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REVIEW

Therapeutic effects of *Clostridium botulinum* C3 exoenzyme

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Abstract C3 exoenzyme from *Clostridium botulinum*, specifically ADP-ribosylates small GTP-binding proteins RhoA, B, and C. ADP-ribosylation causes functional inactivation of Rho proteins resulting in cessation of the complete downstream signaling. Rho proteins are general regulators of a lot of essential cellular functions, among others, the neuronal growth cone. Rho activation, triggered by neuronal injury, inhibits neuronal repair mechanisms. To prevent the detrimental effect of active Rho in the recovery of injured neuronal systems, C3 has become a promising drug to inactivate RhoA in neurons. During the advancement of C3 to a drug candidate, it was found that ADP-ribosyltransferase activity of C3, in fact, is not essential for axonal and dendritic growth and branching. Rather, a peptide fragment of C3 covering the surface exposed ARTT loop from C3 ($C3^{154–182}$ peptide) is sufficient to induce growth and branching of neurons comparable to the effect of full-length C3. Whereas full-length C3 also acts on astrocytes and microglia to induce-at least in an in vitro model-inflammation and glial scar formation, $C3^{154–182}$ peptide is inert and seems only to act on neurons. In addition to its axono- and dendritotrophic effects on cultured primary hippocampal neurons, $C3^{154–182}$ peptide enhanced functional recovery and regeneration in a mouse model of spinal cord injury. Thus, in a proof-of-principle experiment, C3 peptide was shown to be efficacious in post-traumatic neuro-regeneration.

Keywords ARTT loop · Axon · C3 exoenzyme · Growth cone · Neurone · Neuronal injury · Rho

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Introduction

Clostridium botulinum type C co-produces three exotoxins, the well-known neurotoxin C1, the binary ADP-ribosyltransferase C2, and the ADP-ribosyltransferase C3. All three toxins act intracellularly: (1) the neurotoxin C1 (150 kDa) is an A-B-type of protein-toxin harboring endoprotease activity to cleave elements of vesicle fusion machinery thereby inhibiting neurotransmitter release resulting in flaccid paralysis (Bigalke and Shoer 2000). (2) C2 toxin is also an A-B-type of protein toxin composed of two separate components; the enzyme component possesses ADP-ribosyltransferase activity to modify cytoplasmatic monomeric actin; modified actin is incapable of polymerization so that C2 toxin induces destruction of the actin cytoskeleton accompanied by morphological changes (Aktories et al. 1986). (3) C3 (25 kDa) is a mere exoenzyme, also catalyzing ADP-ribosylation reaction (Aktories et al. 1987; Rubin et al. 1988). The target is the low molecular weight GTP-binding protein Rho, belonging to the Ras superfamily (Aktories et al. 2004; Aktories and Just 2005).

C3 from *C. botulinum* ($C3^{bot}$) is the prototype of Rho ADP-ribosylating exoenzymes (Aktories and Just 2005; Vogelsgesang et al. 2007). The C3-like family comprises transferases from *C. limosum, B. cereus*, and also from *S. aureus* (Just et al. 1992a, b; Wilde et al. 2001, 2003). The

C3-related exoenzymes from *S. aureus* are called EDIN (epidermal differentiation inhibitor) (Inoue et al. 1991). All C3-like exoenzymes are devoid of any attributable transport domain, but they reach their intracellular target protein Rho. Therefore, C3 cannot be categorized as classical bacterial protein–toxin. At the moment, it is not clear which entry pathway is used by C3 (Aktories and Just 2005; Vogelsgesang et al. 2007). There is one finding that—at least partially—an acid endosomal compartment is involved, indicating a receptor-mediated endocytosis pathway in macrophage-like cells (Fahrer et al. 2010). The usually mentioned non-specific uptake of C3, in fact, reflects our lack of knowledge.

Mode of action

Except EDIN, C3 exoenzymes from different bacterial strains, exclusively mono-ADP-ribosylate members of the Rho subfamily of low molecular weight GTP-binding proteins. Only the highly homologous RhoA, B and C are modified (Aktories and Just 2005; Vogelsgesang et al. 2007). EDIN additionally ADP-ribosylates RhoE (Wilde et al. 2001). The ADP-ribose moiety is transferred to amino acid residue Asn-41, which resides in the so-called effector region of the GTP-binding proteins (Sekine et al. 1989). The effector region undergoes conformational change induced by binding to GTP, allowing this region to interact with Rho-effector proteins, such as ROCK-kinase or mDia. Binding results in activation of the effector allowing downstream signaling (Aspenström et al. 2004; Etienne-Manneville and Hall 2002; Wennerberg and Der 2004; Heasman and Ridley 2008). The ADP-ribose at Asn-41 has different impact upon Rho-downstream signaling: (1) ADP-ribosylated Rho is entrapped by GDI (guanine nucleotide inhibitor) to form a high affinity complex localized to the cytoplasm. Thus, Rho is stabilized in the inactive state (Fujihara et al. 1997; Genth et al. 2003a). (2) Non-complexed ADP-ribosylated Rho cannot interact with Rho-GEF, a guanine-nucleotide exchange factor, which mediates GTP-binding, and thus, activation. In contrast, inactivation by Rho-GAP, a GTPase-activating protein, catalyzing inactivation of Rho, is not blocked by ADP-ribosylation (Sehr et al. 1998; Genth et al. 2003b). Thus, the inactive GDP-bound form of Rho is stabilized, preventing activation by intra- or extracellular input signals. Rho downstream signaling is essentially disturbed, and regulator proteins with anti-Rho function take over the cellular governance. E. g., RhoA is the master regulator of the actin cytoskeleton by recruiting the effector proteins ROCK and mDia. The highly dynamic actin polymerization and formation of actin–myosin-based stress fibers are regulated through RhoA. Its inactivation by ADP-ribosylation dramatically disrupts the fragile balance. Depolymerizing input prevails, resulting in destruction of the actin cytoskeleton accompanied by cell rounding and (in some cell types) detachment of cells (Chardin et al. 1989; Ridley and Hall 1992 and 1994; Wiegers et al. 1991). This is, in fact, the prominent cellular outcome of C3, especially as it can be easily visualized. However, in primary neurons, C3 does not induce rounding up but elongation of neurites. Many cytoskeleton-associated effects, such as motility, migration, endo-, and exocytic processes, as well as neurite growth, are altered (Jalink et al. 1994; Mackay et al. 1995). However, also non-actin-based cellular functions, such as cell-cycle regulation, cell death, and phosphor-lipid metabolism are changed by C3 (Aktories and Just 2005; Vogelsgesang et al. 2007; Just et al. 2010).

C3 as a tool in cell biology

C3 selectively modifies only RhoA, B, and C out of approximately 180 low molecular weight GTP-binding proteins. This is the basis of its common application as tool in cell biology to study the role of RhoA in cellular functions (Just and Boquet 2000; Aktories and Just 2005). This notion is still true in the era of knockout cells and application of siRNA. Application of RhoA–siRNA is accompanied by massive RhoB expression and activation. This unwanted side effect is based on the fact that the activity of the *rhoB* promoter is suppressed by RhoA, and RhoA inactivation is always associated with RhoB expression (Huelsenbeck et al. 2007). Strong RhoB expression is also observed upon treatment of cells with C3, but RhoB is completely inactivated by C3-catalyzed ADP-ribosylation. Thus, application of C3 represents the only approach to effectively inhibit RhoA without concomitant RhoB activation (Just et al. 2010).

Rho functions in neurons

The three canonical Rho-functions, Rac-mediated formation of lamellipodia, Cdc42-regulated filopodia, and RhoA-governed actin cables are present in the neuron growth cone. The growth cone is essential for neurite and axonal growth. Rac and Cdc42 are positively involved, as their activation mediates growth and repair mechanisms induced by neuronal injury (Dickson 2001; Govek et al. 2005; Watabe-Uchida et al. 2006; Nadif and Van 2008; Linseman and Loucks 2008; Hall and Lalli 2010). RhoA, in contrast, is the antagonist not only by stopping growth, but also inducing growth cone collapse. In the course of neuronal injury, myelin-associated glycoprotein

(MAG), together with ganglioside GT1b, cause activation of RhoA through the receptor p75 (NTR) (Yamashita et al. 2002; Mimura et al. 2006). In addition, serum released by injury also triggers growth cone collapse through RhoA activation (Dubreuil et al. 2003; Dubrueil et al. 2006; McKerracher and Higuchi 2006; Schmandke et al. 2007). These findings were the basis of the concept to apply $C3^{bot}$ as drug to prevent Rho-mediated growth cone collapse and promote neuronal repair (Bertrand et al. 2005 and 2007). In fact, there is a strong requirement for drugs, enhancing the recovery from (central and peripheral) neuronal lesions, as current therapeutic approaches are of poor efficacy. The novel concept exploits the ADP-ribosyltransferase activity of $C3^{bot}$. To improve the poor cell accessibility, $C3^{bot}$ has been applied as cell permeable fusion protein (Lord-Fontaine et al. 2008). The constructed BA-210 (Cethrin®) was tested as drug for the treatment of spinal cord injuries and reached phase IIb of clinical studies.

Effect of C3 on hippocampal neurons

Treatment of cultured murine hippocampal neurons (prepared from the brain of embryonic mice at day 17) with $C3^{bot}$ exoenzyme, results in growth of axons and dendrites (Ahnert-Hilger et al. 2004). In addition, branching is increased. Hippocampal neurons are sensitive at a concentration range of 5–50 nM of $C3^{bot}$, whereas other cell types, such as fibroblasts or epithelial cells, respond to $C3^{bot}$ at concentrations higher than 1 μM (Just et al. 2010). Neurons seem to be the most sensitive cell type studied so far. The related C3-like transferases from the C3-family of ADP-ribosyltransferases, such as C3 from *C. limosum* and *S. aureus*, are not able to cause growth and branching, although they act inside the neurons to ADP-ribosylate cellular RhoA (Ahnert-Hilger et al. 2004). Thus, the Rho-inactivating enzyme activity seems not to be essential. If this notion is correct, enzyme-deficient $C3^{bot}$ should be active upon hippocampal neurons. Mutation of the catalytic amino acid residue (Glu-174) of $C3^{bot}$ cuts down transferase activity and blocks modification of Rho. However, the growth and branching, promoting activity of enzyme-deficient $C3^{bot}$, was comparable to that of wild-type C3. The concentration range is also comparable.

The next step was to check whether the full length of $C3^{bot}$ is essential for the axon growth-promoting activity, or whether a peptide fragment from $C3^{bot}$ is sufficient. To this end, $C3^{bot}$ protein was cut down into fragments, showing that the C-terminal 60 residues harbor the growth-promoting activity. This short fragment was further shortened, and overlapping peptides were tested resulting in the identification of a 29-mer peptide, covering the region of the catalytic amino acid Glu-174 ($C3^{154–182}$ peptide). The identified peptide is the extended ARTT-loop of $C3^{bot}$ (Fig. 1) (Höltje et al. 2009). The catalytic amino acid (Glu-174) and the amino acid responsible for binding to the protein substrate Rho (Phe-169) reside in the ADP-ribosylation-turn-turn motif (ARTT) as can be deduced from the crystal structure of $C3^{bot}$ (Han et al. 2001; Ménétrey et al. 2002 and 2008). But the fragment is devoid of any ADP-ribosyltransferase activity. The ARTT-loop is exposed at the surface of the $C3^{bot}$ molecule, and is thus, accessible to interaction with target structures of the neuronal plasma membrane. All C3 fragments, which harbor the region 154–182, possess the axon growth-promoting activity independently from their lengths. These findings suggest a ligand-like interaction of $C3^{bot}$ and the $C3^{154–182}$-peptide, respectively, with a putative neuronal receptor, which transduces the growth-promoting signal into the intracellular space. Only preliminary findings

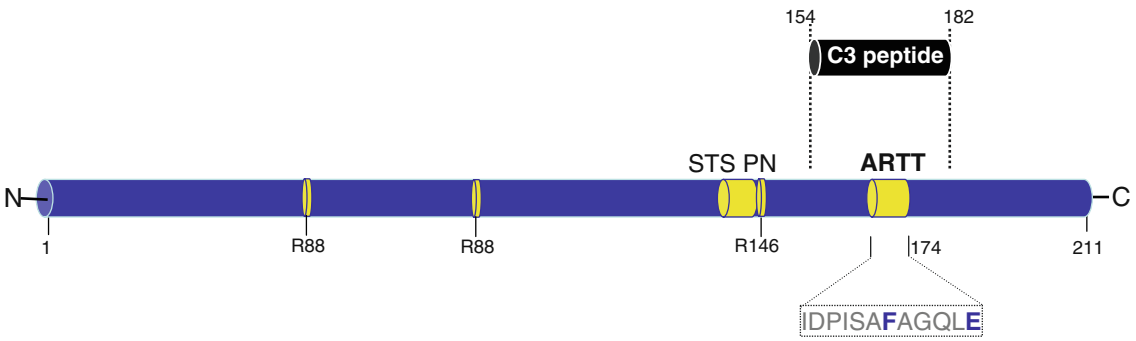

Fig. 1 The primary structure of $C3^{bot}$ from *Clostridium botulinum* is given. Important motifs are depicted: the ADP-ribosylation toxin-turn-turn (ARTT) loop is involved in protein substrate recognition (F169) and harbors the catalytic amino acid residue E174; the PN-loop (phosphate-nicotinamide loop) is involved in the binding of NAD^+; the STS-motif (serine–threonine–serine) is thought to maintain the reaction cavity. Several arginine residues (R51, R88, and R146) are involved in the interaction with the co-substrate NAD^+. The peptide fragment, harboring the axon growth-promoting activity covers residues 154 through 182, including the ARTT-loop. All $C3^{bot}$ fragments, which harbor the region 154–182, possess axonotrophic activity

support this working hypothesis. Expression of GFP-tagged wild-type and mutant $C3^{bot}$ clearly showed that intracellular expression of enzymatically active $C3^{bot}$ caused the opposite, namely reduction of axonal length (Ahnert-Hilger et al. 2004). Enzyme-deficient $C3^{bot}$ was without any effect resembling mock transfection of neurons with GFP alone. Thus, the exclusive presence of $C3^{bot}$ in the cytoplasm is not sufficient for the axon growth-promoting effect. In the case of wild-type $C3^{bot}$, the interaction with the membrane receptor induces a signal, which seems to be dominant to the effect caused by the intracellular (cytoplasmic) signal. In addition, a blot overlay approach of membranes with $C3^{bot}$ identified distinct protein bands binding to $C3^{bot}$ (Just et al. unpublished data).

At the moment, it is not clear how $C3^{bot}$ triggers its promoting effect on neurons. There are at least three working hypotheses: (1) $C3^{bot}$, as well as the $C3^{154–182}$ peptide acts like a ligand on a putative neuronal receptor; secondarily C3 (enzymatically active and inactive) is taken up into the neuron. (2) $C3^{bot}$ and peptide fragment interact with a putative receptor, which only mediates uptake via a non-characterized pathway to act intracellularly. (3) Both full-length C3 and the C3 peptide act intracellularly, but the uptake is different; full-length C3 enters via a receptor-mediated pathway, while $C3^{154–182}$ peptide comes in through a, e.g., peptide transporter.

Are there some hints of the mode of action? Although the $C3^{154–182}$ peptide does not ADP-ribosylate Rho, it alters the activation state of RhoA. Treatment of hippocampal neurons with the C3 peptide-induced inactivation of RhoA is shown by pull-down assays from the neurons (Boato et al. 2010). Thus, C3 peptide is able to down-regulate the signaling competence of RhoA in intact neurons. This finding underscores the relevance of functional inactivation of RhoA in axonal growth and repair processes.

Human neuronal model

So far, all neuronal cell lines tested do not respond to enzyme-deficient $C3^{bot}$, or to the $C3^{154–182}$ peptide. Because cell lines are transformed and differ in cellular functions from differentiated cells, the NT2 cell line was tested. NT2 cells are human embryonic teratocarcinoma cells, which are nice cellular models, as they can be differentiated to postmitotic neurons by treatment with retinoic acid for 6 weeks (NT2-N). Differentiation was visualized by tubulin (for neurons) and by MAP2 (for dendrites) staining. In addition, differentiation was confirmed by immunoblot detection of neurofilament M (NF-M) expression, a differentiation marker for neurons. The original NT2 cells were only sensitive to enzymatically active $C3^{bot}$, whereas enzyme-deficient $C3^{bot}$ and $C3^{154–182}$ peptide were without effect. By contrast, NT2-N (differentiated by retinoic acid treatment) responded to enzyme-deficient C3, as well as to the $C3^{154–182}$ peptide. There was no gross difference in the effective concentration and effectiveness to induce axon growth. A $C3^{bot}$ peptide, covering part of C3 outside the ARTT-loop, was without effect confirming the specific growth-promoting activity of the $C3^{154–182}$ peptide (Just and Mühlenstädt, non published data).

Human neurons do respond to $C3^{bot}$ and $C3^{154–182}$ peptide comparable to rat and mice neurons. To be sensitive to enzyme-deficient $C3^{bot}$ and $C3^{154–182}$ peptide, neurons seem to be at least in a differentiation stage, where the axon is clearly distinguishable from dendrites. Neurons at a differentiation stage, when only neurites are present, are insensitive to $C3^{154–182}$ peptide. The differentiation stage may reflect the presence of membrane receptors or signal pathways receptive to $C3^{154–182}$ peptide, which are not present in nondifferentiated types of neurons.

Effect of $C3^{154–182}$ peptide in an animal model

$C3^{bot}$ has been shown in different in vitro and in vivo models to promote neurite growth and regeneration. In spinal cord injury models in rats and mice, $C3^{bot}$ has been successfully shown to improve the functional recovery (Bertrand et al. 2005; Dergham et al. 2002; Lord-Fontaine et al. 2008). In these studies, $C3^{bot}$ was applied as a membrane-permeable construct to inactivate RhoA by ADP-ribosylation based on the concept: inhibition of RhoA activity inhibits growth cone collapse.

However, enzymatically active $C3^{bot}$ acts on astrocytes to release excitatory glutamate and induce strong proliferation in a scratch wound assay indicative for fostering glial scar formation. Furthermore, $C3^{bot}$ acts on microglia to induce a pro-inflammatory response. $C3^{154–182}$ peptide, however, did not act on astrocytes nor on microglia (Table 1); the peptide is inactive in such cell types (Höltje et al. 2005 and 2008;

Table 1 The activity of three $C3^{bot}$ constructs (wild-type = enzymatically active, enzyme-deficient, and peptide$^{154–182}$) are compared with respect to axon and dendritic growth and length promotion, as well as their impact on astrocytes/microglia

$C3^{bot}$	Neurons		Astrocytes/microglia
	Axon	Dendrites	
Wild-type	+	+	+
Enzyme-deficient	+	−	−
Peptide$^{154–182}$	+	+	−

Hoffmann et al. 2008). Based on the findings that $C3^{154–182}$ peptide is effective in inducing axonal growth and branching in primary hippocampal neurons, it was tested in a murine spinal cord compression injury model and dorsal hemisection model (Boato et al. 2010). The 29-mer amino acid fragment of $C3^{bot}$ significantly improved locomotor restoration in both models. The functional data were supported by enhanced regenerative growth of the corticospinal tract fibers and enhanced axonal outgrowth of alpha-motor neurons. Wild-type $C3^{bot}$, however, was toxic and caused death of some animals. Thus, $C3^{154–182}$ peptide is active in the context of a whole animal; it fosters axonal protection and improves functional recovery.

Conclusion

C3-like transferases from microbial source are a small family of Rho-inactivating exoenzymes, whose role as virulence factors, is still ill-defined. Their extremely high target specificity is the basis for their application as established tool in cell biological research, further playing an important role in the knockout and -down era. The well-characterized mode of action and the target specificity have made C3 a promising drug to treat neuronal lesions, such as spinal cord injury. Poor cell-type/tissue specificity has been solved by local application, and in fact, the cell-permeable $C3^{bot}$ construct was tested in patients with acute cervical spinal cord injury in clinical study phase II.

Surprisingly, the activity of $C3^{bot}$ to promote recovery of neuronal lesions by axonal growth promotion is not based on its inherent enzyme activity. Rather a small surface-exposed peptide fragment encompassing residues 154 to 182 is sufficient to do the job originally assigned to the transferase activity of C3. In contrast to the enzymatically active full-length $C3^{bot}$, the $C3^{154–182}$ peptide acts only on differentiated neurons but not on astrocytes and microglia. At a first glance, C3 peptide possesses a tissue or cell-type specificity, lacking by full-length C3 expecting less side effects as potential drug. In addition, a small peptide is the solid basis for the development of so-called peptide-mimic drugs. Peptide-mimetic drugs have in fact the advantage of increased in vivo stability and decreased immunogenicity compared to peptides. Furthermore, it is conceivable that the peptide-mimetic analogue of the C3 peptide is systemically applicable rather than local, allowing treatment of neurodegenerative diseases.

The therapeutic potential of C3 is not restricted to enhancement of neuroregeneration. At the moment, the enzymatically competent (wild-type) C3 is used to create C3-coated stents, which are implanted into coronary arteries to overcome artery stenosis. Coated stents continuously release C3 to prevent smooth muscle cell proliferation-based restenosis, a major problem in balloon angioplasty/stent implantation (http://www.ibridgenetwork.org/columbia/ir_1224?search_term=C3&x=4&y=11).

References

Ahnert-Hilger G, Höltje M, Große G, Pickert G, Mucke C, Nixdorf-Bergweiler B, Boquet P, Hofmann F, Just I (2004) Differential effects of Rho GTPases on axonal and dendritic development in hippocampal neurons. J Neurochem 90:9–18

Aktories K, Just I (2005) Clostridial Rho-inhibiting protein toxins. Curr Top Microbiol Immunol 291:113–145

Aktories K, Bärmann M, Ohishi I, Tsuyama S, Jakobs KH, Habermann E (1986) Botulinum C2 toxin ADP-ribosylates actin. Nature 322:390–392

Aktories K, Weller U, Chhatwal GS (1987) Clostridium botulinum type C produces a novel ADP-ribosyltransferase distinct from botulinum C2 toxin. FEBS Lett 212:109–113

Aktories K, Wilde C, Vogelsgesang M (2004) Rho-modifying C3-like ADP-ribosyltransferases. Rev Physiol Biochem Pharmacol 152:1–22

Aspenström P, Fransson A, Saras J (2004) Rho GTPases have diverse effects on the organization of the actin filament system. Biochem J 377:327–337

Bertrand J, Winton MJ, Rodriguez-Hernandez N, Campenot RB, McKerracher L (2005) Application of Rho antagonist to neuronal cell bodies promotes neurite growth in compartmented cultures and regeneration of retinal ganglion cell axons in the optic nerve of adult rats. J Neurosci 25:1113–1121

Bertrand J, Di Polo A, McKerracher L (2007) Enhanced survival and regeneration of axotomized retinal neurons by repeated delivery of cell-permeable C3-like Rho antagonists. Neurobiol Dis 25:65–72

Bigalke H, Shoer LF (2000) Clostridial Neurotoxins. In: Aktories and Just (eds) Handbook of Experimental Pharmacology, vol 145 Bacterial Toxins pp 407–443

Boato F, Hendrix S, Hülsenbeck SC, Hofmann F, Große G, Djalali S, Klimaschewski L, Auer M, Just I, Ahnert-Hilger G, Höltje M (2010) C3 peptide enhances recovery from spinal cord injury by improved regenerative growth of descending fiber tracts. J Cell Sci 123:1652–1662

Chardin P, Boquet P, Madaule P, Popoff MR, Rubin EJ, Gill DM (1989) The mammalian G protein rhoC is ADP-ribosylated by Clostridium botulinum exoenzyme C3 and affects actin microfilament in Vero cells. EMBO J 8:1087–1092

Dergham P, Ellezam B, Essagian C, Avedissian H, Lubell WD, McKerracher L (2002) Rho signaling pathway targeted to promote spinal cord repair. J Neurosci 22:6570–6577

Dickson BJ (2001) Rho GTPases in growth cone guidance. Curr Opin Neurobiol 11:103–110

Dubreuil CI, Winton MJ, McKerracher L (2003) Rho activation patterns after spinal cord injury and the role of activated Rho in apoptosis in the central nervous system. J Cell Biol 162:233–243

Dubrueil CI, Marklund N, Deschamps K, McIntosh TK, McKerracher L (2006) Activation of Rho after traumatic brain injury and seizure in rats. Exp Neurol 198:361–369

Etienne-Manneville S, Hall A (2002) Rho GTPases in cell biology. Nature 420:629–635

Fahrer J, Kuban J, Heine K, Rupps G, Kaiser E, Felder E, Benz R, Barth H (2010) Selective and specific internalization of clostridial C3 ADP-ribosyltransferases into macrophages and monocytes. Cell Microbiol 12:233–247

Fujihara H, Walker LA, Gong MC, Lemichez E, Boquet P, Somlyo AV et al (1997) Inhibition of RhoA translocation and calcium sensitization by in vivo ADP-ribosylation with the chimeric toxin DC3B. Mol Biol Cell 8:2437–2447

Genth H, Gerhard R, Maeda A, Amano M, Kaibuchi K, Aktories K et al (2003a) Entrapment of Rho ADP-ribosylated by Clostridium botulinum C3 exoenzyme in the Rho-guanine nucleotide dissociation inhibitor-1 complex. J Biol Chem 278:28523–28527

Genth H, Schmidt M, Gerhard R, Aktories K, Just I (2003b) Activation of phospholipase D1 by ADP-ribosylated RhoA. Biochem Biophys Res Commun 302:127–132

Govek EE, Newey SE, Van AL (2005) The role of the Rho GTPases in neuronal development. Genes Dev 19:1–49

Hall A, Lalli G (2010) Rho and Ras GTPases in axon growth, guidance, and branching. Cold Spring Harb Perspect Biol 2:a001818

Han S, Arvai AS, Clancy SB, Tainer JA (2001) Crystal structure and novel recognition motif of rho ADP-ribosylating C3 exoenzyme from Clostridium botulinum: structural insights for recognition specificity and catalysis. J Mol Biol 305:95–107

Heasman SJ, Ridley AJ (2008) Mammalian Rho GTPases: new insights into their functions from in vivo studies. Nat Rev Mol Cell Biol 9:690–701

Hoffmann A, Hofmann F, Just I, Lehnardt S, Hanisch UK, Brück W, Kettenmann H, Ahnert-Hilger G, Höltje M (2008) Inhibition of Rho-dependent pathways by Clostridium botulinum C3 protein induces a proinflammatory profile in microglia. Glia 56:1162–1175

Höltje M, Hoffmann A, Hofmann F, Mucke C, Grosse G, Van Rooijen N, Kettenmann H, Just I, Ahnert-Hilger G (2005) Role of Rho GTPase in astrocyte morphology and migratory response during in vitro wound healing. J Neurochem 95:1237–1248

Höltje M, Hofmann F, Lux R, Veh RW, Just I, Ahnert-Hilger G (2008) Glutamate uptake and release by astrocytes are enhanced by Clostridium botulinum C3 protein. J Biol Chem 283:9289–9299

Höltje M, Djalali S, Hofmann F, Münster-Wandowski A, Hendrix S, Boato F, Dreger S, Große G, Henneberger C, Grantyn R, Just I, Ahnert-Hilger G (2009) A 29 amino acid fragment of Clostridium botulinum C3 protein enhances neuronal outgrowth, connectivity, and reinnervation. FASEB J 23:1115–1126

Huelsenbeck J, Dreger S, Gerhard R, Fritz G, Just I, Genth H (2007) Up-regulation of the immediate early gene product RhoB by exoenzyme C3 from Clostridium limosum and toxin B from Clostridium difficile. Biochemistry 46:4923–4931

Inoue S, Sugai M, Murooka Y, Paik S-Y, Hong Y-M, Ohgai H, Suginaka H (1991) Molecular cloning and sequencing of the epidermal cell differentiation inhibitor gene from *Staphylococcus aureus*. Biochem Biophys Res Commun 174:459–464

Jalink K, Van Corven EJ, Hengeveld T, Morii N, Narumiya S, Moolenaar WH (1994) Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. J Cell Biol 126:801–810

Just I, Boquet P (2000) Large clostridial cytotoxins as tools in cell biology. Curr Top Microbiol Immunol 250:97–107

Just I, Mohr C, Schallehn G, Menard L, Didsbury JR, Vandekerckhove J et al (1992a) Purification and characterization of an ADP-ribosyltransferase produced by Clostridium limosum. J Biol Chem 267:10274–10280

Just I, Schallehn G, Aktories K (1992b) ADP-ribosylation of small GTP-binding proteins by Bacillus cereus. Biochem Biophys Res Commun 183:931–936

Just I, Huelsenbeck SC, Genth H (2010) Clostridium Botulinum C3 Exoenzyme: Rho-inactivating tool in cell biology and a neurotrophic agent. The Open Toxinology Journal 3:19–23

Linseman DA, Loucks FA (2008) Diverse roles of Rho family GTPases in neuronal development, survival, and death. Front Biosci 13:657–676

Lord-Fontaine S, Yang F, Diep Q, Dergham P, Munzer S, Tremblay P, McKerracher L (2008) Local inhibition of Rho signaling by cell-permeable recombinant protein BA-210 prevents secondary damage and promotes functional recovery following acute spinal cord injury. J Neurotrauma 25:1309–1322

Mackay DJG, Nobes CD, Hall A (1995) The Rho's progress: a potential role during neuritogenesis for the Rho family of GTPases. Trends Neurosci 18:496–501

McKerracher L, Higuchi H (2006) Targeting Rho to stimulate repair after spinal cord injury. J Neurotrauma 23:309–317

Ménétrey J, Flatau G, Stura EA, Charbonnier J-B, Gas F, Teulon J-M et al (2002) NAD binding induces conformational changes in Rho ADP-ribosylating clostridium botulinum C3 exoenzyme. J Biol Chem 277:30950–30957

Ménétrey J, Flatau G, Boquet P, Ménez A, Stura EA (2008) Structural basis for the NAD-hydrolysis mechanism and the ARTT-loop plasticity of C3 exoenzymes. Protein Sci 17:878–886

Mimura F, Yamagishi S, Arimura N, Fujitani M, Kubo T, Kaibuchi K, Yamashita K (2006) Myelin-associated glycoprotein inhibits microtubule assembly by a Rho-kinase-dependent mechanism. J Biol Chem 281:15970–15979

Nadif KN, Van AL (2008) Rho-linked genes and neurological disorders. Pflugers Arch 455:787–797

Ridley AJ, Hall A (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 70:389–399

Ridley AJ, Hall A (1994) Signal transduction pathways regulating Rho-mediated stress fibre formation: requirement for a tyrosine kinase. EMBO J 13:2600–2610

Rubin EJ, Gill DM, Boquet P, Popoff MR (1988) Functional modification of a 21-Kilodalton G protein when ADP-ribosylated by exoenzyme C3 of Clostridium botulinum. Mol Cell Biol 8:418–426

Schmandke A, Schmandke A, Strittmatter SM (2007) ROCK and Rho: biochemistry and neuronal functions of Rho-associated protein kinases. Neuroscientist 13:454–469

Sehr P, Joseph G, Genth H, Just I, Pick E, Aktories K (1998) Glucosylation and ADP-ribosylation of rho proteins: effects on nucleotide binding, GTPase activity, and effector coupling. Biochemistry 37:5296–5304

Sekine A, Fujiwara M, Narumiya S (1989) Asparagine residue in the rho gene product is the modification site for botulinum ADP-ribosyltransferase. J Biol Chem 264:8602–8605

Vogelsgesang M, Pautsch A, Aktories K (2007) C3 exoenzymes, novel insights into structure and action of Rho-ADP-ribosylating toxins. Naunyn Schmiedebergs Arch Pharmacol 374:347–360

Watabe-Uchida M, Govek EE, Van Aelst L (2006) Regulators of Rho GTPases in neuronal development. J Neurosci 26:10633–10635

Wennerberg K, Der CJ (2004) Rho-family GTPases: it's not only Rac and Rho (and I like it). J Cell Sci 117:1301–1312

Wiegers W, Just I, Müller H, Hellwig A, Traub P, Aktories K (1991) Alteration of the cytoskeleton of mammalian cells cultured in vitro by Clostridium botulinum C2 toxin and C3 ADP-ribosyltransferase. Eur J Cell Biol 54:237–245

Wilde C, Chhatwal GS, Schmalzing G, Aktories K, Just I (2001) A novel C3-like ADP-ribosyltransferase from Staphylococcus aureus modifying RhoE and Rnd3. J Biol Chem 276:9537–9542

Wilde C, Vogelsang M, Aktories K (2003) Rho-specific Bacillus cereus ADP-ribosyltransferase C3cer cloning and characterization. Biochemistry 42:9694–9702

Yamashita T, Higuchi H, Tohyama M (2002) The p75 receptor transduces the signal from myelin-associated glycoprotein to Rho. J Cell Biol 157:565–570