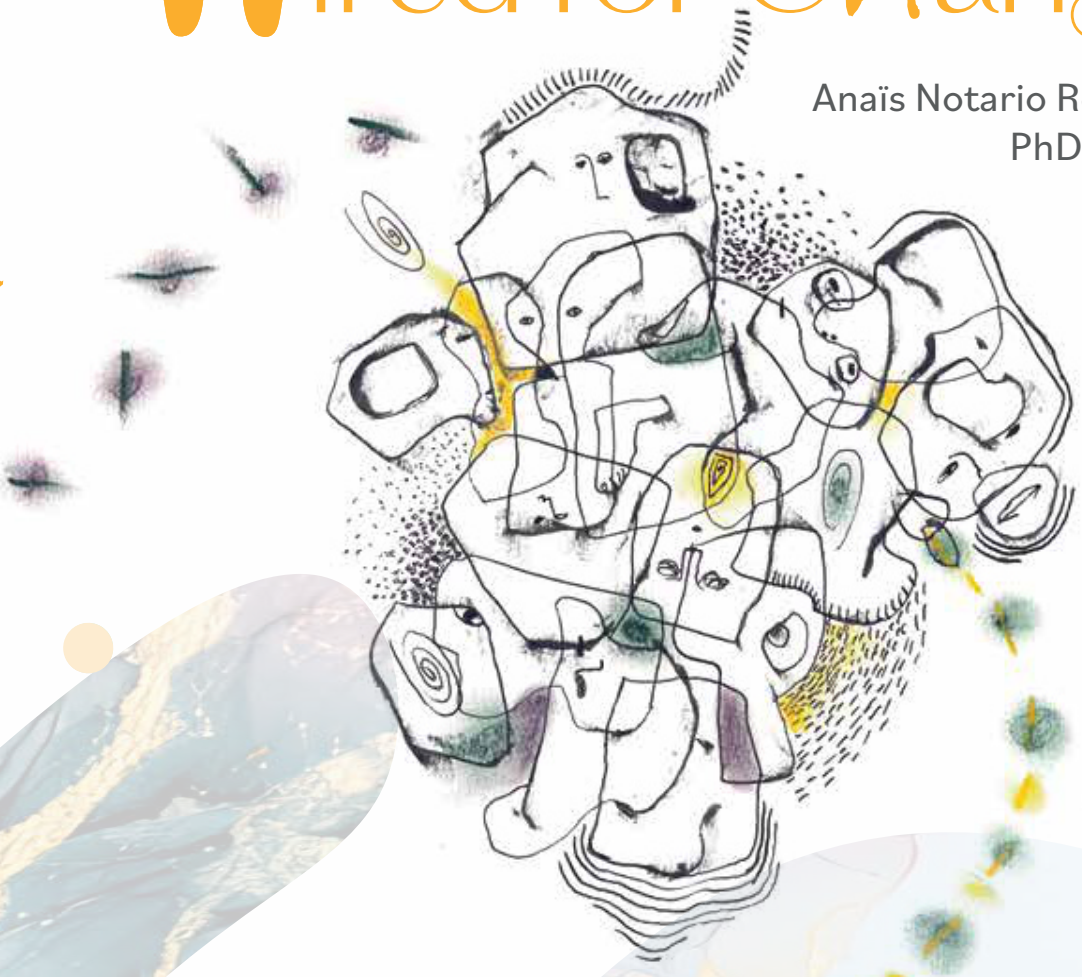
Wired for Change

Anaïs Notario Reinoso

PhD thesis



Cannabinoid
and
GluA3-containing AMPA receptors
in cortical synaptic transmission throughout life

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Wired for Change

Cannabinoid and GluA3-containing AMPA receptors in cortical synaptic transmission throughout life

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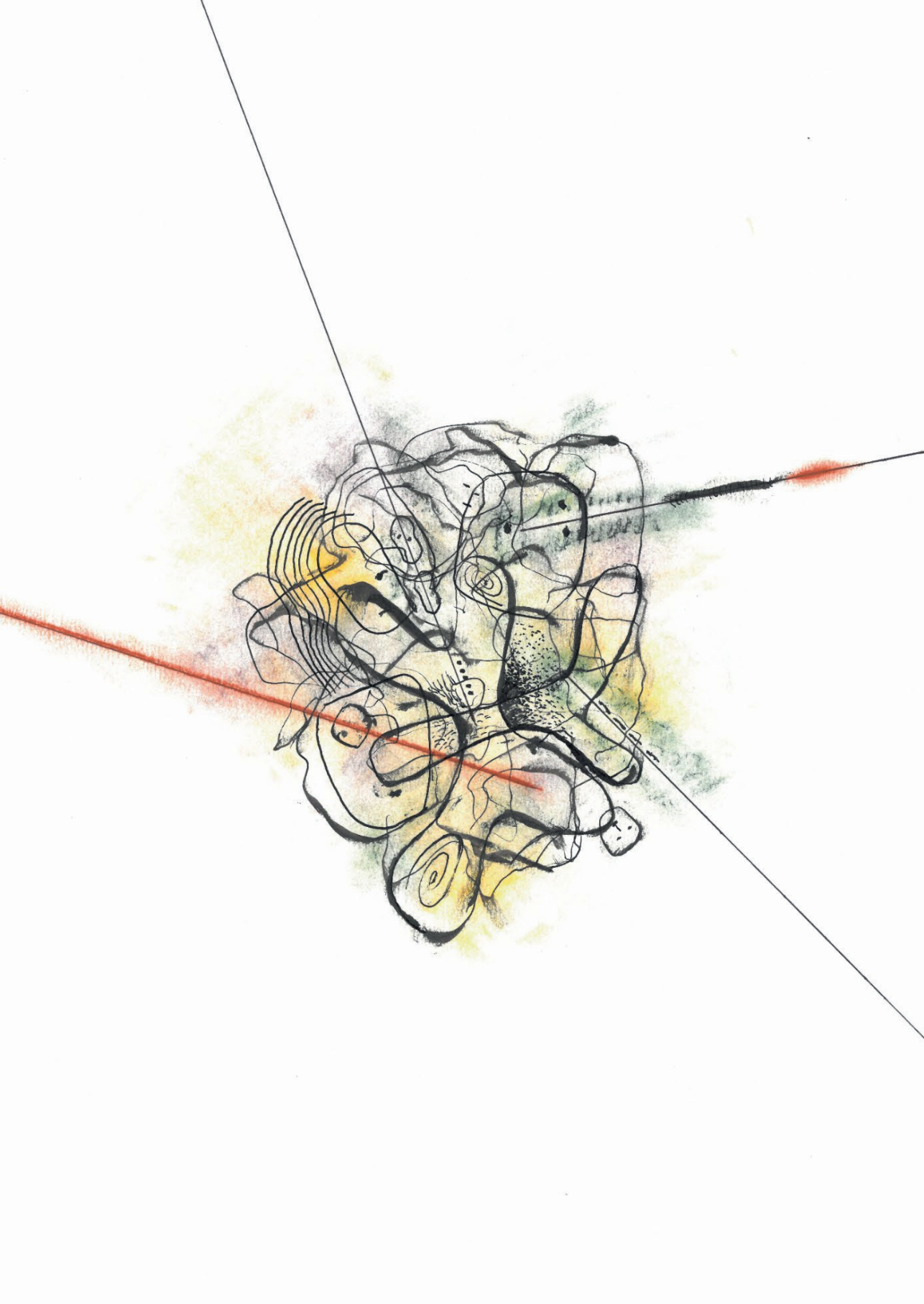
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Change as a constant.

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Chapter 1

General introduction

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1. The brain: importance in complexity

The brain is the most complex organ in the body, comprising tens of billions of cells, each forming thousands of connections with other neurons [1]. The beauty of the brain lies not only in its complexity, but in what emerges from it. From the coordinated activity of molecules confined to nanoscopic compartments arise behaviors of exceptional richness: memory, decision-making, or social interactions. Neuronal signals are continuously integrated, transformed, and conveyed across circuits and brain regions, each layer of organization building upon the next. It is through this tangled hierarchy of interactions that the brain gives rise to functions that far exceed the sum of its parts.

The brain, however, still holds many nooks left to be enlightened. Now it is our job to find them.

As Goriely wrote, “The brain bugs the mind”.

2. Historical landscape

At the end of the nineteenth century, a profound shift in our understanding of the brain began with the work of a revolutionary Spanish neuroscientist. Using Golgi staining, Santiago Ramón y Cajal observed that neurons were contiguous but not continuous, leading him to propose the **neuron doctrine**: the idea that neurons are discrete cellular units rather than part of a continuous reticular network. This view directly challenged the prevailing reticular theory (the nervous system as a continuum) and laid the conceptual foundation for modern neuroscience.

Beyond identifying neurons as individual units, Cajal also proposed that communication between them occurs at specialized points of contact. Building on this idea, Charles Scott Sherrington introduced the term *synapse* to describe “the special connection of one nerve cell with another,” emphasizing its functional relevance for neural communication [2]. Derived from the Greek *synaptein*, meaning “to fasten together,” the term beautifully captures the dual nature of synapses as both structural and functional junctions.

While the neuron doctrine and the concept of the synapse were firmly established by the early twentieth century, the molecular language used by synapses remained elusive for decades. It was not until the 1950s that glutamate was first shown to exert excitatory actions in the brain [3], and only in the 1970s that it was broadly accepted as a neurotransmitter [4]. Subsequent work identified distinct classes of glutamate receptors, including N-methyl-D-aspartate (NMDA) receptors and non-NMDA

receptors, later termed α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPARs) [5]. The cloning and molecular characterization of AMPAR subunits in the 1990s marked a decisive expansion of the field [6]. Four AMPAR subunits, GluA1 to GluA4, were identified, each conferring distinct biophysical and functional properties depending on their combination within the receptor complex. Since then, GluA1-containing AMPARs have dominated the literature, largely due to their central role in synaptic plasticity mechanisms such as long-term potentiation (LTP), and in spatial working memory [7–9]. More recently, these receptors have also been shown to be necessary for the formation of sparse engrams, neuronal ensembles that represent the physical substrate of memory [10]. In contrast, AMPARs containing other subunits, particularly GluA3, have received less attention. This imbalance is gradually shifting, as GluA3 has been increasingly implicated in neurodevelopmental, neurological, and psychiatric disorders [11–16].

In parallel with these molecular and synaptic discoveries, the cerebral cortex emerged as a central focus of neuroscience. Early anatomical work revealed that the cortex is not a homogeneous sheet, but rather a mosaic of distinct regions defined by their cellular organization. Most notably, Korbinian Brodmann used cytoarchitectonic criteria to delineate discrete cortical areas in the human brain, establishing the principle that cortical structure predicts function [17]. This framework was reinforced by classical lesion studies, beginning with Broca's and Wernicke's identification of language-related cortical regions [18] and extending to the landmark case of Phineas Gage, whose frontal lobe injury highlighted the role of the cortex, particularly the frontal cortex, in personality and executive functions [19].

From the first description of neurons as individual units, through the identification of synapses and neurotransmitters, to the recognition of the cortex as a specialized structure, the field has followed a progressive journey, one that continues in this thesis through the study of cortical synaptic transmission. Within this trajectory, certain molecular players have remained relatively in the background, a historical imbalance that shaped the questions addressed here and motivated a closer examination of understudied AMPAR subunits within cortical circuits.

3. The cortex, the canvas

3.1. Cortical functions

The cerebral cortex is a thin sheet of neural tissue, ranging from approximately 1 to 4.5 mm in thickness, lying just beneath the meninges [20]. Despite its modest thickness, the cortex occupies a disproportionate volume of the brain: in humans, it accounts for nearly 77% of total brain volume, whereas in rodents, it comprises roughly 31% [20].

This expansion reflects the cortex's central role in integrating information and supporting complex behavior.

The majority of the cortex is classified as neocortex, a phylogenetically recent structure characterized by a six-layered organization. The neocortex encompasses primary sensory areas, such as the visual, somatosensory, and auditory cortices, as well as association areas, including large portions of the prefrontal cortex (PFC) [20]. Although this laminar organization is conserved across regions, cortical areas differ markedly in cellular composition, layer thickness, and connectivity, a diversity captured by Korbinian Brodmann's anatomical maps of the human brain [21].

Sensory cortices exemplify how cortical structure supports specialized function. In the primary visual cortex (V1), visual information captured by the retina is relayed through the thalamus to the contralateral hemisphere, where it is progressively processed and integrated. V1, the first cortical stage of visual processing, is tuned to fundamental stimulus features such as edge orientation, motion direction, and spatial frequency [22–24]. Information extracted at this stage is then distributed to other cortical (including visual) and subcortical areas. In this way, V1 serves as a gateway through which sensory input is transformed into meaningful neural representations.

On the other hand, the prefrontal cortex (PFC) is an association area dedicated not to processing a single sensory modality, but to integrating information across modalities and contexts. The functional significance of the PFC was first dramatically illustrated by the case of Phineas Gage, whose frontal lesion in the nineteenth century profoundly altered his personality and behavior [19]. Although Gage survived the injury, the resulting changes in his personality regarding planning, emotional regulation, and social conduct highlighted the essential role of the frontal cortex in higher-order cognitive functions.

Today, the PFC is recognized as the substrate for executive processes such as working memory, decision making, cognitive flexibility, and goal-directed behavior [25]. In humans and other primates, it comprises the lateral PFC, including dorsolateral (dl) and ventrolateral (vl) subdivisions, the ventromedial PFC (vmPFC) and the orbitofrontal cortex (OFC) [26–28]. Although cytoarchitechtonically distinct, these subregions do not operate individually. Rather, they form a highly interconnected network in which each region contributes preferentially to specific aspects of cognition, with lesions affecting different prefrontal areas producing characteristic behavioral deficits. For example, damage to the dlPFC is frequently linked to deficits in decision making and working memory, whereas lesions to the vmPFC are more commonly associated with impairments in emotional processing and social behavior [27,29]. The close structural and functional connectivity between the medial PFC (mPFC) and the OFC lead

Carmichael and Price to propose the concept of an "*orbitomedial*" PFC, underscoring that these anatomical distinct regions operate as a coordinated unit [28].

In rodents, the anatomical definition of the PFC is a topic of active debate [30]. Studies most commonly focus on the medial prefrontal cortex (mPFC), which typically encompasses the infralimbic (IL), prelimbic (PrL), and anterior cingulate cortex (ACC), while the orbitofrontal cortex (OFC) is generally considered a neighboring prefrontal subdivision. As in primates, the rodent PFC supports higher-order cognitive processes, including decision making, cognitive flexibility, and social behavior [31,32]. Although these regions are functionally interconnected, each contributes preferentially to distinct behavioral domains. The OFC is the principal neural substrate for reversal learning [31,33,34], whereas the PrL and IL support the flexible updating of behavioral strategies in response to changing contingencies [31,35], and have also been implicated in the regulation of social hierarchy [36,37]. In contrast, the ACC is more prominently involved in emotional processing [38]. As an association cortex, the PFC continuously integrates information from sensory areas, including the visual cortex, with internal states and past experience, enabling flexible, context-dependent behavioral responses.

3.2. Cortical organization

Although the neocortex follows a conserved six-layered blueprint, the organization of these layers varies substantially across cortical regions, reflecting their functional specialization. In sensory cortices such as V1, information flow follows a relatively stereotyped pattern. Thalamic afferents primarily target layer 4 (L4), which serves as the main input layer. From there, signals are conveyed to pyramidal neurons in layers 2/3 (L2/3), where information is integrated and distributed locally or transmitted to deeper layers [39–41]. Neurons in layers 5 and 6 provide output to subcortical targets and other cortical areas, while layer 1 contains apical dendrites of pyramidal neurons and diverse inhibitory interneurons that modulate synaptic integration [42,43].

In contrast, the prefrontal cortex differs from this canonical organization. Most notably, it lacks a clearly defined layer 4, resulting in a more distributed pattern of thalamic and cortical inputs across layers 1, 2/3, 5, and 6 [44]. Projection neurons are correspondingly spread across multiple layers, forming extensive reciprocal connections with cortical and subcortical regions [44,45]. This decentralized architecture is thought to contribute to the integrative and flexible computations that characterize executive function [25,44].

Across the cortical regions, inhibitory interneurons play a central role in shaping how information is integrated and transmitted within local circuits. There are three main interneuron groups in the neocortex, namely Parvalbumin-positive (PV) interneurons, somatostatin-positive (SST) interneurons and VIP interneurons [46], each contributing distinct forms of inhibitory control. PV interneurons, the most abundant inhibitory subtype, innervate somata and proximal dendrites of pyramidal neurons across cortical regions and provide the principal source of fast perisomatic inhibition [47–49]. Receiving the majority of thalamic input to interneurons [50,51], PV cells exert control over pyramidal neuron firing, tuning excitatory activity to keep cortical networks within an optimal balance [52,53]. Somatostatin-positive (SST) interneurons complement this control by targeting distal dendrites and apical tufts, sculpting synaptic integration and playing a crucial role in feedback inhibition [48,54,55], while VIP interneurons preferentially inhibit other interneuron subtypes, particularly SST cells, thereby enabling disinhibitory control of cortical circuits [49,56,57]. Nevertheless, regional differences in the role of inhibitory cells in cortical circuits exist. In primary sensory areas, PV interneurons play a highly relevant role in shaping cortical output and timing, whereas in association cortices such as the PFC, somatostatin-positive (SST) interneurons preferentially regulate dendritic integration and input processing [58]. These region-specific balances between excitation and inhibition further underscore how cortical architecture is tuned to functional demands.

3.3. Cortical development

Across the lifespan, the brain undergoes profound developmental changes that gradually shape immature circuits into the finely tuned networks underlying adult cognition and behavior. Cortical development is central to this process, enabling the refinement of synaptic connectivity and the establishment of efficient information processing. Although sensory and association cortices share many developmental mechanisms, they mature along distinct timelines, reflecting the progressive emergence of increasingly complex functions. Sensory cortices typically reach functional maturity earlier than association areas, consistent with the early necessity of basic perceptual abilities. In contrast, higher-order functions, such as cognitive flexibility and executive control, hallmarks of the PFC, emerge later, following prolonged periods of circuit refinement [59,60]. These functions depend on the integration of multisensory and contextual information, which becomes possible only after sensory pathways have matured.

During embryonic development, both V1 and PFC follow broadly similar programs of neurogenesis, neuron migration and maturation, and gliogenesis, processes that extend into postnatal life (**Figure 1**) [61–63]. However, relevant regional differences

emerge in the timing of circuit assembly and maturation. In the visual cortex, thalamocortical and corticothalamic connections are established prenatally and

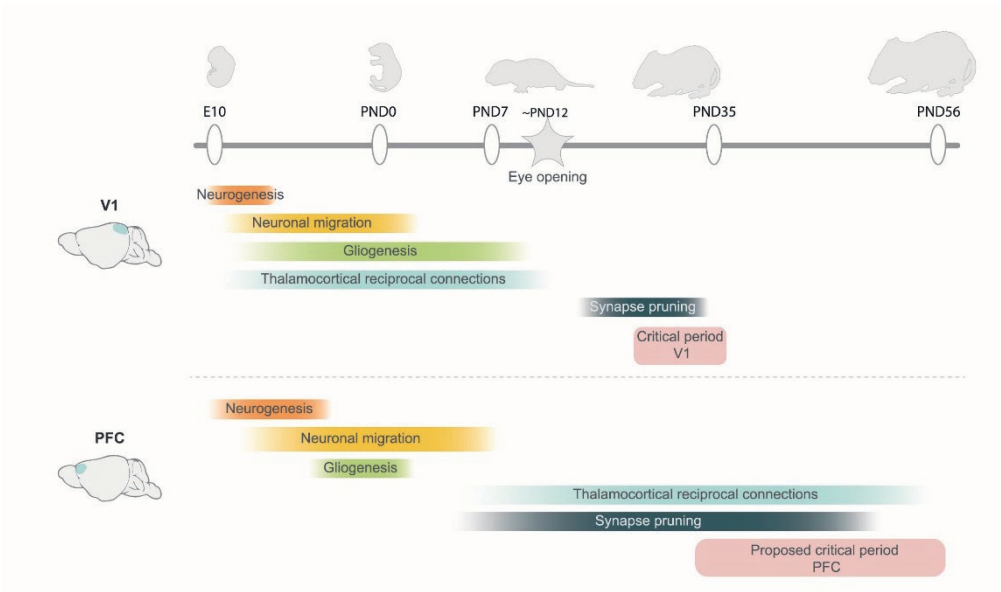


Figure 1. Schematic representation of hallmarks in cortical development. Top: developmental hallmarks in primary visual cortex (V1); bottom same but in prefrontal cortex (PFC). Identical colors are used to indicate the same process in V1 and PFC. Days during embryonic (E) and postnatal (PND) development are shown at the top. Modified from [55-57].

mature around the time of eye opening [64–66]. In contrast, equivalent reciprocal connections in the PFC develop primarily after birth and continue to mature through adolescence (**Figure 1**) [61]. In both regions, thalamocortical projections precede corticothalamic ones [61,64–66]. Synaptic pruning further differentiates these developmental trajectories. In V1, pruning is concentrated around the third postnatal week, coinciding with a well-defined critical period for visual plasticity between postnatal day (PND) 21 and 35 [67,68] (**Figure 1**). In the PFC, synaptic pruning begins later and extends over a much longer period, continuing into adolescence [69,70]. During this time, the PFC undergoes extensive circuit reorganization, reaching a more stable, adult-like state around PND56 in rodents (**Figure 1**) [61].

Adolescence has therefore been proposed as a critical period for prefrontal cortex development [60]. This developmental window shares key mechanistic features with classical sensory critical periods, including increased inhibitory drive, shifts in excitation-inhibition balance, and changes in NMDAR subunit composition that favor experience-dependent plasticity [71–73]. The progressive accumulation of

perineuronal nets stabilizes synaptic architecture and contributes to the closure of this period [74,75]. Notably, adolescence is marked by heightened exploration and risk-taking behaviors, including substance use, and coincides with the onset of neuropsychiatric disorders such as schizophrenia [76–79].

Considering their similarities and differences in structure, development and function, side-by-side examination of V1 and the PFC provides a powerful comparative framework to identify physiological principles conserved across the cortex, as well as mechanisms uniquely shaped by regional specialization.

4. Synaptic transmission: the colors

4.1. AMPA receptors

Adaptation to changing environments requires rapid neural processing, and AMPA receptors (AMPAR) serve as the principal mediators of fast excitatory synaptic transmission in the brain. AMPARs are ubiquitous tetrameric glutamate receptors composed of four subunits, GluA1–GluA4. These receptors are most commonly assembled as heteromers, although homomeric configurations have also been described, with subunit composition conferring distinct biophysical and functional properties [80]. In the mature cortex, GluA2 is the predominant subunit (~45%), followed by GluA3 (~27%) and GluA1 (~21%), with a smaller contribution from GluA4 (~7%) [81]. This distribution suggests that the majority of cortical AMPARs are composed of either GluA1/GluA2 or GluA3/GluA2 heteromers. The presence of the GluA2 subunit renders the AMPARs calcium-impermeable, restricting ion flow primarily to Na^+ and K^+ (**Figure 2**) [82,83].

Interestingly, the incorporation of GluA2-containing AMPARs into synapses emerges around the second postnatal week, coinciding with a brain-wide increase in GluA2 expression and the progressive replacement of GluA2-lacking, Ca^{2+}-permeable AMPARs characteristic from early postnatal development [84,85]. GluA4 expression is largely confined to early developmental stages and can form homomers that are preferentially inserted into silent synapses during the first postnatal week, which are subsequently replaced by GluA2-containing AMPARs, thereby converting previously silent synapses into functional ones [86].

Importantly, these developmental transitions in AMPAR subunit composition are not uniform across the cortex. In frontal cortical regions, the expression of both GluA1 and GluA3 peaks between the second and third postnatal weeks before declining into

adulthood. In contrast, in the occipital cortex, GluA1 expression rises between the second and third postnatal weeks and remains stable thereafter, whereas GluA3 expression reaches a minimum shortly after the first postnatal week and gradually increases into adulthood. Although the original study did not define precise anatomical subdivisions within frontal and parietal cortices, these findings highlight pronounced region-specific trajectories in AMPAR subunit maturation. Distinct developmental expression patterns are also observed in other brain regions, such as the hippocampus and cerebellum, further emphasizing that AMPAR composition is dynamically and regionally regulated throughout development [84].

Since the cloning of AMPAR subunits in the early 1990s and subsequent expansion of the field [6], GluA1-containing AMPARs have dominated the literature, largely due to their well-established role in hippocampal LTP as well as their contribution to spatial memory, social stress and engram formation [7,8,10,87]. In contrast, GluA3-containing AMPAR have received comparably less attention, despite exhibiting distinct synaptic dynamics. GluA1-containing AMPARs are found both synaptically and perisynaptically, while GluA3-containing AMPARs are mostly enriched at the synapse [88,89]. Additionally, GluA1 trafficking into synapses upon LTP-like activity is facilitated by Protein Kinase A (PKA) phosphorylation of its cytoplasmic tail [90,91], whereas GluA3-containing AMPAR are primarily recycled to replace existing AMPARs at synapses [90] and their ion-channel activity is modulated by changes in conductance upon cAMP signaling [92]. In addition, GluA2/3-containing AMPARs have been shown to reside at spines in the absence of synaptic activity, in contrast to GluA1/2 AMPARs [8]. This relative stability of GluA2/3-containing AMPARs at synapses under basal conditions, together with their enrichment following sensory deprivation, suggests a role in homeostatic regulation of synaptic strength rather than in classical activity-dependent forms of plasticity.[8]

Over the last decade, interest in GluA3 within the scientific community has surged. Our lab first described GluA3-dependent plasticity mechanisms in the hippocampus and cerebellum [92,93]. These studies also revealed regional differences in GluA3 physiology. For instance, GluA3-containing AMPARs contribute substantially to basal synaptic transmission in cerebellar Purkinje cells but relatively little in hippocampal CA1 neurons [92,93]. In addition, authors described GluA3-dependent forms of plasticity in these two brain regions. In the cerebellum, GluA3-plasticity relies on cAMP signaling and is mediated by exchange proteins activated directly by cAMP (Epac), leading to LTP and playing a major role in motor learning [93]. While in the hippocampus, GluA3-plasticity is not required for LTP or LTD, and GluA3KO mice exhibit intact learning and memory performance [92,94]. Despite these insights, the contribution of GluA3-containing AMPARs to cortical synaptic transmission and plasticity remains largely unexplored. More recently, GluA3 has been implicated in a

range of neurodevelopmental, neurological, and psychiatric disorders, further highlighting its functional relevance [11,12,14–16,95,96]. Notably, GRIA3 is the only AMPAR subunit gene located on the X chromosome, providing a unique entry point for investigating sex differences in glutamatergic transmission, dysfunction, and associated

pathologies.

Taken together, their central role in fast excitatory transmission, their involvement in cognition and pathology, and their region-specific physiology position AMPARs as critical elements for understanding cortical synaptic function.

4.2. Cannabinoid receptors

Another key component of the molecular landscape found in synapses is cannabinoid receptors (CBR), which are part of the endocannabinoid system (ECS), along with endocannabinoids (2-arachidonoylglyceroland anandamide) and enzymes responsible for the synthesis and degradation of endocannabinoids [97] (**Figure 2**). CBRs are G protein-coupled receptors, with two main types: cannabinoid receptor type 1 (CB1R) and cannabinoid receptor type 2 (CB2R), each fulfilling distinct physiological roles. CB1R are primarily expressed in the central nervous system, and are particularly enriched in both subcortical and cortical structures, including the mPFC [97,98]. Although present in both excitatory and inhibitory neurons, CB1R expression is higher in the presynaptic site of inhibitory interneurons [98,99].

A defining feature of endocannabinoid signaling is its retrograde nature. Endocannabinoid ligands are synthetized postsynaptically in an activity- and Ca^{2+}-dependent manner and travel backward across the synaptic cleft to activate presynaptic cannabinoid receptors (**Figure 2**). Upon activation, CB1Rs decrease neurotransmitter release, thereby weakening synaptic transmission [100–103]. Mechanistically, CB1R activation modulates K^+ and voltage-gated Ca^{2+} channels through Gi/o proteins via $\beta\gamma$ subunits, resulting in presynaptic hyperpolarization and reduced neurotransmitter release, a form of short-term plasticity (**Figure 2**) [104–106]. In addition, CB1R activation can support long-term forms of plasticity by recruiting slower signaling pathways that involve cyclic adenosine monophosphate (cAMP) modulation and PKA signaling, which can induce more persistent changes in synaptic strength [105].

Although expression of CB2Rs was initially considered to be restricted to the peripheral nervous system and immune cells, several studies have demonstrated their functional presence in the central nervous system [107–110]. In mPFC neurons, activation of CB2R leads to reduced neuronal excitability, suggesting a complementary role to CB1R in shaping cortical activity [111,112]. CBRs play a crucial role in various cognitive processes, including learning and memory, social behaviors, such as sociability and social memory, and cognitive flexibility [113–116].

Cannabinoid receptors modulate not only mature synaptic transmission but also play a role during brain development. Components of the endocannabinoid system are expressed from early embryonic stages through adulthood, following tightly regulated temporal and spatial patterns [117]. In line with this broad developmental involvement, recent work from our lab identified a role for CB1Rs in neuronal migration within the PFC, highlighting their contribution to early cortical circuit assembly [118]. Among these developmental windows, adolescence stands out as a period of pronounced endocannabinoid system remodeling, particularly within the prefrontal cortex. For instance, CB1R expression peaks at the beginning of adolescence, especially in the PFC, before gradually declining into adulthood in both humans and rodents [119–121]. A similar developmental trajectory has been reported for several endocannabinoid ligands,

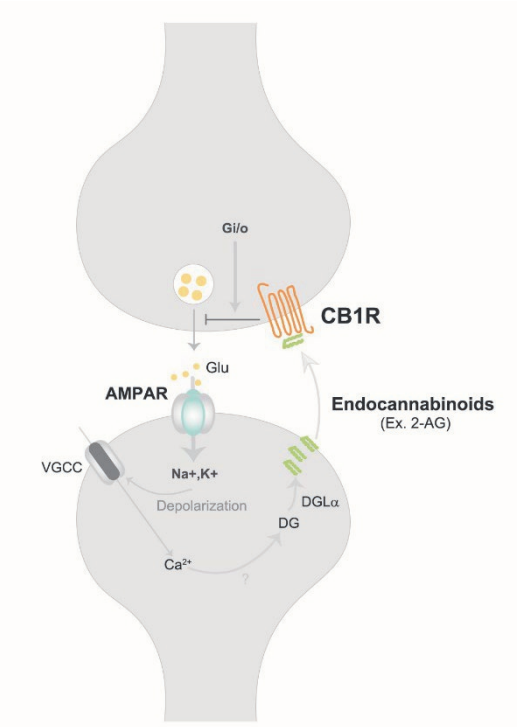


Figure 2. Schematic representation of a glutamatergic synapse from a pyramidal neuron in the cortex. AMPAR are located postsynaptically and activated upon glutamate (Glu) binding, causing an influx of Na+ and K+ and subsequent depolarization. This depolarization leads to the opening of voltage-gated Ca2+ channels (VGCC) and increase in intracellular Ca2+ concentration. Through undescribed pathways, a rise in intracellular Ca2+ increases diacylglycerol, which is converted into 2-arachidonoylglycerol (2-AG) by diacylgylcerol lipase (DGL). 2-AG is then released from the post-synaptic neuron. CB1R are located presynaptically and activated upon post-synaptically-synthetized endocannabinoids, such as 2-AG. Upon activation, CB1R decrease neurotransmitter release through Gi/o proteins. This scheme represents on-demand cannabinoid synthesis.

suggesting coordinated regulation of receptor availability and signaling strength during this period [122].

There is growing evidence indicating that perturbations of cannabinoid signaling during this sensitive period can have long-lasting consequences for brain physiology, particularly in the PFC. Several studies have shown that adolescent exposure to CB1R agonists or to tetrahydrocannabinol (THC), the psychoactive component of cannabis and which binds with high affinity to CB1Rs, caused permanent alterations in neuronal morphology as well as in glutamatergic and GABAergic marker expression in the PFC of adult rats [123–125]. These findings suggest that the functional excitation/inhibition balance within the network may also be affected. In addition, adolescent CBRs activation causes long-term behavioral consequences in adult rodents, including impairments in object recognition, spatial memory, social recognition, motivation, and increased anxiety-like behaviors [126–132]. Collectively, these observations led Tseng & Molla (2025) to propose that adolescence represents a CB1R-sensitive period for PFC maturation and associated functions [122].

Taken together, the region-specific physiology and central role of AMPARs in fast excitatory transmission, alongside the endocannabinoid system's modulatory control over synaptic transmission and its critical influence on cortical development and maturation, position these elements as key regulators of cortical synaptic function.

5. Neuromodulation: the textures

Neuromodulators are essential elements of brain physiology, acting as gain controllers rather than ON/OFF switches. Among these systems, the noradrenergic system occupies a central position in cortical function. Norepinephrine (NE), also known as noradrenaline, is a monoamine neuromodulator implicated in a broad range of cognitive and behavioral processes, including arousal, alertness, attention, and learning [133–135]. NE is synthetized in the locus coeruleus (LC), which provides the main source of noradrenergic innervation to the mammalian neocortex [136]. Both the primary visual cortex and the prefrontal cortex receive dense LC projections, placing noradrenergic signaling in a strategic position to modulate sensory processing as well as higher-order cognition [136–138].

Functionally, NE enhances visual acuity and signal-to-noise ratio in the visual cortex [139], while in the PFC, it plays a crucial role in cognitive flexibility, particularly during

rule shifting [140,141]. Indeed, noradrenergic deafferentation of the mPFC resulted in an overly focused attentional state that impairs rule shifting [140].

The effects of NE are mediated by adrenergic receptors, namely α1-, α2-, and β-adrenergic receptors, each coupled to distinct intracellular signaling cascades. β-adrenergic receptors, in particular, are coupled to Gs proteins and increase cAMP levels through activation of adenylyl cyclases [142]. cAMP subsequently binds to downstream effectors such as Epac and PKA [143]. PKA signaling is a well-established regulator of AMPAR trafficking and function, notably through phosphorylation of the GluA1 subunit, which promotes receptor surface expression and facilitates LTP [144,145].

In the visual cortex, PKA-dependent phosphorylation of GluA1 in layer 2/3 neurons is sufficient to trigger GluA1-trafficking into synapses [146]. In addition, β-adrenergic receptor activation modulates the propensity for LTP and LTD induction [147], underscoring the relevance of β-adrenergic stimulation at synapses of the visual cortex. Similarly, in the medial prefrontal cortex, β-adrenergic signaling potentiates excitatory synaptic transmission in layer 5/6 neurons [148], and recent work has demonstrated that β_2-adrenergic receptor activation promotes GluA1 phosphorylation and surface trafficking via PKA-dependent mechanisms [149]. Beyond V1 and mPFC, noradrenergic modulation of AMPARs extends to other brain regions. For instance, β-adrenergic receptor activation in the hippocampus has been shown to induce GluA3-mediated synaptic potentiation through cAMP- and Ras-dependent changes in ion-channel conductance [92].

These findings position β-adrenergic receptors as critical modulators of AMPAR-mediated cortical transmission. By dynamically tuning synaptic strength, noradrenergic signaling adds a layer of flexibility that is essential for adaptive cortical function.

6. Sex as a biological variable, the frame

Early evidence of sex differences in cortical physiology emerged from studies showing that cortical thickness differs between men and women, with females exhibiting greater thickness in parietal and temporal regions, differences that persist independently of body size and age [150]. Since then, a growing body of work has sought to elucidate how sex-specific features of brain morphology, connectivity, and function may contribute to behavioral diversity [151–153].

Among cortical regions, the prefrontal cortex stands out as a site of pronounced sexual dimorphism. As a structure central to higher-order cognitive functions, the PFC has been repeatedly shown to differ between males and females at functional, morphological, and anatomical levels [154,155]; differences that are also reflected in cognition [156]. At finer scales, sex differences have been reported in neuronal morphology, synaptic density, and synaptic receptor composition within the PFC, suggesting that fundamental properties of synaptic integration and plasticity are sexually differentiated [154,157–159]. Importantly, these differences are dynamic rather than static and are shaped across development. Sex-specific trajectories of prefrontal maturation have been documented in both humans and rodents, with females reaching PFC maturity earlier than males [160,161]. This developmental divergence is particularly pronounced during adolescence, a period already characterized by heightened plasticity and vulnerability.

Beyond cortical architecture, neuromodulatory systems also exhibit marked sexual dimorphism. In the PFC, females have been shown to display stronger glutamatergic transmission [154], as well as differences in the expression and functional properties of cannabinoid receptors. Notably, females exhibit lower levels of CB1 receptor expression but higher receptor binding activity, alongside region-specific differences in endocannabinoid ligand concentrations [162–167]. In addition, behavioral responses to β-adrenergic receptor antagonism in the hippocampus differ between males and females, suggesting that adrenergic modulation may itself be sexually differentiated [168]. Importantly, among AMPAR subunits, *GRIA3* is uniquely located on the X chromosome, positioning GluA3-containing AMPARs as a particularly compelling candidate for sex-dependent regulation of cortical synaptic transmission.

Together, these findings position sex as a fundamental dimension of cortical organization, shaping structure, development, and neuromodulatory control of synaptic function, motivating a framework in which sex and developmental stage must be considered integral to the study of cortical synaptic physiology.

7. The painting: outline and scope of the thesis

Guided by a deep sense of scientific curiosity, this thesis aimed to deepen our understanding of cortical synaptic physiology, with particular attention to sex differences and developmental trajectories. Despite progress in deciphering cortical circuits, important gaps remain in our knowledge of how synaptic receptors and neuromodulatory systems interact across different cortical regions and

developmental stages. Three central questions, therefore, shaped the work presented here:

1. What roles do GluA1- and GluA3-containing AMPARs play in synaptic transmission within sensory and association cortices? (**Chapters 2 & 3**)
2. How do β-adrenergic receptors interact with AMPAR across different cortical structures? (**Chapters 2 & 3**)
3. What are the long-term consequences of adolescent cannabinoid receptor activation on PFC physiology (**Chapter 4**) and GluA3-mediated synaptic transmission? (**Chapter 5**)

To address these questions, in **Chapter 2** I focused on synaptic transmission in a primary sensory area, the visual cortex, and examined the respective contributions of GluA1- and GluA3-containing AMPARs to basal synaptic transmission. To further probe subunit-specific plasticity mechanisms, I also examined how β-adrenergic receptor activation modulates AMPAR-mediated synaptic responses.

Building on these findings, **Chapter 3** extends the investigation to an association cortex, the prefrontal cortex. In that chapter, I examined the contribution of GluA3-containing AMPARs to PFC basal synaptic transmission and its modulation by β-adrenergic signaling, as well as the role of this subunit in reversal learning. Importantly, the protracted development of the PFC motivated the inclusion of a developmental perspective in the study.

Chapter 4 deepens the focus on cortical development, exploring the long-term impact of adolescent cannabinoid receptor activation on adult prefrontal cortex physiology and behavior. Particular emphasis was placed on identifying sex-specific adaptations to this developmental perturbation, reflecting the heightened vulnerability of the PFC during adolescence.

Finally, I integrated the conceptual threads of the previous chapters in a pilot study investigating acute interactions between GluA3-containing AMPARs and cannabinoid receptors (**Chapter 5**). In light of the association of both systems with schizophrenia, I hypothesized a double-hit framework to examine the interaction between genetic vulnerability and environmental exposure, by assessing the long-term consequences of adolescent cannabinoid receptor activation in a GluA3-deficient background.

Together, the work presented in this thesis aims to provide a coherent framework for understanding cortical synaptic physiology across sensory and association cortices, highlighting both conserved principles and region-specific mechanisms. By focusing on understudied components such as GluA3-containing AMPARs, incorporating sex as a biological variable, and exploring interactions between glutamatergic, adrenergic,

and endocannabinoid systems, this thesis seeks to open new perspectives and lay groundwork for future investigations into the complexity of cortical function.

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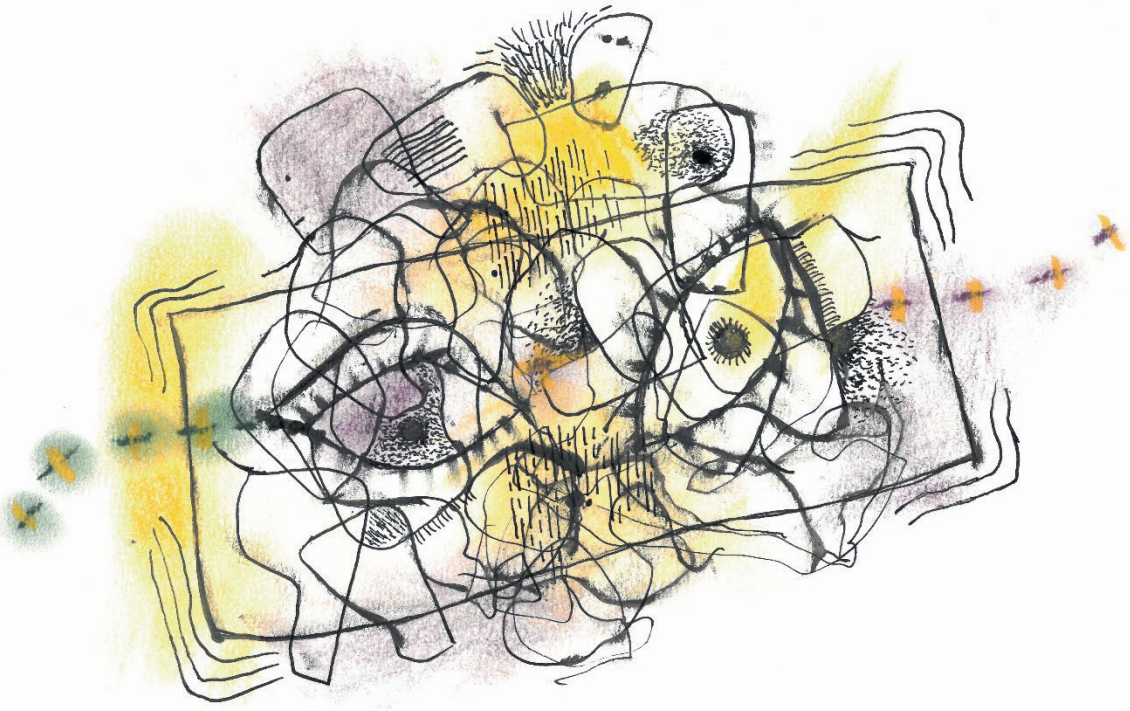
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Chapter 2

Role of GluA3- and GluA1-containing AMPA receptors in synapse physiology of layer2/3 pyramidal cells in primary visual cortex

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Abstract

The regulation of excitatory synaptic transmission in the visual cortex relies heavily on AMPA receptors (AMPARs). Yet, the specific roles of GluA1- and GluA3-containing AMPARs, and their modulation by β-adrenergic signaling, remain incompletely understood. This chapter aimed to determine the contribution of GluA3- and GluA1-containing AMPARs to basal synaptic transmission in layer 2/3 pyramidal neurons of the primary visual cortex (V1), and to define how these subunits participate in β-adrenergic modulation of synaptic transmission. Using patch-clamp recordings in acutely isolated brain slices, we found that although both GluA3 and GluA1 contribute to synaptic transmission in V1 pyramidal neurons, GluA3-containing AMPARs play a more prominent role. β-adrenergic activation potentiated spontaneous presynaptic vesicle release, but not evoked release, and had little effect on both GluA3- and GluA1-dependent AMPAR currents. We also found that PKA is active under basal conditions, and that this protein did not contribute to the synaptic potentiation driven by β-adrenergic activation. In somatostatin interneurons, GluA3-containing AMPARs did not play a role in basal synaptic transmission, and β-adrenergic activation failed to induce potentiation. Together, these findings reveal a cooperative role of GluA3- and GluA1-containing AMPARs in supporting basal synaptic transmission but not in mediating β-adrenergic synaptic potentiation at layer 2/3 pyramidal neurons in V1. These results highlight the AMPAR subunit-specific contributions to synapse physiology in the primary visual cortex.

Introduction

Vision is our dominant sense, and roughly half of the human cerebral cortex is involved in processing visual information [1]. The visual cortex is divided into 5 different areas, V1 to V5 [2]. Among these, each subregion is specialized based on structure and function, with the primary visual cortex (V1) being responsible for receiving input directly from the eyes via the lateral geniculate nucleus (LGN) of the thalamus [3]. This makes V1 the first cortical structure involved in processing visual information. From the LGN, signals are primarily relayed to layer 4 of V1, whose neurons project to layer 2/3 pyramidal neurons, a pathway referred to as the vertical pathway [3]. In addition, intracortical connections exist between layer 2/3 neurons, forming the horizontal pathway. Layer 2/3 neurons also receive associative feedback connections from other brain regions at their dendritic tufts located in layer 1 [4]. Information is then passed to deeper layers for transfer to other brain regions [5]. Layer 2/3, therefore, serves as a hub for integrating input, both direct and indirect, from the thalamus as well as from other local layer 2/3 neurons [3]. In V1, both excitatory and inhibitory cells are present. The inhibitory cell population consists mainly of somatostatin- (SST, ~23%), parvalbumin- (PV, ~39%), calretinin- (CR, ~24%), and vasoactive intestinal polypeptide- (VIP, ~11%) expressing cells [6]. SST-expressing somata are most abundant in layers 2/3 and 5 and play a crucial role in visual processing: not only do they contribute to feedback inhibition in excitatory neurons, but they also inhibit excitatory input to PV+ interneurons [4,7].

The release of noradrenaline is a key factor in survival, as it is one of the main signaling molecules of the “fight-or-flight” response, increasing the body’s alertness to environmental stimuli [8]. One prominent effect of elevated noradrenaline is the enhancement of visual acuity and processing [9], which relies on fast information transmission. AMPA-receptors (AMPARs) mediate fast synaptic transmission at excitatory synapses and are indeed implicated in visual processing, being required for spatial phase-selective responses and for mediating long-term potentiation induced by rapidly repeated visual stimulation [10,11]. AMPARs are composed of four different subunit types: GluA1 to GluA4. The vast majority of AMPARs are heterodimers that contain the GluA2 subunit, which renders them Ca^{2+} impermeable [12,13]. In the cortex, GluA2 is the predominant subunit (~45%), with similar levels of GluA1 and GluA3 (respectively ~21% and ~27%) and a minor portion of GluA4 (~7%) [14], suggesting the majority of cortical AMPARs consist of either GluA1/GluA2 or GluA3/GluA2 combinations. GluA1-containing AMPARs traffic into synapses upon the induction of long-term potentiation (LTP) [15,16], and this GluA1-trafficking is necessary for experience-dependent synaptic potentiation in the visual cortex [17].

Noradrenaline can modify AMPAR transmission at synapses through the activation of adrenergic receptors, which are G-protein-coupled receptors divided into three different subtypes, β, α_1, and α_2 [8]. Upon activation, β-adrenergic receptors, coupled to Gs proteins, increase cAMP levels by activating adenylyl cyclases [8]. cAMP then binds to downstream effectors such as protein kinase A (PKA) or exchange proteins activated directly by cAMP (Epac), initiating signaling cascades [18]. PKA-mediated phosphorylation of GluA1 increases surface expression of GluA1-containing AMPARs, which facilitates LTP and inhibits long-term depression (LTD) [19,20]. In the visual cortex, PKA-phosphorylation of GluA1 in layer 2/3 neurons is sufficient to trigger its trafficking into synapses [21], and β-adrenergic receptor activation, through GluA1 phosphorylation, modulates the propensity for LTP and LTD [22], underscoring the relevance of β-adrenergic signaling in regulating synaptic plasticity in the visual cortex.

The contribution of GluA3-containing AMPARs to synaptic transmission in the visual cortex has remained unknown. Previous research from our lab has shown that at CA1 synapses in the hippocampus, GluA1- and GluA3-containing AMPAR have distinct roles in synaptic transmission. While GluA1-containing AMPARs predominantly contribute to synaptic currents under basal conditions, increased cAMP levels following β-adrenergic activation lead to PKA- and Epac-independent synaptic potentiation through the activation of GluA3-containing AMPARs. This potentiation requires cAMP signaling via the GTPase Ras and is not mediated by synaptic trafficking of these receptors, but rather by an increase in channel conductance of AMPARs already present at synapses [23]. Similarly, in Purkinje cells of the cerebellum, a rise in cAMP increases channel conductance of GluA3-containing AMPAR at synapses, although in this case the mechanism is mediated by Epac activation [24], suggesting this potentiation is mediated by Rap instead of Ras. However, it is unclear whether this type of GluA3-dependent synaptic plasticity is operational in the visual cortex. In this context, the present chapter aims to elucidate the contribution of GluA1- and GluA3-containing AMPARs to synaptic transmission within this region. Given that GluA1- and GluA3-dependent plasticity rely on β-adrenergic activation and intracellular cAMP signaling in other brain structures, this study also investigates the role of β-adrenergic activation in layer 2/3 of the primary visual cortex.

Materials & Methods

Animals

Gria3-deficient (GluA3KO) mice (*Gria3tm1Dgen/Mmnc;* Mutant Mouse Regional Resource Center, Davis, CA) and Gria1-deficient (GluA1KO) mice (kindly provided by R. Huganir; Johns Hopkins University, Baltimore; [25]) were maintained on a C57BL/6

background. Double *GRIA1/GRIA3*-deficient mice (GluA1/GluA3KO) were generated by crossing a GluA1KO homozygous male with a GluA1 homozygous, GluA3-heterozygous female. For the experiments in which GluA3 was specifically knocked-out in SST neurons, SST-flippase (FLP) x floxGria3 mice were used in the study (see Adeno-associated viral vectors). Both sexes were used in this study. All experiments were conducted in accordance with the European guidelines for the care and use of laboratory animals (European Directive 2010/63/EU). The experimental protocol was approved by the Animal Experiment Committee of the Royal Netherlands Academy of Arts and Sciences (KNAW) and by the Animal Welfare Body of the University of Amsterdam (permit number AVD11100202114907).

Acute slice preparation

Mice were killed using cervical dislocation at postnatal day 28-32 (PND28-32) for acute slice preparation. Brains were extracted and placed in ice-cold modified artificial cerebrospinal fluid (mACSF, in mM: 139 choline chloride, 3.5 KCl, 0.5 $CaCl_2$, 6.0 $MgSO_4$, 1.25 NaH_2PO_4 H_20, 10 glucose and 25 $NaHCO_3$, continuously bubbled with 5% CO2/ 95% O2 (pH 7.4)). 350 µm-thick coronal slices of the V1 were cut with a vibrating blade microtome (VT 1200S, Leica Microsystems). Slices were subsequently incubated at 35°C for 20 minutes in ACSF (in mM: 118 NaCl, 2.5 KCl, 26.2 $NaHCO_3$, 1 NaH_2PO_4 H_20, 22.2 glucose, 2 $CaCl_2$, and 1 $MgCl_2$, bubbled with 5% CO2/ 95% O2), and then kept for at least 45 min at room temperature before recording.

Ex-vivo patch-clamp recordings

Whole-cell voltage clamp recordings were made from layer 2-3 pyramidal neurons of the V1. During recordings, slices were submerged in ACSF at 28-30 ° Celsius, bubbled with 5 % CO2/95 % O2 and superfused at a rate of 1.8 ml/min. Glass recording pipettes were pulled from borosilicate glass (Science Products) and had a resistance of 2.5-5 MΩ (Raccess <30 MΩ and Rinput > 9x Raccess) when filled with intracellular solution, containing (in mM): 115 CsMeSO3, 20 CsCl, 10 HEPES, 2.5 MgCl2, 4 Na2-ATP, 0.4 Na-GTP, 10 sodium phosphocreatine, and 0.6 EGTA, at pH 7.25. To isolate excitatory transmission, all recordings were made in the presence of 100 mM picrotoxin (Tocris).

For the recording of miniature excitatory post-synaptic currents (mEPSCs), cells were clamped at -60 mV (uncorrected for liquid junction potential) and 5-minute recordings were made in the presence of 1 mM Tetrodotoxin (TTX, Latoxan, France). Signals were acquired using a MultiClamp 700B amplifier controlled by pClamp 11 software (Molecular Devices), low-pass filtered at 3 kHz, and sampled at 10 kHz. Series resistance ranged from 6–20 MΩ and was compensated to ~65 %. Frequency and amplitude were determined from at least 100 events or 10 min of recording with

MiniAnalysis (Synaptosoft). Individual mEPSCs events above the 5 pA noise threshold were manually selected.

To evoke excitatory postsynaptic currents (eEPSCs), one stimulating electrode (two-contact Pt/Ir cluster electrode; FHC) was positioned in layer 4, while a 1–2 MΩ glass stimulating electrode was placed either in layer 1 (100–200 µm from the soma of the recorded neuron) or in layer 2/3 (200 µm from the soma). Stimulation intensity was adjusted to evoke postsynaptic currents with half-maximal amplitude at a holding potential of −60 mV. Biphasic currents (100-800 µA, 0.1 ms) were injected through the stimulation electrode. For evoked recordings, the internal pipette solution was supplemented with 5mM QX-314 (Alomone labs) to prevent bursting. Neurons were voltage-clamped at -60 mV and +40 mV to record AMPA-mediated and NMDA-mediated EPSCs (respectively). Additionally, current at +0 mV was measured for correction (reversal potential for AMPA and NMDA currents). Thirty sweeps were recorded per holding potential for accuracy. AMPA-mediated EPSC amplitude was measured at the peak of the inward synaptic responses at -60 mV, while the amplitude of the NMDA-mediated EPSCs was measured as the mean of the outward current between 40 and 90 ms after stimulation evoked at +40 mV, and corrected by the current at 0mV. Paired pulse ratios were calculated as the ratio between the second and the first peak amplitude of inward EPSCs evoked at -60 mV by two stimuli with an inter-pulse interval of 50 ms. Signals were acquired using a MultiClamp 700B amplifier controlled by pClamp 11 software (Molecular Devices), low-pass filtered at 3 kHz, and sampled at 10 kHz. Series resistance ranged from 6–20 MΩ and was compensated to ~65 %. Off-line analysis was performed with Clampfit (Molecular Devices).

Pharmacology

Where indicated, the following drugs were added to the perfusion solution: isoproterenol (10 µM; Sigma), IBMX (50 µM; Tocris), PKI (2 µM; Tocris), ESI-05 (10 µM; Biolog), MR-L2 (10 mM, Bio-Connect Diagnostics B.V.). Incubation time was 5 min for isoproterenol and IBMX and ESI-05 and 20 min for MR-L2.

Adeno-associated viral vectors

SST-FLP+/+ × FloxGria3+/+ or SST-FLP+/+ × FloxGria3-/- (=WT) littermate mice received three viral injections: (1) a FLP-dependent Cre AAV vector (pAAV-EF1a-fDIO-Cre), (2) a Cre-dependent GCaMP6f/mRuby AAV vector (pAAV1-hSyn1-flex-mRuby2-GSG-P2A-GCaMP6f), and (3) a GCaMP6f/CaMKII AAV vector (pAAV9-CaMKIIa-GCaMP6f). The first vector expresses Cre specifically in FLP-expressing SST interneurons and simultaneously knocks out the floxed *GRIA3* gene. As a result, *GRIA3* is selectively deleted in SST-FLP+/+ × floxGria3+/+ mice (KO), but remains intact in SST-FLP+/+ × floxGria3−/− mice (WT). Cre expression is also required for the second vector, which

drives expression of red fluorescence (mRuby2) in Cre-positive cells, enabling identification of SST interneurons among other cortical neurons. Finally, the third vector expresses the green fluorescent calcium indicator GCaMP6f under the CaMKII promoter, selectively labeling excitatory neurons. (Calcium indicator GCaMP6f was used in imaging experiments to record neuronal activity by Valentina Riguccini in the Levelt lab.)

Viral injections

Viral vectors were injected in both groups of mice under anesthesia, which was induced with 4–5% isoflurane and maintained at 2–2.5% in oxygen (minimum flow rate 0.5 L/min). Analgesia was provided by subcutaneous injection of Metacam (5 mg/kg) prior to surgery. Mice were head-fixed in a stereotaxic frame, eyes were protected with ophthalmic ointment (Cavasan, AST Pharma), and body temperature was maintained using euthermic pads. Additional analgesia and anti-inflammatory treatment were administered with Buprenorphine (0.1 mg/kg) and Dexamethasone (2 mg/kg). A midline scalp incision was made, and lidocaine was applied locally to the periosteum. Four or five small holes, approximately pipette-sized, were drilled into the skull at approximately AP −2.92 mm, ML −2.25 mm, DV −0.80 mm relative to bregma for viral vector injections. After injections, the scalp was sutured, and mice were placed in a 37°C recovery chamber. Postoperative monitoring included behavioral inspection and body weight measurements over the following two days. Drinking water was supplemented with Rimadyl (0.06 mg/mL) to minimize pain, and mice were housed with separation walls for 3–9 days to facilitate suture healing.

Statistical analysis

All statistical tests were performed in GraphPad version 8 (GraphPad Software, San Diego) or RStudio version 2024 (RStudio Team, Boston). Normality of data distributions was assessed using the Shapiro–Wilk test. For comparisons between two unpaired groups, normally distributed data were analyzed using an unpaired *t*-test, whereas non-normally distributed data were analyzed using the Mann–Whitney test. For comparisons involving more than two unpaired groups, differences were assessed with either a one-way ANOVA followed by Tukey's post hoc test (normally distributed data) or the Kruskal–Wallis test (non-normally distributed data). Reported *p*-values correspond to post hoc contrasts, unless the overall ANOVA did not reach significance. A threshold of $p < 0.05$ was considered statistically significant.

Results

Synaptic transmission onto layer 2/3 pyramidal neurons in V1 depends on GluA1 and GluA3

To examine the contribution of GluA1 and GluA3 to synaptic transmission in the visual cortex, we performed patch-clamp recordings of layer 2/3 pyramidal neurons in the primary visual cortex (L2/3-V1) of GluA3KO, GluA1KO and their corresponding WT littermates (**Figure 1A**). In GluA3KO animals, both mEPSC frequency (-39.15%, U=35, p=0.0008; **Figure 1B**) and amplitude (-13.03%, U=39, p=0.001; **Figure 1C**) were significantly decreased compared with WT littermates. In contrast, L2/3-V1 neurons from GluA1KO mice did not show a reduction in mEPSCs frequency (-1.60%, U=119, p=0.7; **Figure 1D**), whereas mEPSCs amplitude was significantly decreased relative to WT littermates (-8.96%, U=65, p=0.017; **Figure 1E**). When comparing the magnitude of change as a consequence of lacking either of the subunits relative to WT, the reduction in mEPSC frequency was significantly greater in GluA3KO than in GluA1KO, with a mean fold-change difference of 0.37 between genotypes (t(30)=3.20, p=0.0032; **Figure 1F**), whereas mEPSC amplitudes were reduced to a similar extent in both groups (t(30)=0.725, p=0.725; **Figure 1G**).

Together, these data indicate that both GluA1- and GluA3-containing AMPARs contribute to synaptic currents onto L2/3-V1 neurons, but with a greater impact on synaptic currents by the absence of GluA3 than by the absence of GluA1.To assess whether AMPAR subunits other than GluA1 or GluA3 (e.g. GluA4) contribute to synaptic currents of L2/3-V1 neurons, we next recorded from mice lacking both GluA1 and GluA3 (GluA1/GluA3KO). GluA1/GluA3KO mice exhibited delayed growth and increased mortality during development compared with GluA1KO littermates but reached normal size at maturity (unpublished observations). During patch-clamp recordings, mEPSCs were rarely detected in L2/3-V1 neurons from GluA1/GluA3KO mice (**Figure 1H**), resulting in a markedly reduced frequency compared with GluA1KO mice (-99.50%, U=0, p<0.0001). The few remaining miniature excitatory events exhibited amplitudes comparable to those observed in GluA1KO mice (-3.18%, U=113, p=0.829, **Figure 1I**). These findings indicate that approximately 99.5% of synaptic currents in L2/3-V1 neurons are mediated by GluA1- and GluA3-containing AMPARs.

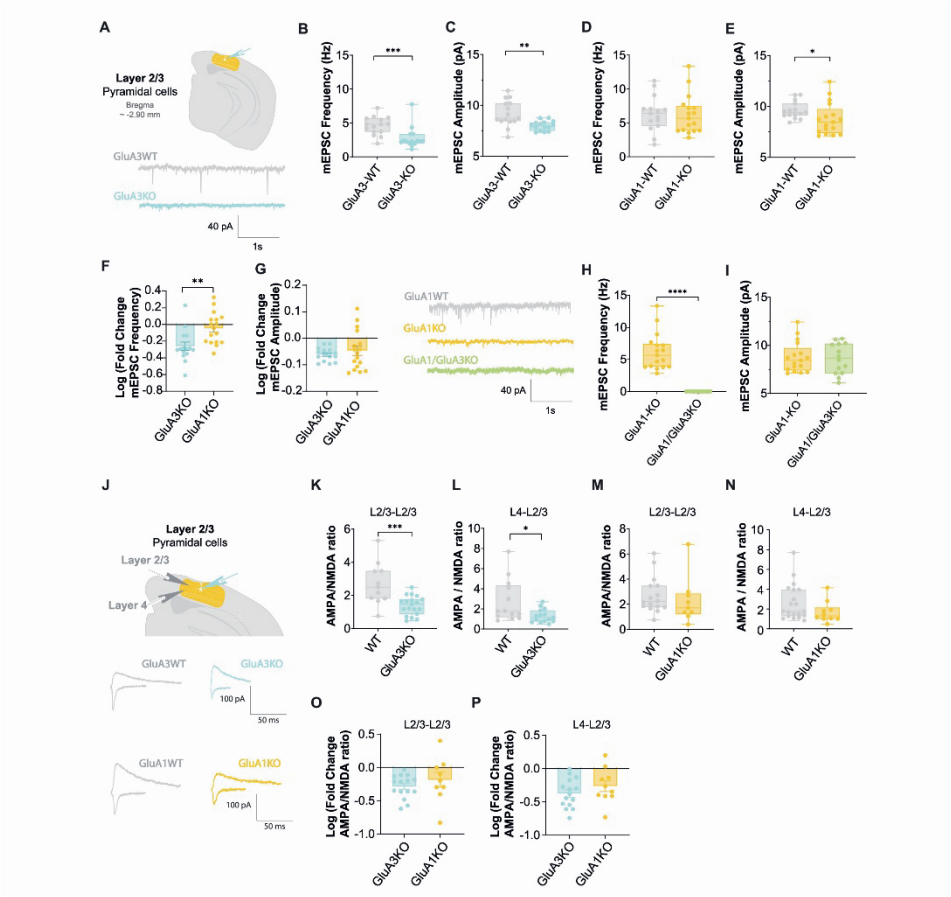


Figure 1. Larger contribution of GluA3-, compared to GluA1-containing AMPARs, to synaptic transmission in V1-L2/3 pyramidal neurons. (A) Top: schematic representation of a coronal slice at ~ -2.90mm relative to bregma (adapted from Paxinos and Franklin atlas 2nd edition), showing the primary visual cortex and cortical layer included in the study. Bottom: example traces of miniature excitatory postsynaptic currents (mEPSCs) from layer 2/3 pyramidal neurons of GluA3KO mice and WT littermates, GluA1KO mice and WT littermates and GluA1/GluA3KO mice. For all panels, colors indicate genotype: grey (WT), blue (GluA3KO), yellow (GluA1KO) and green (GluA1/GluA3KO). (B) mEPSCs frequency and (C) amplitude in GluA3KO (n=15 cells) and WT littermates (n=15 cells). (D,E) Same as B,C but for GluA1KO (n=17 cells) and their WT littermates (n=15 cells). (F) Fold change of mEPSC frequency and (G) mEPSC amplitude in GluA3KO (n=15 cells) and GluA1KO (n=17 cells) compared to their WT littermates. (H,I) Same as B,C but for GluA1KO (n=17 cells) and GluA1/GluA3KO (n=14 cells). (J) Top: schematic representation of the same region shown in A but including stimulating electrodes in either layer 2/3 or layer 4, bottom: example traces of AMPA/NMDA ratios in GluA3KO, GluA1KO and their WT littermates. (K) AMPA/NMDA ratios upon stimulation from L2/3 in GluA3KO (n=11 cells) and their WT littermates (n=19 cells). (L) Same as K but for stimulation from layer 4 (WT n=13 cells; GluA3KO n=15 cells). (M) Same as K but for WT (n= 16 cells) and GluA1KO mice (n=9 cells). (N) Same as L but for WT (n=20 cells) and GluA1KO mice (n=10 cells). (O,P) Same as F,G but for AMPA/NMDA ratio.

In the visual cortex, information flow can follow different pathways, namely horizontal or vertical [3]. To determine whether GluA1- and GluA3-containing AMPAR usage is input-specific, we recorded evoked EPSCs by stimulating either layer 2/3 (horizontal pathway L2/3-L2/3) or layer 4 (vertical pathway L4-L2/3). Evoked currents were measured at -60mV and at +40mV to calculate AMPA/NMDA ratios (**Figure 1J**). L2/3-V1 neurons from GluA3KO mice displayed significantly reduced AMPA/NMDA ratios for both the horizontal (-48.74%, U=30, p=0.0008; **Figure 1K**) and vertical pathways (-52.45%, U=49, p=0.025; **Figure 1L**) compared with WT littermates. In contrast, AMPA/NMDA ratios in GluA1KO neurons were not significantly different from WT for either pathway (L2/3: -19.48%, U=49, p=0.82, **Figure 1M**; L4: -35.74%, U=48, p=0.65; **Figure 1N**). However, when comparing the magnitude of change relative to WT, no significant differences were observed between GluA3KO and GluA1KO mice for either pathway (L2/3: t(22)=0.972, p=0.341, **Figure 1O**; L4: t(23)=1.149, p=0.262, **Figure 1P**). These results indicate that postsynaptic AMPAR currents at L2/3-V1 synapses that receive either horizontal or vertical input are similarly affected by the absence of either GluA1 or GluA3.

β-adrenergic activation potentiates miniature postsynaptic currents in layer 2/3-V1 synapses independently of AMPAR subunit composition

We next investigated whether increasing cAMP levels via β-adrenergic receptor activation combined with phosphodiesterase (PDE) inhibition potentiates AMPAR-mediated transmission in V1. Miniature EPSCs were recorded from L2/3-V1 pyramidal neurons in the presence or absence of isoproterenol, a β1- and β2-adrenergic receptor agonist, together with IBMX, a broad-spectrum PDE inhibitor (**Figure 2A**). In WT

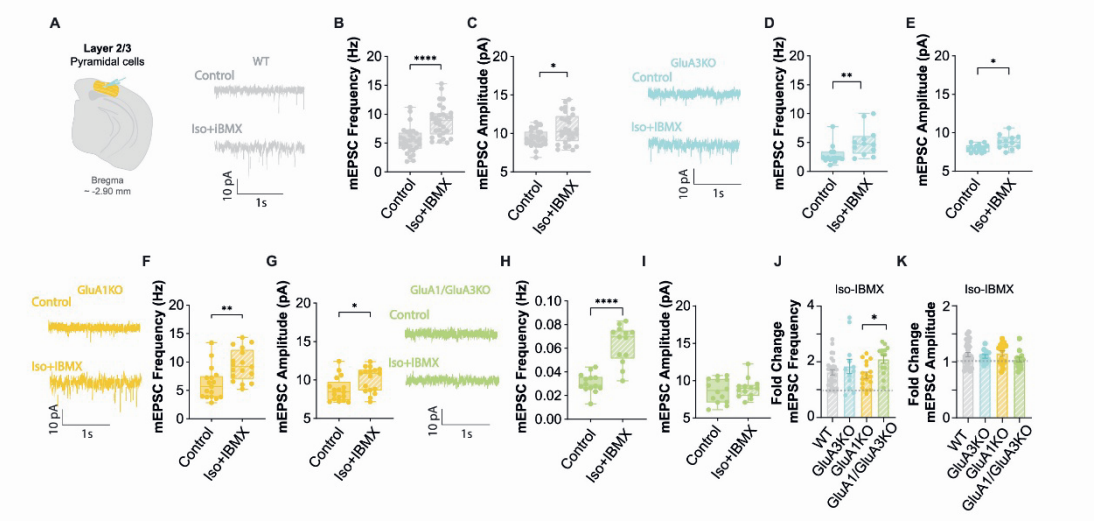


Figure 2. β-adrenergic activation increases miniature postsynaptic currents in V1-L2/3 neurons irrespective of AMPAR subunit composition. (A) Schematic representation of a coronal slice at ~ -2.90mm relative to bregma (adapted from Paxinos and Franklin atlas 2nd edition), showing the primary visual cortex and cortical layer included in the study. For all panels, colors indicate genotype: grey (WT), blue (GluA3KO), yellow (GluA1KO) and green (GluA1/GluA3KO). (B) Frequency of miniature excitatory postsynaptic currents (mEPSCs) from layer 2/3 pyramidal WT neurons in the presence and absence of isoproterenol and IBMX (Iso+IBMX) (control n=13 cells, Iso+IBMX n=9 cells). (C) mEPSC amplitude in the presence and absence of isoproterenol and IBMX in WT mice (control n=13 cells, Iso+IBMX n=9 cells). (D,E) Same as B,C but for GluA3KO (control n=15 cells, Iso+IBMX n=15 cells). (F,G) Same as B,C but for GluA1KO (control n=14 cells, Iso+IBMX n=15 cells). (H,I) Same as B,C but for GluA1/GluA3KO (control n= 14 cells, Iso+IBMX n=13 cells). (J) Fold change of mEPSC frequency and (K) mEPSC amplitude in the presence of Iso+IBMX compared to their control conditions in WT (n=29 cells), GluA3KO (n=12 cells), GluA1KO (n=17 cells) and GluA1/GluA3KO mice (n=13 cells). Dashed line indicates value of 1.

neurons, isoproterenol plus IBMX significantly increased both mEPSC frequency (+62.12%, U= 139, p<0.001, **Figure 2B**) and amplitude (+13.15%, U=282, p=0.020, **Figure 2C**). Similar increases in mEPSC frequency and amplitude were observed in GluA3KO neurons (+83.54%, U=27, p=0.001, **Figure 2D**; +9.73%, U=42, p=0.018, respectively; **Figure 2E**) and in GluA1KO neurons (+52.04%, U= 55, p=0.001, **Figure 2F**; +15.16% increase, U=80, p=0.026, respectively; **Figure 2G**). Although virtually no events were detected in GluA1/GluA3KO L2/3-V1 neurons, β-adrenergic activation increased mEPSC frequency (+108.85%, U=8, p<0.001, **Figure 2H**), while amplitudes remained unchanged (+3.88%, U=82, p=0.684; **Figure 2I**).

To compare the magnitude of contribution of either subunit to the potentiation, we quantified fold changes following isoproterenol plus IBMX application. Fold-changes in mEPSC frequency were substantially increased in all genotypes, with a significant difference detected only between GluA1/GluA3KO and GluA1KO neurons (one-way ANOVA (F(3,67)= 2.869, p=0.04; post-hoc multiple comparison p=0.046; **Figure 2J**). In contrast, the magnitude of mEPSC amplitude increase was on average small and comparable across genotypes (one-way ANOVA (F(3,67)= 1.896, p=0.345; **Figure 2K**). Together, these results suggest that β-adrenergic activation potentiates synapse strength in a manner that is largely independent of postsynaptic AMPAR subunit composition.

Activation of β-adrenoreceptors does not affect evoked transmission in layer 2/3 pyramidal neurons from V1

To further characterize the effect of β-adrenergic activation on information conveyance in V1, we stimulated horizontal and vertical pathways in the presence or absence of isoproterenol and IBMX (**Figure 3A**). AMPA/NMDA ratios and paired-pulse ratios (PPRs) were calculated to investigate potential postsynaptic and presynaptic effects, respectively. In WT neurons, isoproterenol plus IBMX did not significantly alter

AMPA/NMDA ratios at either the horizontal (L2/3-L2/3: +3.82%, U=100, p=0.88; **Figure 3B**) or vertical pathways (L4-L2/3: +22.98%, U=80, p=0.40; **Figure 3C**). To assess whether unchanged AMPA/NMDA reflected a lack of potentiation or an increase in both AMPAR and NMDAR currents, we directly compared AMPAR currents before and after drug wash-in. AMPAR-mediated responses were not increased by isoproterenol plus IBMX at either horizontal (W=3, p=0.81; **Figure 3D**) or vertical pathways (W=-9,

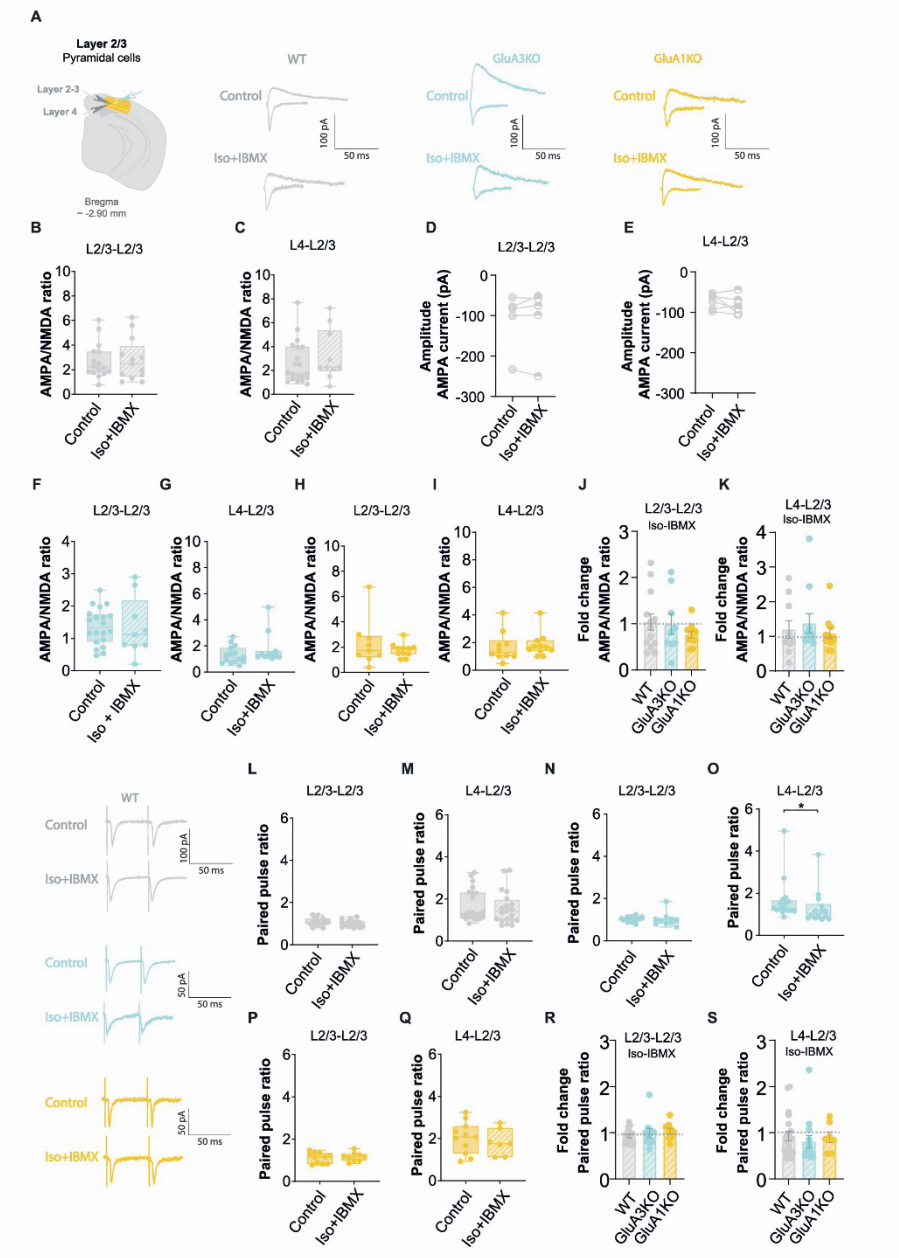


Figure 3. Evoked transmission in V1-L2/3 is not potentiated by β-adrenergic activation regardless of genotype. (A) Left: schematic representation of a coronal slice at ~ -2.90mm relative to bregma (adapted from Paxinos and Franklin atlas 2nd edition), showing the primary visual cortex and cortical layer included in the study. Right: Example traces of +40mV and -60 mV currents from layer 2/3 pyramidal WT, GluA3KO and GluA1KO neurons in the presence and absence of isoproterenol and IBMX (Iso+IBMX). For all panels, colors indicate genotype: grey (WT), blue (GluA3KO) and yellow (GluA1KO). (B) AMPA/NMDA ratio in the presence and absence of Iso+IBMX upon L2/3 stimulation in WT mice (control n=16 cells, Iso+IBMX n=13 cells), stimulated layer is shown on the top of the panel. (C) Same as B but upon stimulation of layer 4 (control n=20 cells, Iso+IBMX n=10 cells). (D) Amplitude of paired AMPA currents before and after wash-in of Iso+IBMX upon L2/3 stimulation in WT slices (n=5 pairs). (E) Same as D but upon L4 stimulation (n= 6 pairs). (F,G) Same as B,C but for GluA3KO mice (L2/3 control n=20 cells, Iso+IBMX n=10 cells; L4 control n=15 cells, Iso+IBMX n=11 cells). (H,I) Same as B,C but for GluA1KO (L2/3 control n=9 cells, Iso+IBMX n=11 cells; L4 control n=10 cells, Iso+IBMX n=11 cells). (J) Fold change of AMPA/NMDA ratio in the presence of Iso+IBMX compared to the control conditions per genotype upon stimulation of layer 2/3 (WT n=13 cells, GluA3KO n=9 cells, GluA1KO n=11 cells). Dashed line indicates value of 1. (K) Same as J but upon stimulation of layer 4 (WT n=10 cells, GluA3KO n=11 cells, GluA1KO n=11 cells). (L,M) Paired pulse ratio in the presence and absence of Iso+IBMX upon stimulation of layer 2/3 (control n=15 cells, Iso+IBMX n=16 cells) and (N) layer 4 (control n=22 cells, Iso+IBMX n=20 cells). (O-Q) Same as M-O but for GluA3KO (L2/3 control n=15 cells, Iso+IBMX n=10 cells; L4 control n=17 cells, Iso+IBMX n=14 cells). (R-T) Same as M-O but for GluA1KO (L2/3 control n=11 cells, Iso+IBMX n=9 cells; L4 control n=11 cells, Iso+IBMX n=7 cells). Iso=Isoproterenol. (U) Same as J but for paired pulse ratio (WT n=16 cells, GluA3KO n=10 cells, GluA1KO n=9 cells). Dashed line indicates value of 1. (V) Same as K but for paired pulse ratio (WT n=20 cells, GluA3KO n=14 cells, GluA1KO n=7 cells).

p=0.437; **Figure 3E**). Similarly, AMPA/NMDA ratios were unaffected by drug application in GluA3KO neurons (L2/3-L2/3: +1.11%, U=82, p=0.728, **Figure 3F**; L4-L4: +37.16%, U=56, p=0.180; **Figure 3G**), and GluA1KO neurons (L2/3-L2/3: -23.03%, U=46, p=0.823, **Figure 3H**; L4-L2/3: +8.33%, U=48, p=0.653, **Figure 3I**). Compared to their control conditions, the magnitude of change did not differ across genotypes for either pathway (L2/3-L2/3: one-way ANOVA (F(2,30)= 2.63, p=0.088, **Figure 3J**; L4-L2/3: one-way ANOVA (F(2,29)= 0.33, p=0.696, **Figure 3K**).

PPRs were also largely unaffected by β-adrenergic activation. In WT neurons, PPRs did not change upon stimulation of either pathway (L2/3-L2/3: -7.10%, U=93, p=0.299, **Figure 3L**; L4-L4: -7.24%, U=192, p=0.492; **Figure 3M**). We note that PPRs were on average close to one in both pathways, suggesting these synapses have a high presynaptic release probability. In GluA3KO neurons, PPRs remained unchanged for horizontal inputs (-0.94%, U=55, p=0.285, **Figure 3N**), but were reduced in the vertical pathway (-19.34%, U=66, p=0.035; **Figure 3O**), indicating an increase in presynaptic release probability at this pathway. No significant differences were observed in PPR in GluA1KO neurons for either pathway (L2/3-L2/3: +3.69%, U=46, p=0.823, **Figure 3P**; L4-L2/3: -10.02%, U=33, p=0.659; **Figure 3Q**). In addition, the magnitude of change in PPR did not differ across genotypes for either pathway (L2/3-L2/3: one-way ANOVA (F(2,32)= 0.621, p=0.543, **Figure 3R**; L4-L2/3: one-way ANOVA (F(2,38)= 0.340, p=0.713, **Figure 3S**). These data suggest that synaptic changes as a consequence of β-adrenergic activation and PDE blockade are more readily detected in miniature EPSC

recordings than in evoked EPSC recordings. To exclude the possibility that β-adrenergic effects were restricted to distal dendrites, we stimulated synapses in layer 1. Isoproterenol plus IBMX similarly failed to affect AMPA/NMDA ratios or PPRs of WT neurons under these conditions (**Figure S2**). Together, these results indicate that β-adrenergic activation and PDE blockade induce only minimal changes in postsynaptic AMPAR currents and presynaptic Ca^{2+}-dependent release probability at L2/3-V1 synapses.

PKA is active under basal conditions in layer 2/3 pyramidal neurons of V1

We next aimed to isolate GluA3-dependent plasticity by manipulating cAMP levels while blocking PKA, given that PKA activity has been shown to increase presynaptic vesicle availability and promote GluA1 trafficking to synapses [21,26], yet it is insufficient to induce GluA3-dependent plasticity [23]. Because PKA can be in an active state under basal conditions in cortical neurons of awake mice [27], we first tested whether PKA is tonically active in L2/3-V1 neurons. In WT mice, inhibition of PKA with PKI did not significantly affect mEPSC frequency (-18%, U=233, p=0.083; **Figure 4A,B**), but significantly reduced mEPSC amplitude (-10%, U=192, p=0.011; **Figure 4C**), indicating that PKA is active under basal conditions in L2/3 V1 neurons to maintain postsynaptic AMPAR currents in a potentiated state. To assess whether this PKA-driven postsynaptic potentiation is AMPAR subunit specific, we repeated this experiment in brain slices from GluA1KO and GluA3KO mice. Similar effects were observed in GluA3KO neurons, where PKI did not affect mEPSC frequency (-7.41%, U=36, p=0.218; **Figure 4D**) but significantly reduced mEPSC amplitude (-20%, U=24, p=0.033; **Figure 4E**). In GluA1KO neurons, neither mEPSC frequency (-6.86%, U=57, p=0.34; **Figure 4F**) nor mEPSC amplitude (-16.05%, U=54, p=0.26) were significantly altered by PKI (**Figure**

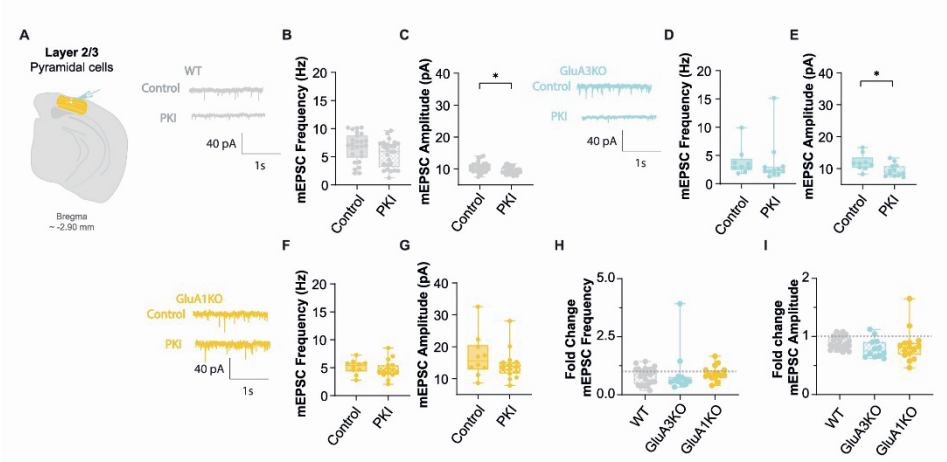


Figure 4. PKA is active under basal conditions in L2/3 neurons from V1. (A) Schematic representation of a coronal slice at ~ -2.90mm relative to bregma (adapted from Paxinos and Franklin atlas 2nd edition), showing the primary visual cortex and cortical layer included in the study. For all panels, colors indicate genotype: grey (WT), blue (GluA3KO) and yellow (GluA1KO). (B) miniature excitatory postsynaptic currents (mEPSCs) from layer 2/3 pyramidal WT neurons in the presence and absence of PKI (control n=26 cells, PKI n=25 cells). (C) Same as B but for mEPSC amplitude (control n=26 cells, PKI n=25 cells). (D,E) Same as B,C but for GluA3KO (control n=9 cells, PKI n=12 cells). (F,G) Same as B,C but for GluA1KO (control n=10 cells, PKI n=15 cells). (H) Fold change of mEPSC frequency and (I) mEPSC amplitude in the presence of PKI compared to their control conditions in WT (n=25 cells), GluA3KO (n=12 cells) and GluA1KO mice (n=15 cells). Dashed line indicates value of 1. PKI= Protein Kinase A Inhibitor.

4G). However, the magnitude of PKI-induced changes did not significantly differ across genotypes neither for mEPSC frequency (H(3) =2.66, p=0.26; **Figure 4H**) nor amplitude (H(3)=4.89, p=0.086; **Figure 4I**). These results indicate that basal PKA activity keeps L2/3-V1 synapses in a potentiated state, largely irrespective of AMPAR subunit usage.

To investigate whether increasing cAMP levels can drive GluA1- and GluA3-dependent plasticity independently of PKA activity, we recorded mEPSC in WT neurons in the presence or absence of isoproterenol plus IBMX, with PKI continuously applied to the bath. In WT mice, isoproterenol plus IBMX increased both mEPSC frequency (+114.29%, U=12, p=0.001; **Figure 5A**) and amplitude (+17.67%, U= 24, p=0.020; **Figure 5B**) despite PKI inhibition. Similar increases in mEPSC frequency were observed in GluA3KO neurons (+60.10%, U=58, p=0.022; **Figure 5C**) although amplitude changes did not reach significance (+11.27%, U=67, p=0.061; **Figure 5D**). In GluA1KO neurons, both mEPSCs frequency (+41.81%, U=27.50 p=0.0004; **Figure 5E**) and amplitude (+12.21%, U=46, p=0.009; **Figure 5F**) were significantly increased. While fold changes in mEPSC frequency differed across genotypes (H(3)=6.254, p=0.043; **Figure 5G**), no significant differences were detected after correction for multiple comparisons. No differences were observed for the fold change of mEPSC amplitude (H(3)=1.454, p=0.483; **Figure 5H**). These findings indicate that, β-adrenergic-induced potentiation of synaptic transmission in L2/3-V1 neurons does not require PKA and is largely independent of AMPAR subunit composition. To assess whether Epac signaling is responsible for β-adrenergic-induced potentiation, we used the Epac inhibitor ESI-05 in GluA1KO slices, to isolate effects on GluA3-containing AMPARs. ESI-05 did not prevent the synaptic potentiation induced by isoproterenol and IBMX (**Figure S2A,B**), suggesting that this potentiation is also not Epac-dependent.

The notion that PKA is active under basal conditions may suggest that cAMP levels are tonically high in L2/3-V1 neurons, potentially at levels sufficient to maintain GluA3-containing AMPARs in an active state. Under such conditions, further increases in cAMP would be unlikely to produce additional GluA3-dependent potentiation. One possible explanation for elevated basal cAMP levels is low PDE activity in these neurons. Consistent with this idea, application of isoproterenol alone, in the absence of PDE-inhibitor IBMX, was sufficient to induce synaptic potentiation in L2/3-V1 neurons (**Figure S2C,D**). We also tested whether reducing cAMP levels by enhancing PDE activity would decrease synapse strength. Brain slices were incubated with MR-L2, a reported activator of PDE4, a PDE family involved in the hydrolysis of β-adrenergic-induced cAMP in the cortex [28,29]. However, MR-L2 failed to decrease mEPSC frequency or amplitude in either WT (**Figure S2E,F**) or GluA3KO slices (**Figure S2H,I**), indicating that PDE4 activation by MR-L2 was insufficient to alter synaptic currents.

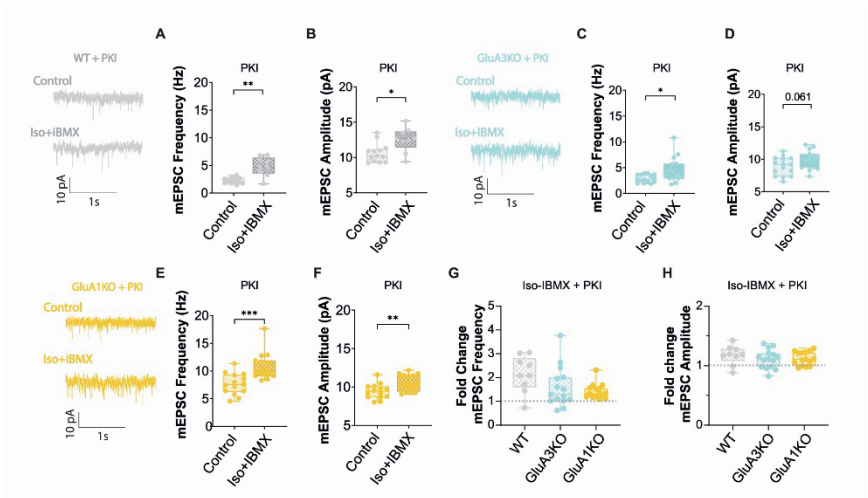


Figure 5. Blocking PKA does not prevent potentiation of L2/3-V1 synaptic transmission upon β-adrenergic activation. For all panels, colors indicate genotype: grey (WT), blue (GluA3KO) and yellow (GluA1KO). (A) Frequency of miniature excitatory postsynaptic currents (mEPSCs) in the presence and absence of Iso+IBMX and in continuous incubation of PKI in WT mice (control n=13 cells, Iso+IBMX n=9 cells). (B) Same as A but for mEPSC amplitude. (C,D) Same as A, B but for GluA3KO (control n=15 cells, Iso+IBMX n=15 cells). (E,F) Same as A,B but for GluA1KO (control n=14, Iso+IBMX n=15). (G) Fold change of mEPSC frequency and amplitude (H) in the presence of Iso+IBMX and incubated with PKI, compared to their control conditions (only PKI) in WT (n=9 cells), GluA3KO (n=15 cells) and GluA1KO mice (n=15 cells). Iso= isoproterenol; PKI= Protein Kinase A Inhibitor.

Basal synaptic transmission in layer 2/3-V1 somatostatin neurons is independent of GluA3 and not potentiated by β-adrenergic activation

Having described that basal synaptic transmission in L2/3-V1 pyramidal cells of V1 is dependent on GluA3-containing AMPARs (**Figure 1**), we investigated whether this AMPAR subunit also contributes to excitatory transmission onto inhibitory neurons, concretely, in SST+ neurons, since this group of interneurons are abundant in V1, specifically in layer 2/3, and perform a key role in visual processing [4,7]. To compare synaptic currents in SST+ neurons with or without the GluA3 AMPAR subunit, we used AAV-mediated gene transfer express Cre-recombinase selectively in SST+ neurons in V1 of 'floxed' GluA3KO mice or control (WT) mice. mEPSCs were then recorded from Cre-expressing neurons (**Figure 6A**).

Both mEPSCs frequency (-15.78%, U=89, p=0.700; **Figure 6B**) and amplitude (+17.67%, U=62, p=0.103; **Figure 6C**) did not significantly differ between WT SST+ and GluA3KO SST+ neurons, indicating GluA3-expression is not required to maintain synaptic strength onto SST+ neurons in V1. Next, we investigated whether β-adrenergic activation leads to GluA3-dependent plasticity in SST+ neurons. To test this, mEPSCs were recorded in both WT- and GluA3KO-SST+ neurons in the presence or absence of isoproterenol and IBMX. In V1-SST+ neurons of WT mice, isoproterenol plus IBMX did not affect mEPSC frequency (-12.07%, U=71, p=0.349; **Figure 6D**) nor amplitude (+5.95%, U=81, p=0.650; **Figure 6E**). Similarly, SST+ neurons lacking the GluA3 subunit did not show any significant differences in mEPSC frequency (+16.18%, U=62, p=0.975, **Figure 6F**) or amplitude in the presence of isoproterenol and IBMX (-6.76%, U=55, p=0.643; **Figure 6G**). These results demonstrate that GluA3-containing AMPARs

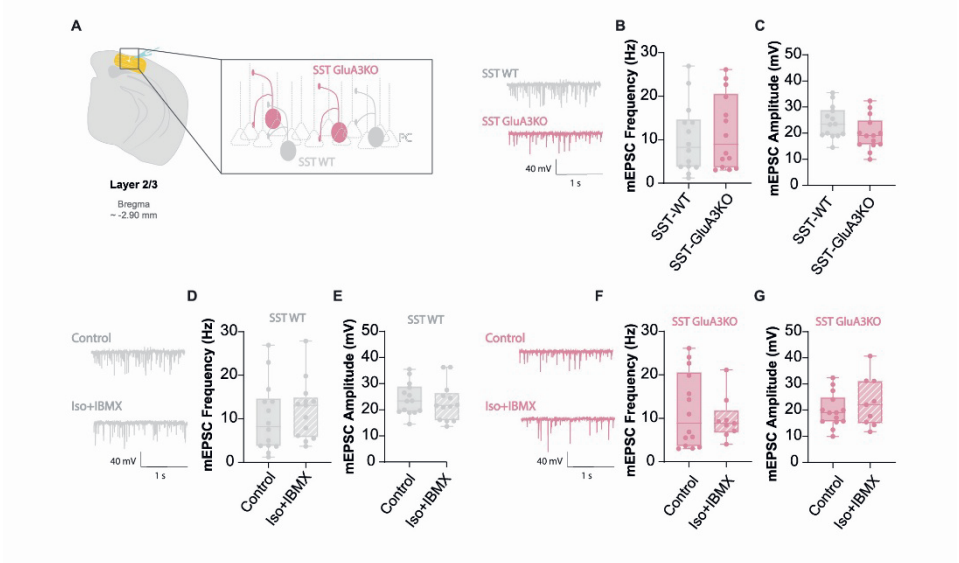


Figure 6. Basal synaptic transmission in V1-L2/3 somatostatin neurons is independent of GluA3 and not potentiated by β-adrenergic activation. (A) Schematic representation of a coronal slice at ~ -2.90mm relative to bregma (adapted from Paxinos and Franklin atlas 2nd edition), showing the primary visual cortex and cortical layer included in the study. SST neurons are filled, either in grey (WT, GluA3 containing) or pink (GluA3KO) while pyramidal cells are represented in dashed lines. (B) Frequency of miniature excitatory postsynaptic currents (mEPSCs) from layer 2/3 neurons either in SST-WT (n=14 cells) or GluA3KO-SST neurons (n=14 cells). (C) mEPSCs amplitude in SST-WT (n=14 cells) and SST-GluA3KO (n=14 cells). (D,E) Example traces, mEPSC frequency and mEPSC amplitude (E) in the presence and absence of isoproterenol and IBMX in WT-SST neurons (control n=14 cells, Iso+IBMX n=13 cells). (F,G) Same as D,E but for GluA3KO-SST neurons (control n=14 cells, Iso+IBMX n=9 cells).

are not involved in mediating basal synaptic transmission or β-adrenergic activation of L2/3-V1 SST+ neurons in V1.

Discussion

In this chapter, we investigated the role of AMPAR subunits GluA1 and GluA3 in synaptic transmission within layer 2-3 of the primary visual cortex. Our results show that 1) in L2/3-V1 pyramidal cells, fast excitatory transmission depends on both GluA3- and GluA1-containing AMPARs, with a greater contribution from GluA3; 2) β-adrenergic activation potentiates basal synaptic transmission and this process is independent of AMPAR subunit composition; 3) PKA is active under basal conditions and does not mediate β-adrenergic-induced synaptic potentiation; and 4) GluA3-containing AMPARs do not contribute to basal synaptic transmission in SST+ neurons, nor does β-adrenergic activation potentiate glutamatergic synaptic transmission in these cells. Together, these findings highlight the role of GluA3-containing AMPARs in synapse physiology of L2/3-V1 pyramidal cells and provide new insights into the mechanisms underlying synaptic transmission in this brain region.

AMPARs have long been recognized as relevant players in visual processing and synaptic transmission within V1 [11,30]. Despite GluA3-containing AMPARs recently gaining attention in various fields of neuroscience [31–34], to our knowledge, no studies have specifically examined the role of this AMPAR subunit in visual processing. A central outcome of this study is the contribution of both AMPAR subunits, GluA3 and GluA1, to basal synaptic transmission, which suggests that the far majority of AMPAR currents at L2/3 synapses of pyramidal neurons are mediated by GluA1/2s and GluA2/3s. GluA1-containing AMPARs only traffic into synapses of cortical neurons upon experience-dependent plasticity, i.e. when LTP-like activity is triggered [15,16]. In the visual cortex, GluA1KO mice were shown to be particularly impaired in ocular dominance (OD) plasticity during the critical period [35]. This implies that LTP-like plasticity will mainly take place during the critical period, and that the contribution of

GluA1 may be more pronounced during this period in comparison to the mature, post-critical period stage. In contrast to GluA1-containing AMPARs, synaptic trafficking of GluA3-containing AMPARs to synapses is independent of activity, and as a consequence, synapses become more enriched for GluA2/3s in the absence of sensory experience, possibly replacing GluA1-containing AMPARs at synapses [36,37]. GluA1-containing AMPARs in L2/3 of V1 are responsive to visual experience in a layer- and depth-specific manner, suggesting that GluA1-containing AMPARs localized in basal versus apical dendrites may contribute differently to visual-dependent transmission [38]. Although we did not examine the specific contribution of GluA3- and GluA1-containing AMPAR in spatially segregated synapses, we found that the relative contribution of GluA1 and GluA3 are similar at synapses when stimulating either vertical or horizontal pathways in L2/3 of the visual cortex.

We here report a decrease in synaptic transmission at L2/3-V1 neurons when PKA activity was blocked, indicating that PKA is active under basal conditions in these neurons from acutely isolated brain slices. This is in contrast to CA1 neurons of the hippocampus, where PKA inhibition has no effect on basal synaptic transmission [23]. In hippocampal and cortical neurons, PKA interacts with MAP2, a microtubule-associated protein localized at dendrites and part of the A-kinase anchoring proteins (AKAPs) [39]. As a consequence, PKA is largely excluded from spines in CA1 neurons [39]. Upon a rise in cAMP, it is released from MAP2 and moves into spines of CA1 neurons [39]. At spines of L2/3 pyramidal neurons of the somatosensory cortex, both in acute slices and in vivo, PKA is similarly enriched at dendrites, but appears to be relatively more located at spines in comparison with CA1 neurons [39]. It will be interesting to examine to what extent PKA is localized at spines of L2/3-V1 neurons under basal conditions and how this localization changes upon a rise in cAMP.

PKA-phosphorylation of GluA1 increases its levels at the cell surface, facilitating its incorporation into synapses upon the induction of LTP [40–42]. In L2/3 neurons of V1, PKA phosphorylation of GluA1 is sufficient to deliver GluA1-containing AMPARs to synapses, even in the absence of LTP induction [21]. Thus, basal PKA activity may maintain a proportion of GluA1-containing AMPARs in a phosphorylated state, keeping synapses in a relatively potentiated state. Consistent with this idea, blockade of PKA would be expected to reduce the pool of phosphorylated GluA1 available for synaptic incorporation, thereby decreasing postsynaptic strength, as observed in our recordings.

There are two likely explanations for PKA being active at rest. First, PKA may be enriched at dendritic spines of L2/3-V1 neurons through anchoring by AKAPs, in contrast to what has been reported in the hippocampus [39]. This possibility raises the question of whether PKA is preferentially localized to spines of L2/3-V1 neurons under

basal conditions. A second explanation is that cAMP levels are constitutively high in L2/3 neurons from the primary visual cortex. Given that cAMP can increase the channel conductance of GluA3-containing AMPARs independently of PKA activity [23,24], elevated basal cAMP levels could maintain these receptors in a high-conductance state, thereby contributing to synaptic strength. Future work employing genetically-encoded cAMP and PKA sensors [43,44] will be important to test these possibilities and further clarify the regulation of GluA3-containing AMPARs in L2/3-V1 pyramidal neurons.

We observed that the pharmacological activation of β-adrenergic receptors markedly increased mEPSC frequency, yet had no detectable effect on evoked EPSCs of L2/3-V1 neurons. One possible explanation for this discrepancy is methodological: while mEPSCs reflect transmission across the entire population of synapses, evoked EPSCs sample only subsets of input, those stimulated by the electrode. However, this scenario appears unlikely, as we consistently failed to detect potentiation when stimulating inputs from either layers 4, layers 2/3 or layer 1. Thus, it is improbable that β-adrenergic–induced potentiation occurred selectively at synapses that were not sampled. Spontaneous release has been proposed to contribute to the homeostatic regulation of synaptic strength [45], and accumulating evidence indicates that spontaneous and evoked transmission are regulated by partly distinct molecular signaling cascades and are partially segregated within presynaptic terminals [45,46]. Consistent with this idea, presynaptic NMDARs have been shown to control evoked vesicle release but not spontaneous vesicle release [47], underscoring the differential regulation of these two transmission modes. Interestingly, postsynaptic AMPARs can respond differentially to spontaneous versus evoked neurotransmission[48], with GluA2-lacking AMPARs preferentially contributing to spontaneous events. In our experiments, however, the absence of GluA1 or GluA3 did not differentially affect spontaneous and evoked EPSCs before or after β-adrenergic stimulation, suggesting that GluA1/2- and GluA2/3-containing AMPARs do not selectively couple to either release mode in L2/3-V1 neurons.

We show that, unlike in CA1 hippocampal synapses, β-adrenergic-induced synaptic potentiation is not mediated by PKA. While PKA is well established as a key player in β-adrenergic-induced plasticity, particularly LTP in the hippocampus [49], studies in cortical areas such as the PFC and perirhinal cortex have reported that β-adrenergic-induced synaptic potentiation is not prevented by PKA inhibition [50,51]. This supports the idea that β-adrenergic-mediated plasticity is regulated differently in the cortex and the hippocampus. We also observed that inhibition of Epac did not prevent β-adrenergic-induced synaptic potentiation in L2/3-V1 neurons. Further experimentation

may reveal whether this potentiation may be driven by a different cAMP-dependent pathway [23] or is independent of cAMP signaling [52].

In this chapter we also examined the contribution of GluA3-containing AMPARs to synaptic transmission in L2/3 SST neurons of the V1. We found that the absence of GluA3 did not alter basal synaptic transmission. Although the *GRIA3* transcript, which encodes GluA3, has been detected in V1 SST neurons (Allen Cell Types Database, VISp Patch-seq dataset, 2018), our results indicate that GluA3 expression is not required for maintaining synaptic currents onto these neurons. However, previous studies did not resolve layer specificity, and there is currently no direct evidence for the presence of GluA3 in L2/3 V1-SST neurons. We cannot exclude the possibility that GluA3-containing AMPARs play a functional role in SST neurons from L4, as SST has been shown to enhance orientation selectivity and L4 represents the first layer to receive information from the LGN [4,7]. In addition to basal transmission, we assessed the effects of β-adrenergic activation in L2/3 SST neurons and the potential involvement of GluA3-containing AMPARs. Despite the reported expression of β-adrenergic receptors in V1-SST neurons [53], activation of these receptors did not induce synaptic potentiation in either WT or GluA3KO SST neurons. These results suggest that GluA3-containing AMPARs do not contribute to basal synaptic transmission in L2/3-SST neurons and that β-adrenergic receptors may not be operational at synapses. Supporting this notion, only one recent study has demonstrated a functional role for β-adrenergic receptors in SST neurons, although focused on β3-adrenoceptors in the prefrontal cortex [54].

Altogether, these results reveal a prominent role of GluA3 to synapse physiology of L2/3 pyramidal neurons in the primary visual cortex. Future studies may reveal how plasticity of GluA3-containing AMPARs contributes to visual processing, such as in ocular dominance plasticity and visual acuity.

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Supplementary Figures

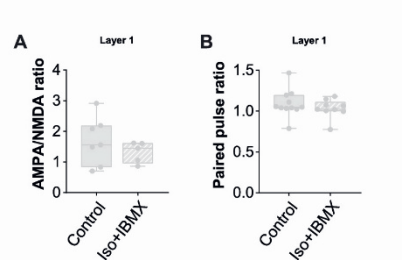


Figure S1. Evoked transmission is not affected by β-adrenergic activation upon stimulation of layer 1. (A) AMPA/NMDA ratio in the presence and absence of Iso+IBMX upon L1 stimulation in WT slices (control n=7 cells, Iso+IBMX n=5 cells). β-adrenergic activation did not alter AMPA/NMDA ratio (U=14, p=0.638). (B) PPR in the presence and absence of Iso+IBMX upon L1 stimulation in WT slices (control n=11 cells, Iso+IBMX n=9 cells). β-adrenergic activation did not alter PPR (U=34, p=0.261). PPR= Paired-pulse ratio.

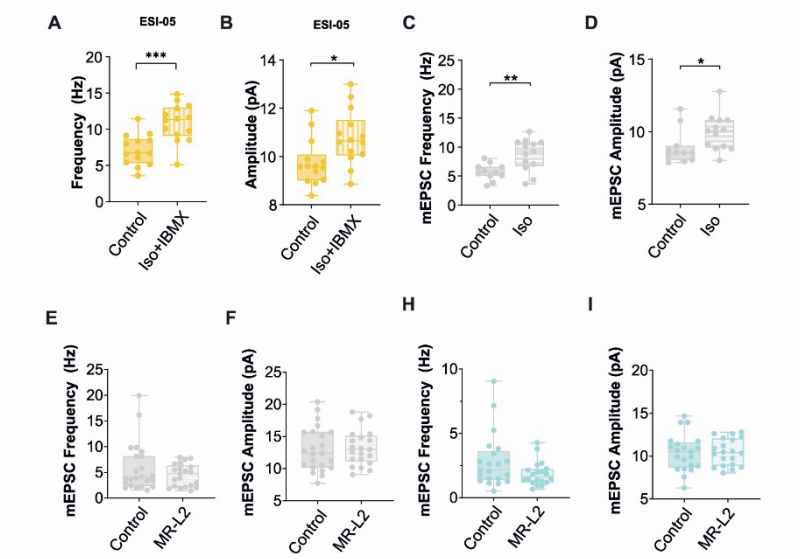
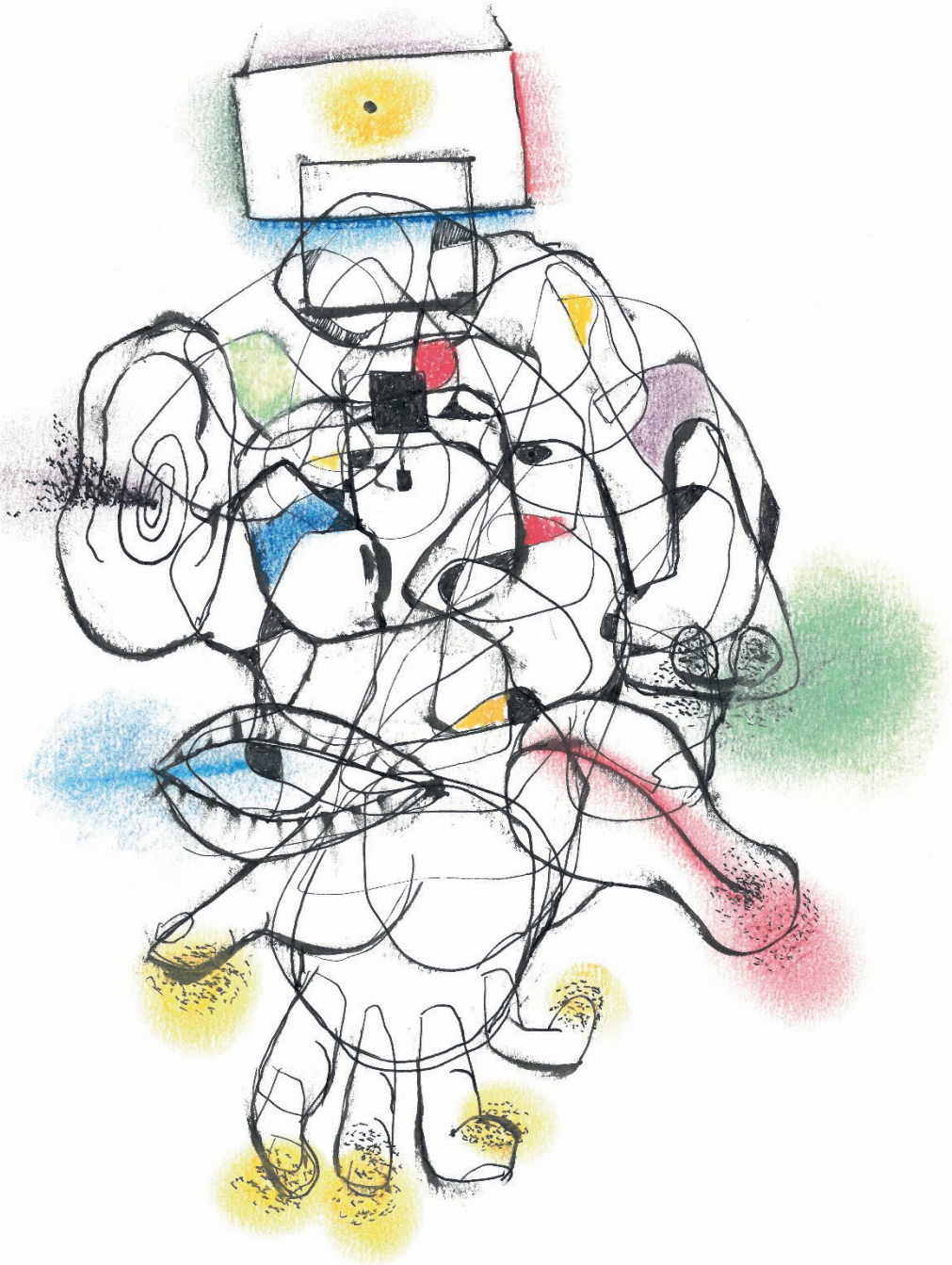


Figure S2. Synaptic transmission in V1-L2/3 pyramidal neurons upon different pharmacological approaches for modulation of cAMP levels. For all panels, colors indicate genotype: grey (WT), blue (GluA3KO) and yellow (GluA1KO). (A) mEPSC frequency in the presence and absence of isoproterenol in WT mice (control n=11 cells, Iso n=13 cells). Iso potentiated mEPSC frequency (U=26, p=0.007). (B) Same as A but for mEPSC amplitude (control n=11 cells, Iso n=13 cells). Iso increased mEPSC amplitude (U=31, p=0.018). (C) Same as A but in the presence of MR-L2 (control n=23 cells, MR-L2 n=21 cells). MR-L2 did not affect mEPSC frequency (U=214, p=0.529). (D) Same as C but for mEPSC amplitude (control n=23 cells, MR-L2 n=21 cells). MR-L2 did not affect mEPSC amplitude (U=226, p=0.721). (E,F) Same as C,D but in GluA3KO mice (control n=21 cells, MR-L2 n=20 cells). Neither mEPSC frequency (U=150, p=0.121) nor mEPSC amplitude (U=200, p=0.806) were affected by MR-L2. (H) mEPSC frequency in the presence of Iso+IBMX and continuous incubation of ESI-05 (control n=14 cells, Iso+IBMX n=14 cells). The increase in mEPSC frequency was not prevented by ESI-05 (U=24, p=0.0003). (I) Same as H but for mEPSC amplitude (control n=14 cells, Iso+IBMX n=14 cells). The increase in mEPSC amplitude was not prevented by ESI-05 (U=45, p=0.014).



Chapter 3

Consequences of GluA3 subunit loss
on mPFC synaptic transmission and behavioral outcomes

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Abstract

Fast synaptic signaling and cognitive flexibility are essential for adaptation to changing environments, processes that depend on proper PFC development. While AMPARs have been previously studied in the context of excitatory transmission and reversal learning, the specific contribution of GluA3-containing AMPARs to medial prefrontal cortex (mPFC) physiology and neuromodulator-induced plasticity remains incompletely understood. This chapter, therefore, examines the role of GluA3-containing AMPARs in excitatory synaptic transmission across distinct stages of mPFC development, evaluates their contribution to β-adrenergic modulation, and investigates whether loss of this subunit affects behavioral flexibility. Using whole-cell patch-clamp recordings from layer 2/3 pyramidal neurons in acute mPFC slices, we show that excitatory synaptic transmission undergoes marked developmental refinement from early adolescence to adulthood and that GluA3KO neurons exhibited an overall lower mEPSC frequency. In addition, β-adrenergic activation potentiated synaptic transmission in L2/3-mPFC neurons independently of AMPAR subunit composition. Finally, behavioral assessment revealed that GluA3KO male mice exhibit intact discrimination learning but age-independent deficits in reversal learning. Together, these findings identify GluA3-containing AMPARs as important contributors to basal synaptic transmission in layer 2/3 neurons of the mPFC and demonstrate that loss of GluA3 results in impairments in cognitive flexibility.

Introduction

In continuously changing environments, organisms must rapidly adapt their behavior to ensure survival. Fast synaptic signaling is therefore essential for efficient information processing in the brain. One of the primary mediators of rapid excitatory transmission are α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs). These ionotropic glutamate receptors are composed of four different subunit types: GluA1 to GluA4 and are most often found as heterodimers, although homomers have also been described [1]. In the cortex, GluA2 is the predominant subunit (~45%), with similar levels of GluA1 and GluA3 (~21% and ~27% respectively) and a minor portion of GluA4 (~7%) [2], which is in line with the notion that the majority of AMPARs consist of either GluA1/GluA2 or GluA3/GluA2 combinations. The presence of the GluA2 subunit renders the AMPARs calcium-impermeable, restricting ion flow primarily to Na^+ and K^+ [3,4]. Notably, the expression of AMPAR subunits follows a developmental trajectory. During the second postnatal week, there is a brain-wide increase in GluA2 expression, coinciding with the incorporation of this subunit into AMPARs [5,6]. In the mPFC, this peak in GluA2 expression levels occurs at the beginning of the third week and rapidly declines afterwards [7]. In addition, mRNA levels of both GluA1 and GluA3 in the frontal cortex peak between the second and third postnatal weeks before declining into adulthood, emphasizing the dynamic regulation of AMPAR composition [5].

An important modulator of AMPAR-mediated transmission is noradrenaline, acting through the activation of adrenergic receptors. Adrenergic receptors are G-protein-coupled receptors divided into three different subtypes, β, α_1, and α_2 [8]. Upon activation, β-adrenergic receptors, coupled to Gs proteins, increase cAMP levels by activating adenylyl cyclases [8]. cAMP then binds to downstream effectors such as protein kinase A (PKA) or exchange proteins activated directly by cAMP (Epac), initiating signaling cascades [9]. For instance, PKA-mediated phosphorylation of GluA1 increases surface expression of GluA1-containing AMPARs, thereby facilitating long-term potentiation (LTP) and inhibiting long-term depression (LTD) [10,11].

Compared with GluA1-containing receptors, GluA3-containing AMPARs have received considerably less attention, likely because they contribute minimally to canonical long-term potentiation, in contrast to GluA1-containing AMPARs [12]. Nevertheless, previous work from our lab demonstrated that at CA1 synapses in the hippocampus, β-adrenergic activation induces GluA3-mediated synaptic potentiation [13]. Similarly, in cerebellar Purkinje cells, a rise in cAMP increases the channel conductance of GluA3-containing AMPAR at synapses [14], underscoring the relevance of this receptor subunit for synaptic physiology. In addition, recent studies have implicated GluA3 in several pathological conditions, including developmental disorders, epilepsy, and

aggressive behavior [15–18]. Magri and colleagues also reported evidence of an association between the X-linked *GRIA3* gene, which encodes the GluA3 subunit, and schizophrenia (SCZ) in female patients [19]. More recently, *GRIA3* was identified among a set of 10 genes carrying ultra-rare variants that confer substantial risk for SCZ, primarily through loss-of-function or high-impact missense variants [20].

The prefrontal cortex (PFC) is a key brain region involved in higher-order cognitive processes, including cognitive flexibility [21,22], a highly important behavior for adaptation to changing environments. Within the PFC, layer 2/3 circuits have been shown to play a critical role in reversal learning, particularly in the encoding and categorization of learnt rules [23]. Interestingly, the PFC exhibits a protracted maturation timeline compared with sensory cortical areas, extending from embryonic stages through early adulthood in both humans and rodents [24]. Nevertheless, similar developmental programs operate in both PFC and sensory cortices [25–27] (see **Chapter 1, Figure 1**).

In this context, this chapter aims to determine the role of GluA3-containing AMPARs in synaptic transmission across different stages of PFC development, as well as their contribution to β-adrenergic-induced plasticity. In addition, we investigate whether loss of GluA3-containing AMPARs leads to behavioral impairments in a reversal learning task. We first analyze excitatory synaptic transmission in layer 2/3 pyramidal neurons of the mPFC across development, revealing that the absence of GluA3 results in reduced excitatory synaptic transmission in these neurons, and suggesting a role for this subunit in synapse maturation. Synaptic transmission followed a developmental trajectory characterized by higher mEPSC frequency and amplitude during early adolescence, which declined into adulthood. We further show that, similar to the visual cortex, β-adrenergic activation induces synaptic potentiation, although this mechanism appears to have a presynaptic origin in the mPFC. Finally, we assess whether the absence of GluA3-containing AMPAR leads to behavioral alterations in reversal learning and identify specific deficits in reversal, but not discrimination, performance. Together, these findings highlight GluA3-containing AMPARs as important contributors to mPFC circuitry, with implications for PFC-dependent behavior.

Materials & Methods

Animals

Gria3-deficient (GluA3KO) mice (*Gria3tm1Dgen/Mmnc;* Mutant Mouse Regional Resource Center, Davis, CA) and their wild-type littermates of both sexes were used in

this study. Gria1-deficient mice (GluA1KO), maintained on a C57BL6/129 hybrid background, were kindly provided by R. Huganir (Johns Hopkins University, Baltimore) (Kim et al., 2005). Animals ranged from 2 to 33 weeks of age at the time of testing. Mice were group-housed under standard laboratory conditions (12-h light/dark cycle; lights on 7:00-19:00) with ad libitum access to food and water, unless otherwise specified. Body weight was monitored once throughout the experimental period. For behavioral testing in the Phenotyper (Noldus, the Netherlands), mice were single-housed one week before the start of the test to allow for habituation.

All experiments were conducted in accordance with the European guidelines for the care and use of laboratory animals (European Directive 2010/63/EU). The experimental protocol was approved by the Animal Experiment Committee of the Royal Netherlands Academy of Arts and Sciences (KNAW) and by the Animal Welfare Body of the University of Amsterdam (permit number AVD11100202114907).

PhenoTyper

The PhenoTyper (Noldus) is an automated home-cage monitoring system equipped with a shelter, water bottle, food dispenser, and a cognition wall. Mice were individually housed in PhenoTyper cages for the entire duration of the test period (7 days) and weight was monitored before and after this period. During the initial habituation phase, that lasted 2.5 days, water and regular chow were provided ad libitum and basal behavior was recorded. Thereafter, regular food was removed from the feeding compartment. At this time (end of the third day) the cognition wall was introduced in the cage and the cognitive testing started. The cognition wall consisted of three entry holes and the mouse passage through each entry was monitored (Figure 1A). Mice were habituated to the cognition wall for 1 hour and 3 free rewards (Dustless Precision Pellets, 14 mg, Bio-Serve) were delivered to facilitate familiarization before the start of the experiment. The multiple-choice reversal learning task consisted of two phases. In the first phase, animals were trained in a discrimination learning task, where only one of the three holes was rewarded. Following 48 hours of discrimination learning, the reward allocation was changed to a different hole, initiating the reversal learning phase, which lasted for 48 hours. During discrimination and reversal learning, one sugar pellet was delivered for every fifth entry through the correct hole. The five entries were not required to be consecutive [28].

Learning during the discrimination and reversal learning tasks was quantified as the total number of entries to reach an 80% or 90% correct performance criterion, calculated using a sliding window of 30 consecutive entries (i.e. at least 24 correct responses out of the last 30 entries for 80%) [28]. The 80% criterion has previously been shown to exceed chance performance and is therefore commonly used as the minimal

criterion to indicate successful learning in the Phenotyper task [28]. In addition, a more stringent 90% criterion was included to assess performance under higher task demands and to reveal more subtle differences in learning and behavioral flexibility. For Kaplan-Meier survival analysis, a cut-off was set to 800 and 1700 entries for reaching the criterion in discrimination and reversal learning, respectively. All entries were automatically registered by the EthovisionXT11 software and video was recorded during the entire course of the experiment. Data was analyzed off-line using AHCODA-DB [29].

Acute slice preparation

Mice were killed using cervical dislocation at 2-3 weeks, 4-5 weeks, 9-10 weeks or >4 months old for acute slice preparation. Brains were removed and placed in ice-cold modified artificial cerebrospinal fluid (mACSF, in mM: 139 choline chloride, 3.5 KCl, 0.5 $CaCl_2$, 6.0 $MgSO_4$, 1.25 NaH_2PO_4 H_20, 10 glucose and 25 $NaHCO_3$, continuously bubbled with 5% CO2/ 95% O2 (pH 7.4)). Coronal slices (300 µm) containing the mPFC (approximately 1.94 mm anterior to Bregma) were obtained using a vibrating blade microtome (VT 1200S, Leica Microsystems). Slices were subsequently incubated at 35°C for 20 minutes in ACSF (in mM: 120 NaCl, 3.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4 H_20, 10 glucose, 2.5 $CaCl_2$, and 1.3 $MgCl_2$, bubbled with 5% CO2/ 95% O2), and then kept for at least 30 min at room temperature before recording.

Ex-vivo patch-clamp recordings

Whole-cell voltage clamp recordings were made from layer 2-3 pyramidal neurons of the mPFC. During recordings, slices were submerged in ACSF at 28-30° Celsius, bubbled with 5 % CO2/95 % O2 and superfused at a rate of 1.8 ml/min. Glass recording pipettes were pulled from borosilicate glass (Science Products) and had a resistance of 2.3-3.5 MΩ (Raccess <30 MΩ and Rinput > 9x Raccess) when filled with intracellular solution, containing (in mM): 115 CsMeSO3, 20 CsCl, 10 HEPES, 2.5 MgCl2, 4 Na2-ATP, 0.4 Na-GTP, 10 sodium phosphocreatine, and 0.6 EGTA, at pH 7.25.

To isolate excitatory transmission, all recordings were made in the presence of 100 mM picrotoxin (Tocris). Where indicated, isoproterenol (10 µM; Sigma), IBMX (50 µM; Tocris) were added to the perfusion solution and incubated for 5 minutes.

Miniature excitatory post-synaptic currents (mEPSCs):

Cells were clamped at -60 mV (uncorrected for liquid junction potential) and 5 minutes recordings were made in the presence of 1 mM Tetrodotoxin (TTX, Latoxan, France) to

block spontaneous action potentials. Signals were acquired using a MultiClamp 700B amplifier controlled by pClamp 11 software (Molecular Devices), low-pass filtered at 3 kHz and sampled at 10 kHz. Series resistance ranged from 6–20 MΩ and was compensated to ~65 %. Frequency and amplitude were analyzed using *Clampfit* (Molecular Devices), and only events exceeding a 5 pA noise threshold were included in the analysis. For charge transfer analysis, the area under the curve was calculated for each event and averaged across all the events within a single recording [30].

Statistical analysis

All statistical tests were performed in GraphPad version 8 (GraphPad Software, San Diego). Data were statistically analyzed using unpaired t-tests, Kolmogorov-Smirnov tests or two-way ANOVA, as appropriate. Post-hoc multiple comparison was done by Tukey's or Dunn's multiple comparison tests. Survival curves were tested for significance with the log-rank (Mantel-Cox) test. Pearson correlation coefficient was used for correlations. Data are presented as bar plots with error bars representing the standard error of the mean (SEM), based on the distribution of the data. Statistical significance was considered as * $p<0.05$, ** $p<0.01$,*** $p<0.001$.

Results

Contribution of GluA3-containing AMPAR to the developmental trajectory of layer 2/3-mPFC excitatory synapses

To examine whether GluA3-containing AMPARs contribute to the strength and maturation of mPFC synapses of layer 2/3 pyramidal neurons , we performed whole-cell patch-clamp recordings from L2/3 pyramidal neurons in the infralimbic (IL) and prelimbic (PrL) subregions of the mPFC (L2/3-mPFC) in WT and GluA3KO mice (**Figure 1A**). Since synaptic remodeling takes place in the PFC across distinct developmental stages [31], we examined three time points relevant to PFC maturation according to previous literature [32–34]: early adolescence (4-5 weeks old), early adulthood or end of adolescence (9-10 weeks old), and late adulthood (older than 4 months). The first two stages capture the adolescent period, which is characterized by synaptic pruning and circuit remodeling [31], whereas the final stage reflects mature PFC physiology.

We first examined developmental changes in the frequency and amplitude of miniature excitatory postsynaptic currents (mEPSCs) in L2/3-mPFC neurons from GluA3KO and WT mice. Miniature EPSC frequencies showed a significant main effect of genotype ($F(1,193)=14.17$, $p<0.001$; **Figure 1B**), with GluA3KO mice exhibiting a lower frequency

than WT mice (19% reduction). In addition, mEPSC frequency showed a significant main effect of age ($F(2,193)=8.880$, $p<0.001$; **Figure 1B**) and post-hoc analyses revealed that mEPSC frequency was significantly higher during early adolescence compared with early adulthood ($p=0.029$; 21% reduction) and late adulthood ($p<0.001$; 33% reduction), with no significant difference between early and late adulthood ($p=0.162$; 6% reduction). The magnitude of change of mEPSC frequency relative to WT neurons did not differ across age groups ($F(2,80)=0.983$, $p=0.378$; **Figure 1C**), indicating that while the absence of GluA3 reduces synaptic events frequency, this effect is not age-dependent.

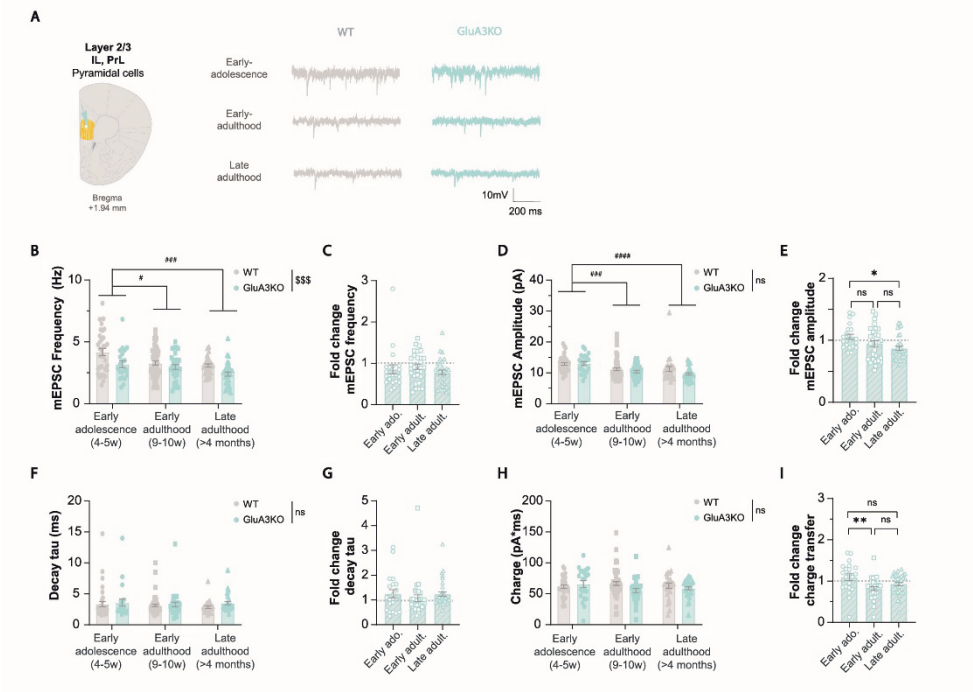


Figure 1. Developmental trajectory of L2/3-mPFC excitatory synapses in WT and GluA3KO male and female mice. (A) Left: schematic representation of a coronal slice at +1.94 mm relative to bregma (adapted from Paxinos and Franklin atlas 2nd edition), showing the IL and PrL regions and cortical layer 2/3 included in the study. Right: example traces of miniature excitatory postsynaptic currents (mEPSCs) from layer 2/3-mPFC pyramidal neurons of WT (left) and GluA3KO (right) male mice at three different ages. For all panels, colors indicate genotype: grey (WT) and blue (GluA3KO) and symbols indicate age groups: early adolescence (circles), early adulthood (squares) and late adulthood (triangles); mEPSCs were recorded from the same set of cells: early adolescence (WT n=35 from 5 mice; GluA3KO n=21 from 3 mice), early adulthood (WT n=54 from 6 mice; GluA3KO n=30 from 4 mice) and late adulthood (WT n=27 from 4 mice; GluA3KO n=32 from 4 mice). (B) mEPSC frequency of layer 2/3-mPFC pyramidal cells from WT and GluA3KO mice. (C) Fold-change of GluA3KO relative to WT for mEPSC frequency. (D,E) Same as B,C but for mEPSC amplitude. (F,G) Same as B,C but for decay tau. (H,I) Same as B,C but for charge transfer. For panels F and G, all events from a single recording were averaged per cell, and the resulting mean value per cell is shown in the graph. Symbols indicate main effects of genotype ($), age (#) or genotype-by-age interaction (*).

Average mEPSC amplitudes were similar between WT and GluA3KO neurons and did not show a significant genotype effect ($F(2,192)=2.759$, $p=0.098$; **Figure 1D**). Similar to mEPSC frequency, amplitudes showed a significant main effect of age ($F(2,192)=11.17$, $p<0.001$; **Figure 1D**), with early adolescence displaying higher mEPSC amplitudes compared to early adulthood ($p<0.001$, 17% reduction),and late adulthood ($p<0.001$, 23% reduction) and no differences between early and late adulthood ($p=0.886$, 3% reduction). The fold-change in mEPSC amplitude relative to WT differed across age groups ($F(2,80)=4.409$, $p=0.015$; **Figure 1E**), with a significant decrease between early adolescence and late adulthood ($p=0.010$; **Figure 1E**), suggesting that GluA3 may contribute to synaptic maturation across development. Overall, these results indicate that synaptic remodeling occurs primarily during adolescence and remains largely stable from early to late adulthood, consistent with the synaptic pruning characteristic of this developmental period. In addition, our findings indicate that GluA3 contributes to synaptic transmission and that it may participate in the maturation of L2/3-mPFC synapses.

To assess whether mEPSC properties changed across development and whether these changes were influenced by the absence of GluA3-containing AMPARs, we analyzed the average decay time of mEPSCs and the average charge transfer, which reflects the net influx of ions through AMPARs. Neither decay time (**Figure 1F,1G**) nor charge transfer (**Figure 1H**), showed significant changes based on genotype or age. However, we change in charge transfer relative to WT differed across age groups ($F(2,80)=5.76$, $p=0.004$; **Figure 1I**), showing a reduction between early adolescence and early adulthood that stabilized thereafter ($p=0.003$; **Figure 1I**).

Together, these results indicate that GluA3-containing AMPARs contribute to excitatory synaptic transmission in L2/3-mPFC neurons and suggest a potential role for this subunit during the transition from adolescence to adulthood, a developmental period characterized by a reduction in synaptic transmission consistent with synaptic pruning.

β-adrenergic-activation potentiates layer 2/3-mPFC excitatory synaptic transmission

In the hippocampus, GluA3-containing AMPARs have been shown to specifically mediate synaptic potentiation following β-adrenergic activation, despite their limited contribution to basal synaptic transmission [13]. In **Chapter 2**, we investigated whether a similar mechanism operates at L2/3 synapses in the primary visual cortex, a sensory cortical area. We found that β-adrenergic activation likewise potentiates

synaptic transmission in this region, although independently of AMPAR subunit composition. Here, we examined whether GluA3-containing AMPARs also contribute to β-adrenergic signaling in the mPFC, an association cortex involved in cognitive flexibility that receives adrenergic innervation [35,36]. To address this question, we performed whole-cell patch-clamp recordings of L2/3 pyramidal neurons in the IL and PrL areas of the mPFC from WT, GluA3KO, and GluA1KO mice at postnatal day (PND) 28 (**Figure 2A**). This age was selected to match the age examined in the visual cortex, thereby enabling direct comparison across brain regions. mEPSCs were recorded under baseline conditions and following β-adrenergic activation using isoproterenol, a β1/β2-adrenoreceptor agonist, and IBMX, a phosphodiesterase inhibitor that enhances cAMP signaling by preventing its degradation.

In L2/3-mPFC neurons from WT mice, β-adrenergic activation significantly increased mEPSC frequency (52% increase, t(20)=3.96, p=0.0008; **Figure 2B**). In GluA3KO neurons, we observed a non-significant trend toward an increase in mEPSC frequency following isoproterenol and IBMX application (48% increase, t(20)=2.006, p=0.058; **Figure 2C**), with no significant effects in GluA1KO neurons (36% increase, t(18)=1.469,

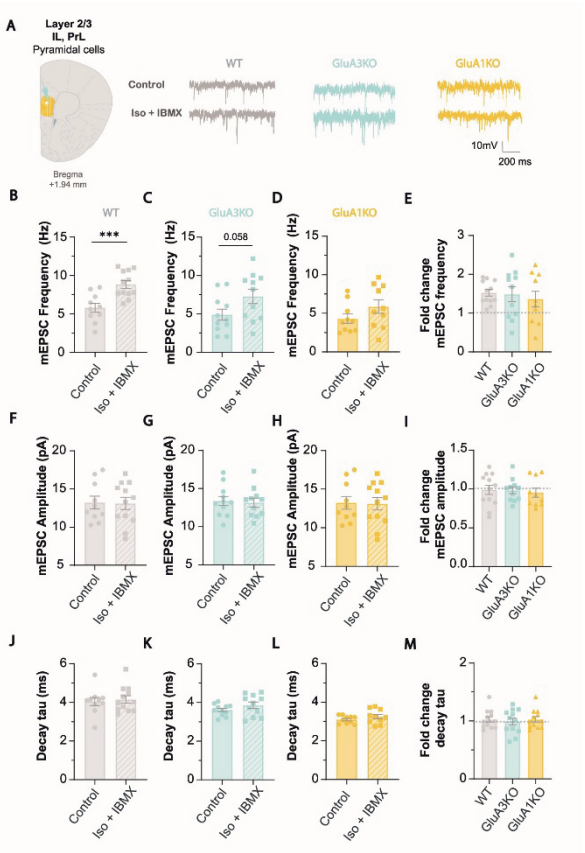


Figure 2. β-adrenergic-activation potentiates L2/3-mPFC excitatory synaptic transmission. A) Left: schematic representation of a coronal slice at +1.94 mm relative to bregma (adapted from Paxinos and Franklin atlas 2nd edition), showing the IL and PrL regions and cortical layer 2/3 included in the study. Right: example traces of miniature excitatory postsynaptic currents (mEPSCs) from layer 2/3 pyramidal neurons of WT (left), GluA3KO (middle) and GluA1KO mice (right) in the presence (bottom) and absence (top) of isoproterenol (Iso) and IBMX. For all panels, colors indicate genotype: grey (WT), blue (GluA3KO) and yellow (GluA1KO). mEPSCs were recorded from the same set of cells: WT control (n=10 from 4 mice), WT isoproterenol and IBMX (n=12 from 4 mice); GluA3KO control (n=11 from 3 mice), GluA3KO isoproterenol and IBMX (n=11 from 3 mice); GluA1 control (n=10 from 3 mice), GluA1 Isoproterenol and IBMX (n=10 from 2 mice). (B-D) mEPSC frequency of WT (B), GluA3KO (C) and GluA1 KO (D) mice in the presence and absence of isoproterenol and IBMX. (E) Fold change of mEPSC frequency in the presence of isoproterenol and IBMX for WT, GluA3KO and GluA1KO mice. (F-I) Same than B-E but for mEPSC amplitude. (J-M) Same than B-E but for decay tau.

p=0.159; **Figure 2D**). However, when comparing the magnitude of change relative to baseline across genotypes, no significant differences were detected (F(2,30)=3.302, p=0.262; **Figure 2E**), indicating that β-adrenergic stimulation induced a comparable overall potentiation in all three groups. Miniature EPSC amplitude remained unchanged in WT (1% decrease, t(20)=0.104, p=0.918; **Figure 2F**), GluA3KO (1% decrease, t(20)=0.253, p=0.802; **Figure 2G**) and GluA1KO mice (5% decrease, t(18)=0.551, p=0.588; **Figure 2H**), with fold changes close to one across conditions (F(2,30)=1.029, p=0.369; **Figure 2I**). Lastly, we examined whether β-adrenergic receptor activation affected AMPAR kinetics by analyzing mEPSC decay time. Decay tau was unchanged following drug application in all genotypes (WT: 3% decrease, t(20)=0.372, p=0.713; **Figure 2J**; GluA3KO: 7% decrease, t(20)=1.322, p=0.201; **Figure 2K**; GluA1KO: 5% decrease, t(18)=1.130, p=0.273; **Figure 2L**), and no differences in fold change were detected across groups (F(2,33)=0.859, p=0.432; **Figure 2M**).

Overall, these findings indicate that β-adrenergic activation potentiates excitatory synapses in layer 2/3 of the mPFC, irrespective of AMPAR subunit composition. Moreover, the selective increase in mEPSC frequency, in the absence of changes in amplitude or decay time, suggests that this potentiation is mediated primarily by presynaptic mechanisms.

GluA3KO mice show age-independent deficits in reversal learning

Having shown that GluA3KO mice exhibited reduced synaptic transmission onto L2/3 neurons of the mPFC (**Figure 1B**), we next asked whether this synaptic deficit is accompanied by behavioral alterations, particularly given that L2/3 from the mPFC is involved in reversal learning [23]. Previous studies have reported that GluA3KO mice do not exhibit deficits in spatial memory tasks despite alterations in aggressive behavior and motor function [37]. However, the contribution of GluA3-containing

AMPARs to higher-order cognitive processes, such as behavioral flexibility, remains unexplored. Therefore, we sought to determine whether GluA3KO mice display behavioral deficits in this cognitive domain. To address this question, male GluA3KO mice and their WT littermates were housed in an automated home-cage monitoring system that allows the assessment of basal behavior, spatial discrimination, and subsequent reversal learning over a period of 1 week (**Figure 3A**) [38]. Only male mice were included due to time constraints.

Measures of basal behavior, including total distance moved and time spent in the shelter, reflected a day-night cycle (**Figure 3B,C**). Whereas no significant differences were observed between GluA3KO and WT mice in total distance moved ($F(68,3128)=1.209$, $p=0.118$, **Figure 3B**), GluA3KO mice spent significantly less time in the shelter compared to WT littermates ($F(1,46)=21.04$, $p<0.0001$, **Figure 3C**), suggestive of decreased resting time.

Following 2.5 days of baseline monitoring, cognitive testing started. A wall containing three ports was placed in the cage, and mice were required to learn that entering the

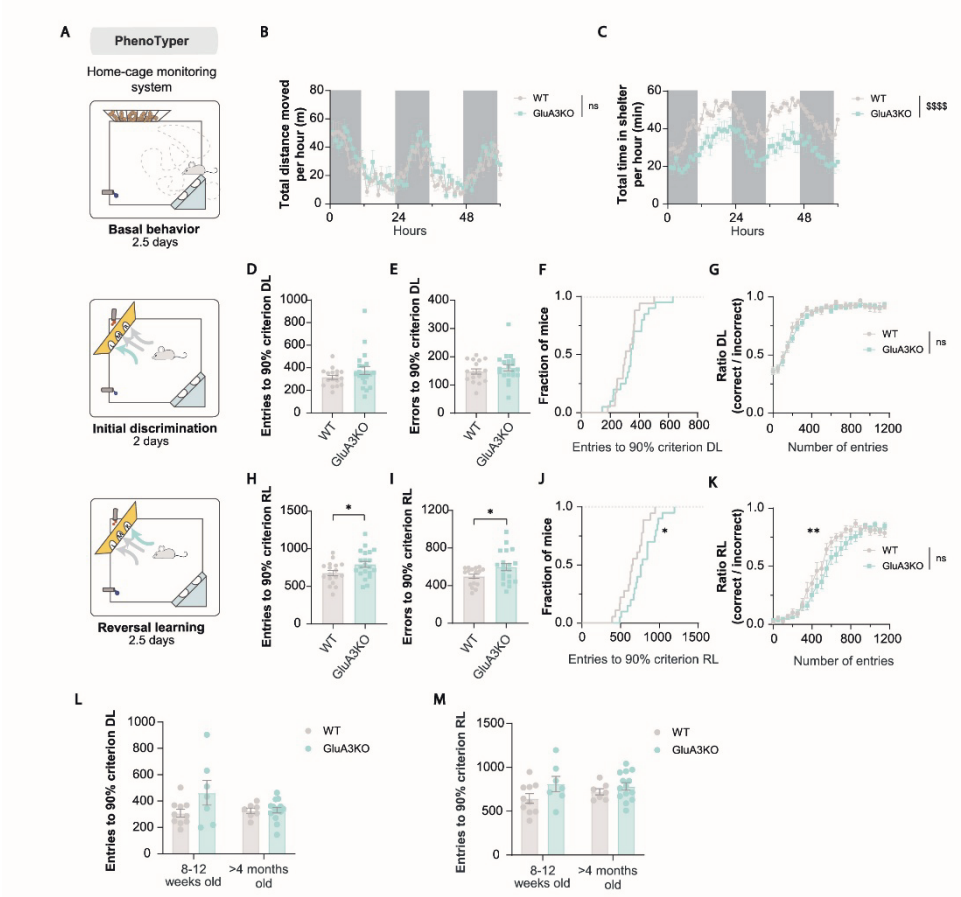


Figure 3. GluA3KO male mice show poorer performance in reversal learning. (A) Schematic representation of the PhenoTyper cage during basal behavior (top panel), initial discrimination (middle panel) and reversal learning (bottom panel). (A) Total distance moved and (B) time in the shelter per hour during basal behavior. WT= 24 mice (grey) ; GluA3KO= 24 mice (blue). GluA3KO mice spent less time in the shelter compared to their WT littermates. Symbols indicate genotype effect ($) or an interaction effect (*). Grey squares indicate the dark phases. For panels D-M: WT= 17 mice (grey), GluA3KO=21 mice (blue). (D) Number of entries and (E) errors to reach the 90% criterion during discrimination learning. (F) Survival plot showing the fraction of WT and GluA3KO mice that reached the 90% learning criterion as a function of the number of entries during discrimination learning. Each square represents one animal that reached the criterion. Dashed grey line indicates 1 (all animals reached the criterion). (G) Performance during discrimination learning as a function of the number of entries. For panels F-G symbols indicate genotype effect ($), number of entries effect (#) or an interaction effect (*). (H-I) same as D-E but for reversal learning. (J) Same as F but for reversal learning. (K) Same as G but for reversal learning. (L) Number of entries required to reach the 90% criterion during discrimination learning in WT and GluA3KO mice. Animals were grouped according to age at testing: young adulthood (8 weeks to 4 months; filled circles, WT n=10, GluA3KO n=7) and late adulthood (>4 months; open circles, WT n=7, GluA3KO n=14). (M) Same as L but for reversal learning. DL: Discrimination learning, RL: reversal learning.

left port 5 times resulted in the delivery of a sugar pellet (**Figure 3A**, middle). Discrimination learning was quantified as the number of entries required to reach the 90% performance criterion, defined as 90% correct choices within a sliding window of 30 consecutive entries. During the initial discrimination phase, WT and GluA3KO mice required a comparable number of entries ($t(36)=1.373$, $p=0.178$; **Figure 3D**) and committed a comparable number of errors to reach the 90% criterion ($t(35)=0.884$, $p=0.382$; **Figure 3E**). All animals successfully reached the criterion, doing so at a similar rate (log-rank test, $\chi^2(1)=1.41$, $p=0.235$; **Figure 3F**) and with overlapping learning curves (entries-by-genotype interaction $F(23,661)=0.791$, $p=0.745$; **Figure 3G**).

Having established that discrimination learning was unaffected in GluA3KO mice, we next assessed reversal learning over 2.5 days by switching the rewarding entry from the left- to the right-sided port (**Figure 3A**, bottom). GluA3KO mice required, on average, a greater number of entries to reach the 90% performance criterion ($t(36)=2.135$; $p=0.039$; **Figure 3H**) and committed more errors compared with WT mice ($t(35)=2.133$, $p=0.04$; **Figure 3I**). All animals ultimately reached this performance threshold, although GluA3KO mice required a higher number of entries to do so (log-rank test, $\chi^2(1)=5.984$, $p=0.0144$; **Figure 3J**). Furthermore, the learning curve of GluA3KO mice was shifted toward a higher number of entries ($F(23,787)=2.06$, $p=0.002$; **Figure 3K**), suggesting a slower learning rate despite reaching a similar maximal proportion of correct over incorrect responses (~80%).

Considering the variability in ages across the cohort (approximately PND50-PND200), we next examined whether age influenced performance in discrimination or reversal learning. Notably, attempts to conduct this task in adolescent mice (~5 weeks old)

were discontinued, as animals at this age were unable to acquire the initial discrimination task. The remaining cohort was therefore divided into two age groups: young adulthood (8 weeks to 4 months old) and late adulthood (> 4 months old). During initial discrimination learning, we observed no significant main effects of age or genotype on the number of entries required to reach the criterion (age: $F(1,34)=1.745$, $p=0.195$; genotype: $F(1,34)=3.433$, $p=0.072$; Figure 3L). Similarly, performance during reversal learning did not show significant effects of age ($F(1,34)=0.181$, $p=0.673$; Figure 3M) or genotype ($F(1,34)=3.971$, $p=0.054$; Figure 3M. Together, these findings indicate that adult GluA3KO mice exhibit deficits in reversal learning that are independent of age.

Discussion

This chapter aimed to determine the role of GluA3-containing AMPARs in synaptic transmission across different stages of PFC development, as well as their contribution to β-adrenergic-induced plasticity. In addition, we investigated whether loss of GluA3-containing AMPARs results in cognitive flexibility impairments. Our findings demonstrate that (1) excitatory synaptic transmission in mPFC-L2/3 pyramidal neurons partly relies on GluA3-containing AMPARs and undergoes developmental refinement during adolescence; (2) β-adrenergic activation does not induce GluA3-specific potentiation in these neurons; and (3) male GluA3KO mice exhibit age-independent deficits in reversal learning. Together, these results support a role for GluA3 in basal synaptic transmission in L2/3-mPFC pyramidal neurons and in reversal learning performance in male mice. In addition, our data suggests a role for this subunit in synaptic maturation while further emphasizing adolescence as a period of synaptic refinement during PFC development. More broadly, our findings identify GluA3-containing AMPARs as important contributors to mPFC circuit function and associated behavior.

In the first part of this chapter, we investigated the role of GluA3-containing AMPARs in excitatory synaptic transmission of layer 2/3 mPFC pyramidal neurons across development. Consistent with a previous study [15], we observed reduced synaptic transmission in L2/3-mPFC neurons of GluA3KO mice compared with WT controls. We found that after adolescence, mice exhibited a marked reduction in mEPSC frequency and amplitude that remained stable from early into late adulthood. The developmental decline in synaptic transmission is consistent with the synaptic pruning that characterizes adolescence, a period during which synapse number peaks and subsequently declines [31,39]. Our data also suggest a role for GluA3-containing AMPAR in synapse maturation. The decrease in fold change of mEPSC amplitude and

charge transfer across development suggests that GluA3 may be relevant during the transition from early adolescence to adulthood, a period during which a shift in AMPAR subunit composition could occur, with synaptic transmission becoming more dependent on GluA3-containing receptors after adolescence. Converging evidence indicates that glutamatergic transmission in the mPFC undergoes developmental refinement during adolescence. For instance, protein levels of the AMPAR subunit GluA2 peak around PND28 and decline into adulthood [7]. Similarly, mRNA levels of GluA1 and GluA3 in the frontal cortex show a transient increase between the second and third postnatal weeks before declining [5], underscoring the dynamic regulation of AMPAR expression and subunit composition during this developmental window. Other components of glutamatergic transmission are also subjected to developmental changes. NMDA receptor-mediated glutamate currents onto layer 5 pyramidal neurons increase from early adolescence (PND25-PND40) to late adolescence/adulthood (PND50-PND80), in a process dependent on postsynaptic PKA and D1 receptor signaling [40]. In humans, glutamate levels in the prefrontal cortex decrease during adolescence [41], further supporting an overall refinement of glutamatergic signaling during this period. As part of synaptic maturation, previous studies have described a developmental acceleration of AMPAR-mediated currents, termed "AMPA speeding", in regions such as the auditory cortex and cerebellum. This process is associated with faster AMPAR decay kinetics and improved temporal precision of excitatory transmission [30,42]. In cerebellar synapses, it is also accompanied by a reduction in charge transfer [30]. By contrast, we did not observe changes in decay tau or charge transfer across the ages examined, suggesting that AMPA speeding may not occur in L2/3-mPFC pyramidal neurons during the transition between adolescence and adulthood.

In the second part of this chapter, we examined the effect of β–adrenergic activation on synaptic transmission in L2/3 pyramidal neurons of the mPFC. Activation of β-adrenoreceptors increased mEPSC frequency, with no differences in the fold change of mEPSC frequency across genotypes, suggesting that this potentiation is independent of subunit composition in mPFC-L2/3 neurons. Notably, the increase in mEPSC frequency was not accompanied by changes in amplitude or AMPAR kinetics, strongly suggesting that β-adrenergic activation exerts mainly a presynaptic effect on synaptic transmission. In agreement with our findings, Ji and colleagues (2008) reported an increase in mEPSC frequency, but not amplitude, in L5/6-mPFC neurons following isoproterenol application [43]. The observed potentiation is consistent with results from **Chapter 2**, where β-adrenergic activation enhanced synaptic transmission in L2/3 neurons from the primary visual cortex, and with findings observed in CA1 hippocampal synapses [13]. In V1, similarly to the mPFC, β-adrenergic potentiation was independent of subunit composition (see **Chapter 2**),

while in the hippocampus, the potentiation was GluA3-mediated [13], underscoring region-specific mechanisms following β-adrenergic activation.

Having established that loss of GluA3-containing AMPAR impairs L2/3-mPFC synaptic transmission, the final part of this chapter examined whether GluA3KO mice display behavioral alterations in cognitive flexibility. Basal behavioral assessment in WT and GluA3KO male mice revealed normal locomotor activity. However, GluA3KO mice spent significantly less time in the shelter than WT littermates, which may reflect an altered need for rest. GluA3KO mice showed intact discrimination learning but impaired reversal performance, requiring a higher number of entries and committing more errors. Importantly, these deficits were independent of age within the adult cohort tested. Although we initially attempted to perform the task in adolescent mice, these experiments were discontinued because animals at this age failed to acquire the discrimination phase. Therefore, the observed age-independence applies specifically to adulthood, as only adult mice were able to successfully engage in the paradigm. Notably, our electrophysiological data revealed pronounced refinement of L2/3-mPFC synaptic transmission during early adolescence, a developmental window preceding the age at which mice reliably acquired the task. This temporal sequence suggests that maturation of excitatory synaptic transmission in L2/3-mPFC neurons may represent a prerequisite for successful task performance. In this context, discrimination and reversal learning abilities may follow a developmental threshold: before adolescence is completed, mice are unable to perform the task, whereas once this functional maturation is achieved, behavioral performance remains relatively stable and age-independent throughout adulthood. However, this hypothesis contrasts with findings from Johnson and Wilbrecht (2011), who reported successful learning in juvenile mice (4 weeks old) in an odor-based discrimination and reversal task [44].

Other studies have also implicated AMPAR function in reversal learning. Mouse lines with disrupted AMPAR endocytosis or impaired GluA1 ubiquitination exhibit reversal learning deficits [45,46], highlighting the importance of proper AMPAR trafficking for behavioral flexibility. Although these studies focused on AMPAR dynamics rather than subunit composition, they support the broader notion that intact AMPAR function contributes to the synaptic mechanisms underlying reversal learning and behavioral flexibility.

Considering the extensive literature describing sex differences in PFC synaptic transmission, morphology, anatomy, and maturation trajectory [47–50], future studies that examine the role of AMPAR plasticity in PFC development should include both sexes in their experimental design. For our analysis of L2/3-mPFC synaptic transmission, sample sizes were sufficient to examine males and females separately, and these sex-specific trajectories are presented in **Appendix I**. When splitting the

data by sex, mEPSC frequency, amplitude, and channel properties of AMPAR displayed distinct patterns characterized by age- and sex-specific contributions of GluA3 to synaptic transmission. Examining sex differences in the context of GluA3 is particularly interesting considering that *GRIA3* is located on the X chromosome and has been implicated in genetic studies of depression, sleep duration, and schizophrenia in women [19,51,52].

In conclusion, our findings identify GluA3 as a contributor to excitatory synaptic transmission in L2/3 neurons of the mPFC. Our findings set the ground for future studies that directly address the role of GluA3 in mPFC maturation and mPFC-dependent behavior. This will require mPFC-specific, inducible GluA3 knockout models in which gene deletion is spatially and temporally restricted, for example, targeting L2/3-mPFC neurons either before or after adolescence, thereby isolating the contribution of this subunit to mPFC development. Although additional experiments are necessary to fully define the role of GluA3 in synaptic maturation and to systematically examine sex-specific effects, the present findings support an important role for GluA3-containing AMPARs in PFC circuit physiology and associated behavior.

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APPENDIX I. Sex-specific developmental trajectory of L2/3-mPFC excitatory synapses in WT and GluA3KO mice.

The PFC has been shown to be sexually dimorphic, with differences between males and females observed at functional, morphological, and anatomical levels [49,50], which are also reflected in cognition [53]. At finer scales, sex differences have been reported in neuronal morphology, synaptic density, and synaptic receptor composition within the PFC, suggesting that fundamental properties of synaptic integration and plasticity are sexually differentiated [49,54,55]. Importantly, these differences are dynamic rather than static and are shaped across development. Sex-specific trajectories of prefrontal maturation have been documented in both humans and rodents, with females reaching PFC maturity earlier than males [47,48]. This developmental divergence is particularly pronounced during adolescence, a period already characterized by heightened plasticity and vulnerability.

In light of this growing literature, we found relevant to describe the developmental trajectories of L2/3-mPFC neurons from male and female WT and GluA3KO mice separated by sex. Sex was not included in the analysis as an independent variable due to restricted sample sizes. In male mice, mEPSC frequency did not show a significant age-by-genotype interaction ($F(2,86)=1.595$, $p=0.208$; **Figure 1A-Appendix I**). However, we observed a significant main effect of age ($F(2,86)=14.06$, $p=<0.001$; **Figure 1A-Appendix I**) and genotype ($F(1,86)=5.129$, $p=0.026$; **Figure 1A-Appendix I**). Post-hoc analyses revealed that mEPSC frequency was significantly higher during early adolescence compared with early adulthood ($p<0.001$, 33% reduction) and late adulthood ($p<0.001$, 47% reduction). No difference was detected between early and late adulthood, suggesting that synapses remained stable across these stages. Miniature EPSC amplitude showed an age-by-genotype interaction ($F(2,86)=3.818$, $p=0.025$; **Figure 1B-Appendix I**); however, this effect was not significant after post-hoc correction for multiple comparisons.

We next assessed whether mEPSC parameters changed throughout development and whether these changes were influenced by the absence of GluA3-containing AMPARs. Decay time exhibited a significant main effect of age ($F(2,86)=3.916$, $p=0.023$; **Figure 1C-Appendix I**), and post-hoc analyses indicated higher decay times during early adolescence compared with late adulthood ($p=0.027$, 53% reduction). No differences were observed between early adolescence and early adulthood or between early and late adulthood. Charge transfer, a reflection of the net influx of ions through AMPARs, showed significant age-by-genotype interaction ($F(2,85)=5.007$, $p=0.008$, **Figure 1D-**

Appendix I). Post-hoc comparisons revealed that GluA3KO males displayed reduced charge transfer during early adulthood compared with WT males (p=0.001, 33% reduction). Together, these results indicate that excitatory synaptic transmission in L2/3-mPFC pyramidal neurons of male mice undergoes developmental reorganization, particularly between early adolescence and late adulthood. In addition, excitatory synaptic transmission was overall reduced in GluA3KO mice, suggesting a role for GluA3-containing AMPAR in synaptic physiology of L2/3-mPFC neurons.

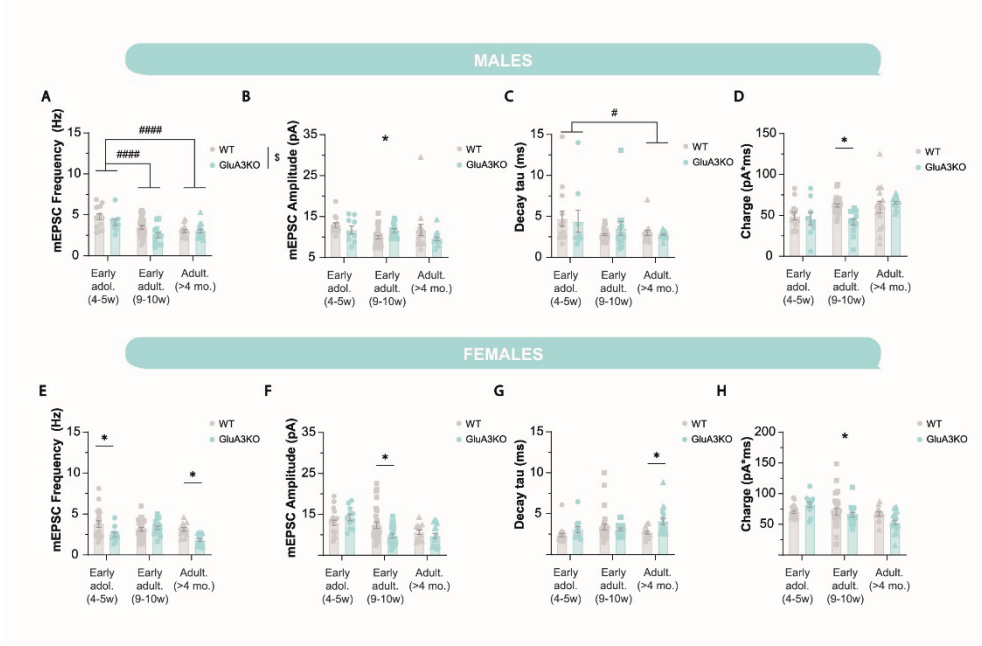


Figure 1 Appendix I. Developmental trajectory of L2/3-mPFC excitatory synapses in male and female WT and GluA3KO mice. For all panels, colors indicate genotype: grey (WT) and blue (GluA3KO) and symbols indicate age groups: early adolescence (circles), early adulthood (squares) and late adulthood (triangles). For panels A-D, mEPSCs were recorded from the same set of cells in male mice: early adolescence (WT n=14 from 3 mice; GluA3KO n=9 from 1 mouse), early adulthood (WT n=26 from 3 mice; GluA3KO n=13 from 2 mice) and late adulthood (WT n=15 from 2 mice; GluA3KO n=15 from 2 mice). (A) mEPSC frequency,(B) amplitude, (C) decay time and (D) charge transfer of layer 2/3-mPFC pyramidal cells from WT and GluA3KO male mice. For panels (E-H) mEPSCs were recorded from the same set of cells in female mice: early adolescence (WT n=21 from 2 mice; GluA3KO n=12 from 2 mice), early adulthood (WT n=28 from 3 mice; GluA3KO n=17 from 2 mice) and late adulthood (WT n=12 from 2 mice; GluA3KO n=17 from 2 mice). (E-H) Same as A-D but for female mice. For panels C,D, G and H all events from a single recording were averaged per cell, and the resulting mean value per cell is shown in the graph. Symbols indicate genotype effect ($), age effect (#) or an interaction effect (*).

In female mice, developmental effects were predominantly genotype-dependent. Miniature EPSC frequency exhibited a significant age-by-genotype interaction ($F(2,99)=5.045$, $p=0.008$; **Figure 1E-Appendix I)**, with GluA3KO females exhibiting a significantly lower frequency than WT females at early adolescence (p=0.011, 34% reduction) and late adulthood (p=0.010, 41% reduction). Miniature EPSC amplitude similarly showed a significant age-by-genotype interaction ($F(2,103)=3.707$, $p=0.027$; **Figure 1F-Appendix I**), driven by reduced amplitudes in GluA3KO females specifically during early adulthood (p=0.010, 21% reduction). Decay tau exhibited an age-by-genotype interaction ($F(2,101)=3.327$, $p=0.039$; **Figure 1G-Appendix I**), with mEPSC from GluA3KO females displaying a significantly longer decay tau compared with WT females during adulthood (p= 0.028, 50% increase). Charge transfer showed a genotype-by-age interaction that was not significant after correction for multiple comparisons ($F(2,100)=3.403$, $p=0.037$; **Figure 1H-Appendix I**). These results indicate that GluA3-containing AMPARs contribute to L2/3-mPFC excitatory synaptic transmission at different stages of PFC development in female mice, suggesting that this subunit may be involved in the maturation of synapses in this brain region.

Overall, these findings indicate that the absence of GluA3-containing AMPARs leads to sex-specific alterations in L2/3-mPFC basal excitatory synaptic transmission. While developmental changes were observed in both sexes, GluA3 deficiency resulted in age-specific disruptions in females, with synaptic deficits spanning adolescence to adulthood. In addition, our results highlight the need to incorporate both male and female mice in the experimental design of future studies.



Chapter 4

Repeated activation of cannabinoid receptors during adolescence induces long-term sex-specific changes to medial prefrontal cortex neurons

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Abstract

Cannabis use has increased significantly over the past decades, particularly among adolescents. This is particularly concerning because adolescence represents a critical window for prefrontal cortex (PFC) development. Although the endocannabinoid system is a key regulator of sex-dependent PFC maturation, the long-term impact of chronic cannabinoid receptor (CBR) activation during adolescence on PFC physiology and behavior later in life remains largely unexplored. We investigated the long-term consequences of repeated administration of the CBR agonist WIN55,212-2 (WIN) during adolescence at the single-cell, local medial PFC (mPFC) network, and behavioral level. Adolescent WIN administration led to altered intrinsic excitability properties of layer 5 pyramidal neurons of the adult mPFC (L5-mPFC) predominantly in males. In males, this effect was accompanied by an increased action potential threshold and a greater propensity for regular spiking over burst firing. Significant synaptic changes were only observed in females, who exhibited increased excitatory and inhibitory synaptic strength of input onto L5-mPFC neurons, without alterations in the excitatory/inhibitory balance. Adolescent WIN administration did not affect behavioral flexibility or social hierarchy, two types of adaptive behavior regulated by L5-mPFC neurons. Despite this absence of detectable behavioral changes in our tests, our findings establish a causal link between repeated CBR activation during adolescence and sex-specific functional alterations in adult L5-mPFC neurons. These results highlight the risk for chronic recreational cannabis use during adolescence to induce long-lasting effects by altering normal PFC development.

Introduction

Adolescence is a critical developmental stage characterized by a transition from subcortical to cortical control of behavior, during which integration of social and environmental cues becomes essential [1]. This period is often associated with increased risk-taking and a drive for intense emotional experiences, including increased vulnerability to drug use and abuse [2,3]. In light of contemporary policies in favor of cannabis legalization, the recreational use of cannabis among adolescents warrants attention, as it poses potential risks for mental health and thus represents a public health concern [4,5].

The prefrontal cortex (PFC) is (one of) the last brain region(s) to mature, remaining under development during adolescence [6]. During this period, the PFC undergoes a nearly two-fold reduction in synaptic density between the ages of 10 and 30 in humans [7]. In rodents, synaptic pruning in the medial PFC (mPFC) occurs between postnatal day (PND) 20 and 45 [8,9]. Notably, the timing and pattern of maturation differ between males and females [8,9]. Between PND 35 and 90, a reduction in the number of basilar spines on layer 5 mPFC (L5-mPFC) neurons was observed in both sexes, whereas a decrease in apical dendritic spine density occurred only in females [9]. Proper development and maturation of the mPFC is essential for adult behavior, as this brain region supports higher-order cognitive functions, including decision making, social cognition, and cognitive flexibility [10,11].

The endocannabinoid system (ECS) consists of cannabinoid receptor type 1 (CB1R), cannabinoid receptor type 2 (CB2R), various endogenous ligands, and the enzymes responsible for their synthesis and degradation [12]. CB1Rs are highly expressed in subcortical and cortical areas of the central nervous system, including the mPFC, with higher expression levels in inhibitory neurons than in excitatory ones [13,14]. Upon activation, presynaptically located CB1Rs reduce neurotransmitter release, resulting in synaptic weakening [15–17]. Although expression of CB2Rs was initially considered to be restricted to the peripheral nervous system and immune cells, several studies have demonstrated their functional presence in the central nervous system [18–21]. In mPFC neurons, activation of CB2R leads to reduced neuronal excitability [22,23]. CBRs play a crucial role in various cognitive processes, including learning and memory, social behaviors, such as sociability and social memory, and cognitive flexibility [24–27]. Notably, the ECS is a key player in the refinement and maturation of PFC networks during adolescence, which may underlie its contribution to the modulation of cognitive processes. Along these lines, Tseng and Molla recently proposed adolescence as a CB1R-sensitive period for frontal cortex development, given that changes in synaptic activity, as well as dopaminergic, GABAergic and glutamatergic tone coincide with

alterations in key ECS components within the PFC [28]. Additionally, several studies have demonstrated that adolescent exposure to CBR agonists or to tetrahydrocannabinol (THC), the psychoactive component of cannabis, disrupts the development and function of rat PFC networks in adulthood. These disruptions lead to altered neuronal morphology and changes in glutamatergic and GABAergic marker expression [29–31]. Although these studies did not directly address it, their findings suggest that the functional excitation/inhibition balance within the network may also be affected. Behaviorally, repeated activation of CBRs during adolescence results in long-lasting deficits, including impairments in object recognition, spatial memory, social recognition, motivation, and increased anxiety-like behaviors [31–37].

Adolescent brain development differs between males and females, with females reaching peak gray matter volume earlier, particularly in parietal and frontal regions [38]. Additionally, sex differences have been identified in the ECS, including variations in the density, desensitization and functional properties of CB1Rs in the PFC between male and female rats [39,40]. Although numerous studies have shown that CBRs activation during adolescence has lasting effects into adulthood, a major limitation in the literature is the underrepresentation of both sexes, limiting insights into sex-specific outcomes. Only a few studies have addressed long-term consequences as a function of sex. For example, following adolescent THC exposure, female rats exhibited impaired novel object recognition, whereas males did not [41]. In contrast, other studies have reported long-term cognitive deficits in male rats following adolescent THC exposure [33,42], although these studies did not include female subjects. Together, these findings highlight sex differences exist both in the ECS and in the long-term consequences of CBR activation during adolescence that remain to be further explored.

In this study, we aimed to elucidate the long-term consequences of repeated CBRs activation during adolescence on adult mPFC physiology in both sexes. To this end, we used the synthetic CBRs agonist WIN 55,212-2 (WIN), which selectively and potently activates CB1Rs and CB2Rs, avoiding off-target effects associated with the use of other cannabinoids such as THC. Neuronal physiology was assessed with patch-clamp recordings, a powerful approach to examine intrinsic neuronal properties as well as synaptic and network functions. We found that adolescent CBR activation induced long-term sex-specific alterations in intrinsic excitability properties and synaptic transmission while leaving the E/I-balance intact. Importantly, these neurophysiological changes were not accompanied by deficits in social hierarchy and reversal learning in adulthood. Together, our findings contribute to the growing body of evidence on the long-term impact of adolescence CBR activation on adult PFC

physiology, and may support science-based policies regarding [29–31]cannabis use during this vulnerable developmental period.

Materials and methods

Animals

C56BL/6J male and female mice were purchased from ENVIGO and delivered at PND23. Mice were left to acclimatize for 6 days before any experimental procedure started. Animals were group housed in tetrads with ad libitum access to food and water under standard conditions unless otherwise stated. Each group consisted of littermates or animals born on the same day, housed together from weaning onwards. Mice were maintained on a 12-h light/dark cycle (lights on 7:00-19:00). Body weight was monitored 3 times a week during the injection period and once a week during the remainder of the experiment. Mice were solitary housed for a week for habituation before being tested in the PhenoTyper (Noldus, the Netherlands) (Figure S1).

All experiments were conducted in accordance with the European guidelines for the care and use of laboratory animals (European Directive 2010/63/EU). The experimental protocol was approved by the Animal Experiment Committee of the Royal Netherlands Academy of Arts and Sciences (KNAW) and by the Animal Welfare Body of the University of Amsterdam (permit number AVD11100202114907).

WIN-treatment

Mice aged between PND30-PND50 were injected intraperitoneally with 1.2 mg/kg R(+)-WIN 55,212-2 (WIN) (MERCK-Sigma Aldrich). WIN was dissolved in 3.5% DMSO and 0.3% Tween-80 and diluted in saline (0.9 %). Animals were randomly allocated to either experimental condition, WIN or vehicle (3.5% DMSO and 0.3% Tween-80 diluted in saline). Additionally, the experimenter was blinded to the treatment group during the administration of injections and following experimental procedures. Adolescent treatment with the CB1-CB2R agonist WIN, or vehicle, lasted 20 days (PND30-PND50). The administration regimen was irregular, mimicking the sporadic pattern of recreational cannabis use, as previously described by others [33,34,43,44]. During this period, WIN-injection dosages were varied and interleaved across days, totaling 10 days of single-dose, 5 days of double-dose, and 5 days without injection, following the pattern described in Figure S1. Mice were socially housed in tetrads. Per cage, 2 mice received WIN injections, while the remaining 2 were administered vehicle injections. A

wash-out period of 20 days was included to avoid withdrawal symptoms from influencing behavioral testing.

Behavioral testing

Three behavioral tests, including the warm spot test, tube test, and the home-cage Phenotyper/reversal learning task, were conducted to assess the consequences of adolescent CBR activation on adult behavior. The warm spot test was performed on the last day of tube testing. Afterwards, animals were single-housed for 7 days prior to Phenotyper testing (7 days), and subsequently used for patch-clamp recordings (Figure S1).

Tube test. The protocol was carried out as described by Fan and colleagues [45]. During the final 5 days of the washout period after WIN-treatment, animals underwent familiarization and habituation to the tube test (Figure S1). During the initial 3 days mice were handled for approximately 3 minutes per day to get acquainted with the experimenter and 15-cm long transparent plexiglass tubes were placed in the home cages for habituation. Next, a 2-day habituation phase commenced, during which each animal walked 10 times (5 times from each side) through the tube in the experimental arena (60 cm width x 40 cm depth x 9 cm height) without opponent. The experimental plexiglass tube was 30-cm long with a diameter of 3cm for males and 2.6cm for females. The testing phase began in young adulthood (PND70) and lasted up to 14 testing days. Mice were acclimated to the experimental room for at least 15 minutes prior to the start of the test. All four animals of a group competed against each other in a round-robin design, with each pair of mice competing twice daily to avoid possible confounding effects due to the starting side of the tube. Each pair was allowed to compete for a maximum of 2 minutes, after which the competition was considered a tie if none of two mice had exited the tube. Animals were returned to their home cage between trials for at least 2 minutes. The arena and the tube were cleaned with water and 95% ethanol after each pair. Males were tested in the behavioral room before females to avoid provoking aggression between males. For quantification of social hierarchy, David's score was calculated for the last 4 days of competitions [46]. Stability was assessed by counting the number of rank transitions (i.e., changes in social rank) per group of 4 over the four-day analysis period. Each animal could undergo a maximum of 3 transitions during this window, adding to a total of up to 12 possible transitions per four-animal group. A higher number of transitions indicates higher instability, with 12 transitions representing 0% stability. To assess changes in stability over time, transitions were calculated separately for two periods: testing days 1–4 and testing days 8–11. Animals were identified with tail marks. Videos were recorded with a top-down camera and analyzed manually with BORIS [47].

Warm spot test. The warm spot test was used as a complementary measure of social hierarchy. Animals were placed for 30 minutes on an ice-cold (~6°C) empty cage of 20 cm width x 26 cm depth x 14 cm height for habituation. Subsequently, they were transferred to the testing ice-cold cage, which contained a warm spot. The warm spot consisted of a nestlet (Ancare, Bellmore) and a heating coil. The nestlet had a diameter of 3 cm for males and 2.3 cm for females and was placed on the cage floor. The heating coil was located directly underneath the cage at the same location as the nestlet, heating the nestlet to a temperature of around 24°C. The testing cage had a transparent lid with holes for transpiration. Testing lasted 20 minutes and mice were subsequently returned to their home cage. Males were tested in the behavioral room before females to avoid provoking aggression among males. Videos were recorded with a top-down camera and analyzed manually with BORIS [47]. For analysis, only visits to the warm spot that lasted longer than 1 second were included; durations of 1 second or less were classified as exploratory behavior. Totem behavior (piling up around the warm spot) was excluded from the total time spent in the warm spot. For each animal, the time spent in the warm spot was normalized to the total warm-spot time of all cage-mates.

PhenoTyper. The PhenoTyper (Noldus) is an automated home-cage monitoring system equipped with a shelter, water bottle, food dispenser, and a cognition wall. Mice were individually housed in PhenoTyper cages for the entire duration of the test period (7 days) and weight was monitored before and after this period. During the initial habituation phase, that lasted 2.5 days, water and regular chow were provided ad libitum and basal behavior was recorded. Thereafter, regular food was removed from the feeding compartment. At this time (end of the third day) the cognition wall was introduced in the cage and the cognitive testing started. The cognition wall consisted of three entry holes and the mouse passage through each entry was monitored (Figure 5A). Mice were habituated to the cognition wall for 1 hour and 3 free rewards (Dustless Precision Pellets, 14 mg, Bio-Serve) were delivered to facilitate familiarization before the start of the experiment. The multiple-choice reversal learning task consisted of two phases. In the first phase, animals were trained in a discrimination learning task, where only one of the three holes was rewarded. Following 48 hours of discrimination learning, the reward allocation was changed to a different hole, initiating the reversal learning phase, which lasted for 48 hours. During discrimination and reversal learning, one sugar pellet was delivered for every fifth entry through the correct hole. The five entries were not required to be consecutive [48].

Learning during the discrimination and reversal learning tasks was quantified as the total number of entries to reach an 80% correct performance criterion, calculated using a sliding window of 30 consecutive entries (i.e. at least 24 correct responses out of the last 30 entries) [48]. For Kaplan-Meier survival analysis, a cut-off was set to 800

and 1700 entries for reaching the 80% criterion in discrimination and reversal learning respectively. All entries were automatically registered by the EthovisionXT11 software and video was recorded during the entire course of the experiment. Data was analyzed off-line using AHCODA-DB [49].

Ex-vivo patch clamp recordings

Animals were killed using cervical dislocation at approximately 4 months of age (Figure S1). Brains were extracted and placed in ice-cold modified artificial cerebrospinal fluid (mACSF, in mM: 139 choline chloride, 3.5 KCl, 0.5 $CaCl_2$, 6.0 $MgSO_4$, 1.25 NaH_2PO_4 H_20, 10 glucose and 25 $NaHCO_3$, continuously bubbled with 5% CO_2/ 95% O_2 (pH 7.4)). 300 µm-thick coronal slices of the medial PFC were cut with a vibrating blade microtome (VT 1200S, Leica Microsystems). Slices were subsequently incubated at 35°C for 20 minutes in ACSF (in mM: 120 NaCl, 3.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4 H_20, 10 glucose, 2.5 $CaCl_2$, and 1.3 $MgCl_2$, bubbled with 5% CO_2/ 95% O_2), and then kept for at least 30 min at room temperature before recordings commenced. Whole-cell voltage-clamp and current-clamp recordings were performed from layer 5 pyramidal neurons of the infralimbic (IL) and prelimbic (PrL) areas of the mPFC. During recordings, slices were kept submerged in ACSF at 28-30° C and superfused at a rate of 1.8 ml/min. Glass recording pipettes were pulled from borosilicate glass (Science Products) and had a resistance of 2-3.5 MΩ when filled with intracellular solution. For current-clamp recordings of intrinsic excitability and voltage-clamp recordings of local recurrent network excitability (evoked synaptic responses to determine the balance excitation /inhibition), glass pipettes were filled with (in mM): K-Gluconate 131.25, KCl 8.75, EGTA 0.5, HEPES 10, Mg-ATP 4 and Na_2-GTP 0.4, (pH=7.3). Voltage-clamp recordings of miniature excitatory and inhibitory post-synaptic currents (mEPSCs and mIPSCs respectively) were performed in the presence of 1 mM tetrodotoxin (TTX, Latoxan, France) and glass recording pipettes were filled with (in mM): 115 CsMeSO3, 3.97 CsCl, 2.5 $MgCl_2$, 10 HEPES, 4 Na_2-ATP, 0.4 Na-GTP, 10 Na-Phosphocreatine and 0.6 EGTA. Recordings of evoked excitatory post-synaptic currents (eEPSCs, single and paired-pulse stimulations) were performed in the presence of 100 µM picrotoxin (MERCK Sigma Aldrich) and glass pipettes were filled with (in mM): 115 CsMeSO3, 20 CsCl, 10 HEPES, 2.5 $MgCl_2$, 4 Na_2-ATP, 0.4 Na-GTP, 10 Na-Phosphocreatine and 0.6 EGTA and 5 QX-314 (Tocris). Signals were acquired using a MultiClamp 700B amplifier controlled by pClamp 11 software (Molecular Devices), low-pass filtered at 4 kHz and sampled at 10 kHz. Series resistance ranged from 6–20 MΩ and was compensated to ~65 %. Signals were corrected for liquid junction potential. Off-line analysis was performed with either Clampfit (Molecular Devices) or in-house software under Matlab (MathWorks, USA).

Electrophysiological properties:

Intrinsic passive and active properties of neurons were determined from current clamp recordings from neurons held at -65 mV by continuous current injection. Resting membrane potential (E_{REST}) was measured immediately after the whole-cell configuration was achieved. Passive properties were estimated from the averaged voltage responses of 50 sweeps (for accuracy) to a 200 ms hyperpolarizing current injection of -10 pA amplitude (pre-pulse) and active properties were estimated from voltage responses to a 1 sec current injection ranging from -100 pA to +400 pA in 20 pA steps. The input resistance (Rin) was determined at the steady state voltage response and the capacitance of the neuron (Cm) was calculated according to the equation: $\tau m = Rm * Cm$ (where Rin estimates Rm). The membrane time-constant (τm) was determined by fitting the voltage response with a mono-exponential function: $V(t) = Iinj * Rin * (1 - e^{-t/\tau m})$, where *Iinj* represents the injected current intensity. Action potential (AP) threshold, peak amplitude, and half-width duration were calculated as the average values of the first four suprathreshold current steps. AP threshold was calculated using the second derivative (dV^2/dt^2) of the somatic membrane potential. AP amplitude was measured relative to the AP threshold. AP half-width was defined as the width of the AP at half of the AP amplitude. Afterhyperpolarization (AHP) amplitude was measured as the amplitude of the AHP peak relative to the AP threshold. The sag ratio, defined as the ratio between the steady state voltage and the maximum decrease in voltage, was calculated from the voltage response to the -100 pA hyperpolarizing current injection. Repetitive action potential firing properties were characterized by the rheobase (the minimal current amplitude initiating an AP) and the mean AP firing frequency as a function of current injection.

Miniature synaptic current analysis:

Cells were voltage-clamped for 5 minutes at -70 mV and 0 mV for recording mEPSCs and mIPSCs, respectively. Recordings were performed in the presence of tetrodotoxin (TTX, 1 μM) to block spontaneous AP. The amplitude and frequency were determined and analysed for every miniature synaptic event over 5 minutes of recording. Ratio excitation/inhibition (E/I) was calculated as the ratio of the total synaptic charge carried by mEPSCs and mIPSCs, obtained by summing the integrals (area under the curve) of all detected events within a fixed recording period [50,51]. For mEPSCs and mIPSCs, at least 100 events were analyzed per cell. Events with an amplitude smaller than 5 pA (detection limit [52]) were excluded from the analysis. To compare mEPSC frequency distributions between cells, cumulative probability distributions were constructed for each individual cell and compared to a control distribution using the two-sample Kolmogorov–Smirnov test. The resulting D value, representing the

maximum distance between the two cumulative distributions, was calculated for each cell and used for subsequent statistical analysis across experimental groups.

Evoked synaptic responses:

To determine the balance between excitation and inhibition, in addition to the miniature synaptic current analysis, we used a method based on the continuous measurement of conductance dynamics of evoked synaptic responses. This method was developed and first described *in vivo* in cat visual cortex and allows the decomposition of the total conductance into its excitatory and inhibitory components [53]. A mono-polar stimulation electrode (glass pipette filled with ACSF) was placed in layer 5 100 to 200 mm away from the recorded neurons for synaptic stimulation. Biphasic currents (100-800 µA, 0.1 ms) were injected through the stimulation electrode. Currents were adjusted to an intensity evoking postsynaptic currents of half-maximum intensity when recorded at a holding potential of -70 mV. Evoked synaptic responses were recorded from layer 5 pyramidal neurons at different membrane potentials (-90 to -50 mV, in steps of 5 mV) and averaged for accuracy (six traces). This allowed for constructing an instantaneous I/V relationship (every ms) and determining the slope conductance from the best linear fit of the instantaneous I/V relationship. The evoked synaptic conductance ($g_{total(t)}$) was calculated by subtracting the slope of the average I/V curve before stimulation (g_{rest}) from the slope of each instantaneous I/V curve. The voltage abscissa of the intersection point between the average rest I/V curve and an I/V curve at time (t) was taken as the reversal potential of the synaptic current ($E_{syn}(t)$). The evoked synaptic conductance (g_{total} (t)) was then decomposed into its excitatory ($g_e(t)$) and its inhibitory ($g_i(t)$) component as initially described by others [53,54] and more recently by us (see [17] for details). The integral of the conductance over a time window of up to 300 ms after synaptic stimulation was used to quantify changes in conductance. The decomposed contributions of the excitatory and inhibitory components were expressed by the ratio of the integral for excitatory conductance or the integral for inhibitory conductance to the integral of the total conductance. Total synaptic conductance was expressed as the summed excitatory and inhibitory conductance normalized to the cell capacitance.

To determine the AMPA/NMDA and paired-pulse ratios, eEPSCs were evoked by electrical stimulation using a glass pipette located in layer 5, as described for determining the balance between excitation and inhibition (see above). Stimulation intensity was optimized using an input-output curve at –70 mV, and subsequent recordings were conducted at half-maximal current. Neurons were voltage-clamped at -60 mV and +40 mV to record AMPA-mediated and NMDA-mediated EPSCs. Additionally, current at +0 mV was measured to isolate the NMDA component. Thirty

sweeps were recorded per holding potential. AMPA-mediated EPSC amplitude was measured at the peak of the inward synaptic responses at -60 mV. NMDA-mediated EPSCs were measured by the mean of the outward current between 40 and 90 ms after stimulation evoked at +40 mV, corrected for the mean inward current between 40 and 90 ms after stimulation at +0 mV. Paired pulse ratios were calculated as the ratio between the second to the first peak amplitude of inward EPSCs evoked at -60 mV by two stimuli with an inter-pulse interval of 50 ms. All the above-mentioned holding potentials included correction for liquid junction potential.

To classify neurons as either regular spiking or burst spiking, inter-spike interval (ISI) histograms were analyzed from rheobase across all current injections [55]. Regular spiking was identified by a Gaussian-like ISI distribution, whereas bursting neurons exhibited a bimodal distribution, with the burst peak having an ISI of ≤20ms (corresponding to ≥50Hz). Visual inspection of neuronal responses to the current-step protocol was also conducted to confirm the ISI-based classification.

Statistical analysis

All statistical tests were performed in GraphPad version 8 (GraphPad Software, San Diego) or RStudio version 2024 (RStudio Team, Boston). Data were statistically tested with the Mann-Whitney test, Fisher's exact test, Friedman test, Kruskal-Wallis test or two-way ANOVA. Post-hoc multiple comparison was done by Tukey's or Dunn's multiple comparison tests. Survival curves were tested for significance with the log-rank (Mantel-Cox) test. Pearson correlation coefficient was used for correlations. Data are represented as median with whiskers indicating minimum and maximum values. Statistical significance was considered as * $p<0.05$, ** $p<0.01$,*** $p<0.001$.

Results

CBR activation during adolescence results in sex-specific changes in intrinsic properties of L5- mPFC neurons in adults

To mimic repetitive recreational cannabis use during adolescence, animals received either vehicle or WIN injections between PND 30 and 50 following an irregular pattern (Figure S1), as described previously [33,34,43,44]. First, we assessed how this drug treatment impacts mPFC neurons later in life by examining their electrophysiological properties in 4 months-old mice. We conducted whole-cell patch-clamp recordings from L5-mPFC neurons located in the infralimbic (IL) and prelimbic (PrL) areas (**Figure**

1A), which are the neurons that constitute the major output source from mPFC to other cortical and sub-cortical areas [56,57]. While no significant differences were found in membrane resistance in neurons from either females (**Figure 1B**) or males (**Figure 1D**), a significant decrease in membrane resting membrane potential was observed in neurons from both female (**Figure 1C**, p=0.03) and male mice (**Figure 1E**, p=0.006). The intrinsic excitability of L5-mPFC neurons, measured by the number of action potentials elicited in response to increasing current injections, was not significantly different between neurons from vehicle- and WIN-treated animals for both female (**Figure 1F**) and male mice (**Figure 1G**). Action potential threshold (**Figure 1H**), sag ratio (**Figure 1I**), and adaptation ratio (**Figure 1J**) were unchanged in L5-mPFC neurons from female

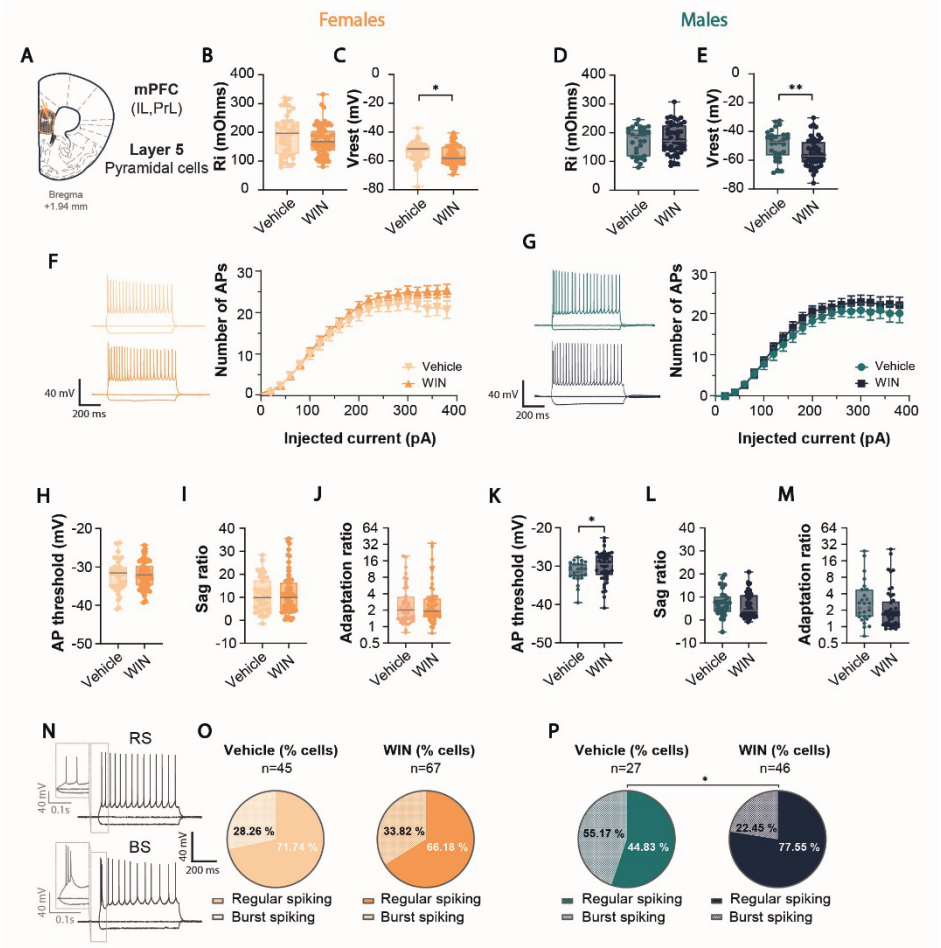


Figure 1. Repeated adolescent WIN injections alters intrinsic excitability of IL and PrL layer 5 pyramidal neurons. (A) Schematic representation of a coronal slice at +1.94 mm relative to bregma (adapted from Paxinos and Franklin atlas 2nd edition), showing the IL and PrL regions and cortical layer 5 included in the study. Data in all panels were obtained from the same set of cells: female vehicle (light orange) n= 45 from 8 mice; female WIN (dark orange) n=67 from 9 mice; male vehicle (light blue) n=27 from 5 mice; male WIN (dark blue) n=46 from 6 mice. Membrane resistance (B) and resting membrane potential (C) of neurons from vehicle and WIN female mice. Membrane resistance (D) and resting membrane potential (E) of neurons from vehicle and WIN male mice. There was a significant difference in resting membrane potential between vehicle and WIN injected female mice (U=1190, p=0.030, Mann-Whitney U test) and vehicle and WIN injected male mice (U=450, p=0.006. Mann-Whitney U test). (F) Left: representative firing response traces from layer IL and PrL pyramidal neurons of vehicle and WIN (groups of female mice under current-clamp conditions at -100 pA, 0 pA and 300 pA.; right: number of action potentials as a function of injected current for vehicle and WIN-injected females. (G) Same as F, but for males. (H-J) Action potential threshold (H), sag ratio (I) and adaptation ratio (J) of neurons from vehicle and WIN female mice. (K-M) Same as H-J but for male mice. There was a significant difference in action potential threshold between vehicle and WIN injected male mice (U=439, p=0.037, Mann-Whitney U test). (N) Representative traces of regular spiking (top) and burst spiking (bottom) neurons. (O) Pie chart showing the proportion of regular and burst spiking cells in layer 5 pyramidal neurons from vehicle (left) and WIN (right) female mice. (P) Same as O but for males. The proportion of regular and burst spiking cells was significantly different between vehicle and WIN injected mice (p=0.046, Fisher's exact test, two-sided). AP=action potential, BS=burst spiking, IL=Infralimbic, mPFC= medial Prefrontal Cortex, PrL=prelimbic, RS=regular spiking, WIN = WIN 55,212-2.

mice. A significant increase in action potential threshold was observed in neurons from male mice (**Figure 1K**, p=0.037), while sag ratio (**Figure 1L**) and adaptation ratio (**Figure 1M**) remained unaffected. Other passive and active properties, including action potential waveform were unchanged upon chronic CBR activation during adolescence (**Table S1**). In our dataset we observed both regular- and burst-spiking neurons during prolonged depolarization (**Figure 1N**). Layer 5 regular-spiking neurons are thought to participate in sensory processing, whereas burst-firing neurons are associated with high-order cognitive functions and flexibility [58] and have been implicated in PFC-dependent cognitive functions such as attention [59]. Given these distinct functional roles, [56,58–61]we quantified the proportion of L5-mPFC neurons exhibiting each spiking mode. [58][59]In vehicle-treated female mice, 72% of recorded neurons exhibited regular spiking, while 28% displayed burst firing (**Figure 1O**). These proportions were not significantly different in WIN-treated females, where 66% of neurons were regular spiking and 34% were bursting (**Figure 1O**). In males, while the percentage of regular spiking neurons in vehicle mice was 45%, this number was significantly larger (78%) in neurons from WIN-injected males (**Figure 1P**). Overall, these findings demonstrate that repeated adolescent CBR activation during adolescence induces lasting sex-specific changes in the intrinsic properties and in the proportions of bursting neurons in L5-mPFC neurons.

Adolescent CBR activation does not alter basal synaptic transmission onto L5-mPFC neurons

Given the role of the ECS in adolescent brain development, particularly in synaptic pruning [62,63], we hypothesized that repeated injections of WIN during this period may disrupt the physiological synaptic remodeling that normally occurs in the mPFC [7,64]. The elimination of weak or unnecessary synapses is typically accompanied by the strengthening and stabilization of the remaining connections, together contributing to the fine-tuning of cortical networks [65]. To understand whether repeated CBR activation during adolescence affected excitatory and inhibitory synapses, basal synaptic transmission was assessed by recording both miniature excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs respectively) onto L5-mPFC neurons within IL and PrL (**Figure 2A**). The frequency of miniature events is a reflection of both presynaptic release probability and the number of synapses with AMPA receptor currents above detection limit [52]. Their amplitude reflects the size of postsynaptic AMPA receptor currents per detected synapse. Neurons from WIN-injected female mice did not exhibit significant differences in mean mEPSC frequency or amplitude compared with controls (**Figure 2B,C**). Consistent with the unchanged mean amplitude, the distribution of mEPSC amplitudes was also unaltered (**Figure 2D**). Similarly, adolescent WIN exposure did not affect mEPSC frequency, amplitude or amplitude distribution in male mice (**Figure 2E-G**). Together, these findings indicate that CBR activation during adolescence does not induce long-term changes in AMPA receptor-mediated synaptic transmission onto L5-mPFC neurons.

We next examined inhibitory synaptic transmission onto L5-mPFC neurons. In female mice, no significant differences were observed in mean mIPSCs frequency, amplitude or amplitude distribution between vehicle- and WIN-treated groups (**Figure 2H-J**). Similarly, these parameters remained unchanged in male mice (**Figure 2K-M**). For all cells both the mEPSCs and mIPSCs were recorded, allowing for the estimation of the E/I balance at the single cell level. Correlation analyses between excitatory and inhibitory synaptic properties revealed different degrees of synaptic E/I scaling that was group- and sex-specific (**Figure S2A-H**). We measured the mEPSC/mIPSC ratio per neuron by calculating the ratio of total charge of excitatory and inhibitory synaptic events (**Figure 2N**) [50,51], which was below one for the majority of neurons (**Figure 2O,P**), suggesting that inhibitory conductance generally outweighs excitatory conductance. No significant differences in this ratio were detected between vehicle- and WIN-injected female (**Figure 2O**), or males (**Figure 2P**), indicating the overall excitatory/inhibitory balance of miniature synaptic events was not disturbed.

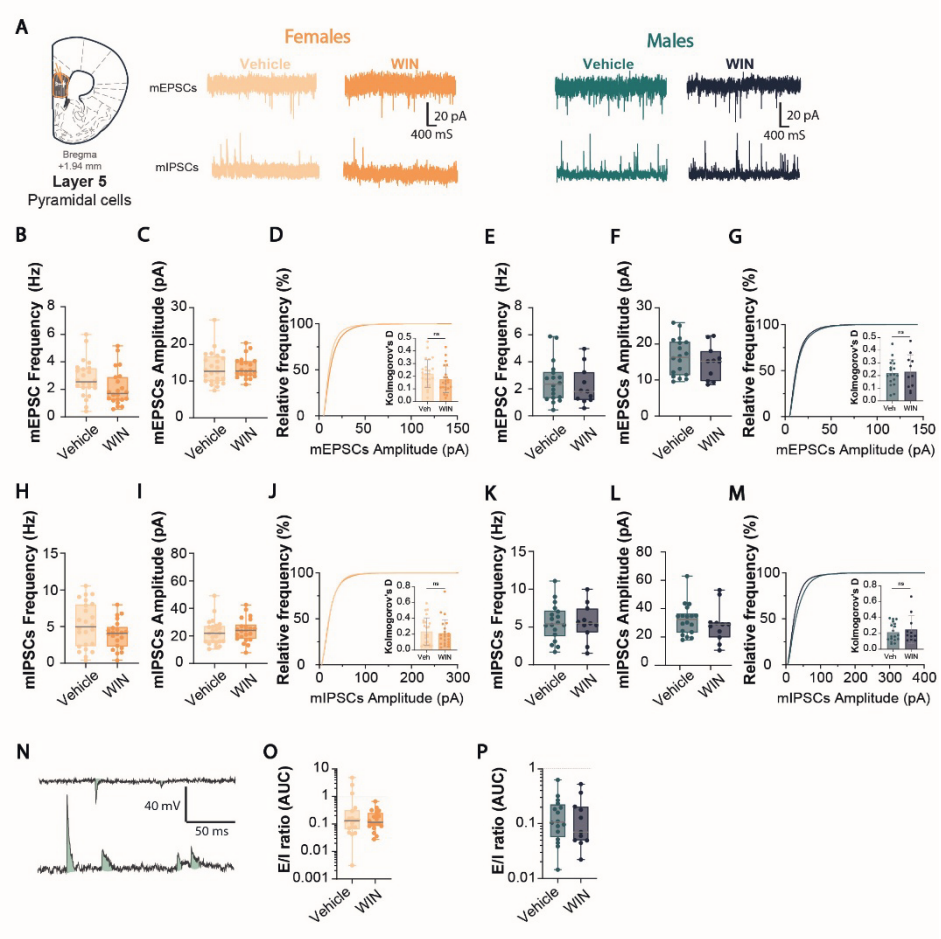


Figure 2. Repeated adolescent WIN exposure altered synaptic strength in IL and PrL layer 5 pyramidal neurons of female mice. (A) Left: schematic representation of a coronal slice at +1.94 mm relative to bregma (adapted from Paxinos and Franklin atlas 2nd edition), showing the IL and PrL regions and cortical layer 5 included in the study. Right: example traces of miniature excitatory postsynaptic currents (mEPSCs, top) and miniature inhibitory postsynaptic currents (mIPSCs, bottom) from layer 5 pyramidal neurons of vehicle and WIN females (left) and vehicle and WIN male (right) mice. For all panels, colors indicate treatment and sex: female vehicle (light orange), female WIN (dark orange), male vehicle (light blue), male WIN (dark blue). Miniature excitatory and inhibitory postsynaptic currents were recorded from the same set of cells: female vehicle (n=24 cells from 3 mice), female WIN (n=22 from 4 mice), male vehicle (n=18 from 4 mice) and male WIN (n=11 from 4 mice). (B) mEPSC frequency and (C) amplitude of layer 5 pyramidal cells from female mice. (D) Cumulative probability of mEPSC amplitude and Kolmogorov's D value (insert) from vehicle and WIN females. (E-F) Same as B-C but for males. (G) Same as D but for males. (H) mIPSC frequency and (I) amplitude for female layer 5 pyramidal cells. (J) Cumulative frequency distribution of mIPSC amplitude and Kolmogorov's D value (insert) from vehicle and WIN females. (K-L) Same as H-I but for males. (M) Same as J but for males. (N) Example traces of mEPSCs (top) and mIPSCs (bottom) with area under the curve of excitatory and inhibitory events colored in grey. (O) mEPSCs/mIPSCs ratio from vehicle and WIN females. Dashed grey line indicates value of 1. (P) Same as O but for males. WIN = WIN 55,212-2.

Synapse strength and network balance in L5-mPFC neurons are affected by adolescent CBR activation

Since adolescence CBR activation altered aspects of miniature synaptic transmission onto L5-mPFC neurons, we next investigated whether the underlying mechanisms involved postsynaptic glutamate receptors or presynaptic release machinery. To address this, local afferents onto L5-mPFC neurons in the IL and PrL areas were stimulated, and evoked synaptic responses were recorded (**Figure 3A**). We measured the ratio of AMPA receptor over NMDA receptor currents, which indicates changes in postsynaptic currents independent of synapse density or presynaptic release probability. L5-mPFC neurons from WIN-injected female mice displayed a significant increase in AMPA/NMDA ratio compared to controls (**Figure 3B**, p=0.047), indicating

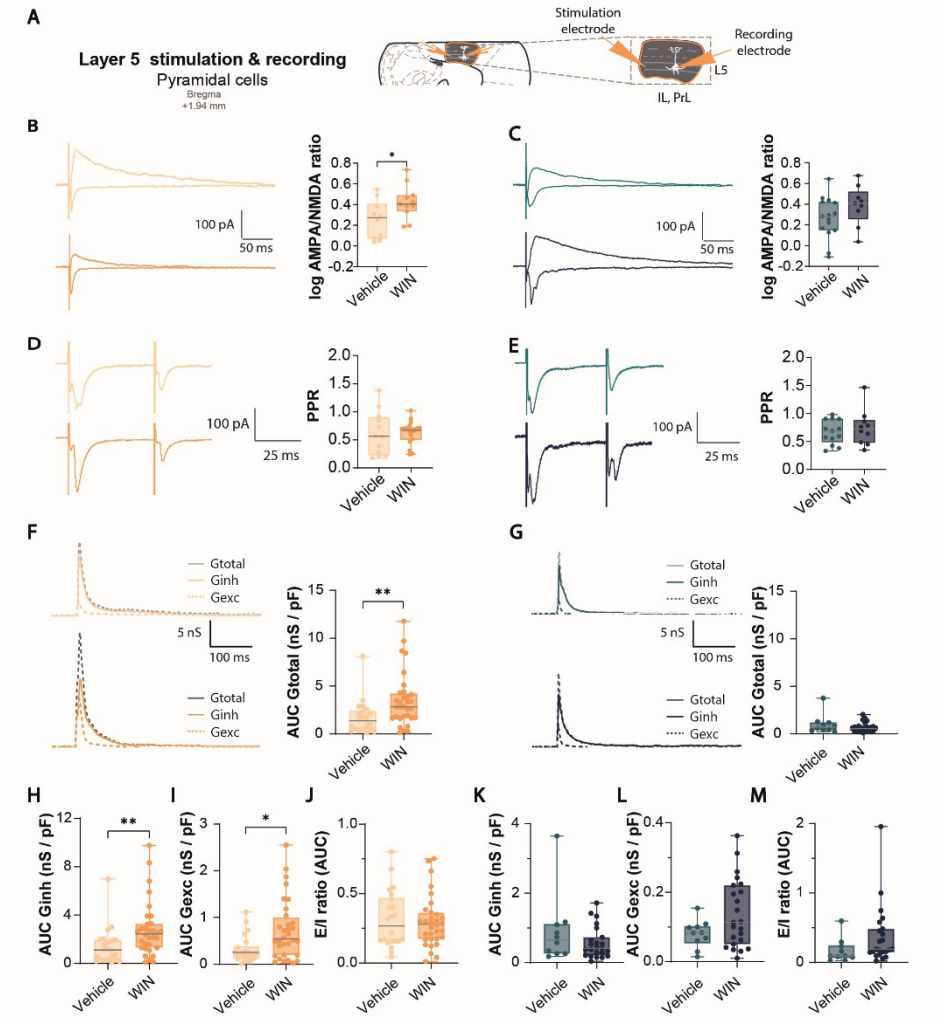


Figure 3. Repeated CBR activation during adolescence alters excitatory and inhibitory transmission on layer 5 IL and PrL pyramidal cells. (A) Schematic representation of a coronal slice at +1.94 mm relative to bregma (adapted from Paxinos and Franklin atlas 2nd edition), showing the IL and PrL regions, cortical layer 5 included in the study and placement of recording and stimulation electrodes. For all panels, colors indicate treatment and sex: female vehicle (light orange), female WIN (dark orange), male vehicle (light blue), male WIN (dark blue). (B) Left: example trace of evoked synaptic responses at -60 mV and +40 mV from female layer 5 pyramidal cells (vehicle top, WIN bottom) to calculate AMPA/NMDA ratio; right: AMPA/NMDA ratio for vehicle (n=10 from 3 mice) and WIN-injected (n=11 from 4 mice) female neurons. Repeated WIN exposure during adolescence increased AMPA/NMDA ratio in females (U=37, p-0.047, Mann-Whitney U test). (C) Same as B but for males; vehicle (n=15 from 4 mice), WIN (n= 9 from 3 mice). (D) Left: example trace of paired-pulse ratio from female vehicle (top) and WIN (bottom) layer 5 pyramidal cells; right: paired-pulse ratio for vehicle (n=12 from 3 mice) and WIN (n=18 from 4 mice) female neurons. (E) Same as D but for males; vehicle (n=13 from 3 mice) and WIN (n=10 from 3 mice). (F) Left: representative traces of the total (dashed grey line), inhibitory (orange squares), and excitatory (solid orange line) conductance of layer 5 pyramidal neurons from female vehicle (top) and WIN-injected (bottom) mice; right: normalized area under the curve (AUC) of the total conductance for vehicle (n=22 from 7 mice) and WIN-injected (n=32 from 8 mice) female neurons. Repeated WIN exposure during adolescence increased total conductance in females (U=26, p=0.008; Mann-Whitney U test). (G) Same as F but for males; vehicle (n=10 from 5 mice), WIN (n=23 from 6 mice). (H-I) Normalized AUC of the (H) inhibitory and (I) excitatory conductance for vehicle and WIN-injected female neurons. Same cells than in F. Repeated WIN exposure during adolescence increased inhibitory (U=185, p=0.002, Mann-Whitney U test) and excitatory conductance (U=212, p=0.013, Mann-Whitney U test) in females. (J) Ratio of the AUC of excitatory and inhibitory conductance for vehicle and WIN-injected female neurons. Same cells than in F. (K) Same as H but for males. (L) Same as I but for males. (M) Same as J but for males. Same cells than in G. WIN = WIN 55,212-2, AUC = Area Under the Curve, Gtotal = Total conductance, Ginh = Inhibitory conductance, Gexc = Excitatory conductance.

post-synaptic change in synaptic currents. In contrast, no significant difference was observed in neurons from male mice (**Figure 3C**). Paired-pulse ratio, an indirect measure of pre-synaptic release probability, was unchanged in neurons from both female (**Figure 3D**) and male mice (**Figure 3E**). These results demonstrate that adolescent CBR activation results in a long-lasting increase in excitatory transmission of post-synaptic glutamatergic currents onto L5-mPFC neurons in female mice but not in male mice.

We next assessed whether this also had an effect on the excitation/inhibition (E/I) balance of the local mPFC circuit. In female mice, neurons from WIN-injected animals showed a significant increase in total conductance compared to controls (**Figure 3F**, p=0.003), an effect not observed in neurons from male mice (**Figure 3G**). When total conductance was decomposed into excitatory and inhibitory components, both inhibitory (**Figure 3H**, p=0.002) and excitatory (**Figure 3I**, p=0.013) conductance were significantly elevated in neurons from WIN-injected female mice, resulting in the excitation/inhibition ratio to remain unchanged (Figure 3J). In male mice, no differences were detected in inhibitory conductance (Figure 3K), excitatory conductance (**Figure 3L**), or E/I ratio (**Figure 3M**). Together, these results demonstrate that repeated activation of CBRs during adolescence induced a sex-specific increase

in both excitatory and inhibitory synaptic drive onto L5-mPFC neurons exclusively in female mice, without altering the E/I balance.

Repeated CBR activation during adolescence does not alter social hierarchy in adulthood

Having established that repetitive WIN-treatment during adolescence produced long-term changes to L5-mPFC neurons, we next aimed to assess whether this treatment alters mPFC-dependent behavior. The mPFC has been identified as a control center for social hierarchical behavior in humans, primates and rodents [11], and studies in mice demonstrate that increased output of layer 5 mPFC neurons promotes winning in social contests [66]. To investigate the long-term effects of repeated CBR activation during adolescence on adult social hierarchy, we used the tube test on 6 groups of females and 5 groups of male mice (**Figure 4A**). Within each group of four mice, two mice were injected with WIN and two received vehicle injections. Four weeks after the last injection, mice were pair-wise exposed to the tube, and ranking was determined by a round-robin competition design over a 3-week period (**Figure S1**). The consistency of tube test results became more stable over time (**Figure 4B,D; Figure S3A,C**), and tube test ranks were calculated based on the David's score over the final 4 days of testing. As a complementary read-out for social status, the warm spot test was

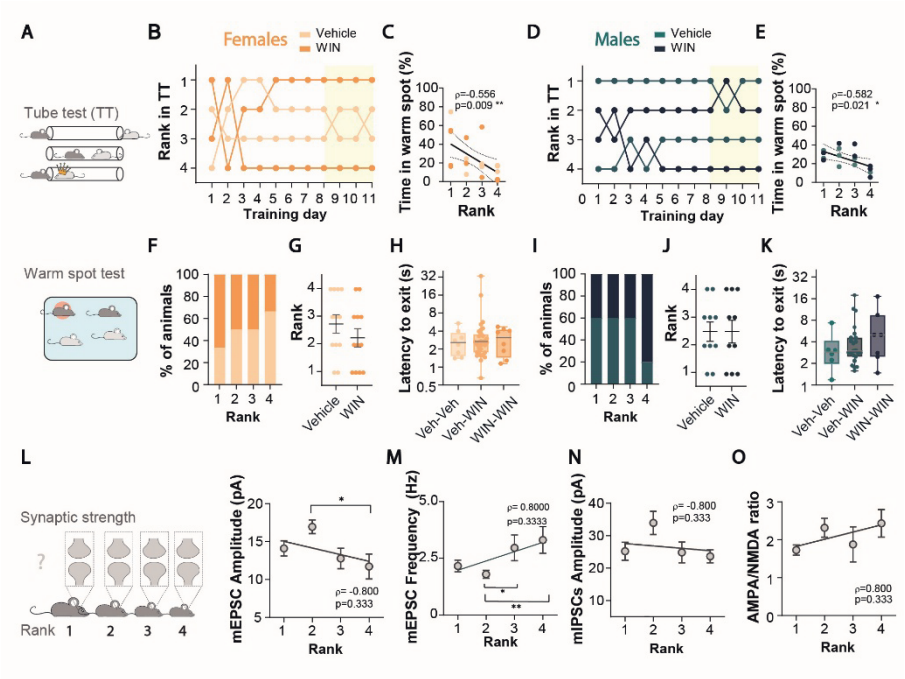


Figure 4. Repeated CBR activation during adolescence does not affect social hierarchy. (A) Schematic representation of the tube test (top) and the warm spot test (bottom). For all panels, colors indicate treatment and sex: female vehicle (light orange), female WIN (dark orange), male vehicle (light blue), male WIN (dark blue). Data in all panels were obtained from the same set of animals: females (n=6 groups of 4 mice), males (n=5 groups of 4 mice). (B) Example of a female hierarchy across training days, with rank positions determined using David's score; the yellow box indicates the days used for score calculation. (C) Relative time spent in the warm spot as a function of tube test rank in female mice. (D) Same as B but for males. (E) Same as C but for males. (F) Proportion of female mice from each treatment group per rank. (G) Average rank (±SEM) for vehicle and WIN females. (H) Latency to exit for different treatment pairs in females (light orange: Vehicle–Vehicle; medium orange: Vehicle–WIN; dark orange: WIN–WIN). (I) Same as F but for males. (J) Same as G but for males. (K) Same as H but for males (light blue: Vehicle–Vehicle; medium blue: Vehicle–WIN; dark blue: WIN–WIN). (L) mEPSC amplitude as a function of social rank, data from all four groups are shown together (male and female, vehicle and WIN). Rank 1 n=21 cells, rank 2 n=11 cells, rank 3 n=10 cells, rank 4 n=10 cells. One-way ANOVA showed a significant effect of rank on mEPSCs amplitude ($F(3,47)= 2.899$, $p=0.044$). Sidak's multiple comparison analysis revealed that animals in second rank showed a significantly higher amplitude compared to animals in fourth rank ($p=0.024$). (M) Same as L but for mEPSC frequency. One-way ANOVA showed a significant effect of rank on mEPSCs amplitude ($F(3,98)= 7.878$, $p=0.0009$). Sidak's multiple comparison analysis revealed that animals in second rank showed a significantly lower frequency compared to animals in third ($p=0.012$) and fourth rank ($p=0.0014$). (N) AMPA/NMDA ratio as a function of social rank, data from all four groups are shown together (male and female, vehicle and WIN). Rank 1 n= 15 cells, rank 2 n= 11 cells, rank 3 n=7 cells, rank 4 n=10 cells. Panels L-N display Spearman correlation analysis. WIN = WIN 55,212-2.

performed on the last day of tube test competitions (**Figure 4A**). The time spent in the warm spot relative to its other group members correlated significantly with the rank determined in the tube test for both female and male groups of mice (**Figure 4C,E**), validating tube test ranks as a reflection of social status [67,68]. These results indicate that both male and female mice established transitive and largely stable social hierarchies. WIN-injected mice were evenly distributed across the four tube test ranks in both females (**Figure 4F**) and males (**Figure 4I**), with no significant differences in the proportion of animals per treatment group attaining each rank. Likewise, the average rank did not significantly differ between vehicle- and WIN-injected mice in either sex (**Figure 4G,J**).

As a further analysis of behavior during tube test encounters, we measured the latency to exiting the tube, which tended to last longer between two high-ranked mice (rank 1 vs. 2) than between the highest and lowest ranked mice (e.g. rank 1 vs. 4) (**Figure S3B,D**), as previously described [45]. The latency to exit was on average similar irrespectively whether a pair consisted of two vehicle-treated mice, two WIN-treated mice, or one vehicle- and one WIN-treated mouse, for both females (**Figure 4H**) and males (**Figure 4K**), suggesting that repeated CBR activation during adolescence did not affect the competition strategy. Overall, these results indicate that repeated activation of CBRs during adolescence does not influence social hierarchy.

Since it was previously shown that high social status is causally related to post-synaptic strength of L5-mPFC neurons [11], we recorded synaptic currents (mEPSCs, mIPSCs and AMPA/NMDA-ratios) from L5-mPFC neurons of mice that were exposed to the social hierarchy test (**Figure 4B-K**). We found that particularly 2nd ranked mice appeared to have on average higher mEPSC amplitude compared with lowest ranked mice (**Figure 4L** p=0.024) and lower mEPSC frequency compared with lower-ranked mice (**Figure 4M**, p=0.012 2VS3; p=0.0014 2VS4). Differences between social ranks were not significantly reflected in AMPA/NMDA-ratios (**Figure 4N**) and other inhibitory synaptic parameters (**Figure S4E,F**). These findings indicate that average mEPSC amplitude at L5-mPFC neurons reflects performance in the tube test.

No long-term impact of adolescent CBRs activation on adult reversal learning

The maturation of the mPFC circuit plays a relevant role in the development of behaviors that rely on cognitive flexibility, such as reversal learning [69,70]. To investigate the long-term consequences of repeated CBR activation during adolescence on reversal learning, mature mice were housed in an automated home-cage monitoring system [71] that allows the assessment of both spatial discrimination learning and subsequent reversal learning (**Figure 5A**). Measures for basal behavior, such as total distance moved and time spent in the shelter, were not different between mice that were either WIN- or vehicle-treated during adolescence (**Figure S4A-J**). In the discrimination learning task, mice learned to earn a reward (sugar pellet) when they passed five times through the left entrance in a wall with three entry holes (**Figure 5A**). All mice showed a gradual increase in their preference for entering the left (correct) entrance compared to right and middle ones within the first 6 hours (**Figure 5B-E**). No significant differences in performance in this task, i.e. reaching the criterion of 80% correct entries, were observed between vehicle- and WIN-injected females (**Figure 5F**) or males (**Figure 5H**). After having been exposed to this task for 48 hours, their ability for reversal learning was tested by switching the delivery of the reward to 5 times passage through the right entrance. Mice changed their preference for left-side entries to right-side entries within the first 6 hours, though the percentage of left entries remained higher than middle entries, suggesting residual information from prior learning (**Figure 5B-E**). Performance in this reversal learning task, as measured by the number of entries required for reaching the 80% criterion, did not significantly differ between WIN-treatment and control groups in either females or males (**Figure 5G, 5I**). Together, these results suggest that repeated CBR activation during adolescence does not produce long-term effects on discrimination learning or reversal learning performance in either sex.

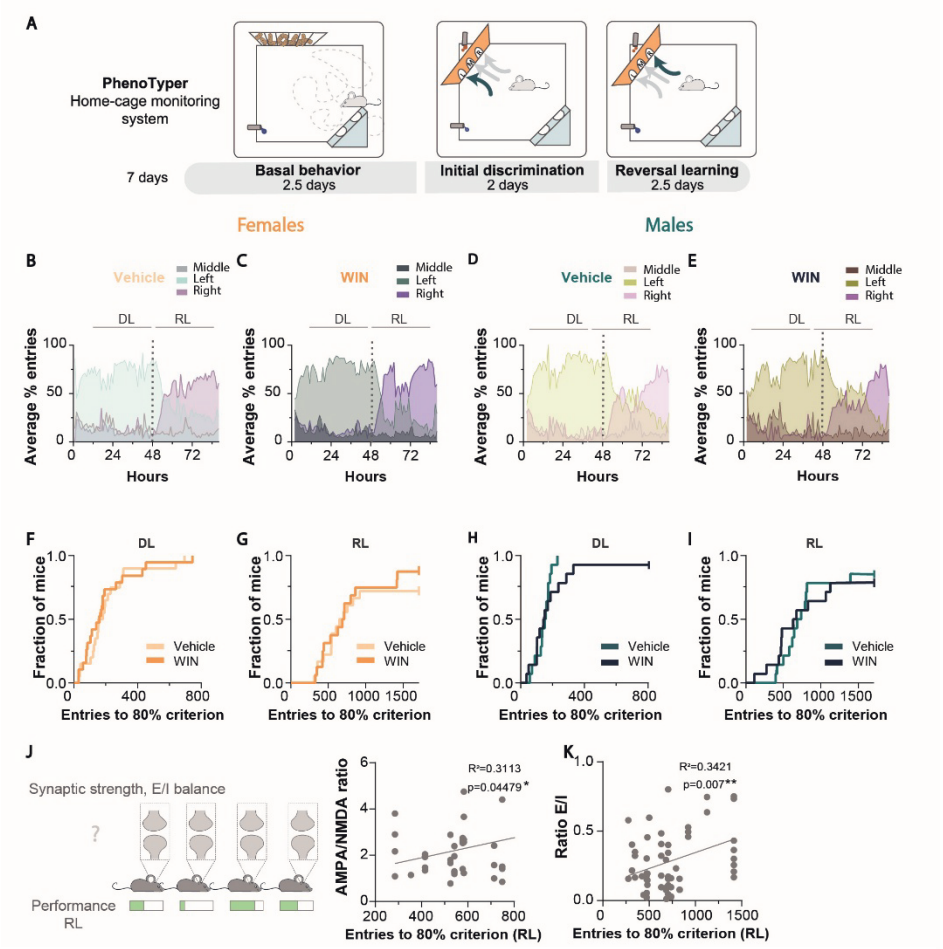


Figure 5. Adolescence WIN exposure does not alter reversal learning performance. (A) Schematic representation of the PhenoTyper cage during basal behavior (left panel), initial discrimination (middle panel) and reversal learning (right panel). (B) Proportion of average entries per hour in the left, middle, or right holes in female vehicle mice. The dashed line represents the beginning of the reversal learning phase. (C) Same than B but for female WIN. (D) Same as B but for male vehicle. (E) Same as B but for male WIN. (F) Survival plot showing the fraction of vehicle (n = 20) and WIN-injected (n = 19) female mice that reached the 80% learning criterion as a function of the number of entries during discrimination learning. Each square represents one animal that reached the criterion. (G) Same as F but for reversal learning. Vehicle n = 13, WIN n= 14. (H) Same as F but for males. Vehicle n=14, WIN n= 13. (I) Same as G but for males. Vehicle n=12, WIN n=11. (J) AMPA/NMDA ratio as a function of entries to 80% criterion during reversal learning, data from all four groups are shown together (male and female, vehicle and WIN). AMPA/NMDA ratio significantly correlates with performance during reversal learning (Pearson's r = 0.31, 95% CI [0.01, 0.56], R^2 = 0.097, p = 0.0448, n=42 cells). (K) Same as J but for network E/I ratio. A significant correlation between network E/I ratio and entries to 80% criterion during reversal learning was observed (Pearson's r = 0.342, 95% CI [0.09, 0.54], R^2 = 0.117, p = 0.007, n=61 cells). DL=Discrimination learning; RL= reversal learning; WIN = WIN 55,212-2.

To assess whether the capacity for reversal learning is related to L5-mPFC function, performance in the reversal learning task was correlated with electrophysiological properties of L5-mPFC neurons. A significant correlation was found between AMPA/NMDA-ratio and the number of entries to reach the 80% criterion for reversal learning (**Figure 5J**). In addition, the strongest correlation was observed for the balance between excitatory and inhibitory conductance (**Figure 5K**), where a higher AMPA/NMDA ratio was associated with poorer performance, suggesting that the E/I balance of L5-mPFC neurons may serve as a predictor of reversal learning performance.

Discussion

In this study, we investigated the long-term consequences of repeated, irregular activation of CBRs by WIN55,212-2 during adolescence on neuronal excitability and synaptic transmission within the mPFC, as well as associated behaviors. We found that WIN exposure produced the following permanent changes to layer 5 pyramidal neurons in IL and PrL areas of mPFC: (1) in males, intrinsic excitability was altered, including a shift in the proportion of bursting neurons; (2) in females, synaptic physiology was altered, showing increased AMPA/NMDA ratio; and (3) also in females, both inhibitory and excitatory conductance were elevated while the overall excitation/inhibition balance was preserved. Taken together, these findings show that repeated adolescent CBR activation induces long-lasting, sex-specific alterations in mPFC physiology at both cellular and synaptic levels. Nevertheless, these physiological alterations did not translate into measurable deficits in two mPFC-dependent adaptive behaviors, social hierarchy and reversal learning, in adulthood. Our results, therefore, provide new insights into how CBR activation during adolescence alters PFC physiology at the cellular and network levels without necessarily impairing adaptive behavior in adulthood.

Our patch-clamp experiments revealed sex-specific alterations in the intrinsic electrophysiological and synaptic properties of L5-mPFC neurons. A main finding was a significant shift in the proportions of RS versus BS neurons, specifically in WIN-treated males. Bursting is a key feature that enhances the reliability of synaptic transmission and increases the coding capacity of neuronal networks by conveying more information than single spikes [72]. In cortical layer 5, neuronal bursting has been described as a coincidence detector for the synaptic integration of inputs from both apical and basal tufts [56,58,60]. Furthermore, burst firing in the PFC plays a crucial role in attentional control by synchronizing the activity between PFC and anterior

cingulate cortex [59,61]. Although a computational model [70] suggests that bursting behavior is modulated by the interplay between excitatory and inhibitory inputs onto basal and apical dendrites [73], our data from male mice showed no detectable changes in evoked excitatory and inhibitory synaptic transmission. Given that synaptic stimulations were evoked by placing the stimulating electrodes in layer 5, we assume that our experimental design specifically recruited the local network surrounding the soma and proximal dendrites of the neurons. Therefore, it may be possible that distal inputs may have been affected by the WIN-treatment. In addition, alternative mechanisms, such as intrinsic properties or morphological changes, rather than changes in synaptic drive, may underlie the shift in the proportions of bursting neurons we observed. Disruptions in the normal activity of the ECS during PFC development are known to induce long-term changes in neuronal morphology and physiology of other cortical cells, ultimately affecting the organization and function of PFC networks. Renard and colleagues [30] demonstrated that daily injections of the CB1/CB2R agonist CP55,940 in male rats during adolescence led to alterations in the dendritic arborization of layer 2-3 neurons, accompanied by an impairment in LTP induction in the hippocampus-PFC circuit. Such disruptions in neuronal morphology may, in turn, alter the firing patterns of pyramidal neurons, as dendritic architecture influences neuronal excitability and the likelihood of burst generation.

Alongside alterations in intrinsic excitability, we observed additional sex-specific patterns in our data. WIN-treated female mice exhibited synaptic changes, reflected by an increased AMPA/NMDA ratio. This strengthening of excitatory drive onto L5-mPFC neurons could result from either an increase in AMPA receptor density or a downregulation of NMDA receptors. Our findings are partially supported by Rubino and colleagues [29], who reported alterations in glutamatergic receptor expression of adult female rats treated with THC during adolescence. Specifically, they observed elevated levels of the AMPA receptor subunit GluA1 in PFC homogenates of THC-exposed animals, along with increased expression of the NMDA receptor subunit GluN2B. In addition, although only males were assessed, recent evidence has shown upregulation of AMPA-subunit-encoding genes (*GRIA1* and *GRIA3*) in L2/3 pyramidal neurons following adolescent WIN exposure, supporting a potential increase in AMPA receptor density. Along with the increase in AMPA/NMDA ratio, we also observed elevated excitatory and inhibitory conductances in WIN-treated female mice, resulting in preservation of local network E/I balance. Notably, these alterations in evoked excitatory and inhibitory synaptic transmission were absent in WIN-treated males, highlighting the relevance of sex as a biological variable.

Taken together, our electrophysiological investigation reveals that repeated CBR activation during adolescence leads to lasting, sex-specific alterations in intrinsic

excitability and synaptic transmission of L5-mPFC pyramidal neurons. These changes may ultimately affect the mPFC output and, consequently, the cognitive functions it supports. Several preclinical studies have shown that repeated activation of CBRs by cannabinoid agonists such as CP55,940 or WIN during adolescence leads to long-lasting behavioral impairments in adulthood, including deficits in object recognition, spatial memory, and social interactions [31,32,34–37,77]. All these studies were conducted exclusively with male rats. In contrast to reports of long-term behavioral impairments, our study found no measurable effects of repeated WIN administration during adolescence on adult social hierarchy or reversal learning in either male or female mice. This suggests that either compensatory mechanisms may occur that nuance the functional impact of the treatment at the behavioral level, or that our behavioral tests did not capture the cognitive deficits that may arise under more challenging environments. Interestingly, we found a correlation between synapse strength in L5-mPFC neurons and social status in mice. It was previously shown that top- ranked mice have stronger L5-mPFC synapses compared to lowest ranked mice [78], although mice in intermediate ranks were not examined. In our study, mice in the second rank exhibited the strongest L5-mPFC synapses, which dovetails our unpublished findings that mice with superior PFC synaptic plasticity preferentially occupy the second rank (Xiong et al., unpublished). Our observation that WIN-treated mice, despite presenting alterations in L5-mPFC neurons, showed no changes in social hierarchy may reflect that these neuronal changes were too subtle to influence behavior. More specifically, among measurements for synaptic connectivity, we only found a significant difference between social ranks for mEPSC amplitudes, which did not show large changes in mice exposed to adolescent CBR activation. Alternatively, changes at L5-mPFC synapses were counterbalanced by alterations in other neuronal populations. For instance, whereas downstream targets of L5-mPFC promote social hierarchy, L3-mPFC neurons have the opposite effect on social status [66]. Similar arguments can be made for the absence of phenotypic consequences of adolescent WIN-treatments on the capacity for discrimination learning and reversal learning. Consistent with our findings, Hernandez and colleagues [79] reported no impairment in reversal learning in a food-motivated task following adolescent cannabis smoke exposure to male rats. Whereas the mPFC has traditionally been described to play a role in cognitive flexibility, a more updated model is that the mPFC only substantially contributes to reversal learning when high attentional levels are required [80]. Nevertheless, our results did reveal a significant correlation between performance in reversal learning and synaptic E/I balance at L5-mPFC neurons. The preserved E/I synaptic and network balance in both male and female WIN-treated mice may explain why repeated CBR activation during adolescence did not translate into observable behavioral alterations.

In conclusion, in this study we showed a causal relationship between repetitive CBR activation during adolescence and alterations in mature mPFC neurons, highlighting sex-differences. Our study has bearing on the recreational use of cannabis during a period in life when the mPFC is still in development. Interestingly, both clinical and preclinical studies indicate that vulnerability to cannabis consumption during adolescence may be higher in females than in males [40,81]. In fact, although CB1R density in PFC is lower in female rats, these receptors were shown to have a higher affinity than in males [40] as well as greater downregulation and desensitization [81]. In humans, cannabis consumption during adolescence has also been associated with altered excitatory and inhibitory neurotransmission [82,83]. A recent epidemiological study found that cannabis use during this critical period, but not in young adulthood, was associated with an over elevenfold increase in the risk of developing a psychotic disorder [84]. Complementing this, other studies have demonstrated sex-dependent effects of regular cannabis use on cognitive outcomes in young males and females [85,86]. Our findings add to this body of work by providing insight into the long-term, sex-specific risks of adolescent cannabis exposure, thereby contributing knowledge that may help inform future legislation and prevention strategies.

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Supplementary Figures

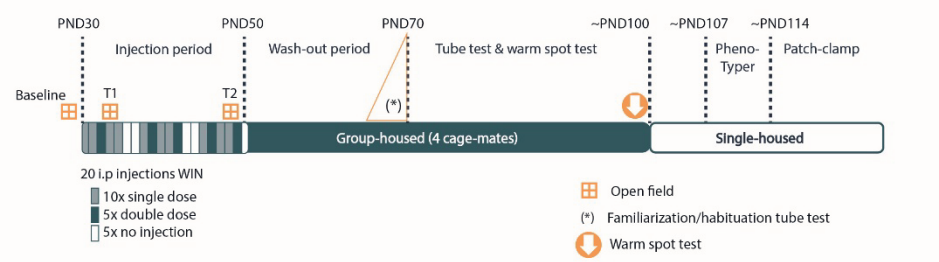


Figure S1. Time-line of the experimental design. Mice were injected between PND30 and PND50 following an irregular administration scheme with 10 times of single dose injection, 5 times of double dose injection and 5 times of no injection. Squares represent the moment of open field testing baseline (before first day of injections), T1 (after first double-dose injection) and T2 (after last double-dose injection). Familiarization and habituation to the tube test set up started one week before the end of the wash-out period. Yellow arrow specifies the last day of tube test, when animals were tested in the warm spot test. PND = postnatal day; WIN = WIN 55,212-2.

Table S1. Intrinsic properties of IL and PrL layer 5 pyramidal neurons after repeated adolescence WIN exposure. Passive and active properties are separated, top and bottom respectively N refers to number of cells while number of animals is shown in brackets. All variables were non-normally distributed. Statistical differences were tested with Mann-Whitney U test.

	Females					Males				
	Vehicle		WIN			Vehicle		WIN		
Variable	Mean (SEM)	n	Mean (SEM)	n	p-value (U)	Mean (SEM)	n	Mean (SEM)	n	p-value (U)
Passive properties										
Membrane capacitance (pF)	92.245 (6.093)	45 (8)	95.882 (3.507)	67 (9)	0.361 (1405)	103.100 (7.209)	27 (5)	97.794 (5.098)	46 (6)	0.658 (667)
Time constant (ms)	25.157 (1.585)	45 (8)	24.080 (1.541)	67 (9)	0.260 (1368)	28.690 (1.828)	27 (5)	24.919 (1.509)	46 (6)	0.065 (532)
Active properties										
Rheobase (pA)	89.604 (8.502)	45 (8)	100.196 (7.721)	67 (9)	0.245 (1362)	115.330 (12.303)	27 (5)	120.769 (8.707)	46 (6)	0.551 (652)
AHP amplitude (mV)	13.057 (0.514)	45 (8)	13.574 (0.349)	67 (9)	0.117 (1243)	13.031 (0.590)	27 (5)	15.413 (1.606)	46 (6)	0.216 (512)
AP amplitude (mV)	86.356 (2.071)	45 (8)	84.962 (1.530)	67 (9)	0.739 (1451)	79.970 (1.498)	27 (5)	73.162 (1.260)	46 (6)	0.395 (546)
AP half width (ms)	1.116 (0.044)	45 (8)	1.228 (0.168)	67 (9)	0.529 (1401)	1.061 (0.068)	27 (5)	0.969 (0.033)	46 (6)	0.462 (556)

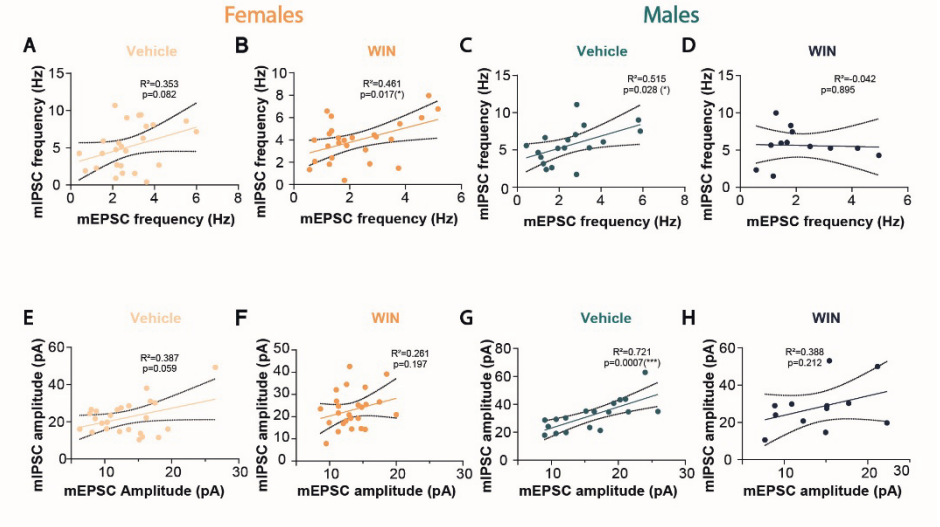


Figure S2. Correlations between excitatory and inhibitory synaptic parameters. For all panels, colors indicate treatment and sex: female vehicle (light orange), female WIN (dark orange), male vehicle (light blue), male WIN (dark blue). Miniature excitatory and inhibitory postsynaptic currents were recorded from the same set of cells: female vehicle (n=18 cells from 3 mice), female WIN (n=19 from 4 mice), male vehicle (n=16 from 4 mice) and male WIN (n=12 from 4 mice). Correlation between mIPSC and mEPSC frequency in neurons from (A) vehicle females, (B) WIN females, (C) male vehicle and (D) male WIN. Positive correlations were found in WIN injected females (Pearson's r = 0.593, 95% CI [0.17, 0.83], R^2 = 0.351, p = 0.009) and vehicle males (Pearson's r = 0.515, 95% CI [0.06, 0.79], R^2 = 0.265, p = 0.028).(E-H) Same than A-B but for mIPSCs and mEPSCs amplitude. A significant correlation was found in vehicle males (Pearson's r = 0.721, 95% CI [0.38, 0.88], R^2 = 0.52, p = 0.0007). All panels display Pearson correlation.

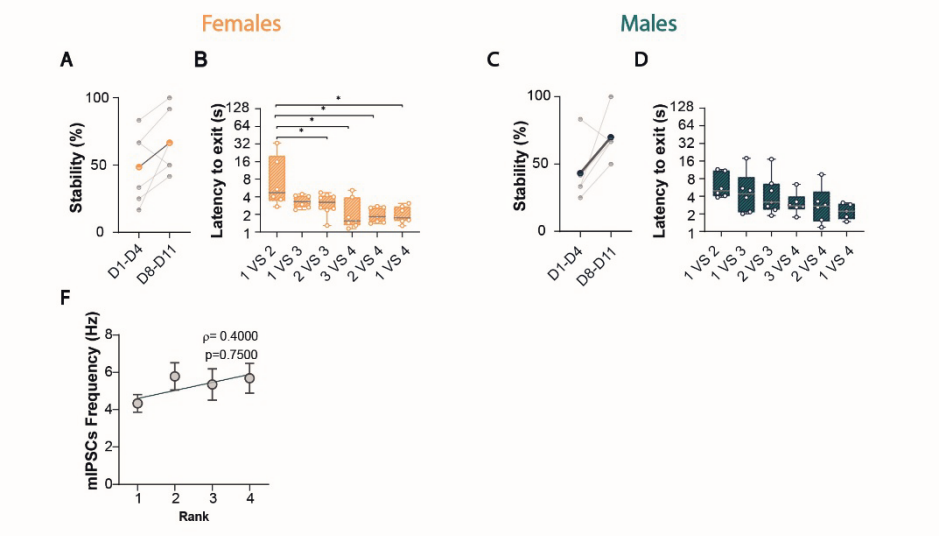


Figure S3. Descriptive parameters of social hierarchy behavior in male and female mice. (A) Percentage stability in females (vehicle and WIN mice are pulled together) between the first (D1-D4) and last (D8-D11) four training days of tube test. Stability was calculated as the percentage of transitions out of the maximum number (12) for all animals of a cage during the four days. Grey lines and dots refer to individual cages, black line and orange dots refer to the average of all cages (n=6). (B) Latency to exit per competition pair in females. Vehicle and WIN are pulled together. One-way ANOVA showed a significant effect on latency to exit ($F(5,36)= 2.548$, $p=0.013$). Dunnett's multiple comparison analysis revealed significant effects on 1VS2 vs 1VS3 ($p=0.026$), 1VS2 vs 2VS3 ($p=0.017$), 1VS2 vs 3VS4 ($p=0.009$), 1VS2 vs 2VS4 ($p=0.006$) and 1VS2 vs 1VS4 ($p=0.006$). (C) Same as A but for males. (D) Same as B but for males. (E) mIPSC amplitude as a function of social rank, data from all four groups are shown together (male and female, vehicle and WIN). Rank 1 n=21 cells, rank 2 n=11 cells, rank 3 n=10 cells, rank 4 n=10 cells. (F) Same as E but for mIPSCs frequency. E-F display Spearman correlation analysis.

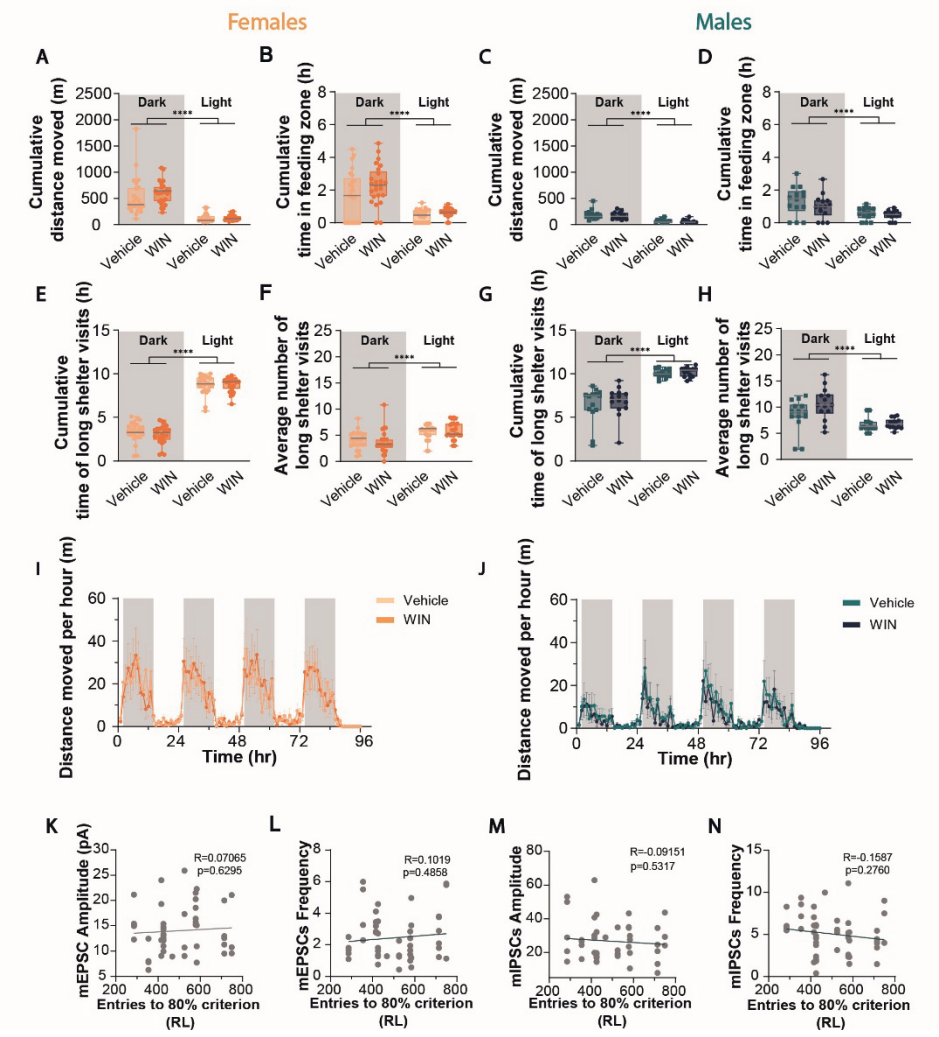
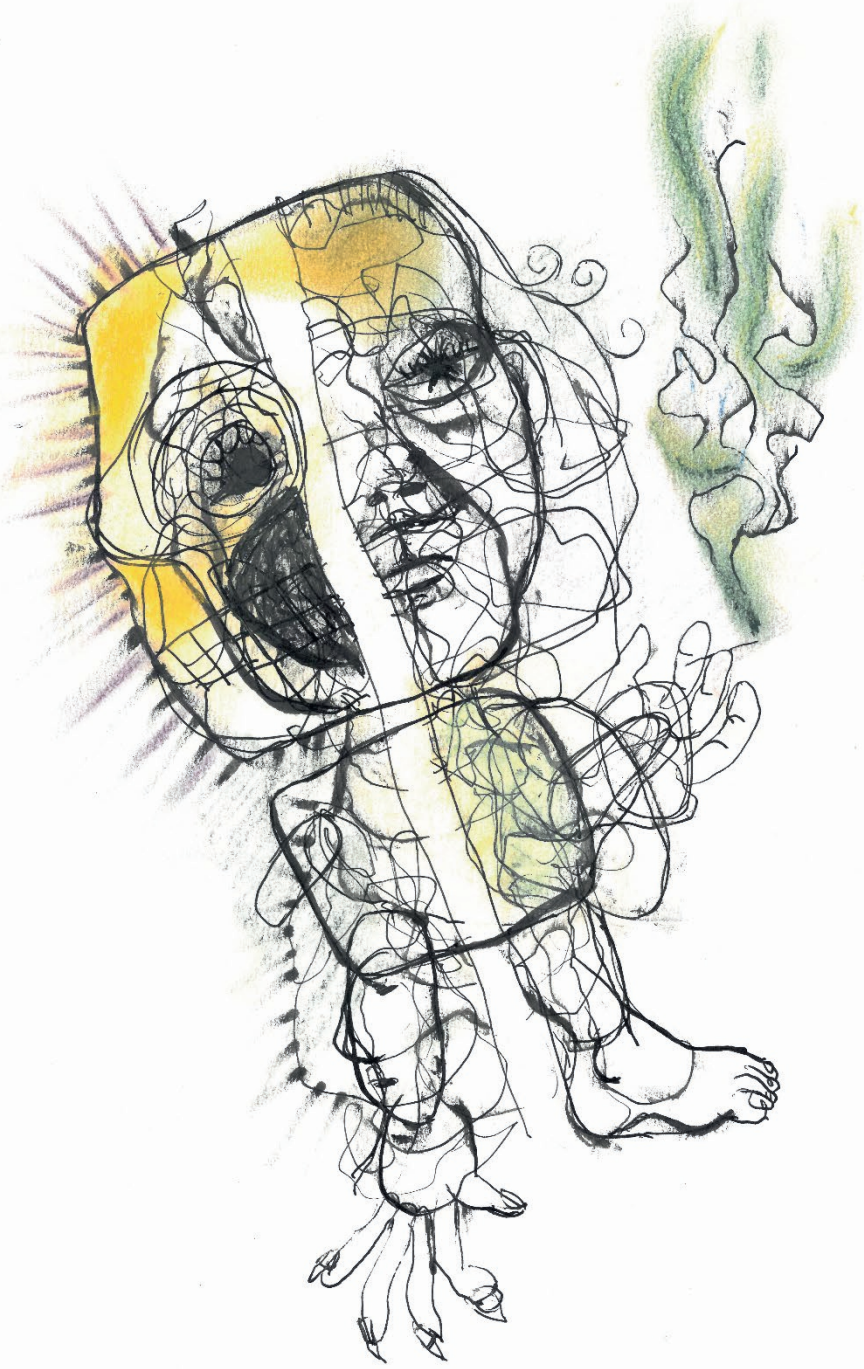


Figure S4. Repeated adolescent WIN exposure does not alter basal behavior during adulthood. For all panels, colors indicate treatment and sex: female vehicle (light orange, n=25), female WIN (dark orange, n=25), male vehicle (light blue, n=14), male WIN (dark blue, n=14). Grey and white squares indicate dark and light periods respectively. Distance moved (A) and time in feeding zone (B) for vehicle- and WIN-injected females during dark and light periods. A significant effect of dark/light time was found on distance moved ($F(1,48)=137$, $p<0.0001$, 2-way ANOVA) and time in feeding zone ($F(1,48)=82.01$, $p<0.0001$, 2-way ANOVA). (C-D) Same as A-B but for males. A significant effect of dark/light time was found on distance moved ($F(1,26)=98.69$, $p<0.0001$, 2-way ANOVA) and time in feeding zone ($F(1,26)=30.59$, $p<0.0001$, 2-way ANOVA). (E) Total time and average number (F) of long shelter visits for vehicle and WIN injected females. A significant effect of dark/light time was found on time ($F(1,48)=1030$, $p<0.0001$, 2-way ANOVA) and number of long shelter visits ($F(1,48)=52.26$, $p<0.0001$, 2-way ANOVA). (G-H) Same as E-F but for males. A significant effect of dark/light time was found on time ($F(1,26)=107.1$, $p<0.0001$, 2-way ANOVA) and number of long shelter visits ($F(1,26)=24.19$, $p<0.0001$, 2-way ANOVA). (I) Total distance moved per hour during basal behavior in vehicle and WIN females (J) Same as I but for males. (K) mEPSC amplitude as a function of entries to 80% criterion during reversal learning, data from all four groups are shown together (male and female, vehicle and WIN). Total n=51 cells. (L) Same as K but for mEPSCs frequency. (M) Same as K but for mIPSCs amplitude (P) Same as K but for mIPSCs frequency. Panels K-P display Pearson's correlation analysis.



Chapter 5

Susceptibility of GluA3-deficient mice to reversal learning deficits following adolescent cannabinoid receptor activation: a pilot study

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Abstract

AMPA receptors (AMPARs), which mediate fast excitatory transmission in the brain, have been implicated in several pathological conditions. In particular, *GRIA3*, the gene encoding the AMPAR subunit GluA3, has been associated with schizophrenia. GluA3-knockout (GluA3KO) mice may therefore represent a model of increased susceptibility to schizophrenia-related phenotypes, including impairments in cognitive flexibility, a domain frequently altered in patients with the disorder. Adolescence represents a critical developmental window in this context, as cannabis use during this period has been reported to increase the likelihood of developing schizophrenia. In this pilot study, we explored a double-hit model of susceptibility, in which the absence of GluA3-containing AMPARs represents genetic predisposition (first hit), and adolescent activation of cannabinoid receptors (CBRs) constitutes an environmental challenge (second hit). To test this, GluA3KO mice were exposed to a cannabinoid receptor agonist during adolescence, and behavioral and electrophysiological recordings were performed in adulthood. Pharmacological activation of CBRs during adolescence did not impair discrimination or reversal learning in adult GluA3KO mice. However, preliminary data suggest a possible increased vulnerability in GluA3KO male mice, which showed a tendency toward reduced reversal learning performance. At the synaptic level, adolescent CBR activation led to reduced inhibitory synaptic transmission in layer 2/3 neurons of the medial prefrontal cortex (mPFC) in adult male mice. Despite the inherent limitations of a pilot study, these findings provide a valuable foundation for future work exploring potential sex-dependent vulnerability to the combined effects of GluA3-deficiency and adolescent CBR activation in the context of schizophrenia.

Introduction

The prefrontal cortex (PFC) plays a central role in higher-order cognitive functions, including planning, social behavior, and cognitive flexibility [1,2]. Cognitive flexibility, the ability to switch between mental processes, is essential for adaptation to changing environments and requires intact PFC function [3,4]. Within the PFC, neurons in layer 2/3 contribute specifically to the encoding and categorization of learnt rules during reversal learning [5]. Developmentally, the PFC shares several maturation mechanisms compared with other cortical regions [6–8]; yet its overall maturation is protracted, spanning from embryonic stages through early adulthood in both humans and rodents [9]. Given the PFC's critical role in executive functions, disruptions in this region are frequently implicated in psychiatric conditions such as schizophrenia, where abnormalities in prefrontal circuitry are well documented [10–13]. Nevertheless, despite the strong association between schizophrenia and impaired cognitive flexibility, the evolution of this impairment during clinical progression remains unclear [10].

α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) are ionotropic glutamate receptors responsible for mediating fast excitatory synaptic transmission [14]. These tetrameric receptors are composed of GluA1-GluA4 subunits, most commonly assembled as GluA1/GluA2 or GluA2/GluA3 heteromers in the cortex, although other configurations may occur [14,15]. Genetic studies have implicated *GRIA3*, which encodes the GluA3 AMPAR subunit, in schizophrenia. Early evidence identified associations in female patients [16], and more recently, rare coding variants of *GRIA3* were found to confer substantial risk for the disorder, primarily through loss-of-function or high-impact missense variants [17]. For these reasons, GluA3KO mice may serve as a model of increased susceptibility to schizophrenia-related phenotypes. Consistent with this, in **Chapter 3** of this thesis we show that GluA3KO mice exhibit reduced synaptic transmission onto layer 2/3 pyramidal neurons of the mPFC, along with a mild impairment in cognitive flexibility, deficits that are also found in patients and animal models of the disease etiology [10–13].

In parallel, cannabis use during adolescence is associated with increased risk of developing psychiatric conditions, including schizophrenia [18–21], and many studies have examined its link to psychosis-related outcomes [22–25]. This association is influenced by factors such as age of onset and cannabis potency, with use during early adolescence and exposure to high-potency cannabis more strongly linked to later psychiatric episodes [18,26]. Cannabis use leads to activation of cannabinoid receptors (CBRs), which are G-protein-coupled receptors that regulate synaptic activity through retrograde synaptic signaling [27,28]. CB1Rs are primarily expressed in

the central nervous system, and are particularly enriched in both subcortical and cortical structures, including the mPFC [29,30]. On the other hand, CB2Rs expression was initially considered to be restricted to the peripheral nervous system and immune cells, although several studies have demonstrated their functional presence in the central nervous system [31–35]. Endocannabinoids are produced on demand in the post-synapse and act on presynaptic CBRs, primarily CB1Rs, to reduce neurotransmitter release and weaken synaptic transmission. Repeated activation of CBRs during adolescence has been shown to have long-term consequences at the behavioral, cellular and network level [36–40]. More specifically, layer 2/3 pyramidal neurons of the mPFC have been reported to exhibit alterations following adolescent CBR activation. In this layer, both neuronal morphology and developmental trajectories differ from vehicle-treated controls [41,42], highlighting the sensitivity of the prefrontal cortex, and of layer 2/3, to CBR activation during adolescence, a critical period for PFC maturation. In addition, in **Chapter 4** of this thesis, we found that repetitive injection of CBR activation during adolescence has long-lasting effects on layer 5 neurons, underscoring the susceptibility of the circuit to this insult, although it did not significantly influence the ability for reversal learning.

In this chapter, we explored the hypothesis that adolescent CBR activation may have a greater impact on reversal learning in GluA3KO mice, a model of increased predisposition for schizophrenia-like phenotypes. Considering that GluA3KO mice exhibit weakened postsynaptic transmission (**Chapter 3**), activation of CBRs during adolescence could further depress presynaptic function in layer 2/3-mPFC synapses, providing a potential “double-hit” scenario for the emergence of schizophrenia-like behavior. To test this double-hit hypothesis, we conducted a pilot study in which GluA3KO mice underwent adolescent CBR activation, and we evaluated its impact on reversal learning and synaptic transmission in layer 2/3 of the mPFC.

Materials & Methods

Animals

Gria3-deficient (GluA3KO) mice (*Gria3tm1Dgen/Mmnc;* Mutant Mouse Regional Resource Center, Davis, CA) and their wild-type littermates of both sexes were used in this study. WT and GluA3KO littermates were group-housed in tetrads from weaning onwards under standard laboratory conditions (12-h light/dark cycle; lights on 7:00-19:00) with ad libitum access to food and water, unless otherwise specified. Body weight was monitored 3 times a week during the injection period and once a week

during the remainder of the experiment. For behavioral testing in the Phenotyper (Noldus, the Netherlands), mice were singly housed one week prior to the start of the test to allow for habituation.

All experiments were conducted in accordance with the European guidelines for the care and use of laboratory animals (European Directive 2010/63/EU). The experimental protocol was approved by the Animal Experiment Committee of the Royal Netherlands Academy of Arts and Sciences (KNAW) and by the Animal Welfare Body of the University of Amsterdam (permit number AVD11100202114907).

WIN-treatment

Mice aged between PND30-PND50 were injected intraperitoneally with 1.2 mg/kg R(+)-WIN 55,212-2 (WIN) (MERCK-Sigma Aldrich). WIN was dissolved in 3.5% DMSO and 0.3% Tween-80 and diluted in saline (0.9 %). Animals were randomly allocated to either experimental condition, WIN or vehicle (3.5% DMSO and 0.3% Tween-80 diluted in saline). Additionally, the experimenter was blinded to the treatment group during the administration of injections and following experimental procedures. Adolescent treatment with the CB1-CB2R agonist WIN, or vehicle, lasted 20 days (PND30-PND50). The administration regimen was irregular, mimicking the sporadic pattern of recreational cannabis use, as previously described by others [43–46]. During this period, WIN-injection dosages were varied and interleaved across days, totaling 10 days of single-dose, 5 days of double-dose, and 5 days without injection, following the pattern described in **Figure 2A**. Per cage, 2 mice received WIN injections, while the remaining 2 were administered vehicle injections. A wash-out period of 20 days was included to avoid withdrawal symptoms from influencing behavioral testing.

PhenoTyper

The PhenoTyper (Noldus) is an automated home-cage monitoring system equipped with a shelter, water bottle, food dispenser, and a cognition wall. Mice were individually housed in PhenoTyper cages for the entire duration of the test period (7 days) and weight was monitored before and after this period. During the initial habituation phase, that lasted 2.5 days, water and regular chow were provided ad libitum and basal behavior was recorded. Thereafter, regular food was removed from the feeding compartment. At this time (end of the third day) the cognition wall was introduced in the cage and the cognitive testing started. The cognition wall consisted of three entry holes and the mouse passage through each entry was monitored (**Figure 2B**). Mice were habituated to the cognition wall for 1 hour and 3 free rewards (Dustless Precision Pellets, 14 mg, Bio-Serve) were delivered to facilitate familiarization before the start of

the experiment. The multiple-choice reversal learning task consisted of two phases. In the first phase, animals were trained in a discrimination learning task, where only one of the three holes was rewarded. Following 48 hours of discrimination learning, the reward allocation was changed to a different hole, initiating the reversal learning phase, which lasted for 48 hours. During discrimination and reversal learning, one sugar pellet was delivered for every fifth entry through the correct hole. The five entries were not required to be consecutive [47].

Learning during the discrimination and reversal learning tasks was quantified as the total number of entries to reach an 80% or 90% correct performance criterion, calculated using a sliding window of 30 consecutive entries (i.e. at least 24 correct responses out of the last 30 entries for 80%) [47]. For Kaplan-Meier survival analysis, a cut-off was set to 800 and 1700 entries for reaching the criterion in discrimination and reversal learning respectively. All entries were automatically registered by the EthovisionXT11 software and video was recorded during the entire course of the experiment. Data was analyzed off-line using AHCODA-DB [48].

Acute slice preparation

Mice were killed using cervical dislocation after behavioral testing (at 13-16 weeks of age) for acute brain slice preparation. Brains were extracted and placed in ice-cold modified artificial cerebrospinal fluid (mACSF, in mM: 139 choline chloride, 3.5 KCl, 0.5 $CaCl_2$, 6.0 $MgSO_4$, 1.25 NaH_2PO_4 H_20, 10 glucose and 25 $NaHCO_3$, continuously bubbled with 5% CO2/ 95% O2 (pH 7.4)). Coronal slices (300 µm) containing the mPFC (approximately 1.94 mm anterior to Bregma) were obtained using a vibrating blade microtome (VT 1200S, Leica Microsystems). Slices were subsequently incubated at 35°C for 20 minutes in ACSF (in mM: 120 NaCl, 3.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4 H_20, 10 glucose, 2.5 $CaCl_2$, and 1.3 $MgCl_2$, bubbled with 5% CO2/ 95% O2), and then kept for at least 30 min at room temperature before recording.

Ex-vivo patch-clamp recordings

Whole-cell voltage clamp recordings were made from layer 2-3 pyramidal neurons of the mPFC. During recordings, slices were submerged in ACSF at 28-30 ° Celsius, bubbled with 5 % CO2/95 % O2 and superfused at a rate of 1.8 ml/min. Voltage-clamp recordings of miniature excitatory and inhibitory post-synaptic currents (mEPSCs and mIPSCs respectively) were performed in the presence of 1 mM tetrodotoxin (TTX, Latoxan, France) and glass recording pipettes were filled with (in mM): 115 CsMeSO3, 3.97 CsCl, 2.5 $MgCl_2$, 10 HEPES, 4 Na_2-ATP, 0.4 Na-GTP, 10 Na-Phosphocreatine and 0.6 EGTA. Signals were acquired using a MultiClamp 700B amplifier controlled by

pClamp 11 software (Molecular Devices), low-pass filtered at 4 kHz and sampled at 10 kHz. Series resistance ranged from 6–20 MΩ and was compensated to ~65 %. Signals were corrected for liquid junction potential.

Miniature excitatory and inhibitory post-synaptic currents:

Cells were voltage-clamped for 5 minutes at -70 mV and 0 mV for recording mEPSCs and mIPSCs, respectively. These holding voltages correspond with the reversal potential for GABA and glutamate in our experimental conditions, allowing for the isolation of excitatory and inhibitory components. Recordings were performed in the presence of tetrodotoxin (TTX, 1 µM) to block spontaneous AP. The amplitude and frequency were determined and analysed for every miniature synaptic event exceeding a 5 pA noise threshold over 5 minutes of recording. Ratio excitation/inhibition (E/I) was calculated as the ratio of the total synaptic charge carried by mEPSCs and mIPSCs, obtained by summing the integrals (area under the curve) of all detected events within a fixed recording period [49,50]. For mEPSCs and mIPSCs, at least 100 events were analysed per cell. Off-line analysis was performed using *Clampfit* (Molecular Devices). Where indicated, 5 mM WIN dissolved in 0.01% DMSO was bath-applied for at least 15 minutes before recordings [51].

For correlation analyses between behavioral and electrophysiological data, each recorded cell was assigned the behavioral performance score of the animal from which it was obtained. These cell-level data were then treated as independent measures. To compare mEPSC frequency distributions between cells, cumulative probability distributions were constructed for each individual cell and compared to a control distribution using the two-sample Kolmogorov–Smirnov test. The resulting D value, representing the maximum distance between the two cumulative distributions, was calculated for each cell and used for subsequent statistical analysis across experimental groups.

Statistical analysis

All statistical tests were performed in GraphPad version 8 (GraphPad Software, San Diego). Data were statistically analyzed using unpaired t-tests, Mann-Whitney tests, Kolmogorov-Smirnov tests or two-way ANOVA, as appropriate. Post-hoc multiple comparison was done by Tukey's or Dunn's multiple comparison tests. Survival curves were tested for significance with the log-rank (Mantel-Cox) test. Pearson correlation coefficient was used for correlations. Data are presented as medians with whiskers indicating minimum and maximum values, or as bar plots with error bars representing the standard error of the mean (SEM), depending on the distribution of the data. Statistical significance was considered as * $p<0.05$, ** $p<0.01$,*** $p<0.001$.

Results

Acute CBR activation decreases inhibitory synaptic transmission onto L2/3 pyramidal neurons in the mPFC

CBRs are predominantly expressed at presynaptic terminals, where their activation reduces presynaptic vesicle release at both excitatory and inhibitory synapses onto L2/3-mPFC neurons [51–53]. To determine whether CBR activation induces synaptic depression at mPFC neurons in our experimental conditions, we performed whole-cell recordings from L2/3 pyramidal neurons in the infralimbic (IL) and prelimbic (PrL) areas of the mPFC in acute slices from WT mice. Miniature excitatory and inhibitory postsynaptic currents (mEPSCs, mIPSCs respectively) were recorded in the presence and absence of WIN, a CB1R/CB2R agonist (**Figure 1A**). In WT neurons, bath application of WIN did not significantly decrease mEPSC frequency (t(18)=1.19, p=0.25; **Figure 1B**) or amplitude (t(18)=1.41, p=0.174; **Figure 1C**). Regarding inhibitory transmission, mIPSC frequency was significantly reduced following WIN application (t(18)=2.591, p=0.018; **Figure 1D**), without a significant change in average mIPSC amplitude (t(18)=2.09, p=0.050; **Figure 1E**). These findings indicate that acute WIN exposure predominantly reduced inhibitory, but not excitatory, synaptic transmission onto L2/3 pyramidal neurons in the mPFC, likely through suppression of presynaptic vesicle release.

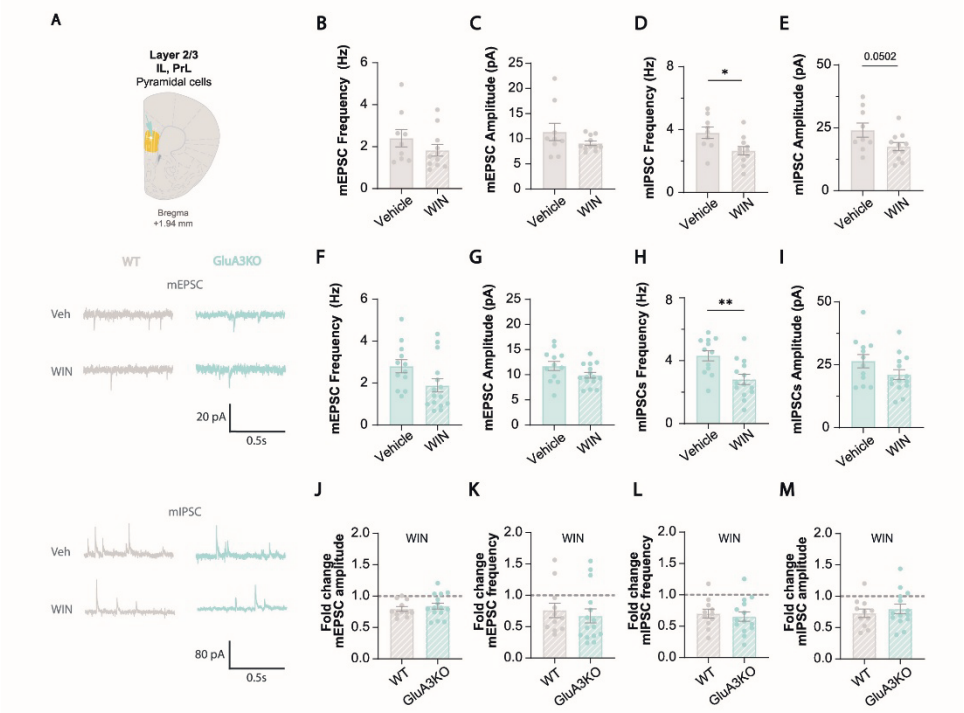


Figure 1. Acute WIN incubation reduces inhibitory transmission in mPFC-L2/3 pyramidal neurons of WT and GluA3KO males. (A) Top: schematic representation of a coronal slice at +1.94 mm relative to bregma (adapted from Paxinos and Franklin atlas), showing the IL and PrL regions and cortical layer 2/3 included in the study. Bottom: Representative traces of miniature excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs) recorded from L2/3 pyramidal neurons of WT (left) and GluA3KO (right) mice under vehicle (0.01% DMSO) or WIN (5 µM) conditions. Colors indicate genotype throughout: grey, WT; blue, GluA3KO. All data were obtained from the same set of cells: WT vehicle (9 cells from 2 mice), WT WIN (11 cells from 2 mice), GluA3KO vehicle (12 cells from 3 mice), and GluA3KO WIN (14 cells from 3 mice). (B–D) mEPSC frequency in vehicle and WIN conditions from L2/3 pyramidal neurons of WT (B) and GluA3KO (C) mice, and corresponding fold change (D). (E-G) Same as B-D but for mEPSC amplitude. (H-I) Same as B-D but for mIPSC frequency. (K-M) Same as B-D but for mIPSC amplitude.

Given that CBRs are expressed presynaptically, whereas GluA3 is postsynaptic, we expected the effects of WIN to be preserved in L2/3-mPFC neurons lacking GluA3. WIN application did not significantly affect mEPSC frequency ($t(25)=2.05$, $p=0.051$; **Figure 1F**) or amplitude ($t(25)=1.804$, $p=0.083$; **Figure 1G**). Moreover, the magnitude of change relative to their control conditions was comparable across genotypes for both mEPSC frequency ($t(24)=0.540$, $p=0.594$; **Figure 1J**) and amplitude ($t(24) = 0.668$, $p = 0.510$; **Figure 1K**). Inhibitory transmission was affected in a similar manner as in WT neurons. WIN application significantly reduced mIPSC frequency in GluA3KO neurons ($t(24)=3.231$, $p=0.003$; **Figure 1H**), without a significant decrease in mIPSCs amplitude ($t(24)=1.645$, $p=0.113$; **Figure 1I**). Accordingly, the degree of reduction did not differ between genotypes for either mIPSC frequency ($t(23)=0.448$, $p=0.658$; **Figure 1L**) or amplitude ($t(23)=0.644$, $p=0.525$; **Figure 1M**). Together, these results confirm that the absence of GluA3-containing AMPARs does not alter the acute effects of WIN on excitatory and inhibitory synaptic transmission onto L2/3 mPFC neurons.

Exploring the behavioral long-term effects of adolescent WIN exposure in GluA3KO mice

In **Chapter 3** we investigated the long-term effects of adolescent WIN exposure in male and female WT mice. Given that *GRIA3* has been associated with increased risk for schizophrenia [16,17], and that adolescent cannabis use has been linked to an over 11-fold increase in the likelihood of developing psychiatric disorders [18,19], we aimed to explore a potential double-hit model for schizophrenia-like phenotypes. In the framework of this double-hit hypothesis, the lack of GluA3-containing AMPARs represents a genetic predisposition (first hit), while cannabis exposure during adolescence constitutes an environmental challenge (second hit). To address this, GluA3KO male and female mice were injected with the CBR agonist WIN between PND30 and PND50, following an irregular administration regime [43–46]. After a wash-

out period, mice were single-housed, tested in the Phenotyper, and subsequently used for ex-vivo patch-clamp recordings at approximately three months of age (**Figure 2A**).

The Phenotyper is an automated home-cage monitoring system that allows the assessment of both discrimination learning and subsequent reversal learning over 1 week (**Figure 2B**) [54]. Both vehicle- and WIN-injected GluA3KO mice were able to learn the discrimination learning phase of the task, as shown by a rapid increase in the percentage of left (correct) entries during the first hours of testing (**Figure 2C,D**). Consistently, all mice reached the 90% criterion for discrimination learning, and performance curves did not differ between vehicle- and WIN-injected GluA3KO mice ($\chi^2(1)=0.19$, p=0.66; **Figure 2E**). After 48 hours, reversal learning was initiated by switching the rewarding port from the left to the right side (**Figure 2B**). Shortly after this shift, most mice increased their percentage of right (correct) entries while reducing left (previously rewarded) entries, indicating task adaptation (**Figure 2C,D**). No significant differences were observed between groups in the proportion of mice reaching the 90% criterion during reversal learning ($\chi^2(1)=0.472$, p=0.492; **Figure 2F**). To further assess potential differences in reversal learning performance, we quantified the ratio of right-to-left entries during the final 12h of the reversal learning phase (dashed square **Figure 2C,D**), used here as a proxy for perseveration of the previously learnt strategy. This analysis did not reveal significant differences between vehicle- and WIN-treated groups ($t(13)=1.496$, p=0.158; **Figure 2G**).

We noticed that among WIN-injected animals, two mice failed to reach the 90% criterion, both of which were males (**Figure 2L**). Although the sample size was insufficient to draw meaningful conclusions from sex-stratified analyses, separate comparisons indicated that WIN-injected females performed comparably to vehicle-injected littermates during both discrimination ($\chi^2(1)=0.015$, p=0.901; **Figure 2H**) and reversal learning ($\chi^2(1)=0.973$, p=0.323; **Figure 2I**), as well as in the right-to-left entry ratio during the final phase of reversal ($t(6)=0.38$, p=0.72; **Figure 2J**). By contrast, WIN-injected males showed a non-significant trend toward reduced reversal performance compared with vehicle-treated males ($t(5) = 2.00$, p = 0.102; **Figure 2M**). The fraction of male mice reaching the criterion during discrimination ($\chi^2(1)=1.852$, p=0.173; **Figure 2K**) and reversal learning ($\chi^2(1)=2.381$, p=0.122, **Figure 2L**) did not significantly

differ between treatment groups. While the present data are preliminary, they provide an incentive to further increase sample sizes and include WT controls to help determine whether male GluA3KO mice may be particularly susceptible to the combined effects of GluA3 deletion and repeated adolescent CBR activation.

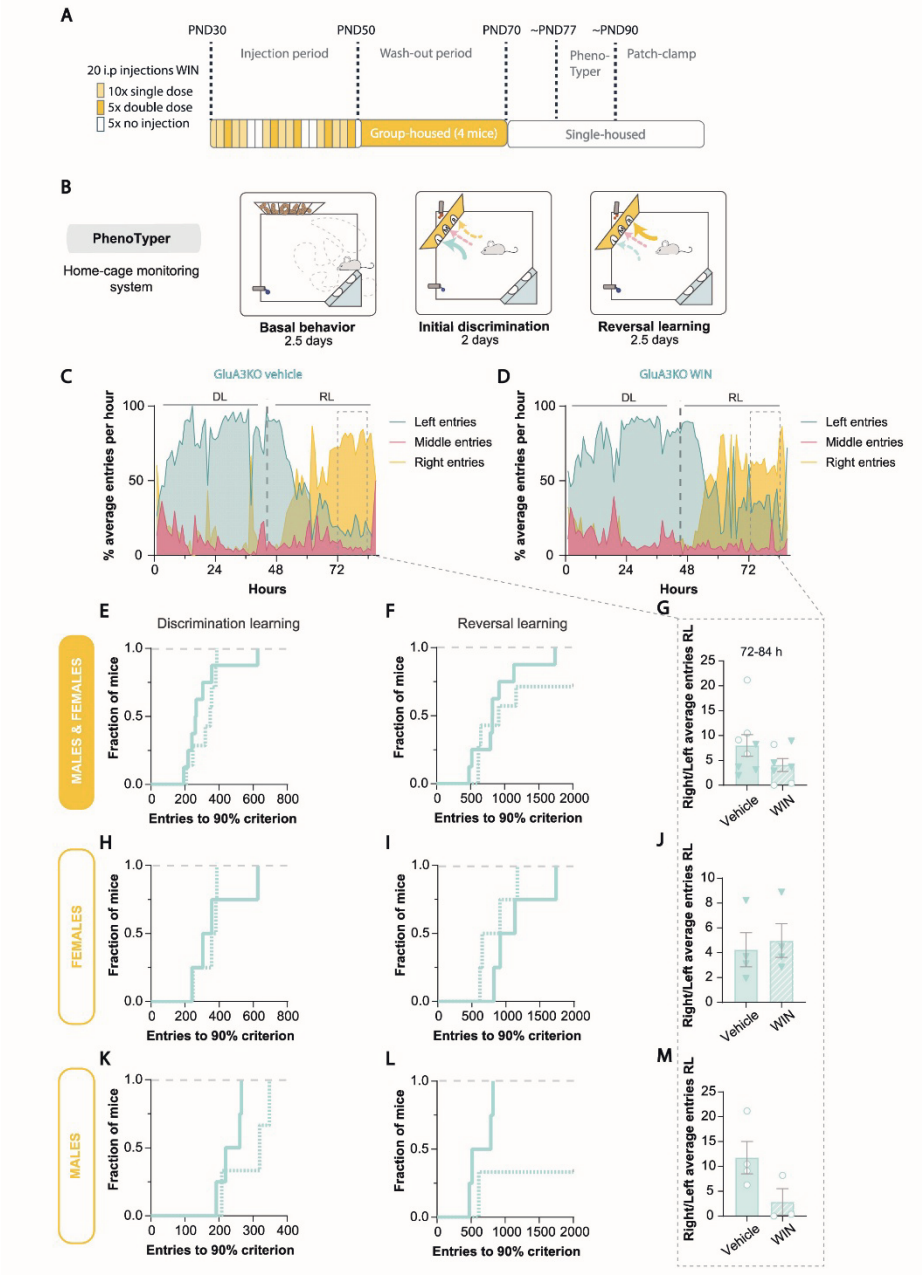

A
PND30
PND50
PND70
~PND77
~PND90
Injection period
Wash-out period
Pheno-Typer
Patch-clamp
20 i.p injections WIN
10x single dose
5x double dose
5x no injection
Group-housed (4 mice)
Single-housed
B
PhenoTyper
Home-cage monitoring system
Basal behavior
2.5 days
Initial discrimination
2 days
Reversal learning
2.5 days
C
GluA3KO vehicle
D
GluA3KO WIN
DL
RL
% average entries per hour
Hours
Left entries
Middle entries
Right entries
E
Discrimination learning
F
Reversal learning
G
72-84 h
Right/Left average entries RL
Vehicle
WIN
MALES & FEMALES
FEMALES
MALES
Fraction of mice
Entries to 90% criterion
H
I
J
K
L
M

Figure 2. Adolescent WIN exposure does not alter discrimination or reversal learning performance in adult GluA3KO mice. (A) Experimental design. Mice were injected between PND30 and PND50 following an irregular administration schedule consisting of 10 single-dose injections, 5 double-dose injections and 5 no-injection days. (B) Schematic representation of the PhenoTyper cage during basal behavior (left panel), initial discrimination (middle panel) and reversal learning (right panel). Thicker solid arrows indicate the rewarded port, while dashed arrows indicate non-rewarded ports. (C) Proportion of average entries per hour in the left (Blue), middle (red), or right (yellow) holes in vehicle-injected GluA3KO mice. (D) Same as C but for WIN-injected GluA3KO mice. The dashed grey square indicates the time window analyzed in panels G, J and M. (E) Survival plot showing the fraction of vehicle-injected (solid line n=8 mice; 4 females and 4 males) and WIN-injected (dashed line, n=7 mice; 4 females and 3 males) GluA3KO mice that reached the 90% learning criterion as a function of the number of entries during discrimination learning. Each step represents one animal that reached the criterion (F) Same as E but for reversal learning. (G) Ratio of right to left average entries between 72-84 hours from the beginning of cognitive testing. Triangles represent female mice while circles represent male animals. (H,I) Same as E,F but for GluA3KO females (solid line n=4; dashed line n=4). (J) Same as G but for GluA3KO females. (K,L) Same as E,F but for GluA3KO males (solid line n=4; dashed line n=3). (M) Same as G but for GluA3KO males. IL: infralimbic, PrL: Prelimbic; PND: postnatal day; DL: discrimination learning; RL: reversal learning.

Long-term effects of repeated adolescent WIN exposure on synaptic transmission in mPFC-L2/3 pyramidal neurons of GluA3KO mice

In **Chapter 4**, we observed that repeated activation of CBRs during adolescence had sex-specific long-term consequences on L5-mPFC synaptic transmission. Based on this and within the framework of a double-hit hypothesis, we explored whether repeated CBR activation during adolescence might induce long-term functional alterations in mPFC synaptic physiology of GluA3KO male and female mice. To address this question, we performed whole-cell patch-clamp recordings from L2/3 pyramidal neurons in the mPFC of GluA3KO mice that had previously undergone behavioral assessment in the Phenotyper (**Figure 3A**).

Adolescent WIN exposure did not alter excitatory synaptic transmission, with no significant differences in mEPSC frequency (U=123, p=0.063; **Figure 3B**) or amplitude (U=808, p=0.936; **Figure 3C**) between vehicle- and WIN-treated GluA3KO mice. On the contrary, mIPSC frequency was significantly reduced in L2/3-mPFC neurons following adolescent CBR activation (U=523, p=0.005; **Figure 3D**), while mIPSC amplitude (U=771, p=0.666; **Figure 3E**) and synaptic excitatory/inhibitory (E/I) input ratio remained unchanged (U=796, p=0.847; **Figure 3F**).

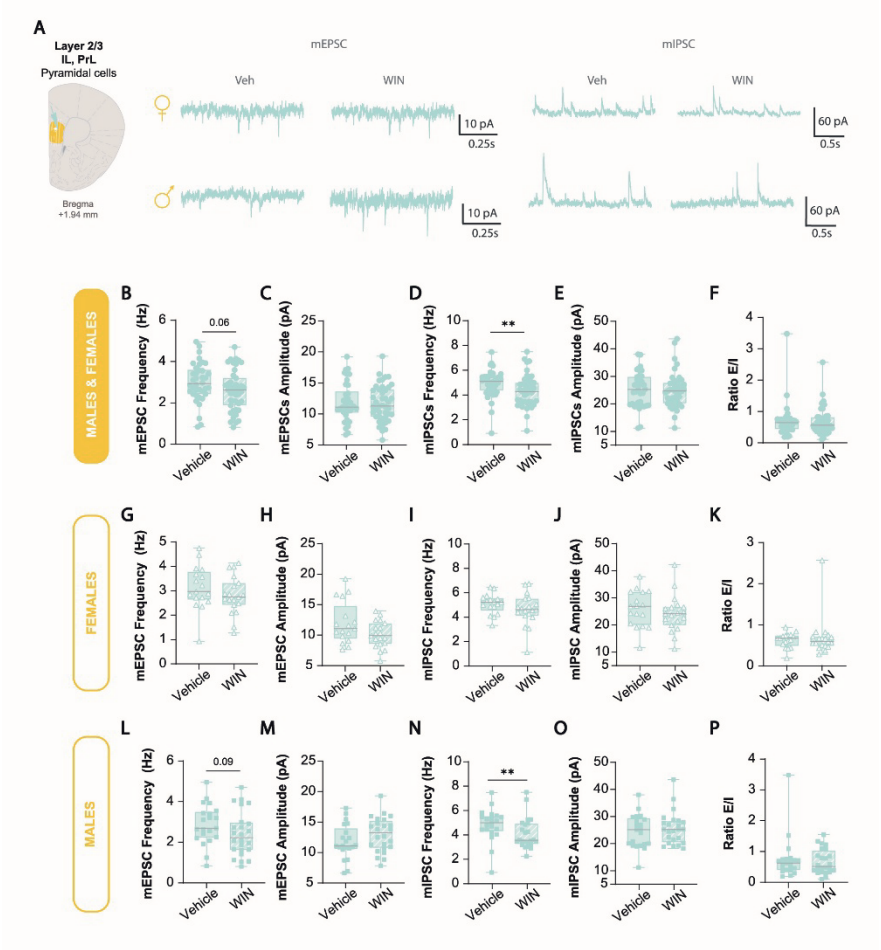


Figure 3. Adolescent WIN exposure decreases inhibitory transmission in mPFC-L2/3 pyramidal neurons of adult GluA3KO males. (A) Left: schematic representation of a coronal slice at +1.94 mm relative to bregma (adapted from Paxinos and Franklin atlas), showing the IL and PrL regions and cortical layer 2/3 included in the study. Right: Representative traces of miniature excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs) recorded from L2/3 pyramidal neurons of vehicle- and WIN-injected male and female GluA3KO mice. All data were obtained from the same set of cells: vehicle-injected GluA3KO females (17 cells from 4 mice), WIN-injected GluA3KO females (19 cells from 3 mice), vehicle-injected GluA3KO males (21 cells from 3 mice) and WIN-injected GluA3KO males (24 cells from 3 mice). (B-F) mEPSC frequency (B), amplitude (C), mIPSC frequency (D) and amplitude (E) and corresponding mEPSC/mIPSC ratio (F) from vehicle- and WIN-injected GluA3KO mice. (G-K) Same as B-F but only for vehicle- and WIN-injected GluA3KO females. (L-P) Same as B-F but only for vehicle- and WIN-injected GluA3KO males. IL: infralimbic, PrL: Prelimbic.

When we separated the data by sex, adolescent WIN exposure did not affect synaptic transmission in GluA3KO females: no significant differences were detected in mEPSC frequency (U=123, p=0.231; **Figure 3G**), mEPSC amplitude (U=114, p=0.137; **Figure**

3H), mIPSC frequency (U=119, p=0.185; **Figure 3I**), mIPSC amplitude (U=129, p=0.314; **Figure 3J**) or E/I input ratio (U=44, p=0.594; **Figure 3K**). Consistent with these findings, the cumulative distributions of mEPSC and mIPSC amplitudes were also unchanged (**Figure S1A,B**). In contrast, GluA3KO male mice exhibited a significant reduction in mIPSC frequency following adolescent WIN exposure (U=138, p=0.008; **Figure 3N**), without significant changes in mEPSC frequency (U=179, p=0.098; **Figure 3L**), mEPSC amplitude (U=190, p=0.163; **Figure 3M**) and mIPSC amplitude (U=237, p=0.739; **Figure 3O**). A shift toward higher mEPSC amplitudes was noted in WIN-injected males whereas the distribution of mIPSC amplitudes remained unchanged (**Figure S1C,D**). Despite the reduction in inhibitory frequency, the overall synaptic E/I ratio was not significantly altered (U=251, p=0.991; **Figure 3P**). Overall, these findings indicate that repeated adolescent WIN exposure caused long-term sex-specific effects on synaptic transmission in L2/3-mPFC neurons of adult GluA3KO mice.

Exploring synaptic correlates of reversal learning

In **Chapter 4**, we showed that reversal learning performance correlated with synaptic and circuit properties of L5-mPFC pyramidal neurons in WT mice. We therefore asked whether synaptic properties of L2/3-mPFC might also relate to cognitive performance in GluA3KO mice. To address this question, we correlated electrophysiological parameters with the right-to-left entry ratio (R/L ratio). No significant correlations were detected between the R/L ratio and either mEPSC frequency or amplitude (**Figure 4A,B**), nor with mIPSC frequency or amplitude (**Figure 4C,D**). Interestingly, in GluA3KO females, we observed a significant negative correlation between mEPSC amplitude and the R/L ratio (**Figure 4F**), an effect that was absent in males (**Figure 4J**). No correlations were found between the R/L ratio and mEPSC frequency, mIPSC frequency, or mIPSC amplitude in either females (**Figure 4E, 4G,4H**) or males (**Figure 4I,4K,4L**). These observations suggest that excitatory postsynaptic strength in L2/3-mPFC neurons may contribute to circuits supporting reversal learning, although its influence may differ between sexes.

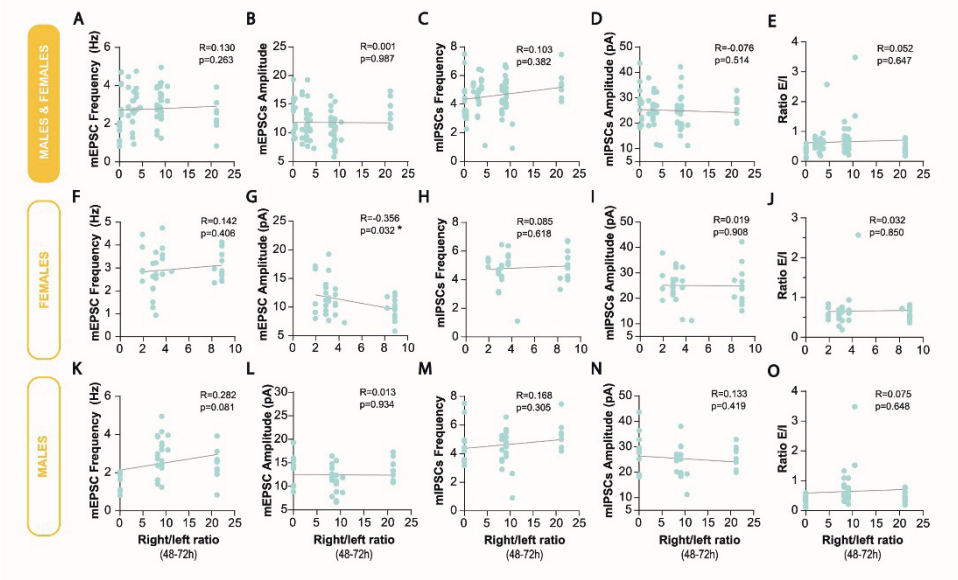


Figure 4. Excitatory postsynaptic synaptic strength in mPFC-L2/3 pyramidal neurons correlates with reversal learning performance in a sex-specific manner. Data for vehicle and WIN-injected mice are shown together. (A) mEPSC frequency as a function of right-to-left entries ratio (n total= 75 cells from 15 mice). (B-D) Same as A but for mEPSC amplitude (B), mIPSC frequency (C) and mIPSC amplitude (D). (E-H) Same as A-D but for data from GluA3KO females (n= 36 cells from 7 mice). (I-L) Same as A-D but for GluA3KO males (n= 39 cells from 6 mice). DL: discrimination learning; RL: reversal learning.

5

Discussion

In this chapter, we explored the effects of adolescent CBR activation in GluA3 deficient mice within the framework of a potential double-hit model of schizophrenia susceptibility. Given the pilot nature of this study, no firm conclusions will be drawn. Nonetheless, the data suggest the following preliminary observations: 1) acute WIN application reduced inhibitory synaptic transmission onto L2/3-mPFC pyramidal neurons, an effect observed in both WT and GluA3KO mice; 2) repeated WIN exposure during adolescence did not alter cognitive flexibility in adult GluA3KO mice, although 3) inhibitory synaptic transmission in L2/3-mPFC neurons was reduced in adult GluA3KO males. Although these findings remain preliminary, they suggest a possible scenario in which male GluA3KO mice may be more vulnerable to the combined influence of adolescent WIN exposure and the absence of GluA3.

In the first part of this chapter we examined the acute effects of CBR activation on synaptic transmission in L2/3-mPFC neurons from WT and GluA3KO mice. We show that bath application of WIN reduced mIPSC frequency by approximately 40% compared with vehicle, consistent with previous findings from our group showing a

similar reduction in inhibitory transmission in the infralimbic cortex of 2-3-week-old rats [51]. Unlike that earlier study, we did not observe a significant reduction in mEPSC frequency. Importantly, a comparable reduction in inhibitory transmission was also observed in GluA3KO neurons, indicating that the absence of GluA3 does not modify the acute effects of CBR activation onto L2/3-mPFC synaptic transmission.

In this chapter, we also examined whether repeated CBR activation during adolescence affects reversal learning performance in adult GluA3KO mice. This approach was designed as a potential "double-hit" model relevant to schizophrenia, given that both *GRIA3* loss-of-function variants and adolescent cannabis exposure have been associated with increased risk for the disorder [16–19]. Consistent with this framework, Wainberg and colleagues (2021) showed that individuals with genetic predisposition to schizophrenia displayed stronger associations between cannabis use and psychotic experiences [55], highlighting the relevance of taking into consideration genetic and environmental factors. In our data, neither male nor female GluA3KO mice exhibited significant behavioral impairments in discrimination or reversal learning after adolescent WIN exposure compared with vehicle-treated GluA3KO littermates. However, two of the three WIN-treated males failed to learn the reversal task and continue to apply the previously rewarded strategy. Although the small sample size prevents conclusive statements, this pattern may suggest that males are more sensitive to the combined impact of genetic predisposition (lacking *GRIA3*) and environmental factors (adolescent cannabis exposure). Expanding the cohort will be necessary to test this possibility. In addition, previous studies have reported sex differences in both *GRIA3*-related pathologies as well as in the regime and consequences of adolescent cannabis use [16,56–59], and therefore a sex-dependent vulnerability remains a plausible scenario.

At the synaptic level, adolescent WIN exposure did not cause long-term changes in excitatory or inhibitory transmission in female GluA3KO mice but led to decreased inhibitory transmission in GluA3KO males, specifically reducing mIPSC frequency. Because CB1Rs are largely expressed in cholecystokinin-positive and basket interneurons in the mouse forebrain [60], this reduction is consistent with CB1Rs predominantly affecting inhibitory synapses. In line with our results, previous studies have shown that adolescent CBR activation disrupts the inhibitory system in the mPFC: repeated CBR activation during adolescence decreases adult GABAergic markers [39] and reduces the frequency of spontaneous inhibitory events in layer 5 [61]. Together, these findings support a long-term reduction in prefrontal inhibitory transmission, a feature consistent with the GABAergic hypofunction proposed to contribute to schizophrenia-like pathology [62].

As a pilot study, this chapter presents important limitations. First of all, the absence of WT controls prevented us from testing whether the absence of GluA3-containing AMPARs alters the long-term consequences of adolescent CBR activation. Thus, the current dataset provides only preliminary insights into the effects of adolescent CBR activation in GluA3KO mice. Secondly, the limited sample size restricts the extent to which conclusions can be drawn regarding sex-specific effects. Despite these limitations, we believe this chapter provides a valuable foundation for future investigations. Recent studies reporting *GRIA3* upregulation following both acute and adolescent THC or WIN exposure [63,64] further highlight the importance of examining potential CBR-AMPAR interactions. Expanding these findings through larger cohorts and integrating molecular and electrophysiological approaches will be essential to further explore potential interactions between cannabinoid signaling and GluA3 deficiency in the framework of schizophrenia.

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Supplementary Figure

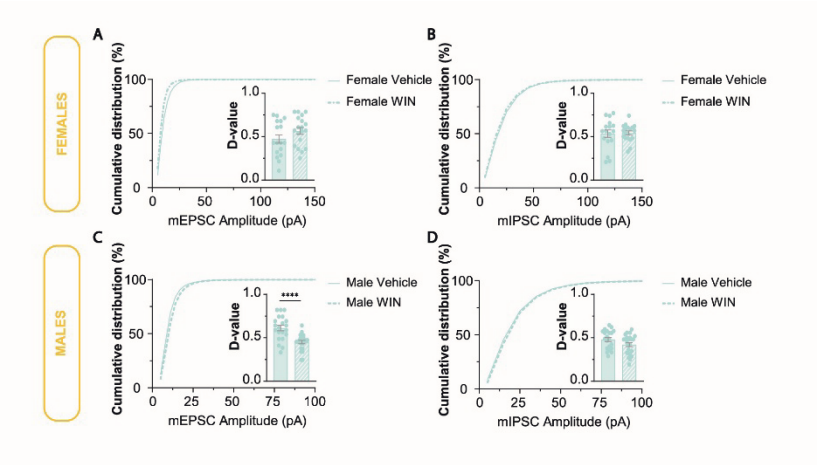


Figure S1. Adolescent WIN exposure shifts the distribution of mEPSC amplitudes in adult GluA3KO male mice. (A) Cumulative probability of mEPSC amplitude and Kolmogorov's D value (insert) from vehicle (solid) and WIN females (dashed) (vehicle n= 17 cells from 4 mice; WIN n= 19 cells from 3 mice). Adolescent WIN exposure did not affect the distribution of mEPSC amplitude. (B) Same as A but for mIPSC amplitude. Adolescent WIN exposure did not affect the distribution of mIPSC amplitude. (C) Same as A but for males (vehicle n= 21 cells from 3 mice; WIN n= 24 cells from 3 mice). Adolescent WIN exposure shifted the distribution toward larger mEPSC amplitudes. (D) Same as B but for males. Adolescent WIN exposure did not affect the distribution of mIPSC amplitude (D=0.241, p=0.366).



Chapter 6

General discussion

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General discussion

The cerebral cortex supports complex cognitive functions through highly dynamic and tightly regulated patterns of synaptic transmission. Disruptions in these processes, whether arising from altered receptor composition, neuromodulatory signaling, or developmental perturbations, can profoundly affect cognition and behavior. From synapses to behavior, this thesis sought to investigate how glutamatergic transmission and neuromodulatory systems shape cortical function across development and how their interaction may contribute to behavioral outcomes.

In **Chapter 2,** we investigated the role of GluA3- and GluA1-containing AMPA receptors (AMPARs) in excitatory synaptic transmission onto layer 2/3 (L2/3) pyramidal neurons in the primary visual cortex (V1). We found that although **both subunits contribute to basal excitatory transmission, GluA3-containing AMPARs play a more prominent role**. Furthermore, we demonstrated that **β-adrenergic receptor activation potentiates excitatory transmission independently of AMPAR subunit composition**.

In **Chapter 3**, we extended this investigation to a cortical region involved in higher-order cognitive processing rather than sensory integration, the medial prefrontal cortex (mPFC), which differs from V1 in structure, cellular composition and neurotransmission [1,2]. Here, we explored the contribution of GluA3-containing AMPARs to excitatory synaptic transmission in mPFC-L2/3 pyramidal neurons, with a particular focus on critical developmental time point such as adolescence, and cognitive flexibility. We showed that **excitatory synaptic transmission in mPFC-L2/3 pyramidal neurons follows a developmental trajectory and that the contribution of GluA3-containing AMPARs varies across development in a sex-specific manner, suggesting a role for GluA3 in the maturation of synapses**. Similar to V1, **β-adrenergic activation in the mPFC potentiated synaptic transmission,** although this process seems to have a presynaptic origin in the mPFC. Cognitively, we showed that **GluA3KO male mice displayed age-independent deficits in reversal learning**, suggesting a role for GluA3 in cognitive flexibility.

Prompted by the developmental perspective on mPFC synaptic transmission, we next turned to another key modulatory system with a well-established role in PFC development, the endocannabinoid system (ECS). In **Chapter 4**, we investigated the long-term effects of repeated cannabinoid receptors (CBRs) activation during adolescence on adult mPFC physiology and associated behaviors. We found that **adolescent CBR activation induced permanent, sex-specific alterations in mPFC-L5 pyramidal neurons; female mice exhibited changes in synaptic transmission**

whereas [3–8]male mice showed alterations in intrinsic excitability. These findings further underscore pronounced sex differences in mPFC physiology. However, **these neurophysiological alterations did not translate into detectable behavioral impairments in social hierarchy or reversal learning**, two behaviors dependent on prefrontal cortical function.

Finally, in **Chapter 5**, we integrated our findings on GluA3-containing AMPARs in mPFC synaptic physiology (**Chapter 3**) together with our study on the long-term consequences of adolescent CBR activation (**Chapter 4**). Given the implication of both systems in synaptic physiology and their association to schizophrenia [3–8], we explored a double-hit model of susceptibility to the disease, in which the absence of GluA3-containing AMPARs represents a genetic predisposition (first hit), and adolescent activation of cannabinoid receptors (CBRs) constitutes an environmental challenge (second hit). Preliminary findings in this pilot study indicate that acute CBR activation induces synaptic depression of inhibitory transmission onto mPFC-L2/3 pyramidal neurons in GluA3KO mice. Notably, **this effect was preserved in adult males following adolescent CBRs activation,** whereas **results on the consequences for cognitive flexibility remained inconclusive**.

In the following sections, I integrate these findings to highlight shared principles, region-specific differences, and emerging questions that arose from this work.

1. Electrophysiological insights of cortical synaptic transmission

1.1. GluA3, a multifaceted AMPAR subunit

In this section, I integrate and contextualize findings from **Chapters 2 & 3,** where we demonstrated the **contribution of GluA3-containing AMPA receptors to excitatory synaptic transmission in cortical L2/3 neurons**. By comparing results across cortical regions, our findings provide further evidence of the versatile physiology of GluA3.

In **Chapters 2 & 3**, we showed that GluA3-containing AMPARs play a prominent role in mediating basal excitatory synaptic transmission in both the V1 (**Chapter 2**) as well as in the mPFC (**Chapter 3**). In the mPFC, this contribution was age- and sex-specific, **further emphasizing the role of GluA3 as a region-specific regulator of synaptic transmission**. Beyond the cortex, GluA3-containing AMPARs have also been implicated in excitatory synaptic transmission in other brain regions. In the cerebellum, for instance, GluA3 contributes to basal synaptic transmission in Purkinje cells and to synaptic plasticity required for motor learning [9]. In contrast to the cerebellum, V1 and mPFC, hippocampal CA1 synapses strongly rely on GluA3-containing AMPARs for

basal synaptic transmission [10–12]. Instead, in the hippocampus, these receptors are required for synaptic potentiation induced by a rise in cAMP, for instance upon β-adrenergic receptor activation [10]. This differs from our findings in V1 and mPFC, where β-adrenergic-mediated potentiation was independent of AMPAR subunit composition (**Chapters 2 & 3**), and appeared to involve a presynaptic component, as indicated by stronger increases in mEPSC frequency than amplitude. Together with our findings, this growing body of evidence demonstrates that GluA3-containing AMPARs play region-specific roles in both synaptic transmission and neuromodulation. We therefore propose **GluA3 subunit as a multifaceted component of AMPAR-mediated transmission**, adding an additional layer of complexity to synaptic physiology and neural computation in the brain (**Figure 1A**).

In the somatosensory cortex, GluA2/3 heteromers have been proposed to participate in homeostatic plasticity, as indicated by the enrichment of these receptors in dendritic spines following sensory deprivation by whisker trimming [13]. Our work establishes that GluA3-containing AMPARs are important for maintaining basal synaptic transmission in L2/3 neurons of both V1 and the mPFC, while not playing a prominent role in β-adrenergic-induced plasticity (**Chapters 2 & 3**). These findings raise the possibility that GluA3-containing AMPARs may serve as homeostatic regulators across cortical circuits. Future studies could address this hypothesis by subjecting mice to dark rearing and analyzing AMPAR subunit composition within spines, where an enrichment of GluA2/3 heteromers would be expected. This would also help elucidate whether the contribution of GluA3-containing AMPAR to synapse physiology is conserved across sensory cortices. Studying homeostatic plasticity in associative cortical structures such as the mPFC is considerably more challenging. Approaches such as thalamic deafferentation or silencing of layer 4 neurons could, in principle, be used to mimic activity deprivation. However, the distributed pattern of thalamic and cortical input across multiple layers in the mPFC [2] complicates the implementation of these strategies.

Additionally, our findings in **Chapter 2** revealed that protein kinase A (PKA) is active under basal conditions in V1 synapses, in contrast to hippocampal CA1 synapses [10]. Although basal PKA activity was not assessed in the cerebellum [9] or in the mPFC (**Chapter 3**), regional differences in PKA signaling between hippocampus and V1 provide evidence that baseline cAMP levels may vary across brain regions. This observation raises the possibility that **baseline differences in cAMP levels could contribute to regional variations in GluA3 physiology**, provided that cAMP regulates GluA3-containing AMPARs in a manner similar to that described in the cerebellum and hippocampus [9,10]. Within this hypothetical framework, low cAMP levels, as suggested from work in the hippocampus [10], would maintain the receptor in a closed

state, preventing its contribution to basal synaptic transmission. Conversely, higher baseline cAMP levels may favor an open receptor state, allowing GluA3-containing AMPARs to participate in synaptic transmission (**Figure 1B**). If this hypothesis holds true, baseline cAMP levels would be expected to be elevated in mPFC-L2/3 pyramidal neurons, V1-L2/3 pyramidal neurons, and cerebellar Purkinje cells. Notably, cAMP levels are likely to be lower in the cerebellum than in the mPFC or V1, since forskolin treatment further increased GluA3 channel conductance [9], indicating that basal cAMP signaling in cerebellar synapses is not yet maximal (**Figure 1B**).

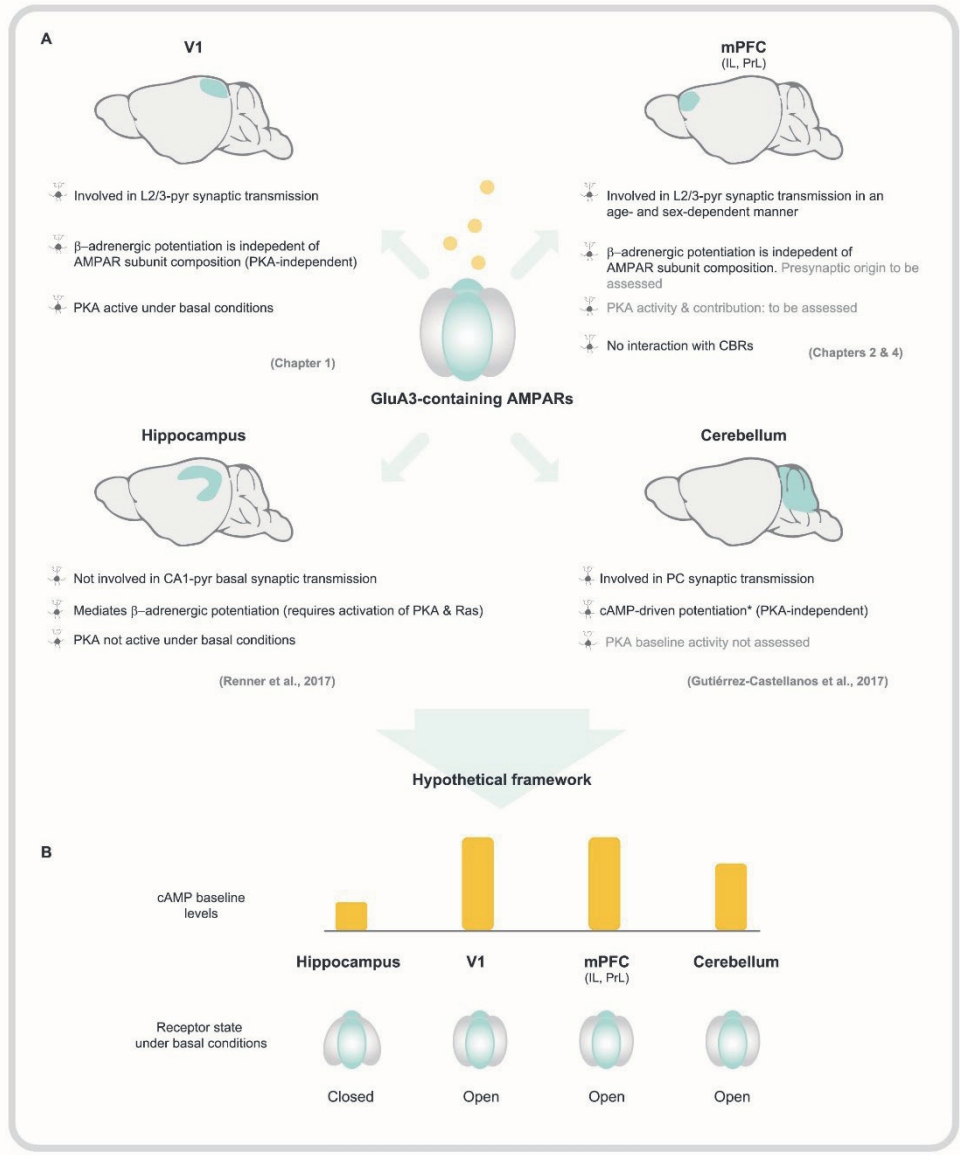


Figure 1. Diverse physiology of GluA3-containing AMPARs across brain regions. (A) Illustration summarizing findings about the contribution of GluA3-containing AMPAR to synaptic transmission in visual cortex (V1), medial prefrontal cortex (mPFC), hippocampus and cerebellum. (B) Hypothetical framework to explain regional differences in GluA3 physiology. (*) In the cerebellum, β-adrenergic activation was not test but rather cAMP was increased with Forskolin.

This hypothesis raises the question of whether regional differences in GluA3 physiology are intrinsic to the subunit itself or instead reflect differences in intracellular signaling environments, particularly cAMP availability. Future work could therefore assess the expression levels of the different AMPAR subunits and investigate their biophysical properties across brain regions. In addition, the use of cAMP sensors such as cAMPFIRE [14] and PKA activity sensors [15], which allow quantitative measurements [16,17], would help address this question and further expand on the physiology of GluA3-containing AMPARs across brain regions. Supporting the concept of regional specificity in cAMP-mediated signaling, several studies have reported differential expression of phosphodiesterase families across brain regions [18–21]. Moreover, previous work has shown opposite effects of cAMP elevation on neuronal firing in L2/3 neurons of V1 versus dorsolateral PFC in rhesus monkeys [1], underlying that the same molecular process, an increase in cAMP, can lead to distinct neurophysiological outcomes across different brain structures.

As discussed above, β-adrenergic activation potentiated spontaneous synaptic transmission in L2/3 pyramidal neurons of V1, although interestingly it did not affect evoked AMPAR-mediated currents in this region (**Chapter 2**). One possible explanation for this discrepancy is methodological and regards the population of synapses that are recruited during stimulation. Spontaneous events reflect transmission across the entire population of synapses, whereas evoked EPSCs sample only subsets of inputs stimulated by the electrode. When stimulating inputs from additional layers, our findings still revealed no potentiation upon β-adrenergic activation, suggesting that this scenario is unlikely in our experimental design. Alternatively, spontaneous release has been proposed to contribute to the homeostatic regulation of synaptic strength [22], and accumulating evidence indicates that spontaneous and evoked transmission are regulated by partly distinct molecular signaling cascades and are partially segregated within presynaptic terminals [22,23]. Consistent with this idea, presynaptic NMDARs have been shown to control evoked vesicle release but not spontaneous vesicle release [24], underscoring the differential regulation of these two transmission modes. Interestingly, postsynaptic AMPARs can respond differentially to spontaneous versus evoked neurotransmission [25], with GluA2-lacking AMPARs preferentially contributing to spontaneous events. Our results provide **additional evidence supporting a partial segregation of synapses involved in spontaneous versus**

evoked synaptic transmission in the primary visual cortex. In this framework, β-adrenergic receptors may preferentially act at synapses or subsynaptic compartments involved in spontaneous, but not evoked, transmission. These findings raise the question of whether similar mechanisms operate in layer 2/3 of the mPFC, particularly given that both structures receive dense projections from the locus coeruleus [26–28] and that β-adrenergic activation in the mPFC appeared to have a strong presynaptic component. Exploring the effects of β-adrenergic activation on evoked transmission in the mPFC may therefore help further elucidate shared and region-specific mechanisms that shape cortical synaptic transmission beyond GluA3-specific processes.

Overall, the findings presented in **Chapters 2 & 3** expand previous literature by demonstrating a role for GluA3-containing AMPAR in synaptic transmission across two distinct cortical regions and by proposing a framework to explain regional differences in the physiology of these receptors.

1.2. A role for GluA3 in synapse maturation?

The primary visual cortex and the prefrontal cortex follow broadly similar developmental trajectories. However, the visual cortex matures earlier than the prefrontal cortex in both humans and rodents [29–40] (see **Figure 1, Chapter 1**). In rodents, the visual cortex exhibits a critical period for plasticity between PND21 and PND35 [37,41], with a particularly sensitive window for ocular dominance plasticity between PND28-PND32 [36]. In contrast, the prefrontal cortex shows a more protracted developmental trajectory and reaches full maturity around PND70-80 in rodents, corresponding approximately to early adulthood in humans (~25 years of age) [42]. Notably, adolescence, the final stage of PFC maturation (~PND30-PND50 in mice), has been proposed to represent a critical period for the development of this brain region [43].

Our findings from **Chapter 3** reveal that in the mPFC, GluA3-containing AMPARs contribute to basal synaptic transmission onto L2/3 pyramidal neurons in an age- and sex-specific manner, with GluA3KO neurons following a distinct developmental trajectory compared with neurons from WT mice. Our results further supported adolescence as a particularly relevant maturational time point. In the primary visual cortex (**Chapter 2**), experiments were conducted at PND28-32, an age that coincides both with the critical period of this brain region [36,37,41] and the onset of adolescence [44–46]. At this age, synaptic transmission in L2/3-V1 neurons was reduced in the absence of GluA3-containing AMPARs. The altered developmental trajectory in GluA3KO neurons in the mPFC, together with the reduced synaptic transmission observed in V1 during the critical period, raises the possibility that this

subunit may contribute to synapse maturation, potentially participating in plasticity processes occurring during cortical critical periods.

Several studies have described the involvement of AMPARs in brain development, however, our data may provide the first evidence of a possible role for GluA3 in brain maturation. In the visual cortex, for instance, the absence of GluA1-containing AMPARs particularly impairs ocular dominance plasticity during the critical period [47]. In the primary motor cortex, developmental changes in AMPAR subunit composition have also been reported, with a decrease in the GluA1:GluA2 ratio at PND30 (early adolescence) compared with PND10, underscoring the importance of subunit composition in synapse maturation [48]. In addition, postsynaptic AMPARs have been implicated in the maturation of functional synapses through retrograde transsynaptic signals mediated by their extracellular domain, which promote presynaptic development [49]. Although this mechanism appears to be independent of AMPAR subunit composition, GluA3 was not examined in these experiments. The association between *GRIA3*, the gene encoding GluA3, and a wide range of neurodevelopmental disorders, further supports a possible role for GluA3 in brain maturation [8,50–53].

Future studies could therefore assess the developmental trajectory of L2/3 synapses in V1 in the presence and absence of GluA3, as well as their contribution to ocular dominance plasticity, providing further insights into the role of this subunit in synaptic and circuit maturation in the cortex. Inducible, region-specific knockdown of GluA3 at defined developmental stages, such as the critical periods for V1 and the mPFC, could also help address this question with greater temporal precision. Overall, the findings presented in **Chapters 2 & 3** expand previous literature and raise the possibility that GluA3-containing AMPARs contribute to synapse maturation during cortical development.

1.3. Converging synaptic receptors: GluA3 & CBRs in Prefrontal Cortex

Besides examining the effect of β-adrenergic receptor activation on synaptic transmission (**Chapters 2 & 3**), we also investigated a potential functional crosstalk between CBRs and GluA3-containing AMPARs (**Chapter 5**). CBRs are predominantly localized presynaptically [54,55], whereas AMPARs are mainly postsynaptic [56,57]. Because CBR activation primarily decreases cAMP levels [55], we wondered whether a reduction in cAMP signaling would lead to a decrease in GluA3-mediated transmission. In addition, recent evidence indicates that GluA3 removal from synapses can be mediated by protein kinase C (PKC) [58], a pathway that can also be recruited following CB1R activation, although it is not the predominant signaling cascade [59].

Thus, two mechanisms downstream of CBR activation could potentially affect GluA3-mediated currents: a decrease in cAMP levels could reduce GluA3 channel conductance and shift the receptor toward a closed state, as described in the hippocampus and cerebellum [9,10], whereas activation of PKC could promote phosphorylation-dependent removal of GluA3 from the synapse [58]. Our data show that both WT and GluA3KO mice exhibited a reduction in inhibitory transmission following application of the CBR agonist WIN, suggesting that the absence of GluA3-containing AMPARs does not alter the effects of acute CBR activation on synaptic transmission in mPFC L2/3 pyramidal neurons (**Chapter 5**). Therefore, our data indicates that the acute synaptic effects of CBR activation occur independently of GluA3-mediated signaling, suggesting that these receptors do not functionally interact under our experimental conditions.

Having established this, we then built on our findings from **Chapters 3 & 4**. In **Chapter 3**, we showed that GluA3KO mice exhibit deficits in excitatory synaptic transmission in L2/3 neurons of the mPFC, together with impairments in reversal learning, phenotypes that resemble alterations described in schizophrenia patients and in models of the disease etiology [60–63]. In **Chapter 4**, we demonstrated that activation of CBRs during adolescence produces long-lasting sex-specific consequences at synaptic and cellular levels in L5-mPFC neurons. Based on these observations, in **Chapter 5** we proposed a double-hit hypothesis for susceptibility to schizophrenia-like phenotypes that incorporates both genetic (lack of GluA3) and environmental factors (exposure to a CBR agonist during adolescence), given that variants in the *GRIA3* gene as well as cannabis use during adolescence have been associated with increased risk for the disorder [8,64–67]. In that pilot study, we explored whether the synaptic and behavioral deficits observed in GluA3KO mice (**Chapter 3**) were exacerbated by repeated CBR activation during adolescence, a critical period for PFC development [43].

Although no firm conclusions can be drawn due to the pilot nature of the study, our results provide initial insights into this interaction. Adolescent WIN exposure did not significantly affect discrimination or reversal learning performance in GluA3KO mice (**Chapter 5**). However, it is notable that two out of three WIN-injected males were unable to complete the reversal learning task, raising the possibility that males may be more vulnerable to the combination of GluA3 deficiency and adolescent CBR activation. At the synaptic level, adolescent WIN exposure resulted in long-term depression of inhibitory transmission in L2/3 mPFC neurons selectively in male mice, further supporting a hypothetical scenario of sex-specific vulnerability to schizophrenia-like phenotypes.

Alterations in inhibitory circuits of the PFC have been widely described in schizophrenia patients and in experimental models of the disorder (see review [68]), including among others reduced parvalbumin (PV) mRNA expression in PV-positive interneurons and decreased levels of the presynaptic GABA transporter GAT-1 [69–71]. In this context, the long-term reduction in L2/3 mPFC inhibitory transmission observed in our experiments may represent a synaptic phenotype reminiscent of alterations associated with the disease. In **Chapter 3** we observed that GluA3KO mice present synaptic and reversal learning deficits that also recapitulate aspects of the disease etiology [60–63]. Future studies should expand on this pilot work by increasing cohort size and including the appropriate WT control groups to directly test the proposed double-hit hypothesis, with particular attention to potential sex-specific effects. This line of research is especially relevant in light of recent evidence showing that prenatal THC exposure increases AMPAR channel conductance in the adolescent hippocampus [72] and that *GRIA3* expression is upregulated in the adult frontal cortex following adolescent CBR activation [73]. In this context, the results presented in **Chapters 3, 4 & 5** open new possibilities for investigating how genetic and environmental factors interact during critical periods of PFC maturation such as adolescence, and support the study of cannabinoid and glutamatergic systems as converging regulators of cortical development.

Overall, the findings discussed in **Section 1** expand our understanding of cortical synaptic transmission by demonstrating **a role for GluA3-containing AMPARs across distinct cortical regions** and proposing a framework to account for regional differences in their physiology. In addition, our data raise the possibility that these receptors contribute to synapse maturation during cortical development. Building on this, we provide evidence supporting the idea that cannabinoid and glutamatergic systems may act as regulators of cortical development. Collectively, these insights provide a foundation for future investigations into the mechanisms that shape synaptic function and cortical maturation.

2. From synapses to behavior

2.1. Contribution of synaptic components to reversal learning

Cognitive flexibility, as measured by reversal learning, enables organisms to adapt their behavior to changing environments. In this context, the PFC has been extensively studied and is known to be essential for the encoding, categorization, and updating of

learned rules [74–76]. In **Chapter 3**, we proposed a role for GluA3 in reversal learning, as GluA3KO male mice exhibit deficits in this task. Other studies have also implicated AMPAR function in reversal learning. Mouse lines with disrupted AMPAR endocytosis or impaired GluA1 ubiquitination show deficits in reversal learning [77,78], highlighting the importance of proper AMPAR trafficking for behavioral flexibility. Although these studies focused on receptor dynamics rather than subunit composition, they support the broader notion that intact AMPAR function is required for the synaptic mechanisms underlying cognitive flexibility. A previous study by Adamczyk and colleagues (2012) reported that mice lacking GluA3 do not display deficits in spatial working memory, despite alterations in motor behavior and increased aggression [79]. The absence of deficits in other cognitive tasks such as the Y-maze or the Morris water maze [79], suggests that the behavioral phenotype of GluA3KO mice is not generalized but rather affects specific cognitive domains, further supporting a role for GluA3-containing AMPARs in reversal learning. Future work addressing this question could employ experimental approaches with higher temporal resolution, such as the dTAG system for selective degradation of GluA3 shortly before behavioral testing (see **Section 5**). In addition, we cannot rule out that the observed deficits in reversal learning may arise from developmental alterations caused by the ubiquitous absence of functional GluA3-containing AMPARs, particularly in light of the evidence presented in **Chapter 3** that suggests a role for GluA3 in synaptic maturation (see **Section 1.2**). Inducible, mPFC-specific knockdown of GluA3 at defined developmental stages would therefore help distinguish between developmental and acute contributions of this subunit to cognitive flexibility.

In **Chapters 4 & 5**, we further examined whether repeated CBR activation during adolescence affected reversal learning performance. In both WT and GluA3KO mice, adolescent CBR activation did not result in measurable deficits in this task, despite the poorer performance of GluA3KO males relative to WT mice (**Chapter 3**). One possible explanation for the preserved behavioral performance is that the excitation/inhibition (E/I) balance remained largely intact in both studies despite alterations in synaptic and cellular physiology. In **Chapter 4**, adolescent WIN exposure did not disrupt synaptic or local network E/I balance in L5-mPFC WT neurons. Similarly, in **Chapter 5**, GluA3KO mice exposed to adolescent CBR activation showed a preserved synaptic E/I balance in L2/3 mPFC neurons. Although these experiments were performed in different cortical layers, which are known to serve distinct functional roles [2], they suggest that compensatory mechanisms may operate to maintain E/I balance at synaptic and network levels, thereby preserving behavioral output. Such compensatory processes could involve changes that were not addressed in the our experimental design, such as alterations in interneuron activity, or adaptations in brain regions such as the thalamus, hippocampus, or other cortical areas that project to the mPFC [2].

In our experimental design reversal learning was assessed using the Phenotyper [80], which constitutes an intra-dimensional reversal learning paradigm in which animals update rules based on a single dimension (in this case spatial location) [81–83]. In contrast, extra-dimensional set-shifting tasks require the integration and switching between multiple stimulus dimensions, such as odor, color, or texture [81–83]. Interestingly, schizophrenia patients present stronger difficulties in extra-, rather than intra-, dimensional tasks [84,85]. Based on these observations, it is possible that deficits following adolescent CBR activation, in WT or GluA3KO mice, may become more evident in paradigms requiring extra-dimensional rule switching. Future studies employing tasks that specifically address this type of rule switching may therefore reveal behavioral impairments not captured by the current paradigm and provide deeper insight into how PFC physiology, and disruptions in its developmental trajectory, contribute to adult cognitive flexibility.

2.2. Synaptic and behavioral deficits in the absence of GluA3: a model?

The PFC exhibits a protracted development, which renders it particularly susceptible to early-life perturbations. Accordingly, several studies have reported structural and functional abnormalities in the PFC of schizophrenia patients, supporting the view that disrupted PFC maturation contributes to the neurodevelopmental origin of the disorder [86–88]. In **Chapter 3** we explored synaptic transmission in L2/3 mPFC pyramidal neurons together with cognitive flexibility in GluA3KO mice. Given the synaptic and behavioral alterations observed in GluA3KO mice, together with the reported association between *GRIA3* and schizophrenia [7,8], a remaining open question is whether this mouse line may serve to model specific aspects of the disorder.

Schizophrenia patients often exhibit deficits in reversal learning performance [60], a finding that has been replicated in rodent models of the disease etiology [61,62]. In parallel, alterations in PFC physiology have been widely reported in both schizophrenia patients and animal models, including disrupted neural oscillations and impaired synaptic plasticity [89–94]. At the cellular level, structural and synaptic abnormalities within L2/3 have been documented. Specifically, reduced spine density on basal dendrites of layer 3 pyramidal neurons in the dorsolateral prefrontal cortex has been observed in postmortem tissue from schizophrenia patients [95–97], accompanied by alterations in neuronal morphology [98]. Consistently, reduced excitatory synaptic transmission in L2/3 of the medial PFC has also been described in mouse models of schizophrenia etiology [63].

In **Chapter 3** we demonstrated that GluA3KO mice exhibit reduced excitatory synaptic transmission in L2/3-mPFC pyramidal neurons. These findings are consistent with those of Nagahama and colleagues (2020), who reported impaired excitatory synaptic transmission in mPFC-L2/3 pyramidal neurons in a mouse line recapitulating behavioral aspects of schizophrenia -like pathology, accompanied by reduced dendritic spine density [63]. Such structural alterations parallel observations from post-mortem studies in patients [95–97]. The decreased mEPSC frequency observed in GluA3KO mice may similarly reflect a reduction in spine number, raising the possibility that structural changes accompany the functional synaptic deficits identified in L2/3-mPFC neurons and motivating future studies to directly address this question. In addition, schizophrenia symptoms are classically grouped into three domains: positive, negative, and cognitive [99]. In rodent models, positive symptoms are typically assessed through measures such as locomotor activity and pre-pulse inhibition (PPI). In **Chapter 3**, we show that locomotion was normal in GluA3KO mice, while PPI was not tested. Negative symptoms were not evaluated either, and future studies should explore these domains using paradigms such as the forced swim test, sucrose preference test, or three-chamber social interaction test [99]. Interestingly, previous reports have shown that GluA3KO male mice display increased sociability and aggression [79,100], suggesting specific alterations in social behavior, although not in line with schizophrenia -like social deficits [99]. With respect to cognitive symptoms, we observed impairments in reversal learning in GluA3KO male mice, a domain that is often affected in schizophrenia patients and has been replicated in models of the disease etiology [60–62]. However, using an extra-dimensional rather than intra-dimensional paradigm like that employed in this study would provide a more translationally relevant assessment of cognitive flexibility deficits [82,83], given that intra-dimensional shifting is less affected in patients [84,85]. Notably, Adamczyk and colleagues (2012) reported elevated dopamine levels in the striatum of GluA3KO male mice [79], consistent with dopaminergic dysregulation observed in schizophrenia [101]. Altogether, our data indicate that GluA3KO mice exhibit synaptic deficits in L2/3-mPFC excitatory transmission and impairments in reversal learning, suggesting they may capture certain aspects of schizophrenia-like pathology. Future studies examining additional features, such as PFC synaptic structure, microglial function, or dopaminergic signaling [95–97,101–103] will be essential to determine the extent to which GluA3KO mice can serve as a model for the disease.

2.3. Bridging synaptic function and behavior

One of the aims of this thesis was to bridge the gap between synaptic function and behavior by identifying putative mechanistic links underlying behavioral output, rather

than characterizing synaptic and behavioral phenotypes independently. To achieve this, whenever possible, electrophysiological and behavioral experiments were performed in the same cohort of mice, allowing cellular measurements to be directly related to individual behavioral performance.

The most direct approach to link neuronal activity and behavior involves in-vivo recordings, that enable the measurement of extracellular electrical signals, calcium dynamics, or neurotransmitter release within task-relevant circuits during behavior. While highly informative, these approaches are less suited for research questions requiring detailed electrophysiological characterization of synaptic and intrinsic properties at the level of individual neurons and defined microcircuits, information that is more readily obtained using patch-clamp techniques. Although in vivo patch-clamp recordings have recently been extended to behaving animals [104], the method remains technically demanding, low-throughput, and experimentally restrictive. In this context, ex-vivo patch-clamp recordings combined with behavioral analysis offer a more accessible complementary approach to linking synaptic function and behavior. While correlation-based analyses do not establish causality, they provide an effective strategy to identify candidate synaptic mechanisms associated with behavioral performance and to generate testable mechanistic hypotheses. In this thesis, we used this approach to reduce the conceptual gap between synaptic transmission and behavior by relating ex-vivo electrophysiological measures to individual behavioral outcomes (**Chapters 4 & 5**).

In **Chapter 4** we found that animals occupying the second rank presented larger mEPSC amplitudes but lower mEPSC frequencies compared with fourth-rank mice, suggesting that excitatory synaptic transmission in L-5-mPFC neurons might contribute to the regulation of social hierarchy. This observation is consistent with previous studies showing stronger excitatory synaptic transmission in L5 mPFC neurons of dominant compared with submissive animals [105]. More recent work further demonstrated that optogenetic activation of dorsomedial L5-mPFC neurons projecting to the dorsal raphe nucleus and periaqueductal gray increases social rank in the tube test [106], providing direct mechanistic evidence that activity in this circuit contributes to social dominance. Together, these findings support the idea that variations in activity within the L5-mPFC influence social hierarchy.

With respect to cognitive flexibility, **Chapter 4** revealed that higher AMPA/NMDA ratio and increased local network excitation/inhibition ratio correlated with poorer performance in reversal learning, suggesting that excessive excitation within the mPFC might be detrimental for cognitive flexibility. In **Chapter 5**, a similar relationship was observed, with increased excitatory postsynaptic strength correlating with poorer

performance during reversal learning. These results converge on the idea that elevated excitatory drive within the mPFC, whether reflected at the synaptic or network level, may negatively affect cognitive flexibility. This interpretation is consistent with previous studies showing that increasing excitatory drive in the mPFC, either by reducing GABAergic signaling or by inhibiting vasoactive intestinal polypeptide (VIP) interneurons, impairs reversal learning [107]. Notably, correlations in **Chapter 4** were obtained from pooled data across sexes, whereas in **Chapter 5** the relationship was observed selectively in females, raising the possibility that females may be more sensitive to alterations in excitatory transmission within the mPFC.

Overall, the correlation-based analyses between electrophysiological and behavioral readouts included in this thesis provide a valuable framework for connecting synaptic physiology and behavior. Although this approach does not establish causality, it allows the identification of candidate mechanisms linking synaptic function to behavioral output and may serve as a foundation for future studies incorporating circuit-specific designs.

3. Sex differences in Prefrontal Cortex synaptic systems

3.1. Sex as a biological variable in Neuroscience Research

Throughout much of the history of scientific research, the description of biological processes and the development of medical interventions have been largely based on male physiology. During the last three decades, efforts from funding agencies such as the National Institute of Health (NIH) have been made to enforce inclusive research [108]. Nevertheless, recent analyses indicate that more than 60% of neuroscience studies still do not consider sex or omit it entirely, although this varies by model organism and journal [109]. Notably, these authors reported no reduction in sex bias in NIH-funded studies compared to non-NIH-funded work, suggesting that policy alone is insufficient to eliminate this imbalance.

One of the most common justifications for limiting studies to male animals has been the assumption that female physiology is inherently more variable due to hormonal fluctuations across the reproductive cycle, thus assuming that larger sample sizes, greater labor and a bigger financial investment is needed to obtain reliable and significant results. However, multiple studies have demonstrated that variability in females is not greater than in males across a range of outcome measures [110–113] including behavior, electrophysiology, histology or neurochemistry [113]. An insightful review by Shansky & Murphy, (2021) highlighted that the publishing pressure, funding limitations and experimental efficiency incentivize researchers to focus on

mechanistic depth in one sex, most often males, rather than extending findings to females [114].

In the present thesis, efforts were made to investigate neuronal physiology and associated behaviors using a representative approach by including both male and female mice. Accordingly, we identified **sex-specific effects in the role of GluA3-containing AMPARs in mPFC synaptic transmission** (**Chapter 3**) as well as in the **long-term consequences of CBR activation during adolescence on mPFC cellular and synaptic physiology** (**Chapter 4**). In addition, preliminary data from the pilot study in **Chapter 5** raise the possibility of further sex-differences in the combined effect of GluA3-deficiency and adolescent CBR activation. However, while both sexes were included in all chapters, sex is undefined in **Chapter 2** and female mice were not behaviorally tested in **Chapter 3**. In addition, direct statistical comparisons between males and females were not performed in **Chapters 3, 4 & 5** due to limited statistical power. Consequently, these studies describe sex-specific effects rather than formally testing sex differences. Despite these limitations, analyzing males and females separately when possible proved informative, as several of the effects described in this thesis were sex-specific. These findings highlight that treating sex as a source of biological insight can uncover mechanisms that would otherwise remain unexplored.

3.2. Sex dimorphism in Prefrontal Cortex glutamatergic transmission

The development of the PFC has been shown to be sexually dimorphic at functional, morphological, and anatomical levels [115,116], differences that are also reflected in cognition [44,117]. In line with this literature, the results presented in **Chapter 3** demonstrated that **excitatory synaptic transmission in mPFC-L2/3 pyramidal neurons follows a sex-specific developmental trajectory**. Notably, GluA3-containing AMPARs contributed in an age-specific manner to mPFC-L2/3 synaptic transmission in female mice, an effect that was not observed in males. Sex differences in mPFC glutamatergic transmission have been previously reported, although these studies have generally not incorporated a developmental perspective. Evidence indicates baseline differences in glutamatergic signaling between males and females. For instance, antagonism of NMDA receptors in the IL and PrL regions increased mPFC dopamine levels in males, while causing the opposite effect in females [111]. In addition, recent evidence suggests that AMPAR trafficking may also differ between sexes, with the AMPAR internalization protein PICK1 appearing to serve a distinct function in females compared to its canonically described role in males [118]. Sex differences in AMPAR physiology are further supported by Knouse and colleagues

(2022), who reported higher spontaneous excitatory postsynaptic current (sEPSC) amplitude and frequency in layer 5 pyramidal neurons from IL and PrL in females compared with males [115]. In the same study, synaptosomal, but not total, levels of GluA1 and GluA2 were higher in females, although GluA3 was not examined. Overall, these data point toward stronger baseline glutamatergic transmission in females, which may render them more dependent on this neurotransmission pathway and consequently more vulnerable to its disruption. Our findings from **Chapter 3** expand on this, where we show that the absence of GluA3-containing AMPARs resulted in more pronounced synaptic depression at multiple stages of mPFC development in female mice.

Interestingly, these sex differences in PFC physiology appear to emerge during development, particularly during adolescence. In humans, there is evidence of earlier cortical maturation in females, since total and frontal gray matter volume peak earlier in females than in males [119]. Preclinical studies have provided mechanistic insights into these sex-specific developmental trajectories. For instance, white matter volume in the PFC increased progressively in males between PND25 and PND90, while in females it followed a similar increase but plateaued around PND60, suggesting that structural maturation of the PFC occurs earlier in females [120]. Interestingly, the same study showed that while the total number of mPFC neurons remained stable across PND25-PND90 in males, females exhibited a decrease in total neuron number between PND35 and PND45, further supporting the existence of sex-specific developmental trajectories in PFC maturation.

The emergence of sex differences during adolescence strongly suggests a role for sex hormones in shaping PFC physiology, particularly given that androgen receptors are expressed in mPFC neurons, with a higher density of receptor-expressing cells in males than in females [121]. Supporting a modulatory effect of sex hormones in mPFC physiology, gonadectomy in males reduced mPFC synapse number, an effect that was reversed by androgen treatment [122]. Similarly, local infusion of 17β-estradiol into the mPFC of females increased apical spine density within two hours [123]. Interestingly, sex hormones have also been shown to influence glutamatergic transmission in the mPFC. In females, during the low-estradiol phase of the estrous cycle (diestrus), sEPSC frequency in IL-L5 pyramidal neurons was higher than during proestrus (high estradiol) and higher than in males, and this change was accompanied by reduced GluN2B-mediated NMDA receptor transmission [124]. This modulatory role of sex hormones also extends to inhibitory circuits in the mPFC. In females, pre-, but not post-, pubertal ovariectomy, prevented the normal developmental increase in inhibitory synaptic transmission in L2/3 neurons of the cingulate cortex [44]. This notion was further supported by findings showing increased inhibitory transmission

following short-term treatment with estradiol and progesterone administered prior to puberty [44].

In **Chapter 3**, we observed synaptic deficits in L2/3-mPFC neurons across developmental stages in female but not male mice lacking GluA3-containing AMPARs, pointing to a possible interaction between GluA3-dependent transmission and sex-specific regulatory mechanisms. One possible explanation is that sex hormones may play a role in regulating GluA3-dependent synaptic transmission. Consistent with this idea, previous work has shown stronger AMPAR-mediated transmission during diestrus compared to proestrus, suggesting a modulatory role of sex hormones on AMPAR function [124]. Although the reproductive cycle was not monitored in the female mice included in our experiments, and we did not directly assess the impact of sex hormones on AMPAR-mediated transmission (e.g. via ovariectomy or pharmacological manipulation of estrogen signaling), the sex-specific phenotype observed here provides ground for further investigation. Future studies will be necessary to elucidate the mechanisms underlying the female-specific synaptic depression observed in GluA3 knockout mice, including the possibility that GluA3-containing AMPARs are regulated by sex hormones. The female-specific effects reported here underscore the need to include both males and females in experimental studies, as functional frameworks derived primarily from male data may not fully translate to female physiology.

3.3. Interactions between sex hormones and the endocannabinoid system

In **Chapters 4 & 5**, we identified sex-specific effects of adolescent CBR activation on mPFC physiology, including changes in both synaptic transmission and intrinsic cellular properties in WT mice (**Chapter 4**), and changes in synaptic transmission in GluA3KO mice (**Chapter 5**). Additionally, preliminary data from **Chapter 5** raises the possibility that GluA3KO males may be more vulnerable to adolescent CBR activation. These findings align with previous reports showing that, under baseline conditions, the rodent ECS exhibits pronounced sex differences at multiple levels, including receptor expression and efficacy, ligand availability, and the expression of metabolic enzymes [125,126].

Across the two experimental chapters, adolescent CBR activation produced long-lasting neurophysiological consequences in both sexes, although with distinct outcomes. In **Chapter 5**, persistent effects on mPFC synaptic transmission were observed exclusively in male mice, whereas in **Chapter 4**, neurophysiological alterations were detected in both sexes, affecting different cellular parameters. In

particular, female mice exhibited more pronounced synaptic changes, while males showed stronger alterations in intrinsic cellular properties. Although these studies differed in the cortical layers examined (L2/3 in **Chapter 5** versus L5 in **Chapter 4**) and in genetic background (WT versus GluA3KO), together they suggest that **adolescence represents a sensitive window during which ECS manipulation affects both sexes, engaging partially divergent mechanisms**, as the effects observed were sex-specific.

Sex differences in ECS organization may underlie these sexually dimorphic outcomes. CB1Rs density has consistently been reported to be higher in males than in females across multiple brain regions, including the mPFC [127–129]. However, the lower-expressed CB1Rs in females display higher binding activity and greater desensitization following activation [128,130], suggesting that despite lower receptor density, CB1Rs may exhibit greater functional efficiency in females. Beyond receptor expression, sex dimorphism has also been reported in the levels of endocannabinoid ligands, as well as in their regulation across the lifespan, although these effects appear to be brain-region specific [131,132]. Importantly, when estrous cycle phases were taken into account, fluctuations in endocannabinoid levels were observed across the cycle, further supporting an interaction between the ECS and sex hormones [131]. Consistent with hormonal regulation of ECS components, higher CB1Rs levels have been reported in the hypothalamus during diestrus (low estradiol) compared to estrus [133]. In males, this interaction appears to be bidirectional: testosterone can modulate ECS signaling [134], and CB1Rs activation has been shown to stimulate testosterone production [121,135].

Taken together, the sex-specific organization of the ECS, its interaction with sex hormones, and the earlier maturation of the PFC in females compared with males [120], are all likely to contribute to the distinct neurophysiological outcomes observed following adolescent CBRs activation. Based on the sex-specific findings from **Chapters 4 & 5**, it is plausible that CB1Rs desensitization and internalization following repeated activation [136] differ between sexes as a consequence of hormonal regulation, which may also influence AMPAR function [124]. Future studies will therefore be important to elucidate the mechanisms through which long-lasting sex differences emerge after adolescent CBR activation, with particular emphasis on the intersection between sex hormones and the regulation of CB1R and GluA3-containing AMPAR.

Overall, the evidence discussed in **Section 3** highlights sex differences as intrinsic players to PFC development, synaptic physiology, and neuromodulatory signaling. Particularly during adolescence, changes in glutamatergic transmission, hormonal regulation and endocannabinoid signaling converge to produce specific developmental trajectories. The findings presented in this thesis reinforce the notion that sex-specific mechanisms shape mPFC function and vulnerability to perturbations, and that interpretations derived from male-centric frameworks may be insufficient to explain female physiology. **Recognizing sex as a biological variable therefore represents not only a methodological consideration, but rather a necessity to uncover mechanistic diversity within cortical circuits**.

4. Lost in definitions: conceptual challenges in Neuroscience

A crucial aspect of scientific dissemination lies in the accuracy with which methods are described, results are reported, and conclusions are drawn. In 2016, Baker highlighted the reproducibility crisis facing the scientific community, identifying selective reporting and publication pressure as the primary contributing factors [137]. Beyond these challenges, I propose that an additional contributor is **the lack of consensus on standardized definitions across studies**. This concern became increasingly evident over the course of the thesis, particularly when searching for relevant literature, comparing findings across studies, reporting results and attempting to interpret their translational relevance. In this section, I discuss how several aspects of the work presented here are influenced by, and in some cases contribute to, this broader issue. By directly addressing ambiguities, this discussion seeks to contextualize the findings within the current conceptual frameworks while emphasizing the importance of clear and consistent definitions for replicability and interpretation.

4.1. Defining the (medial) Prefrontal Cortex

The PFC occupies a central role in this thesis due to its integrative function and protracted developmental trajectory. In humans, the PFC is typically subdivided into

lateral PFC, including dorsolateral (dl) and ventrolateral (vl) subregions, and the ventromedial (vm) PFC, also known as orbitofrontal PFC [138]. These subregions are associated with distinct cognitive processes, and lesions in each area lead to different cognitive impairments. For example, damage to the dlPFC is frequently linked to deficits in working memory, whereas lesions of the vmPFC are more commonly associated with impairments in decision-making, emotional processing, and social behavior [138,139].

While the concept of PFC is relatively well defined in humans, its definition in rodents, one of the main model organisms used in preclinical and fundamental neuroscience research (including this thesis), remains in development [140] . In a review by Laubach and colleagues (2018), a meta-analysis of PFC-related publications across species and a survey of researchers highlighted substantial inconsistencies in how the term "PFC" is applied in rodent studies [141]. For example, the IL, PrL, and anterior cingulate cortex (ACC) are often grouped under different labels, with some studies referring to these regions collectively as mPFC and others as ACC, due to alternative schemes for prefrontal nomenclature [141,142]. More recent work illustrates the diversity in anatomical annotations of mouse prefrontal subregions [143], underscoring the challenges in establishing a unified definition. Importantly, these challenges are being actively addressed through efforts to improve cross-species alignment [140]. Newer editions of rodent brain atlases have adopted Brodmann-based nomenclature to better mirror primate cortical organization [144]. This shift toward cross-species consistency may collaterally promote the establishment of a standardized nomenclature for the rodent prefrontal cortex.

How, then, is the mPFC defined in the present work? Throughout this thesis, the term mPFC is used as a general descriptor for the regions under investigation, namely the L and PrL areas. Electrophysiological recordings were performed in these subregions based on an older edition of the mouse brain atlas (Paxinos & Franklin, 2001), with no data recorded from the ACC. In more recent atlas editions, these areas correspond to Brodmann areas 32 (IL) and 25 (PrL) (Paxinos & Franklin, 2019) (**Figure 2**). While referring to the broader mPFC introduces some anatomical imprecision and conceptual ambiguity, this designation allows for a clear description of the experimental focus, as IL and PrL do not differ in some functional domains such as fast network oscillations despite encoding behavioral information differently [145,146]. Overall, these considerations highlight that the rodent mPFC remains an evolving construct. Recent efforts to standardize anatomical definitions and align them across

species offer a valuable framework, reinforcing the need to clearly specify subregions when interpreting electrophysiological and developmental findings.

4.2. Conceptualizing adolescence in preclinical research

Adolescence is a developmental period during which behavior becomes increasingly dependent on cortical rather than limbic regulation. A major contributor to this

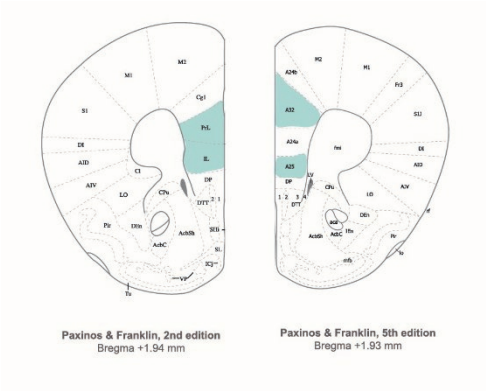


Figure 2. Evolution of the anatomical definition of mPFC subregions across mouse brain atlas editions. Left: Coronal section at +1.94 mm from bregma from the second edition of the Paxinos & Franklin mouse brain atlas, which was used throughout this thesis. Blue shading indicates the regions targeted in these studies (prelimbic and infralimbic cortex). Right: Section at the same coordinates in the fifth edition of the atlas. Blue shading indicates the regions corresponding to the prelimbic (PrL) and infralimbic (IL) areas according to the updated anatomical nomenclature. IL, infralimbic; PrL, prelimbic.

transition is the maturation of the PFC, which undergoes substantial synaptic and local circuit refinement during this stage, as well as long-range connectivity reorganization, particularly with limbic structures [147–149]. In **Chapters 3, 4 & 5**, adolescence was central to our research questions and experimental designs, as this stage has been proposed to constitute a critical window for PFC maturation [43,150].

Across all experiments presented here, adolescence was defined as the period between PND30 and PND50. Although many studies use a comparable age range, there is no consensus on the on the precise beginning and end of this developmental period in either mice [44–46,150] or rats [151–153]. Importantly, studies administering WIN during adolescence have typically used similar ages [154,155], facilitating comparison across studies. The lack of consensus regarding the definition of adolescence raises the question of whether adolescence should be defined not by chronological age but by physiological markers that better reflect the maturational transition from prepuberty to adulthood.

Potential markers include vaginal opening or balano-preputial separation [45,120,156], as well as circulating sex-hormone levels, which fluctuate substantially during this period [156,157]. Notably, sex hormones also exhibit region-specific

developmental trajectories: for example, estradiol and testosterone levels differ across the hippocampus, hypothalamus, and frontal cortex, and these patterns are sex-dependent, although many differences emerge during early postnatal life [158]. Moreover, puberty onset in mice differs markedly between sexes, occurring at approximately PND45 in males and PND35 in females [120], with comparable findings in rats [156]. These observations collectively suggest that if the goal is to match animals at equivalent maturational stages for a certain experimental design, age alone may not be an adequate criterion, particularly when both sexes are included.

Based on this literature, physiological measures could be considered a more accurate and standardized definition of adolescence in rodent research. However, defining adolescence based on physiological markers also presents practical challenges. Such measures are often time-consuming, as they may require repeated measurements, and can be invasive, particular if blood sampling is involved. In addition, reliance on physiological markers complicates experimental design, as it becomes more difficult to initiate interventions simultaneously across animals and to plan experiments in a standardized manner. Importantly, physiological markers also exhibit variability across strains [159,160], further complicating their use as universal criteria for defining adolescence. Lastly, considering that puberty occurs later in males (around PND45) than in females (around PND 35) [120] the pharmacological intervention used in **Chapters 4 & 5** (PND30-PND50), likely coincided with the pubertal period in females but largely preceded puberty in males. This asymmetry may partially account for the sex-specific long-term neurophysiological consequences we observed following adolescent CBRs activation. Overall using age to define adolescence in our studies (**Chapters 3, 4 & 5**) has provided a clear framework for comparing and contextualizing our findings within previous work.

4.3. What defines a valid model?

Understanding of human physiology, and neurophysiology in particular, relies on the use of models. Although studies conducted in humans provide invaluable insights, many mechanistic questions require experimental manipulations that are not feasible or ethical in humans. Models can take many forms, such as genetic, pharmacological or computational approaches, each providing distinct and complementary information. For this reason, the validity and scope of experimental models remain an active and necessary topic of discussion within the scientific community. In **Chapters 4 & 5**, we investigated the long-term consequences of repeated CBR activation during adolescence, with the aim of modelling specific aspects of adolescent cannabis

exposure in humans. In addition, in **Chapter 5**, we examined whether GluA3KO mice could serve as a model to interrogate certain features related to schizophrenia-like etiology and, based on our findings presented in **Chapter 3**, this possibility was also discussed in **Section 2.2** for GluA3KO mice. These approaches provide the opportunity to reflect on what constitutes a *model*, its intended scope, limitations and the conclusions that can be drawn from it.

Based on the experimental design used in **Chapters 4 & 5**, one could argue that our paradigm does not reproduce human adolescent cannabis consumption, as it relies on administration of a synthetic CBR agonist and therefore does not capture the complex pharmacology of cannabis, which contains both Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) and acts on targets beyond CBRs [161]. Nevertheless, the chosen approach was deliberately designed to isolate and interrogate the role of CBRs, the primary targets of THC [161]. This raises a key question: does the inability of a model to fully reproduce a human condition render it without value, or can the generation of fundamental knowledge through modelling be sufficient? For example, in **Chapter 4** we observed long-term changes in L5-mPFC synaptic transmission following adolescent CBR activation in females, but not in males. Rather than considering these results solely in a translational context, they can also serve as a framework for understanding the role of CBRs in shaping PFC development and female physiology.

A similar consideration applies in the context of GluA3KO mice. In **Chapters 3 & 5**, we explored their utility as a partial model for schizophrenia-related mechanisms and for examining interactions between genetic and environmental risk factors. Although GluA3KO mice do not present the specific high-risk loss-of-function variants identified in schizophrenia patients [8], they do offer a useful representation of ubiquitous GluA3 dysfunction. More broadly, modeling psychiatric disorders, and schizophrenia in particular, remains a subject of continued debate within the scientific community, largely due to the difficulty of capturing the full complexity of symptoms, especially positive symptoms, in animal models [162]. Nevertheless, models that reproduce selected aspects of disease physiology can yield valuable mechanistic insight and generate testable hypotheses [163], particularly regarding how specific synaptic or circuit alterations may contribute to disease etiology, vulnerability or progression.

These considerations pose the question of **what minimum criteria render a model informative**. Is the recreation of a single physiological process or behavioral alteration sufficient? While the ultimate aim of modeling is to gain insight into human (patho)physiology, it is essential to recognize that models are, by definition, simplified representations. Rather than limiting their value, this simplification allows the isolation

of defined variables and the investigation of specific mechanisms. In this context, carefully interpretating results and explicit acknowledging the scope of the model will allow meaningful conclusions to be drawn.

In lines with these considerations, the findings of this thesis should be interpreted accordingly. In **Chapter 3**, GluA3KO mice exhibited alterations that parallel specific features reported in schizophrenia patients, such as deficits in reversal learning [60] and impairments in mPFC-L2/3 synaptic transmission [95–97] (see **Section 2.2**). These results do not imply that GluA3KO mice constitute a comprehensive model of schizophrenia, but they support their usefulness for exploring selected mechanisms relevant to the disorder and for generating new hypotheses. Similarly, in **Chapter 4**, adolescent CBR activation induced long-lasting, sex-specific neurophysiological alterations. Although these findings cannot be directly extrapolated to human adolescent cannabis use, employing a controlled WIN administration protocol provides a defined experimental framework to identify potential consequences of CBR activation on mPFC physiology. Such knowledge may contribute to the development of more refined preclinical models and inform future translational studies

Taken together, these considerations highlight that the scientific value of a model depends on how it is defined, applied, and interpreted. Clarifying definitions, whether of developmental stages, brain regions, or model validity, will facilitate comparison across studies and strengthen the integration of experimental findings. In **Box 1** we examine how inconsistencies in definitions within neuroscience influence data interpretation and reproducibility, and how increased conceptual clarity can support the translation and consolidation of experimental knowledge.

BOX 1. The impact of definitions on interpretation and replicability.

In **Section 4**, we have discussed how the definitions of *mPFC*, *adolescence* and *experimental model* remain controversial within the field. Here, we turn to the broader implications of these ambiguities, particularly their impact on replicability (new data, same conclusion), translatability, and, ultimately, the scientific and societal value of research findings.

Using terms such as *mPFC* or *adolescence* in a broader sense offers certain practical advantages. Broad terminology facilitates the clustering of discoveries and simplifies literature searches within a given field. Indeed, some journals explicitly encourage authors to include relevant key words in

titles and abstracts to improve discoverability in databases such as PubMed. From this perspective, an article that includes a general term such as *mPFC* is likely to reach a wider audience than one that specifies narrower subregions, such as the infralimbic or prelimbic cortex. The same rationale applies to the use of terms such as *adolescence* or *model*.

At the same time, the desire for visibility, combined with structural pressures within the academic system, may encourage the use of terms in a looser or less precise manner. This practice carries potential negative consequences for the scientific community, as imprecise terminology can complicate the comparison and integration of findings across studies, particularly when methodological details are not carefully described or closely examined [137].

Many of the issues discussed in this section reflect a mismatch between conceptual definitions, how terms such as mPFC, adolescence, or disease models are understood in theory, and operational definitions, which specify how these concepts are implemented experimentally. In this context, inconsistencies and ambiguity in definitions can undermine replicability and blur meaningful differences between experimental approaches.

Nevertheless, a certain degree of flexibility in terminology may also be beneficial, or even necessary, in specific contexts. For example, in science communication aimed at the general public, simplification is essential, and the use of broader, less technical terminology may facilitate understanding without substantially compromising the intended message. Thus, strict precision in definitions may not always be compatible with accessibility.

I therefore argue for a balanced approach in which scientists remain both critical and reflective when interpreting or communicating research, while still allowing for flexibility when the context demands it. This can be achieved by clearly defining key terms, transparently reporting methods and limitations, critically evaluating assumptions, and adjusting the level of precision to the audience and context. This approach can help keep scientific knowledge both accurate and accessible, supporting broader dissemination without compromising conceptual clarity or scientific integrity.

5. From limitations to opportunities

A central component of scientific progress lies in the careful evaluation of limitations, not only as constraints but also as opportunities to refine hypotheses, improve experimental design, and advance understanding. While addressing fundamental questions regarding cortical synaptic physiology, cognitive flexibility, endocannabinoid signaling and development, several aspects of the work presented in this thesis open new directions for future research. Rather than weakening the conclusions, these considerations provide a framework for more targeted, mechanistic, and translational studies that can build on the findings described here.

First, although this thesis emphasizes the importance of including sex as a biological variable, not all experiments were performed in both male and female mice. In **Chapter 3**, some experiments were restricted to one sex, and in **Chapter 2** the sex of the animals was not specified. Expanding these experimental designs to systematically include both sexes represents an important next step that could further clarify the sex-specific effects observed throughout this work. In addition, the limited cohort size in **Chapter 5** set a foundation for future studies with larger sample numbers to increase statistical power and strengthen the interpretation of the results.

In **Chapter 4**, we demonstrated that repeated activation of cannabinoid receptors during adolescence induces long-lasting alterations in adult mPFC physiology. While these findings identify adolescence as a sensitive window for endocannabinoid modulation, the mechanistic processes occurring during the exposure period itself remain to be explored. Exploring CBR dynamics across adolescence would be particularly informative, given the rapid CB1R internalization following activation [136] and its downregulation and desensitization after repeated exposure [128]. Immunohistochemical analyses of CB1R expression at multiple time points throughout the injection period (PND30-PND50) would provide further insights into the processes that shape the long-term effects observed in adulthood. To further enhance the translatability of our findings from **Chapter 4**, future studies could also incorporate experimental designs that more closely resemble human cannabis exposure. For example, cannabinoid vapor exposure [164] would more closely resemble the route of intake in humans and the use of THC rather than the synthetic agonist WIN would allow assessment of whether the neurophysiological alterations generalize to compounds more representative of cannabis. Supporting the importance of this distinction, Navarri and colleagues (2024) reported that adolescent exposure to THC and WIN induces distinct transcriptomic changes in the frontal cortex, with WIN preferentially affecting pyramidal neurons and THC exerting broader effects on non-neuronal cell populations [73].

Chapters 2 & 3 addressed the contributions of GluA3- and GluA1-containing AMPARs to cortical synaptic transmission. These findings provide a basis for future experiments with increased temporal, spatial, and cell-type specificity. The use of conditional rather than global knockouts would help reduce potential developmental compensations, while Cre-dependent strategies targeting defined brain regions or neuronal populations (e.g., via CamK2a, Pvalb, Sst, Cck, or Dlx-family promoters) would allow a more precise dissection of subunit-specific functions in cortical physiology. Extending this approach to additional cortical regions with earlier developmental trajectories, such as sensory cortices, may also help disentangle whether the maturational patterns observed in **Chapter 3** reflect subunit-specific mechanisms or broader principles of cortical maturation.

Finally, in **Chapter 3** we demonstrated that GluA3KO male mice exhibit deficits in reversal learning and that GluA3-containing AMPARs contribute to basal synaptic transmission in mPFC-L2/3 neurons at different stages of mPFC development in female mice. These results identify GluA3 as a potential regulator of both synaptic maturation and cognitive flexibility, but do not directly establish its causal role in these processes. Addressing this question will require experimental designs with higher temporal precision. The dTAG system for target-specific and reversible protein degradation through ubiquitination provides a promising approach [165,166]. By knocking in a tag ($FKB12^{F36V}$) into the *GRIA3* gene, GluA3 could be selectively degraded within hours following dTAG ligand administration, assuming that the tag does not affect trafficking or function of the receptor. Importantly, recent evidence indicates that GluA3 is naturally degraded through ubiquitination, highlighting the feasibility of this strategy for reducing GluA3 levels [167]. Daily dTAG treatment restricted to adolescence would enable direct testing of the role of GluA3-containing AMPARs in PFC development, while acute degradation prior to reversal learning tasks would allow assessment of their contribution to cognitive flexibility.

Taken together, the considerations described above illustrate how the limitations identified throughout this thesis become opportunities for research questions and experimental designs. By integrating sex as a biological variable, refining developmental and circuit-specific manipulations, and applying emerging molecular tools, future studies can build on the foundations established here to advance our understanding of how cortical synaptic mechanisms shape cognition.

Closing remarks

From synapses to behavior, this thesis investigated how glutamatergic transmission and neuromodulatory systems shape cortical function across development, and how

their interaction may contribute to (patho)physiology. By combining genetic and pharmacological approaches, the work highlights adolescence as a critical period for cortical maturation, during which perturbations in endocannabinoid signaling can induce long-lasting effects on cortical physiology and behavior. Rather than providing definitive answers, the studies presented here explore the contribution of understudied receptors, such as GluA3-containing AMPARs, to cortical synaptic transmission. Based on these findings, we propose that GluA3 acts as a regulator of synaptic transmission and possibly maturation in the developing cortex.

As a fundamental scientist I intended the work presented here as fundamental; **creating knowledge for the beauty of increasing knowledge**.

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General summary

The neocortex confers mammals the ability to perceive, process, and integrate information from the environment, capacities that are essential for adaptation and survival. Such adaptation relies on fast synaptic signaling, a function largely supported by AMPA receptors (AMPARs), the principal drivers of rapid glutamatergic transmission. AMPARs are tetrameric receptors composed of four subunits, GluA1-GluA4, and have been implicated in key cognitive processes. In particular, GluA1-containing AMPARs have been extensively studied and are known to contribute to cortical and hippocampal synaptic plasticity, as well as to learning, memory, and social behavior. In contrast, the role of GluA3-containing AMPARs in synaptic transmission within sensory and association cortices, and their interaction with neuromodulatory systems, remains largely unexplored.

In the first part of this thesis, we investigated the contribution of GluA3-containing AMPARs to synaptic transmission and plasticity in the visual and prefrontal cortices, as well as their modulation by β-adrenergic signaling. Given the protracted maturation of the prefrontal cortex (PFC), we incorporated a developmental perspective into our analyses. To further deepen our understanding of PFC development, the second part of the thesis focused on the role of cannabinoid receptors (CBRs), which are key component of cortical maturation, in PFC maturation and explored potential interactions between endocannabinoid signaling and GluA3-containing AMPARs, both acutely and in the long term.

In **Chapter 1**, we establish the conceptual and historical framework that shaped this thesis. We begin with a brief overview of foundational milestones in neuroscience, from the formulation of the neuron doctrine by Santiago Ramón y Cajal to the identification of AMPA receptors and the recognition of the PFC as a central substrate of executive function and personality. We then outline the organization, function, and developmental trajectories of the two cortical regions examined in this work: the primary visual cortex and the prefrontal cortex. Next, we introduce the principal synaptic and neuromodulatory systems investigated throughout this thesis, given their relevance to synaptic physiology, including AMPA receptors (AMPARs), cannabinoid receptors (CBRs), and adrenergic signaling pathways. Finally, we discuss the importance of incorporating sex as a biological variable, particularly given the sexually dimorphic nature of the PFC and the sex-specific effects observed in the experimental chapters of this work.

In **Chapter 2**, we examined synaptic transmission in a primary sensory area, the visual cortex, and assessed the respective contributions of GluA1- and GluA3-containing AMPARs to basal synaptic transmission and plasticity. Using patch-clamp recordings,

we demonstrated that both subunits contribute to excitatory transmission onto layer 2/3 pyramidal neurons, with GluA3-containing AMPARs playing a more prominent role than GluA1-containing receptors. To further probe subunit-specific plasticity mechanisms, we examined how β-adrenergic receptor activation modulates AMPAR-mediated synaptic responses. We found that β-adrenergic-induced potentiation was independent of subunit composition and occurred independently of protein kinase A (PKA), despite being active under basal conditions in these neurons. Finally, we investigated synaptic transmission in somatostatin-positive (SST) interneurons and showed that GluA3 does not contribute to basal excitatory transmission in these cells, nor is transmission potentiated by β-adrenergic signaling.

In **Chapter 3**, we extended this investigation to an association cortex, the mPFC. Here, we examined the contribution of GluA3-containing AMPARs to basal synaptic transmission in layer 2/3 pyramidal neurons, their modulation by β-adrenergic signaling, and the role of GluA3 in reversal learning. Incorporating a developmental perspective due to the protracted development of this brain region, we showed that GluA3-containing AMPARs play a significant role in mPFC-layer 2/3 synaptic transmission in an age-dependent manner. In addition, β-adrenergic signaling induced potentiation through a mechanism that appeared to have a presynaptic component and was not GluA3-specific. At the behavioral level, GluA3KO mice exhibited intact discrimination learning but displayed alterations in basal behavior and impairments in reversal learning, a well-established PFC-dependent function.

In **Chapter 4**, we further explored prefrontal cortical development by investigating the long-term consequences of adolescent cannabinoid receptor activation on adult mPFC physiology and behavior. We found that repeated CBRs activation during adolescence induced persistent, sex-specific alterations in layer 5 pyramidal neurons of the mPFC. Female mice exhibited changes in synaptic transmission, whereas intrinsic excitability was predominantly altered in males. Despite these neurophysiological changes, adolescent CBR activation did not impair social hierarchy or reversal learning in adulthood, two behaviors commonly associated with PFC function.

Finally, in **Chapter 5**, we integrated the conceptual threads of the previous chapters in a pilot study investigating potential interactions between GluA3-containing AMPARs and CBRs. Given that both systems have been associated with schizophrenia, we proposed a double-hit framework for susceptibility to the disorder, based on the interaction between genetic vulnerability and environmental exposure. To this end, we assessed the long-term consequences of adolescent CBR activation in a GluA3-deficient background. At the synaptic level, acute WIN exposure reduced inhibitory synaptic transmission onto layer 2/3 pyramidal neurons, and effect that was also

observed in adult GluA3KO males following adolescent CBR activation. Behaviorally, GluA3KO mice did not show clear deficits in discrimination or reversal learning although preliminary data suggested a possible increased vulnerability in GluA3KO male mice, which displayed a tendency toward reduced reversal learning performance.

Finally, in **Chapter 6**, we integrate the findings of this thesis and contextualize them within the current literature. We begin by examining the electrophysiological principles of cortical synaptic transmission, synthesizing our results on the role of AMPARs across cortical regions and their modulation and interaction with β-adrenergic and cannabinoid receptors. We then bridge the gap between synapses and behavior by evaluating how alterations in synaptic receptors relate to behavioral outcomes. The second part of this chapter addresses broader conceptual themes that have shaped and influenced this work, including the consideration of sex as a biological variable and the operational definitions of key concepts such as medial prefrontal cortex (mPFC), adolescence, and animal models. Finally, we outline future directions that may help refine and extend the research questions initiated in this thesis.

Together, the work presented in this thesis aimed to provide a coherent framework for understanding cortical synaptic physiology across sensory and association cortices, highlighting both conserved principles and region-specific mechanisms. By focusing on the understudied GluA3 subunit, incorporating sex as a biological variable, and exploring interactions between glutamatergic, adrenergic, and endocannabinoid systems, this thesis aimed to open new perspectives and to lay the groundwork for future investigations into the complexity of cortical function.

Nederlands samenvatting

De neocortex geeft zoogdieren het vermogen om informatie uit de omgeving waar te nemen, te verwerken en te integreren, functies die essentieel zijn voor aanpassingsvermogen en overleving. Dit aanpassingsvermogen is afhankelijk van snelle synaptische signalering, een functie die voornamelijk wordt ondersteund door AMPA-receptoren (AMPARen), de belangrijkste mediatoren van snelle glutamaterge transmissie. AMPARen zijn tetramere receptoren die bestaan uit vier subeenheden, GluA1–GluA4, die in verband worden gebracht met essentiële cognitieve functies. Met name de GluA1-bevattende AMPARen zijn uitgebreid bestudeerd en staan bekend om hun bijdrage aan corticale en hippocampale synaptische plasticiteit, evenals leren, geheugen en sociaal gedrag. Daarentegen is er vooralsnog weinig bekend over de rol van GluA3-bevattende AMPARen in synaptische transmissie in sensorische en associatieve cortices, of over hun interactie met neuromodulaire systemen.

In het eerste deel van dit proefschrift onderzochten we de bijdrage van GluA3-bevattende AMPARen aan synaptische transmissie en plasticiteit in de visuele cortex en prefrontale cortex (PFC), evenals hun modulatie door β-adrenerge signalering. Gezien de langdurige groei van de PFC hebben we ook het aspect van ontwikkeling meegenomen in onze analyse. Om de ontwikkeling van de PFC beter te begrijpen, lag de focus van het tweede deel van dit proefschrift op de rol van cannabinoïde receptoren (CBRen), die een cruciaal onderdeel zijn van corticale groei. Ook werden mogelijke interacties tussen endocannabinoïde signalering en GluA3-bevattende AMPARen onderzocht, zowel op de korte als lange termijn.

In **Hoofdstuk 1** introduceren we het conceptuele en historische kader dat dit proefschrift heeft gevormd. We beginnen met een kort overzicht van de fundamentele mijlpalen binnen de neurowetenschap, van de formulering van de neurondoctrine door Santiago Ramón y Cajal tot de identificatie van AMPARen en de erkenning van de PFC als een centrale speler in executieve functies en persoonlijkheid. Vervolgens beschrijven we de organisatie, functie en ontwikkeling van de twee corticale gebieden die in dit werk worden onderzocht: de primaire visuele cortex en de PFC. Daarna introduceren we de belangrijkste synaptische en neuromodulaire systemen die in dit proefschrift zijn onderzocht, waaronder AMPARen, CBRen en adrenerge signaaltransductieketens, gezien hun relevantie voor synaptische fysiologie. Tot slot bespreken we het belang van het meenemen van sekse als biologische variabele, met name gezien de seksuele dimorfe aard van de PFC en de sekse-afhankelijke effecten die in de experimentele hoofdstukken van dit werk zijn waargenomen.

In **Hoofdstuk 2** onderzochten we de synaptische transmissie in een primair sensorisch gebied, de visuele cortex, en beoordeelden we de bijdrage van GluA1- en GluA3-

bevattende AMPARen aan basale synaptische transmissie en plasticiteit. Met behulp van patch-clamptechnieken toonden we aan dat beide subeenheden bijdragen aan exciterende transmissie naar laag 2/3-piramidale neuronen, waarbij de GluA3-bevattende AMPARen een prominentere rol speelden dan de GluA1-bevattende receptoren. Om subeenheidspecifieke plasticiteitsmechanismen verder te bestuderen, onderzochten we hoe activatie van β-adrenerge receptoren de synaptische activiteit van AMPARen moduleert. We ontdekten dat β-adrenerge potentiëring onafhankelijk was van zowel de subeenheidssamenstelling als van proteïnekinase A (PKA)-activiteit, ondanks dat PKA al actief was in deze neuronen onder basale omstandigheden. Tot slot onderzochten we de synaptische transmissie in somatostatine-positieve interneuronen. We lieten zien dat GluA3 niet bijdraagt aan basale exciterende transmissie in deze cellen, noch wordt hun transmissie gepotentieerd door β-adrenerge signalering.

In **Hoofdstuk 3** breidden we dit onderzoek verder uit naar een associatieve cortex, de mediale PFC (mPFC). Hier onderzochten we de bijdrage van GluA3-bevattende AMPARen aan basale synaptische transmissie in laag 2/3-piramidale neuronen en de modulatie van deze receptoren door β-adrenerge signalering. Ook onderzochten we de rol van GluA3-bevattende AMPARen in het 'omkeerleren'. Gezien de langdurige ontwikkeling van dit hersengebied hebben we ook het aspect van ontwikkeling meegenomen. We vonden dat GluA3-bevattende AMPARen een significante, leeftijdsafhankelijke rol speelden in synaptische transmissie in laag 2/3 van de mPFC. Daarnaast zorgde β-adrenerge signalering voor een potentiëring via een mechanisme dat waarschijnlijk een presynaptisch component bevatte en niet specifiek was voor GluA3. Op gedragsniveau vertoonden muizen zonder GluA3 een intact vermogen tot 'discriminatieleren', maar lieten ze wel veranderingen in basaal gedrag zien, evenals gebreken in het omkeerleren, een bekende PFC-afhankelijke functie.

In **Hoofdstuk 4** verkenden we de ontwikkeling van de PFC verder door de langetermijngevolgen van adolescente CBR-activatie op de volwassen mPFC-fysiologie en gedrag te onderzoeken. We ontdekten dat herhaalde activatie van CBRen tijdens de adolescentie aanhoudende, sekse-afhankelijke veranderingen veroorzaakte in laag 5-piramidale neuronen van de mPFC. Vrouwelijke muizen vertoonden veranderingen in synaptische transmissie, terwijl bij mannetjes voornamelijk de intrinsieke exciteerbaarheid was veranderd. Ondanks deze neurofysiologische veranderingen had adolescente CBR-activatie geen effect op de sociale hiërarchie of het omkeerleren in de volwassenheid, twee gedragingen die vaak geassocieerd worden met de functie van de PFC.

Tot slot hebben we in **Hoofdstuk 5** de conceptuele lijnen van de vorige hoofdstukken geïntegreerd in een pilotstudie die de mogelijke interacties tussen GluA3-bevattende

AMPARen en CBRen onderzocht. Aangezien beide systemen geassocieerd worden met schizofrenie, hebben we een tweeledig model voor de vatbaarheid voor deze aandoening voorgesteld, gebaseerd op de interactie tussen genetische kwetsbaarheid en blootstelling aan omgevingsfactoren. Daartoe hebben we de langetermijngevolgen van adolescente CBR-activatie beoordeeld in een GluA3-deficiënte achtergrond. Op synaptisch niveau verminderde acute blootstelling aan WIN de inhiberende synaptische transmissie naar laag 2/3-piramidale neuronen, een effect dat ook werd waargenomen bij volwassen GluA3KO mannetjes na adolescente CBR-activatie. Gedragsmatig vertoonden GluA3KO muizen geen duidelijke gebreken in discriminatie- of omkeerleren, hoewel voorlopige resultaten een mogelijk verhoogde kwetsbaarheid bij mannelijke GluA3KO muizen suggereerden, die namelijk een neiging tot verminderde omkeerleerprestaties vertoonden.

Ten slotte integreren we in **Hoofdstuk 6** de bevindingen van dit proefschrift en plaatsen we deze in context binnen de huidige literatuur. We beginnen met het onderzoeken van de elektrofysiologische principes van corticale synaptische transmissie, waarbij we onze resultaten over de rol van AMPARen in meerdere corticale gebieden en hun modulatie en interactie met β-adrenerge en CBRen samenvoegen. Vervolgens overbruggen we de kloof tussen synapsen en gedrag door te evalueren hoe veranderingen in synaptische receptoren zich verhouden tot gedragsuitkomsten. Het tweede deel van dit hoofdstuk behandelt de bredere conceptuele thema's die dit werk hebben gevormd en beïnvloed, waaronder de beschouwing van sekse als biologische variabele, evenals de operationele definities van sleutelconcepten zoals de mPFC, adolescentie en diermodellen. Tot slot schetsen we toekomstige richtingen die kunnen helpen om de onderzoeksvragen die in dit proefschrift zijn opgeworpen te verfijnen en uit te breiden.

Samenvattend had dit proefschrift als doel een samenhangend kader te bieden voor het begrijpen van corticale synaptische fysiologie in sensorische en associatieve cortices, waarbij zowel geconserveerde principes als regio-specifieke mechanismen worden benadrukt. Door de nadruk te leggen op de onderbelichte GluA3-subeenheid, sekse als biologische variabele te integreren en interacties tussen glutamaterge, adrenerge en endocannabinoïde systemen te verkennen, beoogde dit proefschrift nieuwe perspectieven te bieden en de basis te leggen voor toekomstig onderzoek naar de complexiteit van corticale functies.

Resumen general

El neocórtex confiere a los mamíferos la capacidad de percibir, procesar e integrar la información procedente del entorno, capacidades esenciales para la adaptación y la supervivencia. Esta adaptación depende de una transmisión sináptica rápida, un proceso mediado principalmente por los receptores AMPA (AMPARs), responsables de la transmisión glutamatérgica excitadora de acción rápida. Los AMPARs son receptores tetraméricos formados por cuatro subunidades, GluA1–GluA4, y participan en procesos cognitivos fundamentales. En particular, los AMPARs que contienen la subunidad GluA1 han sido ampliamente estudiados y se sabe que contribuyen a la plasticidad sináptica cortical e hipocampal, así como al aprendizaje, la memoria y el comportamiento social. En cambio, el papel de los AMPARs que contienen la subunidad GluA3 en la transmisión sináptica de las cortezas sensoriales y de asociación, así como su interacción con otros sistemas neuromoduladores, sigue siendo en gran medida desconocido.

En la primera parte de esta tesis investigamos la contribución de los AMPARs que contienen GluA3 a la transmisión sináptica y la plasticidad en las cortezas visual y prefrontal, así como su modulación por la señalización β-adrenérgica. Dada la prolongada maduración de la corteza prefrontal (PFC), incorporamos una perspectiva del desarrollo en nuestros análisis. Con el objetivo de profundizar en nuestro conocimiento del desarrollo de la PFC, la segunda parte de la tesis se centró en el papel de los receptores cannabinoides (CBRs), componentes clave de la maduración cortical, en el desarrollo de la PFC, así como en las posibles interacciones entre la señalización endocannabinoide y los AMPARs que contienen GluA3, tanto de forma aguda como a largo plazo.

En el **Capítulo 1** establecemos el marco conceptual e histórico que dio forma a esta tesis. Comenzamos con una breve revisión de algunos de los hitos fundamentales de la neurociencia, desde la formulación de la doctrina de la neurona por Santiago Ramón y Cajal hasta la identificación de los receptores AMPA y el reconocimiento de la PFC como un sustrato clave de las funciones ejecutivas y la personalidad. A continuación, describimos la organización, las funciones y la trayectoria del desarrollo de las dos regiones corticales estudiadas en este trabajo: la corteza visual primaria y la corteza prefrontal. Posteriormente, introducimos los principales sistemas sinápticos y neuromoduladores investigados a lo largo de esta tesis, dada su relevancia para la fisiología sináptica, incluidos los receptores AMPA (AMPARs), los receptores cannabinoides (CBRs) y las vías de señalización adrenérgica. Finalmente, abordamos la importancia de incorporar el sexo como variable biológica, especialmente teniendo en cuenta el carácter sexualmente dimórfico de la PFC y los

efectos dependientes del sexo observados en los capítulos experimentales de este trabajo.

En el **Capítulo 2** estudiamos la transmisión sináptica en una región sensorial primaria, la corteza visual, y evaluamos la contribución de los AMPARs que contienen GluA1 y GluA3 a la transmisión sináptica basal y a la plasticidad. Mediante registros electrofisiológicos de patch-clamp demostramos que ambas subunidades contribuyen a la transmisión excitadora sobre las neuronas piramidales de la capa 2/3, aunque los AMPARs que contienen GluA3 desempeñan un papel más destacado que aquellos que contienen GluA1. Con el fin de profundizar en los mecanismos de plasticidad específicos de cada subunidad, analizamos cómo la activación de los receptores β-adrenérgicos modula las respuestas sinápticas mediadas por AMPARs. Observamos que la potenciación inducida por la señalización β-adrenérgica era independiente de la composición en subunidades y ocurría de forma independiente de la proteína quinasa A (PKA), a pesar de que esta se encontraba activa en condiciones basales en estas neuronas. Finalmente, investigamos la transmisión sináptica en interneuronas positivas para somatostatina (SST) y demostramos que GluA3 no contribuye a la transmisión excitadora basal en estas células, ni esta es potenciada por la señalización β-adrenérgica.

En el **Capítulo 3** extendimos esta investigación a una corteza de asociación, la corteza prefrontal medial (mPFC). En este capítulo estudiamos la contribución de los AMPARs que contienen GluA3 a la transmisión sináptica basal en neuronas piramidales de la capa 2/3, su modulación por la señalización β-adrenérgica y el papel de esta subunidad en el aprendizaje por reversión. Incorporando una perspectiva del desarrollo, motivada por la prolongada maduración de esta región cerebral, demostramos que los AMPARs que contienen GluA3 desempeñan un papel relevante en la transmisión sináptica de las neuronas de la capa 2/3 de la mPFC de manera dependiente de la edad. Además, la señalización β-adrenérgica indujo una potenciación mediante un mecanismo que parecía tener un componente presináptico y que no dependía específicamente de GluA3. A nivel comportamental, los ratones GluA3KO mostraron un aprendizaje de discriminación intacto, pero presentaron alteraciones en el comportamiento basal y déficits en el aprendizaje por reversión, una función ampliamente reconocida como dependiente de la PFC.

En el **Capítulo 4** profundizamos en el estudio del desarrollo de la corteza prefrontal investigando las consecuencias a largo plazo de la activación de los receptores cannabinoides durante la adolescencia sobre la fisiología y el comportamiento de la mPFC en la edad adulta. Observamos que la activación repetida de los CBRs durante la adolescencia inducía alteraciones persistentes y específicas del sexo en las neuronas piramidales de la capa 5 de la mPFC. Las hembras presentaron cambios en

la transmisión sináptica, mientras que en los machos la excitabilidad intrínseca fue la propiedad principalmente afectada. A pesar de estas alteraciones neurofisiológicas, la activación adolescente de los CBRs no produjo déficits en la jerarquía social ni en el aprendizaje por reversión en la edad adulta, dos comportamientos comúnmente asociados con la función de la PFC.

Finalmente, en el **Capítulo 5** integramos los conceptos desarrollados en los capítulos anteriores en un estudio piloto destinado a investigar posibles interacciones entre los AMPARs que contienen GluA3 y los CBRs. Dado que ambos sistemas se han asociado con la esquizofrenia, propusimos un modelo de doble impacto (*double-hit*) de susceptibilidad a este trastorno, basado en la interacción entre una vulnerabilidad genética y una exposición ambiental. Con este objetivo, evaluamos las consecuencias a largo plazo de la activación de los CBRs durante la adolescencia en un modelo deficiente en GluA3. A nivel sináptico, la exposición aguda a WIN redujo la transmisión sináptica inhibitoria sobre las neuronas piramidales de la capa 2/3, un efecto que también se observó en machos adultos GluA3KO tras la activación de los CBRs durante la adolescencia. A nivel comportamental, los ratones GluA3KO no mostraron déficits evidentes en el aprendizaje de discriminación ni en el aprendizaje por reversión. No obstante, los resultados preliminares sugieren una posible mayor vulnerabilidad en los machos GluA3KO, que mostraron una tendencia a un peor rendimiento durante el aprendizaje por reversión.

Por último, en el **Capítulo 6** integramos los hallazgos de esta tesis y los contextualizamos dentro de la literatura científica actual. Comenzamos examinando los principios electrofisiológicos de la transmisión sináptica cortical, integrando nuestros resultados sobre el papel de los AMPARs en distintas regiones corticales, así como su modulación e interacción con los receptores β-adrenérgicos y cannabinoides. A continuación, tendemos un puente entre las sinapsis y el comportamiento analizando cómo las alteraciones en los receptores sinápticos se relacionan con los cambios conductuales. La segunda parte de este capítulo aborda cuestiones conceptuales más amplias que han influido en el desarrollo de esta tesis, entre ellas la consideración del sexo como variable biológica y la definición operativa de conceptos clave como la corteza prefrontal medial (mPFC), la adolescencia y los modelos animales. Finalmente, planteamos futuras líneas de investigación que podrían ayudar a refinar y ampliar las preguntas de investigación iniciadas en esta tesis.

En conjunto, el trabajo presentado en esta tesis tuvo como objetivo proporcionar un marco coherente para comprender la fisiología sináptica cortical tanto en cortezas sensoriales como de asociación, poniendo de manifiesto principios conservados y mecanismos específicos de cada región. Al centrarse en la subunidad GluA3, todavía

poco estudiada, incorporar el sexo como variable biológica y explorar las interacciones entre los sistemas glutamatérgico, adrenérgico y endocannabinoide, esta tesis aspira a abrir nuevas perspectivas y sentar las bases para futuras investigaciones dirigidas a comprender la complejidad de la función cortical.

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Por último, a **A.**, en todas sus fases y facetas. A la niña pequeña que decidió ser investigadora con 8 años y que pidió una maleta por reyes porque quería ver el mundo. A la que creció con ansiedad y de adulta ha conseguido (casi siempre) gestionarla. A la que siempre, siempre, dibujó espirales porque el cambio ha sido una parte inherente a ella. A la que no olvidó sus lados creativo y social y los cultivó a pesar de que la Ciencia le dejase poco espacio. A la que siempre dijo que sí y lo disfrutó aunque ahora haya aprendido que a veces hay que decir que no. A la que ha decidido escucharse y reinventarse, orgullosa del camino emprendido. A la que se fue de viaje y encontró lo que buscaba. A todas ellas, gracias por existir y co-existir, este doctorado es nuestra victoria.



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