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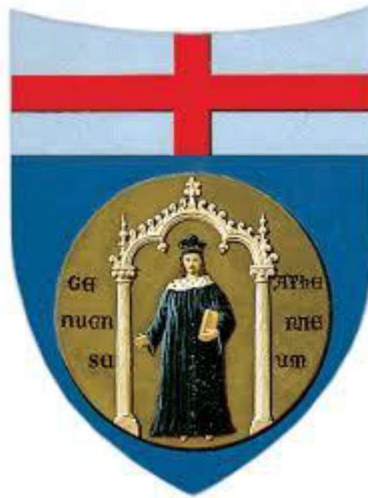

PhD Programme in Experimental Medicine
Pharmacology and Toxicology curriculum
XXXIII Cycle

“Signalling from astrocyte processes in CNS”

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INTRODUCTION

Glial cells in Central Nervous System (CNS) were first studied during the 19th century by the eminent scientists as Santiago Ramon y Cajal, Pio del Rio-Hortega, and Rudolf Virchow. At that times, glia was merely described as a sort of "glue" necessary to maintain the structure of the nervous system: Rudolf Virchow, indeed, used the evocative German word "Nevernkitt", literally meaning "nerve-glue" (Kettenmann and Verkhratsky, 2008). However, since Virchow's observations, glia role in CNS has been widely reconsidered. From the concept of glia as a *"substance… which lies between the proper nervous parts, holds them together and gives the whole its form in a greater or lesser degree"* (Parpura, 2012) new insights have been gathered and, to date, glia is considered more than a mere support for neural cells.

Glial cell population is composed by 3 main cell types: astrocytes, microglia, oligodendrocytes and their precursors (NG2-glia).

Astrocytes have a prominent role in neural development, in maintaining homeostasis and neurons survival, oligodendrocytes ensheath, and neuronal axons myelination, while microglia make up innate immune cells in the brain.

ASTROCYTES: FUNCTIONS AND PHYSIOLOGICAL ROLES

Among all the glial cell populations, astrocytes represent the most abundant component in adult brain (Kettenmann and Ransom, 2005). Given their importance, they are the best studied and characterized glial cell population in CNS.

1. Morphology

The eminent Italian scientist Camillo Golgi described astrocytes-like cells in 1872, even though the term "astrocyte" was first used by Michael von Lenhossek in 1893, evoked by the peculiar morphology of these cells (the term *astron* means, indeed, star, while *kytos* stands for hollow vessel).

It was thank to Ramon y Cajal's impressive efforts that astrocytes complexity was finally comprehended: the development of a specific staining protocol targeting the astrocytic marker glial fibrillary acidic protein (GFAP), indeed, enabled Cajal to observe and describe

astrocytes pleomorphism (Ramon Y Cajal, 1909).
During the last century, astrocytes were classified in two different groups, based on their morphological characteristics. Such classification is currently in use, so that nowadays we can distinguish between fibrous and protoplasmic astrocytes (Ramon Y Cajal, 1909).
The former, mostly widespread in white matter, is characterized by small cell body endowed with numerous cytoplasmatic filaments and few thin processes. Fibrous astrocytes spatial disposition does not seem to follow any precise pattern: in fact, they appear equally distributed. On the other hand, protoplasmic astrocytes are the most common type of astrocytes within rodents and primate brains. Those cells are within the grey matter and seem characterized by a voluminous cell body with abundant granulous cytoplasm and numerous filaments (Ramon Y Cajal, 1909).
Classical and more recent neuroanatomical studies showed that both subpopulations have extensive contacts with blood vessels. Furthermore, electronic microscopy analysis showed that protoplasmic astrocytic filaments can envelope synapses, while the fibrous ones have contacts with the nodes of Ranvier. However, both can form gap junctions at the level of the extremity of the close astrocytic processes (Peters et al., 1991).
Through imaging experiments carried on post-mortem tissues, Oberheim and colleagues provided a high number of details on the morphology and structure of astrocytes, which showed high expression of *glial fibrillary acid protein* (GFAP), increasing according to an age-dependent pattern (Nichols et al., 1993).
Astrocytes are characterized by the presence of thick fibrillar structures within body cell and processes, which are aggregation of thin glial filaments composed by GFAP. GFAP is included into the family of intermediate filaments as vimentin or nestin (Pekny and Pekna, 2004) and its expression is narrowed down to astrocytes and Schwann cells. Many GFAP isoforms generated through alternative splicing pathway have been observed to date, for instance GFAP α, β, γ, δ, and κ, whose expression may vary in CNS due to physiological and pathological conditions.
Observing the single astrocytic cells, it is clear that GFAP is not homogenously distributed in cytoplasm but rather mostly localized in main cell ramifications, while absent in cell body and thin cytoplasmic processes (Andreiuolo et al., 2009; Blechingberg et al., 2007; Roelofs et al., 2005).
Thus, it is clear that GFAP expression represents the gold standard marker for astrocytes. Although other markers, for instance S100β, have been used, their selectivity has never

resulted highly accurate and thus they cannot represent a valid alternative to GFAP (Goncalves et al., 2008; Norenberg, 1979).

Recent genetic analysis led scientists to the identification of a wide number of molecules with higher expression in rodent and human astrocytes in comparison with glial or neuronal cells (Cahoy et al., 2008; Lovatt et al., 2007).

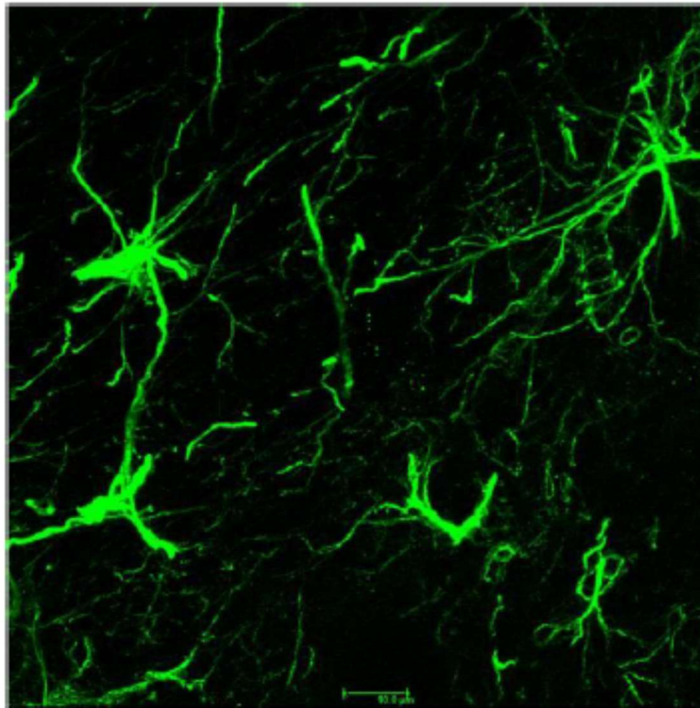

Fig.1 Striatal astrocytes

Immunofluorescence for GFAP on sagittal sections of adult rat striatum (20µm); image acquired under confocal microscope by Cervetto. The adult rat striatum was fixed for 24h at 4°C in 4% PAF, transferred in 30% sucrose solution in PSB and finally included in OCT before freezing in liquid nitrogen. The slices were saturated with blocking solution (containing Triton X-100 0.1% and 10% serum) for 1h at T° environment. The primary mouse anti-GFAP antibody (Sigma; diluted 1:500 in PBS with serum) was incubated for 24h at 4°C. After incubation with secondary antibody conjugated with Alexa Fluor 488, the slices were sealed with coverslip and a 20% glycerol solution in PBS.

2. Gap junctions

Connexins are an important family of membrane proteins which can assemble each other to form six-subunits membrane pores, called connexon. Connexons, in turn, were observed to bind the homologous expressed on sorrounding cells in order to constitute channelswith the subsequent formation a direct line for metabolic and electrical signalling (LeBeau et al., 2003; Herve et al., 2007; Yeager and Harris, 2007).

Astrocytes are also physically connected each other through gap junctions. In details, channels are constituted of several connexon subunits (e.g: Cx30 and Cx43). Those channels mediate astrocytes-astrocytes communication enabling the exchange of important molecules as ions, metabolites, substrates (as glucose) and neuromodulators (Pannasch and Rouach, 2013). It is recognized that gap junctions play a key role in glutamate or potassium uptake, avoiding the potenitally harmful accumulation of those molecules in the extracellular space. This gap junctions-mediated communication pattern significantly supports the traditional signal transmission pathways, constituted by the neuronal synaptic network.

3. Anatomical organization

Astrocytic cells are characterized by a high number of processes which allow astrocytes to set up a well-structured network covering the whole CNS, enveloping neurons and nervous fibers and filling the anatomical spaces between these structures.

Single cells observation showed that protoplasmic astrocytes in gray matter do not present overlapping domini and that only the distal extremities of the processes mutually interact through gap junctions (Bushong et al., 2002; Halassa et al., 2007; Nedergaard et al., 2003; Ogata and Kosaka, 2002). Between 5 to 10 thin cytoplasmatic processes extend from each protoplasmatic astrocyte spreading in the whole grey matter.

According to diverse studies, a single astrocyte process can get in touch with hundreds of neurons dendrites and ensheath more than 100.000 synapses in cerebral cortex and hippocampus (Bushong et al., 2002; Halassa et al., 2007; Ogata and Kosaka, 2002).

Interactions between synapses and astrocytic processes can go towards modifications: during memory development, for instance, astrocytic processes retract and synaptic space become subject to the lymphatic system action (Heller and Rusakov, 2015; Ostroff et al., 2014).

The important role of astrocytes in integrating information is structurally reflected in the tripartite synapses, where astrocytic processes tightly envelop synapses, contributing to regulate several aspects of synaptic signal transmission (Araque et al., 1999; Nedergaard and Verkhratsky, 2012; Perea et al., 2009; Verkhratsky and Nedergaard, 2014).

It is noteworthy to point out that the number of synapses contacted by astrocytes varies according to the brain region and that not all synapses in CNS can be considered "private", due to the presence of astrocytic processes.

For instance, the 90% of synapses formed by climbing fibers in cerebellum are covered by astrocytic processes, as well as the 65% of synapses between parallel fibers and Purkinje cells (Grosche et al., 1999; Xu-Friedman et al., 2001)

On the other hand, 29-56% of excitatory synapses in rat neocortex are ensheathed by astrocytic processes, while around the 90% of synapses within the IV cortical layer of adult mouse get in touch with the membranes of astrocytic perisynpatic processes (Bernardinelli et al., 2014).

3. Physiology

Unlike neurons, which generate action potential causing Ca^{2+}-dependent neurotransmitter release, astrocytes have different excitation pathways, based on the action of ions and cytoplasmatic second messengers (Zorec et al., 2012). Astrocytes can perceive neuronal activity through their membrane receptors, which sense neurotransmitters in the extracellular space. Neurotransmitter as glutamate and purines can activate sodium and potassium channels or metabotropic G-protein coupled receptors (GPCRs, Agulhon et al., 2008; Parpura and Verkhratsky, 2012; Zorec et al., 2012).

The activation of astroglial receptor usually enhances ion-mediated signals and/or second messenger production (e.g: cAMP) (Na^{2+} o Ca^{2+}; Kirischuk et al., 2012; Verkhratsky et al., 2012). Variation of calcium and cAMP concentrations seem to have different timing: while Ca^{2+} increase is phasic, cAMP one is tonic (Horvat et al., 2016).

The significant role of cytoplasmatic ions and second messenger in astrocyte-astrocyte and astrocyte-neuron communication was pointed out by several studies. In particular, the increase of intracytoplasmatic calcium can provoke gap junctions-mediated neurotransmitter release in astrocytes (Halassa et al., 2007; Nedergaard et al., 2003; Perea et al., 2009; Shigetomi et al., 2008; Volterra and Meldolesi, 2005).

The signalling pathways mediated by Gq and Gs can regulate exocytosis in astrocytes. Signalling molecules can in turn interact with neuronal receptors, modulating their excitability (Calegari et al., 1999; Parpura and Verkhratsky, 2012).

4. Role of astrocytes in neuronal development

During neurodevelopment, astrocytes differentiate later than neurons in several brain regions. However, astrocytes presence is determinant for the development and organization of both gray and white matter and for synaptogenesis. In particular, they can form molecular borders gradients which address neuronal axons and provide neuroblasts with the orientation during the migration towards their definitive site (Powell and Geller, 1999). Astrocytes are also involved in the formation of future synapses through the release of several signal molecules as trombospondin (Barres, 2008; Christopherson et al., 2005; Ullian et al., 2001). They seem to be implied in the elimination of synapses through signals which induce the C1q complement expression in the synapses to be eliminated, thus evoking microglia response (Barres, 2008; Stevens et al., 2007).
Finally, astrocytes are also implicated in the development of white matter, since it has been observed that loss or impairment of connexin and gap junctions lead to demyelination (Lutz et al., 2009).

5. Blood-brain barrier and regulation of blood flow

The blood brain barrier (BBB) is a physical barrier mainly composed by endothelial cells of capillaries, connected by tight junctions and forming a continuous barrier surrounding the blood vessels. BBB is interposed between blood and interstitial liquid and avoids to the potentially toxic molecules the access to cerebral parenchyma (Abbott et al., 2006; Ballabh et al., 2004).
Endothelial cells in BBB are surrounded by a basal lamina, perivascular pericytes, and astrocytes endfeet: it was hypothesized that they are an important component of the BBB, regulating molecules transport from blood to the nervous tissue (Abbott et al., 2006; Ballabh et al., 2004; Beck et al., 1984; Savidge et al., 2007).
Since astrocytes get in touch with blood vessels, they can regulate the blood flow to SNC through the release of molecular mediators as prostaglandin (PGE), nitric oxide, and arachidonic acid (AA) causing the modification of capillaries diameter and, subsequently,

of the blood flow (Gordon et al., 2007; Iadecola and Nedergaard, 2007). Furthermore, astrocytes might represent major mediators of blood flow variations in response to changes of neuronal activity (Koehler et al., 2009).

6. Energy and metabolism

Robust proofs suggest that astrocytes are major players in CNS metabolism. Astrocytic processes have contacts with blood vessels, axons (at the nodes of Ranvier level), and synapses, carrying glucose from the circulatory system to the cells within both gray and white matter.

Interestingly, it is widely recognized that astrocytes represent a major glycogen storage in CNS and that this molecule is mainly concentrated in high-density synapses areas (Peter set al., 1991; Phelps, 1972).

Recent studies showed that glycogen storage can support neuronal activity under hypoglycemic conditions and during periods of elevated neuronal activity (Brown and Ransom, 2007; Suh et al., 2007).

Importantly, glycogen storage in astrocytes might be regulated by neurotransmitters, for instance glutamate, as well as by metabolites diffused through gap junctions (Brown and Ransom, 2007).

7. Astrocytes heterogeneity

Given the relevance of astrocytes in nervous system physiology, their variety and functional complexity**,** many studies aimed to further deepen our knowledge of specialization and heterogeneity of these cells (Hewett, 2009). An important astrocyte group presenting both similar and different characteristics in comparison with protoplasmic and fibrous astrocytes was observed and described many years ago. This population includes Muller glia in retina, Bergmann glia in cerebellum, pituitary cells in neurohypophysis.

Those cells express typical astrocyte markers as GFAP, S100B, glutamine synthetase, and have specialized functions depending on the brain region of localization (Sofroniew and Vinters, 2010). Interestingly, heterogeneity of neuron-associated astrocytes is progressively increased during the evolutionary path, since astrocytes-neurons ratio raised is 1:6 in worms, 1:3 in rodent cortex, and 1:4 in human cortex, suggesting that their role

may have acquired higher importance through the evolution of nervous tissue (Nedergaard et al., 2003).

GLIOTRANSMISSION

Nowadays, the idea according to whom astrocytes represent a secondary cell population in CNS has been overcome. Astrocytic cells are recognized as prominent cells in regulating the synaptic activity and brain plasticity (Araque et al, 1999).
The release of transmitter was demonstrated first in neurons and only later in astrocytes. For many years, indeed, these cells were not considered able to secrete transmitters (Araque et al., 1999; Perea et al., 2009).

1. Signals from neurons to astrocytes

Astrocytes have different types of receptors for neurotransmitters, such as, for instance, glutamate and ATP. Several studies suggested that astrocyte processes in the cerebellum can be divided into different regions, each of which is able to interact with a limited number of synapses (Gundersen et al., 2015). These perisynaptic processes in Bergmann's glia are equipped with AMPA receptors for glutamate (Matsui et al., 2005; Saab et al., 2012) which can be directly activated by the glutamate released at the synapse level between parallel fibers and Purkinje cells (Matsui et al., 2005). The activation of receptors generates a local and transient increase of intracellular Ca^{2+} in the astrocyte processes of Bergmann's glia (Grosche et al., 1999). This communication seems to regulate the motor functions of the cerebellum (Saab et al., 2012).
On the other hand, astrocytes of cerebral cortex do not express AMPA receptors: the hypothesis that glutamate NMDA receptors may transmit the synaptic signal to astrocytes was advanced (Lalo et al., 2006). Astrocytes in hippocampus respond to the release of neurotransmitters by activating metabotropic glutamate and ATP receptors, which in turn produce an increase of intracellular Ca^{2+} concentration (Dani et al., 1992; Pasti et al., 1997; Perea and Araque, 2005; Porter and McCarthy, 1996). Furthermore, it was demonstrated that also physiological sensory stimulation can generate similar increase of the intracellular Ca^{2+} levels in astrocytes in diverse CNS regions (Petzold et al., 2008; Schummers et al.,

2008; Wang et al., 2006). It can be therefore supposed that astrocytes are strategically positioned to perceive at local level the synaptic activity of neurons and respond with an increase in intracellular Ca^{2+} (Gundersen et al., 2015).

2. Signals from astrocytes

The concept of astrocytes as secretory cells almost dates back to the discovery of glial cells: in 1895 Michael von Lenhossék introduced the term "astrocyte" (von Lenhossék, 1895), and in 1909 Hans Held observed granular inclusions in the astrocyte processes, interpreted as a sign of active secretion (Held et al., 1909). One year later, Jean Nageotte identified secretory granules in the glial cells of the grey substance (Nageotte, 1910). These granules were later called "gliosomes" by Alois Alzheimer and the hypothesis of an astroglial secretion was also considered by Wilder Penfield (Penfield et al., 1932). The term "gliosomes" coined in the first years of the 20th century should not be confused with the recent use of the name gliosomes to identify the sub-cellular particles derived from glial cells (Nakamura et al., 1993) and containing vesicles charged with transmitters (Stigliani et al., 2006; Cervetto et al., 2015; Cervetto et al., 2018). Both Nageotte and Penfield considered astrocytes as true endocrine components able to release their compounds into the bloodstream from pedicels closely associated with capillaries, or into extracellular space from the astrocyte processes surrounding synapses. In recent years, there was an increasing amount of evidence that astrocytes can release various substances that contribute to the regulation of CNS development and homeostasis, synaptogenesis and cognitive functions.

Therefore, astrocytes can play an active role in the neuroglial secretory network, which, by analogy with the endocrine system, may be defined as the CNS gliocrine system (Vardjan and Parpura, 2015).

Molecules released from astrocytes include: classical neurotransmitters (the "gliotransmitters", including glutamate, GABA, D-serine and ATP; Araque et al., 2014), precursors of neurotransmitter, neuromodulators, hormones, peptides, eicosanoids, metabolic substrates, reactive oxygen species scavengers (ROS), growth factors, factors that might be defined as "plastic" (e.g. substances that regulate synaptogenesis and synaptic connectivity), and finally pathologically relevant molecules such as inflammatory factors. Astrocytes release these signal molecules as a result of the increased intracellular Ca^{2+} in response to neuronal activity through numerous pathways. The same molecules are

usually released via different mechanisms, increasing the complexity/specificity of its action (Verkhratsky et al., 2016). Recently, many research groups focused on the study of the different release mechanisms used by astrocytes, ranging from exocytotic release to the use of channel proteins.

2.1. Vesicular release of gliotransmitters

Exocytotic release appeared very early during the evolution pattern (Spang et al., 2015; Vardjan et al., 2010) and most of the eukaryotic cells can release neurotransmitters. The fusion between the organuli and plasma membrane is fundamental both for intracellular communication and the activation of molecules, as receptors and transporters expressed in cell membrane. Exocytosis is dependent on the intracellular calcium levels and can occur both without stimulation (constitutive secretion) or in response to an exogenous stimulation (regulated secretion) (Kasai et al., 2012). Thanks to their fine regulation of neurotransmitters release, neurons represent the most reliable model for studying exocytotic neurotransmission. Exocytosis occurs also in astrocytes, even though through different molecular and kinetic mechanisms.

Vesicular release is promoted by the SNARE proteins family (Sollner et al., 1993), which, concomitantly with an increase of intracellular Ca^{2+} concentration, assemble in a proteic complex that mediate the fusion process between vesicles and extracellular membrane, causing the release of the content within the extracellular space. Several astrocytes organuli are subjected to regulated exocytosis:

- Synaptic-like microvesicles (SLMVs)

SLMVs have small dimension (30-100 nm of diameter) and are present in astrocytes in groups of 2-15 vesicles (Bezzi et al., 2004; Jourdain et al., 2007; Bergersen et al., 2012; Martineau et al., 2013).

Molecules are stored inside vesicles thanks to vesicular transporter for neurotransmitter (VNT), which present different substrate affinity and specificity in comparison with membrane transporters. To date, six types of transporters are known: the ones for glutamate (VGLUT 1-3), acetylcholine (VAChT), monoamine (VMAT1-2), GABA and glycine (VGAT) and, recently, ATP transporter (VNUT) (Chaudhry et al., 2008; Sawada et al., 2008; Blakely and Edwards, 2012).

D-serine storing in microvesicles is mediated by the VSerT transporter, although its molecular profile remains still unclear (Martineau et al., 2013)

Transporter are essential molecules in chemical transmission and represent the marker for regulated exocytosis. Some transporters, for instance the VGLUT 1-3, have been identified in cultured astrocytes (Fremeau et al., 2002; Bezzi et al., 2004; Crippa et al., 2006; Kreft et al., 2004; Montana et al., 2006).

In situ analysis on astrocytes via Gene Chip microarray or RT-PCR and immunohistochemistry lead to such variable and discordant results that transporters expression in astrocytes (and therefore the gliosis concept) was questioned.

In particular, immunohistochemistry results from many research groups pointed out that VGLUT 1, VGLUT 2 andVGLUT 3 colocalize within thin astrocytic processes which ensheath synapses in CNS (Anlauf and Derouiche, 2005; Bezzi et al., 2004; BowserandKhakh, 2007; Li et al., 2013; Sahlender et al., 2014; Liu et al., 2011; Marchaland et al., 2008; Montana et al., 2004; Ni and Parpura, 2009; Potokar et al., 2009; Stenovec et al., 2007; Zhang et al., 2004). However, Li et al. demosntrated that no expression of glutamate transporters in astrocytes can be detected by confocal imaging (Li et al., 2013). Such results were then confirmed by two other studies: microarray analysis never detected RNA transcripts codifying for VGLUT transporter in astrocytes from mouse brain (Cahoy et al., 2008; Lovatt et al., 2007), on the other hand, previous experiments carried out on isolated astrocytes showed the presence of mRNA for glutamate transporters (Bezzi et al., 2004; Danik et al., 2005; Zhang et al., 2004).

In astrocytes, both glutamate and D-serine are packed in SLMVs (Martineau et al., 2008, 2013; Bergersen et al., 2012). In cultured astrocytes, SLMVs colocalize with D-serine (Mothet et al., 2005; Martineau et al., 2013) and with VGLUT transporters, suggesting that these molecules can be localized within the same organelle (Bezzi et al., 2004; Ormel et al., 2012).

Nevertheless, in situ studies showed that glutamate and D-Serine are packed in different vesicles within the same astrocytic cells (Bergersen et al., 2012). A comparative study between astrocytic microvesicles (Crippa et al., 2006; Martineau et al., 2013) and synaptic vesicles in neurons pointed out that the former contain D-serine and glutamate, while the latter glutamate, glycine and GABA but not D-Serine (Martineau et al., 2013; Sild and Van Horn, 2013). SLMVs are present in astrocytes from different cerebral regions, including hippocampus and cortex and cerebellar Bergmann glia (Bergersen et al., 2012; Ormel et al.,

2012)

Glutamate and D-serine release quantification from astrocytes was carried out mostly on cells in culture.

An important number of studies share the same idea on the mechanisms underlying the vesicular release of these gliotransmitters (Bal-Price et al., 2002; Bezzi et al., 1998; Bezzi et al., 2004; Cali et al., 2014; Cali et al., 2008; Domercq et al., 2006; Görge et al., 2010; Höltje et al., 2008; Kanno and Nishizaki, 2012; Li uet al., 2011; Marchaland et al., 2008; Montana et al., 2004; Pasti et al., 2001; Trkov et al., 2012; Yaguchi and Nishizaki, 2010; Zhang et al., 2004; Zhang et al., 2004). Two important aspects are pointed out in these works:

1) glutamate and D-serine release are both Ca^{2+}-dependent;

2) that mechanism is sensitive to the inhibition of some components of the exocytotic release machine.

In fact, VGLUT and pomp H+-ATPasi inhibitors or tetanic toxins inhibit the release of gliotransmitters in astrocytes (Bezzi et al, 2004; Mothet et al, 2005; Martineau et al, 2008, 2014; Henneberger et al, 2010; Parpura and Zorec, 2010; Kang et al, 2013; Shigetomi et al, 2013; Cervetto et al, 2015).

Taken together, these results provide important proofs supporting the theory according to whom astrocytes use an exocytotic mechanism to release gliotransmitter in the extracellular environment.

- *Dense-core vesicles (DCVs)*

Dense-core vesicles play a key role in packaging and releasing hormones and neuropeptides in neuroendocrine (Burgoyne and Morgan, 2003) and neuronal cells (Klyachko and Jackson, 2002). DCVs can also store ATP, which is probably accumulated via the transporter VNUT, although its expression has never been confirmed. Ultrastructural characteristics of DCVs in astrocytes are similar to the ones in neuroendocrine and neuronal cells, even though their nucleus doesn't appear as dense as the neuroendocrine cells one (Potokar et al., 2008). DCVs presence in astrocytes is limited in astrocytes: VAMP2-positive vesicles cover only the 2% of the total amount of vesicles (Crippa et al., 2006); DCVs have in general greater dimension in comparison with microvesicles, presenting a diameter between 100 and 600 nm range (Calegari et al., 1999; Hur et al., 2010; Prada et al., 2011), DCVs found in medium of cultured astrocytes are endowed with the secretory proteins secretogranine II (Calegari et al., 1999; Paco et al., 2009; Prada et

al., 2011) and secretogranine III (Paco et al., 2010), cromogranine (Hur et al., 2010), ANP (Kreft et al., 2004; Paco et al., 2009), neuropeptide Y (Ramamoorthy and Whim, 2008; Prada et al., 2011) and ATP (Coco et al., 2003; Pangrsic et al., 2007). Dense nuclei vesicles containing secretogranine were observed in astrocytes derived from human brain samples (Hur et al., 2010), confirming the existence of these vesicles in situ.

- *Secretory lysosomes*

In vitro, lysosomes contribute to ATP packing and Ca^{2+}-dependent exocytotic release (Zhang et al., 2007; Jaiswal et al., 2007; Li et al., 2008). Lysosomes dimensions are within the range of 300-500 nm and cohabit with microvesicles in astrocytes. Apparently, these organelles are not endowed with VGLUT transporter and VAMP2 (Zhang et al., 2007; Liu et al., 2011) protein, whilst specific lysosomes markers as catepsin D, LAMP1 (Zhang et al., 2007; Martineau et al., 2008) and VAMP7 (Chaineau et al., 2009) are expressed. Exocytosis from lysosomes in astrocytes mostly depends on the protein VAMP7 which allows both ATP and catepsin β release.

A negative regulation of VAMP7 expression inhibits the fusion of ATP-containing vesicles and the diffusion of ATP-mediated intracellular Ca^{2+} waves Verderio et al., 2012). Secretory lysosomes fusion is due to a slow and local increase of Calcium concentration, while microvesicles fusion is caused by Ca^{2+} peaks (Verderio et al., 2012).

2.1.1. Molecular mechanisms of astroglial exocytotis

Protein SNARE expression in cultured astrocytes was demonstrated 20 years ago (Parpura et al., 1995). Further studied stressed out that astrocytes also express proteins typical of neuronal synaptic vesicles, e.g. VAMP2, or involved in exocytotic trafficking of vesicles in non-neuronal cells as SCAMP and VAMP3 (Parpura et al., 1995; Maienschein et al., 1999; Wilhelm et al., 2004; Mothet et al., 2005; Crippa et al., 2006; Montana et al., 2006; Martineau et al., 2008). Interestingly, the expression of SNARE associated proteins as Synaptotagmine 4 has been demonstrated in vitro (Zhang et al., 2004a).

Immunohistochemical and confocal microscopy studies showed that a considerable number of SNARE proteins, including VAMP2 (Wilhelm et al., 2004) and VAMP3 (Bezzi et al., 2004; Zhang et al., 2004a; Jourdain et al., 2007; Bergersen and Gundersen, 2009; Schubert et al., 2011) are expressed in situ, in addition to other SNARE proteins as TI-

VAMP/VAMP7 (Verderio et al., 2012), SNAP 23 and sintaxin 1 (Schubert et al., 2011). VAMP 2 and 3 colocalize with the transporters VGLUT1-2 on glutamate-carrying vesicles and, presumably, with D-serine; TI/VAMP7 colocalize with the markers of the late endosomial compartments (Verderio et al., 2012) loaded with ATP.

On the other hand, neuronal proteins generally associated to SNARE proteins, such as synaptotagmine 1 and 2, and synaptophysin (Wilhelm et al., 2004), have not been detected in astrocytes in situ.

In brain slices, VAMP2 and/or VAMP2 inhibition mediated by tetanus toxin abolishes glutamate (Jourdain et al., 2007; Perea and Araque, 2007) and probably D-Serine release (Henneberger et al., 2010) in astrocytes. Transgenic mice expressing a SNARE protein with negative dominant mutation in astrocytes (Pascual et al., 2005; Hines and Haydon, 2013) display abnormality in behaviour, in synaptic transmission and in neurons maturation (Pascual et al., 2005; Hines and Haydon, 2013; Nadjar et al., 2013; Turner et al., 2013; Lalo et al., 2014; Sultan et al., 2015). These data suggest that in vivo exocytosis is VAMP2-dependent.

2.1.2.Speed of exocytotis in astrocytes

Although release in neurons occurs 0.5 ms after Ca^{2+} entry in cytosol, exocytotic release in astrocytes is a slower process, occurring later after stimulation (Neher, 2012; Sudhof, 2012).

Furthermore, inhibition of astroglial exocytosis affects only slow electrical activity in cerebral cortex (Fellin Lapchak and Taboada, 2010 et al., 2009), while the fast one is not apparently affected by alterations of gliotransmission (Slezak et al., 2012). The slow cynetic of vesicular release in astrocytes is likely related to the differences of the pathway underlying exocytosis in neurons and astrocytes. For instance, while active zones in presynaptic terminal are characterized by structurally patterned vesicle, electronic microscopy studies have never detected such a structure in astrocytes (Bezzi et al., 2004; Jourdain et al., 2007; Bergersen et al., 2012).

Secondly, SNARE and SNARE-associated proteins responsible for neuronal and astrocytic exocytosis present differences in terms of the proteins SNARE complexes stability.

Different isoforms of VAMP protein share similar properties and may participate in various SNARE complexes (Wilhelm et al., 2004; Montana et al., 2009).

In astrocytes, protein VAMP2/3 or TI-VAMP, SNAP 23 and sintaxin are able to assemble to

compose the SNARE protein complex involved in vesicles fusion (Montana et al., 2009). In addition, R-SNARE protein associated with a single synaptic vesicle contains about 70 molecules VAMP2 (Takamori et al., 2006), while 25 molecules were detected in a single astrocytic vesicle (Singh et al., 2014). That low presence of VAMP2 may lead to a reduction of the density of protein SNARE complex that might cause a delay during the process of vesicle fusion in astrocytes.

2.2. Release of extracellular vesicles

Extracellular vesicles can be divided in two main groups:

- exosomes
- ectosomes

The latter generally transport a wide spectrum of bioactive substances such as cytokines, signalling proteins, mRNA and microRNA (Mause and Weber, 2010).

Exosomes are definded as vesicles with a diameter variable between 40 and 100 nm and are generated from the fusion of multivesicular bodies (MVBs) with cell membrane (Mathivanan et al., 2010).

On the contrary, ectosomes are larger (diameter from 100 and more 1000 nm) and are generated via cell membrane budding (Thery et al., 2009).

Exosomes generation follows the endophytic pathway; during differentiation, MVBs go towards lipid enrichment as cholesterol and sphingomyelin (Kobayashi et al., 1998; Chevallier et al., 2008).

Astrocytes can release both types of ECVs. In particular, ectosomes budding occurs upon activation of purinergic receptors P2X7 and involves fast activation of the acid sphingomyelinase which act on the outer layer of cell membrane.

2.3. Non-vesicular release of gliotransmitters

Gliotransmitters can be released by astrocytes not only through exocytosis-mediate mechanisms but also via non-vesicular pathway, as:

a) release through opening of volume-regulated anionic channels,

b) release through connexins (hemichannels) or pannexine

c) diffusion through purinergic ionotropic P2X7 receptors

d) release through transporters

e) release through molecules exchange via the cystine/glutamate antiporter (Malarkey and Parpura, 2011)
f) release through K+-channels Trek-1 and bestrofina-1 channels (Best-1; Woo et al., 2012).

All the release pathways are mainly Ca^{2+}-independent and apparently occur under pathological conditions (Agulhon et al., 2008; Hamilton and Attwell, 2010). The most common release mechanisms used by astrocytes depend on the opening of specific membrane proteins, as hemichannels and purinergic receptors P2X7.
Hemichannels Cx43 opening enables glutamate (Jiang et al., 2011; Ye et al., 2003) and ATP (Arcuino et al., 2002; Cotrina et al., 1998; Kang et al., 2008; Stout et al., 2002) influx. According to some authors, P2X7 receptors might form a membrane pore which allows glutamate (Duan et al., 2003), ATP (Ballerini et al., 1996; Suadicani et al., 2006) and GABA (Wang et al., 2002) to pass through astrocytes membrane.
Several studies tried to figure out the identity of the channel through which ATP is released: Saudicani and colleagues (2006) showed that ATP is released through P2X7 receptors and not hemichannels Cx43, while Kang (2008) and colleagues formulated diametrically opposed conclusions.
Contreras and colleagues advanced the hypothesis that hemichannels maintain a close position during cell resting switching to an open conformation only after strong membrane depolarization (Contreras et al., 2003). Hemichannels, then, might represent an important tool for gliotransmission under pathological conditions. Interestingly, hemichannels and P2X7 receptors open conformation results to be not Ca^{2+}-dependent (Thompson et al., 2008; Ye et al., 2003) in contrast with the exocytotic pathway.
In conclusion, it is likely that both hemichannels Cx43 and P2X7 receptors mediate a massive efflux of small cytoplasmatic molecules under physiological and pathological conditions.
Several studies described pannexins, proteins constituting hemichannels and gap junctions, as involved in ATP release pathway (Iglesias et al., 2009; Suadicani et al., 2012). Garre and colleagues further suggested that pannexin and connexin-constituted hemichannels might enable ATP release from astrocytes upon ATP-mediated P2X7 receptors activation (Garré et al., 2010).
According to many studies in literature, also volume-regulated anion channels (VRAC) may play a role in astrocytic glutamate release in situ (Abdullaev et al., 2006; Haskew-Layton et

al., 2008; Kimelberg et al., 1990; 2006; Takano et al., 2005; Wang et al., 2013; Benfenati et al., 2009). VRAC-mediated glutamate release is regulated by intracellular Ca^{2+} level (Haskew-Layton et al., 2005; Mongin and Kimelberg, 2005). So far, this pattern release has been demonstrated in astrocytes under pathological conditions, for instance an ischemic stroke (Feustel et al., 2004; Zhang et al., 2008) or in spreading depression condition (Basarsky et al., 1999).

It still remains unclear if neuronal activity is able to generate enough osmotic changes in astrocytes to cause VRAC opening and glutamate release.

Furthermore, VRAC are not a selective way for glutamate release since many others amino acids, such as aspartic acid, taurine and glutamine, can be released through these channels (Kimelberg et al., 1990; Takano et al., 2005).

In conclusion, it seems that physiological glutamate release can occurs in astrocytes through two pathways: channels or vesicular release.

Further investigations will be warranted to figure out whether these two different pathways cooperate or not under physiological or pathological conditions (Gundersen et al., 2015).

2.4. Gliotransmitters regulation of neuronal activity

Synaptic activity and neuron plasticity can be modulated by the gliotransmitters released from astrocytes. For instance, experiments on slices of hippocampus showed that glutamate released from astrocytes can increase or inhibit neuronal activity (Andersson et al., 2007; Di Castro et al., 2011; Fiacco and McCarthy, 2004; Jourdain et al., 2007; Liu et al., 2004; Navarrete et al., 2012; Navarrete et al., 2013; Perea G, Araque et al., 2007; Santello et al., 2011; Wang et al., 2013). Interestingly, Di Castro and colleagues demonstrated that astrocytes can perceive the reduction of neuronal basal activity responding with a rapid increase of the intracellular Ca^{2+} aimed to stimulate glutamate release and strength synaptic transmission (Di Castro et al., 2011).

Astrocytes in somatosensorial cortex are able to regulate the activity of NMDA receptors and the A1 receptor causing, respectively, an increase and a decrease of cortical rhythm (Fellin et al., 2009).

It is now recognized that astrocytes in hippocampus can release not only glutamate but also D-serine, which is thought to bind the glycine site on NMDA receptors, potentiating

the plasticity on glutamatergic synapses (Henneberger et al., 2010; Shigetomi et al., 2013; Yang et al., 2003)

ATP is another gliotransmitter which can control neuronal activity (Koizumi et al., 2003). ATP can have different effects on cells, depending on the cerebral region where it is released. According to Pascal and colleagues, ATP released from astrocytes in hippocampus is rapidly converted in adenosine which, in turn, enhances the inhibition of synaptic activity through the activation of A1 receptors for adenosine (Pascual et al., 2005). Similarly, ATP gliotransmission is important for the homoeostatic processes underlying sleeping (Halassa et al., 2009).

ATP can also activate postsynaptic P2X7 receptors causing a downregulation of $GABA_A$ receptors on dendrites (Lalo et al., 2014).

Furthermore, increase of intracellular calcium level in astrocytes caused by variation of extracellular pH values have been reported. High calcium level causes the release of ATP in extracellular environment, which in turn activates chemoreceptors enhancing an increase of cellular respiration (Gourine et al., 2010).

According to recent studies, single released gliotransmitter is able to generate a wide range of synaptic responses, likely related to the cerebral region where the release occurred. For instance, glutamate can enhance synaptic transmission through the activation of NMDA or metabotropic receptors on presynaptic nerve terminals (Fiacco and McCarthy, 2004; Jourdain et al., 2007; Perea and Araque. 2007). Pathways underlying glutamate action seem to be very similar to the ones of inhibitory synapses, potentiating GABAergic inhibitory power (Kang et al., 1998). In addition, can also bind to NMDA receptors expressed on postsynaptic dendrites increasing, thus, neuronal excitability (Angulo et al., 2004; Fellin et al., 2004; Perea and Araque, 2005). Notably, although the activation of astrocytic receptors leads to intracellular Ca^{2+} signalling not all of them can trigger gliotransmitter release. Volterra and colleagues hypothesized that local intracellular Ca^{2+} concentration variations in ensheathing-synapses astrocytic processes can control gliotransmission and consequently its effects on synaptic activity (Volterra et al., 2014). Since the effects of the released gliotransmitter are strictly related to the kind of neuronal receptor that will be bound, neurons-astrocytes communication scenario appears very complex. For instance, glutamate has a neuro-protective effects when binding synaptic NMDA receptors, whereas it enhances increase of NO and consequent neuronal damaging upon binding extrasynaptic NMDA receptors (Kaufman et al., 2012; Molokanova et al.,

2014).

Notably, astrocytes heterogeneity can contribute to explain different molecular pathways underlying gliotransmitter release. In fact, it is recognized that grey matter astrocytes from different cerebral regions show different morphology and, therefore, a different molecular composition (Emsley and Macklis, 2006).

2.4.1. L-lactate

The idea that lactate, generated from cerebral metabolism, can work as a gliotransmitter was recently formulated. Astrocytes, indeed, can produce and release not “classical” transmitters such as lactic acid, probably released via the so called “volume transmission” in CNS (Bergersen and Gjedde, 2012). Astrocytes synthesize lactate via glycolysis or from glucose from blood stream. Lactate can diffuse following a concentration gradient:

1) in the intracellular environment

2) through gap junctions in astrocytes

3) in the extracellular space through transporters for monocarbossilic acid (MCT)

4) in extracellular space

5) in proximal neurons and in other cellular types through MCT (Gundersen et al., 2015).

Lactate released from astrocytes through the mechanisms listed above might be an autocrine mediator for astrocytes or, alternatively, as a paracrine mediator for neurons sited in other regions than astrocytes.

Lactate in nervous tissue, indeed, can diffuse for long distances.

During acid lactic synthesis from piruvate NAD^+ is generated, which is fundamental for glycolysis. MCT transporter co-transport lactate in exchange with protons, a process enhanced by the concentration gradient generated from the production of lactate (Mookerjee et al., 2015). The variation of pH value due to this reaction can determine effects on a wide range of receptor and ionic channels (Wemmie, 2011), including glutamate receptors (Makani and Chesler, 2007; Traynelis and Cull-Candy, 1991). Changes of pH values can also affect histones acetylation influencing, therefore, gene expression (McBrian et al., 2013). Thus lactate, increasing the $NADH/NAD^+$ ratio, can influence the activity of NMDA receptors, increasing also the activity of NMDA receptors and intracellular levels of calcium, stimulating the expression of genes involved in synaptic plasticity both in vitro and in vivo (Yang et al., 2014).

The G-protein coupled receptors GPR81, receptor for lactate, was found in brain some

years ago (Bergersen et al., 2012; Bozzo et al., 2013; Lauritzen et al., 2014).

However, the role of lactate in brain remains not still clear. Some studies on rodents pointed out that lactate is able to inhibit neuronal activity. For instance, lactate receptor in rat hippocampus slices inhibits forskolin-induced cAMP production (Lauritzen et al., 2013), while in primary culture of mice cortical cells can inhibits the activity of neurons, including the Gabaergic ones (Bozzo et al., 2013). According to these studies, after being released from astrocytes following adrenergic stimulation, lactate activate HCAR1 receptor on neurons (Gundersen et al., 2015).

On the other hand, other studies supported a role of lactate as a positive modulator of norepinephrine release from neurons in locus coeruleus: lactate synthesized from astrocytes provoke, indeed, cAMP up-regulation in these neurons (Tang et al., 2014). Notably, only astrocytes in CNS contain a large quantity of glycogen, clearly representing a source of lactate. According to the astrocyte-to-neuron lactate shuttle hypothesis (ANLS), lactic acid produced during glycolysis (Peters et al., 1991) is carried both outside and inside active neurons (Pellerin and Magistretti, 2012).

Neuronal synaptic transmission (Nagase et al., 2014) and memory formation (Suzuki et al., 2011) seem to be lactate-dependent mechanisms. The role of lactate in synaptic plasticity is further supported by Robinet and Pellerin, who observed that the Brain-derived neurotrophic factor (BDNF) increases the expression of both the lactate transporter MCT2 and postsynaptic proteins (Robinet and Pellerin, 2011). Astrocytes can rapidly uptake and release lactate due to the gap junctions, they can also carry glucose towards neurons (Gandhi et al., 2009).

Astrocytic thin processes completely lack mitochondria but use their glycogen reservoir or containing-phosphate high energy compounds as energy source. Glycogenolisis and glycolysis in astrocytic processes lead to intra and extracellular increase of lactate concentration (through MCT transporter) (Lavialle et al., 2011).

It was reported that Purkinje cells after AMPA receptor activation were able to produce lactic acid from environmental glucose. The activation of these neurons leads to higher levels of extracellular lactate, postsynaptic currents, blood flow, and glucose and oxygen consumption (Caesar et al., 2008). These effects are inhibited by CNQX, AMPA receptor antagonist, with no inhibition of glycogenolisis (Caesar et al., 2008).

In synthesis: lactate released from excitatory synapses or astrocytic processes through volume transmission might be able to activate HCAR2 receptor, and probably other lactate

receptors, expressed on neurons.

The selective localization of lactate transporter MCT2 (Bergersen et al., 2005) and receptor HCAR1 (Lauritzen et al., 2014) on neuronal postsynaptic membrane, and in the intracellular compartment of glutamatergic dendritic spines, stress the role of lactate as a point of union between synaptic and neuroglial transmission (Gundersen et al., 2015).

ASTROCYTES: PATHOLOGICAL ROLES

In general, pathologies (including neurologic diseases) can be defined as the homeostatic imbalance of a tissue, an organ or a system. Despite neuropathologies have been considered for decade exclusively linked to the neuronal cells, this neurocentric vision has been recently questioned. Nowadays, indeed, glial cells, and in particular astrocytes, are considered important players in neuropathology. According to this "astrocentric" vision, astrocytes are the major responsibles for nervous tissues homeostasis and actively contribute to its protection under pathological conditions (Burda and Sofroniew, 2014; Giaume et al., 2007; Nedergaard et al., 2010; Parpura et al., 2012; Sofroniew, 2009).

Astrocytes display a wide range and regionally specialized functions (Anderson et al., 2014; Chaboub and Deneen, 2012; Matyash and Kettenmann, 2010; Oberheim et al., 2012; Parpura et al., 2012; Schitine et al., 2015). For instance, they divide parenchima within grey substance in relatively independent units, known as neurovascular units, characterized by the presence of both neuronal and vascular elements (Bushong et al., 2002; Iadecola and Nedergaard, 2007; Nedergaard et al., 2003). All cells of nervous tissues are involved in CNS cellular pathophysiology: since the multitude of their functions, astrocytes can be involved in all the neuropathological conditions (Burda and Sofroniew, 2014). Being able to synthesize and store glycogen, astrocytes maintain CNS homeostasis. Regardless its aetiology, nervous tissues damaging can compromise CNS homeostasis: through molecular mechanisms, astrocytes can protect neurons from glutamate toxicity, extracellular K+ and ROS action. Furthermore, astrocytic cells provide to under stress neurons energetic substrates.

Loss of these delicate functions can trigger the progression of diverse pathologies, such as ALS and encephalopathies (Verkhratsky et al., 2014a, 2016). Many changes at astrocytic levels could coexists or emerge during the progression of neurological pathology. A

classification of astrogliopathology based on functional responses of astrocytic cells has been proposed:

1) astrocitopathies, characterized by astroglial atrophy or pathological modelling
2) reactive astrogliosis (Pekny et al., 2016).

1) Many neurological pathologies have been associated to astrocytes degeneration, atrophy or functional asthenia. The “gliodegeneration” concept was introduced for the first time by Emilie Croisier and Manuel Graeber in 1996 (Croisier and Graeber, 2006). Astroglial atrophy is generally characterized by loss of function and was described in diverse pathologies, including also psychiatric and neurodegenerative disorders (Hazell, 2009; Rajkowska and Stockmeier, 2013; Rossi et al., 2008; Verkhratsky et al., 2014a, 2014d). Atrophy causes depletion of both neuroprotection and synapses ensheathed by astrocytic processes, with a subsequent decrease of homoeostasis. Such a condition commonly leads to glutamate uptake impairment which in turn disrupts neurotransmission and promotes excitotoxicity. Astrocytes asthenia might also go towards to the failing of active astrogliosis, worsening the neuropathology outcome (Verkhratsky et al., 2014b).

2) Astrocytes can enhance a specific defensive response, defined as reactive astrogliosis. Transcriptomic analysis on reactive astrocytes after treatment with two different stimuli, ischemic ictus or bacterial LPS, showed differences, pointing out that stress signals may draw molecular characteristics of astrogliosis process (Zamanian et al., 2012). Astrogliosis represents a crucial component of cell pathophysiology and its suppression usually leads to a worsening of neuronal damages avoiding tissue regeneration (Sofroniew and Vinters, 2010).

1. Reactive astrogliosis

Astrogliosis is widely used as a characteristic marker of CNS pathology. Basing on experiments on murine models, a new definition of reactive astrogliosis has been recently proposed. Four key characteristics are included (Sofroniew, 2009):

1) Reactive astrogliosis is a range of potential molecular, cellular and functional modifications occurring in astrocytes in response to CNS lesions and pathologies, including mild perturbations;

2) Modifications whom astrocytes are subjected to are related to severity of damage along with a gradual but continuous alteration of molecular expression, a progressive cellular ipertrophy, and cells proliferation with subsequent scar onset;
3) Modification occurring during astrogliosis are regulated by intra and intercellular signals;
4) Such changes can affect astrocytic activity both through gain or loss of functions which can positively and negatively affect neuronal and non-neuronal cells.

1.1. Mild or moderate astrogliosis

The pathological background is characterized by up-regulation of genes such as GFAP (Sofroniew, 2009) and by atrophy of both astrocytes cell body and processes (Wilhelmsson et al., 2006). Mild or moderate astrogliosis is generally due to non-blunt trauma or activation of the innate immune system. When the main cause of astrogliosis is eliminated, astrocytes can assume again a similar phenotype to the one of normal tissue (Sofroniew, 2009).

1.2. Severe diffuse astrogliosis

Such pathological condition is characterized by high GFAP and other genes up-regulation and by considerable cell body and processes hypertrophy. Astrocytic proliferation leading to an extension of cytoplasmic processes was observed (Sofroniew and Vinters, 2010). This causes an overlapping of processes of neighboring astrocytes with a subsequent long-term reorganization of nervous tissue architecture.

1.3. Scar-forming astrogliosis

In addition to the molecular and structural modifications previously described, astrogliosis can lead to the disruption of healing processes, promoting the formation of scars surrounding the lesion. Recent evidences demonstrated that such scar onset emergence typically occur at the edges of important tissues damage, necrosis, infection or inflammatory autoimmune infiltration and represent a defensive barrier against inflammatory cells and infective agents (Bush et al., 1999; Drogemuller et al., 2008; Faulkner et al., 2004; Herrmann et al., 2008; Sofroniew, 2009; Voskuhl et al., 2009).

On one hand, lesion isolation promotes a clinic stabilization and patient survival, on the other hand it might negatively affect the regeneration mechanisms occurring during the subsequent stages (Silver and Miller, 2004).

Interaction between reactive astrocytes with other cells, mostly fibroblasts and meninges, is an important characteristic of glial scars. Furthermore, a dense extracellular matrix mostly composed of collagen and containing diverse molecular signals inhibiting axonal and cellular migration is synthesized.

The role of glial scars in inhibiting axonal regeneration has been well known since scars were first described 100 years ago. For this reason, they have been merely considered as an obstacle for functional recovery of the damaged cerebral region (Ramon y Cajal, 1928). However, the negative role of astrogliosis has been questioned by experiments on transgenic models.

Recent results shed a new light on the protective role of reactive astrocytes through different pathways:

- glutamate uptake (Bush et al., 1999; Rothstein et al., 1996; Swanson et al., 2004);
- protection from NH4+-mediated toxicity and oxidative stress (Che net al., 2001; Shih et al., 2003; Swanson et al., 2004; Vargas et al., 2008);
- Neuroprotection through adenosine release and β-amiloid degradation (Che net al., 2001; Shih et al., 2003; Swanson et al., 2004; Vargas et al., 2008)
- Enhancing BBB recovery (Bush et al., 1999);
- Reduction of vasogenic edema after trauma, storke or obstructive hydrocephalus (Bush et al., 1999; Zador et al., 2009);
- Extracellular liquid stabilization and ions balancing (Zador et al., 2009);
- Limitation of diffusion of inflammatory cells or infective agents from damaged area or healthy parenchima (Bush et al., 1999; Drogemuller et al., 2008; Faulkner et al., 2004; Herrmann et al., 2008; Li et al., 2008; Myer et al., 2006; Okada et al., 2006; Voskuhl et al., 2009).

Several researches demonstrated that astrogliosis is not an all-or-none mechanism due to an on/off regulation, but a finely regulated process environment-dependent and regulated by a multitude of specific molecular signals mediating specific responses (Sofroniew, 2009; Sofroniew and Vinters, 2010).

Numerous molecules can trigger astrogliosis processes or regulate its specific aspects:

- Growth factors and cytokines, as IL6, TNFa, INFγ, IL1, IL10, TGFb, FGF2

- Innate immunity mediator as lipopolysaccharide (LPS) and other tool-like receptors ligands
- Neurotransmitter as glutamate and noradrenaline
- Purines, e.g. ATP
- ROS, e.g. NO
- Glucose depletion and subsequent hypoxia
- Metabolite related to neurodegeneration as B-amiloid
- Molecules associated to systemic metabolic toxicity, as $NH4^+$
- Regulator of cell proliferation as endothelin-1 (Sofroniew, 2009).

In addition, many microRNA (miR), e.g. miR-21 and miR-181, and some miR-regulatory enzimes as Dicer able to modulate astrogliosis and its functions, have been indentified: such results indicate therefore the existence of a further level of astrogliosis regulation (Bhalala et al., 2012; Hutchison et al., 2013). All CNS cells can release molecular mediators of astrogliosis in response to nervous tissue damages (Sofroniew, 2009).
It is increasingly recognized that molecular, morphologic and functional changes in reactive astrocytes are finely regulated by inter and intracellular signals which reflect the context of the specific stimulus and generate an astrogliosis-mediated response (Sofroniew, 2009). Furthermore, reactive astrocytes can in turn produce and release numerous types of pro- and anti-inflammatory factors in response to different kind of stimuli (Eddleston and Mucke, 1993; John et al., 2003) and can exert pro- or anti-inflammatory effects on microglia (Farina et al., 2007; Min et al., 2006). Thus, astrocytes are able to produce a wide range of effects depending on damage etiology and cerebral region where the damage occurred, under control of specific molecular pathways (Sofroniew, 2009).

1.4. Alexander Disease

Alexander disease (AxD) is one of the clearest examples of primary astrocytes pathology. AxD is a rare genetic neurodegenerative disorder, caused by a gain-of-function mutation of the GFAP gene (Brenner et al., 2009; Messing et al., 2012). Mutated GFAP, along with other constitutive proteins, form protein aggregates within astrocytes, called Rosenthal fibres, a distinctive marker of Alexander diseases (Herndon et al., 1970; Iwaki et al., 1989). One of the major modification observed in affected astrocytes is the decrease of membrane glutamate transporter expression. In particular, immunohistochemistry studies

showed a reduction of 75% of EAAT2 in AxD murine models, while a complete or variable EAAT2 depletion was observed in the hippocampus of patients affected by AxD (Tian et al., 2010).

Reduction of astrocytic glutamate uptake causes a risk of glutamate overload in neurons and, thus, the risk of excitotoxicity, that might explain why seizure is common in AxD patients (Messing et al., 2012).

1.5. Neuronal lesions

Traumatic lesions occurring in brain and spinal cord can have different forms (e.g. wounds or concussions), dimension (focal or diffuse), severity and anatomical localization (Verkhratsky et al., 2016). However, reactive astrogliosis is the major CNS response to nervous tissue damage, even though the phenotypes of activated glial cells appear highly heterogeneous according to the pathological context (Burda et al., 2015). Astrocytes responses, indeed, are affected by the distance and severity of the lesion.

The activation of the molecular processes leading to astrogliosis probably involves a massive release of ATP from injured cells, causing a subsequent generation of InsP3-mediated Ca^{2+} signalling in astrocytes (Huang et al., 2012; Roth et al., 2014)

Astrocytes limit injuries caused by focal traumas through the formation of glial scars, which generate a barrier surrounding the injured area. Astrogliosis suppression and lack of glial scar exacerbate pathology consequences increasing the subsequent neurologic deficit (Sofroniew, 2005). Proximal astrocytes respond through hypertrophy and biochemical remodelling (Verkhratsky et al., 2016)

Thus, after neuronal damage, not only astrocytes isolate the injured area through formation of glial scars but they are also able to modulate inflammatory response, providing homeostatic protection to nervous tissues and the regulation of the synaptic network remodelling.

In addition, astrocytes release molecules which can reduce BBB permeability enhancing its recovery.

For all these reasons, astrocytes are essential for post-traumatic regeneration of nervous tissues (Burda et al., 2015)

1.6. CNS infections

CNS infection can be caused by bacteria, virus, fungi, and parasites and classified in meningitis, encephalitis or cerebral abscess. Reactive astrocytes and microglia are often indispensable for damage limitation. Astrocytes responses to pathology depend mostly on the nature of pathogen agent. Infections caused by Gram+ bacteria, as Pneumococcus and Staphylococcus, rapidly trigger astrogliosis insurgence leading to astrocytes hypertrophy and GFAP expression up-regulation (Iovine et al., 2013), secretion of pro-inflammatory factors as TNF-α (Li et al., 2012) and interleukin, and toll-like receptor TLR2 activation (Esen et al., 2004).
It was also reported that bacterial infections can down-regulate the expression of gap junctions-forming connexin (Esen et al., 2007).
Gram- bacteria can trigger an early astrogliosis, which increases with astrocytes proliferation (Chauhan et al., 2008; Dotevall et al., 1996). On the other hand, the astrocytes response to viral infection results more complicated, primarily because astrocytes can be a target for infection, as in HIV dementia. Astrogliosis and degeneration associated with astrocyte death have been associated with those pathological conditions. Loss of astrocytic functionality, thus, might contribute to the cognitive decline through a general decrease of astrocytes homeostatic support to neurons (Churchill et al., 2009).

1.7. Toxic Injury

- Encephalopathy from heavy metal

Astrocytes represent the major target of encephalopathy due to exposition to toxic heavy metal concentration. Accumulation of those metals in astrocytic through membrane transporter may lead to EAAT1/2 glutamate transporter downregulation, compromising physiologic glutamate uptake (Struys-Ponsar et al., 2000; Verkhratsky et al., 2013; Yin et al., 2007).
Such condition elicits excitotoxicity with subsequent neuronal death and neurotransmission disruption.

- Encephalopathy from hyperammonemia and epathic

The increase of ammonium concentration in CNS can provoke several mental and behavioural symptoms as confusion, irritability or consciousness alteration (Brusilow et al., 2010; Butterworth, 2011; Felipo, 2013)

For many decades, it was believed that hyperammonemia affected the astrocytes-specific glutamine synthetase which metabolizes ammonia synthesizing glutamine (Albrecht et al., 2010; Rose et al., 2013)

However, the pathological potential of astrocytes resulted to be more complex: recent studies pointed out that under high ammonia concentration conditions astrocytes go towards a rapid functional remodelling compromising their homeostatic functions.

In details, are included:

- K+ buffer failure (Obara-Michlewska et al., 2014; Rangroo Thrane et al., 2013)
- aberration in Ca^{2+} signal and homeostasis (Haack et al., 2014; Wang et al., 2015)
- abnormal calcium signalling leading to glutamate exocytosis (Gorg et al., 2010; Montana et al., 2014)
- pathological increase of Na^{+} cytoplasmic concentration and H^{+} transport impairment causing alteration of pH regulation (Kelly et al., 2009; Kelly and Rose, 2010).

1.8. Ischemic stroke

Astrocytes role in stroke pathophysiology is multifaceted (Gleichman and Carmichael, 2014). After stroke insurgence and ischemic core formation, astrocytes from the peripheric zone (the penumbra) become key elements in the limitation of the ischemic lesion and the neurological deficit (Verkhratsky et al., 2016).

Astrocytes are the major neuroprotective element in the penumbra zone, through the control of ions and glutamate concentrations and providing nutrient supply to neurons. In fact, an exasperated neuronal death rate can be observed after inducing a stress condition in astrocytes (Sayre et al., 2014). Since astrocytes play a key role in glutamate excitotoxicity control: GLT1 transporter downregulation, indeed, increases the area of the injured lesion (Rao et al., 2001) while its overexpression reduces the area dimension and limit neurological deficits (Harvey et al., 2011). Maintenance of glutathione, ascorbic acid and ROS buffer system plays an important neuroprotective role in post-stroke tissues (Dringen et al., 2000; Dringen and Hirrlinger, 2003).

1.9. Epilepsy

Epilepsy is a CNS pathology characterized by seizures, affecting around the 2% of world population (Hesdorffer et al., 2011). To date, anti-epileptic drug-discovery focused on molecules targeting neuronal cells.

However, several studies demonstrated that astrocytes under epilepsy conditions are subjected to pathological remodelling and might represent a new pharmacological target. Those cells show a phenotype characterized by changes in ion channels, receptors and transporters, which lead to a general disruption of astrocytes-dependent homeostasis in the damaged areas (Bedner et al., 2015; Steinhauser et al., 2015).

Under epilepsy conditions, astrocytes display a phenotype characterized by modification of ionic channles, receptors, and transporters which compromise the astrocyte-dependent homeostasis in the damaged areas.

Studies on patient tissues or murine models revealed the abnormal electrophysiological properties of astrocytes and the complete lack of intercellular coupling between astrocytes mediated by gap junctions (Bedner et al., 2015). Furthermore, alterations of Cx43/Cx30 connexin and increase of GFAP expression have been observed (Steinhauser et al., 2012). Astrocytes role in epilepsy pathophysiology appear related to the dysregulation of K^+-mediated buffering system, in which potassium channels Kir4.1 are involved. In fact, both ion flow density mediated by these channels and the expression of Kir4.1 itself are reduced in a sclerotic area of human hippocampus (Heuser et al., 2010; Hinterkeuser et al., 2010; Steinhauser et al., 2015).

Others pathological changes compromise glutamate uptake (Coulter and Eid, 2012) or decrease GS expression in neurons, with a subsequent decrease of GABA synthesis, favouring the occurrence of epileptic crisis (Benedetti et al., 2011; Wang et al., 2009). Astrocytes can affect neuronal excitability caused by changes of adenosine kinase (ADK) expression with effects on adenosine homeostasis (Aronica et al., 2013; Boison and Aronica, 2015). Li and colleagues showed that an increase of ADK expression in astrocytes reduces adenosine availability, which in turn enhances higher neuronal excitability, potentially responsible of the increase predisposition to epilepsy (Li et al., 2012).

1.10. Neuropsychiatric pathology: Schizophrenia

According to the current view, abnormalities in neuronal network and a general dysregulation of neurotransmission might be the major pathological mechanisms of schizophrenia (Laruelle, 2014; Muller and Schwarz, 2006; Rubinov and Bullmore, 2013). Since astrocytes are fundamental for synaptogenesis and sustainment of glutamatergic transmission, their possible involvement in schizophrenia onset has been increasingly investigated (Bernstein et al., 2015; Sanacora and Banasr, 2013; Verkhratsky et al., 2014; Xia et al., 2014).

A lower number and density of astroglial cells was detected in diverse cerebral region of patients affected by schizophrenia (Rajkowska et al., 2002; Schmitt et al., 2009; Webster et al., 2001). Xia and colleagues detected a significative decrease of the expression of astrocytes-specific proteins, fundamental for CNS homeostasis, such as AQP4 or membrane glutamate transporter. Thus, authors advanced the hypothesis that astrocytes subpopulation localized in cortex might be harmed during schizophrenia progression (Xia et al., 2014).

In addition, the expression of the cistin/glutamate exchanger Sxc-, able to regulate glutamate release and control its extracellular concentration, resulted to be decreased in an animal model for schizophrenia.

Furthermore, the metabolism of the endogenous NMDA receptor positive modulator D-serine presents alterations.

Post-mortem analysis showed lower production of kynurenic acid in brain tissues of psychiatric patients. Kynurenic acid is a tryptophan metabolite mainly produced by astrocytes and acting as endogenous inhibitor of acetylcholine and NMDA receptors.

All these data suggest that astrocytes under schizophrenic conditions present functional deficits in controlling glutamatergic transmission and might therefore a key factor in this pathology (Laruelle, 2014).

1.11. Neurodegenerative pathologies

-Alzheimer's Disease

Alzheimer Disease (AD) is a chronic neurodegenerative pathology whose etiology remains unknown (Verkhratsky et al., 2010). AD is characterized by senile plaques composed of

extracellular B-amiloid accumulation and neurofibrillary tangles constituted of phosphorylated tau protein (Selkoe, 2001).

Morphological and functional modifications occur in astrocytes during AD early stages, mainly characterized by atrophy, while astrocytes number remains constant (Beauquis et al., 2014; Kulijewicz-Nawrot et al., 2012; Olabarria et al., 2010; Verkhratsky et al., 2010; Yeh et al., 2011).

Atrophic astrocytes are not able to accomplish their homeostatic functions, leading to irreversible modifications in brain, disruption of the synaptic network, and early cognitive deficit. Since astrocytes play a key role in synaptogenesis and synapses maintenance (Verkhratsky and Nedergaard, 2014), their alteration can cause to a decrease or loss of synaptic activity, representing the early stage in pathology progression (Verkhratsky et al., 2014a, 2010).

Furthermore, astrocytes atrophy compromise β-amiloid clearance (Kimura et al., 2014). In addition, a progressive reduction of glucose metabolism has been observed during AD early stages, due to modifications of neurons and astrocytes (Mosconi et al., 2008).

Since astrocytes present a significative glicolitic pathway, it is likely that alteration of glucose metabolism results deeply harmful for these cells (Verkhratsky et al., 2016).

The scientist Alois Alzheimer observed for the first time the presence of hypertrophic glial cells surrounding both damaged neurons and senile plaques. Recent post-mortem analysis carried out on brain of patients affected by AD revealed the upregulation of main astrocytes markers such as GFAP and S100B. Many studies demonstrated that high concentration of β-amiloid can modulate the expression of protein involved in Ca^{2+} signalling pathways and homeostasis in cultured astrocytes (Grolla et al., 2013; Lim et al., 2014), eliciting the downregulation of the expression of glutamate transporters (Matos et al., 2008). However, little is known about molecular pathways underlying such mechanisms. Despite therapies against AD resulted to be limited and ineffective, recent results described a prominent role of astrocytes in neurodegenerative pathologies, proposing these cells as a possible pharmacological target in future therapies (Verkhratsky et al., 2016)

1.12. Amyotrophic Lateral Sclerosis (ALS)

ALS is a chronic neurodegenerative and untreatable pathology, with unknown etiology. It is characterized by the loss of muscular functions due to a progressive neuromuscular weakening and spasticity (Verkhratsky et al., 2016). Astrocytes seem to be involved in mechanisms responsible to SLA-related neuronal damaging. In theory, these cells might contribute to neuronal death both directly (releasing neurotoxic substances) and indirectly (through the loss of homeostatic and protective functions) (Verkhratsky et al., 2016). Slower progression of the pathology and longer life expectancy can be observed in transgenic animals subjected to the transplant of wild-type astroglial precursors (Lepore et al., 2008). Papadeas and colleagues demonstrated that transplantation of astrocytes carrying a mutated copy of the gene hSOD1 in spinal cord of wild-type rodents triggers motoneurons degeneration and peculiar SLA symptomatology (Papadeas et al., 2011). *In vitro* experiments confirmed the pathological role of astroglia in ALS: neurons and motoneurons co-culture demonstrated that astrocytes are responsible for the synthesis of molecules harmful for neurons (Bilsland et al., 2008; Di Giorgio et al., 2008; Ferraiuolo et al., 2011; Haidet-Phillips et al., 2011; Marchetto et al., 2008; Nagai et al., 2007; Phatnani et al., 2013). Such factors include: excess of extracellular glutamate and D-serine, increase of prostaglandin E2 secretion, massive interferon-γ release, TNF-β secretion, nerve growth factor (NGF) and lipocalin 2 release (Valori et al., 2014).

1.13. Parkinson's Diseases (PD)

To date, astrocytes involvement in Parkinson's disease pathogenesis has been scarcely studied.

Several toxins play a key role in PD onset and astrocytes might represent a target for that substances (Rappold and Tieu, 2010). For instance, MPTP, a lipophilic substance accumulated in astrocytes, can be converted in the toxic metabolite MPP^{+} by MAO-β. MPP^{+} is then released from astrocytes and up-taken by dopaminergic neurons, where compromises ATP synthesis and increases ROS production causing neuronal death (Meredith et al., 2008).

MAO-β is mostly expressed in astrocytes (Ekblom et al., 1993) and its levels increase proportionally with age, a risk factor for PD. In addition, the expression of such enzymes is

correlated to neuronal death in *substantia nigra* (Mahy et al., 2000; Mandel et al., 2003). Global level of glutathione, mostly produced by astrocytes, are significantly lower (40%) in Parkinson's disease and this could indicate a lack in astrocytic protection against oxidative stress (Rappold and Tieu, 2010).

Astrocytes contain proteins related to ~~the~~ pathologies ~~such~~ as α-sinuclein (AS), parkin and phospho-tau: it was demonstrated that such cells are able to accumulate AS and produce massive Lewy's bodies *in vitro* (Lee et al., 2010).

Interestingly, only protoplasmic astrocytes display high amount of AS in cytoplasm, while no evident changes of AS levels have never been detected in fibrous astrocytes (Song et al., 2009). Since AS accumulation increases astrocytes sensitivity to oxidative stress leading to apoptosis, protoplasmic astrocytes degeneration could occur in all synucleinopathies (Song et al., 2009).

Finally, many researches pointed out the role of the astrocytic protein S100B, which can act both as a cytokine or a protein associated to the inflammation or neurodegenerative-related damage (Iuvone et al., 2007).

AIM OF THE RESEARCH

The aim of this thesis is the investigation of the release pathways in astrocytes and their role in signal transmission in the central nervous system.
The research was addressed towards three research lines:

- release of exosomes from astrocytic processes
- modulation of release of the gliotransmitter glutamate from astrocytic processes by putative receptor-receptor interactions between D2, A2A and Oxytocine (OXT) receptors
- effect of low energy laser light on the release of glutamate from astrocyte processes as compared to the release from nerve terminals

Investigation on these topics might shed a new light, respectively; on

  - the pattern for astrocyte-neuron communication network and the ability of astrocytes to send messages through non-classical signalling;
  - the role of astrocytes in signal integration in CNS, through allosteric interactions between membrane GPCRs and their control on the release of the gliotransmitter glutamate
  - further information on the effect of photons on nervous system cells

MATERIALS AND METHODS

The methods here reported concern the techniques applied in the works described in detail in this thesis.
Further information on material and method can be found in the attached Supplements.

1. Animals

Adult male rats (Sprague-Dawley 200-250 g) and mice (C56Bl6J) were housed at the animal house of Pharmacy Department (DIFAR) of the University of Genova. Water and food were freely available. Temperature (22± 1°C), relative humidity (50%), and light/dark schedule were continuously monitored.
Experimental protocols and animal care observed the European Communities Parliament and Council Directive of 22 September 2010 (2010/63/EU) and with the Italian D.L. n. 26/ 2014, and were approved by the Italian Ministry of Health (protocol number 26768 of November 2012 and protocol number 75F11.N.6JI of August 2018), in accordance with *Decreto Ministeriale 116/1992*. All possible efforts were made to minimize the quantity of animals used and their suffering.

2. Isolation of purified astrocytic processes and nerve terminals

Brain tissues (rat striatum, mouse cerebral cortex) were removed after rat decapitation and quickly placed in ice-cold medium. Purified gliosomes (resealed astrocytic processes) were prepared according to Nakamura's protocol (Nakamura et al., 1993), as previously reported (Stigliani et al., 2006; Cervetto et al., 2015, 2016)
Tissues were homogenized in 10 volumes of 10mM Tris/HCl pH 7.4 with 0.32 M sucrose, using a glass-Teflon tissue grinder (clearance 0.25 mm). After centrifugation (5 min, 1000g 4°C) to remove debris and nuclei, the homogenate was stratified on a discontinuous Percoll gradient (2, 6, 10, 20 % v/v in Tris buffered sucrose) and then centrifuged (5 min, 33500g; 4°C). Gliosomes are purified astrocytic processes containing vesicles loaded with gliotransmitters and are harvested in the interlayer between 2% and 6% (v/v) Percoll layers. During release experiments purified gliosomes were resuspended in a HEPES buffered medium additioned with NaCl 128, KCl 2.4, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.0, and HEPES 10 con glucose 10, pH 7.4 (concentration expressed in mM). Protein determinations were

carried out according to Bradford (1976).

3. Release of endogenous glutamate and [^{3}H]D-Aspartate

Superfusion technique (Raiteri M et al., 1974; Raiteri L. et al., 2000) was applied to investigate the release of endogenouse glutamate or [^{3}H]D-Aspartate from astrocytic processes (Cervetto et al., 2015; Cervetto et al, 2016; Cervetto et al., 2017; Marcoli et al, 2017) and synaptosomes.

One of the major advantages of that techniques was that superfused gliosomes were subjected to a condition defined as “naked” receptor: continuous perfusion, indeed, constantly removes released substances avoiding the instauration of a receptor biophase. Thus, that experimental model could be used for the pharmacological characterization of receptors which can modulate the release of transmitters or the analysis of receptor-receptor interactions

Since previous stuides did not observe differences in the behaviour of tritium or endogenous release from astrocytic processes obtained from rodent brain, [^{3}H]D-Aspartate can be used to investigate glutamate release form perfused gliosomes. Gliosomes were incubated with 0.03 µM for 15 minutes at 37°C and then superfused. For experiment with homocystein, 4mM homocystein was added during tissue homogenizaztion. Gliosomes entrapped homocystein and were then perfused.

Briefly, equal samples of gliosomes (or synaptosomes) were distributed at the bottom of up to 20 parallel chambers of the perfusion apparatus; after 33 minutes of perfusion necessary to stabilize the release processes, the perfusion liquid can be harvested. Liquid collection was divided in fractions lasting 3 minutes each (from b1 fraction, starting at t=33’ and ending at t=36’, to b6 or b7 according to the experimental protocol).

At t=38’, gliosomes were stimulated for 6 minutes with either depolarizing stimulus or agonist. The antagonists were added 8 min before the agonists, when necessary.

During each experiment, at least one chamber was perfused with standard medium as a control.

Resuspended gliosomes or synaptosomes were distributed on parallel chamber and the effects of the single substance on endogenous glutamate release was quantified in triplicate. The endogenous glutamate released in the collected fractions was measured by HPLC analysis (Waters Alliance, Milford, MA, USA): the analysis involves an automatic pre-

column derivatization with o-phthalaldehyde, the separation on a C18 reverse phase chromatography column (Chrompack International, Middleburg, The Netherlands), fluorimetric detection and the amino-acid homoserine was used as the internal standard. The limit of detection was 100 fmol/µL (Marcoli, M et al, 2004).

The amounts of glutamate in the collected samples were expressed as pmol/mg protein. The effect of the compounds (or depolarization) on the glutamate efflux was calculated as overflow by subtracting the basal release in the control chambers from the total amount of glutamate released during stimulation in substance-treated chambers (or in chambers supplemented with 4-AP).

In a subset of laser experiments, synaptosomes were incubated with rose bengal (0.5 µM) 30 minutes at 3 °C in the dark, and then perfused.

4. Immunohistochemistry and confocal microscopy

Gliosomes and synaptosomes (15-20 ug) have been fixed with 2% paraformaldehyde for 15', permeabilized with 0.2 % Triton X-100 for 5' and incubated for 60' with primary antibody diluted in 3% albumin PBS (p/v). The used primary antibodies were the following: mouse anti-synaptophysin (1:1000; Sigma-Aldrich, St. Louis, 61 MO, USA), mouse or rabbit anti-fibrillary acidic protein (GFAP; 1:1000; Sigma-Aldrich); mouse anti-oligodendrocyte (RIP; 1:10000; Merck Millipore Corporation, Darmstadt, Germany), mouse anti-integrin αM (clone OX-42; 1:25; Merck Millipore Corporation), guinea pig anti-VGLUT1 (1:1000; Synaptic Systems, Goettingen, Germany), mouse anti-VGLUT1 (1:500; Synaptic Systems), rabbit anti-A2A (1:200; Alomone Labs, Jerusalem, Israel; AAR-002), mouse anti-D2 (1:200; Merck Millipore Corporation; AB5084P), rabbit anti-D2 (1:200; Atlas Antibodies, Stockholm, Sweden), rabbit anti-D2 (1:200; Alomone Labs; ADR-002) mouse anti-OXTR (1:200; Santa Cruz; sc-515809). Once incubated with primary antibodies (overnight, 4°C), samples were washed with PBS and then incubated for 60 minutes with the matched conjugated secondary antibodies diluted in 3% albumin PBS. A three channel TCS SP2 laser-scanning confocal microscopy was used to acquire images.

The purity of gliosomes and synaptosomes prepared from cerebral cortex was evaluated using specific astrocytic and neuronal markers, anti-GFAP, anti-synaptophysin, and antibodies usually used for labelling oligodendrocytes and microglia, anti-RIP or anti-integrin αM.

5. Preparation and perfusion of slices

Cerebral cortex were chopped to obtain 250 µm slices, which were then transferred to parallel superfusion chambers (37°C) and the constantly superfused with standard medium (mM: 125 NaCl, 3 KCl, 12 $MgSO_4$, 1,2 CaCl, 1 NaH_2PO_4, 22 $NaHCO_3$, 10 glucose, gassed with 95% O_2, 5% CO_2, pH 7,4). Liquid was collected in 5-minutes samples. After 30 minutes, slices were subjected to the laser light stimulation (60 J/cm^2, 1 W, 60s).

6. Laser parameters and experimental design

Slices, synaptosomes or gliosomes were transferred on the superfusion chambers, which we wrapped with aluminium foil to avoid light dispersion, and then exposed to laser stimulation, more precisely a near-infrared diode laser by the AB2799 hand-piece (Doctor Smile, LAMBDA Spa, Vicenza, Italy). Chosen parameters were the following: 808-nm wavelength, 1 or 0.1 W of power, 1 or 0.1 W/cm^2 irradiance in CW (pulse energy of 0 Hz). Ca^{2+}-free medium was used to study calcium role in laser-evoked glutamate release.

7. Vescicle isolation from perfused astrocyte processes

Vescicle released from perfused astrocyte procssese were analyzed. Perfusion experiments were carried out as previously described (See Marcoli et al., 2008; Cervetto et al., 2010). Briefly, astrocyte processes purified from adult male rat cortex were transferred on parallel chambers and perfused with physiological medium at a constant flow (0.5 ml/min). After 5 minutes, perfused liquid was harvested in one 10 minutes long collection. The sample was the ultracentrifuged (110.000g for 70 minutes) to isolate the exosomes (Guescini et al., 2010). The pellet was then resuspended as follows:

- in PBS for DLS (Dynamic light scattering) analysis with the Zeta sizer nano ZS90 (Malvern Instruments) to determine vesicle dimension

- in lysis buffer (Laemli buffer) to carry out Western Blot analysis .

The ultracentrifuged sample was diluted with 0.22 µm filtered PBS to obtain the optimal

parameters suitable with the DLS analysis. The analysis had a 60 seconds duration and was carried out at 25°C. Sample light reflection was scattered 90° in comparison with the incidence line of the laser.

8. Electron Microscopy

Gliosomes and exosomes were studied through ultrastructural analysis performed by negative staining method. In brief, 5µl drops of gliosomes or purified exosomes were put onto formvar and carbon-coated copper grids and adsorbed for 20 min at RT. A filter paper was used to remove the excess of buffer. Then, grids were fixed in 2% PFAin PBS (pH=7.2) for 5 min and rinsed three times with distilled water. Grids were then incubated for 5 min at RT with 1% uranyl acetate aqueous solution. Contrast enhancement was obtained incubating the grids with a mixture of 1% uranyl acetate and 1% methylcellulose for 5 min. After drying, grids with gliosomes or exosomes were immediately observed with a CM10 electron microscope (Philips, Eindhoven, The Netherlands). Digital images were taken with a Megaview II camera.

9. Exosomes labeling

The exosome pellet was resuspended in diluent C and then labelled using the PKH67 dye according to the producer's technical bulletin (Fitzner et al.,2011). After gently pipetting the dye with the sample, and waiting for 5 min at room temperature, the staining reaction was stopped bringing the volume up to 35ml with 10% BSA in PBS. Exosome pellet was then obtained by ultracentrifugation (110.000 g for 90 min) and then resuspended in 150 µl of Neurobasal. As control condition, we prepared a samples with equal volumes of PBS plus the same amount of diluent C, PKH67 dye, 10% BSA and PBS, and by omitting the exosomes, in order to exclude non-specific labeling of cells due to micelles of the aliphatic dye or to the excess of dye itself.

10. Neuron-Astrocyte Co-Cultures

Primary cortical cells were edrived from Sprague–Dawley rat embryonic day 18 (E18). Culture preparation was performed as follow. Briefly, an E18 timed pregnant Sprague–Dawley female rat was euthanized by CO2 and cervically dislocated in accordance with

institutionally approved animal care. Embryos were dissected and cortices isolated in Hank's buffer solution without Ca^{2+} and Mg^{2+}. All tissues were collected and maintained in ice-cold buffer solution and, cerebral cortices were enzymatically digested at 37°C with warm TrypLe Express for 15–18 min in a water bath to obtain a single-cell suspension. The digestion was stopped by adding medium (Neurobasal or DMEM) complemented with 10% FCS (fetal calf serum) for 3 min. After that, medium was carefully removed and the cortices, transferred in Neurobasal/B27 (supplemented with Glutamax and Pen- Strepto), were mechanically triturated with a sterile firepolished Pasteur pipette. Single-cell suspension was well mixed, counted, and finally diluted. Cells were plated on poly-L-ornithine (100 µg/ml) coated-coverslips placed into multiwells plates at the density around 5.0x 104cell/cm2. The primary cultures were kept at 37°C in humidified atmosphere of 5% CO2 in air. The culture medium was changed weekly, until the uptake experiments at 21 DIV. After 3 weeks of development, the percentage composition of the cell population was distributed with 70 ± 15% of neurons and 30 ± 15% of glial cell (the percentage of neurons and astrocytes were estimated in three to five nonoverlapping fields from three different cultures, and are expressed as mean ± SEM). Immunofluorescence assays were performed using rabbit anti-MAP-2 (1:500), mouse anti-GFAP (1:1,000), and DAPI dye.

11. Cellular Uptake of Exosomes

Exosomes were lebelled with the fluorescent PKH67 lipophilic dye and dispersed (16 µl) in the cell culture medium and incubated for 1 h at 37°C in humidified atmosphere of 5% CO2 in air., the same volume (16 µl) of control sample was added in the medium of other coverslips from the same neuronal preparation, as a negative control and incubated for 1 h under the same
conditions as above. After one hour, both the coverslips with the exosomes and the control sample were repeatedly rinse to remove the excess.Uptake capabilities of the exosomes was evaluated by experiments repeated on three different neuronal preparations, developed in vitro for 3 weeks, and the biological sample was subjected to specific staining to confirm both its neuronal/glial morphology and exosomes uptake. Briefly, cells were fixed in 4% PFA, blocked with 3% BSA, and incubated with rabbit or mouse primary antibodies (over-night at 4°C in humid chamber) and then with Alexa Fluor 546 donkey anti-mouse and 633 goat anti-rabbit (1h at room temperature). The following primary

antibodies were used: rabbit anti-β III tubulin (1:500) or rabbit anti-MAP2 (1:500), or rabbit or mouse anti-GFAP (1:1,000). The antibodies in excess were washed out by PBS. The glass coverslips were mounted with antifade mounting medium and observed at the confocal microscopy.

12. Materials

4-aminopyridine (4-AP), homocysteine (Hcy), 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4 triazolo[1,5-c]pyrimidine (SCH 58261), sulpiride, quinpirole and rose bengal were from Sigma-Aldrich. Oxytocin and L- -371,257 were bought from Tocis. When possible, drugs were dissolved in distilled water or in physiological medium. SCH 58261 and sulpiride were dissolved in DMSO, then diluted 1:1000 in HEPES medium. PKH67 dye and homocystein were purchased from Sigma-Aldrich.

13. Statistical analysis

The data related to the study of endogenous release were reported as the mean values ± SEM (standard error of the mean) of n experiments, indicated in the legends of the individual figures. The significance of the differences was evaluated using the one-way ANOVA and then the Bonferroni post hoc test and with a statistical significance placed at $p < 0.05$.

RESULTS

1. LASER STIMULATION ON CORTICAL ASTROCYTIC PROCESSES AND NERVE TERMINALS

Almost all the living organisms can spontaneously emit photons known as biophotons. Although many effects of photon have been reported also in the central nervous system, their ability to determine the release of neurotransmitters or gliotransmitters in CNS has been scarcely investigated. Here, we evaluated whether photons stimulation can determine the release of the gliotransmitter/neurotransmitter glutamate from different models, and investigated the source of the released glutamate and the mechanisms involved.

1.1. Cortical gliosomes and synaptosomes are purified preparations

Immunohistochemistry analysis carried out before the beginning of my PhD showed that synaptosomes and gliosomes are purified preparations expressing, respectively, neuronal and astrocytic markers.

As shown in the figure below, indeed, synaptosomes showed positivity to anti-synaptophysin antibody, while no reactivity was observed for anti-GFAP, RIP or Integrin αM antibodies, indicating the absence of contamination from astrocytes, oligodendrocytes or microglia.

On the other hand, gliosomes were positive to GFAP labelling**,** with neglectable neuronal, oligodendrocytic or microglial contamination (Figure.2)

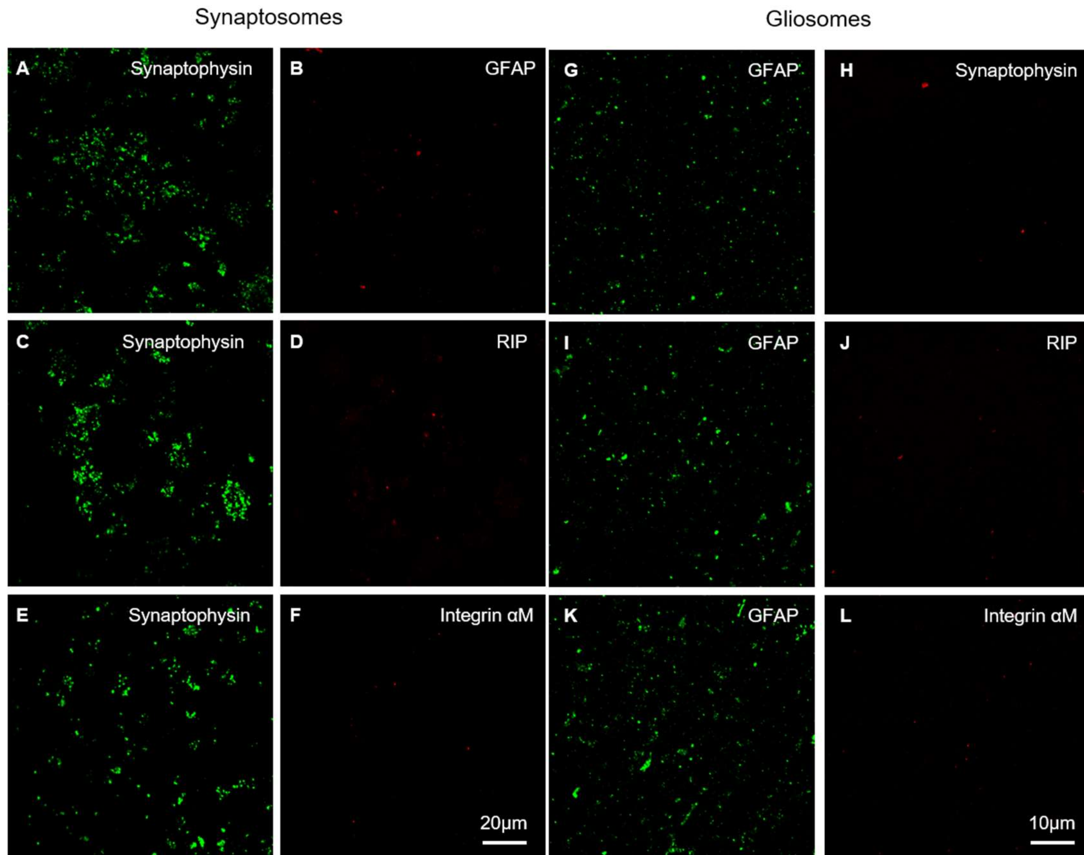

Fig. 2 **Synaptosomes and gliosomes are highly purified preparations**

Cortical synaptosomes and gliosomes were labelled with anti-synaptophysin, GFAP, RIP and Integrin αM antibodies to detect possible contamination. Synaptosomes and gliosomes resulted positive exclusively for neuronal (synaptophysin) and astrocytic (GFAP) markers, respectively. Scale bares are indicated in the images.

1.2. Laser light evokes glutamate release from mice cortex slices

Previous experiments on laser light application were carried out in prof. Marcoli's lab, using cortical slices and nerve terminals.

Perfused cortex slices were stimulated with laser light (See Material and Methods). The amount of endogenous glutamate released during perfusion was significantly higher in comparison with control.

Slices are a suitable model to mimic neuron-astrocytes network communication since they conserve the integrity of the nervous tissue, contrarily to other models (Fig.3)

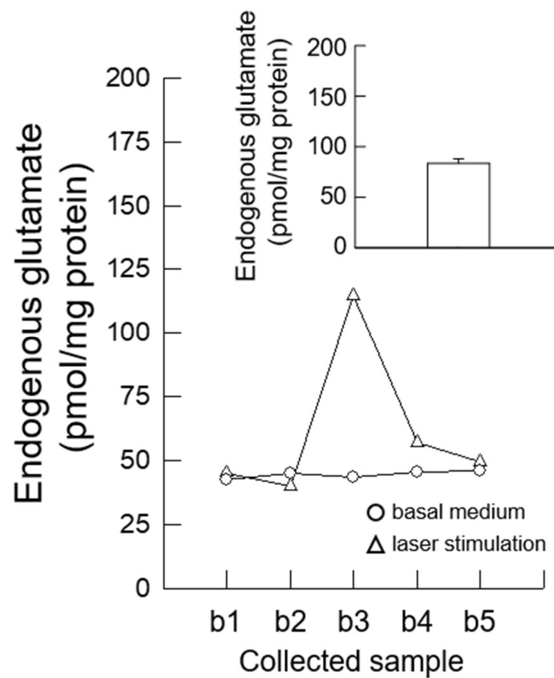

Fig.3 Photons evoke glutamate release from mouse cerebral cortex slices.

Representative time course of endogenous glutamate release from cerebral cortex slices perfused with physiological medium (1.2mM Ca^{2+}; o basal condition; Δ laser stimulation). Laser stimulation (60 J/cm^2, 1 W, 60 secondi) was applied during perfusion. Furhter details in Material and Methods section.

Insert. The column represents the increase of endogenous gluatamte releas evoked by laser light. The result is indicated as the mean ± SEM di n= 3 indipendent experiments.

1.3. Effects of laser light on gliosomes

Since photons stimulation evoked glutamate release from cortical slices, the next step was to figure out which cell population was responsible for this phenomenon.

To this aim, two different model were used: astrocytic processes and nerve terminals. As shown in Figure 4, laser light administration did not provoke any effect on glutamate release. It was possible to rule out, therefore, that the increase in glutamate release observed in cortical slices was due to the action of astrocytic processes.

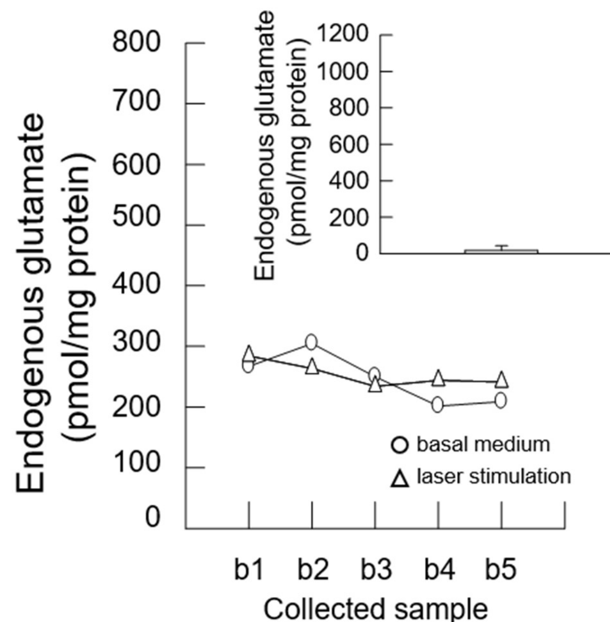

Fig. 4 Effects of photons on glutamate release from astrocytic processes.

Representative time-course of endogenous glutamate released from gliosomes perfused with physiologic medium (1.2 mM Ca^{2+}; o basal; Δ laser stimulation). Laser (60 J/cm^2, 1 W, 60 seconds) stimulation was applied during the perfusion. Further details in Material and Methods section.

Insert,The bar represents the amount of endougenos glutamate released from gliosomes perfused with standard medium after stimulation with laser light. The result is shown as the average ± SEM of n= 3 indipendent experiments.

1.4. Laser light increases glutamate release in nerve terminals

Once excluded that astrocytic processes might be involved in glutamatergic transmission evoked by laser, it was hypothesized that photons stimulation might induce glutamate release from nerve terminals. Synaptosomes stimulation by laser light, indeed, induced synaptosomes to release glutamate, as shown in Figure 5.

Since the fact that the neurotransmitter release was due to the neuronal population, and not to the glial compartment, had been established by previous experiments, we decided to further investigate and characterize the phenomenon in the nerve terminals, to reveal

the mechanisms possibly involved in the absence of responsiveness of the astrocyte processes to photons, and in responsiveness of the nerve terminals. Thus, we wondered whether the release was dependent on extracellular Ca^{2+} concentration and mediated by vesicles.

Laser administration to nerve terminals perfused with Ca^{2+}-free medium (Figure 5, insert) or treated with the V-GLUT-1 inhibitor Rose Bengal determined a significant reduction of glutamate release in comparison with the control (Figure 6, insert), confirming that the laser-evoked glutamate is released through Ca^{2+} and vesicle-dependent mechanisms.

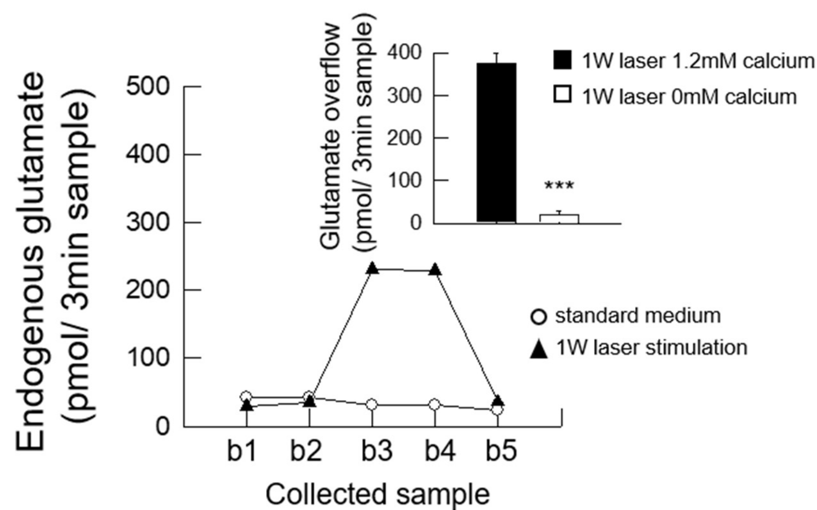

Fig. 5 Laser effects on nerve terminals

Representative time-course of endogenous glutamate released from synaptosomes perfused with physiologic medium (1.2 mM Ca^{2+}; o basal; ▲ laser stimulation). Laser stimulation was applied during the perfusion. Further details in Material and Methods section.

Insert. Bars represent the laser-evoked endogenous glutamate from perfused synaptosomes in normal conditions (black) or Ca^{2+}-free medium (white). The result is shown as the mean ± SEM of n= 3 indipendent experiments.

*** p< 0.001 vs 1W laser in standard medium (1.2 mM Ca^{2+}) one-way ANOVA followed by Bonferroni post hoc test.

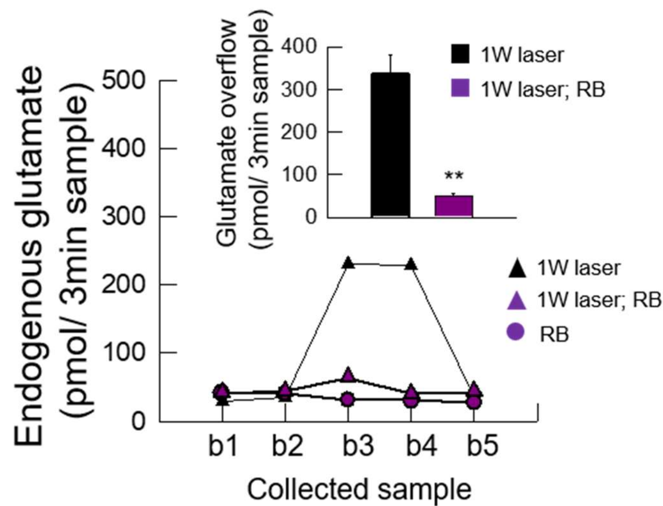

Fig. 6 Laser evokes a vesicular glutamate release from synaptosomes

Representative time-course of endogenous glutamate released from nerve terminals. Control synaptosomes were stimulated with 1W laser (▲), or previously incubated with Rose Bengal (RB) and then perfused (●) or stimulated with laser (▲).

Insert. Bars represent the laser-evoked endogenous glutamate from synaptosomes (black) or Rose Bengal-(dark-pink) treated synaptosomes. The result is shown as the mean ± SEM of n= 3 indipendent experiments.

** p = 0.003 vs 1W laser in standard medium one-way ANOVA followed by Bonferroni post hoc test

1.5. Glutamate release from cortical nerve terminals depends on laser power

Results obtained with 1 W laser suggested that stimulation with photons provoke a large amount of glutamate release from nerve terminals, suggesting that the selected power might not be suitable to study physiologic processes as glutamate release in response to photons in our model. Thus, we wondered whether a 0.1 W laser was able to stimulate lower amount of glutamate release from nerve terminals.

The results confirmed our hypothesis, demonstrating that the release from cortical nervous terminal is dose-dependent (Figure.7). Interestingly, nerve terminals treated with Rose Bengal and then stimulated with 0.1 W laser released significantly lower quantity of glutamate, suggesting that, also under those conditions, the release is vesicle-dependent (Figure 7, insert).

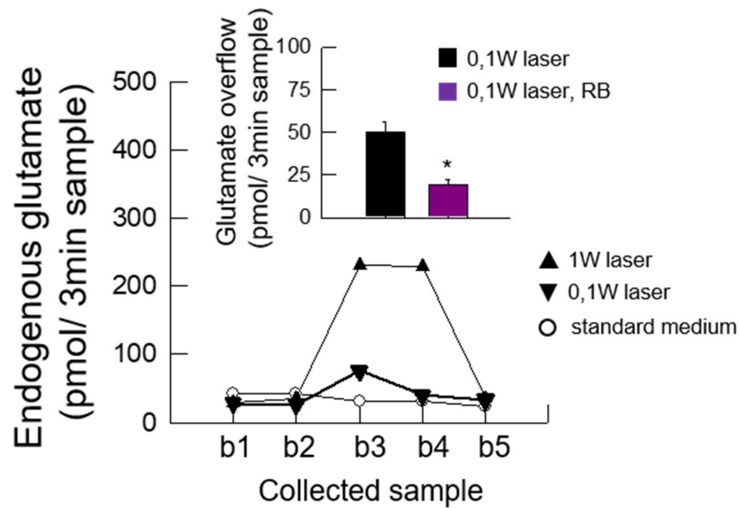

Fig. 7. Glutamate release from cortical nerve terminals depends on laser power Representative time-course of endogenous glutamate released from nerve terminals. Synaptosomes did not received any stimulation (○) or were stimulated with 0.1 W (▼) or 1W laser (▲). *Insert*. Bars represent the laser-evoked endogenous glutamate from synaptosomes treated with only 0.1 W laser (black) or Rose Bengal-treated synaptosomes stimulated with 0.1 W laser (dark-pink). The results are shown as the mean ± SEM of n= 3 indipendent experiments.

** p = 0.003 vs 1W laser in standard medium one-way ANOVA followed by Bonferroni post hoc test

1.6. Glutamate cannot be released from cortical astrocyte process also at lower laser power

Also at 0.1 W, able to evoke a lower more "physiological" glutamate overflow from the nerve terminals, laser light was unable to stimulate any glutamate release from the astrocyte processes (data not shown).

2. MODULATION OF GLUTAMATE RELEASE BY RECEPTOR-RECEPTOR INTERACTIONS IN RAT STRIATAL ASTROCYTIC PROCESSES

A2A-D2 Receptor-Receptor Interaction

A research project directed towards the investigation of the expression of A2A-D2 heterodimers in striatal astrocytes and their effects on the mechanisms controlling the release of glutamate in astrocytic processes led to the demonstration of functional, biochemical and biophysical interaction between the A2A and D2 receptor on stritatal astrocytes.The results were published in the following articles:

Homocysteine and A2A-D2 receptor-receptor interaction at striatal astrocyte processes

C. Cervetto, A. Venturini, D. Guidolin, G. Maura, M. Passalacqua, C. Tacchetti, P. Cortelli, S. Genedani, S. Candiani, P. Ramoino, S. Pelassa, M. Marcoli, L.F. Agnati

J Mol Neurosci (2018)

A2A-D2 Heteromers on Striatal Astrocytes: Biochemical and Biophysical Evidence.

S. Pelassa, D. Guidolin, A. Venturini, M. Averna, G. Frumento, L. Campanini, R. Bernardi, P. Cortelli, GC. Buonaura, G. Maura, L.F. Agnati, C. Cervetto, M. Marcoli.

Int J Mol Sci. 2019 May 17;20(10):2457. doi: 10.3390/ijms20102457

(Supplement 1)

The results published in the two papers abovementioned are here shortly reported:

- Immunofluorescent analysis demonstrated that both D2 and A2A receptors are expressed on striatal astrocytic processes (see also Cervetto et al, 2017), and colocalize with the V-GLUT1 transporter (see Cevetto et al, 2018 and Supplement 1).

Functional interactions were studied using the superfusion method (See Material and Methods). Gliosomes were stimulated with the depolarizing agent 4-AP alone or with D2

and A2A agonists or antagonists (see Cervetto et al, 2018). Results showed that the D2 agonist quinpirole decreases 4-AP-evoked glutamate release from gliosomes in comparison with the control (4-AP alone). The effect was actually mediated by the D2 receptor, as the D2 antagonist sulpiride abolished the quinpirole-evoked inhibitory effect on glutamate release.

Although the administration of the A2A agonist CGS21680 didn't show any effect on the level of released glutamate, the stimulation with both quinpirole and CGS21680 caused the release of amount of glutamate comparable with the control (see Figure 8). Taken together, these data suggest that A2A and D2 receptor are colocalized in astrocytic processes and control glutamate release through functional interactions. Furthermore, gliosomes were treated with D2 synthetic peptide VLRRRKRVN, corresponding to the D2 receptor intracytoplasmatic region involved in electrostatic interactions with the A2A receptor occurring during receptor heteromerization. VLRRRKRVN-gliosomes treated with quinpirole and CGS21680 as previously reported, showed that the synthetic peptide abolished the effect of A2A receptor on D2-mediated inhibition of 4-AP-evoked glutamate release, indicating that A2A and D2 receptors have physical interaction at the level of the intracytoplasmatic tail (see also Cervetto et al, 2017). Intracellular (but not extracellular) homocysteine was able to reduce the D2- mediated inhibition of glutamate release. Even in presence of intracellular homocysteine, astrocytic A2A receptors were still capable of interacting with D2 receptors (see Figure 8). The effect of intracellular homocysteine, able to reduce the D2-mediated inhibition of glutamate release (allosteric action of homocysteine on D2), and not interfering with the ability of the A2A receptor to abolish the D2 effect (therefore maintaining A2A-D2 interaction) indicate the complexity of A2A-D2 receptor-receptor interaction.

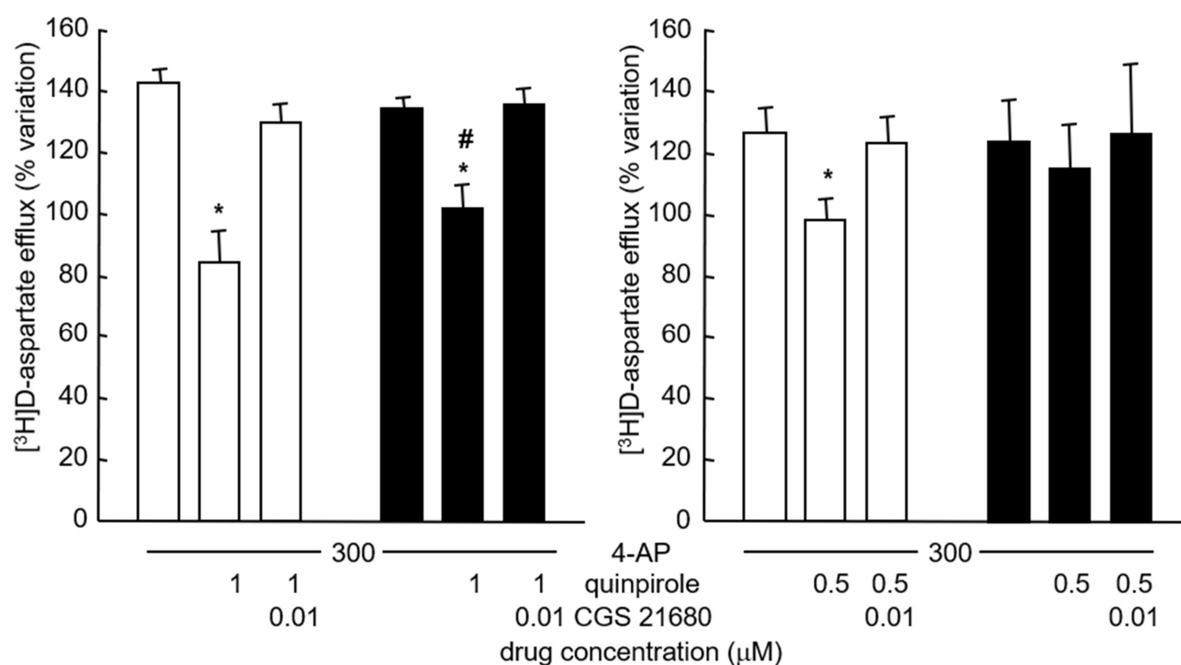

Fig.8 Intracellular Homocystein effect in astrocytic processes

The A2A agonist CGS2168 is able to abolish the quinpirole-mediated inhibition of 4-AP-evoked glutamate release (white bars, black bars). Intracellular homocysteine interferes with the quinpirole-mediated inhibitory effect on the 4-AP-evoked glutamate release (black bars) in comparison with the control (white bats). Bars represent percentage increase of [^{3}H]-D-Aspartate in the presence of the drugs of the concentration indicated.

*p < 0.05 compared with the effect of 4-AP alone. #p < 0.05 compared with the effect of 4-AP plus quinpirole in the absence of homocysteine.

From Cervetto et al, 2018.

Physical interactions between the two receptors were demonstrated by Proximity Legation Assay (PLA) and co-immunoprecipitation (Supplement 1).

Co-immunoprecipitation results, in particular, demonstrated that all D2 receptors expressed in striatal astrocytic processes have a physical interaction with the A2A receptors, while not all the A2A receptor were connected to D2 receptors.

Taken together, all this data demonstrated that A2A and D2 receptors expressed on striatal astrocytic processes form heterodimers with functional and physical interactions. Heterodimers between Oxytocin and D2 receptors have been previously reported in rats striatum (Romero-Fernandez et al., 2013).

After demonstrating A2A-D2 heterodimer existence, we focused on the study of the possible OXT-D2-A2A receptors interactions in astrocytic processes.

2.1. EXPRESSION OF OXT RECEPTORS IN FRESHLY ISOLATED ASTROCYTES FROM RAT STRIATUM

2.1.1. OXT receptor is expressed on astrocyte processes of adult rat striatum

Gliosomes purified from adult rat striatum were labelled with anti-OXTR and anti-GFAP antibodies. Immunofluorescence analysis indicated that oxytocin receptors were expressed in the single GFAP^{+}-astrocytic process (Figure.9)

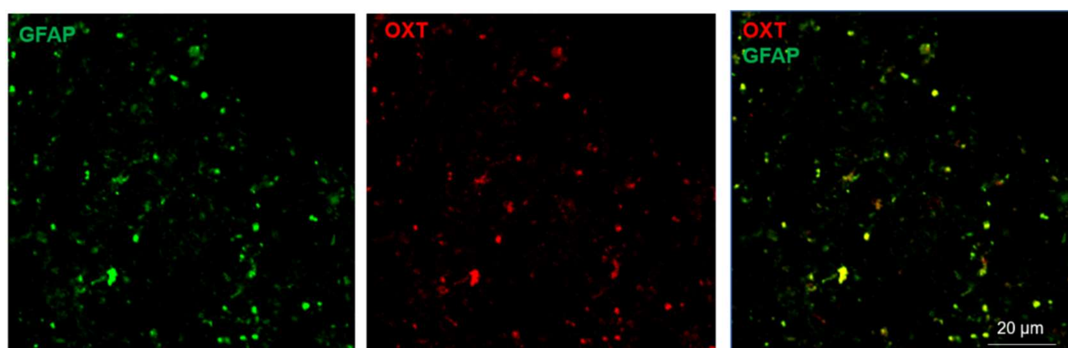

Fig.9 Rat striatal purified astrocyte processes express OXT receptors.

Confocal microscopy analysis. Immunofluorescence for GFAP (green) and for the OXT receptor (red): merge image showing co-expression of the markers. Scale bare is indicated in the image.

Moreover, further immunofluorescence analysis were performed using an anti-VGLUT1 primary antibody to investigate the expression of oxytocin receptors in V-GLUT-1 positive gliosomes.

We obtained the evidence that oxytocin receptors are localized on glutamatergic gliosomes, as shown in Figure 10.

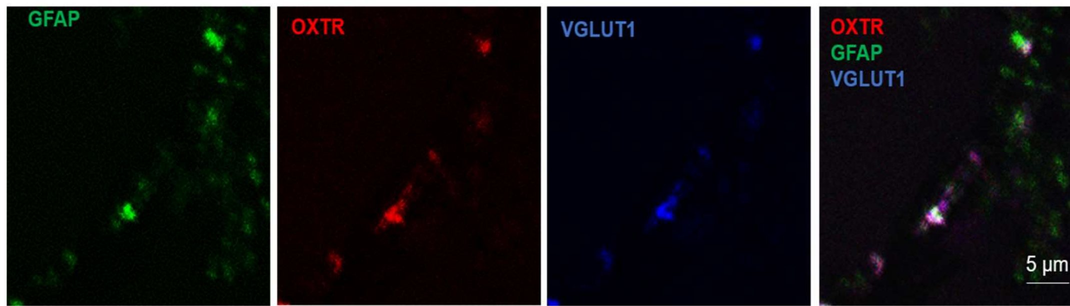

Fig.10 Rat striatal purified astrocyte processes endowed with the V-GLUT1 transporter express OXT receptors.

Confocal microscopy analysis. Immunofluorescence for GFAP (green), OXT receptor (red), and VGLUT1 (blue): merge image showing co-expression of the markers (white dots). Scale bare is indicated in the image.

2.1.2. OXT receptors control glutamate release from striatal astrocytic processes

Since oxytocin receptors are expressed in gliosomes endowed with V-GLUT1, we wondered whether OXT receptors were able to control the mechanisms underlying glutamate release in astrocytic processes.

Gliosomes were then stimulated with the depolarizing agent 4-AP in addition with:

- oxytocin
- oxytocin in presence of the oxytocin antagonist L-371,257

Oxytocin (0.003 µM) inhibited the glutamate release response evoked by the 4-AP; while the OXT antagonist L-371,257 (0.1 µM), per se ineffective on the 4-AP-evoked endogenous glutamate release, prevented the oxytocin inhibition of the release (Figure 11). This data indicates the presence of oxytocin receptors negatively coupled to the release of glutamate from astrocytic processes.

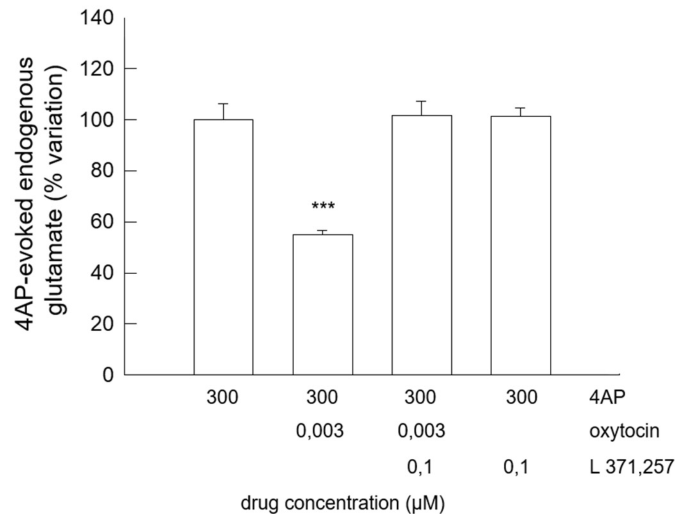

Fig. 11 Oxytocin receptor controls the glutamate release from striatal astrocytic processes

Striatal astrocytic processes in perfusion were stimulated with the depolarazing agent 4-AP (300 μM), Oxytocin (0.003 μM) and oxytocin receptor antagonist L-371,257 (0.1 μM), as indicated in the figure. The results are shown as the mean ± SEM of n= 3 indipendent experiments.

*** p<0.01 one way ANOVA followed by Bonferroni post hoc test vs 4AP.

2.1.3. OXT and A2A receptors are co-expressed in striatal astrocytic processes

In striatal astrocyte processes, the presence of heteromers A2A-D2 was previously demonstrated with physical and biochemical evidence (Pelassa et al., 2019) and fuctional evidence. (Cervetto et al., 2017, 2018). As D2, the OXT receptors were involved in modulating the release of glutamate from striatal processes; by immunofluorescence and release experiments we tested if OXTR had physically or functionally interactions with the A2A receptor, as previously reported by our group for the D2 receptor.

Immunofluorescent analysis showed that OXT and A2A receptors were co-expressed in the same $GFAP^+$ astrocytic process (Figure.12)

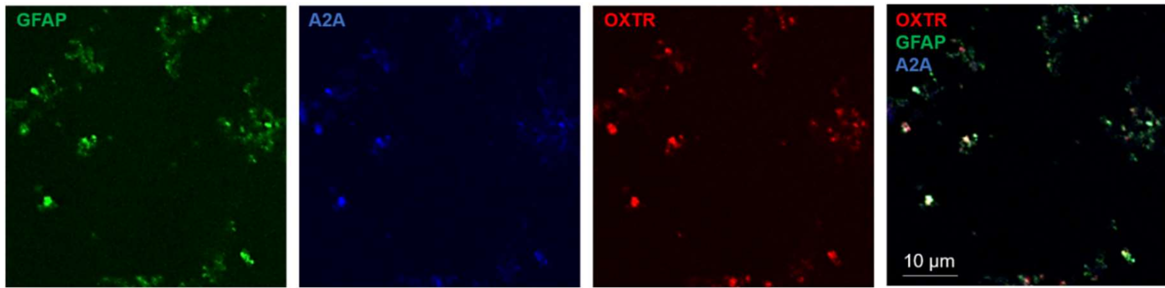

Fig.12 Rat striatal purified astrocyte processes express OXT and A2A receptors

Confocal microscopy analysis. Immunofluorescence for GFAP (green), OXT receptor (red), and A2A receptor (blue): merge image showing co-expression of the markers (white dots). Scale bare is indicated in the image.

2.1.4. OXT and A2A receptors functionally interact in striatal astrocyte processes

As immunofluorescent results demonstrated the existence of both A2A and OXT receptors on the same astrocytic processes, we hypothesized that these receptors may functionally interact. By studying the effects of oxytocin agonist and antagonist, and of A2A receptor agonist and antagonist, we found that oxytocin was able to inhibit the depolarization-evoked release of endogenous glutamate from the astrocyte processes, following a pattern sensitive to the OXT receptor antagonist L-371,257, indicating the presence of release-inhibitory receptors for oxytocin. Activation of the A2A receptor, per se ineffective on the release of glutamate, was able to revert the inhibitory action of oxytocin, in a way sensitive to A2A receptor antagonism (Figure.13).

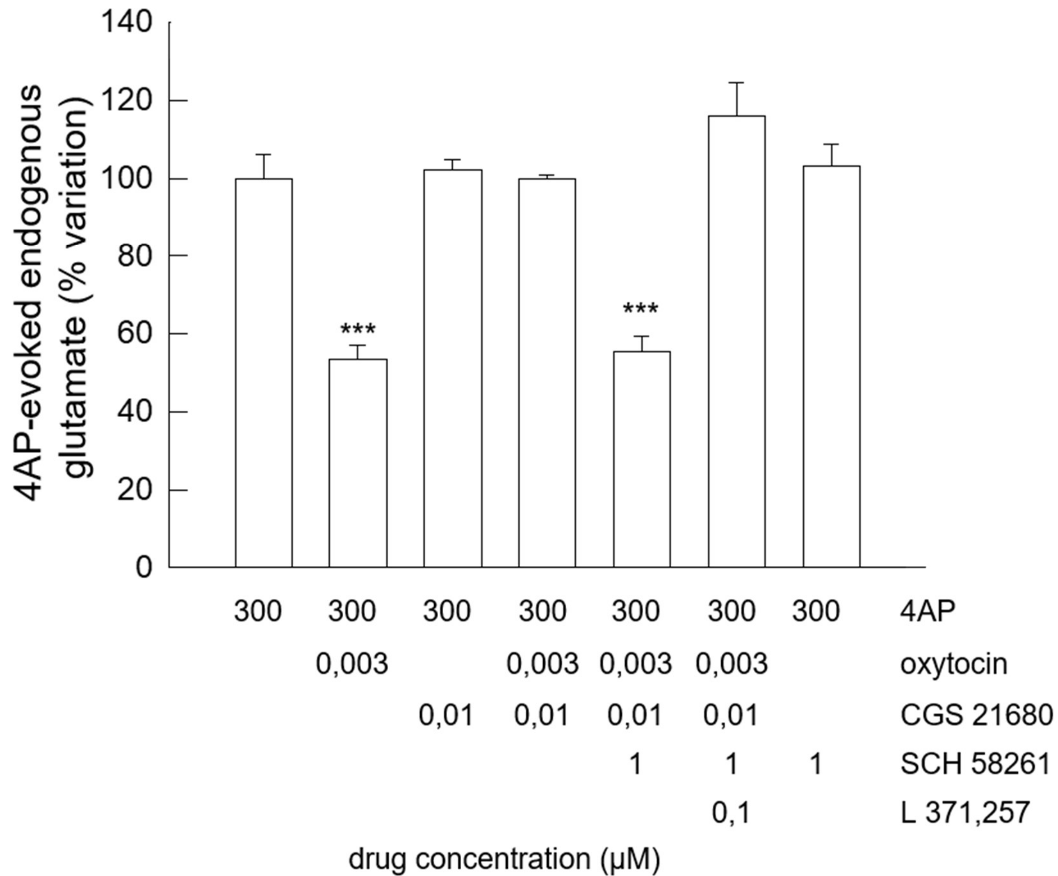

Fig. 13 Functional interaction between Oxytocin and A2A receptors

Striatal astrocytic processes in perfusion were stimulated with the depolarazing agent 4-AP (300 µM), in the presence of the used drugs: oxytocin (0.003 µM), A2A receptor agonist CGS21680 (0.01 µM), A2A receptor antagonist SCH58261 (1 µM) or the OXT antagonist L-371,257 (0.1 µM). The antagonists SCH 58261 and L-371,257 were added 8 minutes before the agonists (see Materials and Methods).The results are shown as the mean ± SEM of n=10 for 4-AP; + oxytocin; + oxytocin & CGS 21680;
n=5 for 4AP + CGS21680; + oxytocin &CGS 21680 & SCH 58261; +oxytocin &CGS 21680 & SCH 58261 & L-371,257; n=3 for 4-AP + SCH 58261.

*** p<0.01 one-way ANOVA followed by Bonferroni post hoc test vs 4AP

Taken together, the results indicate a functional interaction between A2A and OXT receptors in rat striatal astrocyte processes.

2.1.5. OXT and D2 receptors are co-expressed in striatal astrocytic processes

As previously observed, the OXT activation inhibited the 4-AP-evoked glutamate release as the dopamine D2 receptor (Cervetto et al., 2017). Moreover, we recently demonstrated the presence of A2A-D2 heteromers in striatal astrocytes with a functional role in the modulation of glutamate transmission. Here we carried out immunofluorescence analysis to figure out whether OXT, A2A and D2 receptor were colocalized on the same astrocytic process (Figure 12).

Results showed that the three receptors are expressed on the same gliosome, suggesting that they might interact to form a heterocomplex, or receptor mosaic. (Figure 14)

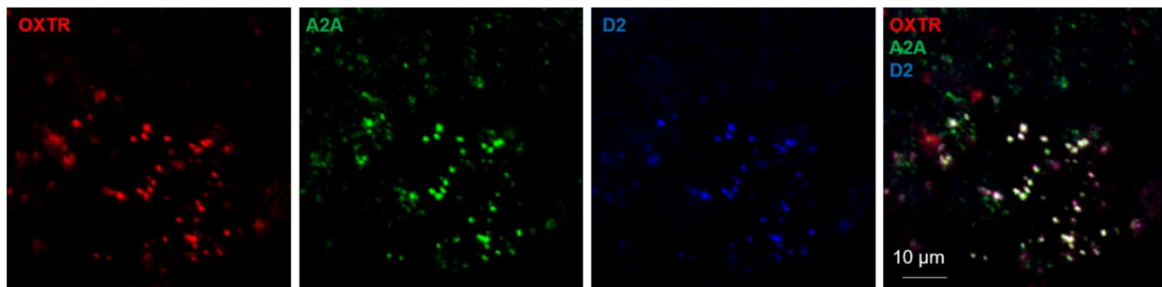

Fig. 14 Rat striatal astrocyte processes express OXT, A2A, and D2 receptors.

Confocal microscopy analysis on striatal gliosomes. Immunofluorescence for OXTR (red), A2A (green), and D2 receptor (blue): merge image showing co-expression of the receptors (white dots). Scale bare is indicated in the image.

2.1.6. Do OXT and D2 receptor have functional interaction in striatal astrocytes processes?

To verify the hypothesis that OXT, A2A and D2 receptors might form heterocomplex in striatal astrocytic processes, we evaluated the possible functional interactions between OXTR and D2 receptors stimulating gliosomes in perfusion with 4-AP in presence of the oxytocin and D2 receptor agonists (oxytocin and quinpirole) and antagonists (L-371,257 and sulpiride). In striatal astrocytic processes, the D2 receptor agonist quinpirole (1 µM) inhibited the glutamate releasing response to 4-AP; the D2 receptor antagonist sulpiride (10 µM), per se ineffective on the 4-AP-evoked glutamate release, prevented the quinpirole-evoked inhibition of the release (Figure 15).

The co-presence of the two agonists, oxytocin and quinpirole, produced a more intense

inhibition than each agonist alone (Figure 15), confirming the reported evidence of the dopamine D2-oxytocin receptor heteromers in striatum with facilitatory receptor-receptor interactions (Romero-Fernadez et al., 2013). Coadministration of sulpiride plus L-371,257 was able to completely prevent inhibition of the glutamate release due to the co-presence of the two agonists.

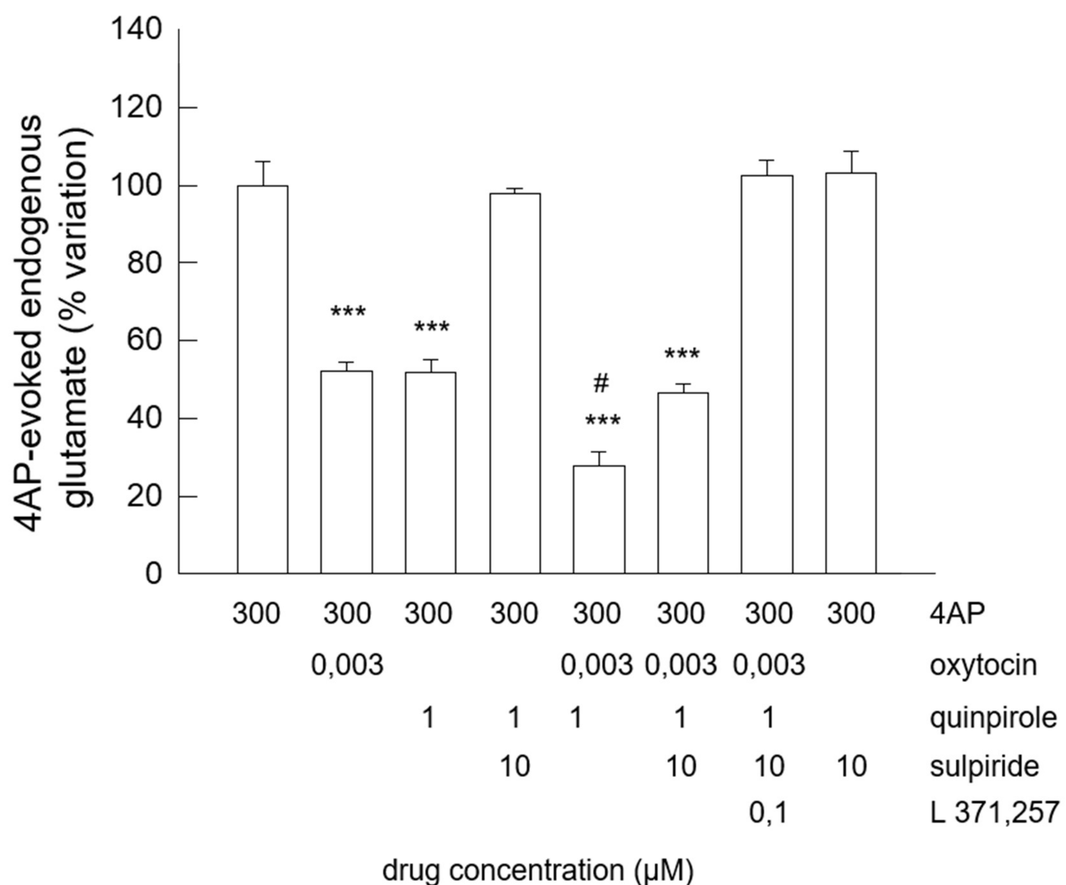

Fig. 15 Oxytocin and D2 receptors control the glutamate release in striatal astrocytic processes

Striatal astrocytic processes in perfusion were stimulated with the depolarazing agent 4-AP (300 µM) in the presence of the used drugs: oxytocin (0.003 µM), D2 receptor agonist quinpirole (1 µM), D2 receptor antagonist sulpiride (10 µM) or the OXT antagonist L-371,257 (0.1 µM). The antagonists sulpiride and L-371,257 were added 8 minutes before the agonists (see Materials and Methods). The results are shown

as the mean ± SEM of n= 8 for 4AP; 4AP + oxytocin; n= 4 for 4AP + quinpirole; n= 7 for 4AP + quinpirole & sulpiride; for 4AP + quinirole & oxytocin; n=3 all the others.

*** p<0.01 one-way ANOVA followed by Bonferroni post hoc test vs 4AP.

<0.01 one-way ANOVA followed by Bonferroni post hoc test vs 4AP & oxytocin or 4AP & quinpirole

As expected, experiments confirmed that individual stimulation of oxytocin or D2 receptor determines a lower amount of glutamate release from gliosomes.

However, simultaneous stimulation of both the receptors leads to a higher inhibition of glutamate release from astrocytic processes. Results from perfusion experiments show that, apparently, D2 and oxytocin receptors could present functional interactions.

Additional experiments are necessary, using a no-effective quinpirole concentration, since is reported that oxytocin is able to increase the Bmax value for D2 receptor antagonist at D2 receptor, without a change in the K_D value of the D2-likeR antagonist [^{3}H]–raclopride-binding sites in striatal membrane preparations (Romero-Fernadez et al., 2013).

In any case, our results indicate that glutamate release from astrocyte processes can be modulated also by activation of oxytocin receptors, and by a receptor-receptor interaction between A2A and oxytocin receptors.

Further experiments will be requested to completely understand whether functional interactions occur not only between OXT and D2 receptors but also among OXT, A2A, and D2 receptors, and if the functional interaction might depend on receptor heteromerization in receptor mosaics.

3. EXOSOMES RELEASE FROM ASTROCYTIC PROCESSES

The release of nanovesicles (exosomes) from astrocytic processes is one of the research lines in prof. Marcoli's lab. Results were published in the following manuscript:

Exosomes From Astrocyte Processes: Signaling to Neurons

Venturini A, Passalacqua M, Pelassa S, Pastorino F, Tedesco M, Cortese K, Gagliani MC, Leo G, Maura G, Guidolin D, Agnati LF, Marcoli M, Cervetto C.

Front Pharmacol. 2019 Dec 2;10:1452. doi: 10.3389/fphar.2019.01452.

(Supplement 3)

Here, we provided a brief summary of the results obtained:

- Electron microscopy analysis showed that astrocyte processes purified from adult rat cortex are endowed with the Multivesicular bodies and could release extracellular vesicles when perfused with buffered physiologic medium. Collected nanovesicles were then analysed using Electronic Microscopy, Dynamic Light Scattering (DLS), and Western Blot.

-

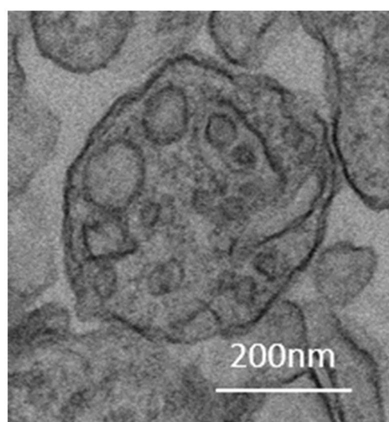

Fig. 16 Electron microscopy image of a single cortical astrocyte process.

In a gliosome, vesicles scattered in the cytoplasm and a multivesicular body are shown. Scale bar: 200 nm. For experimental details, see Materials and Methods.

DLS analysis on purified vesicles showed that their size distribution (range 50-75 nm, peaking at 60nm) was compatible with the exosomes reported size (10-100 nm, Doyle,2019). Imaging analysis showed that vesicles released from astrocytic processes have the typical bell-shape and size as exosomes.

Finally, blot experiments demonstrated that gliosomes-derived vesicles express the typical exosomes markers TGS 101 and Alix19. In addition, they resulted positive even for astrocytic marker GFAP and ezrin. Taken together, these results strongly suggested that vesicles released form rat cortical astrocytic processes are exosomes.

Even though exosomes release from astrocytes had been already demonstrated in culture cells, this is, to our knowledge, the first demonstration that astrocytic processes derived from in vivo mature astrocytes can release exosomes.

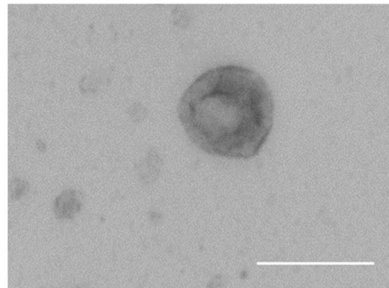

Fig. 17 Characterization of rat cerebrocortical astrocyte processes-released exosomes. Electron microscopy images of a vesicle released from astrocyte processes. The cup shape appearance and its size, consistent with previously reported exosome electron microscopy images characteristics. Scale bar: 100 nm. For experimental details, see Materials and Methods.

Interestingly, exosomes were positive for the protein Neuroglobin (NGB), a protein with anti-oxidant, apoptotic, and inflammatory effects.

- Exosomes from astrocytic processes seem to be able to target neurons in a neuron-astrocytes mixed co-culture. Primary cortical cells from rat embryonic day 18 were cultured and differentiated in neurons and astrocytes. The neuron-astrocytes co-cultures were then exposed to astrocytic-derived exosomes labelled with the PKH67 fluorescent cell linker kit.

Confocal imagines showed that exosomes are up-taken selectively from cultured neuronal cells.

In conclusion, our work demonstrated that astrocytic processes purified from rat cortex can release exosomes which, in turn, can be up-taken from in vitro neuronal cells. It can be hypothesized that exosomes have a protective role in CNS, carrying the NGB protein which could be up-taken by neurons.

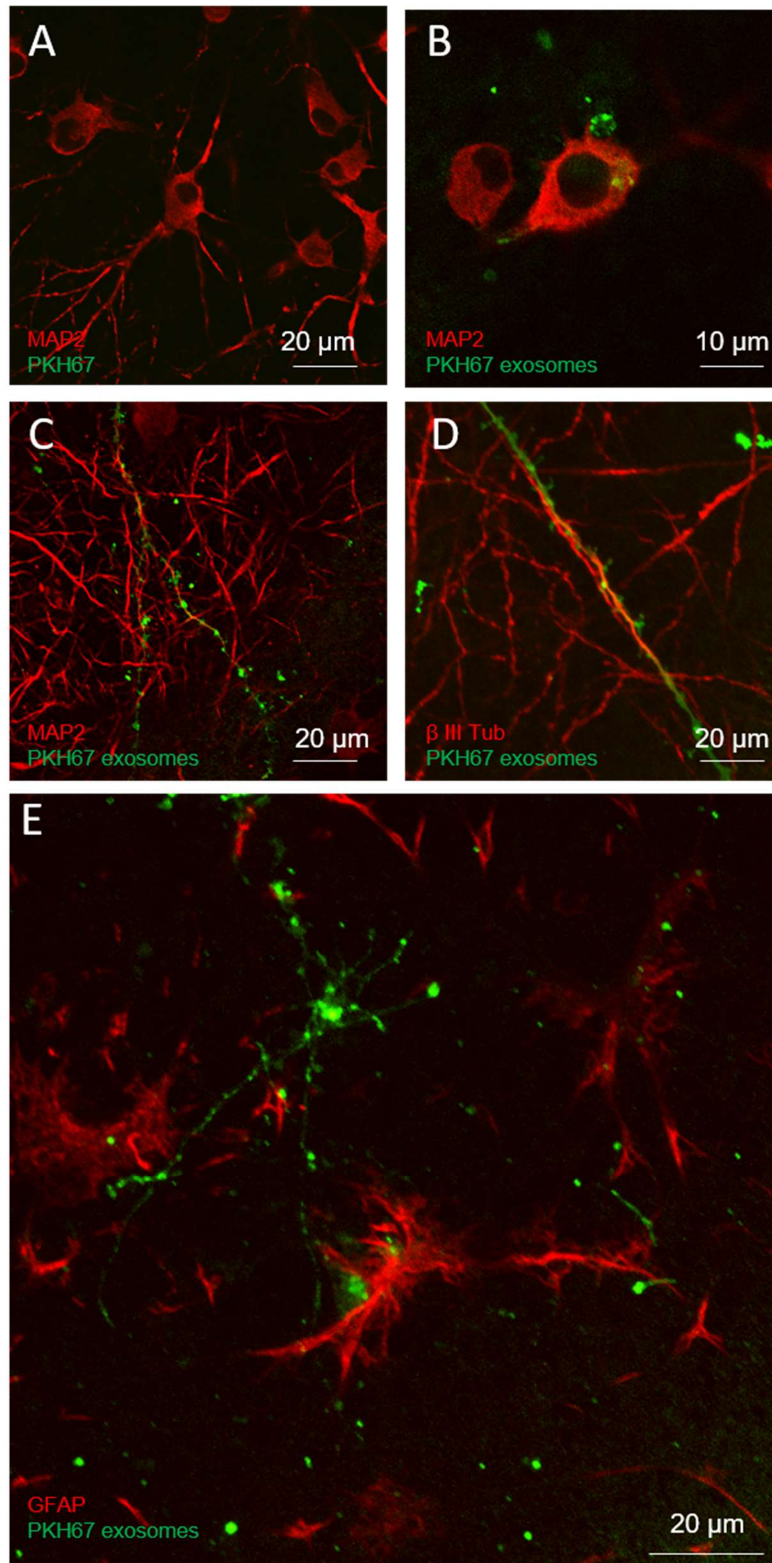

Fig. 18 Neurons as targets for the exosomes.

The confocal analysis showed exosomes targeting neurons when added to a neuron-astrocyte co-culture. Exosomes marked with PKH67 (green) selectively target MAP2 (red, B, C) or β III tubulin (red, D) – positive neurons, while GFAP-positive astrocytes are not targeted (red in E). The showed images are the merge of a single z stack of the two channels (A-D) or representative maximum intensity projections of the

acquired z stacks of the two channels (E). Scale bars are indicated in the images. For other experimental details, see Materials and Methods.

DISCUSSION

1 LASER STIMULATION ON CORTICAL ASTROCYTIC PROCESSES AND NERVE TERMINALS

We here investigated the possible link between photobiomoduation (PBM), a manipulation of cells occurring through the emission of light from a light source, and glutamate-releasing cells in CNS.

In fact, it is recognized that almost all the living organisms can spontaneously emit photons both under physiological or pathological conditions, known as biophotons (Devaraj et al., 1997; Prasad and Pospíšil, 2013). The emission is strictly related to the physiological and pathological conditions of the organisms (Moraes et al., 2012; Bertogna et al., 2013). Bioluminescence phenomenon is well known since a long time, for instance light-emitting fireflies.

Biophotons existence was postulated in 1920 by Gurwitsch (Gurvitch et al., 1926) but, due to the lack of technology, they were never detected. In 1955, Colli (Colli et al., 1955), observed for the first time the biophotons presence in plant thanks to the use of photomultiplier tubes, at that time just invented. During the 60s and 70s, scientists were interested in mechanisms underlying biophotons generation, proposing the hypothesis according to whom that processes might be related to mitochondrial respiration, lipidic oxidation, and other metabolic processes (Vladimirov et al., 1971; Zhuravlev et al., 1973). Numerous experiments demonstrated that free radicals, reactive species and their derivates can play an important role in the regulation of cellular processes and, since their production is the result of well determined processes, it is likely that even biophoton production follows a well-regulated pathway and play a role in cell signalling (Dröge, 2002; Lee et al., 2013).

Thanks to Popp’s studies, DNA is recognized as a source of biophoton. Popp himself proposed the theory of biophotonic coherent radiation, hypothesizing the existence of an electromagnetic field in the cell which might be behind cell communication (Popp et al., 1984; Popp and Belousov, 2003).

Photobiomoduation (PBM) was discovered fifty years ago and its role in human and veterinary medicine has been increasingly recognized (Amaroli et al., 2018). The story of PBM starts thanks to Endre Mester in 1967, the pioneer of photobiomodulation, who first observed electromagnetic field in cells. However, Prof. Karu was the first to propose a model explaining the mechanisms through which laser might interact with target cells

(Karu, 1986). Nowadays, PBM is defined as a manipulation of cells occurring through the emission of light from a light source. Once in cells, photons are able to interact with specific molecules called photoacceptors and induce a photochemical reaction in the cell itself (Hamblin and Demodova, 2006). As a matter of fact, effects of photons in mammals have been known for 50 years, and their effects were associated with increase of intracellular Ca^{2+} and NO synthesis in cells (Amaroli, 2017).

PBM is a therapy based on the administration of non-thermic light from the near infrared (NIR) (600-1000 nm) emitted by a laser or a non-coherent light source. PBM is able to produce beneficial effects in patients affected by many pathologies. The high numbers of reports which, recently, showed PBM positive effects for treatment of lesions and pathology of the nervous system, is a sign of the increasing interest for photobiomodulation and its applications (Huang et al., 2014). Interestingly, PBM application was proposed to be effective for many clinical procedures in murine models (Wu Q, 2012; Henderson 2015). Therapy application on animal model, therefore, demonstrated that PBM is able to enhance memory recovery (Karu et al, 1989), axon growth and nerve regeneration (Byrnes et al., 2005; Rochkind, 2009), and neuron protection from neurotoxic damage (Oron et al., 2006; De Taboada et al., 2006). PBM efficacy on nervous system was further demonstrated by studies carried out on mice affected by stroke event or post-traumatic lesions (TBI): those animals, once therapies was administrated, showed a better neurological and functional recovery (Oron et al., 2006; Lapchak and Taboada, 2010; Oron et al., 2007; Quirk et al., 2012). Moreover, twelve-months CD1 mice treated with PBM presented better emotional (decrease stress) and mnemonic responses (Michalikova et al., 2008).

After applying PBM on humans, encouraging results were obtained in terms of tissue regeneration, wound healing (Albertini et al., 2007), and neuron protection from neurotoxic lesions (Wong-Riley et al., 2005; Liang et al., 2008). Furthermore, PBM applied on subjects who suffered for a trauma resulted to reduce the inflammation (Aimbire et al., 2006), enhance anti-inflammatory effects (Brosseau et al., 2000), reduce the edema (Stergioulas et al, 2004), and restore the issues of blood streaming (Avci et al., 2013), finding the application in diverse field of medicine aimed to the treatment of muscleskeletical lesions, skin diseases, degenerative pathology, and syndrome caused by neuropathic pain (Hopkins et al., 2004; Xuan et al., 2013). In addition, many other data support PBM application in diverse biomedical areas, including endodontia (a branch of the

odontoiatry), oral pathologies treatment, oral surgery, and orthodontia (Milward et al., 2014). PBM application can prevent pain and protect muscles before intensive workout, implying a wide use of of that technology as a preanesthetic assessment before surgical procedures (Agrawal et al., 2014).

On the other hand, some studies suggested that PBM is uneffective or even potentially harmful (Tunér and Hode, 1998; Bjordal et al., 2003). Non significative effects may be explained with non-proper dosimetry, wrong energy level, irradiance, and time of exposition (Tunér and Hode, 1998; Hunag et al., 2009; Ilice t al., 2006).

It is known that the cells that make up the nervous system are able to communicate via chemical or electrical signals. In addition, it has been shown that such cells are able to emit biophotons: this emission can be modulated by depolarizing stimuli (as shown by Artem'ev et al., 1967) or by the presence of glutamate (as shown by Tang and collaborators on mouse bark slices; Tang and Dai, 2014).

To date, only hypotheses exist regarding the production pathways of such biophotons in nervous system. For example, some scholars believe that they could originate from mitochondria and then propagate through nerve fiber microtubules (Tang and Dai, 2014). Many studies in literature described the effects of photons on the physiology of the central nervous system. In fact, several experiments carried out on cultured neurons showed that laser light is able to modify the membrane potential of mitochondria, ATP levels, intracellular Ca^{2+} levels, and the production of reactive species of oxygen and nitric oxide (NO) (Huang et al., 2014; Amaroli et al., 2015 and 2016). It was demonstrated that laser light can modify the redox state of the cell producing ROS or increasing cellular reductive capacity (Oron et al., 2012).

However, the mechanisms through which laser light acts on the functioning of the nervous system have not yet been fully understood. One hypothesis is that laser light might act on neurotransmission processes by modifying the abovementioned mechanisms. In fact, some studies have shown a correlation between changes in mitochondrial membrane potential and ATP synthesis levels and have been linked to the ability of the neuron to release neurotransmitters (Choi et al., 2009; Rangaraju et al., 2014).

Regarding the relationship between photons and neurotransmitter release, we need to focus on two molecules known for their role in cellular signal modulation and involved in the mechanisms that lead to the release of neurotransmitters: calcium and nitric oxide (NO). The former is a second messenger fundamental for the exocytotic release of vesicles

(Barclay et al., 2005) while, according to some studies, NO could play a role in the regulation of neurotransmitter release (Prast and Philippu, 2001; Garthwaite, 2008).
Therefore, for a better understanding of the mechanisms through which laser light acts on the functioning of the nervous system, we investigated on photons effects on the release of glutamate by taking advantage of different preparations from the rodent cerebral cortex, slices - maintaining neuron-astrocyte relationships - and purified astrocyte processes and nerve terminals.

1.1 Cerebral cortex slices release glutamate if stimulated with low-intensity laser light

The major advantage of cerebral cortex slices is that they maintain intact the interactions between cells, efficiently mimicking the in vivo conditions.
During our experiments, perfused slices from rat cerebral cortex were stimulated with laser light (See Material and Methods), determining an increase of the amount of glutamate in comparison with the control, and confirming the ability of PBM to enhance glutamatergic transmission in CNS.
As slices maintain neuron-astrocyte networks, and since both neurons and astrocytes can release glutamate, results from those experiment do not allow to understand which cell population respond to the laser stimulation.

1.2. Laser light did not evoke glutamate release from astrocytic processes but evoked it from nerve terminals

Since cortical slices represent both neuronal and glia cells responses to stimulus, we were interested to understand which cell population was the source of the released glutamate.
Experiments showed that stimulated astrocytic processes did not release glutamate, while nervous terminals did.
This result is consistent with the data reported by Golovynska and colleagues, who demonstrated that the application of laser light at different wavelength (including the 808 nm) determines Ca^{2+} influx and membrane depolarization in neuronal cells (Golovynska, 2020).
On the other hand, astrocytic processes did not show any response to the stimulus. According to our knowledge, administration of low laser therapy was never directly

experimented on astrocytes, except for a study published by Sun and colleague, who applied PBM on murine model of Spinal Cord Injury. According to their study, PBM significantly reduces the activation of astrocytes and the secretion of chondroitin sulfate proteoglycans from the cells (Sun 2020).

We can rule out that glutamate release was due to membrane damage caused by the light because the amount of glutamate levels increases during stimulus administration and then decreases to basal levels after stimulus cessation, as shown in the time-course.

However, we must keep in consideration that PBM targets mitochondrial cytochrome in cells (Poyton,2011). According to ultrastructural analysis, astrocytic processes, differently from nerve terminals, are not endowed with mitochondria. So, we cannot exclude that inability of photons to evoke glutamate release from the astrocyte processes is dependent on the absence of mitochondria in gliosomes.

The exposition of nerve terminals to laser stimulation causes an impressive release of glutamate, much higher than the amount we usually observe using different stimuli (data not shown). Although photons emission was proposed to be involved in CNS signal transmission (Tang,2014; Kumar2016), the amount of glutamate released after laser stimulation was too high for physiologic processes. Thus, we realized that, probably, laser parameters were not suitable for our perfusion model. Accordingly, we modified laser parameters in order to obtain lower power laser.

1.3. Glutamate release at 0.1 W laser

Since protocols for photobiomodulation application are variable and no agreement on the parameters (as laser power, intensity or wavelength) (Zein et al,2018) that should be used has been reached, we wondered whether the release of glutamate from nerve terminals was laser power-dependent. The quantity of glutamate evoked from synaptosomes treated with the 0.1W laser, indeed, was significantly lower than the glutamate released from synaptosomes stimulated with 1 W. Experiments with Rose Bengal demonstrated that also in this case, the glutamate release from the nerve terminals was vesicle-dependent. Taken together, considering that excitotoxicity of glutamate at high concentration, this result might indicate that the choice of parameters for phtobiomodulation application should be accurate in order to avoid release of glutamate possibly harmful for the individuals.

On the other hand, also at 0.1W, laser could not evoke any release of glutamate from the astrocyte processs. Further investigation is required to understand the reason for the ineffectiveness of PBM on astrocytic release. Indeed, by exploring the mechanisms possibly involved in the glutamate releasing effect from the nerve terminals, and specifically by investigating on the possible involvement of different Ca^{2+} channels types, of NO signalling, or of mechanisms related to mitochondria function, it will be possible to get light on the effectiveness and ineffectiveness of PBM on transmitter release from different cell types in the CNS. As a matter of help, these investigations would also help to obtain a greater knowledge and understanding of the pathways affecting the release of glutamate from the astrocyte processes.

2 MODULATION OF GLUTAMATE RELEASE BY RECEPTOR-RECEPTOR INTERACTIONS IN RAT STRIATAL ASTROCYTIC PROCESSES

2.1 G-protein coupled receptors can form heteromers in CNS

G-protein coupled receptors (GPCRs) are important proteins expressed on cell membrane which play a fundamental role in detecting signals from the extracellular space (Rozenfeld et al, 2010).

As known, GPCRs are a key receptor family involved in numerous physiological processes and for this reason they represent the target of many drugs (40-50% of drugs currently on the market).

GPCRs activation causes signal transduction mediated by four Gα-protein subclasses ($G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_o$), leading to the regulation of many cellular events. Receptor stimulation causes changes in the levels of intracellular second messengers (mainly cAMP and Ca^{2+}) and, often, involves Ser/Thr protein kinases and phosphatases (Brugarolas et al., 2014). During the '80s, it was observed that GPCRs do not exist only as individual receptors but can also assembly in oligomers, probably formed through the instauration of allosteric interaction between receptors (Agnati et al., 1980; Fuxe et al., 1983).

Receptor heteromerization was described for many receptors, as for instance, mGLU, dopamine, adenosinergic, cannabinoids, and ocytocin receptors (Gomes et al., 2016, Cervetto et al., 2017, Perez de la Mora et al., 2016).

The major aspect of heteromerization is that the proteins forming the heteromers acquire new properties which are different from the ones of the receptor alone: pharmacological responses and G-protein coupling are two examples (Rozenfeld et al., 2010).

Noteworthy, heterodimers can be involved in pathological conditions (e.g: prostaglandin EP1 receptor-β2-AR heteromer in airway smooth muscles; this heteromerization reduces coupling of β2-AR to Gas, reducing the bronchodilatory potential of the β2-AR agonist isoproterenol), representing thus an important target for pharmacological therapies (McGraw et al., 2006).

In CNS, GPCR heterodimers play a crucial role in the striatum, integrating signals from the dopaminergic and adenosinergic system. For instance, the response of striatal neurons to dopamine can be dependent on the expression of heteromers between adenosine and dopamine receptos. Furthermore, the occurrence of heteromers in models of Parkinson's

disease was previously identified (Brugarolas et al.,2014)

3. Heterodimers on striatal astrocytes

Data previously obtained in Prof. Marcoli's lab provided the evidence of the existence of interactions between the A2A and D2 receptors expressed on astrocytes.
The activation of A2A receptors reduced the D2-mediated inhibition of the 4-AP-evoked ^{3}[H]-D-aspartate release in gliosomes (Cervetto et al., 2017, 2018; Pelassa et al., 2019). Functional evidences suggested that the receptor–receptor interaction was based on heteromerization of native A2A and D2 receptors at the membrane of striatal astrocyte processes. Moreover, biochemical and biophysical evidences confirmed that the receptor–receptor interaction between A2A and D2 receptors in

astrocyte is based on A2A-D2 heteromerization (Pelassa et al., 2019). The co-immunoprecipitation and proximity ligation assay approaches provided the evidence for the ability of native striatal astrocytic A2A and D2 receptors to heteromerize (Pelassa et al., 2019).
Therefore, the interactions between native A2A and D2 receptors on the striatal astrocyte processes might be due to the formation of receptor heterodimers. Here, the experimental evidences supporting the theory:

- A2A and D2 receptors result expressed on same striatal astrocytic processes (Cervetto et al., 2017, 2018);
- A2A and D2 receptors functionally interact in the control of the release of the gliotransmitter glutamate Cervetto et al., 2017, 2018);
- A2A and D2 receptors can form heterodimers on astrocytes through physical interactions (Pelassa et al., 2019).

Notably, the heteromers were able to modulate the release of the gliotransmitter glutamate from the processes, indicating that native striatal astrocytic A2A-D2 receptors via allosteric receptor–receptor interactions could play a role in the control of striatal glutamatergic transmission. Also, the findings indicate the involvement of striatal astrocytes in A2A and D2 receptor signal transmission.
Data here presented suggest that also oxytocin (OXT) receptors are likely involved in oligomeric structures in striatal astrocytes processes purified from adult rats.

2.3 . Oxytocin is a G-protein coupled receptor

Oxytocin is a nonapeptide produced and released in the brain where it works as a neurotransmitter (Perez de La Mora et al.,2016). Oxytocin is involved in many functions, including not only sexual and maternal behavioural but also anxiety, sociability and even drug abuse (Jurek et al,2018). Oxytocin binds and activates its receptor, a G-protein coupled receptor which activate G_q, G_i or G_o proteins when stimulated.

BRET experiments on transfected HEK293 cells demonstrated that OXTR stimulated with Oxytocin interacts with G_q, Gi, and G_o proteins (Busnelli et al., 2010).

2.4 . Oxytocin receptor can form heterodimers

As many GPCRs, even oxytocin receptors might be able to interact with other GPCRs and form oligomeric structures. Perez de la Mora and colleagues, indeed, demonstrated that the dopamine receptor D2 interacts with the oxytocin receptor in HEK293 cells and suggest the existence of D2-OXT heterodimers in rats amydgala playing a role in fear/anxiety control (Perez de la Mora et al., 2016).

Proofs of the existence of D2-OXTR heterodimers in murine striatal neurons were provided by Romero-Fernandez and colleagues through Proximity Ligation Assay experiments. Interestingly, the authors found that oxytocin significantly increased the affinity of the high (KiH) affinity agonist state of the D2R in association with an increase in the D2Rs density in the ventral striatum (Romero-Fernandez et al, 2013).

2.5 . Oxytocin receptors are expressed in striatal astrocytic processes and control glutamate release

Immunochemistry analysis showed that the oxytocin receptors are expressed in astrocytic processes purified from adult rats. Since OXTR colocalized with the glutamate transporter V-GLUT1, we used the perfusion technique to investigate whether oxytocin receptor activation may affect the glutamate release from astrocytic processes.

Gliosomes were stimulated with the depolarizing agent 4-AP in presence of the agonist oxytocin leading to an inhibition in the endogenous glutamate efflux. Thus, this data showed that the oxytocin receptor can actually interact with the pathways underlying gliotransmitter release in astrocytic processes, reducing the amount of glutamate released

from astrocytic processes following depolarization. As previously described for D2 receptor (Cervetto et, 2017), also oxytocin receptor can control and decrease 4-AP-evoked glutamate release.

2.6. Oxytocin receptor colocalizes with A2A and D2 receptors on striatal astrocytic processes

Further confocal imaging analysis demonstrated that OXT receptors colocalize with A2A and D2 receptors. In particular, striatal astrocytic processes co-express OXT, A2A and D2 receptors. Since A2A and D2 are well known to form heterodimers involved in the control of glutamate release from striatal processes (Cervetto et al., 2017, 2018; Pelassa et al., 2019), it was possible to hypothesize that OXTR might be involved in similar functions. Thus, data suggest that receptor-receptor interaction might occur between OXT, D2, and A2A receptor. We thus wondered if functional interactions between OXT and D2 or A2A receptors may occur. As already mentioned, physical interactions between OXT and D2 receptors were reported in rat striatal neurons (Romero-Fernandez, 2013) and hypothesized in amygdala (Perez de la Mora, 2016).
Here, we evaluated whether functional interactions were involved in the modulation of the glutamate efflux the astrocytes processes (Romero-Fernandez W et al., 2013).

2.7. Do OXT-A2A receptors functionally interact in striatal astrocytic processes?

As previously mentioned, A2A-D2 heterodimers are present in striatum astrocyte processes and can control the astrocyte glutamate release. The activation of A2A receptors reduced the D2-mediated inhibition of the 4-AP-evoked ^{3}H]-D-aspartate release in gliosomes (Cervetto et al., 2017, 2018; Pelassa et al., 2019). Functional evidences suggested that the receptor–receptor interaction was based on heteromerization of native A2A and D2 receptors at the membrane of striatal astrocyte processes; co-immunoprecipitation and proximity ligation assay confirmed that the receptor–receptor interaction between A2A and D2 receptors at the astrocyte is based on A2A-D2 heteromerization (Pelassa et al., 2019).
Here, we described that the A2A receptor reduced the OXT-mediated inhibition of the 4AP-evoked glutamate release, which indicates the presence of a functional receptor-receptor interaction between A2A and OXT receptors in rat striatal astrocytes processes. We must highlight that the A2A receptor activation was *per se* unable to affect the glutamate release

from striatal astrocyte processes (see also Cervetto et al., 2017, 2018). Nevertheless, A2A receptor abolished the inhibition of glutamate release depending on the activation of D2 receptors, demonstrating the receptor-receptor interaction through which D2 and A2A receptors regulate release of striatal astrocytic glutamate (Cervetto et al., 2017, 2018). Similarly, here we report the ability of the A2A receptor to abolish the oxytocin-mediated inhibition of the 4-AP-evoked glutamate release from striatal glisomes. Although OXT and A2A receptors seem to have a functional interaction, further experiments are necessary to demonstrate whether they can also physically interact.

2.8. Do OXTR and D2 receptors functionally interact in striatal astrocytic processes?

Physical interactions between OXT and D2 receptors were reported in rat striatal neurons (Romero-Fernandez et al., 2013) and hypothesized in amygdala (Perez de la Mora et al., 2016).

Here, we evaluated whether a functional interaction between OXT and D2 receptors was involved in the modulation of the glutamate efflux from the astrocytes processes.

In superfusion experiments studying the 4-AP-evoked glutamate release from striatal gliosomes, both D2 than OXT receptor activations inhibited the gliotransmitter efflux. The co-activation of the receptors induced a stronger and significant inhibition of the glutamate release than a single receptor agonist, quinpirole (1 µM) for D2 or oxytocin (3 nM) for OXTR. To our knowledge, at the used agonist concentrations, it doesn't seem that D2 and OXTR could interact each other; in fact, the strong inhibition obtained with the contemporary D2 and OXTR activation might be the sum of the single agonist effects. Further experiments are needed to evaluate whether, at a lower and ineffective quinpirole concentration, the OXT receptor activation could behave similarly to what observed in striatal neurons (Romero-Fernandez W et al., 2013). This receptor-receptor interaction could be more in deep investigated, even considering our previous observation: in particular, we found that the D2 receptor expressed in striatal gliosome membrane almost completely immunoprecipitated with the A2A receptor. On the contrary, only a fraction of the A2A receptor immunoprecipitated together with the D2 receptor.

In conclusion, further experiments are warranted to understand whether the oxytocin receptor have physical interactions with A2A or D2 receptors or with both of them.

3. EXOSOMES RELEASE FROM ASTROCYTIC PROCESSES

The main results obtained in this study can be summarized as follows:

- gliosomes perfused with standard physiological medium release MVs positive for the exosome markers ALIX and TSG 101;
- the MVs released by astrocyte processes and purified through ultrafiltration have dimensions that are compatible with those of the exosomes;
- the exosomes released from cerebrocortical gliosomes also carry the characteristic proteins of the cells of origin.

The significance of the data obtained and reported here should be considered in the context of the current state of knowledge on the role of EVs and in particular of exosomes in communication in the central nervous system under physiological and pathological conditions, and in the possible spread of signals of damage or neuroprotection, as well as in their intended use for diagnostic or therapeutic purposes. In particular, the following considerations can be made:

- Although it was already demonstrated that exosomes can be released from astrocytes in culture (Guescini et al., 2012), in vitro astrocytes mimic only marginally the behaviour of the cells in physiologic conditions. The experiments reported here were carried out on astrocyte processes prepared from adult rat cerebral cortex, thus reflecting the characteristics of astrocyte processes in a mature neuron-astrocyte cerebrocortical network. These processes resulted positive for ezrin, an astrocyte cytoskeletal protein and selective marker of perisynaptic astrocyte processes (Derouiche, 2003), necessary for the structural plasticity of astrocyte processes (Lavialle et al., 2011; Bernardinelli et al., 2014). Vesicles positive for exosomes markers, as the proteins of the endosomal-lysosomal complex Alix and Tsg101, were found in the perfusate, indicating that the exosomes can be released from astrocyte processes.

Astrocyte processes might therefore participate in the roamer-type volume transmission through the release of exosomes.

Astrocytes could therefore be able to contribute in many ways to the transmission of signals in the CNS, both by receiving and sending messages of different types, and presumably with different target, such as gliotransmitters (e.g. glutamate, which can be

rapidly recaptured and/or activate non-synaptic glutamate receptors) or molecules that can be carried within nanovesicles. These mechanisms could contribute to a shift towards a neuro-astrocentric view of CNS functioning. In fact, the perisynaptic astrocyte processes could play a key role both for the classical volume transmission, through the release of gliotransmitters, and the non-classical roamer type volume transmission, through the release of exosomes. Through the latter, astrocytes would be able to induce transient phenotypes in the cells receiving the exosomes. Noteworthy, it was demonstrated that G proteins coupled receptors deriving from astrocytes and transported in exosomes can be uptaken and expressed in the receiving cells (Guescini et al, 2014).

- It was found that exosomes can carry neuroglobin. Initially, it was believed that NGB in the mammalian CNS was expressed only in neurons (Laufs et al., 2004); later NGB was also observed in astrocytes and reactive astrocytes (Chen et al., 2005; Della Valle et al., 2010). Interstingly, it was hypothesized that NGB may be produced and secreted from astrocytes, probably as a neuro-protective agent for neurons (Della Valle et al., 2010). In fact, the role of astrocytes is well known as a significant source of neuroprotective mediators for neurons under pathological conditions (Masmoudi-Kouki et al., 2007). The possibility that astrocyte processes can release NGB through exosomes would allow them to send messages to distant cells, transiently modify their vulnerability to trauma, and contribute to the protective effects against ischemic damage (Gleichman and Carmichael, 2014; Verkhratsky et al., 2016).

To date, it is well known that NGB can play several crucial roles in cell defense and resistance to degeneration: in this context, the transport of NGB from astrocytes to neuronal cells could help to protect neurons.

It cannot be excluded that NGB might transferred to other glial cells, such as neighboring astrocytes: that could be a mechanism aimed to increase the resistance of astrocytes to damage.

It has been shown that oestradiol regulates NGB expression in both neurons and astrocytes through molecular mechanisms mediated by ERβ (De Marini et al., 2010; 2013), and this regulation could be part of the neuroprotective mechanisms implemented in astrocytes by oestradiol (De Marini set al., 2013; Estefania Acaz-Fonseca et al., 2014; Guidolin et al., 2016). On the other hand, the production of NGB in astrocytes in response to damage (also considering the possibility of hormonal regulation) and the sending of messages through

NGB could work as a protective mechanism for the neighboring cells belonging to the neuron-astrocyte network.

- Since exosomes are released from a large variety of cell types, they have been proposed as peripheral markers for diagnostic-prognostic purposes for several diseases, including those of the CNS. However, one of the problems affecting their reliability as markers, besides the correct classification of exosomes, is their origin: most of the exosomes recovered in plasma are generated by endothelial cells. Therefore, the discovery that exosomes derived from astrocytes are positive for GFAP and ezrin astrocyte markers allows to hypothesize that the analysis of these markers could give a contribution to the identification of the cellular origin of exosomes from peripheral blood, both under physiological and pathological conditions of the CNS.
- Further experiments demonstrated that exosomes released from astrocytic processes purified from cortex of adult rats can be up-taken by cultured neurons.

Although it is well known that cultured astrocytes can release exosomes able to target the co-cultured neurons, this is, to our knowledge, the first demonstration that *ex-vivo* astrocytes can release nanovesicles which, in turn, can be up-taken by neurons.

Although we did not demonstrate that NGB levels in neurons increased upon exosomes administration, we can speculate that exosomes release from astrocytes can represent a new mode of astrocyte-neurons communication able to transfer molecular messages from astrocytic to neuronal cells.

CONCLUSIONS AND FUTURE DIRECTIONS

In recent years, astrocytes role in CNS has been reconsidered assuming increasingly relevance in biomedical research. This cell population, indeed, is no longer considered merely as a glue or a protection for neurons, but also as key player in synaptic transmission and neuronal activity under both pathological and physiological conditions.

Data presented in this thesis tried to investigated astrocytes functions and modes for signalling from astrocyte processes in different cerebral regions of murine brain (striatum and cortex) and in different contexts.

1. Laser stimulation on cortical astrocytic processes and nerve terminals

Stimulation with low intensity laser is a reality in odontoiatry and its therapeutic use against CNS pathology is currently under investigation. However, to our knowledge, no data about effects of laser light on neurotransmitter or gliotransmitter release in CNS were available.

Photobiomodulation, also known as Low Laser Therapy (LLT), is the application of low intensity light for therapeutic purposes. Interest on photobiomodulation relies on the promise of a drug-free therapeutic approach employing light, which might be used not only to treat pain, promote reduction of inflammation and speed up wound healing, but also to promote tumours and cancer cell inhibition, and regeneration and restoration of various organism functions.

Recently, the transcranial application of irradiation in 600–1070 nm wavelength range became increasingly considered as a new neuroprotective approach to treat neurodegenerative diseases (Golovynska, 2020).

However, application of such therapy on human organisms requires a deep knowledge of the effects of light action.

Our results indicate that photons can evoke glutamate release from mice cortex and, thus, may be involved in the astrocytes-neurons network interactions.

Furthermore, laser application on astrocytic processes and nerve terminals provided the evidence that photon action is effective only on the latter. Notably, laser-evoked glutamate release was dependent on Ca^{2+} availability in the medium, strongly suggesting that the mechanism underlying the release is vesicular exocytotic mediated by vesicles. Vesicles involvement was confirmed by the use of the V-GLUT1-inhibitor Rose Bengal, which

dramatically decreased the glutamate release after photon stimulation.

TTX effectiveness on synaptosomes stimulated with laser demonstrated that photons provoked membrane depolarization with Na^+ channel involvement in nerve terminal.

Mitochondria and ATP production are widely recognized targets for PBM, and presynaptic mitochondria and their capacity for ATP production are known to be related to the efficiency of synaptic vesicle exocytosis. Ultrastructural analysis showed that the nerve terminals are equipped with presynaptic mitochondria, while astrocytic processes are not. Although activation of mitochondrial ATP production might be related to the glutamate-releasing effect of photons, further investigation is required for a better understanding of the ability of photons to activate vesicular glutamate release from the nerve terminals, and its inability to evoke glutamate release from the astrocyte processes. Indeed, we cannot exclude the participation of other mechanisms; for example, NO production - which is reported to be increased by laser light - could also function as a signal to activate vesicular glutamate release from nerve terminals and slices.

It is to be noted that in our experiments the laser light was administered directly on nervous tissues, while in clinical conditions laser light should pass through the skull of the subject, which is not represented in our experimental model. Although these limitations, we believe that these finding can be of interest for the application of low laser intensity on nervous system. Nevertheless, it is to be considered that glutamate is a well-known excitatory neurotransmitter involved in nociceptive signalling in CNS (Pires de Sousa 2016).

Future directions

Future studies are required to deepen the topic and to understand the mechanisms involved in the low intensity laser ability to enhance glutamate release not from astrocytic processes but only from the nerve terminals.

Preliminary data (data not shown here) suggest that photobiomodulation can evoke not only glutamate but also GABA release. Nerve terminals treated with the VGLUT inhibitor rose bengal and then stimulated with laser light, could release GABA. It will be of interest to compare the effect of photons with the effect of different stimuli, such as depolarization or receptor activation, on GABA release. Aa a matter of fact, future experiments will be necessary to investigate this phenomenon as well as the effects of photobiomodulation on the release of different transmitters.

2. Modulation of glutamate release by receptor-receptor interactions in rat striatal astrocytic processes

Receptor oligomers have been thoroughly studied since the 80s, when their existence was demonstrated in neurons. More recently, they were studied in astrocytic cells and the presence of heteromers between A2A and D2 receptors in astrocytic processes was demonstrated by prof. Marcoli's group in previous studies.

Those findings may be interesting for future therapeutic strategies which target the D2 receptor: according to our data, indeed, dopaminergic transmission can be modulated by allosteric interaction with A2A receptor, also at the level of the astrocytic plasma membrane. Therefore, adenosinergic signalling could become relevant in that therapies which target D2 receptor, as anti-Parkinsonian or anti-schizophrenic therapies.

Indeed, our findings indicate that activation of the A2A receptors for adenosine may play a crucial role in the regulation of dopaminergic control of the striatal glutamatergic transmission through astrocytic receptor-receptor interaction. Evidence for A2A-D2 heterodimerization may reveal new possible pathogenic mechanisms, therefore new possible targets for pharmacological intervention in pathological conditions involving the striatal D2 (and A2A) receptors such as schizophrenia as well as the Parkinson's disease.

In fact, high D2 receptor activation at the level of striatum or frontal cortex, and reduced extracellular adenosine level, have been related to schizophrenia (see Lara and Souza, 2000). It is interesting to note that the ecto-nucleotidases involved in adenosine production from ATP and located near A2A receptors seems abundant in striatal gliosomes (Augusto et al., 2013), consistent with important roles of astrocytic A2A receptors in the adenosinergic control of striatal glutamate transmission. Furthermore, a dysfunction of the glutamatergic transmission is increasingly recognized to be involved in schizophrenia, also at the striatal level (Simpson et al., 2010). Evidences for the ability of adenosinergic A2A receptor to impair the D2 receptor control of the striatal glutamatergic transmission therefore might bring together three major neurochemical actors in schizophrenia, namely the dopaminergic, adenosinergic, and glutamatergic transmission.

As far as the Parkinson's disease is concerned, we must consider that the D2 receptors were found to be modulated not only via A2A receptors, but also by intracellular homocysteine. Notably, increased plasma homocysteine levels have been reported in

patients affected by Parkinson's disease, mainly during L-dopa treatment and suggested to play a role in dyskinesias during L-dopa treatment (see discussion and references in Cervetto et al., 2018); L-dopa has been demonstrated to cause production of homocysteine in astrocytes (Huang et al., 2005). It could therefore be hypothesized that reduction of homocysteine production (e.g., by COMT inhibitors) together with A2A receptor antagonists may play synergistic roles in the therapy of PD against the loss of L-dopa effectiveness and in the late side effects of L-dopa treatment in Parkinson's disease. Furthermore, we have to remember that activation of astrocytic D2 receptor was reported to suppress neuro-inflammation (Shao et al. 2013), while reduced signaling of the astrocytic D2 receptors increased the vulnerability of striatal dopaminergic neurons to toxic damage. Indeed, changes of striatal astrocytes, and altered interactions between astrocytes and neurons at striatal glutamatergic synapses with altered control of glutamatergic transmission have been implicated in the pathophysiology of the Parkinson's disease (see Cervetto et al, 2018 and references therein).

Alltogether these data provide new insights into the involvement of molecular circuits at the level of the astrocyte plasma membrane, based on formation of A2A-D2 heteromers, in the dopaminergic control of the striatal glutamatergic transmission. These findings on the regulation of glutamatergic transmission in striatal astrocyte-neuron communications may pave the way to investigations on possible pathogenic mechanisms and to the development of new pharmacological approaches to pathological conditions involving striatal glutamatergic transmission dysregulation.

Moreover, we demonstrated that the oxytocin receptor is colocalized with A2A and D2 receptors in astrocytic processes.

Data here presented suggest the existence of functional interactions between the OXT and A2A receptors in striatal astrocytic processes, while we did not provide evidence for an interaction between OXT and D2, even though D2-OXT heterodimers expression has been previously described in literature. However, confocal imaging showed that OXT, D2, and A2A receptors are co-expressed on the same astrocytic process: it is conceivable that OXT receptors can form oligomeric structures with the A2A and, maybe, D2 receptor. Further experiments via PLA or co-immunoprecipitation will provide new information on biochemical or physical interactions between these receptors.

As previously mentioned, oxytocin is an important regulator of sexual and social behaviour of individuals and its use in therapies against schizophrenia and autism was suggested

(Yamasue 2018; Shilling 2016). Interestingly, elevated levels of glutamate were found in basal ganglia of patients affected by schizophrenia (Uno, 2019).

Thus, the fact that oxytocin signal transmission might be affected by allosteric interaction with A2A receptors should be kept in consideration for the development of therapies targeting oxytocin receptors.

Interestingly, elevated levels of glutamate were found in basal ganglia of patients affected by schizophrenia (Uno, 2019).

According to our data, the pathways leading to glutamate release in astrocytic processes are controlled both by the dopamine and Oxytocin receptors, with functional interactions with the adenosine A2A receptor.

If the expression of the A2A-D2-OXT receptor heteromers in astrocityc processes will be confirmed in the future, a new perspective for the treatment and the development of new drug for diseases or psychiatric disorders would be opened.

Future directions

The topic of receptor-receptor interaction at the level of the plasma membrane of astrocytes is only at the beginning and requires further work for a better understanding of the modes for the interaction between or among different GPCRs, the functional effects of the interactions, and the regional selectivity. In any case, these findings open a new field of investigation, on possible roles for astrocytic receptor-receptor interaction in the astrocyte signalling and in the control of the glutamatergic transmission. Furthermore, evidence for receptor-receptor interaction highlights the roles that astrocytes may play in the decoding of signalling in central nervous system. Altogether, the findings may contribute to the change from a neurocentric to a neuro-astrocentric view of the brain function and of neuropsychiatric disorders.

3. Exosomes release from astrocytic processes

Exosomes extrusion from astrocytic processes can be intended as a volume transmission mode of communication (Agnati and Fuxe, 2000; Vizi, 2000), with a non-classical typology of release.

Exosomes are known to play an important role in communication between CNS cells, carrying molecules (e.g: proteins or mi-RNA) and messages which can have both protective or enhance harmful processes.

Thus, these new findings are relevant in defining the role of astrocytes in CNS not only as cells involved in supporting neurons functions but also able to participate in CNS communication in multiple ways, through the release of glutamate (classical mode of transmission) or exosomes (non-classical mode of volume transmission, the roamer-type volume transmission).

In particular, in the context of the putative roles that exosomes might play under physiological or pathological conditions in the CNS, the results here reported appear relevant since:

- they demonstrate that astrocytic processes can release exosomes. Signal transmission in gliosomes can occur through either vesicular release of gliotransmitters or roamer-type pattern as in case of exosomes. These evidences contribute to elucidate the multiple roles that astrocytic processes may play in the control of signal transmission in CNS.
- shed a new light on exosomes origin. The fact that these nanovesicles express astrocytic markers allows the identification of the cellular origin of exosomes which can be collected from different biological fluids. The involvement of a precise cell population in a physiological or pathological process could thus be established, helping to define the potential role of peripheral exosomes in the pathogenesis, diagnosis, and monitoring of various CNS pathologies.
- According to our data, exosomes carry the protein NGB. Although NGB was thought to be expressed only in neurons, its expression in astrocytes was observed. The hypothesis advanced is that NGB may play a role in neuroprotection for neurons. In that case, exosomes release from astrocytes might have a protective role transferring the NGB protein to neurons through the release of vesicles.

To further investigate this aspect, we added labelled exosomes to astrocyte-neurons co-

culture to figure out whether astrocyte-derived nanovesicles can be up-taken by neuronal cells, our confocal imaging showed that exosomes are actually able to target and enter in neuronal cell.

Future directions

In conclusion, we can state that astrocytic processes can release NGB^+ exosomes which selectively target neurons.

Future studies will establish whether NGB - or any other signal is contained in exosomes - is transferred from astrocytic-derived exosomes to neurons and if, in case, neuronal cells will receive beneficial (or detrimental) effects from the presence of that molecule(s).

ERASMSUS+ PERIOD-BRIEF ACTIVITY DESCRIPTION

During my PhD, I spent six months (from the 1st of July to the 31st of December 2019) in Düsseldorf (Germany) in prof. Ellen Fritsche's lab at IUF-Leibniz Research Institute for Environmental Medicine.

The aim of the period was acquiring the experience in managing induced pluripotent stem cells (iPSCs), using different protocols aimed to obtain adult neurons and astrocyte from progenitors cells.

Results were presented in the following abstract, presented at the 11th 11th World Congress on Alternatives and Animal Use in the Life Sciences (WC11):

*Hartmann J, **Pelassa S**, Pahl M, Klose J, Tigges J and Fritsche E.*

2D Neural Inductions of Human Induced Pluripotent Stem Cells (hiPSCs) followed by 3D Differentiation as Alternative Method for (Developmental) Neurotoxicity Testing

Here, I briefly describe the role of iPS cells in scientific research and the preliminary results obtained during my time in Düsseldorf.

Human induced pluripotent stem cells (h-IPSCs) were generated for the first time in 2006 by the Japanese scientist Shin'ya Yamanaka, who was awarded with the Nobel Prize in 2012 (shared with Jonh B. Gurdon).

h-IPSCs represent a milestone in scientific research, since differentiated cells can be converted into pluripotent stem cells which can be pushed to differentiate towards potentially all phenotypes, opening wide perspective on their use in research and clinical therapies.

Yamanaka obtained ES-like cells through retrovirally introduction of four transcription factors in mice fibroblasts: Oct3/4, Sox2, Klf4 and c-Myc (Takahashi et al., 2006).

However, results were unsatisfying: ES marker were lower expressed in h-IPSCs than in ES and h-iPS were not able to generate adult chimera.

Other groups obtained IPS cells with a ES-like DNA methylation pattern, even though highly subjected to tumour development due to transgene c-Myc reactivation

This issue was solved by Nakagawa and Koyanagi who managed to generate iPSC excluding c-Myc from the procedure and, thus, reducing the risk of cancer in adult chimeras (Aoi et al.,2016).

h-IPSCs can be differentiated in vitro into different fates and thus represent a major tool for biomedical research and personalized medicine, since cells can be obtained from patients.

Nowadays, iPSCs are currently used for modelling neurodegenerative diseases (Parkinson's and Alzheimer's disease), and psychiatric disorders as schizophrenia (Wu,2018) or personalized medicine (Li et al.,2016).

H-IPSC, indeed, enable scientists to obtain human cells not derived from embryos, thus avoiding the bioethical concerns connected with those practices.

Although animal models played a fundamental role for the development of scientific knowledge, nowadays the role of induced pluripotent stem cells is being increasingly recognized.

Our laboratory has been working on animal models (in particular mice and rats) for a long time, as witnessed by publications in peer-reviewed journals (Cervetto et al., 2017; Venturini et al, 2019; Pelassa et al., 2019 and references therein). However, we believe that h-IPSC could provide a new useful model both under the scientific and the ethical profile. For this reason, through the Erasmus+ program, I had the opportunity to spend six-months at IUF.

From July to December 2019, then, I participated to prof. Fritsche's lab activity.

In particular, the aim of my research was understanding the efficacy of the Monolayer Culture Neural Induction Protocol from StemCell Technologies in inducing IPS differentiation towards the astrocytic and neuronal phenotype.

The parameters used to asses h-ipsc differentiation in astrocytes or neurons were:
- marker expression
- electrical activity

1. Marker expression

Mature phenotype can be observed 28 days after the induction of the protocols. However, RT-PCR analysis showed that the 99% and 95% of cells resulted positive, respectively, for PAX-6 and Nestin, (neuroprogenitors makers), 7 days after neural induction.

The expression of pluripotency marker Oct 3/4 was measured only in the 2,74% of cells. Even though PAX6 expression decreases during the time, Nestin and OCT3/4 expressions

remained constant, suggesting that IPS cells were effectively differentiated in Neural Progenitor Cells (NPCs).

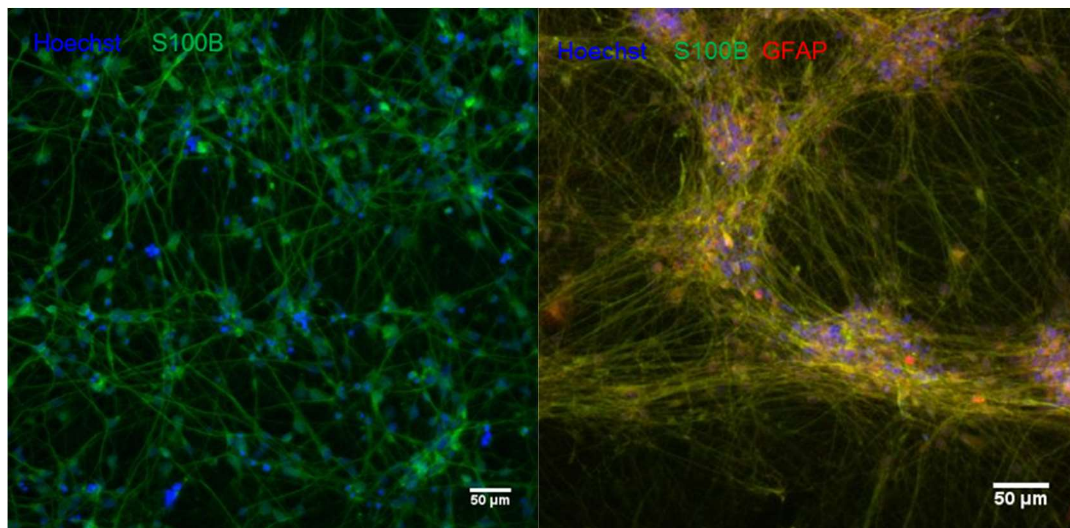

Figure.15 Representative immunohistochemical imagines on IPSC-derived and astrocytes Cells were positive for the astrocytic markers S100β (green, left image) and GFAP.

Mature neurons marker expression was evaluated after culturing NPCs in a differentiation medium for 28 days. Neuronal marker MAP2 and BIII or astrocytic marker S100β and GFAP were used to understand whether NPCs obtained from the Monolayer protocol differentiated in mature neurons and astrocytes.As shown by the images, it seems that the Monolayer Protocol affectively enhance iPSCs differentiation in mature neurons and astrocytes.

2. Electrical activity of Monolayer protocol-derived cells

Since neurons in physiologic conditions are able to set up electrically active network, in vitro cells aimed to mimic neuronal cells are supposed to do the same.

According to the preliminary result obtained, it seems however that neurons differentiated from the monolayer protocol do not show any electrical activity. However, this is a very preliminary results and further investigation is requested for a better understanding of the differentiation procedures allowing cells to acquire a fully-developed mature neuronal phenotype.

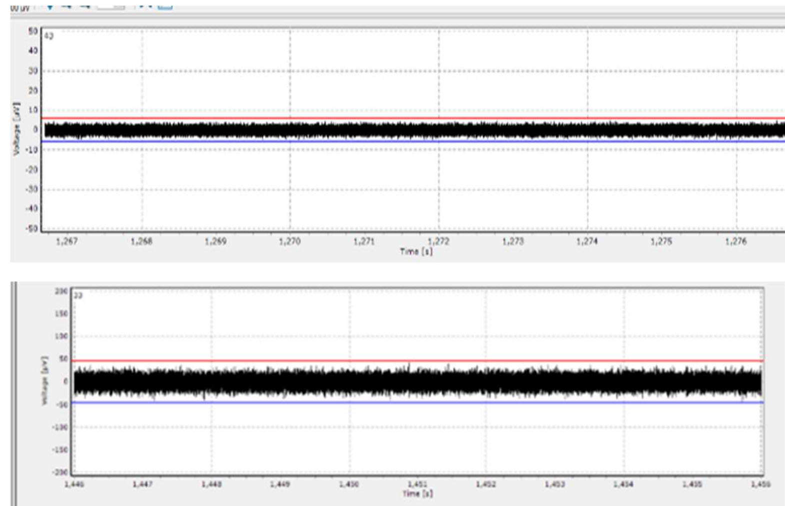

Fig.16 Microelectrode Array alysis on mature cells differentiated from iPCs following the NIM protocol

The image is only a representative screenshot. Apparently, NIM-derived cell are not able to develop electrical activity.

3. Conclusions

Although prof. Marcoli's group has a long and well documented background with animal models, our interest is also addressed to the *in vitro* cutting-edge techniques which can play a key role in modelling human physiologic or pathological conditions.

Therefore, the most important aim of my Erasmus period was acquiring the technical skills necessary for iPSC culturing.

The results here presented, even though partial and preliminary, show that the goal was achieved.

Handling iPSCs culture open new future perspectives to the future activities of our group. Interesting collaborations and projects involving the use of induced pluripotent stem cells, indeed, are already ongoing in our laboratory.

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APPENIDX

List of Supplemental Materials

1. ***A2A-D2 Heteromers on Striatal Astrocytes: Biochemical and Biophysical Evidence.***

   S. Pelassa, D. Guidolin, A. Venturini, M. Averna, G. Frumento, L. Campanini, R. Bernardi, P. Cortelli, GC. Buonaura, G. Maura, L.F. Agnati, C. Cervetto, M. Marcoli.

   Int J Mol Sci. 2019 May 17;20(10):2457. doi: 10.3390/ijms20102457

2. **Exosomes From Astrocyte Processes: Signaling to Neurons**

   Venturini A, Passalacqua M, Pelassa S, Pastorino F, Tedesco M, Cortese K, Gagliani MC, Leo G, Maura G, Guidolin D, Agnati LF, Marcoli M, Cervetto C.

   Front Pharmacol. 2019 Dec 2;10:1452. doi: 10.3389/fphar.2019.01452.

LIST OF PUBLICATIONS, PROCEEDINGS, AND ABSTRACTS

Here is reported a list of full-length papers and proceedings published, and abstracts presented at scientific meetings during my PhD:

Publications

Cervetto C, Venturini A, Guidolin D, Maura G, Passalacqua M, Tacchetti C, Cortelli P, Genedani S, Candiani S, Ramoino P, **Pelassa S**, Marcoli M, Agnati L.F. (2018)
Homocysteine and A2A-D2 receptor-receptor interaction at striatal astrocyte processes
Journal of Molecular Neuroscience 65(4), 456-466 https://doi.org/10.1007/s12031-018-1120-4

S Pelassa, D Guidolin, A Venturini, M Averna, G Frumento, L Campanini, R Bernardi, P Cortelli, G Calandra Buonaura, G Maura, L F Agnati, C Cervetto , M Marcoli (2019)
A2A-D2 heteromers on striatal astrocytes: biochemical and biophysical evidence.
Int J Mol Sci 20, 2457

A Venturini, M Passalacqua, S **Pelassa**, F Pastorino, MT Tedesco, K Cortese, MC Gagliani, G Leo, G Maura, D Guidolin, LF. Agnati, M Marcoli, C Cervetto (2019)
Exosomes From Astrocyte Processes: Signaling to Neurons
Front Pharmacol 10:1452. doi: 10.3389/fphar.2019.01452

B Polini, C Cervetto, S Carpi, **S Pelassa**, F Gado, R Ferrisi, S Bertini, P Nieri, M Marcoli, C Manera (2020)
Positive allosteric modulation of CB1 and CB2 cannabinoid receptors enhances the neuroprotective activity of dual CB1R/CB2R orthosteric agonist.
Life 10, 333; doi:10.3390/life10120333

Proceedings

C. Cervetto, A. Venturini, **S. Pelassa,** L. Campanini, M. Averna, D. Guidolin, G. Maura, L. F. Agnati, M. Marcoli (2019)
Biochemical and biophysical evidence for the A2A-D2 heteroreceptors in striatal astrocytes.
Glia 67 (S1), E707-E708

Marcoli M., Cervetto C., **Pelassa S**., Tedesco M., Maura G., Martinoia S. (2019).
In vitro neurotoxicity testing: Functional neuron-specific endpoints.
ALTEX 8(1), 130

Pelassa S., Martines A, Guidolin D, Maura G, Agnati LF, Marcoli M.
A2A-D2 heterodimers on striatal astrocytes: biochemical and biophysical evidence
BraYn, Genova, Italy, 29-30 June, 2018

Pelassa S, Guidolin D, Maura G, Campanini L, Agnati L.F., Marcoli M., Cervetto C
Striatal astrocytes: biochemical and biophysical evidence of A2A-D2 heterodimerization
More than Neurons, Torino, Italy, 29 November-1 December, 2018

Hartmann J., **Pelassa S**, Pahl M, Klose J., Tigges J and Fritsche E
2D Neural Inductions of Human Induced Pluripotent Stem Cells (hiPSCs) followed by 3D Differentiation as Alternative Method for (Developmental) Neurotoxicity Testing
11th World congress on Alternatives and Animal Use in the Life Sciences, Maastricht, the Netherlands, postponed to August 22-26, 2021

Marcoli M, **Pelassa S**, Amaroli A, Maura G, Benedicenti S, Cervetto C.
Photobiomodulation and release of the neurotransmitter glutamate from the nerve terminals
40° Congresso Nazionale della Società Italiana di Farmacologia SIF. digital edition 9-13 March, 2021

Cervetto C, **Pelassa S**, Venturini A, Passalacqua M, Pastorino F, Tedesco MT, Cortese K, Maura G, Guidolin D, Agnati LF, Marcoli M
Exosomes: a signal from astrocyte processes to neurons
40° Congresso Nazionale della Società Italiana di Farmacologia SIF. digital edition 9-13 March, 2021