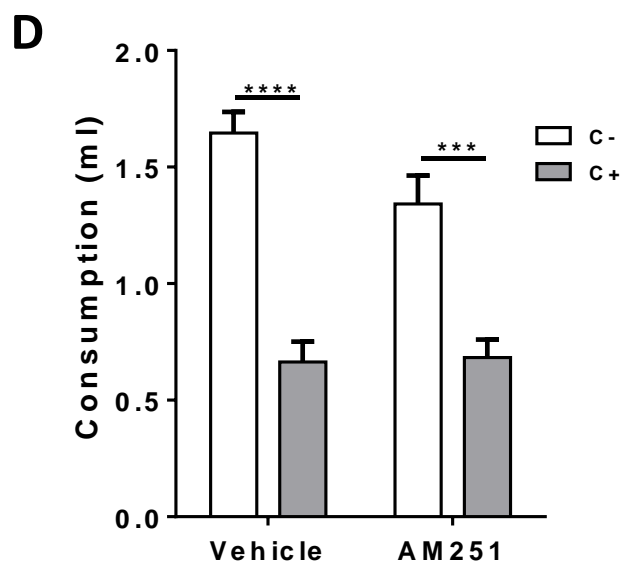
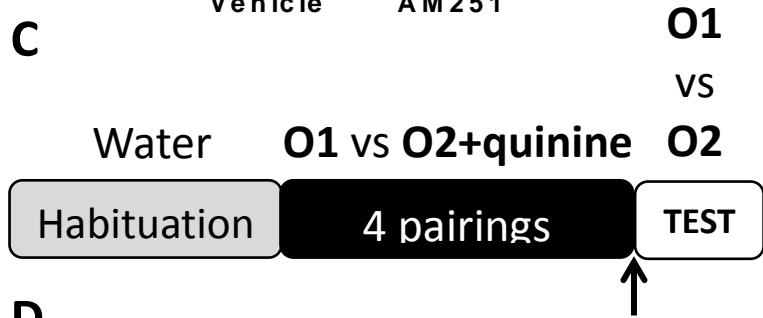
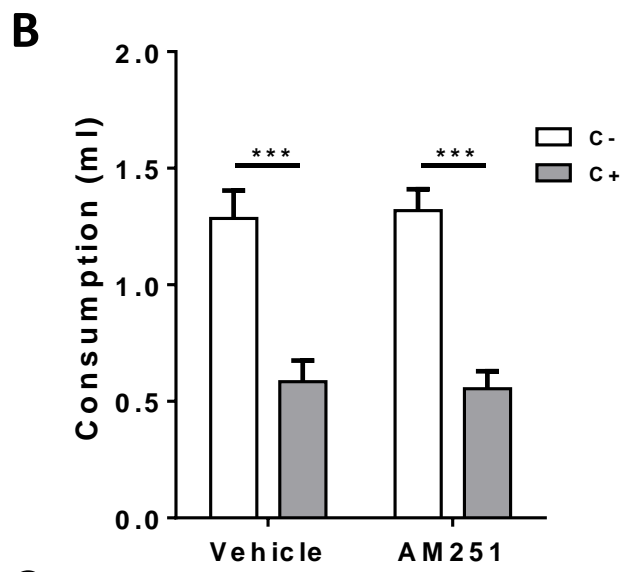
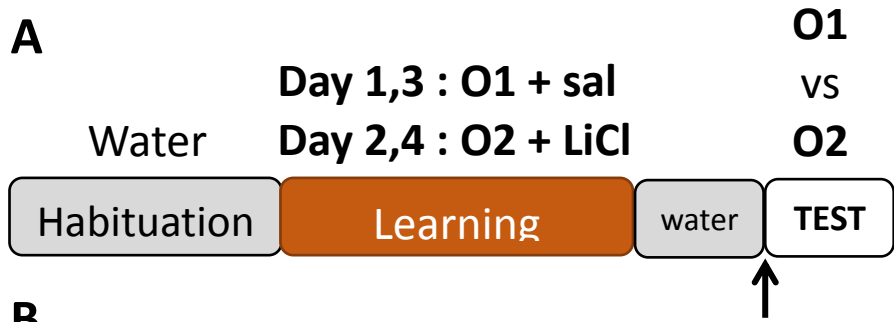


Figure 4. CB1 receptors in the aPC are necessary for memory retrieval of odor preference.

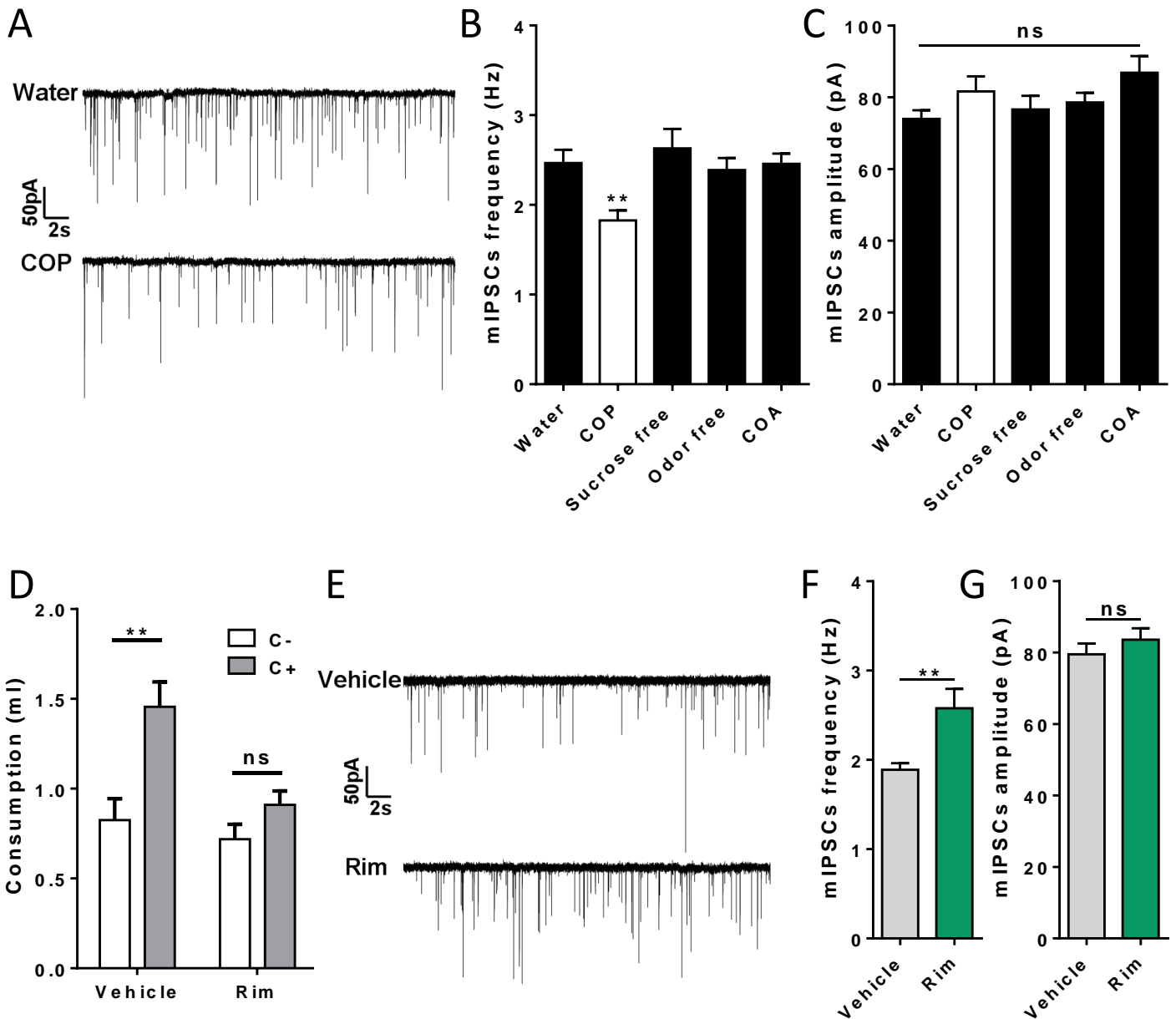


**(A)** Schematic protocol used for conditioned odor preference (COP). Colors indicate the odor associated with the sucrose. **(B)** Consumption during the test of the odorized water previously associated with sucrose (C+) or not (C-). Animals prefer the odor previously associated with the sucrose (C+). Paired-t test. \*\*\*,  $p < 0.001$ ;  $n = 20$ . **(C)** Consumption of C+ and C- during the COP test depending on the odor used as C+ (Almond C+ in blue or Banana C+ in yellow). Similar relative (C+ vs C-, **C**) or **(D)** total consumptions are obtained whatever the odor used as C+. Two-way ANOVA, repeated measures (left) and unpaired t-test (right). \*,  $p < 0.05$ ; C+ vs C- (Almond C+,  $n = 9$ ; Banana C+,  $n = 11$ ). **(E)** Consumption of C+ and C- during the COP test in control mice, mice with CB1 receptors deletion in the aPC (aPC-CB1-KO), mice receiving aPC infusion of the CB1 receptors antagonist AM251 ( $4\mu\text{g}/0.5\mu\text{l}$ ) before each odor-sucrose pairings (AM pairings) or mice receiving AM251 before the test (AM test). Deletion or blockade of aPC-CB1 receptors before the test impair COP. Two-way ANOVA, repeated measures. \*\*\*\*,  $p < 0.0001$ ; C+ vs C- (Control,  $n = 36$ ; aPC-CB1-KO,  $n = 10$ ; AM pairings,  $n = 8$ ; AM test,  $n = 12$ ). **(F)** Consumption of C+ and C- during a second COP test performed after retraining. Mice previously infused with AM before the first test were infused with vehicle before this second test (Veh(AM) test) and those previously infused with vehicle before the first test were now infused with AM (AM(Veh) test). Again, aPC-CB1 receptors blockade abolishes COP. Two-way ANOVA, repeated measures. \*\*\*,  $p < 0.001$ ; C+ vs C- [Veh(AM) test,  $n = 14$ ; AM(Veh) test,  $n = 13$ ]. **(G)** Consumption of sucrose and water after aPC infusion of Vehicle or AM251. AM does not affect sucrose preference. Two-way ANOVA, repeated measures. \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ ; water vs sucrose (Vehicle,  $n = 8$ ; AM251,  $n = 7$ ). Values are represented  $\pm$  SEM.



**Figure 5. CB1 receptors in the aPC are not involved in conditioned odor aversion.**

**(A)** Schematic protocol used for LiCl-induced COA (arrow represents aPC infusion) and **(B)** consumption during the test of the odorized water previously paired with LiCl (C+) or saline (C-). **(C)** Schematic protocol used for quinine-induced COA and **(D)** consumption during the test of the odorized water previously paired with quinine (C+) or not (C-). Note that aPC-CB1 blockade (using AM251, 4µg/0.5µl) does not disturb the retrieval of either LiCl- or quinine-induced COA. O1, odor 1 (almond or banana); O2, odor 2 (banana or almond). Two-way ANOVA, repeated measures. \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ ; C+ vs C- (COA with LiCl, Vehicle,  $n=13$ ; AM251,  $n=11$ ; COA with quinine; Vehicle,  $n=11$ ; AM251,  $n=12$ ). Values are represented  $\pm$  SEM.



**Figure 6. COP retrieval induces pre-synaptic activation of aPC-CB1 receptors at inhibitory synapses.**

**(A)** Representative mIPSCs traces immediately after water consumption (Water) or COP test (COP). **(B)** COP retrieval test decreases mIPSCs frequency but **(C)** do not affect amplitude, in comparison to the other control groups receiving water (Water), only odors (Sucrose free), only sucrose (Odor free) or COA retrieval test (COA). One-way ANOVA.

\* ,  $p < 0.05$ ; \*\*,  $p < 0.01$  (Water,  $n=44$ ; Sucrose free,  $n=17$ ; Odor free,  $n=27$ ; COA,  $n=24$ ; COP,  $n=30$ ). **(D)** Consumption of C+ and C-during COP test after intraperitoneal (ip) administration of the CB1R antagonist Rimonabant (1 mg/kg, Rim) or Vehicle. Rim impairs COP retrieval. Two-way ANOVA, repeated measures. \*\*,  $p < 0.01$  (Vehicle,  $n=16$ ; Rim,  $n=21$ ). **(E-G)** Effect of ip injection of Vehicle or Rimonabant (1mg/kg, Rim) on mIPSCs. **(E)** Representative mIPSCs traces immediately after COP test in group treated with Vehicle (Veh) or Rimonabant (Rim) before the test. Rim reversed COP-induced decrease of mIPSCs frequency **(F)** without affecting mIPSCs amplitude **(G)**. Unpaired t-test \*\*,  $p < 0.01$  (Vehicle,  $n=26$ ; Rim,  $n=21$ ). Values are represented  $\pm$  SEM.

## Supplementary Information

### SI Materials and Methods

**Animals.** All experimental procedures were approved by the local Committee on Animal Health and Care of Bordeaux (authorization number 13693) and Committee of Ethics for Animal Welfare of the University of the Basque Country (CEEA/408/2015/Grandes Moreno, CEIAB/ 213/2015/Grandes Moreno). Two to three months-old naive male CB1-flox (mice carrying the “floxed” CB1 gene (CB1 f/f)) were used (Busquets-Garcia et al., 2018b; Marsicano et al., 2003; Monory et al., 2006). Rescue, stop and knockout lines were generated as described (Gutiérrez-Rodríguez et al., 2017; Marsicano et al., 2002; Ruehle et al., 2013). Briefly, Stop-CB1 mouse line was produced by silencing the endogenous *CB1* gene with a *loxP*-flanked stop cassette in the 5' UTR of the CB1 receptor start codon. To rescue the expression of CB<sub>1</sub> receptor, Stop-CB1 line was crossed with a Cre-deleter mouse line. Conditional rescue mice were obtained by crossing Stop-*CB1* mice with Dlx5/6-CRE mice (gene expressed in differentiating GABAergic neurons) allowing the expression of CB1 in GABAergic neurons, named as “GABA-CB1 rescue”, and with Nex-CRE mice (gene expressed in cortical glutamatergic neurons) allowing the expression of CB1 in cortical glutamatergic neurons, named as “Glu-CB1 rescue”. CB1 receptor knockout (CB1-KO) mice were obtained by crossing CB1 f/f mice with transgenic mice expressing Cre recombinase ubiquitously. All behavioral experiments were performed during the light phase (from 9am to 1pm) and animals were kept in individual cages. At least three separate animals for each of the groups were used for immunohistochemistry, fluorescent *in situ* hybridization and electrophysiology.

**Immunohistochemistry.** Mice were anesthetized with chloral hydrate (400 mg/kg body weight), transcardially perfused with phosphate-buffered solution (PBS 0.1M, pH 7.4) before being fixed with 4% formaldehyde prepared at 4°C. Serial coronal sections were cut at 40µm and collected in PBS at room temperature (RT). Sections were permeabilized in a blocking solution of 10% donkey serum, 0.3% Triton X-100 and 0.02% sodium azide in PBS for 1 hour at RT. Free-floating sections were incubated with a goat polyclonal antibodies against C-terminal sequence of the mouse CB1 receptor (CB1-Go-Af450-1; 1:2000, Frontier Science Co. Shinko-nishi, Ishikari, Hokkaido, Japan) for 48h at 4°C. After several washes, slices were incubated for 2 hours with a secondary antibody anti-goat conjugated to Alexa 488 (1:500, Fisher Scientific) and then washed in PBS at RT. Finally, sections were incubated with DAPI (1:20 000, Fisher Scientific) for 5 minutes before being washed, mounted and coverslipped. The fluorescence was visualized with an epifluorescence Leica DM6000 microscope.

**Immunocytochemistry for electron microscopy.** Coronal Anterior Piriform Cortex vibrosections were cut at 50 mm and collected in 0.1 M phosphate buffer (pH 7.4) at RT. Sections were preincubated in a blocking solution of 10% BSA, 0.1% sodium azide, and 0.02% saponin prepared in 1X Tris-HCl-buffered saline, pH 7.4, for 30 minutes at RT. A pre-embedding silver-intensified immunogold method was used for localization of the CB<sub>1</sub> receptor protein. Briefly, Piriform Cortex sections were incubated with the primary goat polyclonal anti-CB<sub>1</sub> receptor antibody (2 mg/ml Frontier Sciences Institute; goat polyclonal; CB1-Go-Af450, AB\_2571530) in 10% BSA/Tris-HCl-buffered saline containing 0.1% sodium azide and 0.004% saponin on a shaker for 1 day at RT. After several washes

in 1% BSA/Tris-HCl-buffered saline, tissue sections were incubated in a secondary 1.4-nm gold-labeled rabbit anti-goat Immunoglobulin G (Fab' fragment; 1:100; Nanoprobes Inc., Yaphank, NY) in 1% BSA/Tris-HCl-buffered saline with 0.004% saponin on a shaker for 4 hours at room temperature. Piriform Cortex sections were washed in 1% BSA/ Tris-HCl-buffered saline overnight at 4°C and postfixed in 1% glutaraldehyde in Tris-HCl-buffered saline for 10 minutes at RT. After several washes in double-distilled water, gold particles were silver intensified with an HQ Silver kit (Nanoprobes Inc.) for approximately 12 minutes in the dark and then washed in a 0.1 M phosphate buffer, pH 7.4. Stained sections were osmicated (1% osmium tetroxide, in 0.1 M phosphate buffer, pH 7.4, 20 minutes), dehydrated in graded alcohols to propylene oxide, and plastic-embedded in Epon resin 812. Ultrathin sections of 60 nm were collected on mesh nickel grids, stained with 2.5% lead citrate for 20 minutes, and examined in a JEOL JEM 1400 Plus electron microscope. Tissue preparations were photographed by using a digital camera coupled to the electron microscope. Adjustments in contrast and brightness were made to the figures in Adobe Photoshop (Adobe Systems, San Jose, CA).

**Fluorescent *in situ* hybridization.** The procedure was performed as described (Marsicano and Lutz, 1999; Soria-Gómez et al., 2014b)(Marsicano and Lutz, 1999; Soria-Gómez et al., 2014b). Briefly, mice were sacrificed by cervical dislocation. Their brains were extracted, frozen on dry ice and stored at -80 °C until sectioning in a cryostat (14 µm, Microm HM 500M, Microm Microtech). Fluorescein (FITC)-labeled riboprobes against mouse CB1 receptor and digoxigenin (DIG)-labeled riboprobes against mouse GAD65 were prepared as described (Marsicano and Lutz, 1999). After hybridization overnight at



60°C with the mixture of probes, the slides were washed with different stringency wash buffers at 65°C. Then, the slides were blocked with a blocking buffer prepared according to the manufacturer's protocol. Anti-DIG or anti-FITC antibodies conjugated to horseradish peroxidase (HRP) (Roche ; 1 :2000) were applied 2 hours at RT or overnight at 4°C to detect respectively GAD65-DIG or CB1-FITC probes. Probes hybridization was revealed by a tyramide signal amplification (TSA) reaction using Cyanine 3-labeled tyramide (Perkin Elmer; 1:100 for 10 minutes) to detect GAD65 signal or FITC-conjugated tyramide (Perkin Elmer; 1:80 for 12 minutes) to amplify the signal of CB1. The slides were incubated in 4',6-diamidino-2-phenylindole (DAPI; 1:20 000; FISHER Scientific) before being washed, coverslipped and visualized with an epifluorescence Leica DM6000 microscope.

**Numerical evaluation for *FISH*.** Cells expressing mRNAs were quantify as described (Marsicano and Lutz, 1999). CB1 mRNA was classified according to the level of expression, High-CB1 cells were considered to be round-shaped and intense staining covering the entire nucleus whereas Low-CB1 cells were defined with discontinuous shape and/or low intensity of staining (Marsicano and Lutz, 1999). Numerical evaluation of the double *FISH* was performed by evaluating the coexpression of CB1-positive cells with GAD 65 marker.

**Chemical odors and tastes.** The solutions were presented in 50mL drinking bottles in the home cage with either banana (0.05%, isoamyl acetate) or almond odors (0.01%, benzaldehyde) for odors and sucrose (5%) or quinine (0.1mM) for tastes. All compounds

were obtained from Sigma-Aldrich (St. Quentin Fallavier Cedex, France). The concentrations of odors were chosen to be equally preferred (Busquets-Garcia et al., 2017a, 2017b, 2018b).

**Drugs.** For *in vitro* patch-clamp experiment, WIN 55,212-2 (5 $\mu$ M) (Tocris Bioscience) and AM251 (4 $\mu$ M) (Tocris Bioscience) were prepared in Dimethyl Sulfoxide (DMSO) and applied for 10min (mPSCs) or for 30min (ePSCs).

For behavioral experiments, AM251 was dissolved in a mixture of 10% Cremophor-EL, 10% DMSO and 80% saline (NaCl 0.9%). AM251 (4 $\mu$ g/0.5 $\mu$ l per side) or its vehicle was injected bilaterally in the aPC using a peristaltic pump (PHD 22/2000 Syringe Pump Infusion, Harvard Apparatus, Massachusetts, USA, flow rate: 0.5 $\mu$ l/min). Rimonabant (Cayman Chemical) was dissolved in a mixture of 1.25% Tween80, 1.25% DMSO and 97.5% saline (NaCl 0.9%). Rimonabant (1 mg/kg) or its vehicle was injected intraperitoneally (IP) in a volume of 10 ml/kg.

AM251 (4 $\mu$ g/0.5 $\mu$ l per side) and Rimonabant (1mg/kg) were administered 10min and 30min before bottles presentation, respectively. In order to habituate animals to receive aPC infusion, animals were injected with a saline solution (NaCl 0.9%) during the two previous days.

### **Behavioral procedures.**

For all the experiments, data are presented as absolute liquid intake.

#### *Conditioned Odor Preference (COP)*

Mice were water deprived during the whole protocol. During three consecutive days, animals had 1-hour access to two bottles of water. Over the following 4 days, animals received simultaneously (1-hour access) one bottle with an odor-sucrose solution (either banana or almond mixed with sucrose) and one bottle with a different odor-alone solution (either banana or almond in water). Half of the mice received banana-sucrose and the other half almond-sucrose. No differences were observed between both conditions in all the experiments performed. The position of the bottles was changed every day. After this training, a preference test was performed using a 1-hour two bottles choice: each bottle was presented with an odor-alone solution (almond versus banana without sucrose). Subjects showing COP will drink more liquid in the bottle with the odor previously associated with sucrose (C+) than in the other bottle (C-).

### *Sucrose Preference*

All the subjects undergone 3 days habituation to water followed by 3 days with two bottles containing either water or sucrose. Finally, we evaluated the effect of aPC injection of AM251, or its vehicle, on their preference for sucrose over water.

### *Conditioned Odor Aversion (COA)*

#### COA induced by gastric malaise

COA using gastric malaise was adapted from previous studies (Busquets-Garcia et al., 2017a, 2017b, 2018b; Soria-Gómez et al., 2015). Mice followed the same habituation phase as described above. The conditioning phase consisted in 4 days. On days 4 and 6

the subjects received 1-hour access to odorized water (banana or almond) followed by an injection of Saline immediately after the session. On Days 5 and 7 subjects received 1-hour access to the other odor (almond or banana) that they did not receive on Days 4 and 6, followed by an injection of lithium chloride (LiCl, 0.3 M, 1% b.w.) immediately after the session. The different odors were counterbalanced between each group. After this conditioning, the subjects were given a recovery day during which they received water dispensed in two bottles during 1 hour. The following day, a preference test was performed using a 1-hour two bottles choice: each bottle was presented with an odor (almond versus banana). During the test, subjects showing COA will drink less liquid in the bottle with the odor previously associated with LiCl (C+) than in the other bottle (C-).

#### COA induced by quinine

The COA with quinine followed the same procedure as the COP by replacing the sucrose by 0.1mM of quinine. During the test, subjects showing COA will drink less liquid in the bottle with the odor previously associated with quinine (C+) than in the other bottle (C-).

**Surgery.** Mice were anesthetized by IP injection of a mixture of ketamine (100mg/kg, Imalgene 500®, Merial, France) and xylazine (10mg/kg, Rompun®, Bayer, France) and placed into a stereotaxic apparatus (Model 900, Kopf instruments, CA, USA) with a mouse adaptor and lateral ear bars. For local deletion of CB1 receptors (Busquets-Garcia et al., 2018b; Monory et al., 2006) in the aPC, CB1 flox mice were injected with an AAV-cag-CRE or its control AAV-cag-GFP (mixed serotype AAV1/AAV2,  $10^{10}$  Vg/ml) into the aPC (250µl per side, 125µl/min) with the following coordinates: AP +1.6, L  $\pm$  2.5, DV -4.8. For

local pharmacology experiments, mice were bilaterally implanted with 3.5mm stainless steel guide cannulae (Bilaney, UK) targeting the aPC with the following coordinates: AP +1.6, L  $\pm$  2.5, DV -4.5. Guide cannulae were secured in place with dental cement. Mice were allowed to recover for 2 weeks in individual cages before the beginning of the experiments. The correct CB1 deletion and placement of aPC cannulae was verified *post hoc* by Fluorescent *In Situ* Hybridization against CB1 mRNA and injection of 2% pontamine sky blue solution in 0.5M, respectively.

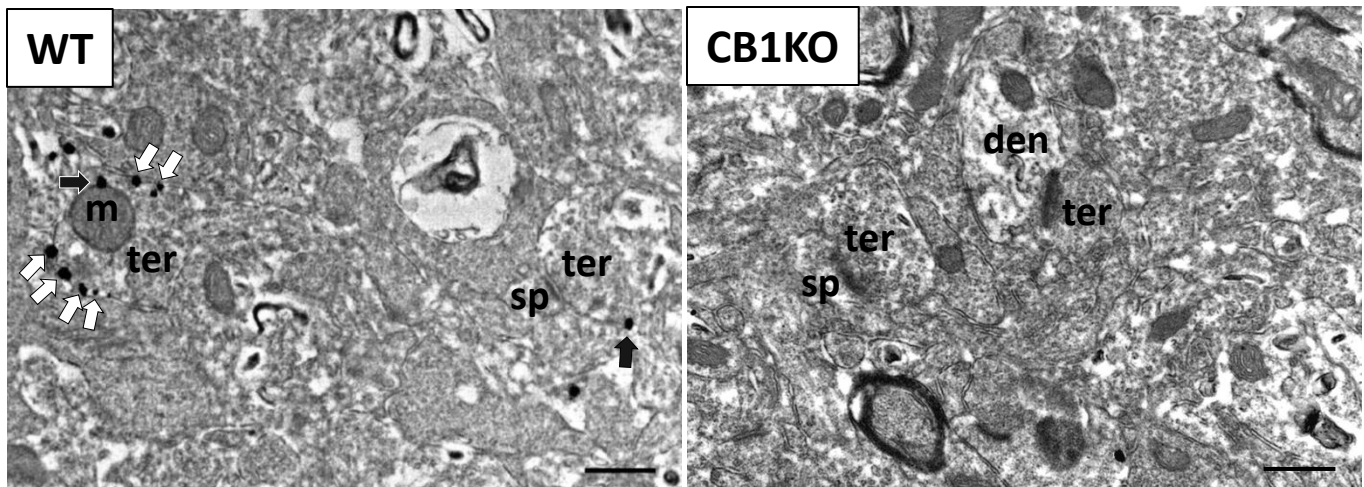
**Electrophysiology.** All the animals were sacrificed by dislocation during the light phase (9am to 12am). The brains were quickly removed and immersed in ice-cold oxygenated cutting solution containing in mM: 180 Sucrose, 26 NaHCO<sub>3</sub>, 12 MgSO<sub>4</sub>, 11 Glucose, 2.5 KCL, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 0.2 CaCl<sub>2</sub>, oxygenated with 95% O<sub>2</sub>/5% CO<sub>2</sub>  $\approx$  300mOsm. Coronal aPC slices (300 $\mu$ m thick) were obtained using a vibratome (VT1200S, Leica, Germany) and transferred for 30min into a 34°C bath of oxygenated ACSF containing in mM: 123 NaCl, 26 NaHCO<sub>3</sub>, 11 Glucose, 2.5 KCL, 2.5 CaCl<sub>2</sub>, 1.3 MgCl<sub>2</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>  $\approx$  305 mOsm. After a minimum of 30min recovery at RT (22-25°C), slices were transferred to a recording chamber in ACSF at 32°C. Whole-cell voltage-clamp recordings were performed using a Multiclamp 700B amplifier (Molecular devices, UK) in semilunar neurons clamped at -70mV with glass pipettes (3-5 M $\Omega$ ). Semilunar neurons were identified on the base of their morphology, location and electrical properties (Suzuki and Bekkers, 2006, 2011). Inhibitory post-synaptic currents (IPSCs) were obtained with an internal solution containing in mM: 130 KCl, 10 HEPES, 1 EGTA, 2 MgCl<sub>2</sub>, 0.3 CaCl<sub>2</sub>, 7 Phosphocreatin, 3 Mg-ATP, 0.3 Na-GTP; pH=7.2; 290mOsm, in presence of NMDA and

AMPA/Kainate receptor antagonists (50 $\mu$ M D-APV and 10 $\mu$ M NBQX) Excitatory post-synaptic currents (EPSCs) were obtained with an internal solution containing in mM: 125 K-gluconate, 5 KCl, 10 HEPES, 0.6 EGTA, 2 MgCl<sub>2</sub>, 7 Phosphocreatine, 3 Mg-ATP, 0.3 Na-GTP; pH=7.25; 300 mOsm, in presence of the chloride channel blocker, picrotoxin (100 $\mu$ M PTX). Miniature PSCs were recorded in presence of the voltage-gated sodium channels blocker, tetrodotoxin (1 $\mu$ M TTX). Bicuculline (10 $\mu$ M) or APV (50 $\mu$ M) and NBQX (10 $\mu$ M) were added to the bath solution to verify the GABA<sub>A</sub>-mediated inhibition or the ionotropic glutamatergic-mediated excitation respectively. Miniature PSCs were collected during the last 5min of recording in vehicle (DMSO), CB1 agonist (WIN 5 $\mu$ M) and CB1 antagonist (AM251 4 $\mu$ M) where both drugs were applied for 10min succinctly. For experiments performed after behavior, animals underwent the two bottles choice test for 15min and were sacrificed 5min later. mIPSCs were collected in the same manner as for naïve animals, for 5 min in presence of vehicle (DMSO).

IPSCs and EPSCs were evoked (eIPSCs and eEPSCs) with a monopolar stimulating patch pipette filled with ACSF placed in layer Ib. Two stimulations with 175ms apart (PPR-eIPSCs) and 50ms apart (PPR-eEPSCs) were delivered every 20s. Effect of WIN (5 $\mu$ M) was assessed by comparing 10min of stable baseline with the average responses 20 to 25min after the application of CB1 agonist.

Signals were filtered at 4kHz by a Digidata 1440A (Molecular devices, UK) and were analyzed using either Clampfit software (pClamp10) or Axograph for ePSCs and mPSCs, respectively.

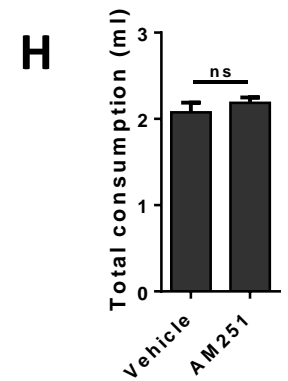
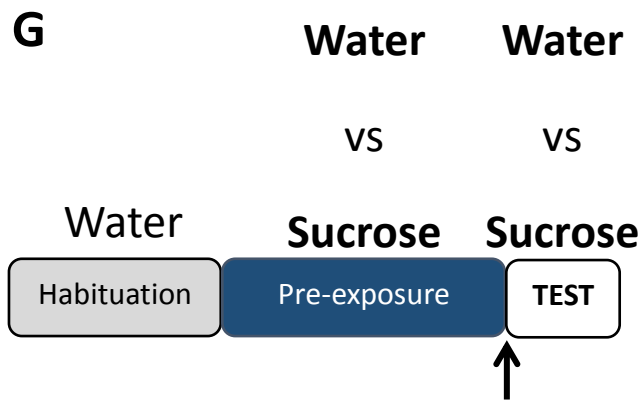
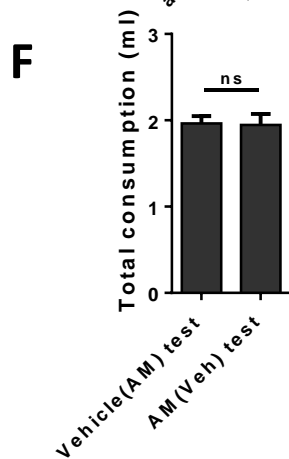
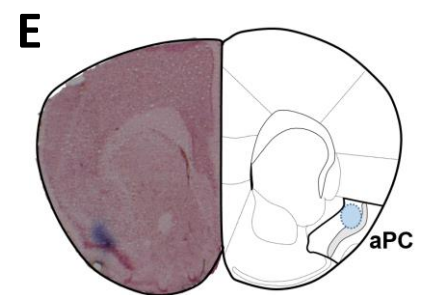
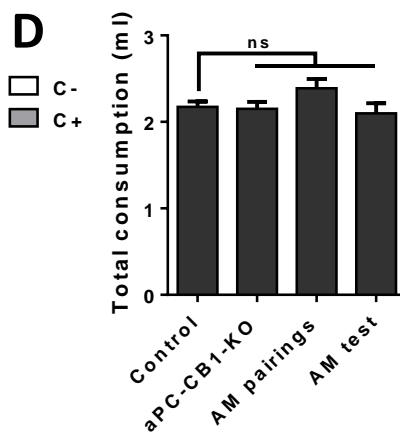
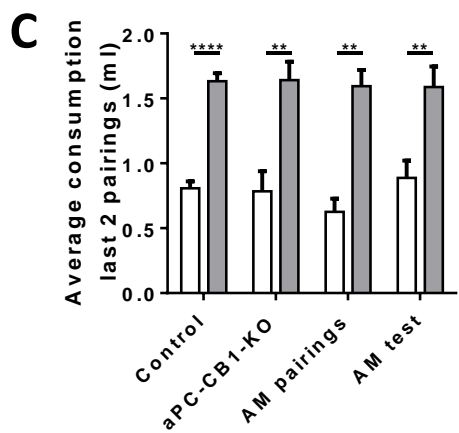
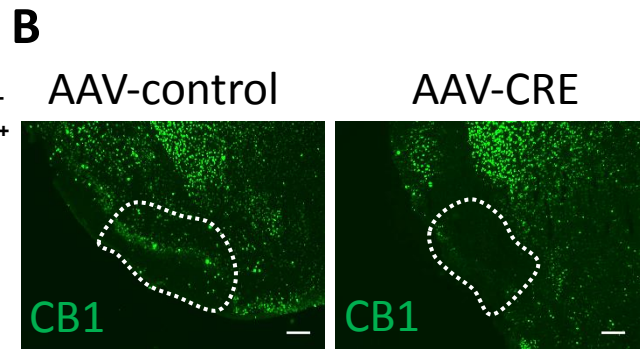
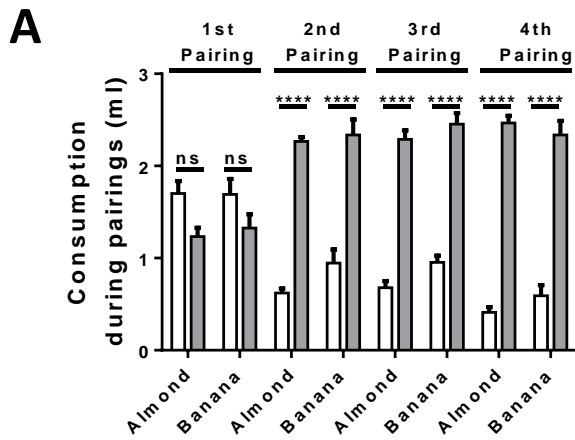
**Statistical analyses.** Electrophysiological and behavioral data analysis were analyzed with Prism 6 Software. Repeated or unpaired statistical analyses were obtained with Student's t-test and ANOVA (one-way or two way) to compare two or multiple groups where appropriate. When ANOVA provided significant main factor effects or significant interactions, Tukey, Dunnett or Sidak post-hoc analyses were performed as appropriate. Types of statistical tests are presented in figure legends. Significance was set at  $p < 0.05$  and data are expressed as mean  $\pm$  SEM.



**Fig. S1. CB1 receptors are mainly present in GABAergic neurons in the aPC.**

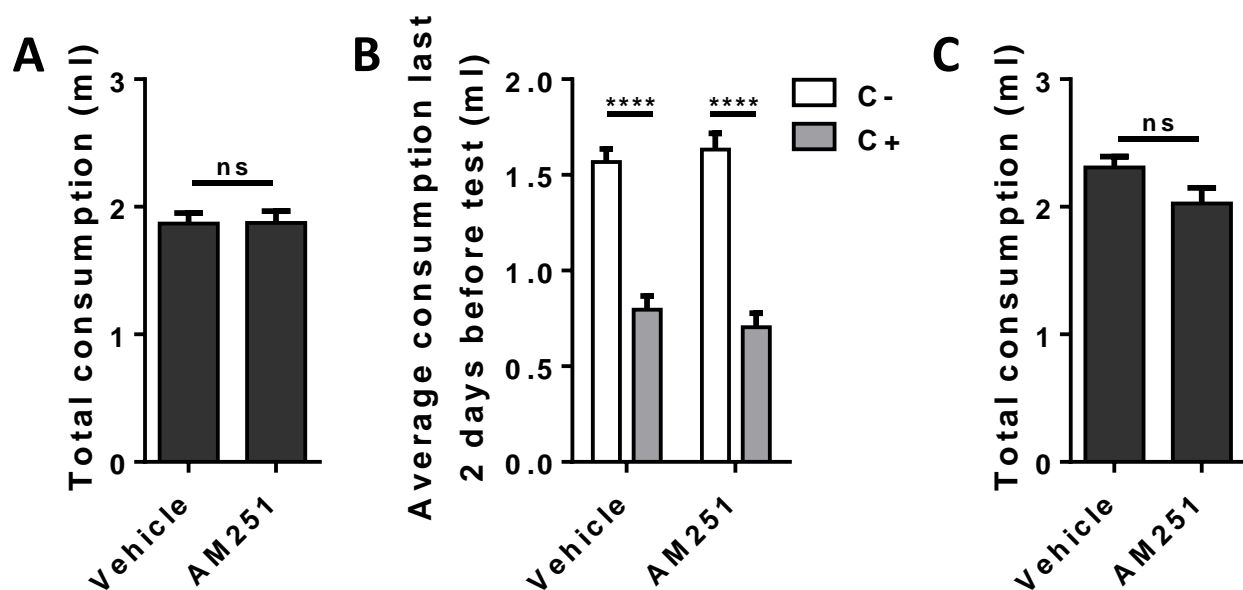
Electron microscopy of immunogold staining for CB1 receptors in the aPC of CB1 wild-type (WT) and knockout mice (CB1-KO). Arrows point to CB1 particles on GABAergic terminals (white arrows), glutamatergic terminals (black arrow) and mitochondria (black arrow with white outline). Note that CB1 receptors are mainly expressed in GABAergic neurons and CB1 receptors are not present in CB1-KO mice. Den, dendrites; ter, terminals; m, mitochondria; sp, dendritic spine. Scale bar, 500nm.





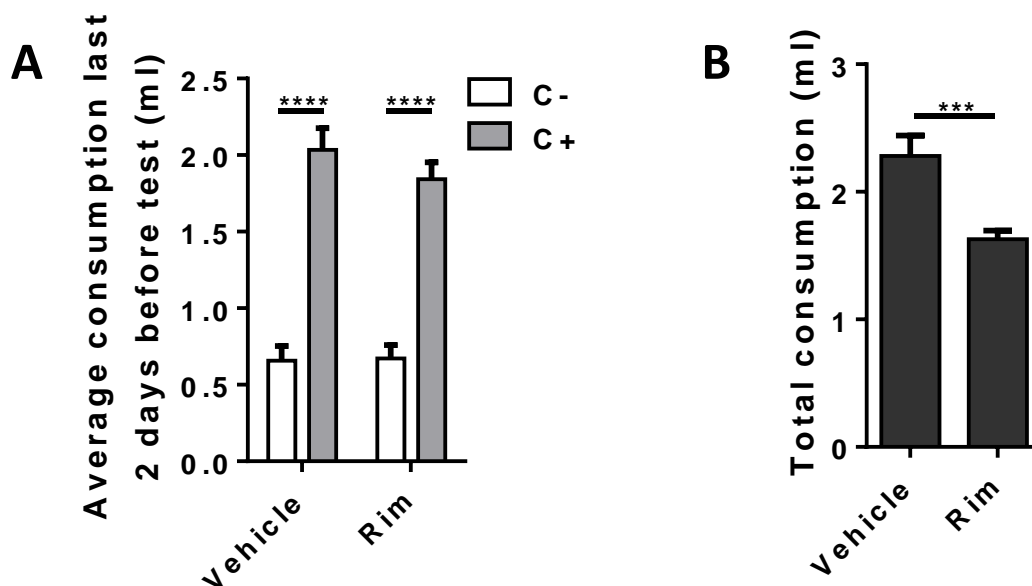
**Fig. S2. The endocannabinoid system in the anterior piriform cortex is necessary for conditioned odor preference retrieval.**

**(A)** Consumption of the almond- and banana-odorized solution during the pairings. Similar preference is observed for sucrose-paired odor after the second pairings whatever the odor paired with sucrose. Two-way ANOVA, repeated measures. \*\*\*\*:  $p < 0.0001$ , C+ vs C- (Almond C+,  $n=9$ ; Banana C-,  $n=11$ ). **(B)** Representative images showing the deletion of CB1 receptors in the aPC using Fluorescent *In Situ* Hybridization against CB1mRNA. **(C)** Average consumption during the last two days of training before the test in the different groups (control, aPC-CB1-KO, AM pairings and AM test). All groups show a preference for the solution containing sucrose (C+). Two-way ANOVA, repeated measures. \*\*\*\*:  $p < 0.0001$ , \*\*:  $p < 0.01$ , C+ vs C- . **(D)** Total liquid consumption during the test in the different groups (control, aPC-CB1-KO, AM pairings and AM test). No difference is observed between groups. One-way ANOVA (Control,  $n=36$ ; aPC-CB1-KO,  $n=10$ ; AM pairings,  $n=8$ ; AM test,  $n=12$ ). **(E)** Representative image showing the injected site (blue) obtained through cannula implanted above the aPC. **(F)** Total liquid consumption during the test day in animals performing the second test after retraining. No difference is observed between groups. Unpaired-t test [Veh(AM) test,  $n=14$ ; AM(Veh) test,  $n=13$ ]. **(G)** Schematic protocol used to evaluate the effect of aPC infusion (arrow) of the CB1 receptor antagonist AM251 ( $4\mu\text{g}/0.5\mu\text{l}$ ) or Vehicle on sucrose preference. **(H)** Total liquid consumption during the test. No difference is observed between groups. Unpaired t-test (Vehicle,  $n=8$ ; AM251,  $n=7$ ). Values are represented  $\pm$  SEM.



**Fig. S3. CB1 receptors in the aPC are not involved in odor-induced aversion learning.**

**(A)** Total liquid consumption during retrieval test of LiCl-induced COA. No difference is observed between groups. Unpaired-t test (Vehicle, n=13; AM251, n=11). **(B)** Average consumption during the last two days of training with quinine and **(C)** total liquid consumption during the test. Mice showed an aversion to the solution containing the quinine during training (C+) and no difference is observed in total consumption during the test. Two-way ANOVA, repeated measures (left) and unpaired t-test (right). \*\*\*\*:  $p < 0.0001$ , C+ vs C- (Vehicle, n=11; AM251, n=12).



**Fig. S4. COP retrieval induces pre-synaptic activation of CB1 receptors at inhibitory synapses.**

**(A)** Average consumption during the last two days of training before the test and **(B)** total liquid consumption during the COP test in animals injected ip either with vehicle or with the CB1 antagonist Rimonabant (1 mg/kg, Rim) before the test. Animals show a preference for the solution associated with sucrose during training (C+) and Rim decreases the total liquid consumption during test. Two-way ANOVA, repeated measures and unpaired t-test. \*\*\*\*:  $p < 0.0001$ , C+ vs C-; \*\*\*:  $p < 0.001$ , Vehicle vs Rim1 (Vehicle,  $n=16$ ; Rim,  $n=21$ ). Values are represented  $\pm$  SEM.

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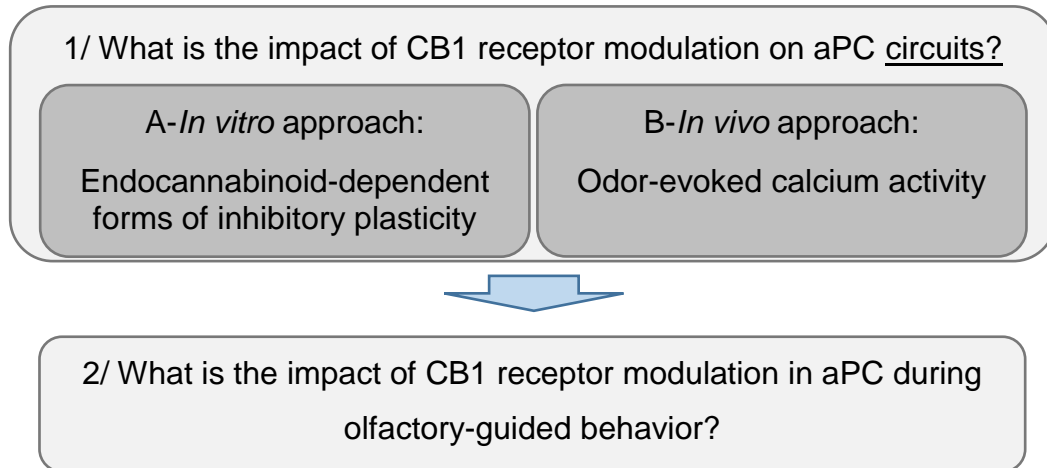
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### III.2 AIM 2 - IMPACT OF CB1 RECEPTOR MODULATION IN APC PROCESSES

In brief summary, in order to answer the second aim, we asked 3 main questions:



We found that, 1/ Both short- (DSI) and long-term forms (iLTD) of CB1-dependent synaptic plasticity are present in the aPC. Whereas DSI expression is independent of the inhibitory inputs' location, the expression of iLTD is layer-dependent and CB1-positive GABAergic interneurons express distinct neurochemical signatures in the layer where iLTD is absent. 2/ CB1 receptor activation and blockade impair CB1-dependent synaptic plasticity, odor-evoked calcium activity and conditioned odor preference retrieval.



# **CB1 receptors dynamically control olfactory processes in the anterior Piriform Cortex**

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## Abstract

The endocannabinoid system (ECS) is involved in the regulation of sensory perception and memory. In the olfactory system, a major structure involved in these processes is the anterior piriform cortex (aPC). However, the impact of ECS signaling in aPC circuitry is still unclear. Using anatomical, electrophysiological, *in vivo* imaging and behavioral experiments, we show that CB1 receptors control inhibitory synaptic plasticity as well as spontaneous and odor-evoked activity in the aPC. Patch clamp experiments revealed that the two major forms of ECS-dependent synaptic plasticity, namely depolarization-dependent suppression of inhibition (DSI) and long-term depression of inhibitory transmission (iLTD) are present in the aPC. Interestingly, iLTD expression depends on layer localization and neurochemical properties of the inhibitory neurons involved, whereas DSI does not seem to be impacted by these factors. Both DSI and iLTD were blocked by pharmacological inhibition and occluded by activation of cannabinoid-type 1 (CB1) receptors, respectively. Consistently, local aPC injections of the CB1 antagonist or agonist blocked spontaneous and odor-evoked calcium responses as well as conditioned odor preference memory retrieval in living mice. These results indicate that either the blockade or the occlusion of the ECS-dependent synaptic modulation in the aPC impairs odor-related functions, suggesting that CB1 receptor signaling dynamically modulate odor processes in aPC circuits.

## Introduction

Neuromodulators play crucial roles in shaping neuronal functions in the brain. The regulation of synaptic transmission is provided by a variety of neuromodulator systems (Avery and Krichmar, 2017; Nadim and Bucher, 2014). G protein-coupled receptors (GPCRs) are the principal effectors of most of these synaptic modulatory activities (Betke et al., 2012; Huang and Thathiah, 2015). Cannabinoid type-1 receptors (CB1) are the major cannabinoid receptors and have been proposed to be the most abundant GPCRs in the brain (Herkenham et al., 1990; Howlett, 2002). They are expressed in various cell types —glutamatergic and GABAergic neurons as well as glial cells— and in different cellular compartments (Araque et al., 2017; Bénard et al., 2012; Busquets-Garcia et al., 2018a; Kano et al., 2009; Marsicano and Kuner, 2008; Rozenfeld and Devi, 2008; Zou and Kumar, 2018). Together with their endogenous lipid ligands (endocannabinoids) and the synthetic and degradative enzymes regulating endocannabinoid levels, CB1 receptors are the main components of the endocannabinoid system (ECS) in the brain (Piazza et al., 2017; Piomelli, 2003). In neurons, activation of pre-synaptic CB1 receptors results in the decrease of neurotransmitter release, inducing several forms of ECS-dependent synaptic plasticity (Araque et al. 2017; Busquets-Garcia, Bains, and Marsicano 2018; Castillo et al. 2012; Kano et al. 2009; Zou and Kumar 2018). Moreover, given the wide expression of CB1 receptors in multiple brain areas, the ECS modulates a large variety of cognitive processes, including learning and memory, anxiety, locomotion, food intake, sensory perception and many others (Chaouloff et al., 2011; Corcoran et al., 2015; Lutz et al., 2015; Morena and Campolongo, 2014; Murillo-Rodriguez et al., 2011; Soria-Gómez et al., 2014a; Wei et al., 2017).

In particular, recent evidence indicates that CB1 receptors tightly control olfactory circuits, thereby modulating olfactory-related behaviors (Breunig et al., 2010; Soria-Gómez et al., 2014b). Olfaction starts with the binding of odorant molecules on sensory neurons of the olfactory epithelium that transduce and transmit the signals into the olfactory bulb. At this stage, information is processed and then relayed in cortical brain areas where the perceptual representation of the odor is formed and stored (Courtiol and Wilson, 2017; Gottfried, 2010; Wilson and Sullivan, 2011). The anatomical and physiological roles of CB1 receptors have been characterized mainly in the initial steps of olfactory processes, such as in the olfactory epithelium and the olfactory bulb, where the ECS modulates olfactory perception (Breunig et al., 2010; Czesnik et al., 2007; Pouille and Schoppa, 2018; Soria-Gómez et al., 2014b; Wang et al., 2012). Interestingly, CB1 receptors are also present in the olfactory cortex, including the piriform cortex (PC) (Gutiérrez-Rodríguez et al., 2017; Marsicano and Lutz, 1999). The PC is the largest region of the olfactory cortex, receiving convergent inputs from the olfactory bulb and from other higher brain regions, such as amygdala, prefrontal cortex, ... (Ghosh et al., 2011; Igarashi et al., 2012; Sosulski et al., 2011), thereby playing key roles in odor perception and olfactory memory (Bekkers and Suzuki, 2013; Gottfried, 2010; Haberly, 2001; Wilson and Sullivan, 2011). This paleocortex can be divided into two anatomically and functionally distinct regions, the anterior (aPC) and the posterior piriform cortex (pPC). The aPC consists of three layers, of which layer II/III contains pyramidal neurons, whereas all layers harbor several types of interneurons (Suzuki and Bekkers, 2006, 2007, 2010a). Particularly, inhibitory interneurons in the anterior Piriform cortex (aPC) have been proposed to play crucial roles in shaping olfactory processes (Bolding and Franks, 2018; Franks et al., 2011; Poo and Isaacson, 2009; Suzuki and Bekkers, 2012; Zhan and Luo, 2010).

In many brain regions, expression of CB1 receptors in specific subpopulations of GABAergic interneurons mediates short- and long-term forms of ECS-dependent plasticity of inhibitory neurotransmission, such as depolarization-induced suppression of inhibition (DSI) (Wilson and Nicoll, 2001) and inhibitory long-term depression (iLTD) (Araque et al. 2017; Castillo et al. 2012; Chevaleyre and Castillo 2003; Kano et al. 2009; Marsicano et al. 2002).

However, the detailed basal distribution of CB1 receptors in PC interneurons, the presence of ECS-dependent forms of synaptic plasticity and their potential consequences on physiological and behavioral odor responses have not been investigated yet.

In this study, we addressed these issues and showed that different forms of ECS-dependent synaptic plasticity are present in the anterior PC (aPC) and are associated with specific anatomical distribution of CB1 receptors. Moreover, we found that selective interference with CB1 receptor signaling in the aPC eliminates these forms of synaptic plasticity, blocks odor-evoked activity and inhibits olfactory-guided behavior. Notably, both pharmacological activation and inhibition of CB1 receptors block these functions, suggesting that the dynamic and temporally-controlled regulation of aPC circuits by ECS activity is required for proper processing of olfactory information in this brain region.

## **Materials and Methods**

### **Animals**

8 to 12 weeks male CB1-flox mice were used for *in vitro* experiments (mice carrying the “floxed” CB1 gene *Cnr1*) (Monory et al., 2006). Conditional knockout mice lacking CB1 receptors in forebrain GABAergic Dlx5/6 positive neurons (GABA-CB1-KO) were obtained as described

before (Monory et al., 2006). Briefly, Dlx5/6-Cre mice were crossed with CB1-flox mice to obtain GABA-CB1-KO mouse line. *In vivo* experiments were performed with C57Bl/6-N (Janvier) and CB1-flox mice (8 to 20 weeks old). All experimental procedures were approved by the local Committee on Animal Health and Care of Bordeaux (authorization number 13693) and the local ethical committee of Institut Pasteur (CETEA #2013.0086). Animals were housed under a 12h-12h light-dark cycle with food and water ad libitum.

## **Electrophysiology**

Brain slices were taken from the anterior piriform cortex as previously described (Terral et al, submitted). Slices were continuously oxygenated with 95% O<sub>2</sub>/5% CO<sub>2</sub> in ACSF containing in mM: 123 NaCl, 26 NaHCO<sub>3</sub>, 11 Glucose, 2.5 KCL, 2.5 CaCl<sub>2</sub>, 1.3 MgCl<sub>2</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub> ≈ 305 mOsm at 32°C during recordings. Whole-cell patch clamp experiments were performed in semilunar neurons, identified by their location, morphology and electrical properties (Suzuki and Bekkers, 2006, 2011), clamped at -70mV (Molecular devices, UK) with glass pipettes (3-5 MΩ). Evoked inhibitory post-synaptic currents (eIPSCs) were recorded with an internal solution containing in mM: 130 KCl, 10 HEPES, 1 EGTA, 2 MgCl<sub>2</sub>, 0.3 CaCl<sub>2</sub>, 7 Phosphocreatin, 3 Mg-ATP, 0.3 Na-GTP; pH=7.2; 290mOsm, in presence of NMDA and AMPA/Kainate receptor antagonists (50μM D-APV and 10μM NBQX). Monopolar stimulating patch pipettes filled with ACSF were placed in layer Ib, II or III to evoked inhibitory currents.

DSI experiments were performed by evoking IPSCs every 3s and depolarizing semilunar neurons from -70mV to 0mV for 5s. DSI magnitude was measured as the average of 3 trials with 2min apart and represented as the percentage of change by comparing the first

3 eIPSCs following the depolarization with the 5 consecutive eIPSCs preceding the depolarization.

iLTD was induced by evoking IPSCs every 20s and 2 trains of High-Frequency-Stimulation (HFS) of 100 pulses at 100Hz were delivered with 20s apart after a minimum of 10min of stable baseline. iLTD magnitude was represented by the percentage of change between the mean of the 10min baseline with the percentage of responses averaged between 20 to 25min after HFS.

Signals were filtered at 4kHz by a Digidata 1440A (Molecular devices, UK) and analyzed using Clampfit software (pClamp10).

### ***Fluorescent in situ hybridization***

The procedure was adapted from previous studies (Marsicano and Lutz 1999; Soria-Gómez et al. 2014b; Terral et al, submitted). Mice were anesthetized with chloral hydrate (400mg/kg body weigh), transcardially perfused with phosphate-buffered solution (PBS 0.1M, pH 7.4) before being fixed with 4% paraformaldehyde and quickly frozen by immersion in isopentane then stored at -80°C. Serial coronal free-floating sections were cut at 30µm in a cryostat (Microm HM 500M, Microm Microtech). Fluorescein (FITC)-labeled riboprobes against mouse CB1 receptor and Digoxigenin (DIG)-labeled riboprobes against mouse GAD65/67 and CCK were prepared as described (Marsicano and Lutz, 1999). The slices were incubated with the hybridization buffer containing the mixture of probes overnight at 62°C. After hybridization, the sections were washed with different stringency wash buffers at 67°C and blocked with a blocking buffer prepared according to the manufacturer's protocol. Anti-FITC or anti-DIG antibodies conjugated to

horseradish peroxidase (HRP) (Roche; 1:2000) were applied 2 hours at room temperature or overnight at 4°C to detect respectively CB1-FITC or GAD65/67-DIG and CCK-DIG probes. Probes hybridization was revealed by a tyramide signal amplification (TSA) reaction using FITC-labeled tyramide (Perkin Elmer; 1:80 for 12 minutes) to detect CB1 signal or Cyanine 3-conjugated tyramide (Perkin Elmer; 1:100 for 10 minutes) to amplify the signal of GAD65/67 or CCK. The slices were incubated in 4',6-diamidino-2-phenylindole (DAPI; 1:20 000; FISHER Scientific, NH, USA) before being washed, mounted, coverslipped and visualized with an epifluorescence Leica DM6000 microscope (Leica, Germany).

Quantitative co-expression data were obtained using ImageJ, by counting CB1 (green) and GAD65/67 or CCK (red) and co-expressing neurons. According to the different level of CB1-expressing cells, High- and Low-CB1 cells were distinguished as previously defined (Marsicano and Lutz 1999; Terral et al, submitted). This numerical evaluation was performed at X10 magnification from 3 different mice.

## **Pharmacology**

WIN 55,212-2 (5µM) and AM251 (4µM) (Tocris Bioscience) used for patch-clamp experiments were prepared in DMSO and applied in the slices a minimum 15min prior DSI recordings.

For *in vivo* experiments, WIN (1µg/0.5µl per side) and AM251 (1µg or 4µg/0.5µl per side) were dissolved in a mixture of 10% Cremophor-EL, 10% DMSO and 80% saline (NaCl 0.9%) and bilaterally injected in the aPC using a peristaltic pump (PHD 22/2000 Syringe Pump Infusion, Harvard Apparatus, Massachusetts, USA, flow rate: 0.5µl/min) 10min before COP and COA test and 15-25min before odor-evoked calcium imaging.

## **Stereotaxic Viral injections and fiber implantation**

For aPC stereotaxic injection, mice were anesthetized (ip injection; Ketamine, 100mg/kg; xylazine, 10mg/kg; buprenorphine 0.05mg/kg) first positioned in a stereotaxic frame. After local anesthesia (lidocaine) followed by skin incision and skull craniotomy, mice were injected bilaterally using pulled glass capillaries connected to a Nanoinjector System (Drummond) in the aPC (From Bregma, AP, +1.6mm; ML,  $\pm$ 2.5; DV, -4mm from brain surface; 250nl in 4min) with GCaMP6f-expressing viral vector (AAV9-CaMKIIa-GCaMP6F-WPRE viral vector provided by the GENIE Project, Janelia Farm Research Campus, Howard Hughes Medical Institute; produced by Upenn Vector Core;  $3.10E+13$  viral genome/ml). Following viral injection, an optic fibers (multimode, 425  $\mu$ m diameter, NA 0.50, LC zirconia ferrule) associated with a guide-cannula were implanted bilaterally above the virus injection site (AP:+1.6; ML, 2.5; DV from brain surface, -3.9) and stabilized with acrylic and dental cement. The stainless steel guide cannulae (26gauge, 7mm long) was positioned  $\sim$ 2mm aside the fiber and 4mm above the tip of the fiber with a  $\sim$ 25° angle so that the tip injection cannula was close to the imaging field. Animal was then moved to its home cage, monitored daily and left to recover for 4 weeks after injection. Postsurgical analgesia (0.05mg/kg buprenorphine) was provided *via* subcutaneous injection over the 48h period post-injection.

## **Calcium imaging using fiber photometry**

A fiber photometry system adapted from (Gunaydin et al., 2014) was used (see Fig. 7A). GCaMP6f was excited continuously using a 473 nm DPSS laser (output fiber intensity, 0.1 – 0.2 mW; Crystal Lasers) reflected on a dichroic mirror (452– 490 nm/505– 800 nm) and collimated into a 425  $\mu$ m multimode optic fiber (NA 0.48) with a convergent lens (f : 30 mm). The emitted fluorescence was collected in the same fiber and transmitted by the dichroic mirror,



filtered ( $525 \pm 19$  nm), and focused on a NewFocus 2151 femtowatt photoreceptor (Newport; DC mode). Reflected blue light along the light path was also measured with a second amplifying photodetector (PDA36A; Thorlabs) to monitor light excitation and fiber coupling. Signals from both photodetectors were digitized by a digital-to-analog converter (Power 1401; CED) at 5000 Hz and recorded using Spike2 software. For drug injection, bilateral acute injections were performed via a pump (PHD Syringe Pump Infusion, Harvard Apparatus) through implanted guide cannulae (injection volume,  $0.5\mu\text{l}$ ; speed,  $0.2\mu\text{l}/\text{min}$  via a 33-gauge cannula connected to a  $10\mu\text{l}$  Hamilton syringe). Animals were left to recover for 15-25min before moving to the recording chamber. For odor presentation, mice were placed in a small, ventilated cage ( $\sim 0.5$  L) coupled to a custom-build air-dilution olfactometer. Pure monomolecular odorants (isoamyl acetate, benzaldehyde; from Sigma-Aldrich) were diluted at different concentration (0.001%, 0.01%, 0.1%, 1%, 10% for both isoamyl acetate and benzaldehyde) in mineral oil (Sigma-Aldrich) in an odorless vial and saturated odor vapor was then mixed with air (dilution 1/5) before delivery into the ventilated cage (exhaust ventilation;  $0.2\text{L}/\text{sec}$ ) at a flow rate of  $3\text{L}/\text{min}$ . Odors were presented sequentially (4sec presentation; exhaust ventilation switched off during odor presentation) from the lowest to the highest concentration (3 consecutive presentation of the same odor) every 60sec. Odor presentation dynamics in the cage were monitored constantly using a mini-PID (Aurora Scientific). To evaluate odor-evoked responses, we extracted the mean fluorescence during odor presentation (4sec period starting 1sec after odor onset) and normalized ( $\Delta F/F$ ) to the fluorescence level during the baseline period (4sec) before odor. The three consecutive odor presentation were averaged per individual. For spontaneous activity, raw fluorescence signals were normalized ( $\Delta F/F$ ) to the mean fluorescence (50sec window), smoothed (0.02sec window), filtered (0.2Hz high-pass filtered). Spontaneous

events above 2 standard deviation were isolated and the mean frequency were calculated. To extract event based on their rise time kinetics, slope of the raw fluorescence signal were also calculated (0.5 sec window) and events above 3 standard deviation were isolated for mean frequency quantification.

### **Conditioned odor preference and aversion**

Conditioned odor preference and aversion protocol was performed as previously described (Terral et al, submitted). Briefly, mice were daily water deprived for 23 hours and had access for 1 hour to water bottles. After 3 days of habituation, animals underwent learning phase during 4 days where one odorized water bottle was associated with 5% sucrose for COP or with intraperitoneal (IP) injection of lithium chloride (LiCl, 0.3 M, 1% b.w.) (C+) and another odorized water bottle with sucrose free or with (IP) injection of saline (NaCl 0.9%) (C-). The preference and aversion test were assessed using a two bottles choice test for 1 hour with the odorized water bottles in absence of sucrose and IP injection. The concentration and the odors used, isoamyl acetate (0.05%) and benzaldehyde (0.01%) (Sigma-Aldrich) were chosen to be equally preferred (Busquets-Garcia et al., 2017a, 2017b, 2018b; Root et al., 2014; Soria-Gómez et al., 2014b; Terral et al, submitted).

### **Statistical analysis**

Data were analyzed with Prism 6 Software. Repeated or unpaired statistical analyses were obtained with Student's t-test and ANOVA (one-way or two way) to compare two or multiple groups where appropriate. When ANOVA provided significant main factor effects or significant interactions, Tukey, Dunnett or Sidak post-hoc analyses were performed as

appropriate. Types of statistical tests are presented in figure legends. Significance was set at  $p < 0.05$  and data are expressed as mean  $\pm$  SEM.

## Results

### Depolarization-induced Suppression in the aPC.

Endocannabinoid signaling mediate several forms of plasticity of inhibitory transmission, which are best characterized in the hippocampus (Castillo et al. 2012). Considering the anatomical and functional similitudes with the hippocampus (Haberly, 2001, 1985), we asked whether aPC-interneurons are able to undergo similar plasticity. Depolarization-induced suppression of inhibition (DSI) is a classic form of ECS-dependent synaptic plasticity (Wilson and Nicoll, 2001). We recorded evoked inhibitory postsynaptic currents (eIPSCs) in layer II pyramidal neurons of aPC slices. To further understand whether DSI features depend on the anatomical location of the inhibitory inputs, the stimulating electrode was placed either in aPC layer I, II or III to generate "layer I, II or III DSI", respectively. Independently of the stimulation site, transient depolarization of the postsynaptic cells induced reliable DSI, which consisted in an approximate 30% reduction of eIPSCs amplitudes (Fig. 1A-C). Interestingly, whereas layer II and III DSI lasted only 30s, DSI induced by fiber stimulation in layer I was still present up to 100s after depolarization (Fig.1 A). Next, we assessed whether aPC DSI depends on activation of CB1 receptors. A specific feature defining ECS-dependent DSI is that its expression is blocked or occluded by application of CB1 receptor antagonists or agonists, respectively (Kano et al., 2009; Wilson and Nicoll, 2001). Importantly, the CB1 receptor antagonist AM251 (4  $\mu$ M) significantly blunted both layer I (from  $-30.6 \pm 2.1\%$  to  $-12.25 \pm 4.6\%$ ;  $p=0.0009$ ) and layer III

DSI (from  $-26 \pm 2.9\%$  to  $-4.49 \pm 3.4\%$ ;  $p=0.0002$ ). Similarly, the application of the CB1 receptor agonist WIN55,512-2 (WIN,  $5 \mu\text{M}$ ) occluded DSI in both layers (layer I,  $1.24 \pm 3.5\%$ ;  $p<0.0001$ ; layer III,  $-1.22 \pm 2.8\%$ ;  $p<0.0001$ ).

CB1 receptors are highly expressed in GABAergic interneurons both in the hippocampus (Gutiérrez-Rodríguez et al., 2017; Marsicano and Lutz, 1999) and aPC (Terral et al. submitted). However, other cell types and afferent fibers might contain CB1 receptors and thereby participate to ECS-dependent synaptic plasticity (Araque et al. 2017; Castillo et al. 2012; Kano et al. 2009). Nevertheless, layer I and III DSI were both virtually absent in conditional mutant mice carrying deletion of the CB1 receptor gene specifically in forebrain GABAergic cells (GABA-CB1-KO mice, Monory et al. 2006; Control:  $-30.6 \pm 2.1\%$  and  $-26 \pm 2.9\%$  vs GABA-CB1-KO:  $-11.8 \pm 3.5\%$  and  $1.79 \pm 8.5\%$ ; one-way ANOVA,  $p<0.0001$  and  $p=0.0002$ , layer I and layer III respectively). Altogether, these results indicate that aPC DSI is a *bona fide* CB1 receptor-dependent form of synaptic plasticity that is due to the endogenous activation of CB1 receptors at GABAergic terminals impinging onto pyramidal neurons. Moreover, DSI expression is independent of the layer location of the inhibitory fibers involved.

### **Long-term depression of inhibitory currents in aPC is layer-dependent**

Whereas short post-synaptic depolarization induces transient short-term DSI, repeated high frequency stimulation (HFS) of afferent fibers results in a long-term form of ECS-dependent synaptic plasticity of eIPSCs in the hippocampus and other brain regions, generally called inhibitory long-term depression (iLTD) (Chevalleyre and Castillo, 2003; Crosby et al., 2011). Thus, we applied two HFS trains to afferent fibers in layer I, II or III, respectively, while recording eIPSCs in layer II pyramidal neurons. HFS failed to produce a significant long-term decrease of eIPSCs when the stimulation was applied to layer I (Fig.2 A,B) ( $14.94 \pm 7.9\%$ , compared to

baseline,  $p=0.1324$ ). However, significant iLTD was obtained when the stimulating electrodes were placed either in layer II (Fig.2 A,C) ( $-41.51 \pm 12.6\%$ ,  $p=0.0214$ ) or in layer III (Fig.2 A,D) ( $-34.67 \pm 12.2\%$ ,  $p=0.0252$ ). Importantly, iLTD was blocked by the application of AM251 both in layer II ( $0.12 \pm 5.3\%$ , compared to baseline,  $p=0.982$ ) and III ( $3.07 \pm 11.7\%$ ,  $p=0.801$ ), indicating that this form of synaptic plasticity depends on the endogenous activation of CB1 receptors. Thus, ECS-dependent iLTD is present in the aPC, where its expression is layer-dependent, being absent when layer I fibers are stimulated.

### **Layer-dependent neurochemical signatures of CB1-positive interneurons in the aPC**

In order to further investigate the reasons of such layer-dependency of aPC iLTD, we next asked whether GABAergic neurons in the different layers display different features. Based on electrophysiological properties, laminar location, morphology and expression of molecular markers, several distinct classes of interneurons have been pointed out in the aPC (Suzuki and Bekkers, 2007, 2010b, 2010a, 2012). Fluorescent *in situ* hybridization (FISH) revealed that, similarly to other brain regions (Marsicano and Lutz, 1999), CB1-positive cells in the aPC contain variable amounts of receptor transcript, ranging from very high to low-to-moderate levels. As revealed by double FISH (D-FISH), all high CB1-expressing cells in the aPC are GABAergic interneurons because they co-express glutamic acid decarboxylase 65KDa and 67KDa mRNA (GAD65/67, see methods), whereas cells containing low levels of the receptor are only partly belonging to this cellular subpopulation (Fig. 3A). Interestingly, independently of the levels of receptor transcript, high CB1-expressing cells from layer I are largely co-expressing GAD65/67 indicating their GABAergic nature. However, layers II and III CB1-positive cells containing low amounts of receptor mRNA are not all GABAergic (Fig. 3A). In the hippocampus, expression of iLTD characterizes GABAergic interneurons belonging to the

family of basket cells containing the neuropeptide cholecystinin (CCK, Basu et al. 2013; Chevaleyre and Piskorowski 2014). Importantly, anatomical data showed that layer I interneurons in the aPC lack typical markers of GABAergic cells, including CCK (Cummings, 1997; Suzuki and Bekkers, 2007, 2010a). D-FISH experiments with CB1 and CCK mRNA (Fig. 3B) confirmed that, independently of the levels of expression, only 1,7% of CB1-positive neurons in the layer I of the aPC contain CCK mRNA (Fig. 3C; 6/359 cells), whereas in the other layers 100% of high CB1-expressing cells (i.e. GABAergic, see Fig. 3A 157/157 cells) are endowed with CCK mRNA with a larger proportion observed in layer II/III (Fig. 3C). Thus, CB1-positive GABAergic interneurons in the aPC are characterized by distinct neurochemical signatures that depend on the anatomical layer location, with iLTD-resistant layer I cells lacking CCK expression.

### **The endocannabinoid system shapes odor-dependent Calcium responses in the aPC.**

To address the potential impact of ECS signaling on aPC spontaneous network dynamics and sensory-evoked responses, we used fiber photometry coupled to local drug infusion to record population activity in aPC pyramidal neurons expressing the calcium reporter GCaMP6f (Mazo et al., 2016) of freely moving mice before and after CB1 pharmacological modulation (Fig. 4A,B). GCaMP6f was excited continuously at low intensity (0.05– 0.1 mW) and the bulk calcium signals was collected using an optic fiber implanted in the layer II/III above the AAV injection site, then spectrally separated using a dichroic mirror, and emission intensity was measured with a femtowatt photodetector (Fig. 4A,B). We first analyzed the effect of local CB1 modulation on spontaneous activity of the aPC network. In awake freely moving animals, aPC imaging showed spontaneous positive fluorescence transients with sharp onset and amplitude in the 2-10% range. Following odor stimulation, we observed strong odor-locked excitatory responses

(fluorescence positive transients of 5-30% of range), with a notable depression of the odor-evoked responses following repeated presentation of the same odor (Fig 4B), as classically observed in the aPC (Best and Wilson, 2004; Linster et al., 2009). Increasing odor concentrations resulted in concentration-dependent increase in odor-evoked responses in the aPC for both isoamyl acetate or benzaldehyde odorants, reflecting a similar increase of neuronal activity (concentration effect,  $p < 0.0001$ ), independently of the nature of the odor (odor effect,  $p = 0.7421$ ; Fig. 4C). In sake of clarity, results from both odorants were merged in the following analysis. Following acute local infusion of CB1 agonists and antagonists at the vicinity of the imaging site, we observed a strong decrease in the frequency of spontaneous positive events after application of the CB1 agonist WIN (-69% compared to vehicle,  $p < 0.0001$ ; Fig. 4D) and a moderate decrease after application of the CB1 antagonist AM251 (AM251 - 1  $\mu\text{g}$ , -36% compared to vehicle,  $p = 0.0012$ ; AM251 - 4  $\mu\text{g}$ , -36% compared to vehicle,  $p = 0.0049$ ; Fig. 4D). Upon odor stimulation at increasing concentration, CB1 receptor blockade significantly blunted aPC odor-evoked responses at high odor concentration (drug main effect,  $p = 0.0096$ , Vehicle vs AM251 - 1  $\mu\text{g}$  and 4  $\mu\text{g}$ ; Fig. 4E) and induced the emergence of odor-evoked fluorescence decrease for low odor concentration, reminiscent of inhibitory responses. Application of the CB1 receptor agonist WIN strongly abolished odor-induced responses in the aPC (drug main effect,  $p = 0.0003$ , Vehicle vs WIN 1  $\mu\text{g}$ ; Fig. 4F) and induced the emergence of inhibitory responses for some concentration. These strong inhibitory effects of WIN were transient and recover to control conditions after wash out of the drug (vehicle post WIN,  $p = 0.5728$ , Vehicle vs Vehicle post WIN; Fig. 4F).

Altogether, the data showed that 1) the ECS in the aPC network is endogenously active in awake animals, 2) both blockade and activation of CB1 receptors in the aPC obliterate odor-

evoked excitatory activity—similar to ECS-dependent synaptic plasticity—and unmasked the contribution of inhibitory responses.

### **Activation of CB1 receptors in the aPC occludes conditioned odor preference**

Given that either activation or blockade of CB1 receptors impair DSI induction and odor-evoked responses in the aPC, we next asked if these mechanisms might correlate with odor-dependent behaviors. To date, the only evidence of the involvement of CB1 receptor in olfactory behaviors related to the aPC is that CB1 blockade in the aPC specifically impairs odor-dependent memory in an appetitive conditioned odor preference (COP) paradigm, without affecting aversive odor-dependent memory (Conditioned odor aversion, COA, Terral et al, submitted). If CB1 receptor activation and blockade have similar effects on aPC functions, local injection of WIN should block COP memory, without affecting COA. Indeed, bilateral aPC injections of WIN (1 µg/side) impaired the preferential consumption of odorized water previously paired with sucrose (C+ vs. C- vehicle,  $p=0.0112$ ; C+ vs. C- WIN,  $p=0.7661$ ; Fig. 5A), without altering total water intake (vehicle vs WIN,  $p=0.3358$ ; Fig. 5B). Interestingly, similar to what observed with AM251, WIN was not able to alter the aversion towards odorized water previously paired with a negative reinforcer, such as LiCl injections (C+ vs. C- vehicle,  $p=0.0002$ ; C+ vs. C- WIN,  $p=0.0003$ ; Fig. 5C), again without changing total water intake (vehicle vs WIN,  $p=0.6089$ ; Fig. 5D). Together, these results indicate that activation of CB1 receptors in the aPC specifically blocks retrieval of positively-, but not negatively-conditioned odor memories, an effect that is similar to the one previously observed with a CB1 receptor antagonist.



## Discussion

In this study, we evaluated the effect of CB1 receptor modulation on aPC processes. We characterized the presence of different forms of ECS-dependent inhibitory synaptic plasticity. Whereas DSI expression is independent of the inhibitory inputs' location, the expression of iLTD together with the presence of GABAergic interneurons containing CCK transcript are layer-dependent in the aPC. Moreover, we found that pharmacological activation and inhibition of CB1 receptors in the aPC eliminates these forms of synaptic plasticity, blocks odor-evoked activity and alters specifically COP retrieval.

CB1 receptors, mainly located on local GABAergic interneurons, mediate typical ECS-dependent forms of synaptic plasticity (DSI and iLTD), which are abolished by both CB1 receptor antagonists and agonists. This apparently counterintuitive phenomenon has been thoroughly investigated in several brain regions, like the hippocampus for DSI and iLTD or other forms of ECS-dependent synaptic plasticity in other brain regions (Castillo et al. 2012; Chevaleyre and Castillo 2003; Kano et al. 2009; Marsicano et al. 2002; Wilson and Nicoll 2001). Indeed, the abilities of CB1 receptor antagonists to *block* and of agonists to *occlude* DSI and iLTD are generally required conditions to ascribe these forms of synaptic plasticity to ECS physiological signaling. In other words, DSI and iLTD require the temporally-restricted availability of presynaptic CB1 receptors at the moment of their induction: if these are occupied by antagonists or agonists, the endogenous release of endocannabinoids is not able to induce synaptic plasticity, independently of the specific effect of the treatments on neurotransmission. This idea implies that both CB1 receptor antagonists and agonists should abolish brain functions or behaviors involving ECS-dependent synaptic plasticity similar to the ones observed in slices. Indeed, together with our previous results (Terral et al. submitted) our data show that

local aPC applications of the CB1 antagonist AM251 or of the agonist WIN block both odor-induced  $Ca^{2+}$  responses and conditioned odor preference memory retrieval, suggesting that DSI- and/or iLTD-like phenomena are required during these processes.

DSI and iLTD do not share identical anatomical constraints in the aPC. Whereas DSI occurs in a layer-independent manner, iLTD on aPC pyramidal neurons is only present when fibers of layers II or III are stimulated. Such a difference in layer specificity between DSI and iLTD has been described also in the hippocampus, where DSI can be induced both when IPSCs are evoked in *stratum radiatum* and in *stratum pyramidale*, whereas iLTD occurs only in *stratum radiatum* (Chevalleyre and Castillo, 2003). Interestingly, our data show that CB1-positive GABAergic interneurons in the layer I of aPC (where no iLTD is observed) barely express CCK, suggesting that, besides the expression of CB1 receptors, this specific neurochemical signature might be a key element for iLTD, but not for DSI. Indeed, CCK-positive basket cells have been shown to be the responsible for iLTD in the hippocampus (Basu et al., 2013; Chevalleyre and Piskorowski, 2014), possibly explaining why this form of synaptic plasticity in the aPC is restricted to layers II and III, where interneurons expressing both CCK and CB1 are present. It is not presently clear why DSI does not show such anatomical restrictions in the aPC. However, the present data suggest that expression of CCK in the CB1-positive interneurons involved is not strictly required for the induction of DSI. Thus, a sort of CCK-dependency would exist for iLTD but not for DSI. Unfortunately, as in other brain regions, CCK is present in both GABAergic and glutamatergic neurons of aPC layer II and III (Cummings 1997; Fig.3B), making the specific targeting of GABAergic-CCK-positive cells very challenging (Busquets-Garcia et al., 2018b; Dimidschstein et al., 2016). Future technological advances such as intersectional strategies (see (Taniguchi et al., 2011)) will hopefully help addressing this interesting question.

Our data show that local pharmacological activation or blockade of CB1 receptors decrease the magnitude of odor-evoked calcium responses in pyramidal aPC neurons. As mentioned above, this suggests that the endogenous activation of the ECS participate in the spontaneous and sensory-evoked aPC network activity and coding. We speculate that blocking ECS-dependent inhibition of GABAergic transmission—and the associated forms of plasticity—may ultimately reformat the excitatory/inhibitory balance in the aPC. In the context of CB1 agonist application, we think that the resulting non-selective activation of CB1 receptors may act on both GABAergic as well as glutamatergic synapses. The occlusion effect of CB1 receptor agonists would induce a general reduction in synaptic activity and impede the activation of the specific circuits required for these responses at the moment of odor presentation. Indeed our data show that basal levels of spontaneous activity in the aPC are strongly decreased by the local application of WIN (Fig. 4D). Both these different effects of CB1 receptor blockade or activation on basal circuit properties finally result in the lack of dynamic regulation. Thus, together with previous literature, the present data suggest that, rather than the absolute levels of activity of specific neuronal types or circuits, the key determinant of physiological brain responses to specific stimuli is the dynamic and relative balance between inhibition and excitation (Denève et al., 2017; Tatti et al., 2017).

Similar considerations can be applied to the effects of CB1 agonists and antagonists on retrieval of conditioned odor preference. In this behavioral task, a CS+ and a CS- odor stimuli are presented. Assuming that appetitive odor conditioning strengthen neuronal responses to specific odors, the lack of dynamic specific synaptic modulation induced by CB1 receptor blockade or activation might explain the loss of encoding and salience of specific odor stimuli, resulting in the loss of odor preference.

Interestingly, together with our previous results, the present data indicate that the ECS signaling in the aPC is clearly involved in appetitive odor conditioning, but not when the salience of the conditioned stimulus is aversive. Two possible explanations exist for this intriguing phenomenon. On one hand, the aPC circuits possibly involved in aversive odor retrieval might simply escape the direct regulation provided by CB1 receptors. However, in light of recent studies, the most likely explanation is that the aPC is specialized in attributing positive values to specific odors, whereas other parts of the olfactory circuits are engaged when odor stimuli are endowed with negative values (Laviolette and Grace, 2006; Otto et al., 2000; Sevelinges, 2004; Soria-Gómez et al., 2015). Future experiments will address the role of CB1 receptors in these other regions (e.g. the posterior PC) in innate or acquired odor aversion tasks.

In conclusion, this study shows that CB1 receptors expressed in specific cells of the aPC are key modulators of the excitatory/inhibitory balance regulating brain odor responses. Interestingly, several neurological and neuropsychiatric disorders are characterized by altered odor processing (Godoy et al., 2014; Philpott and Boak, 2014). For instance, odor-related disturbances are early symptoms of conditions such as Alzheimer's or Parkinson's Diseases or depression. Considering the involvement of the ECS in these pathologies (Basavarajappa et al., 2017; Yin et al., 2018), the present data might suggest novel ways to tackle at least some symptoms of these diseases.

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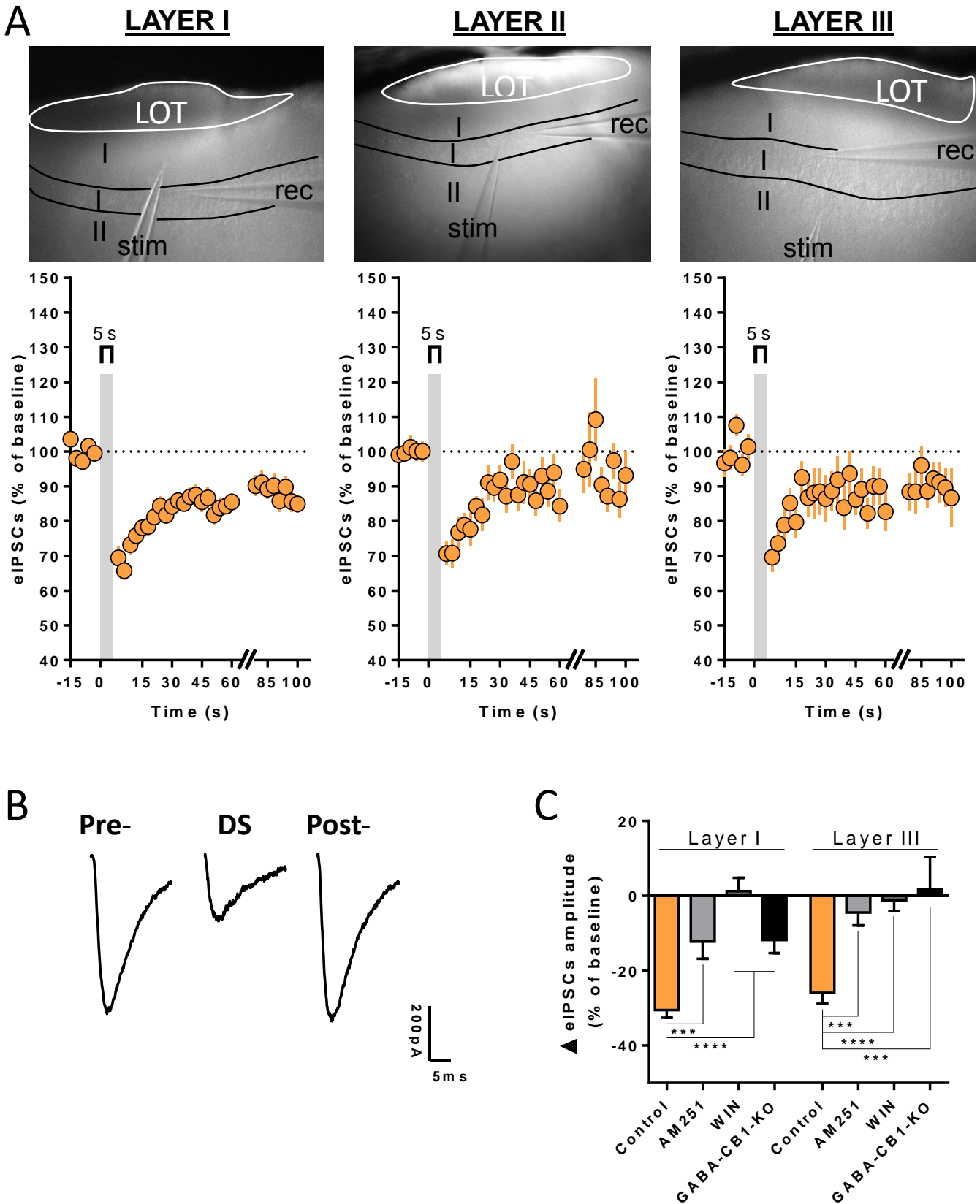
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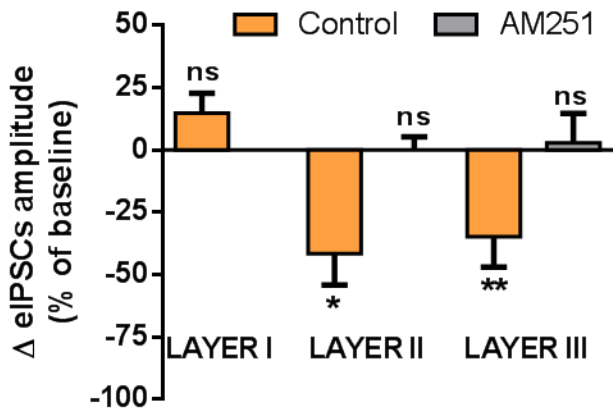
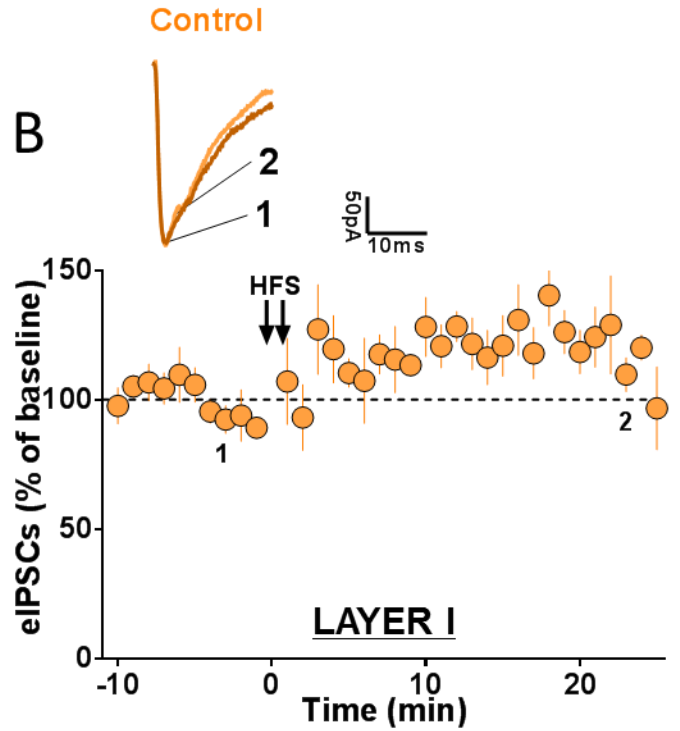
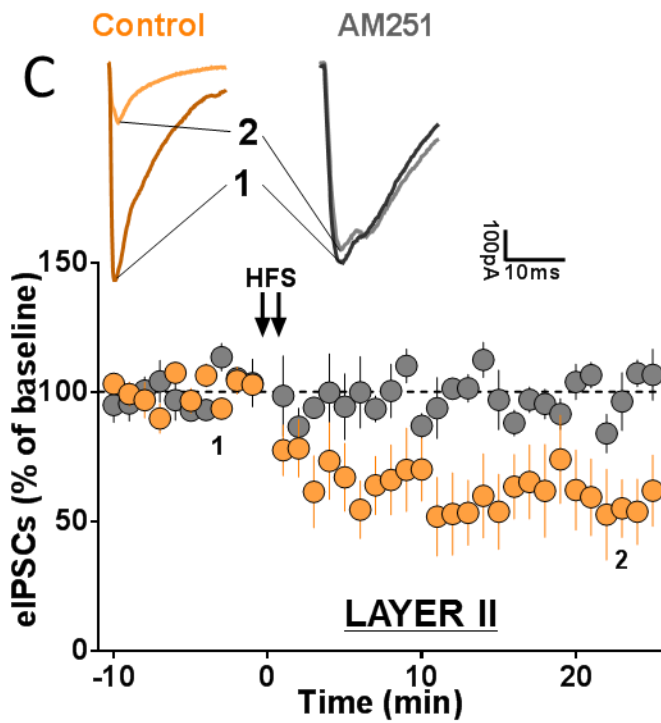
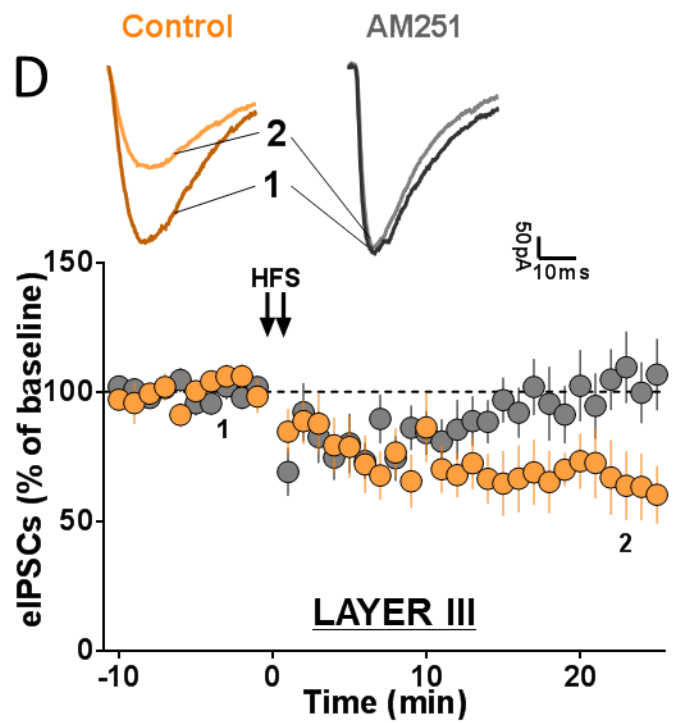
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# Figures



**Figure 1. Depolarization-induced Suppression in the aPC.**

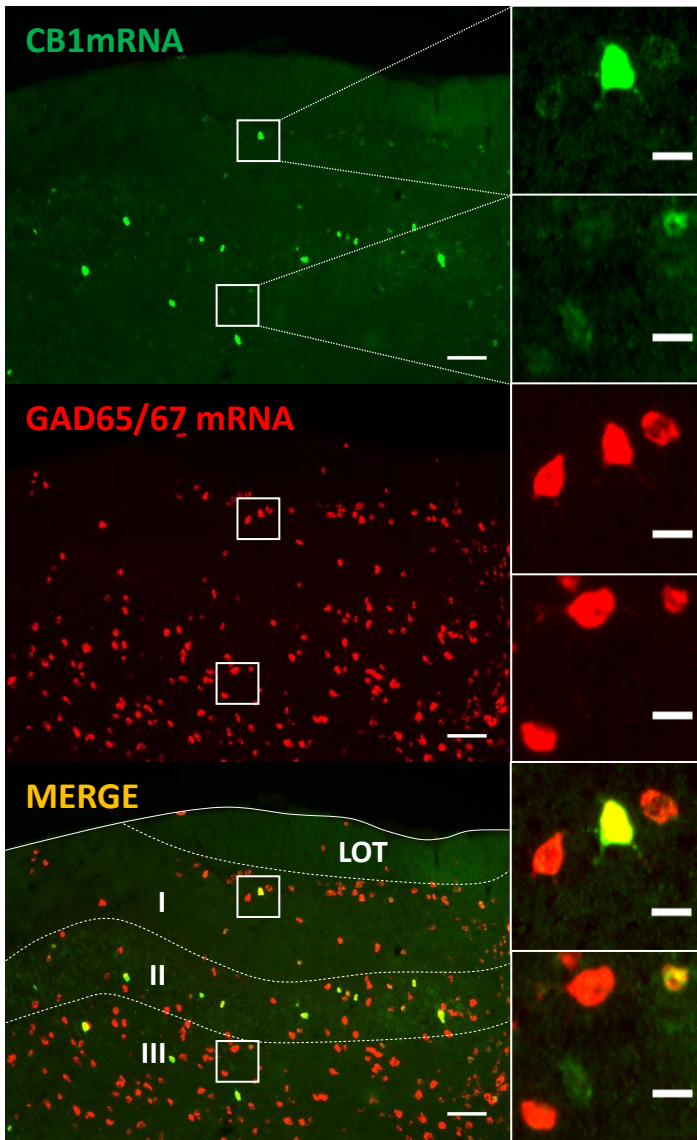
**(A)** Effect of 5s depolarization from -70mV to 0mV by evoking IPSCs in the three layers. Top, representative images showing the position of the stimulating electrodes in the different layers while recording pyramidal neurons in layer II. Bottom, time course average of the eIPSCs during de depolarization. **(B)** Representative traces for a DSI expressed in layer III. Traces were average with the last 5 sweeps preceding the depolarization (Pre-DSI), the first 3 sweeps post depolarization (DSI) and 5 sweeps from 45 to 60s post depolarization (Post-DSI). **(C)** Percentage of reduction normalized to baseline on eIPSCs averaged of the first 3 sweeps. Control (n=40 and n=22), AM251 (n=10 and n=10), WIN (n=8 and n=10), GABA-CB1-KO (n=20 and n=5), by stimulating in layer I and layer III respectively. One-way ANOVA \*\*\*\*,  $p < 0.0001$ ; \*\*\*,  $p < 0.001$ . Values are represented  $\pm$  SEM.

**A****B****C****D**

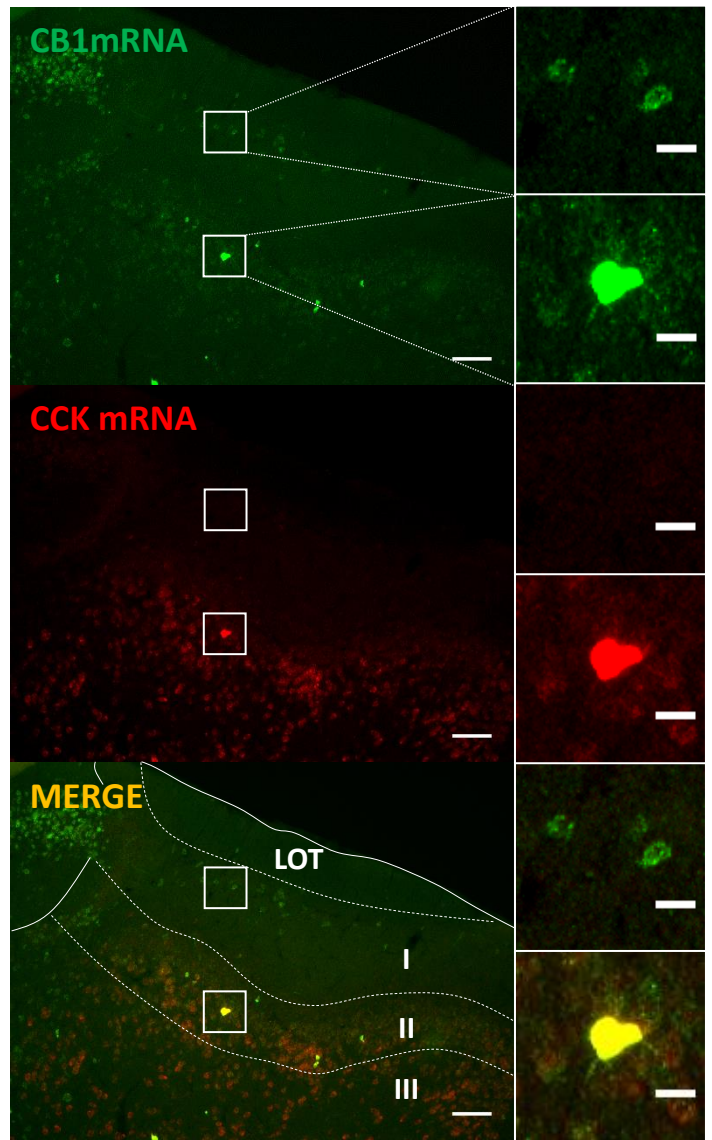
**Figure 2. Long-term depression of inhibitory currents in aPC is layer-dependent.**

**(A)** Average eIPSCs recorded 20 to 25 minutes after High-Frequency Stimulation (HFS) application normalized to baseline. Effect of HFS on eIPSCs in layer I **(B)**, layer II **(C)** and in layer III **(D)**. Top, representative traces average during the last 5 min before HFS (1) and 20 to 25minutes after HFS (2). Bottom, eIPSCs time course.. Layer I (n=5), layer II (n=6), layer III (n=9). One-sample t-test \*,  $p < 0.05$ ; ns, not significant. Values are represented  $\pm$  SEM.

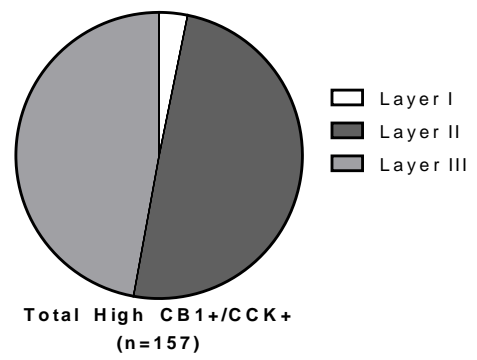
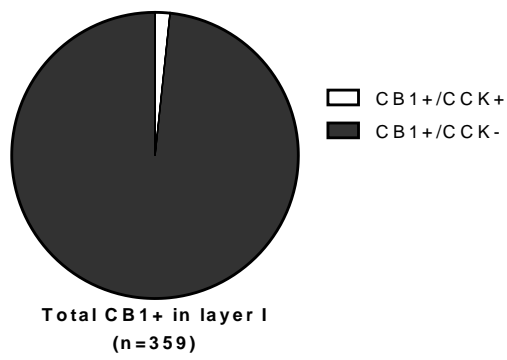
A



B

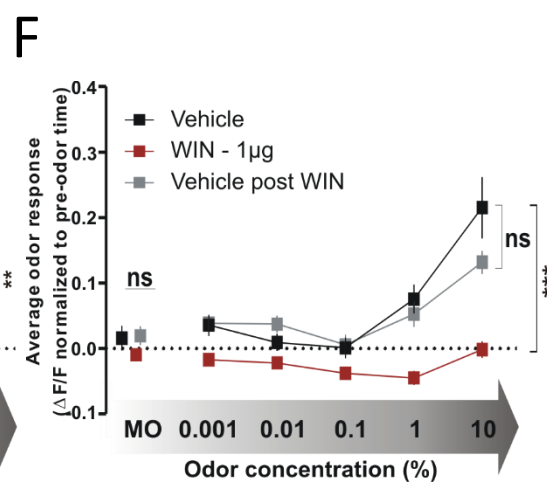
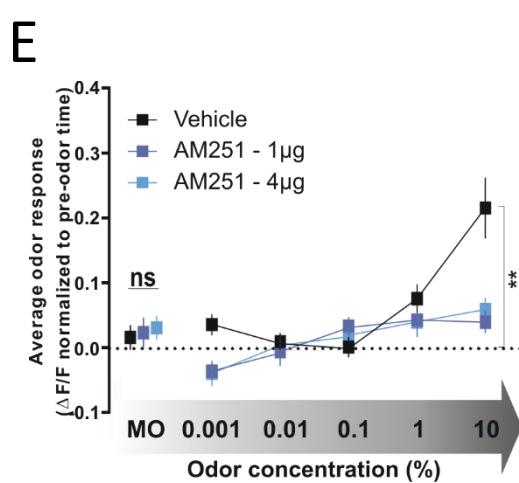
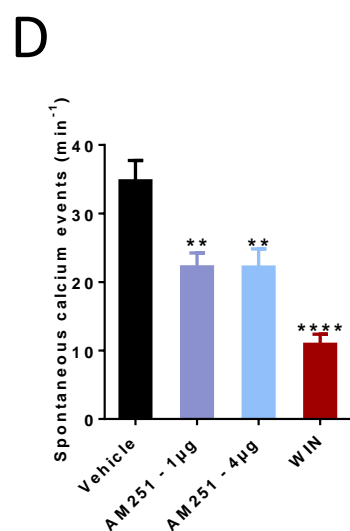
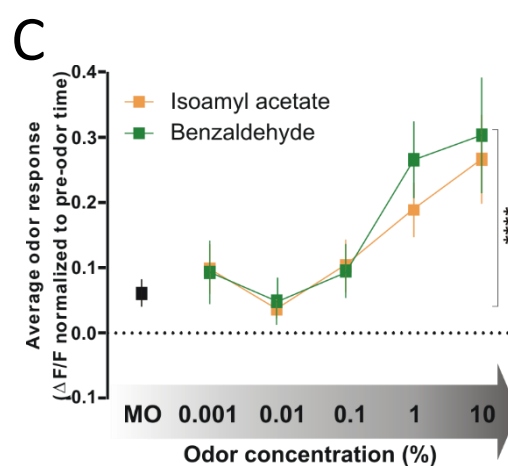
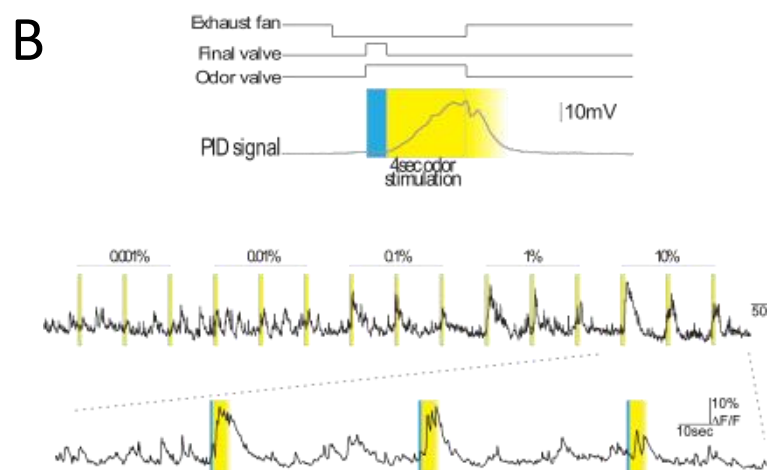
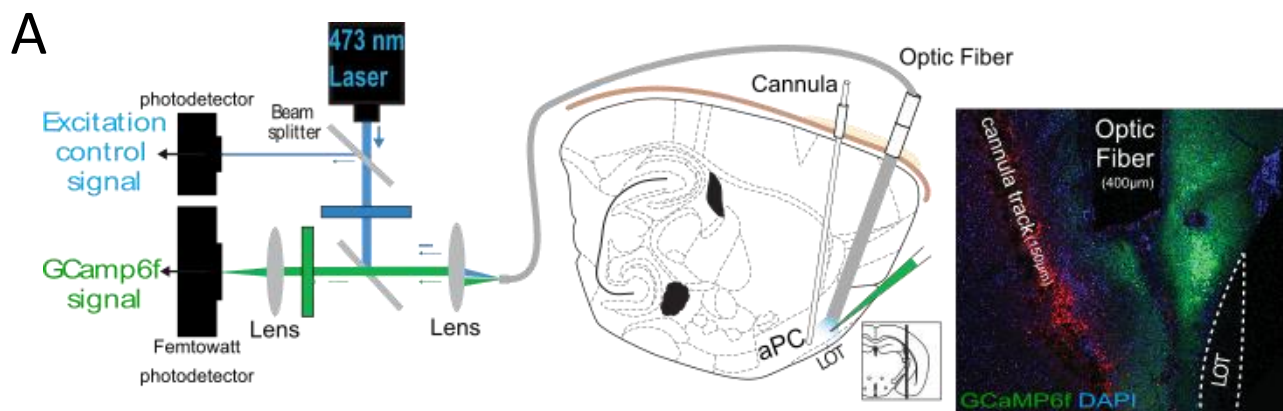


C



**Figure 3. Layer-dependent neurochemical signatures of CB1-positive interneurons in the aPC**

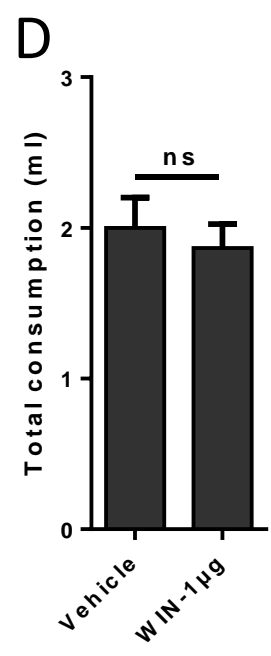
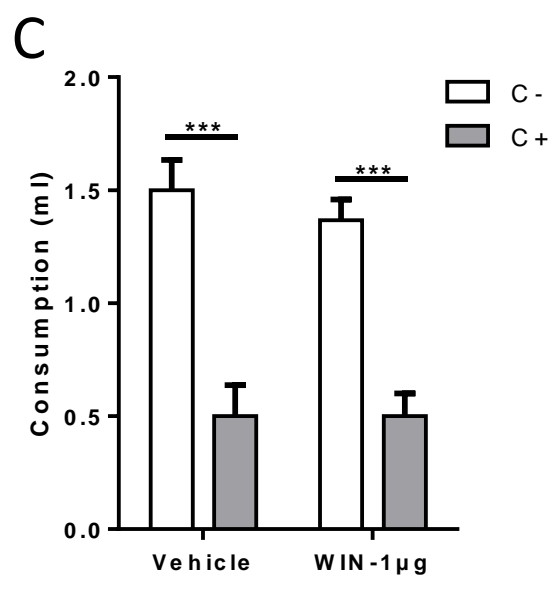
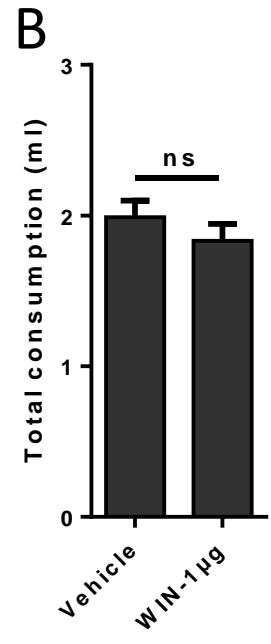
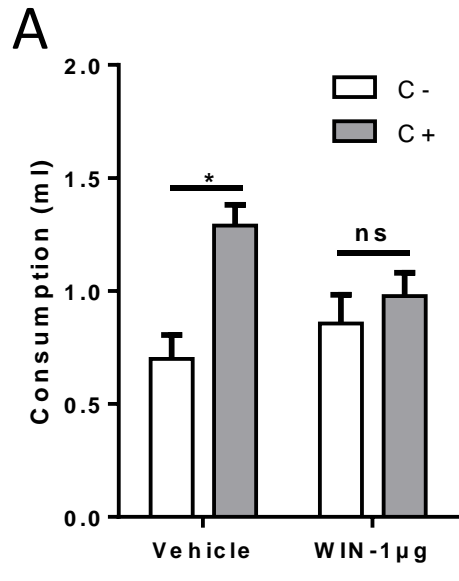
**(A)** Representative images of double Fluorescent *In Situ* Hybridization (D-FISH) against CB1mRNA and GAD65/67mRNA or **(B)** with CCKmRNA. **(C)** Distribution of total cells containing low-to moderate expression of CB1 receptor mRNA (CB1+) with CCK mRNA (CCK+) in layer I (left; CB1+/CCK+, 1.7%) and proportion of cells co-expressing high CB1-levels with CCK marker (right; layer I, 3.2%; layer II, 49.7%; layer III, 47.1%).





**Figure 4. The endocannabinoid system shapes odor-dependent Calcium responses in the aPC.**

**(A)** Schematic experimental design of the *in vivo* fiber photometry used to record aPC pyramidal cells. Right, representative picture of the recording site. Green, GCaMP6f. Red, cannula position. Blue, DAPI. **(B)** Protocol design (top) and example of dynamic of odor-evoked calcium signals across gradient odor concentration (bottom). **(C)** Calcium responses for increasing concentration of isoamyl acetate (orange, n=22) and benzaldehyde (green, n=22). **(D)** Average of spontaneous calcium events in presence of vehicle (black, n=16), CB1 antagonist AM251 at 1 $\mu$ g (purple, n=12) or 4 $\mu$ g (blue, n=20) and CB1 agonist WIN (blue, n=12). **(E)** Effect of vehicle (n=28), AM251 at 1 $\mu$ g (n=28) or 4 $\mu$ g (n=40) on increasing odor-evoked responses. **(F)** Effect of vehicle (n=28), WIN at 1 $\mu$ g (n=16) or vehicle post WIN (grey, n=16) on increasing odor-evoked responses. One-way ANOVA for the spontaneous calcium events and MO analysis; and repeated two-way ANOVA for odor concentration and drug analysis. \*\*\*\*, p<0.0001; \*\*\*, p<0.001; ns, not significant. Values are represented  $\pm$  SEM.



**Figure 5. CB1 receptors antagonist and agonist have similar effect on aPC function**

**(A, B)** Conditioned odor preference (COP). **(A)** Liquid consumption during the test day of odorized bottles, conditioned with the sucrose (C+) or not (C-), after local administration of the CB1 receptor agonist WIN (1 $\mu$ g/0.5 $\mu$ l) into the aPC. **(B)** Total consumption of odorized-water during COP test. Vehicle (n=9), WIN-1 $\mu$ g (n=9). **(C, D)** Conditioned odor aversion (COA). **(C)** Liquid consumption during the test day of odorized bottles, conditioned during COA protocol with LiCl (C+) or vehicle(C-), after local administration of the CB1 receptor agonist WIN (1 $\mu$ g/0.5 $\mu$ l) into the aPC. **(D)** Total consumption of odorized-water during COA test. Vehicle (n=5), WIN-1 $\mu$ g (n=6). Repeated two-way ANOVA for **A, C** analysis and unpaired t-test for **B, D** analysis. \*\*\*, p<0.001; \*, p<0.05; ns, not significant. Values are represented  $\pm$  SEM.

# **PART IV - GENERAL DISCUSSION**

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## **GENERAL DISCUSSION**

In the last decade, numerous studies investigated the involvement of CB1 receptors in olfactory system (Breunig et al., 2010; Czesnik et al., 2007; Ghosh et al., 2018; Hill et al., 2010; Hutch et al., 2015; Laviolette and Grace, 2006; Pouille and Schoppa, 2018; Soria-Gómez et al., 2014a, 2014b; Wang et al., 2012; Zenko et al., 2011). However, how CB1 receptors regulate aPC functions was never been explored. To this aim, we characterized the distribution of CB1 receptors in this brain region and determined their contribution to the control of aPC inhibitory transmission and plasticity. Moreover, we explored the role and the impact of aPC-CB1 receptor modulation *in vivo* in aPC circuits and in odor-related memory. Together, these results contribute to a better understanding how CB1 receptors regulates olfactory functions.

### **IV.1 APC-CB1 RECEPTORS CONTRIBUTION DEPENDS ON THE MEMORY TASK**

Based on previous findings showing the importance of aPC in appetitive behavior (Mediavilla et al., 2016; Roesch et al., 2007), we tested the involvement of the ECS in COP. We found that aPC-CB1 receptors are necessary for COP but not for COA. Although PC neurons have been shown to be able to drive either appetitive and aversive responses (Choi et al., 2011), the involvement of PC in COA remains still controversial. For example, COA has been shown to depend on the basolateral nucleus of the amygdala (BLA) (Bermudez-Rattoni et al., 1983, 1986; Desgranges et al., 2008; Ferry and Di Scala, 1997; Laviolette and Grace, 2006) and growing evidence suggests that pPC but not aPC is important during COA (Gottfried et al., 2002; Hegoburu et al., 2014; Jones et al., 2007; Li et al., 2008; Mediavilla et al., 2016; Sevelinges, 2004). Moreover, CB1 receptors regulate COA behavior in the medial habenula and in the medial prefrontal cortex (Laviolette and Grace, 2006; Soria-Gómez et al., 2015). Therefore, it will be interesting to evaluate whether CB1 receptors in the pPC are required to process negatively motivated olfactory memory. However, even if aPC neurons are potentially involved in aversive memory, CB1

receptors do not modulate COA expression in this brain region. In order to evaluate whether COA retrieval is controlled by CB1 receptors, we injected cannabinoids drugs prior to COA test but not during the acquisition phase. Further experiments are needed to determine if aPC-CB1 receptors play any role in different phases of COA.

Our results raise the question of how CB1 receptors regulate appetitive memory in the aPC. A possible explanation for the aPC involvement in appetitive but not aversive function might result from a difference in olfactory perception. When we are exposed to an appetitive odor (e.g food, flowers, parfum...) our sniffing frequency and/or amplitude increases (Youngentob et al., 1987). Conversely, repulsive odor reduce sniffing frequency and amplitude. These respiratory patterns and the associated activity of mitral/tufted cells can influence aPC neuronal activity (Doucette et al., 2011; Franks and Isaacson, 2006). Indeed, olfactory cues associated with sucrose (leading to COP) activate more aPC neurons than odors associated with quinine (Roesch, Stalnaker et Schoenbaum 2006). Similarly, Gire et al. (2013) demonstrated that positively reinforced odors elicit an increase of aPC neurons' firing. In contrast, odors associated with a negative value do not induce changes in neurons' firing. Accordingly, Choi et al. (2011) showed that repetitive activation of only few ensembles of aPC neurons are capable to elicit similar behavior as when an odor is associated with a reward. Thus, these studies suggest that synchronous activation of aPC neurons is necessary to drive odor preference. Consistent with this explanation, injections of CB1 receptor agonists or antagonists might disturb the temporal window of pyramidal neurons activation that conveys appetitive information for an odor (see "Physiological aPC-CB1 receptors signaling *versus* activation and blockade").

The PC receives projections from other brain structures involved in appetitive behavior such as the orbitofrontal cortex (OFC), the insular cortex, the BLA, the olfactory tubercle and the nucleus accumbens (Calu et al., 2007; Cubero and Puerto, 2000; Gottfried et al., 2002; Schoenbaum and Eichenbaum, 1995; Touzani and Sclafani, 2005; Wesson and Wilson, 2011; Wilson and Bowman, 2005). As CB1 receptors are present in principal neurons of all these brain regions, they might be present at these associative terminals in the aPC and their activation might be involved in COP processing. However, our data obtained by altering CB1 receptor signaling by local genetic deletion and

pharmacological interventions indicate that COP is regulated to a large extent by local endocannabinoid-dependent control of aPC circuits.

Altogether, our results suggest that the expression of COP depends on an increase of aPC neuronal activity, at least partially provided by physiological CB1 receptors activation on local aPC inhibitory circuits. However, how is CB1 receptors signaling responsible for an increase of aPC firing?

#### **IV.2 PHYSIOLOGICAL APC-CB1 RECEPTORS SIGNALING *VERSUS* ACTIVATION AND BLOCKADE**

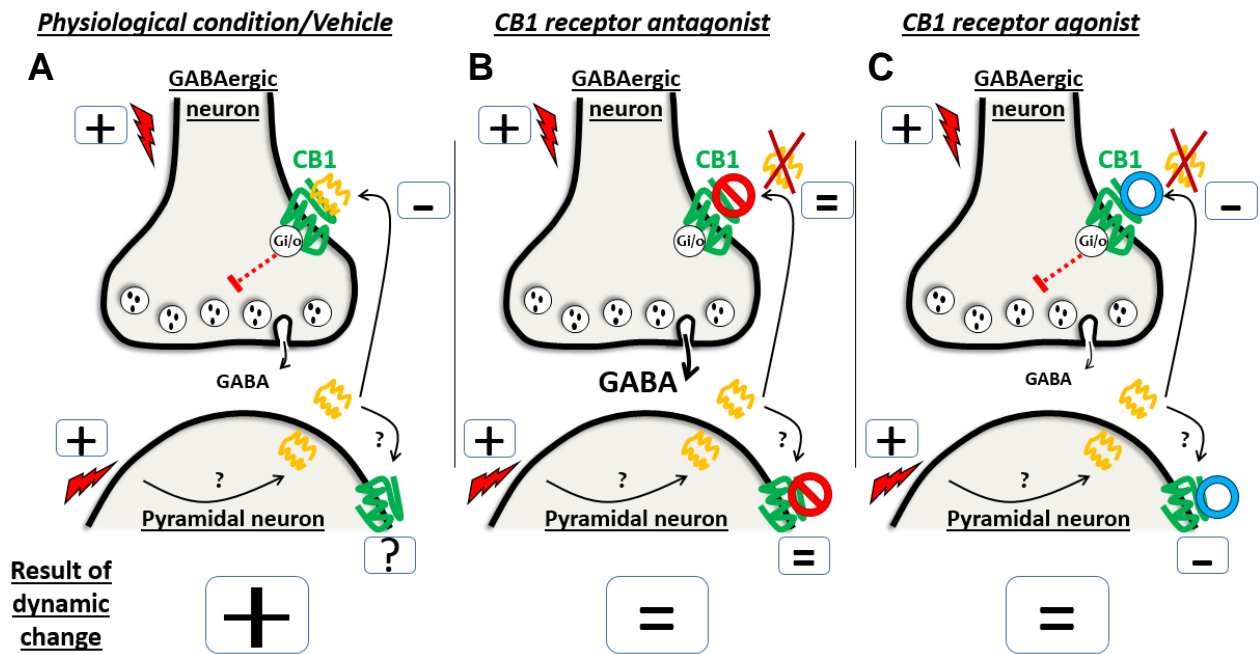
We found that aPC-CB1 receptors are mainly expressed in GABAergic neurons. Interestingly, inhibition is a key determinant for odor processing (Bekkers and Suzuki, 2013; Reuveni et al., 2018; Wilson and Sullivan, 2011). For example, it has been shown that odor exposures induce relatively global and powerful inhibition of pyramidal neurons, suggesting that inhibitory interneurons allow maintaining low and specific aPC excitatory activity (Franks et al., 2011; Poo and Isaacson, 2009; Zhan and Luo, 2010). Consistent with the literature, we found that odors trigger a rise of pyramidal neurons' activity (odor-evoked calcium activity under basal/vehicle condition) (Rennaker et al., 2007; Roland et al., 2017; Stettler and Axel, 2009). Thus, odor presentation in physiological condition stimulates both interneurons and pyramidal neurons. Pyramidal neurons' activation might trigger endocannabinoid release that act retrogradely on CB1 receptors at GABAergic terminals, thereby inducing a decrease of inhibitory transmission. Therefore, pyramidal neurons would be less inhibited and the global effect observed on the circuit would be an excitation (translated into an increase of calcium response in our experiment; **Figure 11A**). However, we can speculate that these physiological dynamics are altered with the use of exogenous cannabinoid ligands. When CB1 receptor antagonist, AM251, is applied to the circuit, the odor would still be able to induce endocannabinoid release but their suppressing action on inhibitory synapses would be prevented by CB1 receptor blockade. As odor exposures might stimulate both inhibitory interneurons and glutamatergic cells, we could hypothesize that this effect block the odor dynamic change resulting in an



absence of pyramidal neurons' calcium modification (**Figure 11B**). Thus, AM251 might lock the balance of excitation/inhibition required for odor-mediated changes of pyramidal neurons' activity.

In absence of applied odors (basal activity) spontaneous spiking activity is observed in pyramidal neurons (Tantirigama et al., 2017). However, CB1 receptor at GABAergic neurons cannot alone explain our observation that the basal activity is reduced in presence of the CB1 agonist, WIN. Indeed, activation of "GABAergic" CB1 receptors alone should drop inhibition and thus should increase the global spontaneous excitation. Although our results indicate that CB1 receptors are mainly present at GABAergic terminals, we found that glutamatergic neurons contain very low amount of proteins. Importantly, the levels of CB1 receptor expression do not reflect their signaling efficiency. Indeed, in the hippocampus, CB1-induced G-protein signaling appears more potent in glutamatergic than in GABAergic neurons (Steindel et al., 2013), suggesting that glutamatergic CB1 receptors might play important roles in the aPC. Thus, CB1 receptors activation on pyramidal neurons might explain the decrease of calcium responses in basal activity. Thus, WIN would suppress the activity of both GABAergic and glutamatergic neurons, causing that the global effect of the odor would be obliterated (**Figure 11C**). Interestingly, CB1 receptor blockade reduces also basal calcium responses, suggesting that endocannabinoid release is involved in the control of spontaneous activity. However, further experiments are needed to validate our hypothesis. For example, it would be interesting to determine if and how exogenous cannabinoids (i.e AM251 and WIN) affect GABAergic activity. To address this, we could repeat the same experiment with odor-evoked calcium responses but by recording aPC GABAergic neuronal activity. In this case, during odor presentation, CB1 receptor blockade should increase the calcium activity of inhibitory neurons more than in physiological condition whereas CB1 receptor activation should decrease it. Moreover, this experiment will allow evaluating whether GABAergic neurons show spontaneous activity (without any odor) and if endocannabinoids are involved in this process. In the other hand, it would be important to characterize how "glutamatergic" CB1 receptors modulate excitatory activity. First, to verify that CB1 receptor agonists are able to decrease excitatory activity, we could repeat similar experiment in mice lacking CB1 receptors in GABAergic neurons. In this

experiment, WIN should still be able to reduce spontaneous activity indicating that both “GABAergic” and “glutamatergic” CB1 receptors are involved in odor processing. Then, patch clamp experiments could allow characterizing the modulatory effect of glutamatergic CB1 receptor (pre- or post-synaptic, on which fibers, endocannabinoid-mediated plasticity).



**Figure 11: Hypothetic synaptic activity during odor presentation.**

Odor stimulates (red flash symbol and +) both pyramidal and GABAergic neurons and allows the production of endocannabinoids (yellow symbol). **(A)**. Retrograde endocannabinoid signaling reduces inhibitory neurotransmitter release, suggesting that odor induces less inhibition resulting in an increase of pyramidal neurons' activity. **(B)**. Retrograde endocannabinoid signaling is blocked by CB1 receptor antagonist, AM251 (red circle symbol). Under these conditions, the reduction of GABA release is not observed; thus, the physiological balance between excitation and inhibition is disrupted, thereby possibly impeding the controlled and synchronous changes of pyramidal neurons' activity. **(C)**. CB1 receptor agonist, WIN (blue circle symbol), occludes the retrograde endocannabinoid signaling and activates glutamatergic CB1 receptors. The odor-mediated increase of GABAergic and pyramidal neurons' responses is obliterated by WIN, thereby impairing the physiological dynamic processes leading to the physiological odor-induced changes in pyramidal neurons' responses.

Interestingly, we found that pharmacological CB1 receptor activation and blockade have similar effects in abolishing/altering endocannabinoid-mediated plasticity, odor-evoked calcium responses (see above) and behavior. On the one hand, consistent with the literature (Chevalleyre and Castillo, 2003; Kano et al., 2009; Kreitzer and Regehr,

2001; Marinelli et al., 2009; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001), our data support the principle of blockade (i.e. AM251) and occlusion (i.e. WIN) of CB1 receptor-dependent synaptic plasticity. Thus, the endocannabinoid-mediated plasticity are not inducible as CB1 receptors are already “occupied” by the exogenous cannabinoids (see I.3.1.6 How to study CB1 receptors contribution, “Pharmacological approach”). On the other hand, we can hypothesize that odor-evoked calcium activity and COP retrieval require similar CB1 receptor temporal availability as observed in DSI and/or iLTD. Indeed, we show that COP retrieval induces CB1-dependent reduction of inhibitory transmission. Thus, in order to make a choice for an appetitive-based odor, CB1 receptor activation by endocannabinoids might increase excitatory responses by suppressing GABAergic release. However, in presence of AM251 and WIN, the dynamic change of the excitatory/inhibitory balance might be disturbed (as explain above), inducing inability to undergo COP expression. Therefore, consistently with other brain structures (Carnevale et al., 2015; Her et al., 2016; Shankar et al., 2011), the temporal dynamics of aPC neurons might be a key determinant for decision-based behaviors related to odor processing.

### **IV.3 MODULATION DURING COP RETRIEVAL**

Understanding how brain circuits store and retrieve associative memory remains a big challenge for neuroscientists (Bocchio et al., 2017; Wang and Cui, 2018). In the PC, odor learning induces synaptic modifications of pyramidal neurons (Barkai, 2014; Chapuis and Wilson, 2012; Kadohisa and Wilson, 2006a; Quinlan et al., 2004; Roesch et al., 2007). Major theories indicate that, during the acquisition of a conditioning task, several associational inputs that convey different features of the task, are integrated into networks of specific neurons (Liu et al., 2017; Wang and Cui, 2018; Wang et al., 2015). For example in COP, aPC neurons likely associate the odor identity (e.g. Almond or Banana) with the positive consequences from its ingestion (sucrose= sweet + energy). Once learnt, partial information (only Almond or Banana) is enough to trigger behavioral choices towards the odor previously associated with the reward. In this context, lack of associative inputs (that convey sweet and energy information) does not impede the retrieval. Thus, this feature

suggests that aPC neurons are able to reconstruct the pattern of odor-sucrose association. Indeed, recurrent properties of aPC pyramidal neurons are believed to play important role for memory recall (Haberly, 2001; Schoenbaum and Eichenbaum, 1995).

In our conditions, we found that aPC-CB1 receptor blockade impairs COP retrieval but not acquisition. Interestingly, the ECS has been reported to play major roles in memory (De Oliveira Alvares et al., 2008; Drumond et al., 2017; Kruk-Slomka et al., 2017; Marsicano and Lafenêtre, 2009). As previously explained, COP expression depends on CB1 receptors in local aPC neurons. Overall, this suggests that associative connections from other brain structures do not express CB1 receptors in the aPC and thus, are not modified by local manipulation of CB1 receptors, leaving the acquisition phase intact. In contrast, perturbation of CB1 receptor signaling might alter the physiological dynamic of local aPC circuits, thereby affecting COP retrieval. Therefore, the functional integrity of aPC circuits might depend on the temporal-restricted dynamic control by endocannabinoid signaling (see above). However, because odor memory recall is still not well understood and that aPC recurrent connections make very complex connectivity with both glutamatergic and GABAergic neurons (Franks et al., 2011), it appears challenging to further explain the exact mechanisms behind the effect of CB1 receptors activation.

#### **IV.4 LAYER-DEPENDENCE OF ENDOCANNABINOID-MEDIATED PLASTICITY**

Similar as revealed in several brain regions (Araque et al., 2017; Chevaleyre and Castillo, 2003; Crosby et al., 2011; Kano et al., 2009; Marsicano et al., 2002; Wilson and Nicoll, 2001), the ECS mediates diverse forms of inhibitory synaptic plasticity in the aPC, including DSI and iLTD. In this brain area, we found a layer difference between DSI and iLTD expression. In agreement with the layer-dependent occurrence of iLTD, we observed that GABAergic interneurons do not contain CCK transcript in layer I, where iLTD is absent. Given that CCK-positive cells have been proposed to be responsible for iLTD induction in the hippocampus (Basu et al., 2013; Chevaleyre and Piskorowski, 2014), we hypothesize that aPC iLTD would require the expression of CCK in the interneurons involved.

Nevertheless, the large majority of hippocampal CB1 receptors in GABAergic interneurons containing CCK marker (Marsicano and Kuner, 2008; Marsicano and Lutz, 1999), suggesting that a relatively homogenous population of CB1 expressing cells exists in the hippocampus. Thus, in order to verify our hypothesis, further investigations are necessary to determine whether other cell types (CB1+/CCK-) are capable of iLTD expression. Indeed, in our conditions, CB1 receptor transcripts were found in layer I interneurons, where typical markers of GABAergic cells are not observed, including CCK (Cummings, 1997; Suzuki and Bekkers, 2007, 2010a). Accordingly, we cannot rule out the possibility that different mechanisms would be involved in iLTD expression. For example, it has been shown that iLTD depends on spontaneous activity of interneurons involved in its induction (Heifets et al., 2008). The authors found that the reduction of spontaneous interneuron firing abolishes iLTD induction, suggesting that iLTD failure might come from low or absence spontaneous activity of aPC GABAergic interneurons present in layer I. Moreover, Younts et al. (2016) demonstrated that iLTD induction (but not DSI) requires pre-synaptic protein synthesis. Thus, we could imagine that the machinery necessary for the expression or the activation of protein synthesis might differ between layer I and layer II/III interneurons. Moreover, it has been shown that activation of pre-synaptic GABAergic interneurons together with CB1 receptor agonists induces “chemical” iLTD that requires similar protein synthesis as “physiological” iLTD (Younts et al., 2016). By considering the decrease of layer I inhibitory transmission that we observed after application of exogenous CB1 receptor agonist (WIN), we can hypothesize that iLTD failure might be due to either an absence of CB1-dependent regulation of protein synthesis in layer I GABAergic interneurons. Finally, in most cases, iLTD has been shown to depend from the activation of metabotropic receptors in different brain regions (Kano et al., 2009). Thus, the iLTD layer-dependence could result from an anatomical constraint of post-synaptic synapses located in layer I. These synapses might lack specific metabotropic receptors required for iLTD induction. If this hypothesis is true, aPC iLTD should be induced by different metabotropic receptors from the ones observed in the hippocampus. Indeed, in the hippocampus, iLTD depends on the activation of post-synaptic group I metabotropic glutamate receptors (mGluRs I; Chevaleyre and Castillo 2003). Nevertheless, layer I aPC stimulation activates post-synaptic mGluRs I located on pyramidal neurons (Sugitani et al., 2002, 2004), suggesting that activation of these receptors is not sufficient to induce iLTD in layer I aPC.

Patch clamp experiments performed after behavior tests indicated that COP retrieval induces pre-synaptic activation of aPC-CB1 receptors at inhibitory synapses. These data indicate a mechanism likely underlying endocannabinoid mobilization. Considering the modification of synaptic transmission occurring in an experience-dependent manner, we could speculate that COP retrieval modify DSI and/or iLTD induction. For example, we can hypothesize that DSI and iLTD would be occluded by the endocannabinoid mobilized during memory retrieval. Preliminary experiments revealed that iLTD is not inducible by stimulating inhibitory inputs in layer II (where naive animals display iLTD) in both “water” (only exposed to water during the same period as COP animals) and COP animals (data not shown). However, our results indicate that Water animals do not show reduction of mIPSCs frequency (compared to naive), suggesting that endocannabinoids are not release and iLTD should still be observed in these animals. This apparently counterintuitive effect could be explained by the individual internal state at the moment of iLTD induction. Indeed, stress has been shown to modify DSI and iLTD occurrence in the hypothalamus (Crosby et al., 2011; Morena et al., 2016). Recently, we also observed similar change in the hippocampus of animals undergoing same hydric restriction as used for COP (Busquets-Garcia et al., 2018b). Thus, different behavioral approaches involving no restriction should be used to test whether COP modify DSI and/or iLTD occurrence.

#### **IV.5 CONCLUSION AND RELEVANCE OF THE STUDY**

Odors are powerful stimuli capable of guiding our actions. In particular, many important behavioral choices are based on the ability to perceive and retrieve olfactory information. Strikingly, the aPC receives both sensory inputs from the olfactory bulb and associational information from other brain structures, placing it at the heart of odor perception and olfactory memory (Bekkers and Suzuki, 2013; Gottfried, 2010; Haberly, 2001; Wilson and Sullivan, 2011). Like in other sensory system (Avery and Krichmar, 2017), odor processing are regulated by neuromodulators. For example, the aPC receives projections from brainstem and basal forebrain neurons that allow modulating various olfactory functions (Linster and Cleland, 2016). However, growing evidence suggests that olfactory processing can be controlled by local neuromodulatory systems such as the ECS

(Breunig et al., 2010; Czesnik et al., 2007; Ghosh et al., 2018; Hill et al., 2010; Hutch et al., 2015; Laviolette and Grace, 2006; Pouille and Schoppa, 2018; Soria-Gómez et al., 2014a, 2014b; Wang et al., 2012; Zenko et al., 2011). In the brain, most of these synaptic modulatory activities are mediated by the activation of CB1 receptors. However, how CB1 receptors regulate olfactory processing in the aPC had never been studied. We addressed this issue by characterizing for the first time the distribution and the functional impact of CB1 receptors in aPC circuits and in olfactory-guided behaviors. We found that CB1 receptors in GABAergic interneurons regulate inhibitory transmission and plasticity. Moreover, alterations of physiological aPC-CB1 receptor signaling lead to inappropriate aPC processing and to the impairment of COP retrieval. Overall, these results contribute to a better understanding of olfactory functions and the involvement of CB1 receptors in the aPC.

Our study also evaluated how exogenous CB1 receptor agonists alter olfactory processes. Cannabinoid intoxication is known since long time to alter sensory perception, including olfaction (Tart, 1970). Nevertheless, very few studies evaluated the impact of THC on olfactory functions in humans (Lötsch et al., 2012). Considering the importance of olfactory system in daily life and the wide use of cannabis (2012), THC-mediated dysregulation of olfactory processes might alter odor-related choices based on appetitive values. For example, it has been described that THC induces an increase of human olfactory perception and disturbs odor discrimination (Walter et al., 2014). Moreover, alterations of ECS functioning have been shown to contribute to the development of neurological and neuropsychiatric disorders in which loss of smell represents early stages of the disease (Basavarajappa et al., 2017; Godoy et al., 2014; Philpott and Boak, 2014; Yin et al., 2018). Interestingly, CB1 receptors are observed in the cerebral cortex of humans (Mato et al., 2003), but they have never been detected in olfactory bulb (Lötsch and Hummel, 2015), suggesting that the PC might be a key region involved in cannabinoid-induced olfactory alterations. Therefore, the present work provides a better understanding of how CB1 receptors control physiological olfactory functions and suggests that interference with these receptors might afford novel frameworks for tackling pathological conditions such as the ones related to olfactory memory.

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