The therapeutic potential of targeting exchange protein directly activated by cyclic adenosine 3′,5′-monophosphate (Epac) for central nervous system trauma

A Brief Overview of Central Nervous System Trauma

Central nervous system (CNS) trauma commonly occurs in the forms of traumatic spinal cord and brain injuries (TSCI and TBI). It is estimated that around 27 million people live with TSCI worldwide (GBD 2016 Traumatic Brain Injury and Spinal Cord Injury Collaborators, 2019). CNS injuries often result in permanent sensorimotor disability. The poor ability of adult mammalian CNS neurons to regrow in response to injuries is known to be associated with their very limited intrinsic growth capacity and the inhibitory environment at the lesion site post-injury (Curcio and Bradke, 2018). This review aims to discuss the importance of exchange protein directly activated by cAMP (Epac) in overcoming these barriers, in conjunction with its role in neural development, regeneration and cell death as well as modulation of the post-injury environment.

The pathophysiology of TSCI

The events following TSCI can be classified into four phases: acute (<48 hours), subacute (48 hours to 14 days), intermediate-to-chronic (14 days to 6 months) and chronic (>6 months) (Badhiwala et al., 2018). During the acute phase, the initial physical trauma causes damage to neural cells and the disruption of vasculature and the blood spinal cord barrier leading to haemorrhages and ischemia (Tator and Koyanagi, 1997; Mautes et al., 2000). Neurons and oligodendrocytes in the injury area undergo necrosis due to haemorrhagic/ischemic events and are also subjected to a further cascade of outcomes, which arise from changes in cell permeabilization and pro-apoptotic signaling (Choo et al., 2007). At the same time, peripheral inflammatory cells including macrophages, neutrophils and lymphocytes infiltrate the injury area and secrete mediators such as tumour necrosis factor-α and interleukin-1β (IL-1β) (Pineau and Lacroix, 2007). These cells...
remain beyond the subacute phase. The exposed myelin from damaged oligodendrocytes is a source of myelin-associated inhibitors, such as Nogo, oligodendrocyte myelin glycoprotein and myelin-associated glycoprotein (MAG), which are known to prevent axonal regrowth after TSCI (Berry, 1982; Caroni and Schwab, 1988). All these molecules signal through the NgR-p75NTR receptor complex via the RhoA protein, which leads to activation of Rho kinase and inhibition of axonal regrowth (Filbin, 2003; Fournier et al., 2003).

In the subacute phase, the secondary SCI cascade is characterized by prolonged inflammation and further death of neurons and oligodendrocytes (Kwon et al., 2004; Badhiwala et al., 2018). The underlying mechanisms for apoptosis include ischemia, which disrupts ionic balance of K+, Na+ and Ca2+ leading to depolarization of cell membranes, ATPase failure and increase of intracellular calcium (Agrawal and Fehlings, 1996; Badhiwala et al., 2018), as well as increased levels of the excitatory neurotransmitter, glutamate (Farooque et al., 1996; McAdoo et al., 1999). Activated microglia, together with infiltrated inflammatory cells, facilitate ongoing apoptosis and produce free radicals, which are formed as by-products of debris clearance by immune cells, caused by lipid membrane peroxidation, DNA oxidative damage and protein oxidation, leading to further apoptosis (Dizdaroglu et al., 2002; Hausmann, 2003). Preclinical studies have shown that neuronal and oligodendrocyte apoptosis can last up to 4 weeks and 1 year, respectively, post-TSCI (Beattie et al., 2002; Huang et al., 2007).

The chronic phase of the injury is characterized by reorganization of neural circuits and vascularization, demyelination and alterations in the composition of the extracellular matrix. In humans and some animal models, the continued cell death during the acute phase leads to the formation of cystic cavities containing cellular debris, extracellular fluid, and macrophages (O’Shea et al., 2017; Braddy and Burnside, 2019). These cavities are a poor substrate for neural regeneration and therefore act as a physical barrier for axonal regrowth (O’Shea et al., 2017). The cavities are lined by scar tissue that is generally classified into two components: fibrotic and glial. The fibrotic, inner component of the scar is primarily a product of fibroblasts migrating to the lesion site from the disrupted vasculature and meningeal layers (Klapka and Muller, 2006; Fawcett et al., 2012). These cells deposit dense collagen depositions forming a condensed structure, which adds up to a physical barrier for axonal regrowth (Klapka and Muller, 2006; Goritz et al., 2011; Fawcett et al., 2012). The glial, outer component of the scar is made up of reactive astrocytes, which proliferate, undergo hypertrophic changes and overlap their processes around the cavities creating a mesh-like array that forms walls of neural tissue from the harmful environment of the cavities (Yuan and He, 2013). During the chronic phase, activated glial cells (e.g., microglia, astrocytes and macrophages), together with oligodendrocyte progenitor cells (OPCs), secrete extracellular matrix proteins that are inhibitory to axonal growth, such as chondroitin sulphate proteoglycans (CSPGs) (McKeon et al., 1991, 1999; Silver and Miller, 2004; Busch and Silver, 2007). CSPGs may also affect OPCs, as they prevent the outgrowth of OPC processes and differentiation, therefore leading to weakened remyelination and an observable decrease in axonal regeneration (Siebert and Osterhout, 2011). The key players and the formation of the glial scar and cavity are demonstrated in Figure 1.

Search Strategy and Selection Criteria

A first broad search was done by AGB in December 2018 using PubMed and key words such as “spinal cord injury”, “CNS trauma”, “axon regeneration”, “cAMP”, “Epac”, “neuroinflammation” and combinations of those words. No limits were used. A second broad search was performed by DD between September 2019 and January 2020 using PubMed with the aim to update information and add new studies. Special emphasis was given to the use of key words such as “cAMP AND Microglia OR astrocytes” and “Epac AND Microglia OR astrocytes”. No limits were used.

**Roles of Cyclic Adenosine 3′,5′-Monophosphate in the Central Nervous System**

Cyclic adenosine 3′,5′-monophosphate (cAMP) is one of the most widely studied intracellular second messengers, as it exists in all types of cells. Over the decades, cAMP signaling has increasingly become a focal point for neural regeneration studies. In the nervous systems, cAMP is known to participate in the regulation of many neuronal behaviors such as axonal growth and guidance as well as neuronal survival and differentiation.

**Role of cAMP signaling in CNS development**

During CNS development, cAMP is vital for the growth of neurons and is expressed at high levels. However, neuronal levels of cAMP decline at postnatal age and reach a minimum in adult animals (Cai et al., 2001; Shewan et al., 2002). It is known that embryonic CNS neurons transplanted into adult CNS tissue grow axons extensively (Li and Raisman, 1993), which contrasts to adult CNS neurons transplanted on to immature CNS tissue, which fail to extend neurites in vitro (Shewan et al., 1995). These events can be attributed to differential cAMP levels in embryonic versus adult neurons, which regulate axonal guidance. Growth cones can read environmental signals which lead to either attraction or repulsion of axons. The variation of cAMP levels in growth cones can determine the cone response to external guidance cues such as Netrin-1 or MAG, which are guidance molecules important during neural development or after injury (Song et
Role of cAMP in CNS regenerative capability

The adult mammalian CNS is characterized by very poor intrinsic neuronal cell regenerative abilities. The so-called ‘conditioning lesion’ is a traditional approach to augment the intrinsic machinery for regeneration in CNS neurons, in which a peripheral injury enhances central regeneration of primary sensory neurons (McQuarrie and Graefstein, 1973; Richardson and Issa, 1984). This phenomenon has been associated with cAMP levels since the injection of a cAMP analogue into the dorsal root ganglion (DRG) can mimic the effects of the conditioning lesion (Neumann et al., 2002; Qiu et al., 2002). These studies led to the understanding of the involvement of cAMP levels in regeneration. Therefore, a major biochemical event linked to the poor regeneration capacity of the CNS is a significant decrease of intrinsic neuronal cAMP levels that occurs postnatally. For example, adult rat DRG neurons that are capable of growth following peripheral nerve lesion have relatively high concentrations of cAMP, while the opposite is observed in adult rat DRG neurons that do not regenerate readily following spinal cord bilateral lesion (Qiu et al., 2002). Notably, the cAMP levels are likely to further decrease after TSCI, as following thoracic contusion injury of the spinal cord of adult rats it was found that cAMP levels decreased by over 60% in the spinal cord as well as in brainstem and cortex (Pearse et al., 2004). Therefore, deteriorating neuronal regenerative abilities after TSCI could be attributed to a further drop in cAMP levels. Previous studies have then focused on the effects of artificially elevating neuronal cAMP levels to promote regrowth after injuries.

In vitro evidence of stimulating cAMP activity to promote neurite outgrowth

The elevation of cAMP using forskolin in cultured adult rat sensory neurons was reported to enhance neurite outgrowth (Neumann et al., 2002). Furthermore, the modulation of cAMP activity by a permeable analogue of cAMP was found to alter the responses of Xenopus spinal axons to several inhibitory axonal guidance cues, shifting growth cone responses from repulsion to attraction (Song et al., 1998). It was previously reported that the upregulation of intracellular cAMP increased neurite outgrowth in cultured embryonic rat motor neurons while antagonizing cAMP resulted in a profound decrease in neurite outgrowth (Aglah et al., 2008). Moreover, cAMP was also shown to positively affect neuronal survival. It was shown that cAMP elevation by forskolin sufficed to promote short term survival of embryonic rat spinal motor neurons in vitro (Hanson et al., 1998). Therefore, it has been suggested that cAMP is important not only in neurite outgrowth but also in neuronal survival.

In vivo evidence of cAMP elevation to promote axonal regrowth

A number of research groups have targeted cAMP and elements of its signaling pathways to promote axonal regrowth. Two studies showed that manipulating levels of cAMP activity, by the injection of the cAMP analogue, dibutyryl cAMP (db-cAMP; Table 1), into adult rat DRG before spinal cord lesioning, resulted in an increase in the number of axons regrowing across the lesion site (Neumann et al., 2002; Qiu et al., 2002). However, neurons in those studies were conditioned prior to lesion, which is not a clinically relevant approach. Subsequently, more clinically relevant experiments used rolipram, which inhibits phosphodiesterase 4 (PDE 4; Table 1) and cAMP elevation by rolipram in combination with the phosphodiesterase 4 inhibitors, however, none of the treatment options such as cAMP analogues or PDE4 inhibitors have been translated to the clinic. Factors to be considered for clinical translation using cAMP elevation manipulation include cAMP’s ubiquitous expression by all types of cells and potential side effects. A more specific indication of cAMP signaling could be pursued by systemic rolipram treatment (Hebenstreit et al., 1989; Scott et al., 1991). Therefore, scientists are actively investigating more specific targets that could limit systemic side effects of cAMP signaling. The potential key molecular mechanisms and pathways of cAMP elevation on neuronal/axonal regrowth are illustrated in Figure 2.

Epac: a Downstream Effector of Cyclic Adenosine 3',5'-Monophosphate

The downstream effectors of cAMP include cyclic nucleotide receptor involved in sperm function, Popeye domain-containing proteins, cyclic nucleotide-gated ion channels, protein kinase A (PKA), and Epac (Brand and Schindler, 2017). In recent years, the latter two have been shown to play major roles in regulating the responses of the nervous system to physical trauma. Epac has been shown to play a role in a wide range of cellular functions such as cell growth, adhesion, differentiation, division, inflammation and neurotransmission (Peace and Shewan, 2011). It is known that there are two mammalian isoforms of Epac, of which Epac1 is expressed...
ubiquitously throughout the body, whereas Epac2 is mainly expressed in postnatal CNS tissue such as brain and spinal cord, but is also found in adrenal gland, heart, pancreas, small intestine and testis (Peace and Shewan, 2011; Ramos and Antonetti, 2017). Furthermore, recent data show that Epac2 has at least 3 different sub-isoforms: Epac2A, 2B, and 2C, which are differentially expressed in brain/heart/adrenal glands, adrenal gland/pancreas, and liver respectively (Hoivik et al., 2013). Moreover, the protein levels of Epac1 and Epac2 might vary accordingly. Thus, Epac2 targeting may potentially result in greater tissue specificity due to postnatal CNS expression leading to greater efficacy. This gives hope for neural tissue-restricted manipulation of Epac2 and resultant localized effects.

Only very recently, specific tools have been developed to specifically discern between Epac1 and Epac2 manipulation (Schwede et al., 2015). For instance, both in vitro and in vivo evidence shows that 8-Br-cAMPS (S-220) is the most potent and selective activator of Epac2 over Epac1, whereas 8-pCPT-2′-O-Me-cAMP (8-Me-cAMP) is a general Epac agonist, and neither activate PKA (Table 1). However, a specific antagonist is currently only available for Epac2 (ESI-05; Table 1). Although, a specific antagonist for Epac1 is not yet available, and neither activate PKA.

### Epac and axonal guidance during neural development

As Epac serves as a major effector of cAMP, its role in positive chemotactic behaviour in growing axons has been investigated. For example, using siRNA against both Epac1 and Epac2 resulted in decreased neurite outgrowth in embryonic rat spinal cord culture model of SCI (Tsalkova et al., 2012; Zhu et al., 2015).

**Table 1** | Drugs manipulating cAMP and its downstream signaling molecules

<table>
<thead>
<tr>
<th>Drug type</th>
<th>Activity</th>
<th>Outcome</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>cAMP analogues</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-Br-cAMP</td>
<td>Cyclic adenosine monophosphate (cAMP) analogue</td>
<td>Mouse neonatal mature astrocytes upregulated antioxidant-related genes and downregulated cell death-related genes after treatment</td>
<td>Paco et al., 2016</td>
</tr>
<tr>
<td>db-cAMP</td>
<td>Injection into adult rat dorsal root ganglion (DRG) before spinal cord injury (SCI) resulted in an increase in the number of axons regrowing across the injury</td>
<td>Neumann et al., 2002; Qiu et al., 2002</td>
<td></td>
</tr>
<tr>
<td>Sp-cAMPS</td>
<td>Improved functional recovery after spinal contusion in adult female rats</td>
<td>Pearse et al., 2004</td>
<td></td>
</tr>
<tr>
<td>Epac agonists</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-Me-cAMP</td>
<td>General exchange protein activated by cAMP (Epac) agonist</td>
<td>Adult rat DRG neurons cultured on spinal cord tissue increased neurite outgrowth after treatment</td>
<td>Enserink et al., 2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adult rat DRG neurons switched their repulsive response to MAG gradients to attraction after treatment</td>
<td>Murray, 2008b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BV-2 cells significantly decreased their phagocytic behaviour after treatment</td>
<td>Steininger et al., 2011</td>
</tr>
<tr>
<td>S-220</td>
<td>Specific Epac2 agonist</td>
<td>Adult rat DRG neurons increased neurite outgrowth after treatment</td>
<td>Wei et al., 2016</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neonatal rat DRG neurons showed a strong turning behaviour towards treatment</td>
<td>Schwede et al., 2015</td>
</tr>
<tr>
<td>Epac antagonists</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESI-05</td>
<td>Specific Epac2 antagonist</td>
<td>Neonatal rat DRG and cortical neurons increased neurite outgrowth after treatment, even in an inhibitory environment</td>
<td>Guijarro-Belmar et al., 2019</td>
</tr>
<tr>
<td>ESI-09</td>
<td>General Epac antagonist</td>
<td>Embryonic hippocampal neurons decreased neurite outgrowth after treatment</td>
<td>Munoz-Llanca et al., 2015</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rolipram</td>
<td>Phosphodiesterase type 4 inhibitor</td>
<td>Improved functional recovery after treatment in spinal contusion in adult female rats</td>
<td>Pearse et al., 2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased axonal plasticity and improved recovery after treatment in spinal hemisection in adult rats</td>
<td>Nikulina et al., 2004</td>
</tr>
<tr>
<td>KT-5720</td>
<td>Protein kinase A antagonist</td>
<td>Improved locomotor abilities after treatment in spinal contusion in adult rats</td>
<td>Costa et al., 2013</td>
</tr>
<tr>
<td></td>
<td></td>
<td>In vitro embryonic rat spinal cord culture model of SCI increased neurite outgrowth after treatment</td>
<td>Boomkamp et al., 2014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Discussion of antagonist specificity</td>
<td>Munoz-Llanca et al., 2015</td>
</tr>
</tbody>
</table>

In vivo rat SCI model

In vitro embryonic rat spinal cord culture model of SCI increased neurite outgrowth after treatment

In vitro embryonic rat spinal cord culture model of SCI increased neurite outgrowth after treatment

In vitro embryonic rat spinal cord culture model of SCI increased neurite outgrowth after treatment
Switched their repulsive response to MAG gradients to attraction, which further supports the notion that Epac is responsible for mediating positive chemotactic behaviour. Furthermore, FRET imaging confirmed that when netrin-1 is added to culture media, embryonic rat DRG growth cones increased selective activation of Epac and not PKA (Murray et al., 2009b). The above evidence, together with existing evidence showing that Epac1 expression is prominent during embryonic/neonatal stages whereas Epac2 expression increases postnatally, suggest that Epac1 mediates attractive axonal guidance during neural development, which is opposite to the repulsive role exerted by PKA (Murray et al., 2009b).

**In vitro evidence of Epac in promoting axonal growth**

Epac activation has been found to promote neurite outgrowth *in vitro*, which can also be achieved by cAMP elevation. The general Epac agonist 8-Me-cAMP was able to induce significant neurite outgrowth of cultured adult rat DRG neurons, comparable to that seen when these neurons where treated with cAMP agonists *in vitro* (Murray and Shewan, 2008; Wei et al., 2016). Moreover, 8-Me-cAMP treatment led to an increase in neurite density and myelination similar to that of Rolipram in an *in vitro* model of SCI using embryonic rat myelinating spinal cord cultures (Boomkamp et al., 2014). Recently, in *in vitro* work has revealed that Epac2 activation by the agonist S-220 significantly enhances neurite outgrowth of neonatal rat cortical and DRG neurons (Guijarro-Belmar et al., 2019) (Figure 3A–C). Moreover, the administration of a cAMP analogue, Sp-cAMPs, to adult DRG neurons that were transfected with Epac1/2 siRNA did not rescue neurite outgrowth (Murray and Shewan, 2008). The above evidence strongly suggests that Epac2 is a key player when it comes to mediating cAMP-induced neurite growth at postnatal and adult stages. The specific involvement of Epac2 in neurite outgrowth was further verified by recent findings in which neonatal rat DRG and cortical neurons transfected with Epac2 siRNA, or cultured with the specific Epac2 antagonist ESI-05, showed significantly reduced neurite outgrowth (Guijarro-Belmar et al., 2019) (Figure 3D–I). Furthermore, treatment of embryonic hippocampal neurons with a general Epac antagonist, ESI-09, also reduced neurite outgrowth ( Munoz-Llancao et al., 2015).

The effect of Epac activation on neurite outgrowth can also be revealed when neurons are cultured in an inhibitory environment. For example, when neonatal and adult rat DRG neurons were cultured on inhibitory adult rat spinal cord slices and received treatment with 8-Me-cAMP, a significant increase in the percentage of neurons with long neurites was observed, which did not occur when adult rat neurons were treated with Rolipram or Sp-cAMPs (Murray and Shewan, 2008). Moreover, after knocking down Epac1/2 expression with siRNA in embryonic DRG neurons that normally grow long neurites on adult spinal cord sections, the number of cells growing long processes was significantly reduced (Murray and Shewan, 2008). In a recent study, it has been shown that CSPGs significantly inhibit neurite outgrowth by 35% of cultured neonatal rat cortical neurons. However, treatment with S-220 induces the neurons to overcome CSPG inhibition and thereby a significantly increased neurite outgrowth was reported (Guijarro-Belmar et al., 2019; Figure 4A–D). These results are similar to what was previously found with cerebellar neurons, in which the use of MAG substrates reduced neurite outgrowth by 40% and was rescued by the addition of the cAMP agonist db-cAMP (Cai et al., 1999). Guijarro-Belmar et al. (2019) used neonatal DRG neurons co-cultured with inhibitory mature astrocytes and showed that, on contact with those astrocytes, less than 20% of DRG neurites grew over the astrocyte surface, with most neurites exhibiting contact-mediated avoidance. These results are consistent with those shown by Adcock et al. (2004) where only 15.1 ± 2.3% of postnatal rat DRG...
measured by the amount of β-tubulin-III protein levels in the brain following neural trauma could provide further confirmation to the in vitro findings where Epac2 activation promotes neurite outgrowth. The potential of Epac2 activation was further explored in a clinically relevant adult rat contusion SCI model, in which the Fmoc hydrogel incorporated with S-220 was directly injected into the lesion cavity 3 weeks after the injury, representing a subacute stage of SCI (Guijarro-Belmar et al., 2019). Over a 4-week period post-gel injection, animals receiving treatment with S-220 delivered by the gel showed significant improvement in locomotor behaviour, reaching on average 3 BBB scales higher than contusion-only animals. Although axonal regrowth was not assessed in that study, it is likely that S-220, locally delivered and released by the gel, promoted axonal regrowth mediated by Epac2 activation. The potential key molecular mechanisms and pathways of Epac2 signaling that aid neuronal/axonal regrowth are illustrated in Figure 2.

**Epac and Neuronal Death**

**In vitro evidence of Epac-mediated neuronal death**

Although convincing evidence shows that Epac activation promotes axonal growth in vitro, ex vivo and in vivo, recent evidence suggests that an increased level of Epac2 activity in cortical neurons after neural trauma might lead to neuronal death (Zhang et al., 2018; Zhuang et al., 2019). Application of OxyHb, a chemical used to induce intracranial cerebral haemorrhage in animal models, has been shown to elevate Epac2 protein levels in cultured embryonic rat cortical neurons, which coincides with increased neuronal apoptosis (Zhuang et al., 2019). In vitro treatment with the Epac2 inhibitor, ESI-05, in the presence of OxyHb resulted in significantly reduced apoptotic death in those cells. However, this study was carried out on embryonic neurons, which were previously shown to express significantly lower levels of Epac2 protein than their adult counterparts (Murray and Shewan, 2008); therefore, the underlying mechanism of this increased Epac2 in OxyHb-exposed embryonic neurons is unclear.

**In vivo evidence of Epac-mediated neuronal death**

In vivo evidence has also suggested that increased Epac2 protein levels in the brain following neural trauma could...
lead to neuronal apoptosis. Following intracranial cerebral haemorrhage or traumatic brain injury in adult rats, Epac2 levels have been shown to increase significantly in cortical neurons, leading to neuronal apoptosis (Zhang et al., 2018; Zhuang et al., 2019). However, treatment with ESI-05 was shown to attenuate Epac2-mediated neuronal apoptosis in these studies and result in improved neurological functions. The underlying mechanism could be via a p38-mediated cell death pathway, as treatment with ESI-05 also led to reduced levels of p-p38 (Zhang et al., 2016). Moreover, our preliminary evidence showed that Epac2 was lost when ESI-05 was applied to culture media, suggesting a protective role of Epac2 in this model (Calderon-Sanchez et al., 2019).

In particular, it is now known that following SCI, there are heterogeneous astrocyte populations in and around the lesion site (Sofroniew, 2014). Therefore, previous literature using cAMP elevation strategies in vivo SCI models as discussed in Section 3 might have also impacted on astrocytes. However, direct evidence of this is lacking.

An elevated cAMP level is crucial for the differentiation of astrocytes from neural precursor cells (NPCs). In cultured embryonic rat cortical NPCs, treatment with pituitary adenylate cyclase-activating peptide increases cAMP levels, which leads to a profound increase in the percentage of cells (~80%) expressing GFAP and S-100B and having a typical astrocyte-like stellate morphology, with no upregulation of neuronal or oligodendrocyte markers (Vallejo and Vallejo, 2002; Figure 6B). Moreover, cAMP has been shown to promote differentiation of astrocytes from C6 glioma cells in the presence of interleukin-6 (Takanaga et al., 2004).

Increased intracellular cAMP levels by treating cultured neonatal rat astrocytes with adrenaline, forskolin or db-cAMP for 30–60 minutes are associated with reduced cell cross-sectional areas and increased cell perimeters (Vardjan et al., 2014). Moreover, Increased intracellular cAMP levels in these cultured cells are also associated with increased sprouts, protrusions, and elongated processes on the membrane surface (Vardjan et al., 2014, 2016). Activation of β-adrenoreceptors with adrenaline in cultured neonatal rat astrocytes can also reduce cell swelling induced by hypotonic conditions, an effect that is also observable with an increase in cAMP level (Vardjan et al., 2016; Figure 6A).

Furthermore, elevation of cAMP levels in astrocytes is known to induce a pro-survival state. After treatment with β-Br-cAMP, a cAMP analogue (Table 1), cultured mature astrocytes from neonatal mouse cortex showed an upregulation of antioxidant-related genes and a downregulation of cell death-related genes (Paco et al., 2016; Figure 6A).

**CAMP and microglia**

Microglia are CNS innate immune cells. They participate in neurogenesis, programmed cell death and synapse elimination, as well as the establishment and remodeling of neuronal circuits during development (Li and Barres, 2018). At adult stages, microglia are known to be responsible for phagocytosis, secretion of growth factors and propagation of immune responses. Microglia, upon activation, can resume similar phenotypes as macrophages, i.e., M1, which is a pro-inflammatory and neurotoxic state, and M2, which is a pro-regenerative phenotype (Colonna and Butovsky, 2017).

Increasing intracellular cAMP levels in cultured microglial cell line BV-2 cells by forskolin, IBMX, or β-adrenergic agonist isoproterenol decreases phagocytic behaviors of the cells (Steinger et al., 2011; Figure 6C). Treatment of BV-2 cells with lipopolysaccharide (LPS) resulted in induction of the M1 phenotype characterized by upregulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 proteins and downregulation of Arg1, transglutaminase-2 and RELM-α (Ghosh et al., 2016). When they were exposed to LPS and treated with IL-4 and db-cAMP simultaneously, cells showed a shift towards a M2 phenotype, which is characterized by iNOS/cyclooxygenase-2 downregulation and Arg1 upregulation (Figure 6C). However, neither IL-4 nor db-cAMP on its own was sufficient to induce the M2 phenotype in LPS-exposed BV2 cells. Moreover, systemic co-treatment with IL-4 and db-cAMP at 15 minutes following a contusion SCI in adult
rats resulted in significantly increased Arg1 in ED1-labelled microglia and macrophages (Ghosh et al., 2016). These findings suggest that cAMP might play an important role in regulating microglia phenotype and its effects on BV-2 cell and microglial activation might require the presence of IL-4.

Epac and astrocytes
Both Epac1 and Epac2 have been implicated in astrocyte biology. In mature astrocytes cultured from neonatal rat cortex, treatment with the Epac general agonist 8-Me-cAMP causes increases intracellular calcium levels (Di Cesare et al., 2006). In mice with global knockout of Epac2, cerebral GFAP expression was decreased at birth (Seo and Lee, 2016), suggesting that Epac2 might be important for astrocytic differentiation. Moreover, when NPCs cultured from Epac2-KO mice were treated with pituitary adenylate cyclase-activating peptide to increase cAMP, these cells failed to increase GFAP expression. Therefore, it is likely that cAMP elevation might act through Epac2 to modulate astrocytic differentiation.

Epac and microglia
Epac1 and Epac2 have also been shown to influence microglial activation. When treated with the Epac general agonist-8-Me-cAMP, BV-2 cells significantly decreased their phagocytic behaviour (Steininger et al., 2011). In vitro activation of Epac2 in LPS-exposed microglia from neonatal rat cortex significantly attenuated their activation by inducing resting morphology and reducing iNOS expression and nitrite release (Ghosh et al., 2016). Notably, regrowing axons accompanied the astrocyte processes, suggesting that S220-treated astrocytes might provide guidance to regrowing axons (Figure 8G and H).

Conclusions
Although cAMP plays an important role in regulating the post-lesion environment after TSCI, as well as neuronal chemotactic behaviour, growth and survival, the direct
modulation of CaMP signalling is unlikely to find a use in the clinic due to the ubiquity of the CaMP pathways in humans. Instead, a downstream effector of CaMP, Epac2, represents a promising target to manipulate the outcome of TSCI. Epac2 is mainly expressed postnatally in the CNS and mediates positive effects of CaMP on neuronal growth and guidance. Epac2 can also modulate microglial/astrocyte activation and astrocyte morphology after SCI to be more supportive to axonal regrowth. Therefore, Epac2 activation is likely to positively affect cells that play crucial roles following TSCI and make the post-injury environment more supportive for the regrowing axons. As Epac2 expression is largely limited to adult CNS and new sub-isosforms are being discovered, it brings hope for CNS tissue-specific effects and reduction of possible side effects, which occur with treatment options that manipulate CaMP levels. Moreover, further combinations with other strategies such as locomotor training (which also enhances CaMP signalling) could maximize and further promote functional recovery. However, further investigation of the molecular events downstream of Epac/Rap1 is essential to reveal the mechanisms that lead to the modulation of the inhibitory environment and axonal regeneration.

References


