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PURINERGIC SIGNALING AND NEUROGENESIS: MODULATION OF ADULT BRAIN SUBVENTRICULAR ZONE CELL FUNCTIONS AND OF PARENCHYMAL PROGENITOR MULTIPOTENCY

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ABBREVIATIONS

2MeSATP	2-methylthio-ATP
5’-GMP	guanosine 5'-monophosphate
AA	arachidonic acid
AC	adenylyl cyclase
Ado	adenosine
ADP	adenosine-5'-diphosphate
ADPβS	adenosine 5'-O-(2-thiodiphosphate)
AP	alkaline phosphatases
AP-1	activating protein 1
ATP	adenosine-5'-triphosphate
BBB	blood brain barrier
BDNF	brain-derived neurotophic factor
bFGF	basic fibroblast growth factor
BMPs	bone morphogenetic proteins
BrdU	5-bromo-2’-deoxyuridine
CC	corpus callosum
CCK	cholecystokinin
CGRP	calcitonin-gene related peptide
CNS	central nervous system
CNTF	ciliary neurotrophic factor
COX-2	cycloxygenase-2
CRMP-4	collapsin response mediator protein 4
Ctr	control
CTX	cortex
cysLTs	cysteinyl-leukotrienes
DA	dopaminergic
DCX	doublecortin
DG	dentate gyrus
DH	dorsal horn
Dlx-2	distal-less homeobox-2
DNase	deoxyribonuclease

EAE	experimental autoimmune encephalomyelitis
ECs	ependymal cells
EdU	5-ethynyl-2’-deoxyuridine
EGF	epidermal growth factor
EL	extracellular loops
E-NPP	ecto-nucleotide pyrophosphatase/phosphodiesterase
E-NTPDase	ectonucleoside triphosphate diphosphohydrolase
eNTs	extracellular nucleotides
Epo	erythropoietin
ERK1/2	extracellular-regulated kinases 1/2
ESCs	embryonic stem cells
ET	endothelin
FAK	focal adhesion kinase
FCS	fetal calf serum
GDNF	glial cell line-derived neurotrophic factors
GFAP	glial fibrillary acidic protein
GL	granule cell layer
GLAST	L-glutamate/L-aspartate transporter
GLT1	Na+-dependent glutamate transporter 1
GPCR	G protein-coupled receptors
Gr	granule neurons
GSDB	goat serum dilution buffer
HBSS	Hanks' balanced salt solution
HDAC	histone deacetylases
ICM	inner cell mass
IFNβ	interferon beta
IFNγ	interferon gamma
IL10	interleukin 10
IP3	inositol trisphosphate
LIF	leukemia inhibitory factor
LW	lateral wall
MAG	myelin associated glycoprotein
MAP	mitogen-activated protein kinase

MBP	myelin basic protein
MBS	meta-binding sites
MCAo	middle cerebral artery occlusion
MCP	monocyte chemotactic protein-1
ML	molecular layer
MRS2179	N^6-methyl-2-deoxyadenosine 3',5'-bisphosphate
MS	multiple sclerosis
NA	noradrenaline
NeuN	neuronal nuclei
NeuroD	neurogenic differentiation
NF-kB	nuclear factor kappa B
NPY	neuropeptides
NS	neurospheres
NSCs	neural stem cells
OB	olfactory bulb
OPCs	oligodendrocyte progenitor cells
OSN	olfactory sensory neurons
P2YRs	P2Y receptors
PBS	phosphate buffered saline
PC	piriform cortex
PC-PLC	phosphatidylcholine-phospholipase C
PDGFαR	platelet-derived growth factor alpha receptor
PG	periglomerular neurons
PI3K	phosphoinositide 3-kinase
PLA2	phospholipase A2
PLC-β	phospholipase C-β
PLD	phospholipase D
PNS	peripheral nervous system
PPADS	pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid
Prox1	prospero homeobox protein 1
PSA-NCAM	polysialylated neural cell adhesion molecule
PTI	post traumatic survival time
PVL	periventricular leucomalacia

RMS	rostral migratory stream
SC	spinal cord
SCI	spinal cord injury
SGZ	subgranular zone
SP	substance P
STAT3	signal transducer and activator of transcription 3
STR	striatum
SV2	anti-synaptic vesicle2
SVZ	subventricular zone
Syt	synaptotagmin
TBI	traumatic brain injury
TEA	tetraethyl-ammonium
TGFβ	transforming growth factor-beta
TNAP	tissue non-specific form of alkaline phosphatase
TNFα	tumor necrosis factor-alfa
TSA	trichostatin A
UDP	uridine-5'-diphosphate
UTP	uridine-5'-triphosphate
UTP-γS	uridine-5'-(γ-thio)-triphosphate
VEGF	vascular endothelial growth factor
VPA	valproic acid
α,β-meATP	α,β-methylene-ATP
βIII-tubulin	βIII-tub

1. INTRODUCTION

1.1 THE PURINERGIC SYSTEM

Adenosine-5'-triphosphate (ATP) has long been recognized only as an intracellular energy molecule, and its acceptance as an extracellular signaling molecule has taken a considerably long period of time. The potent effects of ATP on the heart and blood vessels were first described in 1929 (Drury and Szent Gyorgyi, 1929), while in 1972 Burnstock proposed new roles for ATP as neurotransmitter in non-adrenergic, non-cholinergic nerves in the gut and bladder (Burnstock, 1972). In the following years ATP metabolites deriving from its enzymatic hydrolysis, like the nucleotide adenosine-5'-diphosphate (ADP) and the nucleoside adenosine (Ado), as well as other extracellular nucleotides (eNTs) like the uridine-5'-triphosphate (UTP), uridine-5'-diphosphate (UDP), and sugar nucleotides, were progressively proposed as transmitters not only in sensory nerves, but also in motor nerves and central nervous system (CNS) neurons (Fields and Burnstock, 2006; Inoue et al., 2007). The actions of these molecules in the extracellular environment implicated the existence of post-junctional receptors. Accordingly, numerous subtypes of these receptors were progressively cloned, and scientists gradually coined and accepted the term "*purinergic system*" to describe the system composed by extracellular nucleotides/nucleosides and their receptors. The first classification of purinergic receptors family dates back to 1978, when Burnstock proposed some criteria for differentiating these receptors in two families: the P1 receptors activated by Ado and antagonized by methylxantines, and the P2 receptors, responding to ATP and ADP (Burnstock et al., 1978).

Nowadays, P2 receptors are divided into two families: the ionotropic P2X receptors and the metabotropic P2Y receptors (Burnstock and Knight, 2004). P2X receptors are ligand-activated cationic channels, specifically activated by ATP (Burnstock and Knight, 2004), while P2Y receptors are activated by purine or pyrimidine nucleotides, or by sugar-nucleotides, and couple to intracellular second-messenger systems through heteromeric G proteins (Abbracchio et al., 2006). In 1994, the IUPHAR Subcommittee for Purinoceptor Nomenclature and Classification has approved the new classification and has proposed to substitute the term "*P2*

purinoceptors” with “*P2 receptors*”, in order to take into account the observation that some P2 receptors are preferentially activated by uridine nucleotides (Fredholm et al., 1997). To date, at least seven P2X subtypes (P2X1-7) and eight P2Y members ($P2Y_{1,2,4,6,11,12,13,14}$) have been cloned from different animal species (Abbracchio et al., 2006). The missing numbers in the P2Y series correspond to receptors cloned from vertebrates different from mammals and for which no mammalian orthologs have been identified so far, or to receptors that have not been functionally characterized yet or that were incorrectly classified in the family.

The concept that ATP is an extracellular signaling molecule has been established not only in the rapid signaling involved in neurotransmission, but also in a wide range of other biological processes, including release of cytokines, neurotransmitters and hormones, cell proliferation, differentiation and apoptosis in tissues as diverse as the skin, skeletal muscle, bone, nervous and immune system (Fields and Burnstock, 2006; Inoue et al., 2007). Thus, alterations in purinergic signaling may contribute to the development of disorders of the immune system, inflammation, neurodegeneration, osteoporosis and cancer. On this basis, a better understanding of the roles of purinergic signaling may help identifying novel therapeutic targets for several human diseases.

1.1.1 SOURCES AND METABOLISM OF NUCLEOTIDES

The first clues on the role of eNTs in signal transduction came from the observation that neurons and neuroendocrine cells released ATP packaged with other neurotransmitters (Burnstock, 1972). Moreover, it has been demonstrated that, following mechanical stress, also non secretory tissues can release nucleotides (Lazarowski et al., 2000) which may signal to the same secretory cell (autocrine stimulation) as well as to adjacent cells (paracrine stimulation). Following injury or inflammation, nucleotides can also be pathologically released as a consequence of cell lysis. There is ongoing debate, however, about the transport mechanisms involved in nucleotide release. There are hints for exocytotic release from endothelial and urothelial cells, osteoblasts, astrocytes, and mast and chromaffin cells, but other transport mechanisms have been proposed as well, including ATP-binding cassette transporters, connexin hemichannels and plasmalemmal voltage-dependent anion channels (Fields and Burnstock, 2006). More recently, pyrimidine nucleotides release has been described (Lazarowski et al., 2003).

Once released in the extracellular environment, nucleotides are rapidly degraded by ubiquitous ecto-nucleotidases (Zimmermann, 2000), a family of phosphatases expressed on the cell surface that are able to dephosphorylate different nucleotides. ATP hydrolysis sequentially produces ADP, AMP and adenosine, whereas UTP is degraded to UDP, UMP and uridine; many of these metabolites can act as extracellular signaling molecules. The local response to a specific nucleotide is thus the result of the effects of the nucleotide itself and of its degradation products. Distinct classes of ecto-nucleotidases with different properties and different substrate specificities have been identified so far (Yegutkin, 2008). Some are membrane proteins with an extracellular catalytic domain, but soluble forms released in the extracellular space have also been described. Ecto-nucleotidases can be also released with ATP from sympathetic nerve terminals, representing one of the mechanisms to turn off neurotransmitter signaling (Todorov et al., 1997). A general scheme of nucleotide-hydrolyzing enzymes include: (i) the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family, (ii) the ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) family, (iii) ecto-5'-nucleotidase, and (iv) alkaline phosphatases (AP; Yegutkin, 2008). This complex enzymatic family demonstrates that ATP and other nucleotides can be considered as “classical neurotransmitters” whose signal can be regulated by enzymatic degradation.

Nucleotides released or leaked from both glial cells and neurons play a role in cell-to-cell communication under both physiological and pathological conditions (Burnstock and Knight, 2004). Accordingly, in CNS both neuronal cells and the major classes of glia (i.e. astrocytes, microglia and oligodendrocytes) express a vast variety of purinergic receptors (Fields and Burnstock, 2006). For instance, astrocytes release ATP in response to various stimuli or even spontaneously, and communicate with neurons, microglial cells, and even vascular cells at capillaries (Inoue et al., 2007). Also microglial cells respond to a wide range of ATP receptor agonists, through increases in intracellular calcium, secretion of cytokines, and rapid changes in their morphology and migration capacity (Inoue, 2008). Therefore, eNTs represent a class of signaling molecules that functionally “unite” glia and neurons together. Not by chance neuron-glia or glia-glia communication participates in the control of several pathophysiological mechanisms, including regulation of synaptic transmission (Wieraszko and Ehrlich, 1994), neuroimmune interactions (Inoue et al., 2007), processing of information through the retina (Newman, 2008), Schwann cell proliferation and myelination (Fields, 2006;

Stevens, 2006), cell proliferation (Neary and Zimmermann, 2009), inflammation (Di Virgilio et al., 2009), pain (Burnsock, 2009a) and regulation of neural stem cells (Ulrich et al., 2012; see also 1.4.3).

1.1.2 P2X RECEPTORS

P2X receptors are membrane ion channels that open in response to the binding of extracellular ATP. ATP elicits rapid responses (<10ms) via these ion channels, resulting in selective permeability to Na^+, K^+, and Ca^{2+} cations (North, 2002). In vertebrates, seven genes encode P2X receptor subunits, which are 40-50% identical in their aminoacidic sequence. Each of three putative subunits, proposed to form a homo- or heterotrimeric complex, comprises two hydrophobic transmembrane spanning regions (TM1 and TM2), a large extracellular loop of about 270 amino acids and two intracellularly located terminal tails (amino NH_2- and carboxy COOH-tail). The TM1 and TM2 segments of the trimer are thought to line an integral pore with a diameter of about 8-20 Å, with the selective ion filter (activation gate) consisting of a small polar stretch in the TM2 region. Concerning one subunit, the amino acid residues range from 388 (P2X4) to 595 (P2X7) in human P2X-Rs. The extracellular loop contains the ATP binding site as well as sites for antagonists and modulators (Khakh et al., 2001). The aminoacidic identity between P2X receptor subunits is distributed throughout the extracellular domain, a striking feature of which is the conservation among all known receptors of 10 cysteine residues, which form disulfide bonds to give the correct conformation to the receptor (Vial et al., 2004). All the P2X receptor subunits have consensus sequences for N-linked glycosylation (Asn-X-Ser/Thr), and some glycosylations are essential for trafficking to the cell surface. The extracellular domain carries few conserved glycine (G) and proline (P) residues which are involved in conformational changes subsequent to ligand-receptor binding. Extracellular protons, bivalent cations and some metals are P2X receptors allosteric modulators. In addition, P2X receptors can be modulated via phosphorylation of serine (S) and threonine (T) residues (**Figure 1.1**).

As of today, seven homomeric channels (P2X1-7) have been identified, but functional expression studies have also highlighted the existence of heteromeric P2X1/5, P2X2/3, P2X2/6, P2X4/6, P2X4/7 and P2X5 receptors which can assemble with any other subunit, except P2X7 (North, 2002).

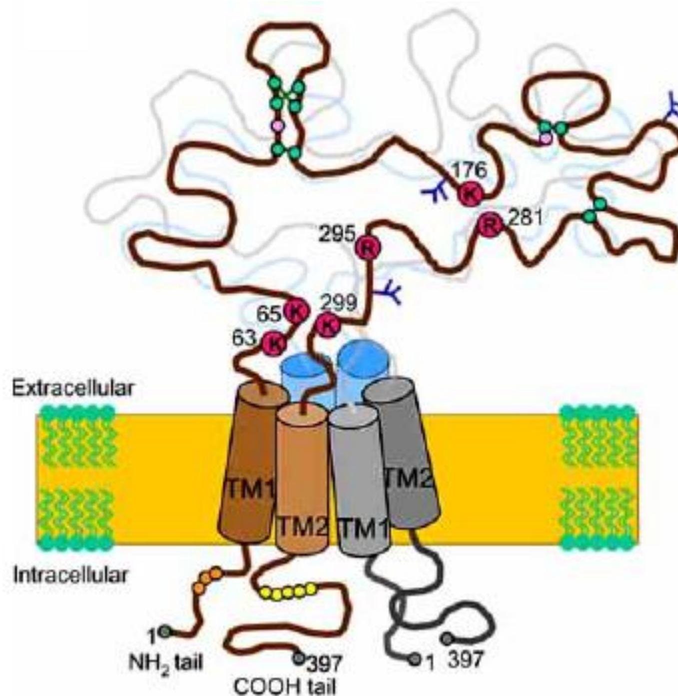


***Figure 1.1: schematic representation of the general features of P2X receptors**. Numbers indicate the position of important residues for agonists binding (i.e., Lys(K)63, K65, K299 may interact with the phosphate tail of ATP; K176 and Arg(R)281 may coordinate adenine binding). In addition, the extracellular loop of all P2X-Rs contains ten conserved cysteine residues forming disulphide bridges (green circles), Zn^{2+} binding sites (pink circles), glycosylated asparagine residues (blue symbols). The intracellular amino- and carboxy-tails are associated with conserved protein kinase C (orange) and trafficking (yellow) motifs, respectively. For the P2X3-R, six putative nucleotide binding sites (highlighted in red) have been postulated by molecular modelling (modified from Fischer and Krugel, 2007).*

1.1.3 P2Y RECEPTORS

P2Y receptors (P2YRs) belong to the superfamily of G protein-coupled receptors (GPCR). GPCR are a family of membrane receptors responding to a wide variety of ligands such as nucleotides, biogenic amines, peptides and other small molecules (Marchese et al., 1999). The binding of the GPCR to its specific ligand results in the activation of the associated heterotrimeric G protein (α, β and γ subunits) that mediates a number of intracellular responses. In particular, ligand binding to its receptor results in

a decreased affinity of the α subunit for GDP, that is thus exchanged for GTP. This binding causes a conformational modification in the G protein and the dissociation of the α subunit from the βγ complex. Both α and βγ subunits can then activate signal transduction pathways (Rebois et al., 1997).

From a phylogenetical point of view, the eight human P2YRs can be subdivided into two distinct subgroups characterized by a relatively high level of sequence divergence. The first subgroup encompasses $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors, whereas the second subgroup encompasses $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors (Abbracchio et al., 2006).

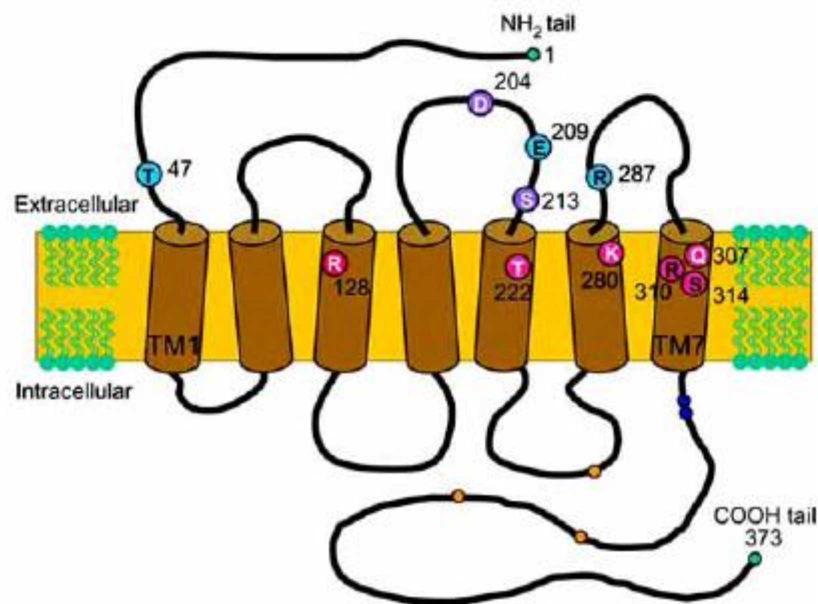


***Figure 1.2: schematic representation of the general features of P2Y receptors**. P2Y-Rs consist of seven transmembrane spanning segments (TM1-TM7) connected by three extracellular and three intracellular loops. The NH2-tail is located outside the cell, while the COOH-tail is located in the cytoplasm, the latter possessing consensus binding motifs for protein kinases. Among the extracellular loop and the outermost parts of the transmembrane segments, the hypothetical binding sites for receptor agonists at the human $P2Y_1$-R are shown. In particular, three different parts responsible for the binding of agonists like ADP were assumed (blue circles: meta-binding site I, white letters: meta-binding site II, red circles: principal binding site). Several potential PKC and G protein-coupled receptor kinase phosphorylation sites are indicated on the third intracellular loop and the COOH-terminus (orange circles). The two arginine residues (R333/R334; dark blue circles) in the COOH-terminus of the human $P2Y_1$-R are thought to be essential for Gq coupling (Modified from Fischer and Krugel, 2007).*

Jacobson and co-workers (Costanzi et al., 2004) presented a detailed model for the binding of nucleotides to the human $P2Y_1R$ (later extended to all P2YRs) by site-

directed mutagenesis combined with molecular modelling analysis. According to their hypothesis, a multistep mechanism for the binding of small ligands like ATP may exist. Initially, the ligand is positioned at the $P2Y_1R$ in an energetically favorable conformation, which is defined by the extracellular loops (EL) and the outermost TM segments by two "meta-binding sites" (MBS). From MBS-I the ligand could progress to MBS-II, located on Arg(R)128, Asp(D)204, Ser(S)213, Thr(T)222, Lys(K)280 and Gln(Q)307. Finally, the ligand reaches the principal binding site located inside the TM bundle consisting of Gln(Q)307, Ser(S)314 for the binding of adenine, and Arg(R)128, Thr(T)222, Lys(K)280, Arg(R)310 for binding of the phosphate groups. Some of the amino acids (R128, T222, K280, Q307) are implicated in both MBS-II and the principal binding site (**Figure 1.2**).

The two P2Y receptor subgroups also differ in their primary coupling to G proteins. In particular, all receptors in the first subgroup (i.e., $P2Y_{1,2,4,6,11}$) couple to Gq/G_{11} and activate phospholipase C-β (PLC-β) with the generation of diacylglycerol and inositol trisphosphate (IP3) and the subsequent mobilization of Ca^{2+} from intracellular stores. The receptors in the second subgroup (i.e., $P2Y_{12,13,14}$) almost exclusively use the Gi/o class of G proteins with subsequent inhibition of adenylyl cyclase (AC) and cAMP formation. The $P2Y_{11}$ receptor subtype also couples to the Gs protein subunit to activate AC followed by an increase in cAMP when stimulated by ATP (**Figure 1.3**; Fischer and Krugel, 2007). Secondary couplings have been also reported, especially for receptors of the first subgroup in heterologous expression systems (Abbracchio et al., 2006). Among receptors of the second group, $P2Y_{13}$ has been also reported to couple to $G\alpha_{16}$ and to stimulate PLC in recombinant systems overexpressing this G protein (Fumagalli et al., 2004). Such "promiscuity" of G protein coupling may depend on the indirect activation of additional G protein subtypes within protein complexes containing the P2Y receptor.

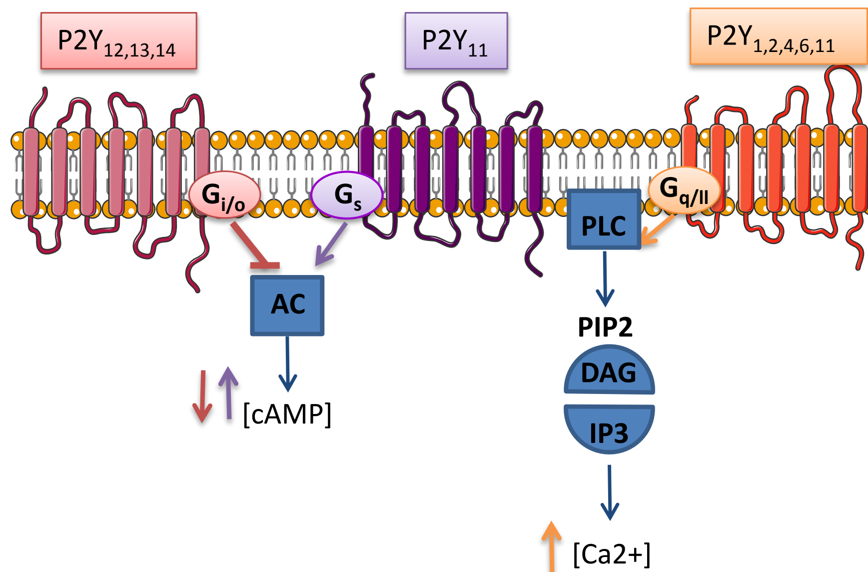


Figure 1.3: second messenger coupling and intracellular signaling pathways of P2Y receptors. *Nucleotides acting at P2YRs cause activation of specific G proteins:* $P2Y_{1,2,4,6,11}$*-Rs are coupled to Gq/G11, which stimulate phospholipase C (PLC) and the mobilization of* Ca^{2+} *from intracellular pools;* $P2Y_{12,13,14}$*-Rs are coupled to Gi/o inhibiting adenylyl cyclase (AC). Finally, the* $P2Y_{11}$ *receptor is coupled also to stimulation of AC.*

From a pharmacological point of view, P2YRs can be broadly subdivided in four groups based on their responsivity to nucleotides: i) adenine nucleotide-preferring receptors, mainly responding to ADP and ATP. This group includes human and rodent $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$, and human $P2Y_{11}$; ii) uracil nucleotide-preferring receptors, including the human $P2Y_4$ and $P2Y_6$ responding to UTP or UDP, respectively; iii) receptors of mixed selectivity, the human and rodent $P2Y_2$, the rodent $P2Y_4$ and, possibly, $P2Y_{11}$; and iv) the $P2Y_{14}$ receptor, responding to both UDP and sugar nucleotides (mainly UDP-glucose and UDP-galactose; Abbracchio et al., 2006; Carter et al., 2009). In this latter group it could be possibly included a receptor, named GPR17, phylogenetically and structurally related to already known P2Y receptors, which has been recently reported to respond to both cysteinyl-leukotrienes and uracil nucleotides (Ciana et al., 2006, see also 1.5.2.2). Unfortunately, although important progress in exploring structure-activity relationships has been achieved, most of the P2Y receptor subtypes are still lacking potent and selective synthetic agonists and antagonists (Abbracchio et al., 2006).

It is widely recognized that purinergic signaling is a primitive system involved in both neuronal and non-neuronal mechanisms, including exocrine and endocrine

secretion, immune responses, inflammation, pain, platelet aggregation, and endothelial-mediated vasodilatation (for a detailed review see Burnstock and Knight, 2004); in this thesis we will focus our attention on the role of eNT in the CNS.

1.1.4 Pathophysiological roles of extracellular nucleotides in the nervous system

The hypothesis that ATP plays a central role in modulating cerebral functions rises from the broad distribution of P2 receptors in the CNS (Abbracchio et al., 2006). ATP is released from nerve terminals and has rapid and direct actions, generally associated to activation, whether Ado has inhibitory effects with a negative feed-back action on the effects of ATP and of other neurotransmitters released at the synapse. The first evidence suggesting a role of ATP as a neurotransmitter in the brain was obtained in 1992, with the demonstration that its stable analogue, α,β-methylene-ATP (α,β-meATP), provokes a current of excitation in rat neurons which is antagonized by P2 antagonists (Edwards et al., 1992).

The roles of eNTs as signaling molecules are now well established. ATP (and maybe other nucleotides) is co–localized with "classical" neurotransmitters at many central and peripheral synapses, from which it can be released upon presynaptic depolarizing stimuli via a regulated secretion pathway. In some brain areas, ATP regulates neurotransmission through the modulation of glutamate release from nerve endings. This effect is mediated by P2 receptors located on the presynaptic as well as on the post-synaptic element (Motin and Bennett, 1995). In particular, it has been suggested that ATP can amplify glutamate post-synaptic action following interaction with a P2Y receptor, or by activating an ecto-kinase responsible for the phosphorylation of membrane proteins associated to glutamate receptors (Chen et al., 1996). These observations have led to the hypothesis that ATP, and maybe also UTP, might participate in long-term synaptic potentiation, memory, and learning processes (Fujii, 2004; Price et al., 2003). The experimental evidence indicating colocalization of ATP with neurotransmitters and neuropeptides in secretory vesicles of most synapses further confirms this hypothesis. Noradrenaline (NA) and ATP are released from sympathetic nervous system endings in variable ratios according to the tissue and to the animal species considered (Burnstock and Verkhratsky, 2010). Despite the demonstration that ATP is stored in the nerve endings together with NA, prejunctional neuromodulation

studies demonstrate that ATP and NA are released independently following different stimuli (Starke et al., 1996). It can thus be hypothesized that different nerve populations containing different ratios of NA and ATP exist in the sympathetic nervous system. Finally, indirect evidence demonstrates that ATP and acetylcholine co-localize in central and peripheral cholinergic terminals and that co-transmission with peptides such as substance P (SP), calcitonin-gene related peptide (CGRP), neuropeptide Y and somatostatin also exists (Burnstock, 1997; Zimmermann, 2008). In recent years, evidence has been accumulating to suggest that nucleotides are also released from non-neuronal cells (e.g., circulating platelets and erythrocytes, inflammatory cells, exercising muscle cells, hypoxic cardiomyocytes) and, within the CNS, from glial cells. It was initially believed that release of nucleotides from glia could only occur under pathological conditions (e.g., brain ischemia and trauma) as a consequence of leakage from the cytosolic nucleotide pool due to loss of membrane permeability under milder stress or hypoxic conditions, or as a result of nucleic acid degradation upon cell death due to persistent or stronger insults. It is now known that significant nucleotide release from glia (astroglia, in particular) can also occur under physiological conditions, suggesting that these molecules not only participate in brain repair and recovery after damage, but may also play key roles in normal brain function. Among the other roles exerted by purinergic system, recent studies also highlighted a direct involvement of eNTs in neurogenesis, that will be discuss in details in the Paragraph 1.4.3.

Following pathological/traumatic events a massive release of purines and pyrimidines at the site of injury is observed and they seem to have a dualistic effect: on one hand, they contribute to lesion worsening, but on the other side they are involved in tissue regeneration and repair. In the next sections the main roles of these molecules during pathological events will be briefly discussed, whereas a detailed dissertation of their role in reactive astrogliosis will be provided in the following paragraphs.

Brain ischemia

During ischemia or hypoxia in the CNS a massive release of nucleotides is observed (Melani et al., 2005; Neary et al., 1996). ATP participates to cell death induction immediately (minutes to hours) following tissue injury, probably as a consequence of a diffuse dysregulation of the release mechanisms and of a pathological activation of P2 receptors. For instance, it has been demonstrated that ATP has a

cytotoxic effect on cerebellar neurons (Amadio et al., 2002), and potentiates hypoglycemia-induced tissue injury (Cavaliere et al., 2002). In the weeks following ischemic injury, when homeostatic control is re-established in the injured area, ATP seems to contribute to differentiation and to promote the long-term functional repair of the damaged area (Abbracchio and Ceruti, 2006; Abbracchio and Verderio, 2006). During ischemic and hypoxic events, high extracellular concentrations of adenosine are also reached, in part as a consequence of ATP degradation. It has been demonstrated that adenosine has a protective role in cerebral ischemia in several experimental models. Accordingly, both adenosine administration as well as inhibition of its degradation reduce injury associated to cerebral or cardiac ischemia and protect from organ loss of function (Fredholm, 2010; Mentzer et al., 1996; Phillis and Regan, 1996). eNTs can also induce cell death, through the formation of P2X7 receptor-associated pore (Di Virgilio et al., 1996; Ferrari et al., 1997). This mechanism could influence neuronal remodeling through the elimination of critically injured cells, in particular during acute brain ischemic events, thus limiting the area of damaged tissue (Neary et al., 1996). Brain damage caused by a transient occlusion of the middle cerebral artery (MCAo) results in a sustained pathologically high ATP outflow; while in the infarct region the release of ATP is low because of disturbed cellular metabolism, in the peri-infarct area its concentration is increased, due both to the deregulation of its enzymatic degradation, and to the concomitantly enhanced outflow of the nucleotide from damaged cells (Melani et al., 2005).

Spinal cord injury

An abnormal purinergic signaling has been also observed following spinal cord injury (SCI). In the peritraumatic spinal cord regions, ATP is released at high levels and causes neuronal cell death through P2X7 receptors. In this respect, P2X7 receptor blockade is associated to improved functional recovery and diminished cell death in the peritraumatic zone (Wang et al., 2011). Analysis of P2X4 receptor expression following SCI also demonstrated a significant accumulation of P2X4-positive microglia/macrophages as early as 24 h after SCI, peaking on day 7 (Schwab et al., 2005). Taken together, these observations confirm the role of the purinergic system in the traumatic degeneration, and possibly in tissue remodeling.

Chronic neurodegenerative diseases

P2 receptor ligands have been proposed as potential neuroprotective agents following neuronal death associated with neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and multiple sclerosis (Burnstock, 2008). For instance, different P2 receptor subtypes are involved in the development and growth of dopaminergic neurons in CNS (Heine et al., 2007), and P2X7 receptors are involved in ATP-mediated necrotic volume increase in substantia nigra, which has been implicated in the pathogenesis of Parkinson's disease (Jun et al., 2007). P2X7 receptors have been also found upregulated in the brain of patients with Alzheimer's disease (McLarnon et al., 2006), and ATP released during neuronal excitation or injury enhances the inflammatory effects of cytokines and prostaglandin E2 in astrocytes, thus contributing to the chronic inflammation observed in Alzheimer's disease (Xu et al., 2003). P2X7 receptor antagonism therefore represents a therapeutic strategy for blocking inflammatory responses associated to neurodegenerative disorders. In the brain, stress or damage cause the release of nucleotides and activation of $P2Y_2$ receptors, leading to pro-inflammatory responses including the stimulation and recruitment of glial cells, which can protect neurons from injury (Peterson et al., 2010). Indeed, $P2Y_2$ receptor expression is increased in glial cells by stimulation with IL1β, a pro-inflammatory cytokine whose levels are elevated in Alzheimer's disease, and this effect has been proposed to be neuroprotective, since it increases the non-amyloidogenic cleavage of the amyloid precursor protein (Camden et al., 2013). Thus, activation of $P2Y_2$ receptors in glial cells can promote neuroprotective responses, therefore indicating this receptor subtype as a novel pharmacological target for the treatment of neurodegenerative diseases.

1.2 STEM CELLS

Strictly defined, stem cells possess the cardinal features of multipotency and self-renewal. Multipotency is the ability to differentiate into multiple functional cell types, whereas self-renewal describes the ability of stem cells to make identical copies of themselves via cell division. Stem cells exhibit extensive proliferation and are often presumed to be able to divide indefinitely, yielding a virtually unlimited supply of cells

(Burns et al., 2009). A stem cells can divide by symmetric cell division, which generate two daughter cells with the same fate, or asymmetric cell division, which generate one daughter cell identical to the mother cell and a second one that is lineage-restricted. A variety of stem cells can be identified that differ in their potency, or in the diversity of cell types they can generate.

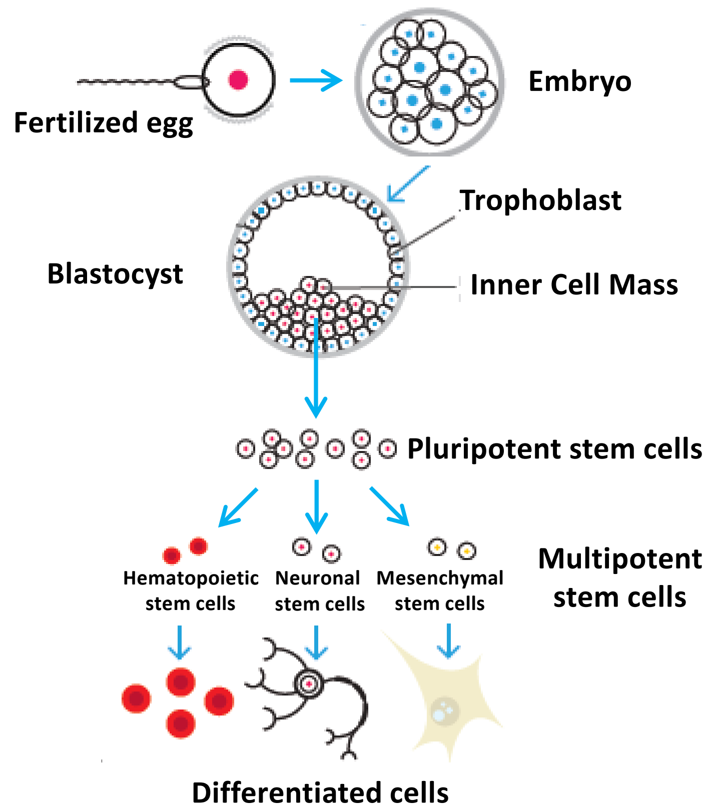


Figure 1.4: stem cell differentiation. *The mass of cells formed four to five days after fertilization is known as a blastocyst. The blastocyst contains a mass of outer cells known as the trophoblast that becomes the placenta and surrounds an inner cell mass (ICM). Pluripotent embryonic stem (ES) cells are derived from the ICM. In adult organs, multipotent stem cells can be found that can only give rise to cells of a single organ or tissue (modified from Stem cells basics for life researchers, www.bio-rad.com).*

Following fertilization, a single totipotent zygote will be formed that is capable of developing into all embryonic and extra-embryonic tissues. However, given that the zygote is transient and does not self-renew, it is not generally regarded as a stem cell. The zygote will subsequently divide many times toward the generation of a sphere-shaped conglomerate of cells, known as the “blastocyst”. This early embryonic structure has an outer layer called trophoblast, which will form the placenta, and an insider side called Inner Cell Mass (ICM), where embryonic stem cells (ESCs) can be isolated.

ICM-derived ESCs are pluripotent with the potential of indefinite self-renewal and ability to differentiate into any cell type and tissue within the body (Olynik and Rastegar, 2012). Stem cells can also be found in adult organ. These cells, known as somatic stem cells, are multipotent since, unlike ESCs, they have a restricted differentiation potential and they can only give rise to cells of a single organ or tissue (**Figure 1.4**).

The best studied tissue-specific stem cell population is the hematopoietic stem cell, which is capable of generating all blood cell types. Other tissue-specific stem cells have been identified in numerous organs including muscle, skin, gut, liver, pancreas, and brain. The most commonly discussed role for stem cells involves replacement of cell types lost due to disease or injury, i.e., "cell replacement therapy". Such replacement may be achieved either by *in vitro* differentiation of stem cells into the desired cell type followed by transplantation of the pre-differentiated cells into the affected region, or by direct transplantation of stem cells followed by spontaneous *in vivo* differentiation of the stem cells into the needed cell types. An alternate cell replacement strategy involves the mobilization of resident tissue-specific stem cells to provide the substrate for cell replacement (Burns et al., 2009).

1.3 THE TWO NEUROGENIC REGIONS IN THE ADULT BRAIN

Since the early 1900s, it has been generally believed that the adult CNS of mammals has very limited regenerative capacity (Ramon y Cajal, 1928). The predominant repair mechanisms in the CNS were thought to be postmitotic, such as sprouting of axon terminals, changes in neurotransmitter-receptor expression, and synaptic reorganization. Joseph Altman and Gopal Das proposed the concept of persistent neurogenesis in the adult brain in the '60 where they used tritiated [^{3}H] thymidine, which is incorporated into the DNA of dividing cells during S-phase of mitosis and can be detected autoradiographically, to suggest the production of new neurons in the cortex (Altman, 1963), hippocampus (Altman and Das, 1965, 1966), olfactory bulb (OB), and subventricular zone (SVZ) of the lateral ventricle (Altman, 1969). However, little attention was given to these studies, perhaps because they were considered to lack any functional relevance. The field was revolutionized by the introduction of 5-bromo-2'-deoxyuridine (BrdU), a synthetic thymidine analogue, as

another S-phase marker of the cell cycle (Gratzner, 1982). Since BrdU can be detected by immunocytochemistry for phenotypic analysis and stereological quantification, this approach remains the most commonly used technique in the field. Before the end of the twentieth century, adult neurogenesis was observed with BrdU incorporation in all mammal samples examined, including those from human patients (Eriksson et al., 1998). Combined retroviral-based lineage tracing (Price et al., 1987; Sanes et al., 1986) and electrophysiological studies provided the most convincing evidence so far that newborn neurons in the adult mammalian CNS are indeed functional and synaptically integrated (Belluzzi et al., 2003). In the early Nineties, Altman's findings were further confirmed by two reports that demonstrated the presence of cells in the striatum of adult murine brain that were able to proliferate *in vitro* in the presence of epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF), giving rise to free-floating clusters of cells called neurospheres (Reynolds et al., 1992).

Neurospheres can be formed from single cells from the lateral wall of the lateral ventricle and passaged several times *in vitro*. Indeed, the undifferentiated cells within neurospheres can either self-renew, giving rise to secondary, tertiary neurospheres and so on, or differentiate to astrocytes, neurons and oligodendrocytes. The neurosphere-forming assay has been used to detect the presence of neural stem cells (NSCs) in embryonic and adult neural tissues (Johansson et al., 1999).

It is now well established that adult neural stem cells actively undergoing neurogenesis and gliogenesis are retained only in two regions of the murine CNS: the SVZ, and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) (Doetsch et al. 1999a; Kriegstein and Alvarez-Buylla 2009; Seri and Garcia-Verdugo 2001). Outside these "typical" germinal niches no constitutive neurogenesis takes place under normal conditions in the mature parenchyma with few exceptions amongst mammals. However, it is now known that the mature nervous parenchyma retains some active proliferation and glial turnover, sustained by persisting glial progenitors (**Figure 1.5**).

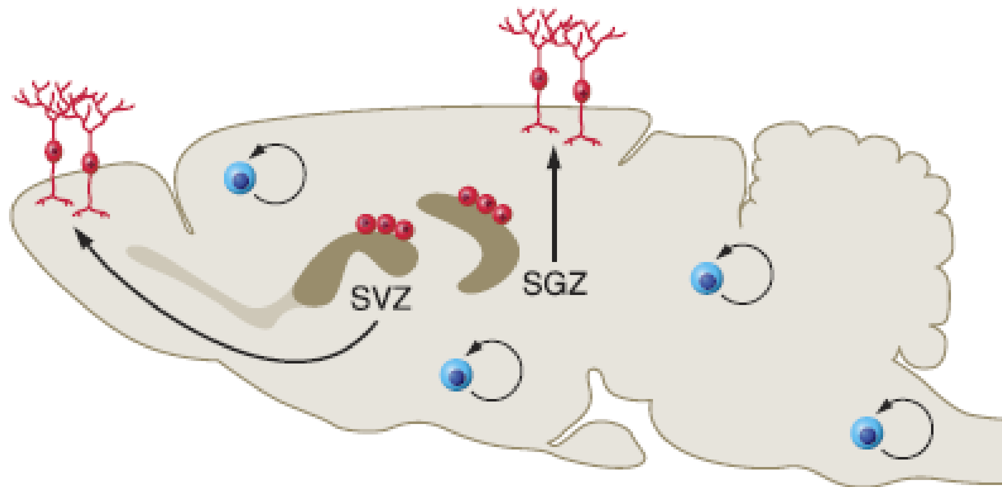


Figure 1.5: schematic representation of adult neurogenesis. *Constitutive neurogenesis, granting continuous renewal of specific neuronal populations, is restricted to germinal layer-derived neurogenic sites (subventricular zone, SVZ; subgranular zone, SGZ). Although retaining some multipotency, progenitors widespread within the parenchyma mainly contribute to the slow renewal of glial cells (Modified from Martino et al., 2011).*

On these bases, the idea emerged that the non-germinal CNS may be endowed with a latent stem cell potential, normally repressed *in vivo*, that, if properly evoked, might be exploited in situ for reparative purposes (Boda and Buffo 2010; see Paragraph 1.5).

1.3.1 THE SUBVENTRICULAR ZONE

The largest germinal region in the adult mammalian brain is the SVZ, which extends along the length of the lateral wall of the lateral ventricle. Here, over 30,000 neuroblasts are estimated to be produced every day in rodents (Alvarez-Buylla et al., 2001). The cellular composition and three-dimensional organization of the SVZ in adult mice was described in great detail by means of electron microscopy, [^{3}H] thymidine autoradiography, and immunocytochemistry (Doetsch et al., 1997). Within the SVZ, three main cell types are morphologically and functionally distinguished: astrocyte-like stem cells (type B cells) give rise to clusters of transit-amplifying cells (type C cells), which in turn generate neuroblasts (type A cells, **Figure 1.6**). In rodents, newborn neurons proliferate and migrate along the rostral migratory stream (RMS) to the olfactory bulbs (OBs). Along the entire wall of the ventricle are also present ependymal cells (type E cells) which separate the SVZ from the ventricular cavity and are

characterized by long and numerous cilia (Doetsch et al., 1997). Type B cells are considered the primary precursors and act as *bona fide* CNS stem cells, both *in vitro* and *in vivo*. Doetsch and colleagues (Doetsch et al., 1999b) demonstrated that subventricular treatment with the antimitotic substance cytosine-beta-D-arabinofuranoside resulted in elimination of type A and C cells, while part of the B cells remained and started dividing soon after the treatment. Two days later, C cells re-emerged, followed by type A cells, showing that type B cells are the primary precursor cells in the SVZ.

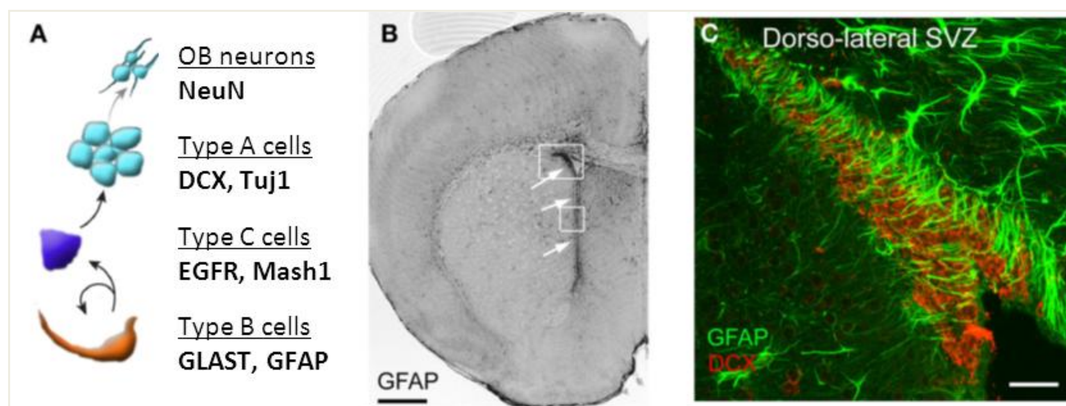


Figure 1.6: subventricular zone (SVZ) cell lineage and antigenic properties. *(A) Diagram illustrating the lineage and antigenic properties of the different SVZ progenitor cells. (B) Photograph of GFAP immunostaining in a coronal section containing the SVZ. The arrows point to the SVZ. (C) Confocal image of GFAP (green) and DCX (red) co-immunostaining in the dorso-lateral SVZ (modified from Lacar et al., 2010).*

Type B cells can be characterized by the expression of L-glutamate/L-aspartate transporter (GLAST), glial fibrillary acidic protein (GFAP) and the intermediate filament nestin. Type B cells give rise to actively proliferating (transit-amplifying) type C cells which are scattered along the network of migrating neuroblasts. They show the strongest proliferative activity among the three types of precursor cells and are the fastest proliferating cells in the SVZ. They are immunopositive for nestin, the homeobox transcription factor distal-less homeobox-2 (Dlx-2) and Mash1. Type C cells are considered to be the precursor cells to type A cells and are not found in the RMS. Type A cells are migrating neuroblasts and correspond to proliferating, migrating neural precursor cells, which show positive immunolabeling for polysialylated neural cell adhesion molecule (PSA-NCAM), doublecortin (DCX) and neuronal class III β-Tubulin (Doetsch et al., 1997, 1999a, 1999b; Garcia-Verdugno et al., 1998; Kim et al., 2011).

As early as 14 days after birth, some of the new neurons have reached the OB and migrate radially to their final positions. It has been shown that type B cells ensheat the chains of type A cells, and provide a trophic support and isolation from electrical and chemical influence from the surrounding parenchyma. Although migrating type A cells still undergo cell division, proliferation rates are reduced compared to the SVZ and their cell cycle time is lengthened. Once the migrating type A cells reach the OB, they disperse and migrate toward the granule cell and the periglomerular layers. Only upon entry in the OB do these cells achieve neuronal maturity, detectable by altered electrophysiological properties, release of neurotransmitters and expression of mature neuronal markers (Lie et al., 2004). Periglomerular and granule cells are the two major types of interneurons in the OBs and are responsible for the modulation of incoming sensory signals. These unusual interneurons lack an axon and instead have reciprocal dendro-dendritic synapses with mitral or tufted cells (Shepherd et al., 2007; **Figure 1.7**).

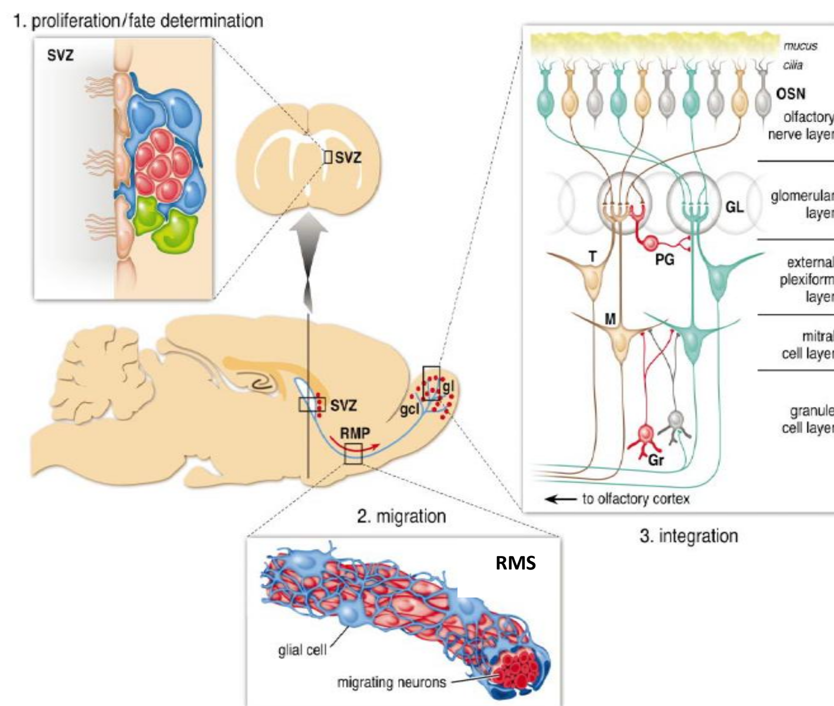


***Figure 1.7: subventricular zone (SVZ)/olfactory bulb (OB) system**. 1. Proliferation and fate determination: stem cells in the SVZ of the lateral ventricle (blue) give rise to transit amplifying cells (green) that differentiate into immature neurons (red). 2. Migration: Immature neurons (red) migrate in chains through the rostral migratory stream (RMS). The migrating neurons are ensheathed by astrocytes (blue). 3. Integration: Immature neurons differentiate into interneurons (red) in the granule cell and the periglomerular layer. Olfactory sensory neurons (OSN); tufted neurons (T); mitral neurons (M); granule neurons (Gr); periglomerular neurons (PG; from* Lie et al., 2004*).*

The unilateral movement of neuroblasts from their birthplace in the SVZ to their integration sites in the granular and periglomerular zones in the OB requires multiple intrinsic and extrinsic cues working coherently. The OB has been postulated to generate a persistent attractive cue for the directed migration of the neuroblasts (Liu and Rao, 2003). However, complete removal of the OB did not affect the continuous proliferation and migration of newborn precursors towards the absent OB (Kirschenbaum et al., 1999). Similarly, surgical disconnection of the RMS pathway from the OB did not block neuronal differentiation of the NSCs, but promoted ectopic positioning of the differentiated neurons in the anterior olfactory nucleus and frontal cortex, as they could no longer migrate into the OB (Jankovski et al., 1998). A number of cell intrinsic and extrinsic factors have been shown to regulate NSC migration. For example, directional migration cues, which are probably more relevant following neural injury, include members of the chemokine family, such as CXCL12/CXCR4 (Dziembowska et al., 2005). There may be also chemoattractive agents secreted from the OB such as sonic hedgehog (Shh) (Angot et al., 2008) or secreted or membrane-bound factors on the astrocytic glial tube that surrounds the NSCs in the RMS (Peretto et al., 2005), which may include molecules such as vascular endothelial growth factor (VEGF) (Wittko et al., 2009), the neuropeptides NPY and cholecystokinin (CCK; Stanic et al., 2008).

1.3.2 THE DENTATE GYRUS OF THE HIPPOCAMPUS

The second region in the mammalian brain with substantial adult neurogenesis is the DG of the hippocampus. Here, precursor cells reside in the SGZ, at the boundary between the granule cell layer and the hilus.

Neurogenesis in this region has been extensively studied, partially due to its major role in the processing and storage of new information. Progenitor cells migrate a short distance from the SGZ into the granule cell layer (GL) and send their dendrites into the molecular layer (ML) and their axons into the CA3 region of the hippocampus (Markakis and Gage, 1999). Adult-born hippocampal granule neurons are morphologically indistinguishable from surrounding granule neurons (Kempermann, 2003), and they develop mature electrophysiological properties (van Praag et al., 2002).

In the SGZ, three types of neural progenitors have been identified based on their morphology and the expression of specific molecular markers. Type 1 hippocampal

progenitors (or B cells) have a radial process spanning the entire granule cell layer and ramifying in the inner molecular layer. These cells express nestin, GFAP and Sox2, but do not express S100β, a marker of mature astrocytes (Fukuda et al., 2003). Type B cells do not give rise to neurons directly but generate intermediate progenitors, which correspond to the small basophilic cells that are darkly stained by hematoxylin, referred to as type D cells or type 2 progenitors. Type 2 hippocampal progenitors have only short processes and do not express GFAP. Immature type 2 cells (type 2a) appear to divide and function as intermediate progenitors while more mature darkly stained type 2 cells have a prominent process and have properties of neurons at different stages of maturation, characterized by the expression of DCX, PSA-NCAM, collapsin response mediator protein 4 (CRMP-4, also known as TUC-4 or Ulip-1), neurogenic differentiation (NeuroD), prospero homeobox protein 1 (Prox1), and neuronal nuclei (NeuN; Seri et al., 2004, **Figure 1.8**).

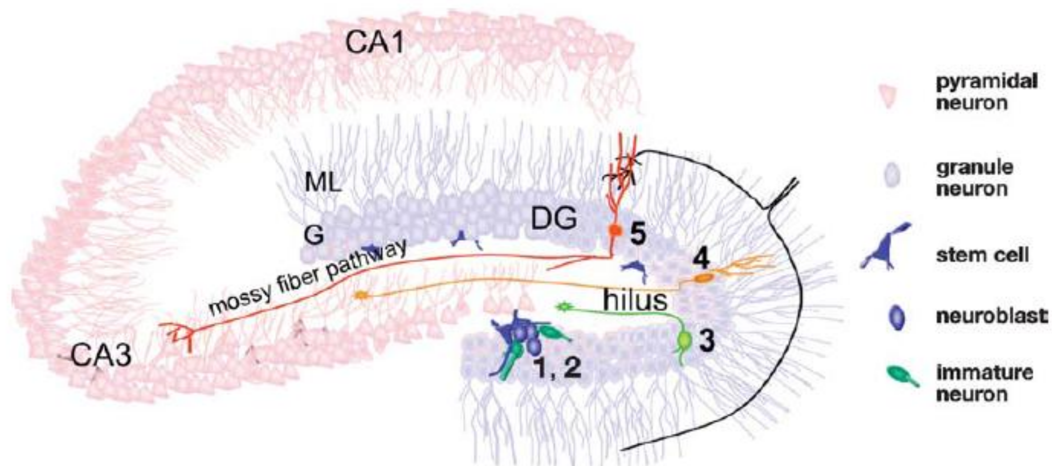


Figure 1.8: generation of new granular neurons in the dentate gyrus of the hippocampus from neural stem cells in the subgranular zone (SGZ)*. Adult neurogenesis in the dentate gyrus of the hippocampus undergoes different developmental stages. Stage 1. Proliferation: stem cells (blue) give rise to transient amplifying cells (light blue). Stage 2. Differentiation: transient amplifying cells differentiate into immature neurons (green). Stage 3. Migration: immature neurons (light green) migrate a short distance into the granule cell layer. Stage 4. Axon/dendrite targeting: immature neurons (orange) extend their axonal projections along mossy fiber pathways to the CA3 pyramidal cell layer. They send their dendrites in the opposite direction toward the molecular layer. Stage 5. Synaptic integration: new granule neurons (red) receive inputs from the entorhinal cortex and send outputs to the CA3 and hilus regions. DG, dentate gyrus region; ML, molecular cell layer; GL, granular cell layer (from Ming and Song, 2005).*

1.4 FACTORS INFLUENCING ADULT NEUROGENESIS

1.4.1 Physiological factors influencing adult neurogenesis

In the adult brain neurogenesis is dynamically regulated by a number of intrinsic as well as extrinsic factors. Endogenous extrinsic factors in the local microenvironment, often referred to as the "neurogenic niche" or "stem cell niche", include neural precursor cells, surrounding mature cells, cell-to-cell interactions, cilia, secreted factors, and neurotransmitters. Microenvironments of the SVZ and SGZ, but not of other brain regions, are thought to possess specific factors that are permissive for the differentiation and integration of new neurons (Faigle and Song, 2013). The importance of the stem cell niche in determining the fate of adult NSCs is highlighted by several different transplantation experiments. SVZ-derived committed neural precursor cells differentiate into glial cells when grafted into ectopic non-neurogenic regions of the brain (Seidenfaden et al., 2006). Furthermore, SVZ precursor cells generate hippocampal neurons when transplanted into the DG of the hippocampus, whereas SGZ precursors generate olfactory interneurons after transplantation into the RMS (Suhonen et al., 1996). Similarly, neural precursor cells derived from the adult spinal cord differentiate into granule cell neurons after implantation into the hippocampus, but fail to generate neuronal phenotypes, and differentiate into glial cells only after transplantation back to their original site in the spinal cord (Shihabuddin et al., 2000). In contrast, Merkle et al. showed that SVZ-derived NSCs maintained their region-specific potential *in vivo*, and that environmental factors at the host graft site were not sufficient to re-specify the grafted cells after heterotopic transplantation (Merkle et al., 2007).

Several **growth factors** have been shown to be involved in regulating neurogenesis in the adult brain, most importantly EGF, FGF and VEGF. Members of the FGF and EGF growth factor families are primary mitogens used to propagate adult neural progenitors *in vitro* and are likely to perform similar functions *in vivo* (Lie et al., 2004). In fact, infusion of EGF or FGF-2 increases cell proliferation in the SVZ and transient amplifying cells of the SVZ appear to express the EGF receptor (Doetsch et al., 2002; Morshead et al. 1994). VEGF receptors are expressed on endothelial cells and neural progenitors in the adult hippocampus and SVZ (Maurer et al., 2003) and the intracerebroventricular (i.c.v.) administration of VEGF increases neurogenesis in the SVZ and the SGZ of adult rat (Jin et al., 2002).

Bone morphogenetic proteins (BMPs) comprise a group of multifunctional extracellular signaling molecules of which over 20 members have been identified to date, and constitute the largest subgroup of the transforming growth factor-beta (TGFβ) superfamily (Kingsley, 1994). In the adult neurogenic niche, BMPs promote glial differentiation and inhibit neuronal fate specification. In the adult SVZ, BMP ligands and their receptors are expressed by the neural stem cell and progenitor cell population, and act as potent inhibitors of neuronal differentiation of Type B and C cells. Furthermore, BMPs were found to be important for promoting survival of neuroblasts migrating along the RMS (Lim et al., 2000). Interestingly, the BMP inhibitor Noggin is produced by ependymal cells of the SVZ and antagonizes endogenous BMP signaling and BMP-mediated premature glial differentiation at the expense of neurogenesis, thus promoting the formation of new neurons from SVZ precursors. In the adult SGZ, endogenously produced **Noggin** had previously been shown to be important for self-renewal and proliferation of adult hippocampal NSCs *in vitro* and *in vivo* (Bonaguidi et al., 2008).

Also **chemokines** have been reported to have a role in the regulation of neural cell proliferation, migration and survival, and can act in a paracrine or autocrine manner (Bajetto et al., 2002; Cartier et al., 2005). Adult NSCs express many chemokine receptors; in particular, neurospheres derived from adult mouse SVZ have been shown to express a range of chemokine receptors, including CCR1-8,10 and CXCR1-6 (Tran et al., 2004). Chemokines are expressed in different brain regions, with the highest levels in the OB, suggesting a role for these molecules in regulating basal adult NSC migration (Turbic et al., 2011). Functionally, specific chemokines can promote NSC proliferation, survival or migration (Dziembowska et al., 2005; Imitola et al., 2004; Krathwohl and Kaiser, 2004) *in vitro*. Following neural damage, NSC migration to the site of injury is promoted by CXCL12 (Imitola et al., 2004; Itoh et al., 2009), and is mediated, at least in part, by induction of metalloprotease expression in the NSCs (Barkho et al., 2008). CCL2, CCL3, and CXCL1 also promote NSC migration to the striatum following quinolinic acid lesion (Gordon et al., 2012).

Signal	*Effect on neurogenesis*	*Neurogenic area*
EXTRINSIC		
Morphogens		
Wnt	*Increases neurogenesis*	*SGZ*
	Required for neuronal differentiation	*SGZ*
	Stimulates NSC proliferation/self-renewal	*SGZ/SVZ*
Notch	*Required for NSC proliferation, maintenance*	*SGZ/SVZ*
	Required for dendritic arborization	*SGZ*
Shh	*Required for progenitor proliferation*	*SVZ*
	Required for NSC maintenance	*SVZ*
	Required for neuroblast migration	*SVZ*
BMP	*Decreases neurogenesis*	*SVZ*
	Promotes neuroblast survival	*SVZ*
Growth Factors		
FGF2	*Increases neurogenesis*	*SGZ*
VEGF	*Increases neurogenesis*	*SGZ/SVZ*
Neurotransmitters		
GABA	*Decreases NSC proliferation*	*SVZ*
	Required for dendritic arborization	*SGZ*
	Required for NSC quiescence	*SGZ*
Glutamate	*Required for survival of migrating neuroblasts*	*SVZ*
	Required for neuronal survival	*SGZ/SVZ*
Dopamine	*Required for progenitor proliferation*	*SGZ/SVZ*
INTRINSIC		
Transcription Factors		
CREB	*Required for neuronal survival*	*SGZ/SVZ*
	Required for dendritic arborization	*SGZ/SVZ*
Pax6	*Promotes neuronal differentiation*	*SVZ*
Sox2	*Required for NSC proliferation*	*SGZ/SVZ*
	Required for neural progenitor cell proliferation	*SGZ/SVZ*
NeuroD	*Necessary for survival, maturation of neuroblasts*	*SGZ*
Epigenetic modulators		
miR-124	*Promotes neuronal differentiation*	*SVZ*
miR-137	*Required for NSC proliferation, maintenance*	*SGZ/SVZ*

Table 1.1: overview of signaling in adult neural stem cells. *(Modified form Faigle and Song, 2013)*

Ciliary neurotrophic factor (CNTF) and **leukemia inhibitory factor (LIF)** activated signal transducer and activator of transcription 3 (STAT3), mitogen activated protein (MAP) kinase and PI-3K/Akt pathways upon ligand binding to their receptors, and have been shown to regulate NSC proliferation and differentiation (Ernst and Jenkins, 2004; Heinrich et al., 2003; Turnley and Bartlett, 2000). Specifically, in the dentate gyrus the activation of STAT3 by CNTF appears to be essential for the formation and maintenance of NSCs (Müller et al., 2009).

Moreover, several soluble and membrane-bound extracellular factors and their intracellular signaling cascades including **Wnt**, **sonic hedgehog (shh)** and **Notch** have been recently identified as determinants of the local microenvironment of the SVZ and SGZ (Balordi and Fishell, 2007; Imayoshi et al., 2010; Kuwabara et al., 2009). Finally, cell-intrinsic mechanisms including transcription factors and epigenetic regulators of neurogenesis have recently been shown to be crucially involved in modulating neurogenesis in the adult brain (for review see Faigle and Song, 2013; **Table 1.1**).

Moreover, although the precise mechanisms that generate new neurons in the adult brain remain elusive, also a range of environmental, behavioral, genetic factors have been shown to be involved in the regulation of adult neurogenesis.

Environmental stimuli can greatly affect the proliferation and survival of newborn neurons in the adult CNS. Exposure of rodents to an enriched environment increases the survival of newborn neurons in the SGZ without affecting SVZ neurogenesis (Brown et al., 2003). Physical exercise, such as running, promotes SGZ neurogenesis by increasing cell proliferation and survival of the new granule neurons (Van Praag et al., 1999a, 1999b). VEGF signaling may be responsible for the increased neurogenesis by both enriched environment and running (Cao et al., 2004). Hippocampus-dependent learning, such as blink reflex or water maze learning, appears to increase the survival of new granule neurons that have been generated only at a particular time window before the training (Leuner et al., 2004). Similarly, enriched odors exposure increases SVZ cell proliferation, but not SGZ neurogenesis (Rochefort et al., 2002). Both physical and psychosocial stress paradigms, as well as some animal models of depression, lead to a decrease in cell proliferation in the SGZ (Duman et al., 2001). Adult neurogenesis in both the SGZ and SVZ is also reduced during aging (Enwere et al., 2004; Kuhn et al., 1996). Adrenal steroids may contribute to the aging-associated decline of neurogenesis in the SGZ (Cameron and Gould 1994). Reducing corticosteroid levels in aged rats can

restore the rate of cell proliferation, which suggests that aged neural progenitors retain their proliferation capacity as in younger adult animals (Cameron and McKay, 1999). Other hormones, including estrogen and prolactin, also regulate adult neurogenesis (Perez-Martin et al., 2003; Shingo et al., 2003).

1.4.2 *PATHOLOGICAL FACTORS INFLUENCING ADULT NEUROGENESIS*

Injury and pathological stimulations not only feature different aspects of adult neurogenesis in neurogenic regions, but also have an impact in otherwise non-neurogenic regions (Arlotta et al. 2003, Parent, 2003). Most brain injuries lead to increased proliferation of progenitors in the SGZ and the SVZ and sometimes cause migration of newborn neurons to injury sites.

Ischemic brain insults potently stimulate progenitor proliferation in both the SGZ and SVZ of adult rodents as shown by BrdU incorporation (Kokaia and Lindvall, 2003; Parent, 2003). In an experimental stroke model immature neurons also migrate from the SVZ to the damaged striatum and follow blood vessels. Most of these new neurons die between two and five weeks after injury (Arvidsson et al. 2002). These studies suggest that the local environment, although providing cues for attracting immature neurons and inducing neuronal subtype differentiation, is not adequate for long-term survival of the newborn cells. Several factors have been shown to promote neurogenesis following ischemia, including bFGF, EGF, BMP7, GDNF (glial cell line-derived neurotrophic factors) and cerebrolysin (Baldauf and Reymann, 2005; Chou et al., 2006; Ninomiya et al., 2006; Jin-qiao et al., 2009; Shen et al., 2010) and the inflammatory mediator tumor necrosis factor alfa (TNFα, Iosif et al., 2008). These factors appear to regulate NSC proliferation or survival and it is not clear whether they also play a role in the migration of the NSCs per se. Importantly, induction of endogenous neurogenesis following ischemia results in neuronal replacement (Parent, 2003) and functional electrophysiological integration (Lai et al., 2008), and is associated with improved functional recovery in rodent models (Bao et al., 2011).

The migration of neurons from the SVZ towards the damaged areas of the brain is not observed only in cases of ischemia, but also in other CNS diseases. For example, studies of adult rodent models of limbic epileptogenesis or acute seizures show that seizure or seizure-induced injury stimulate neurogenesis in both the SGZ and SVZ

(Parent, 2003). These neuroblasts also show more rapid migration to the OB, and some appear to exit the RMS prematurely and migrate into injured forebrain regions.

Moreover adult neurogenesis is significantly altered in chronic degenerative neurological diseases. Increased SVZ proliferation and neuronal production in response to degeneration are observed in patients with Huntington's disease (Curtis et al., 2005). Data from simulated animal models of Huntington's disease agreed with the post-mortem findings in the human Huntington's disease brains, with the SVZ showing lesion-induced upregulation of NSC proliferation, recruitment to the injured striatum, and differentiation into mature neurons (Collin et al., 2005; Gordon et al., 2007; Tattersfield et al., 2004). However, induced neurogenesis is transient and late derived precursors mostly display a glial phenotype (Gordon et al., 2007). Exogenous factors, such as BDNF, enhanced SVZ neurogenesis, promote precursor recruitment, neuronal differentiation and survival at the lesion site (Henry et al., 2007). Similarly, bFGF promotes neurogenesis and increases striatal spiny neuron differentiation, as well as their survival (Jin et al., 2005).

Brains of Alzheimer's disease patients also show increased expression of immature neuronal markers, such as DCX and PSA-NCAM, in the SGZ and the CA1 region of Ammon's horn (Jin et al., 2004a). In a transgenic mouse model of Alzheimer's disease, an approximately twofold increase in BrdU incorporation and expression of immature neuronal markers in the SGZ and SVZ has been observed even before the neuronal loss and deposition of amyloid (Jin et al., 2004b). In the case of Parkinson's disease patients, the proliferation of progenitors in the SGZ and SVZ is impaired probably by a mechanism that also involves a reduced secretion of EGF and CNTF by NSCs caused by the decreased dopaminergic (DA) transmission (O'Keeffe et al., 2009; Yang et al., 2008). Proliferation of transit-amplifying C cells in the SVZ is completely restored by DA agonists (ropinirole and pramipexole) and selective agonists at D2 or D3 receptors (Winner et al., 2009).

Multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE) are demyelinating diseases characterized by multifocal inflammatory lesions, with progressive oligodendrocyte and axonal loss. Although SVZ type B cells generally give rise to OB interneurons, they can also generate NG2-positive oligodendrocyte progenitor cells (OPCs) and give rise to mature myelinating oligodendrocytes (Menn et al., 2006), while PSA-NCAM-positive NSCs can be differentiated into myelin basic

protein-expressing myelinating oligodendrocytes (Cayre et al., 2006; Lachapelle et al., 2002). Focal demyelination in the corpus callosum has been shown to induce SVZ expansion, resulting in mobilization of the newborn progenitors to the lesion sites (Menn et al., 2006), while in EAE, SVZ-derived progenitors migrate into periventricular white matter and exclusively differentiated into astrocytes and oligodendrocytes (Picard-Riera et al., 2002). Consistent with the rodent studies, human active MS plaques frequently show PSA-NCAM-positive progenitor accumulation, accompanied by increased cell proliferation and NSC numbers in the SVZ (Nait-Oumesmar et al., 2007). SVZ stem/precursor cells introduced via intravenous or intracerebral injection have been shown to enter and populate demyelinating lesions in rodent and primate CNS (Oka et al., 2004; Pluchino et al., 2003), and this migration appears to rely on the inflammatory environment (Giannakopoulou et al., 2011). The chemokine CXCL12, which is expressed by astrocytes in active MS lesions (Calderon et al., 2006), plays a role in NSC and oligodendrocyte precursor recruitment (Carbajal et al., 2010; Maysami et al., 2006). Mitogenic factors, such as EGF and bFGF, also assist progenitor cell mobilization, as well as their subsequent differentiation into oligodendrocytes at lesion sites. These findings indicate that an inflamed brain environment can trigger NSC proliferation and migration, with the specific cell types generated depending on the brain region. However, persistent inflammation in the brain can also result in accumulation of non-migratory NSCs in the SVZ, and pro-inflammatory factors such as interferon gamma (IFNγ) have a role in down-regulating their self-renewal capacity (Pluchino et al., 2008). This is consistent with the anti-proliferative effects of IFNγ on NSCs *in vitro* (Wong et al., 2004; Lum et al., 2009). Therefore the timing and balance of pro- and anti-inflammatory agent expression is important in dictating the NSC response to inflammatory CNS damage.

1.4.3 EXTRACELLULAR NUCLEOTIDES AND NEUROGENESIS

Recent evidence suggests an important role of the purinergic system in controlling adult neurogenesis. Mishra and co-workers (2006) were the first to show that the cells constituting secondary neurospheres expressed the mRNA for the $P2Y_{1,2,6}$, but not for the $P2Y_4$ receptor subtype. By measuring intracellular Ca^{2+} concentration they also showed that ATP ($P2Y_2$ agonist), ADP ($P2Y_1$ agonist) and, to a lesser extent, UTP ($P2Y_{2,4}$ agonist) evoked rapid Ca^{2+} transients in secondary neurospheres, while UDP

had no effect. This latter finding suggested that, although expressed by neurospheres, the $P2Y_6$ receptor is not functionally active. The $P2Y_1$ specific antagonist N^6-methyl-2-deoxyadenosine 3',5'-bisphosphate (MRS2179) and the P2 non-selective antagonists, suramin and pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), significantly reduced the response evoked by the agonists; on the contrary, the application of antagonists alone had no effect on the cytosolic concentrations of Ca^{2+} (Mishra et al., 2006). Adenosine 5'-O-(2-thiodiphosphate) (ADPβS, non-selective agonist for $P2Y_{1,12,13}$) and UTP were also able to increase the proliferation of secondary neurospheres kept in culture in the presence of low growth factor (EGF and bFGF) concentrations. Also in this case, the antagonists MRS2179, suramin and PPADS drastically reduced the effect induced by purinergic agonists, while the application of the antagonist alone had no effect. These data led to the conclusion that the nucleotides acted mainly through the action of the $P2Y_1$ and $P2Y_2$ receptors and exerted a synergistic effect on the proliferation induced by growth factors such as EGF or bFGF (Mishra et al., 2006). Later, Stafford and colleagues (2007) demonstrated that also primary neurospheres derived from the SVZ of the adult mouse express mRNA for all the P2YRs (with the exception of the $P2Y_4$), and for the P2X4 and P2X7 receptors subtypes. ATPγS, ADPβS and UTP evoked transient increases in cytoplasmic Ca^{2+} concentration in primary neurospheres while the application of UDP produced no effect, again suggesting that the $P2Y_6$ receptor was not functional. Furthermore, increases in cytoplasmatic Ca^{2+} concentrations induced by ADPβS and ATP were completely inhibited by the $P2Y_1$ selective antagonist MRS2179, further confirming a primary involvement of this receptor in the observed effects (Stafford et al., 2007). Stafford and colleagues also demonstrated that a single administration of ATPγS, ADPβS and adenosine (P1 agonist) was able to reduce the number and size of the resulting primary neurospheres. The administration of specific antagonists (MRS2179 selective for $P2Y_1$, MRS2395 selective for $P2Y_{12}$ and SCH58261 selective for A_{2A}) before the addition of the above-mentioned agonists reduced their effects, whereas the administration of antagonists *per se* had no effects. The same studies were then conducted on secondary neurospheres, and the authors observed that ATPγS and adenosine retained their inhibitory effect while ADPβS enhanced cell proliferation, thus supporting the results obtained earlier by Mishra and collaborators (Stafford et al., 2007). Grimm and colleagues (2009) further investigated the combined effect of growth factors and

nucleotides and demonstrated that treatment of adherent and undifferentiated (nestin positive) cells with ADPβS or UTP induced the phosphorylation of ERK and CREB (Grimm et al., 2009). Grimm and colleagues used a pharmacological approach to understand which receptor subtypes were involved in the effect exerted by the nucleotides on ERK and CREB phosphorylation. Using a selective agonist for the $P2Y_1$ receptor subtype (MRS2365) they observed a slight decrease of ERK and CREB phosphorylation compared to what observed with ADPβS. The use of a $P2Y_{12}$ selective antagonist (MRS2395) showed no effects on the activity of ADPβS, whereas Cangrelor, a selective competitive antagonist at both $P2Y_{12}$ and $P2Y_{13}$ receptors (Fumagalli et al., 2004), and MRS2179 treatment resulted in a significant reduction in the phosphorylation of ERK and CREB. These data suggest that the $P2Y_1$ and $P2Y_{13}$ receptors were the main mediators of the effect of ADPβS on ERK and CREB (Grimm et al., 2009). Upon differentiation of the neural precursors into astrocytes, oligodendrocytes and neurons (induced by removal of growth factors and by the addition of serum), only astrocytes were still able to respond to the application of ADPβS and UTP with an increase in the phosphorylation of ERK and CREB, while oligodendrocytes and neurons lost their ability to respond to agonists, suggesting the absence in these two populations of still functionally active P2YRs (Grimm et al., 2009). Besides the effect on proliferation and differentiation, nucleotides and EGF can also influence cell motility and act as chemotactic signals. In fact, in NSC cultured in a medium lacking growth factors, a short exposure to agonists (ATP, ADPβS and UTP) resulted in significant changes in cell structure. Moreover it has been demonstrated that the addition of agonists to a culture medium with reduced growth factors concentrations stimulates cell migration (Grimm et al., 2009). These effects were due to the ability of ATP, ADPβS and UTP to induce changes in the phosphorylation of AKT and FAK (focal adhesion kinase) mediated by ERK and CREB. FAK is a tyrosine kinase located between the intercellular connections and exerts its action by changing actin polymerization and altering contacts between cells (Mitra et al., 2005). AKT is a kinase implicated in many cellular processes, and its activity is associated with cell motility as it influences the organization of the actin cytoskeleton and the interactions of the cell with the extracellular matrix (Qian et al., 2004). This study demonstrated that EGF and nucleotides are able to act as autocrine or paracrine signals in order to maintain the

cellular structure of the SVZ and to regulate the migration of neuroblasts towards the OB (Grimm et al., 2010).

In the SVZ and in the OB specific ectonucleotidase were found (Hassenklöver et al., 2010). The tissue non-specific form of alkaline phosphatase (TNAP) is expressed by all the neural progenitors of the adult SVZ cells (type B, C and A cells). TNAP is responsible for hydrolysing extracellular nucleoside triphosphates to the respective nucleoside. By contrast, NTPDase2 (which preferentially hydrolyses nucleoside triphosphates to nucleoside diphosphates) is specifically expressed by type B cells (either residing in the SVZ or ensheating type A cells along the RMS, by neural progenitors of the dentate gyrus and by postnatal radial glial cells of the cerebral cortex (**Figure 1.9**).

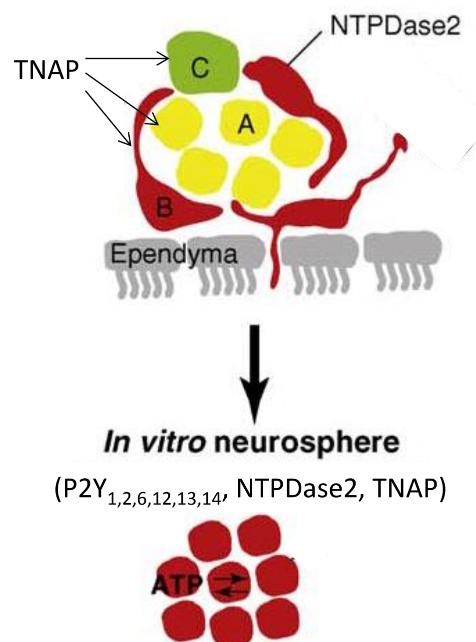


Figure 1.9: purinergic signaling in adult neural development. *The tissue non-specific form of alkaline phosphatase (TNAP) is expressed by all the neural progenitors of the adult SVZ cells (type B, C and A cells). By contrast, NTPDase2 is specifically expressed by type B cells. The NTPDase2- and TNAP-expressing neurospheres derived from the SVZ release ATP and express several P2Y receptors (modified from* Neary and Zimmermann, 2009).

Although the presence of P1, P2X2,4,5,6,7 and $P2Y_{1,2}$ receptors in the murine OB (Hassenklöver et al., 2010) and of P2X1,2,6,7 and $P2Y_{1,2,4}$ in the SVZ of adult mice has been proved, *in vivo* pharmacological studies are almost completely missing, and the physiological relevance of the purinergic system in these brain regions is still elusive. Hassenklöver and collaborators investigated the effects 2-methylthio-ATP (2MeSATP,

$P2Y_1$ agonist) and ATP (P2X and $P2Y_2$ agonist) administration to ex-vivo sections of OB and SVZ of *Xenopus laevis larvae*, and observed a significant increase in $[Ca^{2+}]i$ in both regions. Furthermore, they showed that the non-selective antagonists suramin and PPADS attenuated these responses, confirming that the observed effect were due to the presence of purinergic receptors in both areas (Hassenklöver et al., 2010). To understand which receptor subtypes were involved, Hassenklöver and collaborators evaluated the effect of the removal of calcium in the culture medium of cells derived from the SVZ of *X. laevis*. In these conditions, the increase in $[Ca^{2+}]i$ induced by the two agonists were significantly reduced in OB, while in the cells of the SVZ agonists still retained a significant effect. This difference between the two areas has suggested that in the OB the increase in $[Ca^{2+}]i$ induced by nucleotides was almost completely due to the activation of ionotropic P2X receptors, whereas in the SVZ was the result of the combined effect of ionotropic and metabotropic receptor (P1 and/or P2Y), which are known to be coupled to intracellular cascades linked to calcium release from internal stores (Hassenklöver et al., 2010).

Suyama and colleagues (2012) have conducted further *in vivo* studies to investigate the role of purines on SVZ cells proliferation. ATP, suramin or MRS2179 were chronically infused in the lateral ventricle of adult mice for 3 days. Mice were also treated with BrdU following two different protocols to highlight either the proliferation of rapidly proliferating cells (transit amplifying cells) or of stem cells. ATP, suramin and MRS2179 treatment did not induce changes in stem cell proliferation, but when short BrdU administration was used to mainly label cells in rapid proliferation, ATP increased the number of $BrdU^+$ cells. Furthermore, 38% of $BrdU^+$ cells were also positive for the typical type C cell marker Mash1, suggesting that exposure to ATP promoted the proliferation of this cells type. Authors suggested the $P2Y_1$ receptor subtype as the possible mediator of this effect. In fact, the administration *per se* of both suramin and MRS2179 lowered the percentage of proliferating type C cells. In addition, a similar decrease was also observed in $P2Y_1$ knock-out mice. Finally, Suyama and collaborators demonstrated that the $P2Y_1$ receptor was expressed in 73.2% of DCX^+ cells and 31.1% of $Mash1^+$ cells, but not in BrdU/GFAP double-positive cells (Suyama et al., 2012).

In conclusion, the currently available data on the role of the purinergic system in the OB and SVZ are still limited and further studies are needed to define in detail the role of purines in these brain areas and in the control of adult neurogenesis.

1.5 NEUROGENESIS IN NON-NEUROGENIC REGIONS

As previously mentioned, neurogenesis outside the two classical neurogenic regions appears to be extremely limited in the intact adult mammalian CNS. However after pathological stimulation, such as brain insults, adult neurogenesis occurs in regions otherwise considered to be non-neurogenic, such as the brain parenchyma and the spinal cord. In particular, in the brain parenchyma two glial cell types are considered to retain stem cell properties: astrocytes and polydendrocyets, also known as oligodendrocyte precursor cells (OPCs).

Parenchymal astrocytes react to insults by proliferation and GFAP up-regulation. Reactive astroglia reacquire self-renewal capability and generate astrocytes, neurons and oligodendroglia in a favorable environment *in vitro* (Buffo et al., 2008). Accordingly, *in vitro* transduction of astrocytes with retroviruses containing neurogenic transcription factors (e.g., Pax6, Dlx2 or Ngn2) promoted neuronal fates (Berninger et al., 2007; Heinrich et al., 2011; Heins et al., 2002). Moreover, also *in vivo* transduction of adult reactive glia evoked neurogenic attempts, that, however, remained abortive, as cells did not progress to fully mature neurons (Buffo et al., 2005; Kronenberg et al., 2010).

In basal conditions, OPCs represent the vast majority of actively cycling progenitors outside the germinal niches in the adult CNS (Buffo et al., 2005; Dawson et al., 2003; Horner et al., 2000) and develop into mature oligodendrocytes. However, it has been suggested that, under some conditions, NG2 cells can give raise to astrocytes and neurons, as described in more details below (1.5.2.1). A first sensational demonstration that these cells can be reprogrammed to generate neurons (Kondo and Raff, 2000a) has been followed by only a few *in vitro* confirmations (Liu et al., 2007) and conflicting *in vivo* results (Dimou et al., 2008; Richardson et al., 2011).

In the spinal cord (SC) the cells lining the central canal, namely ependymal cells (ECs), are considered the stem-like cells in this part of the CNS. In the intact tissue, these cells are virtually quiescent, self-renew very slowly and give raise to a very small number of neurospheres *in vitro*. However, after SC injury (SCI) or hypoxia, ECs

proliferate and express GFAP, a marker of multipotency (Ceruti et al., 2009). Activated ECs generate a significantly higher number of neurospheres (Barnabé-Heider et al., 2010), and give raise to astrocytes, oligodendrocytes and, under some conditions, motoneurons when exposed to differentiating agents *in vitro* (Moreno-Manzano et al., 2009). However, fate-mapping analysis of ECs revealed generation of astrocytes and oligodendrocytes only following SCI *in vivo* (Meletis et al., 2008).

1.5.1 ASTROCYTES AND REACTIVE ASTROGLIOSIS

Astrocytes, one of the most abundant cell type in the CNS (Markiewicz and Lukomska, 2006), were for long time believed to mainly provide architectural structure, nutrition and homeostasis in the healthy brain. Nowadays this view has changed and they are also known to control neuronal activity (Araque et al., 1999), to regulate neurogenesis from neural stem cells in the adult brain (Song et al., 2002), or to act as a source of neural stem cells themselves (Buffo et al., 2008).

Since the late nineteenth century, astrocytes have been divided in two main subtypes, protoplasmic or fibrous, based on their anatomical location and cellular morphology. These two main categories retain their validity and usefulness today. Protoplasmic astrocytes are located in the grey matter and possess numerous highly branched fine processes whereas the fibrous astrocytes can be found in the white matter and have less branched and thicker processes (Sofroniew and Vinters, 2010). Electron microscopic analyses revealed that the processes of protoplasmic astrocytes in the grey matter envelop synapses, while the processes of fibrous astrocytes in the white matter contact nodes of Ranvier. Both types of astrocytes form gap junctions between distal processes of neighboring astrocytes (Peters, et al., 1991). Astrocytic processes associated with synapses tightly regulate synapse formation, maintenance and plasticity through the regulation of extracellular K^+ and the uptake and release of many molecules, including neurotransmitters, constituting the so-called tripartite synapse (Halassa et al., 2009). Finally, they also provide to neurovascular coupling and to the formation of blood brain barrier (BBB), integrating glia, neurons and brain vasculature into functional networks (Franke et al., 2012; **Figure 1.10**).

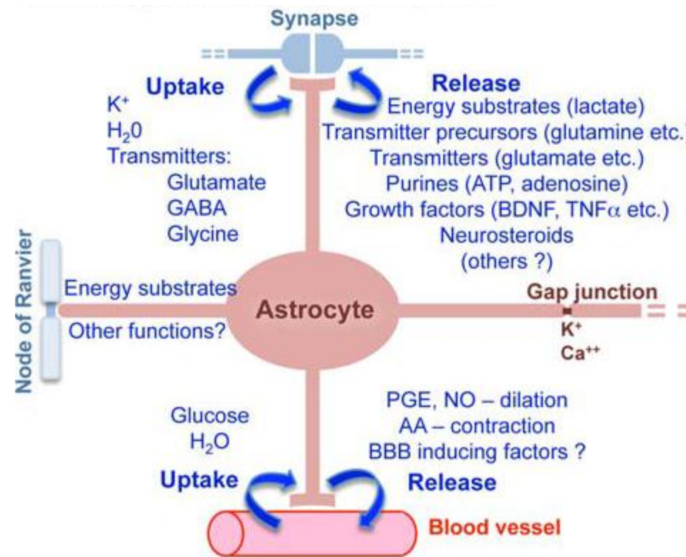


Figure 1.10: schematic representations that summarize astrocyte functions in the healthy CNS. *In the grey matter, astroglial perisynaptic processes ensheath synapses, whereas in the white matter astroglial processes terminate at nodes of Ranvier, the sites of action potential generation. Astrocytes assume also numerous and well-defined supportive functions, including structural support, providing for neurovascular coupling, regulation of extracellular* K^+*, uptake of neurotransmitters, metabolic support of neurons. Finally they are connected via gap junctions into syncytia and communicate through propagated waves of* Ca^{2+} *and other active substances (modified from Sofroniew and Vinters, 2010).*

In addition to play an essential homeostatic role and to contribute to information processing in physiological conditions, astrocytes are capable to respond to any kind of insult to the CNS. In their reaction to injury, named reactive astrogliosis, astrocytes leave their quiescent state and become activated. During this process, they undergo hypertrophy, upregulate intermediate filaments composed of nestin, vimentin, and GFAP, and activate cell proliferation (Pekny and Nilsson, 2005; Buffo et al., 2008). Moreover, in their reactive state astrocytes can continue to divide, migrate to form the glial scar, and release a plethora of factors mediating the tissue inflammatory response and remodeling after lesion (Elmquist et al., 1997; Silver and Miller, 2004; **Figure 1.11**).

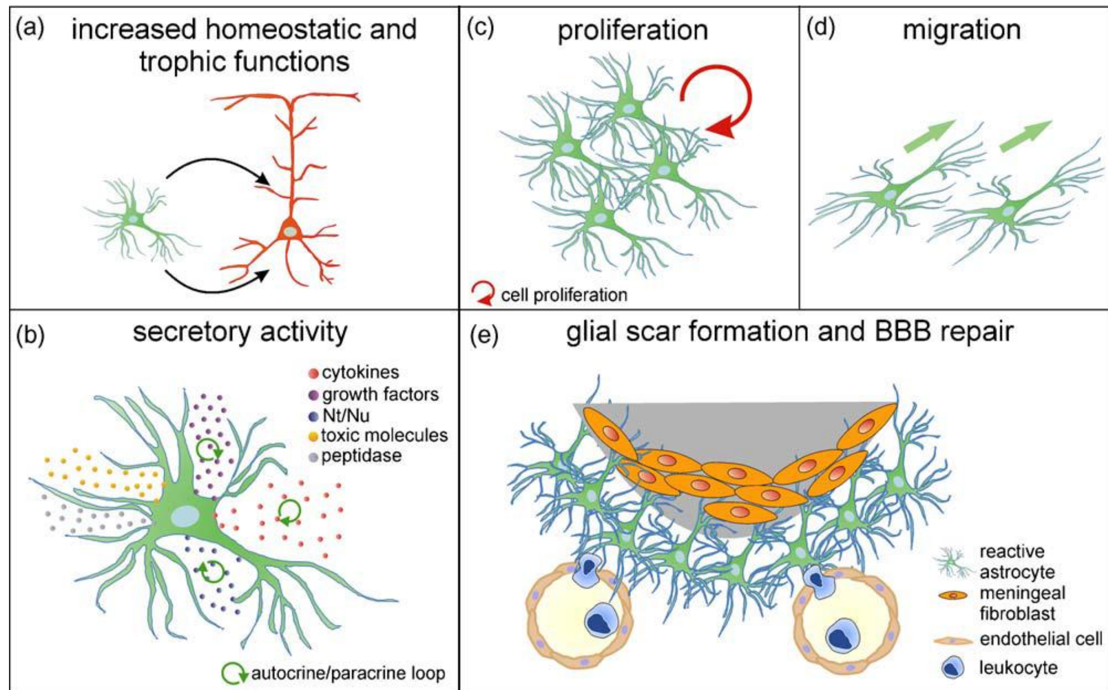


Figure 1.11: hallmarks of astrocyte reactivity. *Upon CNS injury, activated astrocytes increase their homeostatic and trophic functions (a), the production of growth factors and cytokines, as well as the release of nucleotides and toxic compounds (b). Their secretion is regulated via complex autocrine and paracrine loops (b). Astrogliosis includes cell proliferation (c) and migration towards the lesion site (d). Reactive astrocytes participate in glial scar formation, and contribute to the resealing of the damaged blood–brain barrier, thus excluding infiltrating leukocytes and meningeal fibroblasts from the injured tissue (e). Nt/Nu, nucleotides/nucleosides; BBB, blood–brain barrier (From Buffo et al., 2010)*

It is now increasingly clear that the modalities and dynamics of astrocyte response to damage are crucial to the outcome of several brain pathologies and to the degree of neurological damage.

Upon injury, the astroglia response is evoked by several changes occurring in the CNS parenchyma. These changes include the production of a variety of molecular signals, partly derived from plasma extravasation, able to trigger the transition from the quiescent to the activated state or to modulate astrocyte reactivity over time. The distinction between activating and modulating signals remains largely undefined. However, very early triggers such as purines/pyrimidines and pro-inflammatory cytokines (Abbracchio and Ceruti, 2006) are predominantly considered to evoke the initial astrocyte activation (although their release can be also maintained over time thus contributing to later responses). The major role of the later secondary mediators (e.g., endothelin, GFs, inflammatory molecules) may instead be to sustain the long-term

features of astrogliosis until inhibitory molecules, such as interferon beta (IFNβ), interleukin 10 (IL10), and erythropoietin (Epo), predominate over inductive triggers (de Bilbao et al., 2009; Ito et al., 2009; Vitellaro-Zuccarello et al., 2008). At this final stage, resolution of gliosis leads to the reacquisition of quiescent morphological and functional features in astrocytes far from the source of injury (isomorphic gliosis), or to the stabilization of astroglia reactive traits in the form of a permanent glial scar close to the lesion site (anisomorphic gliosis) (see below; Elmquist et al., 1997).

Astrocyte responses to injury are aimed at both protecting the nervous system, and at sealing off the damaged area, leaving the heavily injured zone to its natural degenerative fate, while preserving the less affected tissue. Once activated, these responses seem to proceed in a quite stereotyped way, independently of the initial source of injury. Hence, they may lead to predominant reparative or destructive outcomes depending on the context in which they occur, for example the extent and type of injury (i.e., chronic vs. acute), and the time point after damage. The healing response to injuries of the mature CNS culminates in the formation of a tight barrier, the glial scar, which consists predominantly of reactive astrocytes. Scarring astrocytes have long been considered as a major impediment to regeneration of damaged axons (Davies et al., 1997; Liuzzi and Lasek 1987; Ramon y Cajal 1928) and reactive gliosis regarded as detrimental to nervous tissue repair and functional restoration. Although astrocytes continue and even intensify their trophic activities upon injury, in the condition of extremely severe or prolonged damage, such as in the core of ischemic insults, their energetic metabolism may succumb, and essential functions, such as the activity of Na^+/K^+ ATPase regulating membrane potential, may be lost. The consequent depolarization of astrocyte membrane, together with an increased extracellular Na^+ concentration, can reverse glutamate transport, produce glutamate efflux and transform astrocytes into a source of substances toxic to neurons. Nevertheless, these events are thought to normally occur at a time when neuronal damage is already beyond repair, implicating that they have a limited impact on secondary death (Rossi et al., 2007). Glutamate, however, may also be released by reactive and fully functional astrocytes through their hemichannels, which can be opened by lowering extracellular Ca^{2+} concentration, acidosis, or even through P2X7 receptors activated by excessively high extracellular ATP. All these events occur during lesions such as ischemia, and may account for the spreading of death signals (Rossi et al., 2007). Damage diffusion may

also occur via astrocytic gap junctions, although this form of cell-to-cell communication is globally reduced upon damage (Contreras et al., 2004). A series of studies further revealed that the secretory activity of reactive astrocytes can exacerbate tissue lesion. For instance, released pro-inflammatory cytokines such as TNFα can inhibit neurite growth and kill oligodendrocytes (Neumann et al., 2002; D'Souza et al., 1996). Similarly, reactive astrocytes can produce and release arachidonic acid metabolites, nitric oxide and reactive oxygen species.

The concept of a protective role for astroglial reactivity has only recently been established, emerging from the ability of reactive astrocytes to isolate the damaged core of vascular and traumatic lesions from the surrounding healthy tissue, thus reducing the spreading of toxic substances and metabolites released from dead cells and limiting the development of secondary damage (Rolls et al., 2009). These beneficial effects are related to the capability of astrocytes to produce signals supportive for neuronal and oligodendroglial survival, to preserve and restore altered homeostatic conditions, and, when lost, to re-establish the anatomical barriers (e.g., the BBB) necessary for the correct CNS functioning. Astrocyte protective actions are exerted by scavenging extracellular glutamate, storing energy, supplying neighboring cells with energetic compounds, and neutralizing toxic substances. For instance, one of the major causes of secondary damage in case of ischemia or trauma is the massive release of glutamate (due to direct cell injury or neuronal dysfunction) that triggers secondary excitotoxic events in neurons. Astrocytes are crucial regulators of glutamatergic signaling in normal nervous tissue function by active removal of extracellular glutamate operated by the Na^+-dependent glutamate transporter 1 (GLT1) and GLAST (Danbolt, 2001). When the energy substrates glucose and oxygen are lost (i.e., in ischemia), the persistence of this function in astrocytes is to some extent granted due to the capacity of astroglia to maintain high energy levels upon lesion and to utilize diverse energy sources. For example, astrocytes convert stored glycogen to glucose or lactate to obtain energetic substrates, thereby sustaining their own metabolisms or possibly passing these substrates to neighboring neurons for energy production (Brown, 2004). Moreover, CNTF-activated astrocytes also increase fatty acid beta-oxidation and ketolysis to produce energy, while decreasing glycolytic pathways. This metabolic plasticity confers a remarkable ability to resist to metabolic insults and to support the survival of surrounding neurons (Escartin et al., 2007). Furthermore, the enhanced capability to

metabolize fatty acids has also a detoxificant function when potentially toxic free fatty acids are released from phospholipids in the plasma membrane in several lesion conditions (Phillis and Regan, 2004). Other beneficial aspects of astrocyte reactivity are related to their participation in tissue remodeling after injury. VEGF is produced by reactive astrocytes after traumatic injury, inflammatory lesions, and hypoxic conditions. In addition of being directly neuroprotective, this growth factor strongly promotes angiogenesis, consistent with a role for reactive astrocytes in neovascularization after lesion (Argaw et al., 2009; Carmeliet, 2005; Rosenstein and Krum, 2004). Finally, the wide set of GFs upregulated by astrocytes after lesion conditions provides direct trophic support to neurons and oligodendrocytes to limit secondary damage. Moreover, some of these factors to some extent create a permissive environment for remyelination by their action on oligodendrocyte precursor migration, oligodendrocyte proliferation, and differentiation together with other signals (i.e., IL6) (Williams et al., 2007). In summary, the long-standing concept of astrogliosis as an obstacle to brain repair has now greatly evolved with the discoveries of the many and essential beneficial effects of astrocyte reactivity for damage limitation and tissue preservation. It is also increasingly clear that the inhibitory components of the scar undergo extensive remodeling over time so that the scar becomes progressively more permissive to axon growth.

1.5.1.1 P2 receptors in reactive astrogliosis

eNTs have been implicated as endogenous triggers of reactive astrogliosis. Upon trauma or ischemia, brain cells are exposed to elevated concentrations of nucleotides and nucleosides for prolonged periods as a result of leakage from the nucleotide cytosolic pool (due to loss of membrane permeability) or nucleic acid degradation upon cell death, due to persistent or stronger insults. Thus, during emergencies astrocytes and surrounding cells are exposed to high micromolar nucleotide concentrations. Since astrocytes express the whole panel of P2YRs at the mRNA level, as well as the 7 ionotropic P2X subtypes (Fumagalli et al., 2003), the purinergic system appears as a key modulator of the astrocytic response to injury. Indeed, *in vitro* (Bolego et al., 1997; Brambilla et al., 1999; Neary et al., 1999) and *in vivo* (Franke et al., 2001) evidence indicates that ATP and its analogues promote astrocytic proliferation and emission of long and branched GFAP-positive processes through the activation of specific G protein-coupled P2Y receptors.

The first evidence in this respect dates back to the early 90s when Kim and Rathbone were purifying brain proteins that stimulated proliferation of cultured chick-brain astrocytes (Kim et al., 1991; Rathbone et al., 1992). They isolated a potent mitogenic fraction that was not a protein, and was identified by chemical analysis as guanosine 5'-monophosphate (5'-GMP). Rathbone and his colleagues soon determined that guanosine, adenosine and their respective 5'-mono-, di- and triphosphate derivatives also stimulated proliferation and DNA synthesis of chick astrocytes *in vitro* (Neary et al., 1996). The ability of the hydrolysis-resistant ATP analogue α,βmeATP to influence both proliferation and differentiation of primary rat striatal astrocytes in culture has been also demonstrated (Abbracchio et al., 1994). ATP can act in combination with FGF, EGF, platelet-derived growth factor (PDGF) as well as nerve growth factor (NGF) to stimulate astrocytic proliferation, contributing to the process of reactive astrogliosis and to hypertrophic/hyperplastic responses (Neary et al., 2006). Moreover α,βmeATP, by inducing a concentration-dependent increase of GFAP-positive processes, promoted maturation of astrocytes towards a more differentiated phenotype characterized by longer and thicker processes (Abbracchio et al., 1995). In subsequent studies, similar effects were obtained with other nucleotides, such as ATP, adenosine 5'-O-(2-thiodiphosphate) (ADPβS), 2meSATP, βγmeATP and UTP. Nucleotide-induced astrogliosis was inhibited by pertussis toxin (suggesting the involvement of a G protein-coupled P2Y receptor) and by several typical P2 receptor antagonists, such as suramin and PPADS, but not by P2 receptor antagonists that preferentially block P2X receptors, such as 2',3'-O-(2,4,6-trinitrophenyl)-ATP (TNP-ATP) (Bolego et al., 1997; Brambilla et al., 2002). Importantly, the relevance of these *in vitro* findings were also confirmed *in vivo* by Franke and co-workers (Franke et al., 2001) while investigating the morphological effects induced by local application of P2X and P2Y ligands in the nucleus accumbens of rats. A strong mitogenic effect was induced on astrocytes by ADPβS, 2meSATP, α,βmeATP and, to a smaller extent, uridine-5'-(γ-thio)-triphosphate (UTP-γS). Finally, it has been demonstrated that the selective stimulation of astroglial $P2Y_1$Rs is involved in the secretion of glutamate (Domercq et al., 2006) and in the regulation of several cytokines/chemokines already known to be involved in astrogliosis, such as IL-6 (Fujita et al., 2009), GDNF (Sun et al., 2008) TNF-α, monocyte chemotactic protein-1/chemokine (MCP-1/CCL2) and IP-10/CXCL10 messenger RNA (mRNA) expression (Kuboyama et al., 2011).

P2YRs in astrocytes are linked to a variety of signal transduction mechanisms. This receptor family can activate: (1) the MAP kinase pathway, an obligatory step for the triggering and/or persistence of reactive astrogliosis, (2) the phosphoinositide 3-kinase (PI3K)/Akt pathway, associated with cell growth, fiber regeneration, and inhibition of apoptosis, and (3) the PKC pathway (Brambilla et al., 2003; Burgos et al., 2007; Franke et al., 2009; Jacques-Silva et al., 2004; Neary and Kang, 2005; Neary et al., 2003). In rat primary astrocytes culture, stimulation of P2YRs led to activation of extracellular-regulated kinases 1/2 (ERK1/2) via multiple parallel signaling pathways involving Gi/o protein-dependent and calcium-independent stimulation of phosphatidylcholine phospholipase C (PC-PLC) and/or phospholipase D (PLD), with consequent activation of the Ras/Raf system (Brambilla et al., 2003). Application of a rapid and reversible stretch-induced injury to cultured astrocytes resulted in immediate ERK1/2 activation, which was already maximal after 10 min (Neary et al., 2003). ERK activation was prevented by apyrase and P2 receptor antagonists, suggesting that injury caused the release of ATP, which may in turn autocrinally and heterocrinally signal to astrocytes and stimulate ERK1/2 activity. Studies with various P2 receptor antagonists indicated that, in this experimental model, activation of $P2Y_1$ and P2X2 receptor subtypes might be particularly important for the detected effects (Neary et al., 2003).

The PI3K/Akt pathway is associated with controlling the balance between survival and cell death. Akt activation after trauma, axotomy and ischemia is well established. Purinergic stimulation of Akt phosphorylation in astrogliosis was studied *in vitro* (Burgos et al., 2007; Jacques-Silva et al., 2004; Neary and Kang, 2005) and *in vivo* in the rat brain (Franke et al., 2009). In particular, the involvement of $P2Y_1$Rs in Akt activation *in vivo* suggests that the PI3K/Akt cascade stimulates astroglial proliferation and prevents apoptosis, indicating contribution of specific P2R subtypes during neurodegenerative diseases associated with excessive gliosis (Franke et al., 2009).

Both the ERK and the Akt pathways are known to influence gene expression through phosphorylation/activation of transcription factors, such as c-Fos, c-Jun, Elk1, ATF2 or STAT3. c-Fos is an early gene product that contains a binding site for the activating protein 1 (AP-1) in its promoter and heterodimerizes with c-jun to form an AP-1 complex (Masood et al., 1993; Hope et al., 1994; Perez-Cadahia et al., 2009). Triple-labeling studies demonstrated c-Fos expression in $P2Y_1$R-positive GFAP-labelled astrocytes. Previous *in vitro* experiments reported an extracellular ATP-induced

up-regulation of c-Fos protein and the formation of AP-1 transcription complexes (Bolego et al., 1997). eNTs also increased DNA binding of nuclear factor kappa B (NF-kB), another transcription factor crucially involved in several inflammatory events including reactive astrogliosis. Pharmacological data indicated involvement of distinct P2 receptors in the activation of these two families of transcription factors. In particular, ATP and ADP were equipotent in inducing NF-kB activation (followed by BzATP, with no effect of UTP), whereas ADP was the most potent trigger for AP-1 activation (followed by ATP, with no effect by either BzATP or UTP) (John et al., 2001). Finally, ATP triggers reactive astrogliosis by activation of a P2YR linked to the induction of cycloxygenase-2 (COX-2), and P2YR antagonists might counteract excessive COX-2 activation in both acute and chronic neurological diseases (Brambilla et al., 1999). Transduction signaling to COX-2 induction involves an early activation of phospholipase A2 (PLA2) and arachidonic acid (AA) release; this may, hence, act as an inducer of the COX-2 gene via the PKC/MAPK pathway (Brambilla and Abbracchio, 2001). The different associated intracellular signaling pathways, e.g. STAT3, nuclear NF-κB, signaling molecules suppressor of cytokine signaling 3 (SOCS3), cAMP, JNK/c-Jun, FGF and MAPK (**Figure 1.12**), are implicated in mediating different aspects and/or different degrees of reactive astrogliosis, such as GFAP up-regulation, cell hypertrophy, proliferation and pro- or anti-inflammatory effects (Sofroniew, 2009).

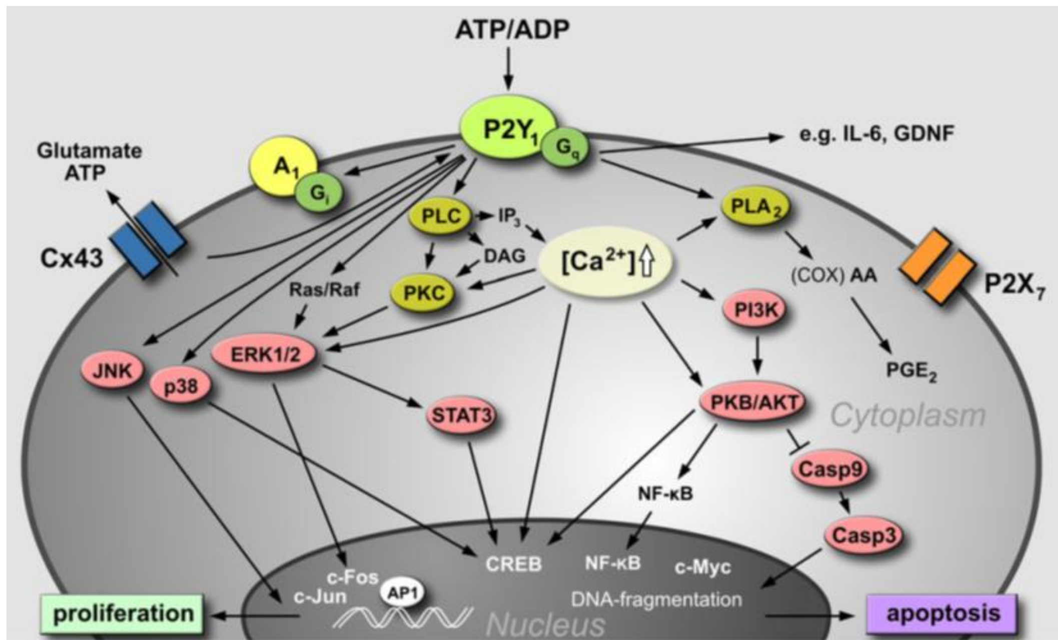


Figure 1.12: schematic illustration of examples of signal transduction pathways in astroglial cells following P2Y$_1$R activation. *Stimulation of P2Y$_1$Rs leads to the activation of phospholipases A2 and C (PLA2, C) and protein kinase C (PKC), as well as to an increase in intracellular calcium ([Ca^{2+}]i). The activation of P2Y1Rs result in the induction of second messenger and enzyme cascades, e.g. activation of the mitogen activated protein kinase (MAPK) pathway proteins [ERK1/2, p38, c-Jun N-terminal kinase (JNK)], and of PI3K/Akt. P2Y$_1$R-mediated signal transducer and activator of transcription 3 (STAT3) signaling may play a role in astrocyte proliferation and reactive astrogliosis. P2Y$_1$R activation appeared to be involved in the activation of caspase (Casp) cascades, in the release of arachidonic acid, and in the increase in prostaglandin E2 (PGE2) levels. In addition, P2Y$_1$R activation induces the activity of transcription factors such as nuclear factor κB (NF-κB), cyclic element-binding protein (CREB) and activator protein 1 (AP-1) which up-regulate the expression of proinflammatory genes, e.g. c-Fos, c-Jun, c-Myc (from Franke et al., 2012).*

1.5.2 Oligodendrocyte precursor cells (OPCs)

OPCs comprise approximately 8% of the glia population in the CNS and they undergo a complex temporally regulated program of proliferation, migration, and differentiation following their appearance at mid-gestation. It was shown that these cells first develop in the ventricular germinal zones of the embryonic spinal cord and brain and disseminate through the developing CNS by proliferation and migration, becoming more-or-less uniformly distributed throughout the CNS soon after birth in rodents (reviewed by Miller, 1996; Richardson et al., 2006). OPCs can be identified by cell-type specific markers including the transcription factor Olig2, the lipid antigen A2B5, the platelet derived growth factor alpha receptor (PDGFαR), and the chondroitin sulfate

proteoglycan NG2. Due to the expression of the latter, they are also known as NG2 cells. During their differentiation into mature oligodendrocytes they lose the expression of these progenitor marker proteins and pass through an intermediate stage, called pre-oligodendrocyte, in which they are O4-positive, towards a mature myelinating stage where they express myelin proteins, including the myelin associated glycoprotein (MAG) and myelin basic protein (MBP) (Nishiyama et al., 2009).

OPCs are not only the primary source of oligodendrocytes during development; they can also generate oligodendrocytes in the mature CNS and represent the primary source of remyelinating cells in demyelinated multiple sclerosis lesions (Nishiyama et al., 2009). However, not all OPCs develop into mature oligodendrocytes and a large number remain as progenitors in the mature white and grey matter. Since these progenitors are more abundant than would be required for the replacement of lost oligodendrocytes, OPCs may have other important yet-to-be understood functions within the CNS. Moreover, OPCs constitute the major dividing population of cells within the adult brain and make up about 70% of the cells labelled following a pulse injection of BrdU (Dawson et al., 2003). Electrophysiologically, NG2 cells show a characteristic ''complex'' pattern, distinct from the passive glial patterns, but also different from neuronal features. In fact, both cultured and *in situ* OPCs express distinct voltage-gated ion channels depending on their maturation stage (Sontheimer et al., 1989), including both inward and outward rectifying K^+ channels (Sontheimer and Kettenmann 1988; Williamson et al., 1997), Na^+ channels (Berger et al., 1992) and different subtypes of Ca^{2+} channels (Verkhratsky et al., 1990). OPCs express also neurotransmitter receptors, proteins usually associated with neuronal activities (Barres et al., 1990, Berger et al., 1992; Sontheimer et al., 1989). Furthermore, there is a synaptic association between OPCs and neurons in the developing and adult CNS (Bergles et al., 2000; Gallo et al., 2008; Jabs et al., 2005). These synaptic contacts between OPCs and axons have been described for neurons in the cerebellum, hippocampus, and corpus callosum (Bergles et al., 2000; Kukley et al., 2007; Ziskin et al., 2007). Excitation of NG2-positive cells can occur via neuronal release of GABA or glutamate which activate GABAA or AMPA receptors, respectively, on OPCs and trigger a calcium signal (Gallo et al., 2008; Hamilton et al., 2010; Paukert and Bergles, 2006). Furthermore some NG2 cells were found to display spiking sodium currents in response to an initial depolarization stimulus (De Biase et al., 2010; Chittajallu et al.,

2004; Clarke et al., 2012; Káradóttir et al., 2008). Thus, NG2 cells appear ambiguous, glial in form (since they do not possess axons), but with some electrical properties akin to neurons, and are now consider as a ‘‘fifth neural cell type’’ after neurons, oligodendrocytes, astrocytes and microglia (Richardson et al., 2011).

1.5.2.1 OPCs as multipotent neural stem cells

Their chimeric nature contributed to the idea that NG2 cells might be more plastic than previously imagined and perhaps capable of transforming into neurons as well as glia; the question of whether OPCs are multipotent progenitor cells, however, is currently a highly debated topic. The first study rising this hypothesis was published by Kondo and Raff (Kondo and Raff, 2000a). In this study they demonstrated that NG2-glia purified from early postnatal (P6) rat optic nerves can be reprogrammed by first treating with fetal calf serum (FCS) or BMPs to generate type-2 astrocytes, followed by growth in bFGF to generate multipotent stem cells that could be grown as neurospheres and could differentiate to oligodendrocytes, astrocytes and even neurons (Kondo and Raff, 2000a). In a later study they also showed that the neuronal fate potential of OPCs was due to epigenetic modification and to the reactivation of Sox2, a transcription factor that is expressed early in the developing neural tube and is essential for the maintenance of the multipotent state in NSCs (Kondo and Raff, 2004). The involvement of epigenetic mechanisms, and in particular the role of the histone deacetylases (HDAC) in the commitment of OPCs has been later further demonstrated. In this respect, it has been shown that HDAC inhibitors, such as valproic acid (VPA) and trichostatin A (TSA), induce neuronal differentiation at the expense of glial differentiation in *in vitro* conditions that favor glia-specific differentiation. Further, HDAC inhibition upregulated the neuron-specific gene NeuroD, a neurogenic basic helix–loop–helix transcription factor (Hsieh et al., 2004), and activated the Ras–ERK pathway (Jung et al., 2008), resulting in the induction and suppression of neuronal and glial differentiation, respectively. All this evidence led to the widespread hope that NG2-glia can be a regenerative resource for neurodegenerative diseases that involve neuronal as well as glial loss. A number of studies have encouraged this hope by describing neuronogenic properties of NG2-glia in the rodent CNS. Supporting this hypothesis, Tamura and colleagues have shown that in the neocortex and piriform cortex NG2-glia express DCX (Tamura et al., 2007). Since antibody-labeling experiments are notoriously difficult and

artifact-prone and genetic labeling should be more predictable, thanks to the advances in this field, transgenic mouse model became a useful tool to investigate the multipotence of this cells. In particular a lot of studies took advantages of the Cre-loxP technologies. This method utilized transgenic mouse lines that express the site-specific recombinase Cre driven by various promoters that are active in OPCs. When these mouse lines are crossed to Cre reporter mouse lines, the expression of a reporter gene (usually a fluorescent protein, i.e. GFP, EGFP or YFP) is activated permanently in cells that express Cre, thereby allowing identification of their progeny by persistent reporter expression. Using NG2creBAC transgenic mice, Zhu et al. demonstrated that NG2 cells generate oligodendrocytes throughout the grey and white matter of the brain and spinal cord (Zhu et al., 2008a, 2008b). In addition, a subpopulation of protoplasmic astrocytes in the grey matter of ventral forebrain and spinal cord appeared to be derived from NG2 cells. Surprisingly, none of the GFAP$^+$ astrocytes in the white matter was generated from NG2 cells under normal conditions, suggesting heterogeneity of the source of astrocytes. Using the same transgenic mouse model, Honsa and colleagues followed the progeny of NG2 cells in mice that were subjected to focal brain cerebral ischemia. The number of EGFP$^+$ cells after ischemia was significantly increased; cells displayed heterogeneous morphology and expressed markers of astrocytes and neuronal precursor cells. Moreover, OPCs multipotency was confirmed by detailed electrophysiological analysis, which also proved the generation of astrocytic and neuronal precursor phenotypes from EGFP$^+$ cells after ischemia (Honsa et al., 2012). Other studies have used inducible Cre lines in which Cre-mediated excision is activated by tamoxifen in transgenic lines that express a fusion protein consisting of Cre and various forms of the mutated ligand-binding domain of estrogen receptor (CreER) engineered to bind tamoxifen with a higher affinity than endogenous estradiol (Metzger and Chambon, 2001). Using PDGFRα–CreER^{T2}: Rosa26-YFP mice, Rivers and co-workers (Rivers et al., 2008) found that, although NG2-glia generate predominantly Sox10-positive oligodendrocyte lineage cells during normal adulthood some Sox10-negative, YFP$^+$ cells appeared in the anterior piriform cortex (PC), acquired NeuN and MAP2 reactivity and morphologically resembled piriform projection neurons. However, using their own independently generated line of PDGFRα–CreER^{T2} mice, Kang et al. (2010) failed to detect production of long-term surviving GFP$^+$ neurons in the PC or elsewhere in the forebrain (Kang et al., 2010). One other study has reported PC neurons from NG2-glia

(Guo et al., 2010). This study used Plp-CreER^{T2}: Rosa26-YFP mice to follow the fate of NG2-glia in the healthy adult CNS. Plp is expressed in differentiated oligodendrocytes as well as NG2-glia, so Guo and co-workers (2010) could not address questions about new oligodendrocyte production; however, similar to the group of River (Rivers et al., 2008), they did observe YFP-labeling of PC projection neurons. Labelled neurons first became apparent 17 days post tamoxifen and increased in number for at least 180 days. In addition, reporter expression was detected in some protoplasmic astrocytes in the ventral forebrain. In one study YFP^{+}-NeuN^{+} neurons were observed for only a few days following tamoxifen injection into NG2-CreER^{T2}: Rosa26-YFP mice (Zhu et al., 2011), suggesting that these neurons were eliminated from the CNS after a short time or else became NeuN negative (or YFP negative). Finally, by using a Olig2-creER™ transgenic mice, Dimou and collaborators (2008) observed that when Cre was induced in adult mice, the reporter gene was expressed almost exclusively in either NG2 cells or mature oligodendrocytes, and in a few protoplasmic astrocytes in grey matter but not in neurons (Dimou et al., 2008). In conclusion, also Cre-lox fate mapping studies have still not completely eliminated the controversy around the multipotency and fate of OPCs. This is likely because, in most studies, the final fate of NG2 cells has been followed under physiological conditions and not after CNS insults that profoundly change cells reactivity and the local extracellular milieu.

1.5.2.2 The G protein-coupled receptor GPR17

GPR17 was originally classified as an orphan G protein-coupled receptor (GPCR) before the discovery of its endogenous ligands. It displays the classical 7 transmembrane domains structure and consists of 399 amino acids (Ciana et al., 2006; Lecca et al., 2008). GPCRs constitute the largest superfamily of membrane receptor and have been involved in a multitude of biological responses in all organs and systems including the central and peripheral nervous system (PNS). Pathological changes in their expression and functions play established role in neurological and neurodegenerative diseases, thus highlighting these receptors as interesting targets for drug development. GPR17 was first identified thanks to a screening of a human cDNA library for P2Y-receptor homologous sequences (Bläsius et al., 1998). Subsequently also the rat and mouse GPR17 orthologous were identified and cloned, showing a 80%

homology in the amino acid sequence to the human GPR17 (hGPR17) (Ciana et al., 2006; Lecca et al., 2008).

GPR17 receptor: structure and pharmacological characteristics

Alignment of rat, mouse and human GPR17 amino acid sequences showed almost complete overlapping of TM3, TM6, and TM7 and conservation of a typical amino acid motif in TM6 (H-X-X-R) that is present in several GPCRs, and is believed to be essential for ligand binding (Ciana et al., 2006). Although GPR17 amino acid sequence is conserved among species, the gene structure is quite different. The human GPR17 gene (*hGpr17*) is located on chromosome 2 and it is composed of four exons, only two of which contain coding sequences. Two distinct transcripts of the hGPR17 receptor were identified to be generated by alternative polyadenylation, lately named short (hGPR17-S) and long isoform (hGPR17-L) (Benned-Jensen and Rosenkilde, 2010). The rat and mouse GPRl7 gene ortholog (*rGpr17* and *mGpr17*, respectively) are both located on chromosome 18. *rGpr17* is composed by only one exon, whereas *mGpr17* has two different exons and only a small portion of the second exon encodes for the protein.

It has been shown that this receptor is a close relative of both already known P2Y receptors (see 1.1.3) and G protein-coupled $CysLT_1$ and $CysLT_2$ receptors responding to cysteinyl-leukotrienes (cysLTs), inflammatory lipid mediators generated by 5-lipoxygenase metabolism of arachidonic acid. Indeed, GPR17 is located at intermediate phylogenetic position between P2Y and CysLT receptors and represents the closest receptor to a common ancestor which also originated $P2Y_{12,13,14}$ receptors and $CysLT_1$ and $CysLT_2$. In line with this, its deorphanisation led to its identification as a new dual uracil nucleotides/cysteinyl-leukotrienes receptor (Ciana et al., 2006) responding to both uracil nucleotides (UDP, UDP-glucose and UDP-galactose) and cysLTs (LTD4 and LTC4) (Ciana et al., 2006). Consistent with this hybrid pharmacology, activation of GPR17 by uracil nucleotides was counteracted by the P2Y receptor antagonists Cangrelor and MRS2179, whereas its activation by cysLTs was antagonized by Montelukast and Pranlukast, two already marketed CysLT receptor blockers. Agonist response profile of GPR17, was different from those of already known CysLT and P2Y receptors as determined *in vitro* by $[^{35}S]GTP\gamma S$ binding, with EC50 values in the nanomolar and micromolar range, for cysteinyl-leukotrienes and uracil nucleotides,

respectively (Ciana et al., 2006). Moreover it has also been suggested that cellular response to receptor activation can be modulated by the independent or simultaneous exposure to uracil nucleotides or cystenil leukotrienes. Bioinformatic analysis indeed showed the presence of two distinct binding sites for the two different classes of ligands. Uracil nucleotides and cystenil leukotrienes can therefore simultaneously bind to GPR17 and, for instance, UDP-glucose can potentiate GPR17 response towards LTD4, through an allosteric mechanism (Parravicini et al., 2010). Exposure of GPR17 to both UDP-glucose and LTD4 leads to receptor desensitization, followed by internalization and re-sensitization upon agonists removal (Daniele et al., 2011). This study also highlighted the effect of UDP-glucose on LTD4-induced GPR17 desensitization, suggesting a fine regulation of receptor pharmacology and function in the presence of one or both endogenous ligands.

In contrast with the above-mentioned results, Benned-Jensen and Rosenkilde (2010) reported that UDP and UDP-glucose, but not cysteinyl leukotrienes, promoted GPR17-dependent GTPγS-binding. In line with these study a very recent publication by Qi and colleagues (Qi et al., 2013) demonstrated that neither nucleotides, nucleotide sugar nor cysteinyl leukotrienes promote activation of GPR17 when assessed by standard and well established signaling assay. The reasons for these discrepancies between different laboratories are still unclear.

Finally, it has also been suggested that GPR17 is a ligand-independent, constitutive negative regulator of the $CysLT_1$ receptor. It is therefore difficult to distinguish between direct or indirect responses to cystenil leukotrienes in those systems where both GPR17 and $CysLT_1$ receptor are expressed, such as peripheral blood cells (Maekawa et al., 2009).

The majority of the pharmacological studies have been performed in transfected cells, cell lines or in primary cultures, where the coupling of receptor with the G protein Gi has been shown (Ciana et al., 2006; Fumagalli et al., 2011). Only in transfected cells a direct correlation between the agonist-induced activation of GPR17 and intracellular increases of Ca^{2+} was observed. This signaling cascade is probably generated by the βy subunits of Gi protein (Benned-Jensen and Rosenkilde, 2010; Ciana et al., 2006). Calcium increases however were not detected in cells endogenously expressing the receptor; this can be explained by tissue or cell specific expression of the βy subunits associated to GPR17 (Maekawa et al., 2009).

Furthermore, electrophysiological studies in 1321N1 astrocytoma cells stably expressing the human receptor GPR17 have shown that activation of the receptor with micromolar concentrations of UDP, UDP-glucose and UDP-galactose and nanomolar concentration of LTD4 leads to an increase of outward rectifying K^+ currents by a Ca^{2+}-dependent intracellular signaling pathway (Pugliese et al., 2009). The same group have recently demonstrated that receptor stimulation by its agonist UDP-glucose enhances delayed rectifier outward K^+ currents without affecting transient K^+ conductance also in an *"in vitro"* native system (purified primary OPCs culture compared to heterologously transfected cells). This effect was observed in a subpopulation of OPCs and immature pre-oligodendrocytes whereas it was absent in mature cells, in line with GPR17 expression. The effect of UDP-glucose on K^+ currents is concentration-dependent, blocked by the GPR17 antagonists MRS2179 and Cangrelor, and sensitive to the K+ channel blocker tetraethyl-ammonium (TEA), which also inhibits oligodendrocyte maturation. In this respect authors propose that stimulation of K^+ currents is responsible for GPR17-induced oligodendrocyte differentiation (see below; Coppi et al., 2013).

GPR17 expression profile

hGPR17 is highly expressed in tissues that undergo ischemic damage, namely brain, heart and kidney. The same pattern of expression has been confirmed in rats and mice (Ciana et al., 2006; Lecca et al., 2008). Interestingly, a recent study has shown that the hGPR17 short isoform is mainly expressed in heart and kidney, whereas the long isoform is present only in the brain (Benned-Jensen and Rosenkilde, 2010). This distinct pattern of expression suggests a specification of GPR17 function related to its localization, at least in human.

A detailed analysis of GPR17 expression profile has been first of all performed in rat and mice brain. GPR17 was detected on SMI-311$^+$/NeuN$^+$ neurons by immunohistochemistry techniques; conversely, no co-localization with cortical astrocytes was found as shown by labeling with the specific marker GFAP. The vast majority of GPR17$^+$ cells co-express the OPC marker Olig2 and NG2; furthermore, no co-localization of GPR17 was found with more mature myelinating oligodendroglial markers, such as CNPase and the myelin-associated glycoprotein MAG. Finally, no GPR17 expression was found in resting microglia detected by anti-Iba1 staining in the intact brain (Lecca et al., 2008; **Figure 1.13**).

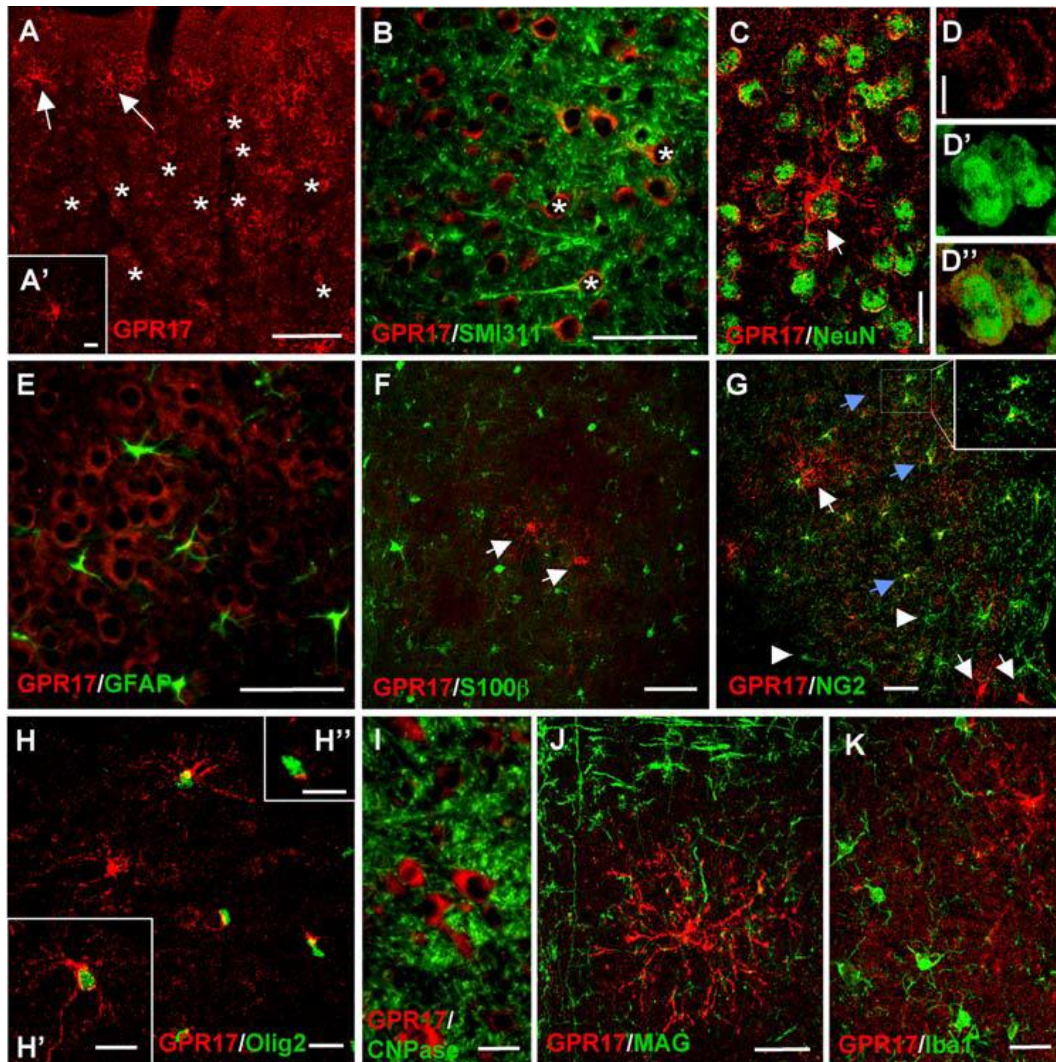


Figure 1.13: cellular localization of GPR17 in the intact mouse brain. *(A) In the intact cortex, GPR17 expression was observed in large rounded cells clustered at high density (asterisks) and in sparse ramified cells (arrows and A' inset). Virtually all GPR17$^+$ large cell bodies displayed co-expression of the neuronal proteins SMI311 (B) or NeuN (C), demonstrating their neuronal identity. (D, D') panels show higher magnification images of two neurons immunopositive for both GPR17 (red channel, D), and NeuN (green channel, D');(D'') merging of the two fluorescence channels. In the cortex, no co-localization of GPR17 with the astroglial markers GFAP (E) and S100β (F; arrows indicate GPR17$^+$ cells) was found. (G) partial co-localization of GPR17 and the oligodendrocyte marker NG2. Indeed, several ramified GPR17-labeled cells co-stained for NG2 (blue arrows in G); however, also single staining for NG2 (arrowheads in G) or GPR17 (white arrows in G) was found. GPR17 co-stained also with Olig2, another oligodendrocyte marker (H, H'). In some cases, NG2$^+$ or Olig2$^+$ cells show immunoreactivity for GPR17 in discrete cell body compartments (inset in G and H'', respectively). I and J depict no co-localization of GPR17 with myelin-related proteins (CNPase and MAG, respectively). Some ''resting'' microglial cells were also found in cortex, as suggested by staining with the specific marker Iba1 (K): in the intact brain, none of these cells expressed GPR17 (from* Lecca et al., 2008).

In the spinal cord the same expression profile was observed, and additionally GPR17 co-localized with CC1-positive mature oligodendrocytes and with the ependymal cells lying the central canal which are considered to be the neuronal stem cells of the spinal cord (Ceruti et al., 2009).

Extensive studies on primary OPCs culture have characterized GPR17 expression during cell differentiation. Receptor expression, almost absent in early OPCs, gradually increases in more mature precursors, reaches a plateau in immature/pre-oligodendrocytes, and then gradually decreases during terminal differentiation. In line with these findings, GPR17 is co-expressed with the early oligodendrocyte marker NG2 and with markers of pre/immature oligodendrocyte phenotype (such as O4), but it is down-regulated in cells expressing myelin proteins, such as myelin basic protein, which is highly synthesized in fully mature cells (Ceruti et al., 2011; Fumagalli et al., 2011).

Other studies showed the expression of GPR17 on human and mouse mastocysts and bone marrow-derived monocytes, where the receptor seems to highly co-localize with $CysLT_1$ receptor (Maekawa et al., 2009, 2010).

Finally, and most interesting, a trascriptome analysis performed on human and mouse adult neural stem cells and fetal embryonic tissue revealed expression of the receptor specifically in adult neuroprogenitors (Maisel et al., 2007). In line with these results strong GPR17 expression has been also detected by RT-PCR in adult rat hippocampus derived NPCs. Here, the antagonist Montelukast significantly and specifically increased the proliferation of NPCs and NSCs *in vitro*. However, it neither affected the cells' fate nor their differentiation potential (Huber et al., 2011).

GPR17 functions

Upon brain injury, the extracellular concentration of nucleotides and cysteinyl-leukotrienes are markedly increased at the site of damage, suggesting that they may act as "danger signals". Moreover, as previously mentioned, GPR17 is highly express in tissues undergoing ischemic damage and in particular in the brain. Due to these two reasons most of the studies on GPR17 were aimed at understanding its function in the CNS and at unveiling its possible therapeutic potential in several pathological conditions.

Role of GPR17 in brain ischemia - The therapeutic potential of GPR17 was first assessed in a rat model, and subsequently in a mouse model, of ischemia (MCAo, middle cerebral artery occlusion). The application of a P2Y antagonist and, more specifically, the use of anti-sense oligonucleotide targeting GPR17 were both sufficient to reduce the penumbra area of the ischemic damage. It is not clear, however, if reduction of the ischemic damage was mainly due to the effect on a specific cell type expressing the receptor or if it was more general. Anyway, in this case GPR17 appeared to be pro-inflammatory and its blockage, by antagonist or anti-sense oligonucleotide application, improved functional recovery (Ciana et al., 2006; Lecca et al., 2008). Recently the role of GPR17 has been also assessed in human injured brain. After traumatic brain injury (TBI), GPR17 underwent profound changes in cells participating to tissue damage and repair according to a precise spatio-temporal pattern. GPR17 was selectively induced in patients deceased from intracranial, but not extracranial complications, and receptor expression negatively correlates with patients' post traumatic survival time (PTI), being very high at short survival periods (2h) and markedly decreased at PTI longer than 16 days (Franke et al., 2013). Strong GPR17 immunoreactivity was found in shrunk dying pyramidal neurons inside or in the immediate vicinity of the traumatic core. This is consistent with rodent data showing early and transient GPR17 upregulation in neurons inside and at the borders of ischemic lesions. Data from TBI patients also showed that GPR17 is specifically induced in IB4-positive activated microglia/macrophages infiltrating the lesion, confirming GPR17 participation to the lesion remodeling that precedes the reconstruction of neuronal circuitries. At variance from rodents, in the cortex of TBI patients GPR17 decorated a subset of reactive astrocytes, suggesting that, in humans, GPR17 may also regulate astrocytic post-traumatic activation. Thus, the role of GPR17 in the injured human brain may be broader than that observed in rodents. Moreover, in several human samples, GPR17 was found in bi-/tripolar or more ramified OPCs, suggesting a role in postinjury-induced OPC differentiation. Some GPR17-expressing OPCs were clearly resuming proliferation. Thus, in both humans and rodents, GPR17 participates to post-injury attempts to generate new myelinating oligodendrocytes (Franke et al., 2013).

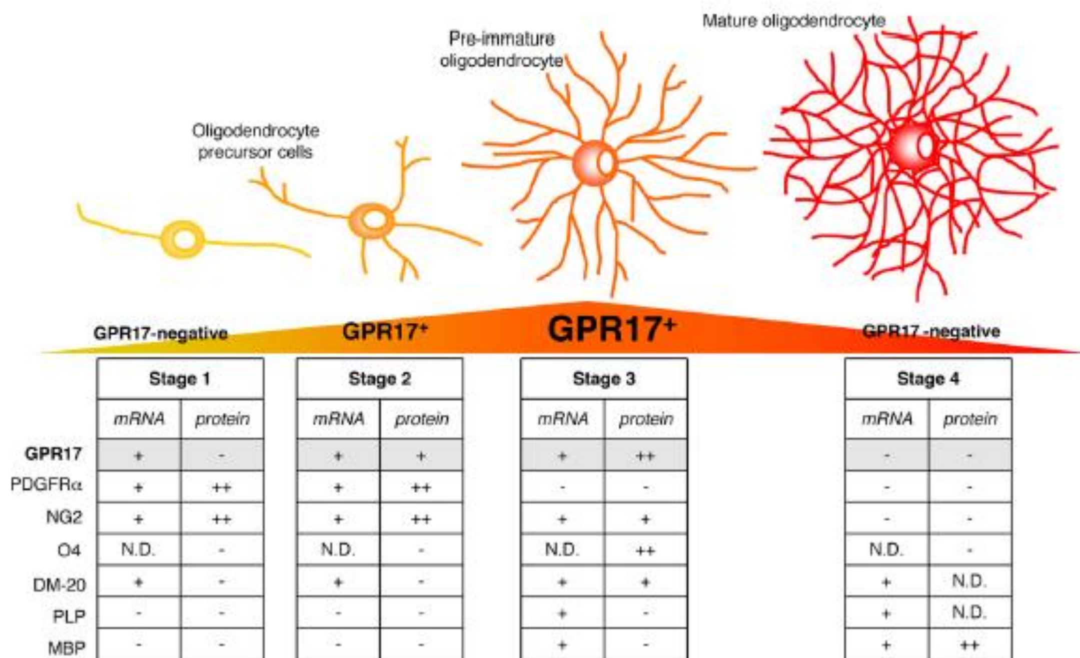
Finally, two recent papers published by the same group (Mao et al., 2012, 2013) also highlighted a role for GPR17 in an experimental model of hypoxic ischemia-induced periventricular leucomalacia (PVL) in neonatal rats. The number of white

matter $NG2^+/GPR17^+/BrdU^+$ and $O4^+/GPR17^+/BrdU^+$ cells was significantly increased within 72 hours and 7 days after induction of PVL, respectively. Moreover, treatment with UDP-glucose improved the limb motor function and coordination of PVL neonatal rats and significantly mitigated the histopathological white matter damage induced by hypoxic ischemia, thus suggesting a role for GPR17 activation in alleviating the deleterious effects of ischemia-induced PVL.

Role of GPR17 in spinal cord injury (SCI) - Induction of spinal cord injury (SCI) by acute compression resulted in marked cell death of $GPR17^+$ neurons and oligodendrocytes inside the lesion followed by the appearance of proliferating $GPR17^+$ microglia/macrophages migrating to and infiltrating into the lesioned area. Moreover, 72 h after SCI, $GPR17^+$ ependymal cells started to proliferate and to express GFAP, suggesting their activation and 'de-differentiation' to pluripotent progenitor cells. The administration of anti-sense oligonucleotides targeting GPR17, gave positive results in terms of damage reduction and functional recovery also in this model, again suggesting an active role of GPR17 in the early phases of tissue damage development (Ceruti et al., 2009). In this scenario the function of GPR17 was described to be temporal and cellular specific; the first response to injury provoked the death of neurons and oligodendrocytes through GPR17 activity; at later times the recruitment of microglia and GPR17-positive macrophages seemed to shed the first steps of tissue remodeling and repair, that was followed by the upregulation of GFAP, as a marker of stem-cell like properties, on $GPR17^+$ ependymal cells. Due to this dualistic pattern of activation, it can be hypothesized that GPR17 represents an interesting target to develop new approaches to the management of secondary damage in SCI aimed at both reducing cell death and fostering tissue repair.

Role of GPR17 in oligodendrocyte differentiation - Several studies performed in our laboratory suggest that GPR17 contributes to OPC differentiation. In mouse cortical primary mixed astrocytes/precursor cell cultures, GPR17 expression was markedly influenced by the culturing conditions. In the presence of GFs, no significant GPR17 expression was found. When cultures were shifted to a differentiating medium without GFs, a dramatic and time-dependent increase in the number of highly branched GPR17-positive cells was observed. Under these conditions, GPR17 was induced in the totality

of O4-positive immature oligodendrocytes (Ceruti et al., 2011). Instead, in cultures originally grown in the absence of GFs, GPR17 was already expressed in morphologically more mature OPCs. Shifting of these cultures to differentiating conditions induced GPR17 only in a subpopulation of O4-positive cells. Under both culture protocols, appearance of more mature CNPase- and MBP-positive cells was associated to a progressive loss of GPR17. GPR17 expression also sensitized cells to adenine nucleotide-induced cytotoxicity (Ceruti et al., 2011). These data are in accordance with the *in vivo* model of ischemia where the knock-down of GPR17 was shown to be protective, suggesting that GPR17 may sensitize cells to cell death in presence of high levels of ATP and ADP also *in vivo*. Another study published by our group reported a detailed molecular characterization of GPR17 during the spontaneous in vitro differentiation and maturation of primary rodent $NG2^+$ OPCs (Fumagalli et al., 2011). In cultured OPCs, the GPR17 transcript was first detected in bipolar $NG2^+$ polydendrocytes. Receptor expression gradually increased along with morphological differentiation in cells with more processes emerging from the cell body, was maximal in immature pre-oligodendrocytes, and then gradually decreased along with terminal maturation (Fumagalli et al., 2011). In line with these findings, and as already demonstrated *in vivo*, the GPR17 receptor protein decorated two subsets of slowly proliferating cells. The first one corresponded to early, morphologically immature and slowly proliferating NG2+ precursor cells that also expressed Olig2, PDGFRα, and the immature PLP isoform DM-20; the second one corresponded to more ramified, still immature pre-oligodendrocytes that were losing NG2 and PDGFRα immunoreactivity and already expressed O4, O1, and the two splicing variants of the myelin protein PLP. After this differentiation stage, GPR17 expression was progressively turned down, and the GPR17 protein was never found in fully mature MAG^+ or MBP^+ oligodendrocytes (**Figure 1.14**).



	Stage 1		Stage 2		Stage 3		Stage 4	
	mRNA	*protein*	*mRNA*	*protein*	*mRNA*	*protein*	*mRNA*	*protein*
GPR17	+	-	+	+	+	++	-	-
PDGFRα	+	++	+	++	-	-	-	-
NG2	+	++	+	++	+	+	-	-
O4	N.D.	-	N.D.	-	N.D.	++	N.D.	-
DM-20	+	-	+	-	+	+	+	N.D.
PLP	-	-	-	-	+	-	+	N.D.
MBP	-	-	-	-	+	-	+	++

F*igure 1.14: scheme showing GPR17 expression as a function of OPC differentiation.* *The drawing illustrates the typical differentiation steps of OPCs, from immature precursors to mature oligodendrocytes. Tables summarize single-cells RT-PCR (mRNA) and immunocytochemical (protein) data for GPR17 and other known markers identifying specific OPC differentiation stages. At early stage (stage 1), bipolar OPCs already express GPR17 mRNA, but receptor protein expression is detectable only when NG2$^+$ cells acquire a more complex morphology (stage 2, polydendrocyte stage). After this stage, GPR17 gradually increases in pre/immature oligodendrocytes, reaching a maximum when O4 is expressed (stage 3). GPR17 expression is then turned down, and mature MBP$^+$ cells no longer express the receptor (stage 4). The graded changes of the GPR17 protein during differentiation are highlighted in a yellow-orange-red triangle, where orange represents the maximum expression level. N.D., not done (from* Fumagalli et al., 2011).

Moreover, administration of the agonist UDP-glucose triggered an increase in the differentiation of precursor cells to myelinating oligodendrocytes (Ceruti et al., 2011; Fumagalli et al., 2011); on the contrary the pharmacological inhibition of GPR17 or its knock-down by siRNAs impaired the normal differentiation program of OPCs. Thus, these data suggest that GPR17 acts as an intrinsic regulator of oligodendrogliogenesis. Indeed, at early differentiation stages the receptor has to be express to keep OPCs in an immature state which is necessary to prepare them for myelination; conversely, at a later stage of differentiation GPR17 has to be turned down to allow the terminal differentiation of immature oligodendrocytes.

However, whether GPR17-mediated signaling positively or negatively regulates this critical process is now highly debated. Indeed, in contrast with the results obtained

by our group, a very recent paper by Hennen and co-workers identified a new small-molecule (MDL29,952), which activated GPR17 and arrested oligodendrocyte in an immature, non myelininating stage (Hennen et al., 2013). We can speculate that these apparently conflicting results could be due to the exposure of OPC cultures to GPR17 agonist at different stages of cell differentiation.

Moreover, in another study by Chen and co-workers, the authors analyzed oligodendrocyte myelination in mouse models of ectopic over-expression and knock-down of GPR17. Interestingly, GPR17 over-expression under the CNPase promoter, at a differentiation stage when it is no longer physiologically expressed, resulted in impaired postnatal myelination and premature death. On the contrary, in a GPR17 knock-out model, myelination started at embryonic stage, much earlier than the physiological process in wild type littermates (Chen et al., 2009). It appears clear that in the transgenic mouse models utilized in this paper, forcing GPR17 transcription in oligodendrocytes at a maturation stage when they physiologically downregulate its expression (i.e., CNPase cells) might have created conflicting intracellular signals leading to cell suicide. Thus, although further confirming the key role played by GPR17 in OPC maturation, the conclusions drawn based on this transgenic mouse model may lead to a misinterpretation of the native function of the receptor.

Taken together these observations led to the conclusion that during development GPR17 is a "cell intrinsic timer" that precisely regulates the onset of myelination. The molecular mechanism that support this hypothesis could stem in the correlation between GPR17 over-expression *in vitro* and the upregulation of the transcription factor Id2 and the translocation of this same factor and Id4 to the nucleus, where they are known to inhibit the expression of genes involved in oligodendrocytes maturation (Chen et al., 2009). In the same line, a trascriptome analysis showed negative regulation of GPR17 by mTOR (mammalian target of rapamycin), another transcription factor that normally promotes OPCs differentiation in myelinating oligodendrocytes (Tyler et al., 2011).

2. AIM OF THE STUDY

For almost a full century, a central dogma of developmental biology declared that the generation of new neurons in the brain concluded shortly after birth (Gross, 2000). This hypothesis was based on evidence from Santiago Ramon y Cajal, who in 1913 sustained that neurons were generated only during the prenatal phase of development (Ming and Song, 2005).

In fact, he stated that “Once development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In adult centres, the nerve paths are something fixed and immutable: everything may die, nothing may be regenerated” (Ramon y Cajal, 1928). Therefore, it has been long believed that no new neurons are generated in the adult brain and that the loss of neurons that accompanied most of central nervous system (CNS) pathologies cannot be restored.

However, this doctrine ended in 1965 when newly generated neurons were found in two specific regions of the adult brain: the subgranular zone (SGZ) in the dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricle wall. As confirmed by subsequent studies, in the SVZ three main cell types can be morphologically and functionally distinguished: $GFAP^+$ precursors (Type B cells) give raise to transit-amplifying $Mash1^+$ Type C cells, which eventually further differentiate to doublecortin $(DCX)^+$ neuroblasts (Type A cells) which migrate towards the olfactory bulb (OB) in rodents where they integrate in existing networks (Doetsch et al., 1997, 1999a, 1999b; Garcia-Verdugno et al., 1998; Kim et al., 2011). Once isolated, cells from the SVZ can grow in vitro as clonal clusters of proliferating cells, called neurospheres (NS), which can either self-renew or differentiate to astrocytes, neurons and oligodendrocytes (Johansson et al., 1999; Reynolds et al., 1992).

Studies carried out during the last two decades revealed that in mammalian CNS parenchyma, which is commonly considered as non-neurogenic, some processes of gliogenesis and, to a lesser extent, neurogenesis also occur. In particular, cells expressing the proteoglycan NG2 (also known as polydendrocytes or oligodendrocyte precursor cells OPCs), represent the vast majority of proliferating cells in the healthy adult CNS (Dawson et al., 2003). They give raise to mature oligodendrocytes during

development and adulthood and can re-establish myelination in the diseased CNS (Fancy et al., 2011). It has been also suggested that, under some conditions, NG2 cells can give raise to both astrocytes and neurons. In particular, NG2 cells lineage plasticity may be markedly widened by insults and epigenetic agents that profoundly change their reactivity and the surrounding local milieu (Honsa et al., 2012). However, more than 10 years ago a first sensational demonstration that these cells can be reprogrammed to generate neurons (Kondo and Raff, 2000a) has been followed by only a few in vitro confirmations (Liu et al., 2007) and conflicting in vivo results (Dimou et al., 2008; Richardson et al, 2011). The possibility to promote and foster the endogenous neurogenic/gliogenic ability of the brain has emerged as a new, safe, and ethically acceptable opportunity for both acute and chronic brain diseases. An in-depth understanding of the molecular basis controlling brain progenitor cells in physiological and pathological conditions would highlight possible pharmacological/biotechnological approaches to help preventing excessive tissue degeneration while enhancing local reparative mechanisms. Nevertheless, despite increased information on lineage specific transcription factors, cell-cycle regulators and epigenetic factors involved in the fate and plasticity of NSCs (Christie and Turnley, 2013), the understanding of extracellular cues driving the behavior of embryonic and adult NSCs is still very limited.

Extracellular adenine, uracil and sugar nucleotides (eNTs) are universal signaling molecules involved in many biological processes acting via specific membrane receptors: the ionotropic P2X receptors and the metabotropic P2Y receptors. P2X receptors are ligand-activated cationic channels, specifically activated by ATP, while P2Y receptors are activated by purine or pyrimidine nucleotides, or by sugar-nucleotides, and couple to intracellular second-messenger systems through heteromeric G proteins (Abbracchio et al., 2006). eNTs play key roles in neurotransmission and neuromodulation and are involved in several cellular functions, including proliferation and migration, apoptosis and cytokine secretion. Moreover nucleotides released from both glial cells and neurons contribute to cell to-cell communication and represent a class of signaling molecules that functionally link glia and neurons together (Abbracchio and Burnstock, 2009; Fields and Burnstock, 2006).

In the adults brain, neurogenesis is dynamically regulated by a number of intrinsic as well as extrinsic factors and also by a range of environmental-, behavioral-, genetic-factors (see Introduction, Paragraph 1.4). Among the other factors already known to be

involved in adult neurogenesis, recently, also a role for eNTs in controlling the functions of stem cells in the subventricular zone SVZ has emerged, although available data are conflicting. In vitro results by the neurosphere assay have shown that ADP or its stable analogue adenosine 5'-O-(2-thio)-diphosphate (ADPβS) promoted the proliferation of SVZ cells, probably acting on the $P2Y_1$ receptor ($P2Y_1R$), but only at low growth factor (GF) concentrations. Conversely, an anti-proliferative effect of various eNTs, including ADPβS, was observed at standard GFs concentrations (around 20 ng/ml; Stafford et al., 2007). Activation of the $P2Y_1R$ also promoted SVZ cell differentiation (Grimm et al., 2009) and migration (Grimm et al., 2010) *in vitro*. *In vivo*, the contribution of eNTs in controlling SVZ cell proliferation is poorly understood. In a recent study, ATP (which non-selectively activates all the P2X and some P2Y receptor subtypes) has been infused in the adult SVZ (Suyama et al., 2012). Upon ATP administration, the proliferation of transit-amplifying cells (type C cells) increased, whereas no effect was observed on proliferation of either type B stem cells or type A neuroblasts. A possible involvement of the $P2Y_1R$ was hypothesized based on the inhibitory effect on $Mash1^+$ type C cell proliferation exerted by the administration of the $P2Y_1R$-selective antagonist 2'-deoxy-N6-methyladenosine-3',5'-bisphosphate (MRS2179) *per se*, and by the reduced number of type C cells in $P2Y_1R$-KO mice (Suyama et al., 2012). In addition to their important physiological actions, eNTs modulate brain functions in pathological conditions, when their extracellular concentrations rise to micromolar levels (Abbracchio et al., 2006). Under these conditions, eNTs trigger and sustain reactive astrogliosis, the astrocytic reaction to brain trauma or ischemia (Abbracchio and Ceruti, 2006), whose protective/detrimental double-edged sword effect is still a matter of debate (Buffo et al., 2010). Activation of the ADP-responsive $P2Y_1R$ promotes astrogliosis, and modulates astrocytic secretion of a plethora of factors, including cytokines/chemokines and GFs (Franke et al., 2012). These molecules might act as autocrine/paracrine signals on surrounding cells, including SVZ, NSCs and progenitors.

Previous studies from our laboratory have identified the purinergic receptor GPR17 as a new marker of early stages of NG2 cell differentiation, showing that GPR17 activation accelerates NG2 cells' oligodendrocyte fate (Fumagalli et al., 2011; Ceruti et al., 2011; Boda et al., 2011). Interestingly, GPR17 is also one of the key genes

expressed by human adult neural stem cells (Maisel et al., 2007), suggesting a possible role in cell fate determination.

Based on these premises, the aim of my PhD project was to investigate the role of purinergic signaling in regulating stem cell proprieties of adult brain subventricular zone and of NG2+ parenchymal progenitors.

In particular, the work presented in the first section of the Results was aimed at investigating the role of ADPβS, acting as agonist at the $P2Y_{1,12,13}$ receptor subtypes, in modulating adult neurogenesis in the mouse SVZ, with a focus on the possible effects exerted by reactive astrocytes. First of all we performed in vitro experiment by the neurospheres assay to test the ability of the ADP analogue to modulate the proliferation and self-renewal capability of neural stem cells and to control the differentiative potential of neurospheres (NS). Moreover, to test whether ADPβS was acting directly on NSCs only or whether reactive astrocytes were involved, we grew NS in the conditioned media derived from control astrocytic cultures or from astrocytes cultured in presence of ADPβS. We also performed in vivo experiments in which ADPβS was chronically infused in the lateral ventricle of adult mice for one week and we then analyzed the effect of the treatment on the three main cell types within the SVZ by immunohistochemistry of specific markers. Finally, to verify whether ADPβS can act directly on NSCs, we took advantage of GLAST::CreERT2;Rosa-YFP transgenic mice in which stem cells expressing the L-glutamate/L-aspartate GLAST and their progeny are permanently labelled by the fluorescent protein YFP upon tamoxifen administration.

The aim of the second part of my PhD thesis was to unveil the stem cell properties of $NG2^+$ precursor cells and to implemant their differentiation towards a neuronal lineage, also through the modulation of the GPR17 receptor.

Indeed, at variance from other proteins expressed by $NG2^+$ progenitors, GPR17 is a membrane receptor that can be easily activated/inhibited with specific ligands, thus allowing the modulation of the final fate of these cells. Thus, we hypothesized that, thanks to the multipotency of $NG2^+$ precursors, it could be possible to generate cell types other than oligodendrocytes (e.g., neurons) if GPR17 is selectively activated or inhibited in cultures grown under different experimental conditions from those utilized in our previous works (see above). Primary OPCs from the cerebral cortex of P2 rat pups were kept in culture for 6-8 days as mixed astrocytes-OPCs cultures and purified by vigorous shaking of flasks, and immunopanning selection. OPCs have been then

cultured according to two protocols able to unveil their stem cell properties (Kondo & Raff, 2000a; Liu et al., 2007). In either protocol, we have verified if and how the exposure to various pharmacological agents (including GPR17 receptor ligands) can modulate OPCs plasticity and their differentiation to neurons. In particular, we have utilized the non-selective GPR17 agonist UDP-glucose (100 μM) and antagonist Cangrelor (10 μM), in parallel to the anticonvulsant agent valproic acid (VPA, 500 μM). Besides being utilized in the therapy of epilepsy, bipolar disorders and migraine (Monti et al., 2009), VPA acts as histone deacetylase (HDAC) inhibitor, thus amplifying the differentiative potential of these cells. *In vivo* and *in vitro* experiments have demonstrated that HDAC inhibition indeed reduces OPCs differentiation towards the oligodendrocytic lineage and increases their maturation towards an astrocytic or neuronal phenotype (Yu et al., 2009).

We believe that the new emerging field of the role of nucleotides in modulating embryonic and adult neural development will contribute to further understanding the interactive role of small signal molecules in the control of neurogenesis. New tools for enhancing neurogenesis by supporting progenitor cell proliferation, migration or differentiation or preventing cell death might eventually lead to therapeutic approaches in neuropsychiatric or neurodegenerative disease associated with impaired neurogenesis or with neuronal loss.

3. MATERIALS AND METHODS

3.1 THE NEUROSPHERE ASSAY

3.1.1 NEUROSPHERE CULTURES

Neurosphere cultures were generated from adult wt CD1 mice. Briefly, 4-6 animals for each experiment were anesthetized with diethyl ether, and then killed by decapitation. The brain was exposed, surgically removed, and placed in an ice-cold sterile Hanks' Balanced Salt Solution (HBSS, Euroclone, Milan, Italy). First of all, to dissect the subventricular zone (SVZ) a coronal section from the whole brain was obtained (about 2 mm in thickness) by cutting the tissue between the rhinal fissure and the hippocampus. The coronal section was then divided into the two hemispheres and, for each of them, a parasagittal cut just lateral to the lateral ventricles, and a horizontal cut above the corpus callosum were made (**Figure 3.1**). Finally, the thin layer of tissue surrounding the ventricles (excluding the striatal parenchyma) was dissected and placed in an ice-cold tube with HBSS.

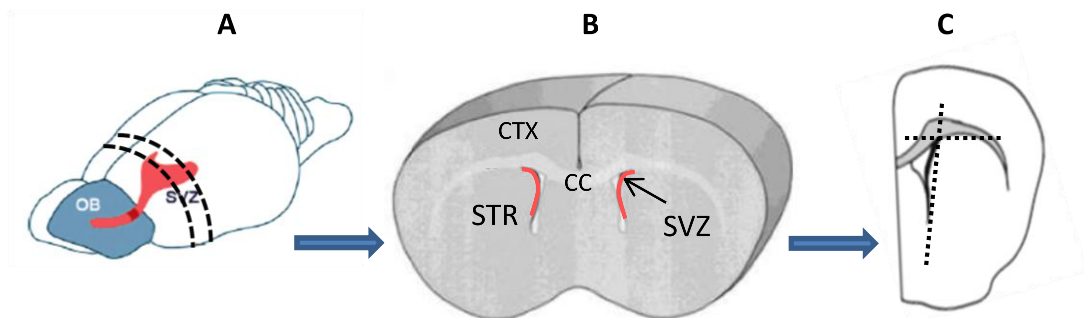


Figure 3.1: schematic representation of the dissection protocol. (*A) Murine adult brain in which the areas of interest are highlighted: blue, the olfactory bulb (OB); red, lateral ventricle and rostral migratory stream; dotted lines show the level of the coronal section (modified from Bonfanti and Peretto, 2007). (B) Coronal section in which the subventricular zone (SVZ), the striatum (STR), the corpus callosum (CC) and the cortex (CTX) are visible. (C) Coronal section of half hemisphere. The dotted lines represent the two cuts that are made to isolate the SVZ (modified from* Garcia-Verdugno et al., 1998).

Upon harvesting the periventricular regions from all brains, HBSS was removed and tissues were incubated in trypsin-EDTA solution at 37°C for 10 min. An equal volume of DMEM/F12 + 10% fetal bovine serum (FBS, Euroclone) was added at the end of the enzymatic incubation to inactivate trypsin. Tissue were then gently triturated through a series of descending-diameter pipettes to make a single-cell suspension. The cells were centrifuged at 800 rpm for 7 min and the pellet was then resuspended in 2 ml of *Neurosphere Medium* (**Table 3.1**) and mechanically dissociated.

Components	Final Concentration
DMEM/F-12 + glutamax^{TM} (Life Technologies, Milan, Italy)	To volume
Penicillin (Euroclone)	100 U/mL
Streptomicin (Euroclone)	100 µg/mL
HEPES (Sigma-Aldrich, Milan, Italy)	8 mM
Heparin (Sigma)	2 ng/mL
D-glucose (Euroclone)	6 mg/mL
B27 supplement (Life Technologies)	1:50

Table 3.1: composition of Neurosphere Medium

The cell suspension was centrifuged again for 9 min at 900 rpm and the pellet was resuspended in 2 ml of *Neurosphere Medium*, mechanically dissociated and filtered with a 70 µm cell strainer (BD Pharmigen). Finally the cell suspension was centrifuged for a third time for 10 min at 1,000 rpm, and the cells plated at low density (20,000 cells/ml) in a six-well plate. Cultures were supplemented with EGF and bFGF (20 ng/mL) every other day (**Figure 3.2**).

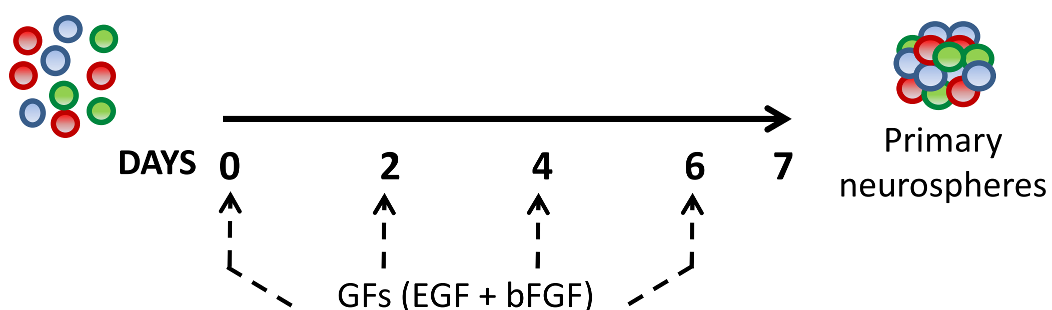


Figure 3.2: schematic representation of the experimental protocol*. Cells obtained from the dissociation of SVZ were plated at low density and kept in culture for seven days till the formation of primary neurospheres. Growth factors (GFs) were added every other day.*

Primary neurospheres were generated: (i) in *Neurosphere Medium*, (ii) in *Neurosphere Medium + ADPβS* (50 µM), or in conditioned media derived from astrocytes cultured (iii) under control condition (*astro CTR*), or (iv) in the presence of ADPβS (*astro ADPβS*) (50 µM, Sigma-Aldrich; see below). This concentration of ADPβS was selected based on previous work showing that: (i) it activates astrocytes *in vitro* by reproducing features of their response to lesion *in vivo* (Franke et al., 2012; Quintas et al., 2011), and (ii) it modulates adult stem cell functions (Grimm et al., 2009; Mishra et al., 2006; Stafford et al., 2007). In selected experiments, the $P2Y_1$ selective antagonist MRS2179 (50 µM, Sigma-Aldrich) was added at the time of SVZ cell plating to the neurosphere medium + ADPβS or to the conditioned medium derived from astrocytes cultured in the presence of ADPβS.

After 7 days in culture, the number of generated neurospheres and their size were evaluated. For each condition, neurospheres included in 5 randomly chosen optical fields at 10X magnification were analyzed under a Zeiss Axiovert 8400 microscope (Carl Zeiss, Milan, Italy), equipped with a CCD camera module. Neurosphere diameters were measured using the ImageJ software (Research Service Branch, National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/).

3.1.2 Passaging or differentiation of neurospheres

In selected experiments, primary neurospheres grown as indicated above were dissociated, and cells re-plated in control *Neurosphere Medium* irrespectively of their original culturing condition to generate secondary neurospheres.

For passaging, neurospheres were spun down and dissociated mechanically using a P200 micropipet tip (30 times up and down). As for primary neurospheres, 20,000 cells/ml were plated in a six-well plate. The number and size of secondary neurospheres were then analyzed after 2 days *in vitro.*

Alternatively, to test cell proliferation and differentiation, 7-days-old control secondary neurospheres were allowed to adhere to the culturing substrate (Lab-Tek II Chamber Slide™ System) previously coated with laminin (0.5 mg/mL stock solution diluted 1:50 in HBSS, Sigma-Aldrich) without being dissociated, and were grown for 7 additional days in *Neurosphere Medium* without growth factors and in the absence or presence of ADPβS (50 µM). In this case, cells migrated from the core of the sphere and underwent differentiation (**Figure 3.3**).

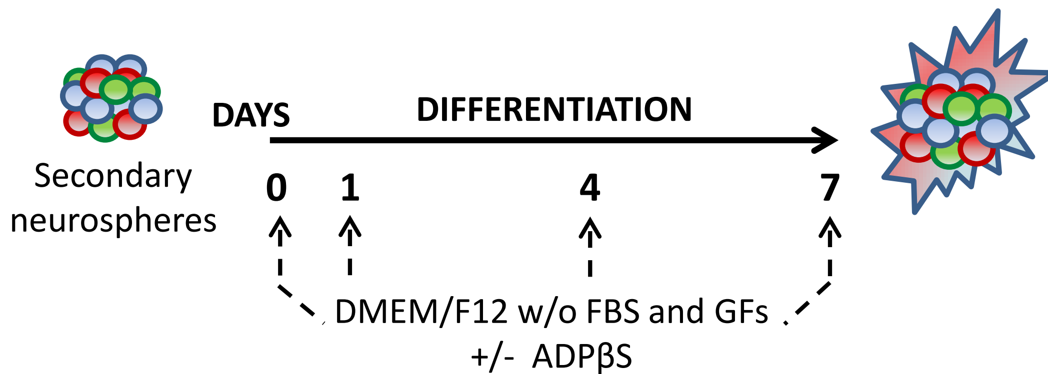


Figure 3.3: schematic representation of the differentiation protocol. *7-days-old secondary neurospheres were plated as adherent cells without being dissociated and grown for 7 additional days w/o FBS and GFs in the absence or presence of ADPβS.*

Differentiation to astrocytes or neurons was evaluated by immunocytochemistry with the specific markers glial fibrillary acidic protein (GFAP) and βIII-tubulin (βIII-tub), respectively. Cell number was evaluated by staining nuclei with the Hoechst33258 dye (see below).

3.2 MIXED GLIAL CELL CULTURES AND PREPARATION OF CONDITIONED MEDIA

3.2.1 PRIMARY MIXED GLIAL CELL CULTURES FROM RAT CEREBRAL CORTEX

Mixed glial cells were obtained from P2 Sprague-Dawley rat cortex and were used to isolate oligodendrocyte precursors cells (OPCs) and astrocytes.

Animals were anesthetized with diethyl ether then killed by decapitation. The brain was exposed and surgically removed. The olfactory bulbs and the cerebellum were cut off and the brain was divided along the midline into two cerebral hemispheres to isolate the cortex. After removal of meninges, cortices were minced with a sterilized razor blade into ~ 1 mm^3 chunks and placed in a 50 ml tube containing HBSS on ice. When all the cortices were isolated, three washes with HBSS without (w/o) Calcium and Magnesum were performed followed by a 30 min incubation at 30 C° in a trypsin-EDTA solution (c.f. 0.5% e 0.2% in HBSS w/o $Ca2^+$ and Mg^+) +1% deoxyribonuclease (DNase; Sigma-Aldrich; c.f. 0.01 mg/mL). At the end of the enzymatic incubation an equal volume of HBSS + 10% FBS was added to stop trypsinization, and the cell pellet was triturated and dissociated until nearly homogenous. The tissue suspension was

passed through a 100 µm cell strainer placed on a 50 ml conical tube to collect the flow-through that was then centrifuged for 10 min at 1,200 rpm. The pellet was then resuspended in *DMEM Medium* (1 ml for each brain) (**Table 3.2**). Finally, 1 ml of the cell suspension was plated on T75 cm^2 tissue culture flask already containing 12 ml of *DMEM Medium* added with 20% FBS. To help cells adhesion, the day before preparation flasks were coated overnight at room temperature with poly-D-lysine coating solution (1 mg/ml stock solution diluted 1:100 in sterile ddH_2O), and then washed three times with sterile ddH_2O and dried completely in a tissue culture hood. The medium was changed every other day with fresh medium. Ten days after plating, mixed glial cultures reached confluence, and phase-dark, process-bearing OPCs appeared on top of a phase-gray bed layers of astrocytes. The culture was therefore ready to be shaken to isolate either OPCs or astrocytes.

Components	Final Concentrations
DMEM high glucose (Euroclone)	To volume
Penicillin (Euroclone)	100 U/mL
Streptomicin (Euroclone)	100 µg/mL
Sodium Pyruvate (Euroclone)	1 mM
Fungizone (Life Technologies)	2,5 µg/mL
L-Glutamine (Euroclone)	2 mM

Table 3.2: composition of DMEM Medium

3.2.2 Astrocyte conditioned media

After removal of overlaying oligodendrocytes and microglia by vigorous shaking of flasks (see 3.4), astrocytes were kept in *Neurosphere Medium* (see above) in the absence or presence of ADPβS (50 µM) for 3 additional days. In parallel, two flasks containing only *Neurosphere Medium* with or without ADPβS (50µM) were kept in the incubator to test for the effect of a prolonged incubation at 37°C on the properties of our media. All the media were then collected, filtered and utilized for the neurosphere assay (see above), and for the cytokine assay.

3.2.3 *CYTOKINE ARRAY*

The expression of cytokines, chemokines and acute-phase inflammatory proteins was determined in neurosphere media and in the conditioned media from astrocytic cultures by means of the Proteome Profiler™ Rat Cytokine Array Panel A (R&D Systems Europe, Abingdon, UK). The array consists of nitrocellulose membranes spotted with antibodies directed against the following 29 proteins (alternative names are shown in parenthesis): CINC-1, CINC-2 α/β, CINC-3, CNTF, Fractalkine, GM-CSF, sICAM-1 (CD54), IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-6, IL-10, IL-13, IL-17, IP-10 (CXCL10), LIX, L-Selectin, MIG (CXCL9), MIP-1α (CCL3), MIP-3α (CCL20), RANTES (CCL5), Thymus Chemokine (CXCL7), TIMP-1, TNF-α, VEGF. The test was performed according to manufacturer's instructions. Briefly, aliquots of the various culture media were mixed with a cocktail of biotinylated detection antibodies, and then incubated overnight at 4°C on separate nitrocellulose membranes included in the array. This allowed the binding of any cytokine/detection antibody complex to its cognate immobilized capture antibody on the membrane. Following a wash to remove unbound material, streptavidin-HRP solution was added to the membrane for 30 min at room temperature. Detection of bound complexes was then performed by ECL (GE Healthcare, Milan, Italy) and autoradiography. The integrated optical density of each spot on the array was quantified by the Image J software.

3.3 ANIMALS, SURGICAL PROCEDURES, AND IN VIVO TREATMENTS

In vivo experiments were performed on wild type (wt) C57BL/6 and on GLAST::CreErt2;Rosa-YFP (Mori et al., 2006; Rolando et al., 2012) adult mice (2-4 months of age). The experimental plan was designed according to the guidelines of the NIH, the European Community Council (86/609/EEC), and the Italian laws for care and use of experimental animals (DL116/92). It was also approved by the Italian Ministry of Health and the Bioethical Committee of the University of Turin. Surgical procedures and perfusions were carried out under deep general anesthesia (ketamine, 100 mg/kg; Ketavet, Bayern, Leverkusen, Germany; xylazine, 5 mg/kg; Rompun; Bayer, Milan, Italy).

In the first set of experiments, ADPβS was chronically infused in the lateral ventricle of adult C57BL/6 mice for one week by osmotic minipumps. Osmotic minipumps (Alzet osmotic pumps 1007D) were implanted into the left cerebral ventricle (coordinates relative to bregma: anterior, 0; lateral, 1 mm; depth, 1.8 mm) to deliver ADPβS (100 µM in phosphate buffered saline, PBS; Sigma-Aldrich) or vehicle. The concentration of ADPβS was chosen based on literature data, to reproduce the massive release of eNTs that is observed following traumatic or ischemic brain injuries (Franke et al., 2012; Melani et al., 2005). To analyze cell proliferation, animals received two i.p./day injections of the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU, Sigma Aldrich; 50 mg/kg in saline/day) on the last 3 days of ADPβS treatment (**Figure 3.4**).

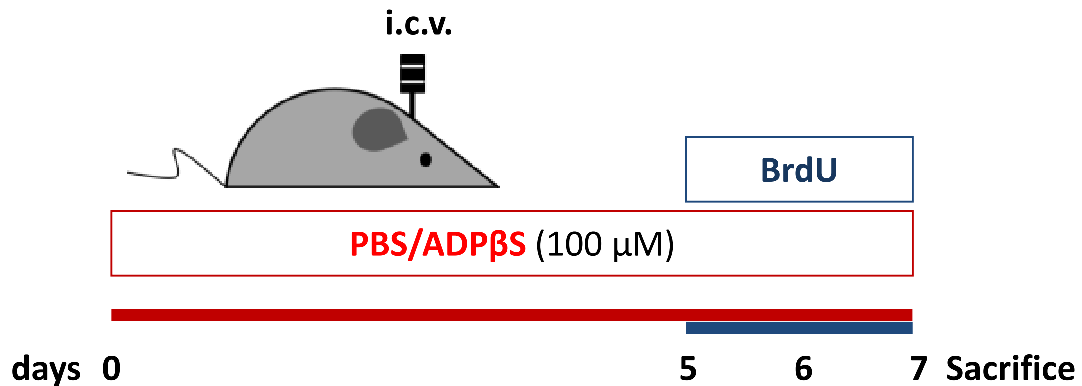


Figure 3.4: experimental design. *ADPβS or PBS were administered i.c.v. by minipumps to 8 weeks-old mice for one week. To primarily monitor the proliferation of transit amplifying cells, animals also received two pulses of BrdU per day on the last three days of agonist administration.*

To test whether ADPβS was acting directly on stem cells we took advantages of the GLAST::CreErt2;Rosa-YFP transgenic mice model. In these mice all the cell expressing the stem cells marker GLAST (astrocyte specific glutamate/aspartate transporter) and their progeny become fluorescence upon tamoxifen administration. Animals received tamoxifen (Tam in **Figure 3.5**) dissolved in corn oil to induce Cre activity and YFP reporter expression (one administration of 5 mg each by oral gavage for two days) before starting the pharmacological treatment with the ADP analogue. ADPβS was again chronically infused in the lateral ventricle of adult mice for one week by osmotic minipumps. In this case, we restricted the analysis to a small pool of cycling cells by injecting animals with another marker of cells proliferation, 5-ethynyl-2'-deoxyuridine (EdU, Life Technologies; 50 mg/kg in saline i.p; Ponti et al., 2013) 2 hours before sacrifice (**Figure 3.5**). In these experiments, EdU was chosen instead of

BrdU since its visualization follows a one-step procedure without DNA denaturation, and was therefore more convenient for triple immunostaining (see below).

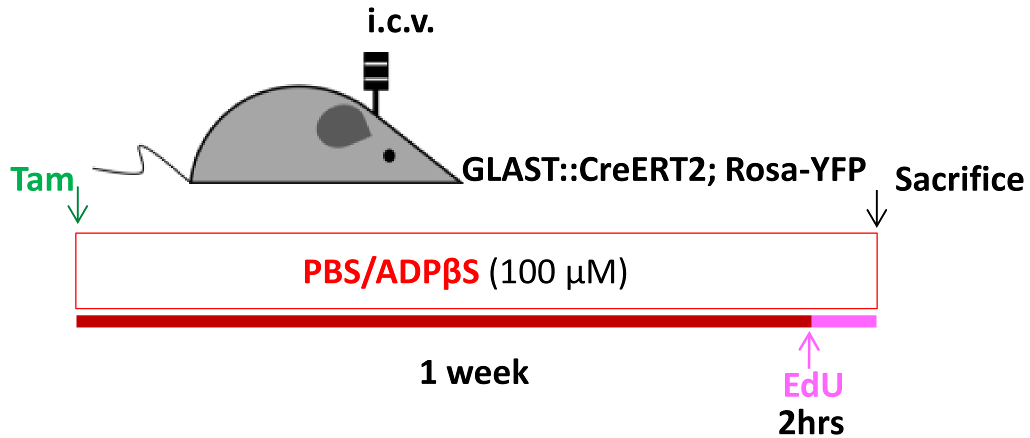


Figure 3.5: experimental design. *ADPβS or PBS were chronically infused in the lateral ventricles of GLAST::CreERT2;Rosa-YFP transgenic mice for one week via osmotic minipumps. Animals were also treated with EdU two hours before sacrifice to stain rapidly dividing cells.*

3.3.1 Immunohistochemistry, image processing and data analysis

For histological analysis, animals were anaesthetized (see above) and transcardially perfused with 4% paraformaldehyde in PBS. Brains were collected, postfixed overnight, cryoprotected, cut coronally in 30 µm-thick slices, and stained according to standard protocols (Rolando et al., 2012). Incubation with primary antibodies (anti-GFAP, 1:1,000, dako, Milan, Italy; anti-doublecortin, DCX, 1:400, Santa Cruz Biotechnologies, Heidelberg, Germany; anti-GFP, 1:700, Life Technologies; anti-BrdU, 1:250, Abcam, Cambridge, UK; anti-Mash1, 1:200, BD Pharmingen Milan, Italy) was performed overnight at 4°C in PBS with 1.5% normal serum and 0.25% Triton-X 100. In the case of BrdU staining, slices were previously treated with 2N HCl for 20 min at 37°C, followed by 10 min in 0.1 M borate buffer (pH 8.5). Sections were then exposed (2 hours, RT) to secondary specie-specific antibodies (all at 1:500 dilution) conjugated to Alexa Fluor® 488, 546, 649 (Life Technologies) or to Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA). Click-It™ EdU Cell Proliferation Assay Kit (Life Technologies) was utilized to detect EdU incorporation, according to the manifacturers' instructions. Nuclei were counterstained with the Hoechst33258 dye (1:10,000 in PBS; 20' at RT; Life Technologies, Milan, Italy). Stained sections were mounted on microscope slides with Tris-glycerol supplemented

with 10% Mowiol (Calbiochem, La Jolla, CA), and analyzed either by an E-800 Nikon microscope (Nikon, Melville, NY) equipped with a color CCD Camera or by a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany). Quantifications were performed by the ImageJ (Research Service Branch, National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/) or the Neurolucida softwares (MicroBrightfield, Colchester, VT). For the evaluation of the number of positive cells for the different markers, comparisons were made between hemispheres ipsilateral to the infusion site of control (Ctr) and treated animals (unless differently stated); data are derived from a minimum of 6 up to 23 sections from 3-4 animals/experimental condition and/or time point. In the Results section and in the Legends to figures, "n" refers to the number of animals analyzed. Results are expressed as either the absolute number of positive cells/section or per lateral wall length (which was similar in the analyzed samples: Ctr mice, 283.62±3.84 µm vs. ADPβS-treated animals, 295.93±6.20 µm), as indicated in the figure legends and in the Results section.

3.4 IN VITRO GENERATION OF PURE OLIGODENDROCYTE CULTURES

OPCs were isolated from mixed glial cultures (see 3.2.1) by the shaking method followed by an immunopurification protocol (Mayer-Pröschel, 2001). This second part consists in a negative selection procedure to remove undesired cells (e.g., astrocytes, meningeal cells, type 1 astrocytes and microglia/macrophages) using the Ran-2 antibody (Bartlett et al., 1980).

Tissue culture-grade plastic was not directly coated with cell surface–specific antibodies used in immunopurification. Instead, a layer of anti-immunoglobulin antibodies was adsorbed to the dish the day before culture shaking, thereby improving the subsequent binding of cell-type specific antibody. In particular, 100µl of anti-IgG stock solution (1 mg/ml, MP Biomedicals, Santa Ana, CA) were diluted with 20 ml of 50 mM Tris HCl, pH 9.5. Two 100 mm tissue culture dishes were then coated with 10 ml of this antibody solution each and incubated overnight at 4°C, taking care to ensure sterility.

The following day, mixed cultures were shaken on an orbital shaker for 3-4 hours at 200 rpm. In the meantime the two IgG coated dishes were rinsed 3 times with PBS and the solution of RAN2 antibody (kindly provided by Prof. Carla Taveggia, Axo-Glia

Unit, Institute of Experimental Neurology Division of Neuroscience, San Raffaele Scientific Institute, Milan) was added (0.5 mL Ab + 6 mL of MEM + 1mg/ml BSA + HEPES 1N for each plate) and incubated at least for 3h at RT.

At the end of the shaking period, the medium containing the detached cells was collected from each flask in 50 ml sterile conical tubes and centrifuged for 10 min at 1,200 rpm. Pellets were resuspended in *NM15 Medium* (**Table 3.3**) + insulin (5µg/ml) and the cell suspension was incubated at room temperature in the first RAN2 antibody-precoated plate. After 20 min, the cells which did not attach to the plate were transferred to the second RAN-2-precoated plate and incubated for additional 20 min at RT. The supernatant was then collected and centrifuged at 1,200 rpm for 10 min. The obtained pellet was resuspended in the appropriate medium in accordance with the protocol applied subsequently (see 3.5).

OPCs were plated onto poly-D,L-ornithine coated 13-mm coverslips (final concentration 5µg/ml; Sigma-Aldrich) for immunocytochemistry.

Components	Final concentrations
MEM (Life technologies)	To volume
FBS (Euroclone)	15%
Glucose (Sigma)	6 mg/ml
Penicillin (Euroclone)	100 U/mL
Streptomicin (Euroclone)	100 µg/mL

Table 3.3: composition of NM15 Medium

3.5 NEUROGENIC PROTOCOLS

To test the ability of OPCs to generate neurons we tested two different protocols already published in literature and claimed to foster neurogenesis (Kondo and Raff, 2000a; Liu et al., 2007).

First of all we reproduced the 3-phases protocol published by Kondo and Raff (Kondo and Raff, 2000a), renamed here **neurogenic protocol #1.** According to this protocol, cells were initially maintained for 5 days in *DMEM Medium* (see **Table 3.2**) +10 ng/ml PDGF + B27 supplement (1:50) to induce OPCs proliferation (phase A). Cells were then shifted to *DMEM Medium* + 10 ng/ml PDGF + B27 supplement (1:50) + 15% FBS to promote their differentiation towards type 2 astrocytes and cultured for 3 days (phase B). Finally, cells were maintained for 5 additional days in *DMEM Medium* + B27 supplement (1:50) +10 ng/ml bFGF (phase C) to induce their differentiation to neurons (**Figure 3.6**).

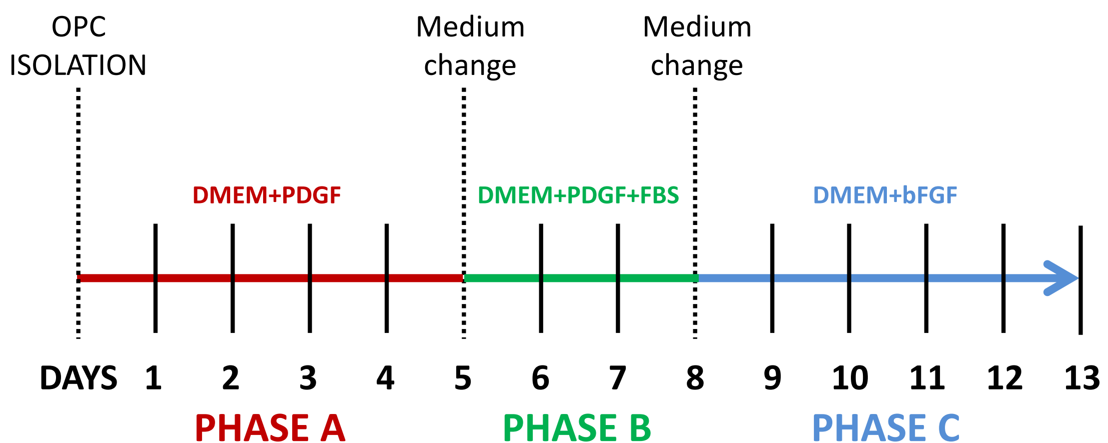


Figure 3.6: schematic representation of neurogenic protocol #1. *Cells were grown according to a 3-phase protocol already published by Kondo and Raff (Kondo and Raff, 2000a). See text for details.*

We then set up an additional successful neurogenic protocol, renamed here **neurogenic protocol #2** (**Figure 3.7**; Liu et al., 2007).

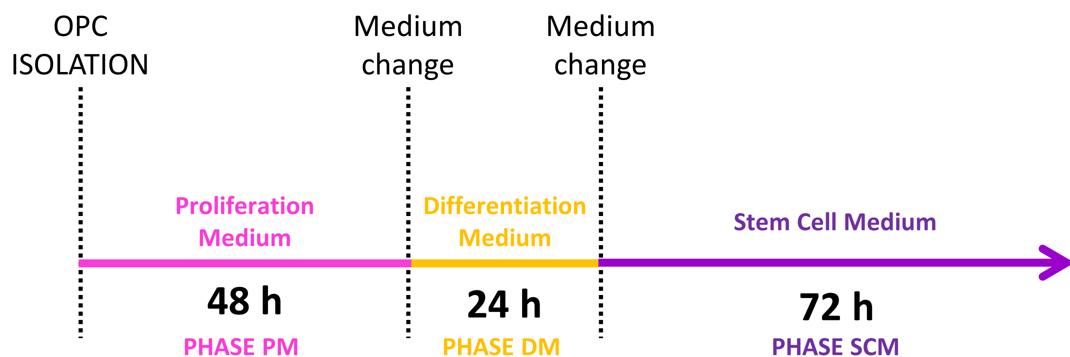


Figure 3.7: schematic representation of neurogenic protocol #2. *Cells were grown according to a second already published neurogenic protocol (Liu et al., 2007).*

In neurogenic protocol #2 cells were exposed for 2 days to *Proliferation Medium* (*DMEM Medium* + 10ng/ml PDGF + 10 ng/ml bFGF, phase PM) and then induced to differentiate into oligodendrocytes by removing the mitogens from the medium for 1 day (*Differentiation Medium, phase DM*). Finally cells were cultured in *Stem Cell Medium* (phase SCM, **Table 3.4**) for 3 days.

Components	Final concentrations
DMEM/F-12 (Life technologies)	To volume
Glutamine (Euroclone)	1 mM
Glucose (Sigma)	25 mM
FBS (Euroclone)	1%
B27 supplement (Life technologies)	1:50

Table 3.4: composition of SCM

In both protocol, cells were fixed with 4% paraformaldehyde and processed for immunocytochemistry (see 3.6) or whole-cell lysates were prepared and analyzed by western blotting (see 3.7) at various time points (see Results and Figures).

3.5.1 Pharmacological treatments

In either protocol, we have verified if and how the exposure to various pharmacological agents (including GPR17 receptor ligands) can modulate OPCs plasticity and their differentiation to neurons. In particular, we have utilized the non-selective GPR17 agonist UDP-glucose (10 μM) and antagonist Cangrelor (10 μM), in parallel to the anticonvulsant agent valproic acid (VPA, 500 μM) for the indicated time periods (see Results and Figures). All reagents were obtained from Sigma-Aldrich, except for Cangrelor that was a kind gift of The Medicines Company, Parsippany, NJ, USA.

3.6 IMMUNOCYTOCHEMISTRY, IMAGE PROCESSING AND DATA ANALYSIS

Immunocitochemical analyses were performed on either adherent neurospheres or cells cultured according to the two neurogenic protocols. In both cases cells were fixed at room temperature for 20 min in 0.1 M PBS (Euroclone) containing 0.12 M sucrose. Cells were subsequently incubated for 20 min at room temperature with Goat Serum Dilution Buffer (GSDB; 450 mM sodium chloride and 20 mM sodium phosphate buffer, pH 7.4, 15% goat serum, and 0.3% Triton X-100), before exposure to the primary antibodies diluted in GSDB. Rabbit anti-GFAP (1:600, Dako), mouse anti-βIII-tub (1:1,000; Promega, Milan, Italy), mouse anti-NG2 (1:200; Abcam) and anti-rabbit GPR17 (1:100; Cayman Chemical Company, Ann Arbor, MI) primary antibodies were used. After an overnight incubation at 4°C, cells were rinsed three times in a high salt buffer solution (500 mM sodium chloride and 20 mM sodium phosphate buffer, pH 7.4) for 10 min, and then incubated (1 hour, room temperature) with secondary goat anti-rabbit and goat anti-mouse antibodies conjugated to AlexaFluor®488 or AlexaFluor®555 (1:600 in GSDB, 1h, RT; Life Technologies). Nuclei were then labelled with the fluorescent dye Hoechst-33258 (1:10,000 in PBS; Life Technologies). Cells were rinsed three times in high salt buffer, once in PBS, and finally once in 5 mM sodium phosphate buffer, pH 7.4. Coverslips were mounted in Dako Fluorescence Mounting Medium (Dako).

Adherent neurospheres were analyzed under a Zeiss Axiovert 8400 microscope (Carl Zeiss, Milan, Italy), equipped with a CCD camera module. To evaluate the intensity of staining, fluorescent images were captured from 10 randomly chosen optical fields/coverslip. Since a direct count of positive cells was technically not feasible, due to their high number and to the low fluorescence signal at the 10x magnification (which better allowed to gain a general view of the effect exerted by the pharmacological treatment), we decided to perform a densitometric analysis of Hoechst33258, GFAP and βIII-tub staining, after splitting the three fluorescence channels and converting the colored fluorescent signals to grayscale values. The mean gray value for each optical field was then evaluated by the ImageJ software. Results represent the mean±S.E.M. of data from 3 coverslips deriving from 2 independent experiments.

Instead, cells subjected to neurogenic protocols #1 and #2 were analyzed by using a fluorescent microscope (Zeiss). In each coverslip, the total number of cells, evaluated by nuclear staining Hoechst dye, and number of GPR17-, βIII-tubulin, GFAP- or NG2-positive cells were counted under a 40x magnification in 20 randomly chosen optical fields.

3.7 WESTERN-BLOTTING ANALYSIS

Whole-cell lysates were prepared and analyzed by Western blotting as previously described (Bianco et al., 2005). Briefly, approximately 30 μg aliquots from each protein sample were loaded on 11% sodium-dodecylsulphate polyacrylamide gels, and blotted onto nitrocellulose or PVDF membranes (Bio-Rad Laboratories, Milan, Italy). Membranes were then saturated with 10% non-fat dry milk in Tris-buffered saline (TBS; 1 mM Tris-HCl, 15 mM NaCl, pH 8) for 1 hour at RT, and incubated overnight at 4°C with mouse anti-synaptic vesicle2 (SV2, 1:2,000) and anti-βIII-tub (1:1,000, Promega) or rabbit anti-synaptotagmin (Syt, 1:2,000; SV2 and Syt are a kind gift of Dr. Claudia Verderio, CNR, Milan) and anti-GPR17 primary antibodies (1:100 home-made monoclonal antibody) all in 5% non-fat dry milk in TBS. Membranes were then washed in TBS-T (TBS plus 0.1% Tween20®), incubated for 1 h with goat anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase (1:4,000 or 1:2,000 in 5% non-fat dry milk in TBS respectively; Sigma-Aldrich). Detection of proteins was performed by enhanced chemiluminescence (ECL, Amersham Biosciences, Milan, Italy) and autoradiography. Non-specific reactions were evaluated by in the presence of the secondary antibodies alone

3.8 STATISTICAL ANALYSIS

Data were analyzed using the GraphPad Prism5 software. Differences between experimental conditions were analyzed using either unpaired, two-tails Student's t test, or one-way ANOVA followed by the Bonferroni correction. P value <0.05 was considered as significant.

4. RESULTS

4.1 PURINES REGULATE ADULT BRAIN SUBVENTRICULAR ZONE CELL FUNCTIONS: CONTRIBUTION OF REACTIVE ASTROCYTES

4.1.1 THE $P2Y_1$ AGONIST ADPβS INCREASES THE PROLIFERATION OF SVZ PRECURSORS AND THEIR LINEAGE PROGRESSION IN VITRO

To examine the ability of non-selective $P2Y_{1,12,13}$ agonist ADPβS to modulate proliferation and multipotency of SVZ cells, we performed the neurosphere assay, in the presence of standard concentrations of both bFGF and EGF (20 ng/ml each; see Materials and Methods, Paragraph 3.1). After 7 days in vitro (DIV), the number and size of primary neurospheres were analyzed. Exposure to 50 µM ADPβS increased the neurosphere forming capacity of SVZ cells, as indicated by their increased number (**Figure 4.1A, A', B**). Yet, ADPβS stimulation determined the generation of neurospheres with a reduced size compared to controls (**Figure 4.1A, A', C**). Furthermore, the total number of cells yielded after the dissociation of neurospheres at 7 DIV decreased upon treatment with the ADP analogue (1,476,562±271,023 in control cultures vs. 686,094±168,364 after exposure to ADPβS; $p<0.05$ Student's t test). Co-exposure to the selective $P2Y_1$ antagonist MRS2179 (50 µM) completely abrogated ADPβS-induced effects on both neurosphere forming capacity and proliferation (**Figure 4.1A'', B, C**), thus demonstrating that the ADP analogue is selectively activating the $P2Y_1$ receptor ($P2Y_1R$) subtype.

These results are in agreement with literature data suggesting a primary role for $P2Y_1R$ in mediating the effects exerted by extracellular nucleotides (eNTs) on SVZ precursor cells (Suyama et al., 2012). Moreover they are also in line with already published data describing an antiproliferative effect of various eNTs, including ADPβS at standard GF concentrations (Mishra et al., 2006; Stafford et al., 2007).

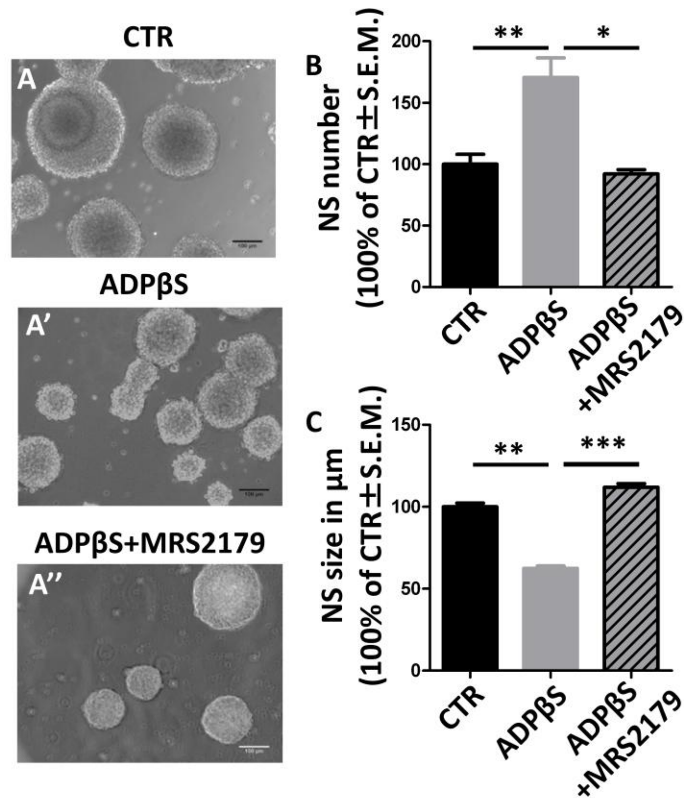


Figure 4.1: ADPβS modulates neurosphere formation from the SVZ in vitro. *(A-A'') Representative micrographs showing 7-day-old primary neurospheres generated under control condition (A), in the presence of ADPβS (50µM) alone (A') or in combination with MRS2179 (50µM) (A''). Quantification of the number (B) and size (C) of primary neurospheres generated under the various experimental conditions (3 replicates from 2 independent experiments; *p<0.05, **p<0.01 and ***p<0.001, One-way ANOVA followed by Tukey post hoc analysis). Scale bars: 100 µM.*

Thus, to assess the effects of $P2Y_1R$ activation in the absence of GFs and to examine the outcome of ADPβS exposure on the lineage progression of SVZ cells, 7-day-old control secondary neurospheres were plated without dissociation and grown for 7 additional days in a medium without GFs (see Materials and Methods, Paragraph 3.1.2), in the absence or presence of ADPβS. The expression of the neuronal and astrocytic marker βIII-tubulin (βIII-tub) and GFAP, respectively, and the mean area of adhering neurospheres were evaluated by immunocytochemistry, in parallel with the staining of cell nuclei by the Hoechst33258 dye (**Figure 4.2A, A'**). Exposure to 50 µM ADPβS led to a moderate, but significant increase in neurosphere area (121.11±17.38% of control neurosphere area, set to 100.00±12.89%; 19-21 optical fields from 3 coverslips/condition; p<0.05, Student's t test). A much higher increase in the mean fluorescence value for Hoechst33258 staining was detected after exposure to the purine analogue (**Figure 4.2B, B', E**), meaning a higher number of cell nuclei per area.

Interestingly, immunoreactivity for both GFAP and βIII-tub clearly increased upon exposure to ADPβS (**Figure 4.2C-D’**). While the increase in GFAP immunoreactivity was proportional to the increased cell number (compare **Figure 4.2E** and **F**), βIII-tub staining in ADPβS-treated cultures exceeded the labeling in control condition by two-fold (**Figure 4.2G**), indicating a prominent effect of ADPβS on neuroblast production.

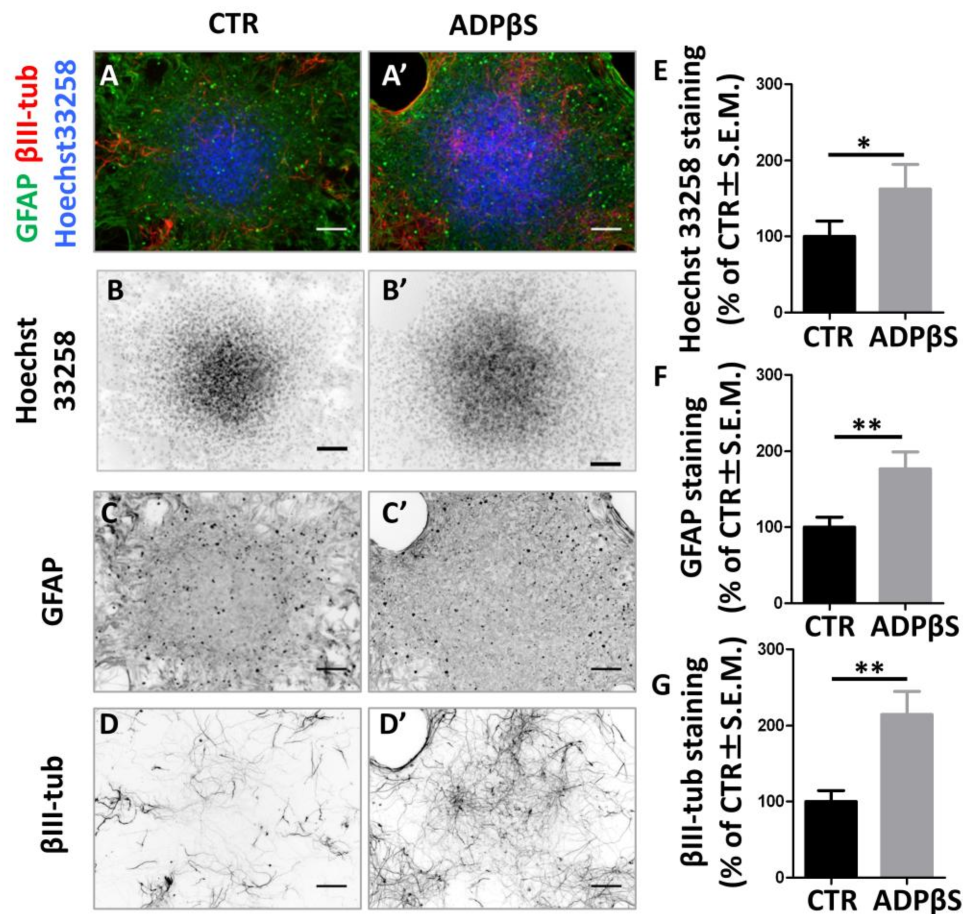


Figure 4.2: ADPβS stimulates neurosphere differentiation and proliferation. *(A, A’) Immunofluorescence staining for βIII-tub (red) and GFAP (green) of adherent undissociated neurospheres (NS) cultured in differentiating conditions in control medium (A) or in the presence of ADPβS (A’). Nuclei were counterstained with the Hoechst33258 dye (blue). (B-D’) The green, red, and blue fluorescent channels in A and A’ were separated and converted to greyscale for densitometric analysis, shown in (E) for Hoechst33258, in (F) for GFAP and in (G) for βIII-tub. Data are shown as the mean percentage±S.E.M. of CTR values set to 100% (19-21 optical fields from at least 3 coverslips/condition, *p<0.05, **p<0.01, unpaired Student's t test). Scale bars: 100 µm.*

Therefore, in vitro results indicate that ADPβS promotes neurosphere formation and stimulates the generation of neurons and astrocytes.

4.1.2 *ASTROCYTES EXPOSED TO ADPβS INFLUENCE THE GENERATION OF NEUROSPHERES FROM SVZ CELLS IN VITRO*

Solid evidence demonstrates that exposure of astrocytes to nucleotides in general, and to ADPβS in particular, induces reactive astrogliosis (Abbracchio and Ceruti, 2006; Franke et al., 2012). Reactive astrocytes release several mediators (see Introduction, Paragraph 1.5.1) which could influence the functionality of surrounding cells, such as SVZ progenitors. To unveil the possible effects of ADPβS-activated astroglia on neural progenitors, we assessed whether astrocyte conditioned media could modify neurosphere formation from SVZ cells. To this aim, we exposed primary astrocytic cultures to conventional neurosphere medium with or without 50 μM ADPβS (see Materials and Methods, Paragraph 3.2.2 for experimental design). Both culture media were then collected after 3 DIV, and utilized to generate neurospheres, as described above (**Figure 4.34**). When SVZ progenitors were grown in medium from Control (Astro ctr) or ADPβS-treated astrocytes (Astro ADPβS), no differences were found in either the number (17.67±2.56 in Astro ctr vs. 14.00±3.36 in Astro ADPβS medium, $p>0.05$ Student's t test) or the size of primary neurospheres at 7 DIV (**Figure 4.3B**). To exclude that the Astro ADPβS medium contained: i) residual ADPβS, or ii) adenine nucleotides released by activated astrocytes, which could influence SVZ cell properties, the $P2Y_1$ antagonist MRS2179 was added to Astro ADPβS medium at the time of neurosphere generation. No significant differences were found in the neurospheres formed in Astro ADPβS medium or in Astro ADPβS + MRS2179 (**Figure 4.3B**), thus ruling out a role eNTs in the conditioned medium from reactive astrocytes.

Interestingly, despite the generation of a similar number of primary neurospheres in Astro ctr and Astro ADPβS compared to standard (formerly indicated as ctr) neurosphere media (not shown), a significantly lower number of cells was yielded from their dissociation. In fact, the total number of cells was 1,476,563±271,023 from ctr neurospheres vs. 157,400±36,603 and 132,500±46,199 from neurospheres grown in Astro ctr and Astro ADPβS media, respectively ($p<0.001$ ctr vs. Astro ctr and ctr vs. Astro ADPβS, Student's t test). Of further interest, these numbers were also lower than those obtained with exposure to ADPβS alone (686,094±168,364; $p<0.05$ ADPβS vs. Astro ctr and ADPβS vs. Astro ADPβS, Student's t test), suggesting a specific negative astrocyte-mediated effect on precursor proliferation.

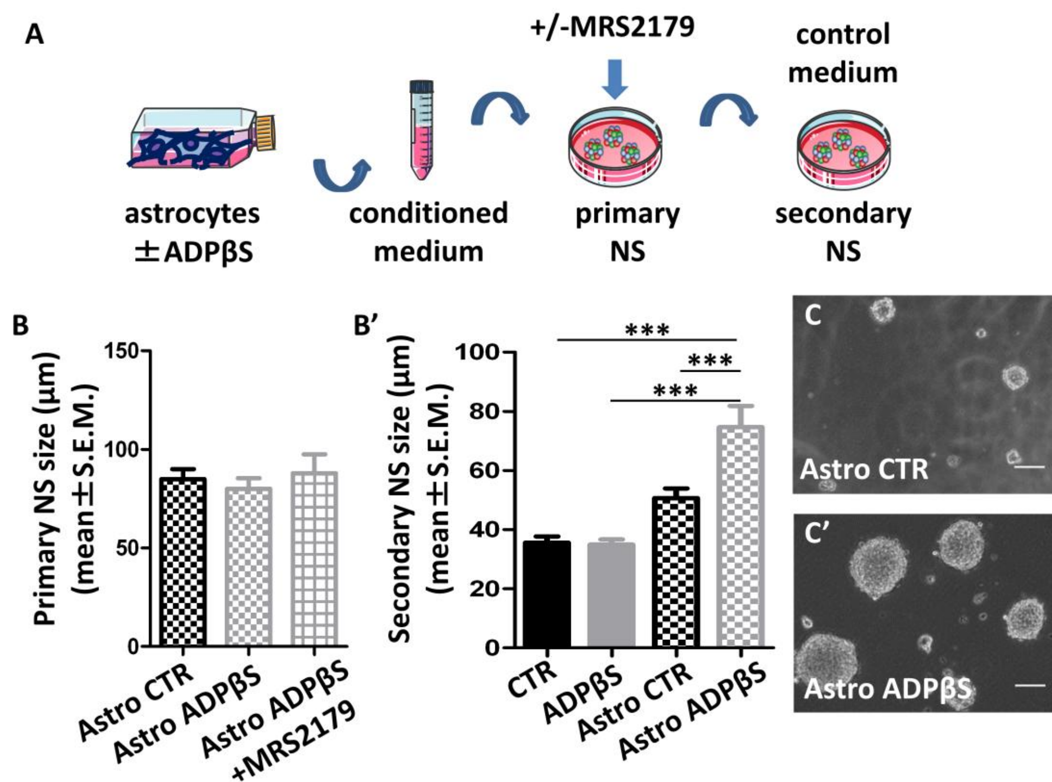


Figure 4.3: ADPβS-treated astrocytes regulate the generation of secondary neurospheres from SVZ precursors in vitro. *(A) Schematic representation of the experimental design (see text for details). (B) Evaluation of the size of primary neurospheres (NS) generated in the conditioned medium from CTR or ADPβS-treated astrocytes. In selected experiments, the $P2Y_1R$ antagonist MRS2179 was added to Astro ADPβS medium at the time of seeding of SVZ cells. (B') Evaluation of the size of secondary neurospheres generated in fresh control medium and derived from cells obtained by the dissociation of primary neurospheres grown under the various experimental conditions (5 coverslips from 2 independent experiments; ***$p<0.001$, One-way ANOVA followed by Tukey post hoc analysis). Representative micrographs are shown in C-C'. Scale bars: 100 µm. The cartoon was produced thanks to "Servier Medical Art" (www.servier.com).*

Primary neurospheres were then dissociated and replated in ctr neurosphere medium, irrespectively of their initial growing condition, to generate secondary neurospheres (**Figure 4.3A**). Surprisingly, fully formed secondary neurospheres were already generated at 2 DIV from cells derived from primary neurospheres grown in Astro ADPβS medium (**Figure 4.3B', C'**). Conversely, very small cell clusters were visible in cultures derived from primary neurospheres grown in Astro ctr medium (**Figure 4.3B'**, compare **Figure 4.3C** with **C'**) or in ctr and ADPβS medium (**Figure 4.3B'**). Numbers of neurospheres were instead similar in all the tested conditions (not shown).

These findings suggest that ADPβS specifically acts on astrocytes by promoting the release of mediator(s) capable to "prime" SVZ precursors inducing them to proliferate. However, this proliferative effect is possibly concomitantly inhibited by other astrocyte-derived factor(s), so that only after their removal from the culture medium the proliferation of SVZ cells is intensively boosted.

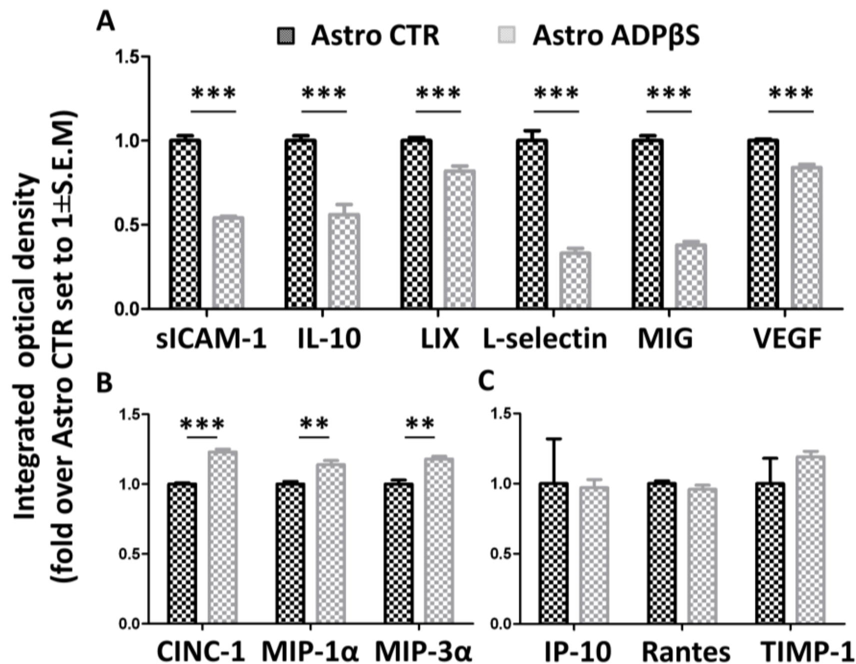


Figure 4.4: ADPβS modulates the release of various signaling molecules from astrocytes in vitro. *Relative expression of various signaling molecules in conditioned medium derived from control or ADPβS-treated astrocytes, as determined by an antibody array (see Methods for details). A and B show molecules whose extracellular concentration decreased or increased after ADPβS exposure, respectively. C shows unchanged molecules. Results are the mean of 2 independent analyses. (**$p<0.01$, ***$p<0.001$, unpaired Student's t test).*

To identify possible mediators released in the astrocyte conditioned medium and responsible for the above observed effects, we took advantage of the Proteome Profiler^{TM} Antibody Array. This approach allows the simultaneous detection of 29 cytokines and chemokines in the tested culture media (see Materials and Methods Paragraph 3.2.3). Interestingly, in control neurosphere medium (see Materials and Methods, Tab 3.1, for the medium composition) and in the neurosphere medium added with ADPβS, the concentrations of all the analyzed molecules were below the detection limit of the array (not shown). However, 12 out of 29 molecules were detected in both Astro ctr and Astro ADPβS media (**Figure 4.4**). Indeed, exposure to the nucleotide

analogue either significantly reduced or increased the astrocytic release of the majority of these mediators (**Figure 4.4A, B**). Only IP-10, Rantes and TIMP-1 concentrations did not change between the two experimental conditions (**Figure 4.4C**).

CINC-2α/β, CINC-3, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-6, IL-13, IL-17, CNTF, GM-CSF, TNF-α, Fractalkine, Thymus Chemokine and IFN-γ were not detected in either conditioned media.

Thus, a combination of various secreted mediators, whose release by astrocytes is modulated by ADPβS stimulation, might account for the observed effects of ADPβS conditioned medium on SVZ cell properties.

4.1.3 ADPβS ACTIVATES BOTH NICHE AND PARENCHYMAL ASTROCYTES AND INCREASES THE PROLIFERATION OF SVZ PRECURSORS IN VIVO

Next we aimed at translating our *in vitro* data to an *in vivo* setting, and at addressing the role of $P2Y_1R$ in the regulation of adult neurogenesis in conditions mimicking those observed after brain injury. Thus, ADPβS (100 μM) was chronically infused for one week into the cerebral ventricles of wildtype mice and cell proliferation was monitored by BrdU administration during the last 3 days (see Materials and Methods, Paragraph 3.3). Incorporation of the thymidine analogue was then evaluated by immunohistochemistry together with the expression of cell specific markers for SVZ populations and parenchymal astrocytes (i.e., GFAP for astrocytes and Type B precursor cells, Mash1 for Type C transit-amplifying cells, and doublecortin, DCX, for Type A neuroblasts).

Notably, ADPβS infusion caused a significant up-regulation of GFAP in both parenchymal astrocytes (arrows in **Figure 4.5A’, B’** and insets **A’’, B’’**), thus confirming our *in vitro* and literature data demonstrating a role for eNTs in reactive astrogliosis (Franke et al., 2012), and in the neurogenic areas of the dorsal horn (DH) and lateral wall (LW) (**Figure 4.5A-A’** and **B-B’**; quantification in **C** and **D**, respectively), where GFAP also labels precursor cells. In parallel, a significant expansion of DH area (**Figure 4.5E**) and a thickening of the LW (**Figure 4.5F**) were observed, confirming a specific effect of ADPβS on $GFAP^+$ type B cells (i.e., stem cells and niche astrocytes).

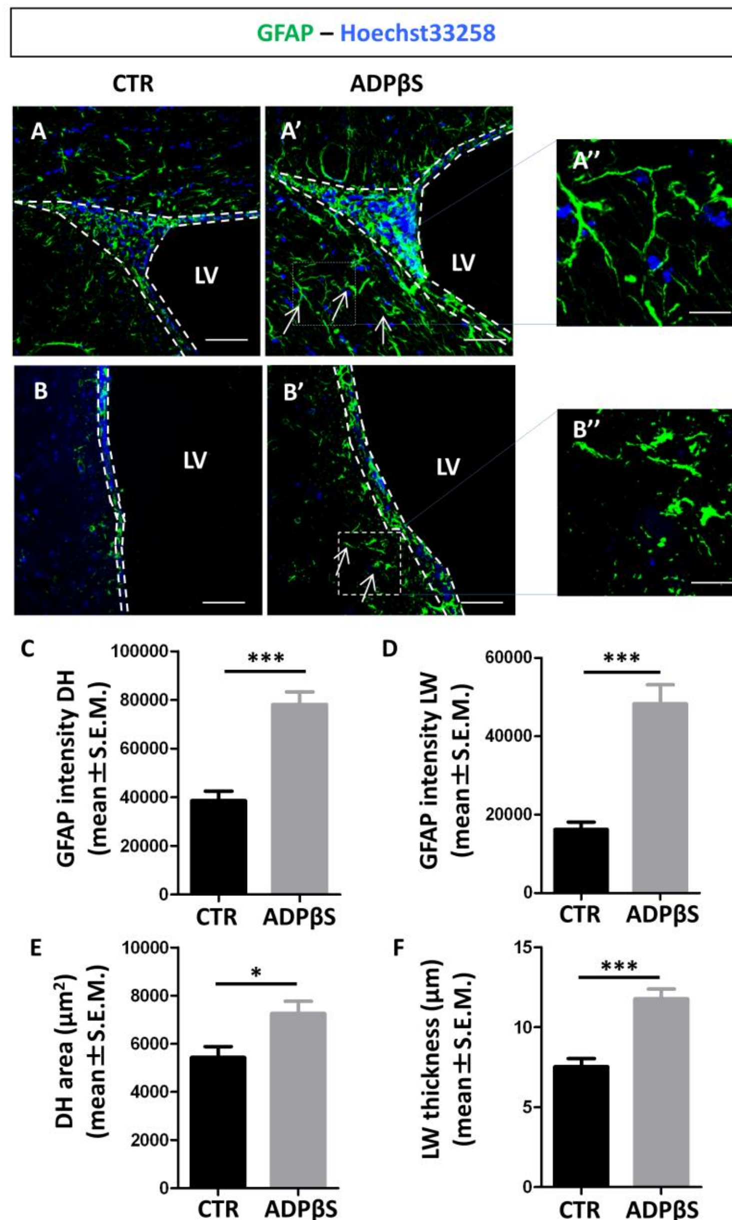


Figure 4.5: a ***7-day-long infusion of ADPβS increases GFAP immunostaining in the SVZ and induces reactive astrogliosis in brain parenchyma.*** *(A-B') Representative images of GFAP staining (green) in the dorsal horn (DH) and lateral wall (LW) of control (A-B) and ADPβS-treated animals (A'-B'). Significant reactive astrogliosis induced by the nucleotide analogue was evident in brain parenchyma (arrows in A', B' and insets A'', B''). (C, D) Quantification of GFAP immunostaining in the DH (C) and LW (D). Values represent the mean fluorescence intensity of GFAP staining/section in arbitrary units. (E, F) Quantification of the DH area (F) and of LW thickness (G), as delimited by the dashed lines in (A, A') and (B, B'), respectively. (*p<0.05, ***p<0.001, Student's t test). Scale bars: 50 μm in A-B' and 150 μm in A'', B''. LV: lateral ventricle.*

In line with these observations, exposure to ADPβS promoted cell proliferation in the SVZ. A significant increase in BrdU incorporation was in fact detected in parallel in

the LW (**Figure 4.6A-B**). Namely, we observed an expansion of Mash1^{+} type C transit-amplifying cell population (**Figure 4.6C-D**), which also incorporated more BrdU (**Figure 4.6E**).

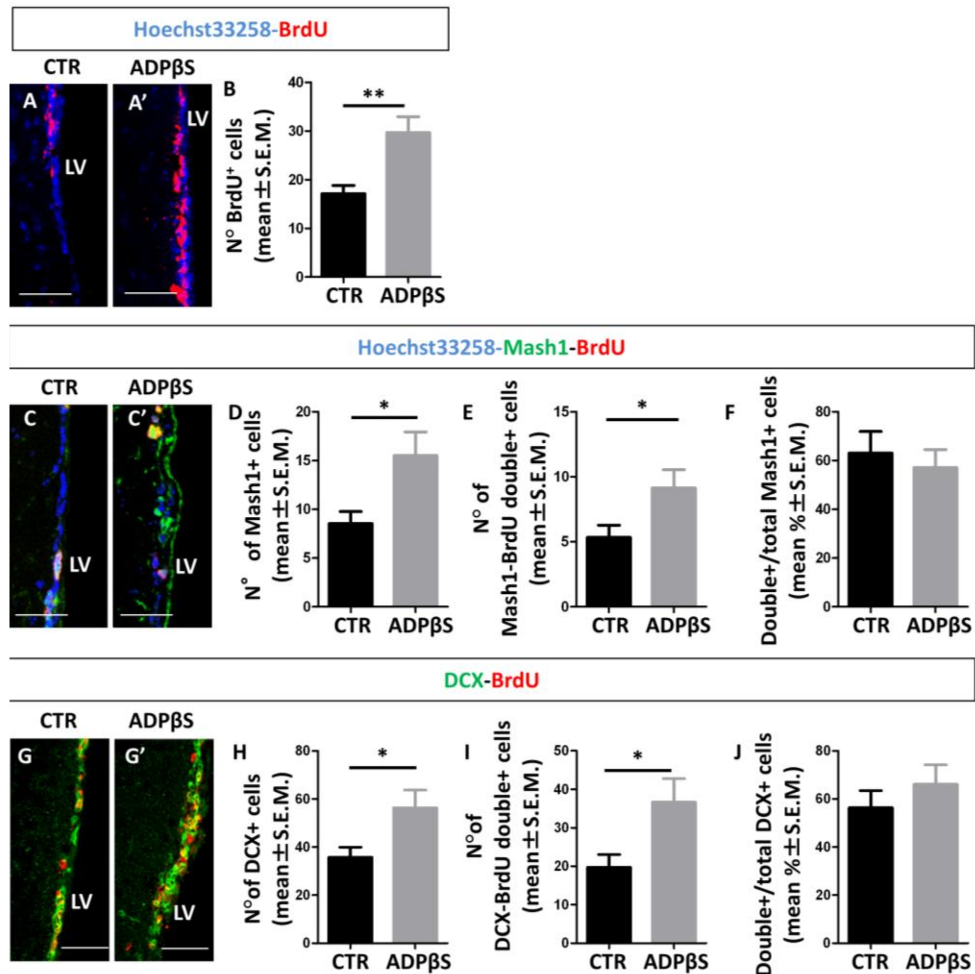


Figure 4.6: a 7 day-long infusion of ADPβS stimulates the proliferation of SVZ cells and increases the number of Mash1^{+}, and DCX^{+} cells. *(A, A') Representative images of the LW from control (A) or ADPβS-treated animals (A') immunelabelled for BrdU (red). (B) Quantification of the total number of BrdU^{+} cells in the LW. (C, C') Representative images of the LW from ctr (C) or ADPβS-treated animals (C') immunelabelled with anti-Mash1 (green) and anti-BrdU (red). (D-F) Quantification of Mash1^{+} (D), of Mash1-BrdU double-positive cells (E), and of the percentage of proliferating cells in the Mash1^{+} population (F). (G, G') Immunofluorescence images of DCX (green) and BrdU (red) staining in the LW of ctr (G) or ADPβS-treated animals (G'). (H-J) Quantification of the total number of DCX^{+} (H), of DCX-BrdU double-positive cells (I), and of the percentage of proliferating cells in the DCX^{+} population (J) (*$p<0.05$, **$p<0.01$, ***$p<0.01$) unpaired Student's t test). Scale bars: 50 µm. In all images nuclei were counterstained with the Hoechst33258 dye (blue). LV: lateral ventricle.*

Moreover, the total number of DCX^{+} neuroblasts and the fraction of DCX/BrdU double-positive cells also significantly increased after ADPβS exposure (**Figure 4.6G-**

I). However, in either cell populations the percentage of proliferating cells (i.e., the number of cells double-positive for both BrdU and the cell population specific marker/the total number of cells for each cell population*100) was not increased by the nucleotide analog (**Figure 4.6F, J**). This suggests that ADPβS does not enhance the proliferation of either one (or both) cell populations, but rather acts by stimulating the proliferation of their parent precursors or by globally overactivating all SVZ populations. Similar results were obtained in the DH (not shown).

4.1.4 ADPβS PROMOTES THE GENERATION OF A PROGENY OF RAPIDLY DIVIDING CELLS FROM GLAST-EXPRESSING STEM CELLS.

Next, to verify whether ADPβS can foster the transition of stem-like precursors towards transit-amplifying cells and neuroblasts, we took advantage of GLAST::CreERT2;Rosa-YFP transgenic mice. In these animals, cells expressing the L-glutamate/L-aspartate GLAST (i.e., SVZ type B cells and parenchymal astrocytes) and their progeny are permanently labelled by the fluorescent protein YFP upon Tamoxifen administration. Animals chronically received either PBS or 100 μM ADPβS in the lateral ventricle for one week (see Materials and Methods, Paragraph 3.3). Two hours before sacrifice, animals received a single administration of the thymidine analogue EdU to label actively dividing cells. In line with previous data, we detected an increased incorporation of EdU upon exposure to the purine analog (**Figure 4.7A-B**). Moreover, the number of YFP/EdU double-positive cells increased by twofold (**Figure 4.7C**), indicating that ADPβS treatment expanded a population of $GLAST^+$ cell-derived progenitors in active proliferation. Although this population was for the most composed of GFAP-negative progenitors (**Figure 4.7D**), likely including $Mash1^+$ and DCX^+ cells (see above), exposure to ADPβS also resulted in a 2-fold increase of actively cycling YFP/EdU double-positive progenitors also expressing GFAP along the ventricle (**Figure 4.7E-F**). Furthermore, a small but significant increase in the total number of YFP/GFAP double-positive cells was detected after treatment (**Figure 4.7G**). These data indicate that ADPβS activates $GFAP^+$ precursors in the SVZ with a consequent expansion of their progeny.

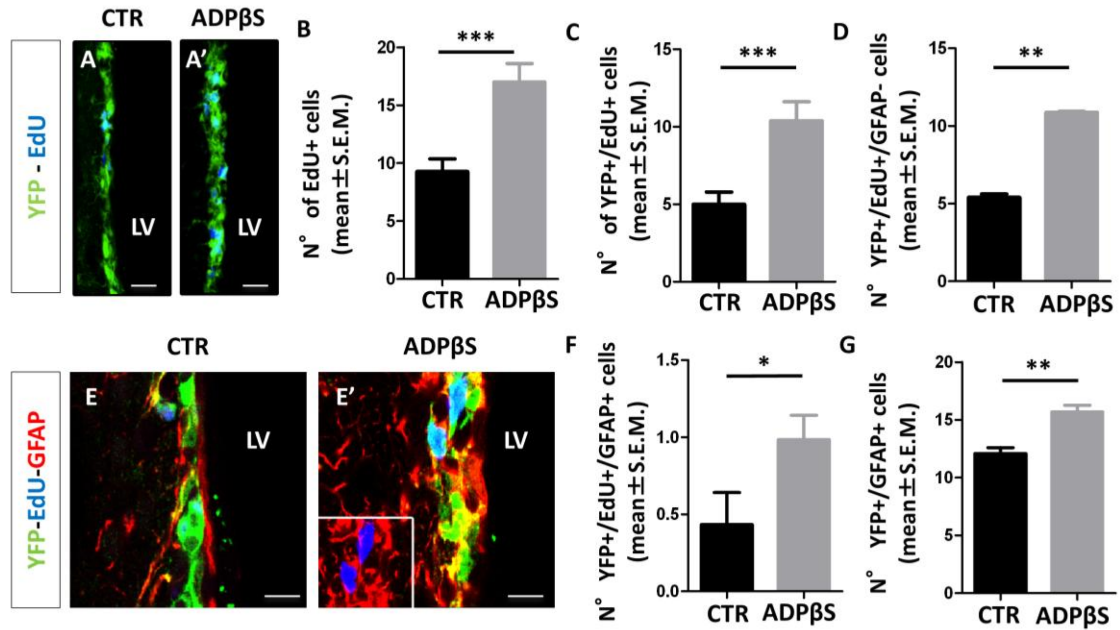


Figure 4.7: ADPβS promotes the proliferation of rapidly dividing transit-amplifying cells and GFAP^{+} precursor cells. *(A, A') Immunofluorescence images of EdU incorporation (blue) and YFP (green) staining in the LW of control (A) and ADPβS-treated GLAST::CreERT2;Rosa-YFP animals (A'). (B-C) Quantification of the number of LW cells incorporating EdU (B) and of EdU-YFP double-positive cells (C). (D) Quantification of YFP-EdU double-positive cells, which do not express GFAP. (E, E') Triple immunostaining (i.e., YFP, green; EdU, blue; GFAP, red) in the LW of ctr (E) and ADPβS-treated animals (E'). Inset shows in detail GFAP/EdU double staining in the triple labelled cells placed at the upper right corner of E'. (F, G) Quantification of YFP-EdU double-positive cells also expressing GFAP (F), and of GFAP-YFP double-positive cells (G) (*$p<0.05$,**$p<0.01$ and ***$p<0.001$; unpaired Student's t test). Scale bars: 15 µm in A-A' and 10 µm in E-E'. LV: lateral ventricle.*

4.2 IMPLEMENTATION OF THE STEM CELL PROPERTIES OF NG2-EXPRESSING NEURAL PRECURSOR CELLS BY PURINERGIC SIGNALING

It is well known that $NG2^+$ precursor cells (also known as “oligodendrocyte precursors cells”, OPCs) can spontaneously differentiate to mature oligodendrocytes both in vitro and in vivo; however, when these cells are cultured upon specific experimental conditions, they also reveal a stem cell potential since they can generate both astrocytes and neurons (for details see Paragraph 1.5.2.1). To test their ability to generate neurons, we isolated OPCs from mixed glial cell cultures from postnatal day 2 rat cortex and grew them according to two already published protocols, renamed here **neurogenic protocol #1** and **neurogenic protocol #2** (Kondo and Raff, 2000a; Liu et al., 2007) (**Figure 4.8**).

In the neurogenic protocol #1, cells were cultured according to a protocol published in 2000 by Kondo and Raff (Kondo and Raff, 2000b). In particular, OPCs were initially maintained in a proliferative medium for 5 days (phase A), then 1% of FBS was added to the culture medium for 3 additional days to induce cells to de-differentiate into a more permissive precursor stage (phase B). Finally, cells were maintained in culture for 5 days in DMEM+10 ng/ml bFGF (phase C) to induce their differentiation to neurons and astrocytes (**Figure 4.8A**).

Neurogenic protocol #2 was based on a protocol published by Liu and colleagues (Liu et al., 2007). OPCs were maintained in proliferation medium for two days (phase PM) and then differentiation was induced by removing mitogens (differentiation medium, phase DM). After 1 day in phase DM, cells were shifted to a defined stem cell medium (SCM, see Materials and Methods, Table 3.3 for composition) for 3 days (phase SCM, **Figure 4.8B**).

We have then verified if and how exposure to various pharmacological agents (including purinergic receptor ligands targeting GPR17) can modulate OPCs differentiation towards neurons in the two experimental protocols. To start the pharmacological manipulation of cultures, we have chosen a non-selective GPR17 antagonist (Cangrelor; Cang, 10 μM), a non-selective GPR17 agonist (UDP-glucose; UDP-glu, 100μM) and the histone deacethylases inhibitor valproic acid (VPA, 500 μM).

In neurogenic protocol #1 cells were treated with the different pharmacological agents during phase C only, whereas in neurogenic protocol #2 cells were treated during both phase DM and SCM (**Figure 4.8**; for details see Materials and Methods, Paragraph 3.5.1).

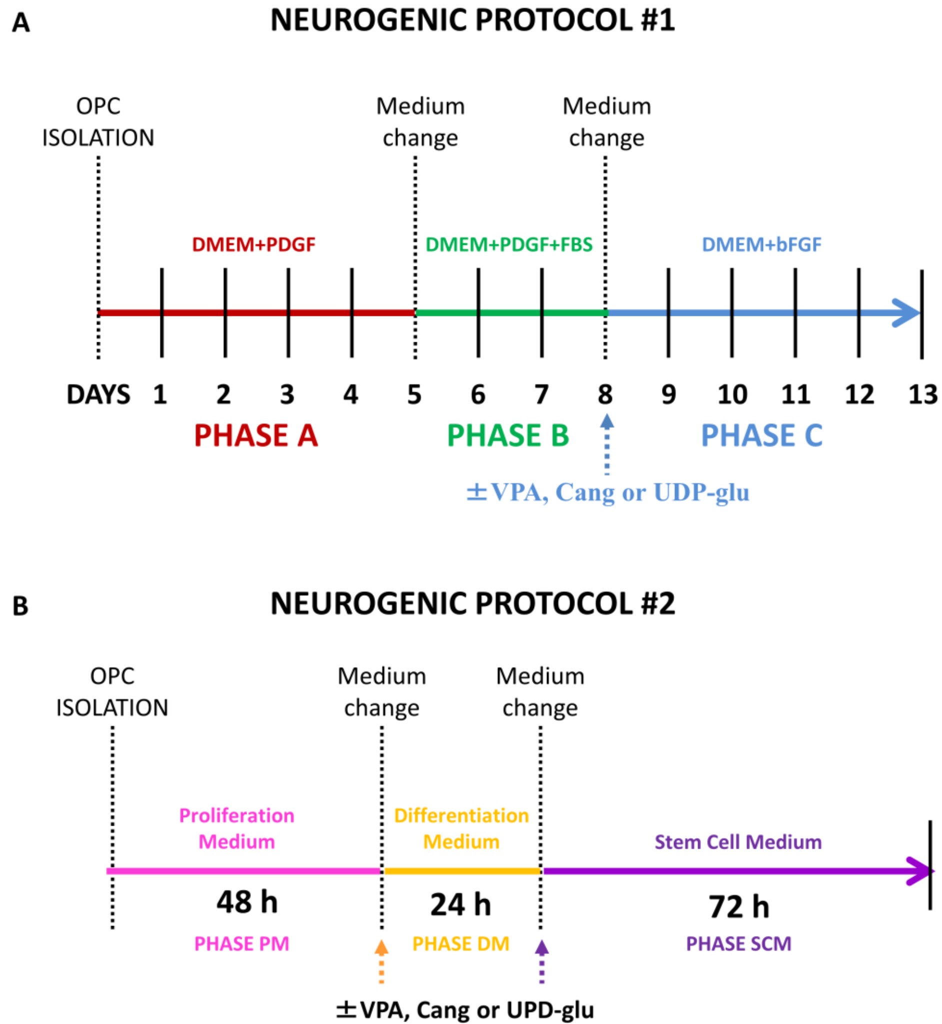


Figure 4.8: schematic representations of the two neurogenic protocols. *A) Experimental design of neurogenic protocol #1. Cells were treated with the anti-convulsant agent and epigenetic modulator valproic acid (VPA, 500 µM), or with a non-selective GPR17 antagonist (Cangrelor; Cang, 10 µM) or with a non-selective GPR17 agonist (UDP-glucose; UDP-glu, 100 µM) during phase C. B) Experimental design of neurogenic protocol #2. Cells were treated with the anti-convulsant agent and epigenetic modulator valproic acid (VPA, 500 µM), or with a non-selective GPR17 antagonist (Cangrelor; Cang, 10 µM) or with a non-selective GPR17 agonist (UDP-glucose; UDP-glu, 100 µM) during both phase DM and SCM.*

4.2.1 NG2+ OPCs ARE MULTIPOTENT CELLS AND EXPRESS GPR17

First of all we tested the ability of both neurogenic protocols to unveil the multipotency of OPCs under control condition. We performed immunocytochemistry analysis to characterize the composition of the cell population at the end of each phase of the neurogenic protocol #1.

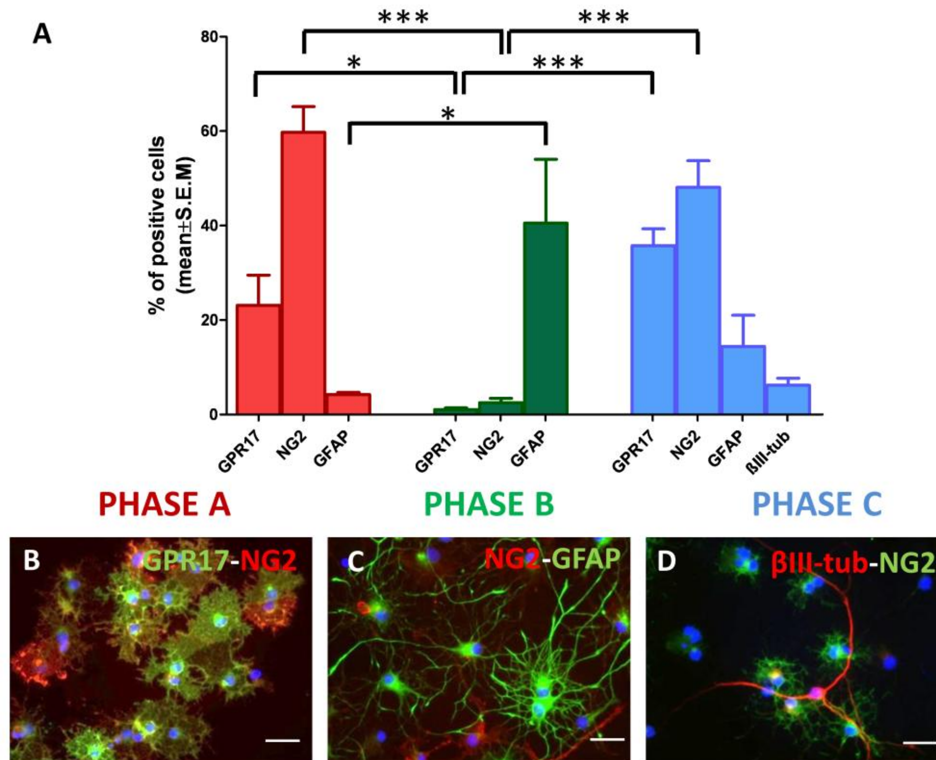


Figure 4.9: generation of βIII-tubulin positive neuron by exposing NG2+ cells to neurogenic protocol #1. *(A) Histograms showing the percentage of cells expressing the different cell markers at the end of phase A (red), B (green) or C (light blue) (n=2-7; *p<0.05, ***p<0.001, unpaired Student's t test). (B-D) Representative images of the cell progeny at the end of each phase of the experimental protocol. Scale bars: 100 µm.*

At the end of Phase A the majority of the cells resulted to be NG2+ OPCs, while only few GFAP+ astrocytes were observed (**Figure 4.9**; red bars in **A** and exemplificative picture in **B**). A significative percentage of cells expressed GPR17 (see below for colocalization). At the end of Phase B, the majority of cells de-differentiated to type 2 astrocytes characterized by strong GFAP immunoreactivity and a branched morphology (**Figure 4.9C**). Moreover, both NG2 and GPR17 expression was dramatically down regulated with respect to Phase A (see **Figure 4.9**; green bars in **A**). At the end of the last phase (phase C), the majority of the cell population re-expressed

NG2 and GPR17, whereas the percentage of GFAP$^+$ astrocytes was significantly decreased with respect to phase B. Interestingly, we also observed the appearance of a small but significant percentage of βIII-tub$^+$ neurons (βIII-tub, light blue bars in **Figure 4.9A**; exemplificative picture in **D**), thus demonstrating that protocol #1 succeeded in redirecting a subpopulation of NG2+ cells towards a neuronal fate.

We also characterized the co-expression of GPR17 with the typical OPC marker NG2 at the end of both phases A and C. In line with the results already published by our group, we observed that the majority of GPR17$^+$ cells also co-expressed NG2, thus confirming that the receptor decorates a subpopulation of pre-oligodendrocyte precursor cells (**Figure 4.10**).

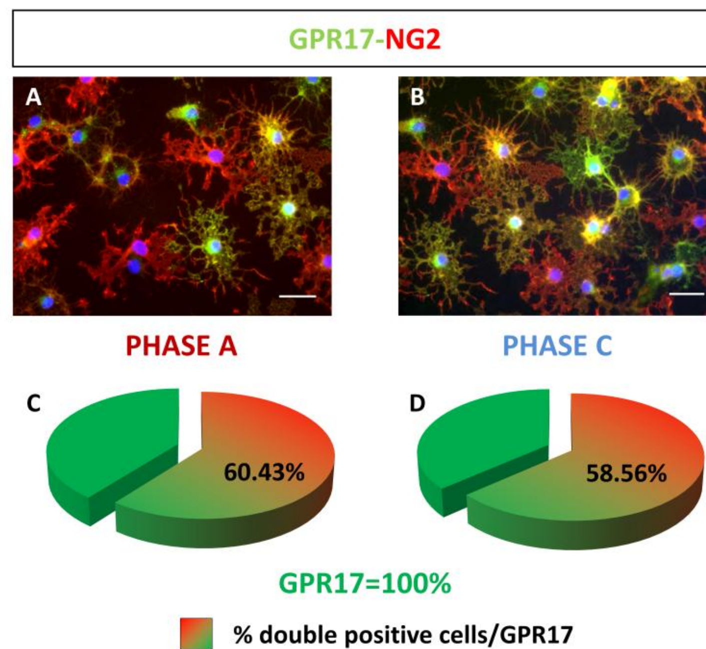


Figure 4.10: at the end of phases A and C the majority of GPR17$^+$ cells co-expresses NG2. *(A, B) Representative images showing the co-localization of GPR17 (green) and NG2 (red) at the end of phase A (A) and phase C (B). (C, D) Pie charts showing the percentage of NG2-GPR17 double-positive cells over the total number of GPR17$^+$ cells at the end of phase A and C. Scale bars: 100 µm.*

We then characterized the progeny of OPCs grown under neurogenic protocol #2 after one day in DM or at the end of the three days in SCM. Also this protocol was able to redirect NG2$^+$ cells towards a neuronal fate. Indeed, we observed a trend to increase in the percentage of βIII-tub$^+$ neurons at the end of SCM phase in comparison to cultures at the end of phase DM (**Figure 4.11A**; phase DM: 3.52±0.65%, phase SCM: 7.36±1.68). On the contrary, the percentage of GPR17-expressing cells was similar at

the end of both phases (**Figure 4.11**; compare orange and violet bars in **A**). Finally, the vast majority of the GPR17$^+$ cells at the end of incubation in DM resulted to be NG2-expressing OPCs (**Figure 4.11B**).

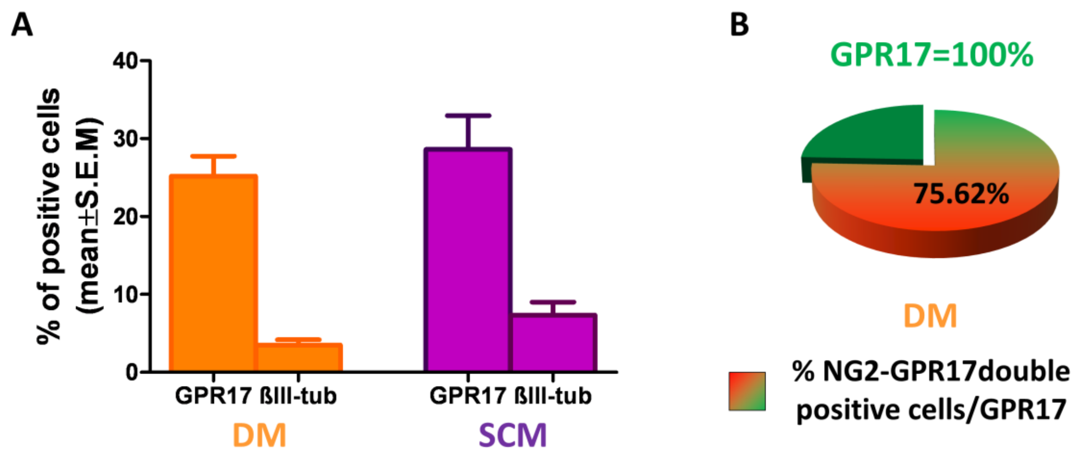


Figure 4.11: incubation in stem cell medium (SCM) stimulates the generation of βIII-tubulin-positive neurons "per se". *(A) Histograms showing the percentage of GPR17- or βIII-tubulin (βIII-tub)-positive cells at the end of 24 hours in Differentiation Medium (DM, orange bars) or after 72 hours in Stem Cell Medium (SCM, violet bars) (n=2-3). See figure 4.8 for details. (B) Pie chart showing the percentage of GPR17$^+$ cells also expressing NG2 over the total number of GPR17$^+$ cells at the end of 24 hours in DM.*

In conclusion, we set up two different neurogenic protocols both suitable to unveil the stem cell properties of NG2$^+$ OPC in vitro, and to redirect them towards a neuronal fate.

4.2.2 VPA IMPLEMENTS THE NEUROGENIC POTENTIAL OF OPCS AND MODULATES THE EXPRESSION OF GPR17

At the end of both neurogenic protocols, two different populations of βIII-tub$^+$ cells were observed under control conditions. The first population was represented by few cells showing the typical morphology of immature neurons, characterized by a small cell body and few long branches (**Figure 4.12A**, upper picture). In addition, βIII-tub was expressed by a second and more abundant population of cells displaying the typical stellate morphology of OPCs (**Figure 4.12A**, lower picture).

When cells were treated with VPA (500 μM) we observed a significant increase in the total number of βIII-tub$^+$ cells at the end of both neurogenic protocols (**Figure 4.12B, C**). The number of βIII-tub$^+$ cells was evaluated as a whole, irrespectively of their neuronal- or OPC-like morphology, since no changes in the ratio between the two cell populations were observed (neuron-like vs OPC-like, data not shown). Moreover, the increase in the number of βIII-tub$^+$ cells was accompanied by a statistically significant reduction in the number of GPR17-expressing cells in neurogenic protocol #1, and by a trend to decrease in neurogenic protocol #2 (**Figure 4.12B, D**). In neurogenic protocol #1 we also evaluated the effect of VPA treatment on the expression of the OPC marker NG2; no differences were observed between control or VPA-treated cells.

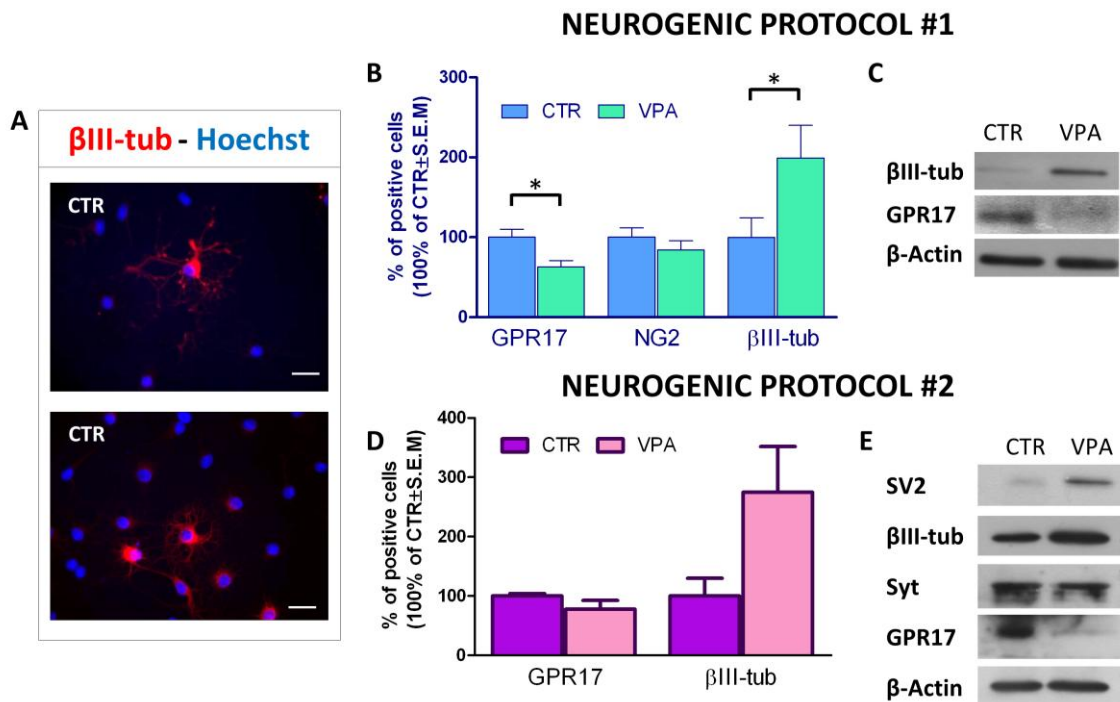


Figure 4.12: VPA treatment increases βIII-tubulin expression, and decreases GPR17 expression in parallel. *(A) Exemplificative images of the two different βIII-tubulin (βIII-tub)-positive populations observed at the end of both neurogenic protocols. (B, D) Histograms showing the effect of VPA treatment on the percentage of GPR17-, NG2- and βIII-tub-positive cells at the end of the neurogenic protocol #1 (B) or on the percentage of GPR17- and βIII-tub-positive cells at the end of neurogenic protocol #2 (D). Data are the mean percentage±S.E.M of control values set to 100% (n= 2-10; *p<0.05, unpaired Student's t test). (C-E) Western Blot analysis of the expression of the neuronal marker βIII-tub and of GPR17 in cell cultures grown under control condition or after VPA treatment in neurogenic protocol #1 (C) and of the neuronal marker βIII-tub, GPR17 and two synaptic proteins in cell cultures grown under control condition or after VPA treatment in neurogenic protocol #2 (SV2, synaptic vesicles 2; syt, synaptotagmin, E). β-actin is used as internal loading control.*

To further confirm these data, we also performed western blot analysis on control and VPA-treated cell lysates at the end of phase C (neurogenic protocol #1) or SCM phase (neurogenic protocol #2; **Figure 4.12C, E**). An increase in βIII-tub expression and a decrease in GPR17 expression were observed in either neurogenic protocols, thus fully confirming immunocytochemistry data. In neurogenic protocol #2, we also investigated the expression of two synaptic proteins (namely, synaptic vesicles 2, SV2 and synaptotagmin, syt). VPA induced a marked increase in SV2 expression compared to control cells, whereas Syt expression was unaffected (**Figure 4.12E**).

Taken together, our results confirm that VPA is able to stimulate the differentiation of OPCs towards neuroblasts that not only express typical neuronal markers, but also display characteristics of mature neurons, as suggested by the presence of synaptic vesicle proteins.

We next analyzed the effects of the pharmacological modulation of GPR17 with the non-selective antagonist Cang (10 μM) or agonist UDP-glu (100 μM). As for VPA, in neurogenic protocol #1 cells were treated during phase C only, whereas in neurogenic protocol #2 cells were treated during both phases DM and SCM.

Exposure to either Cang or UDP-glu did not modify the percentage of GPR17-expressing cells in both neurogenic protocol #1 (Cang: 87.50±11.46% and UDP-glu: 91.34±20.48% with respect to control set to 100.00±9.94%; 20 optical fields from 9-23 coverslips/condition), and neurogenic protocol #2 (Cang: 94.68±8.42% and UDP-glu: 101.70±4.74% with respect to control cells set to 100.00±4.04%; 20 optical fields from 3 coverslips/condition). In neurogenic protocol #1 we observed a trend to increase in the percentage of βIII-tub^{+} cells after either Cang or UDP-glu treatments compared to control cultures (Cang: 142.10±26.68% and UDP-glu: 141.10±38.48% with respect of control positive cells set to 100.00±24.15%; 20 optical fields from 7-19 coverslips/condition), whereas in neurogenic protocol #2 the percentage of βIII-tub expressing cells seemed to be unaffected by GPR17 ligands (percentage of βIII-tub positive cells: Cangr 90.12±29.78% and UDP-glu 70.83±11.33% with respect of control positive cells set to 100.00±29.78%; 20 optical fields from 3 coverslips/condition).

In conclusion, VPA increases the percentage of βIII-tub^{+} cells with the simultaneous down-regulation of GPR17 receptor. On contrary, neither Cang or UDP-glu seem to affect GPR17 expression in both neurogenic protocols, whereas they exert a

complex modulation on βIII-tub expression that differs also between the two neurogenic protocols.

4.2.3 EXPOSURE TO CANGRELOR OR VPA INCREASES THE PERCENTAGE OF GPR17+ CELLS THAT ALSO EXPRESS ßIII-TUBULIN

As previously mentioned, at the end of both neurogenic protocols we observed the expression of the neuronal marker βIII-tub in two morphologically distinct types of cells: the first one presented a typical neuroblast morphology and probably represented immature neurons, whereas the second type of cells bore a OPC-like morphology (**Figure 4.12A**). Interestingly, GPR17 was never expressed by the first cell population. Conversely, in the second population, the appearance of βIII-tub/GPR17 double-positive cells was already observed under control conditions. This is particularly interesting since no expression of GPR17 by neuronal progenitors have ever been observed in vitro. The demonstration that a subset of GPR17$^+$ cells also expresses neuroblast markers further suggests that this receptor may also play a role in the neuronal differentiation of OPCs apart from being important in driving OPCs towards their typical oligodendrocyte lineage.

In this respect, we analyzed the effects of our above-mentioned treatments on the population of GPR17/βIII-tub double-positive cells generated under both neurogenic protocols.

In the neurogenic protocol #1, we observed a tendency to increase in the percentage of the double-positive cells (arrows in **Figure 4.13A-C** and **Figure 4.13D**) over the total number of cell population with respect to control cultures after treatment with either Cang (10 μM) or VPA (500 μM). On the contrary, the percentage of double-positive cells in the UDP-glu (100 μM)-treated cultures was similar to control cells.

When we considered the percentage of double-positive cells over the total GPR17$^+$ cell population, we observed an increase following Cang and, to a greater extent, VPA treatment. Once again, treatment with UDP-glu had no effect on the double-positive cell population (**Figure 4.13E-H**).

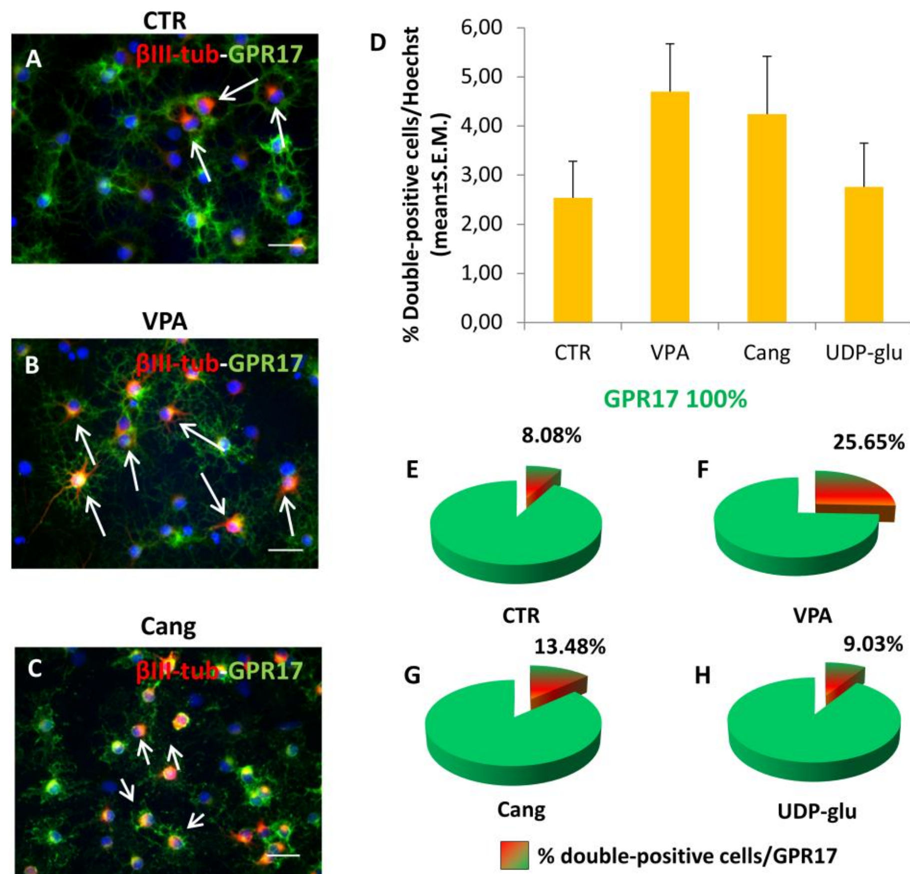


Figure 4.13: Cangrelor and, to a greater extent, VPA expand the percentage of GPR17⁺ cells also expressing βIII-tubulin. *(A-C) Representative pictures of cells grown under control conditions or in the presence of VPA (500 μM) or Cangrelor (Cang, 10μM). Scale bars: 100 μm. (D) Histograms showing the percentage of GPR17/βIII-tub double-positive cells over the total number of cells, as evaluated by Hoechst33258 staining (n=4-9). E-H Pie charts showing the percentage of GPR17⁺ cells also expressing βIII-tub over the total number of GPR17⁺cells at the end of phase C under the various experimental conditions.*

Upon exposure to either VPA or Cang we also observed a trend to a decreased percentage of NG2/GPR17 double-positive cells calculated on the total number of cells (control: 23.28±10.85%, VPA: 8.73±2.11 and Cang: 8.49±2.92%, 20 optical fields from 3 coverslips/condition). Nevertheless, we did not observed any change in the number of GPR17/NG2 double-positive cells over the total number of $GPR17^+$ cell population (control: 58.56±15.75%, Cang: 55.10±18.33% and VPA: 45.62±8.40, 20 optical fields from 3 coverslips/condition).

We next analyzed the GPR17/βIII-tub double-positive cell population also in neurogenic protocol #2 at the end of phase SCM (**Figure 4.14**). VPA exposure significantly increased the percentage of GPR17/βIII-tub double-positive cells with

respect to control cultures either when calculated over the total number of cells, or when expressed over the total number of GPR17-positive cells (**Figure 4.14A, D, E** and arrows in **Figure 4.14B, C**). At variance from neurogenic protocol #1, no effect was observed after Cang treatment, whereas exposure to UDP-glu tends to decrease the percentage of double-positive cells compared with control (**Figure 4.14A, F, G**).

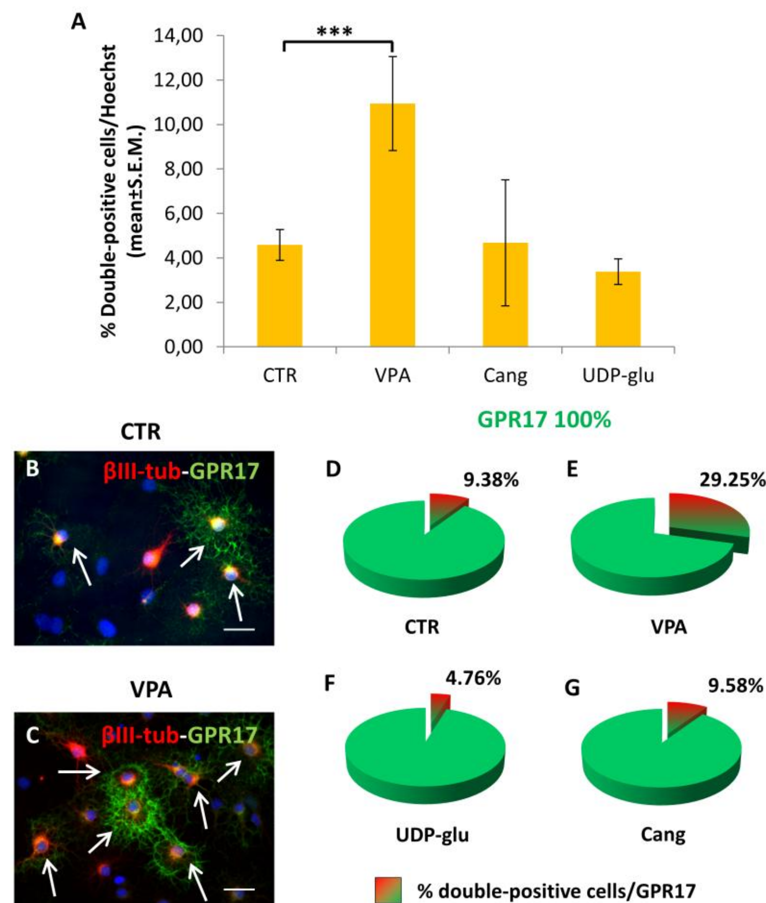


Figure 4.84: VPA treatment in Differentiation Medium and Stem Cell Medium implements the percentage of βIII-tub/GPR17 double-positive cells*. (A) Histograms showing the percentage of GPR17/βIII-tub double-positive cells over the total number of cells, as evaluated by Hoechst33258 staining (n=2 ***p<0.01, unpaired Student's t test). (B, C) Representative pictures of cells grown under control conditions or in the presence of VPA (500 µM). (D-G) Pie charts showing the percentage of GPR17-positive cells also expressing βIII-tub over the total number of GPR17+ cells under the various experimental conditions.*

Taken together our results suggest that VPA and, to a less extend Cang, select a population of hybrid cells with an OPCs morphology that starts acquiring neuronal proprieties. We are currently working on the hypothesis that the modulation of GPR17

receptor on these GPR17/βIII-tub double-positive cells can further favor their differentiation towards neurons.

5. DISCUSSION

Restoration of damaged central nervous system (CNS) is an unresolved challenge of modern medicine, and a great need for additional research in this topic is foreseen, due to the increasing prevalence of CNS disorders and the devastating impact that they have on people life.

It has been recently demonstrated that neural stem cells (NSCs) from the two adult neurogenic areas (i.e., the subventricular zone, SVZ, of the lateral ventricles and the subgranular zone, SGZ, of the hippocampus) take part the brain response upon CNS injuries. A variety of insults, including ischemia and mechanical injury, stimulate NSCs from the SVZ to move towards damaged areas in an attempt to re-establish neuronal connections and to replace damaged neurons (Parent et al., 2002). Furthermore, endogenous NSCs are recruited during chronic neurodegenerative diseases, such as Alzheimer's disease and multiple sclerosis, and migrate into areas of demyelination, where they differentiate into glial cells (Picard-Riera et al., 2002).

Progenitor cells have been also detected in non-neurogenic areas. Besides subsets of parenchymal astrocytes endowed with potential stem/progenitor properties (Buffo et al., 2008), another type of glia bearing progenitor properties has been recently identified in various CNS regions outside typical neurogenic niches (Boda and Buffo, 2010; Nishiyama et al., 2009). Based on their expression of the membrane chondroitin sulphate proteoglycan NG2, these cells are called NG2-positive cells ($NG2^+$; also known as polydendrocytes), and are oligodendrocyte precursor cells (OPCs) that generate oligodendrocytes in the developing and mature CNS, serving as the primary source of remyelinating cells in demyelinated lesions (Nishiyama et al., 2009). As detailed in the Introduction, under specific conditions these cells can also generate neurons and astrocytes (Kondo and Raff, 2000a; Liu et al., 2007).

The discovery that NSCs are maintained in the adult brain is progressively changing the current search for effective neuroregenerative approaches to acute and chronic diseases. In particular, the possibility to promote and foster the endogenous neurogenic/gliogenic ability of the brain has emerged as a new, safe, and ethically acceptable opportunity for both acute and chronic brain diseases. However, to this

purpose, it is imperative to gain a global comprehension of the signaling pathways controlling the survival, differentiation, proliferation, and integration of adult precursor cells. In fact, an in-depth understanding of the molecular basis controlling brain progenitor cells in physiological and pathological conditions would highlight possible pharmacological/biotechnological approaches to help preventing excessive tissue degeneration while enhancing local reparative mechanisms.

Nevertheless, despite increased information on lineage specific transcription factors, cell-cycle regulators and epigenetic factors involved in the fate and plasticity of NSCs (Christie and Turnley, 2013), the understanding of extracellular cues driving the behavior of embryonic and adult NSCs is still very limited. The role of purinergic signaling in regulating the synchronized proliferation, migration, differentiation and death of NSCs during brain and spinal cord development it is now well-known (Neary and Zimmermann, 2009; Ulrich et al., 2012). However, increasing evidence also suggests a role for extracellular nucleotides (eNTs) in controlling adult neurogenesis (Grimm et al., 2009, 2010; Mishra et al., 2006; Stafford et al., 2007; Suyama et al., 2012).

On these premises, our study was aimed at evaluating the influence of purinergic signaling in regulating stem cell properties of adult brain SVZ cells and of $NG2^+$ parenchymal progenitors.

The main finding of the first part of our study is that the purinergic system promotes proliferation and lineage progression of SVZ precursors *in vitro* and *in vivo*, both directly acting on progenitors, and indirectly through the involvement of reactive astrocytes. In fact, eNTs act as local extrinsic factors regulating NSC functions, particularly following traumatic or hypoxic events, when progenitors are in contact with high concentrations of ATP and its metabolites (i.e., ADP, sequentially followed by adenosine), owing to the high activity of ATP-metabolizing ectonucleotidases in neurogenic brain areas (Lin et al., 2007). The high micromolar concentrations of extracellular ATP and its metabolites under these pathological conditions (Abbracchio et al., 2006) also contribute to induction and modulation of reactive astrogliosis (Abbracchio and Ceruti, 2006). Thus, to clarify the role of purinergic system on NSC behavior during brain injury, we used experimental settings that reproduce a pathological environment *in vivo* and *in vitro*. To this aim, we administered ADPβS, a stable analogue of ADP, instead of ATP, mainly for two reasons: i) ATP is highly

instable and undergoes fast hydrolysis (Dunwiddie et al., 1997), and ii) it can activate a vast range of P2Y and P2X receptors subtypes (see also below) differentially from ADPβS which selectively activates the $P2Y_{1,12,13}$ subtypes.

NSCs grown *in vitro* in the presence of growth factors (GFs), like EGF and bFGF, and treated with ADPβS displayed increased neurosphere forming capacity, partially in accordance with previous works where $P2Y_1R$ activation brings to increased neurosphere formation (Mishra et al., 2006; Stafford et al., 2007). Moreover, ADPβS promoted the formation of neurospheres with a reduced size, suggesting that the interaction of purinergic signals with EGF and bFGF inhibited cell proliferation, as already reported (Stafford et al., 2007). Notably, $P2Y_1R$ activation may cause different effects on NSC function that appear dependent on culturing condition (i.e., GFs concentration and neurosphere passages) and may also reflect changes over time in the intrinsic properties of cultured progenitors (Mishra et al., 2006; Stafford et al., 2007). Moreover, exposure to ADPβS under pro-differentiative *in vitro* conditions (i.e., by removing GFs from culturing media) led to stimulation of neuronal and, to a lesser extent, astrocytic commitment of NSCs. Thus, altogether, *in vitro* findings show that purinergic signaling can positively regulate neurosphere formation and neurogenesis under defined culturing conditions.

The above *in vitro* effects were fully confirmed *in vivo* by the i.c.v. infusion of ADPβS at micromolar concentrations (100 μM), with a significant upregulation of GFAP expression in both brain parenchyma and SVZ, showing an ADPβS-mediated activation of astrocytes not only at parenchymal sites (Franke et al., 2004, 2009) but also in the neurogenic area (see also below). Moreover, BrdU incorporation showed that $P2Y_1R$ activation promoted cell proliferation, consistent with a global stimulation of SVZ activity. In line with these results, lineage analysis in GLAST::CreERT2;Rosa-YFP mice exposed to ADPβS showed an increase of both the total number of GFAP/YFP double-positive cells and of their actively cycling fraction. Furthermore, the immediate progeny of $GLAST^+$ precursors (i.e., $Mash1^+$ and DCX^+ cells) was also expanded. Altogether, these findings point to an effect of activation of $P2Y_1R$ on $GFAP^+$ type B cells leading to an expansion of $Mash1^+$ and DCX^+ populations.

Our data partially contrast with a recently published paper. In fact, by administering the natural agonist ATP for 3 days to mice, Suyama et al. observed an increase in the number of both rapidly dividing $BrdU^+$ cells and of $Mash1^+$ type C cells

in the SVZ with no effects on $GFAP^+$ type B cells (Suyama et al., 2012). Differences in the agonist chosen (ADPβS vs. ATP) and in protocols of administration (with a 7-day-long infusion in our study) could explain the differences in the results obtained. Moreover, apart from its fast rate of hydrolysis *in vivo* with a half-life of hundreds of milliseconds (Dunwiddie et al., 1997), ATP can activate a wide variety of purinergic receptors, spanning from the seven P2X ionic channels to some P2Y subtypes (mainly the $P2Y_2$ and $P2Y_4$ receptors; Fischer and Krugel, 2007). In addition to this, ATP is rapidly degraded first to ADP (which stimulates the $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors; Abbracchio et al., 2006) and finally to adenosine, which activates the four types of P1 adenosine receptors. Thus, the different effects exerted by ATP may be related to a more complex pattern of receptor activation with respect to ADPβS that is known as a selective agonist at the $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptor subtypes and to be relatively resistant to ectonucleotidase hydrolysis (Ralevic and Burnstock, 1998).

Literature data suggest that the $P2Y_1R$ is mediating the effects of eNTs on precursor cells (Suyama et al., 2012); this was confirmed by the complete reversal of ADPβS-mediated effects exerted by the $P2Y_1R$ receptor antagonist MRS2179 in our *in vitro* experiments. Moreover, published data and RT-PCR analysis showed that all the three SVZ cell populations expressed the mRNA for this receptor subtype (not shown). Therefore, we are confident that all the observed effects are related to the activation of the $P2Y_1R$. Nevertheless, at present, we cannot completely exclude the contribution of the two additional ADP-sensitive P2Y receptors (i.e., the $P2Y_{12}$ and $P2Y_{13}$), whose mRNA was also detected in SVZ cells.

Since it is well-known that activation of the $P2Y_1R$ is responsible for nucleotide-mediated reactive astrogliosis (Franke et al., 2001) our work also assessed the role of astrocyte reactivity on SVZ cell functions. Reactive astrogliosis virtually occurs in all injuries and pathologies and therefore likely affects NSC activity and neurogenesis, although knowledge on this issue is limited (Buffo et al., 2010). Therefore, we exposed SVZ cells to conditioned media from astrocytic cultures grown under ctr conditions or in the presence of ADPβS, and found a negative effect of both media on the expansion of primary neurospheres. Previous *in vitro* data showed a stimulatory action of cultured astrocytes on the proliferation and neuronal differentiation of NSCs based on both contact-mediated and secreted factors (Barkho et al., 2008; Lim and Alvarez-Buylla, 1999; Song et al., 2002). In another study, reactive astrocytes activated *in vitro* by a

mechanical insult were found to promote astrogliogenesis from NSCs via released factors with no changes in progenitors proliferation compared to non-activated cultures (Faijerson et al., 2006). Our findings unveil a previously unknown inhibitory effect of astrocyte-derived soluble factors on SVZ progenitor activities, in line with an inhibitory action of reactive astrocytes on stem cell maintenance and neuron production *in vivo* (Buffo et al., 2010; Larsson et al., 2004). Moreover, astrocytes can release ATP already under basal conditions, which in turn influences NSC functions in the adult hippocampus via the $P2Y_1R$ (Cao et al., 2013). However, this does not seem the case in our experimental setting, because addition of the $P2Y_1R$ antagonist MRS2179 did not revert the observed effects. A complex pattern of soluble mediators is probably involved in astrocyte-mediated inhibition of NSC functions. Specifically, cytokines/chemokines and adhesion factors have been proposed to control these cells, although their role is far from being fully understood (Christie and Turnley, 2013). In this respect, some interesting hints come from our analysis of cytokine/chemokine/GFs content of conditioned media. IP-10 (also known as CXCL10), TIMP-1, and Rantes were absent in normal neurosphere medium and highly expressed in ctr and ADPβS astrocyte medium at comparable levels, and could therefore account, at least partially, for the observed generalized inhibitory effect of astrocyte-conditioned media on NSCs. So far, these signals have been implicated in the control of differentiation or motility of immature neurons or astrocytes, respectively (Lee et al., 2011; Park et al., 2009). A few studies reported a role for these cues in NSCs specification (e.g., IP-10 in combination with other cytokines; Barkho et al., 2006) and increased motility upon damage (TIMP-1: Ben-Hur et al., 2006; Rantes: Guan et al., 2008). However, their function in SVZ cells remains to be thoroughly investigated. Cues with pro-migratory effects on SVZ cells (CINC-1 and MIP-1α; Gordon et al., 2009) were also detected in astrocyte media, with a slight increase after ADPβS stimulation. By altering cell-to-cell interactions required to sustain NSC activity, these signals, in combination with others and with GFs present in our experimental setting, could lead to a generalized inhibition of NSC properties. In addition to this, a number of astrocyte-secreted molecules, which are known to stimulate stem cell properties also outside the brain (such as VEGF, IL-10, and LIX; Calvo et al., 2011; Choong et al., 2004; Perez-Asensio et al., 2013; Yang et al., 2009), and to be involved in cell adhesion (such as sICAM-1, L-selectin, and MIG), were downregulated upon exposure of astrocytes to ADPβS in astrocytic cultures.

Conversely, the release of molecules with chemoattractive and migration properties was increased by ADPβS. Globally, these data are in line with the pro-differentiative role exerted by the ADP analogue. Interestingly, a significant enhancement in neurosphere formation was detected when secondary neurospheres were generated in standard medium from cells derived from primary neurospheres grown in the supernatant of astrocytes exposed to ADPβS. This suggests that removal of inhibitory mediators triggered upon exposure to the purine analogue boosted the proliferation of SVZ cells during secondary neurosphere culturing. Alternatively, restoration of the stimulatory factors/pathways attenuated by the supernatant of ADPβS-exposed astrocytes could trigger this proliferative compensative response in secondary neurospheres. We are currently working on this hypothesis, which suggests that during acute phases after damage eNTs-mediated reactive astrogliosis does not directly affect the proliferation of SVZ progenitors, but rather modifies their intrinsic properties, leading to a boost toward neurogenesis at more later stages when the concentrations of the various mediators decline. *In vivo*, the situation is likely to be more complicated owing to the presence of reactive microglia, whose functions and properties can be profoundly affected by eNTs (Fumagalli et al., 2011).

In conclusion, the results of the first part of my thesis represent a significant step forward the full understanding of the role of eNTs in controlling SVZ cell functions under pathological conditions, also thanks to their ability to act on a variety of cell populations. Moreover, our data contribute towards the understanding of the complex signaling network regulating neurogenesis in pathological condition and may lead to build the substrate for therapeutic strategies that limit brain damage and promote tissue regeneration

In the second part of our study, we focused on the modulation of the neurogenic properties of parenchymal $NG2^+$ cells by both culturing condition and pharmacological treatments. NG2-expressing OPCs were originally identified as the progenitors of the myelin-forming cells of the brain and spinal cord (Zuo and Nishiyama, 2013); however, recent studies have shown that these cells have the ability to revert to stem-like cells (Kondo and Raff, 2000a, 2000b, 2004), and to generate interneurons (Aguirre et al., 2004; Belachew et al., 2003; Dayer et al., 2005). This seems to be particularly true under pathological conditions. Indeed, to follow the fate of OPCs after CNS pathology, Honsa and co-workers performed permanent middle cerebral artery occlusion (MCAo)

on adult NG2creBAC:ZEG double transgenic mice, in which enhanced green fluorescent protein (EGFP) is expressed in OPCs and their progeny. The number of $EGFP^+$ cells after ischemia was significantly increased; cells displayed heterogeneous morphology and expressed markers of astrocytes and neuronal precursor cells. Moreover, OPCs multipotency was confirmed by detailed electrophysiological analysis, which also proved the generation of astrocytic and neuronal precursor phenotypes from $EGFP^+$ cells after ischemia (Honsa et al., 2012).

The mechanisms underlying the commitment of OPCs towards either myelinating oligodendrocytes or a distinct neural lineage are only partially understood. In these respect, we focused our attention on the possible role exerted by the P2Y-like receptor GPR17, which has been recognized as one of the three genes specifically expressed by adult precursor cells when compared to embryonic ones (Maisel et al., 2007). Moreover our recent data also demonstrate GPR17 expression by OPCs in adult brain parenchyma and its key role in driving cell maturation and reaction to harmful conditions (Boda et al., 2011; Ceruti et al., 2011; Fumagalli et al., 2011).

First of all, we succeeded in reproducing two different published protocols able to unveil the stem cell potential of OPCs and, at least partially, to redirect them towards a neuronal phenotype. NG2 cells from rodent brain have been initially cultured according to a 3-phase protocol, renamed here neurogenic protocol #1 (Kondo and Raff, 2000a). Our data demonstrate an initial regression of NG2 precursors to an intermediate stage where cells lost NG2, and showed massive expression of GFAP (phase B), which was followed by GFAP downregulation, re-appearance of NG2 and, most important, acquisition of the typical neuronal marker βIII-tubulin (βIII-tub, phase C). Interestingly, these phenotypic shifts were accompanied by important changes of GPR17 expression, which was never co-expressed by the intermediate de-differentiated $GFAP^+$ cells, but was highly expressed by $NG2^+$ cells. This is in line with our previous *in vivo* and *in vitro* observations suggesting that GPR17 decorates normally a subpopulation of pre-oligodendrocyte precursor cells (Ceruti et al., 2009; Ciana et al., 2006; Lecca et al., 2008).

A similar pattern of expression was also observed with neurogenic protocol #2 (Liu et al., 2007). At variance from the neurogenic protocol #1, in which OPCs underwent non-oligodendroglial differentiation through an intermediate $GFAP^+$ precursor, cells were cultured in a defined medium (named SCM, stem cell medium)

previously shown to support stem cell growth (Belachew et al., 2003). In accordance with our previous data, the majority of GPR17$^+$ cells resulted to be NG2-expressing OPCs. Moreover, the increased expression of the neuronal marker βIII-tub at the end of the protocol demonstrates that multipotentiality *in vitro* is an intrinsic feature of a subpopulation of NG2$^+$ OPCs. These results emphasize the notion that postnatal progenitor cells expressing the chondroitin proteoglycan NG2 can no longer be considered as mere progenitors restricted to an oligodendroglial fate (Dawson et al., 2000), but are multipotent parenchymal cells.

It is already well known that the oligodendrocyte identity of OPCs is critically dependent on histone deacethylases (HDAC) enzymatic activity. In particular, as the cells progress to mature oligodendrocytes, HDAC activity is high and the final cell fate is established by repressing neuronal and astrocytic genes. On contrary, when HDAC activity is inhibited by pharmacological agents, progenitors are unable to establish an oligodendrocyte-specific program of gene expression, while acquiring a pattern of gene expression consistent with neuronal and astrocytic lineage (Liu and Casaccia, 2010; Marin-Husstege et al., 2002; Shen et al., 2005). In this respect, our data show that the above-mentioned neurogenic shift is markedly implemented by exposure to valproic acid (VPA, 500 μM), an anti-epileptic drug also used as HDAC inhibitor (Monti et al., 2009). In particular, in neurogenic protocol #2 after VPA treatment the increased expression of the early neuronal marker βIII-tub, is accompanied by an higher expression of the synaptic vesicle protein 2 (SV2), an integral membrane protein expressed on all synaptic vesicles (Crèvecœur et al., 2013; Portela-Gomes et al., 2000). This suggests that exposure to the HDAC inhibitor stimulates the differentiation of OPCs towards maturing neurons that are acquiring proteins tipically involved in neurotransmitter release.

Interestingly, VPA exposure also decreased GPR17 expression compared to control culture. This is in accordance with unpublished data from our laboratory showing that inhibition of HDACs with VPA decreases the activity of the *Gpr17* promoter (Lecca et al., unpublished). Since VPA is normally linked to a decreased expression of genes important for oligodendrocyte specification (Hsieh et al., 2004; Liu et al., 2007; Shen et al., 2005), this further supports the hypothesis that GPR17 plays a relevant role in OPCs maturation towards a oligodendrocyte lineage (Boda et al., 2011; Ceruti et al., 2011; Fumagalli et al., 2011)

Very complex effects are instead observed upon modulation of GPR17 receptor with the non-selective antagonist Cangrelor (Cang, 10μM) or agonist UDP-glucose (UDP-glu, 100 μM). In fact, neither Cang or UDP-glu seem to affect GPR17 expression in both neurogenic protocols, whereas they exerted a complex modulation on βIII-tub expression that depended also on the neurogenic protocol applied. It is worth mentioning that for our experiments we used primary cultures, that are usually composed of non-synchronized cells, at different stages of differentiation, and characterized by a different rate of proliferation. The variability among experiments is therefore very high leading to difficulties in obtaining consistent and significant results. Moreover, both neurogenic are composed of sequential and complex culturing conditions that could exert different effects depending on the characteristics of the original cell preparation.

At the end of both protocols, GPR17 was surprisingly co-expressed in a subset of βIII-tub^{+} cells already under control condition. These βIII-tub^{+} cells showed the typical highly-branched OPC morphology, thus probably representing "hybrid" cells still uncommitted to a final oligodendrocyte or neuronal fate. The appearance of this hybrid cell population was further incremented in both neurogenic protocols by the exposure to VPA and, in neurogenic protocol #2, also to Cang. Interestingly, if we consider only the population of GPR17-expressing cells, VPA and, to a lesser extent Cang, promoted the expression of the neuronal marker βIII-tub in these cells. Thus, besides being a trigger for oligodendroglial differentiation (Fumagalli et al., 2011), GPR17 may also be involved in their neurogenic specification. This was already suggested by previous findings (Daniele et al., 2010) that highlighted a role for GPR17 in the neuronal differentiation of PC12 cells, and suggested that activation of this receptor may qualitatively influence the effects induced by classical growth factors. However, we never observed GPR17 expression on βIII-tub^{+} cells displaying the typical neuroblast morphology, suggesting that GPR17 expression is dowregulated at later stages of neuronal differentiation. In this respect, Chen and colleagues (Chen et al., 2009) failed to find expression of GPR17 in neurons of adult mouse brain under physiological conditions. It may well be that GPR17 participates to neuronal specification during development, is turned down during adulthood, and is then re-activated under disease conditions, when endogenous reparative neurogenesis is switched on, as suggested by

markedly increased neuronal expression of GPR17 in the ischemic rat and mouse brain (Ciana et al., 2006; Lecca et al., 2008).

We are currently working on the hypothesis that, by increasing the number of βIIItub/GPR17 double-positive cells, VPA and, partially, Cang are selecting a particular "hybrid" cell subpopulation. Thus, it is tempting to speculate that on these cells, the modulation of GPR17 by its ligands could favor their differentiation towards fully mature neurons. Importantly, GPR17 is a membrane receptor, thus amenable for activation/blockade by signals present in the local CNS milieu, and for exogenous regulation by drugs, at variance from current neurogenic protocols, which mainly exploit genetic manipulations and are therefore less prone to a possible clinical exploitation.

It has already been demonstrated that the systemic administration of VPA in rodents is associated with a reduction of oligodendrocyte generation and a corresponding increase of astrocytes and neurons (Liu et al., 2007). Moreover, VPA has been tested in transient and permanent MCAo rat models and brain trauma (Kim et al., 2007; Ren et al., 2004). Postinjury administration of this HDAC inhibitor decreased blood-brain barrier permeability, reduced neural damage and improved neurobehavioral outcome of the pathology (Kim et al., 2007; Ren et al., 2004). Among the HDACs inhibitors, VPA is one of the most interesting drug, as its HDAC inhibitors action is accompanied by a wide range of biochemical and molecular effects (e.g., regulation of the glutamate excitatory neurotransmission and/or gamma aminobutyric acid (GABA) inhibitory neurotransmission, regulation of several protein kinase signaling pathways and of gene expression), which all appear to cooperate to neuroprotection and cognitive enhancement. Of course, these very exciting perspectives must be confronted with the potential drawbacks linked to the use of such a multitarget drug for safety purposes (Monti et al., 2009).

In vivo treatment of ischemic animals with Cangr markedly prevented damage evolution (Ciana et al., 2006; Lecca et al., 2008) suggesting that activation of GPR17 during ischemia indeed contributes to injury development. However, Cangr acts also as a potent antagonist at the $P2Y_{12}$ receptor subtype which has been shown to be highly expressed in the megakaryocyte/platelet lineage (Burnstock and Knight, 2004). Blockade of this receptor in platelets is associated with a marked antithrombotic effect

(Savi and Herbert, 2005), which should be taken into account when designing future *in vivo* treatments.

Besides pharmacological manipulations, the direct lineage reprogramming is one of the most intriguing strategies recently emerging to replace damaged neurons, consisting in a process where one mature somatic cell transforms into another mature somatic cell without undergoing an intermediate pluripotent state or progenitor cell type. In this approach, the desired cell type can be directly induced from mature somatic cells by overexpression of lineage-specific transcription factors (Graf and Enver, 2009). Anderson and colleagues showed that ectopic expression of Ngn1 in dermomyotome of chick embryo can induce neuronal morphology, and typical neuronal gene expression in these cells (Perez et al., 1999). Subsequently, Götz and colleagues reported neuronal features in mammalian astroglia overexpressing PAX6 (Heins et al., 2002); upon transduction of Ascl1, Ngn2 and Dlx2 in neonatal astroglia, these cells showed neuronal morphology, generated action potentials, and exhibited functional synaptic properties (Heinrich et al., 2010, 2011). In order to accomplish transfection, integrating viral vectors such as lentiviruses and retroviruses, or non-integrating vectors such as adenoviruses, can be used (Patel and Yang, 2010). However, it is worth mentioning that these approaches manipulating the genome could have important drawbacks, such as the potential to cause tumor growth, and to cause mutations. In this respect the pharmacological manipulation of endogenous cells is still a safe and useful strategy to develop a possible clinical application.

Taken together, our results strengthen the evidence that the purinergic system crucially regulates neuronal progenitors, either in a classical neurogenic niche or in the brain parenchyma. The pharmacological modulation of the purinergic system could therefore represent a promising and innovative approach to exploit the intrinsic ability of the adult brain to regenerate in acute and chronic neurodegenerative disorders.

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