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"Studying Glutamatergic Gliotransmission as a Functional Model to Assess Physio-Pathological Conditions and Receptor Cross-Talk"

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SUMMARY

This thesis is divided into two parts; in the first part, we characterized the effect of acute stress on the astrocytic glutamate release in the prefrontal cortex of rat brain. In the second part, we evaluate the interaction of the Oxytocin receptor (OXTR) with both Adenosine receptor A2A and Dopamine D2 receptors in the brain striatum of male rats.

First Part:

Stress is known to disturb the physiological homeostasis of the body (McEwen et al., 2000). The number of studies demonstrated that stress leads to damage the prefrontal cortex the brain and results in the modulation in the secretion of various neurotransmitters (Karats Oreos and McEwen, 2011, Sousa and Almeida, 2012). Stress was recognized as a predominant risk factor for many diseases, together with cardiovascular, metabolic, and neuropsychiatric diseases. Among the latter, stress interacts with the variable genetic background of vulnerability in the pathogenesis of mood anxiety disorder (Laura et al., 2010). It may be acute or chronic, can involve neuropsychiatric components such as stress, depression, mood, and anxiety (Laura et al., 207) and produces many behavioral and neurochemical changes, as determined in human (De Kloet et al., 2005, Kim & Diamond 2002). Studies in the literature truly indicated that acute stress have an effect on glutamatergic neurotransmission in the prefrontal cortex, inducing changes in glutamate release, receptor and glutamate clearance and metabolism (Popoli et al., 2012: Licznerski and Duman 3013). The effects of acute footshock stress on glutamate release and transmission were still unknown. In this study, we investigated the release of glutamate from astrocytes. The analyses have been performed in the prefrontal cortex (PFC) at different time intervals straight away after 40 min of stress and 6 and 24 hours after stress start, to monitor the early and delayed effects of acute stress on glutamate release. After the acute stress, animals were subjected to sucrose test to distinguish vulnerable and resilient rats.

Second Part:

There has been a growing interest in the investigation of the role of astrocytes in neurodegenerative and neuropsychiatric diseases and their complex neuron-astrocytes network function. The receptor–receptor interactions (RRI) can have an important function in the signalling transduction pathways. A previous well established study evaluate the

interactions between the G-protein coupled receptors of adenosine A2A receptors (A2A) and dopamine D2 receptors (D2) in several experimental models (Ferré et al., 2008), while it's barely investigated in astrocytes. Growing evidence shows that adult striatal astrocytes largely express both D2 and A2A receptors (Cervetto et at., 2017). Moreover, the presence of A2A-D2 heteroreceptor complexes has led to a new perspective of molecular mechanisms involved in Parkinson's disease (PD), providing novel drug targets. Therefore in the present study, we investigate the physical and functional interactions of A2A and D2 with another G protein-coupled receptors i.e. OXTR in astrocytes processes from adult rat striatum. We also evaluate the effect of this interaction on the astrocytic glutamate release in rat striatum.

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List of Abbreviation

GFAR	Glial fibrillary acidic protein
K	Potassium ion
GABA	Gama amino butyric acid
TNFα	Tumor necrosis factor alpha
LTP	Long time period
AMPARS	α-amino-3-hydroxy-five-methyl-four-isoxazolepropionic acid receptors
NMDAR	N-methyl-d-aspartate receptors
MEPSC	Miniature excitatory postsynaptic receptors
MIPSC	Miniature inhibitory postsynaptic receptors
EAATS	Excitatory amino acid transporters
MGLURS	Metabotropic glutamate receptors
IP3	Inositol triphosphate
ER	Endoplasmic reticulum
GPCR	G-protein coupled receptors
KO	Knockout
HD	Huntibgton disease
ATP	Adenosin triphosphate
cAMP	Cyclic adenosine monophosphate
AQP4	Aquaporin4
EPSPs	Excitatory postsynaptic potentials
GLAST	Glutamate/aspartate transporter
NCX	Sodium-calcium exchange
DHK	Dihydrokainic acid
DL-TBOA	DL-threo-beta-benzyloxyaspartate
NTFs	Neurofibrillary tangles
SN	Substantia nigra
SOD1	Superoxide dismutase 1
Aps	Amyloid plaques

IPSCi	Inhibitory postsynaptic contemporary
AD	Alzheimer disease
PD	Parkinson disease
MS	Multiple sclerosis
ALS	Amyotrophic lateral sclerosis
MPTP	1-methyl-4phenyl-1,2,3,6-tetrahydropyridine
SPT	Sucrose preference test
FS	Foot shock
PAPs	Presynaptic astrocytic processes
RRI	Receptor-receptor interaction
AT1	Angiotensin type 1
CB1	Cannabinoid 1
LH	Luteinizing hormone
FSH	Follicle stimulating hormone
GHSR	Growth hormone secetagogue receptor
MPTP	1-methy-4phenyl-1, 2, 3, 6-tetrahydro-pyridine
VGLUT1	Vesicular glutamate transporter type 1
NTS1	Neurotensin receptor 1
OXTR	Oxytocin receptor
ER	Endoplasmic reticulum
NKCC	Na-K-Cl co-transport
4-AP	4-Aminopyridene
SR	Sulforhodamine
Aps	Amyloid plaques
NFTs	Neurofibrillary tangles
TM	Transmembrane
ECL	Extracellular loops
ICL	Intracellular loops
CHO	Chinese hamster ovary

RES	Resilient
VUL	Vulnerable
PTSD	Post traumatic stress disorder
PAP	Perisynaptic astrocytic process
EAE	Experimental autoimmune encephalomyelitis

FIRST PART

1. PHYSIOLOGICAL ROLE OF ASTROCYTES

1.1 Astrocyte

Astrocytes are a very important cellular type in the brain system and play basic roles in ion homeostasis, metabolism, neurotransmitter clearance, synapse formation/elimination, neurovascular coupling, and modulating neurotransmission. Astrocytes are involved in the metabolic process as they respond, for that reason to the endeavor-based desires of neurons via the astrocyte-neuron lactate ride (Pellerin et al., 2007b). Lactate is one of the oxidative substrate that's run by means of neurons. while, when neuronal activation occurs, glutamate will increase lactate intake by means of the way of neurons. furthermore, glutamate will increase glycolysis in astrocytes to deliver neurons with lactate so one can preserve neurotransmission (Pellerin et al., 1994a). Astrocytes also control the modulation of cerebral blood flow (Gordon et al., 2008b). Blood vessels are covered by astrocyte present ft, ultimately, which modifies the vasoconstriction and vasodilation of arterioles. Calcium signaling is coupled in astrocytes to alter neural activities, which bring changes in blood motion. As usual, oxygen availability is normally low in the (close to the hypoxic threshold), astrocyte calcium will boom cause astrocyte lactate release and as a result, vasodilation occurs (Gordon et al., 2005a). Astrocytes are responsible for swiftly opening the extracellular space, have particular importance to stop excitotoxicity in some unspecified neurotransmission. (Anderson and Swanson, 2000). similarly, glutamate is also released by way of astrocytes as a gliotransmitter to contribute to the regulation of neural undertakings at the synapse (Anderson and Swanson, 2000). despite that fact, that potassium re-uptake through neurons is just too slow to stop potassium accumulation. while the accumulation of extracellular potassium, which can affect appropriate transmission and channel kinetics. Astrocytes can act by one of these 3 mechanisms; operated potassium-chloride uptake, channel activated potassium chloride uptake, and that they act as an interrelated (hole junctions) spatial buffer that efficaciously confiscates extra potassium away from neurons (Walz, 2000). Astrocytes are ideal to form a tripartite synapse with pre- and post-synaptic neurons. They envelop the synapse and can respond to endeavor at excitatory (glutamate) and inhibitory (GABA) synapses as possess cholinergic, adrenergic, and peptidergic receptors (Hosli and Hosli, 2000). Upon substrate-receptor activation, the changes in astrocyte occur by way of calcium propagation from astrocyte to another astrocyte in a syncytium, which can

generally affect special characteristics. This results in the release of gliotransmitters, together with ATP, D-serine, and/or glutamate (Bezzi and Volterra, 2001). consequently, astrocytes may modulate neuronal venture at many synapses.

1.2 Primary astrocyte morphology

in the late 19th century, astrocytes have been divided into two critical subtypes, one protoplasmic, while other fibrous, the type is based at the variations of their cellular morphologies and anatomical structure (Ramon Y, Cajal S 1909). Protoplasmic astrocytes are found throughout all grey depend and, as first presented the usage of classical silver impregnation approaches, show off a morphology of quite a few stem branches that supply upward push to many finely branching systems in a uniform globoid distribution. while the position of fibrous astrocytes is an unspecified place of all white matter and much-prolonged fibre structure (Ramon Y, Cajal S 1909). Classical and modern neuroanatomical research also additionally suggests that each astrocyte subtypes make a significant interaction with blood vessels. late mid-twentieth century with help of Electron microscopic analyses discovered that the system of protoplasmic astrocytes envelops synapses and that the system of fibrous astrocytes contact nodes of Ranvier, and that every type of astrocytes structure hole junctions between distal side of adjacent astrocytes (Peters A, et al., 1991).

1.3 Astrocyte Physiology

In 1895 Michael lenhossek (1863-1937) described cells with many, enormous big name-fashioned projections "astrocytes" (Lenhosséok, 1895). Contributors of the glial personal family, astrocytes are discovered for the duration of the times and make the high-quality system into synapses, wrap neuronal somata and ship large cellular compartments on top of the blood vessels. Those latter projections are termed end ft and that they enwrap the entire form of the brain's vasculature (Pasti et al., 1997b). Astrocytes are existing in two different types, usually distinguished using their area within the brain. Fibrillary astrocytes, which are in the white, depend and protoplasmic astrocytes, which inhabit the gray, depend (Lee et al., 2014), Protoplasmic astrocytes, with an assessment to be the focus of this, inhabit weird, spherical, non-overlapping areas and uniformly covered grey area (Nedergaard, 1994). At factors, where the quick projections of adjacent astrocytes are available to trace they're

coupled by means of using gap junctions, forming an enormous astrocyte network thru which indicators may be transmitted. An vital structural protein positioned in astrocytes is a glial fibrillary protein (GFAP) that is expressed in glial filaments and responsible for growing those cells' morphology (Eng et al., 1971; Eng, 1985; Fuchs and Cleveland, 1998). Astrocytic gfap, additionally gives a valuable target for staining to turn out to be aware of astrocytes over mature neurons (Kimelberg, 2004a; Lundgaard et al., 2013). One of the hallmark features of the astrocytes is their very own electrically passive nature, which means that they require a competitive cutting-edge injection that permits you to produce a measurable voltage reaction with a direct projection (Hassinger et al., 1996). This element leads astrocytes to be defined as “ohmic” cells (Sontheimer and Waxman, 1993). They work as smooth resistors because they are specific solely to few voltage-gated ion channels. Even although some researchers advise that astrocytes have a specific infinite voltage-gated ion channel, which consists of voltage-dependent l-kind calcium channels, it is miles usually be supposed that mature astrocytes are truely barren of these proteins. An important electrophysiological feature of astrocytes is their low membrane resistance due to the presence of large potassium outflow currents generated with their excessive expression of twik-1 and trek-1 (Zhou et al., 2009), which can be each and every pore-domain potassium (k2p) channels (Heurteaux et al., 2004). Due to their enough expression of those potassium channels, astrocytes keep a resting membrane manageable at approximately the reversal practicable for potassium: ninety mv (Sontheimer, 1992; Kimelberg et al., 2006b; Zhou et al., 2009).

1.4 Synaptic roles of astrocytes

The unique research of astrocytes with the aid of Virchow (1821-1902) and Golgi (1843-1926) assumed that, those cells as having solely a passive characteristic within the brain, offering structural and dietary escort for neurons and, without, filling the place between them (Schatzmann, 1982). This observation indicates that astrocytes include, release and synthesize a range of neuroactive compounds. Astrocytes play very important regulations in the synaptic function; this phenomenon is analyzed by means of a model termed the “tripartite synapse”. In this system, astrocytes preserve the extracellular ion, more mainly k+ (Martin, 1992), and transform synaptic activity thru the reuptake of glutamate from excitatory synapses (Kofuji et al., 2000). The energy of synapses thru astrocytes may additionally now not be particularly unforeseen because they provide prolong projections to the most synapses

(Perea et al., 2009a). This makes them rather located to come across, reply to, and to transmit signals between synapses (Volterra et al., 1999). The common functionality by means of which astrocytes reply to synaptic activity consists of activation in their mGLUR by using way of glutamate launched from excitatory synapses (Colleges and Kimelberg, 1999). This is possibly due to the fact of end result in the discharge of calcium from astrocyte intracellular magnitude, through ip3 (Gordon et al., 2009c), inflicting them to gliotransmitters (Pasti et al., 1997a; Arizono et al., 2012a). Include the compounds of these categories are D-serine (Panatier et al., 2011a), ATP (Gordon et al., 2009c), tumor necrosis element α (TNFα) (Stellwagen and malenka, 2006), glutamate (Volterra et al., 1999), and gamma-aminobutyric acid (GABA) (Vicar et al., 2009). Even though it is not properly understood the mechanism of release of gliotransmitters from astrocytes, the vast calcium transients precipitated with the related by way of synaptic also can facilitate the vesicular release of these compounds through supplying the calcium vital for the binding and activation of snare complexes (Montana et al., 2006). Importantly, these gliotransmitters had been proven by modulating synaptic activity. Long-term potentiation (LTP), for instance, is the extended efficacy of a synapse following excessive frequency stimulation (Bliss and Lomo, 1973). This form of synaptic development is frequently experimentally produced within the hippocampus or neocortex the use of excessive frequency tetani. A gliotransmitter that performs a very critical position inside the enhancement of LTP is D-serine (Henneberger et al., 2010; Larson et al., 2014; Panatier et al., 2011a). D-serine binds to the position up-synaptic N-methyl-D-aspartate receptors (NMDARS), within the co-agonist binding, to facilitate their activation (Hamilton et al., 2010; Panatier et al., 2016b). This is very essential because the quantity of D-serine exocytosed from astrocytes can state-owned the threshold for LTP induction. The Sufficient amount of D-serine from astrocytes have to facilitate the induction of LTP, while on the other hand, low portions of D-serine from astrocytes on the different way may additionally cause the generation of the long-time period (LTP). Although astrocyte obtains glutamate has no longer been located to contribute to LTP, it has been established that NMDA receptor-mediated will increase in neuronal calcium. Mainly, through the production use of bradykinin and elevating inside calcium concentrations in astrocyte, those cells were stimulated to release glutamate and cause the elevations of calcium in neurons (Parpura et al., 1994a). While bradykinin was once applied to neurons co-cultured with the presence of astrocytes, no longer in neuron cultures besides them (Parpura et al., 2012b). Astrocytic release extra

glutamate to produce inward currents in neurons (Fellin et al., 2004). The glutamate, which are derived from astrocyte activated extrasynaptic neuronal NMDARS, and the release of glutamate affects multiple cells simultaneously, generating synchronized. In other varieties of lasting adjustments to synaptic efficacy, consisting of homeostatic plasticity and synaptic mounting, tnfα (Morgan and Teyler, 2001a; Stellwagen and Malenka, 2006) and ATP, respectively, expanded the insertion of synaptic α-amino-3-hydroxy-five-methyl-four-isoxazole propionic acid receptors (AMPARS) besides straight away affecting NMDA receptor activation. The conditions of synaptic mounting, the release of IP3 in astrocytes was once proven to make larger in synaptic electricity with the contribution of using inducing, the release of calcium from intracellular ensuing inside the release of ATP on top of adjacent neurons. This gliotransmitter was proposed to act on postsynaptic purinergic p2x7 receptors to cause an increase in the amplitude of miniature excitatory postsynaptic currents (MEPSC) in neurons with synapses inside the area of the affected astrocyte (Gordon et al., 2009c). Astrocytes are now not nearest able to affecting excitatory synapses, however, the effect of the potentiation of inhibitory synapses (Liu et al., 2006). As an example, direct stimulation of astrocytes or efficacy of baclofen, a GABAβ-receptor agonist, effects inside the potentiation of miniature inhibitory postsynaptic currents (MIPSCS), the effect which is blocked by means of inhibiting calcium signaling inner astrocytes use of the calcium chelator bapta (Kang et al., 1999), By means of keeping synaptic feature and influencing efficacy and potentiation, moreover astrocytes play very important characteristic in affecting neuronal concentration. Acriticalstatement concerning astrocytes' synaptic influence is that they can do so in a tonic way the usage of resting Ca2+concentration (Panatier et al., 2011a; Pascual et al., 2005). Mainly, astrocytes synaptic activity thru practical cubicles along with their processes, and their reaction includes activation of MGLUR5 to extend basal synaptic transmission. Inhibition of basal astrocyte intracellular calcium signaling with the calcium chelator bapta became sufficient to impair this enhancement of synaptic transmission (Panatier et al., 2011a). Moreover, it also becomes established that astrocytes manage the energy of basal synaptic transmission and as a result have been able to modulate the dynamic range of (LTP) (Pascual et al., 2005). Additionally, astrocyte also improves the activity-established heterosynaptic close by unstimulated synapses (Panatier et al., 2011a). Especially, ATP release thru astrocytes used to be hydrolyzed into adenosine inside the extracellular area, and its following binding

to adenosine A1 receptors on presynaptic neurons prevented the release of glutamate (Pascual et al., 2005).

1.5 Crucial astrocyte membrane proteins

The voltage-gated ion channels presence in astrocytes is unlikely, but those cells, which contain many unique important proteins membrane, which includes excitatory amino acid transporters (EAATS) (Hu et al., 2003). connexin hemichannels (Giaume and Mccarthy, 1996; Thompson and Macvicar, 2008), α-1 adrenoreceptors (Duffy and Macvicar, 1995; Gordon et al., 2005a), inward rectifying potassium channels (kirs) (Bezzi et al., 1998), Na+-K+ exchangers (Walz and Hertz, 1984), CIC chloride channels, which might be polytopic membrane proteins that bypass anions down their gradients (Sıokay et al., 2000; Makara et al., 2003), and metabotropic glutamate receptors (MGLURS) (Pasti et al., 1997; Bezzi et al., 1998). Astrocytic EAATS are chargeable for the abolition of glutamate from excitatory synapses, and this brings out by the practice of co-transport of three Na+ ions and 1 H+ ion down their concentration gradients into the cell along with every molecule of glutamate, at the same time as additionally anti-porting 1 K+ ion (Marcaggi and Attwell, 2004). Given that those ion ratios give up bring about the net influx of first-rate value into the cell, EAATS are electronically great and, in reaction to glutamatergic synaptic transmission, generate inward currents that may additionally be measured beneath the voltage clamp prerequisites (Sontheimer and Waxman, 1993). Connexins are substantial pore hexameric ion channels that, at elements, the vicinity astrocytic system are available in trace with one another, pair to form whole junctions, and allow the exchange of cytosol between cells. The foremost structure blocks of whole junctions in astrocytes are connexin 30 and 43, specifically (Thompson et al., 2006; Thompson and Macvicar, 2008). By means of forming Ca2+connections. Between astrocytes, connexins allow the motion of ions and small molecules, which include Ca2+ions and inositol triphosphate (IP3), and permit for rapid transduction between astrocytes. For instance, the astrocyte sequences activation inside the propagation of a wave of open intracellular Ca2+ that spreads distances of 100µm via the syncytium (Cornell-bell et al., 1990; Finkbeiner, 1992; Vicar et al., 2009). Astrocytes unique severa ion channels such as kirs, Na+-K+ and Na+-H+ exchangers, and CIC chloride channels. Those proteins are important for the regulating of intracellular concentrations of sodium, potassium, and chloride ions in astrocytes and their perturbation can cease to result in the impairment of astrocyte function. As an example,

affecting kirs prevents suited potassium switch in astrocytes and therefore prevents glutamate uptake via EAATS in astrocytes in vitro (Kucheryavykh et al., 2007). The capability of astrocytes to respond to close thru synaptic frequently due to the presence of mGLURS on these cells (Gordon et al., 2011d). These proteins are made up of seven transmembrane domains and are classified into group I (MGLURS1, 5), group ii (mGLURS2, 3), and group iii (mGLURS4, 6, 7, 8) (Paquet et al., 2013). In astrocytes, the important mGLURS that have been described are mGLURS1, 3, 5 (Sun et al., 2013; Schools and Kimelberg, 1999; Liu et al., 2013). It is very vital to notice that mGLURS5 might also additionally present within the neocortex until postnatal week three (Sun et al., 2013). The voltage-gated ion channels presence in astrocytes is unlikely, but those cells, which contain many unique important proteins membrane, which includes excitatory amino acid transporters (EAATS) (Hu et al., 2003), connexin hemichannels (Giaume and Mccarthy, 1996; Thompson and Macvicar, 2008), α-1 adrenoreceptors (Duffy and Macvicar, 1995; Gordon et al., 2005a), inward rectifying potassium channels (kirs) (Bezzi et al., 1998), Na+-K+ exchangers (Walz and Hertz, 1984), CIC chloride channels, which might be polytopic membrane proteins that bypass anions down their gradients (Sıokay et al., 2000; Makara et al., 2003), and metabotropic glutamate receptors (MGLURS) (Pasti et al., 1997; Bezzi et al., 1998). Astrocytic EAATS are chargeable for the abolition of glutamate from excitatory synapses, and this brings out by way of the practice of co-transport of three Na+ ions and 1 H+ ion down their concentration gradients into the cell along with each and every molecule of glutamate, at the same time as additionally anti-porting 1 K+ ion (Marcaggi and Attwell, 2004). Given that those ion ratios give up bring about the net influx of first-rate value into the cell, EAATS are electronically great and, in reaction to glutamatergic synaptic transmission, generate inward currents that may be measured beneath the voltage clamp prerequisites (Sontheimer and Waxman, 1993). Connexins are substantial pore hexameric ion channels that, at elements, the vicinity astrocytic system are available in trace with one another, pair to shape whole junctions, and allow the exchange of cytosol between cells. The foremost structure blocks of whole junctions in astrocytes are connexin 30 and 43, specifically (Thompson et al., 2006; Thompson and Macvicar, 2008). By means of forming Ca2+connections. Between astrocytes, connexins allow the motion of ions and small molecules, which include Ca2+ions and inositol triphosphate (IP3), and allow for rapid transduction between astrocytes. For instance, the astrocyte sequences activation inside the propagation of a wave of open intracellular Ca2+ that spreads

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1. 6 Astrocytic Ca2+ signals

Astrocytes have been established to demonstrate two different types of Ca2+ signals; intercellular Ca2+ waves and spontaneous Ca2+ oscillations in individual cells (Matyash and Kettenmann 2009). The initiating occurrence that leads to intracellular Ca2+ waves. In astrocytes shows up to, specifically obtain from the activation of metabotropic G-protein-coupled receptors, activation of phospholipase C, and the production of 2nd messenger inositol 1,4,5- trisphosphate (IP3). Which following IP3 receptor type II (IP3R2) activation leads to Ca2+ release from the endoplasmic reticulum (ER) (Scemes and Giaume 2006). The Ca2+ concentration is 1000 times greater than in the cytoplasm. The Ca2+ waves propagate either through functional gap junctions or by paracrine ATP-release (Verkhratsky, Rodríguez, and Parpura 2012), while the mechanisms at the back of the spontaneous Ca2+ oscillations are more enigmatic. Astrocytic Ca2+ alerts at some point of osmotic swelling have been established to be significantly reduced by way of means of Aqp4 omission, as proven thru (Thrane et al., 2011a), Astrocytic Ca2+ signals are also reduced by standard anesthesia (Thrane et al., 2012b; Ding et al., 2013). Intracellular Ca2+ is strongly regulated in all mammalian cell types, as a fast enlarges in this ion is, amongst other things, used to mediate (Parpura and Verkhratsky 2012a).

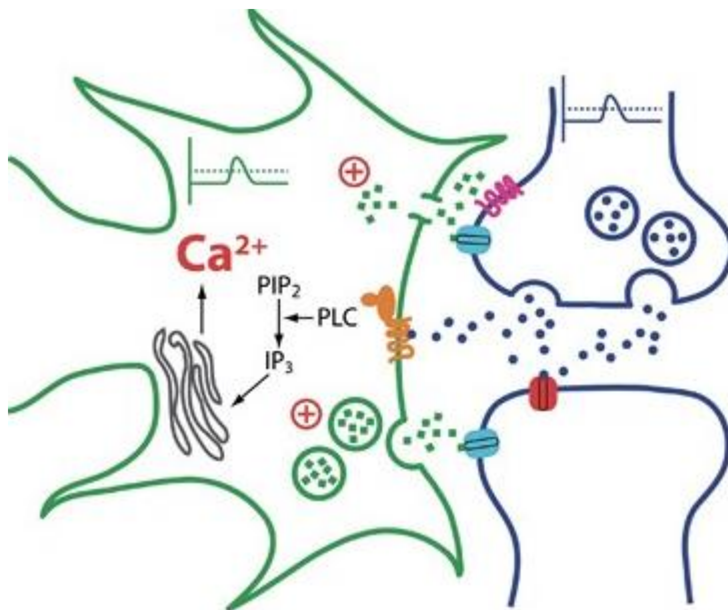

Fig 1 "synchronized Ca2+ transients (Ca2+ oscillations) thru mechanisms of chemical coupling". Until in recent times, Ca2+ transients in astrocytes had been supposed to be entirely dependent on neuronal activities, because astrocytes indicates a large range of receptors for neurotransmitters and surrounded almost all synapses at which neurotransmitters are fell over to stimulate astrocytes. In addition, however, astrocytes have been shown to release diffusible substances, the gliotransmitters, and Ca2+ transients in astrocytes are therefore caused through astrocytic activities, leading to transmission of Ca2+ transients or Ca2+ waves (the figure and legend are taken from Cornell-Bell, Finkbeiner and Smith 1990).

Despite passionate lookup on astrocytic Ca2+ alerts the last decades, the physiological significance of astrocyte Ca2+ signaling is yet poorly understood. Founding discoveries in early 1990 shows that, astrocytes specific glutamate-sensitive ion channels that reply to regionally utilized glutamate with producing a spreading wave of extended intracellular Ca2+ discovering that pointed to an until now an unrecognized role in signaling for these cells (Cornell-Bell, Finkbeiner and Smith 1990). Following these discoveries had been studies showing that cultured astrocytes (Cornell-Bell, Thomas and Caffrey 1992), as well as dye-loaded or genotypically cultured hippocampal slices (Dani, Chernjavsky and Smith 1992) and acute hippocampal slices (Porter and McCarthy 1996), replied to glutamate with elevations of cytoplasmic Ca2+. These propagating of Ca2+waves advised that systems of astrocytes may establish a signaling system within the brain, which talks through the release of Ca2+ion from intracellular and propagates as waves inside the cytoplasm astrocytes and between adjacent astrocytes (Cornell-Bell, Finkbeiner and Smith 1990).

Preliminary research has been carried out in culture preparations for the use of artificial Ca2+ dyes. Subsequently, Ca2+ imaging experiments have been carried out on acute brain slices. In slice experiments, the slices were generally organized the practice of tissue from pups, as slices from mice demonstrated to be hard to load. Recently it used to be shown that the expression of astrocytic receptor supposed to mediate the astrocytic Ca2+ signals, different in younger mice as adversarial to mice (Sun et al., 2013). Furthermore, bulk dye loading as a substitute insufficient method for imagining the astrocytic processes (Reeves, Shigetomi and Khakh 2011). Bulk loading commonly lets in the imagining of the cell soma and the thickest branches, leaving >90% of the astrocytic area unsampled. The complications of artificial Ca2+ dyes have been solved by way of means of the improvement of genetically encoded Ca2+ symptoms (Shigetomi et al., 2013a; Tong et al., 2013). Mice reduced in IP3R2, which is deepened in astrocytes and as soon as stimulated by way of IP3 mediates Ca2+ release from the ER, has been used to study the retribution of getting free astrocytic Ca2+ signaling (Zhang et al., 2014). However, a current study revealed that Ca2+ alerts in the astrocytic system are mostly well-maintained in IP3R2 knockout (KO) mice (Srinivasan R et al., 2015).

1.7 Buffering of K+

Maintaining ion homeostasis is imperative for proper nervous system function. Another way that astrocytes affect synapse characteristics is by the use of buffering of K+ ion. The one system is markedly sensitive to changes in extracellular K+ ion concentrations and consequently, be dependent on astrocytes to buffer K+ ion and strongly adjust extracellular K+ ion concentrations. Even though a small number of K+ ion efflux from neurons can affectedly modify extracellular K+ ion concentrations, which can affectedly change a neuron's resting membrane potential and effect on the activation of voltage-gated channels, electrogenic transport of neurotransmitters and synaptic transmission (Kofuji and Newman, 2004; Noori, 2011). Astrocytes have a specific kind of special transporters and channels that reduced the permeability of K+ ion and contribute to their buffering capabilities. For example, the astrocytic Na+-K+-ATPase pump as appropriately works like Na+-K+-Cl- co-transporter (NKCC) has been made known to mediate K+ion uptake and release the extracellular K+ ion (Wang et al., 2012). Astrocyte Kir 4.1 K+ channels is any other important molecular mediator of astrocytic K+ ion buffering. In assistance with gap junction channels, Kir4.1 K+ ion channels mediate the coordinated transport of K+ between astrocytes in the CNS in so-referred to as

spatial buffering (Butt and Kalsi, 2006; Zhang et al., 2014). In this model, some astrocytes take up K+ ion by way of Kir4.1 channels from areas of excessive K+ ion and then redistribute K+ ion to other astrocytes in the nervous system in areas of low K+ ion by way of the whole junction coupled astrocyte syncytium. Indeed, K+ ion is impaired, when astrocyte whole junction characteristic is disrupted (Wallraff et al., 2006). Disruption of astrocyte mediated K+ ion homeostasis can alter neuronal firing (Melom and Littleton, 2013; Sibille et al., 2014). In mice models of Huntington's disease (HD), reduced astrocyte Kir4.1 channel expression increased extracellular K+ ion points in the Genius striatum. The authors long established that similar elevations in wildtype mice increased neuronal excitability in the way that is similar to neuronal excitability regarded in HD mice. Importantly, viral transport of Kir4.1 channels to astrocytes in HD animals restored extracellular K+ ion levels, decrease neuron excitability, and also reduced motor neuron dysfunction (Tong et al., 2014). Likewise, (Thrane et al., 2012b) confirmed that compromised astrocyte K+ ion buffering might want to adjust the endeavor of neuronal channels and transporters to modify neuronal firing properties. These studies indicate that increased extracellular K+ ion ranges had been associated with over activation of the neuronal NKCC transporter, which led to the accumulation of intraneuronal Cl- ion, which led to neuronal depolarization that eventually resulted in lowered inhibitory indicators (Rangroo Thrane et al., 2013).

1.8 Aquaporin-four

In 1994, cloning and molecular characterization of a brain aquaporin (aquaporin-four; aqp4) used to be mention in independent studies (Hasegawa et al., 1994; Jung et al., 1994). Finally, the aqp4 protein was positioned to be significantly expressed at some degree within the neuropil, localized to glial and ependymal cells (Frigeri et al., 1995; Nielsen et al., 1997). Even though aqp4 is present in all astrocytic area, which includes the processes that envelope synapses, aqp4 has been decided to be particularly centered in astrocyte at the interface among skills tissue and the vital fluid booths of the CNS (Nagelhus et al., 19a). In reality, aqp4 expression is tenfold greater within the perivascular systems adjoin on top of blood vessels and going thru pia limitans externa, than the synaptic glial methods (Nagelhus et al., 2004b). Astrocytes are specially polarized cells both, functionally as well as anatomically. Subsequently, the aqp4 protein became once determined to be broadly expressed in the course of the neuropil, localized to glial and ependymal cells (Frigeri et al., 1995). Even though

aqp4 is current in all astrocytic system, which envelope the synapses, aqp4 has been positioned to be noticeably concentrated in astrocyte at the interface between brain tissue and the essential fluid cubicles of the CNS (Nielsen et al. 1997; Nagelhus et al., 1998). For this reason, astrocytes are alternatively polarized cells, functionally as properly as anatomically. The interplay between aqp4 and α-syntrophin became verified in a take a look at where in it become as soon as verified a sizable loss of aqp4 immunogold labeling in α-syntrophin ko mice (Neely et al., 2001), quantified to 88% in the perivascular membrane (Amiry-Moghaddam et al., 2004).

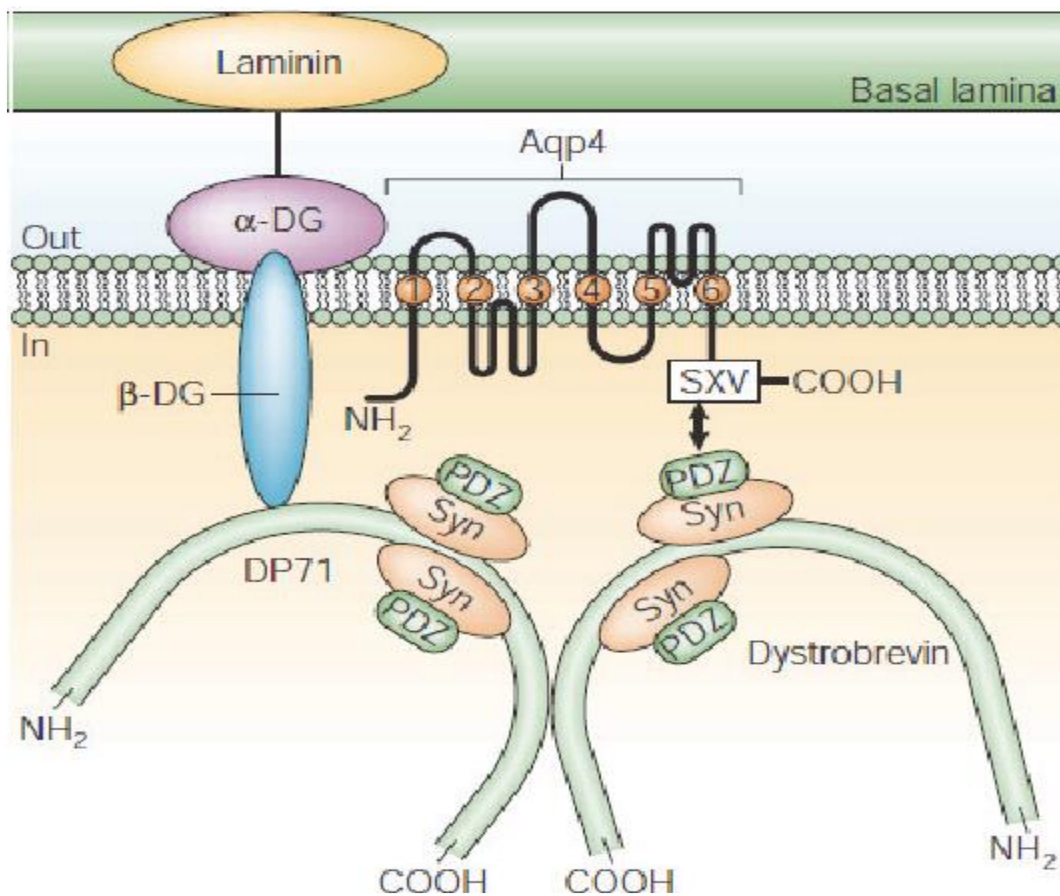

Fig 2." The DAPC binds the transmembrane protein β-dystroglycan (β-DG)", which connects to the Laminin / agrin-binding protein α-DG. On the cytoplasmic side of the complex, dystrophin binds to α- dystrobrevin. Each dystrophin and α-dystrobrevin molecule can bind up to two syntrophins. α-syntrophin is the main syntrophin expressed in astrocytic endfeet, and is concept to be responsible for the clustering of AQP4 in endfoot membranes (the picture and legends is taken from Amiry-Moghaddam and Ottersen 2003).

1.9 A1 and A2 astrocytes

These days, it has been proposed that neuroinflammation and ischemia triggered two important forms of reactive astrocytes, referred to as A1 and A2 reactive astrocytes respectively. A1 reactive astrocytes, induced with the resource of neuro-inflammation, secrete neurotoxins that prompt a lack of existence of neurons and oligodendrocytes; but A2 reactive astrocytes, brought about by way of the use of ischemia, promote neuronal survival and tissue restore. These two forms of reactive astrocytes can be recognized in line with their character's genetic expressions. Additionally, three (C3), cfb, and mx1s are the maximum characteristic and upregulated genes in A1 reactive astrocytes and are now not expressed in A2 reactive astrocytes; thus, those may be used as specific markers of A1 reactive astrocytes (Liddelow SA, et al., 2017). The s100 protein family members s100a10 has been recognized as unique markers of A2 reactive astrocytes (Fujita A, et al., 2018). Activated microglia results in the transformation of naïve astrocytes into A1 reactive astrocytes with the useful reserve release of il-1α, TNF, and c1q cytokines, each of that is essential for inducing A1reactive astrocyte. However, the milk fat globule epidermal boom aspect eight (MFG-E8) regulates A1/A2 reactive astrocytic conversion thru upregulation of the pi3k-Akt pathways and downregulation of the NT-KB pathways (Xu x, et al., 2018). A1 reactive astrocytes lose many normal structures characters, which include the neuronal survival and outgrowth; moreover, A1 reactive astrocytes result in fewer and weaker synapses than healthy naïve astrocytes (Liddelow SA, et al., 2017). A1 reactive astrocytes have also been identified in a selection of human neurodegenerative infections. For example, in Alzheimer’s disease, it has been supposed that almost 60% of GFAP-effective astrocytes within the prefrontal cortex (PF) are C3-fine. This suggests that A1 reactive astrocytes may additionally inspire the development of neurodegenerative diseases. A2 reactive astrocytes exert neuroprotective and re-establish tissue results by secreting numerous trophic factors. The A2 reactive astrocyte-associated gene s100a10 is crucial for cell proliferation, membrane restore, and inhibition of cell apoptosis (Liddelow SA, et al., 2017). Furthermore, A2 reactive astrocytes cell the expression of anti-inflammatory cytokine tgfβ, which participates in synaptogenesis and plays a neuroprotective characteristic (Xu x, et al., 2018).

1.10 A2A, D1 and D2 receptors expressed on astrocytes

Central dopamine and noradrenaline neurons exchange with astroglia by the way of transmission and the existence of astroglial dopamine D1R and D2R receptors. This feature is associated with their communication of a high density of aquaporin-4 (Aqp4) water channels decreasing the resistance to water flow. It is also suggested that GPCRs like A2AR and D2R can modulate the water influx and outflux via Aqp4 channels (Fuxe et al., 2015) present in the plasma membrane of astroglia, thru direct receptor-water channel interactions involving allosteric mechanisms.

Recently it was established that astrocytes from adult striatum that D2R and A2AR receptors coexist in the identical astrocyte using confocal microscopy technique (Cervetto et al., 2017a). Furthermore, A2AR activation was once positioned to inhibit the D2R brought on inhibition of astroglial glutamate release provoked by way of 4-aminopyridine (Cervetto et al., 2018b). By itself, the A2AR agonist lacked outcomes on the glutamate release from astroglia. This indication suggests that receptor-receptor interaction in astroglial A2AR-D2R heteroreceptor complexes since it used to be blocked by means of using a D2R artificial peptide interfering with A2AR-D2R heteromerization (Cervetto et al., 2017a). It looks that this receptor complicated additionally can interact with the astroglial Aqp4 water channels.

Overactivity of astroglial A2AR would play an important role in astroglial glutamate launch seeing that there will be a brake on inhibitory astroglial D2R signaling by means of the A2AR protomer. The influence on synaptic glutamate transmission of enhanced astroglial glutamate release is difficult to predict. Extra-synaptic mGluRs should be in general reached if the important receptor activation thru astroglial glutamate involves the inhibitory mGluR2-4, coupled to Gi/o and located on the glutamate nerve terminals, a reduction of neuronal glutamate release would take place with inhibition of glutamate transmission and the striato-pallidal GABA neurons (Kalivas et al., 2009). Thus, the reduction of toxicity will occur. However, if extra-synaptic and postsynaptic mGluR1 and mGluR5 coupled to Gq are extensively activated by using the way of astroglial glutamate release, glutamate synaptic strength can be increased. This can increase intracellular calcium levels and accelerated inhibition of D2R signaling in A2AR-D2R-mGlu5R complexes (Cabello et al., 2009), the place of A2AR and mGluR5 promoters synergize to inhibit D2R signaling mediated by Gi/o. Under such

a situation, glutamate toxicity is no longer anticipated unless extrasynaptic NMDAR/AMPAR/ kainate receptors occur as significantly activated by way of astroglial glutamate.

The astroglial glutamate transporter-1 is significantly for excitotoxicity. The mechanism includes Na+/K+-ATPase-αii coupled to the astroglial glutamate transporter. It is a way of nature that A2AR can immediately bind to the astroglial Na+/K+-ATPase-αii as indicated by the use of proximity ligation assay and co-immunoprecipitation (Matos et al., 2013). Furthermore, activation of the A2AR inhibits the astroglial glutamate uptake thru inhibition of Na+/K+-ATPase αii activity. This hostile interaction involving the above-designated receptor-protein complexes can prolong the extracellular glutamate degrees that can also reach a fundamental degree to produce excitotoxicity. Through this mechanism, involving A2AR activation ion homeostasis as nicely as the astrocyte-neuron lactate transport requiring astroglial glutamate uptake can deteriorate. The transportability that there is a switch of lactate from astrocytes to neurons. Thus, astrocytes assist as a source of lactate and neurons as a sink for lactate. In this way, lactate can help glucose support oxidative metabolism in neurons made viable by way of astroglial techniques (Pellerin et al., 1998b).

2 GLIOTRANSMISSION

2.1 Glutamate

Astrocytes modulate the temporal and spatial dynamics of neurotransmitter signaling. This rule is a key method in controlling synaptic strength and efficacy in addition to influencing neurotransmitter tone at some factor of brain areas (Huang and bergles, 2004; pannasch et al., 2014; Pita-almenar et al., 2012). As an example, inhibiting glutamate uptake consequences in higher extra-cellular glutamate levels that may exchange put up-synaptic efficacy. Within the rat hippocampus, N-methyl-D-aspartate (NMDA) receptor-mediated put up-synaptic currents had been extended, while glutamate transport occurs as soon as inhibited the use of pharmacological activities (Arnth-Jensen et al., 2002). Extended glutamate amounts additionally mediate long time despair (LTD) through modulating postsynaptic metabotropic glutamate receptor (MGLUR) (Huang et al., 2004; Reichelt and Knöpfel, 2002). Repetitive, high-frequency pre-synaptic exercising can carry about on-going excitatory put up-synaptic currents that are mediated by way of mGLUR and assist LTD. In the rat cerebellum, inhibiting glutamate transport multiplied mGLUR activity and promoted

mGLUR mediated LTD (Brasnjo and Otis, 2001). Moreover, genetic removal of astrocyte glutamate transporters purposes excitotoxic degeneration and neuronal hyperexcitability (Rothstein et al., 1996). The omission of the astrocyte glutamate transporter, GLT-1, in mice ended in spontaneous seizures and eventual loss of existence because of enhanced extracellular glutamate launch levels (Tanaka et al., 1997). Consequently, the regulated assignment and expression of astrocyte neurotransmitter transporters is an imperative mechanism thru which astrocytes from synapse characteristics. Interestingly, astrocyte glutamate transporter expression is dynamically regulated in response to neural activity, By way of astrocyte metabotropic glutamate receptor signaling actions, glutamate transporter expression is each and every single upregulated or downregulated in response to extended or diminished synaptic activity respectively (Benediktsson et al., 2012; Devaraju et al., 2013; Yang et al., 2009). Furthermore, this response is transcriptionally regulated thru the transcription complement, nuclear component-κb (nf-κb) (Ghosh et al., 2011).

2.2 Functional Analysis Using Electrophysiology and Optical Approaches

The impact of synaptically released glutamate on astrocytes cause that, astrocytes can be monitored by electrophysiology and pharmacological tools. While electrophysiological tools very reliable technique to detect ion flux across the plasma membrane, it is valuable to keep the brain that they are generally got from astrocyte somata and consequently solely supply a distant, filtered model of electrical indicators generated in a way far-flung to the soma. This is particularly very important, when the Genius about the low enters resistance of astrocytes, resulting in a loss of modern-day with increasing distance from its site of generation, and bad control of the membrane manageable in voltage-clamp experiments. In addition, astrocytes structure electrically coupled systems by means of whole junctions, in a similar fashion complicating accurate measurement of individual cells (Giaume et al., 2010). Notwithstanding their very low input resistance, glutamate not only excites neurons but also depolarizes astrocytes as discovered in early microelectrode recordings (Bowman and Kimelberg, 1984; Kettenmann et al., 1984).

In practical experiments, astrocytes recognized via their ordinary morphology, by using the way of staining with sulforhodamine (SR) (Nimmerjahn et al., 2004; Kafitz et al., 2008). The expression of fluorescent marker proteins (GFP) beneath the manage of an astrocyte-specific

promoter such as glial fibrillary acidic protein (GFAP) (Zhuo et al., 1997).Application of glutamate or agonists of subtypes of ionotropic glutamate receptors (iGluRs),

such as N-methyl-D-aspartate (NMDA), cis-1-amino-1,3-dicarboxycyclopentane (cis-ACPD),a-amino- 3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or kainate, in mixture with precise antagonists, among them (R)-3C4HPG for NMDA receptors, 6,7-dinitroquinoxaline-2,3- dione (DNQX) for AMPA/kainate receptors or GYKI53655 for AMPA receptors, allowed a greater specified useful investigation of astrocytes' electrophysiological responses to glutamate (Verkhratsky and Steinhäuser, 2000; Zhou and Kimelberg, 2001; Matthias et al., 2003; Zhou et al., 2006; Verkhratsky and Kirchhoff, 2007a). The specificity of such pharmacological processes is now now not sufficient to distinguish between the different types of iGluR subunit compositions, which should be addressed by the way of transgenic knockout mice.

The High-affinity of glutamate transporters is analyzed by using mean of the electrophysiological system such as whole-cell patch-clamp. Electrophysiological system to study glutamate transporter currents in astrocytes and to concurrently neuronal and astrocytic endeavor are sum up by current analyses (Dallérac et al., 2013; Cheung et al., 2015). Actually, in the present days, the activation of glutamate transporters upon agonist application or electrical stimulation of afferent fibers is a reliable, semi-quantitative measure for the useful expression of glutamate uptake in glial cells (Brew and Attwell, 1987; Barbour et al., 1988; Bergles and Jahr, 1997; Bergles et al., 1997; Diamond and Jahr, 1997). Expression of glutamate transporters can be down-regulated thru inhibition of their synthesis of continual antisense oligonucleotide management (Rothstein et al., 1996). A lot of pharmacological agents, such as ceftriaxone, estrogen, tamoxifen, and riluzole expand the expression of glial glutamate transporters at the transcriptional degree by way of the way of activation of nuclear element kB (Karki et al., 2015). Acutely transporter subtypes can be blocked by way of substances like UCPH101 (2amino-5,6,7,8-tetrahydro-4-(4-methoxyphenyl)-7-(naphthalen-1-yl)-5-oxo-4Hchromene3carbonitrile) or dihydrokainic acid (DHK), where DL-threo-beta-benzyloxyaspartate (DL-TBOA) and its high-affinity analog TFB-TBOA are non-specific blockers which can be used to inhibit all accredited transporter isoforms (Shimamoto et al., 1998; Abrahamsen et al., 2013; Tse et al., 2014).

Moreover electrophysiological imaging of intracellular ion transients can be active for detecting the activation of glutamate receptors as properly as transporters in astrocytes. This is particularly applicable for metabotropic receptors for glutamate (mGluRs). For evaluation of other, imaging of related intracellular signaling components, such as calcium or cyclic adenosine monophosphate (cAMP), combined with precise pharmacological tools, is typically employed (Pasti et al., 1995a; Niswender and Conn, 2010). The glutamate-induced calcium signals can be monitored by way of both synthetic and genetically encoded fluorescence-based receptor molecules. A particular description of these ratiometric and single-wavelength sensors is outside the scope of this evaluation (Khakh and McCarthy, 2015; Rusakov, 2015b). The general benefit of imaging-based methods is their spatial resolution, which affords in addition statistics on the site of beginning and the propagation of intra- and intercellular signals.

Imaging also reveals the activation of glutamate transport, which is decided thru cotransport of one kind of ions, between them (Marcaggi and Attwell, 2004; Rose et al., 2016). Its depends on their way of stimulation, sodium signals triggered with the glutamatergic exercise in astrocytes can be nearby or far (Rose and Verkhratsky, 2016), therefore allowing practical activation of glutamate transport in astrocyte microdomains such as presynaptic (Langer and Rose, 2009; Langer et al., 2017). Genetically encoded voltage sensors ought to additionally be influential tools for imaging nearby depolarizations in astrocytes but currently used voltage sensors lack the sensitivity to quantify small voltage adjustments (Yang and St-Pierre, 2016).

While another important technical, assignment is the specific activation of astrocytes (Li et al., 2013). Purely pharmacological approaches, such as the usefulness of receptor agonists, do no longer supply ample studies in situ or in vivo, and even pharmaco-genetic approaches, which have remarkable cell-type specificity (Fiacco et al., 2007), do not increase the specific temporal and spatial elements of astrocyte activation. Electrically evoking neuronal neurotransmitter release, e.g., by means of stimulation of afferent fibers can be used as an indirect, but the physiological capability for astrocyte activation.

Temporally and spatially defined that activation of astrocytes can moreover be received by means of the way of optical techniques, such as photo uncaging of glutamate. For instance, local photo uncaging of 4-methoxy-7-nitroindolinyl- (MNI-) caged glutamate proved to be beneficial for planning the distribution of glutamate transporters on male or female mice

brain astrocytes (Armbruster et al., 2014). Exclusively for astrocytes can be performed with optogenetic methods, i.e., by way of genetically encoded, light-sensitive equipment that can be headquartered to the preferred cell types and brain regions. The expression of channelrhodopsin, a ligand-gated ion channel isolated from inexperienced algae, which can be induced with blue mild to attain depolarization and to induce the influx of calcium. Channel rhodopsin technique is used for a wide variety of studies addressing the physiological function of astrocyte signaling in vivo (Gradinaru et al., 2009; Gourine et al., 2010; Sasaki et al., 2012; Perea et al., 2014). Another optogenetic method, which possibly treasured for controlling astrocyte signaling, is the use of ligand gated glutamate receptors. In this approach, photo-switchable ligands are employed to influence specific iGluRs or mGluRs with excessive precision (Levitz et al., 2013; Reiner et al., 2015; Berlin et al., 2016), mimicking their physiological activation as closely as possible. This method moreover used in cultured astrocytes, where activation of a ligand-gated kainate receptor enabled to induced calcium signals, demonstrating astrocyte to- astrocyte signaling (Li et al., 2012).

The number, density and variability of receptors as well as transporters for glutamate release at synapses (Chaudhry et al., 1995; He et al., 2000; Furness et al., 2008; Zhang et al., 2016).Synaptically, Astrocytes released glutamate with the aid of the way of ionotropic and metabotropic glutamate receptors. Furthermore, astrocytes specific high-affinity glutamate transporters, which characterized by the most essential mechanism for the elimination of glutamate from the ECS. Both mechanisms end result in intracellular signals, by way of direction transport both throughout the plasma membrane and thru induction of store-mediated calcium launch and/or other second messengers.

2.3 Astrocytic Ionotropic Receptors

Expression of ionotropic glutamate receptors by means of astrocytes is incredibly heterogeneous and fluctuates between brain areas (Verkhratsky and Kirchhoff, 2007b; Verkhratsky, 2010c). There is a lot of clear evidence for the functional expression of AMPA receptors on Bergmann glial cells of the cerebellar cortex, which are activated by way of the way of ectopic release of glutamate at parallel fiber as well as ascending fiber synapses (Matsui et al., 2005). These have a tremendously excessive calcium permeability and calcium signals ensuing from AMPA receptor opening have been established to achieve the best

analysis of Purkinje cell synapses by Bergmann glia appendages (Lino et al., 2001). Retraction of Bergmann glial cell action following omission of AMPA receptors or their transformation to calcium-impermeable variabilities decreased the decline of excitatory postsynaptic potentials (EPSPs) in Purkinje cells through delaying the removal of glutamate at the synapse, subsequent in an impairment of great motor coordination (Lino et al., 2001; Saab et al., 2012). Therefore, it seems that AMPA receptors on Bergmann glial cells mediate the interaction between glial calcium signaling shut the enhancement of synapses through perisynaptic glial processes and permission of glutamate in the cerebellar cortex.

In the forebrain, AMPA receptor expression through astrocytes has been defined in the neocortex (Lalo et al., 2006a; Hadzic et al., 2017). They solely mediate a small phase of inward cutting-edge induced by the way synaptic release of glutamate (the majority being carried thru electrogenic glutamate uptake) and their treasured significance is still unclear (Lalo et al., 2011b). Moreover, there is clear evidence for AMPA receptors on processes, but no longer somata of radial-like glial cells in the subventricular area of the dentate gyrus (Renzel et al., 2013). Astrocytes in the hippocampus interestedly lack AMPA receptors, as adverse to NG2 cells, which have been frequently labeled as ‘‘immature’’, ‘‘complex’’ or ‘‘rectifying’’ astrocytes (Jabs et al., 1994; Latour et al., 2001; Zhou and Kimelberg, 2001; Matthias et al., 2003). A comparable image develops for NMDA receptors. These have been identified on the mRNA, protein, and valuable degree in cortical astrocytes (Conti et al., 1996; Schipke et al., 2001; Lalo et al., 2006a). While there is clear evidence for the involvement of NMDA receptors in astroglial signaling in hippocampal astrocytes (Porter and McCarthy, 1995; Serrano et al., 2008; Letellier et al., 2016), However, there is no clear evidence for their expression in these cells and their presence. Therefore, the problems nevertheless need to be discussing (Dzamba et al., 2013).

2.4 Astrocytic Metabotropic Receptors

Compare to iGluRs, the expression of metabotropic glutamate receptors (mGluRs) has been firmly set up for astrocytes in the hippocampus (Tamaru et al., 2001; Aronica et al., 2003). The most well-known position has lengthy been recognized to mGluR5, member of the group 1 mGluRs, which activates Gq and phospholipase C and has acquired specific attention as this

outcome in the generation of calcium signaling through IP3-mediated release from intracellular stores (Porter and McCarthy, 1996; Zur Nieden and Deitmer, 2006).

While this notion interested suitable for the juvenile hippocampus, it used to be these days called for the grownup brain, where the application of mGluR5 agonists failed to persuade calcium signaling in astrocyte somata (Sun et al., 2013). Moreover, in the mature hippocampal mossy fiber pathway, as soon as it was once discovered that mGluR5 was only partly accountable for calcium signals activated by way of axonal glutamate release in astrocyte processes (Haustein et al., 2014). While differences might be related with different variants in experimental approaches (Panatier and Robitaille, 2016b), these effects show that calcium signaling in astrocytes is now no longer completely understood. Apply of extra superior calcium imaging techniques like fast 3D-scanning of astrocytes in situ and in vivo imaging gadget are wished to get to the bottom of this problem (Bazargani and Attwell, 2016; Shigetomi et al., 2016d; Bindocci et al., 2017). In addition, astrocytes express mGluR2/3, the activation of which is coupled to Gi=o and results in inhibition of the adenylate cyclase (AC) (Tamaru et al., 2001; Aronica et al., 2003). This signaling pathway causes the overthrow of cAMP tiers and is normally not saw as being without delay in astrocyte calcium signaling (Sun et al., 2013). Anyhow this, activation of mGluR2/3 has these days been connected to the era of gradual calcium transients in astrocytes in the mossy fiber pathway. The mGluR2/3-induced calcium signaling was recognized to the action of G-protein b/g subunits activating phospholipase C and interplay with IP3 receptors (Haustein et al., 2014), in analogy to GABAB receptors. Here we see the glutamatergic transmation.

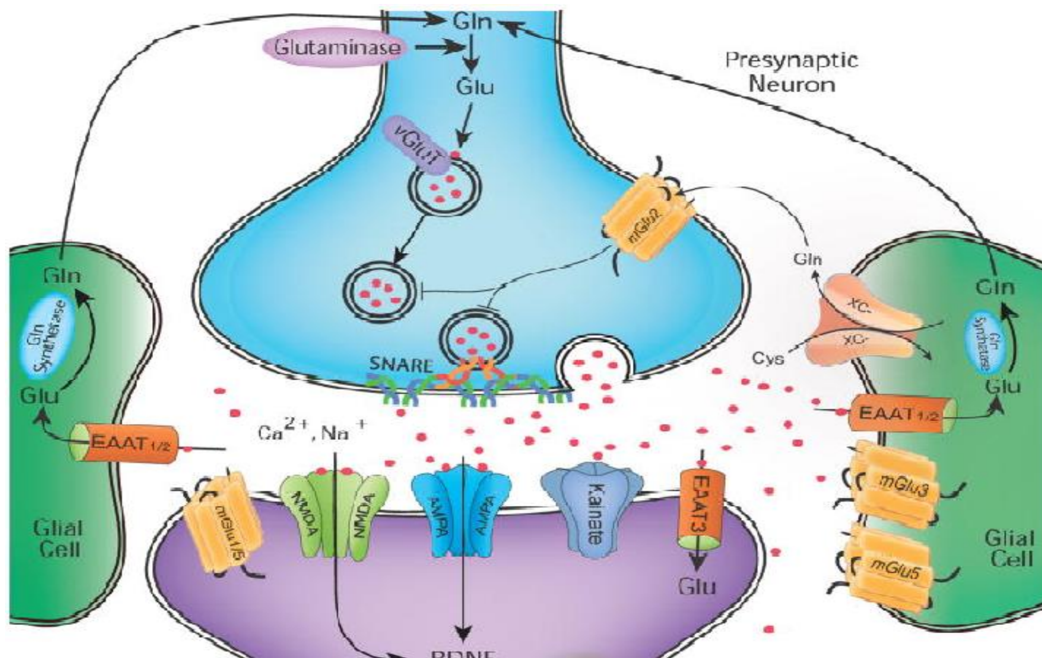

Figure 3 Glutamatergic Neurotransmission

Due to the risk of excitotoxic damage in the wake of excessive glutamatergic stimulation, precise physiological control of glutamate must be maintained in the mammalian CNS. Glutamine (Gln) is converted to glutamate (Glu) by glutaminase [though glutamate may also be derived from the TCA cycle (not shown)]. Glu is packaged into presynaptic vesicles by vesicular Glu transporter (VGLUT) proteins and synaptically released in an voltage-dependent manner through vesicular interactions with SNARE proteins. Synaptically-released Glu is recycled from the extracellular space by excitatory amino acid transporters (EAATs) expressed predominantly on astroglia. In astrocytes, Glu is converted to Gln by Gln synthetase and exported extracellularly to be taken up again by neurons. Additionally, system x-C is a cystine/glutamate antiporter expressed on glia that also contributes to Glu recycling. Glu receptors are present on presynaptic and postsynaptic neurons as well as on glial cells. These include both ionotropic receptors (NMDA, AMPA/KA) and metabotropic receptors (mGluRs). The effect of Glu is determined by the receptor subtype, localization (synaptic, perisynaptic and extrasynaptic), and interactions with various scaffolding and signaling proteins (not shown) in the postynaptic density. Glu receptor stimulation results not only in rapid ionotropic effects but also synaptic plasticity, e.g. long-term potentiation (LTP) and long-term depression (LTD), via cognate signal transduction cascades.(the picture and legends is taken from Niciu et al., 2012)

2.5 High-affinity glutamate transporters in astrocytes

Although receptors are, enable to react synaptically and released glutamate, possibly the largest effect on extracellular glutamate itself is made by way of high-affinity glutamate transporters, EAATs. Not solely do this to inhibit excitotoxicity thru the elimination of glutamate from the cleft and the ECS. However, it additionally acts as a vital aspect of

plasticity and synaptic function. Now a total of five extraordinary mammalian subtypes of EAAT have been recognized (Danbolt, 2001), and these unique expression patterns at some stage in the cortex (Arriza et al., 1994). The different isoforms follow to the equal familiar glutamate uptake stoichiometry; importing one glutamate molecule into the astrocyte through means of energy established from co-transporting three sodium ions and one proton down the electrochemical gradients, and additionally export one potassium ion (Nicholls and Attwell, 1990). While all of these subtypes consequently couple cations to glutamate transport in the equal ratios, they also show off and uncoupled anion conductance, and therefore act as chloride ion channels (Amara and Fontana, 2002; Fahlke and Nilius, 2016). Isoforms are functionally exclusive from every different by using glutamate transport rates, substrate affinities, and accompanying chloride conductance (Maragakis and Rothstein, 2004).

The two major predominant transporter isoforms present in the hippocampus are EAAT1 (rodent analog: GLAST; glutamate/aspartate-transporter) and EAAT2 (GLT-1; glutamate transporter 1). While more than a few Km (Michaelis-Menten constant, reflecting the attention of half-maximal occupancy) and Vmax (maximal transport rate) values have been pronounced for these transporter species depending on the experimental system used, it is usually established on that EAAT2 is the necessary transporter with a greater turn-over rate than EAAT1. Additionally, EAAT3 (also acknowledged as EAAC1) can be found at post-synaptic neuronal membranes, although a lot of decrease density than astrocytic EAAT1 and EAAT2 (Holmseth et al., 2012). Moreover, in the adult hippocampus, 10% of EAAT2 transporters are also expressed on neuronal axon terminals.

Transporter feature is critical for the duration of brain development, and double knockout animals of EAAT 1 and two are not possible and show cortical abnormalities and disorganization (Matsugami et al., 2006). Over-all ablation of solely EAAT2 has life-threatening outcomes and mice die quickly after beginning from essential seizures. Importantly, knocking out EAAT2 specifically in astrocytes mimics these effects, demonstrating the essential magnitude of glial glutamate uptake for glutamate homeostasis in the brain (Petr et al., 2015). Consistent with this assessment, knockout mice for the different transporters, which includes EAAT1, show only vulnerable to moderate impairment (Watase et al., 1998).

In the neonatal hippocampus, astrocytes are still immature and developing their processes that will later outline their outstanding cellular areas (Bushong et al., 2004). The astrocytes of neonatal hippocampus in this segment in a general matter on the subtype EAAT1 for glutamate transport and average transport stages have been established to be lower than in mature astrocytes (Diamond, 2005). This increase will occur in the first two postnatal weeks and then stops (Ullensvang et al., 1997; Schreiner et al., 2014). While later EAAT2 will takes over the bulk of hippocampal glutamate transport capacity, which is more than 90% of whole uptake (Zhou et al., 2014). Its expression is overdue by means of round 10 days, starting to exhibit up at p10–15 and increasing as cells mature until plateauing at p20–25. While this pattern has been established confirmed to be regulated via way of neuronal activity (Swanson et al., 1997), while the cause is unclear, mainly as it is not frequent across brain regions. For example, the identical exchange takes place in the cortex, however is overdue by way of around two weeks (Hanson et al., 2015), while Bergmann glial phone proceeds to specially express EAAT1 into adulthood (Rothstein et al., 1994).

2.6 Ion Signalling related to Glutamate Transport in astrocytes

Astrocytes will take place essential adjustments of their cytosolic ion concentrations in response to glutamate because of the recreation of high-affinity of sodium-dependent glutamate uptake. These ions consist of exceptionally sodium and protons (Rose and Ransom, 1996; Kirischuk et al., 2016), which are collectively transported with glutamate. Moreover, relying on the EAAT isoform expressed, glutamate transporters mediate a detectable flux of chloride (Untiet et al., 2017). The resulting degradation of ion gradients reduces its the usage of pressure, e.g. for the duration of additional disturbance in ion homeostasis, even result in a reversal of glutamate uptake. Ion signaling associated with glutamate transport thereby represents an essential modulator of astroglial manipulate of extracellular glutamate.

Under physiological circumstances, glutamate transporters show a reversal manageable in the far excellentfluctuate>50 mV, (Barbour et al., 1991). Changes in the plasma membrane gradients of the transported ions (NaC, HC, KC) without delay influence glutamate transport activity, and due to the reality of its transport stoichiometry, this type special change is relevant for sodium ions (Szatkowski et al., 1990; Zerangue and Kavanaugh, 1996; Levy et al., 1998). In addition, as glutamate uptake is electrogenic, the depolarization mediated by

glutamate uptake, collectively with the depolarizing impact of activity-related will increase in extracellular KC, will purpose of inhibiting of further transport undertaking (Barbour et al., 1988a).

During this duration, the metabolic inhibition, contrary action of glutamate transport can assist as a source for glutamate and contribute to its accumulation in the ECS as described for neurons below excessive ischemia (Rossi et al., 2000). The same way is followed for reverse glutamate uptake thru glia, which in remoted Müller glial cells has been established to be added on by means of high exterior potassium (Szatkowski et al., 1990). It is very important to emphasize, however, that as compared to uptake of other transmitters such as GABA, glutamate uptake is extremely robust. Reverse glutamate uptake in brain tissue as a mechanism for glia-mediated release of glutamate is frequently proposed however solely viable with immoderate cellular sodium loading collectively with sturdy depolarization, prerequisites usually totally discovered upon entire metabolic inhibition and failure of NaC/KC-ATPase undertaking in ischemic core regions (Gerkau et al., 2017).

While the reverse uptake of glutamate can also exhibit up solely underneath severe ischemic circumstances, less dramatic modifications in astrocyte ion gradients may also still directly feed lower back on pinnacle of extracellular glutamate levels through lowering the driving force for transport. The magnitude of ongoing glutamate uptake is evident from the reality that its pharmacological inhibition reasons an immediate make more in extracellular glutamate accumulation, accompanied by potential of activation of neuronal glutamate receptors (Jabaudon et al., 1999) and rapid, noxious sodium loading of both neurons and astrocytes (Karus et al., 2015). Moderately growing the cytosolic sodium concentration in astrocytes (to 35–40 mM) is at once linked to the glial glutamate transport activity as verified for astrocytes in hippocampal tissue slices uncovered to expanded NH4C/NH3 concentrations (Kelly et al., 2009). The same is real for an average depolarization (by 8–10 mV) of hippocampal astrocytes in situ, which as anticipated based totally on the transport stoichiometry significantly decreased the amplitude of glutamate uptake currents induced with the source of the transporter agonist D-aspartate (Stephan et al., 2012).

These lookup studies indicate that, if intracellular sodium increases in astrocytes, the reversal potential for glutamate transport is shifted in a negative direction, therefor decreasing their glutamate uptake capacity. Particularly, glutamate uptake simultaneously signifies the most

effective pathway for the induction of sodium indicators as confirmed for astrocytes in the stratum of the hippocampal CA1 place, cerebellum (Kirischuk et al., 2007; Bennay et al., 2008), and neocortex (Lamy and Chatton, 2011; Unichenko et al., 2013), and at the Calyx of Held (Uwechue et al., 2012). Activity related to sodium indicators following activation of glutamate uptake is measurable in presynaptic astrocyte processes, and their amplitudes are related to the electricity of synaptic stimulation over an extensive range of stimulation intensities, accomplishing 6 mM with 10 pulses, (Langer and Rose, 2009). If such sodium elevations and the accompanying decrease in transport capacity, the result in the normal glutamate uptake by the way of astrocytes and thereby in the modulation of extracellular glutamate concentrations and synaptic glutamate transients below physiological conditions, stays to be established.

Besides all these the direct negative feedback impact of sodium elevations on glutamate transport capacity, there is additionally gathering proof that sodium alerts in response to transport activation would possibly drive reversal of the sodium-calcium trade (NCX) in astrocytes (Kirischuk et al., 2012c; Boscia et al., 2016). The result of such a reversal is the era of calcium influx into astrocytes (Kirischuk et al., 1997a; Song et al., 2013). Calcium alerts induced thru sodium-driven NCX might, and then result in calcium-dependent release of glutamate, again linking astrocyte sodium indicators to their modulation of extracellular glutamate concentrations.

2.7 Astrocyte calcium signalling at synaptic level

Astrocytes additionally notice synaptically launched glutamate thru the activation of glutamate receptors as described. Their activation regularly leads to the technology of intracellular ion alerts and/or 2d messengers Transient astrocyte calcium boosts in response to glutamatergic recreation had been one of the first intracellular indicators connected in neuron-glia interaction at synapses (Enkvist et al., 1989;Kim et al., 1994; Hassinger et al., 1995). While initial experiments have been carried out in cell culture, it quickly became clear that additionally astrocytes in situ also respond to the neuronal release of transmitters will extend in calcium, as shown in acute hippocampal tissue slices (Dani et al., 1992; Porter and McCarthy, 1996; Pasti et al., 1997). Later on, this brain was once shown to preserve real inside the intact brain by means of demonstrating that hippocampal astrocytes in vivo undergo

calcium signaling in response to neuronal activity (Kuga et al., 2011; Takata et al., 2011; Navarrete et al., 2012). The actual astroglial mechanisms producing calcium transients and their spatial and temporal properties are nevertheless under dialogue regardless of the prosperity of experimental evidence (Agulhon et al., 2010; Volterra et al., 2014; Rungta et al., 2016).

Essential cell response to these calcium transients includes the calcium-dependent release of neurotransmitters from astrocytes concentrated on close-by neurons and their synapses. The big experimental evidence for the existence of such signaling, the concerned mechanisms, the present-day controversies, and open questions have been discussed in detail these days through ourselves and others (Hamilton and Attwell, 2010; Araque et al., 2014; Rusakov et al., 2014a; Verkhratsky et al., 2016f; Bohmbach et al., 2017). The relevance of astroglial calcium signaling for neurotransmitter uptake is a great deal less explored. As mentioned above, calcium and sodium signaling are linked by means of the NCX such that sodium elevations may moreover set off calcium-entry with the NCX. Whether the reverse, sodium-entry as a result of calcium export through NCX, can adjust the riding force of astroglial glutamate uptake (and accordingly its efficiency) significantly.

In addition, calcium increases may want to modify the insertion and internalization of glutamate transporters. Such a direct impact of astroglial calcium ranges on neurotransmitter transport has been verified for the GABA transporters GAT3 in the hippocampus. Chelation of intracellular calcium by way of infusing astroglia with the calcium buffer BAPTA by way of a patch pipette reduced the GAT3 degrees and introduced about a tonic, GABA-receptor-mediated current recorded in interneurons, indicating that lowering astroglial calcium degrees reduces GAT3-mediated GABA uptake (Shigetomi et al., 2011a). Direct evidence for the calcium-dependence of glutamate transport in astrocytes in situ is currently no longer on hand on the other hand several experimental observations make the existence of such a mechanism plausible. First, an make bigger of glutamate uptake by cultured astrocytes can be induced with the resource of essential fibroblast boom issue on a time scale of hours thru a partially calcium-dependent signal cascade (Suzuki et al., 2001). Second, the down-regulation of astroglial glutamate uptake currents in spinal twine slices with the source of interleukin 1beta inside involves astroglial calcium signaling (Yan et al., 2014). Finally, the manipulation of calcium levels in cultured astroglia affected the insertion and removal of

eGFP-tagged GLT-1 into and out of the membrane (Stenovec et al., 2008). Together these research demonstrate that changes of astroglial calcium degrees should directly control glutamate uptake, because astrocyte calcium signaling itself relies upon the neuronal activity, glutamate uptake could be finely tuned by using synaptic undertaking by means of using astroglial calcium signaling.

2.8 Regulation of synaptic transmission by perisynaptic astrocytes structure.

Astrocytes take up the majority of synaptically released glutamate (>90%). However, glutamate is released from the presynaptic terminal at as soon as into the synaptic cleft, which is typically devoid of astrocyte procedures and hence astroglial glutamate transporters. It is, therefore, notice that astrocytes do not manipulate the preliminary spread of synaptically released glutamate inside the synaptic cleft under physiological conditions and that as a substitute diffusion and dilution of glutamate specifically underlie the dissipation of the steep glutamate attention gradients without delay after release (Clements et al., 1992; Scimemi and Beato, 2009). At these nanometer and microsecond scales, intra-cleft glutamate interest transients have escaped direct observation. Instead, numerical simulations have been employed to analyze the intra-cleft spread of glutamate and its escape into the presynaptic space that carries presynaptic astrocyte techniques (Diamond, 2001; Zheng et al., 2008;Allam et al., 2012) ''Influence of Glutamate Transporters on Extracellular Glutamate Homeostasis'' for glutamate binding to astroglial transporters and uptake.

These simulations predicted that presynaptic astrocyte branches and the glutamate transporters located on them decrease the probability of glutamate escaping into presynaptic ECS and activating presynaptic high-affinity receptors such as mGluRs or NMDA receptors. Indeed, glutamate released at one synapse can prompt high-affinity NMDA receptors at close by synapses (a phenomenon is known as glutamate spill-over or synaptic crosstalk) and pharmacological blockade of glutamate transporters drastically exacerbates this procedure (Asztely et al., 1997; Diamond, 2001; Arnth-Jensen et al., 2002; Scimemi et al., 2004a). Thus, astroglial glutamate transporters constrain however do now not stop synaptic crosstalk between hippocampal synapses via high-affinity NMDA receptors.

Because astroglial glutamate transporters restriction activation ratio of EPSCs mediated by means of NMDA and low-affinity AMPA receptors was elevated in recordings from CA1

pyramidal cells in acute slices from GLT-1 knockout mice, suggesting a larger contribution of NMDA receptors. In the same preparation, long-term potentiation of CA3-CA1 synaptic transmission used to be impaired, which was attributed to excessive activation of NMDA receptors (Katagiri et al., 2001). In addition, up-regulation of GLT-1 expression by way of ceftriaxone impaired long-term despair and reduced long-term potentiation at mossy fiber-CA3 synapses (Omrani et al., 2009). A role of glutamate transporters for NMDA receptor-dependent synaptic plasticity has also been hooked up outside of the hippocampus (Massey et al., 2004; Tsvetkov et al., 2004; Valtcheva and Venance, 2016), which suggests that the manage of NMDA receptor-activation and therefore NMDA receptor-dependent synaptic plasticity by way of using astroglial glutamate ought to be a ubiquitous phenomenon in the brain.

Glutamate transporters also manage the activation of different high-affinity glutamate receptors. For instance, mGluR activation of interneurons in response to synaptic glutamate launch was once strongly extended after pharmacological transporter blockade (Huang et al., 2004). To what extent synaptic crosstalk plays a role for low-affinity, the post-synaptic AMPA receptor characteristic is not wholly explored. The net effect of the GLT-1 inhibitor DHK on AMPA receptor-mediated EPSCs varies between small minimize and no have an impact on (Asztely et al., 1997). Similarly, AMPA receptor-mediated EPSCs recorded from CA1 pyramidal cells had been not affected via using transporter blockade using TBOA, irrespective of whether or no longer these were action-potential independent EPSCs (‘‘minis’’) or evoked by minimal-stimulation (Zheng et al., 2008). This correctly guidelines out a role of glutamate transporters in controlling AMPA receptor activation at the releasing synapse. However, it does no longer eliminate that: (1) glutamate may additionally reach AMPA receptors at nearby synapses of different neurons in the densely packed neuropil (not recorded from); and (2) that astroglial transporters control such AMPA receptor-dependent crosstalk. The latter state of affairs is hinted at through our previous experimental findings. The inhibition of astroglial metabolism the use of the gliotoxin fluoroacetate (FAC) is probable to impair astroglial glutamate uptake (Karus et al., 2015). At the equal time, this remedy appreciably accelerated predominantly AMPA receptor-mediated subject EPSP slope, a measurement reflecting all AMPA receptor-activity in the neuropil, by way of about 20% (Henneberger et

al., 2010b). However, a direct measure of the fluctuate of action of glutamate and its dependence on astroglial transporters is still missing.

It is stated here that the frequently used broadspectrum glutamate transporter inhibitors TBOA and TFB-TBOA (Shimamoto et al., 1998) do now not selectively inhibit astroglial transporters however dose-dependently inhibit GLT-1 (expressed often however now not solely via using astrocytes in the hippocampus (Mennerick et al., 1998; Danbolt, 2001), the astroglial GLAST and the neuronally-expressed EAAC1 (Shigeri et al., 2004). However, the extent density of EAAC1 (molecules/mm3) is two orders of magnitude lower than GLT-1 in the CA1 stratum, which raises the question to what extent EAAC1 contributes particularly to glutamate clearance (Holmseth et al., 2012; Scimemi et al., 2009).

3. PATHOLOGICAL ROLE OF ASTROCYTES

3.1 Alzheimer´s Disease (AD)

Alzheimer's disease (AD) is one of CNS neurodegenerative diseases, which is characterized by memory loss and cognitive dysfunction, and have many neurological and psychiatric symptoms and behavioral disorders (Vakalopoulos C 2017). Pathologically, AD is defined is the presence of extracellular amyloid plaques (APs) and intracellular neurofibrillary tangles (NFTs) in the brain. The APs are composed of aggregated β-amyloid peptide (Aβ), whereas the NTFs are composed of atypical phosphorylation and aggregation of protein intracellularly (McGeer PL, & McGeer EG 2002)). The concept of AD is the impairment of the relationship between neuronal and astrocytic features in brain areas related to the memory (such as hippocampus) (Phillips EC et al., 2014)

In the normal circumstances, the precursor protein of amyloid (APP) is cleaved by means of secretase α, producing sAPPα - a soluble neuroprotective fragment and blocking the production of Aβ, thereby its defend the brain cells from the toxic damage of Aβ (Bailey JA et al., 2011). In AD condition, the cleavage is achieved by using secretase β and secretase γ. It's because of Aβ, which can be divided into Aβ40 and Aβ42 by the length of the peptide (Thinakaran G & Koo EH 2008).

The development of AD, Aβpermission can increase Aβ plaque formation, maybe due to the dysfunction of astrocytes and the formation of Aβ (Rossi D &Volterra 2009, Wys Coray T et

al., 2003). To learn more about the AD, mice model found that the Aβ plaques could be removed by the way of transplanting the fluorescent-labeled wild-type astrocytes (Pihlaja R et al., 2011). Astrocytes should play a very important role in the elimination of Aβ peptide in the normal brain. Another find out about also confirmed robust β-site APP cleaving enzyme 1 (BACE1) expression in reactive astrocytes of AD patients, which can contribute to the formation of Aβ (Cole SL & Vassar R 2007). Further study is needed to have a look at the way and amount of Aβ produced in astrocytes compares with neurons.

The atypical homeostasis of calcium and glutamate in reactive astrocytes may additionally lead to the pathogenesis of AD (Acosta C et al., 2017). In a mice model of AD brain, the astrocytic calcium signaling and gliotransmitter releases can be interrupted with the source of Aβ, which proposes that astrocyte dysfunction may also contribute to the initial neuronal deficits in AD (Vincent AJ et al., 2010). The hippocampus of AD mice, excessive GABA released by way of reactive astrocytes can end result in tonic inhibition of dentate gyrus granule cells in hippocampus of AD mice. Remarkably, inhibition of GABA synthesis or pharmacological blockade of GABA transporters restores synaptic plasticity and memory deficits in these mice (Jo S et al., 2014).

Upregulation of transitional filament is assurance of astrocytes reactivity in AD brain (Kamphuis W et al., 2015). Pathological research about AD brain samples and mice models have observed that the APs are surrounded by way of reactive astrocytes, with an accelerated appearance of GFAP and S100β (Nagele RG et al., Li C et al., 2011). Moreover, the wide variation of improved reactive astrocytes is often associated with cognitive decline (Kashon ML et al., 2004). In normal situations, reactive astrocytes are very important regulators in brain's inflammatory response, but under pathological prerequisites in the brain area, reactive astrocytes may also be neurotoxic, when they produced inflammatory cytokines and reactive oxygen species (Farina C et al., 2007). In AD brains, the degrees of pro-inflammatory cytokines produced frequently by reactive astrocytes are excessive in the brain regions the place reactive astrocytes accumulate (Simpson JE et al., 2010). Increased periods of pro-inflammatory cytokines and activated inflammatory production had been additionally discovered in brain tissue of AD sufferer's mice (Salminen A et al., 2008). The transcriptional assessment additionally shows that the levels of these inflammatory components in astrocytes than in microglia (Orre M et al., 2014). Morever, irregular energy metabolism was

also found in AD reactive astrocytes. By blocking the strong metabolism and oxidative stress in AD astrocytes, the effect of Aβ plaque deposition can also be reduced, therefore improving memory and delaying disease development (Allaman I et al.,2010).

The expression of APP has been confirmed in reactive astrocytes, and its upregulated with the resource of multiple pro-inflammatory cytokines (IL-1β and IL-6) in mice brain (Brugg B et al., 1995). The mixtures of pro-inflammatory cytokine significantly increase the expression of APP and secretase β in the main cultured of astrocytes (Zhao J et al., 2011). Interestingly, irritating brain damage has long been connected with the vulnerability of producing AD (Guo Z et al., 2000). Evidence has been shown that acute brain injury can result in APP and PS1 expression in reactive astrocytes (Siman R et al., 1989, Nadler Y et al., 2008). These researches indicate that the reactive astrocytes contribute to Aβdeveloped in AD.

3.2 Parkinson's disease (PD)

Parkinson's disease (PD) is a present-day neurodegenerative disease provoked with the basis of the disorder of dopaminergic neurotransmission in the basal ganglia and neuronal damage in the substantia nigra (SN) of the brain. The pathological assurance of PD is the presence of α-synuclein deposition and protein inclusions, additionally recognized as Lewy our bodies or Lewy neurites in the neuronal cell cytoplasm (Nutt JG, Wooten GF 2005; Albin RL 2006)]. In spite of all this fact, that nowadays the molecular mechanisms of the PD is well known, but there is no clinically effective remedy for this disease. Previous studies have been confirmed that mutations in some of the proteins, along with park2, atp13a2, pten, pink1 and dj-1, can cause PD (Rappold PM, Tieu K 2010; Wang HL et al., 2011). In vivo research shows that intravenous injection of 1-methy-4phenyl-1, 2, 3, 6-tetrahydro-pyridine (MPTP)might result in parkinsonism in mice. All these due to the presence of an enzyme monoamine oxidase inside the cytoplasm of astrocytes, which could switch MPTP to MPP+, whilst MPP+can kill or damage dopaminergic neurons, principal to paralysis agitans (Lieu CA et al 2013; Kohler C et al., 1988). Astrocyte reactivity is identified inside the SN pars compacta (SNPC) of sufferers with PD (Hirsch EC, Hunot S 2009). On the time of PD initiation, α-synuclein accumulate in astrocytes, which because of this fact brought about the recruitment of phagocytic microglia, attacking sure neurons inside the area of the limited skull and causing the scientific signs and symptoms of PD (Halliday GM, Stevens CH 2011). Pathological examinations of PD brains

show that, elevated amount of astrocytes in addition to an advanced degree of GFAP expression (Ciesielska A et al., 2009). Pathological research additionally located that astrocytes may also be activated and accumulated with nonfibrillized α-synuclein at initial levels of PD brain, which allotted better considerably than lewy our bodies (Lee HJ et al., 2010;Barcia C et al., 2012). Curiously, the growing accumulation of α-synuclein aggregates changed into discovered in pre-symptomatic and symptomatic mouse brains and correlated with the growth of reactive astrogliosis. The presence of intracellular aggregates may additionally disrupt astrocytic glutamate transporters and their ability to alter blood-brain barrier, main to non-cellular-autonomously damaging neurons (Gu XL et al., 2010). The above findings endorse that the reciprocal conversation between astrocytes and neurons is of wonderful importance to the health of PD neurons, Just like the behaviors of reactive astrocytes in mice models. The state of activation of stat3 signaling pathway in astrocytes seems like to be a consistent feature in PD. Pharmacological inhibition of jak2 in MPTP mice model of PD can significantly give up result in the restriction of stat3 and GFAP expression ranges and reactivity of astrocytes, suggesting that the jak/stat3 signaling pathway is required to spark off reactivity of astrocytes in the diseased like condition (Sriam K et al., 2004). In regular instances, fzd-1 receptor in mesencephalic neurons is required for astrocyte-mediated neuroprotection. The mesencephalic neurons studies show that the fzd/β-catenin signaling pathway mediated by the way of antagonist can inhibit neuronal survival in SN-triggered reactive astrocytes, which prevented by pharmacological activation of β-catenin within the SN of the brain (L'Episcopo F et al., 2011). Those effects show that the wnt1/fzd-1/β-catenin signaling pathway plays a key functional role inside the interaction between astrocytes and neurons and is of critical significance for retaining the health of PD neurons.

3.3 Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS) is an irreversible motor neuron dysfunction characterized by way of degeneration of motor neurons inside the CNS, which is either characterize like muscle atrophy or even though death induced by respiration failure (Kiernan MC et al., 2011). The most important vital pathological role for ALS remains to be investigated. Infrequently of the ALS cases, there is no clear cut genetic aspect within the system of ALS disorder. However, inside the case of inherited ALS, gene mutations bring about combined classes of proteins (such as SOD1), which can be placed in each neuron and astrocytes (Ferraiuolo L et al., 2011).

Reactive astrocytes are determined by vulnerable areas specific and the reactivity relates to the neurodegeneration in ALS. Furthermore, the entirely determined up to now could be the death of a specific class of motor neurons. ALS astrocytes had been confirmed to contribute to motor neuron death in vivo. While at the same time, these astrocytes are accompanied by a range of abnormalities of signaling pathways, which include adjustments in the neuronal expression of GLUR2 subunit of AMPA receptors (Van Damme P et al., 2007). Impaired lactate delivery, activation of p75-receptor signaling in motor neurons reduction of GLT-1 expression, and persistent Ca2+ release and apoptosis resulted from mGLUR5-mediated glutamate signaling (Martorana F et al., 2012). Once study of mice methods published that transplantation of the precursors of mutant SOD1 astrocytes into the spinal cords can cause degeneration of motor neurons (Papadeas ST et al., 2011), while transplantation of wild type astrocytic precursors into ALS mice models effects within the reduce of motor neurons death, which indicates that ALS astrocytes have insecure or toxic effects on motor neurons in vivo (Lepore AC et al., 2008). While, other in vivo discover shows thatSOD1 (G93A) mice model determined that astrocytes might have defective glutamate uptake, resulting in extracellular accumulation of glutamate that is toxic to motor neurons (Pardo AC et al., 2006). In evaluation, with the valuable resource of growing activation of astrocytes and the expression of immune or inflammatory markers in vivo, the illusory pathological method may be intensified (Chiu IM et al., 2009). In ALS, a major increase inside the transcription of inflammatory molecules, which includes astrocytes derived from each familial and infrequent different varieties of the sickness (Haidet-Phillips AM et al., 2011). The pathological way of ALS is hallmarked through the activation of astrocytes and the expression of immune or inflammatory markers in vivo. Similarly, infinite inflammatory mediators (in precise il-6 and tgfβ) are also observed to contribute in astrocyte-neuron exchange (Allaman I et al., 2011; Phatnani HP et al., 2013). As an example, it has been confirmed that interferon-γ introduced on reactive astrocytes may additionally be neurotoxic, per hazard with the valuable source of the stat3-based signaling pathway, in which activation of stat3 results in the recruitment of reactive astrocytes and the reaction of reactive microglia to motor neurons (Hashioka S et al., 2009). Importantly, each of the recruitment and the neurotoxicity of reactive astrocytes in ALS can be inhibited through stat3 inhibitors (Shibata N et al., 2010).

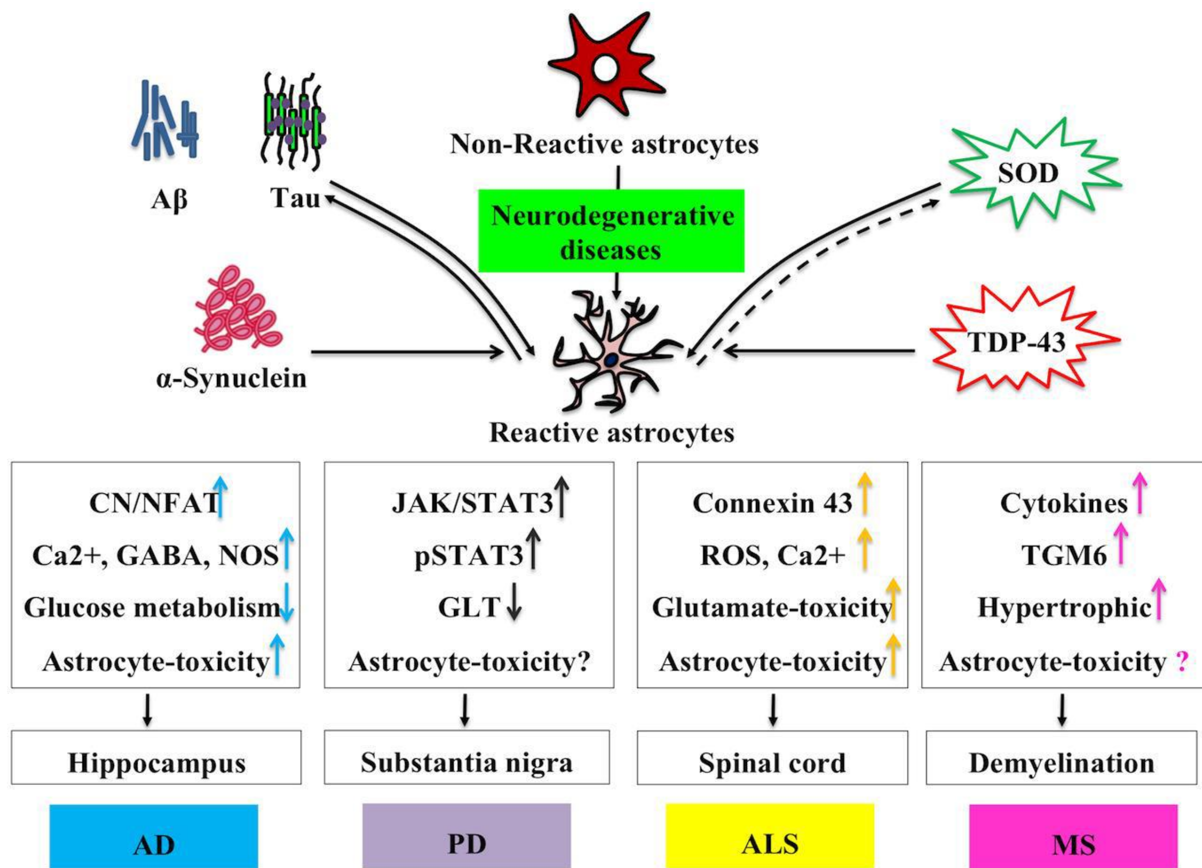

Figure 4. Characteristics of reactive astrocytes in different neurodegenerative diseases. Various molecules can trigger the reactivity of astrocytes, which involves their morphological, transcriptional and functional changes. Different neurodegenerative diseases lead to a variety of changes in reactive astrocytes, which may ultimately cause them to release fewer neurotrophic factors and produce more inflammatory factors. This effect largely depends on different neurodegeneration-related factors, and the molecules they produce and secrete into the microenvironment surrounding the functional neurons in the brain. Aβ, amyloid β; SOD, superoxide dismutase-1; TDP-43, TAR DNA-binding protein 43; CN/NFAT, Calcineurin/Nuclear factor of activated T-cells; NOS, Nitric Oxide Synthase; JAK, Janus Kinase; ROS, reactive oxygen species; TGM6, Transglutaminase 6 (the picture and legends is taken from Li K, et al., 2019)

3.4 Multiple sclerosis (MS)

Multiple sclerosis (MS) is an inflammatory disease, in which the demyelination and axonal damage in CNS. Even though its etiology remains are still unclear, while some proof there that autoimmunity plays a very important role in the pathogenesis of these diseases (McFarland HF, Martin R 2007). The responsibility of astrocytes was commonly blow-out about acute inflammatory lesions inside the MS brain (Brosnan CF, Raine CS 2013). Murine experimental autoimmune encephalomyelitis (EAE) is a set up mice model of one or more than one sclerosis, which shows the activation of astrocytes, followed thru the lack of endfeet around

small blood vessels, the lack of BBB characteristic, as a result, followed by CNS inflammation and perivascular edema. Continuously, pathological examination discovered the presence of reactive astrocytes in affected person with acute MS. It is recommended that genomic amendments can be observed in astrocytes from stroke and inflammatory lesions (Zamanian JL et al 2012). The researchers additionally found that reactive astrocytes lack the expression of MHC-Ⅱ trans-activators, main to the failure to swift CD4+ T cells, which put forward that MHC expression performs a key role inside the activation of T cells in astrocytes (Stuve O et al., 2002). In addition, reactive astrocytes combined with VEGFR-2 can spark off the expression of vascular endothelial boom component and %-γ1, downregulate the expression of claudin-five and blocked, and in the end disruption of the BBB (Argaw AT et al., 2012). In MS brain, reactive astrocytes are also the essential source of inflammatory cytokines (which include IL-10, IL-17a, IL-22 and mip-1α, and so on.). This also causes out of control inflammatory reactions that cause the activation of T cells and the formation of myelin sheath in focal regions and axonal harm (Perriard G et al., 2015; Blazevski J et al., 2013). However, whether or now not special reactive astrocytes are contributing to MS is though undefined, therefore further research is needed.

3.5 The role of astrocytes in the pathophysiology of stress

3.5.1 Definition of stress

Stress is a complex phenomenon that is defined as a “stressor” which may be a predictable or unpredictable environmental change in the system that motivates “stress”, which is described as a multidimensional physiological response that challenges inner stability (Romero 2004 & Dantzer et al., 2014). Animals can fight with stress through “stress responses”, which might be physiological changes or behavioral changes such as secretion of glucocorticoids (Sapolsky, Romero & Munck 2000) and to adjusted the fight or flight response respectively (Romero 2004; Wikelski & Cooke 2006; Stankowich 2008).

Whenever any unpredictable event occurs, and then the body will give physiological responses to maintain the homeostasis (Davis 2006). As we know, Homeostasis helps to maintains Ph, body temperature, glucose utilization, and oxygen (Lupien et al. 2006). This can be achieved through a system known as allostasis, which stabilized through the physiological or behavioral system (McEwen & Wingfield 2003). Allostasis helps to maintain the

homeostasis via adjustments of intrinsic (such as age, body condition, and reproductive status) and extrinsic (such as publicity to weather, predation risk) (Sterling & Eyer 1988; Karlamangla et al., 2002; McEwen 2002; Dantzer et al., 2014). Furthermore, the “allostatic load” every day or month-to-month amount of strength a character needs to fulfill ordinary existence records tasks, such as breeding. When the environment no longer gives the required input, the character goes into “allostatic overload”. In addition, the numbers of studies distinguish between “good stress” (eustress), and “bad stress” (distress); while the former can be adaptive, Allostatic overload has no beneficial purpose and may trigger fitness consequences (McEwen & Wingfield 2003; McEwen 2005). This assessment focuses on distress, hereafter named “stress”.

3.5.2 Acute and chronic stress

Stress mainly are of two types; acute or chronic, which depend upon the duration and reoccurrence (Boonstra 2013; Clinchy, Sheriff & Zanette 2013), GCS can also extend temporarily (acute response) (Dantzer et al., 2014). While sometimes the stressor operates over longer periods, such as recurrent anthropogenic disturbance (Cabezas et al. 2007), the animal may additionally moreover take location continual stress responses (Dantzer et al. 2014), because the physique may additionally now not be successful to deliver returned the physiological stipulations to homeostasis among ordinary stressors.

3.5.3 Physical and psychological stress

The stress may be defined on the basses of the type of stressor, which triggers the stress. However, the stressors can be different throughout species, individuals, existence-levels, and conditions. Extensively talking, animals can show stress responses when they are at risk for his or her comfort (Predation threat, looking, capture, human disturbance), and when the environmental stipulations are sub-most desirable (Horrific trophic sources, insufficient temperatures). Numerous articles are using the term “Stress” to point out specific types of stressors triggering it. Wasser et al separate among physical and mental stress. Physical stress refers to stress delivered “outside” triggers, such as lack of food (Bastille-rousseau et al. 2015), direct disturbance (e. G. Seize-related stress; (Omsjoe et al. 2009) or temperature (Shrestha et al. 2014). on the other hand, mental pressure refers to stress-induced through

the animals' "internal" expertise of the "landscape of fear" (Laundré, Hernández & Ripple 2010).

3.5.4 A way to degree stress

Glucocorticoids (GCS) are a type of corticosteroids known as the "stress hormones", as they provide essential hormonal response sooner or later in both acute and chronic stress (Möstl & Palme 2002; Romero 2004; love et al., 2005; Arnemo & Caulkett 2007). However, high-level release ranges are associated with stress, as the fundamental position of the hormone is straight forward power regulation (Busch & Hayward 2009). Still, GCS concentrations are considerably utilized in ecological studies as proxies of stress (G. Millspaugh et al., 2001; Millspaugh et al. 2002; Ashley et al. 2011), and had been endorsed as "early caution signal" in conservation research (Dantzer et al. 2014). The GCS level measurement provides us with data at specific time scales. Methodological strategies for measuring GCS provided a source of several critiques (G. Keay et al. 2006; Sheriff et al. 2011; Dantzer et al. 2014). Most commonly, GCS are measured within the blood, saliva, excreta (feces and urine), and integumentary structures (hair and feathers). It is very important to underline that at the same time as blood and saliva samples furnish real-time stages of GCS (Sheriff, Krebs & Boonstra 2010a).

Therefore, an appropriate sample is important for extraordinary ecological questions, associated with acute or persistent responses. It's also important to note that methodological techniques yield exclusive effects, however, also the baseline glucocorticoids and stress reaction relies upon on more than a few elements such as species, people, intercourse, age magnificence, reproductive fame, season and ecological context (Huber, Palme & Arnold 2003; Keay et al., 2006; Bonier et al., 2009a; Jaatinen et al., 2013).

3.5.5 Physiological consequence of stress

The literature suggests that the body structure of stress is complex (Sapolsky, Romero & Munck 2000; Wingfield & Romero 2001; Arnemo & caulkett 2007), and therefore it will be summarized very briefly. In a stress situation, the principal hormonal response is the activation of the hypothalamus-pituitary-adrenal axis (HPA axis), which is the fundamental regulator of stress (Reeder & Kramer 2005; Boonstra et al., 2014). The sympathetic

demanding system activates an acute adaption, which initiates energetic conduct. The physiological responses are rapid; within 3 to 5 seconds, the heart rate may be doubled, the arterial blood pressure can extend to two times the normal ranges (Arnemo & Caulkett 2007). The activation induces the secretion of the stress hormones, such as glucocorticoids (GC) in the form of cortisol (e. G. For primates, carnivores, and ungulates) or corticosterone (e. G. Rodents, Birds, and Reptiles) (Romero 2004; Touma & Palme 2005). The GC secretion helps in the physiological and behavioral modifications that facilitate managing the environmental stressor. To maintains the homeostasis, GCS prompts a bad feedback mechanism that inhibits similarly preparation of the stress hormones (Pozzi et al., 2008). the number of animal studies shows that chronic stress and the HPA axis is activated for long intervals of time, to go back to homeostasis position (Boonstra 2004; Dantzer et al., 2014). In chronic stress, the human is characterizing as having: (i) better baseline GCS stages; better frequency of acute GC will increase the amount of time to return GC degrees lower back to baseline (Romero 2004; Wingfield & Romero 2015). If the animal is exposed to a stressor for an extended duration of time, it can additionally be acclimated, i.e. GC secretion might be decreased and the animal will no longer reply to the active stressor. Even though this could additionally appear useful, it might sincerely lead to facilitation. The animal could have a poorer capacity to address a new stressor than a non-acclimated animal. However, in this situation is very important that do not forget that the capability to address stress varies among e.g. Species, reproductive mode, stressor, timing, and duration of the stressor (Sheriff, Krebs & Boonstra 2010; Love, Mcgowan & Sheriff 2013). The prolonged activation of the hpa axis has more than a few physiological outcomes, together with the doable to suppress the immune system, boom, and spark off protein loss (Busch & Hayward 2009). At some stage in the prone maternal and neonatal stage, the non-genetic maternal programming of the offspring takes vicinity (Sheriff, Krebs & Boonstra 2010; Love, Mcgowan & Sheriff 2013). Lab studies on mammals also indicate and verified that maternal and neonatal stress could have a long-lasting hazardous problem by inflicting modifications within the programming and improvement of the offspring (Meaney, Szyf & Seckl 2007; Weinstock 2008; Mastorci et al., 2009) moreover it can be detected in maturity (Romero 2004; Sheriff & Love 2013). A lot of research confirmed that maternal stress led to offspring with more degree of plasma-free cortisol, larger sensitivity to stress, and lower immunity in adulthood (Sheriff, Krebs & Boonstra 2010). Offspring of lab animals exposed to maternal stress are inclined to depression, tension-like behavior, change

in skills improvement, and hpa function (Abe et al. 2007; Meaney, Szyf & Seckl 2007; Kapoor, Leen & Matthews 2008). Better GC degrees can additionally have an impact on metabolic costs and digestive methods, which likely can result in higher protection because of the reality of developing for age time and for that reason reducing resting time (Wingfield et al., 1998; Sapolsky, Romero & Munck 2000). Given the importance of the subject, (Love, Mcgowan, and Sheriff 2013). While for the immune function regulates thru hypothalamic-pituitary-adrenal axis (HPA). When stress is occure than, the hypothalamus releases the corticotrophin-releasing factor (CRF) into the anterior pituitary system, which release the adrenocorticotropic hormone (ACTH) into the blood stream. ACTH stimulates the production of glucocorticoids (corticosterone in mice and cortisol in people) in the cortex of the adrenal medulla (McClennen et al., 1998; Jezova et al., 1999), which are then released into the blood streame. Morever, the nervous system modifies intestinal immunity thru the sympathetic autonomic nervous system by triggering the release of catecholamines (adrenaline and noradrenaline) from the adrenal gland medulla (Kvetnanský, 1995; Jezova et al., 1999)

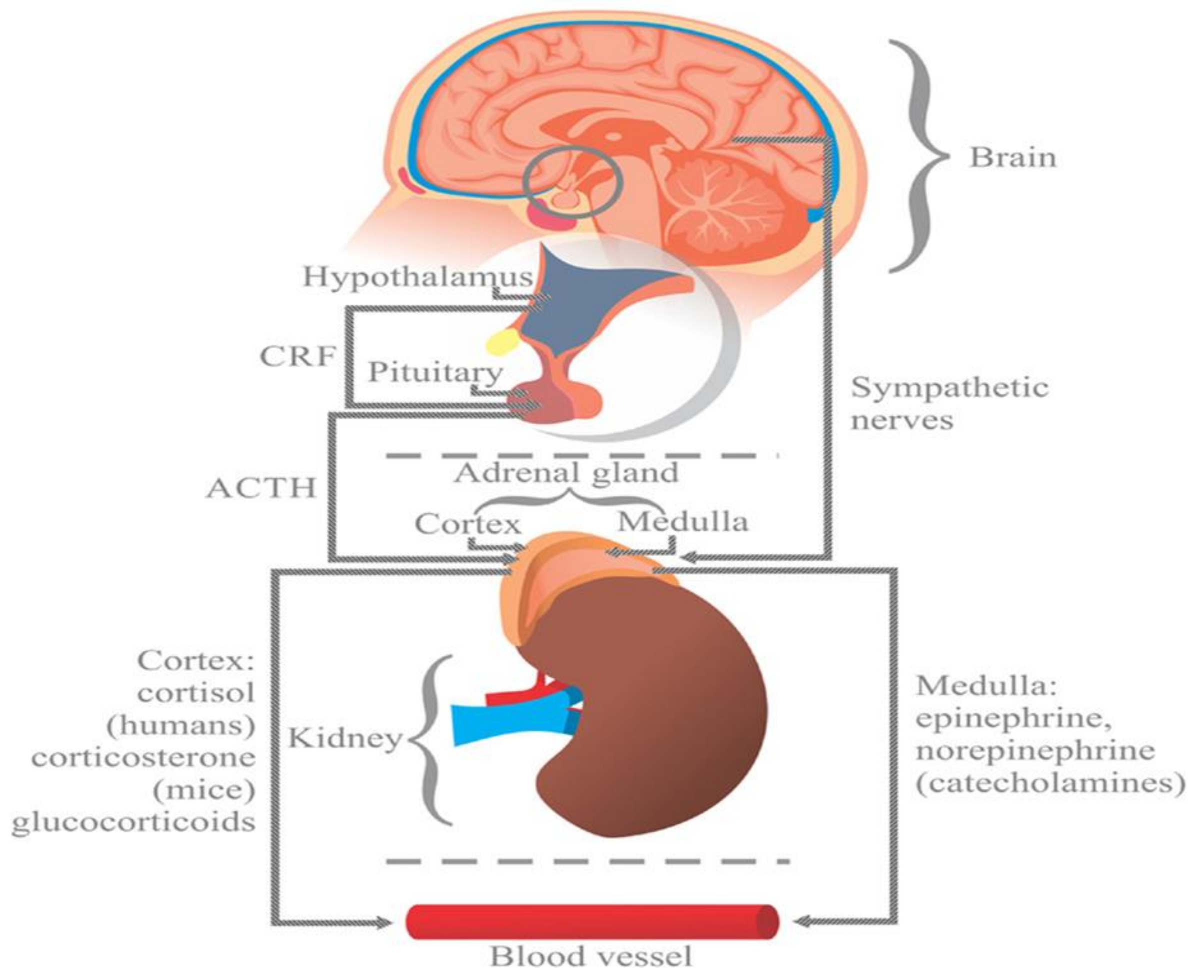

Figure 5 In response to stress, the hypothalamus (H) releases the corticotrophin releasing factor (CRF) into the anterior pituitary (P), causing the release of adrenocorticotropic hormone (ACTH) into the blood flow. ACTH stimulates the generation of glucocorticoids (cortisol in humans and corticosterone in mice) in the cortex of the adrenal gland (A), which are then released into the blood. Stress also activates the autonomic sympathetic nerves in the medulla of the adrenal gland to elicit the production of catecholamines, norepinephrine and epinephrine, which are then released into the blood. Glucocorticoids and catecholamines influence the generation of interleukins, which are involved in the viability and proliferation of immunocompetent gut cells via receptors (the figure and legend is taken from Campos-Rodríguezetal et, al., 2013)

3.5.6 Stress and the brain

The ancient results of stress (Thierry et al., 1968) has been shown that it has many complex effects on the human body system and can be a reason for structural changes in the human brain (Lupien et al., 2009). Chronic stress can cause atrophy and also effect on memory (Sarahian et al., 2014). These structural changes occur in the various brain area in the

response of stress, like cognition and memory (Lupien et al., 2009). The strength and intensity of the stress depend upon the level of stress and period of stress (Lupien et al., 2009).

3.5.7 Stress and Memory

Memory is one of the most important factors of the CNS and it is also categorized as sensory, short term of time, and also a long period. Short-term memory is dependent on the feature of the frontal and parietal lobes, at the same time as long-term memory is predicated upon at the characteristic of the memory (Wood et al., 2000). However, the entire function of memory depends on the hippocampus area (Sco- Ville and Milner, 1957; Asalgoo et al., 2015). Therefore, the connection between the hippocampus and stress had been debated (Asalgoo et al., 2015; Lupien and Lepage, 2001). In 1968, it changed into tested that there were cortisol receptors inside the hippocampus of rats (Mcewen et al., 1968). Later, in 1982, agonists of glucocorticosteroid and mineralocorticoid receptors, the life of these receptors inside the memory and hippocampus of the rat brain become as soon as validated (Veldhuis et al., 1982). It also is noted that the amygdala may be very essential to assessing the emotional reviews of memory (Roozen- daal et al., 2009). Numerous studies also indicate that stress can cause functional and structural adjustments inside the hippocampus a part of the brain (Mcewen, 1999). Those structural changes consist of atrophy and neurogenesis problems (Lupien and Lepage, 2001). Glucocorticosteroids can bring about those adjustments with the useful resource of both affecting the cellular metabolism of neurons (Lawrence and Sapolsky, 1994), or developing the sensitivity of hippocampus cells to stimulatory amino acids (Sapolsky and Pulsinelli, 1985) which increasing extracellular glutamate release (Sapolsky and Pulsinelli, 1985). Animal studies have demonstrated that stress can motive a reversible bargain in spatial memory because of the atrophy of the hippocampus (Luine et al., 1994). In reality, immoderate plasma concentrations of glucocorticosteroids for prolonged duration time can cause atrophy of the hippocampus (Issa et al., 1990). Additionally, human beings with both Cushing syndrome (with an improved secretion of glucocorticosteroids), or people who receive high dosages of exogenous synthetic anti-inflammatory pills, are determined to have atrophy of the hippocampus and associated memory problems (Ling et al., 1981). Several human research has suggested that even frequent healing doses of glucocorticosteroids and dexamethasone can motive issues with express memory (Keenan et al., 1995; Kirschbaum et al., 1996). Moreover, adrenal steroids cause alteration in long-time period potentiation (LTP)

which is a crucial method in memory formation (Bliss and lømo, 1973). The first is noradrenaline, which creates emotional factors of memory inside the basolateral amygdala region (Joëls et al., 2011). Secondly, this method is facilitated through corticosteroids. However, if the release of corticosteroids happens earlier, it inhibits the amygdala and corresponding behaviors (Joëls et al., 2011). Therefore, there may be mutual stability between these hormones for growing a reaction in the memory way (Joëls et al., 2011). Those prerequisites embody nonfamiliarity, non-predictability, and lifestyles-threatening constituents of imposed stimulation. It has been also recommended that stress can sharpen memory in a few situations (Schwabe et al., 2010). As an instance, it has been demonstrated that having to take a written examination can beautify memory for a short period in examination contributors. This condition is related to a restriction inside the level of cortisol inside the saliva (Vedhara et al., 2000). Other studies have confirmed that stress before learning occurs can also cause both an enlarge inside the strength of memory (Domes et al., 2002; Schwabe et al., 2008), or lessen inside the capability for memory (Diamond et al., 2006; Kirschbaum et al., 1996). The technique of strengthening memory is typically strengthened after stress (Schwabe et al., 2012). Diverse studies on animal and human models have shown that the administration of both glucocorticosteroids and stress speedy after studying has come about lets in memory (Schwabe et al., 2012). In addition, it has been confirmed that glucocorticosteroids (no longer mineralocorticoids) are vital to enhancing studying and memory (Lupien et al., 2002). However, the retrieval of activities in memory after stress can be reduced (Schwabe et al., 2012), which can also give up the result from the opposition of updated records for storage in memory in a traumatic country (De kloet et al., 1999). In summary, it's been concluded that the impact of stress on memory is highly based totally at the time of demanding stimulus and, the timing of the imposed stress, the memory may be both higher or worse (Schwabe et al., 2012). Furthermore, current studies have shown that using a selected time desk of exposure to stress now not influence hippocampus-dependent memory, but additionally striatum-dependent memory, which highlights the character of the timing of the imposed annoying stimulus (Schwabe et al., 2010).

3.5.8 Stress and cognition

Cognition is another important factor in brain function. Cognition functionality appreciation of perceived stimuli and its interpretation, which includes learning of, preference making,

interest, and judgment (Sandi, 2013). Stress has an effect on cognition that relies on its intensity, length, and importance (Sandi, 2013). Just like memory, cognition within the hippocampus, amygdala, and temporal lobe (Mcewen and Sapolsky, 1995). In reality, stress activates a few physiological structures, along with the autonomic nervous system, relevant neurotransmitter and neuropeptide system, and the hypothalamus-pituitary-adrenal axis, that have direct results on neural circuits inside the brain concerned with records processing (Sandi, 2013). Activation of stress results in the production and release of glucocorticosteroids. Because of the lipophilic nature of glucocorticosteroids, they can pass through the blood-brain barrier and exert long-time period consequences on processing and cognition (Sandi, 2013). Being uncovered to stress can cause pathophysiologic modifications within the brain, these adjustments can be manifested as behavioral, cognitive, and mood difficulties (Li et al., 2008). Research has shown that continual stress can purpose problems together with improved iL-6 and plasma cortisol, however, reduced quantities of camp responsive factor binding protein and mind-derived neurotrophic issue (BDNF), which may be very similar to what's discovered in people with despair and mood disorders that show a large range of cognitive problems (Track et al., 2006). Additionally, the improved concentrations of inflammatory elements, like interleukins and TNF-α (which play an essential function in growing cognitive problems), proves a physiologic relationship among stress and temper-based cognitive troubles (Solerte et al., 2000; Marsland et al., 2006; Li et al., 2008). The research suggested that cognitive problems resulting from stress can be created because of neuroendocrine, neuro amine factors, and neurodegenerative procedures (Li et al., 2008). However, it should be cited that depression also cannot continually be because of the over activation of the physiological-based stress reaction (Osanloo et al., 2016). Cognitive problems following publicity to stress had been stated in past research (Lupien and Mcewen, 1997). Stress has consequences on cognition both acutely and chronically (Mcewen and Sapolsky, 1995). Acute outcomes are widely speaking prompted with the source of beta-adrenergic consequences, whilst continual consequences are induced in a protected-time period manner through modifications in gene expression mediated via steroids (Mcewen and Sapolsky, 1995). In many mechanisms modulate the results of stress on cognition (Mcewen and Sapolsky, 1995; Mendl, 1999). For instance, adrenal steroids have an effect on the function of the hippocampus in some unspecified time in the future of cognition and memory retrieval in a biphasic manner (Mcewen and Sapolsky, 1995). In chronic stress, these steroids

can break neurons with different stimulatory neurotransmitters (Sandi, 2013). Exposure to stress can moreover cause hippocampus-related cognition; particularly, spatial memory (Borcel et al., 2008; Sandi et al., 2003). Moreover, stress can reduce the genesis of neurons within the dentate gyrus region of the hippocampus (Gould and Tanapat, 1999; Okayöhler et al., 2010). Even though age is a factor that appeared to affect cognition, studies on rats have proven that younger rats uncovered to excessive doses of adrenal steroids show the same degree of decline of their cognition as adult rats with regular plasma concentrations of glucocorticoids (Landfield et al., 1978). Additionally, a lower in the secretion of glucocorticosteroids enhance the spatial memory in adults and has been proven to have neuroprotective results (Montaron et al., 2006). Other studies have proven that stress consequences in numerous outcomes on cognition. As an instance, the injection of hydrocortisone on the time of its most plasma concentration (in the afternoon) improves the cognition and memory (Lupien et al., 2002). In summary, it is believed that slight stress allows an enhancement in cognitive features, particularly in the case of data or verbal memory. However, if the depth of stress passes beyond a predetermined threshold, it motives cognitive disorders, specifically in memory and judgment. The disruption to memory and judgment is because of the effects of stress on the hippocampus and prefrontal cortex (Sandi, 2013). Of path, it has to be found out that factors like age and gender might also play a position in a few cognitive issues (Sandi, 2013). Importantly, it must be emphasized that special human beings might also additionally display off various responses in cognition while uncovered to the very equal stressful stimulus (Hatef et al., 2015).

4 Results

4.1 Identification of vulnerable and resilient population of animals by the sucrose preference test following foot-shock stress

An interesting scientific question related to the dynamics of stress that is still to be answered is the dissection of the short- and long-term outcome of acute stress events to understand how the brain stress response may generate maladaptive conditions and consequently pathological changes.

Most of the pre-clinical studies using animals and experimental stress paradigms generally look at the endpoint of several adaptive changes occurring during the stress response, without understanding how does the physiological system shift into a maladaptive and pathological pathway. Moreover, it would be very important to understand whether and how the brain stress response differently behaves depending on different exposed individuals, thus generating resilient and vulnerable subjects within the stressed population (Musazzi et al., 2017).

Assuming this hypothesis as a first experimental task of the project we decided to investigate the possibility of generating two populations of stress-response subjects after the application of a well-established of acute stress protocol: the foot-shock stress paradigm.

To this aim, we performed the sucrose preference test, before and after the foot-shock protocol, to identify rats that were vulnerable to stress and those that were resilient. The sucrose preference test is a reward-based test using a two-bottle choice paradigm (one containing 1% sucrose solution and the other bottle containing water) to evaluate animals anhedonia that can be induced by stress (Liu et al., 2018).

To this purpose, all rats underwent the preference sucrose test for 7 weeks (one session every 48 hours) to quantify preference baseline values. At the end of this period, rats were subjected to a foot shock stress protocol (total test time 40 min; 0.8 mA electric shock total time 20 min with random inter-shock length between 2 and 8 sec; Bonanno et al., 2005; Musazzi et al., 2010, Treccani et al., 2014) and then trialled again in the sucrose preference test after 6 or 24 hours.

As shown in Fig. 6, six hours after the foot shock stress, 50 out of 70 rats (71%) were found vulnerable, that is they drunk much less sucrose solution than baseline, whereas the remaining 20 rats were resilient (29%), as they did not vary their intake of sucrose solution.

Interestingly, when the test was repeated 24 hours after foot shock stress (Fig. 7), we observed no significant change in the percentage of vulnerable rats (48 out of 70; 69%) and in the percentage of resilient (22 out of 70; 31%), suggesting that results at 6 and 24 hours are consistent.

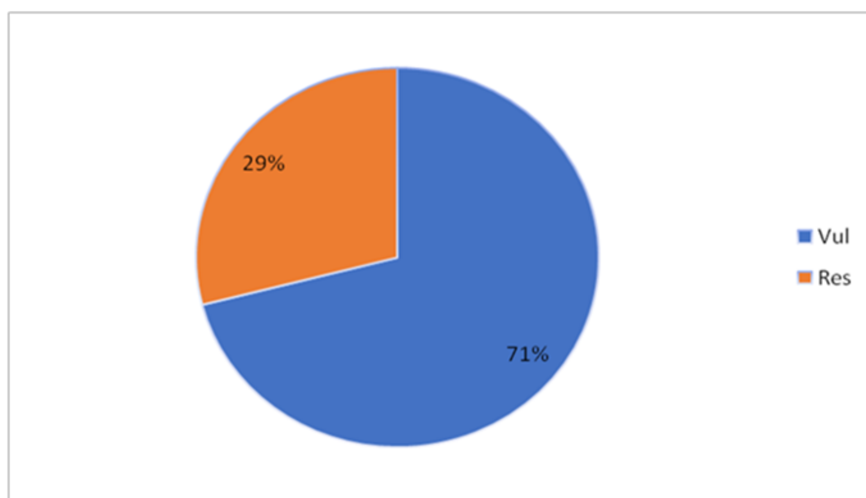

Figure 6: Percent distribution of vulnerable and resilient rats, 6 hours after a session of acute foot shock stress. Vulnerable and resilient rats have been identified using the sucrose preference test before and after a foot shock stress, as detailed in the "Materials and Methods" section.

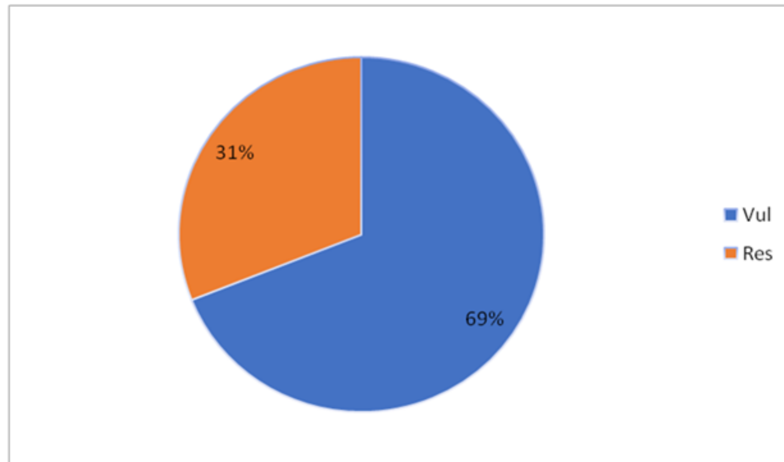

Figure 7: Percent distribution of vulnerable and resilient rats, 24 hours after a session of acute foot shock stress. Vulnerable and resilient rats have been identified using the sucrose preference test before and after a a foot shock stress, as detailed in the "Materials and Methods" section.

4.2 The impact of acute foot shock stress on glutamate release from gliosomes purified form rat prefrontal/frontal cortex

The effects of stress on glial cells are still not well investigated. In fact, although neuroglia represents more than 50% of brain cells and is recognized as having a primary role in modulating CNS activity (Parpura et al., 2012; Fields et al., 2015), so far research on stress neurobiology has only marginally focused its attention on it (Verkhratsky et al., 2014; Yamamuro et al., 2015), although the study of the alterations induced by the acute stress on these cells could be fundamental for understanding the mechanisms responsible for differences in responses as vulnerable or resilient animals.

Based on the above evidence, the second step of the project has been designed in order to investigate the effect of the acute stress paradigm on the glutamate glio-transmission. To verify whether glutamate release in the frontal/prefrontal cortex was affected by foot shock-induced stress, we planned to exploit the superfused gliosomes as an ex-vivo experimental model to study the release of glio-transmitter form subcellular astroglial particles that represent the perisynaptic astroglial processes of the tripartite synapse (Stigliani et al., 2006). Glutamate release from frontal/prefrontal gliosomes was monitored using the metabolism-resistant radioactive marker [3H]D-aspartate that is known to specifically label glutamatergic

nerve terminals. To depolarize gliosomes, we decided to use a medium containing 15 mM KCl, on the basis of previous evidence showing that this concentration was able to induce a significant release of glutamate that was mediated by exocytotic mechanisms (Paluzzi et al., 2007). All the experiments were carried out on rats at 24 hours post-stress. Controls (unstressed animals) were always run in parallel.

Under our experimental conditions, the spontaneous efflux of [3H]D-aspartate (expressed as fractional rate, see Materials and Methods) amounted to 1.57 ± 0.08 (mean ± SEM; n = 13 independent experiments), 1.77 ± 0.16 (n = 18 independent experiments) and 1.63 ± 0.13 (n = 12 independent experiments) in control, vulnerable and resilient rats, respectively.

Statistical analysis revealed that the spontaneous release of [3H]D-aspartate from prefrontal/frontal cortex gliosomes obtained from vulnerable rats was not significantly higher (+13% not significanty) than that measured from frontal/prefrontal gliosomes of control animals (Fig. 8). On the other hand, the spontaneous release of [3H]D-aspartate from frontal/prefrontal gliosomes obtained from resilient rats was not significantly different from controls (Fig. 8)

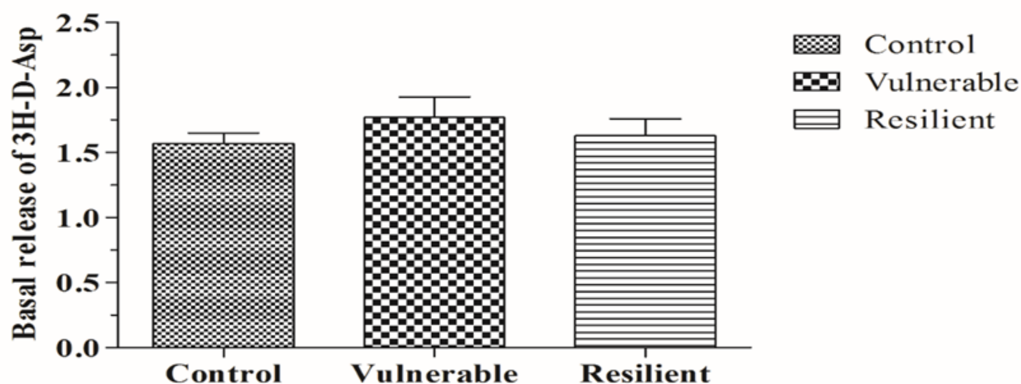

Figure 8: Basal release of [3H]D-Aspartate from purified prefrontal/frontal cortex gliosomes 24 hours after foot-shock stress. Data show that the spontaneous release of [3H]D-aspartate from prefrontal/frontal cortex gliosomes is not significantly changed in the three experimental groups (naïve, vulnerable and resilient rats exposed to foot shock stress. Purified gliosomes have been isolated by centrifugation on a Percoll® gradient, incubated with [3H]D-aspartate at 37 °C for 15 min and then subjected to superfusion at a flow rate of 0.5 ml/min. Spontaneou (basal) release has been evaluated by collecting one fraction of 3 minutes. Bars represent mean ± s.e.m. Data have been analysed by one-wayANOVA followed by Newman–Keuls post hoc test. For further technical details, see Materials and Methods

When gliosomes were challenged with a 90-sec depolarization using a medium containing 15 mM KCl, we observed a significant increase of [3H]D-aspartate release from all gliosomal populations that returned to basal level following repolarization. The calculated overflows (KCl-induced outflow - basal release; see Material and Methods) induced by depolarization in gliosomes of the different experimental groups were the following (mean ± s.e.m.): control rats 2.05 ± 0.15 (n = 10); vulnerable rats 2.65 ± 0.14 (n = 16); resilient rats 1.99 ± 0.17 (n = 10).

The statistical analysis revealed that the [3H]D-aspartate from purified prefrontal/frontal cortex (PFC) gliosomes 24 hours after foot shock stress shows that, the percent overflow induced by the chemical depolarization overflow was significantly higher (+30%; $p < 0.05$) in vulnerable, but not in resilient rats, when compared to controls. [3H]D-aspartate labelled gliosomes were depolarized in superfusion for 90 sec with a solution containing 15 mM KCl and then repolarized. Overflow in resilient rats was significantly lower ($p < 0,05$) than that in vulnerable rats.

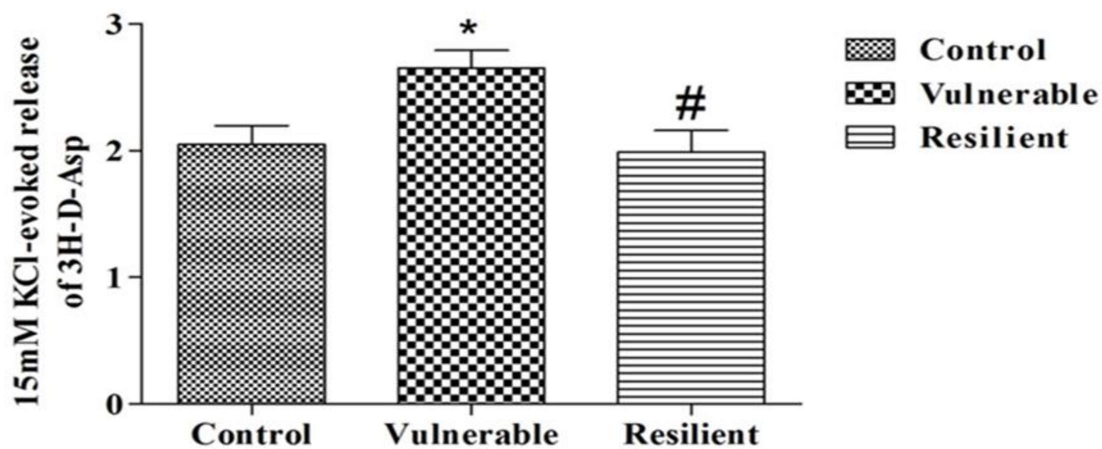

Figure 9. Depolarization-evoked release of [3H]D-Aspartate from purified prefrontal/frontal cortex gliosomes 24 hours after foot-shock stress. The graph shows that the percent overflow of [3H]D-aspartate induced by chemical depolarization from prefrontal/frontal cortex gliosomes is significantly higher in foot-shock stress vulnerable rats but not in resilient ones, when compared to controls. [3H]D-aspartate labelled gliosomes were depolarized in superfusion for 90 sec with a solution containing 15 mM KCl and then repolarized. Data have been analysed by one-way ANOVA followed by Newman–Keuls post hoc test. *p<0.05 vs control; #p<0.05 vs FS-vulnerable. For further technical details, see Materials and Methods.

Overall, our data indicate that foot shock-induced acute stress can produce plastic changes in the release of glutamate from astrocytes in the prefrontal/frontal cortex, as previously demonstrated in glutamatergic neurons of the same area (Musazzi et al., 2010). We followed the protocol of foot shock as acute stress to recognize a distinction presence of vulnerable and resilient strength of the rats and due to this fact we wanted to check whether there was any relationship between the behavioral features (resilience/vulnerability to acute stress events) and modifications of the glio-transmission. According to this protocol, we found, that there was no change in the glutamate release in both vulnerable and resilient rats. While when we depolarized for 90 sec with a superfusion solution containing 15Mm of KCL, the release of glutamate was significantly increased in vulnerable, while unchanged in resilient rats after 24 hours of foot-shock stress.

Moreover, these results are also highlighted the importance of using the sucrose preference test to identify vulnerable and resilient animals before investigating possible effects of stress on the various cellular and molecular processes of central neurotransmission.

5. Discussion

Maladaptive neurobiological responses associated with mood disorders have been studied extensively for decades and it has been shown that the failure to resolve a physiological stress response upon cessation of an acute stressful event may create a deleterious allostatic load, leading to stress vulnerability and enhanced risk of mood disorders (Charney, 2004; Goldstein and McEwen, 2002). On the other hand, adaptive physiological response to stress is crucial for survival in life-threatening situations. The dissection of the response and its readouts at the molecular, cellular, and/or behavioural level, could point out neuronal mechanisms and effectors that undergo pathological changes during the stress response, and could allow the identification of novel targets for treatment. Therefore, the study of the long-term outcome of acute stress may allow the identification of critical effectors and/or targets in the stress response and shed light on the pathophysiology of stress-related disorders. Understanding what distinguishes a vulnerable (maladaptive) from a resilient (pro-adaptive) response is a major goal of research on the pathophysiology of stress-dependent disorders.

Indeed, resilience is an integrated process involving multiple peripheral and central mechanisms that promotes an appropriate, non-pathological response to stress (Charney, 2004; Pfau and Russo, 2015; Russo et al, 2012).

In previous studies, it has been consistently shown that foot shock-induced acute stress is able to increase the release of glutamate from isolated nerve terminals (synaptosomes) purified from rat prefrontal/frontal cortex and subjected to chemical depolarization during superfusion (Musazzi et al., 2010; Tardito et al., 2010; Treccani et al., 2014). Under these experimental conditions, all possible indirect effects on neurotransmitter release (e.g. activation of release-regulating auto/heteroreceptors and re-uptake mechanisms by released neurotransmitters), which occur in other in vitro models such as brain tissue slices, are prevented, thus allowing the study of the direct impact of different pathological mechanisms on the release mechanisms. Different factors, such as hormones, neuroendocrine mediators, peptides, and neurotransmitters are involved in the response to stress especially if the stressor event is severe and prolonged. While animal models of chronic stress are often used to study neuropsychiatric pathology, investigation of the brain response to acute stress may be helpful in dissecting the molecular/cellular mechanisms involved in the stress response.

Indeed, also acute stress event can generate strong alteration the equilibrium of neurological circuits. Independently from the nature of the stress event (acute or chronic) there is always a link between physiological and pathological responses of the body, as in many biological phenomena, and the consequences could be also a function of different individual vulnerability. Thus, investigating the determinants of resilient versus vulnerable trajectories in the brain stress response represent a very interesting issue to be dissected (Musazzi et al., 2017).

During my PhD experience we exploit the foot-shock protocol as an acute stress event in order to experimentally discriminate the presence of vulnerable and resilient population of rats and subsequently we wanted to investigate whether there was a link between the behavioural traits (resilience/vulnerability to acute stress events) and molecular/functional changes at level of the tripartite synapse with particular interest to the glio-trasmission.

As a first step we investigated the effect of an acute stress paradigm, the footshock protocol, on the anhedonic behaviour of adult rats. Acute inescapable footshock stress is a rather simple protocol that is employed not only in one of the most widely used rodent models of depression, Learned Helplessness [Mallei, A. et al. (2011). Daskalakis, N.P. et al. (2013)].

The results coming out from this first part of the project demonstrate primarily that the footshock protocol represents a valid tool for mimicking the conditions of an acute stress event. This paradigm has generated an alteration of behaviour in a significant subset of the animals exposed to the 40 minutes footshock paradigm. On the other hand, our results also show that the sucrose test is a powerful tool to assess the vulnerability (showing anhedonic behaviour) to stress and that vulnerability and reliance can be detected as early as 6/24 hours after the foot shock stress. As expected, only part of the population of rats exposed to stress were vulnerable to the stress event (about 70% of individuals), while a smaller percentage was identified as resilient (about 30% of individuals). An interesting aspect is represented by the fact that the effects, measured by the sucrose test as anhedonic behavior, were already evident starting from 6 hours following the acute event. The sucrose test was also repeated at 24 hours post-stress, obtaining results that are definitely superimposable to the previous time point. The possibility of early identification of subjects who exhibit maladaptive behavior to the acute stress event represents a very important element in order to have a possible

pharmacological time window that can be effective in modulating the damage triggered an acute stress event.

Considering the molecular and functional consequences of acute tress, little is known of the fast changes induced by a stressor at synaptic level, likely mediated by non-genomic mechanisms, that involve not only the neuronal counterpart, but also the glia astrocytic component of the tripartite synapses. On this context, astrocytes have long been thought as essentially unexcitable cells that do not contribute to information transmission and processing in the brain. However, the research of the last twenty years has demonstrated that they actively participate in cell-to-cell signalling in the central nervous system, being equipped with membrane receptors to receive different inputs from neurons and with the exocytotic machinery to release gliotransmitters able, in turn, to modulate neuronal activity (Harada et al., 2016). In this view, glutamate, which is considered the major excitatory neurotransmitter of the CNS, is indeed also a gliotransmitter that is avidly taken up by astrocytes and, upon different types of stimulation, is released in the synapse.

As a matter of fact, a large body of evidence, in particular from the laboratory where I have carried out my doctoral research, has demonstrated that glutamate release can be elicited by different types of stimulation (e.g. chemical depolarization, membrane receptor activation, calcium ionophores, etc.) from gliosomes in superfusion in both physiological and pathological conditions (Stigliani et al., 2006; Pedrazzi et al., 2006; Patti et al., 2007; Bonanno et al., 2007; Paluzzi et al., 2007; Raiteri et al., 2008; Milanese et al., 2009, 2010; Marte et al., 2010; Bari et al., 2011; Salamone et al., 2014; Cervetto et al., 2015). Gliosomes are astrocyte-derived resealed particles that, like synaptosomes, are formed during the conservative homogenization of brain tissues and that can be easily isolated and purified.

Because it has been previously identified the rapid enhancement of depolarization-evoked glutamate release as one of the first consequences of acute stress in synaptosomes of frontal/prefrontal cortex (PFC/FC), we hypothesise that sustained enhancement of glutamate release may also occur at the level of the peri-synaptic astroglial processes that surround the glutamatergic synapse. With this purpose we planned to assess the glutamate release from gliosomes prepared from the PFC/FC. In order to choose the appropriate stimulus, preliminary experiments were conducted to verify the overflow evoked by the 15 and the 25 mM KCl. The 15 mM KCl stimulus was able to induce an exocytotic like release of [3H]D-Asp

from gliosomes with a minimal contribution of the carrier-mediated release (data not shown), thus the 15 mM KCl was the concentration used for the superfusion experiments as a physiological depolarizing stimulus.

To verify, after 24h, the impact of the acute stress on glutamate glio-transmission we measured the basal release and the 15 mM KCl- evoked [3H] D-Asp overflow from gliosomes prepared from vulnerable and resilient rats and the data were compared to those obtained from control rats. As previously demonstrated in synaptic nerve terminals form PFC/FC (Musazzi et al., 2010), also for the gliosomal subcellular fraction obtained from the PFC/FC of rats, the spontaneous release of glutamate was not affected by the footshock event, thus indicating that, although there is a not significant increasing trend, the machinery involved in the basal release of glio-transmitter seems to be not sensitive to the possible plastic changes induced by the acute stress in the group of vulnerable animals. On the other hands, the 15 mM KCl-evoked glutamate release from gliosomes results higher in gliosomes purified form PFC/FC of vulnerable animals compared to control and resilient groups. This data is very interesting since it demonstrates that the vulnerable or resilient behaviour identified by the sucrose test clearly correlates with a synaptic change the involves an unbalanced release of glutamate, as a glio-tranmitter, from the peri-synaptic processes (gliosomes). This is the first time that has been demonstrated the functional involvement of astrocytes with an alteratered glio-transmission subsequent to an acute stress event. Moreover, these preliminary results pave the way for further studies aimed at dissecting the role of astrocytes in the complex scenario of the stress response. For instance, it would have been interesting to show whether vulnerable rats produce an excessive glutamate release, when exposed to a releasing stimulus, also 6 hours after acute stress. These experiments have not performed yet because those aimed at evaluating the effects of ketamine treatment (see below: Futeure directions) has started first, although they strongly deserve our attention.

The evidence obtained until now with acute stress protocols have completely changed the canonical distinction between the effects of acute versus chronic stress. Moreover it appears that a single exposure to stress may have long-term functional (glutamate release) consequences involving not only the neuronal counterpart of CNS, but also the glial cells, in the name of astrocytes with their astroglial processes that compose the tripartite synapse and contribute to the homeostasis of the synaptic cleft. The sustained stress-related changes

may be relevant for the pathophysiology of neuropsychiatric disorders and suggest how acute negative events may affect the stress responses. In the next future, the fine dissection of the short- and long-term effects of acute stress could enable researchers to identify key determinants of positive versus maladaptive responses of single individual. Moreover, it would also be very interesting to test the effect of ketamine and other experimental drug that affects the glutamatergic transmission as suggested approaches to prevent the short and long-term effect of PTSD and other disorders. In the case of effective outcome in a time-dependent way, these pharmacological interventions could represent an attractive alternative for a prophylactic treatment against the onset of psychopathology.

6. Future directions

In future we planned to investigate the following issues:

1] To investigate, at 6 and 24h after the footshock stress paradigm, possible histological changes and the level of reactive astrocytes in the PFC/FC of vulnerable and resilient rats versus control animals and check whether these changes correlate with the vulnerable/resilient phenotype of rats.

2] To test the long-lasting effect of footshock stress, in terms of anhedonic behaviour, 48 and 72 hours after the stressor event.

3] To test the effect of ketamine, administered after 6 or 24h after the acute stress, in preventing the altered glutamate release from gliosomes purified from the PFC/FC of vulnerable rats.

4] To test the effect of ketamine, administered after 6 or 24h after the acute stress, in preventing the possible histological changes induced by the footshock stress paradigm in vulnerable and resilient rats versus control animals.

SECOND PART

1. The concept of RECEPTOR

Thanks to his experiments on salivary secretion in dogs, the physiologists J.N. Langley suggested the existence of substance or substances with which pilocarpine and atropine form 'compounds'. Later, in 1905, he described the effects of nicotine and curare on skeletal muscles using the expression "receptive substance". A.V Hill, student in Langley's lab, anticipated in his paper the concept of theory.

In 1926, Clark and Gaddum published two independent papers on the actions of acetylcholine and atropine on the frog's isolated heart and the actions of adrenaline and ergotamine on the rabbit uterus. They tried to interpret data obtained using the existing physicochemical hypothesis which, however, resulted not predictive. Thus, Clark and Gaddum were the first to introduce the log concentration–effect curve, which has become an icon of pharmacology (Rang HP., 2006)

The concept of receptor implies the idea of formation of precise complexes of drugs with molecules in the target cells, thereby eliciting a cell response. From then, the receptor molecules were biochemically identified, and their structures were studied, in order to enlighten their key role in physiology. More than 4% of the human genome encodes the receptors (Lefkowitz Rj., 2007).

Most important principle 0f the receptor round the ligand-gated ion channels and GPCRs. in the case of ion channels, the use of single-channel recording has been important as an approach for observing the behavior of single receptor molecules in real-time at an excessive degree of temporal resolution. With GPCRs, strategies for gazing receptor function directly are much less properly developed, even though the use of fluorescence techniques to check out agonist-induced conformational modifications may also have considerable potential. In most cases, researchers have to infer what they can from measurements of binding, and a range of downstream functional changes, such as GDP/GTP exchange, modifications in enzyme activity, protein phosphorylation, levels of intracellular 2d messengers, membrane currents, modifications in gene expression, etc, Such research have resulted in many beneficial flow charts representing postulated pathways and interactions, but, assigning values to the variety of price and equilibrium constants to allow quantitative modeling is rarely possible (Rang HP., 2006).

1.2 Families of receptors

There are different type of receptors families (Humphrey PPA, Barnard EA. 1998), which are divided into matrix receptors (e.g., integrin), ligand-gated (76 members in the human genome) and voltage-gated (143 members) ion channels, intracellular receptors (e.g. nuclear hormone receptors, 48 members), enzyme-linked receptors, (e.g receptors tyrosine kinases, 58 members), and the G protein-coupled receptors (GPCRs).

1.3 G protein coupled receptors (GPCR)

In mammals, GPCRs represent one of the most important and largest integral membrane protein families, playing a very crucial role in all physiological processes. These receptors are increasingly attractive drug targets and their role in diseases (including metabolic imbalances, diabetes, obesity, cancer, cardiac disorders, inflammatory disorders, Alzheimer's disease, and psychiatric disorders (Wise A. et al., 2002) is increasingly recognized. Their high druggability provides a valid reason for the continuous efforts in drug discovery and development (Tautermann, 2016).

In humans, more than 800 genes code for this receptor family. Based on sequence, structural and function the GPCRs are subdivided into 6 essential groups: class A (the biggest group, the rhodopsin-like receptors), class B or secretin receptors, class C, the metabotropic glutamate receptors (mGlu), Class D or fungal mating pheromone receptors, Class E or cAMP receptors, and Class F frizzled (FZD) and smoothened (SMO) receptors (Lee et al 2018). GPCRs have a very special unique and highly conserved structure (Farran B. 2017; Guidolin D et al., 2018), with 7 transmembrane domains (TM; α-helixes) that span the plasma membrane and are linked each other through 3 extra- and 3 intracellular loops (ECL and ICL, respectively). Interhelical bonds and hydrophobic interactions between relatively identical sequences provide the stability of the TM region, while the extracellular domain shows several structural differences among the different GPCRs. In the class C, this domain is very long and hosts the ligand-binding pocket (Kunishima N., 2000) while in others classes (most of the class A GPCRs) the ligand-binding site is placed in the extracellular half of the TM bundle (Lee S. M., et al., 2015). Whenever the ligand binding occurs, it induces a structural rearrangement (Manglik and Kruse; Schrage and Kostenis, 2017) and this conformational changes of the TM core allow the GPCR activation or inactivation and coupling to downstream signalling pathways. In fact,

both in vivo and in vitro experiments have confirmed that monomer GPCRs recognize and decode chemical or physical signals (e.g. rhodopsin, β2-adrenergic, and μ-opioid receptors (Bayburt T.H., 2007; Kuszak A.J., et al., 2009). In addition, GPCR monomers have an intrinsic plasticity; in fact, different signal transduction patterns (Goupil E., et al., 2012), such as G protein and/or arrestin pathways (Zidar D.A., et al., 2009).Moreover, many other sites are involved in the control of the receptor activation/inactivation monomers can assume multiple active conformations after the ligand binding, thereby initiating switching addition to the orthosteric, binding sites: allosteric, bitopic, and biased ligand binding sites, but also homo– or hetero-oligomerization of receptors.

1.4 Receptor- receptor interaction (RRI)

GPCRs exist or function not only as monomers, but also as dimers, and/or higher-order oligomers, including homo– or hetero– dimers/oligomers (Guo et al., 2017). X-ray crystallography provided the structural information on GPCR dimers or higher-order oligomers. Fraser and Venter (1982) and Paglin and Jamieson (1982) demonstrated the GPCR dimerization; the discovery of the GABAB receptor heterodimer opened the new field of the RRI (Marshall FH, Jones KA, Kaupmann K, Bettler B. GABAB receptors – the first 7TM heterodimers. Trends Pharmacol Sci. (1999) 20:396–9. 10.1016/S0165-6147(99)01383-8). Liang and colleagues (2003) reported the first higher-order crystal structure of Rhodopsin in native membranes; then several structures of class A GPCRs were elucidated (Lee et al., 2018). In the last years, increasing evidence supported the existence of receptor complexes formed by GPCRs: the number of identified GPCR RRIs is high and continuously increasing (see an update on the physiological and therapeutic relevance of GPCR oligomers. Farran B Pharmacol Res. 2017 Mar; 117():303-327)

At the present time, GPCRs become a basic focal point of research aimed at characterizing RRI, with specific regard to the CNS. In fact, the formation of receptor complexes is considered to play a key role in the neurophysiology (Farran B., 2017), and in the prominent discipline of "connectomics" (Guidolin D., et al., 2017). On the basis of RRIs, the integration of the input signals, already at the level of the plasma membrane, can notably make a contribution to the tuning of synaptic strength and the effectiveness of intercellular communication. Moreover, receptor complexes might play important roles in neuropsychopharmacology (Farran B.,

2017; Guidolin et al., 2015; Borroto-Escuela D.O., et al., 2017; Fuxe K., et al., 2016), and have turn out to be attractive potential objectives for the improvement of novel therapeutic strategies in serious illnesses of the CNS, such as depression and schizophrenia (Fuxe K., et al., 2013; Sahlholm K., et al., 2018), Parkinson's disorder (Fuxe K., et al., 2015), addiction (Gomes I., et al., 2013), eating problems (Kern A., et al., 2012), and neuropathic pain (Bushlin I., et al., 2012). GPCR homomers and heteromers, however, are being discovered also in cell types different from the central neurons, and evidence for receptor oligomerization is now not limited to GPCRs.

1.5 GPCR complexes in Peripheral Cells and Tissues

The GPCR complexes have been given lot of attention in the CNS, where GPCRs play very important roles in physiology and pathology, as compared to the peripheral tissues. Available examples are summarized in Table 1. Many researches show that angiotensin II type 1 receptors (AT1) are of particular interest (Tòth A.D., et al., 2018). AT1 has a key central position in vascular homeostasis, in the structural and functional integrity of the arterial wall; however, it is also implicated in the pathogenesis of hypertension (Daugherty A., et al., 2004; Hunyady L., et al., 2006). AT1 has been suggested to heterodimerize with a variety of other GPCRs (Tòth A.D., et al., 2018), and cross-regulation has been found to arise among angiotensin II and different signalling pathways.

Table 1: Examples of GPCR complexes in peripheral cells and tissues

Cell or Tissue	*Receptor complex*	*References*
Cardiomyocytes	AT1-β2	Barki-Harrington L., et al., 2003
Renal mesangial cells	AT1-β2	AbdAlla S., et al., 2005
Smooth muscle cells	AT1-P2Y6	Nishimura A., et al., 2016
Sympathetic neurons	AT1-α2c	Bellot M., et al., 2015
Stellate hepatic cells	AT1-CB1	Rozenfeld R,, et al., 2011
Gonads	LHR-LHR, FSHR-FSHR, LHR-FSHR	Urizar E., et al., 2005; Mazurkiewicz J.E., et al., 2015
Pancreatic β islet cells	GHSR-SST5A	Park S., et al., 2012
Carotid body	A2B-D2 (putative)	Porzionato A., et al., 2018
Cancer cells	GHSR-NTS1; CB2-GPR55	Takahashi K., et al., 2006; Moreno E., et al. ,2014

From Guidolin D, et al., 2019

The heteromerization of AT1 receptor seems to involve the fourth to seventh TM domains of the receptor (Young BM, et al., 2017), and a specific DRY ligand-binding motif of the receptor AT1 is likely to be involved in the functional activation of signalling from oligomerized AT1 (Szalai B, et al., 2012). Also, the existence of heterodimers between AT1 and β-adrenergic receptors in cardiomyocytes and associated cells lines has been reported (Barki-Harrington L, et al., 2003); antagonists towards a single receptor (AT1 or β-adrenergic receptor antagonist) were proven to inhibit both the receptors. Furthermore, AT1 was proposed to play roles in hypertension by forming complexes with the β2 bradykinin receptor (AbdAlla S, et al., 2005). The receptor proved to interact with purinergic P2Y6 receptors in mouse smooth-muscle cells (Nishimura A, et al., 2016), while the physical interactions with the apelin receptor has been shows to be able to regulate the effects angiotensin II in mouse models of atherosclerosis

(Chun HJ et al., 2008). The receptor AT1 was also reported to form complexes with the A2C adrenergic receptors in sympathetic neurons: dual occupancy of the protomers by the agonists caused abnormality in Gs-cAMP-PKA signalling, promoting NA hyper secretion (Bellot M, et al., 2015). All these finding indicate that receptor complexes involving the AT1 receptor might function as target in cardiovascular diseases (Fuxe K, et al., 2010; see also Guidolin et al 2019) including hypertension and preeclampsia (Quitterer U, et al., 2004; Ariza AC, et al., 2007).

The AT1 receptor not only can contribute to the blood pressure regulation, but also might play important roles in the development of fibrosis in different organs (Tòth AD, et al., 2018). For instance, it is also well-established that AT1 activates hepatic cells (Friedman SL et al., 2008) and increase profibrogenic markers in hepatic stellate cells of rats chronically administered with ethanol; the latter effect is abolished by the cannabinoid CB1 receptor antagonists. The interactions between these two (CB1 and AT1) receptors, and the CB1-AT1 heteromerization in hepatic cells has been validated by co-localization, co-immunoprecipitation and BRET assays (Rozenfeld R, et al., 2011). The study of the heteromer signals has shown that AT1 receptor agonists induced a rapid, dose-dependent increase in ERK1/2 phosphorylation, which is potentiated by mean of CB1 receptor agonists and blocked by CB1 antagonists, suggesting that CB1-AT1 heteromers might play very important roles as target in the treatment of liver fibrosis.

The endothelin and serotonin (5HT) receptors, expressed in different cardiovascular tissues, play roles in the regulation of the cardiovascular system (Kamal M., et al., 2011). In vitro results show that they could be involved in receptor complexes (Evans N.J., et al., 2008; Derange M., et al., 2010). However, their involvement in heteromerization in native cells and tissues needs to be assessed.

Recently, receptor complexes have been hypothesized (Porzionato A., et al., 2018) to exist in the carotid body, and have a role in hypercapnia, hypoxia, hypoglycaemia and acidosis, to trigger the adequate cardiovascular and respiratory responses. This hypothesis is primarily based on the massive repertoire of expressed GPCRs (most of which have been reported to form receptor complexes in other tissues), and on some evidence of the existence of GPCR complexes in the carotid body. Specifically, in the carotid body type I cells the RRI between dopamine D2 and adenosine A2B receptors has been suggested. On the other side, A2B

receptor antagonists counteracted the increased catecholamine release triggered by the D2 antagonists (Conde S.V., et al., 2008; Conde S.V., et al., 2009).

In the endocrine system, where GPCRs have significance roles (Kamal M, et al., 2011; Jonas KC et al., 2017), evidence are provided of GPCR oligomerization (Kleinau G, et al., 2016). For instance, studies show that GPCR heterodimerization play important roles in reproduction, including the secretion of hormones and the growth and maturation of follicles and oocytes (Satake H., et al., 2013). Luteinizing hormone (LH), which is secreted by the adenohypophysis, stimulates testosterone production in Leydig cells in males, and in women triggers ovulation by acting at LH receptor (LHR), a class A GPCR. Biophysical and pharmacological assays have proven that LHR homomers exhibiting negative co-operativity (Urizar E., et al.,2005; Rivero-Muller A., et al., 2010). In the regulation of pubertal maturation and of the reproductive processes, LH acts in collaboration with follicle-stimulating hormone (FSH). The hormone FSH is also produced by the anterior pituitary and binds the FSH receptor (FSHR), a class A GPCR. Crystallographic data shows that FSHR has a dimeric structure and that a FSH dimer bridges the dimeric FSHR (Fan Q.R., et al., 2005): TM of FSHR are involved in the stabilization of constitutive dimers (Guan R,, et al., 2010). The BRET assay and fluorescence correlation spectroscopy also revealed heteromers between LHR and FSHR, in which heteromerization leads to increased ligand dissociation and reduction of cAMP production (Feng X., et al., 2013). LHR-FSHR receptor complexes play a possible physiological significance in females, as during the peri-ovulatory period co-expression of these receptors mainly occurs in granulosa cells (Jonas K.C., et al., 2017).

GPCR heteromers might also affect glucose metabolism, as indicated by FRET-based studies demonstrating heteromerization of growth hormone secretagogue receptor (GHSR) and somatostatin 5A receptor (SST5A) in β islet cells of the pancreas (Park S., et al., 2012). The heteromerization changed the preferred G protein coupling of GHSR from Gaq11 to Gai/0, mediating the inhibition of the glucose-stimulated insulin secretion evoked by ghrelin and somatostatin.

Moreno and co-workers (2014) suggested a GPCR heteromer-based strategy in oncology. This hypothesis based on the discovery that the cannabinoid CB2 receptor and the GPCR55 (GPR55) are overexpressed in most cancer cells and human tumours and that they can form heterodimers showing inhibitory CB2-GPR55 interactions.

Moreover, it has been shown that GHSR and neurotensin receptor 1 (NTS1) can establish direct structural interactions in in vitro model, and neuromedin-U has been indicated as a ligand for this heteromer (Takahashi K., et al., 2006). Indeed, in non-small cell lung cancer, it has been suggested that GHSR-NTS1 heteromers are involved in an autocrine growth-promoting pathway (Takahashi K., et al., 2006). Although these data are preliminary, they may indicate that heteroreceptor complexes may represent novel targets for drug discovery in future cancer studies.

1.6 RRI as an allosteric interaction

Evidence for allosteric interaction in receptors has been provided; as far as GPCR homomers and heteromers is concerned, significantly studies by Kenakin's group and other indicate that the feature for RRI interaction are characteristic of allosteric mechanisms (Kenakin T, et al., 2010; (Smith NJ et al., 2010, Changeux JP, et al., 2017) Here, some primary principles of allosteric interaction will be summarized.

Allostery (Liu J, et al., 2016) is a mode of exchange between different sites in proteins, in which the energy associated with dynamic or conformational modifications at one site can be transported alongside precise pathways within the structure of the protein to other sites, which alter their dynamic or conformational properties for that reason (Liu J, et al., 2017). In this way, receptor molecules are surely "allosteric machines" (Christopoulos A, et al., 2002), if one consider that their activation mechanism entails the binding of an extracellular signal molecule at the ligand-binding domain, and the consequent changes are transmitted to the biologically active site of the protein, which, in transmembrane receptors, can also be located in sites distant from the recognition site forthe ligand. Since adjustments of protein conformation underlie the allosteric processes, for a protein the possibility to be allosterically modulated relies upon its capacity to present new conformations. Therefore, a protein with a rigid shape is less predisposed to be allosterically modulated than one that possesses segments that do not fold into a stable secondary structure, i.e., segments endowed with a high degree of intrinsic disorder (Agnati LF et al., 2008; Wright PE et al., 2015). Intrinsically disordered areas have been identified in all the classes of membrane receptors. Mechanisms of structural exchange from order to disorder (or vice versa), for this instance, have been hypothesized to underlie the activation of receptors of the RTK family (Shan Y, et al., 2012)

and intrinsic disorder of the N-terminal region seems to play a substantial function in the functionality of NHRs (Simons SSJr et al., 2014)]. The GPCRs exhibit disordered segments extracellularly (in the N-terminus) and disordered sequences in the cytosolic region, in most of cases the intracellular loops—particularly ICL3—and in the C-terminal domain (Agnati LF et al., 2008; Tovo-Rodrigues L, et al., 2014).

Structural plasticity and malleability are of significance because they enable conformational fluctuations and intra-receptor interactions to take place, but additionally because they permit the formation and dynamics of receptor complexes. Indeed, when two protomers interact by direct RRI, thereby giving origin to a quaternary structure, the energy associated with a perturbation at one site of one protomer can propagate over the interface between the receptors into the nearby protomers, thus changing their conformation and leading to a cooperative behaviour of the complex (Agnati LF, et al., 2010). Identifying the residues that specially interact to shape the interaction interface is therefore of great interest to a better understanding of receptor oligomerization (Skrabanek L, et al., 2007) as these residues impact on the models of conceivable allosteric interactions between receptor partners.

1.7 Homo- or hetero-dimers between A1 and A2A receptors

Adenosine receptor A1 homodimers have been reported in brain cortex of pigs by radio ligand binding experiments (Ciruela et al., 1995). The ability of the A1 receptors to interact in homodimers has also been demonstrated in Chinese hamster ovary (CHO) cells transfected with human A1 receptors (Gracia et al., 2008) and in transfected human embryonic kidney (HEK-293T) cells expressing comparable levels of A1 receptor as the native tissue (Gracia et al., 2013). In bovine cortex, the existence of homomers could be also additionally shown via PLA (Gracia et al., 2013).

The effects of homo-dimerization on the functioning of the receptors can be suggested by ligand binding assays. When A1 receptor is activated, it inhibits the adenylate cyclase and decreases the cAMP concentration. On the other hand, we know that caffeine is a non-selective adenosine receptor antagonist. At low caffeine concentrations (when caffeine only binds to one protomer of the empty homodimer), it increases the agonist affinity for the other protomer in the A1 receptor homodimer; while, in excessive concentrations (when caffeine fairly saturates both protomers of the homodimer) caffeine behaves like an A1 antagonist

with a reduction of the agonist binding to the receptors. Thus, caffeine modulates A1 agonist-induced inhibition of cAMP in a biphasic manner. Interestingly, this is a pharmacological behaviour explanation for a classical adenosine receptor antagonist like caffeine. Moreover, this pharmacological action is also consistent with the evidence about the biphasic consequences exerted at low and high levels of caffeine on locomotion (Gracia et al., 2013).

A2A homo-dimerization has been investigated in the plasma membrane of transfected HEK-293T cells by way of BRET, FRET, and time-resolved BRET and immunoblotting and biotinylating experiments. Evidences indicate that more than 90% of A2A receptors are existing as homodimers. At the intracellular levels, the presence of a significant level of monomeric species has been reported, that should interact into dimers before the expression on the cell plasma membrane (Canals et al., 2004; Lukasiewicz et al., 2007). In particular, Canals et al. (2004) verified that A2A homodimers are the functional species at the cell surface. Even though A2A homo-dimerization is constitutive, it is further influenced by way of specific ligands: agonists (i.e., CGS 21680) and antagonists (i.e., SCH 58261; caffeine) make bigger and minimize it, respectively (Lukasiewicz et al., 2007). The ability of A1 receptors to hetero-dimerize with A2A receptors was confirmed in transfected cells (Ferré et al., 2008) as well as in vitro and in vivo in rat brain striatal glutamatergic nerve terminals by means of immune gold detection and co-immunoprecipitation (Ciruela et al., 2006). Recently it has been shown that at the presynaptic membrane of cortico-thalamic glutamatergic terminals A1 receptors co-localizes and interacts with A2A receptors, forming receptor heterodimers in the striatum (Fernández-Dueñas et al., 2017). In fact, hetero-dimerization between the adenosine receptors A1 and A2A, which are responsible for opposite signalling (inhibitory and excitatory actions, respectively) (Stockwell et al., 2016), has been advised to exert roles in fine-tuning modulation of brain striatal glutamatergic neurotransmission via adenosine. Since A1 receptor possess a greater affinity for adenosine than A2A, however A1 agonist affinity decreases when A2A is activated, the glutamate release might be inhibited or increase through a change mechanism relying on low and high concentrations of adenosine, respectively (Ciruela et al., 2006; Doyle et al., 2012).

1.8 Homo and hetero dimers between dopamine receptors (D1 and D2)

Convincing evidences are provided for co-expression and co-localization of the D1 and D2 receptors in neurons of human and rat brain (Lee et al., 2004). For instance, a neuronal subpopulation in rat nucleus accumbens co-expresses D1 and D2 receptors, that can interact in a D1/D2 receptor complex (Hopf et al., 2003; Hasbi et al., 2018). Rashid et al. (2007) also provided evidences about co-expression the receptors in human embryonic kidney cells. In parallel, (Rashid et al., 2007;Hasbi et al., 2018) demonstrated the ability of D1 and D2 receptors to oligomerize in vivo in rodent and monkey striatum, respectively, through in situ PLA, in situ FRET and co-immunoprecipitation.

D1 and D2 monomers are coupled to Gs and Gi proteins, respectively, and they are usually considered to exert opposite effects at the cell level (Hopf et al., 2003). Conversely, the heterodimer D1/D2 was reported to couple to Gq/11: In the nucleus accumbens, the activation of this heterodimer-specific pathway causes an increase of calcium/calmodulin-dependent protein kinase IIA, at variance with the effect produced by the activation of the Gs-D1 receptor (Rashid et al., 2007).

1.9 Hetero-dimers between adenosine (A1 AND A2A) and dopamine (D1 AND D2) receptors.

The co-localization of A1 and D2 receptors has been confirmed by means of immunofluorescence in rat cerebral cortex neurons (Gines et al., 2000). Moreover, the presence of A1/D1 complexes was identified in mouse fibroblast Ltk-cells, transfected with human A1, D1, and D2. While the formation of A1/D1 heteromers was validated through co-immunoprecipitation, A1 did not appear to interact in heterodimers with D2. A1 and D1 receptors, separately, also tend to shape homodimers; however, the preferred form of dimerization for these two receptors has not yet been elucidated (Agnati et al., 2003). Additionally, A1/D1 dimerization was once also validated through FRET in HEK-293T cells (Shen et al., 2013).

A2A/D2 heterodimers had been additionally demonstrated in transfected SH-SY5Y (Hillion et al., 2002; Xie et al., 2010) and HEK-293T cells (Navarro et al., 2014), by immunoprecipitation observed with the aid of Western-blotting (SH-SY5Y) and BRET (HEK- 293T). In addition,

A2A/D2 heterodimers have been identified in neuronal cultures of rat brain striatum by means of PLA (Navarro et al., 2014) and cAMP accumulation experiments (Hillion et al., 2002). A2A/D2 heterodimers have been also demonstrated in the mammalian striatum, with the reference to the striatal enkephalin-containing GABAergic neurons that project to the globus pallidus and include the so-called indirect pathway (Fink et al., 1992; Fuxe et al., 1998; Schiffmann et al., 2003; Trifilieff et al., 2011; Doyle et al., 2012; Attack et al., 2014). Thus, A2A/D2 heterodimers appear to play a very important role in the modulation of GABAergic striato-pallidal neuronal feature (Bonaventura et al., 2015). In particular, inhibitory A2A-D2 RRIs take place in the heterodimer, as proven in striatal membrane preparations after incubation with the A2A agonist CGS21680, that reduced the affinity of the high-affinity D2 agonist-binding site (Fuxe et al., 1998; Guidolin et al., 2018). Relationships between A2A and D2 and adenosine/dopamine cross talks had been proposed as suitable new therapeutic strategies for Parkinson's disease, schizophrenia, and drug addiction (Canals et al., 2003; Guidolin et al., 2015).

1.10 GPCR complexes in astrocytes

At the excitatory synapses, neurons and astrocytes interact bidirectional, a finding that has led to the concept of the "tripartite synapse" (Araque A., et al., 1999) in the CNS, where astroglia constitutes the predominant glial population. Astrocytes are equipped with receptors and channels that allow them to sense the extracellular environment (Fuxe K., et al., 2015, Nedergaard M., et al., 2012); the receptor activation can elicit a Ca2+ responses in these cells (Hirase H, et al., 2004); these responses can, in turn, induce the release of gliotransmitters (such as glutamate, D-serine, ATP), thereby actively modulating synaptic transmission (Volterra A., et al., 2005).

Nevertheless, RRIs between GPCRs in astrocytes have been scarcely studied.

Specifically in the striatum, there is evidence that striatal astrocytes present both adenosine A2A receptors (Matos M., et al., 2013) and dopamine D2 receptors (Miyazaki I., et al., 2004). Interestingly, in vivo research has indicated that striatal astrocytic A2A receptor dysfunction disrupts glutamate homeostasis (Matos M., et al., 2015), while the astrocytic D2 receptors modulate immune responses in neuroinflammation and increase the resistance of striatal dopaminergic neurons to toxic damage (Shao W., et al., 2013). Increasing evidence shows

that, when D2 and A2A receptors are expressed on a cell, they can interact and heterodimerize (Canals M., et al., 2003; Fuxe K., et al., 2005). Notably, physical functional and evidence has shown that, in striatal neurons, native A2A and D2 receptors can physical interact in a heterodimer structure (Azdad K., et al., 2009) with an antagonistic interactions in the receptor complex (Diaz-Cabiale Z., et al., 2001). The presence of these receptor also in striatal astrocytes prompted to investigate on the possibility that also in astrocytes they might form receptor complexes as well. The evidence for RRI between the native A2A and D2 receptors in astrocytes was first provided by Cervetto and co-workers (2017). The study shows that A2A and D2 receptors are co-localized in the identical striatal astrocyte process prepared from the adult rat brain, and that the receptors functionally interacted in the control the glutamate release. The outcomes of this interaction also involved the formation of A2A-D2 heterodimers, considering that administration of the synthetic peptide VLRRRRKRVN, which is able to interfere with the D2 receptor domain involved in electrostatic interactions necessary to the receptor heteromerization (Ciruela F., et al., 2004; Woods A.S., et al., 2005), completely prevented the A2A-mediated inhibition of the response to D2 receptor activation.

As a further example, in astrocytes the RRI between GPCRs is also provided for the adenosine A1 and purinergic P2Y1 receptors (Tonazzini I., et al., 2007; 2008): co-localization and reciprocal functional interaction of the two receptors in human hippocampal astrocytes and co-immunoprecipitation experiments demonstrate the existence of A1-P2Y1 heteromeric complexes in the cells.

2. Oxytocin receptor

Oxytocin play very important role in the development of the brain and in social behaviours. Oxytocin interacts with oxytocin receptors, and partially works on vasopressin receptors and therefore it are to be kept in mind the interactions and cross-reactivity between the oxytocin system and other endogenous ligands (Schorscher-Petcu A, et al., 2010; Stoop R, 2012). Oxytocin receptors were recently discovered in the central nervous system and recognized to play very important role in CNS. (Palanisamy A, et al., 2018). Oxytocin contributes to the neuron development and it also takes part in the synapse formation (Ripamonti S, et al., 2017). In the brain produced oxytocin-producing cells appear early and seem to play roles in

the brain development (Madarász E, et al., 1992), in the neuron maturation, and in the formation of neural circuits (Bakos J, et al., 2016).

2.1 Oxytocin Receptors in Neurons

It is will established that receptors for neuropeptides are scattered throughout the brain; they may be present on cell bodies, dendrites, and in axonal terminals (van den Pol A. N, 2012). Oxytocin receptors are found in all the CNS areas, and the neurons, which produce oxytocin, arising from hypothalamus project to the limbic regions and to cortex in the brain (Grinevich V, et al., 2015). Oxytocin receptors are amongst the group of G protein-coupled receptors. Different intracellular pathways are activated by oxytocin receptors according to the specific G proteins (Stoop R, 2012). In neurons, an oxytocin receptor is coupled to Gαi and Gαq, with protein kinase C/phospholipase Cβ as downstream effectors, as a substitute for cAMP (Gimpl G and Fahrenholz F, 2001).

2.2 The Role of Oxytocin in Neural Circuits

In the neuronal circuits function it is very important to maintain the excitation and inhibition balance. Oxytocin can increase directly the excitability of the neurons by modulating the activity of the ion channels in the membrane, and then modulate synaptic transmission (Jo H. Y, et al., 1998). The relevance of oxytocin signalling depends on the brain area, on the type of neurons and their membrane excitability. In vivo and in vitro studies investigated the activity of oxytocin neurons, from the hypothalamus, the hippocampus, the amygdala and the spinal cord (Viviani D, et al., 2010; Harden W. S, and Frazier J. C, 2016). At the beginning, the functions of the neuron which produced oxytocin have been related to parturition or lactation, while it is now generally accepted that physiological functions like analgesia and behaviour and social communication are related with oxytocin (Terenzi G.M and Ingram C.D, 2005); it appears that oxytocin is also involved in the limbic circuit, that is why oxytocin plays important role in memory (Tomizawa K, et al., 2003). Moreover, oxytocin plays important role in anxiolytic activity in the limbic regions and prefrontal cortex (Neumann D. I. and Landgraf R, 2012). On the other hand, it was reported that oxytocin produce anti nociception by reducing the excitatory neurotransmitter release (Hobo S, et al., 2012). These studies hypothesized that oxytocin modulate the voltage-gated calcium channel, mainly of the N-type, to decrease presynaptic glutamate release (Hirasawa M, et al., 20019). A lot of scientific

reports indicate that from the hypothalamus oxytocin is distributed through oxytocin containing fibers to the spinal cord, and results in analgesic effects (Robinson A. D, et al., 2002, Condés-Lara M, et al., 2003).

2.3 Oxytocin: Modulation at Presynaptic level

For the first time presynaptic oxytocin receptors were described in the 1980s (Audigier S and Barberis C, 1985), leading to the suggestion that oxytocin could affect the neurotransmission (Buijs M. R, 1983). In fact, they demonstrated that the synaptic plasma membranes incorporate binding sites for oxytocin and vasopressin. The presence of oxytocin receptors was then found in the central and peripheral nervous system. When these receptors are activated pre-synaptically, as a result of the increase in intracellular calcium concentration they can also increase the secretion of the neurotransmitters into the synaptic cleft. Oxytocin stimulate calcium channel by two way. First, Gαq activate phospholipase C, and the action of inositol-triphosphate (IP3) binding on inositol 1,4,5-trisphosphate receptor (IP3R) increase the release of calcium from intracellular stores (Lambert C. R, et al., 1994). Secondly, it is also stimulated by the inhibition of the potassium Kir7.1 channels, that induces plasma membrane depolarization and calcium entry through the voltage-dependent calcium channel (Osako Y, et al., 2001; York N, et al., 2017). Another mechanism through which oxytocin may result in membrane depolarization of presynaptic membranes, is through activation of Na+/Ca2+ exchanger and the opening of a nonselective cation channel (Yao Y, et al., 2012). The activation of the Na+/Ca2+ exchanger by means of G protein-coupled receptors has been hypothesized in distinct hypothalamic structures (Burdakov D, et al., 2003, Parmentier R, et al., 2009).

2.4 Excitatory Synapses

Activation of oxytocin receptors by oxytocin has complex effects on the regulation of the excitatory synaptic transmission, that may be different in the different brain areas. Recently it has been proven that the activation of presynaptic oxytocin receptors increases the release of glutamate evoked by depolarization in the brain hippocampus (Mairesse J, et al., 2015). Beside this, other brain areas are also the targets for oxytocin effects. It has been confirmed that the activation of oxytocin receptors facilitates glutamatergic synaptic transmission in the spinal cord (Breton D. J, et al., 2008). These findings indicate that oxytocin acts on presynaptic

membrane of subpopulations of glutamatergic neurons and as an end, the result of this action promotes release of the neurotransmitter. This release of glutamate outcomes in excitation of GABA neurons and when connected to further neurons transmits inhibitory message on them. This mechanism is thought to contribute to the nociceptive effects of oxytocin. Nevertheless, it is to be considered that interneurons in the spinal cord may also use the oxytocin as a neurotransmitter, and the synaptic contacts with neurons in the second or third order should be under a distinct oxytocin modulation. Contradictory to the results that describe the enhancement of the excitatory synaptic transmission, there are some reports indicating that oxytocin works in the opposite way as far as glutamate release from the presynaptic membrane is concerned. It has been published that oxytocin release by dendrite decreases the evoked excitatory synaptic transmission through inhibition of the glutamate release from the presynaptic terminals (Kombian B. S, et al., 2002). It was hypothesized that the effect of oxytocin was via modulation of voltage-dependent calcium channels, mainly N-type and to a lesser extent P/Q-type channels, placed on glutamatergic neuro terminals. These evidences indicate the possible existence of two-way communication between the presynaptic terminal and the postsynaptic synaptic membrane. In any case it is proposed that oxytocin acts by means of a mechanism involving the N-type channels and P/Q-type channels inducing neuron outgrowth (Zatkova M, et al., 2018), and it appears that the presynaptic effects of oxytocin are dependent on extracellular calcium (Raggenbass M, 2001).

In conclusion, oxytocin receptors had been observed at the so-called putative excitatory synapses, both at presynaptic terminals and postsynaptic sites; oxytocin receptors have been observed at inhibitory synapses as well (Mitre M, et al., 2016).

2.5 Inhibitory Synapses

The active participation of oxytocin in the function of inhibitory synapses is under discussion. Some reports provide an explanation for the role of oxytocin in inhibitory terminals by suggesting that oxytocin can act through an activation of presynaptic oxytocin receptors resulting in a decrease the GABAergic transmitter release (Mitre M, et al., 2016). In this study, the researchers show that the predominant impact of oxytocin modulation is to reduce the inhibitory neuro transmission, leaving unaffected the excitation. The pathways accountable for the decrease of GABA secretion includes inhibition of voltage-dependent calcium channels

by means of protein kinase C and/or calcium-dependent potassium channels (I. Kruglikov and B. Rudy, 2008). This conclusion was supported by other studies in different brain areas: the auditory cortex, piriform cortex, hypothalamus, and hippocampus (Marlin J. B, et al., 2015; Owen F. S, et al., 2013). Conversely, other study indicated that oxytocin depresses the spontaneous GABA receptor-mediated inhibition by means of an action pre-synaptic at the level of the olfactory blub (Osako Y, et al., 2000). In fact, it was reported that oxytocin exerts inhibitory effects on distinct sets of interneurons in vitro (Maier P, et al., 2016). All together these evidence indicate that the effects of oxytocin on its receptors at the presynaptic membrane are different, depending on the neuron type and the brain area and most probably on the complex presynaptic inputs including astrocyte-originated transmission (Wang F. Y, and Hatton I. G, 2006).

2.6 Oxytocin: Modulation at Postsynaptic level

Regulation of the potential of postsynaptic membrane by oxytocin contributes to the modulation of synaptic function. Oxytocin acts by means of oxytocin receptors on postsynaptic neurons in order to change the neural circuits, which modify social behaviours (Yao S, et al., 2017; Nakajima M, et al., 2014). The presence of oxytocin receptors was discovered in a subpopulation of neurons in the amygdala, which shows the steroid-converting enzyme aromatase. This discovery contributed to understand how the oxytocin receptor stimulation is coupled to specific female-evoked neural responses and behaviours in the male mouse. The impact of oxytocin on membrane excitability of rat dorsal root ganglion neurons has also been investigated (Qiu F, et al., 2014); oxytocin drastically lowered the amplitude of the depolarization, and the number of action potentials induced by acid stimuli; the contribution of the vasopressin receptor V1A was also highlighted. Another study has established that oxytocin increase the frequency of inhibitory postsynaptic currents (Wrobel J. L, et al., 2010); it is important to note that the study also advise that both V1a and oxytocin receptors play an essential role. Another study found that oxytocin induces increased levels of intracellular calcium at the postsynaptic sites, which subsequently depresses inhibitory synaptic transmission (Brussaard B. A, et al., 2000). Oxytocin has also been reported to affect the spontaneous inhibitory and excitatory postsynaptic currents in the olfactory cortex as well (Oettl L. L, et al., 2016), and to depolarize interneurons and enhances synaptic transmission in the hippocampal region of CA1 (Harden W. S, and Frazier J. C, 2016). Other

electrophysiological studies also prove that stimulation of oxytocin receptors reduces spontaneous firing while enhances the excitatory postsynaptic potentials achievable onto pyramidal cells in the hippocampus (Owen F. S, et al., 2013).

2.7 Oxytocin: The Receptors for Oxytocin in Astrocytes and Glial Cells

Astrocytes have been recognized as targets of oxytocin action. Autoradiography studies have proved the presence of binding sites for oxytocin on both the soma and the processes of cells expressing the features of astrocytes (Di Scala-Guenot D, and Strosser T. M, 1992). Furthermore, receptors for oxytocin have been revealed on astrocytoma cell lines (Cassoni P, et al., 1998; Bakos J, et al., 2012), suggesting that the oxytocin receptors might be involved in the modulation of cellular growth. Stimulation of astrocytic oxytocin receptors has been reported to result in the release of calcium from intracellular stores (Kuo J, et al., 2009). It has been reported that the astrocyte oxytocin receptor is coupled to a G protein-coupled receptor that is able to increase intracellular calcium by mobilizing it from IP3-sensitive stores (Di Scala-Guenot D, and Strosser T. M, 1992; D. di Scala-Guenot and Strosser T. M, 1992). On the other hand, electrophysiological recording has shown that oxytocin can evoke depolarization of the astrocytic membrane, and in the context of suckling, oxytocin could be involved in the plasticity and retraction of the astrocyte processes (Wang P, et al., 20179. All these findings appear of particular relevance if one considers the modes of participation of astrocytes in the control of the neuronal activity. In fact, the interactions between astroglial cells and neurons are very complex, and astrocytic function is crucial to the development of synaptic connectivity, and to the formation of synapses.

Recently it was reported that oxytocin affects synapse connectivity and the number of synapses (Ripamonti S, et al., 2017). The research findings suggest that the excitatory

2.8 Oxytocin: Effects on the Formation of Synapses and on the Synapse Stability

Presynapses are increased in cultures obtained from oxytocin receptor knockout mice, while the number of the inhibitory presynapses was barely decreased. Moreover, the study also shows that exposure of cultured mouse hippocampal glutamatergic neurons to oxytocin could result in changes of the neuronal dendrite complexity and in altered numbers of excitatory synapses. On the other hand, the ratio of GABAergic presynapses versus the total number of

presynapses was decreased in hippocampal neurons cultured from oxytocin receptor knockout mice (Sala M, et al., 2011). In addition, the effects of oxytocin on neurofilaments are to be considered as far as the synapse formation is concerned. In fact, oxytocin promotes the formation of filamentous actin (F-actin) networks on the cell membrane of the brain cortex (Wang F. Y, and Hatton I. G, 2006). Furthermore, improved expression of the actin-binding protein drebrin and of the intermediate filament vimentin have been found in response to oxytocin (Lestanova Z, et al., 2016). Therefore, it appears that oxytocin play important roles in the regulation of expression of cytoskeletal proteins associated with the growth of neurites in vitro. Notably, and relevant from a structural point of view, that oxytocin receptor knockout mice exhibited a reduction of postsynaptic density protein (S. Miyazaki, et al., 2016). The findings are consistent with the oxytocin receptors being related to the regulation and modulation of the synapse scaffolding proteins. This conclusion is additionally supported by the effects of oxytocin on the SHANK family of scaffolding proteins (Zatkova M, et al., 2018). Oxytocin has been reported to increase the dendritic complexity (Sánchez-Vidaña I. D, et al., 2016). Oxytocin has been observed to phosphorylate cAMP-responsive element-binding protein (CREB) (Tomizawa K, et al., 2003) and other CREB-regulated genes (Jurek B, et al., 2015). This effect might be a regulatory pathway to many scaffolding proteins, cytoskeleton rearrangement, and synapse formation. Oxytocin signalling has developmental dynamics (Grinevich V, et al., 2015), and the early phases of the neuron development play crucial roles for the development and function of mature oxytocin receptor system. As far as the oxytocin receptor localization is concerned, these receptors have various brain regional localization, including the distribution of the oxytocin receptors in the brain cortex (Duchemin A, et al., 2017).

2.9 Defetcs in Oxytocin Signaling

Oxytocin signal defects have been related to neurodevelopmental problems including autism, and have raised attention on the function of oxytocin and its receptor in the structure of the synapses, in their function, and in neuron connectivity. An early change in oxytocin signalling may disturb the neuronal maturation and may additionally have non-permanent and long-term pathological complication (Muscatelli F, et al., 2017). Autism is a heterogeneous disorder, and its pathology includes variations in cytoskeletal rearrangement, neuritogenesis, and elongation of axons and dendrites resulting in a variety of synaptopathies (Bakos J, et al.,

2015). The impaired synapse formation results in disrupted neuronal connectivity and circuit stabilization, which in turn can contribute to the pathogenesis of the autism (Zatkova M, et al., 2016). Given the growing prevalence of autism, identification of the involved factors and therefore of the possible therapeutic interventions is a key element of the applicable objectives of the current neurobiology research.

In fact, it was proposed that oxytocin could be used as an effective treatment for neurodevelopmental disorders, such as the autism spectrum disorders or the Prader-Willi syndrome (Meziane et al 2015).

2.10 OXTR receptor–receptor interaction

Oxytocin is able to reduce blood pressure and cortisol levels, therefore producing anti-stress-like effects; moreover, it increases the pain threshold, with an anxiolytic-like effects and exhibit a positive effect on the social interactions (Uvnas-Moberg and Petersson, 2005). As NA through α2 adrenergic receptors exhibits comparable actions, the ability of OXTR to interact with α2 adrenergic receptors through RRI has been investigated (see Agnati et al., 1980; Fuxe et al., 1981, 1983, 2012). OXTR α2 adrenergic receptor interaction might play a role in the participation of oxytocin in the cardiovascular control and in the control of food intake; the interaction between OXTR and α2 adrenergic receptors seem to depend on the acute or subchronic oxytocin administration (see Fuxe et al. 2012).

2.2.1 D2-oxytocinR interactions

In female prairie voles, the D2 receptor for dopamine is able to modulate the pair bonding, similarly to OXTR (Young and Wang, 2004). The preference for the partner of the female prairie voles was associated with increased dopamine extracellular levels in the nucleus accumbens; both the preference for the partner and the increased levels of dopamine were blocked by D2 receptor antagonist (see Fuxe et al. 2012). It is hypothesized that co-activation of D2 and OXTRs in the nucleus accumbens is of significance for pair bond formation and maintenance (Aragona et al., 2003; Gingrich et al., 2000; Young and Wang, 2004; Young et al., 2001). The ability of D2 and OXTR to interact in heterodimers has been confirmed in cellular models ((ee Fuxe et al. 2012).

3. Aims and objectives:

In the presence study our aims and objective were:

1. To investigate on the presence of OXTR in freshly isolated astrocytes from rat striatum;

2. To investigate on the presence of receptor-receptor interaction involving OXTR, in particular on A2A-OXTR and D2-OXTR and on their effects on the modulation of glutamate release

4. RESULTS

Aim 1

4.1 Investigation on the presence of OXTR in freshly isolated astrocytes from rat striatum

4.2 OXTR is present on astrocyte processes of adult rat striatum

Striatal gliosomes were labelled with anti-OXTR and anti-GFAP antibodies indicating that single astrocyte processes (positive for GFAP) express oxytocin receptor (Fig.10)

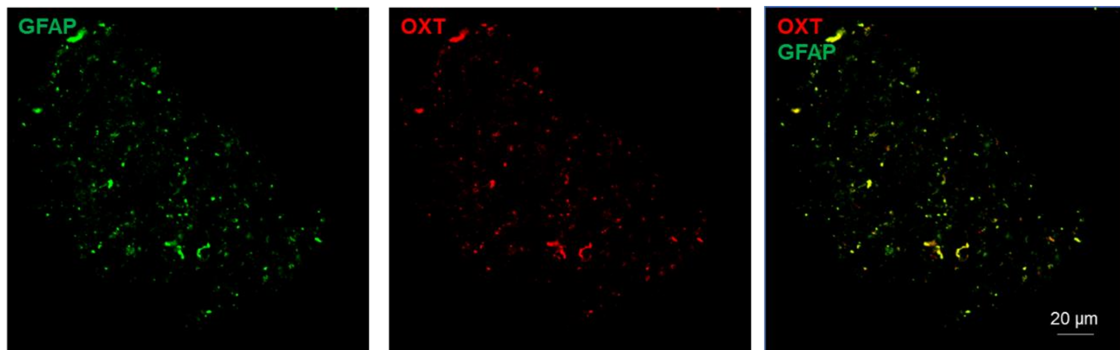

Figure 10: Rat striatal purified astrocyte processes express OXT receptors. Confocal microscopy analysis.

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, the immunofluorescence analysis was performed also using an anti-VGLUT1 primary antibody to investigate the presence of oxytocin receptor in glutamate-releasing gliosomes.

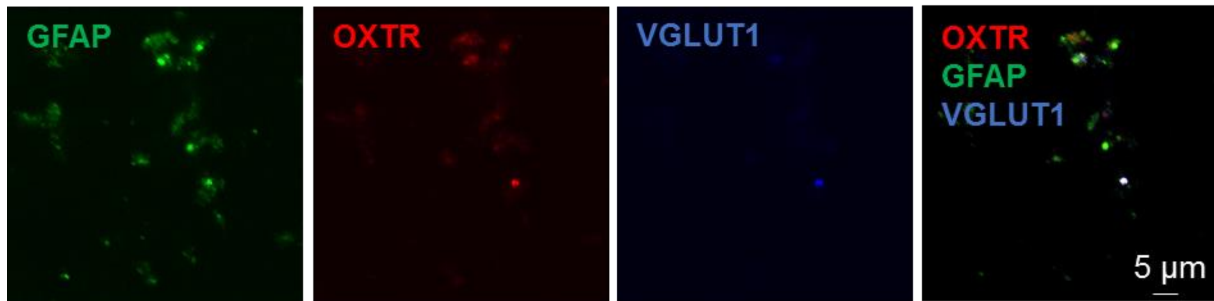

Figure 11: OXT receptors are expressed on VGLUT1 – positive astrocyte processes from rat striatum. Confocal microscopy analysis.

Immunofluorescence for GFAP (green), the OXT receptor (red) and the vesicular glutamate transporter type 1 (VGLUT1; blue): merge image showing co-expression of the markers in a single gliosome. Scale bare is indicated in the image.

We obtained evidence that oxytocin receptors are localized on glutamatergic gliosomes as you can see in Fig. 11.

4.3 OXTR modulate depolarization-evoked glutamate release from striatal astrocyte processes

The astrocyte processes were labelled with [3H] D-aspartate and the glutamate release was evaluated by assessing the tritium efflux during superfusion. Starting after 33 min of superfusion 3-min sample were collected and the basal outflow of [3H]D-aspartate in the first two fractions amounted to 0.60 ±0.04%/min (n = 10). The addition of a depolarizing stimulus as 4-AP (300 µM) increased the [3H]D-aspartate release as reported in Table 2. Previously, we reported that the 4-AP-evoked glutamate release was dependent on the presence of Ca2+ in the superfusion medium, indicating the exocytotic nature of the gliotransmitter release (Cervetto et al 2017).

The D2receptor agonist quinpirole (1 µM) inhibited the glutamate releasing response to 4-AP as reported in Table 2; the A2A receptor agonist CGS21680 was ineffective (0.01µM; Table. 1 and 0.01µM as reported in Cervetto et al 2017, 2018). Moreover, oxytocin (3 nM), the OXTR agonist, inhibited the 4-AP evoked glutamate release. Collectively, the findings indicate that

D2 and oxytocin receptors are negatively coupled to the release of glutamate from the astrocyte processes, while A2A receptors are ineffective on the release of the gliotransmitter.

Previously reported data demonstrate that the quinpirole effect on the 4-AP-evoked [3H]D-aspartate release was reverted by sulpiride, suggesting a D2-mediated effect (Cervetto et al 2016). On the other way, the OXTR antagonist, L-371,257 at the used concentration (1 µM), altered the 4-AP-evoked [3H]D-aspartate release per se (data not shown); other experiments are needed to confirm that the oxytocin effect is mediated to OXTR activation.

Table 2:

	$[^3H]$ D-aspartate efflux (% variation)
4-AP 300µM	144.8± 2.32 (n=4)
4-AP + quinpirole 1 µM	102.8 ± 10.52 (n=4) *
4-AP + oxytocin 3 nM	89.66 ± 7.01 (n=4) *
4AP + CGS 21680 0.01 µM	134.4 ± 5.26 (n=3)

Table 2. Activation of OXT and D2 receptors inhibited the evoked glutamate release from rat striatal astrocyte processes, while the A2A receptor agonist was ineffective.

Quinpirole, a D2 receptor agonist, or oxytocin inhibited the 4-AP-evoked [3H] D-aspartate efflux, while the A2A receptor agonist CGS 21680 was ineffective. The data are reported as the percentage of increase in [3H]D-aspartate efflux in the presence of the drugs at the used concentrations. The percentage of increase in [3H] D-aspartate efflux was measured as the percent variation of the tritium efflux in the presence of 4-AP or 4-AP plus the drugs, calculated by subtracting the area under the curve of percent variations in tritium fractional release in the appropriate control chambers from the area under the curve of the percent variations in drug-treated chambers. The depolarizing stimulus 4-AP was added (6 min) during superfusion experiment: quinpirole, oxytocin or CGS 21680 were added together with 4-AP. Other experimental details in Materials and Methods. Data are means ± SEM of n experiments (indicated in the table) performed in triplicate. Kruskal-Wallis test: $^*p<0.05$ compared with 4-AP.

Aim 2

4.4 Investigation on the presence of receptor-receptor interaction involving OXTR, in particular on A2A-OXTR and D2-OXTR and on their effects on the modulation of glutamate release

4.5 OXT receptors colocalized with A2A and D2 receptors on striatal astrocyte processes

Astrocytic processes were labelled with anti-GFAP, anti-OXTR, anti-A2A and anti-D2 antibodies, indicating that the processes express both the OXTR, A2A and D2 receptors (Fig. 12 and 13).

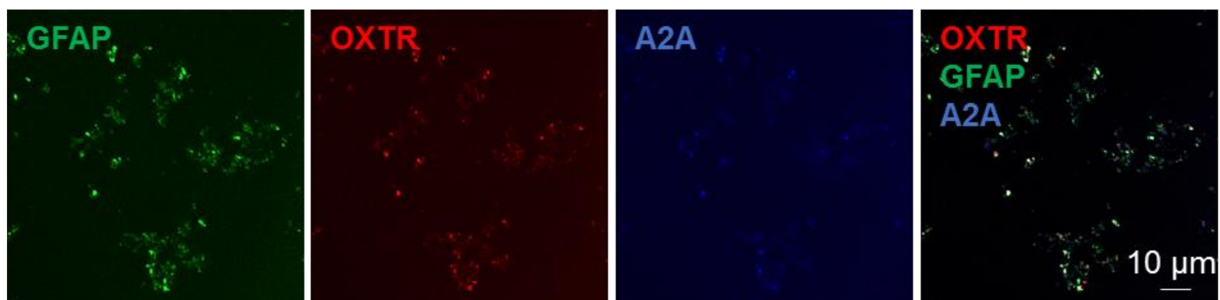

Figure 12: OXT and A2A receptors are co-localized on rat striatal astrocyte processes. Confocal microscopy analysis

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

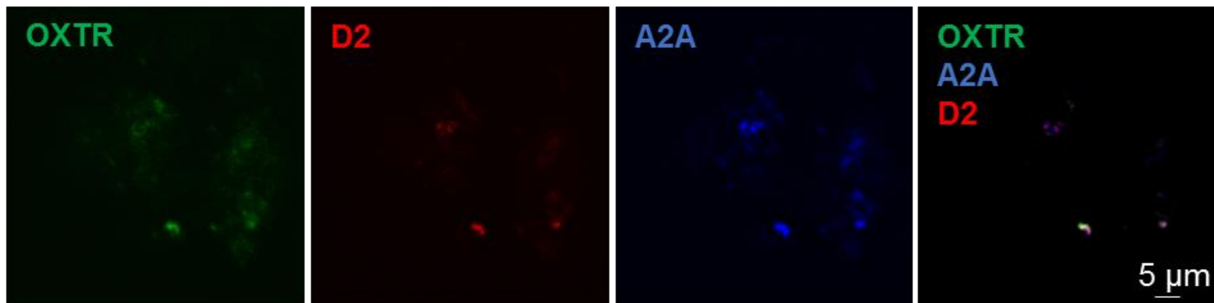

Figure 13: OXT, A2A and D2 receptors are co-localized on rat striatal astrocyte processes. Confocal microscopy analysis.

Immunofluorescence for the OXT (green), adenosine A2A (blue) and the dopamine D2 receptor (red): merge image showing co-expression of the markers. Scale bare is indicated in the image.

Striatal gliosomes, positive for the glial marker GFAP, and the vesicular glutamate transporter type 1 (VGLUT1), express oxytocin receptors (Fig. 2) but also A2A and D2 receptors (Fig. 3 and 4). Also, OXTR were found co-expressed with both A2A and D2. Collectively these findings, also considering the previous data indicating that A2A and D2 receptors physically and functionally interact in heteromers,indicate that A2A, D2 and OXT receptors are co-expressed and that a mosaic receptor complex might be express on striatal astrocytes.

4.6 Does OXTR functional interact with D2 receptors in the control of glutamate release from astrocyte processes?

A facilitatory receptor-receptor interaction between D2 and OXTR was reported in the membrane preparations of ventral and dorsal striatum (Romero-Fernandez W et al 2013). To evaluated if in the astrocyte processes this functional interaction is involved in the modulation of the glutamate release we performed release experiments on gliosomes. The contemporary activation of D2 and OXTR, by quinpirole 1 µM and oxytocin 3 nM, respectively, didn't significant affect the quinpirole or oxytocin mediated inhibition of 4-AP evoked [3H]D-aspartate release when the agonist were used alone and at the indicated concentrations (Table 3). This data might suggest that OXTR and D2 could use the same transduction mechanism to inhibit the glutamate release. Alternatively, the used quinpirole concentration

could be not optimal to assess the facilitatory allosteric receptor–receptor interaction reported as a significant increase in the Bmax value of quinpirole on D2 receptor (Romero-Fernandez W et al 2013). Investigation on the effects of oxytocin on a full concentration-response curve for quinpirole in inhibiting the 4-AP evoked release of glutamate might help to unmask such a facilitatory effect.

Table 3:

	$[^3H]$D-aspartate efflux (% variation)
4-AP 300µM	140.3 ± 3.5 (n=6)
4-AP + quinpirole 1 µM	81.96 ± 1.6 (n=6) *
4-AP + oxytocin 3 nM	80.25 ± 2.4 (n=6) *
4AP + quinpirole 1 µM + oxytocin 3 nM	67.58 ± 6.7 (n=6) *

Table 3: OXT and D2 receptors inhibited the 4-AP evoked glutamate release from rat striatal astrocyte processes.

Quinpirole, a D2 receptor agonist, and oxytocin inhibited the 4-AP-evoked [3H]D-aspartate efflux, but the contemporary activation of D2 and OXTR while did not significant change the effects of either quinpirole or oxytocin alone. The data are reported as the percentage of increase in [3H]D-aspartate efflux in the presence of the drugs at the used concentrations. 4-AP was added (6 min) during superfusion: quinpirole and oxytocin was added together with 4-AP. Other experimental details in Materials and Methods. Data are means ± SEM of n experiments (indicated in the table) performed in triplicate. Kruskal-Wallis test: *p<0.05 compared with 4-AP.

4.7 Activation of A2A receptor inhibits the response to OXTR in striatal astrocytic processes

Previously, we reported evidence for the presence of the A2A-D2 heterodimers on striatal gliosomes and for their functional role in the control of astrocyte glutamate efflux. In fact, the A2A receptor agonist CGS21680 (0.01 µM) counteracted the D2-mediated inhibition of the 4AP-evoked [3H]D-aspartate release in gliosomes (Cervetto et al 2017, 2018; Pelassa et al 2019). We here found that CGS21680 (0.01 µM) abolished the response to oxytocin (3 nM), as reported in the Table 4, and the A2A receptor antagonist, SCH 58261 (1µM), restored the oxytocin-mediated inhibition of the 4-AP-evoked glutamate release. The results suggest a functional interaction between A2A and oxytocin receptors in rat striatal astrocyte processes.

Table 4

	[^{3}H]D-aspartate efflux (% variation)
4-AP 300µM	145.2 ± 1.94 (n=6)
4-AP + CGS 21680 0.01µM	138.4 ± 4 (n=5)
4-AP + oxytocin 3nM	87.06 ± 4.91 (n=6) *
4AP + oxytocin 3nM + CGS 21680 0.01µM	134.9 ± 4.38 (n=6)
4AP + oxytocin 3nM + CGS 21680 0.01µM + SCH 58261 1µM	118.9 ± 4.27 (n=5) *

Table 4: OXT-A2A functional interaction.

Oxytocin inhibited the 4-AP-evoked [3H]D-aspartate efflux. Inhibition by the A2A receptor agonist CGS 21680 of the oxytocin control of 4-AP-evoked efflux of [3H]D-aspartate. The data are reported as the percentage of increase in [3H]D-aspartate efflux in the presence of the drugs at the used concentrations. 4-AP was added (6 min) during superfusion: CGS 21680 and oxytocin was added together with 4-AP. The antagonist was added 8 min before the agonist. Other experimental details in Materials and Methods. Data are means ± SEM of n experiments (indicated in the table) performed in triplicate. *p<0.05 compared with the effect of 4-AP:two-tailed Mann-Whitney test.

5. Discussion

The discovery of receptor-receptor interactions (RRI) has played very important roles in the understanding of the mechanism of action for GPCRs in intercellular communication. The GPCRs not only can operate as receptor monomers but also as receptor complexes, suggesting that several different incoming signals could already be integrated at the plasma membrane level. So far, the research on the topic of RRI among GPCRs has been focused mainly at neuronal level in the central nervous system, where a large number of RRI has been identified. Nevertheless, RRIs have been reported not only in the neurons but also in the astrocytes and area outside the central nervous system, such as endocrine or cardiovascular cells, and in tumors. Moreover, RRIs not only involve the GPCRs, but had also been seen in other receptor families. Thus, RRI appear as a widespread phenomenon and oligomerization has to be considered as a common mechanism for receptor function and regulation. (Guidolin, D et al., 2019).

The RRI can have an important function in the signalling transduction pathway. In particular, the interaction between the G protein coupled receptors of adenosine A2A receptors and dopamine D2 receptors is a well-established phenomenon in several experimental models in neuronal plasma membrane. (Guidolin, D. et al., 2015).

On the other hand, there has been growing interest in astrocytes and in the complex neuron astrocyte network function, and the involvement of astrocytes in neurodegenerative and neuropsychiatric diseases is being increasingly recognized.

Recently, it has been reported that the adenosine A2A receptor and the dopamine D2 receptor were also present in the striatal astrocytes isolated from the adult rat brain . Both A2A and D2 receptors were co-expressed on GFAP-positive astrocytes and astrocytic processes (Cervetto et al., 2017) indicating that native A2A and D2 receptors on striatal astrocytes might interact through A2A-D2 RRI. Especially, the A2A and D2 receptors were co-expressed together with the vesicular glutamate transporter VGLUT1 on a subpopulation of asrocytic processes, and were found able to functionally interact to control the glutamate release from the processes. In fact, the D2 receptor activation reduced the release of glutamate from the processes, while the activation of the A2A receptor, which was per se

ineffective on the glutamate release, prevented the D2 receptor-mediated inhibition (Cervetto et al., 2018).

In the light of above findings, we decided to investigate on: 1. The presence of OXTR in freshly astrocytes isolated from rat striatum; 2. The presence of A2A.OXTR and D2-OXTR receptor-receptor interaction and their effect on the modulation of glutamate release. To this end, we isolated and purified gliosomes from the striatum of adult rat, according to the protocol, which is already mentioned in Material and Method section.

To understand if oxytocin receptors are present in the adult rat striatum astrocyte processes, we performed some experiments on the basis of confocal microscopic analysis. To see the oxytocin receptor, we labelled striatal gliosomes with anti-OXTR and anti-GFAP antibodies and we found that single astrocyte processes (positive for GFAP) express oxytocin receptor. Moreover, to investigate the presence of oxytocin receptor in glutamate releasing gliosomes, we performed immunofluorescence analysis using an anti-VGLUT1 as primary antibody. We obtained evidence that the oxytocin receptors are localized on glutamatergic gliosomes.

Then we used the superfusion method to carry out the release experiments, to understand if the OXYTR localized on the striatal astrocyte processes were able to affect the release of glutamate from the processes. We perfused gliosomes using the depolarizing agent 4-AP, and we found that 4-AP (300 µm) increased [3H]D-aspartate release as shown in Table 2. It was previously found that the 4-AP-evoked glutamate release was dependent on the presence of Ca2+ in the superfusion medium, showing the exocytic nature of the gliotransmitter release (Cervetto et al., 2017).

We found that the OXTR agonist oxytocin (3 nM), inhibited the glutamate release evoked by 4-AP. The ability of the OXTR agonist oxytocin to inhibit the 4-AP evoked release is consistent with the co-localzation of the OXTR on VGlut-positive processes, suggesting the ability of oxytocin to inhibit the vesicular release of glutamate from the astrocyte processes. Notably, the A2A receptor agonist CGS21680, per se ineffective, was able to counteract the oxytocin inhibitory effect on the release, in a way similar to what reported for the D2 receptor-mediated inhibition. In parallel, we here confirmed that the agonist of the D2-receptor quinpirole (1 µM) inhibited the glutamate release evoked by the 4-AP, as also shown in table 2. The quinpirole effect on 4-AP-eoked [3H] D-aspartate release was reverted by sulpiride,

consistent with a D2 receptor mediated effect (see also Cervetto et al., 2017). Also, we confirmed that the A2A receptor agonist CGS21680 was ineffective, as already reported (see Cervetto et al., 2017, 2018).

Altogether, our data shows that functional OXTR are present on the striatal astrocyte processes, that OXTR and D2 receptors are negatively coupled to the glutamate release from the processes, and that the A2A receptor, that per se has no effect on the glutamate release, is capable of preventing the effect of either OXTR or D2 receptor.

We then investigated by confocal imaging if OXTR are colocalized with A2A and D2 receptors on striatal astrocyte processes. We then labelled the astrocyte processes with anti-GFAP, anti-OXTR, anti-A2A and anti-D2 antibodies, and found that the processes express the OXTR, the A2A and the D2 receptors. In particular, striatal gliosomes, positive for the glial marker GFAP and the vesicular glutamate transporter type 1 (VGLUT1), co-express oxytocin receptors but also A2A and D2 receptors. Collectively the findings suggest that coexpression of A2A, D2 and OXTR on the same astrocyte processes might allow receptor-receptor interaction possibly through heterodimers, but also through a mosaic receptor complex that might be expressed on striatal astrocytes.

We then investigated on possible functional interaction between OXTR and D2 or A2A receptors. As a facilitatory receptor-receptor interaction was reported between OXTR and D2 and in the membranal preparation of the ventral and dorsal striatium, we evaluated if a functional interaction is also involved in the modulation of the glutamate efflux the astrocytes processes (Romero-Fernandez W et al., 2013). We carry out release experiments on gliosomes, at the same time we activated both D2 and OXTR respectively by quinpirole (1 µM) and oxytocin (3 nM), but we did not find any significant effect of quinpirole plus oxytocin with respect to either quinpirole or oxytocin alone, as far as inhibition of the 4-AP evoked [3H] D-aspartate release is concerned; see table 3. The data allow to hypothesize that D2 and OXTR were using the same transduction pathway. Further studies to reveal if OXTR might unmask a response to quinpirole at low, per se ineffective, concentrations, are required to a better understanding of a possible facilitatory allosteric receptor-receptor interaction between the OXTR and the D2 receptor also at astrocytic plasma membrane.

Recently, it was reported that A2A-D2 heterodimers are present on striatum astrocyte processes and are able to control the astrocyte glutamate efflux; activation of the A2A receptor reduced the D2 mediated inhibition of 4-AP-evoked [3H] D-aspartate release in gliosomes (Cervetto et al., 2017, 2018; Pelassa et al., 2019). We here found that the A2A receptor agonist also abolished the oxytocin response, which indicates the presence of a functional interaction between A2A and OXTR in rat striatal astrocytes processes. Whether or not OXTR, A2A and D2 receptors are able to physically interact in receptor mosaics remains to be clarified by further biophysical and biochemical approaches.

6. Conclusive remarks

Altogether, the data are suggestive for a functional interaction between OXTR and A2A, but more experiments are needed to a better understanding of the RRIs between OXTR, A2A and D2 receptors. In any case, their co-localization on the same astrocyte process might be the basis for a physical interaction in heterodimers or in receptor mosaics. In fact, physical and functional evidence indicate that almost all the D2 receptors expressed on the plasmamebrane are involved in heterodimers with A2A receptors in striatal gliosomes. We might speculate that in the astrocyte processes freshly prepared form rat striatum OXTR could be involved in an heterodimer with A2A, or possibly in an heterotrimer with A2A and D2, with significant roles in the control of the glial glutamate release.

7. Future directions

1) To antagonize the oxytocin effect on 4AP-evoked glutamate release using the OXTR antagonist L-371,257 (at a minor concentration than 1μM) to demonstrate that its inhibitory effect is receptor mediated

2) To investigate on the potential A2A-OXTR RRI using the A2A receptor antagonist SCH58261 at 10 μM

3) To investigate on the potential D2-OXTR RRI using a minor concentration of quinpirole and the specific D2 receptor antagonist, sulpiride.

4) To conclude the functional evidence on the RRI between OXTR, A2A and D2 and in particular to obtain physical evidence with the co-immunoprecipitation analysis on astrocyte process membrane and the proximity ligation assay on striatal slices.

MATERIAL AND METHODS

1. Animals

Sprague-Dawley male rats (200-250g) wereused. Animals were bred in the animal facility in the Department of Pharmacy (DIFAR) of the University of Genoa and were housed at constant temperature (22 ± 1 °C) and relative humidity (50%) with a regular 12 h–12 h light cycle (light 7 AM–7 PM), throughout the experiments. Food (type 4RF21) standard diet obtained from Mucedola (Settimo Milanese, Milan, Italy) and water were freely available. Experiments were performed in accordance with the European Community Council Directive 2010/63/UE and approved by the Italian legislation on animal experimentation (D.L. 26/2014). All the animal-involving experiments comply with the ARRIVE guidelines, to minimize animal suffering and to use only the number of animals necessary to produce reliable results.

2. Sucrose Preference Test

Sucrose habituation was performed exposing animals (30 post natal day) to two bottles containing 1% sucrose solution, for 2 h. Then, animals were subjected to sucrose preference test (SPT) twice a week, for 4-5 weeks, always in the same day period, in single cages and in absence of food. SPT consisted in presenting rats with two bottles, one containing 1% sucrose and one containing tap water, for 1 h. The position of the bottles was inverted after 30 min. Sucrose preference was calculated as: [sucrose solution intake (ml)/total fluid intake (ml)] x100] (Strekalova et al., 2011). After this period of test, each rat has its own baseline (in term of sucrose intake volume) and preference. The weight of rats was checked weekly.

After the 4-5 weeks necessary to have a baseline, a group of rats was subjected to footshock (FS)-stress. After 6h or 24h from FS-stress, rats were screened for their anhedonia-like behavior by the sucrose preference test. Based on the results, animals were then considered either resilient (RES) or vulnerable (VUL) to acute stress (VUL: subjects with a decrease in sucrose intake > 25% compared to baseline consumption; RES: subjects with a variation < 10%).

3. Footshock Stress protocol

The footshock (FS)-stress paradigm is used to induce an acute dejection status in animals, depending on the duration and intensity of the stimulus. Scientists usually use this protocol to reproduce an anxious status (Vogel et al., 1971) as well as post-traumatic stress disease

(PTSD) (Louvart et al., 2005; Pawlyk et al., 2005). Here, FS-stress protocol was performed essentially according to the following scheme: 40-min total FS-stress at 0.8 mA, including 20 min total of actual shock with random intershock length between 2–8 sec. The FS-stress box was connected to a scrambler controller (LE 100-26, Panlab) that delivers intermittent shocks to the metallic floor. Sham-stressed rats (controls) were kept in the stress apparatus without delivering of shocks (Musazzi et al., 2010, Treccani et al., 2014, Bonanno et al., 2005).

4. Preparation of gliosomes as an ex-vivo experimental model of sub-cellular particles of astrocytic origin

4.1 What are gliosomes?

Traditionally, astrocytes have the role of providing trophic and metabolic support to neurons, regulating blood flow in the CNS and regulating levels of neurotransmitters and ion concentration at the level of the synaptic biophase (Mulligan et al., 2004; Pellerin et al., 1994; Tsacopoulos et al., 1996; Bergles et al., 1998). In particular, astrocytes and neuronal synapses are considered bi-directional partners, beeing active in the modulation of synaptic transmission (Araque et al., 2010). Astrocytes are able to perceive changes in the extracellular environment that are indicative of neuronal activity and, subsequently, they respond releasing neuromodulatory factors, called gliotransmitters, which can act both at presynaptic and postsynaptic level (Santello et al., 2012). This close and specific correlation between neurons and astrocytes is called tripartite synapse (Araque et al., 1999). Furthermore, astrocytes allow the integration and exchange of information even with neurons that are not synaptically connected, using both calcium-dependent and calcium-independent pathways, to regulate synaptic network activity (Araque et al., 2010; Chen et al., 2013; Navarrete et al., 2010; Piet et al., 2004; Serrano et al., 2006). In this context, the example of the astrocytic glutamate transporters GLT-1 and GLAST can be reported, since they are very expressed at the level of the astrocytic processes facing at the active zone of the synapses (Chaudhry et al., 1995). In turn, the physical proximity of astrocytic processes to the synapse affects the clearance efficiency of neurotransmitters, i.e. glutamate, which consequently modulates synaptic transmission (Oliet et al., 2001; Pannasch et al., 2014).

The ezrin protein, a membrane-cytoskeletal cellular linker, has been specifically detected in the brain astrocytes and it is critical for motility of the perisynaptic astrocytic process (PAP) observed during synaptic plasticity (Derouiche et al., 2001; Lavialle et al., 2011).In the regulation of function (Henneberger et al., 2010; Pascual et al., 2005) and synaptic behaviours (Cao et al., 2013; Halassa et al., 2009), PAP are often involved in the release of gliotransmitters.In addition, in this case it is known how astrocytes express the proteins of the SNARE complex, for which there are astrocyte-specific isoforms, for example VAMP3 and SNAP23 (Schubert et al., 2011).

In recent years, hundreds of neuronal proteins belonging to the pre- and post-synaptic membrane have been identified and described (Li et al., 2004; Weingarten et al., 2014), and information about their function in the synapse enormously increased (Rao-Ruiz et al., 2011; Vegh et al., 2012). However, how astrocytic proteins are involved in functional interaction with neurons remain not fully described.

One of the research groups, that I joined during my PhD, previously described and published an experimental method to obtain a subcellular fraction of highly purified particles of astrocytic origin, deriving from perisynaptic astrocytic processes, called gliosomes (Stigliani et al., 2006). Stigliani and collaborators have shown that gliosomes are enriched with subcellular particles expressing typical astrocytic markers, compared to the synaptosomal preparation, that consists of isolated nerve endings. Indeed, gliosomes are enriched with astrocyte-specific proteins such as GFAP and S100, and contain both astrocytic glutamate transporters and components of the exocytotic apparatus. This implies that they also contain molecular structures able to effect a gliotransmission (Paluzzi et al., 2007). As a result, gliosomal preparations have already been widely used to evaluate the release and uptake of different gliotransmitters by the astrocytic cells that characterize and are present in different tissues of the central nervous system, both in physiological and pathological conditions (Pedrazzi et al ., 2006; Raiteri et., 2007; Milanese et al., 2009; Milanese et al., 2010; Cervetto et al., 2017)

4.2 Gliosomes are enriched with proteins associated with the astrocytic membrane

In 2014, one of the research groups with which I carried out my thesis, in collaboration with Prof. Verheijen from the University of Amsterdam, performed a study comparing purified

preparations of hippocampal synaptosomes and gliosomes, isolated by gradient of Percoll®, to establish a more detailed list of astrocytic proteins potentially involved in the neuron-astrocyte interaction. The two purified preparations were compared by means of a triple-label free semi-quantitative mass spectrometry analysis and the results obtained in this work represent the first detailed description, from the proteomic point of view, of the two preparations and at the same time they depict an important source of data for the understanding of astrocytic membrane proteins and their potential role in the function of the tripartite synapse.

The increasing research on the importance of astrocytes for brain function requires advanced techniques to purify astrocytic proteins from carefully isolated brain tissues. Only recently defined methods are available to purify astrocytes from astrocyte neuron cultures (Goudriaan et al., 2014) and to isolate intact astrocytes from brain tissue (Orre et al., 2014; Jungblut et al., 2012). However, there are still no effective methods available to specifically isolate proteins in vivo or ex vivo.

For these studies, a mass spectrometer was used to measure the quantitative level of proteins in gliosomes in order to assess the characteristics of this preparation in terms of astrocytic proteins. A number of studies have shown that gliosomes are highly enriched in proteins involved in the exocytotic machinery (i.e. the protein marker VAMP3; Schubert et al., 2011) as well as of typical proteins of astrocytic perisynaptic processes (i.e. the ezrin protein complex, Derouic et al., 2001; Lavialle et al., 2011) and of proteins associated with the astrocytic membrane (i.e. the astrocytic membrane glycoprotein Basigin; Heller et al., 2003) (Figure 14).

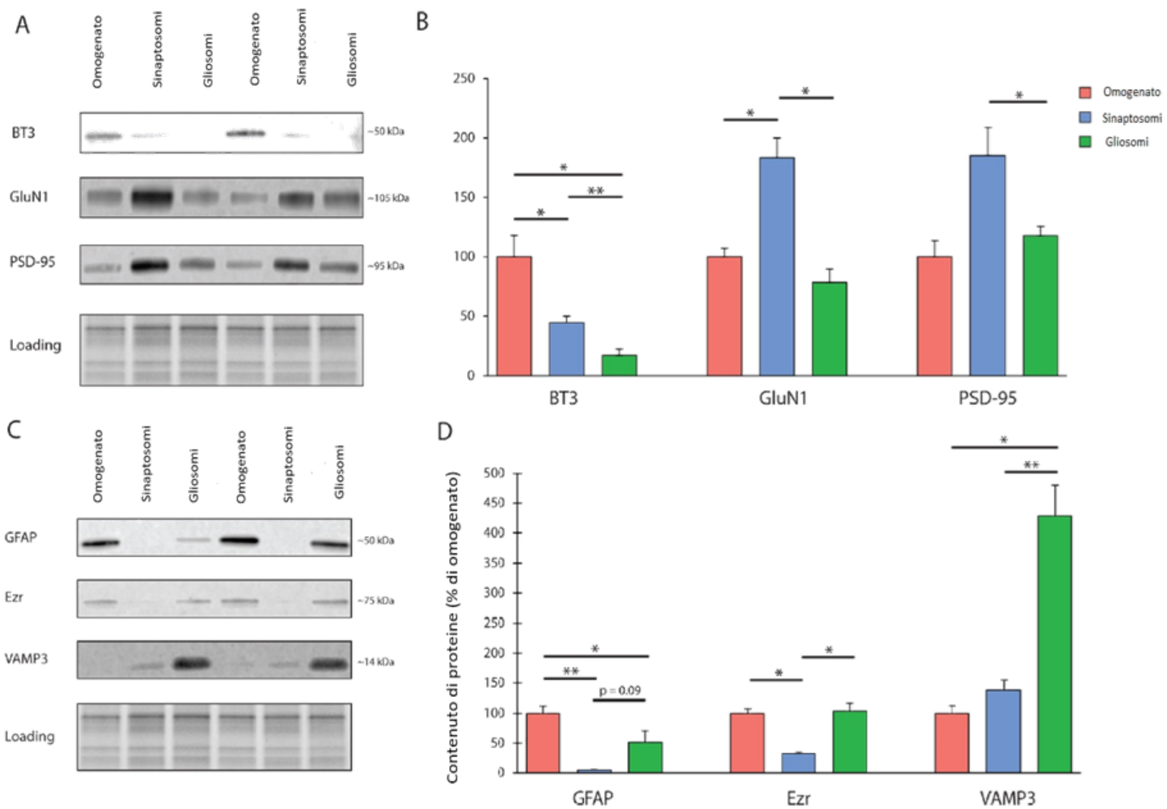

Figure 14: Comparison between hippocampal gliosomes (perisynaptic astrocytic particles) and synaptosomes (isolated nerve endings). Enrichment of specific protein markers for gliosomes and synaptosomes compared to a non-purified homogenate preparation. Immunoblots (A) and quantification (B) for neuronal protein markers: beta-tubulin 3 (BT3), NMDA receptor subunit 1 (GluN1) and postsynaptic density protein 95 (PSD-95). Immunoblots (C) and quantification (D) of protein markers of astrocytic peisynaptic particles: glial fibrillary acidic protein (GFAP), ezrin (Ezr) and vesicle-associated membrane protein 3 (VAMP3). The bar graphs represent the mean ± SEM of 6 independent experiments; * $p < 0.05$, ** $p < 0.005$. (Taken and adapted by Carney et al., 2014).

In line with the functional studies related to the release and uptake of various neurotransmitters previously published using gliosomes from different tissues of the central nervous system (Milanese et al., 2010; Pedrazzi et al., 2006; Raiteri et al., 2007), it has been further demonstrated that gliosomes express glutamate transporters, while they are depleted of synaptosomal proteins, such as SNARE presynaptic proteins and post synaptic proteins NR1 and PSD-95. Further genetic analysis of glisosomal proteins, carried out by Carney and collaborators, revealed that the gliosomes are of membrane origin and are enriched with different subunits of heteromeric and small GTPase proteins (Carney et al., 2014; data not shown). To confirm this, a wide range of G protein-coupled receptors (GPCRs) has been

detected in perisynapticastrocytes (Porter et al., 1997). The activation of these receptors can lead to an intracellular increase in calcium ions, which can subsequently trigger the release of gliotrasmitters thus modulating both excitatory and inhibitory synaptic transmission (Santello et al., 2012; Navarrete et al., 2010; Kang et al., 1998). G protein enriched gliosomes can be components of this complex cascade. It is interesting to note that heterotrimeric G proteins in humans have been genetically associated with cognitive abilities, which suggest involving alterations in the neuronal network of the brain (Ruanoet, 2010). These alterations can involve heteromeric astrocytic G proteins, and gliosomes provide a preparation that very well represents the membrane characteristics and therefore is suitable not only for functional studies, but also for molecular and functional analysis of heteromeric G proteins.

The observation that gliosomes are enriched with PAP markers such as Ezrin and VAMP3 therefore suggests that gliosomes are a preparation that can be exploited to study PAP proteins in general.

These perisynaptic astrocytic processes are extremely mobile and "fine" peripheral synapse processes that form a sheath around synapses in many brain regions, including hippocampus (Ventura et al., 1999), hypothalamus (Lavialle et al. , 2011; Panatier et al., 2006) and the cerebellum (Saab et al; 2012), Moreover, they are functionally suitable for exercising a modulatory control on synaptic activity (Henneberger et al., 2010; Haber et al., 2006).

These peculiar characteristics allow gliosomes to be used for both functional and protein expression analysis regarding astrocytes, in different experimental models, including genetically modified animals, as well as behavioural models aimed to study the effect of pharmacological treatments or to identify new roles of astrocytes in brain function.

4.3 Purification of gliosomes

The animals (rats) were killed and the prefrontal cortices or striatumwere removed. Purified gliosomes were prepared according to Dunkley et al. (1986). Tissue was homogenized in 0.32 M sucrose, buffered to pH 7.4 with Tris HCl, using a homogenizer consisting of a glass tube and a pestle (0.25 mm clearance Potter Elvehjem VWR International). The homogenate was centrifuged (5 min, 1,000 g) to remove nuclei and debris, then the supernatant was withdrawn and centrifuged at 12,000 g for 10 minutes and the pellet was resuspended in

sucrose 0.32 M tris-buffered and stratified delicately on a discontinuous Percoll gradient (Sigma-Aldrich, St Louis, MO; 2%, 6%, 10% and 20% v / v in sucrose 0.32 M tris buffer). After centrifugation at 33.500 g for 5 minutes, the preparation stratified at the interface between the Percoll solution at 2% and at 6% (gliosomial fraction) was recovered. This fraction was finally centrifuged at 20,000 g for 15 minutes with 30 ml of physiological solution (MP) having the following composition (mM): NaCl, 140; KCl, 3; $MgSO_4$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 5; $CaCl_2$,1.2; 4- (2-hydroxyethyl) -1-piperazine than esulfonic acid (HEPES), 10; glucose, 10; pH 7.4. All the above procedures were conducted at 4° C. The gliosomes were resuspended in MP for the release experiments and confocal microscopy or lysate in ultrapure water type II (Milli-Q, Millipore, Billerica, MA) for the endogenous determination of the cytosolic content of glutamate / glutamine. The protein content was measured according to Bradford (1976) using bovine serum albumin (Sigma-Aldrich) as standard.

- interface between 2% and 6% of Percoll solution containing mainly the purified gliosomes.
- interface between 6% and 10% of Percoll solution containing both gliosomes and synaptosomes.
- interface between 10% and 20% of Percoll containing mainly the purified synaptosomes.

Figure 15: Representative image of a discontinuous Percoll gradient reporting the 2%-6%, 6%-10% and 10%-20% interfaces, containing purified gliosomes, gliosomes plus synaptosomes and purified synaptosomes respectively

5 The superfusion technique

The superfusion technique has been set up at the laboratory of Prof. Raiteri from the University of Genoa about 40 years ago (Raiteri M et al., 1974). The original system, the prototype of the current automated system, was composed of four identical blown glass chambers whose bottom was made of sintered porous glass filters. In this system the synaptosomes or gliosomesare stratified on a nitrocellulose filter layed on the bottom of each

chamber and then the experimental protocol can be performed. In the superfusion medium, in contact with the biological preparation, all the neuro- and gliotransmitters are released. From the bottom of the four chambers, kept at a constant temperature, the superfusate was continuously aspirated with a flow of 0.5 ml / min and collected in the vials (Figure 16).

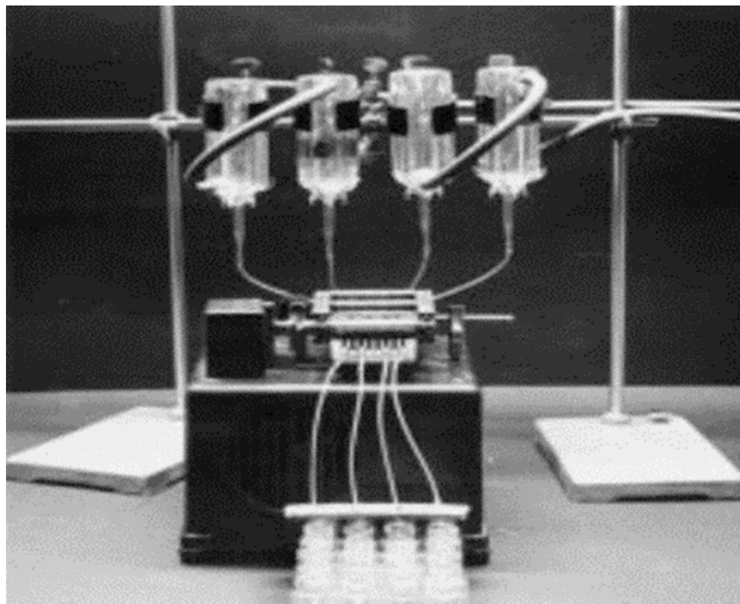

Fig 16: Representative image of the first version of the 4-chamber unit (Raiteri & Raiteri., 2000)

The best way to study presynaptic receptor was started with the superfusion technique. This technique is useful in the identification and the functional characterization of receptors, that are present on the CNS neuronal or glial terminal and regulate the transmitter release. One other positive aspect of this technique is to prevent the reuptake of the release. It also stops to activate the receptor, which are present on the neighbouring terminals.The technique shown remarkable accuracy, when added appropriate ligand, at the desired concentration, to the superfusion medium.

Some of the advantages of the superfusion technique are mentioned in Figure 17

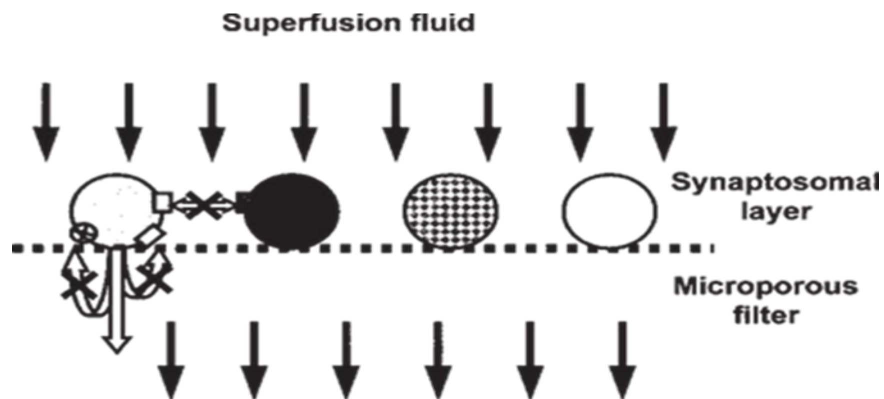

Fig. 17: Distinctive features of the superfusion technique. Endogenous transmitters/modulators released are immediately removed by the superfusion medium before they can be retaken up by transportersand before they can activate autoreceptorsor heteroreceptors present on nerve terminals. Reuptake can therefore not occur and indirect effects are minimized or prevented. During superfusion all the presynaptic targets (transporters, receptors, channels, enzymes, etc.) can be considered virtually free of endogenous ligands; each of these targets can be studied separately by adding the appropriate ligand, at the desired concentration, to the synaptosome thin layer. The effects observed on the release of one transmitter can reasonably be attributed to direct actions at the terminals storing that transmitter (Figure is taken from Raiteri & Raiteri., 2000)

The fundamental principle of this technique, which is based on the 24 chamber, mentioned in Figure 17, is the prevention of any indirect interaction mechanism of the transmitters that are released by synaptosomes or gliosomes on the structures expressed by them, both externally on the synaptic membranes and inside the terminal (receptors, transporters, ion channels etc). All measured changes in release capacity are effects due exclusively to stimuli that are added to the superfusion medium.The response to the stimulus or release of neurotransmitters, thanks to the continuous flow of mediums, does not have the time to perform a feedback on the gliosomes themselves.The continuous flow of the medium from top to bottom takes away what is released, allowing the quantification of the so-called "pure release", a result that cannot be obtained through other in-vitro or in-vivo techniques.

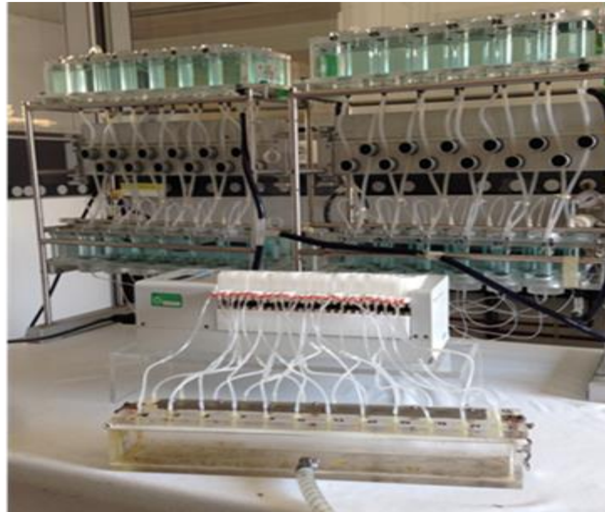

Fig 18: Latest version of the room unit (Raiteri & Raiteri., 2000)

5.1 The superfusion system

The current superfusion system, designed on the basis of the old prototype, produced and marketed by Ugo Basile srl (Superfusion system, Ugo Basile srl, Comerio, Varese, Italy) has undergone several improvements and automations. However, the experimental principle of the technique remained the same. The devices that are now on the market, of which have the following characteristics:

• A number of superfusion chambers (modules of 12 or 24 chambers) at the base of which are arranged seven-sided glass containing porous filters on which to layered the synaptosomes or gliosomes. In detail, synaptosomes or gliosomes are not stratified directly on the filters but on a nitrocellulose membranes resting on them. This offers the advantage of being able to retain synaptosomes or gliosomes throughout the release experiment and to recover and analyze the biological sample at the end of the experiment quantifying their concentration for each chamber.

• The superfusion chambers are connected, by means of manually controlled tubes and electro-valves, to an equal number of upper chambers, which allow the warming of the perfusion medium in advance during the experiment. The superfusion medium may contain various stimuli (i.e. KCl,4-AP)able to evoke or inhibit the neuro or gliotransmitter release or unknown compounds.

• During the experiment, following an established time schedule, the medium in the upper chambers flow down on synaptosomes or gliosomes, thanks to the opening of the electro-valves.

• All the chambers (upper and lower) are thermostat controlled.

• A multi-channel peristaltic pump maintains superfusion at a constant flow of 0.5 ml / min.

• At the base of the superfusion chambers each nitrocellulose filter, containing synaptosomes or gliosomes, is connected to a capillary through which the medium is collected into vials.

• A manual aspiration system allows the elimination of the superfusion medium present in the chambers and the replacement with new medium, thus interrupting i.e. the stimulus effect without interfering with the neuronal or glial preparation.

5.2 Release experiments.

To study the release of glutamate, gliosomes were incubated for 15 min at 37C° in a water shaker bath, in order to activate their physiological functions, in the presence of 0.08 µM [3H] D aspartate, a non metabolizable analogue of Glu which labels the intra-terminal releasable pools of the excitatory amino acid (Fleck et al., 2001). Gliosomes were layered and superfusion was started with physiological medium at a rate of 0.5 ml/min and continued for 48 min.

Regarding FS-experiments: after 36 min of superfusion to equilibrate the system, samples were collected according to the following scheme: two 3-min samples (t = 36 e 39 and 45 e 48 min; basal release) before and after one 6-min sample (t = 39 e 45 min; stimulus-evoked release). Stimulation with a 90 s pulse of 15mM KCl was applied at t = 39 min.Collected samples and superfused gliosomes were counted for radioactivity. The stimulus evoked neurotransmitter overflow was estimated by subtracting the transmitter content in the two 3-min fractions, representing the basal release, from that in the 6-min fraction collected during and after the stimulating pulse.

Regarding the study of the oxytocin receptor:after 38 min of superfusion, gliosomes were depolarized by exposing them to a medium containing 4-AP (300 µM) until the end of the experiments.Superfusion fractions were collected by five 3 min fractions following this time

schedule: 33-36 min (B1), 36-39 min (B2), 39-42 min (B3), 42-45 min (B4), 45-48 min (B5). The agonists (the D2 receptor agonist, quinpirole, 1μM; the A2A receptor agonist, CGS 21680, 0,01μM and oxytocin, OXY, 3nM) were added concomitantly with 4-AP, while the antagonists (the oxytocin receptor antagonist, L-371,257, 1μM or the A2A receptor antagonist, SCH 58261, 1μM) were added starting from 8 min before 4-AP application and till the end of the experiment. At the end of the superfusion, collected samples and superfused gliosomes were counted for radioactivity. The efflux of radioactivity in each fraction was calculated as a percentage of the total radioactivity present at the onset of the fraction considered (fractional release). The mean tritium fractional release in B1 and B2 fractions was taken as the 100% control value for each chamber. Tritium efflux in the other Bn fractions was evaluated as the percentage of variation in tritium fractional release with respect to the corresponding control value. The drug (or depolarization)-evoked tritium efflux was measured by subtracting the area under the curve of percentage of variations in tritium fractional release in appropriate control chambers from the area under the curve of the percentage of variations in drug-treated chambers (or in chambers supplemented with 4-AP).

6. Confocal microscopy

Immunofluorescent confocal microscopy was carried out as previously described (Cervetto et al. 2016, 2017, 2018). Briefly, striatal gliosomes (15 μg) were fixed with 2% paraformaldehyde, permeabilized with 0.05% Triton X-100 in 0.5% albumin (5 min), incubated overnight with primary antibodies diluted in 3% albumin phosphate-buffered saline (PBS). The primary antibodies used are: mouse or rabbit anti-glial fibrillary protein (GFAP; 1:1000; Sigma-Aldrich); guinea pig anti-vesicular glutamate transporter type 1 (VGLUT1; 1:1000; Merck Millipore Corporation); goat anti-oxytocin receptor (OXTR; 1:100; abcam); mouse anti-A2A (1:200; Merck Millipore Corporation) and rabbit anti-D2 (1:200; Atlas Antibodies, Stockholm, Sweden). The following day, gliosomes were washed with PBS, then incubated 1h with Alexa Fluor 488, 568 or 633 conjugated secondary antibodies (1:1000; Life Technologies Corporation, Carlsbad, CA, USA) in PBS containing albumin 0.5%. The images were acquired using a three-channel laser-scanning confocal microscope (TCS SP2; Leica Wetzlar, Germany), equipped with 458, 476, 488, 514, 543, and 633 nm excitation lines.

7. Statistics

The means ± SEM of n, numbers of experiments, are indicated throughout. The significance of the difference was analyzed by the one-way ANOVA plus Bonferroni post hoc test or non-parametric Mann–Whitney test, and statistical significance was taken at $p < 0.05$.

8. Materials

[3H]D-aspartate was from PerkinElmer (Waltham, MA, USA); 4-[2-[[6-amino-9-(N-ethyl-b-D-ribofuranuronamidosyl)-9H-purin-2-yl] amino]ethyl]benzene propanoic acid hydrochloride (CGS 21680) and oxytocin were from Tocris Bioscience (Bristol, UK); 4-Aminopyridine (4-AP), 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH 58261) and quinpirole were from Sigma-Aldrich (Milan, Italy). When possible, drugs were dissolved in distilled water or in physiological medium. SCH 58261 were dissolved in dimethyl sulfoxide and then diluted 1 : 1000 in MP. Dimethyl sulfoxide 0.1% did not affect the [3H]D-aspartate release from gliosomes. All the salts were from Sigma-Aldrich.

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