● **PERSPECTIVE**

Contribution of purinergic receptors to spinal cord injury repair: stem cell-based neuroregeneration

Traumatic spinal cord injury (SCI), defined as physical trauma to the spinal column yielding altered motor, sensory, or autonomic function, is a devastating neurological disease causing major impact at both personal and societal level. SCI is characterized by a primary insult (compression, contusion or laceration) followed by a secondary pathological cascade that propagates further injury disrupting motor, sensory and/or autonomic functions. The primary mechanical trauma produces necrosis, edema, hemorrhage and vasospasm. Afterwards, a cascade of secondary pathophysiological mechanisms is induced, including ischemia, apoptosis, fluid and electrolyte disturbances, excitotoxicity, lipid peroxidation, production of free radicals, and an inflammatory response, resulting in further damage due to swelling and blood flow reduction (Tator et al., 2012). Worldwide incidence of SCI is 15–40 cases per million people annually and affects predominantly to young adults. SCI often involves a lifelong disability including paralysis distal to the lesion, loss of sensation, neuropathic pain, and bowel/bladder dysfunction as a result of axonal damage, demyelination, and death of oligodendrocytes, astrocytes, interneurons, and motor neurons. Unfortunately, to date no effective treatment exists for the major neurological deficits of SCI. However, there are several hopeful neuroprotective agents being currently investigated in ongoing preclinical and clinical trials (Tator et al., 2012). The aim of neuroprotective treatments is not only to reduce cell death and reduce mechanisms of secondary injury, but also to promote regeneration and tissue repair. One of these promising therapeutic strategies consists on cell transplantation to replace dead or damaged cells and provide trophic support. In particular, adult neural stem/progenitor cells (NSPCs) are especially attractive to promote tissue repair after SCI, since they can readily expand *in vitro* to form colonies of undifferentiated cells called neurospheres, and are committed to the neural lineage. Adult NSPCs may have advantages over embryonic or fetal cells: 1) in some cases it will be possible to harvest them in an autologous fashion; 2) they may have less oncogenic potential than embryonic stem cells; and 3) the avoidance of some of the ethical issues surrounding the use of stem cells of embryonic or fetal origin. NSPCs consist primarily of progenitor cells and a small percentage of stem cells. Neural stem cells are multipotent cells that continuously proliferate and divide to self-renew and generate daughter committed to differentiation into neurons, oligodendrocytes, and astrocytes. In contrast, neural progenitor cells are more restricted, with a limited proliferative capacity and differentiation potential (Mothe and Tator, 2012). In the adult brain, NSPCs are found within specific niches including the subventricular zone (SVZ) lining the lateral ventricles of the forebrain and the subgranular layer of the dentate gyrus of the hippocampus (Kriegstein and Alvarez-Buylla, 2009). The periventricular region containing the central canal of the adult spinal cord also contains NSPCs called ependymal progenitor/stem cells (epSPCs), which have the ability to rapidly proliferate, migrate, and differentiate into neurons and glia to regenerate the injured cord in lower vertebrates. In mammals, including humans, proliferation of epSPCs and their progeny is a frequent event during embryonic and early postnatal periods of development. The turnover of epSPCs declines significantly in the postnatal period, but extensive epSPCs proliferation has been observed in response to SCI. SCI induces proliferation of ependymal cells and migration of their progeny towards the site of injury, where they differentiate and give rise mainly to astrocytes as well as myelinating oligodendrocytes (Meletis et al., 2008). Using specific differentiation protocol, 90% of differentiated cultures of epSPCs obtained after SCI stain positive for the motor neuron-specific marker HB9, with 32% of these motor neurons displaying electrophysiological properties that resemble those of functional spinal motor neurons (Moreno-Manzano et al., 2009).

Following SCI, large amounts of ATP and other nucleotides are released by the traumatized tissue leading to the activation of purinergic receptors that, in coordination with growth factors, induce lesion remodeling and repair (Burnstock and Ulrich, 2011). Nucleotides activate two different types of purinergic receptors called P2X and P2Y receptors. The P2X are ion gated channels which lead to a fast calcium influx whereas P2Y are G-protein coupled receptors. To date, seven P2X ionotropic subunits (P2X1–7) and eight P2Y metabotropic receptors (P2Y$_{1,2,4,6,11,12,13,14}$) have been cloned and characterized according to their agonist sensitivity, sequence identities, and signal transduction mechanism. Purinergic receptors are expressed in NSPCs from very early stages of mammalian embryonic development, suggesting their participation in the regulation of proliferation and lineage specification. Neurospheres obtained from fetal rat brain expressed P2X2 and P2X7 receptor subunits, as well as P2Y$_1$, P2Y$_2$, P2Y$_4$, and P2Y$_6$ receptors. Functional purinergic receptors have also been identified in the SVZ of the lateral ventricles and the hippocampal dentate gyrus (Oliveira et al., 2015). Neurospheres derived from the adult mouse SVZ express P2X4 and P2X7 mRNA, and have functional P2Y$_1$ and P2Y$_2$ receptors, whose activation increases cell proliferation in the presence of growth factors and stimulates the migration of neural progenitors isolated *in vitro* and expanded as neurospheres, which may be of relevance for the local movement of cells in the neurogenic niches. Moreover, P2Y$_1$ receptor antagonists reduce neurosphere size and neurosphere-forming frequency of primary neurospheres derived from adult mouse SVZ, allowing the possibility that a proportion of the neural progenitor cell population differentiates into neurons and glia. Recently, we have demonstrated that epSPCs express functional ionotropic P2X4 and P2X7, and metabotropic P2Y$_1$ and P2Y$_4$ receptors, able to respond to ATP, ADP, and other nucleotidic compounds (Gomez-Villafuertes et al., 2015). Furthermore, recent studies demonstrated that acute transplantation of epSPCs isolated from SCI donors 1 week after severe contusion (ependymal stem/progenitor cells after injury, epSPCis) promotes motor recovery with neuroprotective and neuroregenerative effects (Moreno-Manzano et al., 2009). Interestingly, epSPCis proliferate 10 times faster *in vitro* that control epSPCs, display enhanced self-renewal, and are enriched in neuroblasts and oligodendrocyte progenitor cells, thereby minimizing the proportion of astrocytes and migroglia. A comparative genetic profile analysis reveals the upregulation of proinflamatory signalling cascades, including vascular endothelial growth factor (VEGF)/mitogen-activated protein kinases (MAPK) and Jak/Stat pathways, in epSPCis compared to epSPCs derived from healthy donors (Moreno-Manzano et al., 2009). Moreover, a comparative study between epSPCs versus epSPCis reveals that a downregulation of P2Y$_1$ receptor together with an upregulation of P2Y$_4$ receptor occur in epSPCis (Gomez-Villafuertes et al., 2015). P2Y$_1$ receptor downregulation could be facilitating the differentiation of epSPCis into neuronal/glial cells that participate in the improvement of functional locomotor recovery observed after epSPCi transplantation, while an increment in P2Y$_4$ receptor expression in epSPCis could increase the expansion and mitotic index of neural progenitor cells within those neurospheres. These findings suggest that the expression levels of P2Y receptors may play a critical role in the modulation of neural progenitor cell expansion. In support of this idea, some reports demonstrate that purinergic signaling might be required not only for developmental progenitor cell expansion and neurogenesis, but also to maintain NSPCs niches in the adult brain. External addition of ATP or its analogues increases the mitotic index and rate of neural progenitor cells, whereas P2Y antagonists suppress both neurosphere expansion and the mitotic index of cells within those neurospheres (Miras-Portugal et al., 2015). Remarkably, severe traumatic spinal cord contusion induces early and persistent increase in the expression of P2X4 and P2X7 receptors around the injury, which can be completely reversed by acute transplantation of undifferentiated epSPCis, correlating with a functional locomotor recovery in a rat model of SCI (Gomez-Villafuertes et al., 2015). The overexpression of P2X4 receptors following spinal cord lesion was previously reported, identifying the majority of P2X4-positive cells as activated microglia/
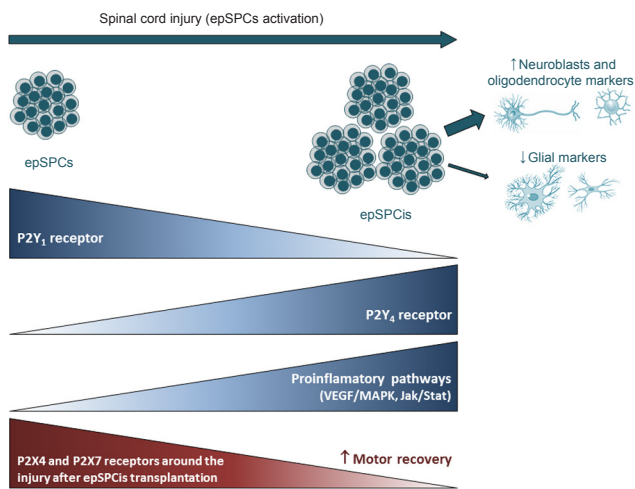
Figure 1 Activation of ependymal stem/progenitor cells profile after spinal cord injury (SCI).
In response to traumatic SCI, ependymal stem/progenitor cells (epSPCs) display enhanced mitotic index and self-renewal, and are enriched in neuroblasts and oligodendrocyte progenitor cells, minimizing the proportion of glia. Moreover, a deep modification in the genetic profile of epSPCs after injury (epSPCis) is observed, a downregulation of $P2Y_1$ receptor expression occurs in epSPCis, whereas $P2Y_4$ receptors and proinflamatory signaling cascades, including vascular endothelial growth factor (VEGF)/mitogen-activated protein kinases (MAPK) and Jak/Stat pathways, are upregulated. Furthermore, SCI induces early and persistent increase in the expression of P2X4 and P2X7 receptors around the injury, which can be completely reversed by acute transplantation of epSPCis.

macrophages and surviving neurons/neurites. P2X4 receptor expression is also enhanced in spinal cord microglia after peripheral nerve injury, and P2X4 knock-out mice have lower levels of neuroinflammation after SCI, resulting in significant improvement in tissue sparing and functional recovery, especially during the first week after injury (Miras-Portugal et al., 2015). Concerning P2X7, post-ischemic, time-dependent upregulation of this receptor on neurons and glial cells has already been reported, but the relationship between its inhibition and the pathogenesis of contusive spinal cord injury remains controversial (Marcillo et al., 2012). P2X7 receptor arouses special interest due to its dual function as an inhibitor of neurogenesis and axon outgrowth and inductor of cell death in other cases. In cultured hippocampal neurons axonal growth and branching was induced following P2X7 receptor inhibition, whereas a strong activation of P2X7 receptor causes necrosis/apoptosis in both embryonic and adult neural precursor cells. Interestingly, it is suggested that the cell death elicited by P2X7 receptor activation may counter-regulate progenitor cell survival after CNS injury, where excessive neuro- and gliogenesis is induced (Miras-Portugal et al., 2015). The expression of P2X7 receptors is transcriptionally regulated by specificity protein 1 factor (Sp1) in basal conditions (Garcia-Huerta et al., 2012). Sp1 is a multifunctional protein expressed constitutively that directly binds with high affinity to GC-rich motifs located in the DNA to modify the expression of a wide variety of genes. At the transcriptional level, Sp1 is not induced in injured neurons, but functions as a scaffolding protein to recruit injury-inducible transcription factors such as c-Jun, ATF3, and STAT3 to switch the expression of regeneration-associated genes (RAGs) on and off according to the regeneration program (Kiryu-Seo and Kiyama, 2011). Interestingly, the activity of Sp1 can be upregulated by interplay with other nerve-injury transcription factors, including p53, c-myc, and AP-2, which may participate in the Sp1/c-Jun/ATF3/STAT3 complex to yield maximal activation when increased gene expression is required in the event of a fatal emergency such as SCI. Considering that the expression of P2X7 receptors is transcriptionally regulated by Sp1, we can speculate that nerve-injury transcription factors could be capable of enhance P2X7 expression under pathological

situations, similarly to what happens with RAGs.

The intrinsic potential of epSPCs to replace some of the cells in the spinal cord after injury opens up the opportunity for developing non-invasive cell replacement therapies restricting the fate of ependymal progeny in order to generate mainly oligodendrocytes and motorneurons to specifically replace the loss of functional units. Receptors for purines or pyrimidines appear early in evolution and are prominently involved in embryonic development. This, together with the recent recognition of the expression of several P2X and P2Y receptor subtypes in NSPCs is indicative that purinergic signaling is an important contributing factor in the earliest steps of stem/progenitor cell proliferation, migration and differentiation. Clearly, full knowledge of the involvement of purinergic signaling in epSPCs biology remains to be elucidated, but our initial results in collaboration with Moreno-Manzano's group in a rat model of spinal cord contusion (Gomez-Villafuertes et al., 2015) strongly support the involvement of the purinergic signaling both in neural progenitor cells proliferation/differentiation and in the control of inflammation-related signaling pathways triggered by SCI (summarized in **Figure 1**). If this is the case, being able to manipulate this purinergic modulation would enable a greater control over the neurogenic capacity of epSPCs.

Rosa Gomez-Villafuertes[*]

Department of Biochemistry and Molecular Biology IV, Veterinary School, Universidad Complutense of Madrid, Madrid, Spain

*Correspondence to: *Rosa Gomez-Villafuertes, Ph.D., marosa@ucm.es.*

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