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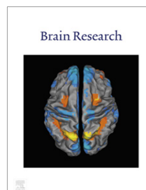

Research report

Density and neurochemical profiles of neuronal nitric oxide synthase-expressing interneuron in the mouse basolateral amygdala

Xiaona Wang [a,b], Chunhua Liu [b,c], Xiaochen Wang [b], Fei Gao [b], Ren-Zhi Zhan [b,*]

[a] Department of Chemistry & Chemical Biology, Indiana University-Purdue University Indianapolis, Indianapolis, USA
[b] Department of Physiology, Shandong University School of Medicine, 44 Wenhua Xi Road, Jinan, Shandong Province, China
[c] Department of Pharmacology, Taishan Medical College, Taian, Shandong Province, China

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A B S T R A C T

Neuronal nitric oxide synthase (nNOS)-expressing interneurons reside in the basolateral nucleus of the amygdala (BLA) of rodents. In the present study, we immunohistochemically analyzed nNOS-positive cells in the mouse BLA by focusing on their density, γ-Aminobutyric acid (GABA)ergicity, and co-localization with calcium-binding proteins and neuropeptides. The density of nNOS-containing neurons was analyzed with unbiased stereology. Experiments were conducted in both adult wild-type C57BL/6 and glutamic acid decarboxylase-green fluorescence protein (GAD_{67}-GFP) knock-in mice, in which GFP is expressed in GABAergic neurons under the control of the endogenous GAD_{67} gene promoter. In the BLA, the density of nNOS-positive cells was 3.92×10^3 cells/mm^3. Immunofluorescence revealed that nNOS-containing neurons constituted almost 26.93 ± 2.36% of the GAD_{67}-GFP neurons. Almost every nNOS-positive cell expressed glutamic acid decarboxylase 65 (GAD_{65}). Proportions of nNOS-positive interneurons that expressed calbindin, calretinin, parvalbumin, somatostatin and neuropeptide Y were approximately 5.20%, 15.63%, 26.50%, 87.50% and 88.00%, respectively; but exhibited no co-localization with vasoactive intestinal polypeptide. By contrast, percentages of calbindin, calretinin, parvalbumin, somatostatin and neuropeptide Y-positive cells that expressed nNOS were approximately 1.93%, 7.25%, 25.25%, 80.25% and 87.50%, respectively. Together, these findings suggest that nNOS-expressing cells are a discrete interneuronal subpopulation in the mouse BLA and may play a functional role in the inhibitory circuitry of this brain region.

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1. Introduction

The basolateral amygdala (BLA), comprising the lateral (LA) and basal (BA) nuclei (Herry et al., 2008), has long been known to critically contribute to the generation of emotional behavior and the formation of emotional memories (Mascagni and McDonald, 2003). Emerging evidence indicates that the BLA actively participates in the acquisition, expression, and extinction of conditioned fear (Ehrlich et al., 2009; Herry et al., 2010). Understanding neuronal mechanisms of fear conditioning in the BLA will require knowledge of the anatomy and physiology of its main cell types. BLA is cortex-like, and contains majority (80–85%) of spiny glutamatergic neurons and a minority (∼20%) of sparsely spiny GABAergic interneurons. Although these cells do not exhibit a laminar organization, their morphology, synaptology, electrophysiology, and pharmacology are remarkably similar to their counterparts in the cerebral cortex (Mascagni et al., 2009). As in the cerebral cortex, subpopulations of GABAergic interneurons in the mouse amygdala contain calcium-binding proteins (parvalbumin [PV], calbindin [CB], and calretinin [CR]), and neuropeptides (vasoactive intestinal peptide [VIP], somatostatin [SOM], as well as neuropeptide Y [NPY]) (Dávila et al., 2008; Guirado et al., 2008; Real et al., 2009; Sims et al., 1980; Wolff et al., 2014).

Nitric oxide (NO) is an important signaling molecule crucial for many physiological and pathological processes such as regulation of neuronal plasticity (Kajitani et al., 2010; Staschewski et al., 2011), enhancement of GABAergic transmission (Lange et al., 2012) in the BLA. It has been shown that synaptic plasticity in the LA during auditory fear conditioning promotes alterations at presynaptic sites via NO-driven "retrograde signaling" (Ota et al., 2010). Additionally, nNOS is the enzyme responsible for the synthesis of NO by neurons. Recent data indicate that nNOS deficiency impairs long-term memory of olfactory fear learning (Pavesi et al., 2013) and genetic functional inactivation of nNOS affects stress-related Fos expression in the BLA (Salchner et al., 2004).

* Corresponding author.
E-mail address: zhan0001@sdu.edu.cn (R.-Z. Zhan).

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Characterization of the density and neurochemical features of nNOS-expressing neurons will contribute to elucidate their function in both physiological and pathophysiological mechanisms in the BLA. In the neocortex, nNOS-expressing inhibitory neurons constitute an average of 20% of GABAergic population (Tricoire et al., 2010, 2011). Previous studies have shown that nNOS-expressing interneurons have been divided into two types based on the level of nNOS expression in the mouse neocortex. Type I nNOS-positive interneurons displaying higher levels of nNOS often express SOM, whereas type II nNOS-positive interneurons displaying lower levels of nNOS express PV, CR and VIP in certain frequencies (Liang et al., 2013; Perrenoud et al., 2012). We have previously shown that virtually every nNOS-positive cell is GABAergic cell in the dentate gyrus (Liang et al., 2013). However, little information is available regarding the density of nNOS-expressing cells in the mouse BLA, or their overlap with interneuronal subpopulations identified using immunohistochemical techniques. In the present study, the density of nNOS-containing cells was quantified used stereological methods. In addition to determining the percentages of nNOS-positive cells that contain each calcium binding proteins and neuropeptides, we also determined the percentages of individual calcium binding proteins and neuropeptides that exhibit nNOS immunoreactivity using immunofluorescence histochemistry combined with confocal laser scanning microscopy.

2. Results

2.1. Stereological analysis and distribution of nNOS-positive neurons

We use stereological method to quantify the density of nNOS-expressing cells in the BLA. Cells defined by nNOS immunolabeling were counted from adult mice approximately between −0.94 and −2.18 mm from Bregma (Franklin and Paxinos, 2007). Counts were normalized by the area of the counted region to provide a measure of density and were repeated on four mice. Our results showed that the overall density of nNOS-positive cells was $(3.92 \pm 0.14) \times 10^3$ -cells/mm^3 in the mouse BLA.

We found that nNOS-immunoreactive cells were particularly distributed sparsely throughout the BLA compared to central nucleus of amygdala (CE). Similar immunoreactive cells were also scattered throughout the entorhinal cortex, but were rare (Fig. 1A). Additionally, in accordance with previous reports in the mouse amygdala (Olmos et al., 2005) and cortex (Kubota et al., 2011; Perrenoud et al., 2012), these cells are defined according to nNOS immunoreactivity. As shown in Fig. 1B and C, nNOS-containing cells displayed well-staining somata.

2.2. GFP-labeled interneurons contain nNOS

Previous work has shown that nNOS cells in the mouse hippocampus and neocortex express markers characteristic of inhibitory neurons (Fuentealba et al., 2008; Jinno and Kosaka, 2002; Kubota et al., 2011). To confirm whether nNOS-expressing cells are restricted to GABAergic neurons in the mouse BLA, we performed immunofluorescence for nNOS in glutamic acid decarboxylase-green fluorescence protein (GAD67-GFP) mice. Staining for GABA confirmed that essentially all GFP-positive cells are GABAergic neurons in the BLA in the GAD_{67}-GFP mouse (Tamamaki et al., 2003). As shown in upper panel of Fig. 2, high magnification images revealed that GFP fluorescence was observed in both somata and unidentified processes of GABAergic interneurons, whereas nNOS fluorescence is mostly confined to the somata. Quantification of nNOS and GAD_{67}-GFP co-localization showed a co-expression level of $67.45 \pm 1.11\%$, whilst significant numbers $32.55 \pm 1.11\%$ of nNOS immunoreactive profiles were not GFP-positive (n = 4). In addition, we also found nNOS-containing cells account for approximately $26.93 \pm 2.36\%$ of GAD_{67}-GFP cells examined in the mouse BLA. Collectively, our results demonstrated that nNOS-expressing cells make up a moderate proportion of GAD_{67}-GFP cells in the BLA.

2.3. Inhibition of axonal transport remarkably improves GAD_{65} labeling

GABA is synthesized by the enzyme glutamate decarboxylase, which exists in two isoforms, GAD_{65} and GAD_{67}. Immunohistochemical and in situ hybridization studies suggest that both these isoforms are present in all GABAergic neurons (Esclapez et al., 1993, 1994). We next examined the colocalization of nNOS with GAD_{65} in the C57BL/6 mice. As shown in Fig. 2, intraventricular injection of colchicine increased GAD_{65} immunoreactivity. At higher magnification, the large numbers of GAD_{65} positive axonal fibers and a few GAD_{65} positive neuronal somata were scattered in the colchicine-untreated mice (Fig. 2, middle-right panel). As anticipated, the colchicine pretreatment increased considerably GAD_{65}-labeling of individual somata (Fig. 2, bottom-right panel). We found that proportions of nNOS-expressing neurons containing

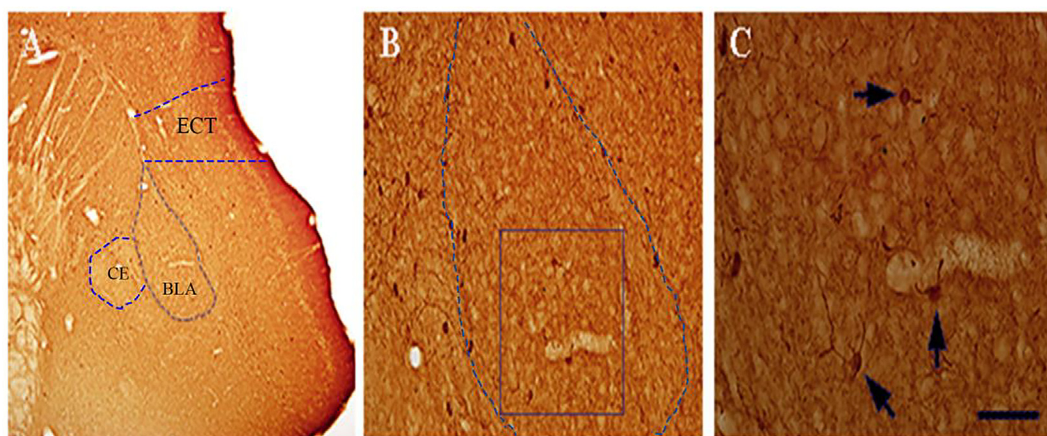

Fig. 1. Photomicrographs of nNOS immunoreactivity in the BLA. A few stained cells were scattered in the BLA (A). Dotted line indicates approximate border of BLA. The areas of BLA outlined by blue rectangle in B is shown at higher magnification in C. Some nNOS immunoreactive neurons have been pointed with arrow. Scale bars: A, 250 μm; B, 50 μm; C, 25 μm.

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