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# Activation of either the $ET_A$ or the $ET_B$ receptors is involved in the development of electrographic seizures following intrahippocampal infusion of the endothelin-1 in immature rats

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ABSTRACT

The period around birth is a risky time for stroke in infants, which is associated with two major acute and subacute processes: anatomical damage and seizures. It is unclear as to what extent each of these processes independently contributes to poor outcome. Furthermore, it is unclear whether there is an interaction between the two processes — does seizure activity cause additional brain damage beyond that produced by ischemia and/or does brain damage foster seizures?

The model of focal cerebral ischemia induced by the intrahippocampal infusion of endothelin-1 (ET-1) in 12-day-old rat was used to examine the role of the endothelin receptors in the development of focal ischemia, symptomatic acute seizures and neurodegeneration. ET-1 (40 pmol/μl) was infused either alone or co-administered with selective antagonists of $ET_A$ (BQ123; 70 nmol/μl) or $ET_B$ receptors (BQ788; 70 nmol/1 μl). Effects of activation of $ET_B$ receptors were studied using selective agonist 4-Ala-ET-1 (40 pmol/1 μl).

Regional cerebral blood flow (rCBF) and tissue oxygenation ($pO_2$) were measured in anesthetized animals with a Doppler-flowmeter and a $pO_2$-sensor, respectively. Seizure development was assessed with video-EEG in freely moving rats. Controls received the corresponding volume of the appropriate vehicle (10 mM PBS or 0.01% DMSO–PBS solution; pH 7.4). The extent of hippocampal lesion was determined using FluoroJade B staining performed 24 h after ET-1 infusion.

Infusion of ET-1 or ET-1+$ET_B$ receptor antagonist reduced rCBF to ~25% and $pO_2$ to ~10% for about 1.5 h, whereas selective $ET_B$ agonist, ET-1+$ET_A$ antagonist and the PBS vehicle had only negligible effect on the rCBF and $pO_2$ levels. Reduction of rCBF was associated with the development of lesion in the injected hippocampus.

In all groups, except sham operated and PBS controls, epileptiform activity was observed after activation of the $ET_A$ or the $ET_B$ receptors. The data revealed a positive correlation between the severity of morphological damage and all the measured seizure parameters (seizure frequency, average and total seizure duration) in the ET-1 group. In addition, the severity of morphological damage positively correlated with the average seizure duration in animals after infusion of ET-1+$ET_A$ receptor antagonist or after infusion of ET-1+$ET_B$ receptor antagonist.

Our results indicate that the activation of $ET_A$ receptors is crucial for ischemia development, however either $ET_A$ or $ET_B$ receptors mediate the development of seizures following the application of ET-1 in immature rats. The dissociation between the ischemic-producing and seizure-producing processes suggests that damage is not necessary to induce seizures, although it may exacerbate them.



## Introduction

The period around birth is a risky time for stroke in infants. Ischemic stroke has been diagnosed in about 1/4000 full-term infants (Nelson and Lynch, 2004) and perinatal stroke represents the second most frequent cause of acute seizures in this age group (Levy et al., 1985). Furthermore, stroke is responsible for long-term cognitive and/or neurological sequelae, including epilepsy (Lynch et al., 2002).

Neonatal stroke is associated with two major acute and subacute processes: anatomical damage and seizures. It is unclear as to what extent each of these processes independently contributes to poor outcome. Furthermore, it is unclear whether there is an interaction between the two processes — does seizure activity cause additional brain damage beyond that produced by ischemia and/or does brain damage foster seizures?

Clinical studies have yielded inconsistent results on these questions. For example, McBride et al. (2000) reported that prolonged or frequent seizures in the context of impaired blood flow may exacerbate ischemic brain damage in the developing brain. Likewise, Shah et al. (2014) have

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demonstrated that a higher seizure burden in neonates with hypoxic/ischemic encephalopathy was correlated with greater damage on MRI. On the other hand, the multicenter study reported by Kwon et al. (2011) came to the conclusion that clinical seizures in neonatal hypoxic/ischemic encephalopathy have no independent impact on neurodevelopmental outcome.

Experimental studies have been equally inconclusive. Wirrell et al. (2001) reported that seizures induced by kainate in P10 rats following carotid ligation significantly exacerbated brain injury. These results contrast with the studies of Cataltepe et al. (1995) in which status epilepticus, induced by bicuculline after hypoxic/ischemic insult, did not exacerbate brain damage in P7 rats. It is unclear what the reasons may be for these differing experimental results, but it is clear that the experimental approaches involved different mechanisms for inducing ischemia and different independent agents for inducing seizures.

An animal model that better mimics the human situation in which his seizures occur as a natural outcome of focal ischemia would provide a more rational experimental approach to answering these questions concerning the relationships among hypoxic/ischemic insult, seizures, brain damage and neurological/cognitive outcome. We believe that the model of focal cerebral ischemia induced by the infusion of the vasoconstrictive agent endothelin-1 (ET-1) into the brain parenchyma satisfies this criterion. In animals, this model has been shown to be highly reproducible and to mimic human stroke (Gilmour et al., 2004). Intracerebral infusion of ET-1 causes a substantial reduction of local blood flow (Fuxe et al., 1997) and ischemia-related changes in aminoacids level concentrations (Van Hemelrijck et al., 2005), induces convulsive behavior (Gross and Weaver, 1993; Nagasaka et al., 1999) and produces morphological damage (Driscoll et al., 2008; Tsenov et al., 2007). Furthermore, unilateral intrahippocampal injection of ET-1 in immature animals leads to the development of electrical seizures with behavioral manifestations and produces morphological damage within the treated hippocampus (Mateffyova et al., 2006; Tsenov et al., 2007).

Simonson and Herman (1993) have shown that ET-1 acts through the G-protein linked receptors $ET_A$ and $ET_B$. Furthermore, these two receptors have been demonstrated to have different localization within nervous tissue. $ET_A$ receptors are located predominantly in cerebrovascular smooth muscle cells (Pierre and Davenport, 1998), while $ET_B$ receptors are distributed on vascular endothelial cells (Stenman et al., 2002), on astrocytes (Baba, 1998) and on microglia (McLarnon et al., 1999).

The consequences of ET-1 injection into the brain parenchyma, as well as the roles of the endothelin receptors, $ET_A$ and $ET_B$, in cerebral ischemia and ischemia-induced changes have been relatively well described in the brains of adult animals (Hama et al., 1997; Schaller, 2006). However, similar studies concerning the role of $ET_A$ and $ET_B$ in ischemia-induced effects in immature brains are largely lacking.

In the present study, we focus on the following questions:

- How is ET-1 induced ischemia mediated in the hippocampus of immature rats? Is there a specific receptor type involved?
- How are seizures induced in the ET-1 model? Is it by the same mechanism as ischemia or are different processes involved?

## Materials and methods

### *Animals*

Experiments were performed on 12-day-old (P12; n = 134) albino male Wistar rats bred by the Institute of Physiology of the Academy of Sciences of the Czech Republic (approval #CZ11760353 and #1398/2014-MZE-17214). The day of birth was defined as day 0. Rats were housed in a controlled environment (temperature 22 ± 1 °C, humidity 50–60%, lights on 06:00–18:00 h) with free access to food and water. Experiments were approved by the Animal Care and Use Committee of the Institute of Physiology of the Academy of Sciences of the Czech Republic and by The Central Committee of the Academy of Sciences of the Czech Republic (approval #095/2010). The Institute of Physiology possesses NIH Statement of Compliance with Standards for Humane Care and Use of Laboratory Animals #1396/2014-MZE-17214, valid until 12/31/2018. Animal care and experimental procedures were conducted in accordance with the guidelines of the European Union directive 2010/63/EU.

### *Drugs*

Focal ischemia was induced by infusion of ET-1 (*#E7764, Sigma-Aldrich, St. Louis, MO, USA*), dissolved in 10 mM phosphate buffer, pH 7.4 (*#79382, Sigma-Aldrich, USA*), in a concentration of 40 pmol and a total volume of 1 μl into the right dorsal hippocampus. Selection of concentration optimal for present study was based on our previously published data (Mateffyova et al., 2006; Tsenov et al., 2007).

To study the role of individual receptors of endothelin, $ET_A$ and $ET_B$, the following specific agonist and antagonists of endothelin receptors were used:

a) Selective $ET_B$ receptor agonist — [$Ala^{1,3,11,15}$]-Endothelin-1 (*#1197, Tocris Bioscience, Bristol, UK*), dissolved in 10 mM phosphate buffer, was used in a concentration of 40 pmol and a total volume of 1 μl.
b) Selective $ET_B$ receptor antagonist — BQ788 (*#1500, Tocris Bioscience, UK*) dissolved in a 0.01% DMSO-phosphate buffer solution (*#W387509, Sigma-Aldrich, USA*) was applied in a concentration of 70 nmol and a total volume of 1 μl.
c) Selective $ET_A$ receptor antagonist — BQ123 (*#1188, Tocris Bioscience, UK*), dissolved in 10 mM phosphate buffer, was given in a concentration of 70 nmol and a total volume of 1 μl.

Selection of optimal concentrations of selective ET receptor antagonists and $ET_B$ receptor agonist were based on previously reported data (Nagasaka et al., 1999) and on our pilot study.

Aliquots of freshly prepared solutions were frozen and stored at −20 °C. On the day of the experiment, the required amount was thawed at room temperature shortly before the infusion. Solutions were used only once after thawing.

Control animals received the corresponding volume of the appropriate vehicle (10 mM phosphate buffer, pH 7.4 or 0.01% DMSO–PBS solution). The infusion speed in all intrahippocampal applications was set to 0.25 μl/min on the perfusion pulse-free pump (*kdS #789200W, WPI, USA*).

*Experiment 1 — Analysis of the role of $ET_A$ and $ET_B$ receptors in local blood flow during focal ET-1-induced ischemia.*

Surgical preparation of animals was performed under 1.5–2% isoflurane anesthesia (*#B306, Abbot Laboratories, Queenborough, UK*). A cannula (*#C315IA/SP, Plastics One Inc., Roanoke, USA*) was stereotaxically inserted into the right dorsal hippocampus for drug application at the level corresponding to AP = 3.7; L = 3.0; H = 3.5 mm relative to bregma in adult animals (Paxinos et al., 1985). Coordinates were recalculated for each animal according to the bregma-lambda distance. In addition, a custom-made needle probe (*#415-346, Perimed, Stockholm, Sweden*) for local blood flow measurement was stereotaxically placed close (850 ± 150 μm) to the cannula used for infusion. Changes in the cerebral blood flow were determined with a laser-Doppler flowmeter monitor (*Periflux 5000, Perimed, Sweden*). The signal was digitalized (*CED Power 1401, Cambridge, UK*) and recorded on a PC. Infusion of drugs was done by an infusion pump after a stable baseline of rCBF was established (approx. 30 min). Due to the high sensitivity of the rCBF sensor to movements related to the cannula extraction–insertion procedure, the infusion antagonist of ET receptors were