



# BoNT/E prevents seizure-induced activation of caspase 3 in the rat hippocampus

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Clinical and experimental studies clearly demonstrate that prolonged seizures and status epilepticus induce neuronal cell death in the brain. Recent evidence suggests that induction of apoptosis greatly contributes to seizure-induced brain damage. We recently demonstrated that intrahippocampal delivery of Botulinum Neurotoxin E (BoNT/E) in the rat hippocampus is able to prevent neuronal loss, which occurs after kainic acid-induced seizures. Here, we investigated the molecular mechanisms of BoNT/

E-mediated neuroprotection. We found that intrahippocampal administration of BoNT/E prevents the upregulation of apoptotic proteins (phosphorylated c-Jun and cleaved caspase 3), which occurs in hippocampal neurones following kainic acid seizures. These results demonstrate that the neuroprotective action of BoNT/E on seizure-injured hippocampal neurones involves the blockade of well-characterized apoptotic pathways. *NeuroReport* 00:000–000  
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**Keywords:** apoptosis, botulinum neurotoxin, caspase, epilepsy, excitotoxicity, kainic acid, neuronal cell death, phosphorylated jun, programmed cell death

## Introduction

Temporal lobe epilepsy (TLE) is one of the most common forms of human epilepsy, characterized by recurrent seizures, which are often resistant to pharmacological treatment. Neuropathological and neuroimaging studies have also shown a clear link between TLE and brain damage. It is calculated that about 70% of drug-resistant TLE patients present temporal lobe damage. Cell loss within Ammon's horn in the hippocampus is the most commonly observed lesion, although other regions may be affected [1]. Experimental studies performed on several animal models of epilepsy allowed to confirm that a prolonged seizure or status epilepticus (SE) can cause neuronal death in the brain. Induction of apoptotic pathways has been shown to contribute significantly to cell death after seizures [2]. We recently demonstrated that delivery of Botulinum Neurotoxin E (BoNT/E) to the rat hippocampus markedly reduces the occurrence of kainic acid (KA)-induced seizures [3]. BoNT/E treatment also prevents neuronal loss associated with KA seizures [3]. It is conceivable to assume that BoNT/E-mediated neuroprotection of hippocampal neurones involves the blockade of apoptotic pathways. In this study, we sought to elucidate the molecular mechanisms through which BoNT/E prevents the death of hippocampal neurones. To this purpose, we evaluated the expression of two proteins involved in the activation of apoptotic pathways after seizures: c-Jun and caspase 3. The induction of the transcription factor c-Jun [4,5] and its subsequent phosphorylation by the Jun N-terminal kinase (JNK) [6,7] are among the first steps of apoptotic cascades triggered by KA seizures. Cleavage of procaspase 3 by caspase 9 determines

the activation of the apoptosis effector caspase 3 [8], and occurs in the rodent brain as a consequence of experimentally induced seizures [2]. Our results show that intrahippocampal delivery of BoNT/E reduces both c-Jun phosphorylation and cleavage of caspase 3, which occur following KA-induced seizures.

## Materials and methods

### Animals and treatments

Sprague–Dawley male rats at postnatal day (P) 35 (150–200 g; purchased from Charles River, Calco, Italy) were used in all experiments. P35 rats were chosen since they display a maximal sensitivity to KA-induced seizures (see in [3] references therein). All procedures were in conformity to the European Communities Council Directive no. 86/609/EEC. Adequate measures were taken to minimize animal pain and discomfort. BoNT/E was obtained by WAKO (Japan), trypsin-activated, purified and tested as described [9]. A total of 24 rats were used in this study. Unilateral stereotaxic infusion of 1.5 µl of BoNT/E (50 nM) or vehicle (2% rat serum albumin in phosphate-buffered saline) was made into the dorsal hippocampus under avertin anaesthesia as described previously [3]. Eight animals were used for each of these two treatment groups. Two days after the injections, animals received a convulsive dose (12 mg/kg, intraperitoneal) of KA (Ocean Produce International, Shelburne, Nova Scotia, Canada). Animals which only received KA (but not BoNT/E or vehicle; *n*=4), as well as control animals (which did not receive any treatment; *n*=4) were also used. Following KA administration, rats were observed for a

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period of 4 h to follow the occurrence of seizures. In keeping with our previous study [3], BoNT/E-treated rats expressed behavioural symptoms of hippocampal focal activity (e.g. gustative and motor automatisms), but never experienced generalized limbic motor seizures nor SE, which were instead observed in rats treated with KA or vehicle+KA. Animals were killed 24 h after KA administration. For immunoblotting, hippocampi were dissected from control, KA, vehicle + KA or BoNT/E + KA rats ( $n=4$  per group). For immunohistochemistry, brains were dissected only from rats treated with vehicle+KA or BoNT/E + KA ( $n=4$  per group).

### Immunoblotting

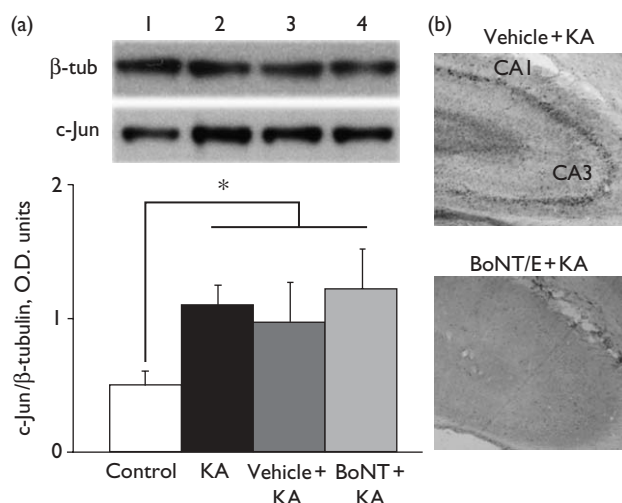
Hippocampal protein extracts were prepared from treated animals according to standard protocols, routinely used in our laboratory [10]. Same amounts of total protein extracts (10  $\mu$ g for c-Jun, 150  $\mu$ g for caspase 3) were separated on 12% polyacrylamide gels by standard sodium dodecyl sulphate-polyacrylamide gel electrophoresis and blotted. Filters were incubated with the following antibodies: rabbit polyclonal anti-c-Jun (Santa Cruz sc45, 1:500 dilution); 8G10 rabbit monoclonal anticaspase 3 (which recognizes both intact and cleaved caspase 3; Cell Signaling Technology, 1:1 000 dilution). Filters were then reacted with peroxidase-conjugated goat antirabbit secondary antibodies (1:3 000 dilution; Bio-Rad) and developed by chemiluminescence (ECL; Amersham, UK). Filters hybridized with c-Jun were stripped and reprobed with anti- $\beta$ -tubulin mouse monoclonal antibody (1:500 dilution; Sigma, St. Louis, MO), which served as an internal standard for protein quantification. Quantification was performed on digitalized images of films. Optical density of the bands was measured with the Image J free software (<http://rsb.info.nih.gov/ij/>). Ratios of c-Jun/ $\beta$ -tubulin or cleaved/intact caspase 3 optical density values (mean  $\pm$  SEM) were calculated and plotted. Statistical analysis was performed by Student's *t*-test.

### Immunohistochemistry

Freezing microtome sections (coronal, 40  $\mu$ m thick) were collected in serial order through the entire dorsal hippocampus of treated rats. Free-floating sections were postfixed in 4% paraformaldehyde, blocked and then reacted *o/n* at 4°C with primary antibodies as follows: phosphorylated c-Jun (Ser63, rabbit polyclonal, 1:200, Cell Signaling Technology); cleaved caspase 3 (Asp175, rabbit polyclonal, 1:1000, Cell Signaling Technology). Bound antibodies were detected by incubation with biotinylated goat antirabbit secondary antibodies (1:200, Vector Laboratories) followed by incubation with ABC kit (Vector Laboratories) and diaminobenzidine (Vector Laboratories) reaction. Manufacturers' indications and our previous studies [11] confirmed the specificity of the used antibodies.

### Results

Previous studies from our laboratory showed that delivery of BoNT/E to the rat hippocampus prevents neuronal loss that occurs following KA-induced seizures [3]. Here, we investigated the molecular mechanisms through which BoNT/E administration prevents the apoptosis of hippocampal neurones in the same experimental paradigm. Immunoblotting and immunohistochemistry experiments were performed to compare the expression of the apoptotic

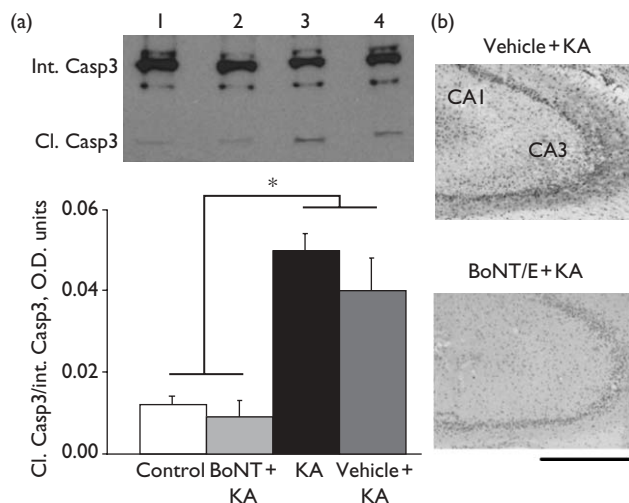


**Fig. 1** Botulinum Neurotoxin E (BoNT/E) prevents c-Jun phosphorylation in the hippocampus after kainic acid (KA) seizures. (a) Top: representative immunoblot showing expression of c-Jun and  $\beta$ -tubulin ( $\beta$ -tub; internal standard for quantification) in the hippocampus of control (lane 1), KA (lane 2), vehicle + KA (lane 3) and BoNT/E + KA (lane 4) rats. Bottom: quantification of immunoblotting experiments. Values are plotted as the mean  $\pm$  SEM ( $n=4$  animals per group) of c-Jun/ $\beta$ -tub optical density (O.D.) ratios. \**t*-test,  $P < 0.05$  (KA, vehicle + KA and BoNT/E + KA vs. control). (b) Representative immunostainings for phospho-Jun on dorsal hippocampus sections from vehicle + KA and BoNT/E + KA rats. CA1 and CA3 hippocampal subfields are indicated. Bar = 500  $\mu$ m.

proteins c-Jun, phosphorylated c-Jun (phospho-Jun) and cleaved caspase 3 in the hippocampus of BoNT/E-treated rats, 24 h after KA administration.

Immunoblotting experiments revealed increased levels of c-Jun expression in the hippocampus of KA-treated rats (Fig. 1a, lane 2), as compared with control animals (Fig. 1a, lane 1). Pretreatment with BoNT/E did not prevent c-Jun upregulation (Fig. 1a, lane 4), which also occurred in the hippocampus of rats treated with vehicle + KA (Fig. 1a, lane 3). Quantification revealed a two-fold increase of c-Jun levels in KA, vehicle + KA and BoNT/E + KA rats (Fig. 1a, graph). Immunohistochemistry was then used to investigate c-Jun activation following KA administration in vehicle- and BoNT/E-treated rats. Cells immunopositive for phospho-Jun were detected in both CA1 and CA3 subfields of the hippocampus of rats treated with vehicle + KA, whereas this labelling was completely absent in the hippocampus of rats treated with BoNT/E + KA (Fig. 1b).

Cleavage of caspase 3 was next investigated to ascertain the level of activation of this apoptosis effector. Immunoblotting experiments performed with an antibody recognizing both intact and cleaved caspase 3 revealed a marked increase of cleaved caspase 3 in the hippocampus of KA- and vehicle+KA-treated rats (Fig. 2a, lanes 3 and 4, respectively), as compared with control animals (Fig. 2a, lane 1). Pretreatment with BoNT/E completely abolished caspase 3 cleavage induced by KA (Fig. 2a, lane 2). Quantification of the bands corresponding to cleaved (17/19 kDa) and intact (35 kDa) caspase 3 isoforms in the different treatment groups revealed a  $\sim$ 400% increase of cleaved caspase 3 in KA and vehicle + KA rats, which was absent in the BoNT/E + KA group (Fig. 2a, graph). Immunohistochemistry experiments performed with an



**Fig. 2** Botulinum Neurotoxin E (BoNT/E) prevents caspase 3 cleavage in the hippocampus after kainic acid (KA) seizures. (a) Top: representative immunoblot showing expression of intact caspase 3 (int. Casp3, 35 kDa) and cleaved caspase 3 (cl. Casp3, 17/19 kDa) in the hippocampus of control (lane 1), BoNT/E + KA (lane 2), KA (lane 3) and vehicle + KA (lane 4) rats. Bottom: quantification of immunoblotting experiments. Values are plotted as the mean  $\pm$  SEM ( $n=4$  animals per group) of cl. Casp3/int. Casp3 optical density (O.D.) ratios. \* $t$ -test,  $P < 0.05$  (KA, vehicle + KA vs control and BoNT/E + KA). (b) Representative immunostainings for cleaved caspase 3 on dorsal hippocampus sections from vehicle + KA and BoNT/E + KA rats. CA1 and CA3 hippocampal subfields are indicated. Bar=500  $\mu$ m.

antibody specific for cleaved caspase 3 confirmed that BoNT/E treatment markedly prevented caspase 3 activation induced by KA in both CA1 and CA3 subfields of the hippocampus (Fig. 2b).

## Discussion

In this study, we showed that intrahippocampal delivery of BoNT/E before KA administration in the rat prevents c-Jun phosphorylation and caspase 3 activation, which occur in hippocampal neurones after KA seizures.

BoNT/E is a bacterial enzyme with a potent blocking activity on neurotransmitter release at the neuromuscular junction *in vivo* [12]. BoNT/E effects have also been demonstrated on central neurons, both *in vitro* and *in vivo* [13]. In particular, previous studies from our laboratory demonstrated that BoNT/E is able to markedly reduce glutamate release and spike activity when injected into the rat hippocampus, resulting in a strong reduction of focal (i.e. hippocampal) and generalized epileptic activity in KA seizure models. KA-induced neuronal loss is also prevented by BoNT/E treatment [3,13].

Our results show that BoNT/E neuroprotective effects involve the blockade of at least two critical steps for neuronal-programmed cell death after seizures: phosphorylation of c-Jun and activation of caspase 3. Seizures elicited by KA in rats and mice rapidly upregulate both *c-jun* mRNA and c-Jun protein in the hippocampus, with a peak of about 6–12 h for *c-jun* mRNA and 6–24 h for c-Jun protein [5,14,15]. Phosphorylation of c-Jun is a consistent response after seizures: levels of phospho-Jun in the hippocampus rapidly (3–24 h) and markedly increase after seizures [14],

depending on the activity of the JNKs [15]. JNK activation and consequent c-Jun phosphorylation are considered an essential step for seizure-induced neuronal death, since mutant mice with altered JNK activity are resistant to KA neurotoxicity [6,7]. In keeping with these findings, we showed a strong increase of c-Jun and phospho-Jun in the hippocampus, 24 h after KA seizures (Fig. 1). In our experiments, we observed a similar c-Jun upregulation in KA, vehicle + KA and BoNT/E + KA rats. This suggests that focal hippocampal activity (such as that observed in BoNT/E + KA rats; see 'Materials and Methods' section and [3]) is sufficient to maximally upregulate c-Jun protein levels. Conversely, BoNT/E treatment was able to completely abolish phospho-Jun upregulation, strongly confirming that prolonged and generalized seizures or SE are required to stimulate JNK-mediated phosphorylation of c-Jun.

Caspase 3 upregulation and activation after prolonged seizures has been clearly demonstrated in the KA model of SE [2,16,17]. Levels of caspase 3 protein are increased in the hippocampus following SE, peaking at 16–24 h after KA administration; after that time, caspase 3 levels remain unchanged, and fragments corresponding to cleaved caspase 3 markedly increase [17]. Moreover, caspase 3 cleavage is considered as one of the essential steps for the execution of seizure-induced programmed cell death, since delivery of a caspase 3 inhibitor into the lateral ventricle was able to prevent neuronal apoptosis following KA-evoked SE in the rat [18]. In keeping with these findings, we observed a strong increase of caspase 3 cleavage 24 h after KA seizures in KA and vehicle + KA rats (Fig. 2). BoNT/E prevented caspase 3 cleavage, thus confirming that activation of this effector caspase requires prolonged and sustained epileptic activity.

## Conclusion

Our results demonstrate that blockade of sustained epileptic activity via intrahippocampal delivery of BoNT/E prevents the induction of the phospho-Jun/caspase 3 apoptotic pathways in hippocampal neurones. The present work supports the notion that prolonged seizures activate apoptosis in susceptible areas of the brain.

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