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ADMINISTRATION OF SECRETONEURIN IS PROTECTIVE IN HYPOXIC–ISCHEMIC NEONATAL BRAIN INJURY PREDOMINANTLY IN THE HYPOXIC-ONLY HEMISPHERE

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Abstract—Neonatal brain injury is a problem of global importance. To date, no causal therapies are available. A substance with considerable therapeutic potential is the endogenous neuropeptide secretoneurin (SN), which has proven to be beneficial in adult stroke. The aim of this study was to assess its effect in neonatal hypoxic–ischemic brain injury models. *In vitro*, primary hippocampal neurons were pre-treated with vehicle, 1 μg/ml, 10 μg/ml, or 50 μg/ml SN and subjected to oxygen–glucose deprivation (OGD) for six hours. Cell death was assessed after a 24-h recovery period. *In vivo*, seven day-old CD-1 mice underwent unilateral common carotid artery ligation and were exposed to 8% oxygen/nitrogen for 20 min. SN plasma concentrations were serially determined by ELISA after insult. One hour after hypoxia, a subgroup of animals was treated with vehicle or SN. SN plasma concentrations significantly decreased 48 h after insult. The number of caspase-3-positive cells was significantly lower in the hypoxic–ischemic hemisphere in the thalamus of SN-treated animals. In the hypoxic-only hemisphere administration of SN significantly reduced the number of caspase-3-positive cells (in cortex, white matter, hippocampus, thalamus and striatum) and inhibited microglial cell activation in the thalamus. SN has neuroprotective potential in neonatal brain injury. Its main action seems to be inhibition of apoptosis in the aftermath of the insult, predominantly in the hypoxic-only hemisphere. This might be explained by the less pronounced injury in this hemisphere, where blood flow and thus nutrient supply are maintained.

Key words: neonatal brain injury, hypoxia–ischemia, hypoxic-only, secretoneurin, neuropeptide, neuroprotection.

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Abbreviations: bw, body weight; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; OGD, oxygen–glucose deprivation; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PI, propidium iodide; SN, secretoneurin.

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INTRODUCTION

Every year, up to six out of 1000 newborns are inflicted with neonatal encephalopathy of hypoxic–ischemic origin (de Vries and Jongmans, 2010). Advances in perinatal care have improved survival rates of affected infants, but long-term morbidity is still substantial (Shankaran et al., 2012). To date, causal therapies are not available. Secretoneurin (SN) is a highly conserved 33-amino acid polypeptide that is generated by proteolytic processing of secretogranin II (chromogranin C) in endocrine, neuroendocrine and neuronal cells (Kirchmair et al., 1993; Fischer-Colbrie et al., 1995; Schmid et al., 1995; Schurmann et al., 1995). Its biological effects are incompletely understood, but a growing body of evidence suggests an implication in the modulation of neurotransmission and neurogenic inflammation (Saria et al., 1993; Storch et al., 1996; Wiedermann et al., 1999; Wiedermann, 2000). Under physiologic conditions, the largest pool of SN is found in the intestine followed by the central nervous system (Leitner et al., 1996). Following insults such as cellular hypoxia, SN expression is upregulated in a tissue-specific way (Fischer-Colbrie et al., 2005). SN levels have been shown to significantly increase in an *in vivo* model of brain ischemia and in adult patients suffering from ischemic stroke (Marti et al., 2001; Shyu et al., 2008). Our study group has previously shown that SN serum levels in asphyxiated term neonates with hypoxic–ischemic encephalopathy are higher in umbilical cord blood and lower 48 h after birth than in healthy controls (Wechselberger et al., 2016). The exogenous administration of SN in adult models of ischemic stroke in the early post-injury phase has revealed neuron-conserving and pro-regenerative properties (Shyu et al., 2008). Whether the administration of SN has therapeutic potential in neonatal brain injury is currently unknown. Thus, the aim of the present study was to a) determine the natural course of SN blood concentrations after an experimental hypoxic–ischemic insult and b) assess its neuroprotective potential in *in vitro* and *in vivo* models of neonatal hypoxic–ischemic brain injury.

EXPERIMENTAL PROCEDURES

Mouse model of neonatal hypoxic–ischemic brain injury

All animal studies were conducted in compliance with current EU legislation (Directive 2010/63/EU revising

Directive 86/609/EEC) and Austrian law. CD-1 mice (Charles River Laboratories, Sulzfeld, Germany) were bred and kept at the Central Laboratory Animal Facility, Medical University of Innsbruck, Austria. Hypoxic–ischemic brain injury was induced by means of a modified version of the Rice-Vannucci model as described previously (Sheldon et al., 1998; Griesmaier et al., 2014). In brief, seven-day-old CD-1 pups were subjected to right common carotid artery ligation under local (lidocaine/prilocaine; AstraZeneca, Wedel, Germany) and general anesthesia (isoflurane in oxygen, 3.0 vol% induction/1.5 vol% maintenance; AbbVie, Vienna, Austria). After a 90-min recovery period, pups were exposed to a hypoxic environment (8% oxygen in nitrogen) for 20 min under normothermic conditions and subsequently returned to their dams until treatment and/or endpoint analysis. Same-aged CD-1 pups kept under standard housing conditions at all times served as controls.

Determination of SN plasma concentrations following hypoxia–ischemia

Baseline SN plasma concentrations were determined in naïve animals not exposed to hypoxia–ischemia on postnatal day 7. For time course analysis of SN concentrations after hypoxic–ischemic injury, animals were sacrificed by decapitation 1, 6, 12, 24, and 48 h after insult. Blood was collected in EDTA tubes supplemented with aprotinin (Cat. No. A1153, Sigma–Aldrich, Vienna, Austria) 5 mg/ml as a protease inhibitor. Plasma was obtained by centrifugation (1600*g* for 15 min at 4 °C), pooled by timepoint to obtain a minimum volume of 150 μl/sample (approximately four animals/sample) and stored at −70 °C until further analysis. SN concentrations were determined in duplicate in at least three independent sample pools per timepoint by means of a competitive peptide enzyme immunoassay (Secretoneurin EIA, Cat. No. S-1307, Bachem Peninsula Laboratories, San Carlos, CA, USA) according to the manufacturer's instructions. Absorbance was measured at 450 nm by means of a multichannel 96-well visible microplate reader (Hidex Sense™, HVD Lifesciences, Vienna, Austria).

Cell culture experiments

Primary hippocampal cultures were prepared from 16.5-day-old embryonic mice as described previously (Obermair et al., 2004; Griesmaier et al., 2014). Oxygen–glucose deprivation (OGD) as a cell culture model of hypoxic–ischemic injury was performed on *in vitro* day 10 (Griesmaier et al., 2014). In brief, primary hippocampal neurons cultivated on glass coverslips were randomly assigned to one of the following treatment groups: (i) healthy control cells receiving no treatment, (ii) OGD + vehicle 1× phosphate-buffered saline (PBS), (iii) OGD + SN 1 μg/ml, (iv) OGD + SN 10 μg/ml or (v) OGD + SN 50 μg/ml. SN was purchased from Anaspec (Secretoneurin, Cat. No. 62673AS, MoBiTec, Göttingen, Germany). Substances were administered 30 min prior to OGD. In treatment groups exposed to OGD, coverslips were switched to a glucose-free salt solution (137 mM NaCl, 5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.22 mM KH_2PO_4, 10 mM HEPES, 2 mM $CaCl_2$) and transferred to an anoxic environment (95% N_2/5% CO_2 at 37 °C, hypoxia chamber, Stemcell Technologies, Cologne, Germany) for six hours. Afterward, they were re-transferred to their respective glial feeder layers for a 24-h reoxygenation period with standard glucose supply. Control cells were cultivated under standard conditions at all times.

Neuronal cell death was assessed after recovery by means of fluorescence imaging using 3.3 μg/ml propidium iodide (PI) and 0.3 μM calcein AM as described previously (Griesmaier et al., 2014). Cell death was determined by a blinded observer calculating a ratio of PI-positive/(PI- + calcein AM-) positive cells in 32 visual fields per treatment using 100-fold magnification.

Evaluation of SN treatment effects *in vivo*

After hypoxic–ischemic injury induction, mouse pups were randomly assigned for a single intraperitoneal injection of (i) SN at a dose of 0.25 μg/g body weight (bw) or (ii) equal amounts of vehicle (1× PBS). On postnatal day 8, mouse pups were sacrificed by decapitation. Brain weight and body weight were determined in each experimental animal by means of calibrated medical precision scales. For histological endpoint determination, brains were harvested, immersion-fixed in 4% formaldehyde for 72–120 h, paraffin-embedded and cut into 10-μm-thick coronal sections.

Neuropathological injury was assessed in Cresyl Violet-stained sections by a blinded observer according to a modified scoring system as described previously (Hagberg et al., 2004). Rating was conducted as follows: 0–4 in cerebral cortex (0, no injury; 1, a few small isolated groups of injured cells; 2, several larger groups of injured cells; 3, moderate confluent infarction; 4, extensive confluent infarction), 0–3 for mild, moderate or severe atrophy and 0–3 for mild, moderate or severe neuronal injury/infarction in hippocampus, striatum and thalamus. A total injury score (0–22) was calculated as the sum of all subratings.

Immunohistochemical slides were pre-treated as published (Posod et al., 2014). In short, paraffin-embedded brain sections were deparaffinized and passed through graded alcohols. Endogenous peroxidase activity was quenched by incubation with 2% hydrogen peroxide in methanol for 30 min. Heat-induced antigen retrieval was performed using citrate buffer. After non-specific blocking with 1% bovine serum albumin (biotin-free) in TBS/0.05% Tween-20, sections were incubated overnight with mouse monoclonal (PC10) anti- proliferating cell nuclear antigen (PCNA) antibody (1:1000, Cat. No. #2586, Cell Signaling Technology, Leiden, The Netherlands) or rabbit polyclonal anti-cleaved caspase-3 (Asp175) antibody (1:250, Cat. No. #9661, Cell Signaling Technology). After rinsing with PBS, sections were incubated for 45 min at 25 °C with biotinylated goat anti-mouse (1:200, Cat. No. 115-065-003, Jackson ImmunoResearch Europe, Suffolk, UK) or anti-rabbit IgG (1:200, Cat. No. 111–065–003, Jackson ImmunoResearch). Detection of activated microglia was performed with biotinylated isolectin B4 from G. simplicifolia (Cat.