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Degeneration of cholinergic basal forebrain nuclei after focally evoked status epilepticus

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ABSTRACT

Status epilepticus (SE) of limbic onset might cause degenerative phenomena in different brain structures, and may be associated with chronic cognitive and EEG effects. In the present study SE was evoked focally by microinfusing picomolar doses of cyclothiazide + bicuculline into the anterior extent of the piriform cortex (APC) in rats, the so-called *area tempestas*, an approach which allows to evaluate selectively the effects of seizure spreading through the natural anatomical circuitries up to secondary generalization. In the brain of rats submitted to SE we analyzed neuronal density, occurrence of degenerative phenomena (by Fluoro-Jade B-FJB-staining) and expression of heat shock protein-70 (HSP-70) in the piriform cortex, the hippocampus and ventromedial thalamus. We further analyzed in detail, the loss of cholinergic neurons, and the presence of FJB- and HSP-70 positive neurons in basal forebrain cholinergic areas, i.e. the medial septal nucleus (MSN, Ch1), the diagonal band of Broca (DBB, Ch2 and Ch3) and the Nucleus basalis of Meynert (NBM, Ch4). In fact, these nuclei are strictly connected with limbic structures, and play a key pivotal role in different cognitive functions and vigilance. Although recent studies begun to investigate these nuclei in experimental epilepsy and in persons with epilepsy, conflicting results were obtained so far. We showed that after severe and long-lasting, focally induced limbic SE there is a significant cell loss within all of the abovementioned cholinergic nuclei ipsi- and contralaterally to the infusion site. In parallel, these nuclei show also FJB and heat shock protein-70 expression. Those effects vary depending on the single nucleus assessed and on the severity of the SE seizure score. We also showed the occurrence of cell loss and degenerative phenomena in limbic cortex, hippocampus and limbic thalamic areas. These novel findings show direct evidence of SE-induced neuronal damage which is solely due to seizure activity ruling out potential confounding effects produced by systemic pro-convulsant neurotoxins. A damage to basal forebrain cholinergic nuclei, which may underlie cognitive alterations, is documented for the first time in a model of SE triggered focally.

1. Introduction

Temporal lobe seizures are by far the most common types of seizures in humans (Panayiotopoulos, 2002), and they originate in the mesial temporal lobe structures, which in most cases correspond to allo- or meso-cortical limbic areas. Often seizures remain limited within limbic structures while, sometimes, they may become generalized. By definition, seizures are self-limiting, lasting < 2–3 min. However, when self-limiting mechanisms fail, seizures can prolong as continuous convulsive activity lasting over 5 min, which characterizes a seizure activity defined as" status epilepticus" (SE)(for more detailed definitions of subtypes of SE, see also Trinka et al., 2015). A major question related to SE is whether SE of focal origin can cause detrimental effects to the brain, and if this occurs, which is the severity and site-specificity of these effects. These questions cannot be fully answered in humans, as in the routine clinical *scenario* one can evaluate SE patients only after they have experienced prolonged SE (whose precise duration can be rarely assessed), and imaging data are obtained only after SE and cannot be compared with pre-seizure ones.

In the past decades several models of limbic SE have been developed

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in rodents: the most widely used consist in systemic administration of kainic acid (KA, an AMPA/KA glutamic acid receptor agonist) and pilocarpine (a muscarinic cholinergic receptor agonist) (for reviews, see Turski et al., 1983; Tremblay et al., 1984; Nitecka et al., 1984). In these models, animals show prolonged limbic seizures, which may evolve into secondarily generalized ones, and SE which can last up to several hours. SE induced by these approaches causes cell loss in different brain regions, including hippocampus, thalamus and neocortex (e.g. see Nitecka et al., 1984). In patients, SE of limbic origin potentially can spread to other limbic or extra-limbic areas through the natural anatomical pathways reciprocally connected with the triggering site. Thus, animal models in which SE is induced focally in the limbic system might be closer to the human condition compared with systemic KA or pilocarpine. In particular, this might allow to decipher what is really produced by the engagement of neurons during seizure and to rule out the potential direct neurotoxic effects of systemically administered kainic acid (Lau and Tymianski, 2010) or the potential haemodynamic alterations caused by systemic pilocarpine (Dage, 1979). In the last decades different models of focally-induced seizures/SE were produced, including focal electrical stimulation protocols (Lothman et al., 1989) or focal chemoconvulsant infusions, such as intrahippocampal (Schwarcz et al., 1978) or intraamygdaloid infusion of Kainic acid (Ben-Ari et al., 1979), or intrahippocampal pilocarpine (Furtado et al., 2002, 2011). In the present study, seizures were evoked focally, from the anterior extent of the deep piriform cortex (APC). This limbic allocortical region was shown to possess a very low threshold to induce seizures when focally microinfused with bicuculline, a GABA-A receptor antagonist (Piredda and Gale, 1985). When bicuculline is injected immediately after cychlothiazide (a desensitization blocker of AMPA glutamatergic receptor) into this region, it induces SE rather than episodic seizures. This SE is self-sustaining and often lasts several hours (Fornai et al., 2000, 2005; Giorgi et al., 2003, 2006, 2008). SE elicited by this approach does not lead to death since it does not impair vital functions; this SE in most cases is self-sustaining but also self-limited (Fornai et al., 2000, 2005; Giorgi et al., 2003).

This model has proven to be particularly useful to detect which brain areas are selectively engaged by limbic seizures. This has been assessed by different in situ techniques: (i) 2-desoxyglucose uptake (Cassidy and Gale, 1998; Dybdal and Gale, 2000; Giorgi et al., 2008); (ii) immediate early gene expression (Maggio et al., 1993; Lanaud et al., 1993; Giorgi et al., 2008); (iii) heat shock protein expression (Shimosaka et al., 1992); (iv) focal pharmacological modulation of seizures within selective brain regions supposed to be involved in seizure circuitry (Maggio and Gale, 1989; Shimosaka et al., 1994; Halonen et al., 1994; Tortorella et al., 1997; Vismer et al., 2015). Such a model was also demonstrated to own a heuristic value for describing the functional anatomy of limbic circuitries. In fact, a functional mapping of anatomical connectivity and the strength of these connections was recently postulated based on these data (Vismer et al., 2015). Thus, the APC, named *area tempestas* by Dr. Gale when she first described its seizure initiating propensity (Piredda and Gale, 1985), provided a tool to explore brain connectivity beyond seizure activity.

Within this frame we were particularly interested to investigate a group of cholinergic neurons in the basal forebrain which are highly connected with limbic structures and play an important role in different brain functions. These cholinergic nuclei of the basal forebrain are classified as Ch 1–4 areas by Mesulam et al. (1983a), corresponding to the medial septal nucleus (Ch1) (MSN), the Diagonal Band of Broca (Ch2-Ch3) (DBB), and the Nucleus basalis of Meynert (Ch4) (NBM). They play a pivotal role in learning and memory (e.g. Everitt and Robbins, 1997; Sarter and Bruno, 1997; Roland et al., 2014), apart from playing an activating effect on EEG (e.g., see Buzsaki et al., 1988; Furman et al., 2015) and a significant role also in regulating vigilance and sleep/waking state (Szymusiak, 1995; Zant et al., 2016; Lagos et al., 2012), which can also indirectly affect cognition. Patients with seizures originating in limbic structures show often subtle cognitive and sleep alterations, which are not fully explained by the occurrence of hippocampal sclerosis (for a review, see Bell et al., 2011). Even more, SE in patients has been associated by several authors with delayed cognitive impairment (e.g. Dodrill and Wilensky, 1990; see also the review by Helmstaedter, 2007) and this seems to be the case especially after generalized convulsive SE (e.g. Power et al., 2018). Less is known about chronic effects on EEG and sleep parameters by SE (Bazil and Anderson, 2001). Similarly to what above mentioned concerning neuronal damage, also the effects of SE on parameters potentially linked with cholinergic functions (cognitive and EEG/sleep), are difficult to assess in patients, mainly because of the potential bias of an effect of the SE- precipitating insult by itself on these parameters, and of the lack of precise knowledge of these parameters in the single patients before SE (e.g. see Helmstaedter, 2007).

Thus, in the present study we wish to disclose whether focally induced limbic SE from APC, apart from altering limbic allocortical and allothalamic regions, may affect NBM, MSN and DBB.

As said, all these cholinergic regions are highly connected with the cerebral cortex and especially with limbic areas. In particular, Ch4 receives direct cortical afferents mainly from the piriform cortex, the medial temporal cortex and entorhinal cortex, orbitofrontal cortex and insula, (Mesulam and Mufson, 1984). Ch1 receives the main cortical afferents from the hippocampus (Haghdoost-Yazdi et al., 2009), but also from the piriform cortex and, to a lesser extent, from the amygdala (Swanson and Cowan, 1979). Ch2 and Ch3 share some afferents from the same areas innervating Ch1 (e.G. *hippocampus*) (Haghdoost-Yazdi et al., 2009) andare interconnected with Ch1 (Swanson and Cowan, 1979). Ch1 sends its efferents mainly to the hippocampus, while Ch4 neurons widely innervate the amygdala, periamygdaloid (perirhinal) cortex and the rest of the cortex (Mesulam et al., 1983a, 1983b). Ch2 seems to be the main source of Ach to the hippocampus and hypothalamus, and Ch3 to the olfactory bulb (Mesulam et al., 1983b), and it also sends efferents to the piriform and enthorhinal cortices (Zaborszky et al., 2012).

We show that all three cholinergic nuclei, although with slight differences, show degenerative phenomena after SE induced focally from the APC, we also describe degenerative phenomena in limbic and thalamic structures.

2. Material and methods

2.1. Animals

50 adult male Sprague-Dawley rats (Harlan Laboratories produced in house) weighing 280–320 g were used. All animals were kept under environmentally controlled conditions (room temperature 22 °C; humidity 40%) with food and water ad libitum and with a regular 12 h light/dark cycle. Rats were handled in accordance with the Guidelines for Animal Care and Use of the National Institutes of Health, and adequate measures were taken to minimize animal pain. (Authorization N° 1204/2015-PR).

2.2. Experimental design and drugs administration

Rats were submitted to surgery and implanted with a guide cannula into the left APC under deep anesthesia. Twenty-four h after recovery from surgery they were submitted to microinfusions of either saline or bicuculline + cyclothiazide into the APC and evaluated behaviorally for seizures occurrence. Cyclothiazide (Hellobio, Bristol, UK), was dissolved in a solution of saline + Dimethilsulfoxide (DMSO 20%) (200 pmol in 120 nL); bicuculline methiodide (Sigma Aldrich, Milan, Italy) was dissolved in saline (118 pmol in 120 nL). For cyclothiazide + bicuculline co-infusions, 120 nL of cyclothiazide solution and 120 nL of bicuculline solutions were injected into the APC, 5 min apart, at the rate of 60 nL/min. Control animals were injected with a vehicle solution (saline alone and saline + DMSO 20%), same volumes and flow rates as