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Review Article

Recent advances in the therapeutic uses of chondroitinase ABC

E. Muir[a,*], F. De Winter[b], J. Verhaagen[b], J. Fawcett[c]

[a] Department of Physiology, Development and Neuroscience, University of Cambridge, UK
[b] Laboratory for Neuroregeneration, Netherlands Institute for Neuroscience, Amsterdam, the Netherlands
[c] John Van Geest Centre for Brain Repair, University of Cambridge, UK

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ABSTRACT

Many studies, using pre-clinical models of SCI, have demonstrated the efficacy of chondroitinase ABC as a treatment for spinal cord injury and this has been confirmed in laboratories worldwide and in several animal models. The aim of this review is report the current state of research in the field and to compare the relative efficacies of these new interventions to improve outcomes in both acute and chronic models of SCI. We also report new methods of chondroitinase delivery and the outcomes of two clinical trials using the enzyme to treat spinal cord injury in dogs and disc herniation in human patients. Recent studies have assessed the outcomes of combining chondroitinase with other strategies known to promote recovery following spinal cord injury and new approaches. Evidence is emerging that one of the most powerful combinations is that of chondroitinase with cell transplants. The particular benefits of each of the different cell types used for these transplant experiments are discussed. Combining chondroitinase with rehabilitation also improves outcomes. Gene therapy is an efficient method of enzyme delivery to the injured spinal cord and circumvents the issue of the enzyme's thermo-instability. Other methods of delivery, such as via nanoparticles or synthetic scaffolds, have shown promise; however, the outcomes from these experiments suggest that these methods of delivery require further optimization to achieve similar levels of efficacy to that obtained by a gene therapy approach. Pre-clinical models have also shown chondroitinase is efficacious in the treatment of other conditions, such as peripheral nerve injury, stroke, coronary reperfusion, Parkinson's disease and certain types of cancer. The wide range of conditions where the benefits of chondroitinase treatment have been demonstrated reflects the complex roles that chondroitin sulphate proteoglycans (its substrate) play in health and disease and warrants the enzyme's further development as a therapy.

1. Introduction

Chondroitinase ABC (ChABC) is a bacterial enzyme from Proteus Vulgaris. It is a lyase that degrades the chondroitin sulphate and dermatan sulphate chains of proteoglycan molecules; it also possesses hyaluronidase activity (Prabhakar et al., 2005; Yamagata et al., 1968). It has been widely used as a strategy to promote repair following spinal cord injury (SCI) (Bradbury et al., 2002; Garcia-Alias et al., 2009; Tester and Howland, 2008; Yick et al., 2000; Zuo et al., 1998c). Most of the therapeutic effects of ChABC can be attributed to its ability to degrade the sugar chains from a class of proteoglycan molecules, the chondroitin sulphate proteoglycans (CSPGs), (Asher et al., 2001; McKeon et al., 1991). This enables the enzyme to degrade molecules inhibitory to nerve regeneration and to break down structures enriched in these molecules, the perineuronal nets (PNNs). These structures act to restrict plasticity (Barritt et al., 2006; Bradbury et al., 2002) and their removal

Abbreviations: ChABC, chondroitinase ABC; CSPGs, chondroitin sulphate proteoglycans; PNNs, perineuronal nets; GAP-43, growth associated protein-43; NGF, nerve growth factor; BDNF, brain-derived growth factor; NT3, neurotrophin 3,GDNF glial derived neurotrophic factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; bPDGF, basic platelet-derived growth factor; PDGF-AA, platelet-derived growth factor; VEGF, vascular endothelial growth factor; TNF-α, tumor necrosis factor alpha,IL1-β interleukin beta; cAMP, cyclic adenosine monophosphate; BBB, Basso Beattie & Bresnahan; SOCS3, suppressor of cytokine signalling 3; PNG, peripheral nerve graft; LLL, low level laser; KLF7, Kruppel-like factor 7; PNS, peripheral nervous system; CNS, central nervous system; CST, corticospinal tract; AAV, adeno-associated virus; 5HT, 5-hydroxytrptamine (serotonin); TH, tyrosine hydroxylase; PTEN, phosphatase & tensin homologue; SH3, src homology domain 3; LTP, long-term potentiation; VP16, virus activation domain 16 (transcription activation domain); ES, embryonic stem cells; BMSC, bone marrow stromal cells; OECs, olfactory ensheathing cells; MSC, mesenchymal stem cells; NSCP, neural stem cell progenitors; iPSC, induced pluripotent stem cells; drNPCs, directly reprogrammed neural progenitor cells.

* Corresponding author.
E-mail address: emm1@cam.ac.uk (E. Muir).

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allows new nerve connections to be made, with beneficial consequences that can be harnessed to obtain therapeutic effects (Banerjee et al., 2017; Garcia-Alias et al., 2009; Gherardini et al., 2015; Pizzorusso et al., 2002). This review focuses on recent advances in therapies using ChABC in animal models of SCI and peripheral nerve damage. However, the potential wider applications of ChABC as a therapy are also summarized.

2. ChABC and SCI

Spinal cord injury (SCI) is a devastating condition for which there is currently no effective treatment (Lee et al., 2014). Permanent paralysis occurs below the site of injury. Patients may also experience urinary and sexual dysfunction, respiratory impairment and neuropathic pain. In the UK alone approximately 50,000 people are affected with a further 1000 new cases per year. The condition arises due to the inability of central nervous system neurons to repair the damage following injury (Cajal, 1928).This is due in part to the presence of molecules in the environment of the damaged spinal cord which block the repair process. One of the major contributory factors is the build-up of a class of molecules called CSPGs (Bradbury et al., 2002; Filous et al., 2014; Lemons et al., 1999; Morgenstern et al., 2002; Zuo et al., 1998c). ChABC breaks down these molecules (Bradbury et al., 2002; Lemons et al., 1999; Zhao et al., 2011), thereby promoting nerve regeneration and functional recovery. It also promotes plasticity, which allows undamaged neurons to sprout and take over the function of the damaged neurons. This is particularly relevant in the case of SCI because most injuries are incomplete. Indeed, when ChABC-treatment is combined with a peripheral nerve graft, restoration of urinary (Lee et al., 2013) and respiratory function have been demonstrated (Alilain et al., 2011). The beneficial effects of the enzyme have been replicated by many independent laboratories and in several animal models (Bradbury et al., 2002; Lee et al., 2012; Mondello et al., 2015). Importantly, as well as being effective in treating acute injuries it has also more recently been shown to be efficacious for chronic injuries (Garcia-Alias et al., 2009; Lee et al., 2012; Mondello et al., 2015; Warren and Alilain, 2019; Warren et al., 2018), greatly extending the number of patients who could potentially benefit.

In the early studies, which first demonstrated the therapeutic effects of ChABC as a strategy to promote nerve regeneration, the enzyme was delivered to the cord via intrathecal injection. However, this approach may require multiple injections for efficacy, as the enzyme is thermally unstable. Therefore, alternative strategies of enzyme delivery have been investigated.

2.1. Strategies of ChABC delivery

2.1.1. Gene therapy

Viral vectors are an efficient method of delivering therapeutic factors to the injured spinal cord. Sustained long-term release is ensured and stability of labile factors is not an issue because transduced cells at the injury site continuously synthesise them. Moreover, this method of delivery is particularly advantageous for large molecules such as ChABC, where diffusion of the protein from an injection site or scaffold will be restricted due to its large molecular size. We have shown that the efficacy of ChABC is significantly enhanced when the enzyme is delivered by gene therapy. Our study revealed for the first time that large scale degradation of CSPGs by ChABC resulted in immunomodulatory effects which limited the secondary damage that normally ensues following the primary insult (Bartus et al., 2014) and this resulted in a large reduction in the cavity size of the lesion. Behavioural improvement was also demonstrated using a ladder walk test. These effects were correlated with the high levels of ChABC achieved by delivery of the enzyme via a viral vector. Virally delivered ChABC also resulted in expression over a much larger area of the cord compared to injections of the bacterial enzyme (Bartus et al., 2014). This is an important finding because the cord dimensions in humans are much larger so a wider spread of the enzyme will likely be required for efficacy. Indeed, intrathecal injections of ChABC did not degrade PNNs in the spinal cord gray matter of larger animals, such as the pig, where multiple intraparenchymal were needed to achieve PNN digestion (Rosenzweig et al., 2019). In a second study we evaluated the gene therapy approach of enzyme delivery in a cervical contusion model of SCI. Again, extensive digestion of CSPGs in and around the lesion site occurred, as well as reduced lesion pathology. This was accompanied by enhanced nerve conduction through the lesion site and behavioural improvements. Rats exhibited better scores for forelimb performance in the ladder walk test and improved grip strength (James et al., 2015).The similar efficacy of virally-delivered ChABC in this model of SCI is important as the majority of patients sustain injuries at the cervical level. Moreover, this study confirms the enhanced efficacy of gene therapy-delivered ChABC, compared to delivery via intrathecal injection of the bacterial protein. In our most recent study we explored the effects of short-term and long-term ChABC delivery. We developed a lentiviral vector containing an immune inert on/off switch. This enables regulated long-term expression of ChABC *in vivo*. This showed that short-term ChABC administration (2.5 weeks) delivery of ChABC produced functional improvements in sensory axon conduction and the ladder walk test whilst long-term ChABC administration resulted in additional improvements. Rats treated with ChABC for 8 weeks exhibited improvements in skilled reaching and grasping tasks indicating enhanced neuroplasticity following long-term administration leads to recovery of motor control (Burnside et al., 2018). These findings suggest that widespread, high levels of ChABC combined with long-term delivery may be required for optimal outcomes. Moreover, the ability to control the levels and timing of ChABC production will enable the optimal levels of ChABC and the therapeutic window to be determined. This is a major step towards clinical application (Burnside et al., 2018).

2.1.2. Synthetic scaffolds and stabilized ChABC

ChABC is unstable at 37° C, most of its activity is lost within 72 h (Tester and Howland, 2008). One of the methods which has been employed to enhance the enzymes' stability is to add co-solvents to the enzyme mixture. One of these, trehalose, has been shown to enhance the enzymes' thermostability (Nazari-Robati et al., 2012) and it has more recently been shown to confer protection against proteolytic degradation and inactivation via oxidation (Nazari-Robati et al., 2016). Trehalose extended the activity of ChABC *in vitro* to 14 days which was sufficient to bring about significant functional improvements in dogs with naturally occurring SCI when delivered in conjunction with lipid microtubes (Hu et al., 2018). Sucrose has also been shown to be effective at stabilizing the enzyme (Raspa et al., 2019). This extended ChABC activity to 14 days which was sufficient to reduce gliosis, enhance axon regeneration and bring about a modest but significant improvement in BBB score (13vs10.5) when combined with rehabilitation. Although the effect is modest, the results are encouraging as the injury was a contusion injury and the enzyme was injected at the chronic stage, 4 weeks post-injury. Moreover, rehabilitation was initiated prior to enzyme delivery, which may not be the optimal strategy, (Marsh et al., 2011).

Delivery of ChABC from scaffolds to enhance the enzyme's stability and attain slow release is another strategy that has been evaluated.

Ni et al. (2015) used poly(propylene carbonate)-chitoscan microfibres to achieve extended release of ChABC and then tested this approach in a rat hemisection model of SCI. The *in vitro* study showed that most of the ChABC was released by 10 days, but only 26% of this was active enzyme. *In vivo* this resulted in an increase in cells staining for GAP-43, a marker for regenerating neurons, and a slight reduction in staining of the lesion site with CS56, an antibody which binds to intact CSPGs. No improvements were seen in the BBB scores. These findings suggest this method of delivery currently results in ChABC levels that are insufficient to have a significant effect on functional recovery.

Table 1
Delivery of ChABC via biomaterials

Author	Biomaterial	SCI injury model	Duration of ChABC release	Outcome
Raspa et al., 2019	Sucrose	Contusion T9/10 Chronic (4 weeks).	14days	Reduced gliosis, improved BBB, 10.5vs13 (when combined with rehab).
Ni et al., 2015	Poly(propylene carbonate)-chitoscan microfibers, electrospun	Hemisection, acute	10 days	↑GAP-43, ↓CS56, no change in BBB score.
Zuidema et al., 2016	Nanoparticles	Hemisection, acute	14 days	↑GAP-43, ↓↓2B6. Enhanced axon outgrowth
Colello et al., 2016	Electrospun scaffold (poly dioxinone 2 violet) with Aliginate beads	Complete transection, acute	Most by 24h, all by 72h.	ChABC alone ND. (with NGF, BBB score 2vs7, at 3 weeks).
Xia et al., 2017	Poly(propylene carbonate)chitoscan, electro spun.	Hemiscetion acute	10.5 days	↑BBB score, 14 vs15 at 4 weeks for ChABC alone.
Pakulska et al., 2017	XMC hydrogel	Compression, acute	Most by 24h, ↓CSPGs for 2 weeks	Improvements in ladder walk test, no change in BBB score.
Zhang et al., 2013	Poly(lactide-co-glycolic acid) microspheres	Transection, acute	ND	↑BBB score 8.95vs 7.46 for ChABC alone at 10 weeks
Hu et al., 2018	Trehalose stabilised ChABC in Lipid microtubes.	Canine, severe, naturally occurring, T & L levels, chronic	15 days	Improvements only seen in ChABC group

Zuidema et al. (2016), evaluated the therapeutic effects of ChABC released from nanoparticles in a rat thoracic contusion model of acute SCI. The nanoparticles stabilized the enzyme, which was released over a period of 2 weeks *in vivo,* and digestion of CSPGs was demonstrated. The pattern of staining was consistent with penetration into the cord, which was associated with enhanced axon outgrowth (GAP-43 staining), evident 5 days post-injury. These results suggest that delivery of ChABC via nanoparticles is superior to that attained with the poly (propylene carbonate)-chitoscan microfibres. However, the extent of digestion produced in and around the lesion site (2.1mm vs 8.5mm) is several orders of magnitude less than that obtained when ChABC is delivered via gene therapy.

Furthermore, it is likely that more longer-term delivery will be required for optimal efficacy; 8 weeks of high level of ChABC are required to regain skilled tasks, such as reaching and grasping following an acute contusion injury to the spinal cord (Burnside et al., 2018).

Manufactured biomaterials have also been employed to deliver ChABC in combination therapies, a summary of the results is given in the table below (Table 1).

2.2. Effect of timing, dose and treatment duration

2.2.1. Acute SCI

The need for substantial levels of ChABC to achieve efficacy is supported by the findings of Cheng et al, They showed that whilst a low dose (1U) of ChABC (delivered 2 weeks post- injury) was without effect, in a complete transection model of rat SCI, a high dose (50 & 100U) promoted axon-outgrowth and a modest amount of functional recovery (BBB score 1vs3) at 10 weeks post-injury. Smaller improvements in BBB scores were evident as early as 2 weeks post-injury. It is of note that when the high dose ChABC was evaluated in a contused rat SCI, no improvements were seen (Cheng et al., 2015). Since virally delivered ChABC results in the significant improvements in a similar contusion model of SCI (Bartus et al., 2014; James et al., 2015), this supports the view that either higher doses, or more long-term delivery of ChABC are required for efficacy. Another interpretation of these results is that it is due to the timing of administration. Lentiviral delivery of ChABC, at the acute stage of SCI, results in delivery of ChABC at the onset of gene expression. This is 24 - 48h post-transduction of the cells at the site of injury, so it is possible that higher levels of ChABC are required for efficacy at the subacute stage, when the glial scar is more established. Another illustration of the effects of timing and dose of ChABC administration is the observation that application of high doses of ChABC, concurrently with SCI, is toxic (Cheng et al., 2015), possibly because early on, CSPGs play an essential role in isolating the injury site from the surrounding tissue, limiting further tissue damage (Rolls et al., 2009).

The Bartus study revealed for the first time the immunomodulatory effects of ChABC, which resulted in a marked reduction in cavity size. This correlated with an influx of wound-resolving M2-type macrophages as a result of upregulation of the ant-inflammatory cytokine, IL10 and downregulation of the pro-inflammatory cytokine IL12-B, in the ChABC-treated animals (Didangelos et al., 2014). Alterations in the extracellular matrix are likely responsible for these effects, which require high doses of the enzyme (Didangelos et al., 2014). The beneficial immunomodulatory effects of ChABC take place early on after injury. They could be demonstrated when ChABC was injected before SCI (24 & 48h) and up to 7 days post-injury (Didangelos et al., 2014). These findings are consistent with the Bartus study where ChABC was delivered in high dose by lentivirus. Expression of transgenes from these vectors is initiated 24 - 48h post-injection. However, it is of note that when ChABC was delivered via an adeno-associated viral vector (AAV) vector, although the extent of CSPG digestion was similar to that seen with the lentiviral vector-delivery, reduction in lesion cavitation, indicative of immune protection, was not observed (Bradbury lab, unpublished observations). Expression from AAV vectors initiates 1 week

post-injection and reaches maximal levels by 3 weeks. This suggests that ChABC delivery within 1 week of injury may be required to fully exploit the immunomodulatory properties of the enzyme. Since delivery of high doses of the enzyme at the time of injury can have detrimental consequences (Cheng et al., 2015), 48h–7days post-injury maybe the optimal time window for the treatment of acute SCI with high doses of ChABC. Indeed injection of ChABC at I week post-injury, resulted in significant improvements in BBB scores (Janzadeh et al., 2017; Liu et al., 2018), supporting the view that timing of delivery plays an important role in efficacy following acute SCI.

Single, low doses of ChABC, injected at the time of injury are tolerated, but in general, result in only modest improvements in terms of functional/anatomical recovery; Pakulska et al., 2017; Wang et al., 2017; Xia et al., 2015; Xu et al., 2015; demonstrated no improvements in BBB scores, Akbari et al. (2017), only a modest improvement, whilst Alilain et al. (2011) and Warren et al. (2018), demonstrated only a modest improvements in breathing when ChABC (alone) was delivered acutely. Nonetheless, there are exceptions; significant functional improvements occur following injection of ChABC concurrently with injury when used in combination with PNG transplant, NT3, or rehabilitation (Alilain et al., 2011; Garcia-Alias et al., 2015; Hunanyan et al., 2013), which may reflect the potency of these combination therapies. It is of note, however, that functional recovery is observed when ChABC is delivered at the time of injury via a controlled release system (Colello et al., 2016; Xia et al., 2017; Zhang et al., 2013) which may be a result of extended duration of administration. Significant improvements in BBB scores were also observed when ChABC is delivered on the day of injury together with MSCs. This was demonstrated in both a transection model of SCI (Xiong et al., 2016) and the more challenging, clinically relevant, contusion model of SCI (Sarveazad et al., 2017).

The importance of the duration of ChABC administration is highlighted in our most recent study (Burnside et al., 2018) and by a study conducted in cats (Mondello et al., 2015). The latter study supports the view that the duration of ChABC administration directly impacts on outcomes. Cats treated with ChABC for 2 weeks showed improvements on the ladder walk test and there was regeneration of rubrospinal axons beyond the lesion site. Cats treated with ChABC for 4 weeks showed additional improvements in the more demanding skilled motor tests (peg and narrow beam). This is consistent with more long-term administration being required for optimal efficacy. Interestingly, the results suggest that short-term ChABC administration can be detrimental, worsening the outcome of some behavioural tasks, likely due to restricted plasticity closing off neuronal circuits which have the potential to lead to further improvements in the more demanding skilled motor tasks. It is of note that their study also showed that whereas in control cats and the 2 week ChABC group there was a direct correlation between tissue sparing and the amount of behavioural recovery, this correlation was not apparent in the group treated for 4 weeks, suggesting that longer term ChABC administration can be effective in treating severe lesions.

2.2.2. Chronic SCI

There are still relatively few studies investigating the effects of ChABC in animal models of chronic SCI. However, Shinozaki et al. (2016) reported that injection of ChABC 6 weeks post-injury for 1 week, followed by rehabilitation for 8 weeks, resulted in a modest but significant improvement in BBB score in rats with thoracic (T10) contusion SCI. Two studies in dogs with chronic SCI also demonstrated the beneficial effects of ChABC on outcomes. Lee et al. (2017) injected ChABC, 3 weeks post–injury, with MSCs, and BDNF, into dogs with SCI compression injuries at L4 and demonstrated improvements in BBB scores. Hu et al. (2018) conducted a clinical trial using dogs with severe chronic (> 3 months) SCI at levels T3-L3. All, dogs in the ChABC group showed improvements and some dogs regained the ability to ambulate unassisted. Nori et al. (2018) assessed the effect of dr NPCs + ChABC 7 weeks post-injury in rats with clip contusion injuries at T7. Improvements in the catwalk test and BBB scores were demonstrated. These findings are encouraging, but not all interventions were successful; no locomotor improvement occurred when ChABC was delivered with NT3, BDNF and NSCP to rats with chronic (13 weeks) contusion SCI at T10 (Jin et al., 2016). ChABC combinations have also been assessed in cervical models of SCI. Suzuki et al. (2017) showed that delivery of ChABC with NSCP 8 weeks post-injury resulted in improvements in forelimb grip and catwalk tests. Warren et al. (2018), showed that injection of ChABC into the phrenic motor pool could restore breathing from 12 weeks up to 1.5years post-injury in a hemisection model of SCI. They also report it is effective in rats with chronic contusion injuries (Warren and Alilain, 2019). Thus, a single injection of ChABC alone may be sufficient to restore function in rats with cervical SCI because of the compensatory rewiring of the spinal circuits which follows injury (Vinit and Kastner, 2009) whilst injuries at lower levels of the spinal cord may require more complex interventions, such as long-term ChABC delivery with cell/PNG transplant, rehabilitation and a cocktail of factors tailored to promote regeneration of the neuronal subpopulations that fail to grow in the chronically injured cord (DePaul et al., 2017).

One of the first studies evaluating the efficacy of ChABC in a primate model of SCI has recently been published (Rosenzweig et al., 2019). Rhesus monkeys with a C7 hemisection, were treated with ChABC delivered by multiple intraparenchymal injections 4 weeks post-injury. The neuronal circuits involved in hand function were targeted (injections C7 to T1). This resulted in significant improvements in pellet retrieval and other hand function tasks. Moreover, this was accompanied by enhancement of corticospinal axon length, density and synapse formation below the lesion site. This is consistent with ChABC mediating recovery by promoting sprouting of spared axons. It is of note that Rhesus monkeys undergo a period of spontaneous plasticity following C7 hemisection. The resulting improvements in hand function occur 5 to 8 weeks post-injury after which no further improvements are observed. In contrast the ChABC treated animals continued to improve for up to 20 weeks post-injury, suggesting that the enzyme extends the window of spontaneous plasticity. At the end of this time the ChABC treated animals recovered 47% of the hand function they had pre-injury as compared to 31% in controls. Interestingly, no increase in number or length of serotonergic fibres were detected. This contrasts with observations in rodents and cats. This may reflect the fact that serotonergic fibres do not undergo spontaneous sprouting following hemisection in Rhesus monkeys which would again be consistent with ChABC acting to enhance and extend a period of natural plasticity. Importantly, this study highlights the differences between rodents and primates. Primates also reflect the human condition more closely with regard to the size of the spinal cord, variability between individuals and immunology. However, in terms of predicting the efficacy and safety of the enzyme for treating human SCI, the results should be interpreted with caution. Most human injuries resemble more closely a contusion model of SCI and furthermore one cannot accurately predict the immunogenicity of the enzyme in humans from results obtained in primates. Thus the enzyme's immunogenicity should be evaluated. Nonetheless, these results are very encouraging, especially as, again, no evidence of adverse effects (microglial response, neuronal toxicity, inflammation or pain) was noted.

2.3. Interventions that target early events following SCI

The effects of early delivery of ChABC in an acute model of thoracic contusion SCI have been assessed (Akbari et al., 2017). The study measured the levels of markers of oxidative stress and inflammation between 4h and 7 days post-injury. ChABC was found to reduce the levels of TNFα, IL1β (an inflammatory cytokine), nitric oxide (an indicator of oxidative stress), malondialdehyde (an enzyme involved in lipid peroxidation) and myeloperoxidase (involved in neutrophil

infiltration). This resulted in improved functional recovery (BBB score 0vs2.8) at 7 days. These results highlight the potency of ChABC as a therapy for SCI. However, since some of these beneficial effects of ChABC occur very early (4h post-injury), translational studies in human patients should aim for intervention as early as medically feasible.

A study by Milbreta et al. (2014), identified two further mechanisms by which ChABC exerts its beneficial effects on repair and regeneration following SCI. They assessed the effects of ChABC on aspects of astrogliosis and revascularisation at the lesion site using a rat hemisection model (T8/9) of SCI. ChABC was given acutely, then two more times for up to 10 days. GFAP staining was reduced, consistent with a reduction in astrogliosis. Additionally, an extended period of astrocyte remodelling from 5 days to 4 weeks was observed. This resulted in more astrocytes with long thin processes aligned towards the lesion. Regenerating axons were found to be associated with these aligned processes, suggesting that they promote axon outgrowth. Moreover, the number of axons penetrating the lesion site was greatly enhanced in the ChABC-treated animals. When the effects on neo-angiogensis were investigated, the beneficial effects of ChABC emerged at a later time point. Revascularization of the lesion site was prolonged and more vigorous in the ChABC-treated animals but this was only evident from 4 weeks onwards. By 10 weeks post-injury, the vasculature more closely resembled that of intact tissue (Milbreta et al., 2014).

2.4. ChABC combination therapies

Over the last 5 years there has been a substantial increase in studies evaluating the outcomes of combination therapies. The pleiotropic actions of ChABC, which simultaneously target multiple aspects of CNS injury, make it unique amongst the technologies currently in use to promote repair and recovery in the CNS. Moreover, these activities do not overlap with the actions of most other therapies used to promote repair following SCI (Zhao and Fawcett, 2013). ChABC will therefore likely form an essential component of any combination treatment. It has been combined with cell transplants, growth factors, transcription factors, ion channels, agents that block myelin inhibitors of regeneration and agents that raise the levels of cAMP. In all cases the effects were synergistic with the actions of ChABC (Zhao and Fawcett, 2013). Since this was written ChABC has been used in conjunction with a number of other therapies which are reviewed here.

2.4.1. New combination therapies with ChABC

A novel combination of low level laser (LLL) treatment and ChABC was investigated by Janzadeh et al. (2017) using rats with clip compression SCI. LLL was used to limit the inflammatory response which ensues post-injury. It was applied 30 minutes post-surgery and continued on a daily basis for 2 weeks. ChABC was administered 1 week later and the rats assessed for functional recovery (BBB score) over 4 weeks. LLL but not ChABC was found to reduce the expression of aquaporin 4 at the chronic stages of injury. However, LLL alone induced a fibrotic scar, which is sub-optimal for axon outgrowth. ChABC, as expected, removed the inhibitory CSPGs from the glial scar but had no effect on cytotoxic edema. This is consistent with its lack of effect on aquaporin levels at the chronic stages of injury. LLL + ChABC caused a reduction in the levels of glycogen synthase kinase-3β, which is upregulated following SCI and has been implicated in demyelination and Wallerian degeneration. Consistent with the down-regulation of glycogen synthase kinase-3β, an increase in axon number and degree of myelination was observed in this group. Cavity size was also reduced and a functional recovery (BBB score) demonstrated. Thus, the combination treatment avoids the deposition of a fibrotic scar and cytotoxic edema, resulting in improved functional recovery, which was evident as early as one week post-injury and continued over the duration of the 4 week experiment (Janzadeh et al., 2017). Similar results were obtained in another study, using hyperbaric oxygen combined with ChABC in the same model of SCI (clip compression followed by ChABC 1 week later). Hyperbaric oxygen reduced lipid peroxidation and the levels of aquaporin 4. Levels of glycogen synthase kinase 3β were reduced and this was accompanied by enhanced myelination and motor function recovery. BBB scores were improved (7.5vs13), (Liu et al., 2018).

In another approach Xia et al. (2015) target the glial scar. They used ChABC-treatment to remove inhibitory CSPGs and antisense vimentin cDNA to modify the phenotype of reactive astrocytes. ChABC and retrovirus encoding antisense vimentin were delivered every other day for 2 weeks. The antisense treatment was successful in reducing vimentin and GFAP expression. Both individual treatments were effective at promoting corticospinal tract (CST) regeneration caudal to the lesion centre of rats with hemisected spinal cords but the combination treatment performed the best. The combination group also had, on average, higher BBB scores than the control group but these differences were not significant.

2.4.2. ChABC and peripheral nerve grafts

DePaul et al. (2015), investigated a combination of peripheral nerve graft (PNG), acidic fibroblast growth factor and ChABC on recovery following complete transection of the spinal cord in mice. Acidic fibroblast growth factor was included, as it has been shown to promote long-distance unbranched axon regrowth, as opposed to the more branched regeneration observed following administration of other neurotrophic factors such as NGF, BDNF and NT3. Here a mouse model of SCI was used because the aim of the study was to assess recovery of bladder function and in this respect the mouse model more closely resembles the pathology of the human condition. This combination was shown to induce nerve regeneration (5HT+ and TH+) through the graft and out into the distal spinal cord. Moreover, this was accompanied by a significant improvement in urinary function (DePaul et al., 2015). In a follow-up study, the efficacy of the same combination therapy on rats with chronic contusion SCI was determined (DePaul et al., 2017). The intervention, carried out 8 weeks post-injury, resulted in regeneration of TH+ but not 5HT axons into the distal spinal cord. Locomotor function (BBB score) and urinary function did not significantly improve but did not deteriorate further. Interestingly, the lack of regeneration of the 5HT neurons correlated with enhanced expression of SOCS3 (DePaul et al., 2017). This raises the intriguing possibility that the intrinsic ability of neurons to regenerate varies with subtype and time post-injury. PTEN expression was similar in both neuronal subtypes suggesting this pathway does not play a significant role in determining the regenerative capacity of these neuronal subsets.

Using a different strategy to promote repair of a transected rat spinal cord, Xu et al. (2015) assessed the ability of axons to regenerate across a ChABC-treated PNG in the presence of a kinesin-5 inhibitor (monastrol). The hypothesis being that by inhibiting Kinesin-5, the microtubule species which promotes axon extension would be favoured over those promoting growth cone collapse. Indeed, *in vitro* studies have shown that inhibition of this kinesin allows axons to partially overcome inhibitory cues and grow on inhibitory substrates like CSPGs (Xu et al., 2015). In this study, monastrol + PNG + ChABC enhanced anatomical regeneration, but this was not accompanied by functional recovery (BBB score).

Wu et al. (2015), combined constitutively active caRheb (which activates mTOR) with thermostabilised ChABC and a PNG to promote regeneration of a completely transected spinal cord (T7/T8). The primary aim of this dual approach is to both boost the intrinsic ability of neurons to regenerate and remove inhibitory CSPGs from the environment. They found that only in the combination treatment group, axons (mostly of propriospinal origin) grew out of the transplanted peripheral nerve graft to form putative synapses with neurons caudal to the graft (Wu et al., 2015).This study was extended to assess the efficacy of the same combination in an incomplete cervical SCI. Again, more axons grew out of the peripheral nerve graft in the combination group and this was accompanied by some functional recovery consistent with the formation of new functional synapses (Wu et al., 2015).

Wang et al. (2017) also employed a strategy both to boost the intrinsic capacity of neurons to regenerate and to remove CSPGs from the lesion site with ChABC. An activated form of KLF7 (KLF/VP16 chimera) a transcription factor associated with regenerating neurons, was introduced into DRGs or cortical neurons following either a dorsal transection injury or a dorsal crush injury. ChABC and KLF both increased the proximity of retracting neurons to the injury site in the transection model of injury but only KLF7 in the crush paradigm. Neither treatment resulted in regeneration across the lesion site in either injury model, suggesting other factors are required to produce long-distance regeneration.

However, using a similar clip compression model of SCI, and gene therapy-mediated delivery of ChABC, we found that regenerating axons were observed not only closer to the lesion epicentre, but also up to 300μm beyond it (Zhao et al., 2011). The discrepancy between our findings and the results described in the Wang study may be due to differences between the two studies in the extent of CSPG digestion achieved, a point raised by the authors. We have found that a wider area of CSPG digestion correlates positively with efficacy (Bartus et al., 2014). The wider area of digestion in the Bartus study could be a result of enhanced viral transduction due to the use of high viral titres and/or improved expression of the enzyme in the spinal cord as a result of the use of PGK rather than CMV-driven ChABC gene expression. It is of note in this regard, that transduction with LVPGKChABC (LVP) resulted in more fibres (~20%vs10%) approaching and crossing the lesion epicentre compared to animals transduced with LVCMVChABC (LVC). Regenerating fibres also extended further past the lesion epicentre, 300μm vs 200μm and this correlated with higher levels of CSPG digestion produced by LVP (Zhao et al., 2011). Differences in protocols for viral production may also play a role. Higher levels of CSPG-digestion were observed in rat brains transduced with LVC (Verhaagen lab) compared to LVD (Yanez-Manoz lab), both vectors contained the same CMV promoter and were of similar titre but whereas LVC is a second generation transfer vector, LVD is a third generation transfer vector (Zhao et al., 2011). Moreover, a comparison of two identical vectors containing a PGK promoter, one commercially produced, the other produced in the Verhaagen lab, showed higher levels of efficacy in spinally injured rats transduced with the latter (Bradbury lab, unpublished results).

2.4.3. ChABC and rehabilitation

Intermittent hypoxia is used as a rehabilitation treatment to promote recovery of respiratory function following SCI. Using a rat hemisection model at C2, Warren et al. (2018) report that when this is combined with ChABC injected at C4 restoration of respiratory function can be achieved long after the initial injury (12 weeks to 1.5 years). The combination treatment was more efficacious than ChABC alone, but the effect was modest and intermittent hypoxia caused over-sprouting of 5-HT neurons in subset of animals causing tonic hemi-diaphragm firing. Thus the observed restoration of respiratory function was attributed mostly to the effects of ChABC. Following injury at the cervical level of the spinal cord some reorganisation of spinal pathways takes place over time as an adaptive strategy to restore function. One of the descending pathways involved is the bulbospinal pathway, which serves the phrenic motor neurons (Vinit and Kastner, 2009). The authors suggest that the recovery they observe is due to enhanced plasticity or unmasking of these anatomo-functionally reorganised pathways by ChABC-treatment. Indeed, a recent study demonstrated the potency of enhancing plasticity to promote recovery of function. Three human subjects with chronic SCI were treated with electrical stimulation, targeted to activate leg muscles involved in ambulation at the correct time. When this intervention was combined with intensive rehabilitation, patients regained control over previously paralysed muscles, even when the electrical stimulation was turned off (Wagner et al., 2018).

Alluin et al. (2014) employed a three pronged approach of ChABC + growth factors (EGF, FGF, PDGF-AA) + treadmill training to promote recovery of rats with clip compression SCI. They found that ChABC + growth factors induced plasticity of the corticospinal and serotonergic tracts but that there was no sustained functional improvement beyond what could be attributed to spontaneous recovery. In this study, rehabilitation was initiated 1 day post-injury and growth factors and ChABC 4 days post-injury, for 1 week. It is possible that a better outcome may have been achieved by promoting plasticity first with ChABC, then initiating rehabilitation (Marsh et al., 2011).

Previous studies have demonstrated that combining ChABC with task-specific rehabilitation is beneficial to functional recovery (Garcia-Alias et al., 2009). This study was extended to investigate the mechanisms underlying the recovery. Rats underwent surgery to ablate the corticospinal and rubrospinal tracts which are required for skilled hand functions. ChABC was administered acutely and rehabilitation started after 1 week. ChABC treated rats recovered skilled hand functions (reaching and grasping) and this was correlated with enhanced plasticity of reticulospinal axons, which normally play a minor role in hand function. The authors conclude that these neurons, which are malleable, take over the function of the damaged corticospinal and rubrospinal axons to restore upper limb function. In view of the difficulty in promoting regeneration of corticospinal neurons this could be used as an alternative strategy to promote recovery of manual dexterity following SCI (Garcia-Alias et al., 2015). In a recent paper, Shinozaki et al. (2016), show that this strategy can improve outcomes in rats with severe (250kdyn contusion) chronic SCI. ChABC was applied 6 weeks post-injury for 1 week, rehabilitation (treadmill training) was also initiated at this time point and continued for 8 weeks. A modest improvement in BBB score was observed after 14 weeks in the combination group compared to the non-treated controls (BBB scores 6vs3.5). Despite the limited amount of recovery this is a very promising finding as obtaining any level of recovery following a severe chronic SCI is a major achievement.

Treadmill training was also used in combination with delivery of thermostabilised ChABC in a clinical trial using dogs with naturally sustained chronic SCI (Hu et al., 2018). Critically, efficacy was only observed in the ChABC-treated animals. The results were variable, but some dogs in the ChABC-treated group showed improved co-ordination of forelimb/hindlimb movement and some animals were able to walk unaided. Importantly, there were no long-lasting adverse effects. This demonstration of ChABC efficacy in a large animal model of SCI, which closely reflects the human condition, is a highly significant finding. Moreover, the results from the first clinical trial using ChABC in humans has recently been completed in Japan. This phase III trial assessed the efficacy of ChABC to treat lumbar disc herniation. This cheminucleolysis approach to treat lumbar disc herniation is an alternative to surgical intervention. The enzyme is injected into the disc to digest the nucleus pulposis. This reduces the ability of the disc to imbibe fluid thereby alleviating leg and back pain. Significant improvements in clinical symptoms were observed in the ChABC-treated patients compared to the placebo group. Leg and back pain were reduced by 13 weeks and these improvements were sustained during the 1 year follow up. The most common adverse events were a reduction in disc height, modic type 1 changes and back pain, but these were without clinical consequences and resolved with time (Chiba et al., 2018). A second study investigated the effects and tolerability of increasing doses of ChABC (Matsuyama et al., 2018).The findings were that the incidence of adverse effects correlated with dose and the lowest dose of ChABC, 1.25U was found to be as efficacious as the highest dose (5U). Encouragingly, the study also found no evidence of anaphylaxis or neurological sequelae, suggesting that the enzyme is well tolerated when injected into the intervertebral discs of patients. These important findings, combined with the confirmed efficacy and lack of toxicity in a primate model of SCI (Rosenzweig et al., 2019) could hopefully pave the way to the first clinical trial using ChABC as a therapy for SCI in humans.

2.4.4. ChABC combined with known therapeutics

Another approach that has recently been employed is to combine known therapeutic agents with new methods of delivery. In one study, ChABC and NGF were incorporated into an electrospun scaffold to direct aligned axon regrowth following SCI. This promoted angiogenesis and aligned axon outgrowth. Some axons were myelinated and significant improvements in BBB scores were reported (Colello et al., 2016).

A scaffold of microspun poly(propylene carbonate) microfibres was used to deliver ChABC and db-cAMP to rats with hemisected SCI. This promoted regenerative sprouting which was accompanied by functional improvements in the narrow beam walk test and BBB scores. Here again, the combination therapy was more effective than ChABC or db-cAMP delivered individually, (BBB score14vs19) for the combined group compared to controls 4 weeks post-injury, (Xia et al., 2017).

Pakulska et al. (2017), combined ChABC with stromal cell derived factor 1α with the aim of promoting endogenous neural stem cell progenitor migration. Both were delivered from a cross-linked methylcellulose hydrogel to accomplish slow-release. Improvements were only seen in the ChABC treated groups (motor subscores and ladder walk tests). No synergistic effects were observed and all improvements were attributed to ChABC.

Another study evaluated a combination of ChABC, GDNF and anti-Nogo A antibody, delivered via delayed-release microspheres. This promoted functional recovery (improved BBB locomotor scores and nerve conduction) following SCI. The BBB scores showed improvements from 5 weeks post-injury. The triple combination group had the best outcomes, BBB scores 10.9vs7.5 for the control group at 10 weeks post-injury (Zhang et al., 2013).

Hunanyan et al. (2013), used an AAV vector to deliver NT3 to a thoracic contusion injury in rats which were also injected with ChABC. This combination induced plasticity which produced detour pathways (derived from the disrupted CST) around the injury site. These formed connections with ventral motor neurons and improvements were seen in the catwalk test 4 weeks post-injury.

The results from these studies suggest the use of scaffolds can improve outcomes when used to deliver small molecules such as NGF and GDNF. They supply a substrate for neurite outgrowth as well as providing a method of sustained release of therapeutic factors incorporated into their structures. Additionally, electrospinning can promote aligned axon-outgrowth. Scaffolds provide a good substrate for cell transplantation and nanoparticles, by virtue of their size, can effectively deliver smaller therapeutic molecules to the spinal cord and other areas of the CNS.

However, for delivery of large molecules such as ChABC, the timing of delivery is relatively short-term (~2 weeks) and the spread of the enzyme restricted by its incorporation into a scaffold compared to the spread of virally-delivered ChABC. Nonetheless, the functional recovery obtained in the canine clinical trial (Hu et al., 2018) where short-term delivery of ChABC via lipid microtubes produced locomotor recovery in some of the dogs suggests that outcomes could be markedly improved by the use of ChABC gene therapy.

New combination therapies incorporating ChABC are being evaluated. One of the most promising is LLL. The anti-inflammatory effects of LLL likely synergise with those of ChABC and the latter's CSPG removal, resulting in significant functional improvements. Moreover, LLL has the advantage that it is non-invasive and could be used early on to limit the inflammatory response which leads to extensive secondary damage (Janzadeh et al., 2017). Rehab also exhibits a powerful synergistic action with ChABC (Alluin et al., 2014; Garcia-Alias et al., 2009). Encouragingly, a combination of ChABC and rehabilitation elicited some functional recovery in two models of chronic SCI (Hu et al., 2018; Shinozaki et al., 2016) (Table 2).

2.5. ChABC and cell transplantation

A rapidly expanding field of research is evaluating the strategy of combining cell transplants with a range of other therapeutic interventions including ChABC. Grafting cells into the injured spinal cord offers many advantages. It provides tissue to replace cells that have been lost as a result of the injury. These cells can then be used to deliver growth and neuroprotective factors to the injury site. Moreover, in addition to providing a more permissive substrate for axon outgrowth, the cells can initiate remyelination of denuded and regenerating axons, thus restoring conduction across the injury site (Assinck et al., 2017). Recent studies (published since Zhao and Fawcett, 2013) involving cell transplant + ChABC are reviewed below.

2.5.1. Olfactory ensheathing cells

Transplantation of autologous olfactory ensheathing cells (OECs) is a promising strategy as a therapy for SCI and clinical trials in humans have not identified any adverse effects to date (Muir and Keynes, 2013). These cells are reported to be neuroprotective and to promote angiogenesis and axon regeneration (Assinck et al., 2017). Migration of these cells is important for their functionality in promoting axon outgrowth. Reginensi et al. (2015), have shown that this is inhibited by CSPGs and myelin associated inhibitors (MAIs). ChABC treatment of CSPG-coated substrates markedly enhanced OEC migration rate and this correlated with an increase in traction force. Similarly, the inhibitory effects of MAIs on OEC migration could be largely overcome by using OECs expressing a truncated form of the NogoA receptor (which neutralises the effects of the MAIs). Critically, these genetically modified OECs migrated further rostral and caudal to the lesion site than the unmodified controls when injected into rats with contusion SCI (Reginensi et al., 2015). This suggests that positive actions of ChABC may also be recapitulated *in vivo*. Consistent with this view, when OECs secreting ChABC are transplanted into the injury site of rats with cervical dorsal crush lesions, degradation of CSPGs is observed, accompanied by enhanced sprouting of CST axons both rostrally and caudally of the lesion site (Carwardine et al., 2017).

2.5.2. Schwann cells

Schwann cells are key to regeneration of neurons of the peripheral nervous system. They act by providing neuroprotection, reducing cyst formation and promoting remyelination (Bunge, 2016). Behavioural improvements have been demonstrated following their transplantation in both transection and the more clinically relevant contusion models of SCI (Bunge, 2016). Schwann cell transplantation has been combined with a number of other therapies (reviewed Bunge, 2016) including ChABC (Kanno et al., 2014). Schwann cells secreting a molecule which mimics the actions of the neurotrophins BDNF and NT3 in combination with Schwann cells secreting ChABC produced better outcomes when transplanted into rats with contusion injuries than any of the therapies individually. Indeed, significant anatomical (enhanced myelination, more neurons entering the graft and rostral and caudal to it) and functional improvements (cat walk test, BBB scores, significant from 5 weeks post-injury) were observed with this group (Kanno et al., 2014). Moreover, a clinical trial using Schwann cell grafts in patients with acute SCI has just concluded and the procedure deemed "safe". A trial on patients with chronic SCI is ongoing (Bunge, 2016).

2.5.3. Mesenchymal stem cells

Mesenchymal stem cells (MSCs) have been used to promote repair following SCI. They have been isolated from various tissues including bone marrow (Muniswami and Tharion, 2018; Xiong et al., 2016) and adipose tissue (Lee et al., 2015; Sarveazad et al., 2017). The rationale for their use is that they are multipotent and can therefore differentiate into a number of different cell types. They secrete neurotrophins, such as BDNF, which is known to promote myelination, growth factors such as hepatocyte growth factor, which is important for wound healing

Table 2
Summary of recent combination therapies using ChABC and outcomes

Author	Treatment	SCI model	Outcome
Janzadeh et al., 2017	LLL,30mins PI for 2 weeks + ChABC,injected 1 week PI.	Compression, T10, acute	Anatomical + functional recovery, enhanced axon number and myelination, reduced cavity size, Improved BBB scores 8vs13 at 4 weeks.
Liu et al., 2018	Hyperbaric oxygen, 2h PI for 2 weeks + ChABC,injected 1week PI.	Clip compression,T13, acute	Anatomical + functional recovery. Enhanced remyelination, improved BBB scores. 7.5vs13 at 4 weeks
Xia et al., 2015	Anti-sense Vimentin + ChABC, injected at the time of injury, then every other day for 2 weeks.	Hemi-section, acute,T7/8.	Anatomical recovery (regeneration) no functional recovery (BBB score).
DePaul et al., 2015	PNG + aFGF + ChABC, injected at time of injury.	Complete transection,T8 acute, Mouse	Anatomical recovery (regeneration) + functional recovery (bladder function).
DePaul et al., 2017	PNG + aFGF + ChABC, injected 8 weeks PI	Contusion, T8 Chronic	Some anatomical recovery (TH axons), no functional recovery (bladder function)
Xu et al., 2015	PNG + monastrol + ChABC(thermostab),injected at the time of injury	Complete transection, T7 acute	Anatomical recovery (regeneration), no functional recovery (BBB).
Wu et al., 2015	PNG + Rheb + ChABC, Rheb, injected at time of injury, PNG, grafted 2 weeks PI, ChABC at 6 weeks PI, at distal end of graft.	Complete transection, cervical, acute	Some functional recovery (forelimb/hindlimb co-ordination)
Wang et al., 2017	KLF7 + ChABC, injected at the time of injury.	Cervical, C4/5, hemi section or Crush, T8. acute	Reduced retraction of fibres from the lesion site with KLF7 or ChABC (hemisection) KLF7 crush only. No combined effect.
Warren et al., 2018	Intermittant hypoxia + ChABC,intervention 12 weeks-1.5 years PI	Cervical, C2 hemisection, Chronic	Restoration of respiratory function. Improvements attributed mostly to the effects of ChABC.
Alluin et al., 2014	Rehab (1day PI) + Growth factors + ChABC (4days PI)	Clip compression, T8, acute	Anatomical recovery (plasticity) no functional recovery (kinematics).
Rosenzweig et al., 2019	ChABC 4 weeks PI. Daily exposure to an environment enriched with objects to encourage fore and hind limb function.	Cervical, C7 hemisection Chronic	Anatomical recovery, (enhanced axon outgrowth,fibre density and synapse formation). Functional recovery (improved hand function)
Garcia-Alias et al., 2015	Rehab(1week, PI) + ChABC, injected at the time of injury.	Cervical C4 complete transection, (ablation of the CST in the medullar pyramids and rubrospinal axons)acute	Recovery of hand function.
Shinozaki et al., 2016	Rehab + ChABC, intervention, 6 weeks PI (ChABC for 1 week, rehab for 8 weeks).	Severe contusion, T10,Chronic	Anatomical recovery (regeneration) + functional recovery (modest improvement in BBB scores, 3.6vs6 at 14 weeks).
Hu et al., 2018	Rehab + ChABC (thermostab via microtubes), intervention > 12 weeks PI, rehab 4 weeks post intervention.	Canine, naturally occuring, Chronic	Functional improvements in locomotion, only seen in ChABC group
Colello et al., 2016	Electrospun scaffold + NGF + ChABC, implanted immediately post surgery.	Complete transection, T8,acute	Anatomical recovery (Angiogenesis, myelination, axon regeneration) + functional recovery (improvements in BBB scores, 2.5vs7 at 3 weeks).
Xia et al., 2017	Microfibres + dbcAMP + ChABC, implanted immediately post surgery.	Hemisection, T8, acute	Anatomical recovery(Regenerative sprouting) Functional recovery (BBB scores & narrow beam walking test) BBB scores 14vs19 at 3 weeks.
Pakulska et al., 2017	XMC hydrogel + stromal cell-derived factor 1α + ChABC, intervention at time of injury.	Moderate compression T1/2, acute	Anatomical recovery (enhanced distribution of neural progenitor cells), Functional recovery, motor subscore, ladder walk test, no improvements in BBB scores. All effects due to ChABC.
Zhang et al., 2013	GDNF + antiNogoA + ChABC, via microspheres, intervention at time of surgery.	Transection,T10, Acute	Functional recovery (Improved nerve conduction and BBB scores 10.9vs7.5 at 10 weeks).
Hunanyan et al., 2013	NT3 + ChABC,intervention at time of surgery.	Contusion, T10, acute	Anatomical recovery (plasticity, detour connections) Functional recovery (improved catwalk after 4 weeks).

SCI models are rat unless otherwise stipulated. PNG+ Peripheral nerve graft, LLL = Low level laser, aFGF = acidic fibroblast growth factor, dbcAMP = dibutyryl cyclic AMP, Rehab = Treadmill, or reaching and grasping training, thermostab = ChABC thermostabilised with trehalose, XMC = Crosslinked methylcellulose, KLF7 = Kruppel-like factor 7.

(Sarveazad et al., 2017) and angiogenic factors. They also produce cytokines, which are known mediators in endogenous neurogenesis and oligogenesis and may also effect beneficial immune modulation by attenuating the production of pro-inflammatory cytokines whilst enhancing the levels of anti-inflammatory cytokines (Assinck et al., 2017)

MSCs derived from adult human adipose tissue were transplanted sub-acutely into rats which had undergone contusion SCI, either alone or in combination with ChABC. The cells remained viable for 8 weeks. All treatment groups showed improvements (enhanced myelination, decrease in CSPG deposition, improved BBB scores) compared to controls but the combination intervention gave the best outcomes. Improvements in the BBB scores were noted as early as 14 days post-injury in the ChABC group, but plateaued thereafter. The combination group showed improvements from 21 days post-injury and this only plateaued after 42 days post-injury. The adipose-derived stem cells differentiated into neurons and astrocytes but the majority of the transplanted cells remained undifferentiated. The authors therefore attributed these beneficial actions to the secreteome of these cells (Sarveazad et al., 2017).

In a canine model of chronic SCI (Lee et al., 2015) MSCs derived from adipose tissue of the dogs were used to provide trophic support and ChABC was used to afford a more favourable environment for cell transplant; the combination produced clinical improvements. A significant difference in BBB score between the control group and the combination group occurred by 4 weeks post-transplant and persisted for 8 weeks. However, the cell transplant was associated with an inflammatory response (Lee et al., 2015). Therefore a second study was performed using MSCs engineered to over-express BDNF (for transplantation) to enhance neuroprotection, together with an intravenous injection of unmodified MSCs (which migrate to the injury site) in order to mitigate the inflammation (Lee et al., 2017). This combination resulted in a modest improvement in BBB score, evident 2 weeks post

transplantation; earlier than seen in their previous study and this continued to improve for up to 8 weeks (Lee et al., 2017).

In another study, MSCs derived from bone marrow, were engineered to over-express BDNF, and then transplanted into a ChABC treated transected rat spinal cord. This resulted in significant functional recovery. BBB scores within the triple combination group (MSCs + ChABC + BDNF) were the best and the improvements were evident from 2 weeks post-injury onwards (Xiong et al., 2016). These regenerative effects were attributed to an up-regulation of NGF expression, which is known to promote neurite outgrowth. Muniswami and Tharion (2018), transplanted a mixture of OECs and MSCs derived from bone marrow into a T10 contusion injury, 9 days post-injury (subacute phase). Improved outcomes, (BBB scores, reduction in cavity size, enhanced nerve conduction across the injury site) were achieved by cell transplant, cell transplant + ChABC and ChABC alone. However, the best improvement in BBB score was obtained with ChABC treatment alone (7vs6). The improvements in motor hindlimb function were evident by 2 weeks post-injury and were maximal by 4 weeks.

2.5.4. Neural stem cell progenitors

Neural stem cell progenitors (NSCP) have also been used for transplantation. These have been isolated from various sources, including embryonic or fetal brain and spinal cord (Jin et al., 2016). They have also been produced from embryonic stem cells (Wilems et al., 2015) and from induced pluripotent stem cells (iPSC) generated by the reprogramming embryonic fibroblasts (Suzuki et al., 2017). They provide trophic support and can differentiate into neurons, astrocytes and oligodendrocytes and can therefore replace lost tissue (Suzuki et al., 2017).They are reported to be neuroprotective, immunomodulatory, and to promote axon regeneration. They can initiate remyelination and promote regeneration of functional neuronal circuits. Cells from fetal tissue can additionally promote re-oxygenation at the injury site (which counteracts the hypoxia which occurs following SCI) and revascularisation (Assinck et al., 2017).

Jin et al. (2016) used NSCPs isolated from the spinal cords of rat embryos to promote repair of rats with chronic (13 weeks post-injury) spinal cord injuries at level T10. Although there was no significant improvement in the BBB and grid walk tests, they found the triple combination of NSCPs + ChABC + neurotrophins (BDNF + NT3) resulted in significant improvements of bladder function. The fact that these authors had previously shown that cell transplantation alone was sufficient to produce significant recovery of locomotor and sensory function when applied at the subacute stage of SCI (7-9 days post-injury), (Mitsui et al., 2005) illustrates the challenge represented by the chronically injured spinal cord.

2.5.5. Glial restricted progenitors

In another study, glial restricted progenitors, isolated from embryonic spinal cord, were transplanted into hemisected spinal cords in combination with lentiviruses expressing ChABC and bFGF. The aim was to increase cell migration with ChABC and to achieve directional migration with bFGF, in order to promote regeneration of axons beyond the graft. No functional studies were carried out in this study, but axons were found to emerge out of the graft into the surrounding spinal cord tissue, suggesting that this is an effective strategy for promoting axon egress from bridge grafts (Yuan et al., 2016).

2.5.6. Embryonic stem cells

Wilems et al. (2015), evaluated the effect of transplanting embryonic stem cell-derived motor neurons with anti-inhibitor therapy. Cells were incorporated into fibrin scaffolds to enhance survival. They differentiated into motor neurons, oligodendrocytes and type II astrocytes. NEP1-40 was used to combat the inhibitory effects of myelin and ChABC to overcome the inhibitory effects of CSPGs. Whilst cell transplantation or anti-inhibitor treatment individually promoted axon outgrowth in a dorsal hemisection model of rat SCI, the combination of cell transplantation and ChABC + NEP1-40 was found to be detrimental, causing enhanced inflammation. Thus not all combinations result in improved outcomes (Wilems et al., 2015).

2.5.7. Induced pluripotent stem cells

More recently, NSCs derived from induced pluripotent stem cells (iPSC) have gained favour as they overcome the ethical issues associated with the use of fetal tissues. Human iPSC-derived neuroepthelial stem cells have been transplanted into rats with SCI induced by clip compression. ChABC was introduced into the lesion site at the time of injury and again 7 days later during cell transplantation (Fuhrmann et al., 2018). Cells survived, proliferated and migrated into the lesion site, reducing the cavity size. However, no functional recovery was observed. Interestingly, more neurons were seen in the cords of animals that had received ChABC compared to those that had received cell transplant alone or controls. The ChABC group also exhibited a wider range of cell types. The authors suggest that this is evidence that CSPGs influence NSCP differentiation (Fuhrmann et al., 2018). With regard to the lack of functional recovery observed in this study, it is of note that these cells were derived from progenitors that were cortically specified and there is evidence that the source of the NSCPs may be important for efficacy. Spinal cord-derived NSCPs are more efficacious at repairing the spinal cord than NSCPs derived from other parts of the CNS (reviewed Nagoshi and Okano, 2017). One of the most promising studies investigates the effect of combining ChABC with NSCP transplantation in a chronic model of SCI. Mice with clip compression injuries at the cervical level were treated with ChABC for I week, then transplanted with iPSC-derived NSCPs 8 weeks post-injury. ChABC-treatment enhanced cell survival and the cells differentiated into all three lineages. Neurons derived from the transplanted cells extended axons which formed functional synapses with the host tissue. Importantly, this was accompanied by significant functional recovery in the catwalk test and forelimb grip strength test (Suzuki et al., 2017). This is one of the first studies showing that it is possible to promote a significant level of recovery in the chronically injured cord. Regaining hand function would greatly improve the quality of life of patients with chronic SCI.

2.5.8. Oligodendrogenic neural progenitor cells (drNPCs)

The use of iPSCs poses the risk of tumor formation, immunogenicity, genetic and epigenetic abnormalities. drNPCs, are directly reprogrammed from somatic cells (human bone marrow cells), avoiding the pluripotent state. These cells, which are biased towards oligodendritic lineage, were transplanted into a clip compression injury at T7, 7 weeks post-injury. ChABC was delivered via affinity-controlled release (methyl cellulose hydrogel) one week prior to cell transplantation. In their study, Nori et al. (2018), show that CSPGs induce the expression of transcription factors which drive the differentiation of transplanted cells towards astrocytic lineage at the expense of oligodendritic lineage, whilst the number of neurons formed is unaffected. Moreover, this is reversed by ChABC. This combination therapy resulted in enhanced cell survival. The cells migrated further and integrated into the lesion site where remyelination of host spared denuded axons was observed. These remyelinated axons formed functional synapses and this is associated with functional recovery (improved BBB scores (9vs11) and catwalk scores), consistent with motor functional recovery (Table 3).

In summary, these studies suggest that combining ChABC with cell transplantation is a promising strategy for promoting repair following SCI. ChABC modifies the micro-environment of the injured cord, thereby making it more favourable for cell survival which is of particular importance in the case of the chronically injured cord. Moreover, ChABC promotes cell migration, which is necessary to aid regenerating axons to exit the graft and integrate into the host parenchyma. The ideal candidate for cell transplantation is not yet apparent: the rationale for and outcomes of using different cell types to promote repair following SCI are discussed in detail elsewhere (Assinck et al., 2017). Adipose-derived MSCs are an attractive option as they are easily

Table 3
Summary of combination therapies using cell transplantation and outcomes.

Author	Treatment	SCI Model	Time of cell transplant	Outcome: anatomical/functional
Reginensi et al., 2015	OECs + NogoA ectodomain OECs + ChABC (in vitro)	Contusion, T8/9, intervention day of injury. N/A	Day of injury N/A	Enhanced cell migration Enhanced cell migration
Carwardine et al., 2017	OECs + ChABC	Dorsal column crush, C4, intervention day of injury.	Day of injury	Enhanced sprouting of CST axons rostral & caudal to the lesion site.
Kanno et al., 2014	SC + NT3 + BDNF + ChABC	Contusion, T8,intervention day 7 PI	Day7 subacute	Enhanced myelination Improved sensory and locomotor function (cat walk gait test, BBB scores 13 weeks PI, 1.5 vs4.5).
Sarveazad et al., 2017	MSCs (adipose) + ChABC	Contusion, T8/9, intervention, day 7 PI	Day 7 subacute	Functional improvement from 2 weeks PT, BBB scores 5vs16, 6 weeks PT.
Lee,S. 2017	MSCs (bone marrow) + BDNF + MSC injection + ChABC	Compression, L4 Chronic, intervention, 3 weeks PI Canine	3 weeks PI	Functional Improvement by 2 weeks PT, BBB scores at 8 weeks PT 8 vs11.
Xiong et al., 2016	MSCs (bone marrow) + BDNF + ChABC	Complete transection, T10, intervention, day of injury.	Day of injury	Functional recovery, improved BBB scores by 2 weeks PI, 4vs14.
Muniswami and Tharion, 2018	OECs + MSCs + + ChABC	Contusion, T10, subacute, intervention day 9 PI.	Day 9 PI	Functional recovery, improved BBB scores, zerovs7, for the triple combination group.
Fuhrmann et al., 2018	NSCP (embryonic cortex) + ChABC	Clip compression, T1/2, intervention, day of injury.	Day 7, PI	Anatomical improvement (reduced cavity size), no functional improvement (ladder walk test, BBB scores).
Jin, Y. 2016	NSCP (embryonic spinal cord) + LVNT3 + LVBDNF+ ChABC	Contusion, T10 Chronic, intervention,14 weeks PI.	13 weeks PI	Poor survival of NSCP, no improvement in locomotor or sensory function but improvement in bladder function.
Yuan et al., 2016	GRP (embryonic spinal cord) + bFGF + ChABC	Hemisection, C4, intervention day of injury	Day of injury	Enhanced directional migration of transplanted cells out of lesion site.
Wilems et al., 2015	ES-derived motor neurons in fibrin + + GFs + antiNogoA (microspheres) + + ChABC (thermostab) via microtubes	Dorsal hemisection, T10, Subacute intervention, 2 weeks PI.	2 weeks PI	Anti-inhibitor or cell transplant therapy individually resulted in improvement (reduced CSPGs) but the combined therapy was detrimental, increased CSPGs, reduced motor neuron survival.
Suzuki et al., 2017	NSCP (iPSC) + ChABC	Cervical clip compression, C6/7 Chronic, intervention 7 weeks PI, mouse	8 weeks PI,	Good NSCP survival, improvement in forelimb grip and cat walk tests.
Nori et al., 2018	drNPCs + ChABC	Clip contusion, T7, Chronic, intervention 6 weeks PI	7 weeks PI,	Improved functional recovery. Improved catwalk and BBB scores 9vs11.

OECs = Olfactory ensheathing cells, SC = Schwann cells, NSCP = Neural stem cell progenitors, ES = embryonic stem cells, GFs = growth factors (NT3, PDGF), PI = Post-injury, PT = Post-transplant, GRP = Glial restricted progenitors, CST = Corticospinal tract, drNPCs = directly reprogrammed neural progenitor cells.

obtainable and allow for autologous transplantation. They can also be expanded quickly to provide sufficient numbers for transplantation and survive well at the lesion site. Indeed, impressive levels of locomotor recovery have been attained with these cells in the clinically relevant contusion model of SCI (Sarveazad et al., 2017). However, MSCs alone may not be sufficient to promote the regeneration of CST axons, which are essential for locomotor function in humans (Kadoya et al., 2016). It is of note that bridges of Schwann cells or glial restricted progenitors also failed to promote regeneration of CST neurons in this experimental model of SCI (Kadoya et al., 2016). NSCP transplantation has produced functional improvements, even in the very challenging environment of the chronically injured spinal cord (Suzuki et al., 2017). Importantly, NSCPs have the potential to promote robust and functional regeneration of CST axons (Kadoya et al., 2016). However, NSCPs derived from primary tissues are more difficult to obtain and viability in the hostile environment of the injured cord can be an issue (Jin et al., 2016). Nonetheless, this can be mitigated to some extent by incorporating the cells in a scaffold. This improves cell survival by shielding them from the hostile environment and also helps prevent loss of the transplanted cells. Scaffolds can also be used to initiate directional growth (Colello et al., 2016) and to pharmacologically modify the lesion site. NSCPs can also be produced from iPSC (Suzuki et al., 2017). The methodology for iPSC production is advancing rapidly. Safety concerns arising from iPSC induction via viral transduction have been largely overcome by the use of transposon vectors to introduce the reprogramming genes. This technology enables exact excision of these genes from the cell's genome once the induced state has been achieved (Woltjen et al., 2009). Embryonic fibroblasts have been identified as a reliable source of the cells from which to derive iPSCs and the inducing signals shown to be conserved across species. Moreover, exposure of the differentiating neurons to caudalising signals (retinoic acid) is important for promoting regeneration of CST axons (Kadoya et al., 2016). The time required to produce iPSCs (6 months) means that autologous transplantation using patient-derived cells is not feasible. Thus, iPSC lines are being developed for transplantation, which allows for screening to remove cells with genetic/epigenetic abnormalities. Significant progress has been made in developing a protocol for generating clinical grade iPSCs (Nagoshi and Okano, 2018) and these cells are now emerging as a feasible option for cell transplantation in the first clinical trials in patients. Directly reprogrammed NPCs have also shown promising results, promoting motor recovery by enhancing the remyelination of spared axons, even in the hostile setting of the chronically injured cord. These studies suggest a combination of drNPCs + caudalised iPSCs + ChABC may be a promising strategy to promote repair of the chronically injured cord.

3. ChABC and peripheral nerve regeneration

3.1. Peripheral nerve regeneration can be enhanced by ChABC

In contrast to the limited regenerative capacity of the CNS, the peripheral nervous system (PNS) exhibits a relatively robust regenerative response after injury. In human patients, however, recovery after PNS injury is almost never complete and nerve injury does often lead to permanent loss of function. Immediately after nerve injury peripheral neurons and nerve Schwann cells convert to a pro-regenerative state. Schwann cells distal to the lesion site radically change their gene expression profile, clear axon and myelin debris, and start to produce neurotrophic factors and cell adhesion molecules which temporarily transform the distal nerve into a neurite outgrowth promoting environment (reviewed in Jessen et al., 2015). Perhaps somewhat unexpected, CSPGs were found to be upregulated in the nerve after experimental nerve injury (Braunewell et al., 1995; Morgenstern et al., 2003; Shum and Chau, 1996; Tona et al., 1993; Zuo et al., 1998b). In vitro studies revealed that neurite outgrowth of peripheral neurons, including dorsal root ganglion or superior cervical ganglion neurons, is reduced on CSPGs enriched substrates (Dou and Levine, 1994; Gardner and Habecker, 2013; Sango et al., 2003; Snow et al., 1996; Snow et al., 1990). The presence of inhibitory CSPGs at the peripheral nerve injury site and in the distal nerve inspired several groups to investigate the therapeutic potential of ChABC treatment after PNS injury.

The severity of the initial nerve injury correlates with the degree of successful regeneration. Following a nerve crush injury the endoneurial tubes remain intact. Injured axons are able to extend through the crush site and follow the trajectory of their original endoneurrial tube. After a complete nerve cut surgical repair is needed to restore continuity between the proximal and distal nerve stumps. However, common surgical repair strategies, like a direct coaptation of the proximal and distal nerve stumps or the insertion of a nerve graft, create nerve junctions that are much more difficult to cross for regenerating axons. The misalignment of proximal and distal endoneurial tubes and the deposition of extracellular matrix molecules like CSPGs, form major obstacles for growing axons at the coaptation site. Several studies have shown that axon crossing from the proximal into the distal nerve stump can be increased by removing CSPGs from the coaptation site (English, 2005; Groves et al., 2005; Tuffaha et al., 2011; Zuo et al., 2002). ChABC treatment significantly improves anterograde crossing of axons and reduces the number of axons that normally displayed aberrant extrafasicular retrograde growth in the proximal nerve stump (Graham et al., 2007). In addition, axons that have crossed the lesion site reach their distal targets faster after ChABC treatment resulting in an improvement of motor recovery compared to non-treated nerves (Sabatier et al., 2012). Unfortunately, ChABC enhanced nerve regeneration does not only lead to an increase in innervation of appropriate targets but ChABC treatment also resulted in misrouting, i.e. innervation of inappropriate targets (English, 2005). Misrouting is caused by the fact that many of the axons that cross the injury site end up in the wrong endoneurial tube guiding them to incorrect targets. In contrast to the improvements observed in the nerve transection model, axon crossing and target reinnervation after a nerve crush injury is not (further) enhanced by ChABC treatment (Zuo et al., 2002). The perfectly aligned endoneurial tubes and the limited scar formation after a crush injury already allow a very rapid undisturbed crossing of axons into the distal nerve, probably leaving little to gain in this model in rodents.

Beneficial effects of ChABC treatment on axon crossing are also observed at other types of barriers. Axons growing from PNS to CNS tissue boundaries, like artificial crossings from peripheral nerve grafts into brain or spinal cord tissue (Tom and Houle, 2008; Tom et al., 2013) or more natural crossings of sensory axons that enter the dorsal root entry zone into the spinal cord benefit from the digestion of CSPGs by ChABC (Steinmetz et al., 2005; Wu et al., 2016). In addition, there are studies that report that ChABC treatment also improves axonal growth into synthetic grafts, like silicone tubes or silk fibroin conduits (Hattori et al., 2008; Sivak et al., 2017). ChABC applied in the synthetic grafts is thought to indirectly improve regeneration through stimulation of Schwann cells in the proximal stump. Degradation of CSPGs increases Schwann cell proliferation, which subsequently migrate quicker and in larger numbers from the proximal nerve stump (through the fibrin bridge) into the silicone tube (Snow et al., 2001). Next, regenerating axons do use these supporting Schwann cell bridges to enter the synthetic graft more easily.

ChABC treatment combined with other strategies to stimulate axonal outgrowth in a peripheral nerve transection-coaptation model are so far unsuccessful. Electrical stimulation of the proximal nerve stump, by itself, is a strong stimulus to increase axon regeneration in experimental animal models (Al-Majed et al., 2000; Brushart et al., 2002; Brushart et al., 2005; English et al., 2007; Geremia et al., 2007) and in human patients (Gordon et al., 2010; Willand et al., 2016; Wong et al., 2015). However, addition of electrical stimulation to the ChABC treatment does not further increase the number and speed of the regenerating axons that cross the coaptation site (Beaumont et al., 2009; English, 2005). Also increasing the cAMP levels in the injured neurons

by subcutaneous application of rolipram has been shown to enhance axon regeneration. But there is no additional effect of increased cAMP levels on ChABC stimulated axon growth (Udina et al., 2010).

ChABC has not only been used to improve axon crossing over nerve junctions, but it is also used to enhance the intrinsic axon growth support of acellular peripheral nerve grafts. This type of nerve grafts can be used to bridge nerve gaps when the gold standard autologous grafts are not available. Acellular nerve grafts are portions of donor nerve that are stripped from their cells by radiation, chemical or thermal treatment to make them less immunogenic. Although, the internal collagen tube and basal lamina structures are largely intact, the strong neurite outgrowth promoting activity of laminin in these grafts is blocked by the presence of CSPGs (Zuo et al., 1998a). ChABC treatment can effectively remove CSPGs from acellular nerve grafts thereby unmasking laminin, resulting in a significantly enhanced support of axon growth. The big advantage of this improvement is that ChABC treated acellular nerve grafts can be used to repair larger nerve gaps (Krekoski et al., 2001; Neubauer et al., 2007, 2010; Yu et al., 2012). ChABC treated acellular nerve grafts can easily be combined with other therapies to further improve their growth supporting properties. In a rat sciatic nerve repair model, addition of the neurotrophic factor NGF (Nerve Growth Factor) to ChABC treated acellular nerve grafts increased the number of neurites in the graft compared to ChABC treated grafts alone (Boyer et al., 2015; Ovalle Jr. et al., 2012). Furthermore, ChABC treatment makes acellular nerve grafts more hospitable for cell transplants, like Bone marrow stromal cells (BMSCs) or differentiated adipose-derived stem cells. BMSCs seeded in ChABC treated acellular nerve grafts survive better than in untreated grafts, allowing them to provide more neurite outgrowth support by the secretion of the neurotrophic factors NGF, BDNF and VEGF. In a rat sciatic nerve gap repair model the combination of ChABC acellular nerve grafts + BMSCs increases the number of fibers that regenerated in the graft and an improved functional recovery (Wang et al., 2016; Wang et al., 2012).

So far, the promising results of ChABC treatment on peripheral nerve injury are obtained in small rodent peripheral nerve injury models. Increased coaptation site crossing and enhanced axonal growth through acellular nerve grafts are, nevertheless, meaningful and are feasible steps to improve peripheral nerve regeneration in human patients in the future. One of the next challenges will be to improve long distance regeneration. Due to the slow speed of axon regeneration, the more distal nerve remains denervated for a prolonged time period resulting in scarring of the nerve, loss of trophic support by Schwann cells and atrophy of target organs. Future studies should reveal whether ChABC treatment can also improve axon growth supporting properties in the chronically denervated distal nerve and can thereby contribute to long distance peripheral nerve regeneration that further improves functional recovery.

4. Other therapeutic applications of ChABC

4.1. ChABC and cancer

ChABC has been utilized as a tool for research purposes since as early as 1945. The first report of a therapeutic effect was made by Takeuchi in 1972, who demonstrated that ChABC inhibited the growth of Ehlrich ascites tumors. Since then additional studies have implicated a wider role for CSPGs in tumor development, angiogenesis and metastasis (Kasinathan et al., 2016). Indeed, treatment of melanoma cells or vascular endothelial cells with ChABC (or AC) inhibited cell proliferation, abolished their ability to metastasize and blocked angiogenesis (endothelial cells) consistent with a role of CSPGs in all three processes (Denholm et al., 2001). CSPGs make up a major proportion of the extracellular matrix stroma, which regulates proliferation and migration of solid tumors. Augmented levels of the CSPG versican, are seen in prostate, breast and lung cancers, whilst brevican expression is up-regulated in gliomas (Kasinathan et al., 2016). Another potential anti-tumor mechanism of ChABC is that it promotes apoptosis of some types of tumor cell (melanoma, pulmonary artery endothelial cells) via activation of caspase-3 (Denholm et al., 2001). Furthermore, in experimental models of glioblastoma, ChABC application enhances the spread of oncolytic viruses (Dmitrieva et al., 2011) and can sensitize cells to tumor-targeting drugs such as temozolomide (Jaime-Ramirez et al., 2017). These data suggest that ChABC may also be a powerful anti-cancer therapeutic for specific types of cancer, as it can target tumors in several different ways. These findings are encouraging, but should be interpreted with caution as there is evidence that ChABC treatment is not beneficial for all cancers. It can facilitate tumor progression in some cases (Silver et al., 2013).

4.2. ChABC, myocardial infarction and stroke

Moreover, recent studies have shown that the enzyme is an efficacious treatment for other conditions where a build up of CSPGs prevents nerve repair, such as myocardial infarction (Gardner and Habecker, 2013) and stroke (Gherardini et al., 2015). In the case of stroke, the neuroprotective actions of ChABC are reported to limit the primary and secondary damage that ensues following a stroke (Chen et al., 2014) and its ability to promote plasticity in the brain and spinal cord is also beneficial (Starkey et al., 2012). Following stroke, a 2 week period of natural plasticity takes place during which time therapies to promote recovery, such as rehabilitation, can be used to improve outcomes. Application of ChABC reopens the window of plasticity extending the time frame that recovery can be accomplished (Tennant, 2014). This can be used to enhance the beneficial effects of training (Wiersma et al., 2017). Critically, ChABC is also efficacious in experimental models of chronic stroke (Soleman et al., 2012; Wiersma et al., 2017). To improve the bioactivity of the enzyme for stroke treatment, Hettiaratchi et al. (2019), enhanced its stability by site-directed mutagenesis (N1000 to G) which doubled the half-life, and PEGlylation which prevents protein unfolding and aggregation. This improved version of ChABC, tagged with Src homology domain 3, was delivered to the injured brain via affinity-controlled release (methyl cellulose hydrogel modified with SH3 binding peptides). A reduction of CSPG levels was observed up to 28days, thus a single dose of ChABC should be sufficient to extend the window of plasticity required to improve outcomes via rehabilitation training.

4.3. ChABC and multiple sclerosis

ChABC may also be a potential treatment for multiple sclerosis since there is strong evidence that CSPGs inhibit oligodendrocyte maturation and remyelination (Lau et al., 2012; Pendleton et al., 2013) and that ChABC can promote remyelination (Colello et al., 2016; Janzadeh et al., 2017; Liu et al., 2018).

4.4. ChABC and skin scarring

CSPGs, in particular versican, are up-regulated in Keloid scars. The findings of an ex vivo study suggest that ChABC would be an effective treatment for these scars which are unsightly and cause considerable discomfort (Kasinathan et al., 2016).

4.5. ChABC and Alzheimer's disease

Very recently, early changes in the extracellular matrix composition have been reported to precede the onset of memory loss in a mouse model of Alzheimer's disease. CSPGs have been implicated and ChABC shown to be beneficial (Vegh et al., 2014). Injection of ChABC into the hippocampus of Alzheime'rs mice restored both LTP and memory performance (Vegh et al., 2014). It also enhances the density of synapses adjacent to the plaques and this is associated with the degradation of the CSPG brevican. The levels of amyloid protein were also reduced in

the ChABC-treated mice in this study (Howell et al., 2015). The results from another study suggest a role for the sugar chains of the CSPG molecules, since administration of ChABC or application of an antibody recognizing 4-sulfated CSPGs delays memory loss in mice with tauopathy-induced neurodegeneration (Yang et al., 2017). In another study Yang et al. (2015), show that removal of PNNs surrounding interneurons of the perirhinal cortex with ChABC restored object recognition and synaptic transmission in two mouse models of tauopathy. ChABC treatment, although without effect on neuronal degeneration or tau aggregation, was able to compensate for neuronal loss by reactivating plasticity. The restoration of memory in the presence of advanced pathology is very encouraging, as it suggests it may be possible to significantly delay memory loss in patients by targeting PNNs in this region of the brain, which is involved in object recognition.

4.6. ChABC and Parkinson's disease

The is also evidence that ChABC is efficacious in rat models of Parkinson's disease, likely a result of CSPG degradation along the nigrostriatal tract (Moon et al., 2001). Delivery of ChABC to the axotomized nigrostriatal-tract of rats facilitated the growth of dopaminergic axons back to the striatum (Moon et al., 2001). Moreover, combining transplantation of dopaminergic neurons into the midbrain with ChABC treatment significantly improved the outcome compared with cell transplants alone (Jin et al., 2011; Kauhausen et al., 2015).

4.7. ChABC and fear memories

A further novel application for ChABC has recently come to light. ChABC degrades PNNs, and these structures are required for fear learning (Banerjee et al., 2017) and are important for protecting fear memories from erasure. Elimination of PNNs has been shown to abolish recall of a fear memory (Thompson et al., 2018) and degradation of PNNs in the amygdala with ChABC allowed inappropriate fear memories to be removed (Gogolla et al., 2009). Thus, ChABC treatment could potentially benefit patients suffering from phobias and anxiety.

5. Concluding remarks and future directions

Substantial progress has been made in the last 5 years towards identifying candidates for SCI combination therapy, with a few studies addressing the important challenge of repairing the chronically injured spinal cord. New mechanisms by which the enzyme promotes recovery following SCI have been identified (Bartus et al., 2014; Didangelos et al., 2014). More details have emerged on the effects of timing and duration of ChABC delivery in different models of SCI (Burnside et al., 2018; Mondello et al., 2015; Warren and Alilain, 2019; Warren et al., 2018), which will aid clinical translation. Moreover, the efficacy of the enzyme has recently been demonstrated in a primate model of SCI (Rosenzweig et al., 2019), adding to the number of large animal models where it confers benefit following SCI. ChABC synergises with almost all the therapeutics tested so far and therefore should form an essential component of any combination therapy. It is also generally accepted that rehabilitation should be included. However, the timing of rehab required to achieve the best outcomes following each intervention will need to be determined.

Cell transplantation (in a scaffold) + ChABC (via gene therapy) + rehabilitation is a promising option. Indeed, a combination of ChABC + rehabilitation without cell transplantation was sufficient to produce a limited amount of functional recovery in two preclinical models of chronic SCI (Hu et al., 2018; Shinozaki et al., 2016). In the case of acute SCI, incorporating LLL (Janzadeh et al., 2017) to reduce the ensuing inflammatory response could be beneficial. Employing scaffolds for delivery small therapeutic molecules such as growth factors and cells seems a promising strategy to enhance repair and aligned axon outgrowth. However, more work is required in this field to identify the ideal material for their construction. For the treatment of acute SCI, high levels and widespread enzyme delivery correlate with efficacy (Bartus et al., 2014) and long-term delivery correlates with enhanced functional recovery (Burnside et al., 2018) which is achieved when the enzyme is delivered via gene therapy. Gene therapy additionally affords a method to tightly control the levels and timing of enzyme release, allowing enzyme delivery to be terminated once treatment is complete. It also results in delivery of the enzyme to a large area of the cord which will likely be required for efficacy in human patients. In rats, continuous delivery of ChABC for up to 8 weeks was not associated with any adverse effects (Bartus et al., 2014; Burnside et al., 2018). However, since the levels of CSPGs will take more time to return to control levels (Pakulska et al., 2017), animals will need to be monitored for any adverse effects over a longer time period. Moreover, for human gene therapy, AAV vectors have been shown to have an excellent safety record (Coura Rdos and Nardi, 2007) and would be the choice of vector for ChABC delivery. To this end, we are developing such a vector, addressing the challenges of expressing a large gene from a vector with limited packaging capacity. Moreover, although preclinical studies have not shown any evidence of an immune reaction to the enzyme and the phaseIII trial carried out by (Chiba et al., 2018) in humans showed no long lasting adverse effects, the possibility of immunogenicity will need to be assessed further using antigen presentation and T-cell proliferation assays such as those developed by proimmune (Oxford). Analysis of toxicology will also need to be conducted for the different conditions. The trial conducted by Chiba et al. (2018) has confirmed that injection of ChABC into intervertebral discs is safe however a different outcome could occur following injection into melanomas in humans or following delivery into different areas of the brain to treat stroke, or Parkinson's disease which also could be technically challenging and have unanticipated side effects.

The use of ChABC in a two preclinical models of chronic SCI (Warren and Alilain, 2019; Warren et al., 2018) and preclinical models of some other (neurodegenrative) conditions suggest a single injection of the bacterial enzyme can restore function. Thus, ChABC released from a scaffold may be sufficient for efficacy in these cases. The reported efficacy of ChABC either alone (Warren and Alilain, 2019; Warren et al., 2018) or in combination with other therapies to treat chronic SCI (Hu et al., 2018; Lee et al., 2017; Nori et al., 2018; Shinozaki et al., 2016; Suzuki et al., 2017) suggests that it may be of benefit to many patients with chronic SCI, especially as these injuries were the clinically relevant contusion/compression type and occurred at all levels of the spinal cord, including cervical (Suzuki et al., 2017) thoracic (Hu et al., 2018; Nori et al., 2018; Shinozaki et al., 2016) and lumbar levels (Hu et al., 2018; Lee et al., 2017).

Nonetheless, other therapies for the treatment of SCI are in development, (reviewed in Bunge, 2016; Muir and Keynes, 2013). One of the most successful therapies is reported in the November issue of Nature last year, (Wagner et al., 2018). It involved 3 patients with chronic SCI, who were treated with spatio-temporal epidural stimulation combined with intensive rehabilitation. After treatment, the patients regained voluntary control over previously paralysed muscles. Moreover, this continued even after the electrical stimulation had been switched off, indicating that the brain and spinal cord had re-established functional connections. This unprecedented restoration of function is impressive, however the treatment may not be suitable for all patients. The patients in this small study all had some residual leg function. Moreover, the treatment is technically complex and expensive. Spinal cord injuries are heterogeneous in nature which warrants the development of alternative strategies to promote repair each of which may be better suited to a particular injury type, for example a PNG for hemisection injuries and ChABC because it is a cheaper alternative to targeted epidural stimulation and preclinical studies have shown it is effective in a wide range of injury models and promotes recovery from chronic injuries at all levels of the cord and thus may benefit a wider range of patients than some of the other therapies in development.

Since the first report of a therapeutic effect of ChABC on cancer cells in 1972, the potential application of this enzyme for the treatment of various pathologies continues to expand. The multiple therapeutic effects of ChABC implicate numerous roles for the extracellular matrix, in particular CSPGs, in health and disease and merit the enzyme's development for clinical application.

Declaration of Competing Interest

The authors declare no conflict of interest

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