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Research paper

# C-type natriuretic peptide functions as an innate neuroprotectant in neonatal hypoxic-ischemic brain injury in mouse *via* natriuretic peptide receptor 2

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ABSTRACT

Neonatal hypoxia-ischemia (HI) is the most common cause of brain injury in neonates, which leads to high neonatal mortality and severe neurological morbidity in later life (Vannucci, 2000; Volpe, 2001). Yet the molecular mechanisms of neuronal death and brain damage induced by neonatal HI remain largely elusive. Herein, using both *in vivo* and *in vitro* models, we determine an endogenous neuroprotectant role of c-type natriuretic peptide (CNP) in preserving neuronal survival after HI brain injury in mouse pups. Postnatal day 7 (P7) mouse pups with CNP deficiency ($Nppc^{lbab/lbab}$) exhibit increased brain infarct size and worsened long-term locomotor function after neonatal HI compared with wildtype control ($Nppc^{+/+}$). In isolated primary cortical neurons, recombinant CNP dose-dependently protects primary neurons from oxygen-glucose deprivation (OGD) insult. This neuroprotective effect appears to be mediated through its cognate natriuretic peptide receptor 2 (NPR2), in that antagonization of NPR2, but not NPR3, exacerbates neuronal death and counteracts the protective effect of CNP on primary neurons exposed to OGD insult. Immunoblot and confocal microscopy demonstrate the abundant expression of NPR2 in neurons of the neonatal brain and in isolated primary cortical neurons as well. Moreover, similar to CNP deficiency, administration of NPR2 antagonist P19 *via* intracerebroventricular injection prior to HI results in exacerbated neuronal death and brain injury after HI. Altogether, the present study indicates that CNP and its cognate receptor NPR2 mainly expressed in neurons represent an innate neuroprotective mechanism in neonatal HI brain injury.

## 1. Introduction

Hypoxia-ischemia (HI) is the most common cause of neonatal brain injury, which results from systemic asphyxia that may occur during the perinatal period, and survivors often suffer from cognitive impairment, seizures, learning disabilities and motor impairment in their later life (Fatemi et al., 2009; Graham et al., 2008; Lee et al., 2013; Vannucci, 2000; Volpe, 2001). To date, only hypothermia treatment has been proven to provide some degree of clinical success in alleviation of neonatal HI-induced brain damage (Koenigsberger, 2000; Zanelli et al., 2009). The HI insult induces a series of neurotoxic events, such as cytotoxic reaction, oxidative stress, proinflammatory response, *etc*., and consequently results in neuronal death. However, the underlying mechanisms and pathways associated with HI brain injury remain largely elusive. Thus, it has become crucial to deepen our understanding of the pathogenesis of neonatal HI brain injury, especially regarding the innate mechanisms of neuronal protection and regeneration, so as to develop novel and effective treatment plans for HI brain injury in neonates.

C-type natriuretic peptide (CNP) is a potent neuropeptide (Kaneko et al., 1993; Stingo et al., 1992), which is released by vascular endothelial cells and brain cells in various regions of the rodent brain such as hippocampal subfields CA1-3, limbic cortices, dorsal endopiriform nucleus, *etc*. (Langub Jr et al., 1995; Maack, 1992; Potter et al., 2006). Considerable amounts of CNP have also been detected throughout the human brain the same as rodents (Komatsu et al., 1991). CNP belongs to a natriuretic peptide (NP) family and works locally as a neuronal growth hormone (Schmidt et al., 2009; Xia et al., 2013; Zhao and Ma, 2009), while other NP family members atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) mainly function as cardiac hormones (Potter et al., 2006). The physiological function of CNP is mediated by its receptors and NPR3 (Bennett et al., 1991; Koller et al.,

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1991; Lumsden et al., 2010; Suga et al., 1992). It has been reported that the CNP/NPR2 system controls axonal development of sensory neurons in the dorsal root ganglion *via* activation of the cGMP-PrkG1 pathway (Zhao and Ma, 2009; Zhao et al., 2009b), as well as regulates neurogenesis in the developing brain (Muller et al., 2009). The brain concentration of CNP is significantly higher than that in peripheral tissues (Minamino et al., 1993), suggesting an important role of CNP in the brain. Indeed, it has been reported that CNP provides neuroprotective effect on retinal ganglion cells by reducing apoptotic damage induced in both *in vitro* and *in vivo* injury models (Ma et al., 2010). However, there is still a lack of evidence supporting a putative neuroprotectant role of CNP in neurological diseases, such as neonatal hypoxic-ischemic brain injury.

Herein, we reveal a novel role of CNP as an endogenous neuroprotectant in neonatal HI brain injury. We demonstrate that CNP deficiency increases the vulnerability of the neonatal brain to HI insult and leads to worsened neurological deficits. In primary cortical neurons, recombinant CNP treatment dose-dependently reduces neuronal death after oxygen-glucose deprivation (OGD) insult. In addition, the neuroprotective effect of CNP is mediated by its cognate receptor NPR2 that is abundantly expressed in neurons. Moreover, we find that NPR2 antagonist increases brain infarct size and neuronal death in response to HI brain injury, demonstrating that CNP/NPR2 system is an endogenous neuronal survival pathway in neonatal HI brain injury.

## 2. Material and methods

### 2.1. Neonatal mouse model of hypoxia-ischemia (HI)

A modified Rice-Vannucci model was produced in postnatal day 7 (P7) C57BL/6J mouse pups modified from the rat model as described previously (Ferriero et al., 1996; Ma et al., 2016; Rice 3rd et al., 1981; Ten et al., 2004). Briefly, mouse pups (Charles River Laboratories) were fully anesthetized with inhalation of 2–3% isoflurane. The right common carotid artery (CCA) in the neck was exposed, double ligated with an 8.0 silk surgical suture, and then cut between two ligation sites. After surgery, pups were recuperated on a heating pad for 1 h at 37 °C, and then placed in a hypoxic incubator containing humidified 8% oxygen balanced with 92% nitrogen for 20 min at 37 °C. At the end of hypoxia, pups were returned to their dams for recovery. Mouse pups of mixed males and females were randomly assigned into each experimental group. There is no significant difference in weights or sex composition in the different groups. $Nppc^{lbab/lbab}$ (*long bone abnormality*; *Nppc*: gene encodes CNP precursor) or wildtype littermate control (WT, $Nppc^{+/+}$) mice were provide by Dr. Zhen Zhao (Zhao et al., 2009b). All procedures and protocols were approved by the Institutional Animal Care and Use Committee of Loma Linda University and followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### 2.2. Intracerebroventricular (i.c.v.) injection of NPR2 antagonist in mice

The stock solution of NPR2 antagonist P19 (Phoenix Pharmaceuticals Inc.) was prepared in 0.1 M phosphate-buffered saline (PBS, pH 7.4) according to the manufacturer's instruction. A total volume of 2 μl NPR2 antagonist solution (500 pmol/pup) were stereotaxically injected into the ipsilateral hemisphere of P7 mouse pups intracerebroventricularly (placement coordinates: 0.8 mm lateral, 1.5 mm below the skull surface) with a flow rate of 0.5 μl/min as described previously (Sadakata et al., 2007). Then the HI brain injury was induced about 2 h after i.c.v. injection. For vehicle group, the same volume of PBS was injected into the mouse brains prior to HI operation.

### 2.3. Measurement of brain infarct size

Brain infarct size was determined 48 h after HI using 2, 3, 5-triphenyltetrazolium chloride monohydrate (TTC, Sigma-Aldrich) staining as described previously (Ma et al., 2016). Briefly, the brain was isolated from each pup, dissected into coronal sections (2 mm thickness, 4 slices per brain), and immersed into pre-warmed 2% TTC in PBS for 5 min at 37 °C against light. Sections were washed with PBS, and then fixed by 10% formaldehyde overnight. The caudal and the rostral surfaces of each slice were photographed using a digital camera, and the percentage of infarct area (average of both sides) in the ipsilateral hemisphere for each slice was traced and analyzed by the NIH Image J software.

### 2.4. Neurobehavioral assay

Rotarod test for locomotor function evaluation was performed one month after neonatal HI as described previously (Hartman et al., 2012; Ma et al., 2016). Briefly, the rotarod (Columbus Instruments) consists of a horizontal cylinder (7 cm diameter) divided into four lanes. Three consecutive block trials were administered, in which the rotarod rotated at a constant speed of 5 RPM for 2 trials, followed by 2 trials of acceleration by 3 RPM every 5 s, and finally 2 trials of acceleration by 5 RPM every 3 s. Latency to fall was recorded as the time of walking on the cylinder.

### 2.5. Primary cortical neuron isolation, culture and treatment

Primary cortical neurons were prepared from early postnatal (P0) mouse pups of either sex as described previously (Beaudoin 3rd et al., 2012). Briefly, the cerebral cortices from P0 mouse pups were removed into HBSS buffer (Fisher Scientific) and dissociated with 0.25% trypsin (Fisher Scientific) and DNAse (Sigma) for 15 min at 37.0 °C water bath. After trituration with fire-polished Pasteur pipettes, dissociated cells were suspended in Neurobasal medium (Invitrogen) supplemented with 2% B27 (Invitrogen), 1% GlutaMAX (100 ×; Invitrogen) and 100 units/ml penicillin/streptomycin (Invitrogen), and run through a 40 μm cell strainer (Fisher Scientific). Cell suspensions plated on poly-D-lysine solution (PDL, 0.1 mg/ml in boric acid buffer; Sigma-Aldrich)-coated 6-well plates (Corning) or 96-well plates (Corning) were used for biochemical assays; Cell suspensions plated on PDL-coated German 12 mm glass coverslips (Fisher Scientific) in 24-well plates (Corning) were used for immunocytochemistry staining at 200–400 cells/$mm^2$. After being seeded, cells were maintained in a $CO_2$ incubator (5% $CO_2$, 21% $O_2$) at 37 °C. At day 2 of *in vitro* culture (DIV2), 2 μM cytosine β-D-arabinofuranoside hydrochloride (Ara-C; Sigma), an inhibitor of DNA replication, was added into culture medium to inhibit non-neuronal cell proliferation. Half of the culture medium was replaced every 2–3 days. Experiments were conducted at DIV 5-7, when cultures consisted primarily of neurons (approximately 90% MAP2-positive cells by immunocytochemistry staining).

Recombinant ANP, BNP and CNP was purchased from Sigma. NPR3 antagonist AP811 was purchased from Tocris. The stock solution was prepared according to the manufacturer's instruction. For the neuroprotective effect study, primary cortical neurons were incubated with CNP, BNP or ANP for 6 h at concentration of 0, 5, 25, 100, or 500 nM. For other experiments, primary cortical neurons were incubated with ANP, BNP or CNP for 6 h at concentration of 100 nM. For the effect of NPR2 antagonist P19 or NPR3 antagonist AP811, primary cortical neurons were incubated with P19 or AP811 alone, or with the presence of CNP for 6 h at concentration of 500 nM.

### 2.6. Oxygen-glucose deprivation (OGD)

Primary cortical neurons were subjected to OGD insult as reported previously (Frantseva et al., 1999; Newcomb-Fernandez et al., 2001; Yin et al., 2002; Zhao et al., 2009a). Briefly, the neuron culture media were replaced with pre-warmed glucose-deprived Neurobasal-A medium (Invitrogen) pre-equilibrated with 1% oxygen, and then