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Clostridial C3 proteins: Recent approaches to improve neuronal growth and regeneration

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SUMMARY

Bacterial C3 exoenzymes are widely used tools to investigate cellular events influenced by small GTPases of the Rho subfamily. In this respect they have gained increasing interest in addressing questions dealing with the neuronal morphogenic program during development and after lesion of the mature nervous system. Since central neurons display only very limited capacity to re-grow their axons after injury, successful strategies to improve regeneration are much sought-after. For a long time exclusively considered to be Rho-inhibiting exoenzymes, there is now accumulating evidence that C3 proteins of clostridial sources exhibit their often beneficial effects on neurite outgrowth by other means than ADP-ribosylation. The current review will outline previous attempts to foster neuronal cell growth by the use of C3 transferases and highlight the more recent approaches to improve regenerative axon outgrowth using enzyme-deficient C3 preparations.

1. The family of C3 transferases

More than 20 years ago, a bacterial ADP-ribosylating enzymatic activity was identified from *Clostridium botulinum* culture supernatants, which was clearly distinct from an already known ADP-ribosylating enzyme (Aktories et al., 1987). The name C3 was proposed to distinguish the newly identified toxin from botulinum neurotoxin (C1) and an enzyme that ADP-ribosylates actin (C2). In subsequent studies, the C3 substrate was identified to represent a *rho* gene product (Aktories et al., 1989). Around the same time it was discovered that the transfer of an ADP-ribose moiety from NAD to the acceptor amino acid asparagine at position 41 of the ras-homologue rho proteins (now named RhoA, B and C) is the specific molecular event in targeting low molecular mass GTPases by C3 transferases (Sekine et al., 1989). As known so far, ADP-ribosylation of RhoA, B or C results in functional inactivation by inhibition of GEF-activity (**g**uanine nucleotide **e**xchange **f**actors, serving as small G protein activators, Sehr et al., 1998) and entrapment of inactivated GDP-bound Rho in the cytosol by inhibitory GDIs (**g**uanine nucleotide **d**issociation **i**nhibitors, Genth et al., 2003). To date, seven members of C3-like transferases have been identified. Besides *Clostridium botulinum* and *Clostridium limosum* also *Bacillus cereus* and *Staphylococcus aureus* are sources of C3 transferases (Just et al., 1992, 1995; Wilde et al., 2001). Noteworthy, apart from exhibiting effects in a strictly enzyme-dependent manner, C3 from *Clostridium botulinum* has proven to elicit growth-promoting effects on neurons that do not involve ADP-ribosylation (Ahnert-Hilger et al., 2004). So far, no specific cell entry machinery of C3 proteins has been described. Unlike clostridial neurotoxins that represent classical AB toxins consisting of a catalytically active (A) and a binding (transport) domain (B) C3 exoenzymes consist of a single chain of around 24 kDa without identified translocation regions. Various modes of cell entry have been proposed over the years, amongst them unspecific uptake when present at higher micromolar concentrations or translocation into the cytoplasm by means of additional pore-forming bacterial cytolysins. At least for *Staphylococcus aureus,* it has been shown that C3 can gain access into the cell when the bacterium itself becomes internalized by invading host cells (Molinari et al., 2006).

Given the plethora of cellular functions in which Rho proteins are involved, interference with Rho signaling may, therefore, result in a magnitude of cellular responses. Amongst the most evident ones are rearrangements in the cytoskeleton since RhoA serves as a master regulator of actin dynamics (Hall, 1998; Hall and Lalli, 2010). This involves the formation of stress fibers, cell growth, cytokinesis, formation of focal adhesions or cell migration.

The present review will mainly focus on different approaches to promote neuronal process outgrowth *in vitro* by means of C3 exoenzymes and, moreover, will discuss the potential of C3bot preparations to overcome the intrinsically limited poten-

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tial of central neurons to regenerate after lesion in the *in vivo* situation.

2. C3 proteins: tools to foster neurite outgrowth

Neuronal process formation resulting in the establishment of unambiguous axons or dendrites, respectively, depends on the action of extracellular attractive and repulsive cues as well as intracellular differentiation programs of both neurons and glia. This also holds true for forms of neuronal plasticity in adulthood under physiological conditions but also after pathological or traumatic events within the nervous system. In earlier studies on a neuroblastoma cell line it has been shown that *Clostridium botulinum* C3 (C3bot) can inhibit lysophosphatidic acid-mediated effects like growth cone collapse and neurite retraction (Jalink et al., 1994a,b). In this line it was demonstrated that microinjection of C3bot results in enhanced neurite formation by N1E-115 cells (Kozma et al., 1997). Morphological effects elicited by C3 could be attributed to abolishment of RhoA signaling pathways. First evidence for beneficial C3bot effects on neurite outgrowth of cultivated primary neurons came from chick sensory neurons that showed an increased neurite extension upon incubation with C3 transferase (Jin and Strittmatter, 1997). Following demonstration of effectiveness in a cell culture model, the next logical step was to test for growth promoting effects *in vivo*. So far, no such studies have been performed to address the effects of C3 proteins on the developing nervous system. Rather, the main focus has been directed at the potential beneficial effects of C3 treatment following traumatic injury to the adult CNS. To investigate this, crush lesions of the rat optic nerve were performed and C3bot administered at the lesion site. By anterograde labeling using cholera toxin it was demonstrated that treatment with C3 indeed improved re-growth of retinal ganglion cell (RGC) axons beyond the lesion site (Lehmann et al., 1999). In a number of both *in vitro* and *in vivo* experiments on the optic system performed by Lisa McKerrachers group involving the use of modified C3bot constructs to facilitate cell entry positive effects were detected on RGC axon regeneration and even survival of RGCs following axotomy (Bertrand et al., 2005, 2007). This might be indicative not only of a regenerative but also a neuroprotective capacity of C3 proteins.

Besides investigations on the optic system rodent spinal cord injury (SCI) has become an intensively used model to study the outcome of C3 treatment on the per se limited ability of central neurons to re-grow their axons after a lesion. Initial experiments were performed on rats and mice. Laminectomized animals underwent dorsal hemisection or contusion injury of the spinal cord at thoracic levels. Following application of C3, animals were allowed to recover and restoration of hind limb motor activity was analyzed by behavioral testing, usually accompanied by histological analysis of the spinal cord at the end of the observation period. According to these studies the application of C3 transferase or Rho-downstream effector kinase inhibitor Y27632 improved the developing locomotor activity (Dergham et al., 2002). The observed improved clinical outcome was attributed to an enhanced axonal regeneration of corticospinal tract (CST) fibers. At the molecular/mechanistic level the authors showed that the observed p75 neurotrophin receptor-dependent RhoA activation following SCI was blocked by cell-permeable C3 protein (Dubreuil et al., 2003). The form of administration as well as the initial dose of C3 transferase turned out to be crucial, since slow delivery of low doses of C3bot by a minipump had no beneficial effect but rather decreased locomotor recovery (Fournier et al., 2003). Upregulation of Rho proteins in various cell types following SCI was confirmed in several additional investigations and claimed to be largely responsible for the growth-inhibitory milieu in the perilesional area (Conrad et al., 2005; Lord-Fontaine et al., 2008). The relevance of RhoA-mediated signaling for regenerative axon growth was also recently demonstrated for spinal sensory axons following dorsal rhizotomy in a genetic ablation paradigm. Rho-associated kinase (ROCK) is a key downstream effector of RhoA. As an alternative to functional inhibition of Rho proteins by C3 transferase, inhibition of ROCK activity might be useful to promote axonal growth (Borisoff et al., 2003). Using brain specific ROCK II knockout mice it was shown that following lesion of the central branches of sensory neurons regenerating axons extend further into the CNS than observed in wild type animals (Duffy et al., 2009).

Overall, various studies have proven the beneficial outcome of C3 transferase treatment for neuronal process formation in both cell culture models and *in vivo* systems aiming to develop strategies to foster regenerative axonal outgrowth. Mechanistic evidence has accumulated that the observed effects are due to inhibition of Rho-signaling leading to alterations in cytoskeletal dynamics and therefore rely on enzymatic, e.g. ADP-ribosylating, activity of C3 proteins. Indeed, there are clear indications that clostridial C3 transferases also exert their effects by Rho-dependent, but not enzyme-mediated, modes of action. This will be reviewed thoroughly in the next chapter.

3. Not only enzymes: development of C3bot-derived neurotrophic peptides

First evidence for non-enzymatic interactions of C3 transferases came from studies on human platelets. Addition of recombinant RalA to platelet lysates blocked the C3bot-mediated ADP-ribosylation of Rho in a direct manner (Wilde et al., 2002). Also C3 transferases from *Clostridium limosum* or *Bacillus cereus* were found to be inhibited in their enzymatic activity by RalA, but to a lesser extent. Structural analysis revealed the molecular details of a RalA(GDP)-C3bot complex (Pautsch et al., 2005; Holbourn et al., 2005). Initial studies applying site-directed mutagenesis had discovered that a common hallmark of all C3-like transferases is a conserved glutamate at position 174 that serves as the catalytic residue for ADP-ribosylation of Rho proteins (Aktories et al., 1995). Exchange of glutamate for glutamine (C3botE174Q), for example, reduced the enzymatic activity more than 1000-fold, resulting in a virtually enzymatically dead protein. However, an unanticipated effect emerged when our group applied these mutant C3bot (C3botE174A and C3botE174Q) at nanomolar concentrations to primary hippocampal cultures. Initially intended to serve as negative controls, enzymatically dead C3bot constructs were found to significantly increase axonal length and branching comparable to wild type C3bot (Ahnert-Hilger et al., 2004). Differential ADP-ribosylation experiments confirmed the absence of remaining ADP-ribosyltransferase activity of the mutant proteins and assured that the observed neurotrophic effects did not result from enzymatic inhibition of RhoA, B or C. Prompted by these unexpected findings we developed a strategy to pinpoint the region of C3bot responsible for enzyme-independent neurotrophic effects. Using recombinant *E. coli* expression systems the full length C3bot amino acid sequence was split into three overlapping "mother peptides" one starting from the amino-terminal end (aa 1–98), one carboxy-terminal (aa 97–211) and a bridging peptide fragment (aa 62–124). Of these peptides all lacking an enzymatic activity only the C-terminal fragment C3bot$^{97–211}$ still exhibited axonotrophic effects on cultivated neurons (Fig. 1). We further truncated this sequence to the amino acids 154–211 that was still capable of exerting growth promoting effects an axons. Finally, we could identify a region spanning the 29 amino acids 154–182 termed C3bot$^{154–182}$ responsible for the enzyme-independent biological activity (Höltje et al., 2009). In contrast to full length mutant proteins C3botE174