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ANTI-EPILEPTIC EFFECT OF BOTULINUM TOXIN E

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INTRODUCTION

Epilepsy is one of the most common neurological diseases, affecting 0.8% of the human population.

The term epilepsy derives from the Greek word "*epilambàno"* that means "to take hold of or to seize". Indeed seizures have fascinated humanity since antiquity, being associated sometimes with evil spirits, sometimes with specials creative powers. The neurophysiologic origin of seizures was recognized only in 1860s by Sir John Hughlings Jackson. He defined an epileptic seizure as "a sudden and excessive discharge of certain nervous arrangements, the cells of which are abnormally highly unstable". Afterwards the development of electroencephalography by Hans Berger in 1929 permitted the analysis of the epileptic phenomena and Jackson's hypothesis was confirmed.

Epilepsy is usually diagnosed after a person had at least two seizures not caused by some known medical condition such as alcohol withdrawal or extremely low blood sugar. However seizures are not the disease in themselves but they are only a symptom of a disorder that affects the functioning of the brain. Therefore each epileptic syndrome has to be considered as a different disease with a set of definable causes and etiologies and characterized by unique electro-clinical manifestations and neurophatological substrates.

Clinical epilepsy can be divided into two main groups according to the International Classification of Epilepsies and Epileptic Syndromes: the *idiopathic (primary) disorders* and *symptomatic (secondary) disorders* (Engel, 1996). The *idiopathic epilepsies* are never associated with neurological disturbances or structural pathology and they are genetically transmitted, age-related and usually benign. Indeed the *symptomatic epilepsies* are disorders in which the epileptic seizures result from a specific cerebral pathological substrate which can be genetic, such as the tuberous sclerosis, or acquired, such as a traumatic scar.

Idiopathic and symptomatic epilepsies can be both further divided into *generalized disorders*, if the brain is diffusely and bilaterally involved, and *partial, localization-related disorders*, if the abnormality is restricted to a part of one hemisphere that represents the seizure focus and that determines the

symptomatology. However partial seizures can progress further and give rise to a secondarily generalized seizures.

The majority of idiopathic epilepsies begin during childhood. The most common types are the *juvenile myoclonic epilepsy*, an idiopathic generalized syndromes characterized by myoclonic jerks, generalized tonic-clonic seizures (GTCSs), and sometimes absence seizures, and the *benign childhood epilepsy with centrotemporal spikes,* a familiar disorder characterized by partial motor and sensory seizures as well as generalized nocturnal convulsions.

Otherwise the majority of symptomatic epilepsies are localization-related and have an adult-onset. They can be etiologically divided into three broad categories:

1) the *mesial temporal lobe epilepsies (MTLE),* associated with hippocampal sclerosis that is the most common structural abnormalities in human epilepsies;

2) the *lesional partial epilepsies,* associated with specific lesions such as tumors, scars, vascular malformations, congenital cysts and a wide variety of neocortical dysplasias;

3) the *cryptogenic partial epilepsies* whose etiology is unknown but that can not be considered idiopathic,

In this thesis I test the efficacy of the bacterial enzyme Botulinum Toxin E (BoNT/E), which is known to block neurotransmitter release and neuronal activity, as a selective drug against intractable MTLE.

1.1 Temporal lobe epilepsy

Symptomatic temporal lobe epilepsy (TLE) is the most common form of human epilepsy (Engel, 1996). This form of epilepsy is also the most medically intractable one, as many TLE patients that initially respond to available anti-epileptic drugs can, despite treatment, experience recurrent seizures and develop an intractable form of the disease.

Animal models that reproduce the unique aspects of TLE disorder as faithfully as possible are important in order to study the pathophysiology of this epileptic disease (Loscher, 1997). Moreover only if the model emulates the features of human syndrome the investigator can be reasonably confident that data obtained from animal experiments have a clinical relevance.

In this chapter I will summarize the clinical and pathophysiological characteristics of human TLE and how these are replicated in animal models.

1.1.1 Human temporal lobe epilepsy

TLE syndrome is characterized by recurrent unprovoked seizures originating from the medial or lateral temporal lobe. The seizures associated with TLE consist of simple partial seizures without loss of awareness and complex partial seizures (i.e., with loss of awareness). The individual loses awareness during a complex partial seizure because the seizure spreads to involve both temporal lobes. The features of temporal lobe seizures can be extremely varied, but certain patterns are common. Dostoevskij, the 19th-century Russian novelist, who himself had epilepsy, gave vivid accounts of apparent temporal lobe seizures in his novel *The Idiot*:

> He remembered that during his epileptic fits, or rather immediately preceding them, he had always experienced a moment or two when his whole heart, and mind, and body seemed to wake up with vigor and light; when he became filled with joy and hope, and all his anxieties seemed to be swept away for ever; these moments were but presentiments, as it were, of the one final second…in which the fit came upon him. That second, of course, was inexpressible.
>
> Next moment something appeared to burst open before him: a wonderful inner light illuminated his soul. This lasted perhaps half a second, yet he distinctly remembered hearing the beginning of a wail, the strange, dreadful wail, which burst

from his lips of its own accord, and which no effort of will on his part could suppress. Next moment he was absolutely unconscious; black darkness blotted out everything. He had fallen in an epileptic fit.

The features of TLE seizures (Kotagal, 1991) depend on the fact that the temporal lobe is a part of the limbic system which controls emotions and memory. A mixture of different feelings, emotions, thoughts, and experiences are distinctive of temporal lobe seizures. In some cases, a series of old memories resurfaces. In others, the person may feel as if everything—including home and family—appears strange. Hallucinations of voices, music, people, smells, or tastes may occur. These features are called "auras" or "warnings." They may last for just a few seconds, or they may continue as long as a minute or two.

Most forms of human TLE are symptomatic, even if some cases of idiopathic TLE has been recently described (Berkovic et al., 1996; Cendes et al., 1998; Gambardella et al., 2000). Symptomatic temporal lobe epilepsies can be further divided into three broad categories according to their etiology:

- mesial TLE, associated with hippocampal sclerosis (HS);
- lesional TLE, associated with specific lesions such as tumors, scars, vascular malformations and a variety of neocortical dysplasias;
- cryptogenic TLE, when the etiology is unknown;

Mesial temporal lobe epilepsy is the most common form of symptomatic TLE. The presence of hippocampal sclerosis (HS) in the brains of patients with TLE was first described by Bratz in 1899. Hippocampal sclerosis involves hippocampal cell loss in the CA1 and CA3 regions and in the dentate hilus. The CA2 region is relatively spared (Mathern et al., 1996). Besides hippocampal sclerosis was associated with TLE more than one century ago, there are still some unresolved issues about it.

1.1.1a Hippocampal sclerosis: cause or consequence?

Whether hippocampal neuron loss is the "cause" or the "consequence" of temporal seizures has been a fundamental question in human epilepsy studies for over a century. This issue is important not only to understand the pathogenic mechanisms of epilepsy but also to determine the best therapeutic goals for patients (Mathern et al., 2002). Indeed, if hippocampal sclerosis generates MTLE and is a result of a pre-epilepsy brain

injury, then preventing the initial pathological process may avert chronic epilepsy. Alternately, if hippocampal neuron loss is the result of repeated seizures, then stopping all seizures at any age becomes an important treatment goal. Unfortunately, the medical literature is inconclusive and rife with controversy regarding this questions since the earliest descriptions of the relationship of hippocampal pathology with epilepsy. Indeed early post-mortem studies established that severe hippocampal damage in a pattern termed "Ammon's horn" was strongly associated with complex partial MTLE, while minor hippocampal neuronal loss in regions such as the end folium were associated with other seizure types (Meynert, 1867; Sommer, 1880; Margerison and Corsellis, 1966). However these studies did not enable to define if the severe hippocampal damage is the cause or the consequence of MTLE.

The advent of pre-mortem surgical resection for MTLE made possible to carry out more detailed pathological studies and compare the surgical findings with the pre-operative clinical data in order to unveil whether hippocampal sclerosis was the cause or the consequence of MTLE. In 1954 Meyer proposed that severe hippocampal sclerosis is the result of early childhood brain injuries, such as febrile convulsions, and that HS is the cause of MTLE (Meyer, 1954). This concept was challenged by epidemiological studies showing that the risk of MTLE after febrile convulsions is very low (Nelson and Ellenberg, 1976). However recent studies (Mathern et al, 2002) revisited Meyer's hypothesis. Mathern and co-workers examined hippocampal specimens from patients with temporal lobe epilepsy or with extra-temporal seizures analyzing both qualitative signs of hippocampal sclerosis and quantitative neuron loss using cell counting techniques. The results obtained are:

- HS is strongly associated with an initial precipitating injury (IPI) both in MTLE and extra-temporal patients;

- IPIs generally occur at young age and involve seizure but hippocampal damage from IPIs is not age or seizure dependent;

- in MTLE patients, chronically repeated seizures add neuronal loss in all hippocampal subfields that require a long time course (i.e. more than 30 years) to be detected;

This clinical-pathological study support the hypothesis that the severe neuronal loss in the hippocampal sclerosis pattern is most likely an acquired pathology consequent to an IPI that affect the brain than a consequence of long-term seizures. Moreover these data validate the original model proposed by Meyer to explain hippocampal sclerosis

and the occurrence of MTLE syndrome. In this model an IPI causes the strong neuronal damage in the hippocampus. Thereafter there is a latent phase, during which progressive anatomical changes occurring in the hippocampus and other limbic circuits produce the necessary conditions to promote or generate spontaneous seizures. These pathological changes may include aberrant excitatory and inhibitory axon sprouting and changes in post-synaptic receptor subunit composition (Mathern et al., 1997; Babb, 1999). Once limbic seizures established, they can induce additional long-term anatomical changes over many years, including cell death that is however only a secondary injury that adds to the substrate of HS (Salmenpera et al., 2001; Kalviainen and Salmenpera, 2002). Therefore, MTLE has to be consider as a pathology that evolves with time, as supported also by neuroimaging and neurocognitive studies (Theodore et al, 2001).

1.1.1b Hippocampal sclerosis: etiology

Another fundamental question about hippocampal sclerosis is its etiology. Indeed, despite intensive effort by many researcher for more than a century, the injury factors that cause hippocampal sclerosis are still on much-discussed. The correlation established by Falconer and collaborators in 1964 between MTLE, hippocampal sclerosis and prolonged febrile seizures in early childhood lead to the concept that prolonged seizures in a age specific time-window can damage the hippocampus. This view was supported by studies showing that experimental focal status epilepticus results in histological changes resembling human ones. However epidemiological studies demonstrated that about half of the patients with MTLE and hippocampal sclerosis lack a history of prolonged febrile seizures. Moreover hippocampal damage is often unilateral or at least very asymmetrically bilateral. These evidences are difficult to conciliate with the hypothesis that prolonged febrile seizure are the only cause of HS. Another theory propose that hippocampal sclerosis is a developmental lesion present prenatally (Raymond et al., 1994; Hardiman et al., 1988). Prolonged febrile seizures are therefore the consequence of this lesion rather than the cause of hippocampal damage. This hypothesis is supported by the observation of focal dysplasia in the temporal lobe of a proportion of cases with hippocampal sclerosis that can be interpreted as dysplastic abnormalities. However MR imaging observations documenting the evolution of hippocampal sclerosis after prolonged febrile seizures have not found preexisting brain lesions in all the patients (VanLandingham et al., 1998). Recently it has been proposed

that there may also be a genetic susceptibility to hippocampal injury. Traditionally hippocampal sclerosis has not been regarded as having a major genetic contribution. Indeed HS has been rarely observed in more than one individual in a family and the twins studies have given negative correlation (Abou-Khalil et al., 1993; Jackson et al., 1998). However a recent study (Kanemoto et al, 2000) has documented a strong association of a polymorphism in the interleukin-1β gene in patients with HS and TLE compared with non epileptic controls and with patients with TLE and no HS. This polymorphism is in the promoter region of the gene and result in increased production of interleukin-1β, a proinflammatory cytokine. Such overproduction might increase the likehood of neuronal damage following hippocampal insult. So this polymorphism may represent a genetic predisposing factor to the development of hippocampal sclerosis. It must be remembered however, that association studies are prone to a variety of subtle and inobvious biases. This important observation requires therefore confirmation in other populations.

Hippocampal sclerosis appears therefore to be determined by multiple and complex causes both within and between the patients. However the unification of the disparate views and apparently inconsistent findings is possible. The proximate cause of hippocampal sclerosis is, in most cases, a brain insult, such as a prolonged febrile seizure or other toxic injury. However hippocampal sclerosis occur after such an insult only if there are also preexisting factors that make the hippocampus vulnerable to injury. Such substrates can be a developmental lesion or a tumor, but also a specific genetic predisposition such as the interleukin-1β polymorphism described previously. This model is able to explain most, but not all, current data about hippocampal sclerosis. Indeed there are cases of HS with no known insult or identified preexisting substrate. Thererefore unveiling the nature and the severity of all potential proximate causes and pre-existing factors that contribute to hippocampal sclerosis will be one of the next goal of epilepsy research.

Neurological examination of MTLE patients reveals memory deficits (Engel 1996) that can be explained by the hippocampal damage. Recent studies (Hermann et al, 2002) established a strong correlation between childhood-onset MTLE, brain damage and reduced memory functions. In particular childhood onset MTLE seems to be associated with an adverse generalized neurodevelopmental impact on the immature brain, characterized by reduced brain tissue volume compared to patients with late onset

epilepsy. This adverse impact on brain structure is associated with a generalized pattern of cognitive impairment, such as neurological difficulties that involve memory as well as more generalized intellectual function. Moreover the increasing duration of epilepsy in childhood onset patients is associated with declining intellectual status as well as memory function, suggesting a progressive cognitive deterioration.

1.1.2 Animal models

Studies of patients with the TLE syndrome have shown a number of clinical-pathological associations that may be important to duplicate in animal model as a way to experimentally test pathophysiologic mechanisms and hypotheses (Loscher, 2002a; Stables et al., 2002; Leite et al., 2002). Among the known features of human disease that must be replicated in animal models we can remember:

- the clinical time course that usually consists of some initial injury to the brain followed by a latent period prior to the onset of recurrent unprovoked seizures;
- the hippocampal sclerosis that consists of severe neuron loss in Sommer's sector (CA1 and prosbiculum), the end folium (hilus and CA4) and CA3;
- hippocampal sclerosis mostly on the epileptic side while less damage must be found in the opposite hippocampus;
- additional hippocampal neuronal loss during recurrent seizures that adds to hippocampal sclerosis but not generate it per se ;
- axon and synaptic reorganization of surviving neurons;

Most of these phenomenological features of human temporal lobe epilepsy are replicated in animal models and I will summarize the characteristics of the most used animal preparations.

1.1.2a Kainic acid model

Limbic status epilepticus in rats can be induced by kainic acid with either local administration (intracerebroventricular or intrahippocampal, at doses of 0.1-0.3 μg per hemisphere) or injected systemically (usually at doses 8-12 mg/Kg). Kainic acid is an agonist of glutamate receptors localized mainly in the CA1 and CA3 hippocampal pyramidal neurons (Monaghan and Cotman, 1982). As such treatment protocols have often been associated with a relatively high mortality rate and a low percentage of rats

becoming epileptics, Hellier and collaborators (1998) proposed a modified treatment protocol using multiple low doses (5 mg/kg, i.p.) of kainate This protocol had a relatively low mortality rate (around 15%) and nearly all kainate-treated rats (97%) had two or more spontaneous motor seizures months after treatment.

Systemic administration of kainate has a strong pro-convulsant effect in rodents. Indeed the animals show initial immobility and staring followed by wet dog shakes and culminating in limbic motor seizures with rearing and falling and forelimb clonus. The pattern of repetitive seizures and status epilepticus induced by parental KA can last for several hours (Ben-Ari et al, 1985). The brain damage induced by status epilepticus in such preparation may be considered an equivalent to the initial precipitating injury events which is commonly found in patients with TLE. A somewhat variable latent period follows status epilepticus and precedes the chronic phase, which is characterized by the occurrence of spontaneous limbic seizures (Stafstrom et al., 1992). Some weeks after kainate administration, massive neuronal loss can be detected in CA1 and CA3 regions. In addition, several interneuron populations in the hilus, such as those expressing parvalbumin, somatostatin and NPY, are vulnerable to KA-induced damage. An extensive reorganization of mossy fibers into the molecular layer of the fascia dentate is also observed (Tauck et al, 1985). This abnormal synaptic reorganization has been suggested to be an anatomical substrate for epileptogenesis (Buckmaster et al., 1997).

With respect to susceptibility of acute kainate-induced seizures to systemic administration of standard anti-epileptic drugs, benzodiazepines and trimethadione are most effective, whereas phenytoin, carbamazepine and valproate have no overt anticonvulsant action in this model (Sperk, 1994). As carbamazepine and phenytoin are the drugs of choice for the treatment of complex partial seizures in humans while benzodiazpines and trimethadione are normally not effective in these patients, there are some doubts on the predictive value of kainate seizures for the development of therapeutic strategies against intractable epilepsy. However the fact that administration of kainate may lead to spontaneous recurrent seizures can be exploited to study the mechanism of chronic epilepsy. Even if the pharmacology of spontaneous recurrent seizures in this model has been poorly investigated, the available data suggest that acute and spontaneous seizures respond differently to AEDs and that the response of spontaneous seizures to AEDs is similar to that of patients with complex partial seizures (Loscher, 1999). Thus the chronic model of spontaneous recurrent seizures is more

predictive of clinical efficacy of AEDs than the acute one and it should be used early in drug development.

1.1.2b Pilocarpine model

In addition to the kainic acid model, status epilepticus can be induced also by intraperitoneally injections of high doses, usually over 300 mg/Kg, of the cholinergic (muscarinic) agonist pilocarpine (Turski et al, 1983; Cavalheiro et al., 1991). Pre-treatment with lithium chloride 24 hr prior to pilocarpine injection, at a dose of 3 mEq/Kg intraperitoneally, potentiates the epileptogenic action of pilocarpine and the amount of drug can be reduced by ten times (Honchar et al, 1983). Acute behavioural manifestation after high doses of pilocarpine and in the lithium-pilocarpine model are very similar. However there are some evidence that antiepileptic compounds respond differentially to status epilepticus, suggesting that distinct biochemical mechanisms control seizures in these two different preparations (Ormandy et al., 1989).

The pattern of neuronal death induced by i.p. administration of pilocarpine is very similar to that induced by KA. Indeed extensive neuronal death can be detected in CA1 and CA3 regions of hippocampus, associated with reorganization of mossy fibers into the molecular layer of the fascia dentate. Moreover spontaneous recurrent seizures develop after a variable latent period that follows status epilepticus.

Local administration of pilocarpine delivered either intracerebroventricularly or directly into the hippocampus has been used in studies assessing the seizure-induced changes in amino acid levels and the effectiveness of some anti-epileptic agents. Intrahippocampal pilocarpine injections (2,4 mg/µl injected in a volume of 1.0 µl) induce status epilepticus with near zero mortality. Spontaneous recurrent seizures and mossy fibers sprouting are also observed in intrahippocampal-pilocarpine injected rats with similar seizure frequency than that observed in systemically injected animals (Furtado et al., 2002).

In the pilocarpine model, AEDs that are efficient to suppress status epilepticus are not necessarily the same that are effective on controlling spontaneous recurrent seizures. Indeed benzodiazepines, phenobarbital, valproate and trimethadione protect against pilocarpine-induced status epilepticus while carbamazepine and phenytoin are ineffective. Otherwise, in the chronic phase, carbamazepine and phenytoin are effective against spontaneous seizures, while valproate is effective only at high doses (600

mg/kg) and ethosuximide is ineffective (Turski et al., 1989; Leite and Cavalheiro, 1995). This difference can be easily explained by the fact that the mechanisms of pilocarpine-induced acute seizures certainly differ from those of spontaneous seizures. Moreover responses to different AEDs in pilocarpine chronic model are very similar to clinical outcome and this validates the use of this model for screening drugs against partial seizures.

Both in the kainate model and in the pilocarpine model, the duration of the status epilepticus is critical for the subsequent development of epilepsy. When status epilepticus is interrupted by diazepam and pentobarbital after 30 min, no spontaneous seizures develop (Lemos and Cavalheiro, 1995) and at least 90-120 min of status epilepticus are necessary to chronic epilepsy to develop.

1.1.2c Kindling model

Kindling is a widely used model of TLE, because the clinical phenomenology of the complex-partial seizures that progress further to secondary generalize and the pharmacology of these seizures are very similar to the clinical conditiond (Sato et al., 1990; Loscher, 1999)

As the name kindling suggests, a small spark applied to tinder will ignite a flame that eventually can grow into a roaring bonfire. Similarly, a small electrical stimulus, just large enough to trigger a brief "afterdischarge" or burst of epileptiform activity, if repeatedly applied can generate seizures that lead to fully generalized behavioural convulsions. Indeed animals chronically implanted with stimulation and recording electrodes in one structure of the limbic system or in other brain areas (the amygdala being among the most responsive structure) develop seizures upon a period of electrical stimulation with an initially subconvulsive current.

The first electrographic and behavioural characterization of kindling as a model of epilepsy was made by Racine in the amygdala. Indeed the initial stimulus applied to the amygdala is able to elicit focal paroxysmal activity (the so-called after-discharges recorded in the EEG) without overt clinical seizure activity. Subsequent stimulations induce the progressive development of seizures, generally progressing into 5 distinct behavioural stages from motor arrest accompanied by facial automatisms (Stage 1) to fully kindled seizures accompanied by forelimb clonus and hindlimb tonus identified by

rearing and bipedal instability (Stage 5) (Racine, 1972 II). All of these stages are associated with reduced responsiveness to sensory stimulation in comparison to the normal waking state. Moreover the behaviour observed in stage 1 and 2 mimics that found in human complex partial limbic seizures, while the behaviour in the latter three stages is consistent with secondary generalized motor seizures, the most devastating and difficult to treat form of epilepsy in adults (Loiseau, 1986). In addition to seizures becoming more severe during kindling acquisition, the paroxysmal EEG alterations, i.e. the afterdischarges, increase in duration and amplitude, while the electrical threshold for induction of after-discharge decreases (Racine, 1972 I). The increased convulsive sensitivity after kindling established, evidenced by stage 5 seizures, persists for months, thus suggesting that kindling involves permanent changes in brain function (Sato et al., 1990).

Kindling is often considered a model of elicited (stimulation-induced) seizures. However kindling has to be considered also as a model of chronic epilepsy, as it replicates some characteristics of chronic epilepsy such as increase in seizures severity and duration and decrease in focal seizure threshold. Indeed, since its introduction in 1969, kindling was widely used to investigate every possible facet of epilepsy research, ranging from membrane physiology involving brain slices to electroclinical phenomenology of seizures (Sato et al., 1990). The kindling model has thus provided a clearer insight into the nature of chronic epilepsy. Indeed the study of hippocamapal kindling demonstrated that seizures induce structural and electrophysiologic alterations in hippocampal pathways that may lead to increased excitability and could play a role in the development and progression of temporal lobe epilepsy. These alterations include mossy fiber synaptic reorganization, induction of NMDA-mediated synaptic transmission, but very little neuronal degeneration. As some of these structural alterations have also been observed in the human epileptic temporal lobe, the study of the mechanisms operative in kindling may help to elucidate the pathogenesis of human temporal lobe epilepsy (Sutula, 1993).

Moreover kindling stimulations can be continued until the animals develop spontaneous seizures (Pinel and Rovner, 1978; Racine and Burnham, 1984; Corcoran, 1988), demonstrating that kindling can be used also as a model of epileptogenesis. In this contest kindling is useful in order to model the latent period in a very controlled way, because the experimenter knows exactly where the epileptogenesis is beginning (at

the electode tip) and can track by numerous methods the progressive changes (electrographic, anatomical, biochemical, genetic) that occur during the latent period.

The pharmacology of elicited kindled seizures in fully kindled rats is very interesting, as it is very similar to that of spontaneous recurrent seizures in post-status pilocapine model. Indeed, in both model, carbamazepine, phenobarbital, phenytoin are effective, while ethosuxymide is not (Leite and Cavalheiro, 1995; Loscher, 1999). This is important, as determining anticonvulsant effects is much easier in the kindling model compared with chronic seizure and EEG recording needed in post-status epilepsy models. Thus, for determination whether a drug acts against partial seizures or not, testing against elicited partial seizures in fully kindled rats is certainly a more convenient method than drug testing in TLE models with recurrent spontaneous seizures.

1.2.1 Pharmacotherapy of epilepsy

The study of the pathogenesis of epilepsy has made much progress during the past decades. However the cellular basis of human epilepsy remains a mystery. In the absence of a specific etiological understanding, approaches for drug therapy of epilepsy must necessarily be directed at the control of symptom, i.e. the suppression of seizures by chronic administration of anti-epileptic drugs or AEDs. However, seizures remain uncontrolled in at least 30% of all epilepsies despite adequate AED therapies (Regesta and Tanganelli, 1999). Furthermore none of the current AEDs represents a 'cure' for epilepsy or an efficacious means for preventing epilepsy or its progression. Therefore new concepts and original ideas for developing AEDs are urgently needed.

1.2.1 Neurobiology of antiepileptic drugs

Antiepileptic drugs (AEDs) are intended to prevent epileptic seizures while permitting normal functioning of the nervous system (Rogawski and Loscher, 2004). AEDs act on diverse molecular target in order to selectively modify the excitability of neurons so that seizure-related firing is blocked without disturbing non-epileptic activity. The target molecules of AEDs in the brain are therefore ion channel, neurotransmitter transporters and neurotransmitter metabolic enzymes in order to modify the bursting properties of neurons and reduce synchronization in localized neuronal ensembles. Moreover AEDs inhibit the spread of abnormal firing to distant sites, which is required for the expression of behavioural seizure activity.

Each AED acts through a unique combination of the following three mechanisms :

- modulation of voltage-gated channel;
- enhancement of synaptic inhibition;
- inhibition of synaptic excitation;

The *modulation of voltage-gated sodium channels* is believed to account, at least in part, for the ability of several AEDs to protect against seizures. These AEDs include phentoin, lamotragine, carbamazepine, oxcarbazepine (Schmutz et al., 1994; Ambrosio, 2001) and possibly felbamate (Taglialatela et al., 1996), topiramate (Taverna et al., 1999) and valproate (Van der Berg, 1993). Normally, the brain sodium channel are able to rapidly cycle through resting, open and inactivate states, in order to allow neurons to fire high-frequency trains of actions potentials, as it is require for normal brain

functioning and for the expression of epileptic activity. The sodium channel-blocking AEDs preferentially bind to inactivated conformations of the channel (Remy et al., 2003b; Ragsdale et al., 1991). They therefore act mainly on action potential firing, blocking high-frequency repetitive spike firing, which is believed to occur during the spread of seizure activity, without affecting ordinary ongoing neuronal activity.

The *regulation of calcium-gated sodium channels* is another potential mechanism through which AEDs can exert their anti-epileptic activity. Calcium channels can be broadly grouped into two groups the high-voltage activated and the low-voltage activated. The high-voltage activated calcium channels require strong membrane depolarization for gating and they are largely responsible for the regulation of calcium entry and subsequent neurotransmitter release from presynaptic nerve terminals. These channels are the target of the action of many AEDs, as the blockade of these channels inhibits neurotransmitter release (Turner, 1998). Gabapentin and their analogues, such as pregabalin, act by blocking one subunit of the multimeric protein channel (Marais et al., 2001). Other AEDs, such as Phenobarbital (Ffrench-Mullen, 1993), lamatrogine (Stefani et al., 1996) and levetiracetam (Lukyanetz et al., 2002) can inhibit calcium channels even if their anti-epileptic effect is probably due to other mechanisms.

The *enhancement of synaptic inhibition* is a key mechanism of AEDs action. The inhibitory neurons represent only a small fraction of cells in regions key to epileptic activity, such as the neocortex and the hippocampus, but they are very important in restraining the natural tendency of recurrently connected excitatory neurons to undergo the transition into synchronized epileptiform discharges (Miles et al., 1987). The main inhibitory neurotransmitter of the central nervous system is the γ-aminobutyric acid, or GABA, that acts through fast chloride-permeable IONOTROPIC $GABA_A$ receptors and also through slower METABOTROPIC G-protein-coupled $GABA_B$ receptors. Many AEDs influence $GABA_A$ receptor-mediated inhibition, either by interacting with $GABA_A$ receptors or by modifying the activity of enzymes and transporters important for GABA homeostasis and altering it. The antiepileptic activity of benzodiazepine-like agents, such as diazepam, occurs though positive allosteric modulation of $GABA_A$ receptors. Indeed benzodiazepines bind to $GABA_A$ receptor and increase the frequency of its GABA-mediated openings (Rudolph et al., 1999). Also barbiturates, such as phenobarbital, act as positive allosteric modulators of $GABA_A$ receptors, but with a different mode of action from benzodiazepines. Indeed they act by shifting the relative proportion of openings in favour of the longest-lived open state (MacDonald and Olsen,

1994). Other AEDs act by increasing the extracellular level of GABA, and consequently the tonic GABA-mediated inhibition, through the modification of the activity of enzymes involved in GABA metabolism. For example vigabatrin irreversibly blocks the GABA transaminase enzyme, resulting in increased intracellular levels of GABA that shift its equilibrium in favour of extracellular GABA (De Biase et al., 1991). Instead tiagabin is a potent and selective competitive inhibitor of GABA transporter that binds with high affinity to the transporter, thus preventing GABA uptake and favouring its extracellular accumulation (Suzdak and Jansen, 1995).

The *inhibition of synaptic excitation* represent a very attractive strategy to use in order to suppress seizures. Unfortunately it revealed unsuccessful by itself, even if many AEDs act partly through inhibition of excitatory activity (Kuo et al., 2004). Glutamate is the main excitatory neurotransmitter of the CNS and its ionotropic receptors can be subdivided in three groups based on their pharmacology: the AMPA subtype (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid), the NMDA subtype (N-methyl-D-aspartate) and the kainate subtype. Blockade of the NMDA and AMPA subtypes has revealed effective in protecting against seizures both in *in vitro* and *in vivo* animals models. Despite this, clinical trials with selective NMDA antagonists have failed in the chronic treatment of epilepsy. A more interesting target for AEDs is represented by kainate receptors. Indeed these receptors are present in pre-synaptic axon terminals where they are able not only to modulate glutamate release from excitatory afferents but also to suppress GABA release from inhibitory interneurons. The blockade of these receptors would therefore enhance synaptic inhibition and reduce synaptic excitation. Indeed selective kainate receptors antagonist have proved effective in protecting against seizure in brain slice and animal models. and have now to be tested in humans (Smolders et al., 2002).

The development of AEDs have provided immeasurable benefits for those afflicted with seizures disorders of all kind. However several AEDs suffer from substantial problems with toxicity, particularly neurotoxic side effects and idiosyncratic reactions such as skin rush (Brodie, 2001). Thus new AEDs with better safety, less toxicity and higher anticonvulsant efficacy are needed (Loscher, 1998; Loscher and Schmidt, 2002). Most clinically effective AEDs have been found by screening in animal models or by structural variation of known drugs. However some AEDs were developed according to a rational strategy based on knowledge of pathophysiologic processes involved in seizures or epilepsy. Among these, the GABA-mimetic drugs vigabatrin and tigabine,

developed according to the 'GABA hypothesis' of epilepsy, that is the idea that impaired GABAergic inhibitory neurotransmission is critically involved in the pathogenesis of several types of epilepsy. Several other rational strategies failed to produce efficacious AEDs. For example drugs developed to counteract exaggerated excitatory activity by blocking the NMDA subtype of glutamate receptors showed no antiepileptic activity, even if there are many evidence that excessive activity of glutamatergic neurotransmission can contribute to various types of epilepsy. Therefore the lesson to be learned from the failure of rationale strategies in developing new AEDs is that absolute selectivity of one target is not desirable in a multifactorial disease such as epilepsy. Moreover the preclinical AEDs development should involve chronic models of epilepsy that mimic more closely the structural and functional brain alterations associated with epilepsy than the simple seizure models, such as the maximal electroshock seizure.

1.2.2 Strategies for developing drugs for pharmacoresistant epilepsy

As I told before, almost 30% of patients with epilepsy are drug-resistant. Drug resistance can be defined as the continuing occurrence of seizures despite trial of at least three appropriate AEDs at maximum tolerated doses. Resistance may be classified as either primary (an intrinsic component of the disease process) or secondary (an undesirable component of the disease process).

The major problem in developing new therapies for drug-resistant epilepsies is that the mechanisms of pharmacoresistance are poorly understood (Kwan and Brodie, 2000). Moreover refractory epilepsy is likely to be a multifactorial and complex process (Loscher and Potschka, 2002) to which many factors, both primary and secondary, contribute. Ontogenetic abnormalities in brain maturation that cause aberrant morphology and distribution of local-circuit neurons have been associated with intractable focal epilepsy (Awad et al, 1991). Genetic factors may also be important and explain why two patients with the same type of epilepsy or seizures can differ in their response to AEDs. Finally, as epilepsy is a progressive disorder, disease-related factors, such as seizure induced synaptic reorganization, alteration in drugs targets or in drug-uptake into the brain, have to play a pivotal role in the development of intractable epilepsy.

1.2.2.a Pharmacoresistance: the role of seizure-induced modifications.

Some recent studies have investigated the role of seizure-related factors in the genesis of pharmacoresistance and have uncovered their importance. Among these, the work of Remy and co-workers (Remy et al, 2003a) demonstrated that the resistance to carbamazepine treatment in epilepsy due to hippocampal sclerosis is associated with the insensitivity of dentate granules neurons to the effects of this drugs. Cabamazepine, like many other antiepileptic drugs, acts partly by inhibiting sodium influx via voltage-sensitive brain-expressed sodium channels in a use-dependent fashion. Carbamazepine blocks sodium channels by binding to them in their inactivated state and slowing reactivation. Blocking of sodium channels with carbamazepine is more effective when the neuronal membrane is depolarized repetitively at high frequency, a property that is thought to underlie the anti-seizure effect of the drug. In their study Remy and co-workers isolate dentate granule neurons from hippocampi of patients with epilepsy who had been treated with cabamazepine and were refractory to its effects. They report that dentate granule cells from these patients were completely insensitive to the effects of carbamazepine. In vitro studies of repetitive stimulation of hippocampal slices from these patients support the hypothesis that the insensitivity of dentate granule cells is likely to cause carbamazepine resistance.

This study is the first demonstration of the hypothesis that changes in the target of AEDs are responsible of pharmacoresistance. Moreover this research raises many interesting questions, mainly the acquired or inherited nature of the disfunction. Indeed an inherited dysfunction could predispose patients to refractory disease and may enable the prediction of such resistance while an acquired patholoy would suggest that early treatment of seizures is necessary.

1.2.2.b Pharmacoresistance: the role of multi-drug transporter proteins.

An important characteristic of pharmacoresistant epilepsy is that most patients with refractory epilepsy are resistant to most, and often all, AEDs (Regesta and Tanganelli, 1999). As a consequence, patients not controlled on monotherapy with the first AD have a chance of only 10 % or less to be controlled by other AEDs, even when using AEDs acting by different mechanisms. This argues against epilepsy-induced alterations in specific drug targets as a major cause of pharmacoresistant epilepsy but rather points to

non-specific and possibly adaptive mechanism. One of these mechanisms could be the decreased drug uptake into the brain caused by seizure-induced overexpression of multidrug transporters in the blood-brain barrier. Indeed, unlike endothelial cells in most tissues, brain capillary endothelial cells are joined by tight junctions and lack intercellular pores and pinocytotic vesicles. Process from pericapillary astrocytes (the glial endfeet) terminate on the capillary and contribute to the barrier function. The blood-brain barrier (BBB) passively excludes strongly ionized (polar), hydrophilic drugs, but non polar, highly lipid-soluble drugs (like most AEDs) penetrate easily into the brain by simple diffusion. As an active defence mechanism, ATP-dependent multi-drug transporters, located in the apical cell membrane of capillary endothelial cells of the BBB, act as outwardly directive active efflux pumps and transfer drugs back into the blood after they have entered endothelial cells from the blood. These transporters are therefore important to limit the penetration of many lipophilic drugs into the brain parenchyma. The main ATP-dependent multi-drug transporters are the P-glycoprotein, PGP, and the family of multi-drug-resistance associated proteins, MRPs, first identified in pharmacoresistant cancer cells and subsequently found in various normal tissue. Overexpression of these transporters might play a significant role in pharmacoresistant epilepsy by limiting access of AEDs to their targets in the brain. Tishler and co-workers were the first to report that brain expression of multi-drug resistance gene *MDR1*, which encodes for PGP, is markedly increased in patients with medically intractable epilepsy (Tishler et al, 1995). Immunohistochemstry for PGP showed increased staining both in capillary endothelium and in astrocytes. Subsequently it was shown that also MPR2 is overexpressed in brain tissue from pharmacoresistant patients (Sisodiya et al., 2002) and that it is localized mainly in the BBB endothelial cells. However it is still nor clear if multi-drug transporters overexpression in epileptic brain tissue is a consequence of seizure or if this defect is present before the onset of epilepsy. Because pharmacoresistant patients have the same extent of neurotoxic side effects under AEDs treatment as patients who are controlled by AEDs, it has been supposed that the overexpression of drug transporters in refractory patients is most likely restricted to the epileptic focus or circuit. Indeed patients in whom the epileptic focus has been resected during epilepsy surgery may re-experience seizures after cessation of AEDs treatment and become pharmacoresistant again, thus suggesting that a ‘secondary focus’ has become activated and drug-resistant. Moreover experimental studies have demonstrated that in rats there is a transient overexpression of PGP in capillary endothelial cells and

in astrocytes after kainate-induced seizures (Zhang et al., 1999), supporting the hypothesis that seizures are responsible for the overepression of drug transporters. These data would explain also the finding that the severity of pharmacoresistance is correlated with a high number of seizure prior to initiation of treatment.

In conclusion there are increasing evidence that genes encoding multi-drug transporters such as PGP and MRPs are involved in the generation of pharmacoresistance in epileptic patients. In this case either systemic or local administration of inhibitors of these drug transporters or novel AEDs that are not substrates for these transporters could prove useful in pharmacoresistant epilepsy. Inhibitors of PGP and, more recently, MPRs are being evaluated clinically for either the reversal or prevention of intrinsic and acquired multi-drug resistance in human cancer (Litman et al., 2002) and might soon be available for clinical trials in epilepsy.

In addition to studies from drug-resistant patient, experimental animal models should be used to study the mechanisms of drug-resistant epilepsy and eventually to discover new drugs effective in patients not controlled on current antiepileptic medication. The main characteristics of models suitable for studying the mechanisms of pharmacoresistance are:

- the type of seizures should be similar in its clinical phenomenology to seizures occurring in human;

- the seizures in the model should be associated with paroxysmal activity in the electroencephalogram (EEG) in order to allow evaluation of drug effects on both behavioural and electrographic seizures manifestations;

- standard antiepileptic drugs should be inactive or weakly active to block seizures. Ideally the same model should comprise subgroup of animals differing in their response to standard drugs (responders vs non-responders) thus simulating the clinical, where patients with the same type of seizure or epilepsy may differ in their response to antiepileptic drug treatment. Models with seizures that are easily suppressed by standard antiepileptics (e.g. all of the commonly used models of primarily generalized seizures) are not likely to detect new drugs with higher efficacy than standard drugs in intractable epilepsy;

- the model should allow long-term studies on anticonvulsant drug efficacy, i.e. the animals should survive the seizures and the maintenance of effective drug concentration should be possible during chronic treatment;

The progressing in studies on brain tissue from pharmacoresistant patients and the improvement of models of intractable epilepsy should therefore enhance the understanding of the mechanisms of pharmacoresistance in epilepsy and allow the development of new effective AEDs.

1.2.3 Strategies for developing drugs that prevent epileptogenesis

The future goal of pharmacotherapy of epilepsy is to develop anti-epileptogenic and disease-modifying drugs, that is drugs that prevent the development of epilepsy after an initial precipitating injury and drugs that block the progression of epilepsy to chronic, often difficult to treat, epilepsy (Loscher amd Schmidt, 2002; Schmidt and Rogawski, 2002).

A number of AEDs, including phenytoin, carbamazepine, valproate and phenobarbital, have been evaluated in clinical trials to test whether they prevent post-traumatic epilepsy after brain injury. Unfortunately results have been disappointing, as none of the drugs have exerted any significant anti-epileptogenic effect (Schachter, 2002; Hernandez, 1997; Temkin et al., 2001). Interestingly, in apparent contrast to clinical trials, valproate has been successful in inhibiting epileptogenesis in two animals models, the kindling and the kainate models of temporal lobe epilepsy (Silver et al., 1991; Bolanos et al., 1998). This may indicate that the mechanisms responsible for epileptogenesis differ after different initiating events such as status epilepticus or brain injury.

The understanding of the mechanisms that promote the development of epilepsy and its evolution to a chronic state is extremely important in order to discover anti-epileptogenic drugs. A wide variety of approaches is used to discover the basic mechanisms of epilepsy, including both clinical (e.g. brain imaging, studies of human brain tissue, studies on gene mutations in familial epilepsies) and experimental approaches, in which new or improved animal models have an important role (Loscher, 2002). These studies indicated a large number of factors are involved in the epileptogenic process, including an imbalance between excitatory and inhibitory neurotransmission, alterations in neurotransmitter receptor expression and function, development of epileptic ion channel (channelopathies), functional changes of neurons, development of epileptic networks within and between brain regions, morphological changes such as hippocampal sclerosis and axonal sprouting (leading to aberrant

neuronal synchronization) and also genetic causes (McNamara, 1999; Pitkanen and Sutula, 2002). The targeting of one or more of these mechanisms should be a good strategy to develop anti-epileptogenic drugs. Currently there are very few studies addressing the effectiveness of interfering with epileptogenic mechanisms in preventing the development of permanent epileptic state. As neuronal loss is one of the major abnormalities in the epileptogenic and epileptic brain, a recent work of Ebert and co-workers (Ebert et al., 2002) investigated if the sparing of neurons is able to counteract the occurrence of epilepsy. To address this question, different neuroprotective drugs, including the NMDA antagonist MK-801 (dizocilpine), have been administrated to rats after kainate-induced status epilepticus during the latent period and before the development of spontaneous recurrent seizures. The massive sclerosis in the hippocampus and piriform cortex induced by status epilepticus was prevented by the administration of MK-801. Despite this, all rats developed spontaneous recurrent seizures, indicating that damage in the limbic brain regions is not critically involved in the epileptogenesis.

The future challenge of pharmacotherapy of epilepsy will therefore be the development of drugs preventing the process of epileptogenesis and their testing both in animal models and in clinical trials. The further elucidating of the molecular and cellular mechanisms of epileptogenesis will be helpful to discover these new drugs.

1.2.4 Treatment options to conventional pharmacotherapy of epilepsy.

A number of treatment procedures are being used or under development for clinical use in order to get therapies for epilepsy alternatives to systemic (usually oral) administration of AEDs.

One of the most interesting approach in this direction is the delivery of the anti-epileptic medication directly into the focus through a biosensor device that anticipates seizures and subsequently applies the treatment via a minipump into the site of seizure origin in order to block it. Recent clinical studies have indeed demonstrated the possibility to predict seizures occurrence from standard EEG recordings. Pre-seizures changes in brain dynamics can be detected from recordings of scalp-EEG activity 1 hour before the onset of the clinical seizure (Le Van Quyen et al., 2000; see also Le Van Quyen et al., 2001 for review). Moreover intracranial EEG recordings permit the identification of quantitative EEG changes corresponding to prolonged bursts of

complex epileptiform discharges 7 hours before seizures (Litt et al., 2001). The predictability of seizure occurrence permit to design implantable anti-seizure devices that, while continuously monitoring EEG in the area of a epileptic focus, can infuse drugs in this area when the probability of an incoming seizure exceeds a predetermined threshold value. Such a device has been tested in an animal model of focal epilepsy (Stein et al., 2000). In this study a computerized system for the detection for seizures was linked to a programmable infusion pump to deliver diazepam directly into the seizure focus. Focal seizures were created in rats using bicuculline, a strong GABA antagonist. After the detection of the first seizure, the computerized system sent a signal to the infusion pump triggering the delivery of vehicle or diazepam though the cannula. The animals receiving diazepam showed cessation of seizures, whereas the animals receiving vehicle generated additional ictal events. Therefore intra-focus delivery of diazepam is effective in suppressing electroencephalographic seizures. This experiment provides therefore proof-in-principle of a closed loop, automated drug delivery system for the treatment of focal epilepsy. Development of clinically useful systems will require attention to a number of issues, such as the efficacy of seizure detection, the choice of appropriate medication and other engineering problems, but it could represent an effective strategy for epilepsy intractable with standard medications.

1.3 THE BOTULINUM TOXINS

Many thousand of living species produce toxins that are used to modify the physiology of other species with the general aim of increasing their chance of survival. Given the essential role of nervous system in animal physiology, it is not surprising that most known toxins are selective for molecules of the nervous system. In general, neurotoxins act by blocking the transmission of the nerve impulse. As a neurotoxin is the product of a long-term coevolution of the toxin-producing species with the target species, every neurotoxin is very specific for it target and the study of its mechanism of action can reveal important features of nerve physiology (Rappuoli and Montecucco, 1997).

Protein neurotoxins produced by bacteria of the genus *Clostridium* act by inhibiting neurotransmitter release through the specific cleavage of a group of proteins integral to the exocytotic process. Indeed the seven botulinum neurotoxin (BoNT) serotypes (A-G) produced by *Clostridium botulinum* bind to and enter the cholinergic terminals from which they inhibit the release of acetylcholine with ensuing flaccid paralysis (Kerner, 1817).

The remarkable specificity of BoNTs is exploited in the treatment of human disease characterized by a hyperfunction of cholinergic terminals. However the further elucidation of BoNTs structure and function can be useful to discover new therapeutic exploitations of botulinum toxin.

1.3.1 The structure

To understand the components of BoNTs responsible for its mechanism of action, it is necessary to describe the main characteristics of their structure. BoNTs are synthesized in the bacterial cytosol without a leader sequence and they are released to the culture medium after bacterial lysis as a single polypeptide chain of 150 kDa. The inactive form of the protein can be cleaved by different bacterial and tissue proteinases within a surface-exposed loop (Krieglstein et al, 1994; Turton et al., 2002) to form the active di-chain neurotoxin (Fig. 1A). The heavy chain (H, 100 kDa) and the light chain (L, 50 kDa) remain associated via both non-covalent protein-protein interactions and the conserved interchain S-S, the integrity of which is essential for neurotoxicity (Schiavo et al., 1990).

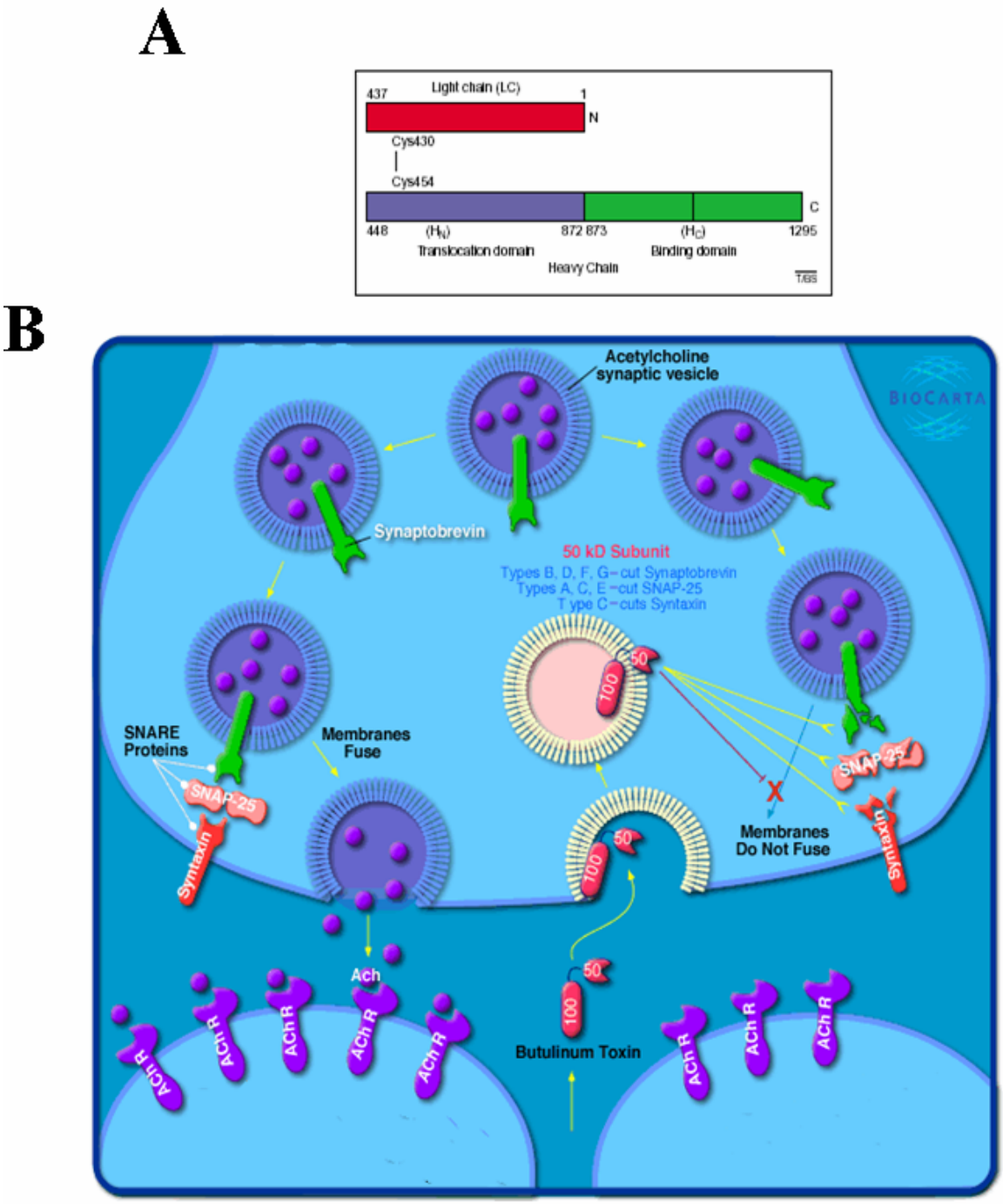

Figure 1

Structure and mechanism of action of BoNT/E

(A) BoNTs are formed by a light (50 kDa) and a high chain (100 kDa) associated by a di-sulfide bond. The light chain present the zinc-endopeptidase domain, while the heavy chain has the binding and the translocation domain. (B) Mechanism of BoNTs action. BoNTs intoxicate peripheral neurons through a four-steps mechanism consisting of binding to target cell, internalization in endosomial vesicles, translocation to cytosol and enzymatic cleavage of one component of the SNARE complex.

The 3D crystal structure of BoNT/A has been recently determined (Lacy et al., 1998). The toxin is subdivided into three 50 kDa domains:

- the zinc-dependent endopeptidase domain of the LC;
- the translocation domain in the N-terminal half of the HC;
- the binding domain in the C-terminal half of the HC;

These three functional domains are structurally distinct and arranged in a linear fashion, such that there is no contact between them.

1.3.2 The function

The structural organization of BoNTs meets nicely their ability to intoxicate neurons via a four-step mechanism (summarized in Fig. 1B) consisting of:

- binding;
- internalization;
- membrane translocation;
- enzymatic target cleavage;

1.3.1a Binding to target cell

BoNTs diffuse in the body fluids from the site of adsorption to the pre-synaptic membrane of cholinergic terminals where they bind. Available evidence indicates that the C-terminal half of the HC plays a major role in neurospecific binding (Shone et al, 1985). However, it appears that additional regions of BoNTs are involved in binding, as immunization with the C-terminal fragment of HC shows only a partial protection from intoxication with the intact BoNT molecule (Poulain et al.,1991).

Identification of pre-synaptic receptors of BoNTs has been attempted by several investigators. Beginning with the seminal work of van Heyningen on TeNT (Van Heyningen, 1974), a large number of studies have established that polysialogangliosides are involved in binding BoNTs (Kitamura and Sone, 1980; Montecucco et al., 1988). Binding to polysialogangliosides well accounts for an unsaturable low-affinity binding of BoNTs to nerve cells and to nerve tissue membranes. However it is unlikely that polysialogangliosides are the sole receptors of these neurotoxins and there are many evidence that proteins of the nerve cell surface have a part in the process. It has therefore been proposed a double-receptor model (Montecucco, 1986) where BoNTs

bind strongly and specifically to the presynaptic membrane because they display multiple interaction with sugar and protein binding sites. Indeed recent experiments demonstrated that both BoNT/B and BoNT/E binds strongly to the synaptic vesicle protein synaptotagmin II in the presence of polysialogangliosides (Nishiki et al., 1994; Li et al., 1998).

The further study of BoNTs receptors will lead not only to important progresses in neurosciences but also contribute to improving present therapeutic protocols based on BoNTs.

1.3.1b Internalization

All available evidence indicate that BoNTs do not enter the cell directly from the plasma membrane but they are endocytosed inside acid cellular compartments. Indeed electron microscopic studies have shown that, after binding, BoNTs enter the lumen of vesicular structures through a temperature and energy-dependent process (Black and Dolly, 1986 I, II). However the nature of the vesicular structures in which BoNTs are internalized is not known. BoNTs activity is influenced by nerve stimulation (Simpson et al., 1980) with paralysis occurring more rapidly when nerve are stimulated at high frequency. The simplest way to account for this is that the neurotoxins enter the synaptic terminals inside the lumen of SSV following the process of vesicle recycling. As I will discuss later, experiments on hippocampal neurons indicate that this is not the case, as BoNTs are internalized via endocytic vesicular structures in these CNS neurons (Verderio et al., 1999). However the mechanism of BoNTs entrance at the motor nerve terminals may be different. Therefore experiments on peripheral motorneurons are needed to evaluate such possibility.

1.3.1c Translocation

Once the neurotoxins have reached the vesicle lumen, their L chain needs to cross the hydrophobic barrier of the vesicle membrane to reach the cytosol where it can display its proteolytic activity. Indirect, but compelling evidence indicates that BoNTs have to be exposed to a low pH step for nerve intoxication to occur (Simpson 1982; Adler et al., 1994). Acid pH does not induce a direct activation of the toxin via a structural change. Rather, it is required in the process of transmembrane translocation of the L chain.

Studies with model membrane systems have shown that BoNTs undergo a low pH driven conformational change from a water soluble neutral structure to an acid structure characterized by the surface exposure of hydrophobic patches. This hydrophobicity enables the penetration of both the H and L chains in the hydrocarbon core of the lipid bilayer. Following this low pH-induced membrane insertion, BoNTs form ion channels in the planar lipid bilayer that regulate the transmembrane translocation of the L domain from the vesicle membrane to the nerve terminal cytosol.

1.3.1d Zinc-endopeptidase activity

The catalytic activity of BoNTs was discovered following the sequencing of the corresponding genes (Minton, 1995). Indeed sequence comparison revealed the presence of a highly conserved 20-residues-long segment, located in the middle of the L chain, containing a zinc-binding motif typical of zinc-endopeptidases. Subsequently, the substrates of BoNTs enzymatic activity were identified through assays of proteolysis performed on synaptic proteins (Söllner et al., 1993).

The BoNTs act through specific cleavage of a group of proteins integral to the exocytotic process, the SNARE proteins (i.e. soluble NSF-attachment protein receptors). Indeed the docking and fusion of synaptic vesicles involve the interaction between specific integral proteins of the synaptic vesicles membrane (the v-SNARE) and receptor proteins of the target membrane (the t-SNARE). In the brain two t-SNARE have been identified: synthaxin, a nerve terminal integral membrane protein, and SNAP-25, a peripheral membrane protein of 25 kDa mass. In the synaptic vesicle the integral membrane protein VAMP (or synaptobrevin) has been identified as the v-SNARE. The ternary complex of VAMP, syntaxin, and SNAP-25 is extremely stable. For efficient recycling to occur, this complex must be disassembled by the binding of two soluble cytoplasmatic proteins: the N-ethylmaleimide-sensitive fusion (NSF) protein and the soluble NSF attachment protein (SNAP). The v-SNAREs and the t-SNAREs serve as receptors for SNAP (hence their name SNAP receptors) which then binds NSF, that unravels the SNARE assembly utilizing energy released upon hydrolysis of ATP.

The seven BoNTs are very specific proteases. Indeed BoNT/B, /D, /F and /G cleave VAMP, each at a single site (Schiavo et al., 1992; Schiavo et al., 1993; Schiavo et al., 1994); BoNT/A and /E cleave SNAP25, each at a single site (Schiavo et al., 1993) while

BoNT/C cleaves both syntaxin and SNAP-25 (Schiavo et al., 1993). Recombinant VAMP, SNAP-25 and syntaxin are cleaved at the same peptide bonds and at the same rate as the corresponding cellular proteins, indicating that there is no additional endogenous factors involved in the proteolytic activity of BoNTs. Analysis of the primary and secondary structure of the neurotoxins reveal that they are very similar. Moreover the variable cleavage sites and flanking regions do not account for the specificity of the three SNARE proteins. These considerations suggest that the SNARE targets could have a common structural element that would serve as a recognition motifs for the neurotoxins. Comparison of the sequence of the neuroexocytosis-specific SNARE proteins of different species has revealed the presence of a nine-long residue motif, characterized by three carboxylate residues alternated with hydrophobic and hydrophilic residues, termed thereafter the SNARE motif (Rossetto et al., 1994). Several experimental evidence support the hypothesis that the SNARE motif is the major determinant of the of BoNTs specificity for the three SNARE proteins (Foran et al.,1994; Shone et al.,1993). The further interaction with regions specific of each SNARE contribute to the selectivity and the strength of neurotoxin binding.

The regions of BoNTs involved in substrate binding are still unknown. It is tempting to suggest that the strongly conserved 100-residue-long NH_2-terminal region is involved, as the removal of more than eight residues from the NH_2 terminus leads to complete loss of activity. However further investigations on the role of this domain are needed.

1.3.2 The neuromuscular junction

The target of BoNTs action is the neuromuscular junction (NMJ), where they cause a selective blockade of regulated exocytosis of acetylcholine (Ach), thereby triggering a profound, albeit transitory, muscular paralysis.

The first electrophysiological investigation of the effect of BoNTs on NMJ was conducted by Burgen (Burgen et al., 1949) on the rat hemidiaphragm preparation. The results of this seminal study can be summarized as follows:

- large and persistent blockade of the end-plate potential (EPP), responsible for the impaired synaptic transmission at intoxicated synaptic terminals;

- reduction of the frequency, but not of the amplitude, of miniature end-plate potential (mEPP);

- no impairment of the processes of neurotransmitter synthesis, uptake, and storage or of the propagation of nerve impulse and Ca^{2+} homeostasis;

- increasing of the frequency of spontaneous quantal events characterised by a prolonged time-to-peak and called “Giant-mEPP (G-mEPP)”;

These data can be well explained by the activity of BoNTs. Indeed each BoNT cleaves a specific SNARE protein that is essential for the neuro-exocytosis process. The neurotoxin-impaired exocytosis apparatus is therefore able to mediate some spontaneous residual synaptic activity, but with reduced efficacy with respect to the amount of neurotransmitter released and to the rate of the overall process. Moreover nerve-evoked Ach release is strongly impaired, thereby the EPP can not reach the appropriate membrane potential level to trigger an action potential in the muscle fiber.

The long-term blockade of regulated Ach exocytosis by BoNTs is able to induce sprouting of nerve terminals, as suggested by the increased frequency of G-mEPPs. Indeed G-mEPPs are considered indicators of immature or pathological states of the synapses and they result from “constitutive” Ach secretion of endosomal compartments precursors of the synaptic vesicles. Their increased frequency at intoxicated NMJ suggests that motorneurons terminals are able to sprout new processes and form new, immature synapses in response to the paralysis induced by BoNTs. Further investigations have confirmed this hypothesis. Immunohistochemical analysis of neuromuscular junction after one month from the treatment with BoNT/A reveal an extensive sprouting network from the paralysed nerve terminal. Sprouts display many key proteins required for exocytosis, as SNAP-25, VAMP/synatobrevin, syntaxin, synaptogamin II, synaptopysin, voltage-activated Na^{+}, Ca^{2+} and Ca^{2+}-activated K^{+} channels (Angaut-Petit et al., 1990). Electrophysiological recordings of original motor nerve endings and newly formed sprouts reveal an extensive propagation of action potentials over most of the nerve terminal arborisation, the presence of Ca^{2+} influx upon active membrane depolarization, and Ca^{2+}-dependent K^{+} currents in the terminal sprout membrane. Interestingly, during the first month after BoNT/A injection, a switch in the nature of voltage-gated Ca^{2+} channels coupled to neurotransmitter release occur. In untreated adult mouse motor nerve terminals, only P/Q-type Ca^{2+} channels are normally present. However, after BoNT/A injection, L, N and P/Q-type channels are found to be functional associated with neurotransmitter release. Thus all these findings indicate that nerve terminal sprouts possess the molecular machinery for Ach release and they

support previous suggestion that terminal sprouts could play a role in replacing parental terminals during the recovery of neuromuscular transmission.

The recent development of fluorescent dyes that allows quantification of synaptic activity in living nerve terminals has been very important in unravelling the unique role of sprouting in implementing functional recovery from paralysis (de Paiva et al., 1999). Upon stimulation, living motor nerve terminals bathed with the fluorescent dye FM1-43 accumulate this marker in synaptic vesicle membrane, while in BoNT/A injected preparation this uptake is clearly inhibited. This technique permitted to demonstrate that, during BoNT/A-induced paralysis and subsequent recovery of neuromuscular transmission, sprouts could establish functional synaptic contacts mediating appropriate exo-endocytosis in vivo. Indeed 28 days after BoNT/A injection, when twitch of intoxicate muscle can be elicited by direct electrical stimulation of the nerve, only the newly formed sprouts and not the original terminals exhibit activity-dependent uptake of FM1-43-labeled vesicles. At this time a clear reorganization of the postsynaptic nAChRs has taken place, mostly at the extremities of the sprouts. The sprouts go on growing for the next 2 weeks and only by 42 days there is a gradual, but marked, diminution in their activity-dependent staining by FM1-43. This recession of vesicle turnover in the outgrowths is concomitant with an enhanced FM1-43 staining in the original terminals that increases further over the next 4 weeks, while the previously extensive sprouts receded. Three months after BoNT/A injection the original endplate has regained morphology and pattern of depolarization-stimulated FM1-43 staining that are indistinguishable from those visualized before poisoning

The muscle atrophy induced by BoNTs in animal models and in humans is therefore largely reversible, even after repeated BoNTs injections, and this is important for therapeutic application as I will discuss later.

1.3.3 The central nervous system

Though BoNTs do not reach the CNS in significant amounts during botulism, there are evidence that they are able to intoxicate CNS neurons in vitro (Habermann and Dreyer, 1986).

To investigate the mechanism of action of BoNTs in the CNS, Verderio and coworkers intoxicated cultured embryonic hippocampal neurons and astrocytes with BoNT/B and BoNT/F and examined the route of entry and the proteolytic activity of

these neurotoxins (Verderio et al., 1999). Both BoNT/B and BoNT/F are able to enter hippocampal neurons and perform extensive cleavage of their targets, i.e. synaptobrevin/VAMP, in the large majority of nerve terminals. No difference in the cleavage of synatobrevin/VAMP could be detected when neurons are incubated with BoNT/B or /F upon depolarization, which suggests that BoNTs internalization is not mediated by a depolarization-dependent mechanism. In line with this, it has been shown that BoNTs enter hippocampal neurons via endocytic processes not mediated by SSVs recycling. Indeed incubation of fluorescent labelled BoNT/B or BoNT/F with fluorescent dextran, a widely used marker for fluid-phase endocytosis, indicate that the two dyes co-localize inside endocytic vesicular structures. Further experiments demonstrated that BoNT/B and BoNT/F are internalized also in astrocytes though the endocytotic route where they cleave the nonneuronal homologue of synaptobrevin/VAMP, i.e. cellubrevin.

Studies performed on rat cerebrocortical synaptosomes demonstrated that BoNT/A and BoNT/E are able to inhibit Ca^{2+}-dependent K^{+}-evoked release of several transmitters, including glutamate, acetylcholine, noradrenaline and dopamine (Ashton and Dolly, 1988; Foran et al., 1996). Interestingly intoxication of cerebral synaptosomes with BoNT/E is found to inhibit GABA release much less than glutamate release (40% decrease of GABA versus 90% decrease of glutamate). These data can be explained by the presence of BoNT/E and BoNT/A target, i.e. SNAP-25, only in the terminals of hippocampal glutamatergic terminals (Verderio et al., 2004). Indeed immunocytochemical studies performed on slices from adult rat hippocampus demonstrated that SNAP-25 is present in the glutamatergic terminals in stratum oriens and radiatum, while it is largely excluded from inhibitory terminals of type III neurons impinging on pyramidal cells in CA1 region.

At a electrophysiological level, treatment with BoNT/A or BoNT/E prevents the occurrence of spontaneous/miniature excitatory postsynaptic currents (mEPSCs) and greatly reduces the amplitude of evoked EPSCs in hippocampal slices (Capogna et al., 1997). The neurotoxins have no effect on post-synaptic glutamate sensitivity, as indicated by their lack of effect on the amplitude of inward currents elicited by application of the glutamate receptors agonist AMPA. Actually the main action of BoNT/A is pre-synaptic and consists of a strongly reduction of neurotransmitter release probability, as indicated by the small amplitude of unitary EPSCs between pairs of CA3 pyramidal cells and by decreased paired-pulse facilitation. Indeed, when two action

potentials are elicited in close succession in a single cell, the second action potential triggers the release of more vesicles than the first, as the release probability is transiently elevated because of the residual Ca^{2+} that remains in the nerve terminal after the first action potential (Katz and Miledi, 1968). The paired-pulse ratio (PPR) can be defined as the amplitude of the second EPSCs divided by the amplitude of the first EPSCs and it is therefore inversely dependent on release probability. In control hippocampal culture, the PPR is very low, consistent with an increase in release probability after the second action potential. In BoNT/A or BoNT/C treated cultures, the PPR is greater than in control cultures, indicating that the release probability is strongly affected by the treatment with BoNTs.

These bulk of data indicate therefore that BoNTs activity in the CNS is very similar to their activity at the neuromuscular junction. However further studies are needed to better characterize the specificity of BoNTs activity and their time-course.

1.3.4 Therapeutic uses

The demonstration that the inhibition of the nerve-impulse is followed by a functional recovery of NMJ provides a scientific basis for the rapidly growing use of BoNTs in the therapy of a variety of human diseases caused by hyperfunction of cholinergic terminals. Injection of BoNTs is currently recognized as the best available treatment for focal and segmental dystonias and for certain types of strabismus, and it is now being extended to several other human pathologies (Jankovic et al., 1994). Indeed injections of minute amounts of BoNTs into the muscle to be paralyzed led to a depression of the symptoms lasting few months. So far, BoNT/A is the most used serotype. However, to overcome the problem of immunization against BoNT/A, other BoNTs serotypes have been tested. BoNT/B, BoNT/F and BoNT/E demonstrated very effective in causing a strong paralysing effect (Eleopra et al., 1998). However their effect is short lasting and hence they are not a valid alternative to BoNT/A, while encouraging results have been obtained with BoNT/C (Eleopra et al., 1997). The short lasting effect of BoNT/E came as a surprise because that toxin cleaves the same substrates as BoNT/A. It is possible that the removal of a long segment from the carboxyl-terminal of SNAP-25 (25 residues by BoNT/E as compared to 9 residues removed by BoNT/A) leads to a different impairment of tSNARE functions, leading to a more rapid removal of the fragment and a consequent more rapid remodelling of the end plate.

The use of BoNTs have now extended also to disorders that have not a neuromuscular basis such as the axillary hyperhidrosis (Heckmann et al., 2001), the myofascial pain (Porta et al., 2000) and tension and migraine headache (Silberstein et al., 2000).

1.4 AIM OF THE THESIS

Substantial evidence indicates that the excessive release of glutamate during seizures is the primary cause of seizure-induced brain damage (Meldrum, 1995; Holmes, 2002). Thus drugs that are specifically targeted to block glutamate release in temporal lobe structures might arrest seizures and the neuronal death in TLE. As the most effective blockers of glutamate release known are botulinum toxins (BoNTs), I decided to investigate whether one of them, i.e. BoNT/E, can block limbic seizures and the ensuing anatomical rearrangements and memory deficits in rodent models of TLE.
In this PhD thesis, I will therefore pursue four major aims:

1) characterizing BoNT/E activity in the CNS *in vivo*. Indeed BoNTs are able to block glutamate release through the cleavage of proteins that are essential components of the neurotransmitter release machinery (Schiavo et al., 2000). However their action is well-characterized only in (PNS) and there are few data reporting their activity in the CNS. As the prolonged blockade of neuronal activity in the CNS can have detrimental effects, I decided to use first BoNT/E, that shows a shorter neuroparalytic duration with respect to other serotypes (Foran et al, 2003). In the first part of my PhD thesis I therefore present the data reporting BoNT/E activity after its injection into rat hippocampus. In particular, I analyzed the effectiveness of BoNT/E proteolytic activity in the hippocampus, the time-course of its action and BoNT/E effects on glutamate release and hippocampal spiking activity.

2) investigating the anti-epileptic effect of BoNT/E in animal models of TLE. In this set of experiments, I used two well-characterized models of TLE in order to prove the anti-epileptic activity of BoNT/E. The first model is represented by the intrahippocampal administration of the glutamate analog kainic acid (KA). Indeed focal application of KA to rat hippocampus induces acute seizures that can be detected through electroencephalographic (EEG) recordings (Vezzani et al., 1999, 2002). The second model utilized is the intraperitoneal (i.p.) injection of KA. Actually, rats receiving parental KA undergo status epilepticus of limbic origin and the severity of seizures can be scored according to a defined rating scale (Lothman and Collins, 1981; Ben-Ari, 1985). In both models I analyze the prophylactic effect of BoNT/E injection into hippocampus on epileptic seizures induced by the subsequent administration of KA.

3) investigating the long-term effects of BoNT/E administration on cognitive functions and on neuronal survival in the i.p. KA model of TLE. Indeed animals treated with KA i.p. develop, some weeks after status epilepticus, cognitive deficits and massive neuronal loss in many limbic structures such as the hippocampus, the lateral septum and the piriform cortex (Ben-Ari and Cossart, 2000). To examine whether BoNT/E injection into hippocampus is able to prevent cognitive deficits and neuronal loss induced by seizures, I test the performance of rats, previously injected with BoNT/E, in the Morris water maze (MWM), a classical hippocampal-dependent memory task, five weeks after KA i.p. administration. Moreover I analyze the anatomy of limbic structures, particularly the hippocampus, in order to evaluate the neuroprotective effects of BoNT/E.

4) investigating the anti-epileptogenic effect of BoNT/E in the kindling model. TLE is a progressive disease that often results from an early insult able to induce rearrangements in hippocampal circuitry that lead, after a latent period, to chronic epilepsy (Pitkanen and Sutula, 2002). This process, called epileptogenesis, is strongly activity-dependent. I therefore assess whether the long-term blockade of neuronal activity through BoNT/E injection is able to prevent or slow down the epileptogenic process in the model of rapid electrical kindling of the ventral hippocampus. I used this model of rapid kindling in order to avoid repeated injection of BoNT/E in the hippocampus. Indeed, in classical kindling, animals need to be electrically stimulated for a period that may exceed the time-window of BoNT/E effect (Rogawski et al., 2001).

MATHERIALS AND METHODS

2.1 ANIMAL TREATMENT

Long-Evans hooded rats were used in this study. Animals were housed in a 12 hr light/dark cycle with food and water available *ad libitum*. All experimental procedures were in conformity to the European Communities Council Directive n° 86/609/EEC.

2.1.1 BoNT/E INJECTIONS

BoNT/E was obtained by WAKO (Japan), trypsin activated, purified and tested as previously described (Schiavo and Montecucco, 1995). Its potency was evaluated with the mice phrenic nerve-hemidiaphragm test. Two unilateral stereotaxic infusions of 1.5 μl of BoNT/E (50 nM) or vehicle (2% rat serum albumin in PBS) were made into the dorsal hippocampus under avertin anaesthesia (tri-bromo-ethanol) at postnatal day (P) 35. P35 rats were chosen since they display a maximal sensitivity to KA-induced seizures (Ben-Ari, 1985; Stafstrom et al., 1993). Coordinates in mm from bregma were (nose bar –2.5): for CA1, AP -2.4, L -1.8, H 2.1 below dura; for CA3, AP -2.4, L -3.3, H 3 below dura.

2.2 HISTOLOGY

2.2.1 DETECTION OF CLEAVED SNAP-25

Rats received hippocampal injections of BoNT/E (n = 10) or vehicle (n = 5) and their brains were dissected 1-4 days later, after transcardial perfusion with 4% paraformaldehyde (Caleo et al., 2003). Coronal sections (40 μm thick, cut on a freezing microtome) were blocked with 10% normal goat serum in PBS and then incubated overnight with the antibody recognizing the BoNT/E-cleaved form of SNAP-25 diluted 1.300 in a PBS solution containing 1% serum and 0.3% Triton X-100. On the following day, sections were reacted with a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) followed by avidin-biotin-peroxidase complex (ABC kit, Vector Laboratories) and diaminobenzidine (DAB) reaction. The antibody recognizing BoNT/E-cleaved form of SNAP-25 is a peptide-affinity purified polyclonal antibody

specifically raised against the BoNT/E truncated C-terminal peptide of SNAP-25 (CDMGNEIDTQNRQIDR). This antibody recognizes specifically cleaved SNAP-25 but not the whole protein.

2.2.2 *In situ* HYBRIDIZATION

Coronal cryostat sections (20 μm thick) were collected on slides in serial order through the entire hippocampus and fixed in 4% paraformaldehyde. Non-radioactive *in situ* hybridization was performed according to standard protocols (http://www.roche-applied-science.com) using a digoxigenin labeled c-*fos* riboprobe (Bozzi et al., 2000). Signal was detected by alkaline phosphatase-conjugated anti-digoxigenin antibody followed by alkaline phosphatase staining. To quantify the level of c-*fos* mRNA, digital images of three matching sections per animal (n = 5 rats per group) were analysed using the MCID-M4 software (Imaging Research, St. Catharines, Ontario, Canada). For the hippocampus, the profile of CA1, CA3 and DG was outlined in each section, and signal intensity within each sector was measured. Background level was calculated in the corpus callosum of each section and subtracted from signal intensity values. The obtained values were averaged and used to calculate the average value per animal. These values (n = 5 per group) were used to calculate the mean (± S.E.) for each group. Statistical analysis was performed by Student's t-test.

2.2.3 EVALUATION OF NEURONAL LOSS

Hippocampal neuronal loss was evaluated in control and BoNT/E-infused rats treated with KA, at the end of Morris water maze experiments (P77). Rats were perfused with 4% paraformaldehyde and coronal sections through the dorsal hippocampus were processed in serial order for immunohistochemistry with mouse anti-NeuN monoclonal antibody (1:500 dilution; Chemicon, Temecula, CA). Neuronal damage was scored in areas CA1 and CA3 of the hippocampus according to the following scale (Bozzi et al., 2000; Cilio et al., 2001): 0, no damage; 1, minimal damage (small spots of degeneration); 2, evident loss of pyramidal neurons; 3, complete disruption of hippocampal architecture. An average number of 10 sections per animal were analysed by an investigator unaware of the treatment. A separate score was initially assigned to CA1 and CA3 regions of both sides of each section, and these values were used to

calculate the mean damage score for each section. These values were averaged and the obtained damage scores for each animal were plotted

2.3 IMMUNOBLOTTING

Rats (n = 13) received hippocampal injections of BoNT/E at P35 and dorsal and ventral hippocampi (ipsilateral and contralateral to the injected side) were dissected after 1, 14, 21 and 35 days. Proteins were extracted (Viegi et al., 2002) with lysis buffer (1% Triton X-100, 10% glycerol, 20 mM Tris-HCl, pH7.5, 150 mM NaCl, 10 mM EDTA, 0.1 mM Na_3V0_4, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1mM PMSF). Protein extracts (10 mg) were separated by electrophoresis and blotted, and filters were incubated with the antibody recognizing the BoNT/E-cleaved form of SNAP-25 (1:50 diluition) or with anti-SNAP-25 (1:1,000 dilution; Synaptic Systems, Germany) polyclonal antibody, reacted with HRP-conjugated goat anti-rabbit secondary antibody (Bio-Rad) and developed by ECL (Amersham, UK). Filters were stripped and re-probed with anti-β-tubulin monoclonal antibody (1:500 dilution; Sigma, St Louis, MO), which served as an internal standard for protein quantification.

2.4 SUPERFUSED SYNAPTOSOMES

Glutamate release measurements were performed on superfused hippocampal synaptosomes from normal rats (n = 4) and from vehicle- and BoNT/E-treated rats (n = 7 per group), one day after intrahippocampal KA. Preparation of synaptosomes was as described by Gobbi et al. (2002). Rats were killed by decapitation and their hippocampi were rapidly dissected out and homogenized in 40 volumes of ice-chilled 0.32 M sucrose, pH7.4, in a glass homogenizer with a Teflon pestle. The homogenates were centrifuged at 1000 *g* for 5 min and the supernatants centrifuged again at 12000 *g* for 20min to yield the crude synaptosomal pellets (P2). The P2 pellets were resuspended in about 20 volumes of Krebs-Henseleit buffer with the following composition (mM): NaCl (125); KCl (3); $CaCl_2$ (1.2); $MgSO_4$ (1.2); NaH_2PO_4 (1); $NaHCO_3$ (22); glucose (10), gassed with 95% O_2 and 5% CO_2, pH7.2 - 7.4. 5-mL samples (5 mg of initial tissue) were distributed onto cellulose mixed ester filters (0.65 μm pore size, Millipore Corporation, Millipore, Milano, Italy) in a 16-chamber superfuion apparatus held thermostatically at 37°C (Raiteri et al., 1974). The synaptosomes were layered onto

filters by aspiration from the bottom under moderate vacuum. Superfusion was started (t=0 min) at a rate of 0.5 mL/min with standard medium and, after a 44-min equilibration period, 4-min fractions were collected from each chamber. The first one, collected from t=44 to t=48 min), was used to determine the basal release. The depolarising impulse (5 mM KCl) was added at t=46.5 and its effect was measured in the second fraction (from t=48 to t=52 min; the fluid takes about 1.5 min to flow from the filters to the collecting vials). The third fraction (from t=52 to t=56) corresponds to the basal release after stimulation. The same synaptosomes were after perfused without Ca^{2+} in order to evaluate the Ca^{2+}-dependent fraction of K^{+}-evoked release. Glutamate overflow was measured by a Waters Alliance HPLC analysis system. The analytical method involved automatic precolumn derivatization with o-phthalaldehyde followed by separation on C18 reverse phase chromatography column and fluorimetric detection (Di Stasi et al., 2002).

2.5 ELECTROPHYSIOLOGY

2.5.1 RECORDINGS OF SPIKE ACTIVITY

Rats received unilateral injections of BoNT/E (n = 6) or vehicle (n = 3). Extracellular multi-unit recordings of spike activity were performed in the injected hippocampus 1-2 days after BoNT/E or vehicle injection as described in Caleo et al. (2003). Animals were anesthetized with urethane (Sigma; 20% solution in saline; 0.7 ml/100 g body weight, i.p.) and placed in a stereotaxic frame. Body temperature was continuously monitored and maintained at 37°C by a thermostat-controlled electric blanket. After exposure of the cerebral surface, a micropipette (tip resistance = 2 MΩ) filled with 3M NaCl was inserted into the brain to reach the dorsal hippocampus. Two to five penetrations per hemisphere were made to map spike activity in CA1 and CA3 sectors. Location of the recording sites was determined using histological controls (Caleo et al., 2003). Signals were amplified 25,000 fold, band-pass filtered (500 – 5,000 Hz) and conveyed to a computer for storage and analysis.

2.5.2 EEG ANALYSIS

P35 rats were unilaterally infused into the right dorsal hippocampus with BoNT/E (n = 8) or vehicle (n = 8) as described above under avertin anaesthesia. At the end of the infusion procedure, one screw electrode was placed over the parietal cortex ipsilateral to the injected hippocampus together with a ground lead over the nasal sinus. Two depth bipolar electrodes made of insulated nichrome wire (60 μm) were implanted bilaterally into the dorsal hippocampus (nose bar -2.5; mm from bregma, AP, -2.4; L±1.8; H 3.0 below dura) and a guide cannula was glued to the right side depth electrode and positioned on top of dura for the intrahippocampal injection of KA. Surface and depth electrodes were connected to a multipin socket and secured to the skull together with the injection cannula by acrylic dental cement. Two days after surgery, freely-moving rats injected with BoNT/E or its vehicle received a unilateral injection of 40 ng KA into the right hippocampus using a needle protruding of 3 mm below the guide cannula (Vezzani et al., 1999). To compare the effect of BoNT/E with that of conventional antiepileptic drugs, three additional groups of rats (not previously treated with BoNT/E) received carbamazepine (CBZ; 10 mg/kg, i.p.; n = 6 rats) or phenytoin (PHT; 50 mg/kg, i.p.; n = 7 rats) dissolved in propylene glycole, or vehicle (n=13), 60 min before focal delivery of 40 ng KA to the hippocampus. EEG recordings on freely-moving animals were performed using a four-channel EEG polygraph, by an investigator who was unaware of the treatment of the animals. An initial 15-30 min recording was made to establish basal activity, then EEG recordings were made continuously up to 4 hours after KA administration. The KA dose (40 ng) was previously shown to induce EEG seizures recurring for about 180 min in 100% of the rats without mortality (Vezzani et al., 1999). Our EEG analysis was based on visual inspection of tracings to detect and quantify ictal activity. This analysis does not allow to detect differences in basal EEG patterns. EEG seizures (ictal episodes) were defined by the occurrence of discrete episodes consisting of the simultaneous occurrence of at least two of the following alterations in cortical and hippocampal leads of recording: high frequency and/or multispike complexes and/or high-voltage synchronized spike or wave activity. The quantitative parameters chosen to quantify seizure activity were the latency to the first EEG seizure (onset), the total number of seizures occurring during the whole period of recording, and the total time spent in seizures which was reckoned by adding together the duration of all ictal episodes (Vezzani et al., 1999). Behavioral correlates of EEG seizures were the

following: during EEG seizures the rats had the typical “frozen” appearance and apparently lost their reaction to external stimuli. “Wet dog shakes” were often observed at the end of seizure episodes. These behavioural sequelae were not quantified or considered in this study. Statistical analysis was performed by two-way ANOVA followed by post-hoc Tukey test.

2.6 BEHAVIOURAL TESTS

2.6.1 SEIZURES RATING SCALE

Behavioral, but not EEG analysis of seizures, was carried out in rats systemically injected with KA. We used P36 rats that are fully responsive to KA (Tremblay et al., 1984; Berger et al., 1984; Nitecka et al., 1984).

Thirty rats received hippocampal injections of BoNT/E at P35. Control animals of the same age (n = 39) were injected with vehicle. One day after injections, animals received a convulsive dose (8 mg/kg, i.p) of KA (Ocean Produce International, Shelburne, NS, Canada). Naïve animals which only received KA at P36 (n = 20) were also used to control for possible effects of hippocampal injections. Rats were observed by an investigator unaware of the treatment. For each animal, behaviour was scored every five minutes for a period of 4 hours, according to a previously defined seizure rating scale (Schauwecker and Steward, 1997, Bozzi et al., 2000): stage 0: normal behaviour; stage 1: immobility; stage 2: stereotypies; stage 3: wet dog shakes, head bobbing; stage 4: rearing and falling; stage 5: continuous rearing and falling (status epilepticus); stage 6: death. Animals that reached stage 6 were excluded from the computation of the mean seizure score in each experimental group. Statistical analysis was performed by two-way ANOVA followed by post-hoc Tukey test.

2.6.2 MORRIS WATER MAZE

To evaluate the cognitive performance of rats we tested the animals in the hidden paltform version of the Morris Water Maze. Rats were given five trials a day for a period of four consecutive days. The time to reach the platform (i.e. the escape latency) was measured for each trial. First of all, we analyzed spatial learning during the time window of action of BoNT/E. Eight animals that received BoNT/E at P35 were tested in

the Morris water maze beginning from 3 to 7 days after treatment. These animals were compared to vehicle-injected animals of the same age (n = 8). A second group of rats (n = 5) injected with BoNT/E at P35 were allowed to recover for five weeks before the spatial learning test and their performance was compared to that of age-matched normal rats (n = 10) which did not receive any treatment.

We also evaluated the cognitive performance of BoNT/E-injected and control rats treated with KA. Behavioural tests were begun on P70 (five weeks after treatment) in both BoNT/E treated (n = 13) and control (n = 26; n = 19 vehicle-injected and n = 7 naïve) rats that received KA at P36.

Experiments were performed according to Mikati et al. (2001) and Cilio et al. (2001). Briefly, a circular tank (200 cm diameter) was filled with opaque water (22±1°C), and a wooden platform (10×10 cm) was positioned in the centre of one quadrant of the pool 2.5 cm below the water surface. On day 1 of testing, rats were placed in the pool for 60 s without the platform present, to become habituated to the training environment. Rats were trained for four days (five trials a day) to locate and escape onto the submerged platform. The latency from immersion into the pool to escape onto the platform was recorded for each trial. On mounting the platform, rats were given a 30 s rest period. Rats which did not find the platform in 120 s were placed on the platform for 30 s. Rats experiencing a spontaneous seizure during testing were allowed to recover for 60 min before resumption of test. All experiments were conducted in a blinded fashion. Statistical analysis was performed by two-way ANOVA followed by post-hoc Tukey test.

2.7 RAPID KINDLING

A total number of 11 rats at P35 were unilaterally infused in the right ventral hippocampus with BoNT/E (n = 5) or vehicle (n = 6) as described above, under avertin anaesthesia (nose bar, -2.5; mm from bregma: CA1, AP -4.7; L -5.5; H 6.0 below dura; CA3, AP -4.7; L -5.0; H 5.0 below dura). After the end of the infusion procedure, twisted bipolar stimulating-recording electrodes were stereotaxically implanted bilaterally into the rat ventral hippocampal CA3 region (nose bar: -2.5; mm from bregma: AP -4.7; L ± 5.0, H 5.0 below dura), and bilateral screw electrodes were placed over the parietal cortex. The electrodes were connected to a multipin socket and secured to the skull with acrylic dental cement. Kindling was started after a postoperative period

of 2 days. Experiments were performed by an investigator unaware of the treatment. The rats were allowed to acclimatize in a plexiglas cage and an EEG recording was made for at least 15 min to assess basal activity. Rapid kindling was induced by delivering constant current stimuli (50 Hz, 10 sec trains of 400 μA, 1 msec bipolar square waves) unilaterally to the right ventral hippocampus through a bipolar electrode with a 5-min interval for 200 min (Kopp et al., 1999; Richichi et al., 2004), thus summing up to a total of 40 stimuli. Behavior was observed and scored according to a modified Racine's classification (Racine, 1972 a, b, c). Stage 2 was defined by unilateral forelimb retraction and mouth stereotypies, stage 3 by head nodding and dorsal muscle twiching and stage 4-5 consisted of generalized clonic seizures without (stage 4) or with (stage 5) postural loss. The duration of the primary and secondary afterdischarge was measured in the stimulated hippocampus after each stimulation in every animal. Twenty four hours after the end of the stimulation period, fully kindled rats received 5 further electrical stimulations (re-test day) as above, to confirm kindling acquisition and maintenance. At the end of the stimulation protocol, kindled rats were sacrificed and the location of the stimulating electrode and the injection cannula was histological verified in each animal.

RESULTS

3.1 Characterization of BoNT/E action in the hippocampus

The activity of all BoNTs in the CNS have been poorly investigated. In order to use BoNT/E as an anti-epileptic agents, it is first of all necessary to characterize the effects of BoNT/E injections in the hippocampus. In particular it is necessary to establish:

- the effectiveness of BoNT/E proteolytic activity in the hippocampus and its duration;
- the effects of BoNT/E on neurotransmitter release;
- the effects of BoNT/E on hippocampal spiking activity;

3.1.1 Cleavage of SNAP-25 by BoNT/E in the hippocampus: efficacy and time-course.

All the animals were injected with a solution of 50 nM BoNT/E in a volume of 1.5 µl. This BoNT/E dose was chosen on the basis of a preliminary survey that indicated that this was the highest dose of BoNT/E not causing mortality or systemic intoxication. At this dose, BoNT/E cleaves specifically SNAP-25 and not SNAP-23 (Sadoul et al, 1997).

In order to investigate the effective cleavage of SNAP-25 by BoNT/E in the hippocampus, I performed Western blot analysis on protein extracts from rat hippocampi of P35 rats that received a unilateral injection of BoNT/E or vehicle into the hippocampus. Western blot for cleaved SNAP-25, performed 1 day after BoNT/E injection, demonstrated that SNAP-25 is efficiently proteolysed *in vivo* by BoNT/E (Fig. 3A). This effect was mainly restricted to the injected side and cleaved SNAP-25 was almost undetectable in the contralateral hippocampus (Fig. 2A). Immunostaining for cleaved SNAP-25 confirmed the regional specificity of the BoNT/E effect, with very limited spread to the contralateral hippocampus (Fig. 2B) or to other cortical areas. The anteroposterior spread of staining around the injection site was of about 3 mm, which means that a single injection of BoNT/E in one hippocampus is enough to get the cleavage of SNAP-25 in the whole hippocampus.

Another important point to determine is the duration of the BoNT/E effects. In the PNS, the recovery of function at the neuromuscular junction after BoNT/E intoxication

takes three weeks. To establish the duration of BoNT/E effect, I decided to analyze the time course of SNAP-25 cleavage after a single BoNT/E injection into the hippocampus. Cleaved SNAP-25 was detected in both the dorsal and ventral hippocampus 1 day after BoNT/E injection. The band was slightly reduced at 14 days, persisted up to 21 days and was no longer detectable at 35 days (Fig. 2C). In keeping with the expression profile of cleaved SNAP-25, intact SNAP-25 was completely absent 1 day after BoNT/E, began to reappear at 14 days and was completely replenished by 35 days (Fig. 2D). Therefore BoNT/E activity persists in the hippocampus for at least three weeks before being reversed.

These data are the first demonstration that BoNT/E effectively cleaves SNAP-25 in the hippocampus *in vivo* and that its effect can persist for at least 3 weeks.

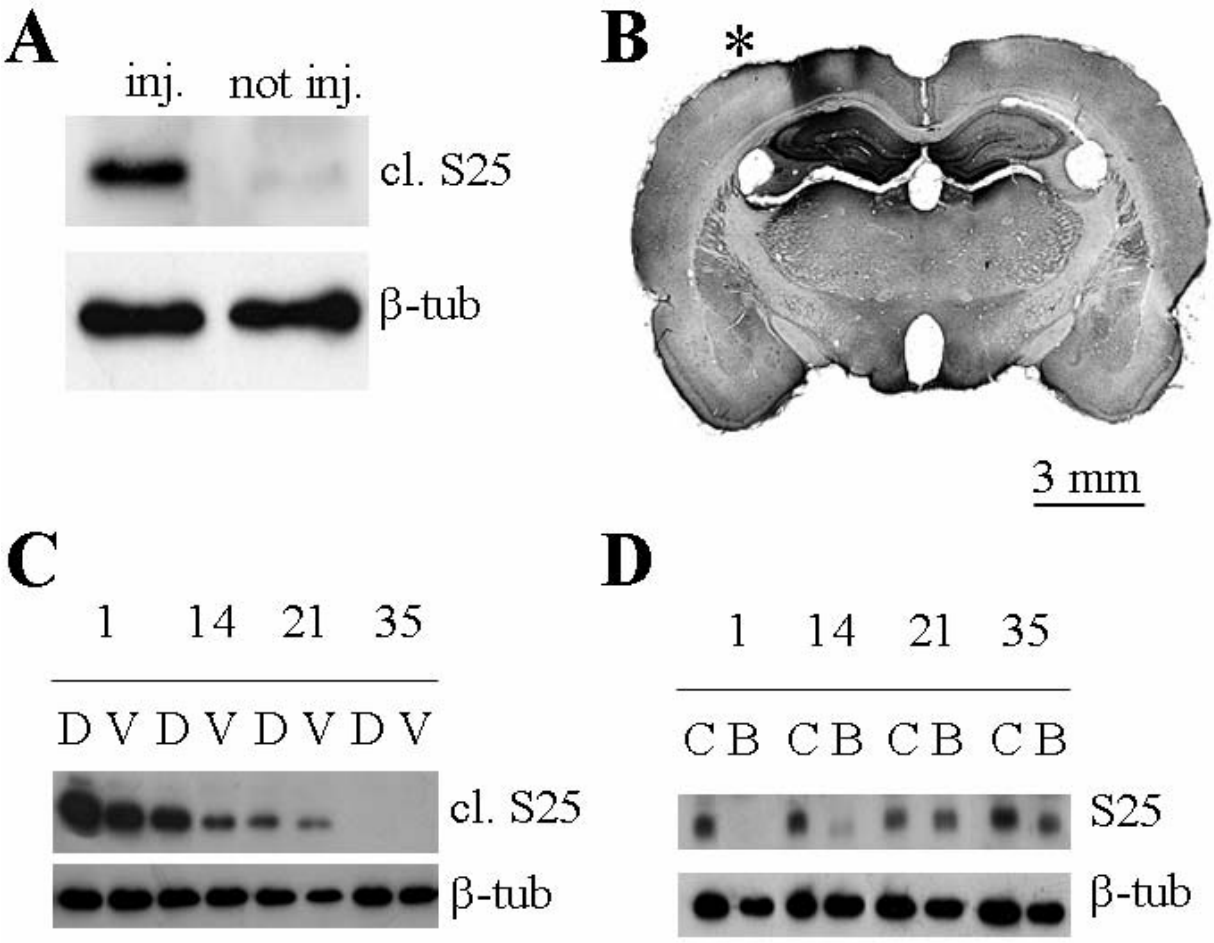

FIGURA 2

Effectiveness and time-course of the proteolytic activity of BoNT/E in the rat hippocampus

(A) Representative immunoblotting for cleaved SNAP-25 (cl. S25) on protein extracts from BoNT/E-injected (inj) and controlateral, not injected (not inj.) hippocampus of a P36 rat, one day after BoNT/E injection. β-tub (β-tubulin) is the internal standard. (B) Forebrain coronal section showing the hippocampus of a P36 rat, one day after unilateral BoNT/E injection. Immunostaining for cleaved SNAP-25 (dark labeling) is mainly restricted to the injected hippocampus, indicated by an asterisk. (C) Immunoblotting for cleaved SNAP-25 (cl. S25) on protein extracts from hippocampi of BoNT/E-treated rats at different times after BoNT/E injection. D, dorsal; V, ventral hippocampus. (D) Immunoblotting for the intact SNAP-25 (S25) on protein extracts from hippocampi of BoNT/E-treated (B) and age-matched control (C) rats at different times after BoNT/E injection.

3.1.2 Effect of BoNT/E on neurotransmitter release in the hippocampus: the superfused synaptosomes.

The superfused synaptosomes are the choice technique to study the mechanisms of neurotransmitter release and how these can be impacted by different drugs (Raiteri, 2000).

The simplest apparatus for studying the release of neurotransmitter from superfused synaptosomes consists of four identical superfusion chambers having at the bottom filter holders of porous glass. The synaposomes fraction is plated as a very thin layer on microporous filters and up-down superfused. Thereafter four collecting tubes gather the fluid from each chamber. The apparatus for superfusion of synaptosomes was originally devised to distinguish between drugs able to enhance release directly and drugs that can do it indirectly, i.e. preventing transmitter re-uptake. Indeed both types of drugs augment the synaptic concentration of the transmitter, but their pharmacological and therapeutic effects are not the same. For instance pure inhibitors of serotonin re-uptake are excellent antidepressants, whereas direct serotonin releasers, such as fenfluramine or *ecstasy*, display completely different biological activities. To solve this problem, true releasers can be distinguished from re-uptake inhibitors by superfusing up-down a very thin layer of synaptosomes and monitoring transmitter release in the presence of the drug under study. The transmitter released is indeed removed by superfusion medium quickly enough to escape re-uptake and therefore only true releasers, and not pure re-uptake inhibitors, can increase neurotransmitter release.

To assess the effects of BoNT/E on neurotransmitter release, I performed experiments on superfused hippocampal synaptosomes and I established that BoNT/E effectively inhibits the Ca^{2+}-dependent fraction of potassium-induced glutamate release (Fig 3).

Glutamate released from synatosomes can be determined in basal condition or after a depolarizing impulse, such as KCl 5mM, both in the presence and in the absence of Ca^{2+} (Fig 3A). The release of glutamate from hippocampal synaptosomes is not influenced by Ca^{2+} in basal condition but it is strongly dependent by Ca^{2+} in the presence of a depolarising impulse such as an high concentration of K^+ (Fig 3A). Indeed the release of glutamate evoked by high potassium concentration is increased by almost two-fold in the presence of Ca^{2+} and this difference represent the fraction of potassium-induced glutamate release that is Ca^{2+}-dependent (Fig 3A).

The effects of BoNT/E on glutamate overflow were assessed on superfused synaptosomes obtained from rats that were injected with BoNT/E (or vehicle) in the hippocampus and that received a single dose of intrahippocampal kainic acid (40 ng) two days later. Therefore the hippocampal synaptosomes were prepared on the third day after BoNT/E. The administration of BoNT/E does not influence the glutamate release in basal condition (data not shown) but it strongly reduces the Ca^{2+}-dependent fraction of potassium-induced glutamate release (n = 7 animals per group; t-test, $p< 0.01$; Fig. 3B). This result can be well explained by the mechanism of action of BoNT/E. Indeed BoNT/E cleaves SNAP-25 that is essential for the release of neurotransmitter from synaptic vesicles. The vesicular release of neurotransmitter is regulated by Ca^{2+} and therefore the cleavage of SNAP-25 by BoNT/E strongly affect the calcium-dependent neurotransmitter release.

Preliminary data suggest that BoNT/E does not affect GABA release from hippocampal synaptosomes. Indeed the Ca^{2+}-dependent fraction of potassium-induced GABA release is not significantly different in vehicle and BoNT/E-injected animals (n = 4 animals per group; t-test $p> 0.05$; Fig 3C). This is in line with evidence that suggest that the target of BoNT/E proteolytic activity, i.e. SNAP-5, is absent in GABAegic neurons where it is replaced by its isoform SNAP-23 (Verderio et al., 2004). However these data require further validation.

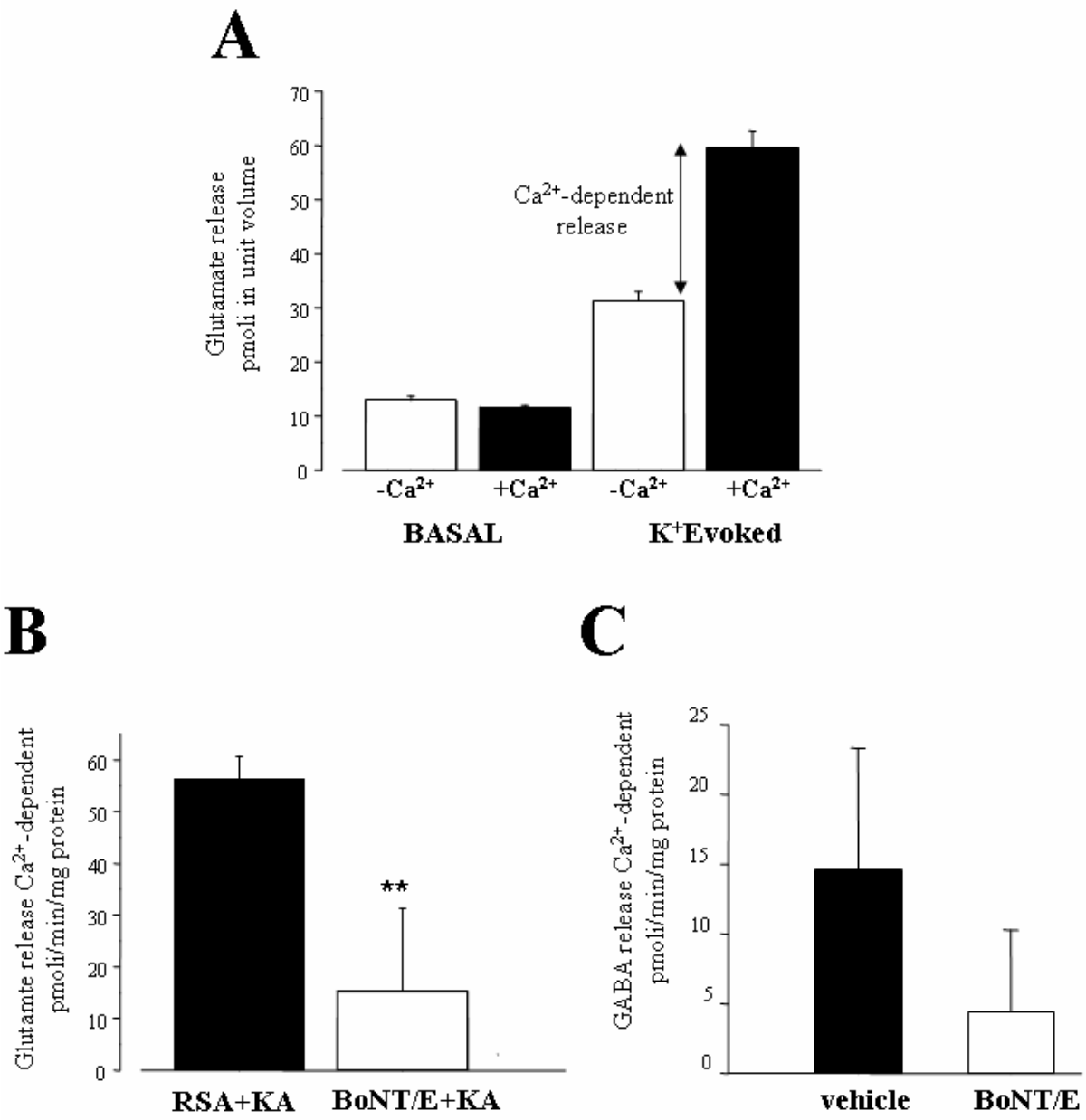

FIGURE 3

Effect of BoNT/E on neurotrasmitter release from hippocampal superfused synaptosomes

(A) Glutamate release from hippocampal synaptosomes, in basal condition or evoked by 5mM KCl, both in the presence and in absence of Ca^{2+}. (B) Effects of BoNT/E on Ca2+-dependent K+-evoked glutamate overflow from hippocampal synaptosomes. Release of glutamate is inhibited by about 80%. Data are expressed as mean ± S.E. **, t-test, $p < 0.01$. (C) Effects of BoNT/E on Ca2+-dependent K+-evoked GABA overflow from hippocampal synaptosomes. Release of GABA is not significantly inhibited.

3.1.3 Effect of BoNT/E on hippocampal spiking activity.

To characterize the effects of BoNT/E in the CNS at the electrophysiological level, I decided to perform *in vivo* multi-unit recordings of spike activity from hippocampal pyramidal neurons in normal rats and in rats that had received a single hippocampal injection of BoNT/E or vehicle 1-2 days before.

In naïve rats the recording of spontaneous hippocampal activity reveals the presence of action potentials of high amplitude both in the CA1 and in the CA3 sectors of hippocampal formation (Fig 4A). This spike activity is not affected by infusion of vehicle solution (Fig 4B) which indicates that, at the electrophysiological level, the functioning of the hippocampus is not impaired by the injection. Instead BoNT/E treatment is able to completely silence spontaneous hippocampal activity, potently inhibiting the high amplitude spikes (Fig. 4C). This strong inhibition of hippocampal spontaneous action potentials by BoNT/E can be easily explained by its capacity to reduce the release of glutamate, as demonstrated in the superfused synatosomes. Moreover the inhibition of spontaneous action potentials is specific to the BoNT/E-treated hippocampus and no effects can be found in the contralateral, uninjected side (Fig 4D). This confirms, from a electrophysiological point of view, the Western blots and immunohistochemical data that show that there is very few diffusion of BoNT/E to the contralateral hippocampus.

These data strongly indicate that BoNT/E is able to impair the excitatory transmission in the hippocampus and that the net electrophysiological effect of BoNT/E treatment is the silencing of spike activity of pyramidal neurons.

I did not perform electrophysiology at the neuromuscular junction in BoNT/E-injected animals to check the diffusion of the toxin through the blood-brain barrier into the body. However none of the animals ever showed any sign of systemic intoxication (i.e., muscular paralysis) after BoNT/E treatment.

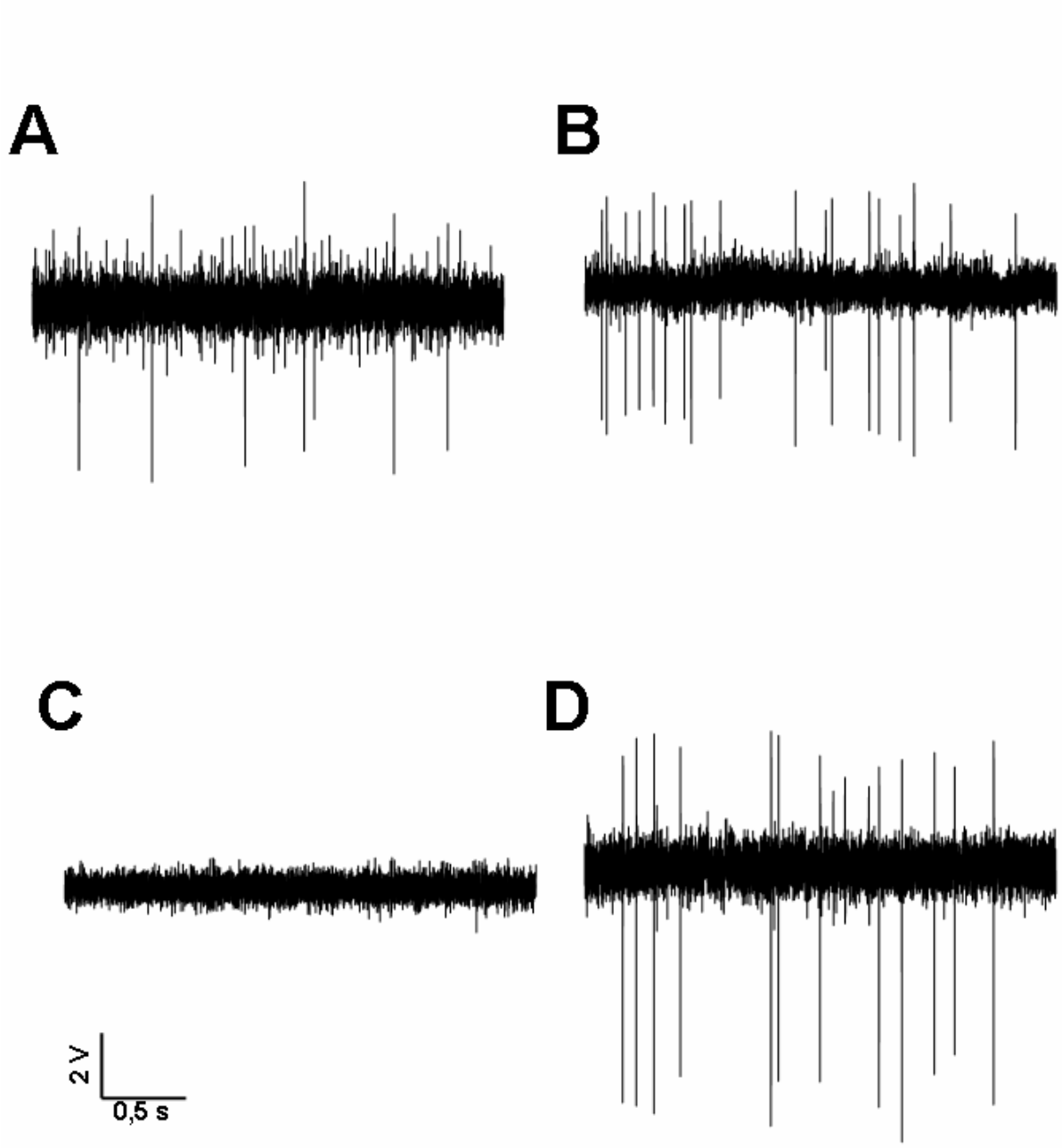

FIGURE 4

Effects of BoNT/E on hippocampal spiking activity

Representative recordings of spontaneous action potentials from CA1 region in normal (A) vehicle-injected (B) BoNT/E-injected (C) and controlateral not injected hippocampus (D) *in vivo*. The high amplitude action potentials characteristic of CA1 are completely suppressed by BoNT/E injection.

3.2 Anti-ictal properties of BoNT/E on EEG seizures induced by intrahippocampal KA

The reduction of glutamate release and the strong inhibition of hippocampal spike activity mediated by BoNT/E prompted me to investigate whether the toxin can display anti-ictal properties.

I started examining the effects of BoNT/E injection on acute electroencephalographic (EEG) seizures triggered by focal unilateral application of 40 ng KA to the hippocampus (Vezzani et al., 1999, 2002). Previous experiments demonstrated that this is the lowest convulsant dose able to cause reproducible EEG seizures in 100% of rats. The EEG seizures induced by intrahippocampal KA are characterized by discrete episodes of epileptic activity lasting 2.5 min on average and consisting of the simultaneous occurrence of at least two of the following alterations in cortical and hippocampal leads of recording: high frequency and/or multispike complexes and/or high-voltage synchronized spike or wave activity. Such events are typically associated with behavioural manifestations such as "wet dog shakes" and occasional retraction of a forelimb. These discrete episodes of epileptic activity last about 180 min from their onset and occur simultaneously in all leads of recordings.

Rats were injected with BoNT/E (n = 8) or vehicle (n = 8) into the hippocampus and the same day they were implanted with two intrahippocampal electrodes one of which was glued to an infusion guide cannula (Fig 5 A,B). Two days later, seizures were induced by delivery of 40 ng KA and the EEG were recorded through all the electrodes for a period of four hours. The EEG pattern of epileptic activity induced by intrahippocampal KA was not altered by vehicle injection, as shown in a representative EEG recording in Fig 5C. Instead in BoNT/E-treated rats the epileptic activity was strongly inhibited, as shown in Fig 5D. Indeed in BoNT/E-treated rats discrete episodes of epileptic activity could be recorded mainly in the left, KA-injected hippocampus but not in the right hippocampus and, importantly, never in the cortex, which means that there is no generalization of epileptic activity.

Quantification of seizure activity demonstrated that the onset time to seizures was delayed by four-fold in BoNT/E-treated animals (t-test, $p < 0.001$; Fig. 6C). BoNT/E-injected rats showed a highly significant reduction of the number of EEG seizures (t-test, $p = 0.005$; Fig. 6D), as well as a 80% decrease in total time spent in seizure activity

(t-test, $p < 0.001$; Fig. 6E). The duration of individual ictal episodes was also reduced by the toxin (min ± S.E., vehicle, 2. 4 ± 0.5; BoNT/E, 1.06 ± 0.1, t-test, $p<0.01$) .

In order to validate BoNT/E as an anti-epileptic treatment, I compared the anticonvulsant effect of BoNT/E with that of standard anticonvulsant drugs. Carbamazepine (CBZ; 10 mg/kg, i.p.; n = 6 rats) or phenytoin (PHT; 50 mg/kg, i.p.; n = 7 rats) administered 60 min before intrahippocampal KA induced an average two-fold delay in the onset of EEG seizures (t-test, $p < 0.01$) and a 2.5-fold reduction in the time spent in seizure activity ($p < 0.01$). The number of EEG seizures was also reduced by almost two-fold with respect to controls (Fig. 7A-C). Hence BoNT/E is significantly more effective in reducing KA-induced EEG seizures than these classical anticonvulsant drugs (Fig. 7A-C; one way ANOVA, $p < 0.01$, post hoc Tukey test, $p < 0.01$ for onset and time spent in seizures; one way ANOVA, $p < 0.05$, post hoc Tukey test, $p < 0.05$ for number of seizures).

These data suggest that BoNT/E can be a valuable treatment for blocking the spread of epileptic activity that occur during seizures.

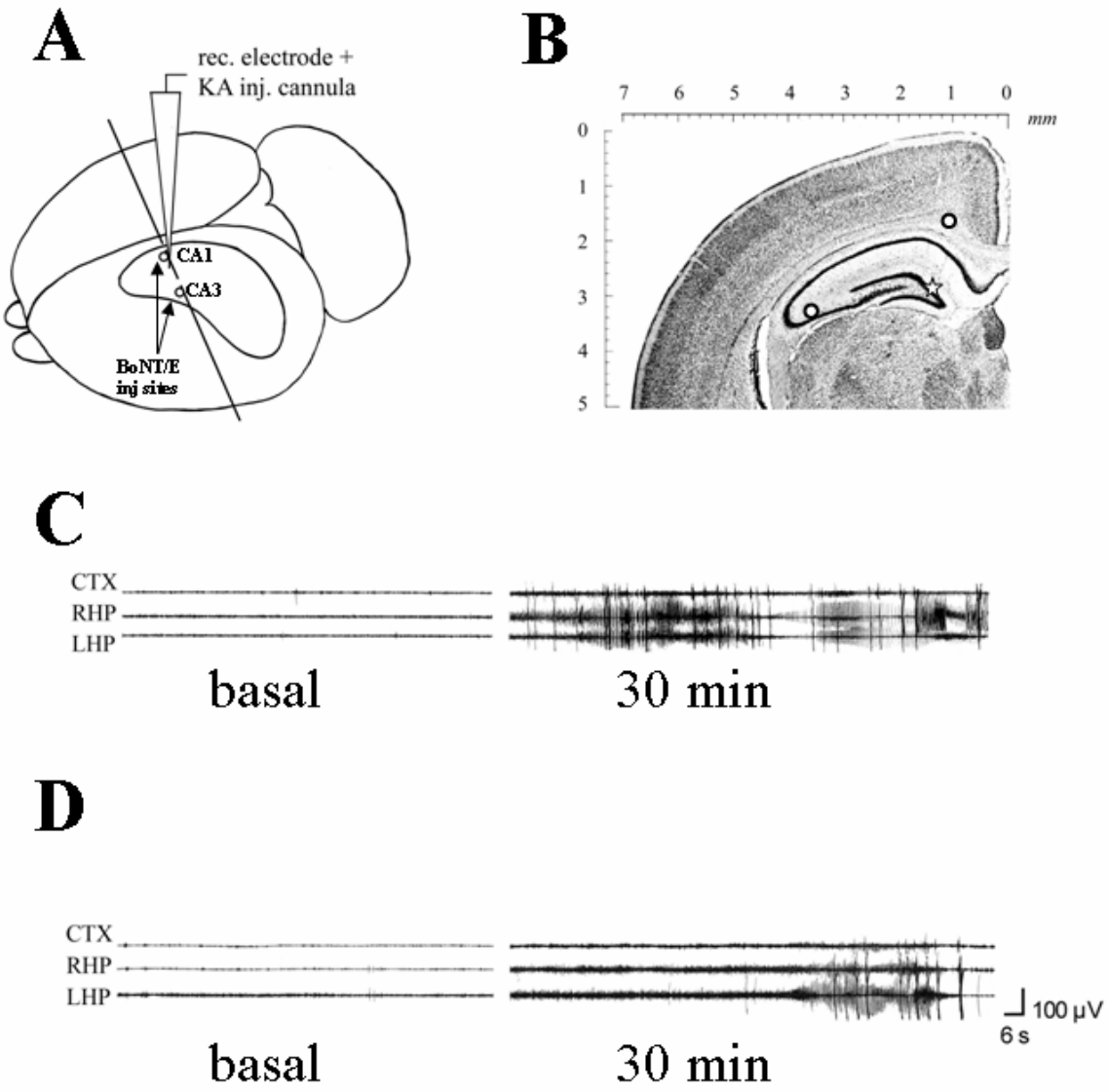

FIGURA 5

Anti-ictal effect of BoNT/E against seizures induced by intrahippocampal KA

(A) Three-dimensional drawing of rat brain illustrating the location of BoNT/E injection sites (open circles) and the placement of KA injection cannula and one depth bipolar recording electrode. (B) Nissl-stained coronal section through the hippocampus indicating the sites of BoNT/E injection (open circles) and the position of the KA infusion cannula glued to the recording electrode (star). (C) Classical EEG pattern of epileptic activity that can be recorded 30 minutes after KA administration both in the hippocampi and in the cortex in vehicle-treated animals. (D) In BoNT/E-treated animals epileptic activity is strongly suppressed in injected hippocampus and in the cortex and it can be recorded only in the controlateral, not-injected hippocampus.

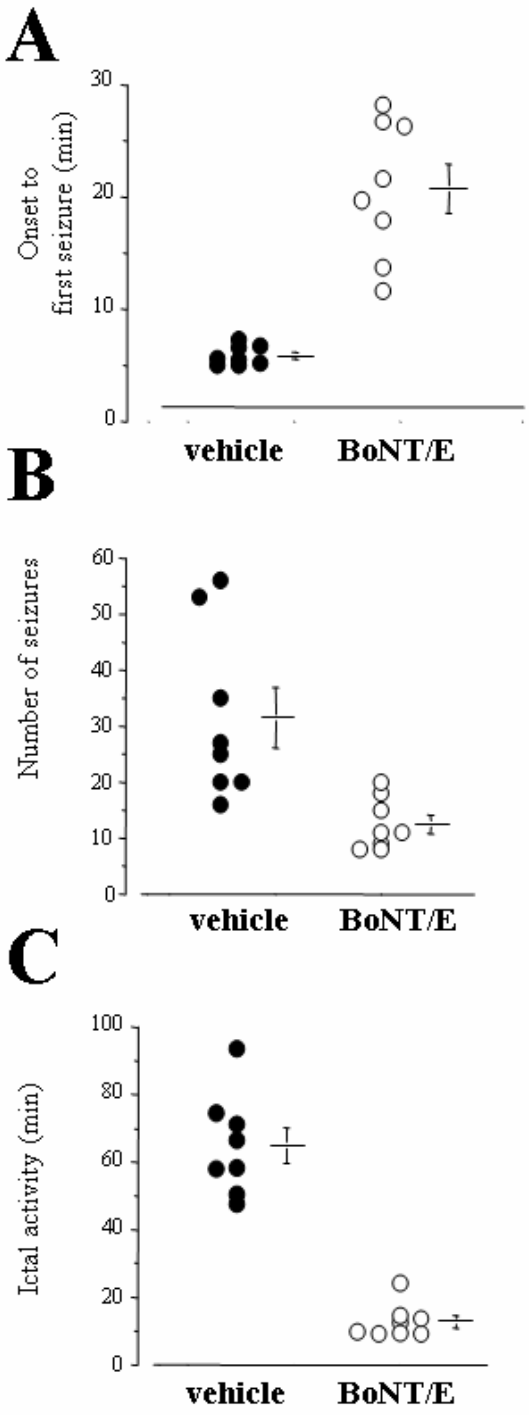

FIGURE 6

Anti-convulsant properties of BoNT/E on EEG seizures induced by intrahippocampal KA

(A-C) Quantification of EEG seizures in control (black circles) and BoNT/E-injected (open circles) animals (n = 8 per group). The onset to the first seizure, the total number of seizures and the total time spent in ictal activity are reported in (A), (B) and (C), respectively. Each point indicates one animal and horizontal bars represent mean values (± S.E.). EEG recordings were performed for a period of four hours.

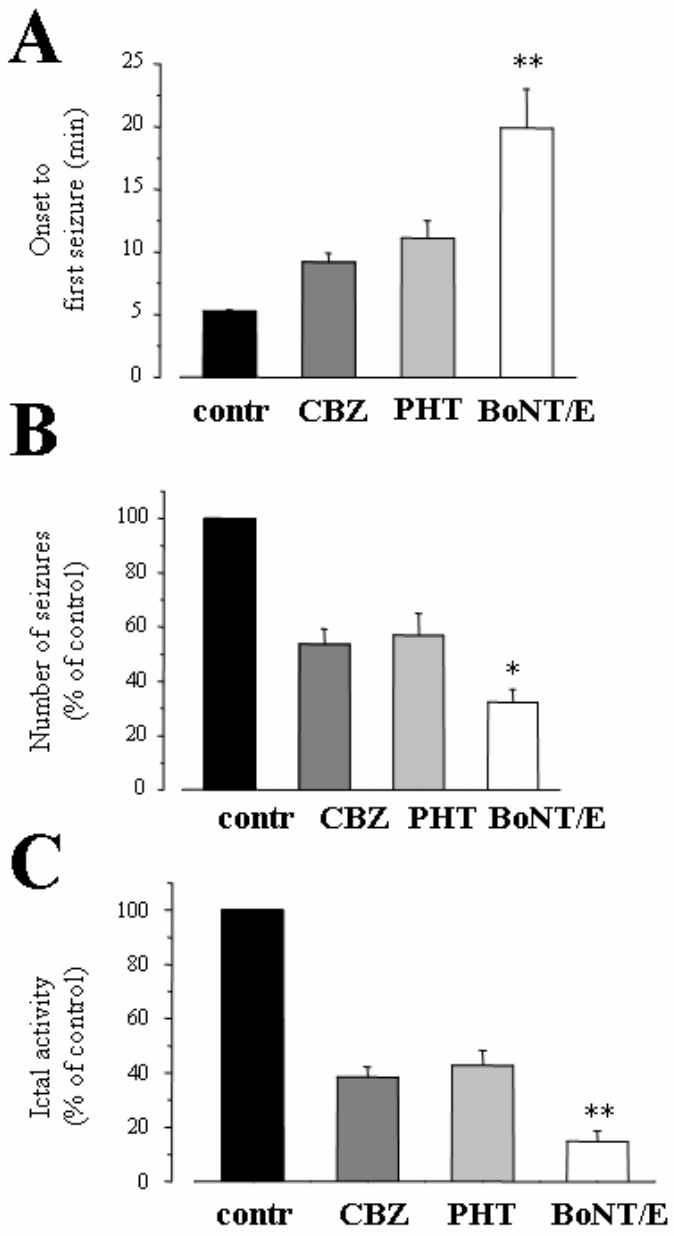

FIGURE 7

Comparison of the anti-ictal effect of carbamazepine (CBZ) phenitoin (PHT) and BoNT/E.

Drugs were given 60 min (CBZ, 10 mg/kg, i.p.; n = 6 rats and PHT, 50 mg/kg, i.p.; n = 7 rats) or two days (BoNT/E intrahippocampal; n = 8 rats) before intrahippocampal KA. The graphs report the onset to first the seizure (A), the total number of seizures (B) and the total time spent in ictal activity (C) scored by the different groups, as compared to control animals (contr; n=21) which received only intrahippocampal KA and the respective vehicles. *, $p < 0.05$; **, $p < 0.01$; one way ANOVA followed by Tukey test.

3.3 Unilateral BoNT/E blocks behavioural seizures induced by systemic KA

The strong anti-ictal effect of BoNT/E on EEG seizures induced by intrahippocampal KA encouraged me to assess the behavioural response of BoNT/E-injected rats to systemic administration of KA, which is known to induce tonic-clonic seizures of limbic origin (Lothman and Collins, 1981; Ben-Ari, 1985). Indeed kainate is able to activate the hippocampus by means of specific receptors present in this structure. This activation is associated with the appearance of limbic motor signs such as wet-dog shakes, facial myoclonia and paw tremor. Afterwards there is a spread of activity from hippocampus to other structures of the limbic system, such as the amygdaloid complex, the medio-dorsal thalamic nuclei, the piriform, entorhinal and rostral limbic cortices and the areas of projection of the fornix. This widespread activation of limbic system produces the tonic-clonic limbic motor seizures.

P35 rats received unilateral stereotaxic injections of BoNT/E (n = 30) or vehicle (n = 39) into the hippocampus. One day later, the animals received a single systemic injection of a convulsive dose of KA (8 mg/kg, i.p.). As a control, 20 uninjected rats were treated with the same dose of KA. KA treatment had a similar pro-convulsant effect in both naïve and vehicle-injected animals (two-way ANOVA, $p > 0.05$; Fig. 8A). These rats showed initial immobility and staring followed by wet dog shakes and culminating, after about 90 minutes from KA injection, in limbic motor seizures with rearing and falling and forelimb clonus. This progression of clinical signs was dramatically different in BoNT/E-injected animals (Fig. 8A). Indeed, these animals displayed pre-convulsive behaviour that only in few cases evolved toward limbic motor seizures. As I described in the M*ethods* section, the behaviour of animals after systemic KA can be described according to a seizure rating scale. This analysis clearly pointed out that the trajectory in behaviour score of BoNT/E-treated rats was dramatically different from that of control rats starting from 80 min following KA administration (two-way ANOVA, $p < 0.001$; post hoc Tukey test, BoNT/E vs. vehicle and uninjected rats, $p < 0.01$).

In Figure 8B, the results of the behavioural analysis are summarized as the maximum seizure rating scale value assigned to each animal during the 4 hours of observation following KA administration. KA triggered typical limbic motor convulsions in 17 out of 20 (85%) naïve rats and 31 out of 39 (79%) vehicle-injected rats. In contrast, the vast majority of the BoNT/E-injected rats showed only pre-convulsive behaviours, and only

5 out of 30 (16%) experienced limbic seizures upon KA administration. Analysis of variance demonstrates that the anticonvulsant effect of BoNT/E is highly significant (one way ANOVA, $p < 0.001$; post hoc Dunn's test, $p < 0.01$), while naïve and vehicle-injected rats do not show significant differences ($p > 0.05$). Lethal toxicity induced by KA (8-20% in control groups) was also abolished by BoNT/E injection (Fig. 8B).

To further support these behavioural data, I performed activity mapping studies with c-*fos* mRNA *in situ* hybridization. A strong bilateral activation was observed in the hippocampus, thalamus and cerebral cortex of control animals treated with KA ($n = 5$), whereas a dramatic decrease of c-*fos* induction was detected throughout the entire brain in BoNT/E-treated rats ($n = 5$; Fig. 9A). Quantification of signal intensity revealed a strong reduction of c-*fos* mRNA within the injected hippocampus of BoNT/E-treated rats as compared to the injected hippocampus of vehicle-treated animals. c-*fos* mRNA labelling in the uninjected contralateral hippocampus was also slightly, but not significantly, reduced in BoNT/E-treated rats (Fig. 9B). In keeping with the inhibition of generalization of seizure activity, c-*fos* mRNA labelling was completely absent in the cerebral cortex of both hemispheres in BoNT/E-treated rats, except for a small area corresponding to the injection track (Fig. 9A).

It is well known that the susceptibility to KA-induced seizures markedly depends on the genetic background (Golden et al., 1995), and KA doses of 8 mg/kg were used to avoid unacceptably high (> 25%) mortality rates in Long-Evans hooded rats. To exclude that suppression of seizure development by BoNT/E could be ascribed to the dose of KA employed, behavioural observations were repeated in Sprague-Dawley rats treated i.p. with KA at 12 mg/kg. We found that status epilepticus occurred in six out of seven rats preinjected with vehicle solution. By contrast, none of the ten rats treated with BoNT/E showed status epilepticus following parenteral KA, confirming the powerful anti-ictal effect of BoNT/E.

Experimental modulation of the inflammatory response is known to affect seizure development following KA administration (Vezzani et al., 2000; De Simoni et al., 2000). We therefore controlled the inflammatory response in a subset of BoNT/E- and vehicle-injected animals 1-2 days following the injection, i.e. the time of seizure induction via KA. Staining for OX-42, an antibody that reveals microglia and neutrophils, revealed no differences between the two experimental groups in both CA1 (Fig 10 A,B) and CA3 (Fig 10 C,D) region. These data suggest that modulation of inflammation is not involved in the anticonvulsant effects of BoNT/E.

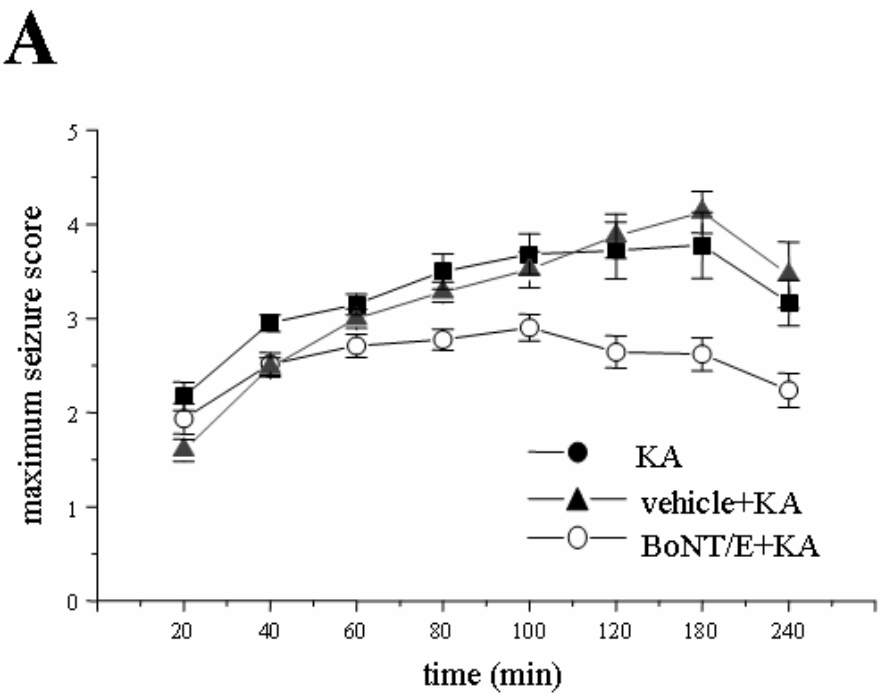

FIGURE 8

BoNT/E prevents seizures induced by systemic KA.

(A) Progression of behavioural changes after systemic KA administration (8 mg/kg, i.p.) in the various treatment groups, scored every twenty min over a 4 hr observation period (see *Materials and Methods* for details). Data are mean seizure scores (B) Scatter plot reporting the maximum seizure score assigned to each experimental animal during a 4 hr observation period following KA administration. The majority of the control animals reached status epilepticus (seizure stage 5), while only 1 out of 30 BoNT/E-injected rats did so. **, $p < 0.01$; one way ANOVA followed by Dunn's test.

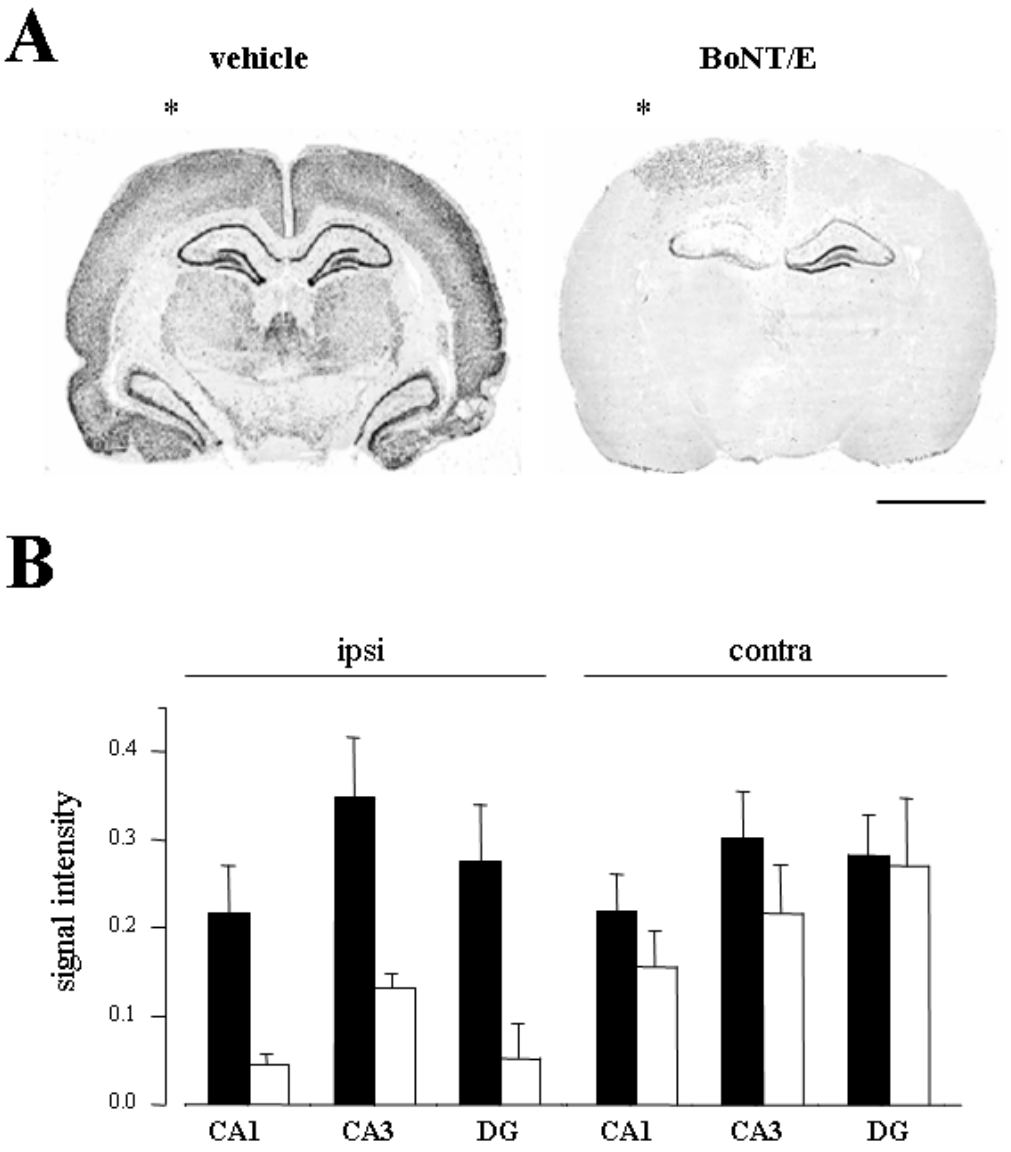

FIGURE 9

BoNT/E markedly decreases KA-induced c-*fos* mRNA expression in the brain.

(A) In situ hybridisation analysis of c-*fos* mRNA in coronal brain sections at the level of the dorsal hippocampus from a vehicle- and a BoNT/E-injected rat, 2.5 hr following systemic KA. The vehicle-injected rat reached status epilepticus while the BoNT/E-injected animal showed only pre-convulsive behaviours (stage 3). Asterisks indicate the injected sides. Scale bar = 3 mm. (B) Quantification of KA-induced *c-fos* mRNA expression in the CA1, CA3 and dentate gyrus (DG) sectors of the hippocampus from vehicle- (black columns) and BoNT/E- (white columns) treated rats. Separate quantifications were made for the hippocampus ipsilateral (ipsi) and contralateral (contra) to the injected side. Signal intensity is expressed as integrated optical density ± S.E. (n = 5 animals per group; *, $p < 0.05$, t-test).

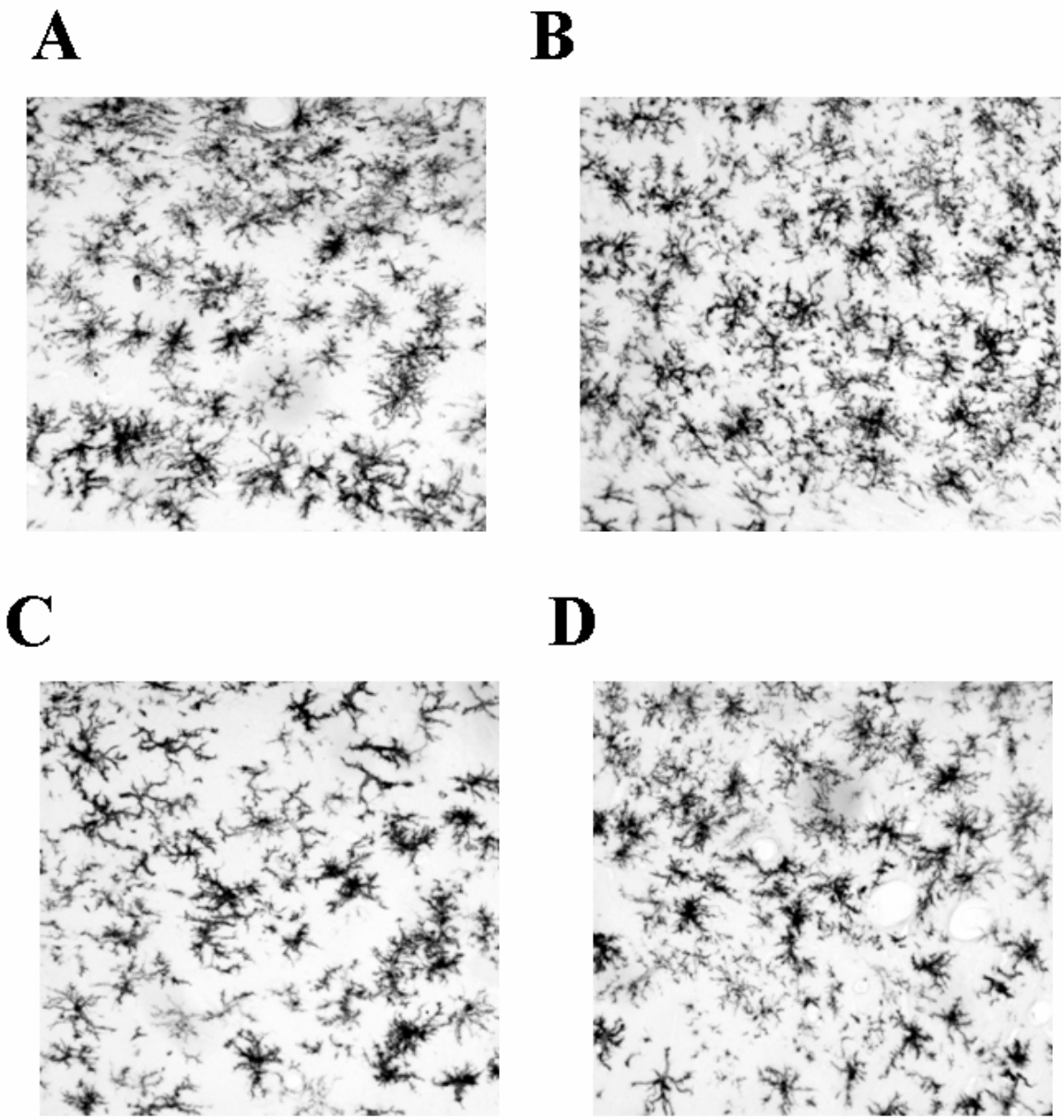

Figure 10

Inflammatory response in hippocampi of BoNT/E- and vehicle- injected animals

Immunostaining for OX-42 one day after BoNT/E (A, C) or vehicle injection (B, D) in the hippocampus. There is no difference in the pattern and in the intensity of OX-42 staining in the CA1 (A, B) and in the CA3 region (C, D) of BoNT/E- or vehicle-injected animals.

3.4 BoNT/E prevents spatial learning deficits induced by KA

To determine whether BoNT/E prevents the cognitive deficits induced by KA, I tested the animals in the Morris water maze, a well-established hippocampus-dependent behavioural task.

In the Morris water maze the animal is released in a circular tank filled with opaque fluid that contains a submerged escape platform in one quadrant. The animal has to find this platform and climb into it in order to avoid to remain in the water. As the animal is released at random locations around the pool, he has to use contextual (i.e. spatial) cues, usually represented by markings on the walls of the room in which the pool is located, to find the platform. During a number of trials, the animal learns to find the platform and escape from the pool. The learning ability of the animal is therefore measured by the time employed to find the submerged platform. This task is considered a test of hippocampal integrity (Morris et al., 1982) since hippocampal lesions, but not lesions of nearby structures, impair this spatial learning. In a non-contextual (i.e. non-spatial) version of the task the platform is raised above the surface or marked with a flag so that it is visible, permitting the animal to navigate to the platform directly. This task does not require the hippocampus and it is used to measure the general behavioural performance abilities of the animal.

Many studies (Stafstrom et al., 1993; Mikati et al., 2001) demonstrated that animals that underwent KA-induced status epilepticus had a severe deficit in the Morris water maze, that is they displayed significantly longer escape latencies with respect to normal animals. Moreover these adverse effects were long-lasting, since they were observed both 20 and 60 days after status epilepticus.

In order to determine if BoNT/E is able to prevent the deficits in the Morris water maze induced by KA, I first examined whether intrahippocampal injection of BoNT/E *per se* has an effect on cognitive performance. According to the requirement of both hippocampi in order to perform the Morris water maze, clear deficits in acquisition of this task were evident in rats tested 3-7 days after BoNT/E, i.e. during the time window of action of the toxin (Fig. 11A). Indeed if these animals are allowed to recover for five weeks, a time at which the effect of BoNT/E is extinguished (see Fig. 3C,D), their learning ability is indistinguishable from that of age-matched normal rats (two-way ANOVA, $p = 0.38$; Fig. 11B). These animals also showed normal exploratory behaviour

in the open field and no evidence of neuronal loss in the brain as assessed by Nissl and NeuN staining (data not shown).

I next analyzed the learning ability of vehicle and BoNT/E-injected animals treated with KA. Control rats treated systemically with KA at P36 and tested five weeks later performed poorly in the Morris water maze. Indeed, they had significantly longer mean escape latencies than normal rats on each of the four test days (two-way ANOVA, $p < 0.01$), consistent with previous studies (e.g. Stafstrom et al., 1993; Mikati et al., 2001). Conversely, rats pre-injected with BoNT/E and treated with KA at P36 showed absolutely normal spatial learning abilities when tested five weeks later. In fact their mean escape latencies was indistinguishable from that of normal rats (two-way ANOVA, $p = 0.4$, BoNT/E + KA vs. normal rats) and their performance was significantly superior to that of control KA rats (Fig 12, two-way ANOVA, $p < 0.001$). Differences in performance could not be attributed to differences in swim speed, which resulted similar across the various experimental groups (one-way ANOVA, $p > 0.05$; data not shown).

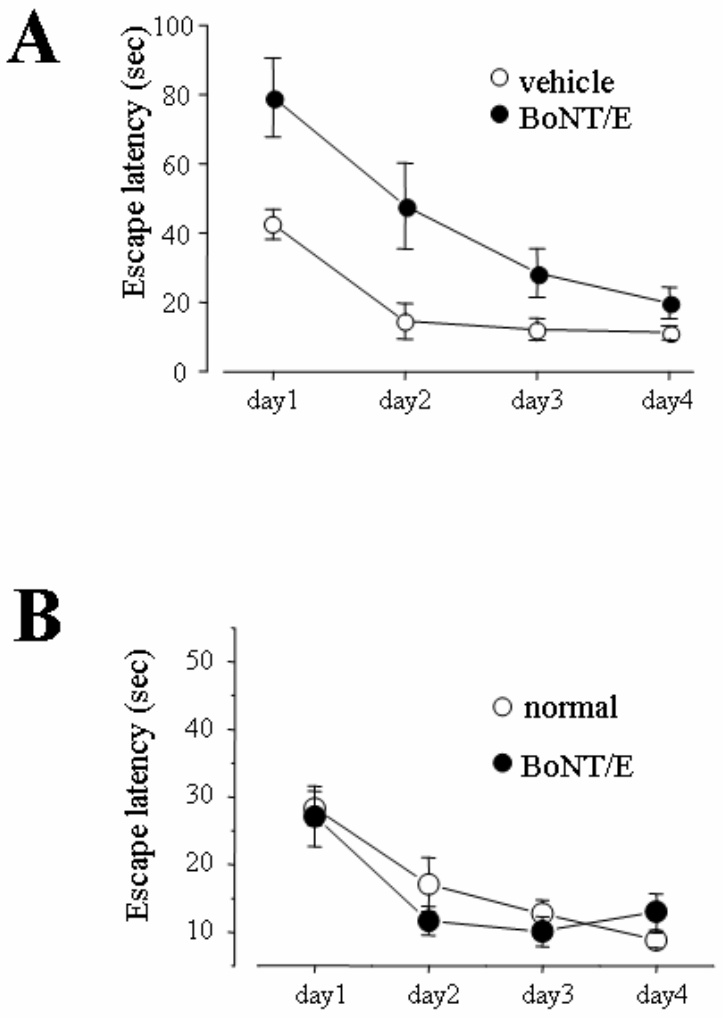

Figure 11

BoNT/E impairment of spatial learning in the Morris water maze is reversible.

(A) Spatial learning in the Morris water maze (MWM) during BoNT/E action. Time required to find the submerged platform (escape latency) is indicated in seconds and plotted as mean values across days of testing. Rats injected with BoNT/E into the hippocampus at P35 and tested 3-7 days later (n = 8; black circles) perform significantly worse (two-way ANOVA, $p<0.001$) than vehicle-injected, age-matched rats (n = 8; open circles). (B) Spatial learning in the MWM after recovery from BoNT/E effects. Performance of BoNT/E-injected rats five weeks after the injection (n = 5; black circle) is superimposable to that of of naïve, age-matched rats (n = 10; open circle). No statistically significant difference is observed between the two groups (two-way ANOVA; $p=0.38$)

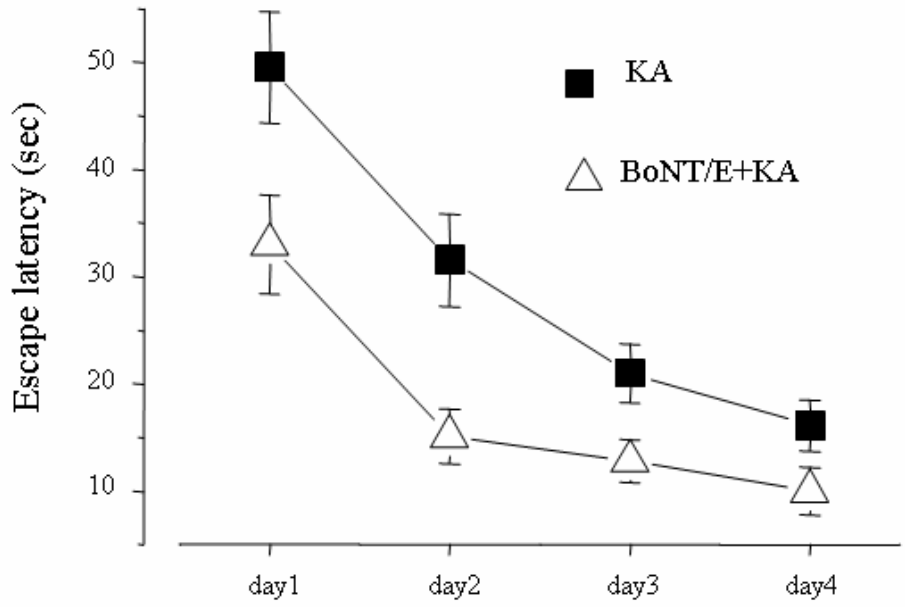

Figure 12
BoNT/E prevents spatial learning deficits induced by KA.

Acquisition of spatial learning in the MWM for rats injected with BoNT/E at P35 and treated with KA at P36 (n = 13; open triangles). Time required to find the submerged platform (i.e. escape latency)is indicated in seconds and plotted as mean values across the days of testing. Performance of control KA rats (n = 26) is shown by black squares and cumulates data from both naïve and vehicle-injected rats treated with KA at P36, since these two groups did not differ (two-way ANOVA, p=0.38). Behavioural testing began for all rats five weeks after KA. Control KA rats were slower to learn the task and never reached the performance of the BoNT/E-injected group (two-way ANOVA, $p<0.01$

3.5 BoNT/E prevents neuronal death induced by KA

The parental administration of kainic acid induces a severe damage in many structures of the limbic system, such as the hippocampal formation, the lateral septum, the amygdaloid complex and the piriform cortex (Ben-Ari, 1985; Zhang et al., 2002) The massive neuronal death is usually accompaigned by other abnormalities such as hypertrophy and swelling of satellite oligodendroglia, proliferation of hypertrophic microglia and of astrocites and hypertrophy of endothelial cells in the capillary wall. In the hippocampal formation the most vulnerable regions are the CA1 and the CA3, where a severe destruction of the pyramidal layer can be detected. Notably the severe brain damage induced by i.p. administration of kainic acid is always associated with the occurrence of limbic motor seizures.

Since BoNT/E injection prevents the occurrence of status epilepticus after KA i.p. administration, I decided to examine if this treatment is also effective in sparing hippocampal neurons from death. Stereological counts in CA1 and CA3 regions of the hippocampus are very difficult, because their pyramidal neurons are too heavily packed. Therefore, as described in the *Methods* section, I expressly developed a scale to quantify neuronal damage. This scale is illustrated in Fig 12, where the whole hippocampus is represented together with details of CA1 and CA3 regions. According to this scale, the degree of neuronal damage can be classified as 0, that is absence of damage (Fig 13A), 1, corresponding to the presence of minimal damage, mainly small spots of degeneration (Fig 13B), 2, corresponding to evident loss of pyramidal neurons (Fig 13C) and 3, represented by the complete disruption of hippocampal architecture (Fig 13D).

BoNT/E injection resulted effective in preventing hippocampal neuronal loss of CA1 and CA3 regions after KA i.p. administration. Indeed five weeks after KA treatment, an abundant neuronal loss can be detected in CA1 and CA3 regions of vehicle-injected rats, while in BoNT/E-injected rats there is a complete rescue of vulnerable hippocampal cells (Fig 14A). The plotting of the mean damage score for each animal reveals a spread of values for vehicle-injected animals, according to the variability of neuronal death induced by KA i.p. treatment, while most of BoNT/E-injected animals have score number 0, which means that they do not display neuronal loss (Fig 14B).

These data therefore demonstrate that prevention of the initial status epilepticus by BoNT/E is able to spare vulnerable hippocampal neurons from death.

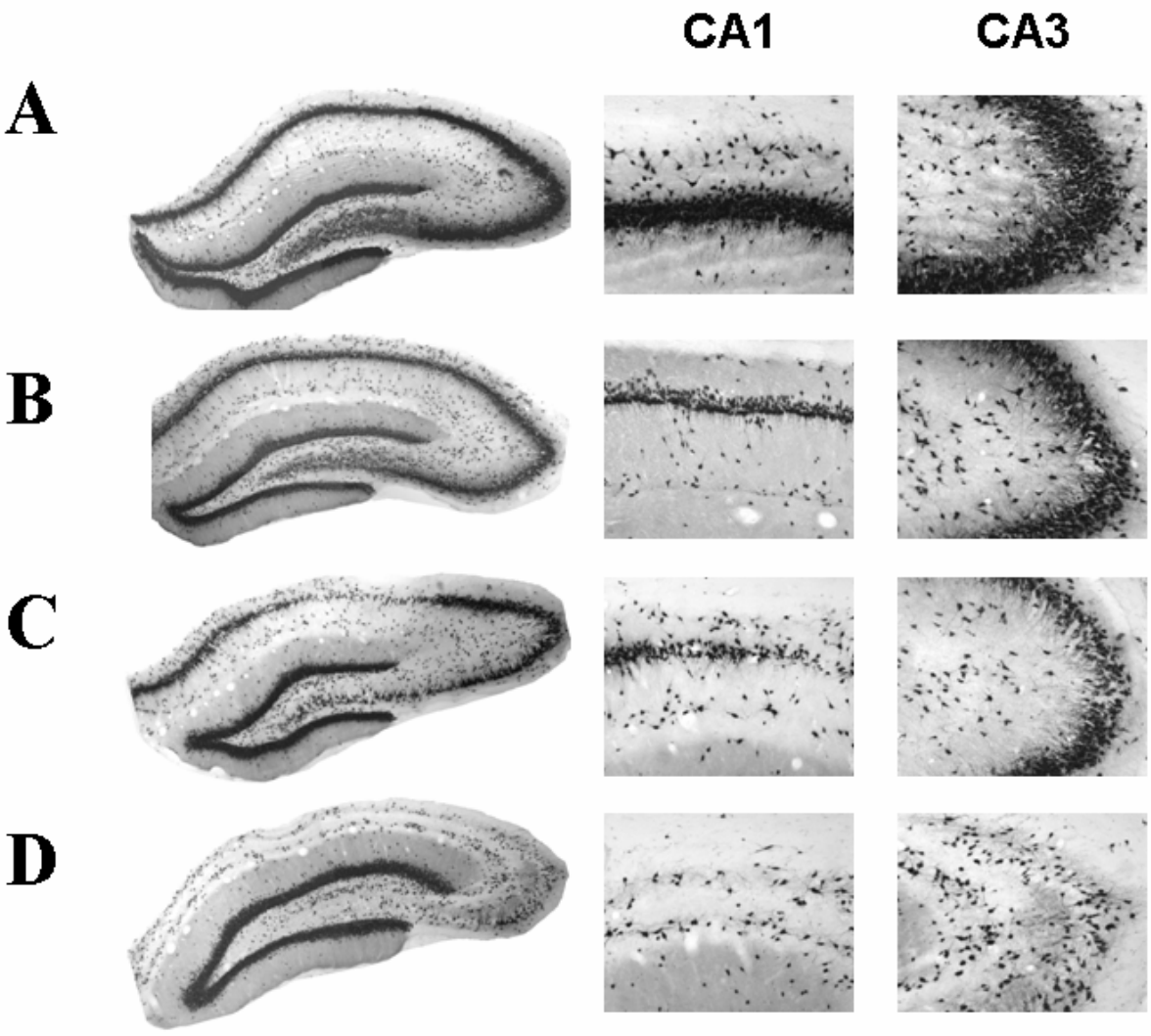

Figure 13

Rating scale used to quantify neuronal damage in the hippocampus

(A, B, C, D) Coronal sections representing the whole hippocampus together with details of CA1 and CA3 regions and illustrating the rating scale used to quantify neuronal damage. (A) Absence of damage, score 0. (B) Presence of small spots of degeneration, score 1. (C) Evident loss of pyramidal neurons, score 2. (D) Complete disruption of hippocampal architecture, score 3.

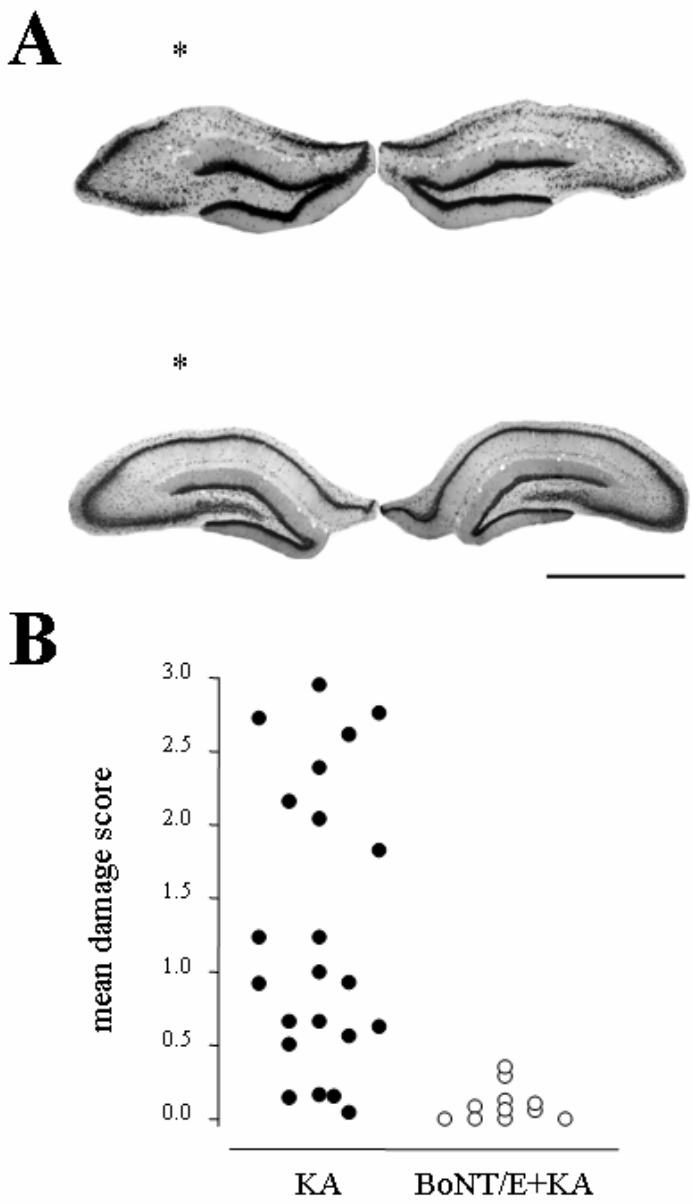

Figure 14

Quantification of BoNT/E neuroprotective effects on neuronal loss induced by KA administration

(A) Representative coronal sections through the dorsal hippocampus of P77 rats that received vehicle (top) or BoNT/E (bottom) at P35 and KA at P36. Sections were immunostained for the neuronal marker NeuN. Note preservation of hippocampal neurons in BoNT/E-injected rats, while widespread neuronal loss was observed in CA1 and CA3 regions of vehicle-injected rats. The injected side is indicated by an asterisk. Scale bar = 2 mm. (B) Quantification of histological lesions in the dorsal hippocampus of control (black circles; n = 22) and BoNT/E-injected (open circles; n = 11) rats treated with KA. The control KA group pools data from both naïve and vehicle-injected rats since these two groups did not differ. The extent of the loss of hippocampal pyramidal neurons was determined for each animal as the mean damage score value in CA1 and CA3 sectors of both hemispheres (see *Materials and Methods* for details). Each point represents one animal. **, $p < 0.01$, Mann-Whitney rank sum test.

3.6 Effects of BoNT/E on kindling epileptogenesis

Finally, I tested if BoNT/E, in addition to its anti-ictal effects, also displays antiepileptogenic-like actions in the model of rapid electrical kindling of the ventral hippocampus.

Kindling is a model of complex partial seizures in which repeated electrical stimulation of limbic structures triggers progressive intensification of epileptiform responses. When the animal has exhibited several generalized convulsions, it is said to be kindled and it will retain abnormal excitability thereafter (Dennison et al., 1995). In the rapid kindling of ventral hippocampus (Richichi et al., 2004) two bipolar stainless steel electrodes are implanted bilaterally in the ventral hippocampus. On the first day of stimulation the threshold for eliciting focal epileptiform activity (after discharge, AD) is determined and this current is used for the next kindling stimulations. The intensity of seizures was scored behaviourally, as described in the *Methods* section. The animals are considered fully kindled after they experienced five seizures of 5° grade. This type of kindling is particularly rapid as 40 stimulations with 5 minutes interval are sufficient to get fully kindled animals.

The anti-epileptogenic effects of BoNT/E was therefore tested in adult rats that received intrahippocampal injections of either vehicle (n = 5) or BoNT/E (n = 6). In this same surgical session, the animals were implanted unilaterally in the ventral hippocampus with bipolar stainless steel electrodes to deliver constant-current stimuli. First of all, the threshold current for eliciting focal epileptiform activity in the stimulated hippocampus was determined. This current was higher, but not significantly different, in BoNT/E-injected rats with respect to the vehicle-injected group (BoNT/E: 185 ± 29 [S.E.] μA; vehicle: 132 ± 7 μA; t-test, $p > 0.05$). However the behavioural progression of kindling in BoNT/E-injected animals showed a marked retardation. Indeed BoNT/E-injected animals required always more stimulations to get the same behavioral stages of vehicle-injected animals. In particular they required significantly more stimulations to exhibit the first generalized motor seizure (Fig 15A, stage 4-5 seizure; two-way ANOVA, $p < 0.05$; post hoc Tukey's test, $p < 0.01$). BoNT/E-injected animals also experienced a significant minor number of stage 4-5 seizures than vehicle-injected ones (vehicle, 5.6 ± 0.5; BoNT/E, 1.7 ± 0.2, t-test, $p < 0.01$). However all rats showed stage 4-5 seizures 24 hr after kindling acquisition and during the re-test day. BoNT/E treatment affect also the duration of the primary afterdischarge (AD). Indeed

primary AD is significantly shorter in BoNT/E-injected animals (cumulative AD, min, mean ± S.E.: vehicle, 18.9 ± 1.1; BoNT/E, 11.9 ± 1.9; t-test, $p < 0.01$; Fig. 15B). The duration of secondary AD was also significantly reduced in BoNT/E-injected animals (vehicle, 10.2 ± 1.8; BoNT/E: 6.6 ± 1.2; t-test, $p < 0.05$; Fig. 15C).

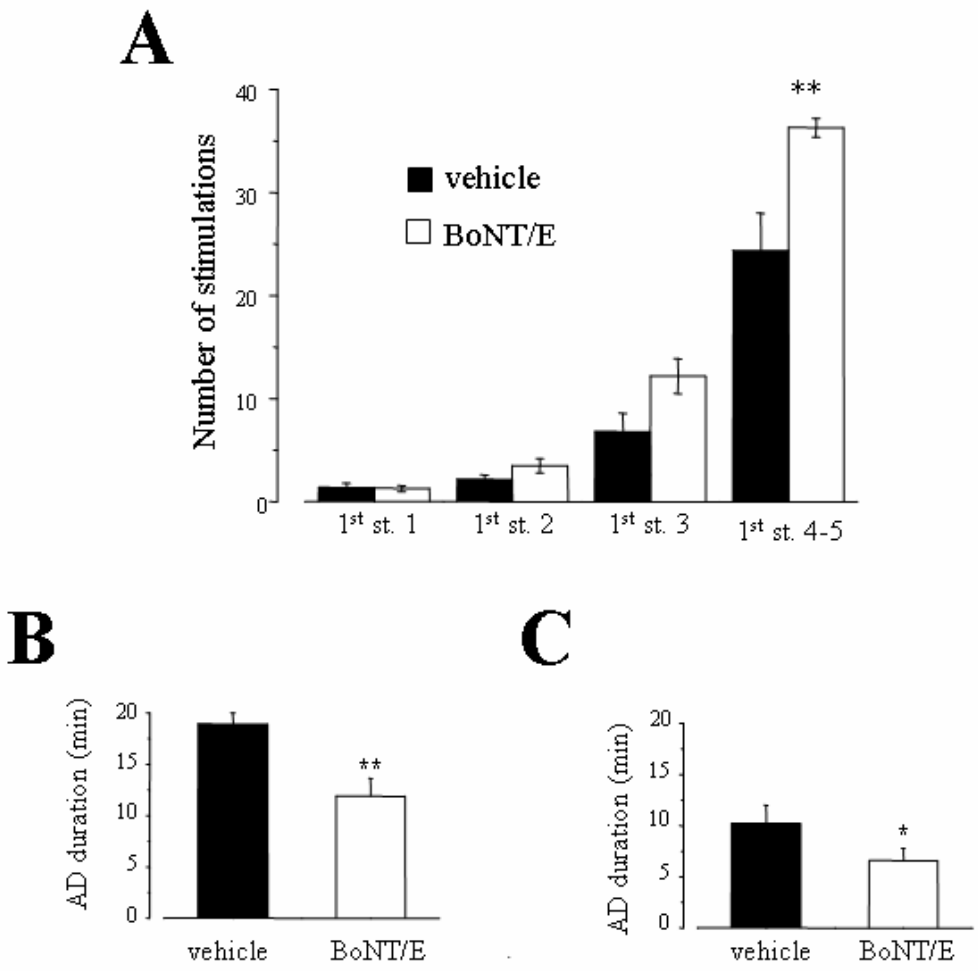

Figure 15

Effects of BoNT/E on kindling epileptogenesis

(A) Behavioural progression of seizures during kindling in vehicle- (black columns) and BoNT/E-injected rats (white columns). BoNT/E-treated animals required more stimulations to reach stages 4 and 5 of kindling. Data represent mean ± S.E. (**, $p < 0.01$; two way ANOVA followed by post-hoc Tukey test). Behavioural stages are scored according to Racine's classification (Vezzani et al., 2002). (B, C) Duration of primary (B) and secondary (C) afterdischarge during kindling in BoNT/E and vehicle-injected rats. Cumulative afterdischarge (AD) was reckoned in each rat by adding together the durations of the single ADs induced in the hippocampus during kindling stimulations. Data represent the mean ± S.E. of cumulative AD. *, $p < 0.05$; **, $p < 0.01$, t-test.

DISCUSSION

In the present study I characterized the effects of intrahippocampal injections of BoNT/E and I evaluated the anticonvulsant and antiepileptogenic properties of this treatment using both two models of acute seizures and the model of kindling epileptogenesis in rats.

I provide the first description of the effects of BoNT/E on CNS neurons *in vivo*. In addition, I present electrographic and behavioural evidence of a pow

erful anticonvulsant action of BoNT/E on both focal and generalized seizures induced by KA. Finally, I show that BoNT/E displays antiepileptogenic-like effects since it delays the development of hippocampal kindling.

4.1 Effects of BoNT/E in the hippocampus

BoNT/E interferes with neurotransmitter release in a very specific manner through its interaction with the SNARE protein SNAP-25. Consistent with previous studies *in vitro* (Bigalke et al., 1981 a,b; Ashton and Dolly, 1988; Verderio et al., 2004), I demonstrated *in vivo* that BoNT/E dramatically impairs hippocampal glutamatergic transmission but has little effect on GABAergic one. Indeed I showed that BoNT/E is able to strongly reduce the Ca^{2+}-dependent K^{+}-evoked release of glutamate from hippocampal synaptosomes but it does not significantly affect GABA-ergic release. The low sensitivity of inhibitory transmission to BoNT/E is due to absence of SNAP-25 at GABAergic synapses, where it is replaced by its functional homologue SNAP-23 (Verderio et al., 2004). BoNT/E, at the concentration we used in our experiment (50nM), retains a high degree of specificity and indeed it is unable to cleave SNAP-23 (Sadoul et al., 1997). BoNT/E at higher doses, i.e. 300 nM; can however proteolize SNAP-23 (Matteoli personal communication).

At the electrophysiological level, treatment with BoNT/E prevents the occurrence of spontaneous excitatory postsynaptic potentials (EPSPs) and greatly reduces the amplitude of evoked EPSPs in hippocampal slices (Capogna et al., 1997; Sutton et al., 2004). Consistent with this reduction of excitatory synaptic responses *in vitro*, our *in vivo* data indicate that BoNT/E potently inhibits the firing of pyramidal neurons in the hippocampus.

The effects of BoNT/E persist for about three weeks after one single intrahippocampal injection, as determined by immunodetection of cleaved SNAP-25

and loss of intact SNAP-25. Blockade of neural activity in one hippocampus by BoNT/E produced, in the first week after treatment, a marked deficit in the acquisition of spatial learning in the Morris water maze, a classic hippocampus-dependent task. This finding is in keeping with previous evidence showing that unilateral lesion of the hippocampus impairs performance in this task (van Praag et al., 1998). However, the deficits displayed by BoNT/E-treated rats were transient since, when the animals were allowed to recover for five weeks after BoNT/E injection, they showed normal spatial learning abilities in the Morris water maze. Thus, intrahippocampal BoNT/E treatment in adult rats does not result in neuronal dysfunction in the long term. Accordingly, reversible inhibition of neural activity via TTX infusion in the adult rat hippocampus has no subsequent deleterious effects on animal behaviour (Lipska et al., 2002). Future studies will determine whether BoNT/E treatment can cause more subtle permanent functional changes, such as alterations in basal EEG patterns.

In addition to normal performance in the Morris water maze after recovery from BoNT/E effect, the animals also displayed normal gait, posture and exploratory behaviour in the open field. Moreover staining for neuronal (NeuN) and glial markers (OX-42, GFAP) revealed no differences between hippocampi of rats injected with BoNT/E or vehicle.

These data allows us to conclude that intrahippocampal BoNT/E treatment in the adult rat is safe and does not result in neuronal dysfunction in the long term. This validates the use of BoNT/E in cases in which reversible silencing of neuronal activity in selected brain areas may be of therapeutic value.

It will be of interest in the future to characterize the effects of other BoNTs serotypes at the level of CNS neurons. In particular it would be intriguing to investigate the action in the CNS of BoNTs acting on SNAREs other than SNAP-25. Indeed different BoNTs may show distinct or overlapping actions with respect to BoNT/E, depending on the pattern of expression of their target SNAREs.

4.2 Anti-ictal effects of BoNT/E

I clearly demonstrated on both EEG and behavioural analysis of kainic acid-induced seizures that a single intrahippocampal BoNT/E injection is able to perform a powerful anticonvulsant action.

Indeed I showed a highly significant effect of BoNT/E on onset, number and duration of EEG ictal episodes in the model of acute seizures triggered by focal delivery of KA. The anticonvulsant efficacy of BoNT/E is underlined by the comparison with conventional, systemically administered antiepileptic compounds. I actually found that BoNT/E is more effective than CBZ or PHT in suppressing electrographic seizure activity. However CBZ and PHT are not the drugs of choice for KA-induced seizures. Indeed benzodiazepines are the most effective ones. Future studies will therefore be necessary in order to compare the anti-epileptic effect of BoNT/E with that of benzodiazepines. Finally, the behavioural analysis demonstrated that unilateral hippocampal injection of BoNT/E is able to prevent limbic status epilepticus normally triggered by parenteral KA in two rat strains, demonstrating that its anti-epileptic actions are not dependent on a specific genetic background.

According to previous data (Pitkanen, 2002; Leite et al., 2002), the prevention of status epilepticus was associated with preservation of hippocampal neurons in BoNT/E-injected rats and sparing of cognitive performances.

The finding that unilateral BoNT/E blocks seizures induced by systemic KA may appear surprising. This phenomenon can be explained by mapping studies of neuronal network activity obtained using *in situ* hybridization analysis of c-*fos* mRNA. These mapping studies demonstrated limited c-*fos* induction within the BoNT/E-injected hippocampus but a nearly normal activation of the contralateral one. Remarkably, c-*fos* mRNA labelling was absent from both cortices and thalamic nuclei of BoNT/E-treated rats, whereas a widespread bilateral activation was evident in these areas in vehicle-treated animals. I can conclude therefore that the propagation of epileptiform activity away from the hippocampus and the emergence of generalized limbic convulsions require the recruitment of hippocampi on both hemispheres. The reverberating paroxysmal activity between hippocampi is hence a critical factor for the generalization of seizure activity and the commissural fibers that connect the hippocampi play an important role in this process. Indeed these fibers represent a preferential way for the transmission of epileptic activity and can also promote the formation of a epileptogenic focus, as demonstrated recently in an *in vitro* preparation (Khalilov et al., 2003). The

authors of this elegant experiment used an *in vitro* system composed of three independent chambers able to accommodate two intact hippocampi and their connecting commissural fibers. This particular preparation permit therefore to apply a convulsive agent, i.e. the kainic acid, to one hippocampus, allow the propagation of a given number of seizures to the other side and then blocking the connections reversibly by applying tetradotoxin (TTX) to the commissural chamber. It was therefore demonstrated that the propagation of seizures from the kainate-treated side to the naïve side transformed the latter into an independent epileptogenic focus that was capable of generating spontaneous and evoked seizures. This is the first *in vitro* demonstration that paroxysmal activity propagating through the commissural fibers is able to generate an epileptogenic focus. It would be very interesting to test whether the blockade of seizures activity generalization by BoNT/E could prevent the formation of this epileptogenic focus.

It might be argued that the effects of BoNT/E are too short-lasting (three weeks) to envisage any possible application as antiepileptic therapy. Other BoNTs are available, however, that produce a more prolonged inhibition of exocytosis at peripheral and central synapses (Eleopra et al., 1998; Foran et al., 2003). For example, BoNT/A cleaves the same molecular target (SNAP-25) as BoNT/E but its effects persist for several months (Eleopra et al., 1998; Foran et al., 2003; Meunier et al., 2003). One possible caveat of this approach is that long-term suppression of glutamatergic activity can induce spontaneous withdrawal seizures (Tandon et al., 1996). Although this aspect awaits further investigation, I never observed spontaneous behavioural seizures in BoNT/E-treated rats during daily cleaning and handling or during our behavioural testing

On the other end, there are evidence that a long-term suppression of activity could have a beneficial effect and provide long-lasting anticonvulsant effects. It is well-known that seizures in intractable epilepsy can remit after a period of months or years following surgery. Indeed seizures can propagate from the original primary focus to distant sites and can transform the latters into permanent secondary epileptogenic foci, that are responsible of the enduring seizures after surgery. These secondary epileptogenic areas are however not completely autonomous in generating seizures and therefore, after some time from the resection of the primary area, they stop. The remission of epileptic activity in the secondary areas is called running-down phenomenon and it is due to the long-term blockade of epileptic input activity from the primary zone after its resection.

This suggest therefore that also the long-term suppression of epileptiform activity by BoNT/E or BoNT/A could provide an anti-convulsant effect that can outlast the temporary inhibition of neuronal activity in the epileptic focus.

4.3 Retardation of kindling epileptogenesis

Kindling is a widely used model of epileptogenesis. Indeed this model displays some characteristics typical of the epileptogenic process, such as the progressive increase in seizure severity and duration, the decrease in focal seizure threshold and the neuronal degeneration in limbic brain regions (Corcoran et al., 1988). Therefore kindling is the model of choice for the first screening of potential anti-epileptogenic treatments, as it is relatively simple and rapid.

In this work, I used a modified protocol of kindling, that is the rapid kindling of ventral hippocampus. The rapid kindling differs from normal one in that repeated suprathresold stimulations are delivered with short (usually 5 min) interstimulus intervals, which give rise to generalized seizures within 1-2 h. Elmer and collaborators (Elmer et al., 1996) have recently shown that following 40 seizures episodes, triggered by rapid hippocampal kindling stimulations during about 3 h, hyperexcitability develops in two phases: enhanced responsiveness is already present after 6-24 h, but it increases gradually from one week up to four weeks post-seizures. The acquisition of the kindled state requires structural rearrengements, while the enhanced responsiveness during the first day post-seizures are due likely to be due to functional short-term changes. Thus the rapid kindling model is very useful for studies both on mechanisms regulating the severity of rapidly recurring seizures, and on chains of events triggeres by the initial epileptic insult, which leads to the development of the permanent epileptic syndrome. Even if the evolution of rapid kindling is different from that of traditional kindling, the two models probably share common mechanisms, such as the induction of mossy fiber sprouting in the supragranular zone of the dentate gyrus.

The effectiveness of BoNT/E in retarding rapid kindling suggests therefore that BoNT/E may be an anti-epileptogenic drug. In particular it is interesting that BoNT/E treatment delays kindling progression from stage 3 onwards, and particularly the acquisition of stages 4 and 5. Indeed these behavioural stages correspond to a generalization of epileptic activity and their retardation by BoNT/E injection indicates that the toxin selectively inhibit seizures generalization from their site of onset. The shortening of the secondary AD in the stimulated hippocampus, which reflects a decreased reverberating activity within the limbic circuit, can also be explained by the inhibition of the generalization of epileptic activity.

Electrophysiology recordings *in vivo* demonstrated that BoNT/E affect glutamatergic transmission. This has been confirmed also in the kindling preparation, as the decrease of primary AD can be accounted for by the reduction of local hippocampal excitability.

Thus, intrahippocampal BoNT/E not only suppresses seizures, but also delays kindling, suggesting that this treatment may have anti-epileptogenic properties in models of status epilepticus evolving to recurrent spontaneous seizures.

4.4 Concluding remarks

In conclusion, I shown that local delivery of BoNT/E to the hippocampus is both anti-ictal and anti-epileptogenic in experimental models of epilepsy. The ability of BoNTs to disrupt neurotransmission at the neuromuscular junction for prolonged periods of time has been exploited in several medical applications and these agents are the therapeutics of choice for the treatment of selected disorders, such as the dystonias, that benefit from a functional inhibition of peripheral nerve terminals (Rossetto et al., 2001; Turton et al., 2002). The present work indicates a possible therapeutic exploitation of BoNTs in the central nervous system. The inhibitory effects of BoNT/E on seizures and epileptogenesis in experimental models, as well as the evidence for neuroprotection from seizure-mediated cell death and sparing of hippocampal physiological functions, opens the possibility of developing novel therapeutic strategies for the treatment and management of seizures with focal onset.

The reversible long-term blockade of hippocampal activity by BoNT/E could also be exploited as a tool for testing the opportunity of the surgical treatment of TLE. Indeed some TLE patients may develop secondary epileptogenesis at sites distant from the original focus, as a consequence of the propagation of paroxysmal activity outside the focus region that transform a naïve structure into one that is capable of generating spontaneous and evoked seizures. The presence of such secondary epileptogenic sites reduce the likelihood of successful surgical treatment of epilepsy. The prolonged blockade of hippocampal activity by BoNT/E could be useful in order to establish the origin of seizures. This will allow to avoid the surgical treatment of epilepsy in not successful cases, such as those where a secondary epileptogenic focus has developed.

4.5 Future perspective

The kindling experiment suggests that BoNT/E, in addiction to be anti-ictal, can display also anti-epileptogenic activity. Since a battery of models has to be used to validate the anti-epileptogenic properties of new drugs (Loscher , 2002), I plan to study the effects of BoNT/E on epilepsy development in post-status epilepticus models of MTLE.

Pilocarpine model

As I told in the Intoduction, the parental administration of the cholinergic muscarinic agonist pilocarpine resuls in chronic behavioural state similar to human MTLE (Turski et al., 1989). Indeed rats receiving pilocarpine undergo status epilepticus of limbic origin and spontaneous recurrent seizures arise following a latent period of several weeks after status epilepticus. Histopathologically, massive neuronal loss can be observed wihin 3-5 days in the granule layer of dentate gyrus and in CA1 and CA3 pyramdal cell layers (Turski et al., 1989).

First of all, I have to confirm, in the pilocarpine model, the anti-ictal and neuroprotective effects of BoNT/E that I have already demonstrated in the KA model. I will therefore investigate whether BoNT/E injection in the hippocampus one day before pilocarpine administration is able to prevent status epilepticus and the consequent neuronal loss. If this is confimed, I will test whether intrahippocampal injection of BoNT/E can affect epileptogenesis following pilocarpine-induced status epilepticus. Adult rats will therefore receive BoNT/E (or vehicle) injections into the hippocampus 1 day after pilocarpine and will be implanted with depth electrodes for the chronic recordings of hippocampal activity. Spontaneous seizures frequency in BoNT/E and vehicle-treated rats will be followed at both the electrophysiological and behavioural level for several eeks after status epilepticus, well after the end of BoNT/E effects. These analyses will explore whether BoNT/E treatment after pilocarpine-induced status epilepticus can prevent the occuenc of recurrent limbuc seizures.

As the rat pilocarpine model is also widely for studying the molecular actors that contribute to the process of epileptogenesis (Elliott et al., 2003), I will also investigate whether BoNT/E administration after status epilepticus can interfere with the molecular rearrengements occurring during the latent phase of epileptogenesis. I plan to study the

expression of a battery of genes whose up-regulation has been correlated to different stages of post-status epileptogenesis. In detail I will peform an *in situ* hybridization study to evaluate whether the hippocampal up-reulation of several such genes is blocked by BoNT/E treatment. The expression of immediate arly genes (Arc), NMDA receptor subunits, neuropeptides (NPY), neurotrophins (BDNF), axon guidance molecules (GAP-43) will be studied at different times after status epilepticus and following BoNT/E or vehicle admnistration.

Unilateral intrahippocampal injection of KA in adult mice

Unilateral intrahippocampal injection of KA in adult mice reproduces most of histopathological changes of human MTLE, including neuronal loss, gliosis, mossy fiber sprouting and granule cell dispersion (Suzuki et al., 1995; Bouilleret et al., 1999). Remarkably in this model, focal spontaneous recurrent seizures begin after a latent period of only two weeks and persist for several months, at an average frequency of about twenty seizures per hours (Riban et al., 2002; Gouder et al., 2003). This rapid development of chronic epilepsy will allow to assess the occurrence of spontaneous seizures both during anf after the time window of BoNT/E action (about 3 weeks). In addition, due to the high frequency of spontaneous ictal events, this model will facilitate the assessment of anti-epileptogenic properties of BoNT/E.

Adult mice will be implanted with intrahippocampal electrodes glued to an infusion cannula and KA will be delivered to freely-moving animals through the cannula one week after surgery (Vezzani et al., 2000). The occurrence of status epilepticus will be assessed by EEG recordings (Riban et al., 2002). On the following day, the animals that reached status epilepticus will be injected with BoNT/E or vehicle into the hippocampus. EEG analysis of the frequency of spontaneous seizures will be started two weeks post-status and continued for an additional four weeks period. At the end, the animals will be perfused and brain sections processed for Timm and Nissl stains in order to assess mossy fiber sprouting and hippocampal cytoarchitecture.

The evaluation of BoNT/E effects in these post-status models of MTLE is important because there are currently no means to interfere with epileptognic mechanisms occurring during the latent period and new therapeutic approaches must necessarily be tested in animal models of progression of epilepsy (Loscher et al., 2002).

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APPENDIX

EFFECTS OF LONG-TERM BLOCKADE OF ACTIVITY ON ADULT AND YOUNG VISUAL CORTEX

INTRODUCTION

Neuronal activity plays a very important role in the formation and in the refinement of anatomical and physiological features of the mammalian visual system.

One paradigm of choice for studying the involvement of afferent neuronal activity in visual cortical development is the segregation of geniculocortical afferents into ocular dominance (OD) bands in layer 4 of the primary visual cortex. Initial studies supported an instructive role for visually evoked activity in the segregation process (LeVay et al., 1978). Indeed geniculocortical terminals representing the two eyes are intermingled at the time of eye-opening and only subsequently achieve the adult-like OD pattern. However recent studies (Crair et al., 1998; Crair et al., 2001) demonstrated that OD bands are already present at time of eye-opening, suggesting that visual experience is not necessary for OD segregation. Since spontaneous activity in the retina is spatially and temporally patterned with neighbouring neurons showing high degree of correlation (Galli and Maffei, 1988), this spontaneous activity can instruct segregation of geniculocortical terminals. Nevertheless total removal of retinal input early in visual system development does not affect the segregation process (Crowley and Katz, 1999), as OD bands develop normally in binocularly enucleated ferrets. Therefore patterns of neuronal activity, evoked or spontaneous, are not necessary for the segregation of geniculocortical afferents in layer 4 of primary visual cortex. Instead, correlated neuronal activity might play an important role in the generation of cortical orientation selectivity. Orientation columns are present in the primary visual cortex at the time of eye-opening (Chapman et al., 1996), but the majority of neurons are still weakly orientation selective. The introduction of artificially correlated activity into the visual pathway through synchronous activation of retinal ganglion cell axons in the optic nerve weakens the orientation selectivity of neurons in the visual cortex without altering the layout of orientation domains (Weliky and Katz, 1997). This demonstrates that afferent activity has an instructive role in shaping orientation tuning properties of visual cortical neurons.

The importance of intrinsic cortical activity in visual system development has been investigated in the process of orientation selectivity maturation. Infusion of TTX into visual cortex during the period of orientation selectivity maturation significantly reduces orientation tuning of cortical neurons (Chapman et al., 1996).

As the emergence of orientation tuning properties of visual cortical neurons depends strongly from local intracortical connections (Somers et al., 1995), the clustering of horizontal connections is also reduced by TTX infusion (Ruthazer and Stryker, 1996). Therefore spontaneous activity in the cortex is important for the establishment of local and long-range intracortical connections that lead to maturation of orientation selectivity.

An important input to visual cortex comes from the controlateral hemisphere throught callosal fibers. Visual callosal connections originate from and terminate on similar classes of cells in supragranular and infragranular layers of primary visual cortex, are reciprocal and exhibit topological specificity (Innocenti et al., 1986). The development of visual callosal connections is strongly activity-dependent. Indeed callosal axons are initially imprecise and exuberant and attain their adult specificity by elimination of ectopic axon terminals (Innocenti and Caminiti, 1980). The removal of retinal input, dark rearing from birth or monocular deprivation lead to preservation of ectopic connections (Berman, 1991; Frost and Moy, 1989; Frost et al., 1990), suggesting that neuronal activity has at least a permissive role for the proper development of callosal connectivity.

The role of callosal connections in the development of visual system have never been addressed. In this study I investigated the role of extrinsic cortical activity coming through callosal fibers on the development of visual system. I induced a long-term blockade of neuronal activity in the visual cortex of one hemisphere through BoNT/E injections and I analyzed visual acuity development in the opposite hemisphere. Visual acuity is strongly reduced both in the injected and in the controlateral visual cortex, suggesting that extrinsic cortical activity is an important factor for visual system development.

MATHERIALS AND METHODS

Animal treatment and experimental protocol

The procedures used in this study were approved by the Italian Ministry of Health. Long-Evans adult (320-450 g body weight) and young rats have been used.

For adult experiment four animals were left untreated and used as normal controls. Twenty-one animals were injected with BoNT/E in the visual cortex. Of these six were recorded the day after the BoNT/E injection and their cortex were dissected for immunoblotting assay. Three were perfused the day after the injection and used for immunohistochemistry analysis of SNAP-25 cleaved. Three were kept in the dark for three days after the injection, re-exposed to light for two hours and then perfuse to perform Fos immunohistochemistry. Three were decapitated fourteen days after BoNT/E injection and their cortices dissected for immunoblotting. Six were recorded twenty-one days following BoNT/E injection and their cortices dissected for immunoblotting.

Twenty young rats were used in the other experiments. Four pups were left untreated and recorded at P45. Fifteen P14 were injected with vehicle (n = 5) or BoNT/E (n = 10). Vehicle-injected animals were all recorded at P45. Five BoNT/E-injected rats were recorded the day after injection and their cortices were dissected for immunoblotting. Other five BoNT/E-injected animals were recorded at P45 and their cortices dissected for immunoblotting.

BoNT/E injection in the visual cortex: BoNT/E was obtained by WAKO (Japan), trypsin activated, purified and tested as previously described (Schiavo and Montecucco, 1995).. For injection animals were placed in a stereotaxic apparatus under deep avertin anesthesia (1 ml/100 g body weight, i.p.). In adult animals a U-shape hole was made in the skull overlying visual cortex and three injection of 0.5 μl of BoNT/E (50 nM) were made at the following coordinates from lamba AP -1, L 5, H 1 below dura; AP 0, L 4.7, H 1 below dura; AP 0, L 5.3, H 1 below dura. In young animals three holes were performed with a subtle needle at the sites of injection with the following coordinates AP -1, L 2.5; AP 0, L 2.5; AP 1, L 2.5. An amount of 0.5 μl of BoNT/E (50 nM) or vehicle (2% rat serum albumin in PBS) were then injected.

Electrophysiological analysis

Animals were anesthetized with urethane (Sigma; 20% solution in saline; 0.7 ml/100 g body weight, i.p.) and placed in a stereotaxic frame. Additional doses of urethane (0.07 ml/hg) were given to keep the level of anesthesia stable. Body temperature was monitored and maintained at 37°C by a thermostat-controlled electric blanket. Oxygen was also continuously administered. Both eyes were fixed by means of adjustable metal rings surrounding the external portion of the eye bulb. After exposure of the cerebral surface, a micropipette (tip resistance = 2 MΩ) filled with 3M NaCl was inserted into the visual cortex. In most adult experiments, microelectrodes were inserted 4.7-5.3 mm lateral to lamba, while for most young experiments microelectrodes were inserted 4.5-4.8 mm lateral to lamba. For VEPs recordings the signal was amplified (10,000 fold), band-pass filtered (0.1 - 120 Hz), digitized and averaged (60-200 events in packs of 10-20 events each). Partial averages from single packs were used to establish response reliability (Pizzorusso et al., 1997; Porciatti et al., 1999). Visual stimuli were gratings of various spatial frequencies and contrast generated by a VSG2/5 card (Cambridge Research Systems, Rochester, England) on a display (Sony Multiscan G500) that was positioned 20-30 cm in front of the rat's eyes to include the central visual field. The mean luminance was 15 cd/m^2. Contrast was defined as C = Lmax-Lmin / Lmax+Lmin, where Lmax and Lmin are the maximum and minimum luminance, respectively.

Steady-state VEPs: VEP recordings in steady-state mode were used to measure spatial resolution. Steady-state VEPs were recorded in response to gratings with sinusoidal modulation of contrast at different temporal frequencies. Visual response was measured as the amplitude (μV) of the second harmonic of the stimulation frequency, calculated after Fourier analysis of the signal (Fagiolini et al., 1997; Pizzorusso et al., 1997). Noise was the average of amplitudes of at least three VEP responses with both eyes closed. Visual acuity was assessed by presenting gratings of variable spatial frequencies alternating at 4-6 Hz (90% contrast). Acuity was taken as the highest spatial frequency that evoked a VEP response greater than the mean value of the noise.

Transient VEPs: We recorded transient VEPs to estimate latency of visual drive in the visual cortex. Transient VEPs were recorded in response to the abrupt contrast reversal of a square-wave grating (spatial frequency 0.1 c/deg, contrast 90%) at the frequency that evokes maximal VEP amplitude in the rat (0.5 Hz; Pizzorusso et al., 1997). At least 60 responses were averaged.

Immunohistochemistry

For immunohistochemical analysis, each animal was perfused through the heart with PBS followed by fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (Caleo et al., 2003). Brains were dissected, post-fixed for 2 hr in the same fixative, rinsed in buffer and cryoprotected in 30% sucrose. Coronal brain sections (50 μm thick) were cut on freezing microtome and collected. For cleaved SNAP-25 detection, coronal sections were blocked with 10% normal goat serum in PBS, incubated overnight in a solution with anti-BoNT/E-cleaved SNAP-25 antibody 1:300, 1% serum and 0.3% Triton X-100. and then reacted with a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) revealed by avidin-biotin-peroxidase complex (ABC kit, Vector Laboratories) and diaminobenzidine (DAB) reaction. For SNAP-25 immunostaining, sections were blocked with 10% normal donkey serum, incubated overnight in a solution with anti SNAP-25 antibody 1:200, 1% serum and 0,1% Triton X-100 and then reacted with biotinylated secondary antibody revealed with Avidin FITC 1:200. For Fos immunostaining sections were blocked with 10% normal goat serum, incubated overnight with a solution with anti-Fos antibody (rabbit polyclonal Oncogene Science Ab-5) 1:3000, 1% serum and 0,1% Triton X-100, reacted with a biotinylated secondary antibody and processed for DAB reaction.

Immunoblotting

For immunoblotting, proteins (Viegi et al., 2001) were extracted from dissected cortices with lysis buffer (1% Triton X-100, 10% glycerol, 20 mM Tris-HCl, pH7.5, 150 mM NaCl, 10 mM EDTA, 0.1 mM Na_3V0_4, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1mM PMSF). Protein extracts (10 mg) were separated by electrophoresis and blotted, and filters were incubated with the antibody recognizing the BoNT/E-cleaved form of SNAP-25 (1:50 diluition) reacted with HRP-conjugated goat anti-rabbit secondary antibody (Bio-Rad) and developed by ECL (Amersham, UK).

Statistical analysis

Differences between two groups have been assessed with t-test. Level of significance $p < 0.001$.

RESULTS

Effects of BoNT/E on adult visual cortex

Botulinum neurotoxin E (BoNT/E) inhibits glutamate release and blocks neuronal activity in the periferal nervous system (PNS) through the cleavage of the synaptic protein SNAP-25 (Schiavo and van der Goot, 2001; Turton et al., 2002). One day after BoNT/E injection in the visual cortex of adult rats, cleaved SNAP-25 is clearly detectable by immunostaining in the infused region (Fig 1A). Accordingly, the intact form of SNAP-25 is absent in the synaptic terminals of visual cortical neurons in the injected region (Fig 1B). In visual cortical slices of normal adult rats, the pattern of SNAP-25 labelling is typical of a pre-synaptic vesicle marker, with an high density of labelled neuropil punctate structures likely corresponding to presynaptic terminals and absence of label in cell bodies. This pattern of labelling is completely absent in visual cortical slices of BoNT/E-injected rats, indicating that BoNT/E effectively cleaves SNAP-25.

To evaluate the responsiveness of visual cortical neurons after BoNT/E injection, we performed electrophysiological recordings *in vivo*. BoNT/E injection completely suppresses visual evoked potentials (VEPs) recorded intracortically in response to a sinusoidal grating of optimal spatial resolution (0.1 c/deg) that reverse abruptly at 0.5 Hz (Fig 1C). Indeed the waveform of intracortical transient VEPs in normal adult rats consists of an early negative wave, peaking between 100 and 200ms, and a late positive wave, peaking between 600 and 800 ms (Fagiolini et al., 1994). This classical waveform can not be recorded in BoNT/E-injected animals, demonstrating that BoNT/E is able to block visually-evoked neuronal activity.

The extent of neuronal activity blockade in BoNT/E-injected animals has been determined through Fos immunohistochemistry (Kaczmarek and Chaudhuri, 1997; Chaudhuri, 1997). Indeed, when animals kept in the dark for three days are re-exposed to light, a strong expression of Fos protein can be detected in their visual cortex. This activity-induced expression of Fos protein is not detected in BoNT/E-injected visual cortex, as shown by interruption of the typical Fos nuclear labelling (Fig 1D). This is consistent with the blockade of visually evoked activity by BoNT/E injection.

The duration of BoNT/E effects in adult visual cortex has been established by analysing the time course of SNAP-25 cleavage after a single BoNT/E injection. Cleaved SNAP-25 is clearly detected one day after BoNT/E-injection, the band is slightly reduced at 14 days and it is no longer detectable at 21 days (Fig 2A). We can therefore conclude that BoNT/E effects in the adult visual cortex are over three weeks after the injection.

To determine if long-term blockade of visual cortical activity by BoNT/E can have detrimental effects on visual system function, we measured its spatial and contrasr response once BoNT/E effects are extinguished. The spatial resolution of visual system, i.e. visual acuity, can be determined by measuring VEPs amplitude in response to sinusoidal gratings of various spatial frequencies alternated in phase at 4 Hz. In adult pigmented rats, the curve relating VEP amplitude to stimulus spatial frequency is approximately low-pass-shaped for spatial frequencies >0.1 c/deg (Fagiolini et al., 1994). Visual acuity is determined as the spatial frequency that gives VEPs response amplitude above the noise (Fig 2B). The prolonged blockade of visual cortical activity by BoNT/E does not modify the curve relating VEPs amplitude to stimulus spatial frequency (Fig 2B). Moreover visual acuity determined after recovery from BoNT/E effects is not significantly different from normal one (1.025 ± 0.047 cycle/deg in normal rats; 1.083 ± 0.047 cycle/deg in BoNT/E-injected rats after recovery; t-test, P>0.1; Fig 2C). We also recorded transient VEPs in response to abrupt reversal (0.5 Hz) of the same stimulus gratings in BoNT/E-injected animals after recovery. Indeed latency of the early negative peak of response in these animals is not affected by prolonged blockade of activity thought BoNT/E (107.5 ± 5.6 ms in normal rats; 112 ± 6.5 ms in BoNT/E-injected rats after recovery; t-test. P>0.1; Fig. 2D). We can therefore conclude that the prolonged blockade of cortical activity through BoNT/E during adulthood has no detrimental effect on visual system function.

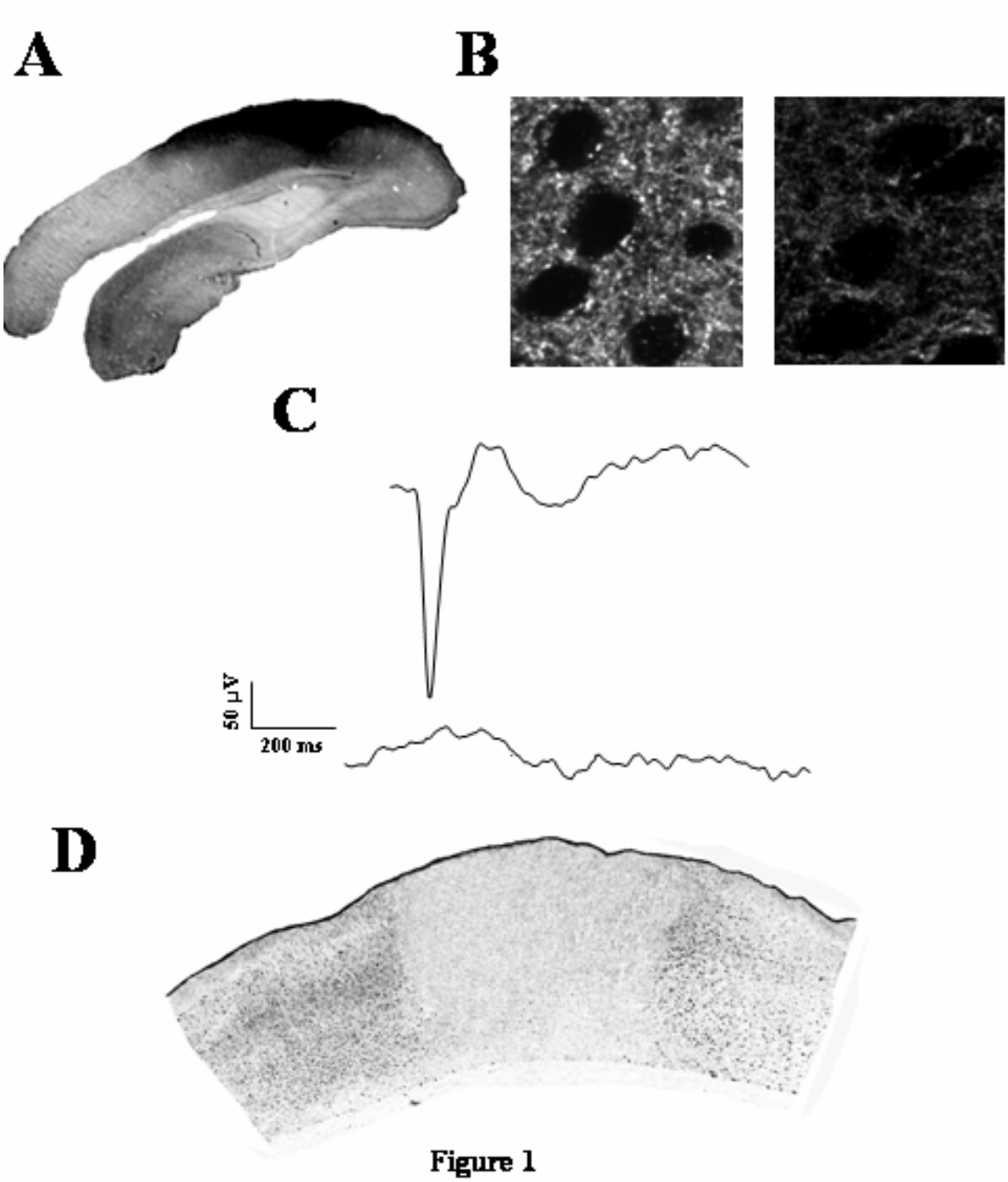

Figure 1

Characterization of BoNT/E activity in adult visual cortex

(A) Coronal section of rat visual cortex one day after BoNT/E injection. Immunostaining for cleaved SNAP-25 (dark labeling) is restricted to the injected region. (B) SNAP-25 immunostaining in normal (left) and BoNT/E-injected (right) adult visual cortex. The dense puncta ring labelling typical of SNAP-25 immunostaining is abolished by BoNT/E injection. (C) VEPs recording from visual cortex of normal (up) and BoNT/E-injected (bottom) rats. BoNT/E injection completely suppresses VEPs response. (D) Distribution of Fos immunostaining in a coronal section from BoNT/E-treated animal. Fos labelling is clearly absent in the BoNT/E-injected region.

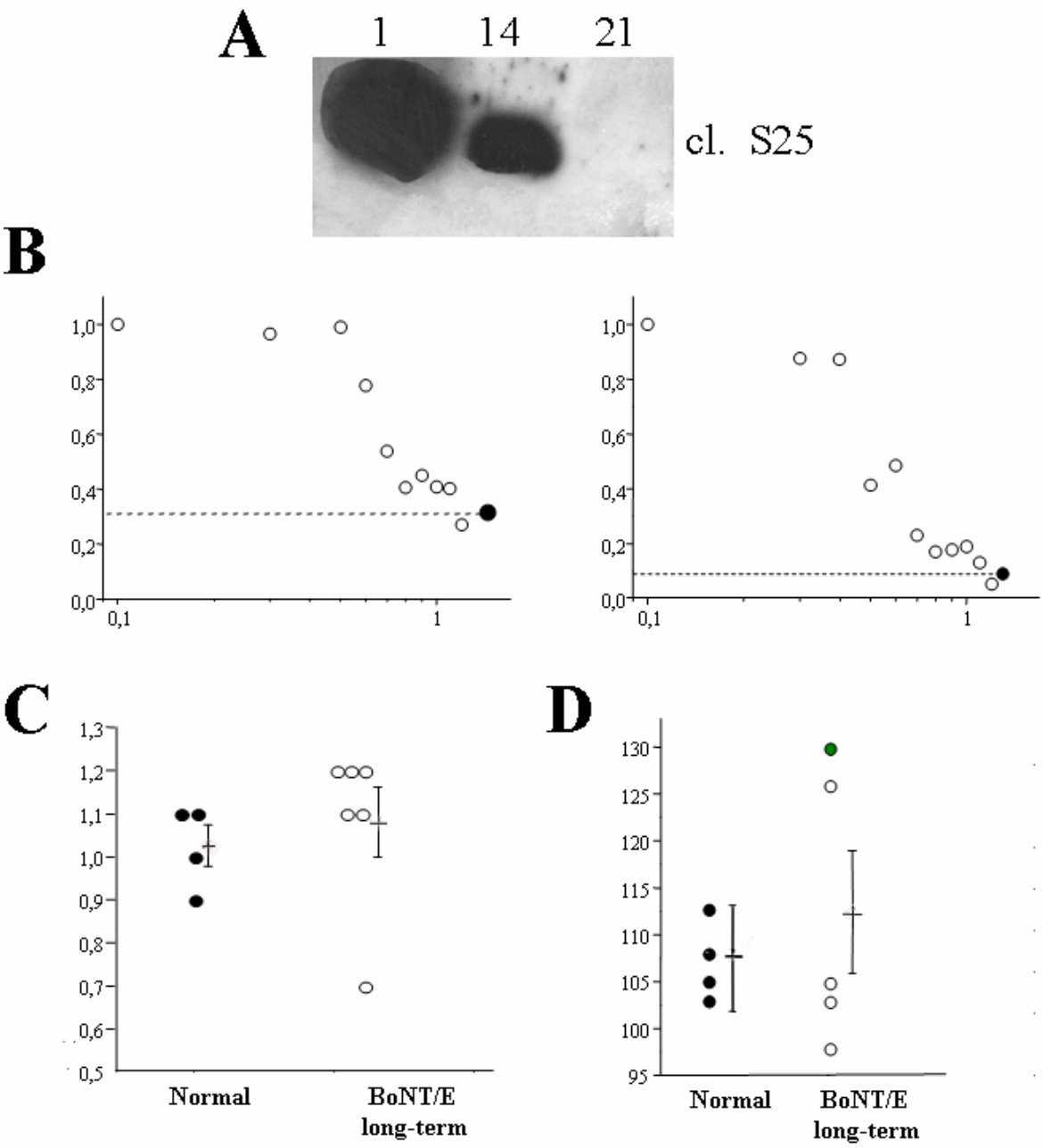

Figure 2

Time-course of BoNT/E activity in adult visual cortex and its effects of on visual system functional properties

(A) Immunoblotting for cleaved SNAP-25 (cl. S25) on protein extracts from visual cortex of adult rats at 1, 14 and 21 days after BoNT/E injection. (B) Visual acuity curve for a normal adult rat (left) and for a rat injected with BoNT/E 21 days before (right). (C) Visual acuity in normal animals (solid circle) and in animals injected with BoNT/E 21 days before (empty circle). Each point indicates one animal and horizontal bars represent mean values (± S.E). (D) Latency of the first negative peak of response to transient VEP in the same experimental groups.

Effects of BoNT/E on young visual cortex

As sensory stimulation strongly influences the maturation of neuronal system by altering the firing pattern in the developing brain (Grubb and Thompson, 2004), we decided to inject BoNT/E in the visual cortex of P14 rats, i.e. just before eye opening. To rule out the diffusion of BoNT/E in the controlateral, not-injected cortex, we recorded flash VEPs one day after BoNT/E injection in both cortices. VEP response to flash is completely absent in the BoNT/E-injected visual cortex, while a clear VEP response can be recorded in the controlateral, not-injected cortex with a latency of (Fig 3A). Moreover cleaved SNAP-25 is present in BoNT/E-injected visual cortex but not in the controlateral, not-injected cortex (Fig 3B). This demonstrates that BoNT/E does not diffuse in the controlateral, not-injected visual cortex that therefore receives a normal, visually-driven activity.

To determine the duration of BoNT/E effects in young visual cortex, we analyzed the time-course of SNAP-25 cleavage after BoNT/E injection (Fig 3C). Indeed cleaved SNAP-25 is clearly detected one day after BoNT/E infusion in young visual cortex and the band persists well after one week. At 14 days cleaved SNAP-25 is absent in half of the animals and it is surely no longer present at 21 days. This allowed us to estimate that BoNT/E effects on young visual cortex last for about 2 weeks and they are fully extinguished at 3 weeks when we performed electrophysiological analysis of visual system functional properties.

The functional properties of visual system develop gradually during postnatal development and dark rearing (DR) from birth prevents this normal maturation (Fagiolini et al., 1994). To establish if the prolonged blockade of visual cortical activity in young animals has the same effect of dark-rearing on visual system development, we assessed visual acuity by VEP recordings in rats injected with BoNT/E at P14. Recordings were made from BoNT/E-injected cortex at P36, a time when the effects of BoNT/E are extinguished and visual acuity has reached its normal value. Vehicle injection at P14 did not influence the postnatal development of visual acuity. Indeed vehicle-injected rats has the same visual acuity of age-matched rats at P36 (1.05 ± 0.05 cycle/deg in vehicle-injected rats versus 1.025 ± 0.047 cycle/deg in normal age-matched rats; t-test, $P>0.1$; Fig 3D). Instead prolonged blockade of visual cortical activity through BoNT/E from P14 to P36 significantly impairs visual acuity development (0.65 ± 0.1 cycle/deg in BoNT/E-injected rats versus 1.05 ± 0.05 cycle/deg in vehicle-injected rats; t-test, $P<0.01$; Fig 3D). The latency of response to transient VEP is also affected

by BoNT/E administration. Indeed latency is significantly higher in rats treated with BoNT/E at P14 with respect to rats injected with vehicle solution (147 ± 9.8 ms in BoNT/E-injected rats versus 115.4 ± 8.2 ms in vehicle-injected rats; t-test. $P<0.01$; Fig 3E). We can therefore conclude that silencing of visual cortical neuronal activity from P14 to P45 by BoNT/E is able to prevent the normal maturation of visual system.

To establish the role of extrinsic cortical activity coming through the callosal fibers on visual system development, we assessed visual acuity through VEP recordings in the visual cortex controlateral to the one injected with BoNT/E. Visual acuity measured in the controlateral cortex resulted significantly lower than normal one (0.591 ± 0.08 cycle/deg in controlateral cortex versus 1.025 ± 0.047 cycle/deg in normal cortex; t-test, $P>0.01$; Fig 3D) and it is not significantly different from that measured in the BoNT/E-injected cortex (t-test, $P>0.01$). Moreover latency of response to transient VEP is abnormally high in controlateral BoNT/E-injected cortex (145.2 ± 11.1 ms in controlateral cortex versus 115.4 ± 11.2 ms in normal cortex; t-test, $P>0.01$; Fig 3E). We can therefore conclude that extrinsic cortical activity coming through callosal fibers is essential for the proper maturation of visual system functional properties.

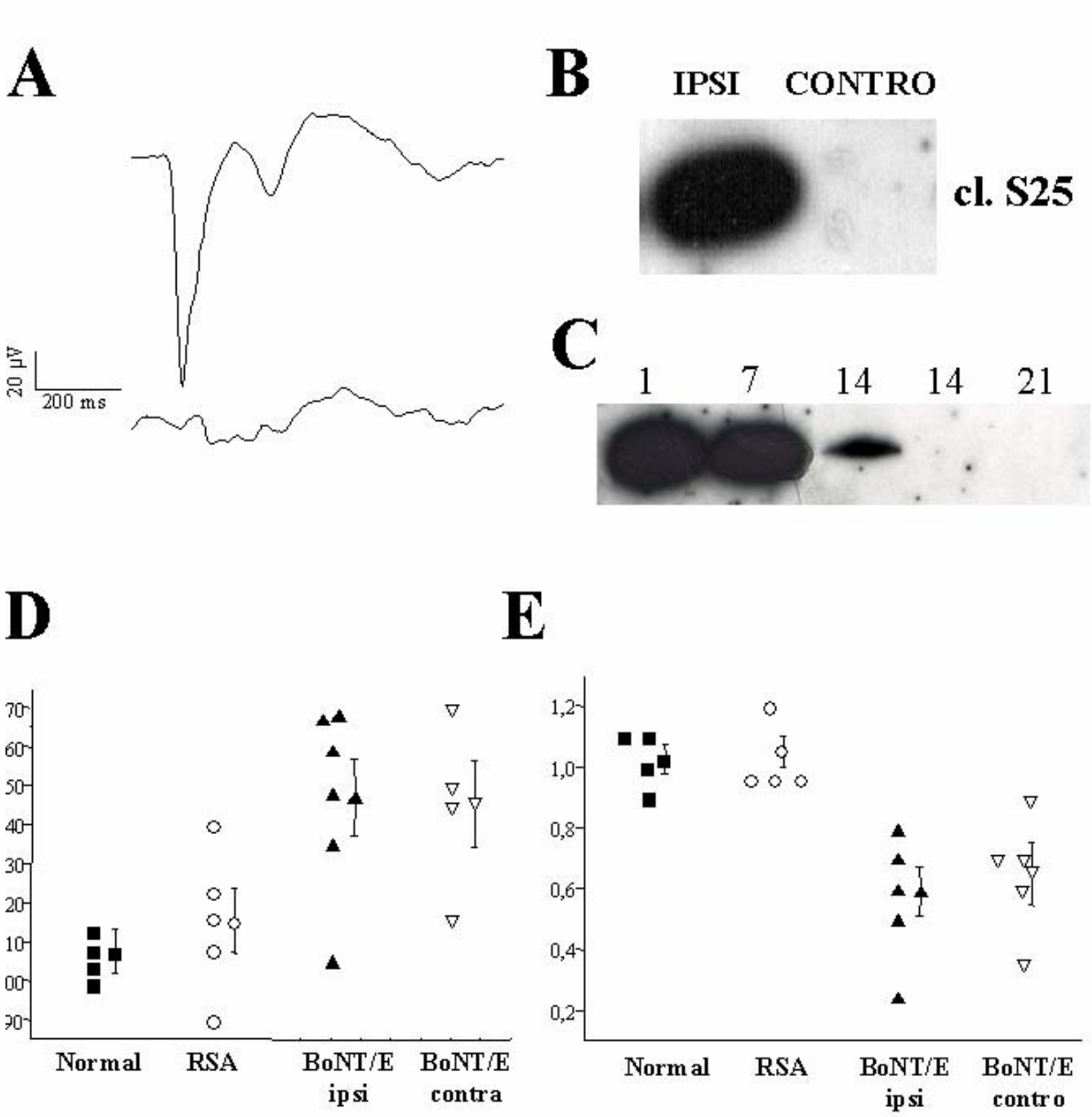

Figure 3

Effects of BoNT/E administration on young visual cortex

(A) VEPs response to flash stimulus recorded from BoNT/E-injected cortex (down) and from controlateral, not-injected one (up). (B) Immunoblotting for cleaved SNAP-25 (cl. S25) on protein extracts from BoNT/E-injected and controlateral, not-injected visual cortex one day after BoNT/E administration (C) Immunoblotting for cl.25 on protein extracts from BoNT/E-injected cortex at different times after injection. (D) Visual acuity in P45 rats determined through VEPs recording from normal (black square), RSA-injected (empty circle), BoNT/E-injected (solid up-triangle) and controlateral, not-injected (empty down-triangle) cortex. All injections were performed at P14. Each point indicates one animal and horizontal bars represent mean values (± S.E) (D) Latency of the first negative peak of transient VEP in the same experimental group.

DISCUSSION

In this study we demonstrated that long-term blockade of cortical activity through BoNT/E injection does not affect visual system function in the adult while it strongly impairs visual system development in young animals. Indeed long-term blockade of visual cortical activity before eye-opening prevents the physiological maturation of visual cortex, maintaining it in a state similar to a young cortex. Moreover also the development of controlateral, not-injected cortex is strongly impaired, suggesting that extrinsic cortical activity carried by callosal fibers is essential for visual system development.

BoNT/E in adult visual cortex

In the PNS BoNT/E is able to block neurotransmitter release and subsequently neuronal activity through its specific interaction with the SNARE protein SNAP-25. In our study we demonstrated that BoNT/E effectively cleaves SNAP-25 once injected in adult visual cortex. The SNAP-25 cleavage is associated with inhibition of visually-evoked neuronal activity in the infused region, as essayed both with electrophysiological and with immunohistochemical techniques. We can therefore conclude that BoNT/E, injected in adult visual cortex, is able to silence neuronal activity through the cleavage of SNAP-25.

BoNT/E effects persist for at least two weeks after one single injection in adult visual cortex. The duration of BoNT/E activity has been estimated through the immunodetection of cleaved SNAP-25, as previous studies (Ashton and Dolly, 1988) demonstrated that inhibition of neurotransmitter release has the same temporal pattern of SNAP-25 cleavage.

The long-term blockade of cortical activity through BoNT/E injection has no permanent deleterious effects on visual system function. Indeed, after recovery from BoNT/E effects, i.e. three weeks after injection, BoNT/E-injected animals display normal visual acuity and latency of response. Of course we did not examine more subtle parameters, such as dendritic spines, that could be influenced by prolonged blockade of activity. However no gross neuronal dysfunctions were detected after

recovery from intracortical BoNT/E treatment in the adult. This validates the use of BoNT/E in cases in which reversible silencing of neuronal activity in selected brain areas may be useful for therapeutic or research purposes.

BoNT/E in young visual cortex.

The long-term blockade of visual cortical activity through BoNT/E before eye-opening affects the development of visual system in a manner similar to dark-rearing. Indeed dark-rearing from birth prevent the normal postnatal maturation of visual system and visual cortical functions determined in adult rats dark-reared from birth are similar to those of young animals (Fagiolini et al., 1994). Likewise functional properties of BoNT/E-injected cortex after recovery are very immature. Visual acuity and response latency are strongly impaired and resemble those of P19-P21 rats. Dark-rearing affects visual system development by strongly reducing the afferent activity to the cortex. In this study we clearly demonstrated that intrinsic cortical activity is essential for the proper postnatal maturation of visual system.

The long-term blockade of activity in BoNT/E-injected cortex in young rats strongly affects also the development of visual cortical properties in the controlateral, not-injected cortex. Indeed visual acuity and response latency in the controlateral, not-injected cortex are typical of an immature cortex and are similar to those of BoNT/E-injected cortex. This is in line with a previous study that demonstrated that callosal sectioning in cats at 1, 2, and 3 but not at 4 postnatal weeks permanently reduced visual acuity threshold (Elberger, 1988). In our study we found that prolonged activity blockade of one cortex during postnatal period affects the development of visual functions in the controlateral cortex. We therefore clearly demonstrated that the correct anatomical and physiological development of visual system depends on extrinsic cortical activity coming from the controlateral hemisphere through the callosal connections. This is in line with another study of our laboratory that demonstrated that visual experience *per se* is not critical for the correct development of visual system. Indeed Bartoletti and co-workers showed that environmental enrichment is able to prevent the deleterious effects of dark rearing on visual system development, as enriched animals dark-reared from birth show a normal maturation of visual acuity (Bartoletti et al., 2004). This clearly points out that factors not under the control of visual experience may contribute to visual

cortical development and the authors suggest that over-expression of factors important for visual cortical plasticity induced by enriched environment can play an important role in this processes. However also afferent activity coming from areas other than visual ones can influence visual system development. Our results showing that visual acuity maturation in one hemisphere depends also on the presence of a proper pattern of activity in the controlateral cortex support this hypothesis.

Our study also suggests that the different cortical "modules" do not mature on their own in a parallel way but they strongly interact during development probably through inter-cortical connections (Shimojo and Shams, 2001; Pallas, 2001). Indeed many recent animal and human studies of cross-modal plasticity reveal that sensory modalities in early stages of development are not as inherently distinct and independent as was previously once thought. Actually in humans that have had sensory deprivation in one modality starting from an early period of life the cortical area normally devoted to that modality is used by some other modality. For examples in early deaf individuals, non-auditory stimuli, including American sign language, can activate language cortex (Neville et al., 1998) and visual stimuli can activate putative auditory areas (Neville, 1990). In the early blind, improved auditory performance (Roder et al., 1999) and auditory and somatosensory activation of occipital cortex have been reported (Weeks et al., 2000; Cohen et al., 1997). Indeed in congenitally blind subjects the right occipital cortex participates in a functional network for auditory localization and the occipital activity seems to arise from connections with posterior parietal cortex (Weeks et al., 2000). These studies therefore demonstrate that the brain has a significant degree of neuronal plasticity in early stages of life so that to compensate the loss of one sensory modalities with changes in the remaining modalities. This argues against the most stringent version of brain segregation and modularity of sensory modalities and our results further support this as we demonstrated that development of visual functions in one hemisphere strictly depends on extrinsic activity coming from the controlateral hemisphere through callosal fibers.

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