Review

Intertwining extracellular nucleotides and their receptors with Ca^{2+} in determining adult neural stem cell survival, proliferation and final fate

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In the central nervous system (CNS), during both brain and spinal cord development, purinergic and pyrimidinergic signalling molecules (ATP, UTP and adenosine) act synergistically with peptidic growth factors in regulating the synchronized proliferation and final specification of multipotent neural stem cells (NSCs) to neurons, astrocytes or oligodendrocytes, the myelin-forming cells. Some NSCs still persist throughout adulthood in both specific 'neurogenic' areas and in brain and spinal cord parenchyma, retaining the potentiality to generate all the three main types of adult CNS cells. Once CNS anatomical structures are defined, purinergic molecules participate in calcium-dependent neuron-to-glia communication and also control the behaviour of adult NSCs. After development, some purinergic mechanisms are silenced, but can be resumed after injury, suggesting a role for purinergic signalling in regeneration and self-repair also via the reactivation of adult NSCs. In this respect, at least three different types of adult NSCs participate in the response of the adult brain and spinal cord to insults: stem-like cells residing in classical neurogenic niches, in particular, in the ventricular–subventricular zone (V-SVZ), parenchymal oligodendrocyte precursor cells (OPCs, also known as NG2-glia) and parenchymal injury-activated astrocytes (reactive astrocytes). Here, we shall review and discuss the purinergic regulation of these three main adult NSCs, with particular focus on how and to what extent modulation of intracellular calcium levels by purinoceptors is mandatory to determine their survival, proliferation and final fate.

This article is part of the themed issue 'Evolution brings Ca^{2+} and ATP together to control life and death'.

1. Introduction

Extracellular nucleotides and their receptors have pivotal roles from the very beginning of life. P2 receptors are expressed by oocytes, sperm and Sertoli cells, and adenosine triphosphate (ATP) is essential for sperm movement. In the majority of living organisms, ATP acts as a key sperm-to-egg signal in the process of fertilization and, immediately after zygote formation, time-specific release of ATP from cells of the developing organism together with their transient expression of P2 receptor subtypes orchestrate embryological, fetal and postnatal development. This suggests the involvement of nucleotides (and of the intracellular calcium rises generated by their receptors) in the synchronized proliferation, differentiation, migration and death of cells during these complex events (for review, see [1,2]).

In a similar way to other organs, in the central nervous system (CNS), purinergic and pyrimidinergic signalling molecules (ATP, UTP and adenosine) act synergistically with peptidic growth factors during both brain and spinal cord development in regulating the synchronized proliferation and final specification of multipotent neural stem cells (NSCs) to neurons, astrocytes or oligodendrocytes, the myelin-forming cells, which are formed by intermediate precursors known as either oligodendrocyte precursor cells (OPCs) or NG2-glia [3].
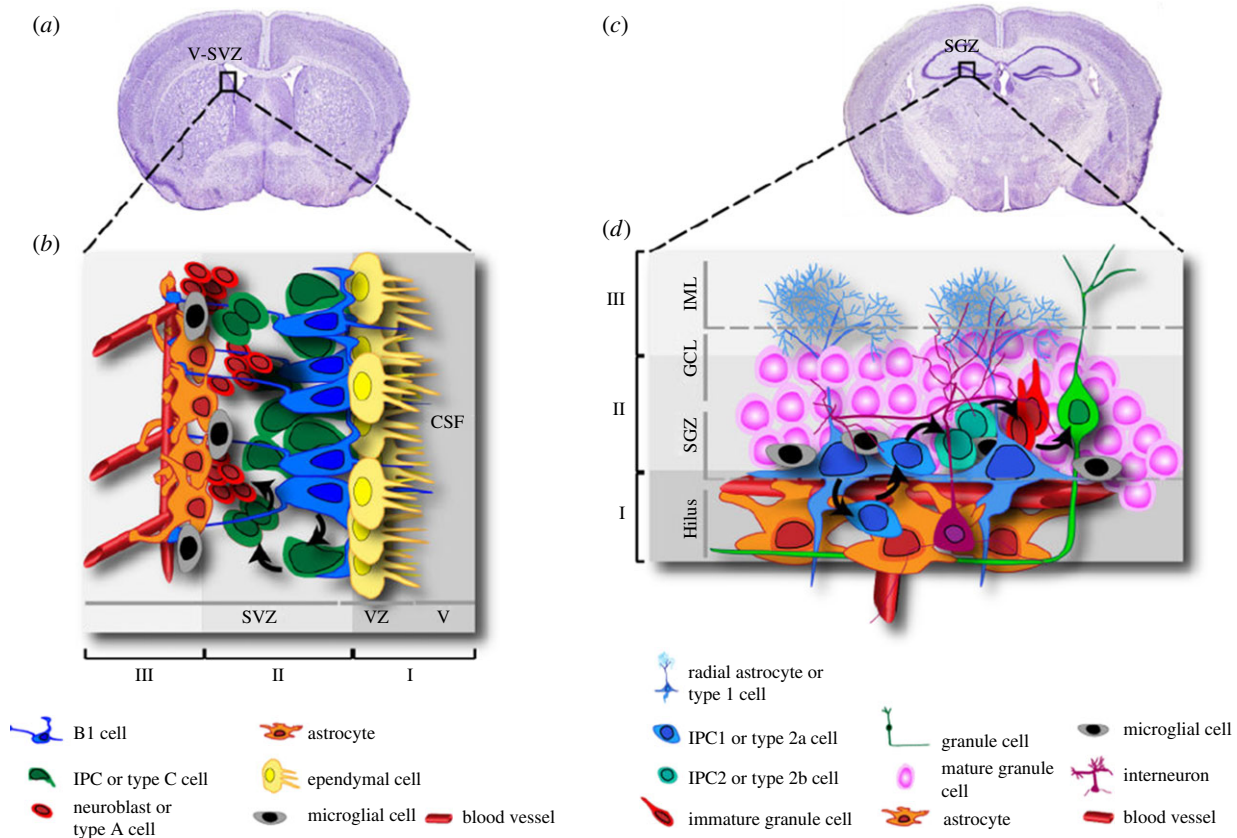
Figure 1. Schematic of the V-SVZ and SGZ neurogenic niches in the adult brain. (a,c) Coronal sections of the adult rodent brain showing the localization of the V-SVZ and of the hippocampal SGZ, respectively. (b,d) Schematic drawing of the organization and relationships of the different cell populations residing in the V-SVZ (b) and in the SGZ (d). See text for details. IPC, intermediate progenitor cells. Reprinted from [5] under a Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0).

Some NSCs, however, still persist throughout adulthood in both specific 'neurogenic' areas and in parenchyma, retaining the potentiality to generate all the three main types of adult CNS cells (see also later sections).

Once CNS anatomical structures are defined, puringergic molecules participate in calcium-dependent neuron-to-glia communication and also control the behaviour of adult NSCs. After development, some purinergic mechanisms are silenced but can be resumed after injury, suggesting a role for purinergic signalling in regeneration and self-repair also via the reactivation of adult NSCs [4].

In this respect, recent data highlight the importance of at least three different types of adult NSCs in the response of the adult brain and spinal cord to insults: (i) stem-like cells residing in classical neurogenic niches, in particular in the ventricular–subventricular zone (V-SVZ), (ii) parenchymal NG2-glia and (iii) parenchymal injury-activated astrocytes (reactive astrocytes).

Here, we shall review and discuss the purinergic regulation of these three main adult NSCs, with particular focus on how and to what extent modulation of intracellular calcium levels by purinoceptors is mandatory to determine their survival, proliferation and final fate.

2. 'Classical' neurogenic areas: the ventricular–subventricular zone and the subgranular layer of the hippocampus

The more extended neurogenic area in the adult mammalian brain is the V-SVZ, located along the entire length of the walls of the lateral ventricles (figure 1a; [6]). Formerly simply known as SVZ, its current denomination as V-SVZ has integrated the concept of the contribution of the ependymal cells (ECs) contacting the ventricle with the physiology of the neurogenic niche.

Within the V-SVZ, three main cell types are morphologically and functionally distinguished: astrocyte-like stem cells (type B1 cells) give rise to clusters of transit-amplifying cells (type C cells or intermediate progenitor cells, IPCs), which, in turn, generate migrating neuroblasts (type A cells; figure 1b). In rodents, newborn neurons proliferate and migrate along the rostral migratory stream (RMS) to the olfactory bulbs (OBs), where they differentiate and integrate in existing neuronal networks [5,7]. It is worth mentioning that, despite the presence, in humans, of a lateral ventricular extension connecting the SVZ to the OB [8], in the adult human brain the RMS remains elusive, possibly owing to the limited importance of the olfactory system in our species. Rather, in the human brain new interneurons are postnatally generated in the corpus striatum, thus identifying a unique pattern of adult neurogenesis [9].

By releasing a plethora of mediators, other cells residing in the close proximity of the V-SVZ, like astrocytes, microglia and cells of the vessel wall (figure 1b), contribute to the modulation of the properties of this neurogenic niche (see later sections).

Type B1 cells are the actual NSCs; they express typical astrocyte markers, like the L-glutamate/L-aspartate transporter (GLAST), glial fibrillary acidic protein (GFAP) and the intermediate filament nestin [7]. Type B1 cells inherit the apical–basal polarity from their precursors, the embryonic

radial glia, with their primary cilium directly contacting the cerebrospinal fluid within the lumen of the ventricles (figure 1*b*). This structure is considered as essential for the reception of growth factors and morphogens and for the subsequent transduction of signals [10]. Their basal side is in close connection with blood vessels at particular sites where V-SVZ vasculature lacks coverage by both astrocytes and pericytes [11], thus being able to receive regulatory signalling molecules released from endothelial cells or circulating in the blood. Although type B1 cells mostly generate new neurons, they are endowed with pluripotency and can also give rise to young migrating oligodendrocytes through intermediate type C cells that move out of the V-SVZ into the corpus callosum, neighbouring striatum and fimbria fornix to differentiate into both non-myelinating and myelinating oligodendrocytes [12]. Moreover, it has been recently shown that type B1 cells can physiologically also generate astrocytes located in the corpus callosum and RMS, but not in the cortex or striatum. This ability decreases progressively with age and sustains a slow, but continuous turnover of astrocytes that normally undergo apoptosis in the corpus callosum [13].

Type C cells are scattered along the network of migrating neuroblasts, they are immunopositive for nestin, the transcription factor distal-less homeobox-2 (Dlx-2) and Mash1, and proliferate at high rate. They are considered as intermediate precursors for type A cells and are not found in the RMS [14].

Type A cells correspond to proliferating, migrating neural precursor cells, showing immunopositivity for typical neuronal markers, like polysialylated neural cell adhesion molecule (PSA-NCAM) and β-Tubulin class III, along with the marker of immature neurons doublecortin (DCX) [7]. Once they reach the OB, type A cells differentiate into periglomerular or granular cells following a specific regional identity [14].

The second region in the adult mammalian brain retaining substantial neurogenesis is the dentate gyrus (DG) of the hippocampus (figure 1*c*), where neurogenesis has been extensively studied over the years, mainly owing to its major role in learning and memory processes. In particular, neural progenitors in this area are located in the subgranular zone (SGZ), a very thin (i.e. only one to three nuclei wide) layer of cells [15]. Three types of neural progenitors have been identified, based on their morphology and on the expression of specific molecular markers (figure 1*d*). Type I (or radial astrocytes) represents the real NSCs, which rarely enter into the cell cycle and have a radial process spanning the entire granular cell layer and ramifying in the inner molecular layer (IML; figure 1*d*). These cells express nestin, GFAP, Sox2 and the brain lipid-binding protein, but do not express S100β, a marker of mature astrocytes [15]. Type I cells generate type II IPCs, which divide faster and are irregularly shaped, and in turn differentiate in postmitotic, immature granule cell neurons positive for DCX and the neuronal marker NeuroD1 (neurogenic differentiation 1) [15]. Immature granule cells are generated in excess and most of them die within the first two weeks whereas the few surviving neurons migrate into the IML, where they elongate their dendrites and axons, and integrate into the DG circuitry [16]. Interestingly, the generation of new DCX positive immature neurons in hippocampal DG has been recently shown to be controlled by the P2Y-like GPR17 receptor [17], whose activation can be counteracted by both purinergic antagonists like cangrelor and by montelukast, a classical antagonist of

cysteinyl-leukotriene receptors [18] that is already marketed as an anti-asthmatic agent. In detail, montelukast was shown to be able to revert the cognitive decline observed in old rodents via an implementation of the proliferation of DCX immature neurons in the DG. This effect was specifically related to blockade of GPR17 on these cells, as demonstrated in neurospheres from both GPR17 KO mice and FoxO1 null mice, where this transcription factor controlling GPR17 expression had been deleted.

Birth of new neurons that integrate locally in functional circuits underlying hormone production and release has been observed in the hypothalamus in response to insulin-like growth factor 1 (IGF-1) administration, leading to the identification of a specific 'neurogenic niche' in the walls of the third ventricle [19], which will not be analysed in this paper. As mentioned, precursors in neurogenic areas derive from embryonic radial glial cells, whose proliferation and differentiation are tightly controlled by intercellular calcium waves. ATP release and $[Ca^{2+}]_i$ transients are connected by autocrine loops, where secretion of ATP-containing vesicles depends on $[Ca^{2+}]_i$ transients and activation of exocytosis through the v-SNARE system [20]. Following its hydrolysis to ADP, ATP activates metabotropic P2Y$_1$ receptors, linked to intracellular calcium mobilization; calcium waves are then propagated into neighbouring cells by intercellular signalling through connexin hemichannels [4], resulting in cell cycle synchronization of migrating neural progenitors and radial glia cells in the V-SVZ for cortex development [21]. This provides an additional link between calcium and the purinergic system in progenitor cells. In fact, spontaneous calcium oscillations in neural progenitors have been shown to depend on the activation of P2R, probably owing to the release of ATP. Reduced calcium transients evoked by P2Y$_1$R were observed in precursor cells isolated from connexin43 (Cx43)-null mice [22]. Blockade of P2Y$_1$R in precursors from wild-type (WT) mice did not alter their differentiation, as measured by the ratio of nestin : GFAP expression levels, but reduced their proliferation rate and their migration distance to distances similar to those observed in Cx43-null cells. Conversely, forced overexpression of P2Y$_1$R in Cx43-null neurospheres led to the generation of spontaneous calcium oscillation and restored the migration pattern observed in WT neural progenitors [22]. Overall, these data provided some of the first evidence linking the purinergic system with calcium mobilization in precursor cells, which, in turn, profoundly affect their functions.

To confirm the existence of a basal fine-tuning of NSC functions and fate by purines and pyrimidines, receptors and metabolizing enzymes for nucleotides and nucleosides are widely expressed in the SVZ and SGZ (reviewed in [23]). Specifically, high levels of the ectoenzyme nucleoside triphosphate diphosphohydrolase 2 (NTPDase2), which hydrolyses extracellular nucleoside diphosphates and triphosphates, have been detected in both neurogenic areas in the adult CNS [24–26]. Studies in NTPDase2-null mice, where increased ATP levels are foreseen, revealed increased progenitor cell proliferation in both V-SVZ and SGZ, with an expansion of intermediate progenitors but no changes in the number of newly generated neurons that died from apoptosis [26]. This suggests the possible recruitment of the P2X7R (see below).

Generation of calcium waves was observed in NSCs isolated from neurogenic areas and grown *in vitro* as floating neurospheres through activation of the ADP-sensitive

G protein-coupled $P2Y_1R$ (with a contribution of $P2Y_{13}R$) and UTP-responding $P2Y_2R$, while no functional $P2Y_4Rs$ or $P2Y_6R$ have been identified [27]. Modulation of calcium transients by $P2Y_1Rs$ exerted opposite functional effects, with reduction of the proliferation rate and of the number of generated primary neurospheres, in contrast with an overall increased proliferation of secondary neurospheres (see also below). This is possibly owing to the enrichment in NSCs that is observed over successive passages in culture and to the presence of growth factors [27,28]. Confirmation of a most prominent role in neurogenesis played by the $P2Y_1R$ came from the demonstration that its activation promoted NSC differentiation [28] and migration [29] *in vitro*, whereas a dual effect on proliferation was observed depending on the presence of growth factors. In fact, stimulation of proliferation was observed in their absence or at low concentrations [30,31], while an anti-proliferative effect was achieved at standard concentrations (around $20 \, ng \, ml^{-1}$) [27]. Caution should be taken when trying to translate results obtained *in vitro* to an *in vivo* setting, because cultured stem cells can express all purinergic receptors and may consequently reveal a broader spectrum of responses than corresponding cells *in vivo*. Nevertheless, it has been clearly confirmed that activation of $P2Y_1R$ controls V-SVZ cell functions *in vivo*. In fact, upon ATP administration, the proliferation of type C cells increased, with no changes in proliferation of either type B stem cells or type A neuroblasts. To further strengthen the role of $P2Y_1R$, an opposite effect was observed upon administration of the selective $P2Y_1R$ antagonist MRS2179 *per se* and a reduced number of type C cells was detected in the V-SVZ of $P2Y_1R$-KO mice [32]. The interpretation of *in vivo* results is further complicated by: (i) difficulties in identifying the exact localization of purinoceptors on a specific cell population within the neurogenic niches (that do not allow us to discriminate whether agonists and antagonists exert direct effects on stem cells or whether the contribution of surrounding cells like astrocytes and microglia is needed) and (ii) by the possible localization of purinoceptors either on the somata or on the processes of cells contacting precursors in the neurogenic niches (see figure 3*d*).

Another purinergic receptor involved in neural differentiation is the $P2Y_2R$ subtype, which activates PLC-β, leading to increased $[Ca^{2+}]_i$ and generation of intercellular Ca^{2+} waves in NSCs *in vitro* [30]. Moreover, neural progenitor proliferation is modulated by an autocrine loop, with cells releasing ATP, activating P2Y receptors for proliferation maintenance. Blockade of proliferation and induction to neural differentiation occurred only when purinergic receptor activity had been antagonized and $[Ca^{2+}]_i$ transients had diminished [33].

The effects of $P2Y_1R$ activation are counterbalanced by the ionotropic P2X7R subtype, which is highly expressed by NSCs in the SVZ, and modulates intracellular calcium spikes [34]. Its prolonged activation by ATP led to caspase-independent lysis/necrosis of NSCs, as demonstrated by cell membrane disruption accompanied by loss of mitochondrial membrane potential. Surprisingly, activation of P2X7R in NPCs causes cell death in the absence of pore formation [35]. These observations might have opposite outcomes following pathological events: in fact, high levels of extracellular ATP in inflammatory CNS lesions may delay the successful graft of newborn NPCs and therefore impair

tentative repair of the damaged tissue. On the other hand, recruitment of P2X7R can inhibit excessive neuro- and gliogenesis, thus reducing the risks for development of tumours.

Apart from the physiological control of neurogenesis, it can be foreseen that the above-mentioned mechanisms involving calcium and extracellular nucleotides become increasingly prevalent upon traumatic and/or ischaemic events, when extracellular purine and neurotransmitter concentrations increase several fold, leading to an amplification of their signalling pathways under emergency conditions [36]. As elevated extracellular nucleotide concentrations are known to be responsible for astrocyte and microglia activation (see also below), it is conceivable that these reactive cell populations residing within the neurogenic niche (or in its close proximity) can participate to drive NSC final destiny and neurogenic potential. In line with that, ATP secretion by astrocytes even at basal level can promote NSC proliferation in the adult hippocampus through $P2Y_1R$ activation [36]. Moreover, *in vivo* intracerebroventricular infusion of high concentrations of a hydrolysis-resistant ADP analogue, thus resembling high pathological nucleotide concentrations, promotes the proliferation of type B progenitors and sustains their progression toward the generation of rapidly dividing progenitors, both directly and indirectly, through the contribution of surrounding reactive astrocytes [31].

Interestingly, traumatic and ischaemic events not only dramatically modify NSC neurogenic environment, but can also recruit them outside their 'natural' migratory pathway towards the site of injury, in an often unsuccessful attempt of regeneration [37], thus profoundly affecting the fate of newborn cells. It cannot be excluded that the increased concentrations of extracellular nucleotides are also involved in this effect.

(a) Ependymal cells lining spinal cord central canal

ECs lining the spinal cord central canal are considered the real stem-like cells in this part of the CNS. They stain positive for markers of immature neural cells, such as nestin, vimentin and the transcription factor Sox2, and of ciliated cells such as Fox1 and Crocc [38]. Moreover, a very recent paper showed for the first time that spinal cord ECs express ionotropic P2X4Rs and P2X7Rs, and metabotropic $P2Y_1Rs$ and $P2Y_4Rs$, all functionally coupled to $[Ca^{2+}]_i$ transients [39]. They also stain positive for the dualistic receptor GPR17 [40], which responds to both extracellular nucleotides and cysteinyl-leukotrienes and plays a fundamental role not only in the differentiation of NG2-glia to mature myelinating oligodendrocytes (see also below), but also in their alternative switch towards a neurogenic fate [41]. In the intact tissue, spinal cord ECs are virtually quiescent, self-renew very slowly and give rise to a very small number of neurospheres *in vitro*. However, after spinal cord injury (SCI) or hypoxia, ECs proliferate, migrate towards the injury site and start expressing GFAP, a marker of multipotency [38,40]. Interestingly, ECs reveal downregulation of $P2Y_1Rs$ in parallel with upregulation of $P2Y_4Rs$ one week after SCI, suggesting that the panel of expression of P2YRs may play a critical role in the modulation of neural progenitor cell expansion [39]. Activated ECs generate a high number of neurospheres and give rise to astrocytes, oligodendrocytes and, under some conditions, motoneurons when exposed to differentiating agents

in vitro [42]. However, fate-mapping analysis of ECs following SCI *in vivo* revealed generation of astrocytes and oligodendrocytes only [43] (see also §3). Although no generation of new neurons has been observed, acute transplantation of undifferentiated ECs fully reverted the pathological increase in P2X4Rs and P2X7Rs observed after SCI and led to a partial recovery of locomotor activity [39], thus suggesting a possible bystander role played by ECs.

3. Oligodendroglial precursor cells (NG2-glia)

In the CNS, mature myelinating oligodendrocytes are formed from OPCs, also termed as NG2-glia for their expression of the membrane-spanning proteoglycan NG2. These cells derive from less differentiated NPCs and constitute a ubiquitous population of glial progenitors in the mammalian CNS, able to generate mature oligodendrocytes during early postnatal development [44]. OPCs remain abundant in grey and white matter regions of the adult CNS parenchyma [44,45] and retain the ability to proliferate and differentiate into oligodendrocytes to sustain basal myelin turnover [46,47]. More importantly, these cells promptly react to injury and demyelination by increasing their proliferation, migration and differentiation rate to repopulate the lesioned area with new myelinating oligodendrocytes [48–50]. Recently discovered properties of OPCs also point to additional neuromodulatory and neuroprotective actions of this cell population [51]. OPCs have indeed the capability to receive and respond to electrical activity [52]; moreover, it has been proposed that the expression of growth factors, cytokines, chemokines and receptors for various signalling molecules may confer to these cells the same reparative bystander actions that germinal neural progenitors exert in the lesioned CNS [51,53–55].

In early studies, changes in intercellular calcium waves have been detected in OPCs [56,57], suggesting that intracellular calcium transients can fine-tune their development. Several studies have later addressed the importance of Ca^{2+} signalling in process extension and OPC migration [58–62], in their differentiation, myelination and re-myelination capacity [63–66], and in retraction of membrane sheaths and cell death in mature mouse oligodendrocytes [67].

(a) Extracellular nucleotides and calcium signalling in oligodendrocyte precursor cells

Several neurotransmitters contribute to the homeostasis of OPCs and it is now well accepted that purinergic signalling is actively involved in the regulation of their physiological behaviour and reaction to injury [68]. Extracellular ATP is one of the main activity-dependent axonal signalling molecules that activate P2 receptors on neighbouring OPCs [69]. Both ATP and uracil nucleotides interact with other extracellular signals impinging on common intracellular pathways in regulating OPC proliferation and migration, various steps of OPC maturation and myelination and axonal activity [70–73]. Key roles in various OPC processes are also carried out by adenosine deriving from ATP metabolism through activation of A_1 or A_2 receptors [74–76]. A_1 receptors are downregulated during oligodendrocyte differentiation, whereas $P2Y_1$ and P2X7Rs are expressed at all differentiating stages and by mature myelin-forming oligodendrocytes [77].

Of interest, nucleotide-dependent mechanisms are implicated in OPC calcium oscillations, which in turn regulate neuronal integrity and stimulate myelin production by oligodendrocytes [74]. Elevation of intracellular basal calcium in these cells occurs via a number of routes by favouring calcium entry across the plasma membrane through: (i) ligand-operated channels (such as the P2X, and glutamate receptors) [78–80], (ii) voltage-operated Ca^{2+} channels (VOCs) activated in response to membrane depolarization [60,65], (iii) other routes, such as the opening of store-operated membrane Ca^{2+} channels following depletion of Ca^{2+} stores in the endoplasmic reticulum [81–83]. Release of calcium from internal stores of the endoplasmic reticulum can occur following activation of a variety of membrane G protein-coupled receptors, including P2YRs coupled to Gq via the inositol-1,4,5-triphosphate (IP3) pathway (figure 2*a*).

The first *in vivo* evidence that, upon release during axonal action potential, ATP evokes rapid and transient Ca^{2+} rises in single OPCs was attested by confocal calcium imaging on optic nerves from a transgenic reporter mouse line in which adult NG2-glia were identified by the expression of the red fluorescent protein DsRed [85]. The predominant mechanism was the activation of metabotropic $P2Y_1R$, because response was abolished by the pre-incubation with the $P2Y_1R$ antagonist MRS2179, but also the P2X7R induced significant calcium increases [85]. The Ca^{2+} signals increased in both amplitude and duration with increasing stimulus strength, indicating that responses in these cells matched with axonal activity; however, it was clearly demonstrated that ATP is not only released by axons during action potentials, but also comes from astrocytes, in response to axonal activity through vesicles, reverse transport, hemichannels and the P2X7R [86]. The close connections between OPCs and astrocytes was demonstrated by direct enwrapment of short segments of astroglial processes, thus enabling the local passage of Ca^{2+} signals from astrocytes to OPCs and vice versa [85]. These results strongly favour the view of a close interdependence among OPCs, astrocytes and neurons in synaptic transmission [87]. It has been proposed that $P2Y_1Rs$ may have a major role in the physiological signalling of OPCs, as it is activated by nanomolar concentrations of ATP in optic nerve glia, whereas P2X7R becomes activated when extracellular ATP is in the micromolar range [73], i.e. under pathological conditions. Thus, low levels of ATP would activate a transient self-limiting $P2Y_1R$-mediated release of Ca^{2+}, whereas at higher levels of signalling, Ca^{2+} influx through P2X7 would prolong the ATP-evoked Ca^{2+} signals for hundreds of seconds [85]. However, this fascinating hypothesis has not been demonstrated yet.

During neuron-to-glia signalling, $P2Y_1Rs$ are also responsible for reduced expression of the calcium-permeable AMPA receptors (CP-AMPARs), which play important roles in OPC proliferation, migration, differentiation and neuron–glia signalling [88,89]. This has led to the hypothesis that, under physiological conditions, ATP primes OPCs for differentiation into myelinating cells by both reducing the expression of CP-AMPARs and promoting differentiation via activation of adenosine receptors [90]. On the other hand, CP-AMPARs also make these cells susceptible to ischaemic damage, with important implications for cell survival [91]. While $P2Y_1R$ activation and consequent release of
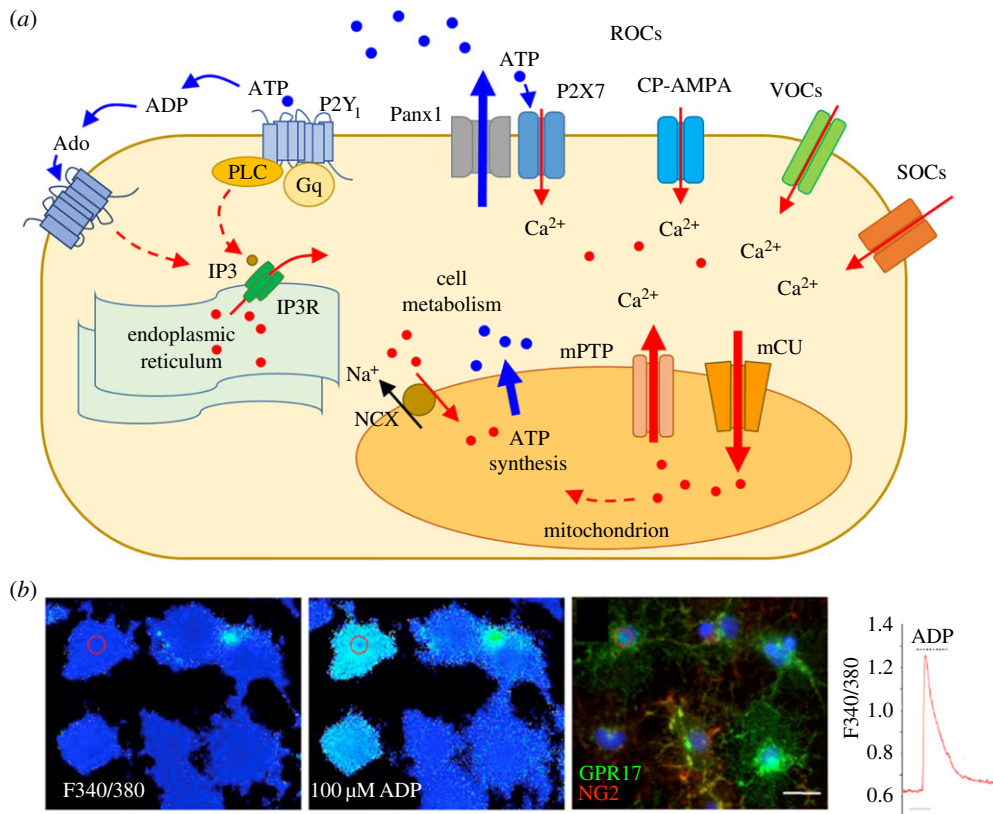
Figure 2. Calcium signalling in OPCs. (*a*) Schematic of the main routes activating Ca^{2+} signalling in OPCs. Calcium enters OPCs through ROCs (receptor-operated channels, such as P2X7 and CP-AMPA), VOCs (voltage-operated channels) and SOCs (store-operated channels). IP3 receptors (IP3Rs) on the endoplasmic reticulum promote the release of Ca^{2+} from the intracellular stores via Gq-coupled receptors, mainly P2Y$_1$ (activated by ATP and ADP) and in part A$_1$ and A$_{2A}$ receptors (primarily Gi- and Gs-coupled, respectively, but secondarily coupled to Gq) activated by Ado (adenosine). In disease, activation of P2X7Rs promotes the opening of a non-selective pore, namely pannexin1 (Panx1), which contributes to massive release of ATP in the extracellular milieu. Mitochondria take up intracellular Ca^{2+} through the mCU (mitochondrial calcium uniporter) and maintain the Ca^{2+} homeostasis by both the mPTP (mitochondrial permeability transition pore) and NCX (Na$^+$/Ca^{2+} exchanger) localized on the inner membrane. ATP synthesized by the mitochondrial respiratory chain is then available for cell metabolism. (*b*) Series of pseudocolour images of the same Fura-2 loaded cells. First micrograph from the left shows a field of cells at F340/380; the second image shows response of the same group of cells after the application of 100 μM ADP (at peak of Ca^{2+} response); changes of fluorescence from dark to light blue indicates increases of intracellular calcium concentrations $[Ca^{2+}]_i$. Post-fixation staining of the same field of cells with NG2 and GPR17 indicates that several NG2$^+$ and GPR17$^+$ cells responded to ADP (see cell highlighted with circle as an example). On the right, representative trace of Ca^{2+} increases recorded from pre-oligodendrocytes after application of ADP. Adapted from [84].

Ca^{2+} from intracellular stores induced rectification of the glutamate I-V receptors, suggesting that P2Y$_1$R may promote OPC survival under stress or ischaemia/hypoxia conditions, this has not yet been proved unequivocally.

Intracellular calcium transients elicited by several purinergic agonists have been also recorded in OPC cultures demonstrating the *in vitro* functionality of P2Y$_1$, P2Y$_2$, and P2Y$_6$Rs ([84]; figure 2*b*). The P2Y-like receptor GPR17, activated by UDP, UDP-sugars and cysteinyl-leukotrienes [18,92], is expressed at an intermediate stage of OPC differentiation. While GPR17's molecular mechanism of action in oligodendrocyte physiology is still unclear and probably involves both modulation of cAMP levels and of K$^+$ conductance [93], its stimulation promotes the transition from early OPCs to intermediate oligodendrocytes [84,93–95]. Conversely, at later differentiation stages, GPR17 plays an inhibitory role and has to be downregulated to allow cells' terminal differentiation [95,96]. Although GPR17 is primarily coupled to a Gi protein, in transfected systems Ca^{2+} fluxes via Gq were also reported using the endogenous ligands UDP-glucose and LTD$_4$ [18] and the synthetic non-specific agonist, MDL29,951 [97]. However, whether and how Ca^{2+}

signalling contributes to the pathophysiological roles of GPR17 in native systems still remain to be established. Single cell calcium imaging showed that adenosine receptors also contribute to the robust Ca^{2+} responses induced by electrical stimulations of axons in both OPCs and immature oligodendrocytes, suggesting that intracellular calcium dynamics may underlie the positive effects of adenosine on OPC maturation and myelination [74]. These data reveal that not only ATP, but also the whole purinergic system is involved in calcium signalling, with different and synergic contributions to OPC proliferation, differentiation and migration.

(b) Adenosine triphosphate and calcium in oligodendrocyte death

As mentioned above, ATP-gated P2XR channels equal NMDA receptors in their calcium permeability [98]. In OPCs, P2X7R seems to be the only functionally active P2X receptor subtype [73], but its physiological role is still elusive [99]. Instead, the role of P2X7Rs in pathological conditions has been well described. P2X7R overactivation opens the
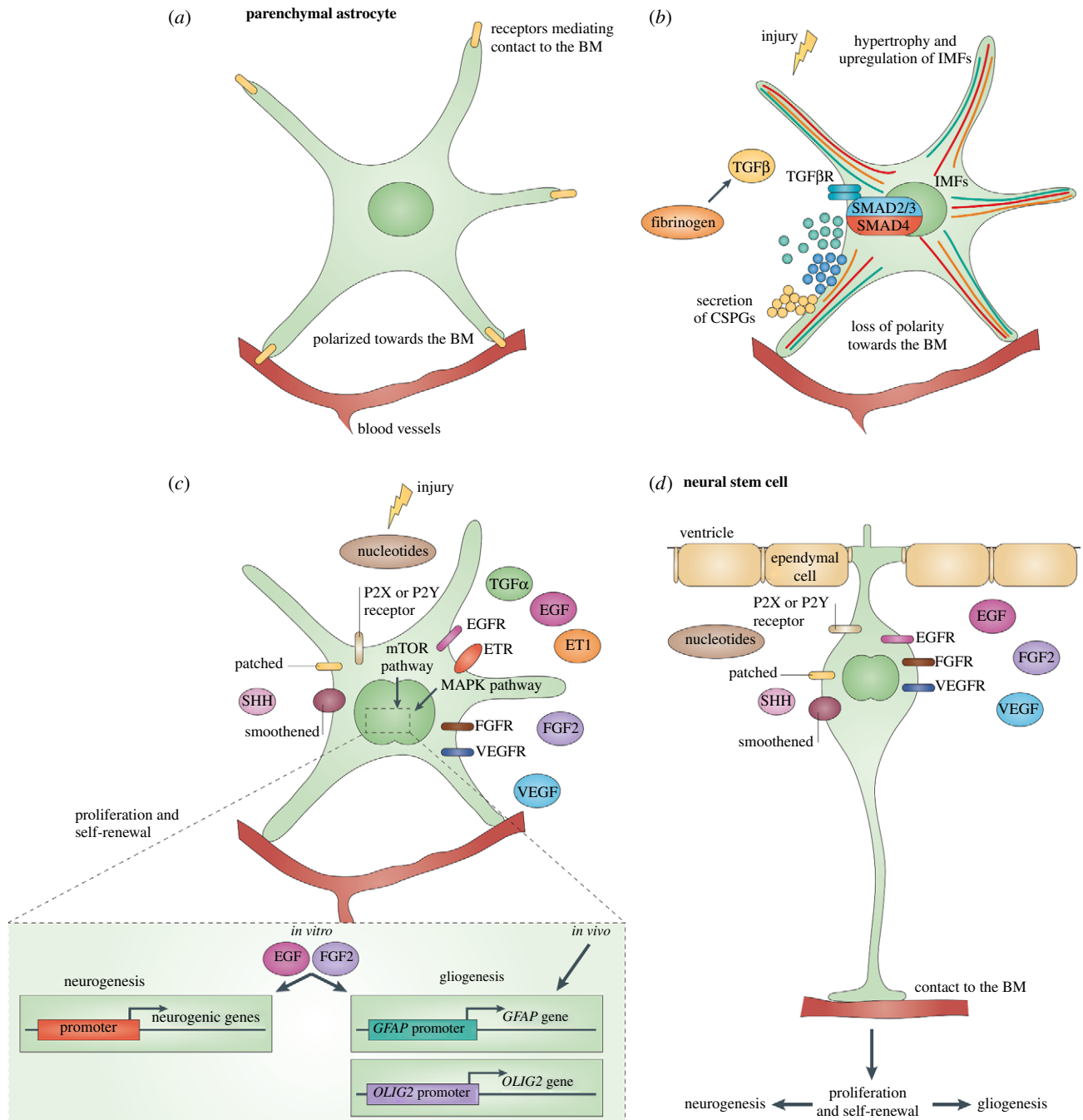
Figure 3. Signals shared after brain injury and in the NSC niche. (*a*) Mature astrocytes in the healthy brain parenchyma are polarized towards (and make contact with) the basement membrane (BM) surrounding blood vessels and do not divide. (*b*) Loss of signalling from the BM as seen after brain injury leads to reactive astrogliosis, including loss of the polarized expression of endfeet proteins, hypertrophy and upregulation of intermediate filaments (IMFs), and secretion of chondroitin sulfate proteoglycans (CSPGs). Secretion of CSPGs is triggered by fibrinogen, which activates transforming growth factor beta (TGFβ) signalling. This pathway also promotes upregulation of glial fibrillary acidic protein (GFAP), which may inhibit axonal regeneration. (*c*) Signals triggering astrocyte proliferation after injury are fibroblast growth factor (FGF), epidermal growth factor (EGF) and transforming growth factor alpha (TGFα), which act via the mitogen-activated protein kinase (MAPK) or the mammalian target of rapamycin (mTOR) pathway. The latter is also activated by purinergic signalling following the release of ATP after brain injury. Proliferating reactive astrocytes do not generate other glia or neurons *in vivo*. However, exposure to further EGF and FGF2 signalling *in vitro* confers the capacity to generate neurons, astrocytes and oligodendrocytes *in vitro*. (*d*) These factors, as well as vascular endothelial growth factor (VEGF) and sonic hedgehog (SHH), are also active in the adult neurogenic niches, like the SVZ, *in vivo*, where they also promote proliferation of neural stem and progenitor cells. EGFR, EGF receptor; ET1, endothelin 1; ETR, ET receptor; FGFR, FGF receptor; OLIG2, oligodendrocyte transcription factor 2; TGFβR, TGFβ receptor; VEGFR, VEGF receptor. Reprinted by permission from Robel *et al.* [114]. (Copyright 2011 Macmillan Publishers Ltd).

non-selective channel pannexin1 (Panx1), which becomes permeable to ATP, allowing massive calcium entry into cells, thus contributing to cell death [100]. OPC cultures exhibit high sensitivity to oxygen–glucose deprivation (OGD) toxicity, which can be attenuated by Brilliant Blue G (BBG), a P2X7R-preferring antagonist [101]. In SCI, high levels of extracellular ATP in spinal cord resulted in massive cell death, whereas the P2X7R antagonist oxidized ATP (oATP) significantly diminished death of OPCs in both grey and white matter [102].

Of relevance, during neuroinflammatory diseases such as multiple sclerosis, activated immune system cells, astrocytes, dead oligodendrocytes and neurons release high amounts of ATP and other nucleotides, which may evoke excitotoxic degeneration of cells [77]. Panx1-mediated ATP release contributes to development of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis; Panx1 knockout (KO) mice, in which ATP release was found to be decreased and P2X7R upregulated in spinal cord of chronic EAE phase (probably owing to a compensatory mechanism), displayed a delayed onset of EAE clinical signs and decreased mortality compared to WT animals [103].

All these data, obtained in several models of disease, suggest that P2X7R contributes to damage exacerbation; however, it has also been reported that the expression of P2X7R was downregulated in OPC cultures and in ischaemic cerebral cortex, subcortical white matter and hippocampus in a paradigm of perinatal hypoxia-ischaemia, probably in an attempt to prevent the excessive receptor stimulation in the early stages of damage [99].

As previously mentioned, during injury, OPCs can also migrate to the lesion site following an ATP gradient to promote repair; although it has not been demonstrated *in vivo*, P2X7R stimulation *in vitro* showed that this receptor can indeed mediate chemotaxis of OPCs via receptor coupling to Fyn kinase [104].

In recent years, adult OPCs have also been implicated in glioma formation. According to the recent proposed 'cancer stem cell' hypothesis, mutated OPCs have been found to be responsible for the growth of the tumour and its resistance to therapies [105–107]. Of relevance in this context, recent data pointed to intracellular Ca^{2+} as a key positive regulator of tumourigenesis in glioma, through influence on processes involved in the quiescence, maintenance, proliferation or migration of adult OPCs [106]. Moreover, several data obtained in the C6 glioma cell line showed that purinergic signalling participates in the development and progression of glioma through the release of ATP. P2X7R, $P2Y_1R$, $P2Y_{12}R$ and A_{2B} activation by ATP, ADP and adenosine result in increased production of chemokines, augmented cell proliferation and tumour invasiveness (for review, see [108]). In line, purinergic-dependent calcium signalling has been highlighted to be crucial for the behaviour of transformed glioma cells [109]. Understanding whether and how the manipulation of purinergic-dependent Ca^{2+} events in mutant OPCs could affect the transformation of these cells into gliomas would greatly help the set-up of novel antitumoural strategies.

Importantly, mitochondria are also involved in OPC Ca^{2+} homeostasis (figure 2a). In basal conditions, mitochondrial calcium is generally maintained at low concentrations, but these organelles can take up and store high Ca^{2+} concentrations. Of note, a proper function of mitochondria has been reported to be required for correct OPC maturation and myelination [110,111]. *In vitro* evidence has shown that cytokine-induced OPC injury involves mitochondrial dysfunction [112,113], while inhibition of OPC differentiation by tumour necrosis factor alpha (TNF-α) is accompanied by alterations in mitochondrial calcium uptake, mitochondrial membrane potential and respiratory complex I activity [113]. However, an evaluation of the purinergic contribution to mitochondrial calcium transport in OPCs has not been defined yet.

4. Parenchymal astrocytes

Astrocytes residing in both brain and spinal cord parenchyma have long been recognized to play key roles in the regulation of neural network activity through modulation of extracellular transmitter levels and ion homeostasis and by directly participating in synaptic activity in the now called 'tripartite synapse' [114]. Notably, these astrocytes do express a wide panel of both P2X and P2YRs, most of which are linked to mobilization of intracellular calcium levels [115]. It has also been known for a quite long time that as a result of injury parenchymal astrocytes become activated, a phenomenon characterized by increased expression of GFAP and marked enlargement of astrocyte cell body and fibres (reactive astrogliosis) [116,117].

For many years, this phenomenon has been viewed as both a positive and a detrimental event for CNS recovery. On one side, reactive astrocytes were recognized to be responsible for the formation of the glial scar, which physically separates the damaged tissue from the surviving one, thus impeding axonal regeneration; on the other side, reactive astrogliosis was soon shown to be important for functional recovery owing to release of beneficial cytokines and growth factors and other trophic actions favouring tissue remodelling and repair in the lesioned area (reviewed in [118]).

However, a seminal breakthrough in reactive astrogliosis was made when evidence was provided that, after injury, reactive astrocytes re-acquire properties similar to those typical of the astrocyte-like cells found in the adult V-SVZ (see §1; [114] and references therein) and in spinal cord central canal [40]. In these niches, astrocyte-like cells indeed behave as progenitor/stem-like cells that, under some circumstances, are able to differentiate to all the three main types of CNS cells, i.e. adult quiescent astrocytes, neurons and oligodendrocytes (see §1). This behaviour is, in turn, reminiscent of that of radial glia, the ubiquitous glial cell type during development, also acting as stem and progenitor cells, which are the source of many, if not most, neurons in vertebrate brains (see §1).

The concept that, among other features, reactive astrocytes re-acquire 'stemcellness' was strongly suggested by work performed in mice subjected to either brain trauma or ischaemia owing to middle cerebral artery occlusion [119]. Specifically, it was demonstrated that, when placed *in vitro*, only reactive astrocytes from the ischaemic side of the brain could generate neurospheres characterized by both self-maintenance and multipotency, as shown by their ability to proliferate and generate astrocytes, neurons and oligodendrocytes under specific differentiation protocols. Astrocytes obtained from the contralateral healthy hemisphere of the same animals were not able to generate neurospheres, to confirm that it is only after injury that astrocytes dedifferentiate to multipotent precursor cells re-expressing stem cell properties that are normally repressed in the adult brain under healthy conditions. In a similar way, upon injury, ECs lining spinal cord central canal were found to be activated, started proliferating and expressing GFAP, suggesting a shift to astrocyte-like progenitor cells [40]. Unfortunately, as already pointed out in §1, generation of new neurons from injury-reactivated adult stem-like cells is strongly hampered by the local unfavourable environment typical of the inflamed CNS, which prevents these cells from fully

expressing their regenerative properties. However, very recent evidence suggests that parenchymal reactive astrocytes may be more prone to express these properties compared with V-SVZ precursors, because, following ischaemic brain injury, some striatal astrocytes were indeed shown to trans-differentiate into functional mature neurons [120]. Obviously, elucidation of the molecular pathways involved in the transition from quiescent to reactive astrocytes would greatly help the set-up of novel neuroreparative strategies.

In this respect, comparison between signals orchestrating the shift of normal parenchymal astrocytes to reactive cells after injury and signals that are active in the endogenous NSC niches have revealed a striking degree of overlap, as shown by involvement of the same molecules (summarized in figure 3). Normally, adult astrocytes do not divide and are polarized towards, and in contact with, the basement membrane (BM) surrounding blood vessels (figure 3a). Leakage of the blood–brain barrier as a result of injury disrupts signalling from the BM to astrocytes, leading to loss of polarization, upregulation of intermediate filaments (IMFs) and hypertrophy. Blood-borne fibrinogen induces secretion of chondroitin sulfate proteoglycans (CSPGs), which activates transforming growth factor beta (TGF-β) signalling (figure 3b). In addition to this, owing to damage, a number of peptide growth factors, such as fibroblast growth factor (FGF), epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) are released in the extracellular milieu to induce astrocytic proliferation via ERK1/2 and the mitogen-activated protein kinase (MAPK) or the mammalian target of rapamycin (mTOR) pathway. Purinergic signals are one of the first and most potently activated after injury, as ATP is immediately released by damaged cells (for a review, see [121]) and may also interact with growth factor signalling in this context [116,117] to phosphorylate the immediate early gene products c-FOS and c-JUN and increase astrocyte proliferation and GFAP expression (figure 3c). These same factors, as well as vascular endothelial growth factor (VEGF) and sonic hedgehog (SHH), are also active in the adult neurogenic niches in vivo, where they promote proliferation of neural stem and progenitor cells (figure 3d). However, while in neurogenic niches the proliferating effects of ATP have been univocally related to P2 receptor-mediated elevation of cytosolic Ca^{2+} concentrations in a synergistic manner with mitogenic growth factors ([30]; see also §1), the involvement of intracellular calcium in the effects mediated by ATP in adult reactive astrocytes is far less defined.

In early studies, exposure of cultured rat cortical astrocytes to ATP and the ATP derivative α,β-methyleneATP resulted in significant elongation of GFAP-positive astrocytic processes, suggesting induction of reactive astrogliosis [122]. In line with the concepts discussed above, this was preceded (and probably mediated) by a very early ERK1/2 activation in the absence of any apparent mobilization of intracellular calcium levels, suggesting this effect to be independent of calcium signalling. Data with a number of P2 receptor antagonists and with pertussis toxin, a G-protein inhibitor, suggested this effect to be mediated by a P2YR subtype that is at variance with the majority of known P2YRs [123] did not seem to be linked to increases of intracellular calcium levels. To investigate in more detail the role of calcium in α,β-methyleneATP-mediated reactive astrogliosis, the intracellular calcium chelator BAPTA-AM was used. BAPTA-AM induced a marked reduction of basal ERK1/2

phosphorylation compared to cultures maintained in standard medium, indicating that basal ERK1/2 phosphorylation in control unstimulated astrocytes is owing to both calcium-dependent and -independent mechanisms. Nevertheless, challenge of cultures with α,β-methyleneATP in the presence of BAPTA-AM resulted in a 7.9-fold stimulation of ERK1/2 phosphorylation that was very similar to that obtained in standard calcium-containing medium. On this basis, authors concluded that the purine analogue could activate intracellular pathways leading to reactive astrogliosis even in the absence of calcium [122]. However, it has to be said that, since then, detection of calcium signalling has undergone significant technical improvements and that lack of sufficiently sensitive methods at those times has prevented the measurement of small, but biologically significant, early astrocytic calcium increases contributing to induction of reactive astrogliosis. In this respect, using transgenic mice that express an ultrasensitive genetically encoded Ca^{2+} indicator, YC-Nano50, in an astrocyte-specific manner, Kanemaru and co-workers [124] have recently reported the in vivo visualization of spontaneous subtle and localized astrocytic Ca^{2+} signals (Ca^{2+} twinkles), which are preferentially displayed in fine astrocytic processes in living mice brain.

In addition to this, the same authors have shown that astrocytic calcium signalling and the downstream function of N-cadherin, a calcium-dependent cell–cell adhesion glycoprotein, play indispensable roles in the reactive response to a neocortical stab wound injury (SWI) and, highly relevant to this review, they have linked these mechanisms to ATP signalling [124]. Specifically, they first demonstrated that brain injury induces inositol 1,4,5-trisphosphate (IP3)-dependent Ca^{2+} signalling in astrocytes and that this is required for SWI-associated astrogliosis. Moreover, type2 IP3 receptor knockout (IP3R2KO) mice, deficient in astrocytic Ca^{2+} signalling, showed impaired reactive astrogliosis and increased injury-associated neuronal death. Next, they explored the mechanisms of Ca^{2+}-dependent reactive astrogliosis by comparing the gene expression profiles of Ca^{2+} signal-silent and -active astrocytes in culture and found that Ca^{2+} signal-active astrocytes have very low levels of the translational repressor pumilio 2 (Pum2). They then reasoned that proteins whose translation is regulated by Pum2 are expected to be Ca^{2+}-dependently upregulated during astrogliosis and found that N-cadherin was indeed markedly increased in reactive astrocytes. In previous studies, N-cadherin and its family proteins were initially characterized by their function in cell–cell adhesion, but were subsequently found also to regulate other cellular functions, including differentiation, proliferation, cell polarization and migration, which are likely to be at the basis of the detected reactive astrogliosis [124]. Interestingly, these authors also found that the Ca^{2+}-mobilizing agonists ATP and ET1 induced downregulation of Pum2 and concomitantly upregulated N-cadherin in cultured astrocytes, thus linking astrocytic calcium mobilization by purinergic signalling to reactive astrogliosis. These findings are consistent with previous studies demonstrating that N-cadherin expression was increased when astrocytes were subjected to rapid and reversible mechanical strain and that this effect was reproduced in a time- and concentration-dependent manner by treatment of cultures with ATP [125]. Globally, these data support ATP as an initiation signal in reactive astrogliosis and suggest that mobilization of intracellular calcium by classical IP3-linked P2Y receptors plays

a major role in mediating the transition of quiescent to activated astrocytes.

Authors' contributions. All authors equally contributed to this manuscript.

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