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Attack of the nervous system by *Clostridium perfringens* Epsilon toxin: From disease to mode of action on neural cells

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ABSTRACT

Epsilon toxin (ET), produced by *Clostridium perfringens* types B and D, ranks among the four most potent poisonous substances known so far. ET-intoxication is responsible for enterotoxaemia in animals, mainly sheep and goats. This disease comprises several manifestations indicating the attack of the nervous system. This review aims to summarize the effects of ET on central nervous system. ET binds to endothelial cells of brain capillary vessels before passing through the blood–brain barrier. Therefore, it induces perivascular oedema and accumulates into brain. ET binding to different brain structures and to different component in the brain indicates regional susceptibility to the toxin. Histological examination has revealed nerve tissue and cellular lesions, which may be directly or indirectly caused by ET. The naturally occurring disease caused by ET-intoxication can be reproduced experimentally in rodents. In mice and rats, ET recognizes receptor at the surface of different neural cell types, including certain neurons (e.g. the granule cells in cerebellum) as well as oligodendrocytes, which are the glial cells responsible for the axons myelination. Moreover, ET induces release of glutamate and other transmitters, leading to firing of neural network. The precise mode of action of ET on neural cells remains to be determined.

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1. Introduction

The bacterial genus *Clostridium* comprises Gram-positive anaerobic bacteria, which are present in all kinds of environments. About 13 clostridia species are major pathogens exerting their deleterious actions through a number of toxins, which include the most toxic substances known so far. Clostridial diseases are not rare in humans (e.g. antibiotic associated pseudomembranous colitis caused by *Clostridium difficile*, intoxications due to food contamination by *Clostridium perfringens*, gangrene and tetanus due to colonization of a wound by *C. perfringens* or *Clostridium tetani*, respectively). Also, they cause considerable loss in domestic and wild animals.

Epsilon toxin (ET) produced by *C. perfringens* types B and D is one of the most potent clostridial toxins. Very high lethality of ET (~400,000 mouse LD_{100}/mg protein, *i.p.*) ranks it among the four most potent poisonous substances known so far (reviewed by Gill, 1982). Infection by ET-producing bacteria occurs via food, water, animal litter or

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soil, and causes severe, often fatal enterotoxaemia mainly in sheep, goat and cattle. Unfortunately, high stability of ET, together with the possibility to express it as recombinant protein into *Escherichia coli* as well as the lack of relevant therapeutics, led to the recognition of ET as a potential biological weapon (Anderson and Bokor, 2012; Greenfield et al., 2002).

Overall, information on the way(s) by which ET kills the infected hosts remains scarce. In animals, enterotoxaemia develops per acutely in most cases, leading to sudden death without any prior signs of disease. Over-proliferation of *C. perfringens* in intestines produces large amounts of ET, which increases the permeability of the intestinal mucosal barrier and therefore enters into the bloodstream. Then, ET diffuses through all organs and accumulates preferentially into the brain and kidneys (Nagahama and Sakurai, 1991). ET induces elevation of blood pressure (Buxton et al., 1978; Nagahama et al., 1993; Sakurai et al., 1983) associated with an increase in the permeability of the cerebral blood vessels (Gardner, 1973c; Morgan et al., 1975). However, the question whether the major neurological disorders observed in ET-intoxicated animals result from neural tissue damage ensuing brain oedema (Barker et al., 1993; Buxton and Morgan, 1976; Finnie, 2003; Finnie et al., 1999; Uzal and Kelly, 1997; Uzal et al., 1997) or direct attack of neural cells constituting brain tissue remains matter of debate.

This review aims to summarize ET effects on the nervous system (mainly the central nervous system) and focuses on the causal linkage between symptoms or manifestations expressed by intoxicated animals as well as structures affected, and the potential direct effect of ET on neural cells.

2. *C. perfringens* and Epsilon toxin

C. perfringens (also termed *Clostridium welchii*) is a Gram-positive, anaerobic and spore-forming bacillus. *C. perfringens* is a ubiquitous environmental bacterium that can be found as normal microflora component of soil, dust and sediments. As many other *Clostridia*, it grows in cadavers and litter. Spores are ingested and can reach intestines of various vertebrates (McClane and Chakrabarti, 2004). Overall, *C. perfringens* is considered as a normal inhabitant of the gastro intestinal tract. Typically, perturbation of microbial balance in the gut (for instance by a rapid change in diet) induces overgrowth of *C. perfringens* leading to production of high level of toxins. Proliferation of types B and D in gut causes enterotoxaemia in sheep and goat and more rarely in cattle (Uzal et al., 2002; McClane and Chakrabarti, 2004). The bacterium has also been found in pig (Bergeland, 1972; Bergeland et al., 1966; Cho et al., 1991) and smaller renting animals, such as rabbit and chicken (Heikinheimo and Korkeala, 2005; Sting, 2009). ET-producing strains of *C. perfringens* have been isolated from human intestine (Gleeson-White and Bullen, 1955; Kohn and Warrack, 1955) and upon the occasion of a case of gas-gangrene (Morinaga et al., 1965). However, it remains unclear whether the strains isolated had a role in the disease observed in man. 17 different toxins including alpha, beta, iota and epsilon toxins are produced by various strains of *C. perfringens*. According to produced-toxins, *C. perfringens* have been divided into five main groups, from A through E (Finegold, 1977; Fisher et al., 2006; Niilo, 1980). Only two groups of *C. perfringens* (types B and D) produce ET. *C. perfringens* type B produces ET together with alpha- and beta-toxins whereas type D produces ET, alpha-toxin and perfringolysin-O (reviewed by Alouf and Popoff, 2006; McClane et al., 2006; Uzal and Songer, 2008).

ET is a single-chain protein synthesized as a protoxin of 32–33 kDa (McDonel, 1980). Removal of the 11 N-terminal (or the 13 N-terminal) and of the 29 C-terminal residues amino-acids by proteases (notably the α-chymotrypsin, trypsin and λ-protease) converts the inactive protoxin (proET) into a fully active form (i.e. the toxin, ET 28.6 kDa), with a lethal dose (LD) of about 70 ng kg^{-1} in mice (i.e. 400,000 mouse-LD per mg protein) (Minami et al., 1997; reviewed by Popoff, 2011a). Proteases involved in conversion of proET into ET are synthesized by *C. perfringens* (Minami et al., 1997) as well as by the host (Bhown and Habeerb, 1977; Worthington and Mülders, 1977). The 3D structure of ET has been resolved and presents similarities with the pore-forming toxin aerolysin produced by *Aeromonas hydrophila* species (Cole et al., 2004; Gurcel et al., 2006; Parker et al., 1994). For further details concerning ET mode of action see §6 and recent reviews written by Popoff (2011a) and by Bokori-Brown et al. (2011).

3. Symptoms manifestations during naturally occurring disease or after administration of ET

Proliferation of *C. perfringens* type D in the intestinal tract and ensuing production of toxins causes enteric disease termed enterotoxaemia in sheep and goats, whereas *C. perfringens* type B is associated with dysentery (sheep) and haemorrhagic enteritis (goats) and signs of enterotoxaemia (reviewed by McClane et al., 2006; Uzal and Songer, 2008). Enterotoxaemia caused by *C. perfringens* type D in sheep is a worldwide problem. The disease is most commonly observed in lambs (Barker et al., 1993; Songer, 1996), frequent in goats (Blackwell and Butler, 1992; Blackwell et al., 1991; Uzal and Kelly, 1996, 1997; Uzal et al., 1994) and adult sheep and calves (Buxton et al., 1981; Munday et al., 1973) but less frequent in adult cattle (Radostits et al., 2000), and has been reported in deers, domesticated camels, horses (Stubbing, 1990). Recently, a suspicious case has even been reported in a tiger (Zeira et al., 2012).

Naturally occurring enterotoxaemia is commonly depicted according to 3 grades of manifestations (per-acute, acute and sub-acute or chronic); the severity of the disease being correlated to the amount of ET produced by *C. perfringens*. In per-acute form, sudden death of animals occurs without premonitory signs. In sheep, the acute form is characterized by a combination of severe neurological (as convulsions) and respiratory troubles; diarrhoea is infrequent. The recovery from the acute form of the disease is rare. In sheep, systemic lesions are observed (such as petechiae, brain and lung oedemas) with minor changes in the intestine (Fernandez-Miyakawa et al., 2003; Uzal and Songer, 2008). At variance, the chronic form is rarely observed in sheep suggesting very mild manifestations while the brain tissue displays signs of focal symmetric encephalomalacia (see below, §4) (Uzal

and Songer, 2008). Contrary to what is observed in sheep, in goats, the acute form provoked by *C. perfringens* intoxication affects mostly young animals while chronic form of the disease is more frequent in adults. In goats, diarrhoea is the most frequent manifestation (Uzal and Songer, 2008; Oliveira et al., 2010).

Symptoms and manifestations observed either in the naturally occurring disease or after experimental intoxication (i.e. either by injecting *C. perfringens* in the gastrointestinal tract or ET in the duodenum, intraperitoneally or intravenously) can be sorted into groups according to the altered-physiological system: intestinal, renal, pulmonary and nervous systems. These numerous manifestations are summarized in Table 1 (in which are quoted the corresponding references). A fifth category of manifestations regroups a number of heterogeneous behavioural alterations, including reluctance to suck, haphazard roaming, anorexia and weight loss (Table 1). The multiple manifestations observed in enterotoxaemia caused by *C. perfringens* type D (which produces high amounts of ET) reveal a prominent alteration of the nervous system. For instance, opisthotonus or hypotonus, which are extrapyramidal motor symptoms, indicates functional impairment of central structures involved in the control of body postures and movements, such as putamen, thalamus, caudate nucleus and globus pallidus, or from alteration of the tracts connecting these structures. Manifestations that belong to the fifth group (Table 1) indicate some decline of cognitive function, either due to direct alteration of central nervous physiology or to pain. Diarrhoea and tenesmus are clinical signs of an ET action on the intestinal system, which may be, in part, a consequence of an effect of the toxin on the enteric nervous system. Indeed, there are increasing evidence indicating that some enterotoxins mediate diarrhoea not only by acting directly upon enterocytes, but also by interfering with the enteric nervous system (Berkes et al., 2003; Farthing, 2004, 2000; Popoff and Poulain, 2010). Elevated blood pressure (Sakurai et al., 1983) can be caused by renal damage and/or overstimulation of the ortho-sympathetic part of autonomic nervous system as suggested by observations of an increase in circulating monoamines levels (Buxton, 1978b; Nagahama and Sakurai, 1993; Worthington et al., 1979).

Several bodies of evidence support the notion that ET is the main etiological cause for the various manifestations of enterotoxaemia. Indeed, *in vivo* intoxication experiments performed in sheep, goats, lambs (Buxton and Morgan, 1976; Griner, 1961; Uzal and Kelly, 1997) and cattle (Uzal et al., 2002) leads to similar clinical signs as observed during the naturally occurring disease (see Table 1). Thus administration of ET can recapitulate the natural disease.

Table 1
Summary of literature-reported symptoms and manifestations occurring after intoxication with *Clostridium perfringens* Epsilon toxin.

System affected	Symptoms and manifestations	Species	References
Intestinal system	Haemorrhagic diarrhoea	Calve, Lamb, Goat, Sheep	Lewis, 2000; Munday et al., 1973; Smith and Sherman, 1994
	Abdominal discomfort	Goat	Smith and Sherman, 1994
	Tenesmus	Calve	Munday et al., 1973
Pulmonary system	Laboured breathing	Calve, Goat	Uzal and Kelly, 1998; Uzal et al., 2002
	Dyspnoea	Sheep	Uzal et al., 2004
Renal system	Glycosuria	Sheep	Niilo, 1980
Central Nervous system	Convulsion	Calve, Goat, Mouse, Sheep	Blackwell and Butler, 1992; Blackwell et al., 1991; Fernandez-Miyakawa et al., 2007b; Gay et al., 1975; Lewis, 2000; Miyamoto et al., 2000; Munday et al., 1973; Niilo, 1980; Smith and Sherman, 1994; Songer, 1998, 1996; Uzal and Kelly, 1998; Uzal et al., 2002
	Opisthotonus	Sheep, Goat, Calve, Mouse	Blackwell and Butler, 1992; Fernandez-Miyakawa et al., 2007b; Gay et al., 1975; Hartley, 1956; Lewis, 2000; Munday et al., 1973; Niilo, 1980; Smith and Sherman, 1994; Uzal and Kelly, 1998
	Incoordination	Mouse, Rat, Sheep	Griner, 1961; Miyamoto et al., 1998; Finnie, 1984a, 1984b; Uzal et al., 2004
	Tremor Pleurothonus	Mouse, Rat, Sheep	Miyamoto et al., 1998; Finnie, 1984a, 1984b; Uzal et al., 2004
	Coma	Calve, Sheep	Munday et al., 1973; Uzal et al., 2004
	Nystagmus	Sheep	Gay et al., 1975; Pimentel et al., 2010
	Strabismus	Sheep	Pimentel et al., 2010
	Ptyalism	Calve, Sheep	Lewis, 2000; Munday et al., 1973
	Bruxism	Sheep	Gay et al., 1975
	Paralysis	Sheep, Goat, Calve, Rat	Gay et al., 1975; Lewis, 2000; Miyamoto et al., 1998; Pimentel et al., 2010; Uzal and Kelly, 1998; Uzal et al., 2004, 2002
	Hypotonus	Sheep, Rat	Miyamoto et al., 1998; Pimentel et al., 2010
	Rigidity of limbs	Goat, Lamb, Rat	Barker et al., 1993; Barron, 1942; Miyamoto et al., 1998; Songer, 1998
	Retraction of the head	Sheep	Niilo, 1980; Popoff, 1984
	Allodynia	Calve	Munday et al., 1973; Uzal et al., 2002
Behavioural alteration	Bleating	Sheep	Lewis, 2000
	Anorexia	Goat	Blackwell and Butler, 1992; Smith and Sherman, 1994
	Weight loss	Goat	Blackwell and Butler, 1992; Smith and Sherman, 1994
	Depression	Lamb, Goat, Mouse	Barker et al., 1993; Barron, 1942; Fernandez-Miyakawa et al., 2007b; Songer, 1998, Uzal et al., 2004
	Staggering	Calves	Munday et al., 1973
	Haphazard roaming	Sheep	Gay et al., 1975; Hartley, 1956

Many of the gross manifestations of enterotoxaemia can be reproduced in rodents by inoculating the bacteria or the toxin intragastrically (Fernandez-Miyakawa et al., 2007b) or into the duodenum (Blackwell et al., 1991; Fernandez-Miyakawa and Uzal, 2003; Uzal et al., 2002), as well as by administrating ET intravenously (Uzal et al., 2002) or intraperitoneally (Fernandez-Miyakawa et al., 2007a; Finnie, 1984a, 1984b; Finnie et al., 1999; Miyamoto et al., 2000, 1998). Studies in mice clearly show that the lethality of different *C. perfringens* strains is directly correlated with their ability to produce high levels of ET (Fernandez-Miyakawa et al., 2007a, 2007b). This further supports the notion that ET is the causative virulence factor of all symptoms and lesions caused by *C. perfringens* type D (Sayeed et al., 2005).

C. perfringens type B produces ET together with variable amounts of alpha and beta toxins, and causes animal illnesses characterized by sudden death or acute neurological signs, with or without intestinal damage (reviewed by Finnie, 2004; Uzal and Songer, 2008; Uzal et al., 2004). Since the enterotoxaemia due to *C. perfringens* types B and C share similar neurological signs while type B produces both beta-toxin and ET whereas the type C synthesizes only the beta-toxin (reviewed by McClane et al., 2006), the question of whether other toxin(s) produced together with ET may explain some of the neurological aspects of the disease was raised. Experiments performed in mice demonstrated that none of the *C. perfringens* type B or D toxins, except ET, is indispensable for inducing illness. However, the other toxins seem to play a synergistic/potentiating role together with ET (for the contribution of beta-toxin to the pathogenesis of *C. perfringens* type B, see Fernandez-Miyakawa et al., 2007a; for the potentiating role of alpha-toxin and perfringolysin-O, see Fernandez-Miyakawa et al., 2008). Sialidases from *C. perfringens* type D may play a role (Li et al., 2011), see also below. However, the mechanism underlying the potentiating role of the other toxins of factors is still unclear. Possibly, they may favour dissemination of ET by increasing vascular permeability (Fernandez-Miyakawa et al., 2008, 2007a).

To summarize, administration of ET mimics the naturally occurring disease produced by *C. perfringens* types B or D. The observed clinical manifestations (Table 1) indicate prominent alterations in the central nervous system functions. Sudden death may result from severe brain damage; however, it can be caused by blood pressure elevation or heart failure. In the next paragraphs we summarize how ET can pass from the intestine to the brain and generates damage in the central nervous system.

4. From intestine to brain tissue

Since ET is produced into the gut lumen, it should first cross the intestinal barrier before being disseminated in the whole organism. Many studies have addressed this step (for reviews see Finnie, 2004; Popoff, 2011a). ET binds to mucosal epithelium of small intestine (Goldstein et al., 2009). ET induces decrease in the trans-epithelial resistance in a time- and dose-dependent manner (Fernandez-Miyakawa et al., 2003; Goldstein et al., 2009). Since no histological and ultrastructural changes in the intestinal epithelium have been observed (except paravascular oedema and presence of apoptotic cells in the *lamina propia*, Goldstein et al., 2009) ET may cross the intestinal barrier by passing through the paracellular pathway, possibly by opening the mucosa tight junctions (reviewed by Popoff, 2011a, 2011b). However, despite ET decreases trans-epithelial resistance in cultured confluent renal epithelial cells, as the MDCK (Madin–Darby Canine Kidney) cells (Petit et al., 2003) or mpkCCD$_{c14}$ immortalized mouse kidney cells (Chassin et al., 2007), no alteration of the tight junctions is detected between the renal cells. Therefore, severing of tight junctions is not a general effect of ET on epithelia. The ET-induced alterations of intestinal barrier permit bidirectional passage of proteins, including ET, between the intestinal lumen and the plasma compartment, as assessed using Horse Radish Peroxidase or Evans blue bound to plasma proteins (Goldstein et al., 2009). Thus, by altering the intestinal permeability, ET facilitates its own passage in the circulatory fluids (Fernandez-Miyakawa and Uzal, 2003; Losada-Eaton et al., 2008). To summarize, whereas the mechanisms in which enterotoxin from *C. perfringens* opens tight junctions is well known (reviewed by Berkes et al., 2003; McClane et al., 2006; Popoff, 2011b), the way in which ET toxin modulates the tight junctions remains unclear.

Following haematogenous dissemination, ET reaches central nervous system. The second step is the passage of ET through the blood–brain barrier. The latter consists of endothelial cells stitched together by tight junctions that restrict the passage of large molecules from blood to brain. After intraperitoneal ET injection in mice, many capillaries are reduced to a thin electron dense band, indicating major changes in endothelial cells (Finnie, 1984b). Following intravenous injection of protoxin or toxin tagged with Green-Fluorescent-Protein (proET-GFP or ET-GFP) in mice, both proET-GFP and ET-GFP can be detected bound onto the luminal surface of the vascular endothelium (Soler-Jover et al., 2007). Studies performed using EBA (endothelial barrier antigen) to assess the integrity of blood–brain barrier in rats, have revealed severe alteration of the barrier following intraperitoneal administration of proET (Zhu et al., 2001). However, consistent with lack of biological activity of proET, others have found that proET remains bound onto the luminal surface of the vascular endothelium, whereas ET-GFP induces blood–brain barrier disorganization and passes through (Soler-Jover et al., 2007). Therefore, the observation that endogenous albumin extravasation occurs after proET application (Zhu et al., 2001) is likely due to the conversion of proET into fully active ET by the plasma and tissue proteases. With this respect, note that a major difference between the above mentioned studies resides in the delay between proET injection and animal sacrifice: 1 h to 14 days post-injection (Zhu et al., 2001) vs. 7 min post-injection (Soler-Jover et al., 2007). This delay may allow significant activation of proET into ET by the body proteases. In mouse, rat or lamb brains, severing of the blood–brain barrier leads to passage of proteins, like serum albumin (endogenous, coupled to Alexa-677, or ^{125}I human serum albumin) as well as Horse Radish Peroxidase or ^{125}I-polyvinyl-pyrrolidone (Buxton, 1976; Finnie et al., 2008, 1999; Griner and Carlson, 1961;

Nagahama and Sakurai, 1991). Spreading of ET in neural tissue has been found more diffused than that of albumin, which remains confined around the damaged vessels (Soler-Jover et al., 2007). Interestingly, the property of ET to open the blood–brain barrier has been exploited to allow delivery of bleomycin, an anti-tumour agent, in rats (Hirschberg et al., 2009).

5. Tissular and cellular lesions in the central nervous system

Penetration of fluid into cerebral parenchyma causes formation of oedema (for references see Table 2), the spread of which depends on both ET concentration and the time between ET application and observation. When high doses of ET cross the blood–brain barrier, the disease is very severe leading to quick death. As compared to the observed generalized vasogenic oedema, other brain lesions appear tiny. By contrast, when low doses of ET are applied, fatal issue is strongly delayed, allowing numerous brain lesions to develop, which are preferentially located in structures such as the basal ganglia, cerebellum, internal capsule, thalamus, and, at a lesser extent, hippocampus (see Table 2). The cerebellum is a predilection site for the induction of early central nervous system damage (Finnie, 1984a, 1984b; Finnie et al., 1999; reviewed by Finnie, 2004). Overall, the observed lesions are fully consistent with the neurological manifestations observed during enterotoxaemia (see Table 1). For instance, opisthotonus results mainly from lesions of the basal ganglia; seizures may be related to damage in hippocampus as well as thalamus; ataxia may result from attack of cerebellum or thalamus, notably. These lesions may result from direct action of ET on neural cells (see below and §5) or indirectly caused by excessive glutamate release (see §6).

Brain tissue lesions are characterized by dark perivascular oedema, haemorrhagic foci, degeneration or distortion of the white matter, and brain necrosis (for references see Table 2). Since these alterations are mainly bilateral and symmetrical, they were collectively termed Focal Symmetrical Encephalomalacia. It is unclear whether the symmetry of the lesions is due to higher ET susceptibility of the neural tissue in certain brain bilateral structures, or due to a regional and bilateral susceptibility of the brain vasculature for disruption of the blood–brain barrier. Similar symmetrical lesions have been reported in the case of naturally occurring disease in different species including goats, sheep and lambs, and have been reproduced experimentally in sheep, goats, calves, and rodents (rats, mice) (for references see Table 2). Note that in goats, reports of histological changes in brain are scarce (Barker et al., 1993; Songer, 1996), possibly due to less severe symptoms expressed in this animal species (reviewed by Songer, 1996; Uzal and Songer, 2008; Uzal et al., 2004). Another sign of suffering brain is the coning of cerebellum (protrusion of

Table 2
Summary of literature-reported tissue-damage occurring after intoxication with *Clostridium perfringens* Epsilon toxin.

Damage/effects	Species	Structures	Occurring	References
Vasogenic Oedema	Goat, Lamb, Mouse, Rat, Sheep,	Basal ganglia[a], internal capsule, thalamus, cerebellum	Experimentally	Barker et al., 1993; Buxton and Morgan 1976; Finnie, 2003; Finnie et al., 1999; Uzal and Kelly, 1997; Uzal et al., 1997
Coning	Mouse, Sheep	Cerebellum	Experimentally	Buxton, 1978a, 1978b; Buxton and Morgan, 1976; Fernandez-Miyakawa et al., 2007b; Pimentel et al., 2010;Uzal, 2004; Uzal and Kelly, 1998; Uzal and Songer, 2008
Microangiopathy	Sheep	Brain	Naturally Experimentally	Uzal et al., 1997
Dark haemorrhagic foci[c]	Lamb, Sheep	Basal ganglia, thalamus, cerebellum, cerebellar whiter matter cores, cerebral cortex, pons, medulla, cerebellar peduncles, occipital pole, cortex[b], hypothalamus[b]	Experimentally	Buxton and Morgan, 1976; Buxton et al., 1978; Hartley, 1956
	Goat			Uzal et al., 1994
Perivascular proteinaceous oedema[c]	Lamb, Sheep	Basal ganglia, thalamus, cerebellum, cerebellar whiter matter cores, cortex[b], hypothalamus[b]	ND	Buxton, 1978a, 1978b; Buxton and Morgan 1976; Buxton et al., 1978; Uzal et al., 2004
	Goat	Internal Capsule, Colliculus superior	Experimentally and Naturally	Uzal and Kelly, 1998, 1997; Uzal et al., 1997, 1994
	Mouse	Brain	Experimentally	Fernandez-Miyakawa et al., 2007b; Finnie, 1984a, 1984b; Finnie and Hadjuk, 1992; Morgan et al., 1975
	Calves	Cerebellum, internal capsule, thalamus	Experimentally	Uzal et al., 2002
Degeneration/ Distortion of white matter[c]	Sheep	Basal ganglia, thalamus, internal capsule, midbrain, cerebellar peduncles and cerebellar white matter cores	Experimentally	Buxton and Morgan 1976; Buxton et al., 1978; Hartley, 1956
	Goat	Brain	Naturally	Uzal et al., 1997
	Mouse	Cerebellum, cerebral cortex	Experimentally	Finnie, 1984a, 1984b

[a] The main components of the basal ganglia are the striatum (caudate and putamen nuclei), the globus pallidus, the substantia nigra, and the subthalamic nucleus.
[b] Less frequently.
[c] These chronic manifestations of ET neurotoxicity are called Focal symmetrical Encephalomalacia.

the vermis) reported in sheep and mice but not in other species (reviewed by Uzal, 2004). This manifestation may be related to an increase in the intraventricular or blood pressure (Sakurai et al., 1983).

Altered neurons are found scattered among apparently normal nerve cells in the cerebral cortex, hippocampus, thalamus, basal ganglia and cerebellum. Cells damage observed in neural tissue following exposure to ET (Table 3) can be sorted into two categories: i) cellular swelling with microvacuolation, and ii) presence of hyperchromatic cells, also called dark cells, (possibly being post-mortem histological neuronal artefacts resulting from brain manipulation, Jortner, 2006), and shrunken cells with nuclear pyknosis. Tissular localization and severity of cells damage depend on ET doses, on the delay between ET injection and animal sacrifice (Finnie, 1984a, 1984b; Finnie et al., 1999; Miyamoto et al., 2000, 1998) as well as on the repetition of ET injection (Finnie, 1984b; Uzal et al., 2002), but not on the way of its administration (natural disease, intravenous or intraperitoneal injection of ET); see Table 3. Some swelling and pyknotic granule cells have been observed in mouse cerebellum (Finnie, 1984b) but not (or to a lesser extent) in rat cerebellum (Finnie et al., 1999; Miyamoto et al., 1998). In rat, injection of ET at a sub-lethal dose (50 ng/kg) seems to cause neuronal damage predominantly in the hippocampus (Miyamoto et al., 1998). Overall, this suggests that ET may have different mode of action or different consequences depending to the cells or the animal species.

6. Neural cells are ET targets

Post-mortem observations of severed neural cells do not allow discriminating between direct and indirect cellular actions of ET. On the one hand, cell alteration in brain tissue may be an indirect consequence of vasogenic oedema: reduction of parenchyma perfusion leads to hypoxia and cell necrosis. On the other hand, the bilateral symmetry of the damage caused by ET (Table 2, and any sign of Focal Symmetrical Encephalomalacia), notably in the brain stem (Finnie et al., 1999) suggests a nerve-tissue or neural cells vulnerability to ET.

Brain tissue is comprised of different types of neural cells, including many sub-types of neurons, and glial cells notably astrocytes (velimentous astrocytes, radial glia, etc.) and oligodendrocytes (which are responsible for myelination of certain neuronal axons and therefore contribute to the formation of the cerebral white matter). In the peripheral nervous system, Schwann cells, which are related to oligodendrocytes, ensure myelination of peripheral axons.

The observed cellular manifestations (binding, cell damage or death) caused by ET, and the identification of cell types affected by this toxin depend on the actual concentration of ET in the neural tissue. The local concentration of ET is likely depending, in part, on the way by which the toxin is administered. Indeed, during the *in vitro* studies (i.e. when neural tissue slices or primary cultures are used) concentration of ET is likely to be homogenous while, during the *in vivo* studies (i.e. using gastro-enteric, intraperitoneal or intravenous injections) tissular concentration of ET depends of the limiting steps due to passage of ET through the different epithelial barriers so that the actual concentration of ET in neural tissue remains unknown and may be non-homogenous depending on the damage caused to the blood–brain barrier.

The possibility that ET binds on specific subsets of neural cells has been addressed by analysing ET cell binding, either using ET-GFP, ET tagged with Alexa 488as well as ^{125}I-ET or by the aid of immunolabeling techniques. Overall, ET binding on neural tissue is observed in the same gross structures as those displaying tissular lesions following *in vivo* exposure to ET (naturally occurring- or experimental disease). For instance, ET binds to the cerebellum, hippocampus, thalamus, cerebral white matter and commissures, and basal ganglia (Dorca-Arévalo et al., 2008; Lonchamp et al., 2010). However, as discussed below, there is no perfect matching between cellular binding and the observed cellular damage.

6.1. ET binds to a subset of neurons

Using slices of mouse cerebellum submitted to ET (ET being applied on acute slices or after fixation of the slices), examination of the cellular localization of ET immunostaining has revealed that the toxin binds to the cell body of cerebellar granule cells, which are glutamatergic neurons (Fig. 1A and C). This identification is confirmed by the observation that ET colocalizes with specific granule cells markers such as the alpha-6-GABA$_A$ receptor subunit or potassium channel subunit Kv3.1b (Lonchamp et al., 2010). In the granule cells layer of the cerebellar cortex, ET colocalizes with MAP-2 (microtubules-associated protein-2) denoting that ET decorates not only the somata but also the dendritic trees of granule cells. In primary culture,

Table 3
Summary of literature-reported cell damages occurring after intoxication with *Clostridium perfringens* Epsilon toxin.

Cellular damages	Species	Structures	Occurring	References
Cellular swelling	Rat	Basal ganglia[a], cerebellar cortex[c] and white matter[c]	Experimentally	Finnie et al., 1999
	Mouse	Cerebellum	Experimentally	Finnie, 1984a, 1984b; Gardner, 1973b; Morgan and Kelly, 1974
Hyperchromatic and shrunken cells[b]	Mouse	Cerebellum, hippocampus	Experimentally	Finnie, 1984a,1984b; Miyamoto et al., 2000
	Rat	Cortex Cerebral, hippocampus, corpus callosum[c], striatum[c], cerebellum[c], thalamus	Experimentally	Finnie et al., 1999; Miyamoto et al., 1998; Zhu et al., 2001

[a] The main components of the basal ganglia are the striatum (caudate and putamen nuclei), the globus pallidus, the substantia nigra, and the subthalamic nucleus.
[b] Often called dark cells.
[c] Mild and patchy, only observed with an increasing of ET concentration or the time after ET administration.

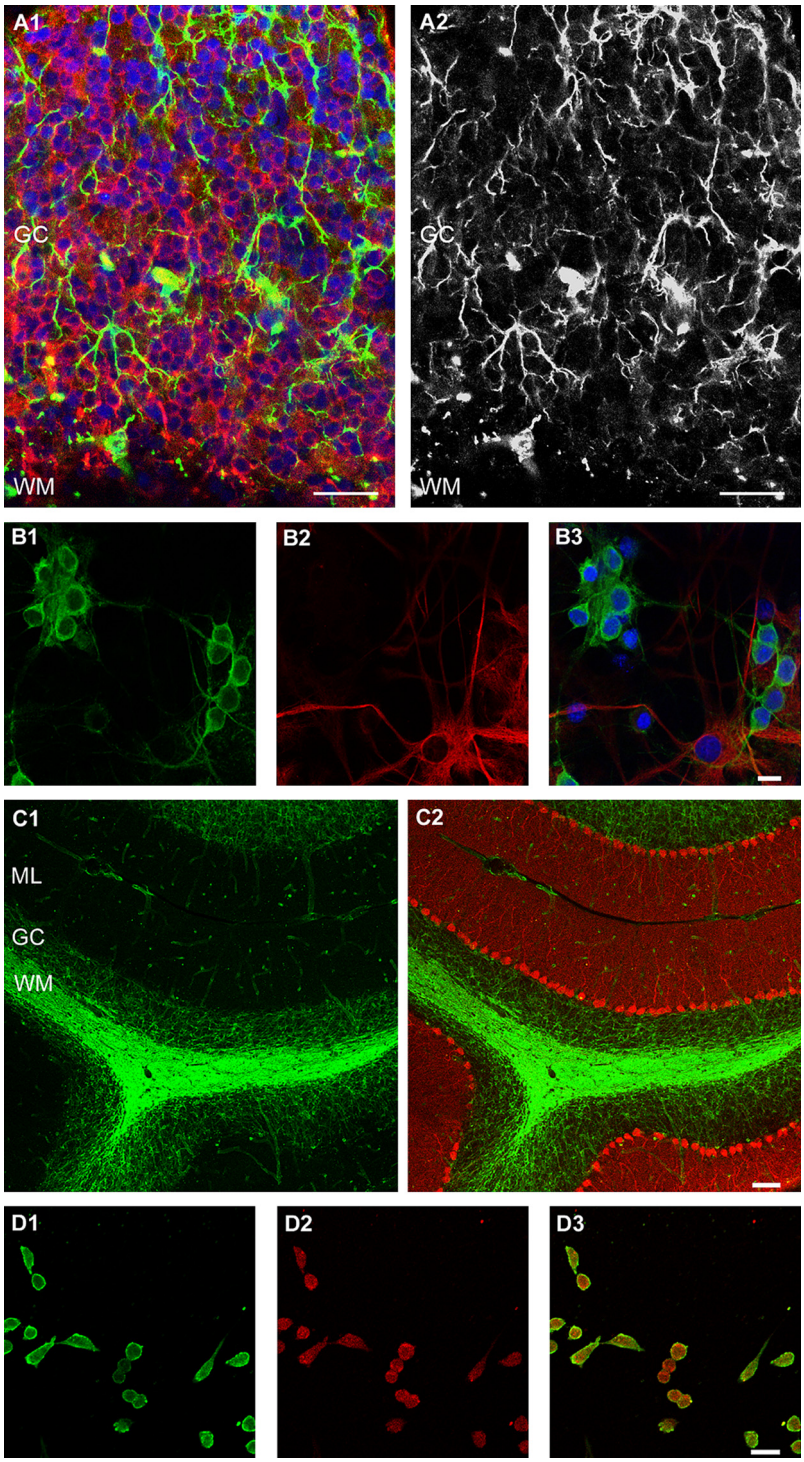


Fig. 1. Identification of the cell types labelled by ET. ET (10^{-7} M) was applied for 5 min after fixation (paraformaldehyde 4%) in the different preparations quoted in A, B, C and D. ET labelling was identified using an immunoaffinity purified rabbit anti-ET primary antibody (Lonchamp et al., 2010). ET binds to oligodendrocytes (C–D) but not to astrocytes (A–B). A. Acute cerebellar slices. A1: merge of ET (in red) and specific astrocytes-marker GFAP (in green) immunoreactivities, and Draq5 DNA labelling (in blue). Note that astrocytes, which are the GFAP positive cells, are not labelled by ET. B2: GFAP immunofluorescence in isolation. Scale bar: 25 μm. GC: granule cells, WM white matter. B. Primary culture of cerebellar cells. B1: ET staining (in green), B2: specific astrocytes-marker immunoreactivity GFAP (in red). B2: merge of ET and GFAP immunoreactivities; Draq5 DNA labelling (blue). Scale bar: 10 μm. C. Acute cerebellar slices. C1: ET staining (in green), C2: merge of ET and specific Purkinje cells- marker Calbindin (in red) immunoreactivities. Note the strong ET immunoreactivity in the white matter where myelinated fibres are concentrated. Scale bar: 50 μm. ML: molecular layer, GC: granule cell layer, WM white matter. D. Oligodendrocytes cell lines 158N (a generous gift from Dr M.S. Ghandour). D1: ET staining (in green). D2: specific astrocytes-marker CNPase immunoreactivity (in red), B: merge of ET and CNPase immunoreactivities. Scale bar: 20 μm.

ET binds to mouse and rat granule cells, too (Lonchamp et al., 2010, and Fig. 1B). In a sharp contrast, studies performed by incubating sections of mouse cerebellum with ET-GFP have not shown significant labelling of granule cells (Dorca-Arévalo et al., 2008). Perhaps the discrepancy between these studies is related to the use of ET *vs.* ET-GFP, or to the timing in the tissue fixation. Indeed, when ET is applied onto cerebellar slices, intensity of ET labelling in white mater and oligodendrocytes increases greatly with incubation time (unpublished data), possibly occluding signal from granule cells. In the mouse cerebellum, not all neurons are recognized by ET: Indeed, this toxin is detected neither onto the GABAergic interneurons like the basket cells, stellate cells and Golgi cells nor onto the large Purkinje cells (which are GABA-ergic) (Lonchamp et al., 2010). Therefore, ET is able to bind to a subset of neurons. The question of whether neurons targeted in other brain regions are glutamatergic remains to be addressed.

Importantly, there is no clear correlation between manifestation of cellular damage and susceptibility to ET. Indeed, a possibility to consider is that the cellular and tissular alterations observed in brain tissue (Tables 2 and 3) in the context of enterotoxaemia may result in part from indirect action of ET. As discussed below into details (see §7), ET induces release of large amounts of glutamate, and when the homeostasis of this excitatory amino-acid is positively dis-balanced, glutamate can induce cell death in many cell types including neurons (like Purkinje cells, reviewed by Slemmer et al., 2005), and glial cells (astrocytes and oligodendrocytes; reviewed by Matute et al., 2006). Therefore, observations of hyperchromatic Purkinje cells after *in vivo* exposure of rats to ET (Finnie et al., 1999), while ET does not bind onto these cells in mice (Lonchamp et al., 2010), might be re-read as a manifestation of glutamate-induced excitotoxicity rather than a direct action of ET on Purkinje cells.

6.2. Does ET bind onto nerve terminals?

Since ET can trigger the release of neurotransmitters (see Section 7 below), several studies have addressed its binding onto nerve terminals leading to controversial results. Indeed, on the one hand ^{125}I-ET has been reported to bind to rat synaptosomes (Miyata et al., 2002, 2001; Nagahama and Sakurai, 1992), but on the other hand, ET-GFP has been found unable to bind to mouse and rat nerve terminals (Dorca-Arévalo et al., 2008). The discrepancy between the conclusions of these studies is likely residing in the contamination of the synaptosomal preparations with resealed myelin debris, which is a common artefact when preparing synaptosomes. This possibility is supported by the demonstration that ET-GFP binds to myelin structures present in mouse brain synaptosomal preparation (as demonstrated by co-staining of ET with myelin basic protein; Dorca-Arévalo et al., 2008). The lack of ET binding onto nerve terminals is also supported by analysis of ET-immunostaining in cerebellum slices. In this preparation, ET has not been detected in the cerebellar molecular layer, which contains the granule cells nerve terminals making synapse with the Purkinje cells (100,000 synaptic contacts per Purkinje cells) or inhibitory interneurons. Also, in the granule cells layer, there is no colocalization of ET with synaptic vesicles markers like synaptotagmin or synaptophysin indicating that ET does not bind to the large glutamatergic nerve terminals of the mossy-fibres making synapse with the granule cells (Lonchamp et al., 2010). From the data obtained in cerebellum slices, ET binding looks compartmentalized onto the neurons that respond to the toxin: ET stains primary dendrites and somata, but not axons or nerve terminals. This suggests that ET receptor is not ubiquitously expressed at the neuronal surface. However, such a compartmentalization is loss in primary culture (Lonchamp et al., 2010).

6.3. Binding of ET to oligodendrocytes

The white matter in central nervous system is the prominent component labelled by ET in several species (sheep, cattle, mouse, and human) (Dorca-Arévalo et al., 2008). This is consistent with post-mortem alterations of white-matter observed in intoxicated animals (Table 2). Whatever is the manner it is administered (ET-GFP intravenous injection or ET application onto acute brain slices), ET co-localizes with myelin markers as MBP (myelin basic protein) or CNPase (2′,3′-Cyclic Nucleotide 3′-Phosphodiesterase) (Dorca-Arévalo et al., 2008; Lonchamp et al., 2010; Soler-Jover et al., 2007). In addition, ET binds to myelinated axons in peripheral nerves (Dorca-Arévalo et al., 2008). Taken together, these data indicate that ET binds to oligodendrocytes, which are the glial cells forming myelin sheath around the axons. The identification of oligodendrocytes as ET targets is supported by our preliminary observations that ET binds to cell line Oligo-158N derived from rat oligodendrocytes, as well as to rat oligodendrocytes in primary culture (Fig. 1D, Wioland et al., 2012).

6.4. Binding of ET to astrocytes

The question of whether ET can target members of the astrocyte lineage (which are glial cells, too) has been addressed. In cerebellar cortex, large radial astrocytes termed Bergmann glia are present in the molecular layer. However, no ET binding has been observed in this layer. In the granule cells layer, ET staining does not colocalize with GFAP (Glial Fibrillary Acidic Protein) that is a specific marker for astrocytes. Similar results have been found using either acute or fixed cerebellar slices, or primary cultures containing both granule cells and astrocytes (Fig. 1A and B; Lonchamp et al., 2010). By contrast, ET-GFP injected intraperitoneally has been reported to bind to astrocyte perivascular end-feet (Soler-Jover et al., 2007). The origin of the difference mentioned above remains unclear. Perhaps ET-GFP binds to capillary endothelial cells that are tightly apposed to the astrocyte perivascular endfeet, leading to the appearance that ET was bound to the astrocytes. Also, one cannot exclude the possibility that ET may target a specific subclass of astrocytes.

7. The mechanisms by which ET acts on neural cells

ET is a member of a large group of cytolysins, the cytotoxicity of which is believed to be related to their ability to

bind to target cell, assemble into oligomers and form large transmembrane pores (for recent general review, see Dunstone and Tweten, 2012). Few reports address the mechanisms by which ET acts on individual neural cells. However, insights gain from experiments performed using brain or neural preparations suggest commonalities with the ET mechanisms established using renal cells. Therefore, in the following paragraphs we will discuss ET mechanisms in neural and renal cells. We will address separately the steps of binding and oligomerization, and the pore formed by ET. Then we will discuss the role played by the cholesterol in these several steps. Finally, we will briefly comment several data that are not fully consistent with the notion that the cytotoxicity is exclusively related to the pore-forming action of ET.

7.1. Binding to its receptor

Immuno-labelling studies have shown that ET binds to a subset of neural cells including certain neurons, and oligodendrocytes (see previous Section 5). Studies performed using ^{125}I-ET and ^{125}I-proET have revealed that both peptides share the same receptor. Their binding onto rat brain homogenates and synaptosomal membrane is saturable, with a single dissociation constant in the nanomolar range (Nagahama and Sakurai, 1992). Very similar findings have been made using mpkCCD$_{c14}$ mouse kidney cells (Chassin et al., 2007) or MDCK cells (with a dissociation constant in the nanomolar range, too; Dorca-Arévalo et al., 2012). Taken together these observations suggest that ET binds to single receptor type, possibly expressed by both neural and renal cells (but see below). However, since ET can form pores (see §6.3) into artificial membrane bilayers (Nagahama et al., 2006; Petit et al., 2001) that are devoid of specific receptor for ET, ET binding to its receptor is not absolutely indispensable for pore formation.

ET binding to isolated membranes from rat brain (Nagahama and Sakurai, 1992) or to white matter mice cerebellum slices (Dorca-Arévalo et al., 2008) is inhibited by treatment with pronase. On the contrary, ET binding to target cells is not or weakly affected by phospholipase C, glycosidases, or neuraminidase (Dorca-Arévalo et al., 2008; Nagahama and Sakurai, 1992). Therefore, ET receptor on neural cells (including certain neurons and oligodendrocytes) is likely to be a protein or a glycoprotein. This corroborates prior deduction on the protein nature of ET receptor on renal cells (Petit et al., 1997). Differences in molecular weight of ET-binding proteins (i.e. receptor candidates) in renal and brain cells suggest that distinct proteins may be implicated into ET binding (reviewed by Popoff, 2011a). Hepatitis-A virus cellular receptor 1 (HAVCR1, also termed KIM-1 for Kidney injury molecule-1) has been shown contributing to ET binding (Ivie and McClain, 2012; Ivie et al., 2011). However no role is known for this protein in the nervous system as yet.

Contribution of ganglioside moiety to ET binding onto the cell membrane is supported by early observation that treatment with neuraminidase decreases ET-binding on rat brain homogenates or synaptosomal membranes, leading to the proposal that ET-receptor might be a sialoglyprotein (Nagahama and Sakurai, 1992) or an O-glycoprotein (Dorca-Arévalo et al., 2008). Treatment by sialidase can modify the ganglioside content in membrane and has been shown modulating ET binding on MDCK cells (Shimamoto et al., 2005). Inhibition of sphingolipids and glycosphingolipids synthesis increases susceptibility of MDCK cells to ET, whilst inhibition of sphingomyelin decreases it. The presence of GM1 decreases the effects of ET, while GM3 does the contrary (Shimamoto et al., 2005). Above observations are compatible with ET binding to a double receptor comprised of a protein and ganglioside(s), as it has been described for clostridial neurotoxins (reviewed by Binz and Rummel, 2009).

7.2. Oligomerization

After binding to its receptor, ET but not proET oligomerizes (reviewed by Bokori-Brown et al., 2011; Popoff, 2011a) to form a large membrane complex of 155 kDa–200 kDa in rat synaptosomes (Miyata et al., 2002, 2001), mouse brain homogenates (Nagahama et al., 1998), liposomes (Nagahama et al., 2006), as well as in MDCK cells (Miyata et al., 2002; Petit et al., 1997) or in mpkCCD$_{c14}$ mouse renal cells (Chassin et al., 2007). By their size, the observed complexes correspond to ET heptamers. Studies made using artificial membranes revealed formation of oligomers of intermediate sizes (Nagahama et al., 2006) indicating that heptamers are formed by progressive addition of monomer to oligomer of smaller size. The question of whether ET oligomerizes before membrane insertion or heptamerization process occurs with ET-monomers already incorporated to membrane remains matter of debate. When studies are performed using cell membranes, no oligomer of intermediate sizes is observed into membrane (Chassin et al., 2007; Miyata et al., 2002) suggesting either that ET monomers inserted into membrane assemble very quickly to form heptamers, or that heptamers are inserted into membrane as a whole. Importantly, ET oligomers formed at 4 °C in MDCK cells display greater sensitivity to pronase treatment than those formed at 37 °C: this supports the notion that ET assembles as a pre-pore complex onto the membrane surface before heptamers insertion into the bilayer in a temperature-sensitive manner (Robertson et al., 2011). Thus, ET looks behaving similar as many other pore-forming toxins (Dunstone and Tweten, 2012). Since a single class of saturable ET binding sites has been detected on synaptosomes and renal cells (Dorca-Arévalo et al., 2012; Nagahama and Sakurai, 1992), the toxin oligomer incorporated to plasma membrane is likely to remain attached to ET receptor; otherwise oligomers inserted into membrane should have been detected as an additional non-saturable binding component.

7.3. The pore formed by ET

Direct information on ET pores is scarce. The pore formation has been deduced from observation that propidium iodide can cross plasma membrane in MDCK cells under condition enabling ET to form oligomers (Lewis et al., 2010; Petit et al., 2003, 2001). Moreover ET induces an early efflux of K^+ ions and influx of Na^+ and Cl^-

ions in MDCK cells (Petit et al., 2001) suggesting formation of ET-pore in plasma membrane. In artificial bilayers, ET pores have been recorded; they are characterized by a large conductance of 480–550 pS and low selectivity for ions ($Cl^- > K^+$) (Nestorovich et al., 2010; Petit et al., 2001). ET pore is highly asymmetric, with a cut-off size of polymers entering the pore from the cis side about 500 Da, whereas the one entering from the trans side is about 2300 Da (Nestorovich et al., 2010). Altogether, these data indicate that when inserted into membrane, ET heptamers forms general diffusion pores allowing passage of rather large compounds (about 1 kDa). Consistent with the formation of ET pores in target cells membrane, a dramatic decrease in individual cell transmembrane resistance has been detected using single cell recording of renal collecting duct mpkCCDcl4 cells (Chassin et al., 2007) and of cerebellar granule cells (Lonchamp et al., 2010). Appearance of abrupt steps in trans-membrane currents recorded in granule cells using the whole cell configuration (Lonchamp et al., 2010) supports the notion that ET pores are maintained open in a long lasting manner. Cell-attached recordings, during which ET has been applied inside the recording patch-clamp pipette, have shown that ET induces large transmembrane unitary currents on granule cells in organotypic cerebellar slices (Lonchamp et al., 2010). The corresponding unitary conductance of which has been estimated around ~270 pS. Such a conductance is larger than that of most endogenous channels in neuron, except the Ca^{2+}-dependent K channels (also termed big K) that may reach 150 up to 250 pS. However, at variance of most endogenous ionic channels, no voltage dependence has been detected in ET-induced currents (Lonchamp et al., 2010). The conductance of ~270 pS induced by ET in granule cell is compatible with that determined in bilayers membrane (~480 pS, Nestorovich et al., 2010; ~550 pS, Petit et al., 2001).

7.4. Role of cholesterol

Similar as for many cytolysins of bacterial origin, lipidic environment in plasma membrane impacts the effects of ET. Overall, the integrity of the plasma membrane is needed for ET to exert its effects (Dorca-Arévalo et al., 2012; Nagahama and Sakurai, 1992; Petit et al., 1997). Studies made using liposomes devoid of specific receptor have suggested that membrane fluidity plays an important role in the interaction of ET with liposomes, insertion in the membrane bilayer, and assembly into complex process in the bilayer (Nagahama et al., 2006; Petit et al., 2001). Reminiscent of data obtained using renal cells (Chassin et al., 2007; Miyata et al., 2002; Petit et al., 1997) the cholesterol sequestration by methyl-β-cyclodextrin (mβCD) does not prevent ET binding onto target neural cells as assessed by immuno-staining of ET on cerebellum slices or cultured granule cells (Lonchamp et al., 2010). Note, however, that a decrease in ^{35}S-ET binding on rat synaptosomes has been reported (Miyata et al., 2002). These results are consistent with single-molecule tracking experiments made on ET at the apical membrane of MDCK cells, which have shown that the ET binding onto plasma membranes does not require presence of cholesterol (Türkcan et al., 2012). Therefore, the cholesterol is dispensable for ET binding to its receptor. This is not the case for the subsequent steps. In the one hand, pre-incubation of renal cells with mβCD prevents ET-oligomerization and ET-induced cytotoxicity (reviewed by Popoff, 2011a), and mβCD prevents ET-oligomerization in synaptosomal membranes fractions (Miyata et al., 2002). In the other hand, the oligomerization process and the pore formation (see below) can occur in artificial membrane in absence of cholesterol (Nagahama et al., 2006; Petit et al., 2001). The contradiction between these different insights is only apparent, and has recently received an explanation. Indeed, the cholesterol plays an essential role in the plasma membrane organization into microdomains (i.e. DRM; detergent-resistant membrane) that confine lateral membrane diffusion of ET monomer or ET monomer bound to its receptor within small zones (of mean area ~0.40 mm^2 on MDCK cells (Türkcan et al., 2012)). This confined diffusion is likely to greatly enhance interactions between ET monomers, thus facilitating their ensuing oligomerization into heptamers. Several types of cholesterol-rich lipid rafts domains exist including planar lipid rafts and caveolae, which are caveolin-dependent invaginations of the plasma membrane (reviewed by Allen et al., 2007). ET heptamers are detected in membrane fractions containing caveolin (Miyata et al., 2002) and expression of caveolins greatly potentiates ET-induced cytotoxicity in human kidney cell line ACHN (Fennessey et al., 2012). Thus caveolae allow confinement of ET into restricted membrane areas (i.e. DRM) thereby favouring ET oligomerization and ensuing steps. To date, no experiment suggests that the cholesterol is indispensable for the membrane insertion of the ET pre-pore complex formed onto the surface of target cells.

7.5. Causal linkage between formation of ET-pore and cytotoxicity

Until now, there is no evidence that ET needs to enter into target cells to induce cytotoxicity (reviewed by Bokori-Brown et al., 2011; Popoff, 2011a, 2011b). Overall, it is believed that flux of ions and leakage of small molecules through ET pores is the unique cause for ET-induced cell death. In mpkCCDcl4 cells, ET induces fall in transmembrane resistance, rapid depletion of cellular ATP, and stimulates the AMP-activated protein kinase, which is a sensitive indicator of reduced cellular energy status. ET also induces mitochondrial membranes permeabilization and mitochondrial-nuclear translocation of apoptosis-inducing factor. The cell death is caused by caspase-independent necrosis characterized by a marked reduction in nucleus size without DNA fragmentation; however this form of cell death is not triggered by the abrupt increase in cytosolic Ca^{2+} detected in these cells (Chassin et al., 2007). There is a good correlation between the kinetics of fluorescent dye entry, supposedly via ET-pores, and the loss of MDCK cell viability (Lewis et al., 2010; Petit et al., 2003, 2001). Site-directed mutagenesis of amino acids within the putative channel-forming domain resulted in changes of cytotoxicity in MDCK cells (Knapp et al., 2009). Moreover, treatments with mβCD prevent the loss of the plasma membrane resistance and the rise in intracellular Ca^{2+} concentration induced by ET in renal collecting duct

mpkCCDcl4 cells (Chassin et al., 2007) as well as the change in intracellular Ca^{2+} concentration and the induction of glutamate efflux caused by ET in granule cells (Lonchamp et al., 2010). Note, however, it remains unclear whether the preventive effect of mβCD is due, as expected, to the cholesterol sequestration from target cells membrane, or direct blockade of ET pore, as recently reported for pores formed by *Bacillus anthracis* lethal toxin and *Staphylococcus aureus* α-haemolysin (Yannakopoulou et al., 2011).

Several data are not fully consistent with a strict causal linkage between formation of ET pore and cellular effects, especially for the early cellular manifestations of ET. Indeed, ET can cause ATP depletion and oncosis in renal collecting duct mpkCCDcl4 cells despite ET heptamerization is prevented by pre-treating cells with mβCD (Chassin et al., 2007). Thus the cytotoxic effects of ET in mpkCCDcl4 cells appears dual and comprised of a pore-forming cholesterol-dependent phase that occurs in DRMs, and an ATP depletion induced oncosis that is almost completely resistant to the removal of cholesterol. Pre-treatment of cerebellar granule cells with mβCD prior to ET application inside the recording pipette does not abolish appearance of ET-induced transmembrane currents, but delays them and reduce their amplitude (Lonchamp et al., 2010). Are these current due to activation of endogenous membrane conductance? Altogether, the emerging picture is that some of the early cellular effects of ET may not be caused by formation of ET pore. This is in line with recent proposal that certain pore-forming toxins act on host cells by another way than forming pores, as recently reported for a staphylococcal toxin (Jover et al., 2013).

8. ET as an excitatory neurotoxin

Several of the manifestations associated with *C. perfringens* type B and D enterotoxaemia (seizure, opisthotonus, convulsion… see Table 1) indicate hyperexcitability of the central nervous system, possibly resulting from an imbalance between excitatory (i.e. glutamate) and inhibitory (i.e. GABA) transmission. Thus, numerous studies have investigated whether release of transmitters is increased following ET administration, and may explain some of the observed ET-induced manifestations.

The intraperitoneal administration of antagonists of the ionotropic glutamate receptors (as MK801 to block NMDA subtype glutamate receptors, or CNQX to antagonize AMPA receptors) prior intravenous injection of ET in rat decreases the number of pyramidal dark cells in the hippocampus (Miyamoto et al., 1998) pinpointing these damage are due to dramatic increase in ambient glutamate concentration in neural tissue (i.e. dark cells manifest glutamate-induced excitotoxicity). Accordingly, direct evidence for induction of increased glutamatergic transmission has been obtained using micro dialysis in the hippocampus in rat and mice submitted to ET (Miyamoto et al., 2000, 1998). Moreover, depletion in zinc ions – which has been shown contained into glutamate-containing synaptic vesicles – in the mossy layers of the hippocampal CA3 region has suggested that the excess of glutamate was due to its vesicular release by the nerve terminals (Miyamoto et al., 1998). Importantly, these effects were demonstrated not due to brain ischemia. In cultured cerebellum slices, the frequency of excitatory (glutamatergic) spontaneous responses in Purkinje cells is strongly increased (Lonchamp et al., 2010). However, in mice anesthetized with ketamine, intravenous injection of ET-GFP does not produce the expected convulsive episodes (Soler-Jover et al., 2007). This is does not necessarily in contradiction with the observations commented just above; indeed, ketamine, which is a well-known glutamate NMDA receptor antagonist, may have minimized the manifestations caused by ET-induced increase in excitatory transmission. In granule cells cultures, ET induces glutamate release as assessed using the Amplex red assay (Lonchamp et al., 2010); but it remains unclear whether glutamate release is due to stimulation of vesicular exocytosis by the ET-induced rise in intracellular Ca^{2+} or reversion of membrane glutamate transporter following ET-induced membrane depolarization.

Several evidence support the view that the increase in neurotransmitters release is not due to direct effect of ET on nerve terminals. Indeed, in cerebellar slices, ET-induced increase in glutamatergic synaptic events in Purkinje cell is abolished by TTX (Tetrodotoxin, a blocker of Na^+ channels) well-known to prevent propagation of action-potentials (Lonchamp et al., 2010). In hippocampus, ET-induced glutamate efflux is greatly attenuated by riluzole (Miyamoto et al., 2000), which is a blocker of TTX-sensitive Na^+ channels, too (Lamanauskas and Nistri, 2008). TTX has been found also to abolish ET-induced contraction of ileum, indicating contribution of propagated action potentials between the site of action of ET (enteric neurons) and acetylcholine secretion (Sakurai et al., 1989). Overall, the emerging picture is that ET depolarizes the somatic membrane of certain neurons, thereby initiating burst of action potentials that propagate along the axons up to the nerve terminals where they stimulate vesicular neurotransmitter release. This proposal may explain the paradoxical situation that ET is able to induce glutamate release (see previous paragraph) despite it does not bind on nerve terminals (Dorca-Arévalo et al., 2008; Lonchamp et al., 2010) or induce glutamate release from purified mouse and rat brain synaptosomes (Dorca-Arévalo et al., 2008).

The stimulatory effect of ET on neurotransmitter release is not restricted to the glutamatergic pathways. Indeed, stimulation of dopamine, noradrenaline and adrenaline release has been reported in mice and sheep (Buxton, 1978b; Nagahama and Sakurai, 1993; Worthington et al., 1979). In ileum preparations, ET stimulates acetylcholine release (Sakurai et al., 1989). However, it is not clear whether these observations are due to direct action of ET on non-glutamatergic neurons, or are secondary consequences of the stimulation of glutamatergic system, which is excitatory. Such a possibility is supported by the observation that in the cerebellar network, ET induces an increase in GABA transmission that can be completely prevented by inhibiting glutamatergic transmission (Lonchamp et al., 2010). Overall, by acting on glutamatergic neurons, ET induces increase in glutamate levels, which in turn triggers firing of the whole nervous system, leading to many indirect manifestations. For example, the ET-induced rise in circulating catecholamine (indicating overstimulation of sympathetic system) activates adenylate

cyclase pathways resulting in plasma cyclic-adenosine-3′, 5′ monophosphate (cAMP) rise after ET injection (Buxton, 1978b; Worthington et al., 1979), an effect that may explain hyperglycaemia (Bullen and Scarisbrick, 1957; Gardner, 1973a).

9. Conclusion

ET has the fundamental structure of a pore-forming toxin, and accordingly it is expected to interact with many various cell types. Indeed, pore-forming toxins recognize ubiquitous membrane components as receptors, such as cholesterol, glycosylated proteins and therefore they can indiscriminately damage membranes from different cells. Consistent with such a notion, the action of ET is not restricted to the neural cells: it acts on epithelial cells in intestine and kidney, and vascular endothelial cells. Therefore, the neurotoxin properties of ET may result from the fact that same molecules and signalling cascade participates in the biology of all ET target cells. However, despite in the pathophysiological condition the actual concentration of ET in brain is likely far lower than that in the periphery; the prominent effects of ET are due to the nervous system attack. Does this mean that ET is more a neurotoxin than a cytolysin? Perhaps! One should consider that ET is singular among the other bacterial toxins because its ability to interact with vascular endothelial cells makes it able to enter the brain tissue by crossing the blood–brain barrier. Since the nervous system is the central coordinator for metazoan, any attack on it produces severe symptoms and manifestations. Acting on neurons and, possibly the oligodendrocytes, amplifies the highly potent systemic action of ET. This may explain why ET lethal activity is 100-fold higher than that of other structurally related pore-forming toxins. Prominence of the neural effects (as in the acute form of the disease) should not distract our interest from more discrete manifestations that may allow identifying new target cells for ET, and may help to anticipate long-term effects of sub-lethal doses of ET.

Ethical statement

This contribution is a review and does not deserve ethical statement.

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Conflict of interest

The authors state they have no conflict of interest.

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