UNIVERSIDADE D
COIMBRA

Maria Luísa Dias Pereira Félix Amado

Deciphering the Molecular Mechanisms that Mediate Postsynaptic Maturation

Dissertação no âmbito do Mestrado de Biologia Celular e Molecular, com especialização em Neurobiologia orientada por Ben Verpoort e pela Professora Doutora Ana Luísa Carvalho apresentada ao Departamento de Ciências da Vida da Universidade de Coimbra

Setembro de 2022

Faculdade de Ciências e Tecnologia
da Universidade de Coimbra

Deciphering the Molecular Mechanisms that Mediate Postsynaptic Maturation

Maria Luísa Dias Pereira Félix Amado

Dissertação no âmbito do Mestrado de Biologia Celular e Molecular, com especialização em Neurobiologia, orientada por Ben Verpoort e pela Professora Doutora Ana Luísa Carvalho apresentada ao Departamento de Ciências da Vida da Universidade de Coimbra

Setembro de 2022

UNIVERSIDADE D
COIMBRA

Affiliation:

The present work was developed in the Lab of Synapse Biology
of the VIB-KU Leuven Center for Brain & Disease Research

Acknowledgements/Agradecimentos

First of all, I would like to express my gratitude to Professor Doctor Joris de Wit for the opportunity to join the Synapse Biology Lab and develop my master's thesis project. This experience was crucial for my growth as a (neuro)scientist, both technically and most importantly, personally.

I deeply thank Ben Verpoort, the self-entitled "coolest supervisor", for teaching me everything I know. It was extremely gratifying to be working side by side with someone so hardworking and invested in science. I realize now how much my thinking process has matured thanks to our discussions and experimental planning, not to say that I learned how resilient I can be, even when nothing seemed to go our way. I will always look up to you over my next steps!

To the Synapse Biology Lab members, I sincerely thank you all for the time spent inside and outside the lab. It was privilege to learn more about your projects and to see you work in such an incredible environment.

Por fim, e agora em Português, gostaria de agradecer aos meus pais por me apoiarem sempre, mesmo quando isso implica estar longe, ao meu Avô Zé por ter sido a figura que me fez interessar pela natureza e pela ciência, e a toda a família por serem parte de mim. Agradeço ao Tomás por ser o meu companheiro de todas as alturas e aos meus amigos por estarem presentes em cada etapa, sem vocês e o vosso entusiasmo, as minhas alegrias não tinham com quem ser partilhadas.

Resumo

No córtex somatossensorial primário de ratinho, também conhecido como córtex em barril, a informação sensorial proveniente dos bigodes é transmitida por projeções talamocorticais, as quais estabelecem sinapses exclusivamente com neurónios excitatórios da camada cortical IV de forma topográfica e organizada em módulos conhecidos como "barris". Este circuito neuronal encontra-se extensivamente caracterizado na literatura, no entanto, os mecanismos moleculares que medeiam a conectividade sináptica entre projeções talamocorticais e neurónios da camada IV não são completamente conhecidos. O desenvolvimento dos barris corticais depende, em grande medida, da maturação do compartimento pós-sináptico, a qual permite o refinamento da organização dos barris e a plasticidade das conexões sinápticas. O recetor acoplado à proteína G, GPR158, foi previamente caracterizado pelo laboratório anfitrião como sendo importante para a formação e funcionamento de sinapses no hipocampo. No entanto, este recetor é também expresso especificamente por neurónios da camada cortical IV durante o desenvolvimento do córtex em barril. Nesta região, GPR158 contribui para a maturação de espículas dendríticas, mas os mecanismos de sinalização celular envolvidos neste processo não são conhecidos. Através de um ensaio de interação entre proteínas, o laboratório anfitrião identificou uma nova interação intracelular entre GPR158 e PLCXD2, uma fosfolipase do tipo C bastante atípica e sem função conhecida no cérebro. Neste projeto de dissertação de mestrado, caracterizámos a expressão espaciotemporal de *Gpr158* e *Plcxd2* no córtex em barril e demonstrámos que ambos são co-expressos em neurónios da camada IV. No período de desenvolvimento caracterizado por intensa génese de espículas dendríticas, a expressão de *Gpr158* aumenta profundamente, enquanto a expressão de *Plcxd2* atinge um *plateau*. Posteriormente, usámos uma estratégia mediada por CRISPR/Cas9 para marcação de epítopos em neurónios pós-mitóticos, a qual revelou a localização pós-sináptica de PLCXD2 expressa de forma endógena. Por fim, avaliámos a contribuição de PLCXD2 no desenvolvimento de espículas dendríticas e observámos uma tendência para a presença de espículas em forma de cogumelo, consideradas mais maduras, em neurónios da camada cortical IV que não expressavam PLCXD2. Coletivamente, estas observações revelam a existência de um novo complexo de sinalização pós-sináptico, GPR158-PLCXD2, envolvido na regulação da maturação de espículas dendríticas.

Abstract

In the mouse primary somatosensory cortex or barrel cortex, thalamocortical axons (TCAs) conveying sensory information from the whiskers selectively synapse onto excitatory layer IV neurons in a topographic fashion as discrete neuronal modules or "barrels". Despite its well-characterized circuitry, the molecular mechanisms mediating synaptic connectivity between TCAs and layer IV neurons are poorly understood. Importantly, proper maturation of the postsynaptic compartment is of key importance for critical period plasticity and fine-tuning the orderly organisation of cortical barrels.
The host lab showed that the postsynaptic G protein-coupled receptor GPR158, a recently identified key regulator of hippocampal synapse formation and function, is strikingly enriched in layer IV neurons of the developing barrel cortex. Here, GPR158 contributes to dendritic spine maturation, but the signalling mechanisms employed by GPR158 to instruct postsynaptic maturation are not known. Using an unbiased protein-protein interaction screen, the host lab identified PLCXD2, an atypical phospholipase C with no known function in the brain, as a novel intracellular GPR158 interactor. In this thesis project, we characterized the spatiotemporal expression profile of *Gpr158* and *Plcxd2*, showing co-expression in individual layer IV pyramidal neurons of the barrel cortex. Interestingly, during a well-defined window of spinogenesis, *Gpr158* expression increases profoundly while *Plcxd2* expression reaches a plateau. Next, using sparse CRISPR/Cas9-mediated epitope tagging in postmitotic neurons we reveal the postsynaptic localization of endogenous PLCXD2. Lastly, we assessed the contribution of PLCXD2 to dendritic spine development and observed a trend towards more mature, mushroom-type spines in barrel neurons lacking PLCXD2. Together, these findings uncover a novel postsynaptic GPR158-PLCXD2 signalling complex regulating dendritic spine maturation.

Table of Contents

List of Figures

List of Tables

List of Acronyms and Abbreviations

AD	Alzheimer's Disease
AMPA	A-Amino-3-Hydroxy-5-Methyl-4 Isoxazolepropionic Acid
APP	Amyloid Precursor Protein
Arp2/3	Actin Related Protein 2/3 complex
ASD	Autism Spectrum Disorder
AAV	Adeno associated virus
BAI	Brain Angiogenesis Inhibitor
BDNF	Brain-Derived Neurotrophic Factor
CAM	Cell Adhesion Molecule
cAMP	Cyclic Adenosine Monophosphate
DA	Dopaminergic
DAG	Diacylglycerol
DGK	Diacylglycerol kinase
DHPG	(S)-3,5-Dihydroxyphenylglycine
DRS	Donor Recognition Site
EGF	Epidermal Growth Factor
ER	Endoplasmic Reticulum
FAD	Familial Alzheimer's Disease
fEPSCs	Field Excitatory Postsynaptic Currents
FLP	Flippase
FLRT	Fibronectin Leucine-Rich Transmembrane Protein
GABA	Gamma-Aminobutyric Acid A
GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GEF	Guanine Exchange Factor
GFP	Green Fluorescent Protein
GPC4	Glypican 4
GPCR	G Protein Coupled Receptor
gRNA	Guide RNA

GTP	Guanosine Triphosphate
HA	Influenza Hemagglutinin
HAP1	Huntingtin-Associated Protein 1
HD	Huntington's Disease
HTT	Huntingtin
Ig	Immunoglobulin
IP_3	Inositol-1,4,5-Triphosphate
IRES	Internal Ribosome Entry Site
KO	Knock Out
Lphns	Latrophilins
LRR	Leucine-Rich Repeat
LRRTMs	Leucine-Rich Repeat Transmembrane Proteins
LTD	Long Term Depression
LTP	Long Term Potentiation
MDD	Major Depressive Disorders
MCU	Mitochondrial Calcium Uniporter
mEPCS	Miniature Excitatory Postsynaptic Currents
mGluR	Metabotropic Glutamate Receptor
mHTT	Mutant Huntingtin
mPFC	Medial Prefrontal Cortex
MPP+	1-Methyl-4-Phenylpyridinium
NCX	Na^+/Ca^{2+} Exchangers
NHEJ	Non-Homologous End Joining
nlgns	Neuroligins
NMDAR	N-Methyl-D-Aspartate Receptor
nrxns	Neurexins
N-WASP	Neural Wiskott-Aldrich Syndrome Protein
OCN	Osteocalcin
PA	Phosphatidic acid
PAK	P21-Activated Kinase
PBS	Phosphate Buffered Saline

p-cofilin	Phosphorylated Cofilin
PD	Parkinson's Disease
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PLC	Phospholipase C
PFA	Paraformaldehyde
PSCs	Postsynaptic Currents
PSD	Postsynaptic Density
PrV	Principal Trigeminal Nucleus of the Brainstem
RER	Rough Endoplasmic Reticulum
RGS	Regulator of G Signalling
ROR	Retinoic Acid-Related Orphan Receptor
RyR	Ryanodine Receptor
SA	Spine Apparatus
sEPSCs	Spontaneous Excitatory Postsynaptic Currents
SER	Smooth Endoplasmic Reticulum
SERCA	Sarco-Endoplasmic Reticulum Calcium-ATPase
SL	Stratum Lucidum
smFISH	Single Molecule Fluorescent in Situ Hybridization
SNc	Substantia Nigra Pars Compacta
SOCE	Store Operated Calcium Entry
SP	Synaptopodin
STEP	Striatal-Enriched Protein Tyrosine Phosphatase
STIM	Stromal Interaction Molecule
TCAs	Thalamocortical Axons
Tg	Thapsigargin
Thsd7a	Thrombospondin 7a
TRE	Tetracyclin Response Element
tTA	Tetracycline-Controlled Transactivator
VGLUT	Vesicular Glutamate Transporter
VPM	Ventral Posteriomedial Nucleus
WT	Wild Type

Chapter 1 - Introduction

Proper brain function relies on the correct assembly of precise neuronal circuits via synapses that are established within complex neuronal networks. Synapses are highly specialized intercellular junctions that mediate communication between neurons and require the coordinated assembly of distinct protein complexes at the pre- and postsynaptic side. During development, axon pathfinding, target recognition and synapse maturation are exquisitely orchestrated so that synapses with specific characteristics are formed between the appropriate neuronal populations at their designated target location. Despite a general understanding of the events that occur during neuronal circuit development, the molecular mechanisms involved in synapse formation and maturation are poorly understood. Synaptic cell adhesion molecules (CAMs) have been proposed to mediate axon target recognition and synapse formation by engaging in transsynaptic interactions. These interactions in turn may trigger a variety of bidirectional signalling mechanisms involving pre- and postsynaptic regions that ultimately lead to synaptic maturation and shaping of synaptic properties (Südhof, 2021). However, the identity of these signalling mechanisms is largely unknown. Here, I provide an overview of the development of the barrel cortex and its widespread use a model system to study the development of cortical circuits. I further highlight the key role of synaptic CAMs during synapse development and address the need for a better understanding of their signalling mechanisms. Finally, I elaborate on the increasingly recognized importance of store operated calcium entry (SOCE) during synapse development.

1.1 The barrel cortex

In 1970, Thomas Woolsey, under the supervision of Hendrik Van der Loos, was the first to discover the correspondence between the mystacial vibrissae or whiskers on the mouse's snout and their representation in barrel-like structures in the cortex (Woolsey & van der Loos, 1970). The observation that injury and removal of whiskers at birth led to the absence of the corresponding barrels in the cortex, gave rise to the idea that the sensory periphery can also contribute to the developmental anatomy of cortical regions (van der Loos & Woolsey, 1973). The mouse primary somatosensory cortex or barrel cortex is involved in processing sensory information originating from their whisker follicles. Whisker deflection provokes a sequence of activities in the trigeminal ganglion primary sensory neurons, the brainstem, and the thalamus (Petersen, 2019). Projections from the ventral posteriomedial (VPM) nucleus of the thalamus synapse specifically onto layer IV cortical neurons in discrete units called "barrels" and relay whisker-related sensory information. Each barrel unit is the representation of a particular whisker on the mouse's snout and the spatial distribution of the barrels is similar to the spatial distribution of the whiskers (Figure 1). Interestingly, this somatotopic organization is maintained not only at the level of the cortex, but also throughout all levels of this somatosensory circuit. Each whisker, or peripheral unit, corresponds to a "barrelette" in the ipsilateral principal trigeminal nucleus (PrV) of the brain stem, to a "barreloid" in the contralateral VPM nucleus of the thalamus, and lastly to a "barrel" in the contralateral somatosensory cortex (Gaspar & Renier, 2018; Martini et al., 2018).

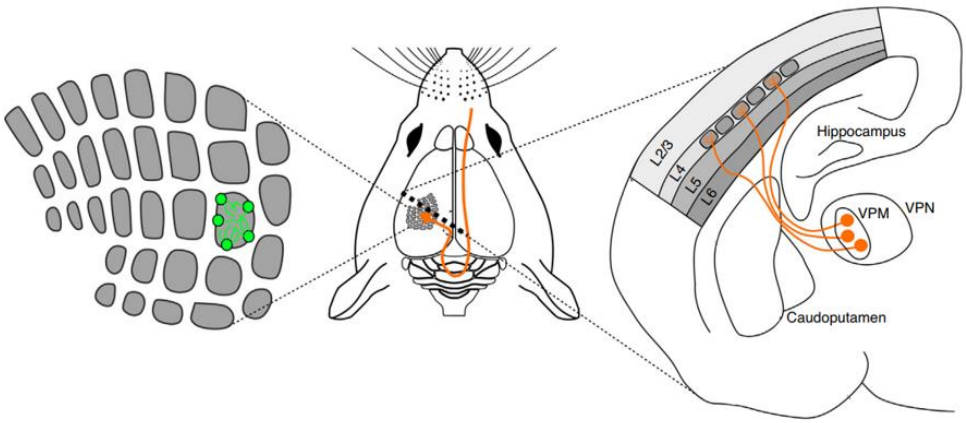


Figure 1 – Schematic representation of the barrel cortex and the thalamocortical projections in cortex layer IV. Projections from the VPM nucleus of the thalamus extend to the barrel cortex where synapses with layer 4 neurons are established. Abbreviations: L1, layer 1; L2/3, layer 2/3; L4, layer 4; L5, layer 5; L6, layer 6. Adapted from Simi & Studer, 2018.

The mouse barrel cortex provides a great model system to study cortical circuit development due to its easily recognizable cytoarchitecture, well-characterized organization and experimental accessibility (Erzurumlu & Gaspar, 2020; Martini et al., 2018). Cortical circuit development, particularly in the barrel cortex, has sparked interest in the field of neuroscience, as the signalling mechanisms required for the development of these thalamocortical synapses are not fully understood.

1.1.1 Thalamocortical circuit development

The mouse neocortex is developed during embryonic and early postnatal stages. It presents a complex laminar structure, consisting of 6 layers that are formed by the chronological migration of newborn neurons from the inside-out. First, layers VI and V are formed, which later project to subcortical areas. Then, layer IV and layer II-III are formed, and both cortico-cortical and thalamocortical projections are established (Alfano & le Studer, 2012; Dwyer et al., 2016; Simi & Studer, 2018). The neocortex is also subdivided tangentially into functional regions with specific characteristics, a process termed “arealization”, which distinguishes four primary areas: primary motor cortex, primary somatosensory cortex, primary auditory cortex, and primary visual cortex (Simi & Studer, 2018).

The thalamus is responsible for processing sensory information and relaying it to the cortex. Thalamocortical projections originating from the VPM nucleus are established with layer IV neurons and corticothalamic projections, originated in layer VI, send information back to the thalamus. These intricate thalamo-cortico-thalamic loops reflect the interdependence between the thalamus and the cortex. In fact, thalamic and cortical developmental programs occur simultaneously and overlap in space (Antón-Bolaños et al., 2018).

Thalamic neurons are generated between E10.5 and E16.6 in mice. Thalamocortical axons (TCAs) from the VPM nucleus cross the diencephalon-telencephalon border and project towards the developing cortex. TCAs reach the subplate around E15.5, when only the inner cortical layers are formed (layers V and VI) and remain here for a few days (Iwasato & Erzurumlu, 2018). During the first postnatal days (P3-P4), TCAs invade the cortical plate, branch, and contact layer IV stellate neurons, the most abundant excitatory cell-type present here. The dendrites of layer IV neurons become progressively oriented towards TCAs in the "hollows" of the barrels, and their cell bodies delimitate their "walls" around P7. The complexity of the contacts established between TCAs and stellate neurons will continue to increase as the circuit matures, and by P14 the barrel cortex is fully developed (Figure 2). Maturation of barrel cortex circuitry is also reflected by the onset of whisking behaviour (Antón-Bolaños et al., 2018; Iwasato & Erzurumlu, 2018; Simi & Studer, 2018).

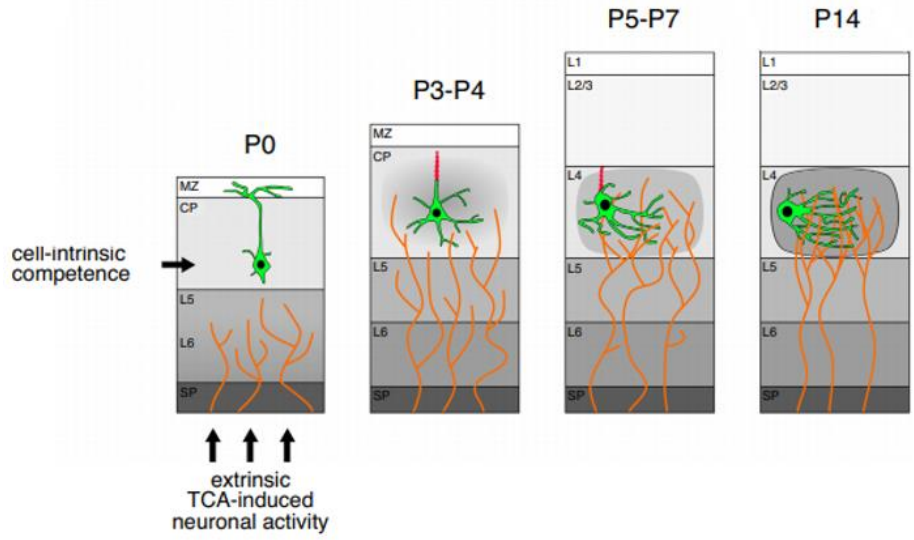


Figure 2- Schematic representation of the somatosensory barrel cortex and the remodelling of layer IV stellate neurons (green) throughout early postnatal development. Abbreviations: MZ, marginal zone; CP, cortical plate; SP, subplate; L1, layer 1; L2/3, layer 2/3; L4, layer 4; L5, layer 5; L6, layer 6. Adapted from Simi & Studer, 2018.

1.1.2 Molecular mechanisms shaping thalamocortical circuit assembly

The formation of neuronal circuits depends on several crucial steps that include axon pathfinding, target recognition and synapse formation. During thalamocortical circuit development, several mechanisms ensure the successful migration of TCAs to the cortex and the establishment of synapses with layer IV neurons (Lokmane & Garel, 2014). Regulation of gene expression by several transcription factors during development plays an important role in specifying neuronal circuits. For example, the retinoic acid-related orphan receptor alpha (RORα) was shown to be important for barreloid and barrel organization (Vitalis et al., 2018). Knocking out *RORα* lead to TCA branch defects and loss of barrel cortex cytoarchitecture. These findings were associated with a loss of RORα transcriptional control over genes that are relevant during neurite outgrowth, such as *Semaphorin 7A*, previously shown to be required for proper TCA segregation and stellate neuron maturation (Carcea et al., 2014). Another member of the retinoic acid-related orphan receptor family, RORβ, is highly expressed during development by layer

IV neurons in the barrel cortex (Jabaudon et al., 2012; Nakagawa & O'Leary, 2003). Interestingly, ectopic expression of RORβ in deep layer cortical neurons is sufficient to induce barrel-like periodic clustering of neurons and RORβ expressing neurons are preferentially targeted by TCAs, further indicating an instructive role for RORβ in thalamocortical circuit assembly (Jabaudon et al., 2012). Recently, RORβ expression in the cortex was shown to be required for barrel wall formation and segregation of TCAs. In the absence of RORβ, part of the layer IV gene expression profile is shifted towards a more layer V-like profile. Lack of RORβ transcriptional control during development also interferes with layer IV neuron synaptic function as evidenced by a reduced frequency of miniature excitatory postsynaptic currents (mEPSCs). Thrombospondin 7a (*Thsd7a*), identified as a RORβ downstream target, was proposed to maintain TCA structure throughout adulthood, since loss of *Thsd7a* mainly disrupted TCA segregation in adult mice (Clark et al., 2020).

Thalamocortical circuit refinement is also crucially dependent on neuronal activity. Focusing specifically on glutamatergic neurotransmission, there are several examples indicating the importance of neuronal activity during barrel cortex formation and function. Presynaptically, it has been shown that in thalamus specific *Vglut1* and *Vglut2* double knock out (KO) mice, barrels failed to form. TCAs did not segregate into barrels and the characteristic placement of layer IV neurons within walls was absent. Additionally, layer IV neuron morphology was affected with most neurons presenting an abnormal pyramidal morphology rather than the expected stellate morphology with retraction of the apical dendrite (H. Li et al., 2013).

As for the postsynaptic region, different strategies were developed to assess the cell-autonomous contributions of metabotropic glutamate receptor 5 (mGluR5) and N-methyl-D-aspartate (NMDA) receptor during barrel cortex development (Ballester-Rosado et al., 2016; Mizuno et al., 2014). The generation of an mGluR5 genetic mosaic mice, in which only a small subset of layer IV neurons lacked mGluR5, allowed to assess *mGluR5* KO neurons and their interactions in a wild type (WT) environment thus excluding the influence of other external factors that may underlie the aberrant phenotypes observed in full *mGluR5* KO models (Wijetunge et al., 2008). In this mosaic model, placement of *mGluR5* KO neurons within layer IV was altered, as a higher percentage of *mGluR5* KO neurons localized to the barrel septae rather than to the barrel walls, where WT neurons are found. Additionally, mGluR5 contributed to dendritic morphology, as the dendrites of *mGluR5* KO neurons showed a decreased polarization and an increased dendritic length comparing to WT neurons. Furthermore, a significantly larger fraction of *mGluR5* KO neurons presented apical dendrites, resembling the morphology of pyramidal neurons and not the stellate morphology characteristic of most layer IV neurons (Ballester-Rosado et al., 2016). Lack of mGluR5 expression also affected dendritic spine development and lead to an increase in immature filopodia and stubby spines. Moreover, mosaic deletion of *mGluR5* caused an increase in the frequency of spontaneous EPSCs (sEPSCs) and mEPSCs (Ballester-Rosado et al., 2016). Taken together, these findings show that mGluR5 plays an important role in the proper maturation of layer IV stellate neurons and the modulation of their excitatory inputs (Ballester-Rosado et al., 2016).

The cell-autonomous contributions of NMDA receptor signalling to layer IV neuron maturation were explored using the recently developed "Supernova" system, which allowed for the simultaneous removal of the essential NMDA receptor NR1 subunit in a sparse population of layer IV neurons and their visualisation. Here, the authors used *in vivo* time-lapse imaging of dendrites from layer IV barrel neurons in neonates. Loss of NR1 caused excessive dendritic extension, both inside and outside the barrels, leading to reduced biased dendritic orientation towards the barrel hollows. Based on these results, it was proposed that NMDA receptor signalling is important to suppress dendritic dynamics in a way that it limits the growth of outer dendrites and allows inner dendrites to be extended and become polarized towards the barrel hollows. Additionally, dendritic spine density of *NR1* KO neurons inside the barrel hollow was lower, suggesting that NMDA receptor signalling also regulates dendritic spine refinement (Mizuno et al., 2014).

Overall, these findings suggest a key role for activity-dependent mechanisms in shaping cortical whisker map formation. However, little is known about the molecular mechanisms mediating synaptic connectivity between TCAs and layer IV stellate neurons. How neuronal activity and molecular cues interact to give rise to the remarkable wiring specificity and architecture of thalamocortical synapses is not understood and remains largely unexplored. Growing evidence indicates that synaptic CAMs, which display cell-type specific expression patterns, mediate cell-cell recognition via transsynaptic interactions, and recruit key components of the synaptic machinery, are prime candidates involved in the specification of neural circuits (de Wit & Ghosh, 2016).

1.2. Synaptic cell adhesion molecules

In recent decades, a growing number of synaptic CAMs that include neurexins (nrxns), neuroligins (nlgns), immunoglobulin (Ig)-domain containing proteins, leucine-rich repeat (LRR) containing proteins, and receptor phosphotyrosine kinases and phosphatases have been identified (Figure 3). Synaptic CAMs are cleft-spanning proteins that engage in heterophilic and/or homophilic interactions to orchestrate synaptogenesis by mediating cell-cell recognition and the initial establishment of synaptic contacts. Furthermore, a subset of synaptic CAMs regulates pre- and postsynaptic differentiation by recruiting scaffolding proteins and neurotransmitter receptors (Giagtzoglou et al., 2009; Missler et al., 2012). Mutations and copy number variations (CNVs) in numerous genes encoding synaptic CAMs were found to be associated with several neuropsychiatric disorders such as autism spectrum disorders (ASDs) and schizophrenia, highlighting the functional importance of appropriate synaptic connectivity for normal brain function (Bourgeron, 2009; Lin et al., 2016; Südhof, 2008).

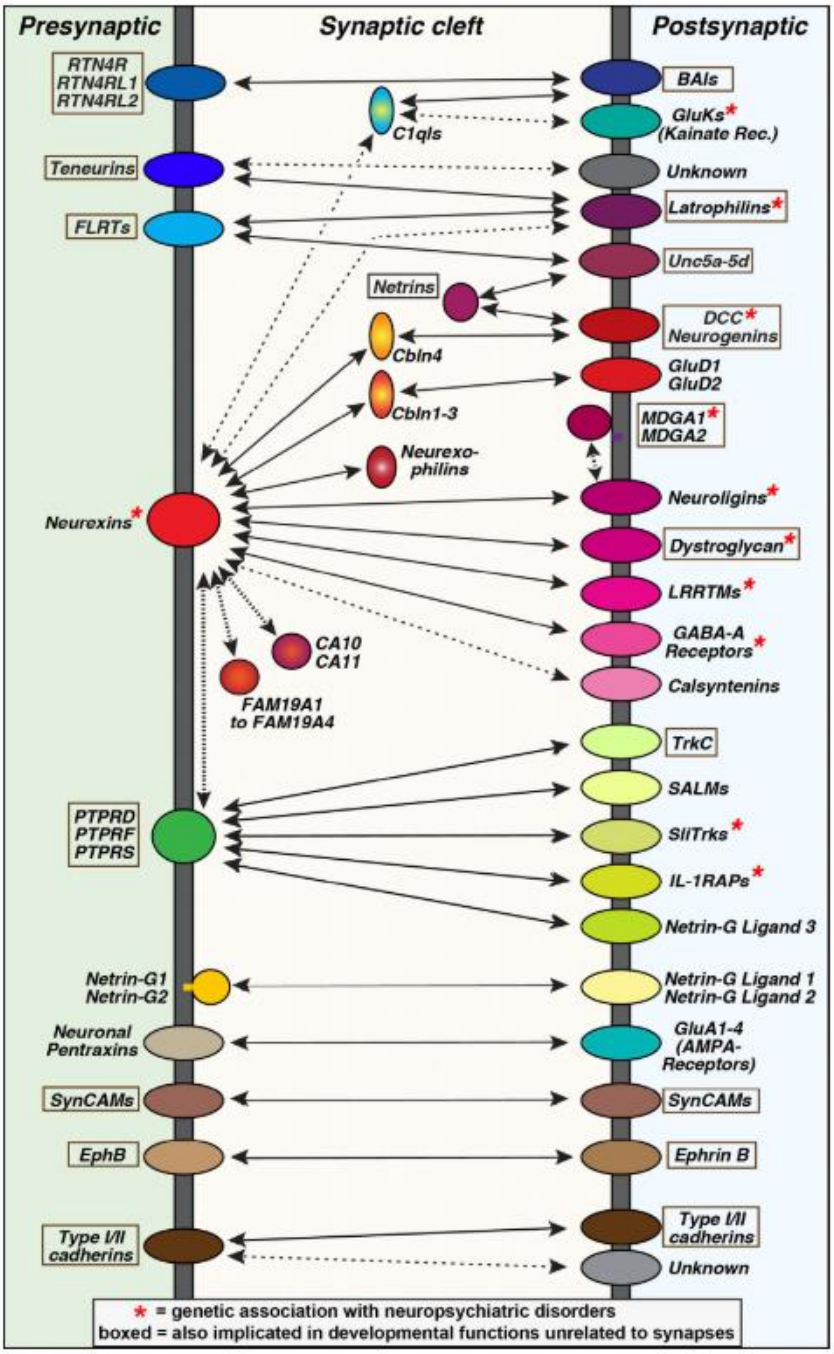


Figure 3 - Schematic representation of interactions between synaptic CAMs involved in synaptic assembly. Lines and arrows represent trans-interactions and dotted lines represent cis-interactions. Dashed lines represent less validated interactions. Reprinted from Südhof, 2021.

1.2.1 A multitude of synaptic cell adhesion molecules shape synapse development

Arguably, the transsynaptic interaction between the presynaptically localized nrxns and postsynaptically localized nlgns represents one of the best-characterized protein complexes regulating both excitatory and inhibitory synaptogenesis (Krueger et al., 2012). Initial insights into the bidirectional synapse organizing function of the nrxn-nlgn

complex were derived from pioneering studies using artificial synapse formation assays. In these assays, neurons are cultured together with non-neuronal cells expressing a CAM of interest and tested for their ability to induce the formation of synapses onto contacting neurites. Surface expression of nlgns in non-neuronal cells induces clustering of presynaptic proteins in contacting axons by locally aggregating nrxns on the axonal surface (Dean et al., 2003; Scheiffele et al., 2000). Conversely, when nrxns are presented to neurons on the surface of non-neuronal cells, postsynaptic components are recruited in dendrites via aggregation of nlgns (Graf et al., 2004; Nam & Chen, 2005).

The aforementioned studies suggest that bidirectional signalling via trans-synaptic nrxn-nlgn interactions is sufficient to induce synapse formation *in vitro*. However, *in vivo* studies indicate that both synaptic CAMs are essential for the functional maturation of synaptic contacts, but not for their initial formation. Specifically, synapse numbers in α-nrxn triple KO mice are only moderately affected with fewer symmetric (presumptive inhibitory) synapses whereas the number of asymmetric (presumptive excitatory) synapses is unaltered. Instead, loss of all three α-nrxns dramatically reduces the frequency of spontaneous and evoked postsynaptic currents (PSCs) at both inhibitory and excitatory synapses due to reduced Ca^{2+}-channel function (Dudanova et al., 2007; Missler et al., 2003), indicating they are essential for Ca^{2+}-triggered neurotransmitter release. Similarly, mice lacking all three nlgns exhibit normal synapse numbers but a strong impairment of glutamatergic and gamma-aminobutyric acid A (GABA)ergic synaptic transmission (Varoqueaux et al., 2006).

Redundancy within individual families of synaptic CAMs and multiple trans-synaptic adhesions systems acting in parallel at a given synapse may provide a possible explanation for the apparent discrepancy regarding synapse formation between *in vitro* and *in vivo* studies. Indeed, nlgns and LRR transmembrane proteins (LRRTMs), a structurally unrelated family of postsynaptic CAMs that also interact with nrxns, seem to cooperate in an additive or synergistic matter to promote excitatory synapse development (Siddiqui et al., 2010). More recently, Quinn and colleagues sought to further clarify the role of nrxns in synapse formation using time-lapse imaging in cultured hippocampal neurons that lacked all nrxn isoforms. Interestingly, pan-nrxn knockdown did not affect the rate of synapse formation but increased the elimination of pre-existing synapses. These results are consistent with a model where nrxns are co-expressed with other families of synaptic CAMs during synapse formation in a highly redundant manner. During synapse maturation, loss of one of these families may confer synapses with a disadvantage and promote their elimination (Quinn et al., 2017).

1.2.2 Signalling by synaptic cell adhesion molecules

The signalling mechanisms employed by synaptic CAMs to instruct synapse development are mostly unknown. However, there is a small subset of synaptic CAMs with well described intracellular signalling mechanisms. For the scope of this thesis, I will focus on the protein interactions and signalling pathways triggered by postsynaptic CAMs that are involved in postsynaptic maturation.

1.2.2.1 Scaffold and neurotransmitter receptor clustering

Nlgns are expressed mainly in the brain and bind nrxns via their esterase-like domain. Nrxns and nlgns both contain a PDZ domain allowing them to interact with synaptic scaffolding proteins (Dalva et al., 2007). Nlgns have been shown to contribute to the clustering of neurotransmitter receptors. Whether nlgns recruit neurotransmitter receptors indirectly through interactions with scaffolding proteins, or directly via interactions with neurotransmitter receptor subunits is not fully clear (Jang et al., 2017). Nlgn1, which is present at excitatory synapses, was found to interact with the scaffolding protein PSD-95 (Irie et al., 1997). The interaction between nlgn1 and PSD-95 was found the be necessary to immobilize laterally diffusing α-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor containing GluA2 subunits at the synapse (Mondin et al., 2011). In addition, nlgn1 was shown to recruit NMDA receptors to the synapse through direct extracellular interaction with GluN1 subunits independently of PSD-95 (Budreck et al., 2013). In contrast, nlgn2 is selectively present at inhibitory synapses and was found to interact with and recruit the inhibitory scaffolding protein gephyrin (Choii & Ko, 2015).

In these past few years, the role of LRRTMs has generated great interest in the scientific community. These synaptic CAMs have been identified more recently and have been shown to be key organizers of excitatory and inhibitory synapses (de Wit & Ghosh, 2014; Roppongi et al., 2017). LRRTM1 was found to contribute to excitatory synapse organization by inducing the clustering of NR1, PSD-95 and SynGAP at postsynaptic sites (Linhoff et al., 2009). LRRTM2 contributes to the postsynaptic organization of excitatory synapses by recruiting NR1 and PSD-95 to the synapse. LRRTM2 was also able to interact with GluA1 or GluA2 subunits via its extracellular LRR domain. However, the exact mechanism through which LRRTM2 recruited neurotransmitter receptors was not elucidated. It was proposed that LRRTM2 recruits neurotransmitter receptors either directly or indirectly via interactions with PSD-95 (de Wit et al., 2009).

Collectively, these results show that synaptic CAMs, through their structural domains, interact with important synaptic proteins, such as scaffolding proteins and neurotransmitter receptors, which supports their role in the regulation of synaptic properties.

1.2.2.2 Actin cytoskeleton dynamics

The interactions between EphB and ephrin-B have been extensively studied and implicated in various synaptic functions. The signalling mechanisms triggered by EphB activation are well-characterized and some have been found to participate in the regulation of the actin cytoskeleton. EphBs are postsynaptically localized and contain different protein domains, including a PDZ domain, a kinase domain and juxtamembrane tyrosines that mediate its interactions with a variety of proteins involved in downstream signalling mechanisms. The EphB-kinase activity is particularly relevant in dendritic spine morphogenesis as it enables EphB to activate several RhoGTPases involved in signalling pathways that lead to actin cytoskeleton remodeling (Dalva et al., 2007).

Activation of EphB has been shown to recruit kalirin, a guanine exchange factor (GEF), to dendritic spines which in turn activates Rho GTPase Rac1 and its effector P21-activated kinase (PAK) (Dalva et al., 2007; Ethell & Pasquale, 2005; Sanderson & Acqua, 2011). This was shown in hippocampal neuron cultures stimulated with ephrins, showing that EphB activation and downstream signalling, lead to quick alterations in dendritic spine morphogenesis. This effect was proposed to result from actin rearrangements regulated by activation of Rac1 and PAK (Penzes et al., 2003). In addition, EphB-kinase-dependent phosphorylation was found to activate Syndecan-2. Studies in hippocampal cultures revealed that the phosphorylation of Syndecan-2 at two specific tyrosine residues is important for its clustering and regulation on dendritic spine maturation. Transfection of neurons with a Syndecan-2 double tyrosine mutant showed almost complete loss of mushroom spines, while mostly immature spine types such as filopodia-like protrusions were observed (Ethell et al., 2001). Finally, EphB2 was found to interact with and activate the GEF intersectin in cooperation with neural Wiskott-Aldrich syndrome protein (N-WASP) which, in turn, leads to Rho GTPase Cdc42 activation. Cdc42 was proposed to contribute to spine morphogenesis by regulating the actin binding complex Arp2/3, which is involved in actin polymerization (Irie & Yamaguchi, 2002).

Besides Eph receptors, other synaptic CAMs have been implicated in regulating actin cytoskeleton dynamics, including cadherins. Cadherins are a large class of CAMs that require Ca^{2+} to form homophilic bonds at intercellular junctions. In neurons, Neuronal (N)-cadherins can be found at both pre- and postsynaptic sites. The intracellular domain of N-cadherin interacts with catenin molecules (αN-, β- and p120 catenins) and provides a link with the actin cytoskeleton (Dalva et al., 2007; Ethell & Pasquale, 2005). N-cadherin signalling impacts dendritic spine density and morphology through the regulation of Rho GTPases downstream of catenin activation (Dalva et al., 2007). For instance, at cadherin mediated junctions, p120 catenin was shown to regulate the regulate the activity of the Rho GTPases Rac1 and RhoA. Deletion of *p120* catenin was found to cause a decrease in the density of dendritic spines and inhibit their maturation both *in vitro* and *in vivo*. *P120* KO mice exhibit reduced levels of N-cadherin and a dysregulated activation of Rac1 and RhoA was suggested to underly the observed effects on dendritic spine density and morphology (Elia et al., 2006).

Recently, nlgn1 was shown to regulate dendritic spine development and synaptic plasticity by mechanisms involving the actin binding protein cofilin (Liu et al., 2016). Cofilin is required for the reorganization of actin filaments and contributes to actin severing and depolymerization in its dephosphorylated state. Levels of phosphorylated cofilin (p-cofilin) are widely used as an indicator of cofilin activity, as phosphorylation renders cofilin inactive (Huang et al., 2006). Levels of p-cofilin were found to be lower in *nlgn1* KO neurons compared to WT neurons (Liu et al., 2016). The cytoplasmic domain of nlgn1 was sufficient to induce cofilin phosphorylation and increase the density of dendritic spines and frequency of mEPSCs (Liu et al., 2016). The mechanism linking nlgn1 and cofilin phosphorylation was proposed to involve proteolytic release of the cytoplasmic domain and its binding to and inactivation of SPAR, a regulator of the actin cytoskeleton. Consequently, Rap1/Rac1 and LIMK1/2 are activated, leading to cofilin phosphorylation (Liu et al., 2016).

1.2.2.3 Other mechanisms

Latrophilins (lphns) are postsynaptic adhesion G-protein coupled receptors (GPCRs) which engage in transsynaptic interactions with presynaptic partners such as nrxns, teneurins, fibronectin and fibronectin leucine-rich transmembrane proteins (FLRTs). This class of synaptic CAMs has gained a lot of attention, since currently only latrophilins and brain angiogenesis inhibitors (BAIs) are considered to be strictly necessary for the actual formation rather than specification of synapses (Sando & Südhof, 2021; Südhof, 2021). In a recent study, expression of lphn2 and lphn3 in HEK293T cells were found to increase cAMP levels and this effect was attributed to GPCR activity, since lphn2 and lphn3 G-protein-binding deficient mutants failed to produce the same result. Both *in vitro* and *in vivo*, loss of lphn3 lead to a reduction in the density of excitatory synapses and a decrease in the frequency of mEPSCs. WT lphn3, but not lphn3 G-protein binding deficient mutants were able to fully rescue these phenotypes, suggesting that lphn3 regulates excitatory synapse formation and function in a GPCR-dependant manner. Since cAMP is a second messenger involved in a multitude of classical signalling pathways, further studies should address how it may be involved in synaptogenesis (Sando & Südhof, 2021).

1.3 Dendritic spine development

Excitatory synapses are typically formed at dendritic spines, very specialized dendritic protrusions. Maturation of dendritic spines relies on several mechanisms to accommodate for an array of postsynaptic proteins, including scaffolding proteins, neurotransmitter receptors, ion channels and intracellular signalling proteins (Hlushchenko et al., 2016). Spine morphology is generally associated with its function and maturity, and are often categorized as mushroom, thin, stubby or filopodia type spines (Ebrahimi & Okabe, 2014). It is commonly accepted that mushroom spines are the most mature type of spines, as they present the longest lifetime and engage in stronger synaptic connections.

Synaptic transmission largely depends on Ca^{2+}-dependent processes, involved in excitability, synaptic vesicle release and gene expression. At the presynaptic terminal, neurotransmitter release is crucially dependant on Ca^{2+}. Postsynaptically, Ca^{2+} influx occurs mainly through NMDA receptors and voltage gated calcium channels which triggers the activation various of signalling pathways involved in neuronal plasticity, some with long lasting effects (Majewski & Kuznicki, 2014). The compartmentalization of dendritic spines is achieved due to the presence of a narrower “neck”, that separates spines from the dendritic shaft, as opposed to their bulky “head” where the postsynaptic density (PSD) is found. This allows tight control of Ca^{2+} signalling and prevents its spreading to adjacent spines (Ebrahimi & Okabe, 2014). In spine heads, calcium ions activate a variety of Ca^{2+}-sensitive proteins, the most studied being calmodulin, CAMKII, calcineurin and calpains, all involved in various aspects of postsynaptic development (Higley & Sabatini, 2012).

1.3.1 Actin Dynamics

In dendritic spines, actin is present in large concentrations, both in its soluble monomeric form (G-actin), but also as polymerized filaments (F-actin). Actin filaments structurally support dendritic spines and are typically longitudinal in the core of the spine head and in the spine neck. In the periphery of the spine head, actin filaments are organized in a fine and dynamic meshwork. Regulation of the actin cytoskeleton greatly influences spine morphology (Ethell & Pasquale, 2005).

Rho GTPases are key regulators of actin dynamics. Guanine exchange factors (GEFs) activate Rho GTPases by exchanging GDP for GTP. In contrast, GTPase-activating proteins (GAPs) inhibit them by accelerating hydrolysis of GTP to GDP. The function of some classic members of this family, particularly RhoA, Cdc42 and Rac1 have been studied in dendritic spine development (Ethell & Pasquale, 2005; Schubert & Dotti, 2007). Rac1 is associated with actin polymerization, and dendritic spine formation and maintenance. Constitutively active forms of Rac1 have been shown to cause increased synaptic density and abnormalities in spine morphology and size (Tashiro et al., 2000). RhoA activity seems to negatively regulate actin polymerization, causing spine shrinkage and elimination (Tashiro et al., 2000). It is worth noting that regulating the activity levels of each Rho GTPase is required to ensure proper spine function since they present distinct but intertwined contributions to actin filament properties (Ethell & Pasquale, 2005; Saneyoshi & Hayashi, 2012).

Signalling pathways downstream of Rho GTPases include mechanisms that control the activity of actin binding proteins, which are directly involved in the regulation of actin dynamics. The actin-related proteins 2 and 3 (Arp2/3) complex, once activated, induces nucleation and branching of actin filaments and is thought to have an important role in spine head enlargement. Cofilin, in contrast, induces depolymerization of actin filaments which is associated with spine head reduction and thicker spine necks (Ethell & Pasquale, 2005; Saneyoshi & Hayashi, 2012).

1.3.2 Calcium signalling

Influx of calcium, actin cytoskeleton remodelling and alterations in spine morphology are all closely associated, as several mechanisms involved in the regulation of actin filament production are Ca^{2+}-dependent (Oertner & Matus, 2005). For instance, the localization of profilin II (the most common profilin isoform in neurons) within dendritic spines is Ca^{2+}-dependant. Profilin II is an actin binding protein involved in the stabilization of mature dendritic spines following sustained elevation of postsynaptic Ca^{2+} levels that occurs upon NMDA receptor activation (Ackermann & Matus, 2003). Tight control over Ca^{2+} is crucial for proper dendritic spine function and maintenance.
Even though the intracellular Ca^{2+} concentrations are low, some organelles contain very high internal Ca^{2+} levels and thus are commonly referred to as “calcium stores”, including the endoplasmic reticulum (ER) (Segal & Korkotian, 2014).

The ER plays a crucial role in neuronal function by contributing to the generation of cytosolic Ca^{2+} signals. Several mechanisms lead to the release of calcium from the ER, including activation of inositol-1,4,5-triphosphate (IP_3) receptors and ryanodine receptors (RyRs) (Segal & Korkotian, 2014). Following Ca^{2+}-activated signalling, re-establishment of resting cytosolic Ca^{2+} levels are achieved by collaboration of plasma membrane Na^+/Ca^{2+} exchangers (NCX) that lead to the efflux of calcium to the extracellular medium, the mitochondrial calcium uniporter (MCU) that drives Ca^{2+} accumulation within the mitochondria, and store operated calcium entry (SOCE) that replenishes ER Ca^{2+} levels (Majewski & Kuznicki, 2014).

In neurons, the ER can be found in the soma, axons, and dendrites. In some, but not all dendritic spines, a specialized form of the ER can be found. The so-called spine apparatus (SA), an extension of dendritic ER, invades preferentially mushroom spines. The formation of the SA in dendritic spines requires synaptopodin (SP), an actin associated protein with a key role in synaptic plasticity (Deller et al., 2003; Jedlicka et al., 2009; Jedlicka & Deller, 2017). Experiments involving flash photolysis of caged glutamate in cultured hippocampal neurons showed that SP-containing spines were much more likely to expand than spines lacking SP. In the presence of thapsigargin, a SERCA blocker that depletes ER stores, this effect was abrogated indicating that calcium stores are required in the regulation of spine plasticity following glutamate stimulation (Korkotian et al., 2014). This is in accordance with previous findings showing increased GluR1 clustering in SP-positive spines following the induction of long-term potentiation (LTP) (Vlachos et al., 2009). SP-containing spines, particularly mushroom spines, exhibit increased levels of stromal interaction molecule (STIM1) and ORAI1 compared to spines lacking SP, suggesting that SP is important to direct STIM1 and ORAI1 to dendritic spines. Since these proteins are key players involved in SOCE, this could provide a possible mechanism linking SP and the spine apparatus to Ca^{2+} store regulation (Korkotian et al., 2014).

1.3.2.1 Endoplasmic reticulum calcium stores

The ER is functionally subdivided in two domains: smooth ER (SER) and rough ER (RER). The RER contains ribosomes and is mainly localized to the soma, while the SER consists of a complex interconnected network of cisterns and tubules, extending from the soma to all neuronal compartments, including the axon, dendrites, and a subset of dendritic spines. The ER dynamic properties are associated with its wide range of functions, which include protein and lipid biosynthesis, as well as acting as an internal (and releasable) calcium store (Ramirez & Couve, 2011; Shibata et al., 2006). The continuity of the SER is thought to play an important role in protein trafficking and overall neuronal function, for example, through allocation of proteins synthesized in the soma to distal regions of the cell, such as the dendritic shaft and axonal compartment. Additionally, mRNAs are also transported and translated locally in the dendritic ER. The mechanisms underlying protein trafficking in the dendritic ER are still not fully understood but its continuous tubular structure hints the possibility that it could function as an intracellular transport system (Ramirez & Couve, 2011).

The ER present in the dendritic shaft has been reported to undergo reversible fusion and fission events in organotypic hippocampal slices. These events were dependent on membrane depolarization induced by potassium ions, on extracellular calcium, and on NMDA receptor activation (Kucharz et al., 2011). In a later publication, ER fission and fusion events were characterized *in vivo* for the first time. ER dynamics were assessed in the somatosensory cortex with two photon microscopy upon whisker stimulation and cortical spreading depolarization. ER fission was shown to depend on NMDA receptor activation and the subsequent increase Ca^{2+} concentration, as well as on the downstream activation of CAMKII. ER fusion, in turn, was found to be mediated by dynamin GTPases (Kucharz & Lauritzen, 2018). The physiological role of ER fission and fusion events remains unknown. However, the aforementioned results suggest that these events are possibly regulated by synaptic transmission and could eventually impact protein trafficking and calcium dynamics within the dendritic shaft or even in dendritic spines (Kucharz et al., 2013).

Initial studies using primary mouse hippocampal cultures aimed to understand the structural properties of the ER in dendritic spines and revealed that its localization is highly dynamic (Toresson & Grant, 2005). Over time, the ER transiently enters most dendritic spines but remains present only in a minority of them (Perez-Alvarez et al., 2020). Generally, smaller spines contain just a single tubule of smooth ER (Spacek & Harris, 1997), and in larger spines the ER can form a spine apparatus. Interestingly, a high percentage of spines that show stably inserted smooth ER contain a spine apparatus (Perez-Alvarez et al., 2020). These observations prompted the study of the regulation and functional role of ER dynamics in spines. For instance, activation of mGluR1 with agonist DHPG caused a reduction of the number of spines containing ER. This effect was reflected in an increase of ER exits from spines and decreased ER-entry times (Ng & Toresson, 2011). Recently, mGluR-LTD was shown to induce selective loss of mushroom spines that do not contain synaptopodin, an effect that was mediated specifically by mGluR1, and not mGluR5. Interestingly, mGluR-LTD lead to proteasomal degradation of dendritic synaptopodin and, at the same time, contributed to the stabilization of synaptopodin already present in dendritic spines, an effect that was mGluR1 dependent, as shown in experiments performed in mGluR1 KO mice (Speranza et al., 2022). These results suggest that mGluR1 activation could serve a dual role by, on one hand, stabilizing the mushroom spines already containing synaptopodin and, on the other hand, preventing weaker spines from recruiting dendritic synaptopodin, thus keeping them susceptible to being lost during mGluR-LTD.

Increasing the synaptic activation of NMDA receptors by eliminating striatal-enriched protein tyrosine phosphatase (STEP), an endogenous negative regulator of NMDA receptors, resulted in enhanced ER growth. (Ng et al., 2014). Later studies seem to corroborate these findings as excitatory synaptic activity was shown to be associated with increased number of ER visits in dendritic spines as well as with increased visit time. In experiments using hippocampal organotypic slices, LTP induction via glutamate uncaging increased the chance of ER visits in stimulated spines but not in neighbouring spines. Additionally, blocking synaptic transmission with AMPA and NMDA receptor antagonists reduced ER dynamics. These results suggest that ER visits are not random, but more likely to occur following synaptic stimulation (Perez-Alvarez et al., 2020).

The role of ER dynamics in synaptic plasticity is still the subject of debate. Conflicting evidence has emerged regarding LTP and the dependence on dendritic spine ER for its mechanistic induction. While various independent studies report that spines containing ER undergo more significant enlargements following LTP induction compared to spines lacking ER (Borczyk et al., 2019; Chirillo et al., 2019), other results suggest that LTP induction does not require dendritic spine ER and that, in fact, spines already containing ER do not experience any enlargement following LTP (Perez-Alvarez et al., 2020). According to the latter study, strongly activated spines are more likely to be visited by the ER, suggesting that spines already containing ER have been previously strengthened, and cannot be further potentiated after LTP induction. This is supported by the observation of higher GluA2 surface expression in spines of MyoV DN expressing neurons, in which ER motility is blocked, compared to control neurons. This change in AMPA receptor surface expression was reflected in higher AMPA receptor currents following uncaging evoked AMPA-EPSCs and rendered impossible the induction of LTP in these neurons.

The purpose of this mechanism could be to limit spine potentiation and it was proposed to rely on mGluR1 and LTD, which was shown to still occur in spines where ER motility was blocked, despite their inability to undergo LTP (Perez-Alvarez et al., 2020). In contrast, the former publications suggest a different sequence of events, in which the presence of ER in dendritic spines represents a relevant factor in synaptic plasticity following LTP induction, as seen by a more significant spine enlargement when compared to spines without ER in the same conditions (Borczyk et al., 2019; Chirillo et al., 2019). Additionally, LTP was reported to lead to the transformation of the smooth ER into a spine apparatus (Chirillo et al., 2019).

The mechanisms underlying ER motility in spines are still unclear but in the past few years several studies have contributed to our understanding of the topic. In Purkinje neurons, smooth ER is found in almost all dendritic spines, but no spine apparatus is formed. Myosin Va, an actin-based motor, is necessary for ER movement into Purkinje neuron spines. The motor activity of myosin allows the ER to be transported along actin filaments into the dendritic spines of Purkinje neurons (Wagner et al., 2011). Recently, myosin V was shown to be responsible for insertion of smooth ER into active dendritic spines of hippocampal neurons (Perez-Alvarez et al., 2020). Additionally, myosin V was identified as a synaptopodin interactor and found to be necessary for its clustering in dendritic spines. Inhibition of myosin V through expression of double negative constructs in hippocampal neurons negatively affected the number of spines containing a spine apparatus, which suggested that myosin V is probably important for spine apparatus formation as well (Konietzny et al., 2019).

Caldendrin acts as a calcium sensor and is a brain specific homolog of calmodulin, a previously known interactor of myosin V. In hippocampal neurons, overexpression of caldendrin increased the percentage of stable smooth ER found in dendritic spines (Konietzny et al., 2021). As mentioned before, the presence of stable ER in spines is associated with formation of a spine apparatus (Perez-Alvarez et al., 2020), which seems to be in line with the observation that caldendrin overexpression increased the spine

localization of synaptopodin. In this study, caldendrin is proposed to inhibit myosin V motor activity independently of its association with actin filaments which in turn leads to an inhibition of ER motility within the dendritic spine. Subsequent accumulation of synaptopodin then allows formation of the spine apparatus in a subset of spines (Konietzny et al., 2021).

1.3.2.2 Store operated calcium entry and synapse development

SOCE is the mechanism responsible for sensing reductions in ER calcium levels and subsequently activating Ca^{2+} influx through the plasma membrane. It is currently accepted that STIM1 and ORAI1 constitute the core molecular machinery involved in SOCE. STIM1 is an ER integral membrane protein with an EF-hand motif on the luminal side. Reductions in ER Ca^{2+} levels cause the dissociation of Ca^{2+} from the EF-hand motif, the oligomerization of STIM1 proteins, and their translocation towards ER-plasma membrane junctions where they bind and activate ORAI1 channels (Figure 4). ORAI1 is a plasma membrane channel, which once activated, allows the influx of Ca^{2+} to the intracellular space. The ER calcium levels are then re-established through the activation of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Wegierski & Kuznicki, 2018). Importantly, SOCE not only aims to replenish ER Ca^{2+} levels but has been shown to create localized Ca^{2+} signalling microdomains that aid in a variety of physiological functions (Prakriya & Lewis, 2015).

Different STIM and ORAI isoforms have been identified (STIM1, STIM2, ORAI1, ORAI2 and ORAI3). Particularly in the mouse cortex, both STIM mRNAs can be found, and both contribute to calcium homeostasis (Zhang & Hu, 2020). However, ORAI isoform distribution is more controversial, and the functional role of ORAI2 and ORAI3 is not completely understood (Moccia et al., 2015). SOCE has been extensively studied in non-excitable cells, where it was first described (Vig et al., 2006) but it was also found to exist in neurons, which overall present more dependence on calcium signalling and exhibit a larger array of calcium activated proteins (Majewski & Kuznicki, 2014).

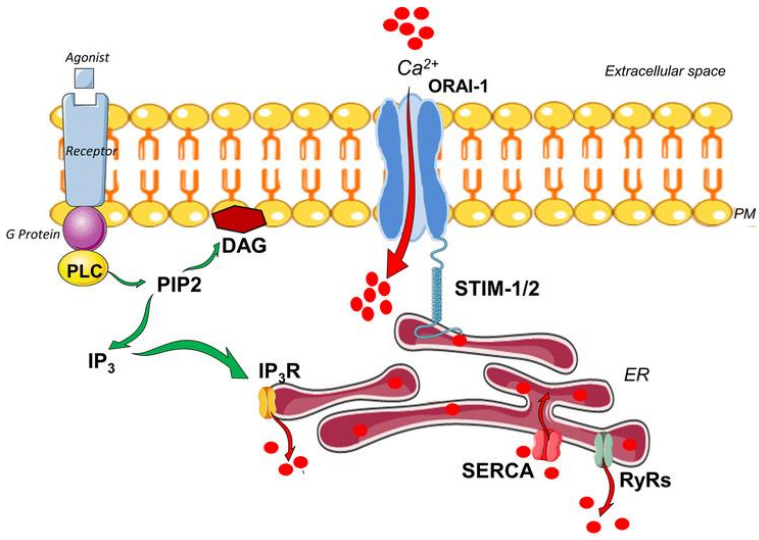


Figure 4 - **Schematic representation of the mechanisms involved in store operated calcium entry**. Abbreviations: PLC – phospholipase C; PIP_2 - phosphatidylinositol 4,5-bisphosphate; DAG – diglyceride; IP_3 - Inositol trisphosphate; SERCA – Sarco-Endoplasmic Reticulum Ca^{2+}-ATPase; RyRs – Ryanodine Receptor; ER – Endoplasmic Reticulum; STIM1/2 – Stromal Interaction Molecule 1 and 2; PM- Plasma Membrane. Adapted from Stanzione et al., 2022.

As mentioned before, dendritic spines are functionally dependent on several Ca^{2+} signalling mechanisms and consequently require tight regulation of their intracellular Ca^{2+} levels. STIM1, STIM2, and ORAI1 have been identified in dendritic spines belonging to different brain regions, including the cortex, hippocampus, and cerebellum (Moccia et al., 2015). SOCE and its components have been implicated in a variety of processes that are important for synapse formation and function. However, many aspects regarding the contribution of SOCE to dendritic spine Ca^{2+} are still unclear and have only recently started to be addressed.

During development, axon pathfinding and growth cone navigation ensure that neurons reach their designated location to engage in synaptic contacts. Studies have shown that STIM1-mediated SOCE is important for growth cone motility. Growth cone turning towards BDNF, which commonly functions as an attractive cue in the developing brain, was shown to be dependent on SOCE. This was proposed to be due to its influence on growth cone calcium signalling dynamics (Mitchell et al., 2012).

After target recognition, maturation of pre- and postsynaptic regions is crucial for proper synaptic function. ORAI1 and STIM2 were implicated in the regulation of dendritic spine maturation (Korkotian et al., 2017; Sun et al., 2014). Knockdown of ORAI1 expression significantly impacts the development of dendritic spines and the maturation of synaptic contacts *in vitro*, observed by the decrease in mushroom spines and an increase in filopodia protrusions in rat hippocampal neurons (Korkotian et al., 2017). Moreover, despite the common belief that only the actin cytoskeleton was present in spines, studies have proven that microtubules with B3 capped plus-ends transiently enter spines and contribute to spine head enlargement (Gu et al., 2008). STIM2 is able to interact with EB3 and this impacts spine morphology. Disruptions of STIM2-EB3 interaction causes loss of mushroom spines and EB3 knock down significantly reduced spine SOCE, as shown in studies using primary hippocampal cultures (Pchitskaya et al., 2017).

Stim2 deletion in hippocampal neuron conditional knock out models, greatly decreased the amplitude of synaptic SOCE and reduced the fraction of mushroom spines (with a correspondent increase in thin spines). In this study, the authors propose that synaptic SOCE is important for stabilizing mushroom spines by allowing a constant Ca^{2+} influx which in turn allows continuous activation of CAMKII. This hypothesis was supported by experiments involving genetic deletion of *Stim2* or pharmacological inhibition of SOCE which both caused a reduction of CAMKII activity (Sun et al., 2014). CAMKII is highly expressed in mushroom spines and is necessary for their stabilization. Activation of CAMKII is associated with changes in the actin cytoskeleton of dendritic spines through different mechanisms, which essentially converge in the activation of members of the Rho family of small GTPases such as RhoA, Rac1, or Cdc42, that have been associated with the regulation of postsynaptic maturation and synapse number (Cornelia Koeberle

et al., 2017; Hlushchenko et al., 2016; Okamoto et al., 2009). All in all, these studies highlight the contribution of SOCE components to the maturation and stabilization of mushroom spines.

Synapse function relies on synaptic plasticity mechanisms, namely long-term potentiation LTP and long-term depression (LTD). LTP is associated with synaptic contact strengthening and enlargement of dendritic spine heads. LTD, in contrast, leads to weakening of synaptic contacts and spine head shrinkage. In an elegant study, ORAI1 was shown to contribute to synaptic plasticity. Experiments involving glutamate uncaging, which elicits activation of NMDA receptor, and calcium sensors to report the subsequent spine Ca^{2+} elevations, showed that ORAI1 amplifies glutamate receptor evoked Ca^{2+} signals. Loss of ORAI1 expression, mutated pore ORAI1 forms, or ORAI1 inhibitors, lead to impairments in Ca^{2+} signals following glutamate exposure (Maneshi et al., 2020). Direct phosphorylation of AMPA receptors or interference with the mechanisms involved with receptor recycling and exocytosis are some of the processes regulated by CAMKII that contribute for postsynaptic maturation and plasticity (Lisman et al., 2012). Loss of ORAI1 diminished CAMKII activation, which was reflected by decreased presence of AMPA receptor containing GluA1 subunits in the postsynaptic region, following stimulation. As insertion of AMPA receptors in the membrane is a hallmark of LTP, the authors proceeded to assess field excitatory postsynaptic currents (fESPCs) in CA1 hippocampal neurons, to find out that ORAI1 conditional knock out impairs LTP in these neurons (Maneshi et al., 2020). LTP, particularly in the hippocampus, is considered to underlie learning and memory formation. Behavioural studies with mice lacking hippocampal ORAI1 expression, further supported these previous findings, as they showed impairments in tasks involving working and associative memory (Maneshi et al., 2020).

Despite the growing number of publications related to this matter, there are limitations regarding the study of synaptic SOCE, mostly explained by the fact that calcium conductance through ORAI channels is relatively modest, when comparing with the conductance of voltage gated calcium channels, for example (Moccia et al., 2015). This complicates identifying the state of activation of SOCE and also the measurement and isolation of currents that are undoubtedly induced by SOCE. There are some strategies to circumvent these issues, namely adding blockers of other Ca^{2+} channels to the system to measure calcium release activated currents, or genetically altering ORAI channels by fusing a genetically encoded Ca^{2+} sensor to the C-terminus of ORAI to observe optical recording changes according to its state of activation (Maneshi et al., 2020). Nevertheless, it is not an easy task to assess the physiological role of SOCE and its components in different relevant contexts.

Taken together, these studies have implicated synaptic SOCE proteins in processes and functions that are very important to ensure proper maintenance and function of neuronal circuits. Indeed, some KO mouse models for SOCE proteins are either not viable or have really limited lifespan (Oh-Hora & Lu, 2018), and impairments in SOCE have been associated with neurodegenerative disorders.

1.3.2.3 Dysregulated store operated calcium entry and neurodegenerative disorders

Neuronal function is dependent on the tight control of calcium-dependent processes. Recent studies have highlighted that neuronal calcium signalling is impaired in several neurodegenerative diseases, namely Alzheimer's, Huntington's, and Parkinson's disease. SOCE in particular has been the focus of intense research in the past few years and has been implicated in these pathologies (Pchitskaya et al., 2018).

a. Alzheimer's disease

Alzheimer's disease (AD) is the most common neurodegenerative disease and affects memory formation and storage. In the majority of the cases, AD is sporadic and has a late onset (~60 years old), but a very small percentage of cases (1-6%) have early onset and are caused by inheritable mutations. Familial AD (FAD) is associated with mutations involving the genes that encode presenilins (*PS1, PS2*) and amyloid precursor protein (*APP*). γ-Secretase is an enzymatic complex comprising PS1 or PS2 that produces different proteolytic variants of the Aβ peptide, being Aβ40 and Aβ42 the most common isoforms produced. A shift towards increased presence of Aβ42 is linked to the formation and deposition of Aβ oligomers, a hallmark of AD (Pchitskaya et al., 2018; Wegierski & Kuznicki, 2018).

Two different AD mouse models, a PS1 mutation knock in mouse model (PS1-M146V KI) and an amyloid precursor protein knock in mouse model (APPKI), showed significantly reduced STIM2 expression levels, particularly at dendritic spines (Sun et al., 2014; Zhang et al., 2015). In WT animals this STIM isoform was found to be highly expressed in mushroom spines (Sun et al., 2014) and the decrease in STIM2 expression observed in both AD models has been linked to mushroom spine number reduction and significantly decreased synaptic SOCE calcium signals (Sun et al., 2014; Zhang et al., 2015). Overexpression of STIM2 in PS1 KI neurons and APPKI neurons only partially rescued synaptic SOCE but was very effective in rescuing mushroom spine morphology (Zhang et al., 2015). Mushroom spines are considered to be "memory spines" as they engage in stable and long-lasting synaptic contacts which are thought to underlie memory storage (Pchitskaya & Bezprozvanny, 2020). For this reason, alterations of the ratio between mushroom and thin spines could contribute to the memory deficits that are observed in AD. These two studies demonstrate that for different mutations involved in AD, SOCE mechanisms seem to be associated with defects in spine morphology and ER calcium regulation.

In a neuroblastoma cell line (SH-SY5Y cells) expressing a different PS1 mutation associated to FAD (PS1-M146L), γ-secretase was found interact with STIM1 and to cleave it causing a significant decrease in SOCE due to impairments in STIM1 clustering and ORAI1 recruitment. This was attributed to the similarity between Aβ and STIM1 transmembrane domains. Possibly, in physiological conditions, PS1 also contributes to SOCE regulation by controlling the presence of STIM1 at the ER membrane. This mechanism could be dysregulated in FAD caused by PS1 mutations, leading to SOCE impairments and ultimately to loss of mushroom spines. However, the mechanisms

involved in this hypothesis have not been explored yet and further studies in primary neuronal cultures need to be conducted (Tong et al., 2016).

b. Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disease. It is characterized by loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc) and some of its common clinical presentations include problems in motor control, displayed as bradykinesia and rigidity (Zhai et al., 2018). The selective vulnerability of these dopaminergic neurons is thought to be related to their autonomous pacemaking activity, which is known to be highly dependent on the activity of L-type calcium channels ($Ca_v1.3$). This suggests that abnormal calcium homeostasis could be a major component in PD and that stress of internal calcium stores, like the mitochondria and the ER, could explain their selective loss (Surmeier et al., 2011).

Experiments using PC12 cells treated with 1-methyl-4-phenylpyridinium (MPP+), a well-established *in vitro* model of PD, showed that inhibition of STIM1 protected these cells against MPP+ injury, which was reflected as higher cell viability and reduced apoptosis. This effect was likely due to SOCE inhibition, suggested by calcium imaging experiments that showed an increase in intracellular Ca^{2+} concentration following MPP+ treatment, which was not observed when *Stim1* was knocked down. The molecular mechanism by which SOCE inhibition exerted protective effects was proposed to involve the upregulation of Homer1a expression (Li et al., 2013).

The contribution of TRPC1, a calcium channel, and SOCE for the regulation of $Ca_v1.3$ channels and pacemaking activity was studied in mouse dopaminergic neurons from the SN. DA neurons from *Trpc1* KO mice presented increased pacemaking activity and application of thapsigargin (Tg, a SERCA blocker) in combination with TTX (voltage gated sodium channel blocker), or of a nonspecific TRPC1 blocker, inhibited pacemaking activity in WT neurons, but not in *Trpc1* KO neurons. These results suggest that TRPC1 inhibits pacemaking activity in DA neurons (Sun et al., 2017). TRPC1 knockdown inhibited SOCE currents elicited by store depletion, which possibly means that TRPC1 acts as a SOCE channel in DA neurons (Sun et al., 2017). This would be in accordance with reports showing that store depletion induces STIM1 aggregation and promotes STIM1 interaction with TRPC1, that in turn activates TRPC1 channels that are able to suppress L-type Ca^{2+} channel currents (Huang et al., 2006). Indeed, it was shown that interactions between the STIM1-TRPC1 and $Ca_v1.3$ channels in DA neurons increased upon ER store depletion, which was not observed in *Trpc1* KO neurons. Taking these results into account, TRPC1 was proposed to function as a scaffold for STIM1 binding that subsequently allowed inhibition of $Ca_v1.3$, which prevented dysregulation of DA neuron pacemaking activity (Sun et al., 2017).

Overall, these studies assess the role of SOCE in different models of PD and in distinct contexts. Inhibition of SOCE seems to be beneficial to protect cultured neuron-like cells from MPP+ damage (Li et al., 2013), but in mouse DA neurons SOCE activation seems to inhibit $Ca_v1.3$ and to prevent DA neuron excitotoxicity (Sun et al., 2017). Despite the

apparently conflicting results, it is worth noting that regulation of SOCE is not linear and that perhaps they could all be integrated in a wider picture of PD mechanisms.

c. Huntington's disease

Huntington's disease (HD) is an inheritable neurodegenerative disease caused by CAG trinucleotide expansions in exon 1 of the huntingtin (*HTT*) gene. Mutant huntingtin (mHTT) causes early corticostriatal synaptic dysfunction and loss of medium spiny neurons (MSNs) through poorly understood mechanisms.

Several studies have showed that SOCE is elevated in MSN cultures from a HD transgenic mouse model, YAC128 (Czeredys et al., 2017; Wu et al., 2016). Mutant huntingtin (mHTT) contributes to an excessive activation of IP_3 receptors, causing Ca^{2+} efflux from the ER lumen. In cultures of YAC128 MSNs, huntingtin-associated protein-1 (HAP1) was found to promote the effect of mHTT on IP_3 receptor activation (Czeredys et al., 2018). The presence of polyglutamine expansions in mHTT, facilitates the interaction between HAP1 and mHTT, which becomes enhanced in HD (Czeredys et al., 2017, 2018). Exacerbated release of calcium from the ER is somehow compensated in this HD model by increased synaptic SOCE, possibly as an attempt to re-establish ER Ca^{2+} levels, which is also reflected by an upregulation of STIM2 in these neurons. However, the upregulation of STIM2 is not beneficial, as it causes spine loss in YAC128 MSNs. This was initially observed in YAC128 MSNs and then further validated by observing a reduction of dendritic spines after overexpression of STIM2 in WT MSNs (Wu et al., 2016).

Currently, there are no available disease-modifying therapies for AD, PD or HD. The complexity of these pathologies and the extent of alterations that they provoke has challenged greatly the scientific community. Since some of the most common neurodegenerative diseases show impairments in neuronal calcium signalling, understanding SOCE could be interesting to find meaningful missing links with previously described disease mechanisms. It is worth noting that, as described before, SOCE dysregulation either by exacerbation or reduction, has very negative consequences in synaptic function and plasticity. For these reasons, it is crucial to study SOCE control mechanisms in order to understand how its modulation could potentially be beneficial in pathological contexts.

Taken together, the observations gathered in this introduction highlight the complexity of processes that take place during development and, at the same time, the enormous lack of knowledge that we still have regarding those processes. Understanding more about the mechanisms that are necessary to establish synaptic contacts and neuronal circuits, and the mechanisms that later contribute to proper brain function will be helpful to understand the defects that may lead to developmental disorders or neuronal diseases. The study of brain development using meaningful models such as the mouse barrel cortex, will certainly continue to unravel the mysteries that underlie synaptic formation and maturation and two certainly promising research fields in this area are the study of signalling pathways activated by synaptic CAMs and synaptic calcium regulation by store operated calcium entry.

Chapter 2 - Objectives

Synaptic cell adhesion molecules (CAMs) are considered crucial for synaptogenesis, being involved both in the establishment of initial synaptic contacts, but also in the maturation of the pre- and postsynaptic compartments. Despite thorough characterization of the transsynaptic interactions engaged by CAMs, surprisingly very little is known about the signalling mechanisms employed by CAMs to mediate synapse formation and development (Südhof, 2021). Previous work from the host lab identified the postsynaptic CAM GPR158 as an important regulator of hippocampal synapse morphology and function. GPR158 was shown to localize to spines present on the proximal part of CA3 pyramidal neuron dendrites which receive inputs from mossy fiber (MF) axons emanating from the dentate gyrus. Ultrastructural analysis and electrophysiological assessment demonstrated that GPR158 is of key importance for the proper maturation of MF-CA3 synapses (Condomitti et al., 2018). These results suggest that GPR158 plays a key role in synapse maturation. However, the intracellular signalling mechanisms employed by GPR158 to instruct postsynaptic maturation are mostly unknown.

Unpublished data from Ben Verpoort shows that in addition to the CA3 region of the hippocampus, *Gpr158* is strikingly enriched in excitatory layer IV neurons of the developing barrel cortex and is similarly required for the maturation of their dendritic spines. Using an unbiased protein-protein interaction screen, Ben Verpoort and colleagues identified PLCXD2, an atypical phospholipase C with no known function in the brain, as a novel intracellular GPR158 interactor. Employing a combination of live-cell imaging and intracellular Ca^{2+} recordings in heterologous cells, they demonstrated that GPR158 prevents PLCXD2-induced SOCE inhibition. These findings suggest the involvement of a novel postsynaptic signalling complex in dendritic spine maturation considering that SOCE has previously been implicated in mushroom spine stabilization and regulation of Ca^{2+}-dependent signalling in developing dendritic spines (Sun et al., 2014; Wegierski & Kuznicki, 2018).

We hypothesize that PLCXD2 also critically contributes to the postsynaptic maturation of layer IV barrel neurons. In this thesis project we aimed to characterize the localization and expression profile of GPR158 and PLCXD2 throughout development in the barrel cortex and to further explore the contribution of PLCXD2 to postsynaptic maturation. In order to do so, we delineated the following objectives:

1) Characterize the spatiotemporal expression profile of *Gpr158* and *Plcxd2* in the developing barrel cortex.

2) Confirm the postsynaptic localization of GPR158 in layer IV neurons of the barrel cortex

3) Examine the subcellular distribution of endogenous PLCXD2 in neurons of the barrel cortex.

4) Assess the impact of loss of *Plcxd2* in layer IV neurons of the barrel cortex on dendritic spine maturation.

Chapter 3 - Materials and Methods

3.1 Animals

All animal experiments were conducted according to the KU Leuven ethical guidelines and approved by the KU Leuven Ethical Committee. Mice were maintained in a specific pathogen-free facility under standard housing conditions with food and water at disposition. The health and welfare of the animals was supervised by a designated veterinarian. The KU Leuven animal facilities comply with all appropriate standards (cages, space per animal, temperature, light, humidity, food, water), and all cages are enriched with materials that allow the animals to exert their natural behaviour. P0 indicates the day pups were born. Mice used in the study were up to a month old and were maintained on a diurnal 12-hour light/dark cycle. For euthanasia, animals were injected with an irreversible dose of ketamine-xylazine. Both males and females were used for all experiments.

Experiments were conducted in C57BL/6J mice and in different transgenic lines. Rorb-IRES2-Cre-D (B6.129S-*Rorb*$^{tm1.1(cre)Hze}$/J, strain 023526), Emx1-Cre (B6.129S2-*Emx1*$^{tm1(cre)Krj}$/J, strain 005628) and H11-Cas9 mice (*Igs2*$^{tm1.1(CAG-cas9*)Mmw}$/J, strain 027650) were acquired from Jackson Laboratories. Layer IV-specific *Gpr158* or *Plcxd2* cKO and WT littermate controls were obtained from crossing *Rorb-Cre*$^{+/-}$;*Gpr158*$^{f/+}$ or *Rorb-Cre*$^{+/-}$;*Plcxd2*$^{f/+}$ mice. Excitatory forebrain-specific *Plcxd2* WT and cKO littermates were obtained from crossing *Emx1-Cre*$^{+/-}$;*Plcxd2*$^{f/+}$ mice.

3.2 Stereotactic injections

For sparse labelling of layer IV neurons, P0 mice were subjected to ice-induced anaesthesia for 10 minutes. 50nL of virus were injected bilaterally in the cortex approximately at the intersection between the vertical and the horizontal medial lines of each hemisphere, at z=-0.8, to target the developing barrel cortex. Virus delivery was performed using a programmable nanoliter injector (Nanoject III, Drummond Scientific) equipped with a sharpened glass capillary. After injections, P0 mice were placed under a heating lamp and were closely monitored to ensure they regained consciousness before being placed back into a cage. The Supernova vector system was used and a solution containing the controller vector (1:500) and the amplifier vector (1:15) was injected. In Cre positive cells, leakage of TRE drives the weak expression of a Flipase and, subsequently, tTA in a very small population of neurons that carry both vectors. Thus, only in these cells, the expression of GFP is facilitated by the positive feedback of the tTA-TRE cycles.

For endogenous tagging of PLCXD2, stereotactic injections were performed at P7. One hour before starting the surgery, 0.05mg/kg of Buprenorphine (Vetergesic®0.6mg/ml) was intraperitoneally injected. Animals were anaesthetized with 5% isoflurane in an induction box and afterwards placed in the stereotactic frame. The concentration of isoflurane was then reduced to 2.5%. Duratears® was applied prior to surgery to prevent the eyes from drying. The head of the animal was disinfected with 70% ethanol, shaved

and afterwards 6mg/kg Lidocain (Xylocain®1%) was administered subcutaneously on the head. The programable injector (Nanoject III Drummond) was placed at predetermined coordinates and bevelled capillaries that penetrate skull and skin were used to inject 300nL of an adeno-associated virus (AAV) encoding antigenic protein tag "spaghetti monster" containing multiple copies of an influenza hemagglutinin (HA) epitope tag at 1 nL/s. After a 5 min recovery period, the capillary was slowly withdrawn. Surgical glue was applied (Millpledge Veterinary). The isoflurane concentration was reduced to 0% and the oxygen concentration increased to 100% for 2 minutes. The animal was then placed under a heating lamp before being returned to its cage. After 6 h, mice were examined again and injected with 0.1 mg/kg buprenorphine.

3.3 PLCXD2 endogenous tagging

H11-Cas9 knock-in mice present constitutive expression of Cas9 endonuclease directed by a CAG promoter. The strategy to endogenously tag PLCXD2 combines the principle of previously described methods (Gao et al., 2019; Suzuki et al., 2016) and involves the presence of a viral construct (donor AAV2/9) encoding a targeting guide RNA (gRNA) with the following sequence ACC GTA GTC AGA GCT CGA TCC TCT, that directs Cas9 to the C-terminal of the *Plcxd2* gene, causing a double break in the DNA strand at this location. The viral construct also contains a cassette that undergoes Cas9 excision directed by a second universal gRNA at donor recognition sites (DRS). This cassette is inserted into the genome by non-homologous end joining (NHEJ) and encodes the antigenic protein tag "spaghetti monster" containing multiple copies of HA (sm-HA). In post mitotic cells infected with the AAV, this will lead to the expression of PLCXD2 tagged with sm-HA. Stereotactic injections at P7 of an AAV encoding *Plcxd2*-smHA were performed to deliver the virus to the barrel cortex. At P28 the animals were perfused with 4% (w/v) PFA, and their brains were sectioned into sixty micrometre sections using a vibratome (7000smz-2, Campden Instruments). Free floating immunohistochemistry was performed to label HA epitopes (rat anti-HA antibody, 1:250, Sigma Aldrich).

3.4 Sparse labelling of layer IV neurons

Sparse labelling of layer IV neurons was achieved by using the Supernova viral vector system, which relies on two viral vectors, the controller, and the amplifier, both AAV2/9. The controller vector contains a Tetracyclin Response Element (TRE) and an inverted Flippase (FLP) sequence flanked by loxP sites which are recognized by Cre recombinase. The amplifier vector contains a TRE element and the inverted sequences of GFP, an Internal Ribosome Entry Site (IRES) and a Tetracycline-Controlled Transactivator (tTA), flanked by FRT sites recognized by the Flippase.

Stereotactic injections of both the controller and amplifier vectors were performed at P0 in *Rorb-Cre*;*Plcxd2*$^{f/f}$ and littermate control *Rorb-Cre*;*Plcxd2*$^{+/+}$ pups, as described above. At P14, the animals were injected with an irreversible dose of ketamine-xylazine and transcardially perfused with 4% (w/v), 2% (w/v) sucrose diluted in 0.1M phosphate buffer (PB), and their brains were sectioned into sixty micrometre sections using a vibratome (7000smz-2, Campden Instruments). Free floating immunohistochemistry

was performed to stain GFP (chicken anti-GFP antibody, 1:500, Aves) and VGLUT2 (rabbit anti-VGLUT2, 1:1000, Synaptic Systems).

3.5 Single-molecule fluorescent in situ hybridization

P4, P7, P9 and P14 C57BL/6J mice were injected with an irreversible dose of ketamine-xylazine and transcardially perfused with 4% (w/v) PFA in PBS. Brains were dissected and post fixed in 4% (w/v) PFA at 4˚C overnight. On the following day, brains were embedded in a 4% (w/v) agarose solution and sectioned on a vibratome (7000smz-2, Campden Instruments) to obtain sixty micrometre coronal sections containing the barrel cortex and the hippocampus. *Emx1-Cre*;*Plcxd2*$^{f/f}$ and littermate control *Emx1-Cre*;*Plcxd2*$^{+/+}$ mice were anaesthetized and perfused at P14, as described above, and their brains were collected and post-fixed in 4% (w/v) PFA at 4˚C overnight. The day after, brains were submerged in a 30% (w/v) sucrose solution for two days at 4˚C and afterwards embedded in OCT (Thermo-Fisher) and frozen in 2-Methylbutane (Sigma Aldrich) at -60˚C. Frozen brains were sectioned on a cryostat (NX70, Thermo Fisher) and eighteen micrometre coronal sections containing the barrel cortex and the hippocampus were collected on SuperFrost glass slides (Thermo-Fisher).

mRNA was visualized using the RNAscope® Multiplex Fluorescent Reagent Kit v2 (Advanced Cell Diagnostics) according to the manufacturer's instructions. The sections were treated with pre-treatment solutions and then hybridized with RNAscope probes (*Gpr158*, Cat. No. 524851; *Plcxd2*, Cat. No. 480591-C2; Slc17a7, Cat. No. 416631). RNAscope was combined with immunohistochemistry to label VGLUT2 (rabbit anti-VGLUT2, 1:1000, Synaptic Systems). DAPI was used as nuclear stain. Coverslips were mounted using Prolong Gold Antifade (ThermoScientific).

3.6 Immunohistochemistry

P14 C57BL/6J mice were injected with an irreversible dose of ketamine-xylazine and perfused with 4% (w/v) PFA. Whole brains were collected and post fixed in 4% (w/v) PFA overnight at 4˚C. On the following day, brains were embedded in a 4% (w/v) agarose solution and afterwards sectioned in a vibratome (7000smz-2, Campden Instruments) to obtain sixty micrometre coronal sections containing the barrel cortex and the hippocampus. For the experiments concerning the synaptic localization of GPR158, heat induced antigen retrieval was additionally performed during 15 minutes in sodium citrate buffer. Sections were transferred to PBS for 30 minutes at room temperature before proceeding with the protocol. Sections were permeabilized for 40 minutes in 0.5% (w/v) Triton X-100 in PBS and blocked for overnight at room temperature in a PBS-0.2% (w/v) gelatine solution containing 10% (w/v) normal horse serum, 0.5% (w/v) Triton X-100 and 0.5M glycine. Incubation with primary antibodies was performed for two days. Primary antibodies were the following: rabbit anti-GPR158 N-terminal (1:250, Sigma-Aldrich), guinea pig anti-VGLUT1 (1:1000, Millipore), guinea pig anti-VGLUT2 (1:1000, Synaptic Systems). Sections were subsequently washed in 0.5% (w/v) Triton X-100 in PBS and incubated overnight with the secondary antibodies at 4˚C. Fluorophore-conjugated secondary antibodies were from Jackson ImmunoResearch or Invitrogen. For the experiments concerning the postsynaptic localization of GPR158, a camelid

nanobody anti-PSD95 conjugated with a 647 fluorophore (1/500, FluoTag-X2 anti-PSD95 Fluorophore-conjugated, Synaptic Systems) was incubated simultaneously with secondary antibodies. Hoechst was used as a nuclear stain. Coverslips were mounted using Mowiol mounting media (Sigma Aldrich).

For validation of the *Gpr158* cKO mouse model, brains from P14 *Rorb-Cre*;*Gpr158*$^{f/f}$ and littermate control *Rorb-Cre*;*Gpr158*$^{+/+}$mice were quickly dissected, embedded in OCT (Thermo Fisher) and frozen in 2-Methylbutane (Sigma Aldrich) at -60˚C. Frozen brains were sectioned on a cryostat (NX70, Thermo Fisher) and eighteen micrometre coronal sections containing the barrel cortex and the hippocampus were collected on super frost glass slides (Thermo Fisher). Sections were postfixed for 10 min at -20˚C in a methanol-acetone 1:1 solution, washed in PBS and permeabilized for 20 minutes in 0.5% (w/v) Triton X-100 in PBS. Sections were then blocked for 2 hours at 4˚C in PBS-0.2% gelatine containing 10% (w/v) NHS and 0.5% Triton X-100 and incubated with a GPR158 primary antibody overnight at 4˚C (rabbit anti-GPR158 N-terminal, 1:250, Sigma-Aldrich). Sections were subsequently washed in PBS and incubated for 2 hours with the secondary antibody at room temperature. Hoechst was used as a nuclear stain. Coverslips were mounted using Mowiol mounting media (Sigma Aldrich).

3.7 Image acquisition and analysis

All images were acquired using a Zeiss LSM 880 – Airyscan microscope with 10X or 63X objectives. This microscope is equipped with an Airyscan module, which allows for “airyscanning”, a technique based on confocal laser scanning microscopy with increased signal-to-noise ratio which retrieves high resolution information. Airyscan processing was used in images captured using the 63X objective, particularly to distinguish synapses in the experiments for assessment of postsynaptic localization of GPR158, and to distinguish dendritic spines in the experiments for PLCXD2 endogenous tagging in cortical neurons and sparse labelling of layer IV neurons. For overview images, the 10X objective was used with regular confocal settings. For single-molecule fluorescent in situ hybridization experiments, regular confocal settings were used for image acquisition both with 10X and 63X objectives.

To quantify *Plcxd2* and *Gpr158* mRNA expression during development, Z-stacked images of the barrel cortex were acquired with the 63X objective, using the VGLUT2 labelling pattern to localize barrels. Afterwards, ImageJ was used to select areas and plan intervals containing individual cells that could be observed throughout Z, based on the presence of nuclear stain DAPI. These cropped Z-stacks were converted into a maximal projection for quantification. Quantification of single dots was performed using NIS-Elements software (Nikon, version 5.4). First, the nucleus of each cell was manually delineated using DAPI staining as a reference. This area was expanded by 7 pixels and all single dots intersecting or contained within this area were quantified. To calculate mRNA puncta density, area measurements (pixel^2) were converted into µm^2 and used to normalize number of puncta per area. We observed that mRNA puncta were frequently distributed around the nucleus, without necessarily overlapping with DAPI staining. For this reason, we enlarged the quantification area and used it to normalize the results, considering also that cell size increases throughout early post-natal

development. The same steps were followed for quantification of *Plcxd2* and *Vglut1* mRNA in *Plcxd2* WT and *Plcxd2* cKO sections.

For dendritic spine reconstruction and classification, we used Neurolucida software (MBF Bioscience). Dendrite reconstruction and spine detection were performed in z-stacks acquired with the 63x objective. Dendritic spines were classified into four different categories (filopodia, thin spines, stubby spines, and mushroom spines) according to predetermined parameters of the software.

3.8 Statistics

To compare statistically significant differences between multiple groups a two-way ANOVA was used. This test was followed by Sidak's multiple comparison test to correct for multiple testing. All graphs represent average values ± SEM.

Chapter 4 – Results

4.1 *Gpr158* and *Plcxd2* are co-expressed within individual layer IV neurons during barrel cortex development

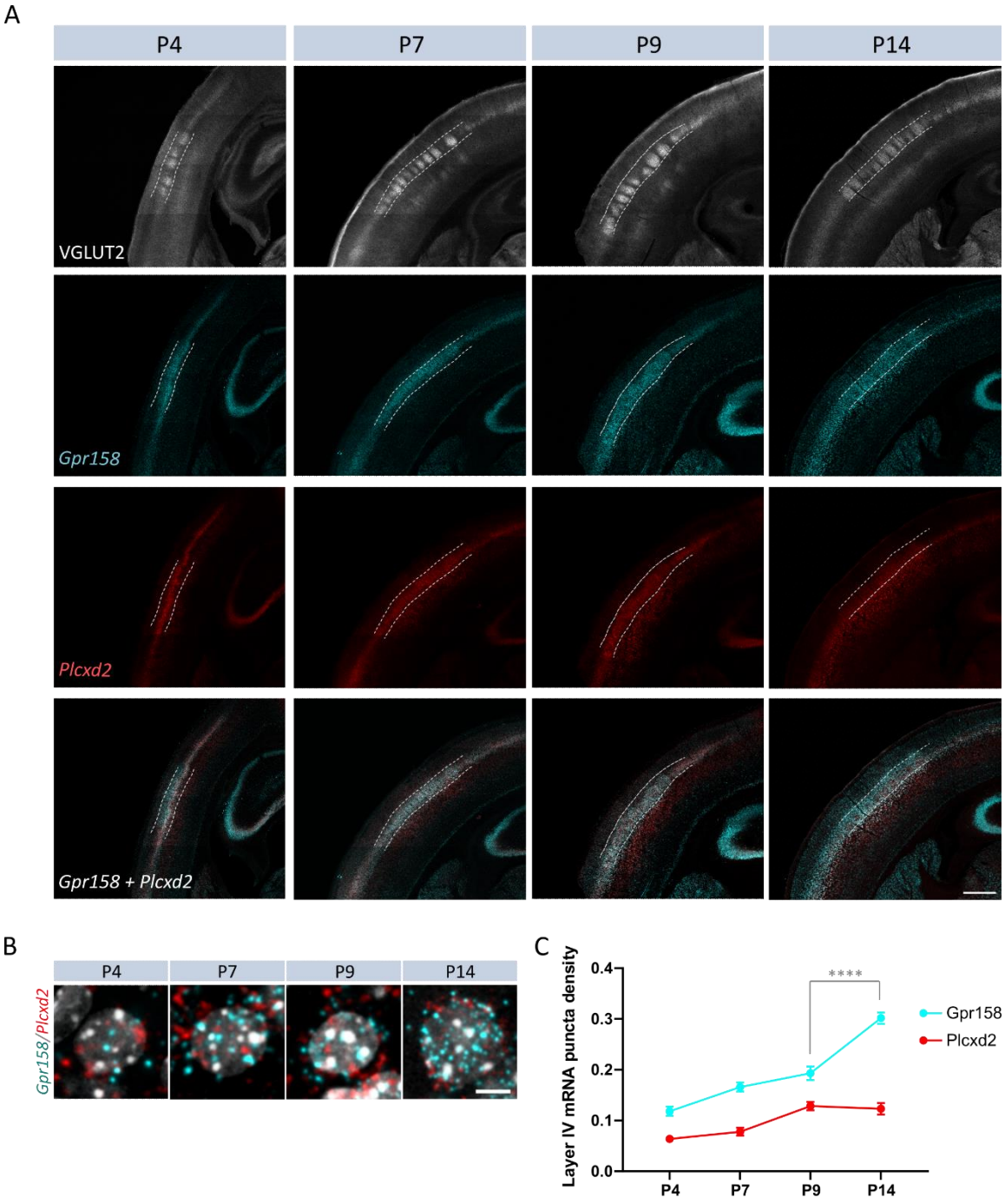


Figure 5 - *Gpr158* and *Plcxd2* are expressed within individual layer IV neurons during barrel cortex development. **(A)** *Gpr158* and *Plcxd2* single molecule hybridization in situ at P4, P7, P9 and P14 WT. *Gpr158* (cyan), *Plcxd2* (red). Dashed lines delineate layer IV. **(B)** Representative examples of P4, P7, P9 and P14 WT layer IV barrel cortex neurons. *Gpr158* (cyan), *Plcxd2* (red). **(C)** Quantification of *Plcxd2* and *Gpr158* mRNA puncta density. Data is presented as the mean ± SEM. For P4, n=34 cells from 3 mice; for P7, n=34 cells from 3 mice; for P9 n=34 cells from 3 mice and for P14, n=33 cells from 3 mice. ****p < 0.0001 by two-way ANOVA followed by Sidak's multiple comparison test. Scale bar in (A) represents 500 μm and in (B), 5 μm.

Prior to this thesis project, a protein-protein interaction screen conducted by Ben Verpoort and colleagues, identified PLCXD2 as a novel GPR158 intracellular interactor. However, this assay was performed and validated in heterologous cells. Given that unpublished data by Ben Verpoort revealed that *Gpr158* is strongly enriched in layer IV neurons, we reasoned that *Plcxd2* could be expressed in these same neurons. We thus assessed the spatiotemporal expression profile of both *Gpr158* and *Plcxd2* in the barrel cortex at four key developmental time points (see chapter 1.2 above).

Using single-molecule fluorescent hybridization in situ (smFISH) combined with VGLUT2 immunolabelling to delineate layer IV, we demonstrate that *Gpr158* and *Plcxd2* are co-expressed within individual layer IV barrel neurons as early as P4 (Figures 5A and 5B). Interestingly, between P9 and P14, *Gpr158* expression shows a dramatic increase, while *Plcxd2* expression remains unchanged (Figures 5B and 5C).

4.2 GPR158 localizes to the postsynaptic compartment at layer IV corticocortical and thalamocortical synapses

Given that RNA and protein expression not always necessarily correlate, we immunostained barrel cortex sections for GPR158, revealing enriched expression in layer IV and demonstrating a barrel-like pattern (Figure 6A). The barrel-like distribution of GPR158 was absent in *Gpr158* layer IV cKO (*Rorb-Cre*;*Gpr158*$^{f/f}$) mice, demonstrating specificity of GPR158 labelling (Figure 6C). Using the *Rorb-Cre* driver line, *Gpr158* expression is selectively removed in layer IV neurons.

Different types of excitatory synapses are found in layer IV, namely intracortical synapses, established between layer IV neurons, and thalamocortical synapses, established between TCAs and layer IV neurons. This prompted us to investigate the presence of GPR158 in these different synapse types which can be distinguished by virtue of the different glutamate transporters expressed by them. We thus immunolabeled GPR158 together with pre- and postsynaptic markers. Vesicular glutamate transporter 1 (VGLUT1) and VGLUT2 are presynaptically localized and mark corticocortical and thalamocortical synapses, respectively (Fremeau et al., 2001; Singh et al., 2016). Postsynaptic density protein 95 (PSD95) is a postsynaptic scaffold present in the postsynaptic density of excitatory synapses. We imaged regions of interest within barrels under high magnification and observed clear colocalization of GPR158 and PSD95 puncta, but juxtaposition to both VGLUT1 (Figure 6A) and VGLUT2 puncta (Figure 6B). Together, these findings demonstrate that GPR158 is expressed in the postsynaptic compartment of layer IV synapses, both corticocortical and thalamocortical.

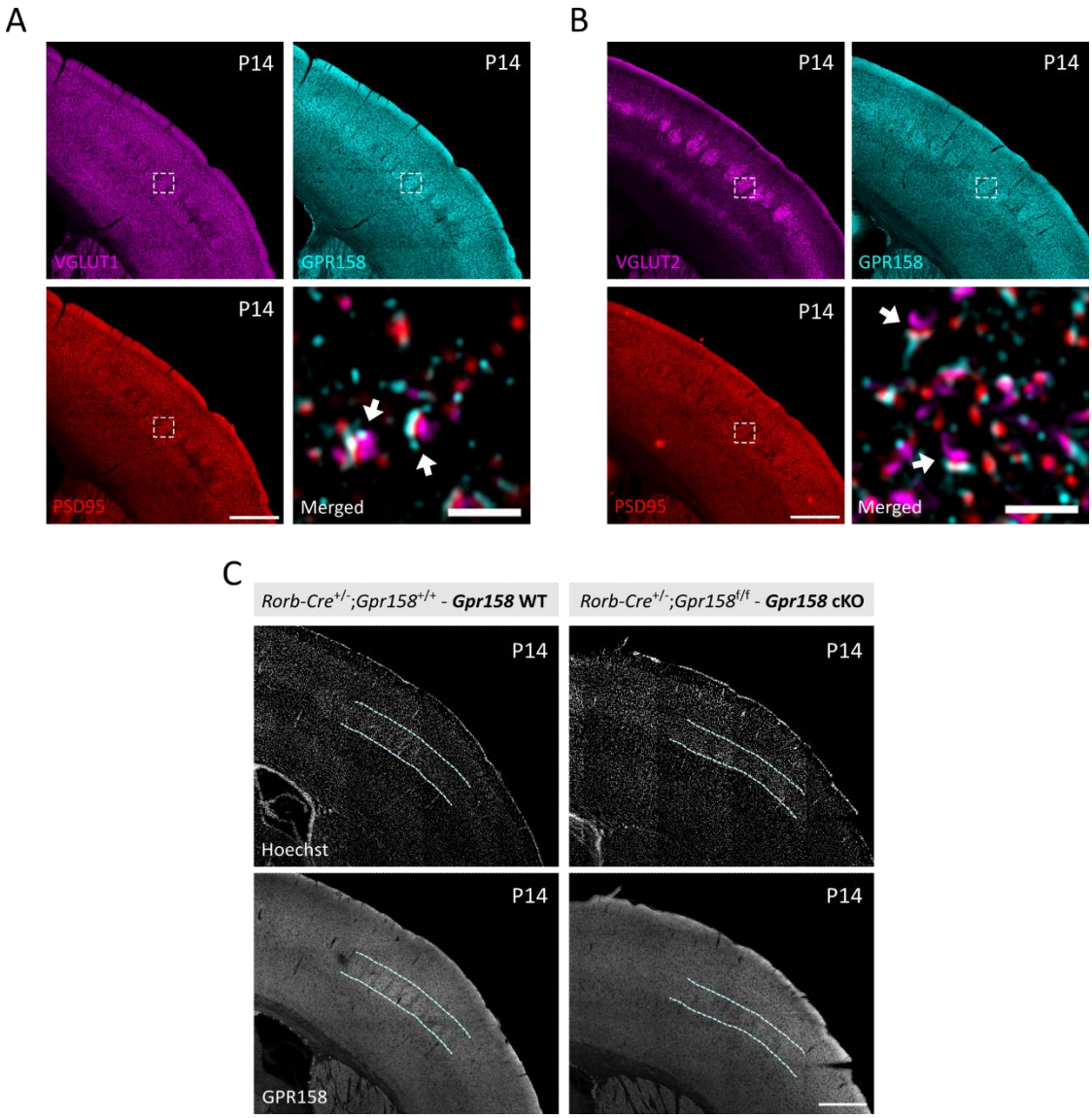


Figure 6 – GPR158 localizes to the postsynaptic compartment at layer IV corticocortical and thalamocortical synapses. (A,B) GPR158 immunoreactivity (cyan) in P14 WT somatosensory cortex. Merged images represent super-resolution imaging of synapses in the barrels. VGLUT1 **(A)** or VGLUT2 **(B)**, presynaptic markers (magenta), PSD95, postsynaptic marker (red). Arrows indicate representative examples of synapses where GPR158 is observed in the postsynaptic compartment. **(C)** Validation of the GPR158 antibody *in vivo* in P14 *Gpr158* WT and *GPR158* cKO mice. Dashed lines delineate layer IV. Scale bar in bottom left images in **(A,B)** represents 500 μm; in bottom right images in **(A,B)**, 2μm; in **(C)**, 500 μm.

4.3 PLCXD2 localizes to dendritic spines *in vivo*

Since *Gpr158* and *Plcxd2* are expressed in the same layer IV barrel neurons with GPR158 being present in the postsynaptic compartment, we reasoned that PLCXD2 should be localized here as well. Many commercially available antibodies were tested in *Plcxd2* cKO (*Emx1-Cre*;*Plcxd2*$^{f/f}$) mice where *Plcxd2* expression is removed from excitatory forebrain neurons (Gorski et al., 2002). Probing barrel cortex sections for *Plcxd2* and *Vglut1* using smFISH, we indeed demonstrate an almost complete absence of *Plcxd2* mRNA, but not *Vglut1* mRNA in these mice (Figures 7A-7D). Unfortunately, all antibodies turned out to be aspecific.

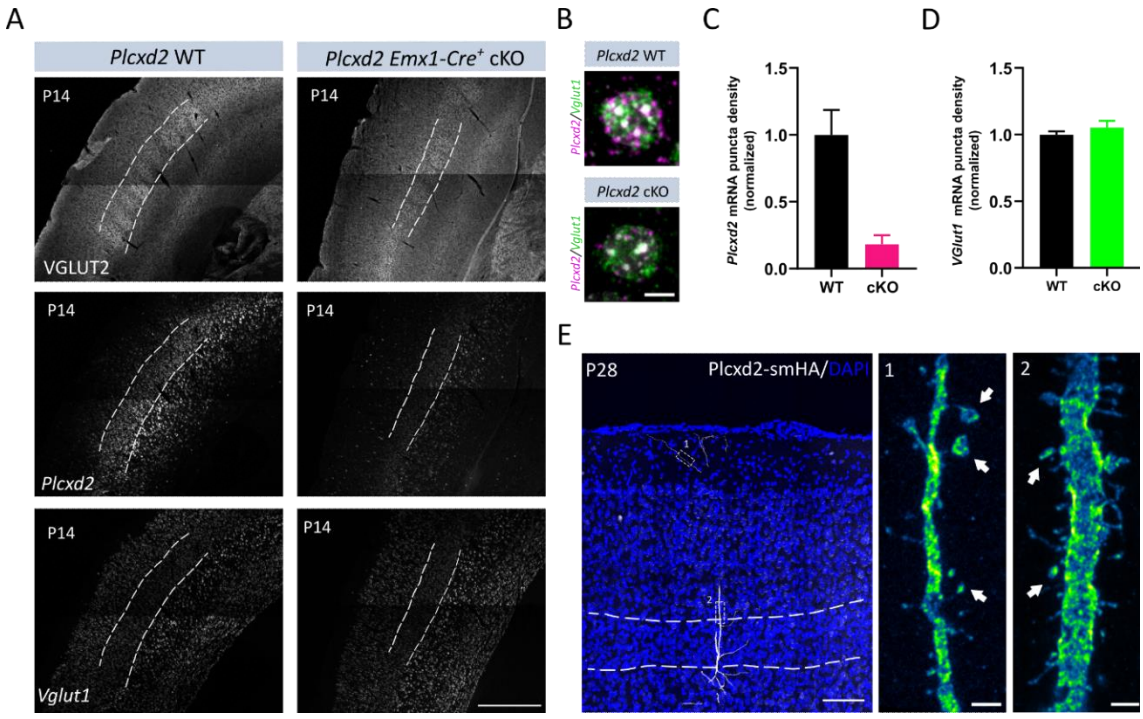


Figure 7 – **PLCXD2 localizes to dendritic spines in vivo. (A)** *Plcxd2* and *VGlut1* single molecule hybridization in situ in combination with VGLUT2 immunolabelling. **(B)** Examples of *Plcxd2* WT and *Plcxd2* cKO barrel cortex cells. *Plcxd2* (magenta), *VGlut1* (green). **(C)** Quantification of *Plcxd2* mRNA puncta density in *Plcxd2* WT and *Plcxd2 Emx1-Cre*$^{+}$ cKO barrel cortex cells (n=10 cells from 1 mouse). **(D)** Quantification of *VGlut1* mRNA puncta density in *Plcxd2* WT and *Plcxd2 Emx1-Cre*$^{+}$ cKO barrel cortex cells (n=10 cells from 1 mouse). **(E)** PLCXD2 spaghetti monster-HA endogenous tagging in P28 H11-Cas9 knock-in mice, counterstained with DAPI (blue). Dashed lines delineate cortex layer IV. Boxes delimitate the portions of the dendrites that are presented in higher magnification on the right **(1** and **2)**. Arrows indicate PLCXD2 expression in dendritic spines. Data is presented as the mean ± SEM. Scale bar in **(A)** represents 500 µm; in **(B)**, 5 µm; in **(E)**, 100 µm (left) and 2 µm (middle and right).

Since characterizing the global distribution of PLCXD2 in the barrel cortex was not possible, we focused on characterizing its expression at the subcellular level as we suspected that PLCXD2 could be expressed in dendritic spines, where GPR158 is found. To do so, we took advantage of an endogenous tagging strategy that was designed to target neurons in a sparse manner, thus allowing for observation of isolated neurons and dendritic spines in detail. This way we were able to circumvent the lack of suitable antibodies and, at the same time, the limitations of their use since it would be challenging to assign a particular labelling pattern to a specific neuron if all were simultaneously immunolabelled in the cortex. PLCXD2 was C-terminally tagged with spaghetti monster-HA (PLCXD2-smHA) (Figure 7E) which consists of a protein scaffold, containing multiple HA epitopes (Viswanathan et al., 2015). H11-Cas9 knock-in mice were injected at P7 in the barrel cortex with an AAV encoding a *Plcxd2*-specific gRNA and smHA, allowing for Cas9-directed double breakage of the *Plcxd2* gene and integration of smHA at its C-terminus via non-homologous end joining (NHEJ). These animals were sacrificed at P28 for immunohistochemistry. In successfully tagged neurons, HA immunolabelling revealed expression in the soma (data not shown) and dendrites in a pattern resembling membrane labelling. Strikingly, PLCXD2 was also found in dendritic spines, often at the base of spine heads (Figure 7E - 1, 2).

4.4 Loss of PLCXD2 in layer IV barrel neurons favors dendritic spine

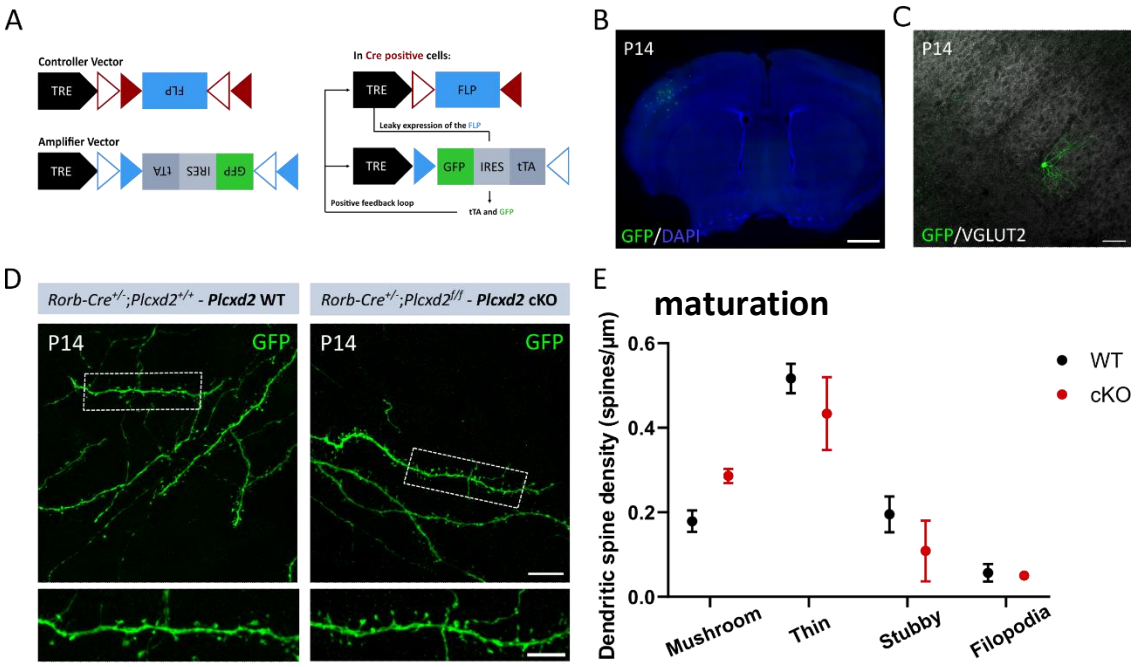


Figure 8 – Loss of PLCXD2 in layer IV barrel neurons favors dendritic spine maturation. (A) Schematic representation of the Supernova system vectors, the controller and amplifier. TRE – Tetracycline Control Element, FLP – Flipase, tTA - Tetracycline Transactivator, IRES – Internal Ribosome Entry Site, GFP – Green Fluorescent Protein. **(B)** Sparse neuron labelling in cortex layer IV using the Supernova vector system. GFP (green), DAPI (blue). **(C)** Layer IV barrel neuron brightly labelled with the Supernova vector system in combination with VGLUT2 immunolabelling. GFP (green) **(D)** Dendrites of Supernova labelled layer IV neurons in *Plcxd2* WT and *Plcxd2* cKO mice. Boxes delimitate the regions of the dendrites showed in higher magnification bellow. GFP (green). **(E)** Quantification of dendritic spine density according to different spine classes (thin, stubby, mushroom and filopodia) in *Plcxd2* WT and *Plcxd2* cKO layer IV neurons (n=2 neurons from 1 mouse). Data is presented as the mean ± SEM. For *Plcxd2* cKO and *Plcxd2* WT, n=2 neurons from 1 mouse . Scale bar in **(B)** represents 1 mm; in **(C)**, 50 µm; in **(D)**, 10 µm (top) and 5 µm (bottom).

Thus far, our findings demonstrate that endogenous GPR158 and PLCXD2 localize to the postsynaptic compartment where they likely interact and influence dendritic spine development. Unpublished work by Ben Verpoort shows that GPR158 is required for the proper maturation of dendritic spines in layer IV barrel neurons. However, whether PLCXD2 also play a role in dendritic spine development is not known. To address this question, we removed PLCXD2 specifically in layer IV barrel neurons and analyzed dendritic spine morphology. We employed an AAV based approach termed Supernova that allows for sparse, but very bright labelling in a Cre-dependent manner.

In *Plcxd2* cKO (*Rorb-Cre*;*Plcxd2*$^{f/f}$) and *Plcxd2* WT (*Rorb-Cre-Plcxd2*$^{+/+}$) animals, Cre recombinase is present in layer IV Rorb-expressing neurons. Cre dependent recombination of loxP sites in the controller vector, leads to inversion of the FLP sequence. Since the TRE is leaky, some initial and weak expression of the FLP will be sufficient for the recombination of FRT sites in the amplifier vector, leading to expression of tTA and GFP. tTA in turn binds TRE elements in both the controller and amplifier vectors, leading to their activation and consequently initiating a positive feedback loop that results in very high expression of GFP in these neurons (Figure 8A). Sparseness of labelling is achieved by adjusting the proportion of controller and amplifier vectors that are co-injected. Additionally, the positive feedback loop depends

on the presence of both vectors in a given cell. *Plcxd2* WT and cKO mice were injected at P0 in the barrel cortex and sacrificed at P14 for immunohistochemistry.

Using this approach, we were able to only label a handful of layer IV barrel neurons (Figures 8B and 8C) Interestingly, we observed that the absence of PLCXD2 indeed appears to impact dendritic spine maturation, as seen by a decrease in the density of thin spines and an increase in the density of mushroom spines compared to WT littermates (Figure 8E). Since mushroom spines are considered to be the most mature type of spines, these preliminary findings suggest that absence of PLCXD2 favours dendritic spine maturation.

Chapter 5 – Discussion and conclusion

5.1 GPR158 and PLCXD2 – a new signalling mechanism?

In this thesis project we sought to gain more insight on the expression pattern of GPR158 and PLCXD2 in the somatosensory cortex, particularly in the barrel cortex, as well as their possible role in layer IV dendritic spine maturation. We showed that *Plcxd2* and *Gpr158* transcripts are co-expressed in layer IV cells (Figure 5B) during early postnatal stages (Figure 5A), corresponding to the period of synapse establishment between thalamocortical axons and layer IV neurons.

We confirmed the specific enrichment of GPR158 in layer IV of the barrel cortex and although we couldn't withdraw any conclusions regarding the global protein expression pattern of PLCXD2, the expression of *Plcxd2* mRNA was specifically confined to layers IV and V, which could indicate that its protein expression follows the same pattern of enrichment (Figures 5A). GPR158 localizes to the postsynaptic compartment of layer IV excitatory neurons, clearly demonstrated by the co-localization of GPR158 puncta with PSD-95 puncta, a scaffolding protein found exclusively in the postsynaptic compartment of excitatory synapses (Figures 6A and 6B - merged images). Endogenous tagging of PLCXD2 was successful in multiple neurons from cortex layer V and results showed that PLCXD2 is present in dendritic spines, in two independent experiments (Figure 7E - 1, 2). The observations gathered in this thesis demonstrate that GPR158 and PLCXD2 are expressed in the postsynaptic compartment, but they are not sufficient to observe their co-localization in dendritic spines *in vivo*. However, we speculate that this might be the case as unpublished results by Ben Verpoort show that GPR158 and PLCXD2 co-localize in dendritic spines *in vitro*.

Previously, GPR158 was showed to be important for maturation of the hippocampal MF-CA3 synapse, and its absence caused synaptic transmission deficits and lack of proper dendritic spine maturation (Condomitti et al., 2018). Unpublished work by Ben Verpoort demonstrated that *Gpr158* is enriched in layer IV of the barrel cortex and that here it also contributes for postsynaptic maturation, as its loss in layer IV neurons caused impairments in dendritic spine maturation, reflected as a decrease in mushroom spines, considered more mature, and an increase in less mature thin spines. Conversely, in this thesis, we performed a similar assay but in neurons from layer IV lacking PLCXD2 expression (Figure 8) and these preliminary results suggest that absence of PLCXD2 is associated with the opposite phenotype, pointing to a higher density of mushroom spines and a reduction of thin spines. However, more experiments must be conducted to withdraw definitive conclusions regarding this potential phenotype.

5.2 GPR158 at specific synapse types of layer IV neurons

Specification of synapse types has been proposed to depend on synaptic CAMs, important for partner recognition through transsynaptic interactions and for shaping synaptic properties via activation of specific signalling pathways (Sando & Südhof, 2021; Sanes & Zipursky, 2020). In the hippocampus, GPR158 localization is restricted to MF-

CA3 synapses, which are established in the proximal part of CA3 pyramidal neuron dendrites. However, GPR158 is not expressed in neighbouring synapses of the same dendrite. For instance, in the medial portion of dendrites from these neurons (Stratum Radiatum), synapses with other CA3 pyramidal neurons are established and here GPR158 loss did not affect spine density or maturation, showing that this effect is input specific (Condomitti et al., 2018).

Pyramidal neurons in the hippocampus and cortex share commonalities, namely possessing extensive dendritic trees that receive input from different presynaptic partners in each anatomical layer, or lamina. These inputs can be considerably different but found in close proximity within the same dendrite (O'Rourke et al., 2012). This suggests that combinations of synaptic CAMs can contribute to synapse specification by limiting which pre- and postsynaptic partners successfully interact in particular regions of the same dendrite (de Wit & Ghosh, 2016). Particularly in barrel cortex layer IV there are two types of excitatory neurons, spiny stellate and star pyramidal neurons, distinguishable by the absence or presence of an apical dendrite, respectively (Staiger et al., 2004; Woolsey et al., 1975). In layer IV, stellate neurons, by far the most abundant type of excitatory neurons, are densely innervated by thalamocortical afferents from the VPM nucleus of the thalamus, particularly in polarized distal dendrites projecting to the barrel hollows, proposed to acquire this morphology to maximize contact with such thalamic afferents (Lübke & Feldmeyer, 2007; Simons & Woolsey, 1984). Additionally, stellate neurons establish intralaminar synapses with each other, which have been suggested to be preferentially observed in more proximal regions of the dendrite and probably only between stellate neurons of the same barrel (Feldmeyer et al., 1999).

Synapse specification observed in layer IV makes us wonder whether GPR158 also follows a differential expression pattern in synapses established in different regions of stellate neuron dendrites, like what was observed previously in the hippocampus (Condomitti et al., 2018). In this thesis, we immunolabeled GPR158 in conjugation with presynaptic VGLUT1 and VGLUT2, which are expressed in corticocortical and thalamocortical synapses, respectively. We found that GPR158 localizes to both VGLUT1 and VGLUT2 expressing synapses, with no obvious preference towards a specific type. Despite this observation, it would be interesting to repeat these immunolabelling experiments and simultaneously label VGLUT1 and 2 in combination with GPR158 and postsynaptic marker, PSD95. Due to incompatibility of primary antibody host species, we were not able to conduct the experiment in this way, but this would allow to determine the fraction of GPR158-containing synapses for each synapse type. Perhaps GPR158 can be found in both types of synapses but it's expressed in a higher fraction of thalamocortical synapses compared to corticocortical synapses, or vice-versa. One could also speculate that GPR158 is present in both types of synapses but presents differential expression levels within each type of synapse. To assess this, single molecule hybridization in situ could be performed using probes against *Gpr158*, *Vglut1* and *Vglut2*. Potential differences in expression levels could account for changes in the balance of GPR158- and PLCXD2-dependent signalling mechanisms activated upon dendritic maturation.

It has been proposed that intracortical synapses are less complex and function as feedback amplifiers of synaptic signals within the neocortex. Indeed, layer IV thalamocortical inputs have been described as relatively weak despite convergence of a large number of TCAs into each individual stellate neuron (Lübke & Feldmeyer, 2007). In the hippocampus, MF-CA3 synapses, where GPR158 is specifically present, are far more complex than CA3-CA3 synapses. If GPR158-PLCXD2 dependent signalling mechanisms are specifically important to a subset of synapses characterized by higher complexity and maturity, then maybe this could also be the case in synapses established in stellate neuron dendrites in layer IV. Assessing the impact of GPR158 loss specifically in different synapse types or compare it in different stellate neuron dendrite regions (more proximal versus more distal) could contribute to answer this question although more studies are necessary to characterize the localization and composition of thalamocortical and corticocortical synapses in layer IV.

5.3 Loss of GPR158 causes opposing phenotypes in different brain regions

In the past few years, GPR158 has gained some attention due to its association with stress induced depression. GPR158 was found to be upregulated in the medial prefrontal cortex (mPFC) of mice subjected to a paradigm of chronic stress. These results were corroborated by the observation of depressive-like behaviours upon overexpression of GPR158 in the mPFC and the contrasting antidepressant effect of GPR158 knock out. Interestingly, the increase in GPR158 protein levels in the mPFC was also observed in post-mortem brain samples of patients with major depressive disorders (MDD) (Sutton et al., 2018).

GPR158 is expressed mostly by glutamatergic neurons and some subpopulations of GABAergic neurons in layers 2/3 of the mPFC. However, the chronic stress-mediated upregulation of GPR158 was restricted to glutamatergic neurons. Absence of GPR158 caused synaptic alterations in these neurons, namely an increase in synaptic density and AMPA function (Sutton et al., 2018). This phenotype contrasts with the phenotype observed in GPR158 cKO in the hippocampal CA3 region. Here, impairments in synaptic transmission, namely a significant reduction of AMPA and NMDA function, were observed as well as a reduction in PSD size, assessed at an ultrastructural level (Condomitti et al., 2018). Curiously, synaptic density was also reported to be increased in this model, but it was accompanied by a shift towards less mature dendritic spines, which was also observed by Ben Verpoort in layer IV barrel cortex neurons lacking GPR158.

This interesting phenotype disparity between loss of GPR158 in the mPFC versus loss of GPR158 in CA3 and barrel cortex layer IV could possibly be explained by differences in the signalling pathways regulated by GPR158 in different brain regions. For instance, *Plcxd2* is particularly enriched in barrel cortex layer IV and hippocampus CA3 (Figure 5), where *Gpr158* is also expressed, but it is not expressed in layers 2/3 of the mPFC (Supplementary Figure 1). This suggests that perturbation of GPR158-PLCXD2 signalling pathways underlies the phenotype resulting from GPR158 cKO in CA3 and layer IV barrel cortex, and that other signalling mechanisms are responsible for the phenotype observed in the mPFC. To confirm that this is the case, PLCXD2 could be overexpressed

in L2/3 glutamatergic neurons of the mPFC in the GPR158 KO mouse model to see if the immature dendritic spine phenotype observed in the hippocampus and barrel cortex could be mimicked in the mPFC.

For instance, GPR158 and regulator of G protein signalling 7 (RGS7) act as a complex in the mPFC to regulate stress induced behaviour via regulation of Gαo signal termination occurring downstream of other GPCRs (Orlandi et al., 2019). It has been proposed that recruitment of RGS7-Gβ5 and inhibition of G protein signalling could occur in response to certain stimuli, making the signal termination more efficient in the post synaptic compartment where GPR158 is expressed (Watkins & Orlandi, 2021). It is reasonable to presume that the GPR158-RGS-Gβ5 complex could be involved in the regulation of relevant signalling pathways in the mPFC in contexts other than stress induced behaviour. Following this rationale, characterizing and comparing the GPR158 interactome in the mPFC with the CA3/ barrel cortex layer IV interactome could unravel specific interactors and shed a light on the type of signalling pathways regulated by GPR158 in each region. This could be achieved by endogenously tagging the C-terminal of GPR158 with BioID, a biotin ligase. This method relies on the biotinylation of interacting proteins by BioID, subsequent microdissection of regions of interest and mass spectrometry analysis to identify such interactors (Qin et al., 2021).

5.4 GPR158-PLCXD2 signalling during barrel cortex synaptogenesis bloom

In a previous study assessing layer IV stellate neuron connectivity during development, it was found that specifically between P8 and P13 there is a striking ≈250-fold increase in spine number (Ashby & Isaac, 2011). Upon assessment of the developmental expression of *Plcxd2* and *Gpr158* in the barrel cortex, we observed a very significant increase of *Gpr158* expression precisely between P9 and P14 (Figure 5C, Supplementary Table 1), whilst *Plcxd2* expression did not present any significant differences. We speculate that *Gpr158* upregulation is associated with the synaptogenesis bloom happening in the barrel cortex at around P9 and that it could be involved in a drastic inhibition of PLCXD2 signalling, to ensure timely maturation of the growing number of dendritic spines. Indeed, it was proposed that dendritic spine maturation is associated with a developmental switch occurring in this time period from synchronous (P9) to sparse spontaneous activity (P11) in layer IV neurons of the barrel cortex. Spine density was reported to more than double between P9 and P11 in dendrites of barrel edge spiny stellate neurons and this was dependent on small GTPase Rac1, a key regulator of actin skeleton dynamics. This transition to sparse and heterogeneously distributed spontaneous activity is crucial for precise information processing and is exhibited by the adult neocortex (Nakazawa et al., 2020). Active whisking and exploratory behaviours start after P14, and this drastically increases the complexity of information processed by barrel cortex neurons (Iwasato & Erzurumlu, 2018; Simi & Studer, 2018). GPR158 and PLCXD2 could be two key players in the signalling mechanisms that contribute for this functional shift, particularly by regulating dendritic spine maturation, possibly via calcium-dependent signalling pathways converging into GTPase Rac1 actin skeleton remodelling in dendritic spines.

Since no GPR158 endogenous ligands have been previously characterized in the literature, identification of PLCXD2 as a new intracellular interactor by Ben Verpoort and colleagues, and its apparent involvement in dendritic spine maturation (Figure 8), suggest that both proteins could be part of an undescribed signalling mechanism relevant for the regulation of dendritic spine maturation, in the barrel cortex. PLCXD2 has no known function in the brain but unpublished work by Ben Verpoort has unravelled certain features of this unusual type C phospholipase. First it was demonstrated that despite its atypical domain organization, PLCXD2 is able to reduce steady-state plasma membrane PIP_2 levels. Next, it was shown that PLCXD2 localizes adjacent to endoplasmic reticulum-plasma membrane (ER-PM) contact sites which function as spatial platforms for store-operated Ca^{2+} entry (SOCE), a critical Ca^{2+} signalling mechanism required for the maturation and plastic properties of dendritic spines. Using a combination of live-cell imaging and intracellular Ca^{2+} recordings, Ben Verpoort showed that PLCXD2 inhibits ER-PM contact site formation, and as a result impedes SOCE. Binding of GPR158 to PLCXD2 prevents PIP_2 hydrolysis, rescues ER-PM contact site formation and restores SOCE. Removal of GPR158 in excitatory layer IV barrel neurons and thus unconstraint PLCXD2 activity leads to the appearance of more immature spines with a concomitant loss of mature spines.

Since IP_3 and diacylglycerol (DAG), secondary messengers resulting from PIP_2 hydrolysis, are involved in a vast array of signalling mechanisms in dendritic spines, their generation needs to be precisely regulated. IP_3 is involved in the release of calcium from internal stores such as the ER, which can trigger the activation of SOCE. The other product of PIP_2 hydrolysis, DAG, can activate downstream effectors, such as PKCε, that inhibits dendritic spine development (Schaffer et al., 2018). Enzymes such as diacylglycerol kinase (DGK) are associated with DAG signal termination by converting DAG into phosphatidic acid (PA), eliciting the activation of signalling pathways associated with dendritic spine maintenance (Kim et al., 2010). Collectively, the aforementioned observations give rise to the idea that inhibition of PLCXD2 by GPR158 plays a role in the regulation of interconnected signalling mechanisms involving PIP_2 hydrolysis and SOCE activation in developing spines.

5.5 Presynaptic partners of GPR158

During synapse formation, synaptic CAMs mediate recognition of synaptic partners by engaging in a multitude of transsynaptic interactions. Synapse specification relies on the collaboration with other elements including components of extracellular matrix present in the synaptic cleft. Previous work by the host lab demonstrated that GPR158 is enriched in CA3 pyramidal neurons and interacts with presynaptic Glypican 4 (GPC4), a heparan sulfate proteoglycan (HSPG) enriched on hippocampal granule cell axons (mossy fibers) (Condomitti et al., 2018). Unbiased protein interaction screens confirm this interaction and reveal that GPR158 can interact with many other HSPGs, namely other glypicans (GPC1, GPC2, GPC3 and GPC6) (Orlandi et al., 2018).

In barrel cortex layer IV, presynaptic interactors of GPR158 remain unknown but it is possible that GPR158 also interacts with presynaptic HSPGs in layer IV. Since synapses between TCAs and layer IV neurons are established during early postnatal development,

further studies must be conducted to characterize the spaciotemporal expression of HSPGs and assess which ones are potentially expressed in the thalamic VPM during development. In situ hybridization data available in the Allen Mouse Brain Atlas reveals that, unlike other glypicans, *Gpc1* is highly expressed in the thalamic VPM, placing this HSPG as an interesting candidate for a GPR158 presynaptic binding partner (Supplementary Figure 2). However, this data is referent to adult animals and expression patterns of HSPGs in early postnatal stages may differ. Future experiments in the host lab will resolve some of these questions. Single molecule hybridization in situ of multiple HPGs will contribute for the characterization of their expression pattern in different developmental time points and reveal which ones are expressed in the VPM nucleus of the thalamus. Additionally, conditional knock out of GPC1 in the thalamus will be performed to assess if there are any changes in dendritic spine development of layer IV neurons.

In conclusion, in this thesis project we studied two proteins, GPR158 and PLCXD2, and confirmed their postsynaptic localization in neurons of the somatosensory cortex. Our findings show that *Gpr158* and *Plcxd2* are expressed during early postnatal development, and that *Gpr158* expression strikingly rises in a period characterized by intense spinogenesis. We hypothesize that this change in *Gpr158* expression is associated with regulation of PLCXD2 signalling during dendritic spine maturation in the barrel cortex. Finally, we explored weather loss of PLCXD2 in dendritic spines of layer IV cortical neurons impacted their maturation and our preliminary results suggest that loss of PLCXD2 could favour dendritic spine maturation. Further studies are necessary to comprehend what signalling mechanisms, particularly calcium dependent, are controlled by PLCXD2 and how is GPR158 contributing for their regulation during establishment of synapses in barrel cortex layer IV.

References

Ackermann, M., & Matus, A. (2003). Activity-induced targeting of profilin and stabilization of dendritic spine morphology. *Nature Neuroscience*, *6*(11), 1194–1200. https://doi.org/10.1038/nn1135

Alfano, C., & le Studer, M. (2012). Neocortical Arealization: Evolution, Mechanisms, and Open Questions. *Inc. Develop Neurobiol*, *73*, 411–447. https://doi.org/10.1002/dneu.22067

Antón-Bolaños, N., Espinosa, A., & López-Bendito, G. (2018). Developmental interactions between thalamus and cortex: a true love reciprocal story. *Current Opinion in Neurobiology*, *52*, 33–41. https://doi.org/10.1016/j.conb.2018.04.018

Ashby, M. C., & Isaac, J. T. R. (2011). Maturation of a Recurrent Excitatory Neocortical Circuit by Experience-Dependent Unsilencing of Newly Formed Dendritic Spines. *Neuron*, *70*(3), 510–521. https://doi.org/10.1016/j.neuron.2011.02.057

Ballester-Rosado, C. J., Sun, H., Huang, J. Y., & Lu, H. C. (2016). mGluR5 exerts cell-autonomous influences on the functional and anatomical development of layer IV cortical neurons in the mouse primary somatosensory cortex. *Journal of Neuroscience*, *36*(34), 8802–8814. https://doi.org/10.1523/JNEUROSCI.1224-16.2016

Borczyk, M., Śliwińska, M. A., Caly, A., Bernas, T., & Radwanska, K. (2019). Neuronal plasticity affects correlation between the size of dendritic spine and its postsynaptic density. *Scientific Reports*, *9*(1), 1–12. https://doi.org/10.1038/s41598-018-38412-7

Bourgeron, T. (2009). A synaptic trek to autism. *Current Opinion in Neurobiology*, *19*(2), 231–234. https://doi.org/10.1016/j.conb.2009.06.003

Budanova, I., Tabuchi, K., Rohlmann, A., Südhof, T. C., & Missler, M. (2007). Deletion of α-neurexins does not cause a major impairment of axonal pathfinding or synapse formation. *Journal of Comparative Neurology*, *502*(2), 261–274. https://doi.org/10.1002/cne.21305

Budreck, E. C., Kwon, O. bin, Jung, J. H., Baudouin, S., Thommen, A., Kim, H. S., Fukazawa, Y., Harada, H., Tabuchi, K., Shigemoto, R., Scheiffele, P., & Kim, J. H. (2013). Neuroligin-1 controls synaptic abundance of NMDA-type glutamate receptors through extracellular coupling. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(2), 725–730. https://doi.org/10.1073/pnas.1214718110

Carcea, I., Patil, S. B., Robison, A. J., Mesias, R., Huntsman, M. M., Froemke, R. C., Buxbaum, J. D., Huntley, G. W., & Benson, D. L. (2014). Maturation of cortical circuits requires Semaphorin 7A. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(38), 13978–13983. https://doi.org/10.1073/pnas.1408680111

Chirillo, M. A., Waters, M. S., Lindsey, L. F., Bourne, J. N., & Harris, K. M. (2019). Local resources of polyribosomes and SER promote synapse enlargement and spine clustering after long-term potentiation in adult rat hippocampus. *Scientific Reports*, *9*(1), 1–14. https://doi.org/10.1038/s41598-019-40520-x

Choii, G., & Ko, J. (2015). Gephyrin: a central GABAergic synapse organizer. *Experimental & Molecular Medicine*, *47*, e158. https://doi.org/10.1038/emm.2015.5

Clark, E. A., Rutlin, M., Capano, L., Aviles, S., Saadon, J. R., Taneja, P., Zhang, Q., Bullis, J., Lauer, T., Myers, E., Schulmann, A., Forrest, D., & Nelson, S. B. (2020). Cortical RORβ is required for layer 4 transcriptional identity and barrel integrity. *ELife*, *9*, 1–45. https://doi.org/10.7554/ELIFE.52370

Condomitti, G., Wierda, K. D., Schroeder, A., Rubio, S. E., Vennekens, K. M., Orlandi, C., Martemyanov, K. A., Gounko, N. v., Savas, J. N., & de Wit, J. (2018). An Input-Specific Orphan Receptor GPR158-HSPG Interaction Organizes Hippocampal Mossy Fiber-CA3 Synapses. *Neuron*, *100*(1), 201-215.e9. https://doi.org/10.1016/j.neuron.2018.08.038

Cornelia Koeberle, S., Tanaka, S., Kuriu, T., Iwasaki, H., Koeberle, A., Schulz, A., Helbing, D. L., Yamagata, Y., Morrison, H., & Okabe, S. (2017). Developmental stage-dependent regulation of spine formation by calcium-calmodulin-dependent protein kinase IIα and Rap1. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-13728-y

Czeredys, M., Maciag, F., Methner, A., & Kuznicki, J. (2017). Tetrahydrocarbazoles decrease elevated SOCE in medium spiny neurons from transgenic YAC128 mice, a model of Huntington's disease. *Biochemical and Biophysical Research Communications*, *483*(4), 1194–1205. https://doi.org/10.1016/j.bbrc.2016.08.106

Czeredys, M., Vigont, V. A., Boeva, V. A., Mikoshiba, K., Kaznacheyeva, E. v., & Kuznicki, J. (2018). Huntingtin-Associated Protein 1A Regulates Store-Operated Calcium Entry in Medium Spiny Neurons From Transgenic YAC128 Mice, a Model of Huntington's Disease. *Frontiers in Cellular Neuroscience*, *12*. https://doi.org/10.3389/fncel.2018.00381

Dalva, M. B., McClelland, A. C., & Kayser, M. S. (2007). Cell adhesion molecules: Signalling functions at the synapse. *Nature Reviews Neuroscience*, *8*(3), 206–220. https://doi.org/10.1038/nrn2075

de Wit, J., & Ghosh, A. (2014). Control of neural circuit formation by leucine-rich repeat proteins. *Trends in Neurosciences*, *37*(10), 539–550. https://doi.org/10.1016/j.tins.2014.07.004

de Wit, J., & Ghosh, A. (2016). Specification of synaptic connectivity by cell surface interactions. *Nature Reviews Neuroscience*, *17*(1), 22–35. https://doi.org/10.1038/nrn.2015.3

de Wit, J., Sylwestrak, E., O'Sullivan, M. L., Otto, S., Tiglio, K., Savas, J. N., Yates, J. R., Comoletti, D., Taylor, P., & Ghosh, A. (2009). LRRTM2 Interacts with Neurexin1 and Regulates Excitatory Synapse Formation. *Neuron*, *64*(6), 799–806. https://doi.org/10.1016/j.neuron.2009.12.019

Dean, C., Scholl, F. G., Choih, J., DeMaria, S., Berger, J., Isacoff, E., & Scheiffele, P. (2003). Neurexin mediates the assembly of presynaptic terminals. *Nature Neuroscience*, *6*(7), 708–716. https://doi.org/10.1038/nn1074

Dwyer, N. D., Chen, B., Chou, S. J., Hippenmeyer, S., Nguyen, L., & Ghashghaei, H. T. (2016). Neural stem cells to cerebral cortex: Emerging mechanisms regulating progenitor behavior and productivity. *Journal of Neuroscience*, *36*(45), 11394–11401. https://doi.org/10.1523/JNEUROSCI.2359-16.2016

Ebrahimi, S., & Okabe, S. (2014a). Structural dynamics of dendritic spines: Molecular composition, geometry and functional regulation. *Biochimica et Biophysica Acta - Biomembranes*, *1838*(10), 2391–2398. https://doi.org/10.1016/j.bbamem.2014.06.002

Ebrahimi, S., & Okabe, S. (2014b). Structural dynamics of dendritic spines: Molecular composition, geometry and functional regulation. *Biochimica et Biophysica Acta - Biomembranes*, *1838*(10), 2391–2398. https://doi.org/10.1016/j.bbamem.2014.06.002

Elia, L. P., Yamamoto, M., Zang, K., & Reichardt, L. F. (2006). p120 Catenin Regulates Dendritic Spine and Synapse Development through Rho-Family GTPases and Cadherins. *Neuron*, *51*(1), 43–56. https://doi.org/10.1016/j.neuron.2006.05.018

Erzurumlu, R. S., & Gaspar, P. (2020). How the barrel cortex became a working model for developmental plasticity: A historical perspective. *Journal of Neuroscience*, *40*(34), 6460–6473. https://doi.org/10.1523/JNEUROSCI.0582-20.2020

Ethell, I. M., Irie, F., Kalo, M. S., Couchman, J. R., Pasquale, E. B., & Yamaguchi, Y. (2001). EphB/syndecan-2 signaling in dendritic spine morphogenesis. *Neuron*, *31*(6), 1001–1013. https://doi.org/10.1016/S0896-6273(01)00440-8

Ethell, I. M., & Pasquale, E. B. (2005). Molecular mechanisms of dendritic spine development and remodeling. *Progress in Neurobiology*, *75*(3), 161–205. https://doi.org/10.1016/j.pneurobio.2005.02.003

Feldmeyer, D., Egger, V., Lübke, J., & Sakmann, B. (1999). Reliable synaptic connections between pairs of excitatory layer 4 neurones within a single “barrel” of developing rat somatosensory cortex. *Journal of Physiology*, *521*(1), 169–190. https://doi.org/10.1111/j.1469-7793.1999.00169.x

Fremeau, R. T., Troyer, M. D., Pahner, I., Nygaard, G. O., Tran, C. H., Reimer, R. J., Bellocchio, E. E., Fortin, D., Storm-Mathisen, J., & Edwards, R. H. (2001). The expression of vesicular glutamate transporters defines two classes of excitatory synapse. *Neuron*, *31*(2), 247–260. https://doi.org/10.1016/S0896-6273(01)00344-0

Gao, Y., Hisey, E., Bradshaw, T. W. A., Erata, E., Brown, W. E., Courtland, J. L., Uezu, A., Xiang, Y., Diao, Y., & Soderling, S. H. (2019). Plug-and-Play Protein Modification Using Homology-Independent Universal Genome Engineering. *Neuron*, *103*(4), 583-597.e8. https://doi.org/10.1016/j.neuron.2019.05.047

Gaspar, P., & Renier, N. (2018). Constraints on somatosensory map development: mutants lead the way. *Current Opinion in Neurobiology*, *53*, 43–49. https://doi.org/10.1016/j.conb.2018.04.028

Giagtzoglou, N., Ly, C. v., & Bellen, H. J. (2009). Cell adhesion, the backbone of the synapse: “vertebrate” and “invertebrate” perspectives. *Cold Spring Harbor Perspectives in Biology*, *1*(4). https://doi.org/10.1101/cshperspect.a003079

Gorski, J. A., Talley, T., Qiu, M., Puelles, L., Rubenstein, J. L. R., & Jones, K. R. (2002). Cortical excitatory neurons and glia, but not GABAergic neurons, are produced in the Emx1-expressing lineage. *Journal of Neuroscience*, *22*(15), 6309–6314. https://doi.org/10.1523/jneurosci.22-15-06309.2002

Graf, E. R., Zhang, X., Jin, S. X., Linhoff, M. W., & Craig, A. M. (2004). Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins. *Cell*, *119*(7), 1013–1026. https://doi.org/10.1016/j.cell.2004.11.035

Gu, J., Firestein, B. L., & Zheng, J. Q. (2008). Microtubules in dendritic spine development. *Journal of Neuroscience*, *28*(46), 12120–12124. https://doi.org/10.1523/JNEUROSCI.2509-08.2008

Higley, M. J., & Sabatini, B. L. (2012). Calcium signaling in dendritic spines. *Cold Spring Harbor Perspectives in Biology*, *4*(4). https://doi.org/10.1101/cshperspect.a005686

Hlushchenko, I., Koskinen, M., & Hotulainen, P. (2016). Dendritic spine actin dynamics in neuronal maturation and synaptic plasticity. *Cytoskeleton*, *73*(9), 435–441. https://doi.org/10.1002/cm.21280

Huang, G. N., Zeng, W., Kim, J. Y., Yuan, J. P., Han, L., Muallem, S., & Worley, P. F. (2006). STIM1 carboxyl-terminus activates native SOC, Icrac and TRPC1 channels. *Nature Cell Biology*, *8*(9), 1003–1010. https://doi.org/10.1038/ncb1454

Huang, T. Y., Dermardirossian, C., & Bokoch, G. M. (2006). Cofilin phosphatases and regulation of actin dynamics. *Current Opinion in Cell Biology*, *18*(1), 26–31. https://doi.org/10.1016/j.ceb.2005.11.005

Irie, F., & Yamaguchi, Y. (2002). EphB receptors regulate dendritic spine development via intersectin, Cdc42 and N-WASP. *Nature Neuroscience*, *5*(11), 1117–1118. https://doi.org/10.1038/nn964

Irie, M., Hata, Y., Takeuchi, M., Ichtchenko, K., Toyoda, A., Hirao, K., Takai, Y., Rosahl, T. W., & Südhof, T. C. (1997). Binding of neuroligins to PSD-95. *Science*, *277*(5331), 1511–1515. https://doi.org/10.1126/science.277.5331.1511

Iwasato, T., & Erzurumlu, R. S. (2018). Development of tactile sensory circuits in the CNS. *Current Opinion in Neurobiology*, *53*, 66–75. https://doi.org/10.1016/j.conb.2018.06.001

Jabaudon, D., J. Shnider, S., J. Tischfield, D., J. Galazo, M., & MacKlis, J. D. (2012). RORβ induces barrel-like neuronal clusters in the developing neocortex. *Cerebral Cortex*, *22*(5), 996–1006. https://doi.org/10.1093/cercor/bhr182

Jang, S., Lee, H., & Kim, E. (2017). Synaptic adhesion molecules and excitatory synaptic transmission. *Current Opinion in Neurobiology*, *45*, 45–50. https://doi.org/10.1016/j.conb.2017.03.005

Kim, K., Yang, J., & Kim, E. (2010). Diacylglycerol kinases in the regulation of dendritic spines. *Journal of Neurochemistry*, *112*(3), 577–587. https://doi.org/10.1111/j.1471-4159.2009.06499.x

Konietzny, A., González-Gallego, J., Bär, J., Perez-Alvarez, A., Drakew, A., Demmers, J. A. A., Dekkers, D. H. W., Hammer, J. A., Frotscher, M., Oertner, T. G., Wagner, W., Kneussel, M., & Mikhaylova, M. (2019). Myosin V regulates synaptopodin clustering and localization in the dendrites of hippocampal neurons. *Journal of Cell Science*, *132*(16). https://doi.org/10.1242/jcs.230177

Konietzny, A., Grendel, J., Kadek, A., Bucher, M., Han, Y., Hertrich, N., Dekkers, D. H. W., Demmers, J. A. A., Grünewald, K., Uetrecht, C., & Mikhaylova, M. (2021). Caldendrin and myosin V regulate synaptic spine apparatus localization via ER stabilization in dendritic spines. *The EMBO Journal*, 1–24. https://doi.org/10.15252/embj.2020106523

Korkotian, E., Frotscher, M., & Segal, M. (2014). Synaptopodin regulates spine plasticity: Mediation by calcium stores. *Journal of Neuroscience*, *34*(35), 11641–11651. https://doi.org/10.1523/JNEUROSCI.0381-14.2014

Korkotian, E., Oni-Biton, E., & Segal, M. (2017). The role of the store-operated calcium entry channel Orai1 in cultured rat hippocampal synapse formation and plasticity. *Journal of Physiology*, *595*(1), 125–140. https://doi.org/10.1113/JP272645

Krueger, D. D., Tuffy, L. P., Papadopoulos, T., & Brose, N. (2012). The role of neurexins and neuroligins in the formation, maturation, and function of vertebrate synapses. *Current Opinion in Neurobiology*, *22*(3), 412–422. https://doi.org/10.1016/j.conb.2012.02.012

Kucharz, K., & Lauritzen, M. (2018). CaMKII-dependent endoplasmic reticulum fission by whisker stimulation and during cortical spreading depolarization. *Brain*, *141*(4), 1049–1062. https://doi.org/10.1093/brain/awy036

Kucharz, K., Wieloch, T., & Toresson, H. (2011). Potassium-induced structural changes of the endoplasmic reticulum in pyramidal neurons in murine organotypic hippocampal slices. *Journal of Neuroscience Research*, *89*(8), 1150–1159. https://doi.org/10.1002/jnr.22646

Kucharz, K., Wieloch, T., & Toresson, H. (2013). Fission and Fusion of the Neuronal Endoplasmic Reticulum. *Translational Stroke Research*, *4*(6), 652–662. https://doi.org/10.1007/s12975-013-0279-9

Li, H., Fertuzinhos, S., Mohns, E., Hnasko, T. S., Verhage, M., Edwards, R., Sestan, N., & Crair, M. C. (2013). Laminar and Columnar Development of Barrel Cortex Relies on Thalamocortical Neurotransmission. *Neuron*, *79*(5), 970–986. https://doi.org/10.1016/j.neuron.2013.06.043

Li, X., Chen, W., Zhang, L., Liu, W. B., & Fei, Z. (2013). Inhibition of store-operated calcium entry attenuates MPP +-induced oxidative stress via preservation of mitochondrial function in PC12 cells: Involvement of Homer1a. *PLoS ONE*, *8*(12). https://doi.org/10.1371/journal.pone.0083638

Lin, Y. C., Frei, J. A., Kilander, M. B. C., Shen, W., & Blatt, G. J. (2016). A subset of autism-associated genes regulate the structural stability of neurons. *Frontiers in Cellular Neuroscience*, *10*(NOV2016). https://doi.org/10.3389/fncel.2016.00263

Linhoff, M. W., Laurén, J., Cassidy, R. M., Dobie, F. A., Takahashi, H., Nygaard, H. B., Airaksinen, M. S., Strittmatter, S. M., & Craig, A. M. (2009). An Unbiased Expression Screen for Synaptogenic Proteins Identifies the LRRTM Protein Family as Synaptic Organizers. *Neuron*, *61*(5), 734–749. https://doi.org/10.1016/j.neuron.2009.01.017

Lisman, J., Yasuda, R., & Raghavachari, S. (2012). Mechanisms of CaMKII action in long-term potentiation. *Nature Reviews Neuroscience*, *13*(3), 169–182. https://doi.org/10.1038/nrn3192

Liu, A., Zhou, Z., Dang, R., Zhu, Y., Qi, J., He, G., Leung, C., Pak, D., Jia, Z., & Xie, W. (2016). Neuroligin 1 regulates spines and synaptic plasticity via LIMK1/cofilin-mediated actin reorganization. *Journal of Cell Biology*, *212*(4), 449–463. https://doi.org/10.1083/jcb.201509023

Lokmane, L., & Garel, S. (2014). Map transfer from the thalamus to the neocortex: Inputs from the barrel field. *Seminars in Cell and Developmental Biology*, *35*, 147–155. https://doi.org/10.1016/j.semcdb.2014.07.005

Lübke, J., & Feldmeyer, D. (2007). Excitatory signal flow and connectivity in a cortical column: Focus on barrel cortex. *Brain Structure and Function*, *212*(1), 3–17. https://doi.org/10.1007/s00429-007-0144-2

Majewski, L., & Kuznicki, J. (2014). SOCE in neurons: Signaling or just refilling? *Biochimica et Biophysica Acta - Molecular Cell Research*, *1853*(9), 1940–1952. https://doi.org/10.1016/j.bbamcr.2015.01.019

Maneshi, M. M., Toth, A. B., Ishii, T., Hori, K., Tsujikawa, S., Shum, A. K., Shrestha, N., Yamashita, M., Miller, R. J., Radulovic, J., Swanson, G. T., & Prakriya, M. (2020). Orai1 Channels Are Essential for Amplification of Glutamate-Evoked Ca2+ Signals in Dendritic Spines to Regulate Working and Associative Memory. *Cell Reports*, *33*(9), 108464. https://doi.org/10.1016/j.celrep.2020.108464

Martini, F. J., Moreno-Juan, V., Filipchuk, A., Valdeolmillos, M., & López-Bendito, G. (2018). Impact of thalamocortical input on barrel cortex development. *Neuroscience*, *368*, 246–255. https://doi.org/10.1016/j.neuroscience.2017.04.005

Missler, M., Südhof, T. C., & Biederer, T. (2012). Synaptic cell adhesion. *Cold Spring Harbor Perspectives in Biology*, *4*(4), 1–18. https://doi.org/10.1101/cshperspect.a005694

Missler, M., Zhang, W., Rohlmann, A., Kattenstroth, G., Hammer, R. E., Gottmann, K., & Südhof, T. C. (2003). α-neurexins couple Ca 2+ channels to synaptic vesicle exocytosis. *Nature*, *423*(6943), 939–948. https://doi.org/10.1038/nature01755

Mitchell, C. B., Gasperini, R. J., Small, D. H., & Foa, L. (2012). STIM1 is necessary for store-operated calcium entry in turning growth cones. *Journal of Neurochemistry*, *122*(6), 1155–1166. https://doi.org/10.1111/j.1471-4159.2012.07840.x

Mizuno, H., Luo, W., Tarusawa, E., Saito, Y. M., Sato, T., Yoshimura, Y., Itohara, S., & Iwasato, T. (2014). NMDAR-regulated dynamics of layer 4 neuronal dendrites during thalamocortical reorganization in neonates. *Neuron*, *82*(2), 365–379. https://doi.org/10.1016/j.neuron.2014.02.026

Moccia, F., Zuccolo, E., Soda, T., Tanzi, F., Guerra, G., Mapelli, L., Lodola, F., & D'angelo, E. (2015). STIM and Orai proteins in neuronal Ca2+ signaling and excitability. *Frontiers in Cellular Neuroscience*, *9*(APR), 1–14. https://doi.org/10.3389/fncel.2015.00153

Mondin, M., Labrousse, V., Hosy, E., Heine, M., Tessier, B., Levet, F., Poujol, C., Blanchet, C., Choquet, D., & Thoumine, O. (2011). Neurexin-neuroligin adhesions capture surface-diffusing AMPA receptors through PSD-95 scaffolds. *Journal of Neuroscience*, *31*(38), 13500–13515. https://doi.org/10.1523/JNEUROSCI.6439-10.2011

Nakagawa, Y., & O'Leary, D. D. M. (2003). Dynamic patterned expression of orphan nuclear receptor genes RORα and RORβ in developing mouse forebrain. *Developmental Neuroscience*, *25*(2–4), 234–244. https://doi.org/10.1159/000072271

Nakazawa, S., Yoshimura, Y., Takagi, M., Mizuno, H., & Iwasato, T. (2020). Developmental phase transitions in spatial organization of spontaneous activity in postnatal barrel cortex layer 4. *Journal of Neuroscience*, *40*(40), 7637–7650. https://doi.org/10.1523/JNEUROSCI.1116-20.2020

Nam, C. I., & Chen, L. (2005). Postsynaptic assembly induced by neurexin-neuroligin interaction and neurotransmitter. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(17), 6137–6142. https://doi.org/10.1073/pnas.0502038102

Ng, A. N., Doherty, A. J., Lombroso, P. J., Emptage, N. J., & Collingridge, G. L. (2014). Rapid regulation of endoplasmic reticulum dynamics in dendritic spines by NMDA

receptor activation. *Molecular Brain*, *7*(1). https://doi.org/10.1186/s13041-014-0060-3

Ng, A. N., & Toresson, H. (2011). Endoplasmic reticulum dynamics in hippocampal dendritic spines induced by agonists of type I metabotropic glutamate but not by muscarinic acetylcholine receptors. *Synapse*, *65*(4), 351–355. https://doi.org/10.1002/syn.20887

Oertner, T. G., & Matus, A. (2005). Calcium regulation of actin dynamics in dendritic spines. *Cell Calcium*, *37*(5 SPEC. ISS.), 477–482. https://doi.org/10.1016/j.ceca.2005.01.016

Oh-Hora, M., & Lu, X. (2018). Function of Orai/Stim Proteins Studied in Transgenic Animal Models. *Calcium Entry Channels in Non-Excitable Cells*, 107–126. https://doi.org/10.1201/9781315152592-6

Okamoto, K., Bosch, M., & Hayashi, Y. (2009). The roles of CaMKII and F-Actin in the structural plasticity of dendritic Spines: A potential molecular identity of a synaptic tag? *Physiology*, *24*(6), 357–366. https://doi.org/10.1152/physiol.00029.2009

Orlandi, C., Omori, Y., Wang, Y., Cao, Y., Ueno, A., Roux, M. J., Condomitti, G., de Wit, J., Kanagawa, M., Furukawa, T., & Martemyanov, K. A. (2018). Transsynaptic Binding of Orphan Receptor GPR179 to Dystroglycan-Pikachurin Complex Is Essential for the Synaptic Organization of Photoreceptors. *Cell Reports*, *25*(1), 130-145.e5. https://doi.org/10.1016/j.celrep.2018.08.068

Orlandi, C., Sutton, L. P., Muntean, B. S., Song, C., & Martemyanov, K. A. (2019). Homeostatic cAMP regulation by the RGS7 complex controls depression-related behaviors. *Neuropsychopharmacology*, *44*(3), 642–653. https://doi.org/10.1038/s41386-018-0238-y

O'Rourke, N. A., Weiler, N. C., Micheva, K. D., & Smith, S. J. (2012). Deep molecular diversity of mammalian synapses: Why it matters and how to measure it. *Nature Reviews Neuroscience*, *13*(6), 365–379. https://doi.org/10.1038/nrn3170

Pchitskaya, E., & Bezprozvanny, I. (2020). Dendritic Spines Shape Analysis—Classification or Clusterization? Perspective. *Frontiers in Synaptic Neuroscience*, *12*. https://doi.org/10.3389/fnsyn.2020.00031

Pchitskaya, E., Kraskovskaya, N., Chernyuk, D., Popugaeva, E., Zhang, H., Vlasova, O., & Bezprozvanny, I. (2017). Stim2-Eb3 Association and Morphology of Dendritic Spines in Hippocampal Neurons. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-17762-8

Pchitskaya, E., Popugaeva, E., & Bezprozvanny, I. (2018). Calcium signaling and molecular mechanisms underlying neurodegenerative diseases. *Cell Calcium*, *70*, 87–94. https://doi.org/10.1016/j.ceca.2017.06.008

Penzes, P., Beeser, A., Chernoff, J., Schiller, M. R., Eipper, B. A., Mains, R. E., & Huganir, R. L. (2003). Rapid induction of dendritic spine morphogenesis by trans-synaptic ephrinB-EphB receptor activation of the Rho-GEF kalirin. *Neuron*, *37*(2), 263–274. https://doi.org/10.1016/S0896-6273(02)01168-6

Perez-Alvarez, A., Yin, S., Schulze, C., Hammer, J. A., Wagner, W., & Oertner, T. G. (2020a). Endoplasmic reticulum visits highly active spines and prevents runaway potentiation of synapses. *Nature Communications*, *11*(1). https://doi.org/10.1038/s41467-020-18889-5

Perez-Alvarez, A., Yin, S., Schulze, C., Hammer, J. A., Wagner, W., & Oertner, T. G. (2020b). Endoplasmic reticulum visits highly active spines and prevents runaway potentiation of synapses. *Nature Communications*, *11*(1), 1–10. https://doi.org/10.1038/s41467-020-18889-5

Petersen, C. C. H. (2019). Sensorimotor processing in the rodent barrel cortex. *Nature Reviews Neuroscience*, *20*(9), 533–546. https://doi.org/10.1038/s41583-019-0200-y

Prakriya, M., & Lewis, R. S. (2015). Store-operated calcium channels. *Physiological Reviews*, *95*(4), 1383–1436. https://doi.org/10.1152/physrev.00020.2014

Qin, W., Cho, K. F., Cavanagh, P. E., & Ting, A. Y. (2021). Deciphering molecular interactions by proximity labeling. *Nature Methods*, *18*(2), 133–143. https://doi.org/10.1038/s41592-020-01010-5

Quinn, D. P., Kolar, A., Wigerius, M., Gomm-Kolisko, R. N., Atwi, H., Fawcett, J. P., & Krueger, S. R. (2017). Pan-neurexin perturbation results in compromised synapse stability and a reduction in readily releasable synaptic vesicle pool size. *Scientific Reports*, *7*. https://doi.org/10.1038/srep42920

Ramirez, O. A., & Couve, A. (2011). The endoplasmic reticulum and protein trafficking in dendrites and axons. *Trends in Cell Biology*, *21*(4), 219–227. https://doi.org/10.1016/j.tcb.2010.12.003

Roppongi, R. T., Karimi, B., & Siddiqui, T. J. (2017). Role of LRRTMs in synapse development and plasticity. *Neuroscience Research*, *116*, 18–28. https://doi.org/10.1016/j.neures.2016.10.003

Sanderson, J. L., & Acqua, M. L. D. (2011). Synaptic cell adhesion molecules. In *Advances in Experimental Medicine and Biology* (Issue April). https://doi.org/10.1007/978-3-7091-0932-8

Sando, R., & Südhof, T. C. (2021). Latrophilin gpcr signaling mediates synapse formation. *ELife*, *10*, 1–22. https://doi.org/10.7554/eLife.65717

Sanes, J. R., & Zipursky, S. L. (2020). Synaptic Specificity, Recognition Molecules, and Assembly of Neural Circuits. *Cell*, *181*(3), 536–556. https://doi.org/10.1016/j.cell.2020.04.008

Saneyoshi, T., & Hayashi, Y. (2012). The Ca2+ and Rho GTPase signaling pathways underlying activity-dependent actin remodeling at dendritic spines. *Cytoskeleton*, *69*(8), 545–554. https://doi.org/10.1002/cm.21037

Schaffer, T. B., Smith, J. E., Cook, E. K., Phan, T., & Margolis, S. S. (2018). PKCε Inhibits Neuronal Dendritic Spine Development through Dual Phosphorylation of Ephexin5. *Cell Reports*, *25*(9), 2470-2483.e8. https://doi.org/10.1016/j.celrep.2018.11.005

Scheiffele, P., Fan, J., Choih, J., Fetter, R., & Serafini, T. (2000). Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell*, *101*(6), 657–669. https://doi.org/10.1016/S0092-8674(00)80877-6

Schubert, V., & Dotti, C. G. (2007). Transmitting on actin: Synaptic control of dendritic architecture. *Journal of Cell Science*, *120*(2), 205–212. https://doi.org/10.1242/jcs.03337

Segal, M., & Korkotian, E. (2014). Endoplasmic reticulum calcium stores in dendritic spines. *Frontiers in Neuroanatomy*, *8*(JULY), 1–7. https://doi.org/10.3389/fnana.2014.00064

Shibata, Y., Voeltz, G. K., & Rapoport, T. A. (2006). Rough Sheets and Smooth Tubules. *Cell*, *126*(3), 435–439. https://doi.org/10.1016/j.cell.2006.07.019

Siddiqui, T. J., Pancaroglu, R., Kang, Y., Rooyakkers, A., & Craig, A. M. (2010). LRRTMs and neuroligins bind neurexins with a differential code to cooperate in glutamate synapse development. *Journal of Neuroscience*, *30*(22), 7495–7506. https://doi.org/10.1523/JNEUROSCI.0470-10.2010

Simi, A., & Studer, M. (2018). Developmental genetic programs and activity-dependent mechanisms instruct neocortical area mapping. *Current Opinion in Neurobiology*, *53*, 96–102. https://doi.org/10.1016/j.conb.2018.06.007

Simons, D. J., & Woolsey, T. A. (1984). Morphology of Golgi-Cox-impregnated barrel neurons in rat SmI cortex. *Journal of Comparative Neurology*, *230*(1), 119–132. https://doi.org/10.1002/cne.902300111

Singh, S. K., Stogsdill, J. A., Pulimood, N. S., Dingsdale, H., Kim, Y. H., Pilaz, L. J., Kim, I. H., Manhaes, A. C., Rodrigues, W. S., Pamukcu, A., Enustun, E., Ertuz, Z., Scheiffele, P., Soderling, S. H., Silver, D. L., Ji, R. R., Medina, A. E., & Eroglu, C. (2016). Astrocytes Assemble Thalamocortical Synapses by Bridging NRX1α and NL1 via Hevin. *Cell*, *164*(1–2), 183–196. https://doi.org/10.1016/j.cell.2015.11.034

Spacek, J., & Harris, K. M. (1997). Three-dimensional organization of smooth endoplasmic reticulum in hippocampal CA1 dendrites and dendritic spines of the immature and mature rat. *Journal of Neuroscience*, *17*(1), 190–203. https://doi.org/10.1523/jneurosci.17-01-00190.1997

Speranza, L., Inglebert, Y., de Sanctis, C., Wu, P. Y., Kalinowska, M., McKinney, R. A., & Francesconi, A. (2022). Stabilization of Spine Synaptopodin by mGluR1 Is Required for mGluR-LTD. *The Journal of Neuroscience*, JN-RM-1466-21. https://doi.org/10.1523/jneurosci.1466-21.2022

Staiger, J. F., Flagmeyer, I., Schubert, D., Zilles, K., Kötter, R., & Luhmann, H. J. (2004). Functional diversity of layer IV spiny neurons in rat somatosensory cortex: Quantitative morphology of electrophysiologically characterized and biocytin labeled cells. *Cerebral Cortex*, *14*(6), 690–701. https://doi.org/10.1093/cercor/bhh029

Stanzione, R., Forte, M., Cotugno, M., Bianchi, F., Marchitti, S., & Rubattu, S. (2022). Relevance of stromal interaction molecule 1 (STIM1) in experimental and human stroke. *Pflugers Archiv European Journal of Physiology*, *474*(1), 141–153. https://doi.org/10.1007/s00424-021-02636-w

Südhof, T. C. (2008). Neuroligins and neurexins link synaptic function to cognitive disease. *Nature*, *455*(7215), 903–911. https://doi.org/10.1038/nature07456

Südhof, T. C. (2021). The cell biology of synapse formation. *The Journal of Cell Biology*, *220*(7), 1–18. https://doi.org/10.1083/jcb.202103052

Sun, S., Zhang, H., Liu, J., Popugaeva, E., Xu, N. J., Feske, S., White, C. L., & Bezprozvanny, I. (2014). Reduced synaptic STIM2 expression and impaired store-operated calcium entry cause destabilization of mature spines in mutant presenilin mice. *Neuron*, *82*(1), 79–93. https://doi.org/10.1016/j.neuron.2014.02.019

Sun, Y., Zhang, H., Selvaraj, S., Sukumaran, P., Lei, S., Birnbaumer, L., & Singh, B. B. (2017). Inhibition of L-type Ca2+ channels by TRPC1-STIM1 complex is essential for the protection of dopaminergic neurons. *Journal of Neuroscience*, *37*(12), 3364–3377. https://doi.org/10.1523/JNEUROSCI.3010-16.2017

Surmeier, D. J., Guzman, J. N., Sanchez-Padilla, J., & Schumacker, P. T. (2011). The role of calcium and mitochondrial oxidant stress in the loss of substantia nigra pars compacta dopaminergic neurons in Parkinson's disease. *Neuroscience*, *198*, 221–231. https://doi.org/10.1016/j.neuroscience.2011.08.045

Sutton, L. P., Orlandi, C., Song, C., Oh, W. C., Muntean, B. S., Xie, K., Filippini, A., Xie, X., Satterfield, R., Yaeger, J. D. W., Renner, K. J., Young, S. M., Xu, B., Kwon, H., & Martemyanov, K. A. (2018). Orphan receptor GPR158 controls stress-induced depression. *ELife*, *7*, 1–27. https://doi.org/10.7554/eLife.33273

Suzuki, K., Tsunekawa, Y., Hernandez-Benitez, R., Wu, J., Zhu, J., Kim, E. J., Hatanaka, F., Yamamoto, M., Araoka, T., Li, Z., Kurita, M., Hishida, T., Li, M., Aizawa, E., Guo, S., Chen, S., Goebl, A., Soligalla, R. D., Qu, J., … Belmonte, J. C. I. (2016). In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. *Nature*, *540*(7631), 144–149. https://doi.org/10.1038/nature20565

Tashiro, A., Minden, A., & Yuste, R. (2000). Regulation of dendritic spine morphology by the Rho family of small GTPases: Antagonistic roles of Rac and Rho. *Cerebral Cortex*, *10*(10), 927–938. https://doi.org/10.1093/cercor/10.10.927

Tong, B. C. K., Lee, C. S. K., Cheng, W. H., Lai, K. O., Foskett, J. K., & Cheung, K. H. (2016). Familial Alzheimer's disease-associated presenilin 1 mutants promote γ-secretase cleavage of STIM1 to impair store-operated Ca2+ entry. *Science Signaling*, *9*(444). https://doi.org/10.1126/scisignal.aaf1371

Toresson, H., & Grant, S. G. N. (2005). Dynamic distribution of endoplasmic reticulum in hippocampal neuron dendritic spines. *European Journal of Neuroscience*, *22*(7), 1793–1798. https://doi.org/10.1111/j.1460-9568.2005.04342.x

van der Loos, H., & Woolsey, T. A. (1973). Somatosensory cortex: Structural alterations following early injury to sense organs. *Science*, *179*(4071), 395–398. https://doi.org/10.1126/science.179.4071.395

Varoqueaux, F., Aramuni, G., Rawson, R. L., Mohrmann, R., Missler, M., Gottmann, K., Zhang, W., Südhof, T. C., & Brose, N. (2006). Neuroligins Determine Synapse Maturation and Function. *Neuron*, *51*(6), 741–754. https://doi.org/10.1016/j.neuron.2006.09.003

Vig, M., Peinelt, C., Beck, A., Koomoa, D. L., Rabah, D., Koblan-Huberson, M., Kraft, S., Turner, H., Fleig, A., Penner, R., & Kinet, J. P. (2006). CRACM1 is a plasma membrane protein essential for store-operated Ca 2+ entry. *Science*, *312*(5777), 1220–1223. https://doi.org/10.1126/science.1127883

Viswanathan, S., Williams, M. E., Bloss, E. B., Stasevich, T. J., Speer, C. M., Nern, A., Pfeiffer, B. D., Hooks, B. M., Li, W. P., English, B. P., Tian, T., Henry, G. L., Macklin, J. J., Patel, R., Gerfen, C. R., Zhuang, X., Wang, Y., Rubin, G. M., & Looger, L. L. (2015). High-performance probes for light and electron microscopy. *Nature Methods*, *12*(6), 568–576. https://doi.org/10.1038/nmeth.3365

Vlachos, A., Korkotian, E., Schonfeld, E., Copanaki, E., Deller, T., & Segal, M. (2009). Synaptopodin regulates plasticity of dendritic spines in hippocampal neurons. *Journal of Neuroscience*, *29*(4), 1017–1033. https://doi.org/10.1523/JNEUROSCI.5528-08.2009

Wagner, W., Brenowitz, S. D., & Hammer, J. A. (2011). Myosin-Va transports the endoplasmic reticulum into the dendritic spines of Purkinje neurons. *Nature Cell Biology*, *13*(1), 40–47. https://doi.org/10.1038/ncb2132

Watkins, L. R., & Orlandi, C. (2021). In vitro profiling of orphan G protein coupled receptor (GPCR) constitutive activity. *British Journal of Pharmacology*, *178*(15), 2963–2975. https://doi.org/10.1111/bph.15468

Wegierski, T., & Kuznicki, J. (2018). Neuronal calcium signaling via store-operated channels in health and disease. *Cell Calcium*, *74*(June), 102–111. https://doi.org/10.1016/j.ceca.2018.07.001

Wijetunge, L. S., Till, S. M., Gillingwater, T. H., Ingham, C. A., & Kind, P. C. (2008). mGluR5 regulates glutamate-dependent development of the mouse somatosensory cortex. *Journal of Neuroscience*, *28*(49), 13028–13037. https://doi.org/10.1523/JNEUROSCI.2600-08.2008

Woolsey, T. A., Dierker, M. L., & Wann, D. F. (1975). Mouse SmI cortex: qualitative and quantitative classification of Golgi impregnated barrel neurons. *Proceedings of the National Academy of Sciences of the United States of America*, *72*(6), 2165–2169. https://doi.org/10.1073/pnas.72.6.2165

Woolsey, T. A., & van der Loos, H. (1970). The structural organization of layer IV in the somatosensory region (S I) of mouse cerebral cortex. The description of a cortical field composed of discrete cytoarchitectonic units. *Brain Research*, *17*(2), 205–242. https://doi.org/10.1016/0006-8993(70)90079-X

Wu, J., Ryskamp, D. A., Liang, X., Egorova, P., Zakharova, O., Hung, G., & Bezprozvanny, I. (2016). Enhanced store-operated calcium entry leads to striatal synaptic loss in a huntington's disease mouse model. *Journal of Neuroscience*, *36*(1), 125–141. https://doi.org/10.1523/JNEUROSCI.1038-15.2016

Zhai, S., Tanimura, A., Graves, S. M., Shen, W., & Surmeier, D. J. (2018). Striatal synapses, circuits, and Parkinson's disease. *Current Opinion in Neurobiology*, *48*, 9–16. https://doi.org/10.1016/j.conb.2017.08.004

Zhang, H., Wu, L., Pchitskaya, E., Zakharova, O., Saito, T., Saido, T., & Bezprozvanny, I. (2015). Neuronal store-operated calcium entry and mushroom spine loss in amyloid precursor protein knock-in mouse model of Alzheimer's disease. *Journal of Neuroscience*, *35*(39), 13275–13286. https://doi.org/10.1523/JNEUROSCI.1034-15.2015

Zhang, I., & Hu, H. (2020). Store-Operated Calcium Channels in Physiological and Pathological States of the Nervous System. *Frontiers in Cellular Neuroscience*, *14*. https://doi.org/10.3389/fncel.2020.600758

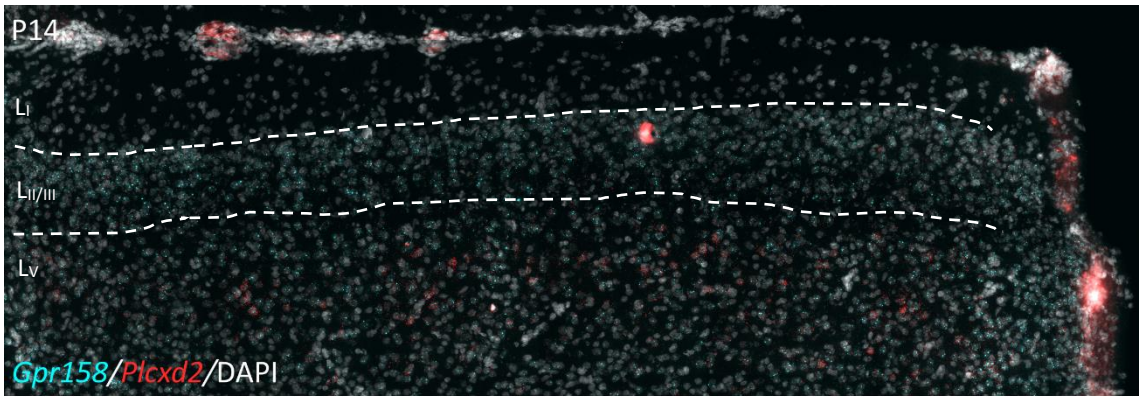
Annexes

Supplementary Table 1 – Two-way ANOVA followed by Sidak's multiple comparisons test to compare the expression of each probe, *Gpr158* or *Plcxd2*, between different time points.

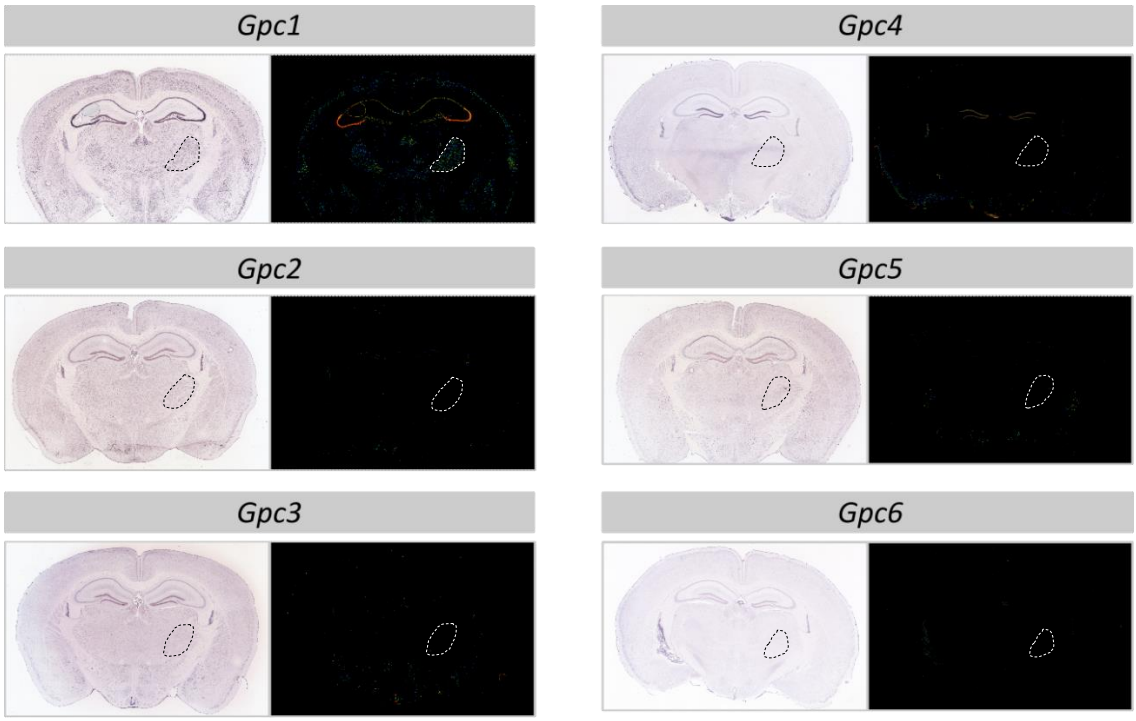
Sidak's multiple comparisons test	Mean Diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P Value	Mean 1	Mean 2
Gpr158							
P4 vs. P7	-0,04739	-0,08139 to -0,01338	Yes	**	0,0027	0,1183	0,1657
P4 vs. P9	-0,07495	-0,1226 to -0,02734	Yes	***	0,0006	0,1183	0,1933
P4 vs. P14	-0,1834	-0,2274 to -0,1393	Yes	****	<0,0001	0,1183	0,3017
P7 vs. P9	-0,02756	-0,06761 to 0,01248	No	ns	0,322	0,1657	0,1933
P7 vs. P14	-0,136	-0,1719 to -0,1001	Yes	****	<0,0001	0,1657	0,3017
P9 vs. P14	-0,1084	-0,1468 to -0,07004	Yes	****	<0,0001	0,1933	0,3017
Plcxd2							
P4 vs. P7	-0,01402	-0,04633 to 0,01829	No	ns	0,7968	0,06392	0,07794
P4 vs. P9	-0,06462	-0,09605 to -0,03319	Yes	****	<0,0001	0,06392	0,1285
P4 vs. P14	-0,05919	-0,09831 to -0,02008	Yes	**	0,0011	0,06392	0,1231
P7 vs. P9	-0,0506	-0,07963 to -0,02157	Yes	***	0,0002	0,07794	0,1285
P7 vs. P14	-0,04517	-0,07981 to -0,01054	Yes	**	0,0054	0,07794	0,1231
P9 vs. P14	0,005428	-0,02678 to 0,03764	No	ns	0,9978	0,1285	0,1231

Supplementary Table 2 – Two-way ANOVA followed by Sidak's multiple comparisons test to compare, for each time point, the expression of *Gpr158* with the expression of *Plcxd*2.

Sidak's multiple comparisons test	Mean Diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P Value	Mean 1	Mean 2
Gpr158 - Plcxd2							
P4	0,05441	0,02527 to 0,08355	Yes	****	<0,0001	0,1183	0,06392
P7	0,08777	0,05713 to 0,1184	Yes	****	<0,0001	0,1657	0,07794
P9	0,06474	0,02405 to 0,1054	Yes	***	0,0006	0,1933	0,1285
P14	0,1786	0,1377 to 0,2195	Yes	****	<0,0001	0,3017	0,1231



Supplementary Figure 1 - ***Gpr158* and *Plcxd2* expression is spatially segregated in the mPFC**. *Gpr158* (cyan), *Plcxd2* (red), DAPI (grey). Dashed lines delineate different cortical layers, L_I - Layer I, $L_{II/III}$ - Layers II/III, L_V – Layer V.



Supplementary Figure 2 – RNA expression pattern of the glypican family members. For each glypican, images on the left represent RNA expression pattern and images on the right represent false colouring by object pixel intensity. Delineated areas correspond approximately to the VPM nucleus of the thalamus. Hybridization in situ data retrieved from the Allen Mouse Brain Atlas.