Excess dopamine D2R activation accounts for PV+ basket cell and learning alterations in mouse model of schizophrenia

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
Der Universität Basel

von

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aus Brasilien

Basel, 2017

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf

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Basel, den 19.04.2016

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Acknowledgements

I want to thank several important persons, who contributed directly and indirectly to my thesis. First of all, I want to thank my family, specially my father and my grandma, who were always there for me, giving me love and cheering for everything to go well. Above all, I want to thank my mom, who was and always will be my example of human being, with her limitless love and attention to people, which I try so much to copy and apply in my life.

I want also to thank my PhD advisor, Pico Caroni. Certainly, I learned a lot from him and for that I'm hugely in debt. The four/five years in his lab were very intense, but I sure enjoyed the time and the lessons I learned here will be important for the rest of my life.

I want to thank my thesis committee, Prof. Botond Roska and Prof. Andreas Lüthi, who spend their time helping me in the development of my thesis. I'm very grateful for their commitment and sure both of them are among my highest appraisal as scientist. I wanted to thank in special Prof. Botond Roska, for the time and patience as the co-referee and also for being so kind in sending the reference letters.

I want to thank all the Caroni lab members, from past and present. Thank you Mike, Kerstin, Melanie, Ana Carolina, Annapoorani, Arghya, Maria Lahr, Maria Spolidoro, Roberta, Matteo, Komal, Manxia, Lan, Lisa, Olga and Sebastian for the good scientific environment. Especially, I want to thank Melissa and Ananya for not only being my colleagues, but more than special friends; you two were there in the good and bad moments and a lot of my PhD I owe to your friendship; thanks for the trips, talks, hugs and extending me the hand when I needed; not only you were good friends, but amazing colleagues that gave me all the scientific stimulation any top class scientist would beg for. I want also to thank specially to Francesco and Flavio, who were exceptional colleagues and friends and helped me discussing the results and always giving me nice inputs.

I owe a lot to my friends, for being my family in Switzerland. I want to thank specially Noelia and Aya for the advices, laughs, beers and trips. A big "thank you" to the Italian group: Cristiana, Cristina, Serena, Maria Anna, Paolo and Giacomo. Also, thank you my friends, Shany, Yann, Julia, Julia, Lisa and Hassana. Also, to my friends in Brazil, thank you, Juliana, Patrícia and Kris.

I want to thank my Swiss family, Christine and Hanspeter for "adopting" me as their fifth kid!

Preface

To date, the field of learning and memory has gathered an enormous amount of data concerning how the memory is acquired, stored, transferred from different regions in the brain and retrieved when necessary. Several regions are implicated in this process, the hippocampus and frontal cortices being the most obvious regions. However, very few advances were made in the understanding of the impairments observed in several psychiatric diseases, especially in schizophrenia.

In my doctoral thesis, I have used chemogenetics, pharmacology and animal behaviour to understand which region in the brain orchestrate the deficits observed in a mouse model of schizophrenia and which signalling pathway is involved in the dysfunction of a particular type of neuron, the parvalbumin-positive basket cell. I describe how the impairments are associated with a specific problem in a subtype of those interneurons and how it is possible for those neurons to be temporarily rescued by pharmacological treatments. Moreover, I demonstrate how this mechanism of rescue can be reverted and applied to wild type animals and induce the same type of impairments faced by the schizophrenic mutants.

This work implicates one of the mechanisms by which antipsychotic medication work alleviating positive symptoms. It also brings to light which region of the brain is involved in the rescue of the impairments and it confirms that the dopamine type 2 receptor is intimately involved in the pathogenesis of the disease.

Abbreviations

22q11DS – 22q11 deletion syndrome

AAV – adeno-associated virus

ADHD – attention deficit/hyperactivity disorder

ASD – autism spectrum disorder

BrdU – 5-bromo-2'-deoxyuridine

CA(1-3) – corpus ammonis (1-3)

cAMP – cyclic adenosine monophosphate

C22orf29 – chromosome 22 open reading frame 29

cFC – contextual fear conditioning

CGE – caudal ganglionic eminence

CLTCL1 – clathrin heavy chain-like 1

COMT – catechol-O-methyltransferase

D(1-5)R – dopamine D(1-5) receptor

del22q11 – monoallelic microdeletion of the chromosome 22q11

DGCR6L - DiGeorge syndrome critical region-6-like

DGCR8 – DiGeorge syndrome critical region 8

dHP – dorsal hippocampus

DISC1 – disrupted in schizophrenia 1

DREADD – designer receptor exclusively activated by designer drug

FOR – familiar object recognition

GABA – gamma-aminobutyric acid

GAD67 – glutamate decarboxylate (isoform 67 kDa)

KO – knockout

LCR – low copy repeat

LgDel – Large Deletion

LGE – lateral ganglionic eminence

MAM – methylazoxymethanol

Mb – megabase

MGE – medial ganglionic eminence

miRNA – microRNA

NMDA – N-methyl-D-aspartate

PBS – phosphate buffered saline

PDE-4 – phosphodiestarase-4

PFA – paraformaldehyde

PNN – perineuronal net

POMC – pro-opiomelanocortin

PKA – protein kinase A

PPI – prepulse inhibition

PrL – prelimbic cortex

PSAM – pharmacologically selective actuator module

PSEM – pharmacologically selective effector molecules

PV – parvalbumin

pri – primary

RFP – red fluorescent protein

RSG – granular region of retrosplenial cortex

SST – somatostatin

vHP – ventral hippocampus

VIP – vasoactive intestinal polypeptide

VTA – ventral tegmental area

WT – wild-type

1. Introduction

1.1. Schizophrenia

Schizophrenia is a heterogeneous syndrome, which do not present a particular and exclusive defining symptom. Traditionally, it is diagnosed by exclusion and when the patient presents psychotic phenomena, as hallucinations, delusions as well as thought disorder. By definition, the disease presents three main symptoms: positive (hallucinations, delusions), negative (anhedonia, social withdrawal) and cognitive symptoms (disorganised speech and thought). Schizophrenia is a highly heritable disease, with a heritability score of 0.8 (the score ranges from 0-1, where 0 indicates that none of the variability between people in a study are due to genetic factors, and 1 the opposite) and, affecting around 1% of the population worldwide (reviewed in Ross et al., 2006).

The term schizophrenia was coined by the Swiss psychiatrist Paul Eugen Bleuler in the early twentieth century, substituting the previous term *dementia praecox* coined by the German psychiatrist Emil Kraepelin, in the late nineteenth century. The term derives from Greek roots, which can be translated as “splitting of the mind”, which Bleuler intended to describe as the four ‘a’s-disturbances of association, affect, ambivalence and autistic isolation. With the split of neurology and psychiatric, for most of the twentieth century, influenced by the psychoanalytic theory, the concentration of studies on the mind ignored the study of the brain. In contrast to most of the other psychiatric diseases, schizophrenia presents a severe lag in its neurological treatment.

The disease typically presents late onset and the abnormal mental functions and disturbed behaviour, characteristically appearing in the late second and third decades of life. Schizophrenia presents 1% lifetime incidence, independent of genre, culture and racial groups (Bromet and Fennig, 1999). However, schizophrenic patients tend to be more prevalent in lower

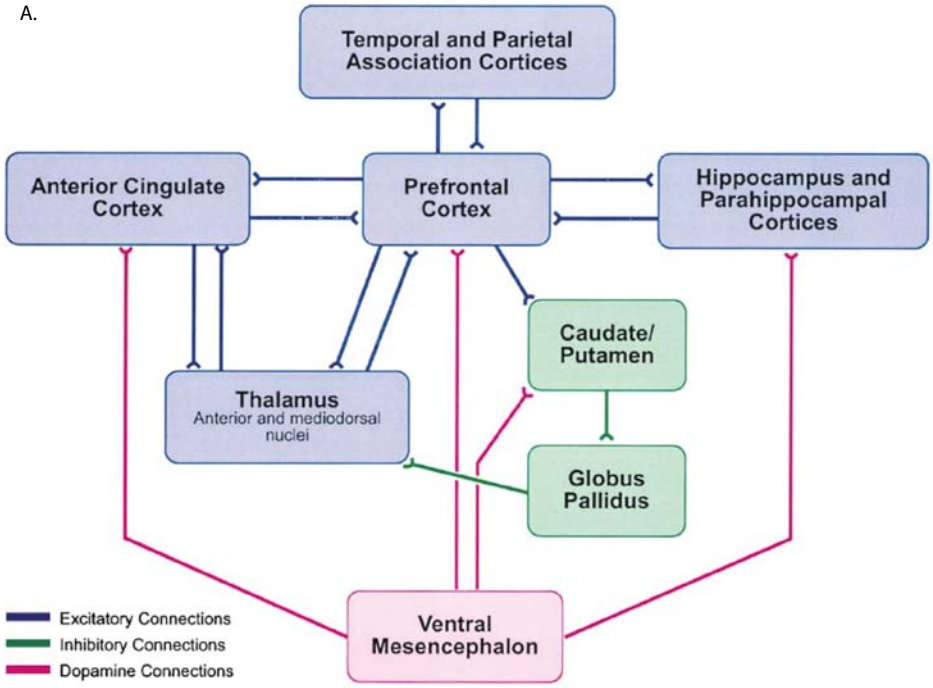
socioeconomic strata and in urban areas (Mortensen et al., 1999). Before the disease fully develops, individuals present a prodromal phase, which are usually attenuated positive, negative and cognitive symptoms. However, it is still elusive how a syndrome with high heritability score presents such delay in eliciting clinical symptoms. Only recently, initial evidence pointed out to the role of two-hit stress component in the development of neuropsychiatric diseases (Giovanoli et al., 2013). In this study, the authors provided evidence that infections during pregnancy, followed by stress insults during adolescence, gave rise to consistent behavioural deficits similar to those observed in model mice for schizophrenia.

Structural brain abnormalities have also been documented in patients with schizophrenia, such as enlargement of the ventricles and reduced volume of cortical grey matter. These changes are, however, restricted to certain structures in the brain, especially the prefrontal cortex, and limbic areas, such as the hippocampal formation and the anterior cingulate cortex (Figure 1A). Additionally, independent groups observed that cell body size and synaptic/dendritic markers are decreased in several regions of the hippocampus (Arnold et al., 1995; Zaidel et al., 1997; Weinberger, 1999).

The structural changes in the prefrontal cortex are less robust then the ones observed in the hippocampal formation. Studies using both functional magnetic resonance imaging and electrophysiological techniques begin to elucidate the cellular physiology of the abnormal cortical activation in schizophrenia. Patients with schizophrenia present increased noise in prefrontal cortical information processing circuits (Figure 1B). Although not conclusive, those data suggest that the greater cortical response variability, i.e. noise, would be reflected as less focused activity or circuit inefficiency (Callicott et al., 2003). Several studies focused on this structure, motivated by the observation that

subjects with schizophrenia present poor performance in cognitive tasks. Working memory in these patients is particularly impaired, as evidenced by the decreased performance in the Wisconsin Card Sort Task (Weinberger et al., 1986). Since the cognitive deficits seem to be correlated to the long-term prognosis of the disease, the study of the disturbances in the prefrontal cortex is paramount, specially due to the lack of efficient treatments for the disease.

A.



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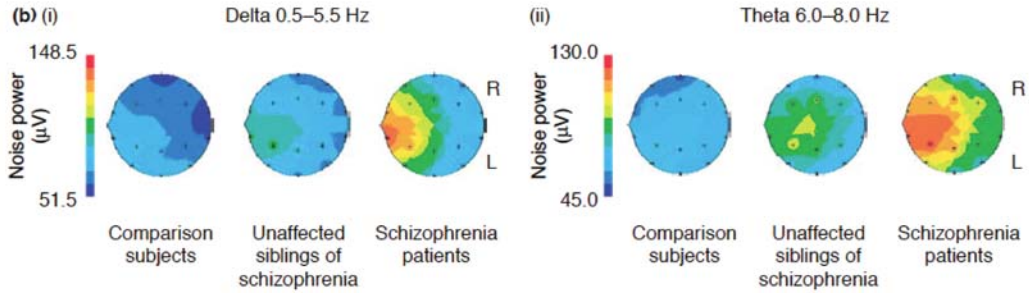

Figure 1 – Structural and functional brain abnormalities in schizophrenia.
A. Main affected brain regions in schizophrenia. Hippocampal formation and prefrontal cortex present decreased markers of synaptic connectivity; dysregulation in dopaminergic transmission, especially in the prefrontal cortex. Adapted from Lewis and Lieberman, 2000.
B. Abnormal cortical signal-to-noise pattern in schizophrenia. Increased prefrontal response variability, evidenced by eletroencephalogram. Topographic maps of event-related eletroencephalogram during an auditory oddball task, showing increased noise in schizophrenic patients in delta and theta frequency bands. Adapted from Winterer and Weinberger, 2004.

1.2. The 22q11 deletion syndrome

The most common human genetic deletion syndrome is the monoallelic microdeletion of the chromosome 22q11 (del22q11), affecting one in 2,000-4,000 live birth (Jonas et al., 2014). This syndrome is highly variable, but most of the defects observed are due to developmental defects of the pharyngeal apparatus. During embryogenesis, the pharyngeal arches are symmetrical structures, which develop in a segmented fashion, following the anterior-posterior axis of the embryo. In the 22q11 deletion sydrome (22q11DS), approximately 75% of the patients present congenital heart defects, thymic hypoplasia, velopharyngeal dysfunction (with or without cleft palate), hypocalcemia (due to hypoparathyroidism). However, for the sake of this thesis, I will focus on the problems in the 22q11DS related to behavioural and psychiatric disorders.

The 22q11DS is associated with an elevated risk for several neuropsychiatric diseases, especially psychosis. Approximately 25-35% of the patients diagnosed with this syndrome develop schizophrenia or other psychotic illnesses, making the 22q11DS the highest known risk factor for schizophrenia (Jonas et al., 2014). Although schizophrenia appears in much higher frequency among 22q11DS relative to other neurogenetic and developmental disorders

associated with intellectual disability, one third to half of the children are diagnosed with attention-deficit/hyperactivity disorder (ADHD), anxiety and mood disorders, and autism spectrum disorders (ASDs).

The monoallelic deletion is typically of 3 megabases (Mb; corresponding to 90% of the patients) or 1.5 Mb (8% of the patients); the typical human deletion encompasses 60 genes and the smaller 1.5 Mb deletion, 35 genes (Karayiorgou et al., 2010; Figure 2). Of those genes, half (51.1%) are protein-coding and most of those genes are expressed in the brain (89.1%; Guna et al., 2015). These genes are known to affect early neuronal migration and cortical development (Guna et al., 2015). Additionally, 90% of the cases of 22q11DS are *de novo*, whereas 10% of the cases are inherited in an autosomal dominant fashion. *De novo* cases are due to mispairing of low copy repeats (LCRs) during meiosis (Emanuel and Shaikh, 2001), which are regions of great instability in the genome and particularly enriched in the 22q11 locus (Emanuel and Shaikh, 2001). The mouse syntenic region of the human proximal deletion (1.5 Mb deletion) is located in the chromosome 16 (MMU 16qA13) and contains 27 of the 30 human protein-coding genes; the exception being clathrin heavy chain-like 1 (*CLTCL1*), chromosome 22 open reading frame 29 (*C22orf29*), and DiGeorge syndrome critical region-6-like (*DGCR6L*).

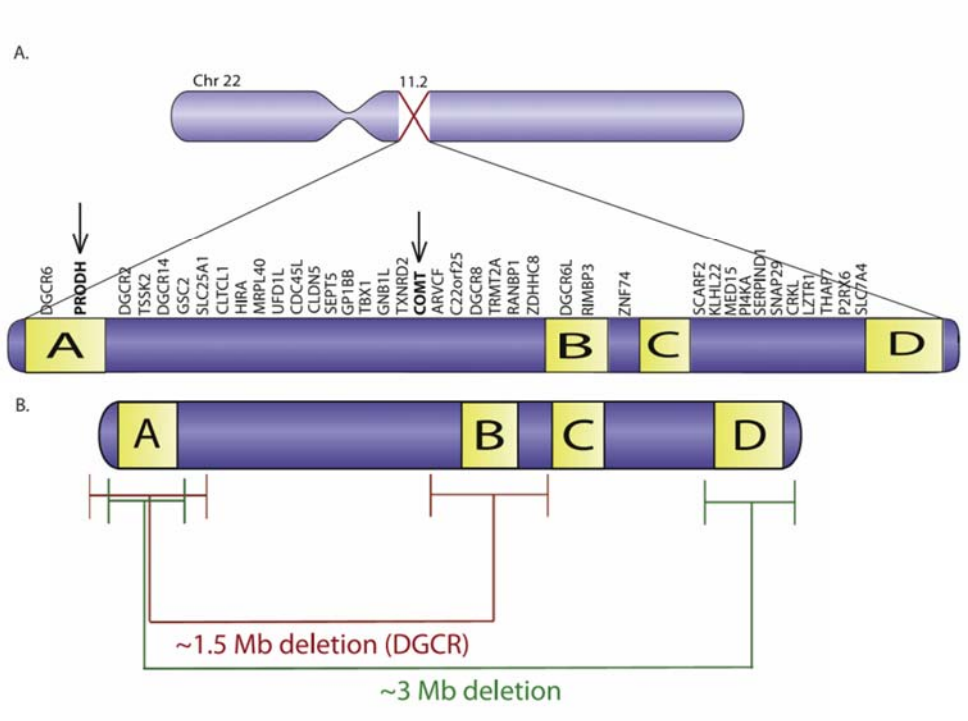

Figure 2 – The 22q11 hemyzygous deletion.

A, B, C and D are common breakpoints on the chromosome.

A. Genes deleted in the 22q11.2 locus. Prodh and COMT, two main genes, are highlighted in bold.

B. The 1.5 Mb deletion, present in the *LgDel/+* model, and the 3 Mb deletion, common in humans. Adapted from Jonas et al., 2014.

In the 1.5 Mb region, syntenic with the mouse models for 22q11DS, are present important protein-coding genes, thought to be related with schizophrenia. Catechol-O-methyltransferase (*COMT*) is a strong candidate, as it encodes for the enzyme necessary for catecholamine degradation and the main genetic variant being associated with schizophrenia is functional. COMT exists in a soluble and a membrane-bound form, and catalyses the methylation of catechols, such as dopamine, norepinephrine, and catecholoestrogens (Myöhänen et al., 2010). In the brain, the membrane-bound COMT predominates, and in the periphery is the soluble form. The brain-enriched form of COMT presents increased affinity for catechols, but low capacity, suggesting a role in neurotransmission. Although initial studies identified COMT as mainly a glial

enzyme, recent studies suggest that it is expressed primarily in neurons and with more abundantly in the prefrontal cortex and hippocampus (Myöhänen et al., 2010). COMT seems to be implicated in cortical interneuronal monoaminergic signalling, especially dopamine (Kimoto et al., 2012).

Another important gene in this locus is the DiGeorge syndrome critical region 8 (*DGCR8*) gene, which is a key component of the microprocessor complex of microRNA (miRNA) production, encoding for the miRNA-processing protein Pasha. MiRNAs are small non-coding RNAs that regulate gene expression at the posttranscriptional level through translational inhibition and destabilisation of their target mRNA. In brief, miRNAs are originated from primary (pri)-miRNAs and this processing is done by the RNAse III Drosha. Drosha is insufficient for substrate binding, and Pasha presents a RNA recognition function, forming henceforth the microprocessor (Han et al., 2009). *Dgcr8* knockouts (KO) fail in producing miRNAs in ES cells, resulting in defects in proliferation and differentiation, and the KO embryos arrest in the development (Wang et al., 2007). Importantly, postmortem analysis of brains of schizophrenic patients demonstrated altered miRNA expression profiles. Moreover, the 22q11 locus presents a high density of miRNAs in which three of the seven miRNAs are expressed in the brain (MIR185, MIR1306, MIR1286).

Finally, another important gene in the del22q11 is Zdhhc8, a palmitoyltransferase. Protein palmitoylation is the addition of the saturated 16-carbon palmitate lipid at specific cysteine residues by a liable thioester bond and has emerged as a key reversible posttranslational protein modification involved in the protein trafficking and the regulation of diverse membrane and cytosolic proteins, especially in neurons. Recently, it was demonstrated that Zdhhc8 palmitoylates several important proteins for axonal development, and its

deficiency is linked to decreased axonal arborisation and connectivity. Those deficits were shown to impair prefrontal-hippocampal synchrony and thus disrupting spatial working memory (Mukai et al., 2015). Those results corroborate to an understanding that the connectivity deficiency observed in the 22q11DS, along with the other problems aforementioned, may be the result of an impairment in protein palmitoylation, leading to decreased short- and long-term connectivity between neurons.

Taken together, the genes of the deleted site of del22q11 are very important to the development of the neurons and to control several synaptic and cellular events (Figure 3). It was observed that the children with this syndrome present a lower intelligence quotient than typical for the ages analysed, and in most cases present verbal delay and decreased attention. The attention deficit seems to impair other functions, as visual-spatial memory and space navigation, and is also important for inhibiting the processing of irrelevant stimuli. Patients with the syndrome present deficits in the inhibitory function, observed by pre-pulse inhibition test (the patient need to suppress the startle after an almost imperceptible warning tone before the aversive noise).

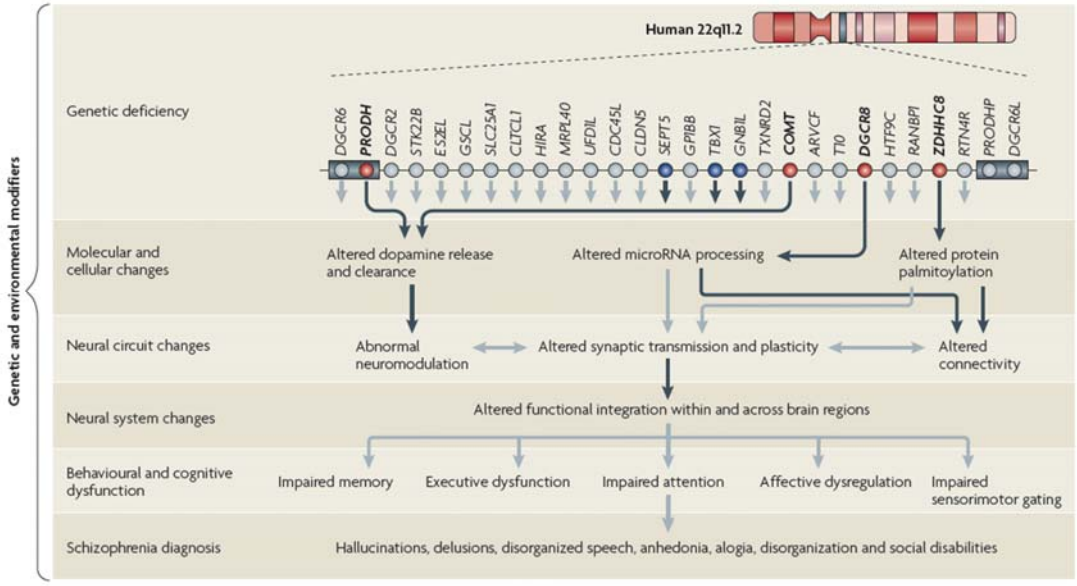

Figure 3 – Pathogenesis and pathophysiology of 22q11.2DS.
Decreased dosage of the genes in the 22q11.2DS and their relation with brain function. Black arrows represent experimentally determined effects; grey arrows represent inferred effects. Genes marked in red have been characterised at molecular, cellular and behavioural levels. Genes marked in blue have been characterised only in the behavioural level.
Adapted from Karayiorgou et al., 2010.

1.3. Dopaminergic system

After the discovery of neuroleptic drugs, the focus on the disturbances in the brain chemistry related to the disease became the emphasis. The *dopamine hypothesis of schizophrenia* became the main explanation for the disease, which was at the time considered impairment in dopamine regulation. The psychotomimetic effects of dopamine-releasing drugs and the countering effects of anti-psychotic drugs that blocked dopamine D2 receptor helped this hypothesis to gain momentum. However, few advances were made in drug development to treat the symptoms of schizophrenia, which traditionally can remediate the positive symptoms, being inefficient against the other symptoms. When compared to haloperidol and chlorpromazine, the recent drugs present fewer

extrapyramidal side effects, but they fail to be more efficacious and to treat the debilitating negative and cognitive symptoms (Dayalu and Chou, 2008).

Based on the improvements in the positive symptoms by the treatment with antipsychotic medications, the dopamine hypothesis of schizophrenia became the main explanation for the positive symptoms. For example, hallucinations and delusions, with a typical onset in the late adolescence and early adulthood, correlate with the hyperactivity of dopamine transmission.

The dopaminergic system is mainly localised in the midbrain, where most of the neurons are located in the ventral tegmental area and in the *substancia nigra* pars compacta. These neurons send extensive projections to several forebrain areas, but especially to the nucleus accumbens and caudate putamen. They also project to the hippocampus, amygdala and other cortical areas. Dopaminergic functions are traditionally associated with motor control and reward system, based on two main observations: the movement impairments observed in Parkinson's disease due to dopamine depletion, and the modulation of behavioural responses to motivated stimuli when interfering in the dopaminergic signalling. Dopaminergic neurons present a phasic firing pattern that coincides with reward presentation, whether predicted or unpredicted. Those neurons work during reward-prediction and, by consequence, in associative conditioning. This firing pattern is reduced, however, when the event is fully predictable. The difference in the expected reward and the present reward gives rise to the reward-prediction error, which is essential for reward-driven learning (Cohen et al., 2012; Schultz, 2007; 2013).

During aversive learning, however, the dopamine firing rate decreases. This is due to increased firing rate of inhibitory GABAergic interneurons in the ventral tegmental area (Cohen et al., 2012). This body of data suggests that

phasic dopaminergic activity is elicited mostly by non-expected rewards and is inhibited in aversive stimulus. Some dopaminergic neurons are activated during aversive stimulus, although they appear to be a nonspecific responding population, as they also fire during reward presentation. However, sustained aversive stimuli or stressors increase the number of dopamine neurons firing over longer periods of time (Lisman et al., 2011).

Alterations in the temporal firing pattern of dopaminergic neurons are correlated to information coding. The tonic and phasic firing are respectively synonymous of single-spike and burst firing patterns. They are related to temporal changes in firing activity, but not to firing patterns. Hence, phasic firing can be related to rapid fluctuations in dopamine concentration, which takes place in seconds, and tonic, to slow changes that occur in minutes or hours (Marinelli & McCutcheon, 2014).

The actions of the dopamine are exemplified by its actions on the medium spiny neurons of the striatum. Two subtypes of those neurons, expressing two different dopamine receptors, are embedded in a network involving distinct types of interneurons and are influenced by dopamine signalling. The dopamine receptors are found in the dendrites of those cells and can be divided in two systems: the D1, direct striatonigral pathway; and the D2, indirect striatopallidal pathway. The striatonigral medium spiny neurons present high expression of dopamine D1 receptor coupled with a $G_{s/olf}$ protein, which activates adenylyl cyclase. When activated, the levels of cyclic adenosine monophosphate (cAMP) are increased, leading to the activation of protein kinase A (PKA).

In contrast, the stratopallidal medium spiny neurons present high expression of dopamine D2 receptors, which are coupled to a $G_{i/o}$ protein, thus inhibiting adenylyl cyclase via $G\alpha_i$. The dopamine D2 receptors (D2R) present an

extensive list of possible targets, as the remaining Gβγ subunit can stimulate phopholipase Cβ, which leads to protein kinase C activation (Beaulieu and Gainetdinov, 2011).

The D2R are part of the D2-like receptor family and are present in both pre- and post-synapse. This receptor can be present in homomers or in heteromers with D1R and D5R (Hasbi et al., 2010), D3R (Maggio & Milan, 2010), somatostatin SST_5 receptor (medium aspiny neurons in striatum and pyramidal cells in cortex; Rocheville et al., 2000) and adenosine A_{2A} receptor (in GABAergic enkephalinergic neurons of striatum; Ferré et al., 2010). Additionally, this receptor presents two alternate splicing isoforms, the short and the long. The two isoforms present different roles in the D2R physiology. The long isoform appears to modulate the signalling pathways involving PKA, while the short isoform is a *bonafide* autoreceptor, which mediates dopamine synthesis and release, as being required for the motor and rewarding effects of cocaine (De Mei et al., 2009; Bello et al., 2011).

1.4. Antipsychotic drugs and schizophrenia

Typical antipsychotic agents vary in the D2R affinity, being classified as high or low potency. Haloperidol, a prototypical high potency typical neuroleptic, is still one of the most used antipsychotic drugs in the therapy of patients with both acute and chronic schizophrenia. In the clinic is largely used as an acute treatment for schizophrenia, for immediate control of patients with psychosis-related violent behaviour. Haloperidol is extensively metabolised in the liver and it presents its peak plasma concentration 20 minutes after administration in healthy individuals and 33.8 minutes in patients with schizophrenia and has a long-lasting effect, with a half-life of about 20 hours (Kudo & Ishizaki, 1999).

Haloperidol produces rapid effects on locomotion in the rats, being catalepsy the most common, which is associated with antagonism of dopamine receptors in the forebrain (Campbell et al., 1982). The effects are long-lasting, specially due to the fact that haloperidol has a long half-life in the brain of rats (at 1 mg/kg the half-life is 6.6 days); neuroleptics present favourable distribution in the brain, being up to 40 times more concentrated than in the blood (Cohen et al., 1992). Based on positron emission tomography data, D2R in human brain become unoccupied at a rate of approximately 10% per day following a near-saturating single dose of haloperidol (Cohen et al., 1992). In schizophrenic patients, the clinical response of antipsychotic is given when the D2R occupancy reaches 65%, and the side-effects (extra-pyramidal side effects) take place when the occupancy reaches 78% (Kapur et al., 2000).

The actions of haloperidol are not completely understood, although for more than sixty years the drug is being used in the treatment of schizophrenia and managing aggressiveness of frontotemporal dementia. Haloperidol is known to elicit changes in cFos mRNA and protein expression in the caudate-putamen, which is supposed to subserve the extra-pyramidal side effects observed during haloperidol treatment (Deutch et al., 1996), but not the therapeutic antipsychotic effects. Extra-pyramidal side effects are bothersome and often debilitating symptoms caused by typical and some atypical antipsychotic drugs. The main symptoms in the acute syndrome are dystonia (sustained muscle activity), akathisia (restlessness) and parkinsonism (tremors, rigidity, bradykinesia, and postural instability) and in the tardive syndrome are tardive dyskinesia (choreic movements of the mouth, limbs, trunk and face) and tardive dystonia (Dayalu and Chou, 2008). Conversely, the ventral striatum and limbic areas extensively innervated by dopaminergic neurons (such as the entorhinal cortex), would be

related to the antipsychotic effects. Comparing haloperidol, which presents both antipsychotic effect and extra-pyramidal side-effect, with metoclopramide, which presents only extra-pyramidal side effects, it was shown activation of caudate-putamen with both drugs, but cFos increment in the limbic areas only by haloperidol treatment (Deutch et al., 1996).

Recently, the glutamatergic hypothesis of schizophrenia gained momentum, influenced by human studies with the N-methyl-D-aspartate (NMDA) receptor antagonist ketamine. Ketamine produces psychotic symptoms and negative symptoms, thought disorder and cognitive impairments, consistent with the core symptoms of schizophrenia (reviewed in Moghaddam and Krystal, 2012). The effects of NMDA non-competitive antagonists to impair cognitive function in rodents and monkeys have been intensively studied as an animal model of the cognitive deficit in schizophrenia and hypoglutamatergic activity has been implicated as a major cause of the cognitive impairment in the disease. However, to date, no antipsychotic medication was developed based on the premise of countering the effects of NMDAR hypofunction in schizophrenia.

1.5. Circuitry associated with the pathogenesis of schizophrenia

The firing of dopaminergic cells is modulated by GABAergic inputs from the ventral pallidum, which leads to the majority of the dopaminergic neurons be silent at baseline. Activation of ventral subicullum, leads to excitation of nucleus accumbens, inhibiting the ventral pallidum. The result is reduced inhibition of dopaminergic neurons and thereby increased number of tonically firing cells. Tonically firing neurons, but not silent neurons, can be driven into a bursting mode by stimuli such as those associated with reward (Lisman et al., 2011). Thus, the effect of electrical or chemical stimulation in the ventral hippocampus

leads to increased extra-synaptic dopamine throughout the nucleus accumbens, a principal target of dopaminergic neurons.

The hippocampus is consistently implicated in the progression of schizophrenia. Imaging studies provided evidence that patients cannot recruit the hippocampus during a task (memory recall; Heckers et al., 1998; Weiss et al., 2003). This study showed that hippocampal baseline activity at rest is increased, but not upon recall of the memory. Interestingly, in healthy individuals several areas are recruited during the task, such as retrosplenial cortex, parahippocampal area and hippocampus. In patients with schizophrenia, only the right prefrontal areas are recruited (Heckers et al., 1998). The CA1 field in the hippocampus present increased activity at rest in chronic patients with schizophrenia, as well as in prodromal patients, and it is directly correlated with clinical measures of psychosis (Schobel et al., 2009). Some authors propose the hippocampus as the source of the impairment in dopaminergic regulation in schizophrenia (Lisman et al., 2008; Lodge and Grace, 2011).

Postmortem studies confirm decreased hippocampal volume. In a large study about morphological changes in the medial temporal structures, patients with chronic schizophrenia presented bilateral reduction in hippocampal volume, but not in the amygdala (Nelson et al., 1998; Velakoulis et al., 2006). Interestingly, the reduction in volume is specific to grey matter, but not white matter, suggesting loss of neurons in the hippocampus (Gur et al., 2000). However, in the biggest case study to date, using more than 30,000 patients across several countries, did not find a causal relationship between subcortical volumes and schizophrenia risk (Franke et al., 2016).

In agreement with the findings in humans, a model of schizophrenia in rodents uses ventral hippocampus lesions to emulate the disease. Neonatal

hippocampal lesions involve ibotenic acid lesions of the ventral hippocampus and subicullum, regions that project to the prefrontal cortex (Lipska and Weinberger, 2000). In this model is observed frontal lobe abnormalities, dopaminergic system dysregulation and changes in molecular markers in the prefrontal cortex (decreased GAD67 and brain-derived neurotrophic factor mRNAs). In line with this model, the methylazoxymethanol (MAM) model present further evidence for the involvement of the medial temporal function in the onset of psychosis (Modinos et al., 2015). The methylating agent MAM further evidenced the role of a hippocampal-midbrain-striatal circuit, ratifying the concept that subcortical dopaminergic function is elevated due to the descending medial temporal lobe connection (Modinos et al., 2015).

Taken collectively, the results shed light on the role of ventral hippocampus in the regulation of the dopaminergic system. This regulation does not present a direct innervation of the two main structures (from ventral hippocampus to ventral tegmental area; Figure 4). The hippocampus is thus related to detecting novelty and then engaging the burst firing of cells in the VTA (Lodge and Grace, 2005; 2007). According to this model the impairments in the hippocampus would lead to the hyper-responsive dopamine system of the schizophrenic patients. The main player appears to be a specific type of interneuron, the parvalbumin (PV)-positive basket neurons.

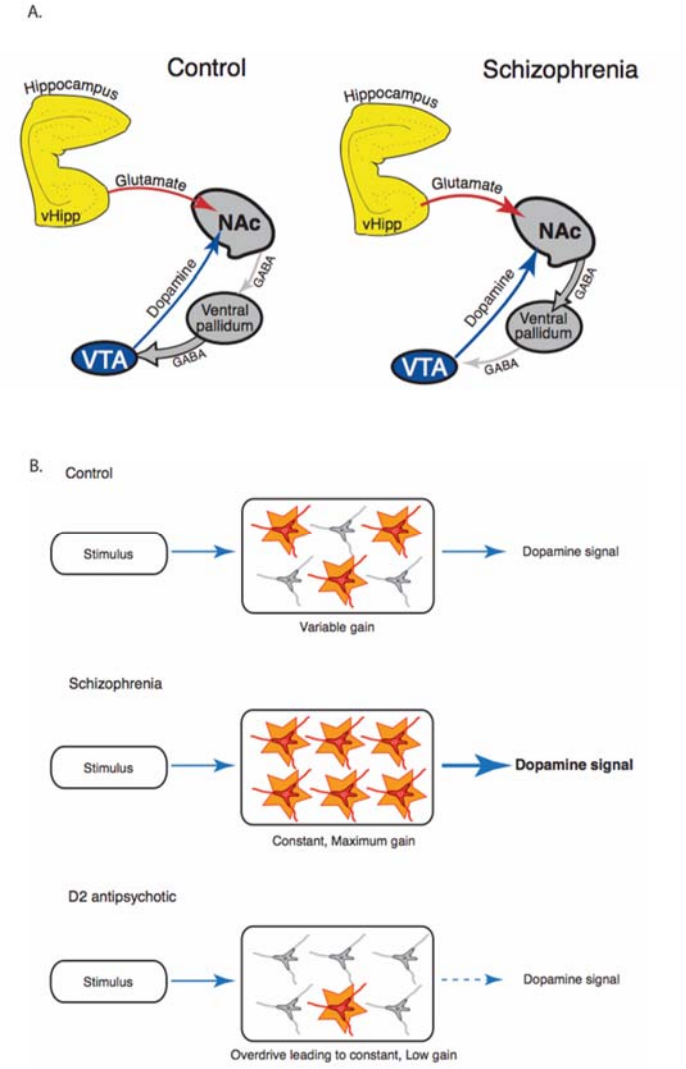

Figure 4 - Ventral hippocampus regulation of VTA and dopamine neuron activation.

A. The main hypothesis in the field suggest that the control of VTA dopaminergic activity is given by a polysynaptic projection from ventral hippocampus to nucleus accumbens, which in turn inhibits the ventral pallidum. This structure then inhibits VTA. Consequently, activation of vHP leads to increased dopamine neuron activity. In schizophrenia, an abnormal increment in activity of vHP leads to increased dopaminergic activity.

B. In the upper part of the panel, in healthy individuals, a number of dopamine neurons will be recruited during a given stimulus. In schizophrenia, the same stimulus elicit the recruitment of higher numbers of dopaminergic neurons, enhancing the dopamine signal. Due to depolarization block action of antipsychotic drugs, decreasing the final dopamine signal.

Adapted from Lodge and Grace, 2011.

1.6. Development of interneurons and role in schizophrenia

Interneurons are a highly diverse neuronal population, arising from structures in the subpallium in the developing telencephalon. They migrate tangentially through long distances and in multiple streams, to reach their destinations, whether to the neocortex, striatum, olfactory bulb, and to the hippocampus. Postmitotic interneurons arise from the ventricular zones of the ganglionic eminences; the progenitor pools are the lateral GE (LGE), medial GE (MGE), caudal GE (CGE), ventral pre-optic area and the septal anlage of the subpallium. After proliferating in the ventricular zones, the postmitotic neurons migrate dorsally to the neocortex, ventrolaterally to the striatum, rostrally to the olfactory bulb and caudally to the hippocampus (Guo and Anton, 2014).

The MGE is the sole site of generation of PV-positive fast-spiking basket neurons (Wonders and Anderson, 2006; Guo and Anton, 2014). In the cortex, an early stream of interneurons (~E11.5 in the mouse) from the MGE migrate dorsolaterally onto the top of the preplate, where many become layer I Cajal-Retzius neurons (a heterogeneous population of glutamatergic interneurons in layer I of neocortex, expressing *reelin*; Hevner et al., 2003). Those cells are important for the secretion of reelin, a glycoprotein involved in the maintenance of the radial glia phenotype. Patients with schizophrenia were shown to present decreased levels of reelin, in multiple brain regions (Folsom and Fatemi, 2013). Additionally, in rodent models, knockdown of reelin in medial prefrontal cortex during puberty or adulthood was shown to impair prepulse inhibition (PPI); the knockdown during puberty only, lead to impairments in spatial working memory and object recognition (Brosda et al., 2011).

Later during corticogenesis (~E13-E15), a second and more prominent stream of interneurons, mainly from the MGE, rapidly migrates into the neocortex,

through the intermediate zone. Those neurons follow a lateral to medial gradient to colonise the cortex, with the younger neurons arriving in lateral portions earlier than in medial domains (Guo and Anton, 2014). Parvalbumin, somatostatin and calbindin positive interneurons arriving from MGE and pre-optical areas show a time-dependent, inside-out pattern, similar to projection neurons. CGE-derived calretinin-positive interneurons present, however, an outside-in pattern.

In the hippocampus, the main source of interneurons to CA3 and CA1 is the CGE (Nery et al., 2002; Yozu et al., 2005). The CA1 region receives also interneurons derived from the MGE, but the hippocampus lack LGE-derived interneurons (Witcherle et al., 2001). To some authors, however, CGE is not properly a structure, since it does not present a sulcus separating it from the LGE and from MGE; it also presents both markers of MGE and LGE. In the "*Large Deletion"* (*LgDel/+*) model of 22q11DS it was observed decreased proliferation of basal progenitors of pyramidal cells and impaired migration of PV-positive interneurons to the cortex (Meechan et al., 2009). According to this study, *LgDel/+* animals presented decreased number of PV-positive interneurons in medial regions (but not lateral) and delayed or impaired interneuronal migration (Meechan et al., 2009).

Recently, some evidence point to the dispersion of interneurons from the MGE to be less coordinated than imagined. The interneurons are generated by asymmetric divisions of the progenitors in the ventricular zone of MGE, giving rise to other progenitors, in the subventricular zone. Those progenitors undergo rapid clonal expansion by symmetric divisions, exiting this region and migrating tangentially, and dispersing throughout several structures, irrespective of their siblings. Therefore, sibling interneurons can populate neocortex, hippocampus, striatum, and globus pallidus (Harwell et al., 2015; Mayer et al., 2015).

Interneurons are responsible for important steps in the development. During an extended period postnatally, GABA present a depolarising effect on principal neurons (Marchionni et al., 2007). A model of schizophrenia, the Disrupted in Schizophrenia 1 (DISC1) knockout mouse, presents decreased duration of the depolarising GABA period and consequent decrement of AKT/mTOR signalling pathway activation and arborisation of principal neurons (Kim et al., 2012). This study sheds light on the convergence of GABAergic control during development and the impact on a model of schizophrenia.

Postmortem studies on patients with schizophrenia provided the first evidence linking PV basket neuron impairment and the disease. In those initial studies, decrement in the number of those cells was observed in several regions of the brain (Figure 5; reviewed in Lewis, 2014). The mRNA levels of both GAD67 and PV are decreased in schizophrenic patients (Straub et al., 2007). How those observations relate to the pathophysiology of schizophrenia and the symptomatology is still elusive.

Recent data point to synaptic deficits in the PV basket neurons in schizophrenia. It was observed an important relation of schizophrenia and Erbb4, a tyrosine kinase receptor expressed majorly in PV basket neurons, which mediates the function of neuregulin-1. Neuregulin-1 knockout mice present decreased number of functional NMDAR, defective short-term synaptic plasticity and long-term potentiation. Behaviourally, the animals also present deficits in PPI and hyperactivity (reviewed in Rico and Marin, 2011). Interestingly, specific deletion of Erbb4 in PV-positive interneurons present decreased excitatory synapses onto them and schizophrenia-related behavioural deficits, especially in PPI, and increased gamma-power oscillations in the hippocampus (Del Pino et al., 2013).

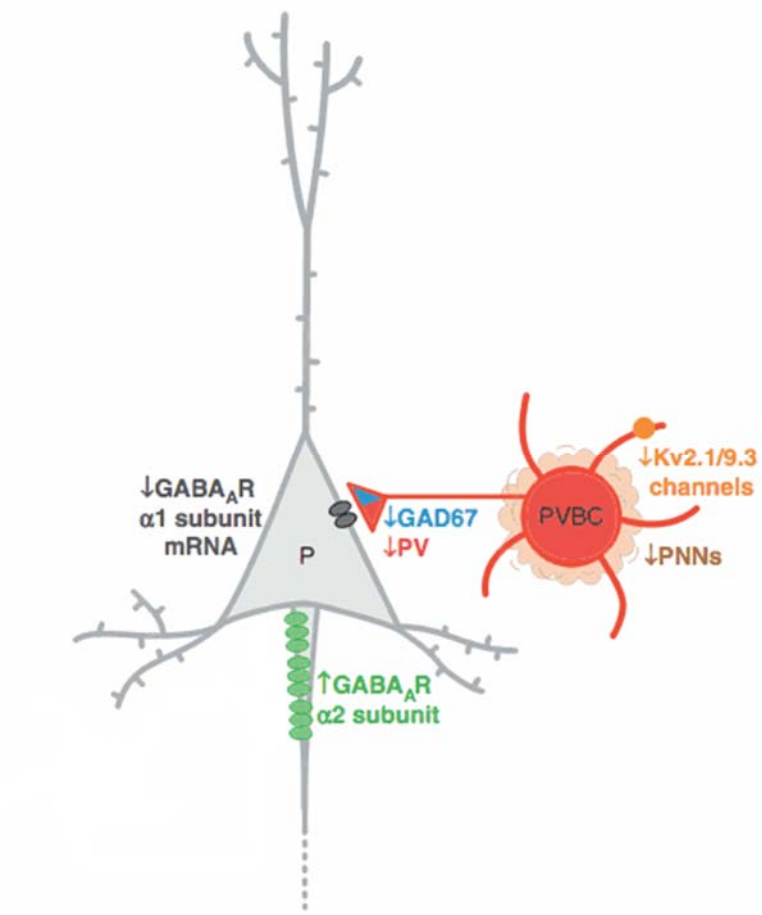

Figure 5 - Changes in PV basket and pyramidal neurons in schizophrenia.
It is reported lower levels of GAD67 enzyme and PV in PV basket neurons, alongside with decrease in the perineuronal nets (PNNs) and in the heteromeric potassium channel Kv2.1/9.3. Pyramidal neurons also present lower levels of $GABA_AR$ alpha1 subunit.
Adapted from Lewis, 2014.

1.7. Regulation of plasticity and learning by PV+ interneurons

Hippocampal and cortical interneurons are responsible for the inhibition of principal cells and also to other interneurons. Different classes of interneurons target distinct compartments in the pyramidal cells, such as proximal or distal dendrites, soma and axonal initial segment (Klausberger and Somogyi, 2008). PV basket neurons target specifically proximal dendrites and soma of pyramidal cells and are involved in feedforward inhibition of the network. Markedly, PV basket neurons fire in bursts after the peak of theta oscillations, in almost opposition to

the activity of pyramidal cells, after the trough of those oscillations (Klausberger and Somogyi, 2008). Theta oscillations are present during memory tasks and spacial navigation, being important for network synchrony.

The action of PV interneurons on pyramidal cells is well described, but only recently, unequivocal data correlated activity of the PV network and learning (Donato et al., 2013). The findings described in this work demonstrate that direct activation or inactivation of PV basket neurons is direct correlated to the outcome of learning paradigms. Moreover, this work led to the finding that PV basket neurons are organised in subpopulations, defined by their birthdate (Donato et al., 2015). More recently, interesting findings from Wolff and colleagues, demonstrated that somatostatin-positive neurons are inhibited by PV basket neurons during conditioned stimulus presentation (Wolff et al., 2014). These findings highlight the function of the PV interneurons as a central player in learning.

Other interneurons PV basket neurons are targeted by vasoactive intestinal polypeptide (VIP) positive interneurons. These interneurons present a pivotal role during learning, since they disinhibit the network, by inhibiting both PV basket neurons and somatostatin-positive neurons (Pi et al., 2013). VIP interneurons are recruited specifically during reinforcement signals and play special role in incremental learning (Donato et al., 2013; Pi et al., 2013).

Pyramidal neurons are composed of different populations of cells. In the hippocampus, the pyramidal cell layer is composed by neurons situated in the deep layers and in superficial layers, which present distinct targets and are generated in different waves of neurogenesis. Lee and colleagues observed that PV basket neurons present preferential connectivity to deeper pyramidal cells in CA1 (Lee et al., 2014). They observed that the neurons in this layer receive more

inhibitory boutons specifically from PV basket neurons, and that neurons projecting to certain areas in the brain are particularly target of PV neurons (Lee et al., 2014).

Along with the different pyramidal neuron subpopulations, PV basket neurons present distinct populations specified during development (Donato et al., 2015). The cells born during early phases (early-born PV neurons) presented higher ratios of excitatory-to-inhibitory synaptic puncta densities, higher levels of PV and GAD67, and targeted preferentially deeper cells (Figure 6). Conversely, cells born during later phases of development (late-born PV neurons) exhibited lower levels of PV and GAD67, higher inhibitory-to-excitatory synaptic puncta ratios, and target especially superficial cells (Figure 6). Functionally, those neurons also presented distinct roles. Early-born PV neurons were specifically plastic during consolidation of validated rules and late-born PV neurons presented plasticity during acquisition of new information (Donato et al., 2015). Taken together, subpopulations of pyramidal cells and PV neurons are differentially involved in learning and in hippocampal functions, possibly being recruited specifically during behaviour.

The early-born subpopulation presents specific roles in the learning process. Those cells are engaged during consolidation, which is evidenced by a shift towards high PV and GAD67 configuration, increased excitatory puncta density and decreased levels of Mef2a, which is a transcription factor that regulates negatively synapse number (reviewed in Caroni, 2015a; b). Those results are in accordance to the previous view that putative populations of basket cells were derived in distinct epochs in the embryo (Ciceri et al., 2013), and establishing niches in the developing neocortex. In this extensive work of Oscar Marín group, the authors observed that interneurons generated in the MGE would

cluster differentially in the deeper or superficial layers, depending whether they were early- or late-born, respectively.

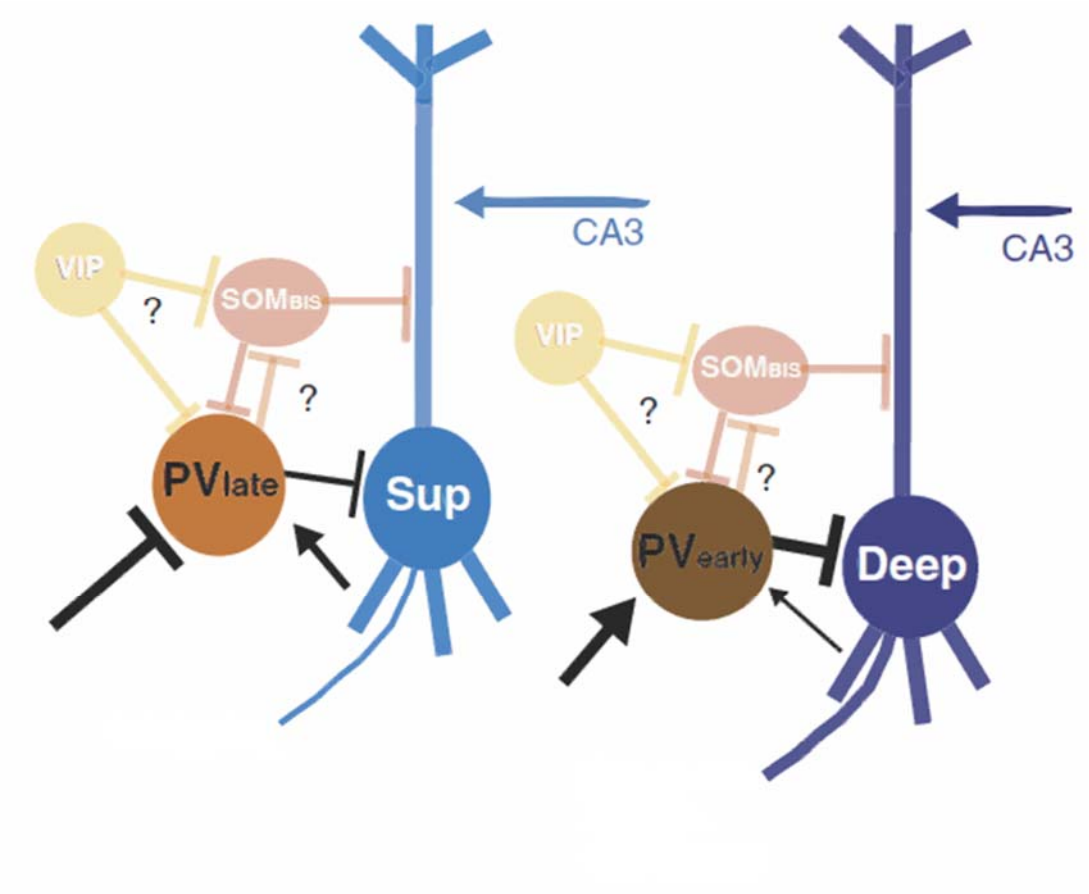

Figure 6 - Microcircuits and subpopulations of hippocampal CA1.
PV basket cells preferentially inhibit specific subpopulations of pyramidal cells. Deep cells are targeted by early-born PV neurons and superficial cells are the main targets of late-born PV neurons. The latter has its plasticity regulated by changes in inhibition. In contrast, plasticity of early-born PV neurons is elicited by changes in excitation. Other interneurons, specially VIP interneurons and somatostatin-positive interneurons play significant role in learning, but still elusive whether they present differnt subpopulations. Adapted from Caroni, 2015.

1.8. Aim and rationale of the thesis

Deletions of the 22q11.2 locus are the most common genetic deletion syndrome in humans and linked to elevated risk for neuropsychiatric diseases.

The role of GABAergic interneurons, especially PV-positive basket neurons, were shown to be affected in patients and in animal models for the disease, and now recognised as the core clinical feature of the disorder. In this thesis, I addressed how the network of PV-positive basket neuron is affected in the *LgDel/+* model. The recently discovered pivotal role of PV neurons in learning (Donato et al., 2013) paved the way to understand how those neurons correlate to the disturbances observed in the model. Few advances were made in the understanding of how these neurons are related to the behavioural impairments observed in the animal models of schizophrenia and how the antipsychotic treatments interfere and act in the hippocampal network. Taking advantage of the recent discoveries on the PV basket neuron network function and their role in rule learning, I investigated the PV network of *LgDel/+* animals in naïve animals and during hippocampal dependent learning paradigms. This analysis led to the observation of an abnormal baseline of the PV network in the *LgDel/+* model, which presented limited plasticity upon learning. The mechanisms involved in the regulation of the PV levels in the *LgDel/+* model were addressed, revealing one main mechanism that allows the rescue of the PV network and the behaviour of the animals, related to excess of D2R activity. The results obtained from the above-mentioned experiments are demonstrated in detail in the following sections.

2. Results

2.1. Parvalbumin network in the *LgDel/+* model

In order to assess whether the impairments observed in schizophrenia are in relation to interneuronal dysfunction, I have used a reliable model for schizophrenia, the *LgDel/+* mouse model. This model presents a hemyzygous deletion from the gene *Idd* to *Hira*, in a syntenic region of the human 22q11.2 (MMU16), encompassing 32 genes (described in Long et al., 2006).

Changes in the PV network configuration reflect plasticity regulated by experience in a given system; high PV shifts are related to increased consolidation of memories and low PV shifts in increased plasticity. In order to understand the fundamental changes in the circuit, hippocampi of *LgDel/+* and WT animals were analysed for PV staining. The hippocampus of the *LgDel/+* animals did not present differences in the gross structure and number of PV interneurons. However, a decrement in the immunostaining for PV was observed, evidenced by an increment in the low PV fraction compared to WT counterparts (Figure 7A, B). This increased fraction of low PV in the CA3 is also observed in other brain structures, such as the PrL and granular region of retrosplenial cortex (RSG; Figure 7C and D). The modified PV baseline in several brain structures points to a different plasticity state of the system. Thus, it was important to observe how the behaviour of the *LgDel/+* animals correlates to the low PV baseline.

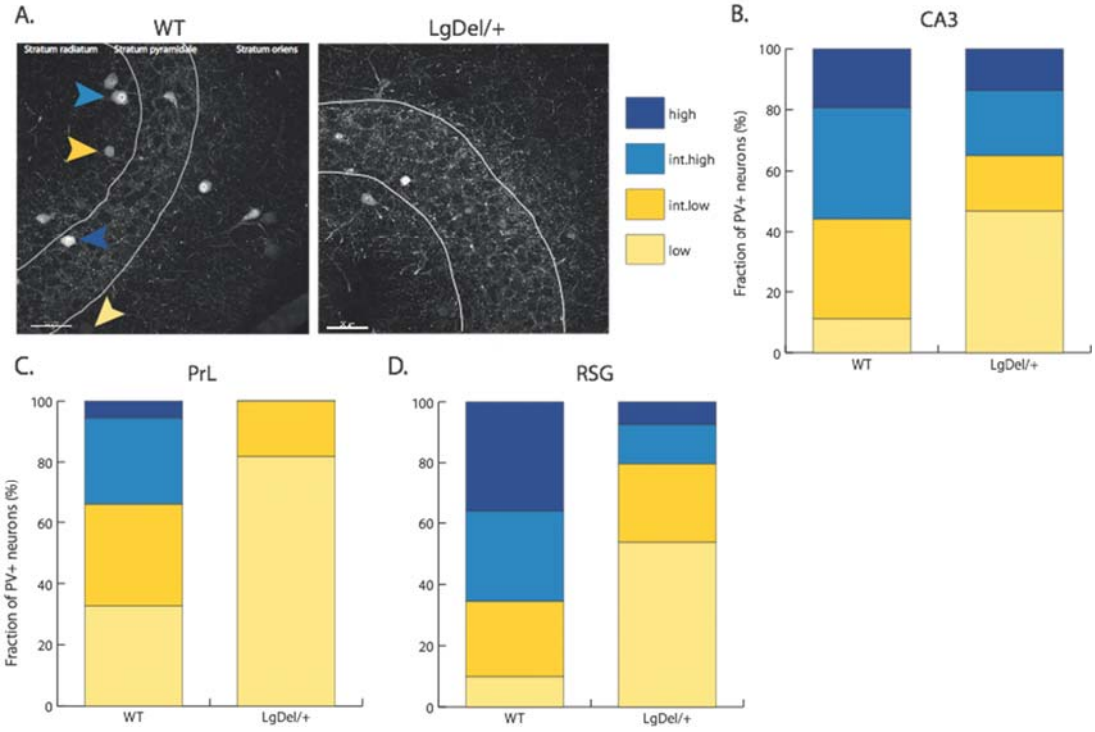

Figure 7 – Analysis of the PV network in hippocampus and cortical regions of *LgDel/+* animals - increment of low PV fraction.

(A) PV immunoreactivity in hippocampal CA3 of WT and *LgDel/+* animals. Scale bar 50 um. Colour-coded arrowheads indicate PV neurons.

(B) PV baseline in CA3. Relative contents of PV neurons of WT and *LgDel/+* animals. n=3

(C) PV baseline in PrL. Relative contents of PV neurons of WT and *LgDel/+* animals. n=3

(D) PV baseline in RSG. Relative contents of PV neurons of WT and *LgDel/+* animals. n=3

2.2. *LgDel/+* animals present impairment in long-term consolidation

LgDel/+ animals present increment in the low PV fraction in several regions observed. Based on the previous findings from the laboratory (Donato et al., 2013; 2015), increased low PV is correlated to higher plasticity and enhanced performance in the familiar object recognition (FOR) test. In this test, memory of the animals is assessed by presenting two identical objects, and in a second moment, five minutes or 24 hours later, a novel object replaces one of the objects (Figure 8A). In order to assess whether the changes in the PV network were correlated to a strengthened memory of the objects, WT and *LgDel/+* animals

were submitted to the FOR test. Importantly, the FOR is a test that does not elicit changes in the PV network; therefore, it reflects the intrinsic state of the PV network prior to the test (Donato et al., 2013).

The *LgDel/+* animals present decreased discrimination of the objects, when compared to their WT counterparts (Figure 8B). In the first day of the test, both WT and *LgDel/+* animals present interest to both objects. When a new object substitutes the familiar 24 hours later, WT animals present preference for the novel object (Figure 8B), while *LgDel/+* animals still present no preference to either objects. This result points to an abnormal function of the low PV in the *LgDel/+* animals. It suggests that the increased lower staining profile is not related to increased plasticity to the network. Conversely, in WT animals with increased low PV baseline, such as those exposed to enriched environment, it is observed enhanced discrimination in the FOR test (Donato et al., 2013).

In order to understand whether the decreased discrimination of familiar and novel objects are related to changes in consolidation of memories in the *LgDel/+* mutants, short-term memory was assessed by testing the animals after five minutes from the acquisition phase. Both WT and *LgDel/+* animals presented similar behaviour, represented by discrimination of the objects and increased time spent investigating the novel object (Figure 8C). This result suggests that *LgDel/+* animals present decreased consolidation of memories after one day. Since consolidation and PV network plasticity are closely related (Donato et al., 2013), I aimed to understand whether this specific correlation is impaired in the model of schizophrenia. Changes in consolidation in schizophrenia have been reported and are supposed to be related to hippocampal impairments (Genzel et al., 2015).

To investigate the high-PV induction in the *LgDel/+* model, I performed contextual fear conditioning, in order to assess the role of hippocampus. Contextual fear conditioning (cFC) is a Pavlovian type of learning, which an otherwise harmless conditioned stimulus (context) is paired with noxious unconditional stimuli, i.e. five consecutive footshocks (reviewed in Tovote et al., 2015). Re-exposure to the same context elicits freezing in mice. Previously, Donato and collaborators observed that cFC evokes plasticity in the PV network, inducing a shift to high PV configuration (Donato et al., 2013). I observed that after cFC, *LgDel/+* animals present freezing to the context in the recall 24 hours post-acquisition (Figure 8E), consistent with previous reports (Long et al., 2006). Although, the *LgDel/+* animals do not present uniform behaviour in the group, statistical analysis based on the amount of time spent on freezing indicates no difference in comparison to WT animals. However, I observed no change in the PV network of those animals, suggesting two possible scenarios (Figure 8G). In the first, cFC evokes a transient change in the network, eliciting changes in the behaviour, although it is not sufficient to induce changes in the PV network. In this case, the animal would have an inconsistent behaviour in the following days, since it did not induce long-lasting plasticity required for consolidation, which is a PV-positive basket cell dependant event. In a second scenario, sensory stimuli coming from the periphery fail to induce proper response in the *LgDel/+* model. This case, however, seems improbable, since the animals freeze to the context in the recall session.

Donato and collaborators provided strong evidence that both low and high PV fractions can be co-induced, when animals are either trained in Morris water maze (which in the first days induce low-PV fraction in the hippocampus) and followed by cFC (inducing high PV); and also by enriched environment (low-PV

configuration induced) followed by cFC (high-PV configuration induced; Donato et al., 2015). However, the *LgDel/+* model case is denoted lack of plasticity to high-PV configuration upon cFC.

In WT animals, cFC produce long-lasting and consistent fear to the specific context, which can last for weeks and months, in rodents. To understand whether the plasticity induced by cFC is transient or long-lasting in the *LgDel/+* model, the long term memory needed to be assessed. I tested the conditioned animals one week after the acquisition and observed WT animals freeze to the context, indicating that the memory persists. In *LgDel/+* animals, the freezing behaviour towards the context is decreased in comparison to WT animals, suggesting a role of the lack of high PV induction after acquisition (Figure 8F). In order to observe changes in the PV network, the perfusion of the animals was performed 24 hours after the recall session.

Taken together, this data suggest that specifically long-term consolidation is impaired in *LgDel/+* animals. One of the main players in this process is the PV network, and one of the hallmarks of the engagement of this circuitry is the shift towards high PV. Recently, it was observed that certain subpopulations of PV basket cells present distinct functions and connectivity (Ciceri et al., 2013; Donato et al., 2015). The cells generated during distinct time-points in the embryogenesis, present specific placement in the cortical layers and in the hippocampal strata. The specific subtypes of cells are related to excitation- or inhibition-driven learning.

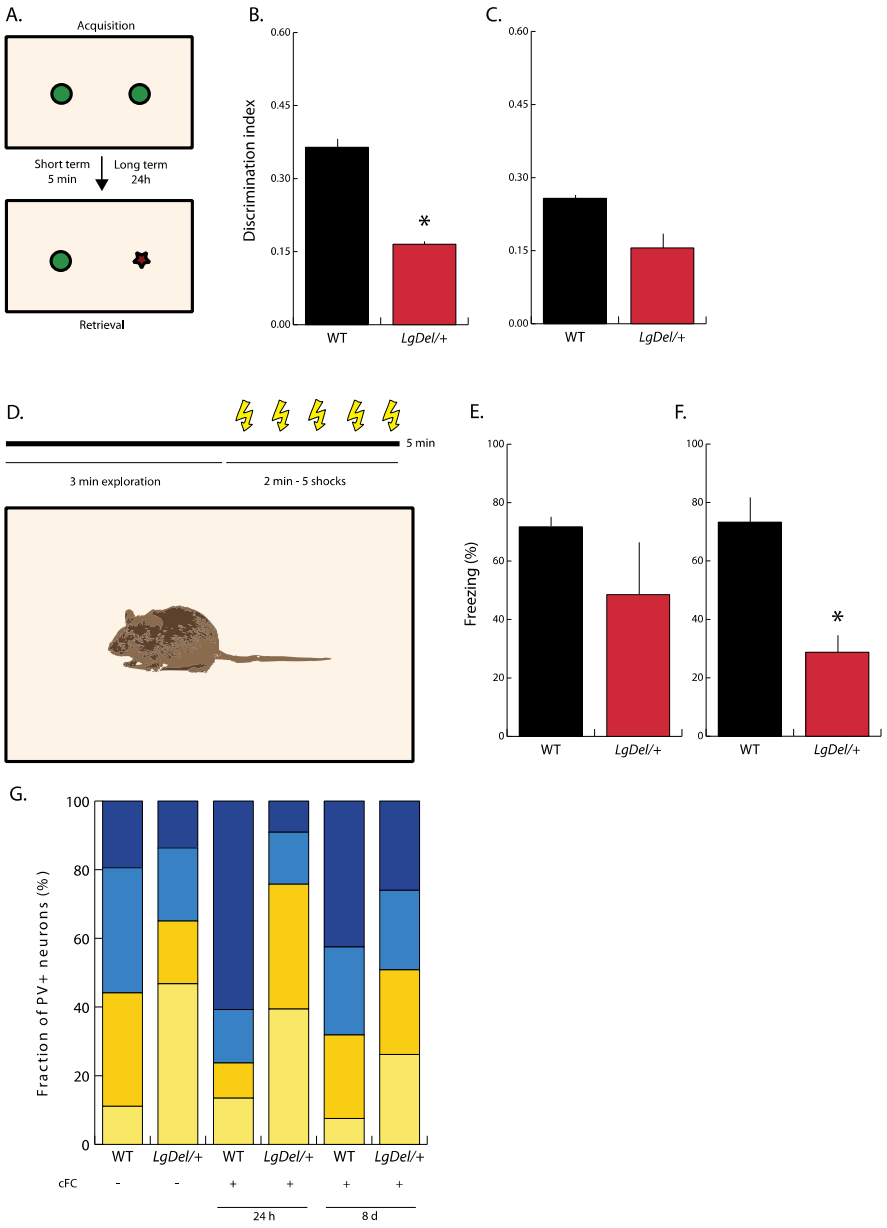

Figure 8 – Analysis of behaviour of *LgDel/+* animals - impairment in long-term consolidation and high PV network shift.

(A) Scheme of behavioural paradigm of FOR.

(B) Decreased long-term memory in *LgDel/+* animals. p<0.05; n=5.

(C) Short-term memory not impaired in *LgDel/+* animals. n=4.

(D) Scheme of behavioural paradigm of cFC.

(E) Freezing behaviour 24 hours after acquisition. n=4 and n=3.

(F) Freezing behaviour 7 days after acquisition. p<0.05; n=3.

(G) PV shift in WT animals upon cFC, 24h post-acquisition (24h); and, 7 days post acquisition, 1 day post-recall (8d). CA3 of *LgDel/+* animals fail to induce high PV shift. n=3.

Error bars represent ±SEM. p values were calculated using t-test.

2.3. *LgDel/+* animals present impairments in early-born PV-positive basket cell subpopulation

The two subpopulations characterised previously in the lab (Donato et al., 2015) are consisted of an early-born, excitation-driven population, and a late-born, inhibition-driven population. The two subpopulation express similar markers of PV-positive basket cells, although their baseline level vary; they also present different targets, not only in hippocampal CA3, but putatively in other areas of the brain (Donato et al., 2013; Caroni, 2015).

In order to understand the changes observed in cFC in the *LgDel/+* model, I traced cells born in two different ages, E11.5 and E13.5. As demonstrated previously, the early- and late-born subpopulations are majorly formed in those two ages. In order to allow unambiguous labelling of proliferating cells, the mitotic marker 5-bromo-2'-deoxyuridine (BrdU), an analogue of the nucleotide thymidine, was injected in timed-pregnant females. I found that the subpopulations targeted during the definite timepoints present abnormal increment of low-PV cells in the adult (Figure 9). Nonetheless, I observed that the PV cells produced during the late-born period although also enriched in low-PV cells, present similar pattern of WT animals (Figure 9).

Early-born PV cells are fundamental for several functions in the network, and were shown to be specifically regulated by cFC learning (Donato et al., 2015). The increment in the low PV fraction of *LgDel/+* animals, which does not present the same functions of low PV cells of WT animals, therefore, possibly is related to this abnormal formation in the early-born period. Based on this result, it is important to understand whether excitation driven directly in the PV cells is able to induce a shift in the early-born PV cells.

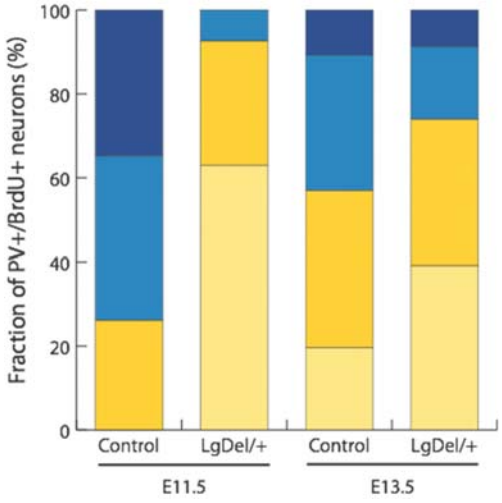

Figure 9 – Analysis of the developmental schedule of neurogenesis in the *LgDel/+* model- baseline of PV levels in the adult reflects increased low PV generation in both analysed birth-dates.
Relationship between schedule of neurogenesis (BrdU labelling time) and PV levels in adult dCA3 of *LgDel*/+ and WT animals.

2.4. Chemogenetic activation of PV cells fails to induce high PV shift in *LgDel/+* animals

Early-born PV cells are key players in the process of rule consolidation (Donato et al., 2015; Caroni, 2015). According to these findings, early-born PV cells receive several different inputs arriving from either excitatory or inhibitory synapses. However, only excitation is able to drive the shift towards high PV in early-born cells. Conversely, only increment in inhibition can modulate the shift to low PV in late-born cells. The monosynaptic system of DG-CA3 represents a clear system of activation of PV cells, where granule cells innervate specifically CA3 pyramidal cells via mossy fibre terminals and interneurons in CA3 via filopodia. In order to understand the impairment in the shift to high PV in cFC, *LgDel/+* animals were crossed with pro-opiomelanocortin-alpha (*POMC*)-Cre

animals. The mouse *Pomc* promoter drives expression of cre primarily in the granule cells of the dentate gyrus subregion of the hippocampus. It was injected floxed pharmacologically selective actuator module (PSAM) carrying AAV9 bilaterally in dorsal DG. In virus-transduced granule cells, chemogenetic activation by the ligand, pharmacologically selective effector molecule (PSEM), delivery was sufficient to induce high PV shift in CA3 of WT animals. In *LgDel/+* animals, however, no induction was observed (Figure 10B).

Given that the main input of PV cells in the CA3 is unable to elicit changes in the PV network of *LgDel/+*, I aimed to understand whether direct activation of those cells would evoke changes in the levels of PV. Therefore, *LgDel/+* animals were crossed with PV-Cre animals. Floxed PSAM carrying AAV9 was injected bilaterally in dorsal CA3, and ligand delivery was sufficient to induce high PV shift in WT animals. As previously shown, PV neuron activation is sufficient to induce high-PV network configuration (Donato et al., 2013). In *LgDel/+* animals, however, no induction was observed (Figure 10D). This result highlights the impairment in the shift to high PV in the PV cells of *LgDel/+* animals. Chemogenetic activation via ligand delivery is sufficient to depolarise the transduced cell, and it is coincidental with a shift towards high PV in WT PV cells. Some mechanism triggering the PV shift may be either missing or dysregulated in PV cells of *LgDel/+* animals.

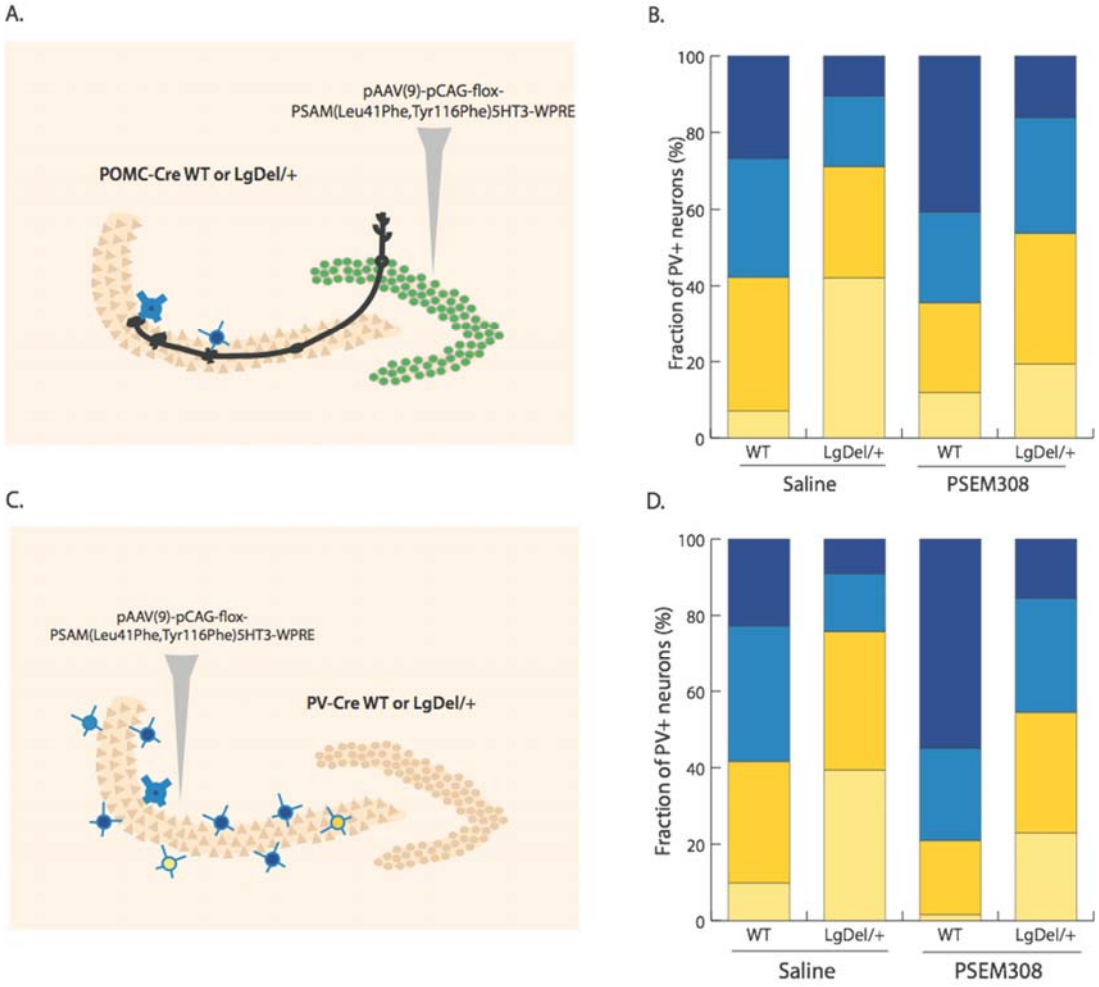

Figure 10 - *LgDel/+* hippocampal CA3 PV network fail to induce high PV upon chemogenetic activation – analysis of indirect or direct PV activation denotes impairment in high PV shift.

(A) Scheme of injection of PSAM virus in dDG of POMC-Cre WT and *LgDel/+* model.
(B) Induction of high PV in WT hippocampal CA3, but not in *LgDel/+* animals. n=3.
(C) Scheme of injection of PSAM virus in dCA3 of PV-Cre WT and *LgDel/+* model.
(D) Induction of high PV in WT hippocampal CA3, but not in *LgDel/+* animals. n=3.

2.5. Chemogenetic activation of cAMP-PKA signalling pathway induces high PV shift in *LgDel/+* model

As discussed previously, some genes deleted in the 22q11 region are related to catecholaminergic activity, such as COMT. I aimed to understand whether the activity of one of the main pathways involved in the regulation of

dopaminergic activity, the G-protein-cAMP signalling is involved in the impairments observed.

The cAMP signalling pathway is a target of several neurotransmitter actions, including metabotropic activity of glutamate, GABA, and especially dopamine. Dopamine receptors lead to either activation of adenylyl-cyclase (via activation of D1R) or inhibition (via activation of D2R). When the [cAMP] is increased via inhibition of the main enzyme responsible for its degradation, the phosphodiesterase-4 (PDE-4), I observed a shift to high-PV in the PV network of WT animals (Figure 11A). However, in *LgDel/+* animals, the PDE-4 inhibition does not elicit shift to high PV network. This result suggests that the levels of adenylyl-cyclase are constitutively low and the inhibition of PDE-4 does not facilitate the increment of [cAMP].

Given that a pharmacological intervention in the hippocampus can elicit changes primarily in other cells, and what I observed could be solely an indirect consequence of cAMP signalling modulation in other cells, I aimed to regulate cAMP signalling only in PV neurons. Floxed Gs-DREADD carrying AAV8 was injected bilaterally in ventral CA3. Upon activation of the designer receptor with clozapine-N-oxide, I observed increased high PV levels in PV cells of both WT and *LgDel/+* animals (Figure 11C). This result highlights the notion that the cAMP signalling is pivotal for the activity of PV neurons and that it constitutes one, if not the main, pathway that is impaired in the *LgDel/+* model. This result suggests that the activity of the G-protein or its activation is impaired in the *LgDel/+* model and it fails to recruit the increment in the activity of adenylyl-cyclase. When provided with DREADDs, activation of this receptors and its signalling cascade is sufficient to induce increment in the high PV.

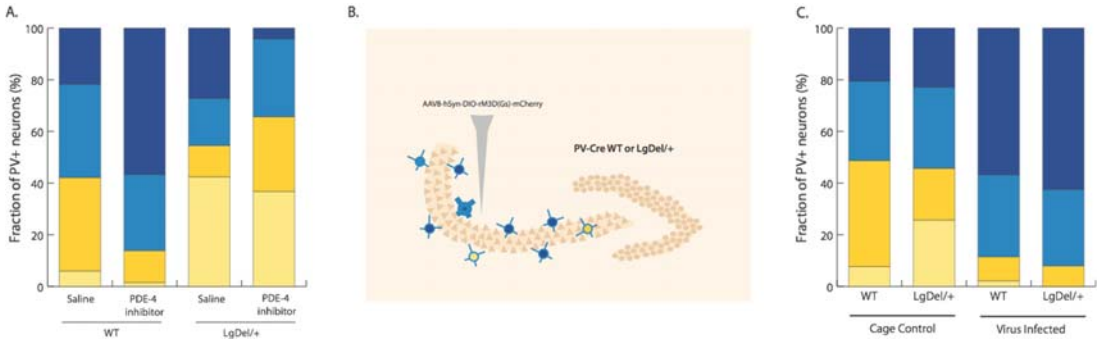

Figure 11 – Analysis of cAMP signalling activity in the *LgDel/+* – chemogenetic activation of cAMP evokes high PV shift in the *LgDel/+*.

(A) PDE-4 inactivation induces high PV shift in WT hippocampal CA3, but not in *LgDel*/+ animals. n=3.

(B) Scheme of injection of DREADD Gs virus in dCA3 of PV-Cre WT and *LgDel*/+ model.

(C) Chemogenetic activation of cAMP signalling pathway leads to shift to high PV in transduced cells in both WT and *LgDel*/+ animals. n=3.

2.6. D2R inhibitor haloperidol rescues PV network in *LgDel/+* animals

Schizophrenia is long associated to changes in the concentration and activity of D2R, one of the main players in the regulation of cAMP signalling. In face of the results obtained in the activation of this pathway and the induction of high PV in the network of *LgDel/+* animals, the regulatory mechanisms of [cAMP] became important to be investigated. Neuroleptics, a class of drugs that act in the regulation of D2R, were used in the *LgDel/+* animals.

In order to understand how the D2R activity is putatively involved in the maintenance of low PV in the *LgDel/+* model, I delivered a single dose of haloperidol intraperitoneally in WT and *LgDel/+* animals. A fast and reversible normalisation of the PV network of *LgDel/+* animals was observed upon haloperidol treatment (Figure 10A). Conversely, the PV network in the WT presented an increment in the low-PV fraction. Additionally, I found a time-dependant effect of haloperidol. The PV normalisation appears after six hours of

the delivery, maintaining its effect at 24 hours; after 48 hours of the treatment, the PV network returns to its baseline in the *LgDel/+* model (Figure 12A).

Haloperidol presents several side effects when used chronically, extrapyramidal side effects being the most common and impairing problems. In order to understand whether acute and chronically delivered haloperidol is capable of eliciting changes in the PV network, I implanted matrix-driven delivery pellets of haloperidol subcutaneously in *LgDel/+* mice. I observed that chronic delivery of haloperidol is efficient in eliciting changes in the PV network (Figure 12A), suggesting that the active effect of haloperidol has a direct impact on the PV levels. It also further suggests that PV network is probably related to positive symptoms of the disease, since chronic use of haloperidol is efficient in alleviating only those symptoms. However, this kind of delivery presented several side effects on the mice, the most obvious being the increased weight gain.

Taken together, these data suggest that the antipsychotic drug, haloperidol, can regulate the PV network. Since haloperidol is involved in alleviating the positive symptoms, it could act via the normalisation of the PV network. Since it is not possible to infer this through any possible experiment on rodents, I used ketamine, which in human produce all the three core symptoms of schizophrenia. Ketamine was injected in WT animals in sub anaesthetic doses and it was observed increased low-PV fraction in the hippocampus; when the animals were submitted to haloperidol treatment, the PV network was again normalised (Figure 12B). Additionally, I have observed that the NMDA antagonist induced motility, observed in the open field, is reverted by the D2R antagonist. This data ratify the notion that the remediation of the positive symptoms possibly works via normalisation of the PV network.

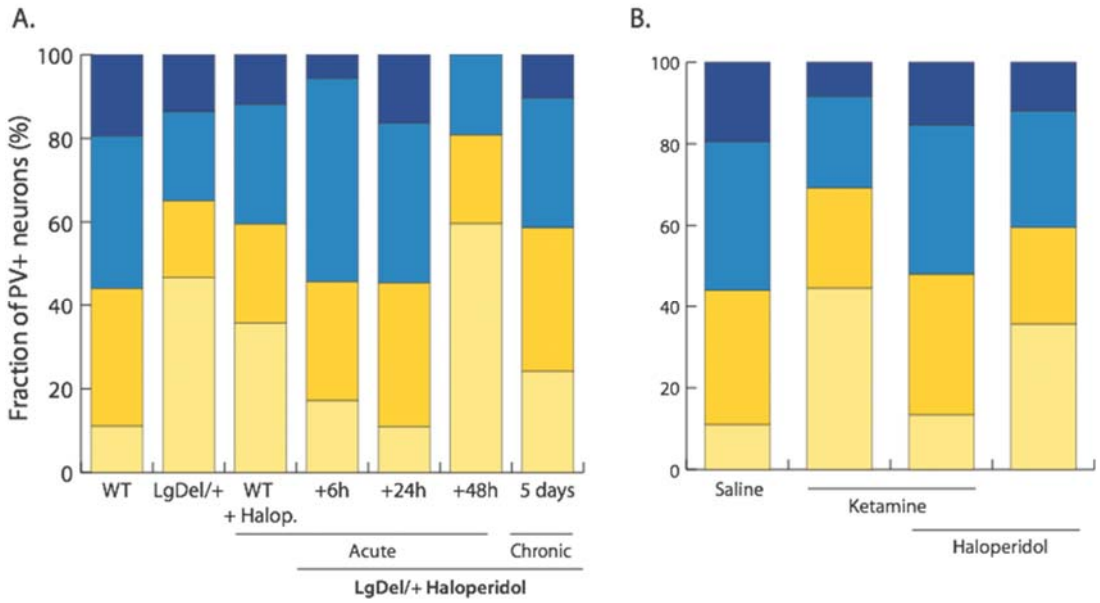

Figure 12 – Analysis of PV network upon haloperidol treatment – D2R antagonist-mediated rescue in *LgDel/+* and ketamine schizophrenia models.

(A) Time-dependent haloperidol rescue of PV network in *LgDel/+* animals. Effect of rescue is reverted after 48 h.

(B) NMDAR antagonist ketamine induces low PV configuration in dCA3 of WT animals. D2R antagonist rescues PV network to baseline levels. n=3.

2.7. Local and long-range effect of haloperidol rescue

In order to understand whether the effect observed in the hippocampus is specific or general to other areas of the brain, I locally injected haloperidol in the CA3, unilaterally. Haloperidol acts in the hippocampus locally, since the effect of the rescue is only in the CA3 of the injected side; the saline treated contralateral side was unaffected by the action of haloperidol (Figure 11B). This result highlight the specificity of haloperidol in rescuing the PV network, and that although other mechanisms may influence this effect, the drug *per se* can act on the PV network locally.

The hippocampus is one of the brain structures implicated in the changes observed in schizophrenia, especially the volume of CA1 and subicullum and activation of pyramidal cells. The prefrontal cortices are closely related to both

positive and cognitive symptoms of schizophrenia. Since this structure is densely innervated by dopaminergic inputs coming from the VTA, I performed injections specifically to this area.

I observed that bilateral injections of haloperidol in the prelimbic cortex (one subdivision of the prefrontal cortex), was sufficient to rescue the PV network locally, but most interestingly, it normalised the hippocampal PV network (Figure 11D). The data suggest that the prefrontal cortex presents an important role in the maintenance of the low PV profile observed in the hippocampus. This indicates that once a normal PV network level in the prelimbic cortex is re-established, it is sufficient to elicit an effect on other areas of the brain.

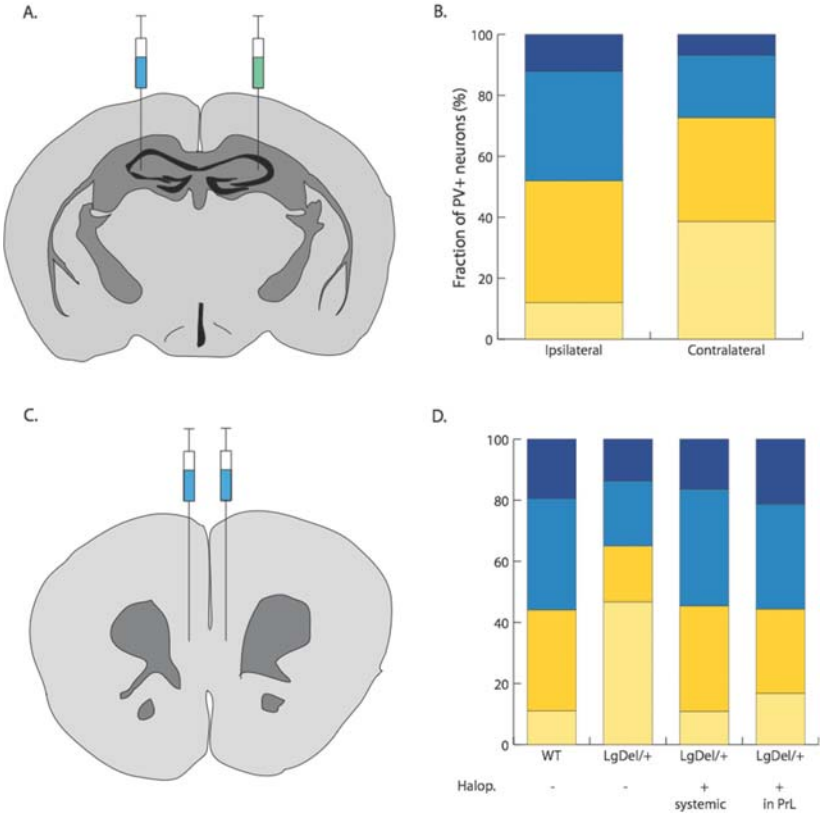

Figure 13 – Analysis of the haloperidol-promoted rescue in local or remote circuitries – PrL as the main regulatory structure for the PV network rescue.

(A) Scheme of injection of haloperidol (blue) and saline (green) in dorsal CA3.
(B) Haloperidol-mediated rescue in injected side, but not in contralateral side. n=3.
(C) Scheme of injection of haloperidol (blue) in PrL.
(D) PV network rescue in dCA3 by systemic or PrL injection. n=3.

2.8. Haloperidol-induced PV network rescue improves learning and memory deficits

One of the main findings concerning the impairments on the *LgDel/+* model is the lack of induction of high PV upon learning. Previous works presented no deficit in learning (Long et al., 2006), since after cFC, *LgDel/+* animals present freezing upon context presentation. My findings indicate a particular problem in long-term retention of the memory and lack of induction of high PV in the hippocampus in this model for schizophrenia.

Based on the results demonstrating rescue of the PV levels to similar levels of a WT baseline upon haloperidol treatment, I have treated *LgDel/+* animals with this drug before the acquisition phase. In order to understand how the normalisation of the PV network prior to the test would influence memory, I treated *LgDel/+* animals with haloperidol six hours before cFC acquisition and exposed them to a recall session seven days after. Six hours after the treatment is the minimum and sufficient time to induce PV level modulation in both WT and *LgDel/+* animals. Importantly, after six hours of the treatment, the effects on motility are absent and, in the dosage used, other active effects are neglectable (Aguilar et al., 1994). Upon this treatment, I have observed an increment in the memory, as the animals freeze longer to the context, compared to saline controls (Figure 14A). Moreover, cFC induced high PV after the rescue of the baseline of PV levels in the *LgDel/+* animals by haloperidol (Figure 14D). Haloperidol treatment also rescued the memory in the FOR (Figure 14B), as the *LgDel/+* animals pre-treated with the D2R antagonist presented improved discrimination.

Rule consolidation is intimately related to the function of early-born PV cells (Donato et al., 2015). In order to understand whether the rescue observed by haloperidol treatment was specifically or generally targeting the subpopulations of PV cells, I have injected BrdU at E11.5 and treated the animals when they reached the age of P60. I have observed that the D2R antagonist rescues the early-born PV cells, inducing shift high PV mainly in this subpopulation (Figure 14C).

Since I previously demonstrated that the prefrontal cortex is the main hub for PV levels regulation in the *LgDel/+* mouse, I next asked how the rescue in this region influences learning and memory mechanisms in those animals. I injected haloperidol either in PrL, in vHP, or intraperitoneally. Through this method, small

differences raised from the action of haloperidol in the distinct regions of the brain (or in all of them at the same time, in the case of intraperitoneal delivery) are observed. Animals were injected six hours prior to acquisition with D2R antagonist and five footshocks were delivered. It was observed that the animals that received the injection in the PrL presented the highest levels of PV staining in the vCA3, compared to the animals, which were injected in vHP (Figure 14D). This result corroborates the idea that the PrL is the main regulatory centre of high PV induction in the *LgDel/+* model.

In order to understand whether the induction of the high PV is a *bona fide* consolidation event and if the network is being recruited, I checked the activation of the early-gene cFos. cFos activation is correlated to the recruitment of a particular region upon a task and is a marker of activated neurons. It was observed that in the vCA1 region of *LgDel/+* animals, at baseline in unconditioned animals, an increased percentage of $cFos^+/NeuN^+$ is present in comparison to WT counterparts. By haloperidol treatment, vCA1 does not present increment in the $cFos^+$ cells; which further indicates that after six hours haloperidol is no longer active, since previous reports demonstrate that D2R antagonists elicit increment in the number of $cFos^+$ cells. Upon cFC, vCA1 of saline injected *LgDel/+* animals present no change in $cFos^+/NeuN^+$ cells. However, the pre-treatment with haloperidol locally elicit induction of cFos in the region, comparable to the induction observed in WT animals upon cFC (Figure 14E; F).

Taken together, the results present an important role for PrL in the regulation of the PV network, both locally and especially in the hippocampus. The early-born PV cells were specifically rescued by the D2R antagonist, suggesting that high PV shift is impaired due to impairments in this subpopulation. The results also shed new light on the reduced activation of the hippocampal

microcircuit in the *LgDel/+* animals, which is reverted when previously treated with D2R antagonist. Additionally, PV network rescue in the PrL facilitates consolidation and high PV induction in the vHP. However, very little is known about the role of D2R activation in the outcome of the impairments observed here.

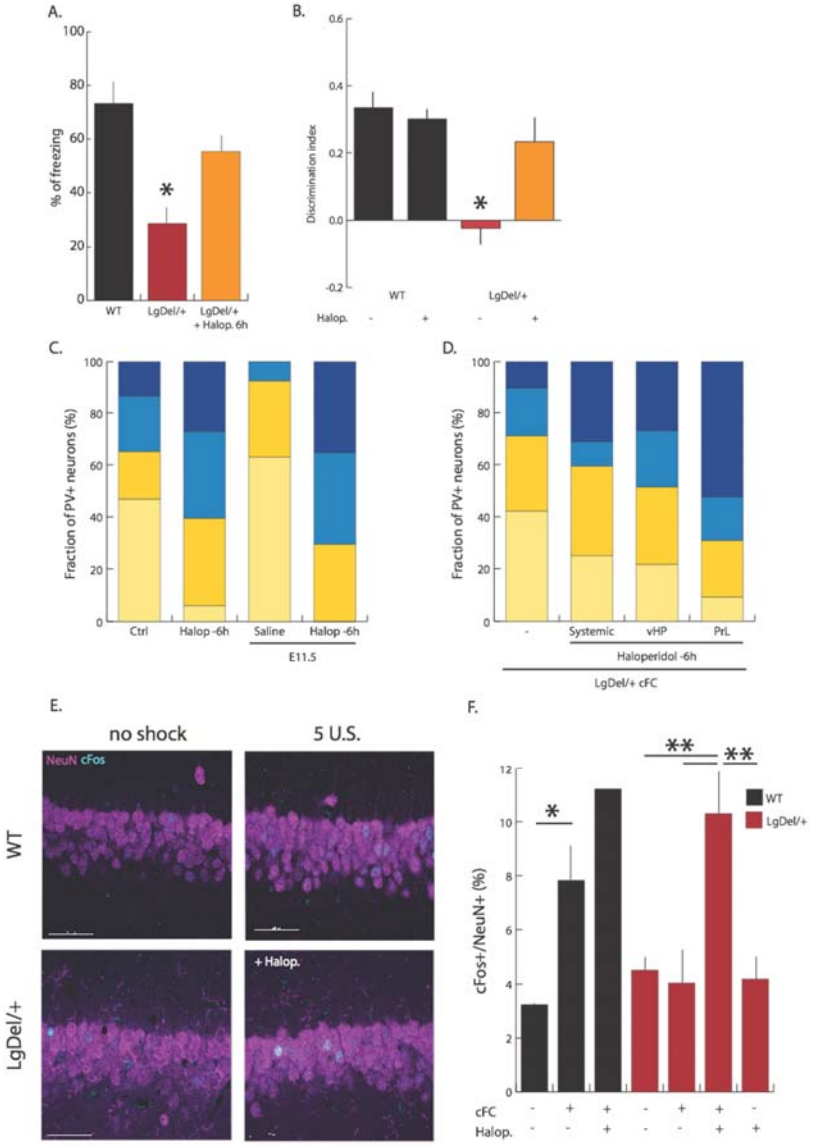

Figure 14 - Rescue of early-born subpopulation by haloperidol and effect on behaviour – analysis denoted high PV and cFos induction.

(A) Freezing behaviour during recall, 7 days after cFC acquisition. Haloperidol was injected 6 h before acquisition of cFC. $p<0.05$; n=3.

(B) Long-term memory in FOR rescued in *LgDel/+* treated prior to acquisition with haloperidol. $p<0.05$. n=5 WT; n=3 others.

(C) Early-born subpopulation is rescued by haloperidol treatment in *LgDel/+* model. General population and E11.5 population of *LgDel/+* animals. n=2.

(D) PV network of haloperidol-treated *LgDel/+* animals 24 h after acquisition. n=5 PrL; n=3 others.

(E) cFos immunoreactivity 90 min after acquisition of cFC. Scale bar 50 um.

(F) Increased number of cFos-positive cells in the neuronal population after cFC in WT; failure of cFos increase upon acquisition in *LgDel/+* model. Rescue of induction of cFos with haloperidol pre-treatment. * $p<0.05$; ** $p<0.001$. n=4 *LgDel/+* Ctrl and *LgDel/+* cFC; n=3 others.

Error bars represent ±SEM. p values were calculated using t-test or one-way ANOVA.

2.9. D2R activation impairs learning and high-PV shift in WT animals

My previous results suggest an important role for D2R activity in the pathogenesis of this schizophrenia model. In order to understand whether activation of the D2R is an important step in the impairments observed, I injected an agonist of D2R, quinpirole, in the vCA3 of WT animals. The activation of the receptor in naïve animals leads to no significant effect on behaviour or in the PV network (Figure 15A). It is possible to conclude that D2R activity *per se* does not influence shifts in either low- or high-PV. Since most of the impairments observed were related to lack of plasticity of the network during behavioural paradigms, as exemplified by cFC, I injected quinpirole six hours before acquisition in WT animals, mimicking the state of the network of the *LgDel/+* model in the activation of D2R. In this case, the WT animals presented decreased freezing upon recall of the memory and an anomalous increment in low-PV, in contrast to saline injected animals (which presented increased high-PV; Figure 15A, B).

In order to understand whether the high-PV shift is impaired upon D2R activation, I injected floxed PSAM carrying AAV9 bilaterally in dorsal CA3, and ligand delivery was sufficient to induce high PV shift in WT animals. However, in animals pre-treated with the D2R agonist, low-PV was induced (Figure 15C). This result corroborates to a modulation of PV levels by D2R activity, indicating that the levels of PV labelling in the *LgDel/+* animals are in part due to the action of D2R. The presence of the agonist of D2R is innocuous in naïve animals, it suggests that some other factors play a role in the low state of the PV network. Yet, the shift to high PV is specifically impaired by the activity of the receptor.

One hypothesis of action of D2R is that high PV shift dependent tasks are impaired by D2R activity. To test this hypothesis, WT animals were injected with quinpirole or saline six hours before the FOR test in the ventral CA3, and no

difference was observed in the discrimination of the familiar object in the animals treated with D2R agonist prior to acquisition (Figure 15D). This result suggests that D2R activity impairs tasks that are dependent on PV shifts. Additionally, D2R activation does not impair cFos increment elicited by cFC (Figure 15E). This result suggests that the main effect of D2R is related to the PV network activity in high-PV dependent tasks.

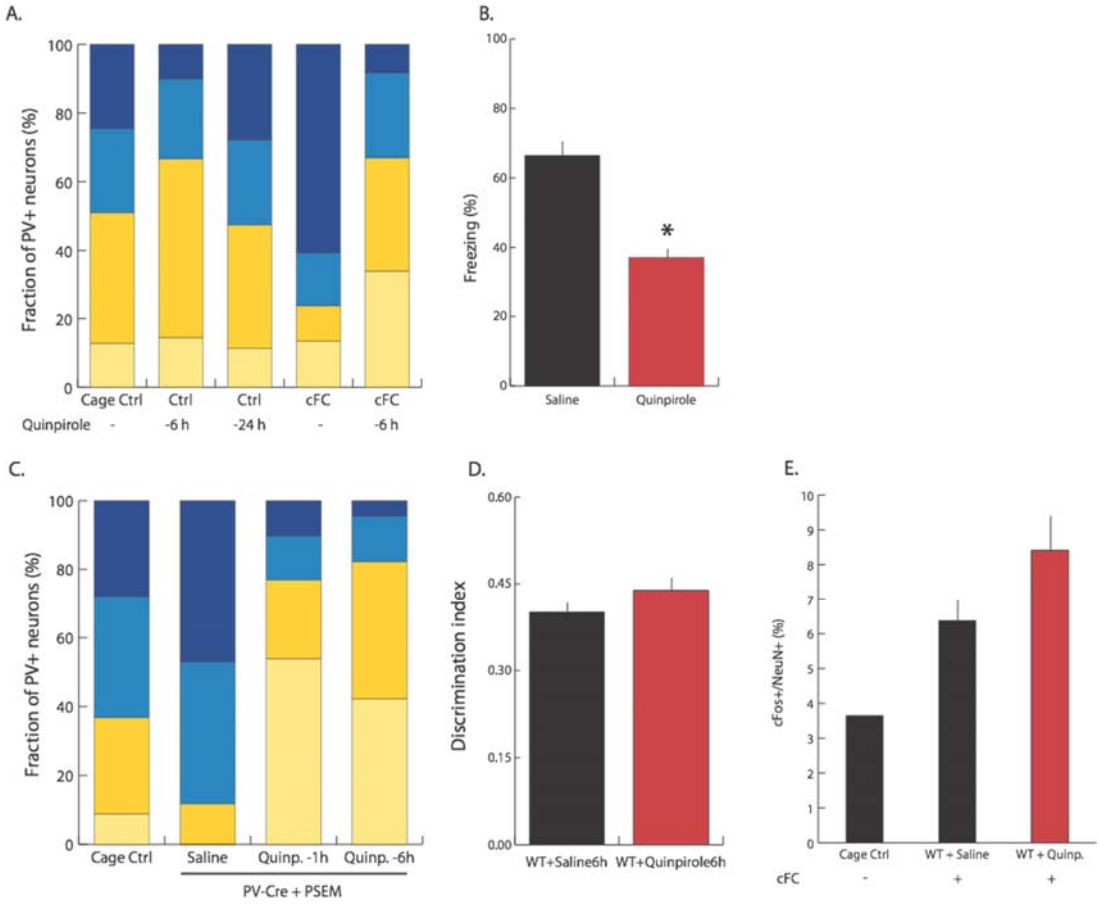

Figure 15 - Effect of D2R signalling in the PV network of WT animals.

(A) PV network of CA3. D2R agonist injection in naive WT does not elicit changes. Injection 6 h prior to acquisition of cFC induce increase in low PV.

(B) Freezing behaviour 24 h after acquisition of cFC. $p<0.05$. n=3.

(C) PV network of CA3. D2R activity prevents increase in high PV of PSAM infected WT PV-Cre treated with PSEM.

(D) D2R activity does not impair FOR. n=3.

(E) D2R activity does not interfere in cFC induced cFos increment.

Error bars represent ±SEM. p values were calculated using t-test.

3. Discussion

The results presented in this thesis shed light on important mechanisms underlying dysfunctions observed in schizophrenia, especially concerning impairments in memory consolidation and hippocampal function (Figure 16). My work provides evidence supporting the involvement of the abnormal PV network of *LgDel/+* animals in the behavioural dysfunctions observed, elucidating the mechanism involving both D2R activity and NMDAR function and that the early-born subpopulation is impaired in the *LgDel/+* model. Moreover, I show that prelimbic cortex is an important hub for the regulation of the PV network remotely, as the rescue of this structure also leads to rescue in other regions of the brain. Finally, my results demonstrate that once the PV network is rescued to a baseline, the animals can learn and consolidate the memory. Taken together, the data provided in this thesis corroborates the three main hypothesis of schizophrenia, as I showed that the PV network is a main player in the impairments observed (GABAergic hypothesis), the early-born PV cells are the subpopulation affected (glutamatergic hypothesis), and the D2R activity propitiate the low level of the PV network and the learning impairments.

The association of interneuronal network dysfunctions and schizophrenia spreads for more than four decades, first observed in schizophrenic patients with reduced concentration of GABA and activity of GAD (reviewed in Nakazawa et al., 2012). However, very little is known about the direct activity of D2R on interneurons, or whether they express this receptor. In my approach, I provided evidence for an abnormal resting baseline of PV network in the *LgDel/+* animals, which is modulated by D2R activity. Therefore, my results point to a regulation through this receptor, but whether this is a direct action in basket cells or indirectly via the activity on pyramidal or granule cells is not addressed here. Nonetheless, the results suggest that the low PV network baseline across

different regions of the brain are linked to the main behavioural impairments observed in the model. I demonstrate that the PV network, once rescued in the *LgDel/+* animals, present similar functions of the WT animals. An important observation was that the deletion is sufficient to disturb the high PV shift in the PV basket neurons, even when those cells were specifically infected, expressed the chemogenetic PSAM receptor, and the receptor was activated. This result is contrary to what takes place in the WT cells, where the activation by the otherwise inert ligand induces consistent increase in PV immunostaining.

These experiments also support the notion that the PV basket neuron is a central player in schizophrenia. In hippocampal dependent tasks, I observed that *LgDel/+* animals perform poorly compared to their WT counterparts. Donato and collaborators provided evidence that PV basket neurons are intimately related to learning processes (Donato et al., 2013). Here, I provided evidence that the abnormal PV network baseline of the model is related to the impairments in hippocampal learning. My results indicate that upon antipsychotic treatment, most of the impairments are normalised. In this sense, my work is pioneer in showing the link between the beneficial effects of an antipsychotic drug and the rescue of the PV network, and subsequent improvement in behaviour.

The impairments observed in the *LgDel/+* animals are the result of the hemyzygous deletion of 32 genes in the MMU16, a syntenic region of the 22q11.2. My results show that to most behavioural paradigms and to the PV network, the blockade of D2R activity is sufficient for their functional rescue. This result suggests that most of the impairments observed in this specific model are the result of dopaminergic signalling imbalance. This imbalance can be better understood when observing one of the main sites of dopaminergic activity, the prefrontal cortex. My results strongly suggest the prefrontal cortex as a main hub

for the effects observed in the hippocampus. Application of haloperidol in the PrL not only rescued the PV network locally, but also in the hippocampus and other areas. In the prefrontal cortex, dopamine exerts a predominantly inhibitory effect, decreasing the spontaneous firing of single pyramidal cells recorded extracellularly *in vivo*. Accordingly, D2R agonists were more effective than D1R agonists in replicating the dopamine-mediated inhibition of spontaneous firing (Thierry et al., 1998). In addition, the enhanced dopaminergic activity in schizophrenia may explain a possible inhibition of prefrontal cortex, which leads to a PV network baseline different from healthy individuals. In this case, prefrontal cortex would provide a top-down effect on other areas of the brain, in particular, the hippocampus.

The hippocampus in schizophrenia is markedly disrupted. Several groups provided evidence that the gross anatomy of regions in the hippocampus are decreased, cell size is decreased and functional imaging provided clues that activity is paradoxically enhanced at rest (but not upon testing; Schobel et al., 2009). The connection between prefrontal cortex and hippocampus is not direct. Two possible routes are thorough thalamus (via nucleus reuniens) or via connection to perirhinal and lateral entorhinal cortices. From the hippocampus, there are specific projections from the ventral hippocampus to the prefrontal cortex (reviewed in Preston and Eichenbaum, 2013). Therefore, the rescue in the hippocampus promoted by haloperidol injections in the PrL is probably an indirect effect from the rescue in the frontal structure. In this scenario, I hypothesise that the excess of D2R activity in the prefrontal cortex decreases the firing rate of pyramidal cells in this brain area and consequently, the connection to the aforementioned areas. Therefore, perirhinal and lateral entorhinal cortices receive less of any given information coming from the prefrontal cortex. This in turn would

lead to less activation of those areas that project to hippocampus. Interestingly, this would be involved in what some authors call the “what” stream (in contrast to a “where” stream, defined by the connection from parahippocampal and medial entorhinal cortices to the hippocampus; Preston and Eichenbaum, 2013), which would create contextual representations that linked related memories. The high PV shift represents the amalgamation between the contextual representation and the learning induced plasticity (only when a “what” is relevant enough to result in plasticity, the high PV is induced; in contrast, the “where” stream does not elicit changes in the PV network, such as placing an animal in a new environment, without any punishment or reward, as in an open field, no plasticity in the PV network is elicited). This would explain why the *LgDel/+* animals freeze to context (functional “where” stream), but do not present the appropriate high PV shift (defective “what” stream), which relies on PrL activity. In this sense, the structures related to the “where” stream would feed to the hippocampus information regarding the context representation. However, the link between the given context and the related memory, which is given by the “what” stream, is impaired in the *LgDel/+* model.

Upon recovery of the prefrontal cortex with haloperidol, the activity is likely restored to WT levels, and the hippocampus now receive activation of the “what” stream. In this sense, rescue of the PrL 6h before acquisition of contextual fear conditioning, an ultimate hippocampal dependent task, allows rescue of not only the PV network in the PrL, but also in the hippocampus. Finally, this leads to high PV shift in the ventral and dorsal hippocampi 24h later. But, how does the rescue in a remote area elicit changes in a non-rescued hippocampus? This question can be answered in two parts. Firstly, injections of haloperidol in the ventral hippocampus of *LgDel/+* animals 6h before acquisition of fear conditioning lead to

less pronounced shift in high PV in the hippocampus, when compared to injections performed in the PrL. Secondly, injections of D2R agonist quinpirole in the ventral hippocampus 6h before acquisition of fear conditioning lead increase in the low PV fraction and no induction of high PV. Taken together, these data suggest that the D2R component most probably is not the central problem in the hippocampus, but in the PrL. If the message from the PrL is arriving in the hippocampus intact (D2R agonist injections in the ventral hippocampus), plasticity occurs, but inducing low-PV shift. More experiments in this particular content will elucidate if the increment of D2R activity in the PrL is the main impairment to learning in the model.

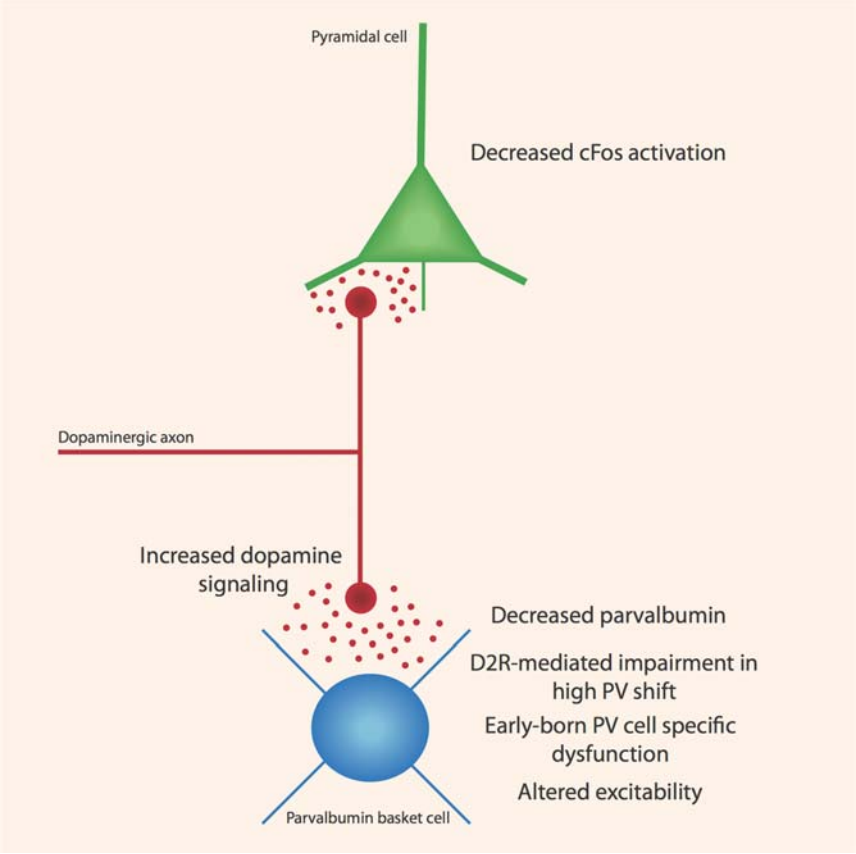

Figure 16 - Scheme of the impairments in the *LgDel/+*.

The main observations of this thesis are depicted. In the upper part, in green, a pyramidal neuron; in the lower part, in blue, a PV basket cell; in the middle, in red a dopaminergic axon.

The recruitment of the prefrontal cortex in the regulation of fear expression based on previously learned information fits with known roles of this region in cognitive control and flexibility. The prefrontal cortex coordinates action through the integration of mnemonic inputs and exerts top-down regulation of specific brain circuits (reviewed in Gilmartin et al., 2014). An important result from a collaboration with my project is regarding another type of rule learning, the trace fear conditioning, which also requires prefrontal cortex function. Unpublished data from the collaborator (Mukherjee and Caroni) provide evidence of impairment in rule learning in *LgDel/+* animals. In his work, he observed that WT animals in P150 persevere in their freezing behaviour during tone presentation for longer periods, when compared to younger animals (P60). *LgDel/+* P150 animals, however, behave more similarly to P60 animals, suggesting that the maturation of the circuits of the prefrontal cortex is impaired. Further analysis demonstrated that chronic chemogenetic stimulation of the PV network in PrL of *LgDel/+* during P60 is sufficient to evoke long-lasting changes in the behaviour of those animals.

In accordance to findings from this collaborative part of the project, the haloperidol-driven rescue of PrL was sufficient to rescue PrL-dependant behaviours. *LgDel/+* animals perform poorly in most parts of the attention set-shifting task, except the simple discrimination, which is not prefrontal cortex dependant. This test relies on plasticity of the PV network, particularly in regions of the prefrontal cortex (infralimbic and PrL), to learn rules and, in a posterior step, modify this rule. It requires contribution from working memory, reversal

learning, attentional set-shifting and sustained attention (unpublished results, Mukherjee and Caroni). However, when *LgDel/+* animals were treated with haloperidol during the PrL sensitive period (P60-70), the animals presented long-lasting and consistent recovery of PrL functions in the test. This result provides not only an important prospect to the clinical treatment of patients with DiGeorge Syndrome during a critical window, but also confirms that the D2R activity in PrL impairs the acquisition of new rules, further supporting the hypothesis described beforehand.

Besides the role of D2R in PV network, NMDAR hypofunction plays an important role in the onset and establishment of schizophrenia (Korotkova et al., 2010; Belforte et al., 2012). Belforte and collaborators provided strong evidence linking the role of NMDAR during late development, especially in adolescence. This work showed that the ablation of NR1 subunit in postnatal ages in a fraction of the corticolimbic interneurons elicits dramatic changes in the behaviour, as anhedonia and anxiety, but most importantly, impairments in spatial working memory and prepulse inhibition were impaired. Moreover, the group showed that this deletion led to increased disinhibition in the cortex and hippocampus. Similarly, Korotkova and collaborators presented important evidence for the role of NR1 subunit in PV neurons in hippocampus (Korotkova et al, 2010). PV neuron-restricted NR1 knockout impaired theta and gamma oscillations in the hippocampus and . My results provide some evidence in favour of those findings, as I have observed increased disinhibition in the hippocampus of WT animals when treated with subanesthetic doses of the NMDAR antagonist ketamine. Additionally, I observed that the main affected subpopulation in the *LgDel/+* animals is the early-born PV subpopulation, which present excitatory-driven shift in the PV staining.

Selective impairment of early-born population explains the increased low PV network in the *LgDel/+* animals. The high PV cells are mostly generated during the early phases of MGE development, but in the *LgDel/+* it is observed an abnormal fraction of low PV cells. Furthermore, my results suggest that antagonists of D2R activity rescue the early-born PV cells. Once the PV network is rescued, *LgDel/+* animals improve in learning. However, how D2R acts on the PV network remains elusive. The D2R is not present in PV-positive basket cells (Gangarossa et al., 2012), but in mossy cells of the DG. Mossy cells are glutamatergic neurons of the hilar region of the DG, which present important role in the activity of basket cells and granule cells in the DG. It is thought that those cells subserve as a relay station for the activation of distant granule cells and inhibit local granule cells, propitiating pattern separation in the DG (reviewed in Scharfman and Myers, 2013). Dopaminergic inputs arrive in the hippocampus from various regions of the brain, including midbrain nuclei, the substantia nigra, the ventral tegmental area and the retrorubral field (Gasbarri et al., 1994a; b; 1996). The main findings of those articles suggest that dopamine, arriving from centres involved in distinct and variable functional roles, target especially the CA1 and subicullum, which are the main projection fields. The authors also highlight the CA3 and the hilus as targets, especially from the VTA and retrorubral field. Those papers, however, present outdated anatomical techniques for tracing and therefore should be revisited in the future with refined viral injections.

The results described in my work were mainly collected in the CA3, which is targeted preferentially by the VTA. The VTA projects to several other areas in the brain, especially to the striatum. Recently, it was demonstrated that the type of firing from the VTA to striatum is impaired in a model of schizophrenia, but not from other dopaminergic inputs (i.e. from substantia nigra; Krabbe et al., 2015). In

brief, the increased D2R activity in the striatum, similar to what is present in schizophrenic patients, is accompanied by a decrement of frequency in tonic and phasic firing from the VTA. If this model holds true for the hippocampus, a decrement in the firing of VTA neurons would in part explain the learning disorders observed in the *LgDel/+* model.

According to one hypothesis of schizophrenia (Lisman and Grace, 2005; Lisman et al., 2008), the communication between VTA and hippocampus play a major role in the establishment of the disease. The hippocampus, according to this hypothesis, is a driving force in detecting novelty and evoking firing of VTA neurons (Lisman and Grace, 2005). Consequently, the increment in the firing of dopaminergic neurons in the VTA leads to increased long-term potentiation in the hippocampus and subsequent memory formation. My results suggest that long-term memory is reduced in the *LgDel/+* animals, when they are tested in the FOR test. However, the short-term memory is present; this indicates that the hippocampus is indeed recognising the novelty, according to this hypothesis. The second step, which involves VTA firing is dysregulated and the memory is not formed.

My results demonstrate that cAMP signalling pathway is directly correlated to the deficits observed in the PV network of *LgDel/+* animals. The modulation of cAMP signalling pathway improved the high PV shift in LgDel/+ animals. Furthermore, the administration of D2R antagonist rescued the PV network of hippocampi of *LgDel/+* animals to WT levels. This suggests that imbalances in the cAMP signalling are related to the abnormal levels of PV in the model. Additionally, injections of PDE-4 induce increment of high PV in the WT hippocampus, which fails to happen in the hippocampus of *LgDel/+* animals. Therefore, I hypothesise that cAMP signalling dysregulation is central in the

impairments of the PV network in the *LgDel/+* model. This data also suggest that early-born PV cells may present higher levels of cAMP signalling activity in WT animals, but not in the *LgDel/+* model, since haloperidol acts specifically in this subpopulation to elicit the network rescue. However, the data presented here only points to a different regulation of the two subtypes in this model of schizophrenia.

PDE-4 is one of the key enzymes related to cAMP metabolism, inactivating intracellular cAMP. Knockout mice for this enzyme present learning and memory deficits (Rutten et al., 2008), while acute inhibition of this enzyme increases long-term memory and improves memory deficits elicited by NMDAR antagonists (Zhang et al., 2000). My results demonstrate that PDE-4 inhibition in WT animals shifts the network to high PV, but *LgDel/+* animals present no network change. Since PDE-4 sole known action is in inactivating cAMP, the result provides further evidence for a decreased cAMP concentration. I hypothesise that the cAMP impairment is a central problem in the *LgDel/+* model. PDE-4 presents interaction with DISC1, a gene implicated in familiar cases of schizophrenia and DISC1 binds specifically to de-phosphorylated, low-activity PDE-4. Upon cAMP-PKA activation, PKA phosphorylates PDE-4, leading to release from DISC1 (Millar et al., 2005). Taken together, those findings shed light on the role of cAMP signalling pathway in schizophrenia.

D2R presents higher affinity for dopamine than D1R - at normal concentration, dopamine would activate preferentially D2R (Surmeier et al., 2007). Since the *LgDel/+* model presents decreased dopamine clearance, due to the hemizygous deletion of *COMT*, it is likely that D2R activity is increased in the model. Therefore, an increment in the Gi signalling would interfere with Gs activity and further decrease the concentration of cAMP. Although I did not express DREADDs to increase Gi activity in the WT to further confirm this

hypothesis, the data presented here indicates that D2R activity is closely related to the impairments in the PV network regulation and learning.

My results indicate that D2R activity in the hippocampus of naïve WT animals does not promote changes in the PV network. This data indicate that other dysregulation is probably present in the *LgDel/+* hippocampal circuit. In D2R knockout animals, it was observed increased number of PV-positive interneurons in the anterior cingulate cortex (Graham et al., 2014). This result suggests that D2R activity may be involved in the fate of PV interneurons. My data suggest that PV interneurons present impaired function once D2R are activated concomitantly with high PV learning, presenting abnormal shift to low PV. In the WT animals injected with D2R agonist, learning processes that do not require high PV shift, such as FOR, do not present impairments. This result contrasts with the impairments in FOR observed in the *LgDel/+* model, which are rescued by D2R antagonist treatment. However, it is not possible to exclude intrinsic differences of activity in the *LgDel/+* model and the effect of the different classes of drugs.

In summary, my results shed new light on the impairments of cAMP signalling on PV-positive basket cells in the *LgDel/+* model, resulting from an overactivity of D2R signalling, especially in early-born PV cells. The results described here also support the notion that the impairments in those interneurons are central in the evolution of the disease, and the rescue of this network is sufficient to improve learning in several behavioural paradigms. Moreover, my results also suggest that PV interneuronal network impairments are related to positive symptoms, since those are treated by D2R antagonists in patients.

4. Materials and methods

4.1. Mice

LgDel/+ and WT mice were generated and genotyped according to Merscher et al., 2000. Animals were genotyped after weaning of the animals. The mice that were used in the present study were in a mixed genetic background (129Sv/C57BL/6/129SvEvTac/FVB/N/SJL) with a significant contribution of C57BL/6J genetic background because they were backcrossed more than five generations into this strain, as in Long et al., 2006. The animals were a kind gift from Dominique Müller, Geneva.

LgDel/+ animals were crossed with PV-Cre line (a kind gift from Silvia Arber, Basel) and with POMC-Cre line (Jackson laboratories). In both cases, animals were genotyped after weaning, controlling for *LgDel/+* mutation and Cre.

Mice were kept in temperature-controlled rooms on a constant 12h light/dark cycle. Before the behavioral experiment, mice were housed individually for 2–3days and provided with food and water ad libitum. All experiments were in accordance with institutional guidelines and were approved by the Cantonal Veterinary Office of Basel Stadt, Switzerland.

4.2. Genotyping by PCR

The following primers were used for genotyping of the mice:

PGK1 – GCTAAAGCGCATGCTCCAGAC

Neo5F – ACCGCTATCAGGACATAGCGT

Idd-KO1 – CTGTTGTTGACACAGCACATG

6x32t3 – AACTCTACCTGTTCCTACTG

Idd-KO2 – CACGTTGTCATTCTCAGACATG

HiraR1 – GTGATGCTAGTCTCTAGCTG

HiraF1 – TCTTGCAACTCTGAGAGGTC

CreF – GGACATGTTCAGGGATCGCCAGGCG

CreR – GCATAACCAGTGAAACAGCATTGCTG

Idd-KO2/Neo5F or Idd-KO1/PGK1 for identification of a homologous recombination event at the Idd locus, Idd-KO1/6x32t3 for amplification of the Idd wild-type allele, HiraF1/Neo5F for identification of a homologous recombination event at the Hira locus, and HiraF1/HiraR1 for amplification of the Hira wild-type allele. To amplify the junction fragment of the Idd and Hira target vectors after a deletion event, the primers PGK1/Neo5F were used. Cre-F/Cre-R primers were used for amplification of the Cre transgene. The PCR conditions were 94°C for 4 min, one cycle; 58°C for 45 s, 72°C for 45 s, and 94°C for 30 s (35 cycles); 72°C for 1 min, one cycle.

4.3. Behavioral experiments:

All behavioral experiments were carried out with mice that were 2-3 months old at the onset of the experiment.

4.3.1 Contextual fear conditioning

The contextual fear conditioning experiment was carried out as described (Ruediger et al., 2011). Briefly, the conditioning chamber (rectangular in shape) was cleaned with 2% acetic acid before each session. Once placed inside the fear-conditioning chamber, mice were allowed to freely explore the apparatus for 2.5-3 min and then received five foot shocks (1 second duration and 0.8 mA each, inter-trial interval of ~30s). To test for contextual fear memory (recall), mice were re-introduced to the same conditioning chamber 24 hours later for 5 minutes but with no shock.

All the experiments – acquisition and recall were digitally recorded and fear retention was measured as the percentage of time spent freezing excluding the

first 1 minutes of exposure. Freezing was defined as the complete absence of somatic mobility, except for respiratory movements.

4.3.2 Familiar object recognition (FOR)- Incidental memory task:

Mice explored two identical objects placed in a 30 × 50 cm arena (10 min exploration) on day one, returned to their home cage immediately after training and were tested for FOR 24 h later, when one of the two objects had been replaced with a new one (5 min exploration). Discrimination indices were calculated as $(t_{novel} - t_{familiar})/(t_{novel} + t_{familiar})$ where t_{novel} and $t_{familiar}$ are time spent with novel and the familiar object respectively. To avoid discrimination of the objects based on odor, both the arena and the objects were thoroughly wiped with 70% ethanol before and after each trial.

4.4. Fixed tissue preparation:

Mice were transcardially perfused with cold 4% PFA (pH7.4) and the brains were collected and kept in 4% PFA overnight at 4^0C.For c-fos analysis mice were perfused 90 minutes after the behavioral protocol.

For all other immunostaining, the brains post overnight fixation was kept in PBS 1X. 40μm coronal sections were then prepared using vibratome Leica VT1000S.

4.5. Immunohistochemistry:

Antibodies and its concentration used are as follows: primary antibodies Rabbit anti-RFP (Rockland) 1:1000 Goat anti-PV (Swant biotechnologies) 1:5000; rabbit anti c-Fos (Santa Cruz), 1:10000; mouse anti-NeuN (Millipore),1:5000; rat anti-Brdu (Abcam)1:500.

The standard immunohistochemistry procedure was as follows: free floating transverse / coronal sections were blocked for an hour at room temperature with

10% donkey serum in PBS-0.3%Triton X-100 (PBS-T) followed by incubation in primary antibody solution in 5% donkey serum, PBS-T overnight at 4°C, washed 3 times in PBS-T for 20 minutes each, further incubated in secondary antibody solution in 3% BSA, PBS-T at room temperature for 2-3 hours and subsequently washed 3 times in PBS for 20 minutes each. Later, sections were mounted in Prolong Gold antifade reagent (Molecular probes), coverslipped and kept at room temperature overnight, sealed with transparent nail polish and stored at 4°C until imaging.

BrdU labeling in vivo was as described (Wojtowicz and Kee, 2006). Mice were injected with 0.1 mg of BrdU at defined times during embryonic development, and hippocampal sections were analyzed for BrdU labeling in the adult. Only strongly BrdU-labeled cells that did not undergo further rounds of DNA replication and cell division subsequent to BrdU incorporation were included in the analysis.

4.6. Drug delivery:

Haloperidol (4-[4-(4-Chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone, 4-[4-(4-Chlorophenyl)-4-hydroxypiperidino]-4′-fluorobutyrophenone, 4-[4-(p-Chlorophenyl)-4-hydroxypiperidino]-4′-fluorobutyrophenone; Sigma-Aldrich) was injected intraperitoneally at 0.1 mg/kg bodyweight; haloperidol was diluted in 0.1 M HCl.

Haloperidol pellets (Innovative Research of America) were implanted subcutaneously at 0.01 mg per pellet on the lateral side of the neck of the animal. Pellets were controlled after perfusion.

Rolipram (*4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone*; Sigma-Aldrich) was injected in the hippocampi via stereotaxic surgeries at 7.5 ug per hippocampus. Rolipram was diluted in saline 0.9%.

Quinpirole ((–)-quinpirole monohydrochloride, *trans*-(–)-(4aR)-4,4a,5,6,7,8,8a,9-Octahydro-5-propyl-1H-pyrazolo[3,4-g]quinoline monohydrochloride, LY-171,555; Sigma-Aldrich) was injected in the hippocampi via stereotaxic surgeries at 0.3 mg per hippocampus; quinpirole was diluted in saline 0.9%.

Ketamine (± ketamine hydrochloride, Narketan, Vétoquinol) was injected intraperitoneally at 30 mg/kg bodyweight; ketamine was diluted in saline 0.9%.

4.7 Stereotactic surgeries

The drugs mentioned were delivered topically to the dorsal CA3b (bregma −1.7 (posterior), 2.0 (lateral), 1.75 (down)), ventral CA3b (bregma −3.0 (posterior), 3.0 (lateral), 3.5 (down)) or in PrL (bregma +1.8 (anterior), 2.2 (lateral), 0.4 (down)). Maximum volume was 200 nl per injection.

4.8. Chemogenetic in vivo

4.8.1. PSAM chemogenetics

For direct local PV- or POMC-neuron control in vivo, a chemogenetic approach aiming for selective activation in hippocampal CA3 was taken. Floxed PSAM-carrying AAV9 (pAAV(9)-pCAG-flox-PSAM(Leu41Phe,Tyr116Phe)5HT3-WPRE was delivered bilaterally in dorsal or ventral hippocampus (maximum volume, 200 nl per injection) in 60- to 70-day-old WT and *LgDel/+* PV–Cre or POMC–Cre male mice. As verified upon anti-bungarotoxin visualization, PSAM expression was confined to the hippocampus, where it was localized at distal CA3, CA2 and proximal CA1. The PSAM constructs were expressed in 75% of PV neurons in CA3 and CA2, in 10–20%of PV neurons in CA1, and in no PV neurons in the dentate gyrus or in any structure adjacent to the hippocampus. Mice were then kept under control conditions for 7–9 days before any experiment, to allow for

transgene expression. The agonist PSEM[308] was injected intraperitoneally 6h before perfusions at the concentration of 5 mg/g of animal weight.

4.8.2. DREADDs chemogenetics

The method to DREADDs chemogenetic was as for the PSAM chemogenetics. Floxed AAV8-hSyn-DIO-rM3D(Gs)-mCherry was delivered bilaterally in dorsal or ventral hippocampus (maximum volume, 200 nl per injection) in 60- to 70-day old WT and *LgDel/+* PV-Cre male mice. As verified upon mCherry visualization, DREADD expression was confined to the hippocampus, where it was localized at distal CA3, CA2 and proximal CA1. Mice were then kept under control conditions for 7–9 days before any experiment, to allow for transgene expression. The agonist clozapine-N-oxide (8-Chloro-11-(4-methyl-4-oxido-1-piperazinyl)-5*H*-dibenzo[*b*,*e*][1,4]diazepine; Tocris) was injected intraperitoneally 6h before perfusions at the concentration of 5 mg/kg of animal weight.

4.9. Imaging

Samples belonging to the same experiment (for example, from the mice of a given time point, with their controls) were acquired in parallel and with the same settings (laser power, 2%; Optical Slice, 1.28–1.35 air units; GaAsP detectors implemented) on an LSM710 confocal microscope (Zeiss) using an EC Plan-Neofluar X40/1.3 oil-immersion or X63/1.4 oil immersion objective (Zeiss). For the PV-intensity analysis, the dynamic range was set during the acquisition of adult (P60) cage control samples. The zero value was set at CA3 pyramidal neurons somas, and the highest threshold so that <20% of the pixels belonging to the brightest PV cells were saturated (ZEN2010 acquisition software, Zeiss). Normalisation and recalibration across different experiments was achieved by

using internal control animals, which were included in each experiment, and were processed using the same criteria mentioned above.

4.10. Image analysis and data quantification:

All the image analysis was done using Imaris (Bitplane). XUV tools was used in order to stitch the images. Parvalbumin intensity analysis was done as in Donato et al., 2013. Briefly, the soma of PV neurons with optimal staining (dampening of intensity between the first and last confocal plane <15%) were isolated in three dimensions (Imaris). Three-dimensional isosurfaces (smoothness: 0.5 µm) were created around each PV-neuron soma and labeling intensities were quantified automatically in arbitrary units as the mean of all isolated pixels. The PV cells were then classified as low, intermediate low, intermediate high and high based on their intensities.

For cFos analysis, cFos+ cells were selected according to signal intensities using an automatic detection (spot detection in Imaris: expected radius,10µm) and medium (>550<750) and high (>750) cFos cells were considered in the quantification (as in Ruediger et al., 2012). The number of cFos+ cells were normalised to total NeuN+ cells in the same section.

4.11. Statistical analysis:

All statistical analyses were performed using GraphPad Prism 6 (GraphPad Softwares). Unless otherwise stated, statistical groups were compared using unpaired, nonparametric Student's *t*-test (Mann–Whitney test). Average values are expressed as means ± SEM.

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