Carolina Cardoso Gonçalves

THE MOLECULAR MECHANISMS UNDERLYING THE CONTROL OF DOPAMINE HOMEOSTASIS BY ASTROCYTES IN THE PREFRONTAL CORTEX

Dissertação no âmbito do Mestrado em Biologia Celular e Molecular orientada pela Doutora Paola Bezzi e pela Doutora Paula Cristina Veríssimo Pires e apresentada ao Departamento de Ciências da Vida da Universidade de Coimbra

Setembro de 2018

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Abstract

For several years astrocytes were considered just support cells for neurons and to have a secondary role in physiologic processes in the brain. Over the last 20 years, accumulating evidences have shown that astrocytes can influence many aspect of synaptic transmission, network activity, and cognitive functions by controlling the extracellular homeostasis of ions and neurotransmitters. However, whether and how astrocytes participate in regulating the homeostasis of dopamine (DA) has never been investigated in detail. Indeed, in the prefrontal cortex (PFC), a brain area responsible for many cognitive and emotional functions, alteration in the normal DA homeostasis can lead to different neuropsychiatric disorders, such as schizophrenia and autism. Here we show that a subset of cortical astrocytes express the organic cation transporter 3 (OCT3), the plasma membrane monoamine transporter (PMAT) and also the astroglial vesicular monoamine transporter 2 (VMAT2). The functionality of these transporters was assessed using a fluorescent false neurotransmitter (FFN200) that mimics de dynamic of DA. We found that OCT3 and PMAT are responsible for DA uptake into astrocytic cytoplasm and VMAT2 for DA storage in specific astrocytic intracellular organelles. Indeed, using in vitro and in vivo deletion of VMAT2 in astrocytes we also found a significant reduction of DA uptake. Moreover, pharmacological inhibition of OCT3 and PMAT reduce significantly the level of false neurotransmitter suggesting that these plasma membrane transporters are fundamental for the DA uptake from the extracellular space. Our results strongly suggest that cortical astrocytes can regulate the homeostasis of DA in PFC and this may have an important role in the physiopathology of mental disorder such as schizophrenia and autism.

Keywords: Astrocytes; Prefrontal cortex; Dopamine homeostasis; VMAT2; FFN200; Uptake 2

Abbreviations: artificial cerebrospinal fluid (aCSF); attention-deficit hyperactivity disorder (ADHD); central nervous system (CNS); catechol-O-methyltransferase (COMT); decynium 22 (D22); Dopamine (DA); dopamine transporter (DAT); 3,4-dihydroxyphenylacetic acid (DOPAC); fluorescence activated cell sorting (FACS); fluorescent false neurotransmitter (FFN200); Gamma-aminobutyric acid (GABA); GABA transporters (GATs); Glial Fibrillary Acidic Protein (GFAP); L- glutamate/L-aspartate transporter (GLAST); Glutamine Synthetase (GS); homovanillic acid (HVA); inward-rectifier potassium channel 4.1 (Kir 4.1); monoamine oxidase B (MAO-B); metabolic glutamate receptors (mGluRs); organic cation transporter 3 (OCT3); postnatal day 40 (P40); phosphate-buffered saline (PBS); phenylalanine (PEA); plasma membrane monoamine transporter (PMAT); potassium (K+); prefrontal cortex (PFC); room temperature (RT); Tamoxifen (TAM); Tyrosine Hydroxylase (TH); vesicular monoamine transporter 2 (VMAT2); ventral tegmental area (VTA)

Resumo

Durante vários anos os astrócitos foram consideradas células que apenas forneciam suporte aos neurónios, tendo um papel secundário nos processos fisiológicos do cérebro. Nas ultimas duas décadas, várias evidências mostraram que os astrócitos influenciam inúmeros aspectos da transmissão sináptica, comunicação e funções cognitivas através do controlo da homeostase de iões e neurotransmissores no meio extracelular. No entanto, se e como os astrócitos participam na regulação da homeostase da dopamina nunca foi estudado em detalhe. Na verdade, no córtex pré-frontal, área do cérebro responsável por várias funções cognitivas e emocionais, a desregulação dos níveis normais de dopamina pode levar a diferentes doenças neuropsiquiátricas, como a esquizofrenia e o autismo. Neste estudo mostramos que, uma parte dos astrócitos corticais expressam o transportador orgânico de catiões 3 (OCT3), o transportador membranar plasmático de monoaminas PMAT e o transportador vesicular de monoaminas VMAT2. A funcionalidade destes transportadores foi testada usando um falso neurotransmissor fluorescente (FFN200) que mimica a dinâmica da dopamina. Descobrimos que o OCT3 e o PMAT são responsáveis pelo transporte de dopamina para o interior dos astrócitos e que o VMAT2 pelo armazenamento da mesma em organelos intracelulares específicos. Usando modelos *in vitro* e *in vivo* onde havia a deleção doo VMAT2 especificamente em astrócitos vimos uma redução significante do transporte de DA para o interior dos astrócitos. Para além disto, a inibição por meios farmacológicos do OCT3 e do PMAT reduz significativamente os níveis de FFN200 sugerindo que estes transportadores membranares são fundamentais para o transporte de DA do espaço extracelular para o intracelular. Os nossos resultados sugerem que os astrócitos corticais têm a capacidade de regular a homeostase de dopamina no córtex pré-frontal e que esta função dos astrócitos poderá ter um papel importante na psicopatologia de doenças mentais como a esquizofrenia e o autismo.

Palavras-Chave: Astrócitos; Córtex Pré-frontal; Homeostase da Dopamina; VMAT2; FFN200; Uptake2

1. Astrocytes

1.1. History of Neuroglia and astrocytes morphology features

The name "neuroglia" means glue of the neurons and emerged in the 1850's when Rudolf Virchow first described what he called a connective tissue that was present between the neurons (Virchow, 1856; Virchow, 1858). Years later, Otto Deiters showed for the first time a real picture of glia cells, while, Camillo Golgi, described that these cells interact through their processes with blood vessels. In 1890's Michael von Lenhossek suggested the name "Astrocyte", because the shape of these glia cells is similar to those of the stars. In fact, a general morphology of one single astrocyte consists in one cell body, 4-10 major branches and more then thousand little processes (Bushong et al., 2002; Sofroniew and Vinters, 2010). However, pioneer studies by Ramon y Cajal in the early nineteenth century evidenced that astrocytes are a heterogeneous cell population. In particular, the initial division of the glial family proposed by Rudolf Albert von Kölliker and William Lloyd Andriezen that separated glia into two groups, fibrous glia and protoplasmic glia, was further refined by Ramon y Cajal, who adopted the term astrocyte for both populations (Somjen, 1988; Kettenmann and Verkhratsky, 2008; Verkhratsky 2012).

The fibrous astrocytes are usually located within white matter, have a relatively few organelles, and exhibit long unbranched cellular processes. This type of astrocytes often has "vascular feet" that physically connect the cells to the outside of capillary. The protoplasmatic are the most prevalent and are found in grey matter tissue, possess a larger quantity of organelles, and exhibit short and highly branched tertiary processes (Wang and Bordey, 2008; Kettenmann & Ransom 2013).

The chronological history of astrocytes it is described in Figure 1.

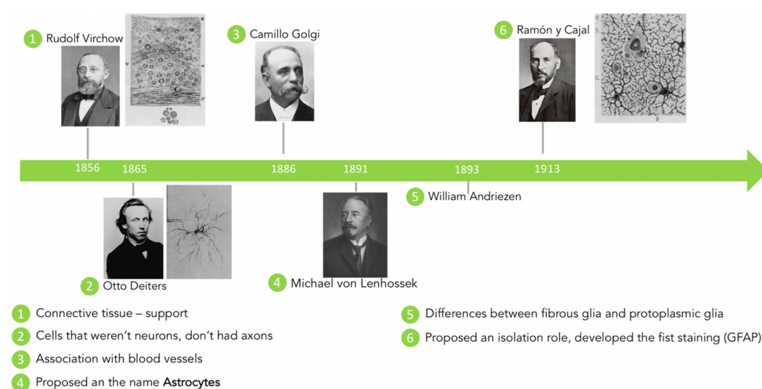

Figure 1. Chronological History of Astrocytes.

1.2. Morphology and Physiology

In the last 10 years, different evidences have demonstrated that astrocytes display an array of morphological and functional features that reflect the brain circuitry in which they surround. Astrocytes are the most abundant glia cells of central nervous system (CNS) and perform essential functions in the brain including H^+, pH and extracellular volume Homeostasis regulation (Benfenati et al., 2011; Kettenmann & Ransom 2013); Blood Brain Barrier

formation and maintenance (Abbott et al., 2006; Cabezas et al., 2014); metabolic regulation (Pellerin et al., 2007; Wang and Bordey, 2008; Kettenmann & Ransom 2013); neuron survival and adult neurogenesis maintenance (Banker 1980; Wagner et al. 2006); synaptogenesis (Baldwin and Eroglu, 2017) and synaptic activity modulation (Araque et al., 2014; Allen and Eroglu, 2017).

1.3. Astrocytes: Synaptogenesis and Synaptic Activity

A single astrocyte can influence over 100 000 synapses in the mouse and over one millions in human brain (Bushong et al., 2002). This close connection allows astrocytes to participate in the formation, maturation and function of synapses by secreting different factors such as thrombospondins, hevin, TGF-β1, BDNF, D-Serine, Glypican, ATP. The astrocytes have the capacity to induce the formation of both excitatory and inhibitory synapsis (Allen, 2013; Baldwin and Eroglu, 2017; Allen and Eroglu, 2017) and to eliminate them (Chung et al., 2015). So, overall, astrocytes modulate and monitor synaptic function.

Astrocytes are associated to the pre- and post-synaptic structures being part of the so called tripartite synapse (Araque et al., 1999) (Figure 2). This leads to a bidirectional communication between astrocytes and neurons. For example, the glutamate released by neurons can activate astrocytes through metabolic glutamate receptors (mGluRs) (Perea and Araque, 2005; Panatier et al., 2011). The activation of these receptors cause an increase of intracellular Ca^{2+} that triggers the release of several substances (called gliotransmitters) such as glutamate (Parpura et al., 1994; Bezzi et al., 1998; Bezzi et al., 2004), Gamma-aminobutyric acid (GABA) (Lee et al., 2010; Jo et al., 2014), adenosine (Penatier et al., 2011; Cao et al., 2013), glycine (Eulenburg et al., 2010; Shibasaki et al., 2017) and D-Serine (Allen and Eroglu, 2017)

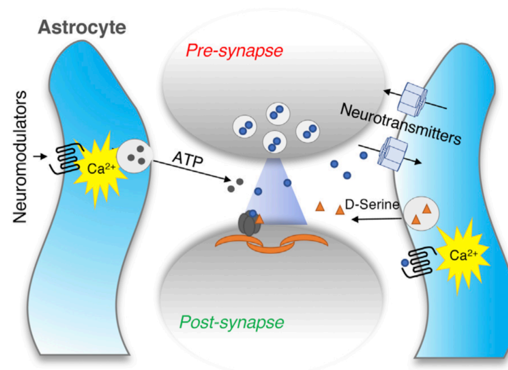

Figure 2. Representation of the tripartite synapse (Allen and Eroglu, 2017)

This gliotransmitters are enable to modulate synaptic function and network activity by acting in neurons. These substances act in different type of targets, being the same substance able to active both excitatory and inhibitory synapses (Araque et al., 2014; Petrelli and Bezzi, 2016). Some experiments proved that defects in astrocytic exocytosis leads to neurologic impairments (Pascual et al., 2005; Halassa et al., 2009), showing the importance of the release of this so-called gliotransmitters.

Another way to modulate synaptic activity is by potassium (K^+) buffering. After the cease o neuronal activity, the concentration of K^+ is quickly restored by the channels present in the astrocytes that allow the clearance of K^+ from the extracellular space (Syková and Chvátal, 2000; Kettenmann & Ransom 2013). The inward-rectifier potassium channel 4.1 (Kir 4.1) it is only expressed only astrocytes, being specific for this kind of cells. The dysfunction of this channel

leads to an alteration in K^+ buffering and the increase or decrease of K^+ in extracellular space will influence the astrocyte-neuron interaction. This can lead to neuropsychiatric disorders, namely depression-like symptoms, if the levels of Kir4.1 are increased, the K^+ clearance enhanced and occurs neuronal hyperpolarization (Cui et al., 2018). In other hand, if there is loss of Kir4.1, the accumulation of K^+ in the extracellular space increase neuronal excitability contributing to neuronal dysfunction in Huntington's disease (Khakh et al., 2014). Thus, Kir4.1 can be an important target for pharmacological treatments for neuropsychiatric disorders.

Contrarily to the capacity of releasing gliotransmitters, the ability of uptake neurotransmitters (Glutamate and GABA) to maintain the extracellular levels is a well-accepted point about astrocytes. The uptake of GABA by astrocytic GABA transporters (GATs) is an important mechanism that allows the clearance of this molecule from the synaptic cleft, the reuptake and the modulation of GABAergic signaling. The transporters GAT-1 and GAT-3 were found in astrocytes, being GAT-3 mainly expressed in astrocytes (Boddum et al., 2016; Ghirardini et al., 2018). A recent paper showed that in particular, the deletion of GAT-3 leads to an increase of extracellular GABA, suggesting that this transporter, that are mainly expressed in astrocytes, have a crucial role in the maintenance of GABA homeostasis (Shigetomi at al., 2011; Muthukumar et al., 2014).

Glutamate uptake in astrocytes occurs mainly through two transporters: L-glutamate/L-aspartate transporter (GLAST) and glutamate transporters Type-I (GLT-1) (Schousboe, 2003; Malarkey and Parpura, 2008). Like in GABAergic synapses this permits the modulation of the signaling, Rothstein and colleagues were one of the firsts to prove that GLAST and GLT-1 were the main responsible for the clearance of glutamate from the extracellular space and that this could prevent excitotoxicity. In their experiments they saw that the inhibition of this two transporters leads to an increase of the extracellular glutamate but the loss of a neuronal glutamate transporter (EAAC1) didn't had effect on the glutamate levels (Rothstein et al., 1994; Rothstein et al., 1996). Overall, the astrocytic glutamate transporters have a major role in the maintenance of low extracellular glutamate levels and avoidance of excitotoxicity. So, they can have an important role to prevent and/or treat diseases such as epilepsy and neurodegeneration (Ferrarese et al., 2000; Schousboe and Waagepetersen, 2005; Petr et al., 2015)

2. Astrocytes and dopaminergic synapses

In the last 20 years, a wide number of data showed how astrocytes are fundamental to regulate and modulate synaptic activity of glutamatergic and GABAergic synapses but which is the role of astrocytes in the regulation of dopaminergic synapses, it is never being investigated in details. Even if decades of research have shown that astrocytes express proteins involved in dopamine (DA) uptake and metabolism suggesting the potential role of these cells in the regulation of DA homeostasis.

2.1. The dopaminergic nature of astrocytes

Growing evidences demonstrated that astrocytes express enzymes and plasma membrane transporters that could have an important role in the regulation of dopaminergic

homeostasis.

In particular, astrocytes express two key enzymes for the metabolism of dopamine and also other catecholamine such as monoamine oxidase B (MAO-B) (Levitt et al., 1982; Carroll et al. 1983) and catechol-O-methyltransferase (COMT) (Karhuen et al. 1995). The role of this two proteins is to catalyze the degradation of DA into homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC). These products enter in the cerebral spinal fluid (CSF) and are transported out of the brain into the bloodstream (Gnegy, 2012). Although these discoveries were done long ago, the importance of these proteins in the dopaminergic synapsis was never studied in detail.

Moreover, astrocytes express proteins involved in the dopamine uptake from extracellular space inside to cytoplasm (Wu et al., 1998; Hayer-Zillgen et al., 2002; Wang, 2016). In particular, these glia cells express specific plasma membrane transporters such as organic cation transporter 3 (OCT3) and plasma membrane monoamine transporter (PMAT). These two transporters are Na^+/Cl^- independent transporters (Uptake 2), with low affinity and high capacity for catecholamine that have as driving force the hyperpolarization of the membrane. Cui and colleagues have demonstrated that astrocytes in the striatum express OCT3 plasma membrane transporter, and that the loss of this transporter in astrocytes leads to an increase of extracellular DA (Cui et al. 2009). Another important transporter that was found in astrocytes is PMAT (Yoshikawa et al., 2013). Mounting evidences suggest that these transporters play a significant role in the clearance of dopamine and other catecholamines in the brain. In particular, the affinity of these two transporters for a dopamine is completely different: OCT3 has a K_m= 1033 ± 127 μM and a V_{max}= 22,676 ± 484 μM, while PMAT has a K_m= 406±4 μM and a V_{max}= 22,402±3,166 μM (Duan and Wang 2010). This suggests that both can uptake dopamine from extracellular space but PMAT transport seems to be more efficient then OCT3.

Also, some evidences showed that astrocytes in cell cultures express uptake 1 transporters, DAT (dopamine plasma membrane transporter) and NET (norepinephrine plasma membrane transporter) (Pelton et al., 1981; Inazu et al., 1999; Takeda et al., 2002). These transporters are Na^+/Cl^- dependent and have high affinity for the substrate. They are the main responsible for dopamine uptake in the dopaminergic system (Giros and Caron, 1993; Amara and Kuhar, 1993; Carboni and Silvagni, 2004).

Finally, Romero and coworkers discovered that *drosophila Melanogaster* contain a variant glial form of vesicular monoamine transporter (VMAT) capable to mediate the storage of histamine (Romero-Caldero et al. 2008). Vesicular monoamine transporter 2 (VMAT2) is the form present in mammals. This is a vesicular monoamine transporter that uptake dopamine (with a K_m = 0.82 - 0.95 μM) (Gasnier et al., 1986; Liu et al., 1992) and also other catecholamines from the cytoplasm inside to specific intracellular organelles. The concentration of catecholamines inside the vesicle is very high, so to contradict the gradient the VMAT2 must be coupled to an H^+- ATPase pump (Dunnett 2005; Chaudhry et al., 2008; Harsing, 2008; Gnegy, 2012).

Zhang and colleagues created a transcriptome database for all cell types of the CNS in mice and also in humans, where they analyzed more the 20000 different genes during brain development. (http://web.stanford.edu/group/barres_lab/brainseq2/brainseq2.html) (Zhang et al. 2014). They showed that astrocytes contain different mRNAs for proteins involved in the control of the DA homeostasis (Figure 3) such as MAOB, COMT, OCT3, PMAT and VMAT2.

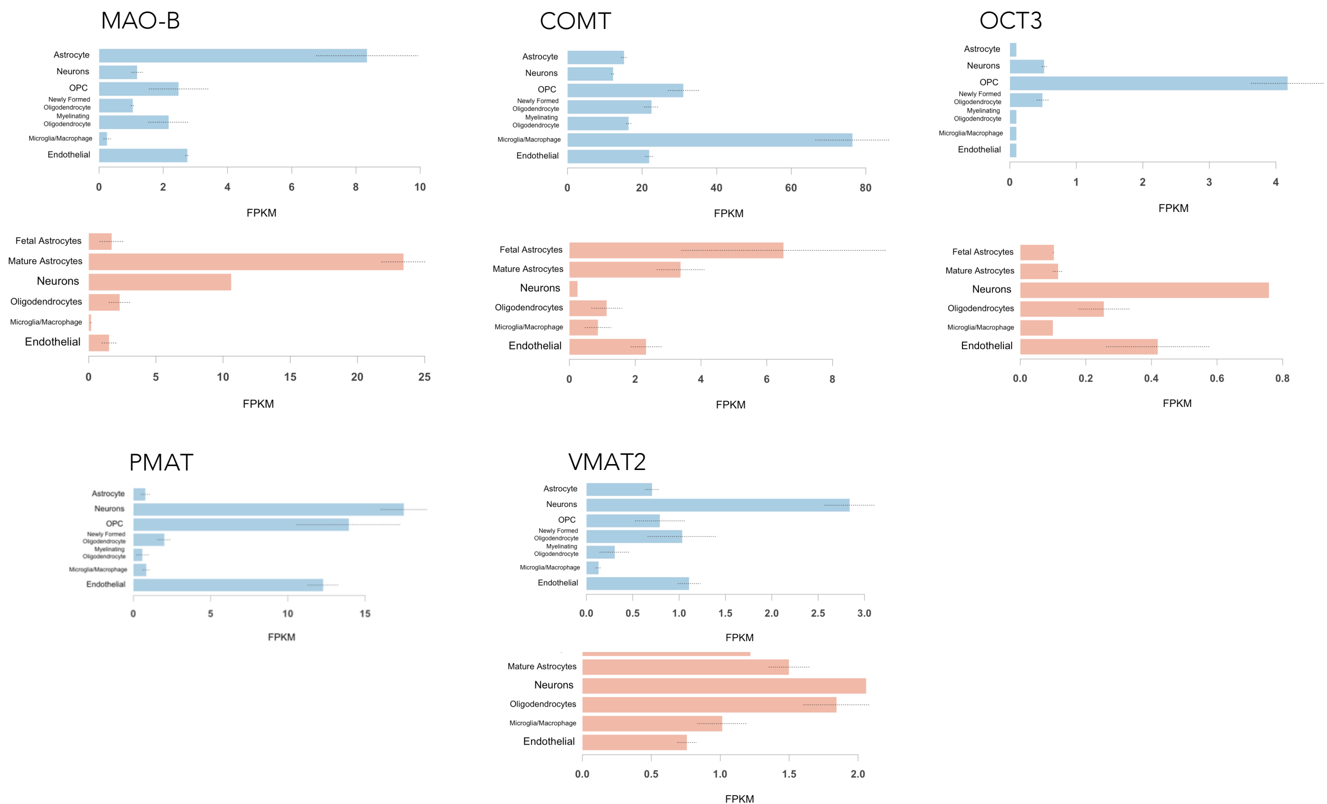

Figure 3. Distribution of mRNAs for some dopaminergic proteins in mouse (blue) and human (pink) (transcriptome data base)

3. Dopamine Homeostasis in the Brain

Long time ago, in 1957, Arvid Carlsson identified DA as a neurotransmitter, this discovery and studies about DA earned him a Nobel Prize for Medicine/ Physiology in 2000 (Carlsson et al., 1958; Carlsson, 1959). This neurotransmitter is a crucial molecule since it has an important role in several main functions such as sleep, walking (Monti and Jantos, 2008), reward and pleasure pathway (Bressan and Crippa, 2005; Schultz, 2002), motivation, emotion control, movement control, cognition and attention (Nieoullon, 2002; Harsing, 2008).

3.1. Structure of the Dopaminergic System in the Brain

In order to simplify the complexity of the dopaminergic system, it is often considered that the mesencephalon contains two major DA neuron subtypes: the nigral A9 neurons projecting to the striatum along the nigrostriatal pathway and the A10 neurons of the ventral tegmental area (VTA) projecting to limbic and cortical areas along mesolimbic and mesocortical pathways. In addition, the DA neurons of the VTA project to the ventral striatum and the ventro-medial part of the head of the caudate-putamen in rodents (equivalent to nucleus caudatus in primates). The A8 cell group that forms a dorsal and caudal extension of the A9 cell group contains cells that project to both striatal, limbic and cortical areas (Björklund and Dunnett, 2007). In particular, the dopaminergic neurons of VTA project directly to

glutamatergic pyramidal and GABAergic neurons of the Prefrontal Cortex (PFC) (Goto and Gace, 2007; Björklund and Dunnett, 2007; Fuster, 2008). In this brain region, DA concentrates mainly in layers III and V, but the terminal of the dopaminergic enervations is primarily in the deepest layers (V and VI). The effects of DA occur through receptors that can be divided into two families: D1 family (D1 and D5 receptors) and D2 family (D2, D3 and D4 receptors). Both are G protein coupled receptors, the D1 family leads to an increase of cAMP levels while the D2 family leads to a decrease (Ranganath & Jacob 2016; Fuster, 2008; Xing et al. 2016). This shows how dopamine can modulate both excitatory and inhibitory synaptic transmission.

All this system is fundamental for the proper regulation of DA levels in PFC, in fact, alterations of this system leads to dysregulation of DA homeostasis in this brain area. Moreover, this brain region has been implicated in planning complex cognitive behavior and executive functions, a set of high level cognitive processes including working memory, attention and mental flexibility. Alterations of this system have been found in different mental disorders such as schizophrenia (Winterer et al., 2004), depression, attention-deficit hyperactivity disorder (ADHD) (Seidman et al., 2005) and autism (Nguyen et al., 2014)

3.2. Dopamine Homeostasis

However, the cellular and molecular mechanisms that govern the homeostasis of dopamine in the brain are not yet defined. Even if decades of researches suggest that dopamine transporter (DAT) is fundamental to maintain the proper concentration of DA in extracellular space (Giros et al., 1996). Indeed, the diffusion of DA in extracellular space, called volume transmission, is fundamental for the proper activation of extrasynaptic dopaminergic receptors and for the functionality of neuronal circuits. DAT is often considered the key transporter limiting the dopaminergic transmission (Rice, M.E, 2000). However, in dopaminergic area like PFC, where the expression of DAT is really low (the density of DAT is around 175-300 molecules per μm^3), this transporter can not block the escape of dopamine from the site of release and the diffusion of dopamine in extracellular space (Cragg and Rise, 2004). Moreover, different studies have showed that the deletion of DAT in mouse do not altered the extracellular levels of DA in PFC (Giros et al., 1996; Shen et al., 2004). This suggests that the homeostasis of DA in the PFC is not controlled only by DAT, but other cellular and molecular mechanisms could influence the homeostasis of dopamine in PFC. In particular, Gogos and colleagues have showed that the lack of MAO-B or COMT in mice leads to an increase of the extracellular dopamine levels in the PFC (Gogos et al., 1998; Kaenmaki et al., 2010). Indicating that these two enzymes are crucial for the maintenance of the DA homeostasis in the PFC. The fact that same of these proteins are express also in astrocytes suggests that these cells can have an important role in the control of dopamine homeostasis in the PFC.

The equilibrium of DA levels in the PFC is essential to a heathy brain, because too much or too little of dopamine can lead to the impairment of PFC functions (Arnsten, 2009; Arnsten, 2011). So, it becomes important to understand the mechanism by which the cells can maintain the homeostasis of DA in the PFC.

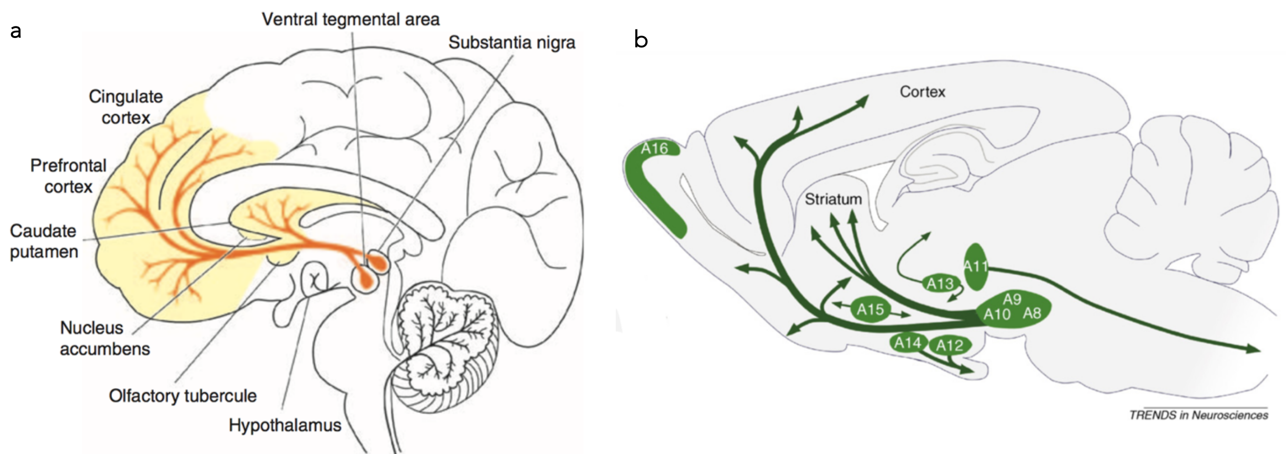

Figure 4. The dopaminergic projections in the human (a) and mouse brain (b) (Björklund and Dunnett, 2007; Fuster, 2008)

4. Prefrontal Cortex

4.1. Anatomy and Physiology

The simplest division of each hemisphere of the brain can be done into 4 major areas: frontal, temporal, parietal and occipital lobes. The Prefrontal Cortex is located in the most anterior part of the frontal lobe before the motor cortex (Figure 5). It can be subdivided in lateral-dorsal, which is more related to motor functions, medial and orbital regions that are more involved in emotional control and the autonomic system (Jacobson and Marcus 2011).

The dominant afferent projections in the PFC come from the mediodorsal nucleus in the thalamus. They were documented in several mammals including mice, rats, monkeys and humans. These projections are in their majority part of the dopaminergic system and their main aim is to transmit information from the limbic system to the PFC (Hendelman 2006; Fuster, 2008). Regarding the architecture of the cortex, it can be divided in five layers in mice: layer I, II, III, V and VI (Raizada and Grossberg, 2003; Zhang, 2004).

The prefrontal cortex is important to cognitive and executive functions such as working memory, where the attention is focused on an internal representation for a purposive action in the proximate future, planning and decision-making. It also has functions related with the limbic system, which can be called the emotional system (Wilson et al., 2010; Euston et al, 2012)

Lesions in the PFC have been documented as leading to alterations of the personality, emotions, learning abilities and behavior of the subject. Of course the effects will depend on which region of the PFC is affected (Wise 2008; Jacobson and Marcus 2008).

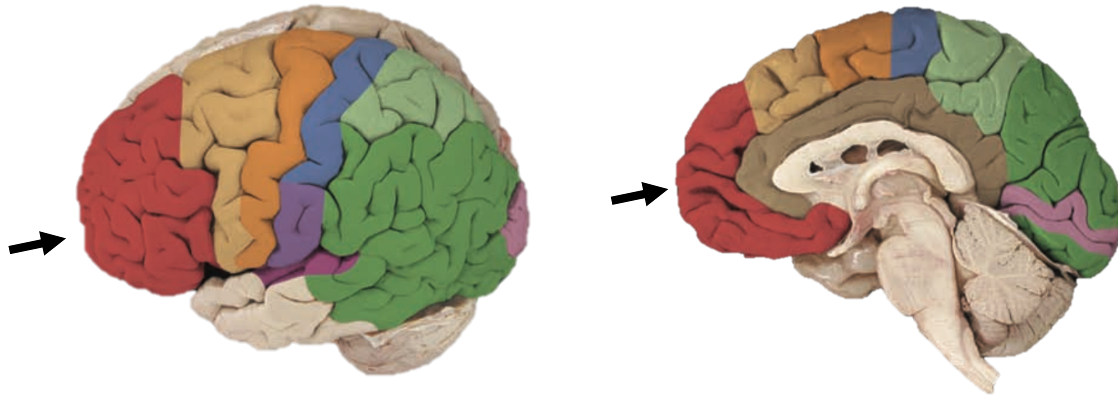

Figure 5. Sagittal plan of the human brain with the PFC in red. (Hendelman 2006)

4.2. Dopaminergic related Diseases in the Prefrontal Cortex

The dysfunction or degeneration of the dopaminergic projections to and from the PFC can lead to several mental diseases.

ADHD is a disease characterized by symptoms as inattention, hyperactivity and impulsivity. These manifestations are caused by dysfunctions in working memory, inhibitory control and visual attention that are typical functions of the PFC (Caylak 2012; Ranganath & Jacob 2015). It was found low levels of phenylalanine (PEA), a stimulator of the release of DA, in the urine of children with ADHD, suggesting lower levels of dopaminergic activity (Baker et al. 1991).

PFC is connected to the limbic system, so mood diseases as Depression are affected by imbalances of the neurochemistry in the PFC. Nowadays, it is known that drugs that prevent the degradation and reuptake of monoamines are efficient in most forms of depression (Nestler & Carlezon 2006; Russo & Nestler 2013; Fuster 2008). These evidences suggest a connection between the dopaminergic system in the PFC and depression.

Schizophrenia is a mental illness that has its outset in the early adulthood and it is characterized by positive symptoms (e.g., paranoid delusions, auditory hallucinations), negative symptoms (e.g.,social withdrawal), and cognitive deficits (e.g., disorganized thoughts, impairment of attention and memory). The DARPP-32, a phosphoprotein essential in DA metabolism is deficient in the PFC of patients with schizophrenia (Albert et al. 2002). Also, the psychomimetic drug Phencyclidine (PCP) that induces similar symptoms of schizophrenia, when administrated to monkeys leads to the reduction of DA in the PFC and deficit in working memory (Jentsch et al. 1997).

All these evidences suggest, that modifications in DA mechanisms, especially in the PFC, are crucial to the development of psychiatric disorders. Given the implications of astrocytes in the regulation of several neurotransmitters levels they must have a fundamental importance not only for the understanding of the mechanisms guiding astrocyte-neuron interaction, but also for developing therapeutic strategies to combat psychiatric disease.

Aim and Hypothesis

The aim of the present project is to understand if astrocytes can participate in the regulation of dopamine homeostasis in the prefrontal cortex. To answer this question, we used different approaches *in vitro* and *ex-vivo*. First, using immunohistochemistry analysis and fluorescence activated cell sorting (FACS) isolated astrocytes we checked if these cells express proteins involved in dopamine uptake and metabolism in PFC. Then, taking advantage of a False Fluorescent Neurotransmitters, the well characterized FFN200 (Pereira et al., 2016), we analyzed if astrocytes can uptake and store dopamine. The understanding of cellular and molecular mechanisms underlying the dopamine homeostasis in the PFC is fundamental for the discovery of new targets for the pharmacological treatment of neuropsychiatric diseases such as schizophrenia and autism.

Materials and Methods

1. Animals

All procedures were approved by the "Service de la consommation et des affaires vétérinaries du Canton Vaud". The animals used in these project were C57BL/6 background. GFAP-ECFP (Hirrlinger et al., 2005), hGFAPcre^{ERT2} (Frank Kirchhoff, University of Saarland, Germany), VMAT2lox/lox (Narboux-Nême et al., 2011), here referred as LoxTAM, from Bruno Giros (Douglas Mental Health University Institute, Canada) and cre^{ERT2}XVMAT2XR26-tdtomato were group housed with littermates in standard housing on a 12:12 h light/dark cycle and kept with a controlled temperature of 23 ± 1 °C. All animals had access to food and Water ad libitum.

The hGFAPcre^{ERT2} sequence was identified from phalange biopsies using the following primers: 5'-CAGGTTGGAGAGGAGACGCATCA-3', 5'-CGTTGCATCGACCGGTAATGCAGGC-3'. PCR reaction product coupled with SYBR Safe DNA gel staining (Invitrogen, S33102) migrates in a 1,5% agarose gel then bands are revealed by UV lights.

All efforts were made to minimize animal suffering and to reduce the number of animals used. Mice were scarified by decapitation 35-45 days after birth.

2. Cell Culture

Enriched frontal astrocytic cultures were prepared from cortices of 0- to 3- day C57BL/6, LoxTAM and cre^{ERT2}XVMAT2XR26-tdtomato pups. Briefly, dissociated neural cells from the frontal cortex were initially plated into 25 m^2 flasks and maintained in minimum essential medium (MEM, Gibco, 21090-022) supplemented with Fetal Bovine Serum (FBS, 10%, PAA cell culture company, A15-101), L- glutamine (2mM, Gibco, 25030-024), D-glucose (20mM), penicillin/streptomycin (100 U/ml, 100µg/ml, Gibco, 15140-22) and kept at 37°C in humidified 5%CO2 / 95% air. After 20-25 days in culture cells were used for the experiments.

3. Tamoxifen treatments

In vivo – Tamoxifen (TAM) (Sigma-Aldrich, with a final concentration of 20mg/ml was made fresh weekly by dissolving in 90% sunflower seed oil / 10% ethanol solution by bath sonication for 20–30 min at 4 °C with intermittent vortexing. P20 mice were injected intraperitoneally with 100 mg/kg with TAM once daily for 8 days (Lioy et al., 2011, Nature).

In vitro – The Tamoxifen treatment (4-hydroxytamoxifen, Sigma-Aldrich) was carried out after 5-10 days in culture with 1 µM daily during 8 days.

4. Fluorescent Activated Cell Sorting (FACS)

Mice from GFAP-ECFP transgenic litter (TgN(hGFAP-ECFP) (Hirrlinger et al., 2005), were used (n=3 mice). The frontal part of the brain was isolated and the olfactory lobes were removed by crude dissection from P40 mice. The tissue was diced with a curved-blade surgical scalpel. To make a suspension of single cells, the tissue was enzymatically dissociated via the incubation at 33°C for 70 minutes. The solution was prepared in 5 ml of dissociation buffer completed with EDTA (0.5mM, pH=8), L-cysteine-HCL (1mM, Sigma-Aldrich, C7477), papain (20U/ml, Sigma- Aldrich, P3125) and DNase (125U/ml, Sigma-Aldrich, D4527). Prior is necessary to activate the papain during 30 min at 33°C. Dissociation buffer contained Earle's balanced salt

(EBSS, Sigma- Aldrich, E7510), D(+)-glucose (22.5mM), NaHCO3 (26mM) and requires equilibration with 5% CO2 and 95% O2 gas before use and during papain treatment (Cahoy et al., 2008). After papain treatment the tissue was washed with 3 x 2 ml dissociation buffer containing BSA (1mg/ml, Sigma-Aldrich, A9647), ovomucoid (also known as Trypsin inhibitor, 1mg/ml, Sigma-Aldrich, T9253) and DNase (125U/ml) (inhibitor solution) and then mechanically dissociated by gentle sequential trituration using a 1mL pipette with 3 x 2 ml fresh inhibitor solution to yield a suspension of single cells. Dissociated cells were layered on top of 12ml of concentrated inhibitor solution (5mg/ml BSA, 5mg/ml ovomucoid, 125U/ml DNase) and harvested by centrifugation (140 x g for 5min) (Cahoy et al., 2008). Cells were resuspended in a solution containing PBS 1% (phosphate-buffered saline), 0.1% BSA, EDTA (0.5mM) and 4',6-diamidino-2-phenylindole (DAPI, 1µg/ml, Invitrogen, D1306) for FACS purification. CFP positive astrocytes were purified by FACS using a MoFlo AstriosEQ High speed cell sorter (Beckman Coulter Life Sciences). Dead cells were gated out using high DAPI staining and forward light scatter. Astrocytes were identified based on high CFP fluorescence and size.

5. RT-PCR

Total RNA from sorted cells was isolated with RNeasy Mini Kit (Qiagen, 74104)zymo and RNA concentration was determined using a NanoDrop 1000 spectrophotometer (Witec AG, Switzerland). Reverse transcription was performed with 400/600 ng of DNase-treated total RNA using M-MLV reverse transcriptase (Promega, M3683). The quantitative real-time PCR (RT-PCR) was done on C1000T Thermal Cycler (CFX96 real-time PCR system, Bio-Rad) using SYBR Select Master Mix for CFX (Applied Biosystems – Life Technologies) (Buscemi et al., 2017). The mRNA levels were normalized to the levels of β-actin (Chai et al., 2017). The primer sequences used were are described in table 1. For quantification, ΔΔCT method was used (Livak and Schmittgen, 2001).

Gene	Primer Sequences	
	Foward	Reverese
β-actin	5′-GGCTGTATTCCCCTCCATCG-3′	5′-CCAGTTGGTAACAATGCCATGT-3′
Calb1	5'-GCTGCAGAACTTGATCCAGGA-3'	5'-TCC GGT GAT AGC TCC AAT CC-3'
Mog	5′-CCTGGTTGCCTTGATCATCTGCTAC-3′	5′-TCTACTCGGTATCCAGAATGTGTCTG-3′
OCT3	5′-CTATGCAGCGGACAGATATGG-3′	5′-AGCGGAAAATCACAAACACAGAA- 3′
PMAT	5′-GCCACATCTGACCAGAGTGT-3′	5′-CTCCTCTACCGCAGAGTCTGTG-3′
Syt1	5′- GCTTTGAAGTTCCGTTCGAG-3′	5′-AGCATGTCTGACCAGTGTCG-3′
VMAT2	5′-GCGAGCATCTCTTATCTCATTGG-3′	5′-AAATGCTGATCCCAACAACTATCA-3′

6. Immunohistochemistry

In order to identify some transporters and metabolic proteins present in astrocytes immunohistochemistry was performed on coronal brain slices of rat's (P35) prefrontal cortex. Sprague Dawley rats were deeply anesthetized with sodium pentobarbitone (6mg/100g body wt, i.p.) and immediately perfused intracardiacally with fresh 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4). Brains were post fixed in PFA 4% for 2h and after equilibrated overnight in sucrose 30% at 4°C. Coronal sections (30 µm) were cut at -20°C using a cryostat (Microm, HM400) and stored in ethylene glycol at -20°C.

First, the slides were washed two times in phosphate-buffered saline (PBS1x) to clean the ethylene glycol. Then, permeabilized for 45 min in PBS 1x containing 0.3% Triton X-100 (PBS-T), and 15% donkey or goat serum at room temperature (RT). The primary antibodies were diluted in a solution composed by PBS-T and 1.5% of donkey or goat serum. The sections were incubated overnight at 4°C using the following primary antibodies: rabbit-VMAT2 (Synaptic System, 1:500); mouse-GS (Chemicon, 1:1000) (Shubert et al., 2011), rabbit-GFAP (Chemicon 1:1000), rabbit-OCT3 (Alpha Diagnostics, 1:100) (Cui et al., 2009), mouse-PMAT (Abcam 1:100). The brain sections were then washed again three times in PBS1x for 10 min and incubated for 2h at RT with fluorescent secondary antibodies (AlexaFluor, Invitrogen, Molecular probes, Eugene, Oregon, goat anti-mouse 488, 555, and 633; goat anti-rabbit 488, 555, and 633; 1:300 and 1:400) diluted in PBS. Finally, nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) (Invitrogen, Molecular Probes, Eugene, Oregon, 1:10000) and then washed before mounting with a reagent FluorSave (Calbiochem).

All images were collected on a Leica confocal imaging system (TCS SP5) with a 40× (1.4 NA) or with 63x (1.4 NA) oil immersion objectives. Images were analyzed using Imaris 7.6.3 (Bitplane AG, Zurich, Switzerland) or FIJI (ImageJ)

7. Immunocytochemistry

Cultured astrocytes were briefly washed with PBS and fixed with PFA 4% at 4°C for 10 minutes. After three washes with PBS cells were incubated for 20 min at RT in phosphate-buffered saline (PBS) containing 4% of goat serum or donkey serum and then immunolabeled 1h at RT in a PBS solution with Saponine (0.5mg/mL, Sigma-Aldrich, 47036) using the following primary antibodies: mouse-Lamp1 (Millipore, 428017, 1/200), rabbit-VMAT2 (Synaptic System, 138302, 1/1000), goat-VMAT2 (Santa Cruz, sc-7721, 1/1000), rabbit-VAMP7 (Termo Fisher Scientific, 1/1000), rabbit VAMP8 Abcam, ab6186, 1:50) and rabbit-cathepsine B (Upstate Biotechnology 06-480, 1:200).

After the incubation with primary antibodies cells were washed three times in PBS for 5 min and incubated 1h at RT with fluorescent secondary antibodies (AlexaFluor, Invitrogen, Molecular probes, Eugene, Oregon, goat anti-mouse 488, cy3; goat anti-rabbit 488, Cy3; 1:300) diluted in PBS. Finally, the cells were washed before mounting with a reagent FluorSave (Calbiochem). All images were collected on a Leica confocal imaging system (TCS SP5) with a 63x (1.4 NA) oil immersion objective.

8. Dose/Response Experiments

Primary astrocytes isolated from C57BL/6, LoxTAM and cre^{ERT2}XVMAT2XR26-tdtomato pups were seeded in a 96-well plate at 0.02-0.03*10^6 cells per well and placed in the incubator at 37 °C and 5% CO_2 (n=3/4 mice per genotype). After reaching the confluence (at least 3 days), the cells were pre-incubated during 30 min with reserpine (1µM) or deprenyl 22 (D22) (5µM) in Krebs Ringer Hepes (KRH) buffer (125mM NaCl, 25 mM Hepes, 5.6 mM glucose, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, pH 7.4). Cells were then incubated with different concentrations of FFN200 in presence or in absence of reserpine (1µM) or D22 (5µM) in KRH buffer (pH 7.4) at 37 °C for 30 min. To have the effects of VMAT2 deletion, astrocyte cultures of LoxTAM and cre^{ERT2}XVMAT2XR26-tdtomato had TAM treatment prior to the experiment.

The cells were washed two times with KRH and the fluorescence was measured by fluorimetry (Hidex, Labgene) (λEx/Em = 355/460 nm).

9. Uptake experiments

Primary astrocytes isolated from C57BL/6, LoxTAM and cre^{ERT2}XVMAT2XR26-tdtomato pups were used to study the uptake by astrocytes of FFN200 *in vitro*. The frontal astrocytic cultures were prepared as described above, briefly, the cells were seeded in 5 mL petri dishes at 0.05-0.075*10^6 cells per well and placed in the incubator at 37 °C and 5% CO_2. When ready (3-5 days after), the cells were pre-incubated for 30 min with reserpine (1μM) or D22 (5μM) in KHR buffer. Then, FFN200 (500μM) was added for 30min. The cells were submitted to an additional incubation, for 10 min with KRH buffer, in order to eliminate the excess of FFN200. After 2 washes with KRH buffer the cells were observed in the confocal microscope (TCS SP5).

Leica LAS AF (Leica microsystems, Wetzlar, Germany and FIJI (ImageJ) software were used to analyze the results.

10. Acute Brain Slices

The uptake of FFN200 into astrocytes, *ex-vivo*, was tested by using the acute brain slices method and the confocal microscope (TCS SP5). cre^{ERT2}XR26-tdtomato and cre^{ERT2}XVMAT2XR26-tdtomato mice (fluorescent astrocytes) of 7-8 weeks old, were decapitated and the brain was taken and put in cold artificial cerebrospinal fluid (aCSF) enriched with $MgSO_4$ (10 mM) and with 5% CO_2 and 95% O_2. By using the Vibratome (Thermo scientific) the frontal part of the brain was cut in 250 μm coronal slices always in the aCSF with 5% CO_2 and 95% O_2 and magnesium. After this passage, the slices were put into a chamber at 34 °C containing normal aCSF plus 5% CO_2 and 95% O_2 and left 1h in order to recover. Recovered slices were incubated with FFN200 (500 μM) during 15min, at 37 °C into the aCSF (5% CO_2 and 95% O_2). Then, the slices were incubated during 10 min in aCSF (5% CO2 and 95% O2) to wash. The slices were observed with the confocal microscope (TCS SP5) with a 63x immersion objective. During the observations the slices were put into a chamber where the flux of oxygenated aCSF was maintained. When the mice were not tdtomato sulforhodamine 101 (2 mol, 15min, 37 °C) was used.

Leica LAS AF (Leica microsystems, Wetzlar, Germany) Imaris 7.6.3 (Bitplane AG, Zurich, Switzerland) and FIJI (ImageJ) software were used to analyze the results.

11. Statistical Analysis

All analyses were performed using GraphPad Prism 6.0 software. Then, a two- way ANOVA was performed followed by Bonferroni's post hoc tests. For two sample comparisons, unpaired t-test was used. All data are presented as mean ± SEM.

3.Results

In this work, we assessed the presence and function of different proteins involved in dopamine homeostasis in prefrontal cortex astrocytes. To achieve our objectives, first we have checked whether astrocytes in PFC express specific plasma membrane and vesicular transporters, respectively. Then, taking advantage of the fluorescent probe FFN200 we analyzed whether astrocytes are able to uptake dopamine from extracellular space and storage this molecule in specific intracellular organelles.

3.1. Astrocytes contain mRNAs for dopamine transporters

The presence of genes involved in the uptake and storage of dopamine were assessed in purified astrocytes, from the prefrontal cortex of postnatal day 40 (P40) GFAP-ECFP (Hirrlinger et al., 2005) mice (n=3 mice), sorted by fluorescence activated cell sorting (FACS) followed by Reverse Transcription polymerase chain reaction (RT-PCR) analysis. We found significant levels of mRNA for the plasma membrane transporters PMAT and OCT3 and also for the vesicular monoamine transporter VMAT2 (Figure 6a).

Astrocytes were sorted with a purity of >90% positive cells. The purity was validated by checking the mRNA levels of markers for neurons (Synaptotagmin 1 – Syt1), dendrites (Calbindin 1 – Calb1) and oligodendrocytes (Myelin Oligodendrocyte Glycoprotein – MOG) (Figure 6b-c)

The presence of mRNA from DA transporters in FACS sorted astrocytes suggests that astrocytes in PFC may express proteins involved in the homeostasis of dopamine.

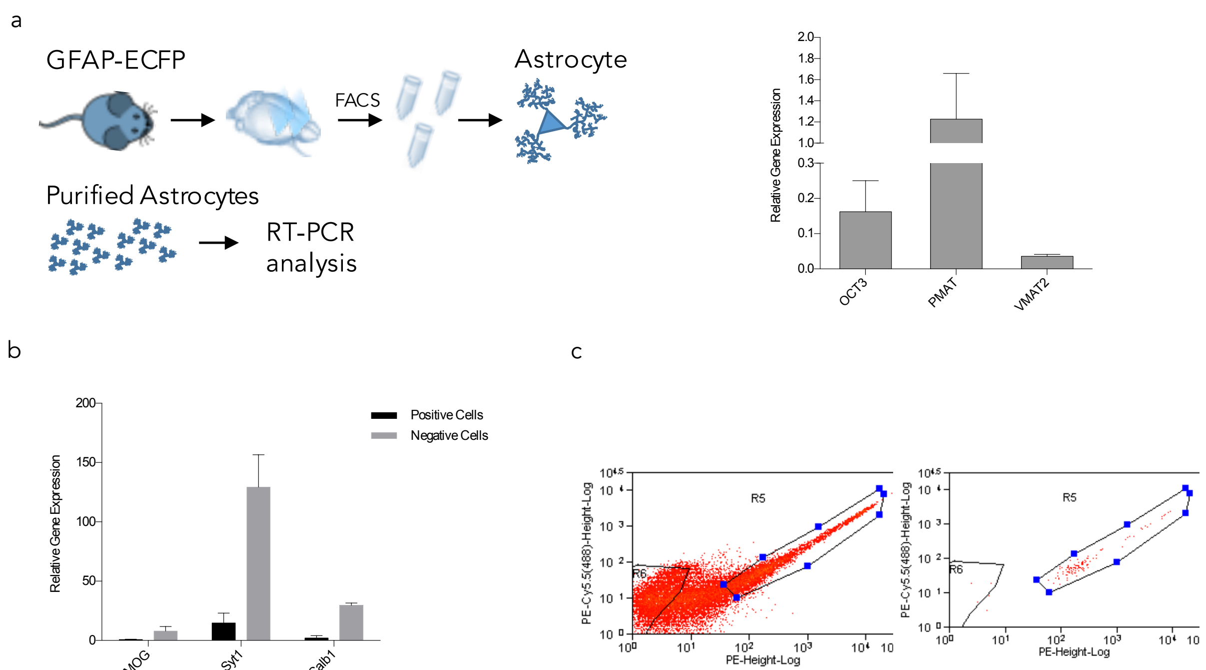

Figure 6 – FACS-sorted astrocytes express mRNA for dopaminergic transporters
(a) Representative image of the RT-PCR analysis and quantification of the relative expression of OCT3, PMAT and VMAT2 mRNA in FACS-sorted astrocytes in comparison with β-actin. The error bars indicate the SEM. **(b)** RT-PCR analysis of the relative expression of MOG, Syt1 and Calb1 mRNA in FACS-sorted astrocytes in comparison with β-actin. The error bars indicate the SEM. **(c)** PFC astrocytes were isolated by means of FACS-sorting, and some were re-analysed to reveal >90% purity.

3.2. Dopaminergic Transporters are expressed in PFC astrocytes

In order to verify the localization and distribution of the dopamine transporters *in vivo* astrocytes, we performed immunolabelling experiments in rat tissue. Coronal brain slices (30 µm) from rat (P40) were stained with antibodies raised against Glial Fibrillary Acidic Protein (GFAP), Glutamine Synthetase (GS), two specific markers for astrocytes (Patel et al., 1985) and OCT3, PMAT and VMAT2. Immunostaining images of PFC showed that astrocytes contain the signal for these transporters (Figure 7). In particular, OCT3 and PMAT signal was detected mainly around the cell body of astrocytes (Figure 7a-b). Moreover, the VMAT2 signal was identified in cell bodies and large process of GS positive cells (Figure 7c). These data were confirmed by single plane images.

These data confirm that OCT3, PMAT and VMAT2 are expressed in PFC astrocytes and they may play an important role in DA homeostasis.

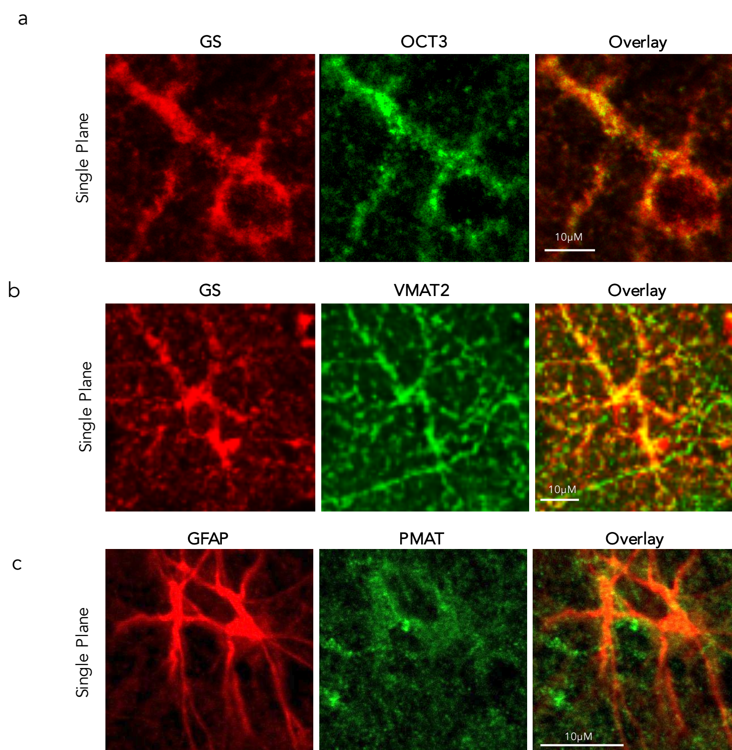

Figure 7 – Astrocytes express OCT-3, VMAT-2 and PMAT

Confocal immunofluorescence imaging of astrocytes in rat prefrontal cortex (PFC) of postnatal day P40 showing: **(a)** Astrocytes stained with glutamine synthase (GS) (red), OCT3 (green) co-localization between the GS and OCT3 signal in astrocytes (yellow). **(b)** Staining of GS (red), VMAT2 (green) and co-localization between both in yellow. **(c)** Glial Fibrillary Acidic Protein (GFAP) (red), PMAT immunolabeling (green) and colocalization (yellow).

3.3. Uptake 2 inhibition causes decrease in intracellular FFN200 levels in Astrocytes

FFN200 is a fluorescent false neurotransmitter and substrate of VMAT2 used to trace dopaminergic axons (Pereira et al., 2016). Taking advantage of its proprieties we used FFN200 to analyze the functionality of dopaminergic plasma transporters present in astrocytes.

Using RT-PCR we assessed the mRNA levels for dopamine transporters OCT3, PMAT and VMAT2 in cell cultured astrocytes from prefrontal cortex (Figure 3a). Similar to the results

obtained in FACS sorted cells (Figure 8a) we found significant levels of mRNA from PMAT and VMAT2, the levels of OCT3 mRNA were under detection.

In order to analyze the FFN200 uptake in astrocyte, we incubated different concentrations of FFN200 (100 µM, 250 µM and 500 µM) in cultured astrocytes (Figure 8b). The results showed that astrocyte can uptake FFN200 in dose dependent manner with a peak at 500 µM. Moreover, we incubated cultured astrocytes with 500 µM in presence or in absence of uptake 2 inhibitor, decynium 22 (D22) (Wang, 2016), the D22 significant reduces the fluorescence of FFN200 puncta in astrocytes (83,9 ± 19,3 %) compared with control cells (n=3 cells, Student's t test, ***p<0.001, Figure 8c)

Finally, we determinate the K_m for FFN200 by initial rates experiments (K_m of 2224 ± 908,2 µM). The results are showed in representative Michaelis-Menten plots for FFN200 (Figure 8d). At 500 µM we start to observe a reduction of FNN200 uptake (30,8 ± 9,5 %) in the presence of D22, the reduction of FFN200 uptake increases proportionally to the concentration at 1000 µM is 53,3 ± 14,5 % and at 1500 µM is 67,7 ± 8,16 %.

Overall, cultured astrocytes were found to accumulate puncta FFN200 at higher concentrations (500µM) and decrease FFN200 accumulation when OCT3 and PMAT are blocked. These data show that these transporters are important to uptake FFN200 in astrocytes *in vitro*.

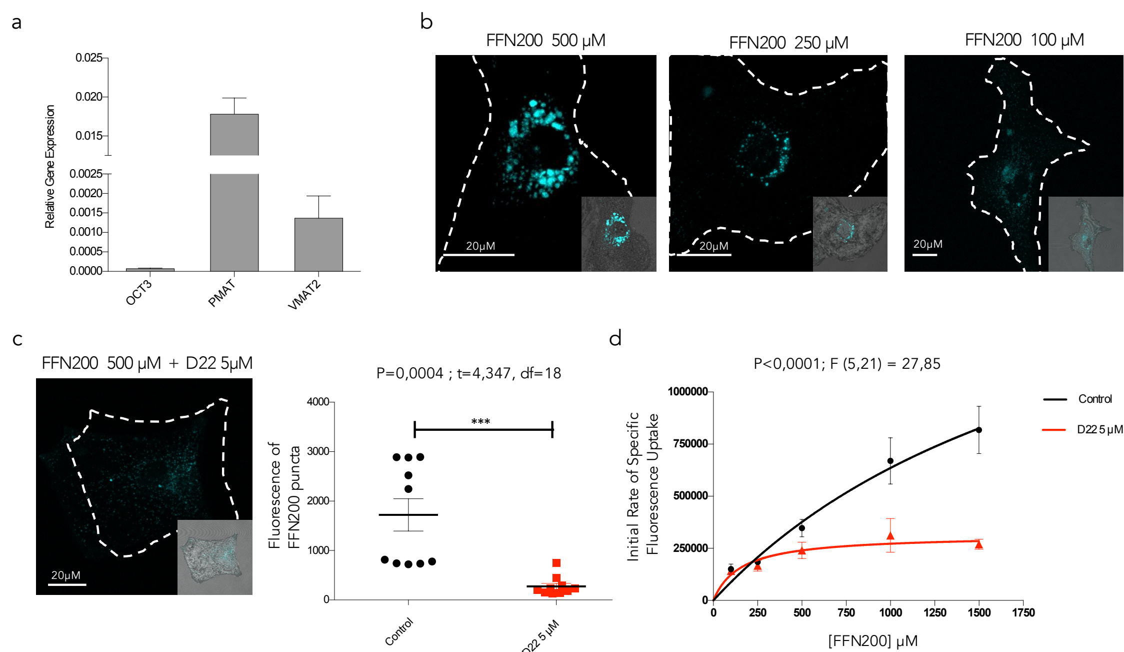

Figure 8 – Dose-dependent uptake of FFN200 by cultured astrocytes
(a) RT-PCR analysis of relative expression of OCT3, PMAT and VMAT2 in cultured astrocytes in comparison with β-actin. The error bars indicate the SEM. **(b)** Confocal fluorescence images of cultured astrocytes after incubation with different concentrations of FFN200 (cyano) (500 µM, 250 µM and 100 µM) **(c)** Confocal fluorescence image of FFN200 (cyano) and respective fluorescence FFN200 puncta analysis of cultured astrocytes incubated with 5µM D22 (Uptake2 inhibitor) versus control. **(d)** Initial rates of specific uptake of FFN200 in the absence (black curve) and presence of D22 (red curve).

3.4. Astrocytes express VMAT-2 in specific late endosome/lysosome like compartments

The storage and release of DA in neurons is mediated by VMAT2 (Henry et al., 1994; Pothos et al., 2000), which storages dopamine in specific small vesicles. Since we found VMAT2 in astrocytes (Figure 2c) the next step was to understand if the inhibition of VMAT2 with reserpine

(Pletscher, 1977) had an effect in FFN200 accumulation/storage. In fact, in the presence of reserpine we had a more spread distribution of FFN200 and a significant decrease of FFN200 puncta intensity (88,4 ± 19%) (n=3 cells, Student's t test, ***p<0.001, Figure 9a).

Previous work in Bezzi's Lab demonstrated the origin of the compartments where VMAT2 is located in in vitro astrocytes. Basically, they found the VMAT2 is express in VAMP7/8 (Advani et al., 1999), cathepsin B (Mohamed and Sloane, 2006) and LAMP1 (Cook et al., 2004) positive organelles, specific late endosomes/lysosome markers (Figure 9b). To understand if VMAT2 is crucial for the uptake and accumulation of FFN200 in these lysosome like compartments, we incubated cultured astrocytes with LysoTracker (50 nM), a fluorescent dye that preferentially labels lysosomal compartments (Chazotte, 2011; Padamsey et al., 2017) in presence of FFN200 (500 µM) (Figure 9c). We found a specific colocalization between FFN200 and the LysoTracker of 86,33 ± 8,62 % suggesting that the majority of FFN200 accumulates in acidic/ lysosome like compartments.

These data indicate that VMAT2 in astrocytes is fundamental to uptake and store FFN200 in lysosome like compartments.

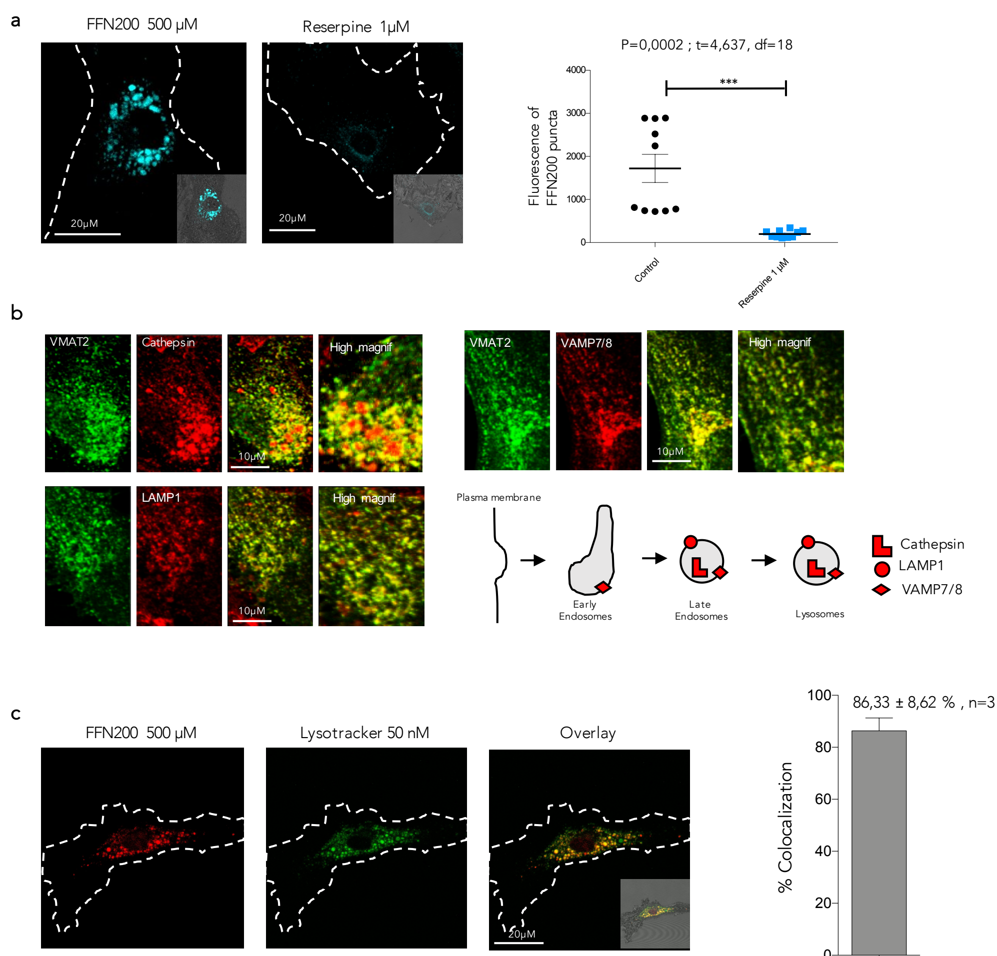

Figure 9 – VMAT2 function and expression in lysosome like compartments
(a) Confocal fluorescence image of FFN200 (cyano) and respective fluorescence FFN200 puncta analysis of cultured astrocytes incubated with 1µM Reserpine (VMAT2 inhibitor) versus control. **(b)** Intracellular distribution of endogenous VMAT2 in cultured astrocytes (Scale bars: 10 µm). The confocal images show double immunolabelling of VMAT2 (green) and markers of intracellular organelles (red): late endosomes, lysosomes (cathepsin); sorting endosomes, late endosomes, lysosomes (LAMP1); late endosomes, lysosomes (VAMP7/8). Photo credit: Julie Marchaland. Draw illustrating the intracellular distribution of the markers used. **(c)** Confocal images showing double immunolabeling of FFN200 (red) and LysoTracker (green) in cultured astrocytes and quantification of the colocalization (yellow).

3.5. FFN200 accumulation in vitro depends on VMAT2

In order to investigate the role of role of VMAT2 in astrocytes we used an inducible knock-out mouse line in which the protein could be specifically deleted in astrocytes in a temporally controlled manner. This specific transgenic mouse model expresses the endogenous VMAT2 gene between two cre-excisable loxP sequences and TAM-inducible cre^{ERT2} recombinase under promoter of human astrocytic glial fibrillary acid protein (hGFAP) (here referred to as cre^{ERT2}XVMAT2XR26-tdtomato). We isolated primary astrocytes from control $VMAT2^{loxP/loxP}$ (here referred to as control Loxtam) and Cre^{ERT2}XVMAT2XR26-tdtomato pups. RT-PCR analysis confirm the decrease of VMAT2 mRNA in Cre^{ERT2}XVMAT2XR26-tdtomato cells (86,4 ± 18,2 %) (n=2 cells, Student's t test, *$p<0.05$, Figure 10a).

In Cre^{ERT2}XVMAT2XR26-tdtomato cells there is a significant decrease in the fluorescence of FFN200 puncta (71,5 ± 10,5 %) compared with Control LoxTAM (n=5 cells, Student's t test, ****$p<0.0001$, Figure 10b).

Thus, Cre^{ERT2}XVMAT2XR26-tdtomato showed a similar effect of reserpine confirming that FFN200 is a subtract for VMAT2 also in astrocytes and indicating that this transporter is the key element for FFN200 storage.

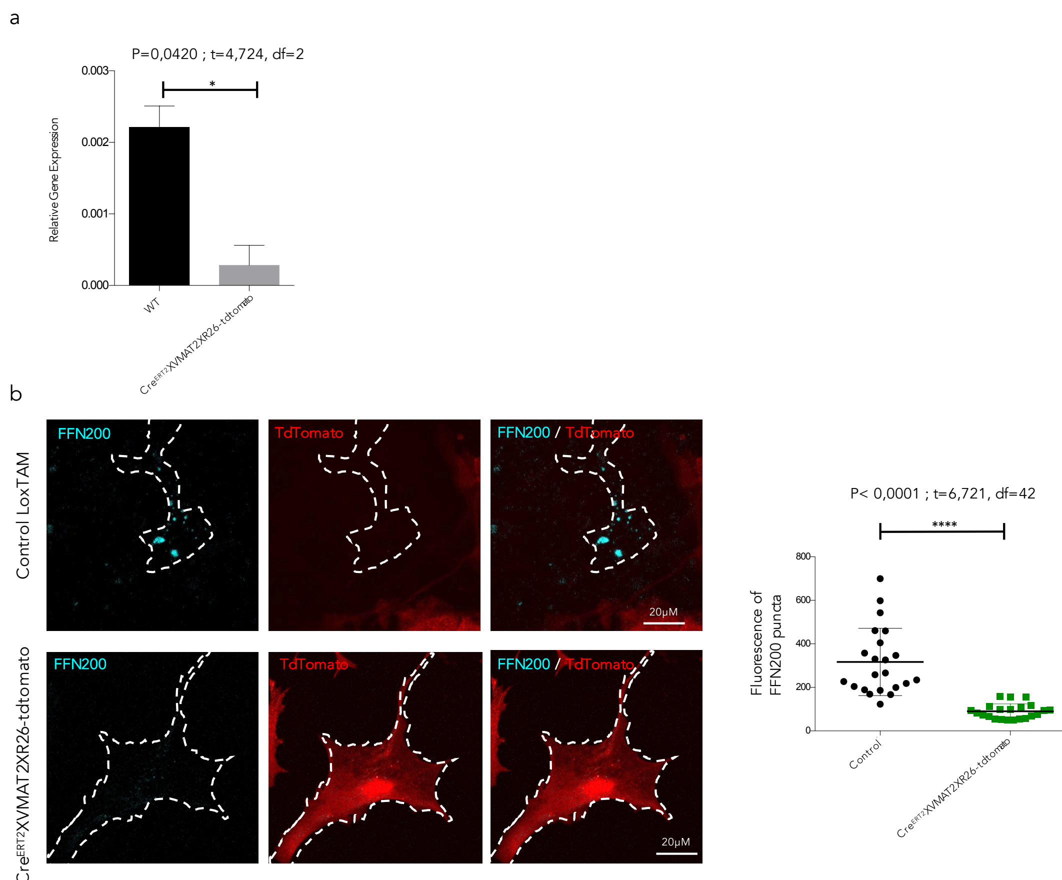

Figure 10 – FFN200 accumulation in cre^{ERT2}XVMAT2XR26-tdtomato mouse model

(a) RT-PCR analysis of relative expression of VMAT2 in cultured astrocytes (WT and cre^{ERT2}XVMAT2XR26-tdtomato) in comparison with β-actin. The error bars indicate the SEM.

(b) Confocal fluorescence image of FFN200 (cyano) in a cre^- cell (Control LoxTAM) and a cre^+ cell (creERT2XVMAT2XR26-tdtomato) in creERT2XVMAT2XR26-tdtomato mouse. At the right panel analysis of the fluorescence FFN200 puncta analysis of Cre^{ERT2}XVMAT2XR26-tdtomato cultured astrocytes versus control. **(b)** RT-PCR analysis of relative expression of VMAT2 in cultured astrocytes (WT and Cre^{ERT2}XVMAT2XR26-tdtomato) in comparison with β-actin. The error bars indicate the SEM.

3.6. VMAT2 in astrocytes controls the FFN200 accumulation ex vivo

To understand the FFN200 accumulation, we investigate the putative role of Plasma membrane transporters (OCT3 and PMAT) and vesicular monoamine transporter 2 ex vivo using control hGFAP-tdtomato and Cre^{ERT2}XVMAT2XR26-tdtomato mice. The incubation of FFN200 (10 µM) in control Cre^{ERT2}XR26-tdtomato acute slice from the PFC showed the labeling only in dopaminergic axons, no signal in astrocytes was found (Figure 11a). Moreover, the FFN200 puncta intensity was increased in astrocytes incubated with 500µM (Figure 11b). Interestingly, the incubation of acute slices with FFN200 (500 µM) in the presence of D22 or Reserpine significant decrease the number of FFN200 puncta intensity (81,1 ± 8,4 % with D22 and 92,8 ± 7,8% with Reserpine) (n=6 cells, Student's t test, **** $p<0.0001$). Finally, using acute slices of Cre^{ERT2}XVMAT2XR26-tdtomato where VMAT2 was genetically deleted in astrocytes, we found a significant reduction of FFN200 intensity puncta in recombinant astrocytes (91,3 ± 7,6%) (n=6 cells, Student's t test, **** $p<0.0001$).

These results indicate that the data obtained *in vitro* are reproducible in brain slices (*ex vivo*) and confirm that OCT3 and PMAT are crucial for the uptake of FFN200 into the intracellular space. These data also confirm that VMAT2 is the main responsible for the storage of FFN200 inside astrocytes.

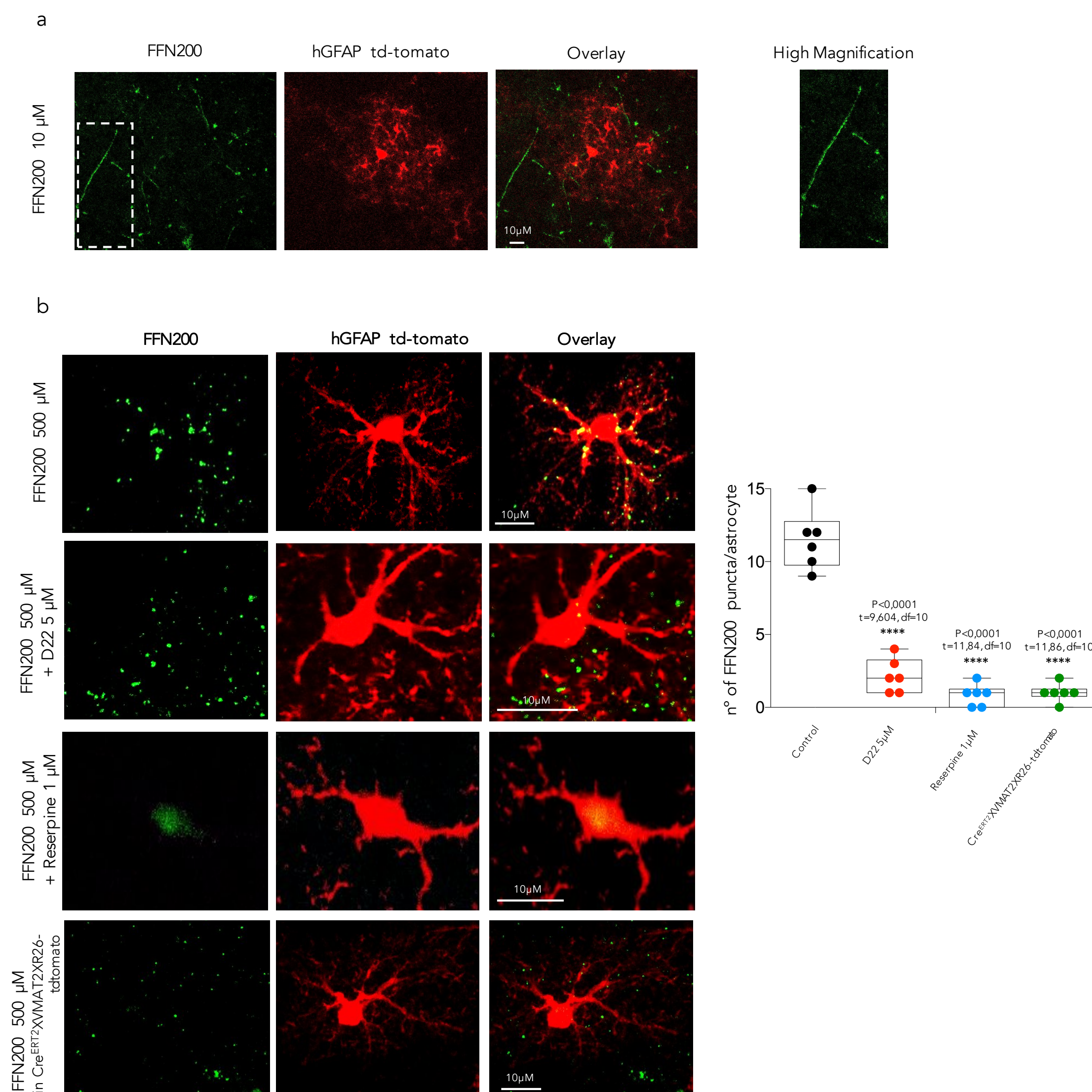

Figure 11 – FFN200 accumulation in acute brain slices
(a) Confocal fluorescence image of FFN200 10 μM (green) in acute slices from hGFAP-tdtomato mice (red) and overlay (yellow). In the right panel a high magnification image showing a fiber (in green). **(b)** Confocal fluorescence image of FFN200 500μM (green) in hGFAP-tdtomato mice (red) and in cre^{ERT2}XVMAT2XR26-tdtomato mice (red). Number of FFN200 puncta per astrocytes analysis of acute slices from hGFAP-tdtomato mice with treatments (D22 or Reserpine) and cre^{ERT2}XVMAT2XR26-tdtomato mice.

3.7. VMAT2 in astrocytes is fundamental to uptake FFN200 in specific intracellular organelles

In order to confirm that VMAT2 in astrocytes is fundamental to uptake and storage FFN200 in specific intracellular acid compartments, we measured the diameter of this puncta (average is around 1 μm) (Figure 12a), the same average size of late endosome/lysosomes organelles (Yu et al., 2010). The incubation of acute slices with FFN200 and LysoTracker (Figure 12b) leads to similar results as *in vitro*. High magnification confocal analysis showed the colocalization between FFN200 and the LysoTracker signal (Figure 12c, white arrows). Interestingly, no colocalitation in neuronal fibers was detected (grey arrow) indicating the presence of FFN200 inside these intracellular acid organelles only in astrocytes (white arrow).

Taken together these data suggest that VMAT2 in astrocytes is fundamental to uptake and store FFN200 inside lysosome like compartments.

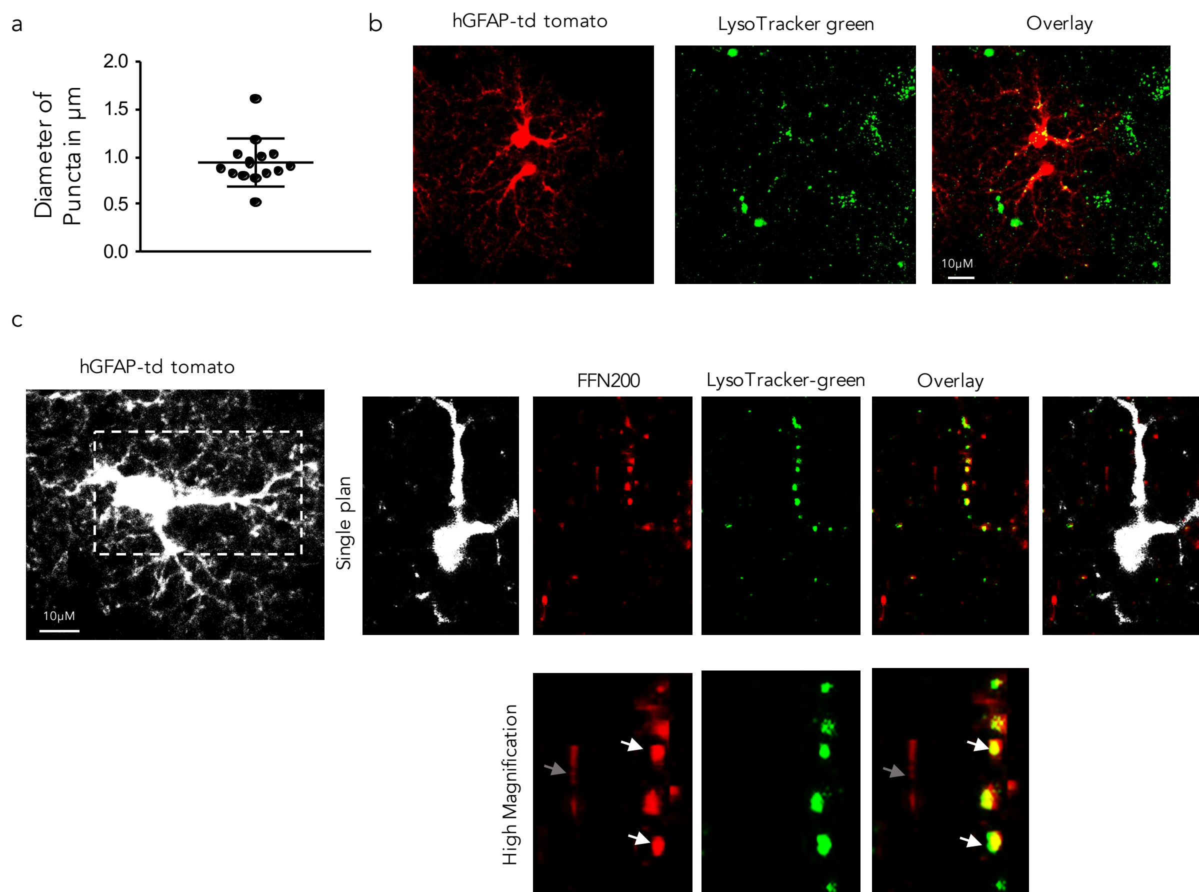

Figure 12 – FFN200 and LysoTracker accumulation in acute brain slices
(a) Analysis of the diameter of FFN200 puncta from acute brain slices. **(b)** Confocal fluorescence image of hGFAP-tdtomato (red), LysoTracker (green) and overlay (yellow) **(c)** Confocal fluorescence image of FFN200 500 μM (red), LysoTracker (green) and overlay (yellow). In the bottom part of the panel, the images are represented in high magnification. White arrows show the co-localization between the FFN200 and LysoTracker and the grey arrow shows a fiber.

4. Discussion and Conclusion

The PFC plays an important role in cognitive and executive functions (Wilson et al., 2010; Euston et al, 2012). In this brain region we have dopaminergic projections that release DA in order to control the the neuronal network. Indeed, DA homeostasis in the PFC shows peculiar features, since this dopaminergic area contains drastically less high-affinity dopamine transporter (DAT) than the striatum or Nucleus Accumbens (Sesack et al., 1998), and several studies have shown that DA uptake through DAT plays a crucial role in clearing extracellular levels (Giros et al., 1996). So a possible alternative is that the uptake of DA in the PFC is made by astrocytes through uptake2 transporters like OCT3 and PMAT. The cellular and molecular mechanisms that control DA homeostasis are not well understood. Recent studies using RNAseq of purified astrocytes showed the presence of dopamine transporters responsible for uptake2 (OCT3 and PMAT) and vesicular transporters (VMAT2) in human and rodent astrocytes (Zhang et al., 2016), withal the physiological role of these proteins in astrocytes from the prefrontal cortex has never been investigated in detail.

We verified the presence of plasma membrane uptake 2 transporters and vesicular transporter VMAT2 in PFC astrocytes and then, we examined whether and how these three transporters may control the dopamine homeostasis. To achieve our objectives, we took advantage of a Fluorescent False Neurotransmitter (FFN200) that consists in a dopamine molecule associated with a fluorescent dye, in order to mimic the dynamic effect of "real dopamine" (Pereira et al., 2016).

Interestingly, we found that some of the proteins that play an important role in DA homeostasis such as OCT3, PMAT and VMAT2 are expressed in the PFC astrocytes. Although these uptake2 transporters are mainly expressed in neurons, OCT3 and PMAT have been reported in astrocytes of adult rodents, where they regulate the extracellular clearance of amines including DA (Vialou et al., 2008; Cui et al., 2009; Naganuma et al., 2014). Our data show that astrocytes are able to uptake FFN200 in dose dependent manner and this uptake is mediated by OCT-3 and PMAT transporters. Thus our results suggest that the plasma membrane transporters OCT3 and PMAT may play an important role in the extracellular clearance and consequently homeostasis of DA in the PFC.

Furthermore, our group found that astrocytes in PFC express VMAT2 and provided evidences that this vesicular transporter acts in concert with OCT3 to control the level of dopamine in PFC. In particular, they showed that VMAT2 is fundamental to store dopamine in astrocytes. Alteration of this system causes unbalance of dopamine in PFC (Petrelli et al, 2018, in press). To understand the molecular mechanism through which astrocytes uptake and store dopamine we performed different experiment using FFN200 in the presence of a VMAT2 inhibitor (Reserpine) or in the absence of a VMAT2 (genetically deleted in Cre^{ERT2}XVMAT2XR26-tdtomato mice). We found that the pharmacological inhibition or genetic deletion of VMAT2 in astrocytes causes a significant decrease the cytosolic levels of FFN200 taken up by the plasma membrane transporters and the number of FFN200 positive puncta. These data showed that the extracellular uptake and the storage of FFN200 in specific organelles in astrocytes is fully dependent of VMAT2. It is likely that the control of dopamine homeostasis by astrocytes is fully dependent by this bi-dynamic control between VMAT2 and OCT3/PMAT. In fact, in absence of VMAT2, this dynamic equilibrium is lost, and the cytoplasmatic dopamine taken up by OCT3/PMAT decreases. This could be the effect of

dopamine metabolic enzymes that quickly metabolize the dopamine in astrocytic cytoplasm. Further experiments are still necessary to understand this molecular mechanism.

Finally, our lab found that astrocytes in vitro express VMAT2 in specific secretory organelles that contain also exocytic machinery to promote the fusion of organelles with plasma membrane (Petrelli and Bezzi, 2016; Verkhratsky et al., 2016). Lysosomes can be more than simple organelles intended to degrade molecules. Besides other functions, lysosomes are important regulators of cell homeostasis by storing molecules and then secreting them when needed (Luzio et al., 2007; Appelqvist et al., 2013). We discovered that the FFN200 had a colocalization of about 83% with a lysosome/late endosome marker (LysoTracker), suggesting that dopamine accumulates in late endosome/lysosome like organelles in astrocytes by VMAT2. Moreover, the role of astrocytic VMAT2 in the regulation of extracellular levels of DA may also arise from a direct release of DA-containing organelles. The release of neurotransmitters by astrocytes in vivo is still debated in neuroscience. For this reason, the demonstration of dopamine from astrocytes in vivo requires the development of new technological tools that allow us to answer at this very important question.

In conclusion, this study highlights the existence of a previously unknown pathway by which VMAT2 in association with OCT3 and PMAT control the dopamine homeostasis and allows the DA storage in lysosome/late endosomes like compartments in PFC astrocytes. OCT3 and PMAT were both founded in astrocytes (Cui et al., 2009; Yoshikawa et al., 2013). Very recently VMAT2 has been reported in astrocytes from the PFC by our group (Petrelli et al., 2018, in press). These discoveries support our hypothesis that these three transporters are crucial to the proper uptake and storage of DA in astrocytes. Therefore, strategies to control the OCT3, PMAT and VMAT2 activity in the PFC may lead to new pharmacological treatments for neuropsychiatric diseases such as schizophrenia and autism.

5. Future Perspectives

In this project we analyzed how astrocytes participate in the dopamine control in PFC. However, more experiments are needed in order to understand the cellular and molecular mechanisms of action. In the future, it will be interesting to perform electromicroscopy analysis to study the distance between astrocytic processes and Tyrosine Hydroxylase (TH) positive boutons, in order to understand the morphological and functional interactions between them. The encouraging results in the VMAT2 role in storing DA, open the doors to the possibilities to occur DA release from astrocytes. To test this, we could stimulate astrocytes pre-incubated with FFN200, and then assess the FFN200 release. This experiment could be done by using ionomycin, that raise the intracellular level of Ca^{2+} (Morgan and Jacob, 1994) or with metabotropic glutamate receptors agonists and increasing by an indirect pathway the intracellular Ca^{2+} (Schwartz and Alford, 2000). Other strategy to increase the levels of Ca^{2+} is using a viral strategy, we could express in astrocytes the hM3Dq DREADD, an engineered receptor that upon stimulation with Clozapine-N-oxide (CNO) activates the cell (Covelo and Araque 2018; Adamsky et al, 2018) leasing to a possible release of FFN200. These experiments are fundamental to understand ifdopamine can act like gliotransmitter.

For a long term experiment it will be curious to test the compound on human cells and human tissue and in a more advanced step to test in human Induced Pluripotent Stem Cells model of schizophrenia to see if there is any modification in DA uptake and storage compared to control cells.

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