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Comparisons of terminal densities of cardiovascular function-related projections from the amygdala subnuclei

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ABSTRACT

The amygdala is important in higher-level control of cardiovascular functions. In this study, we compared cardiovascular-related projections among the subnuclei of the amygdala. Biotinylated dextran amine was injected into the central, medial, and basolateral nuclei of the amygdala, and the distributions and densities of anterograde-labeled terminal boutons were analyzed. We found that the medial, basolateral, and central nuclei all had projections into the cardiovascular-related areas of the hypothalamus. However, only the central nucleus had a significant direct projection into the medulla. By contrast, the medial nucleus had limited projections, and the basolateral nucleus had no terminals extending into the medulla. We concluded that the medial, central, and basolateral nuclei of the amygdala may influence cardiovascular-related nuclei through monosynaptic connections with cardiovascular-related nuclei in the hypothalamus and medulla.

1. Introduction

The amygdala has been implicated in various autonomic functions, including cardiovascular control. Mogenson and Calaresu (1973) found that electrical stimulation of the medial (Me), central (CeA), and basolateral (BLA) nuclei of the amygdala elicited cardiovascular changes. In several studies, cardiovascular responsive regions were extensively searched in the amygdala and found these regions were located in the Me, CeA, and BLA nuclei of the amygdala (Gelsema et al., 1987; al Maskati and Zbrozyna, 1989). Other studies reported that electrical (Galeno and Brody, 1983; Cox et al., 1986; Iwata et al., 1987; Gelsema et al., 1989) and chemical (Galeno and Brody, 1983; Cox et al., 1986; Iwata et al., 1987; Gelsema et al., 1989; Ohta et al., 1991; Soltis et al., 1998; Roder et al., 1999; Goren et al., 2000; Salome et al., 2001; Chiou et al., 2009) stimulation of these nuclei lowered the blood pressure (BP) of anesthetized animals and raised the BP of conscious rats. However, in the study of Gelsema et al. (1987), stimulations produced both raised and lowered BP responses. In addition, in the results of al Maskati and Zbrozyna (1989), stimulations produced raised BP responses in the anesthetized rats.

In lesion studies, bicuculline injected into the BLA in conscious rats elicited blood pressure and heart rate changes (Soltis et al., 1998). Several researches showed that lesioning the CeA impaired the cardiovascular performance in fear conditioning tasks (Kapp et al., 1979; Iwata et al., 1986; Zhang et al., 1986; Sananes and Campbell, 1989; Roozendaal et al., 1990; Chachich and Powell, 1998). On the other hand, Me was found to play an important role in unconditioned fear responses (Chen and Herbert, 1995; Dayas et al., 1999; Dayas and Day, 2001; Kubo et al., 2004; Li et al., 2004; Ma and Morilak, 2004; Mantella et al., 2004; Beijamini and Guimaraes, 2006; Muller and Fendt, 2006). Based on these stimulation and lesioning experiments, it was clear that amygdaloid subnuclei are involved in the control of the cardiovascular functions, but whether functional differentiation exists for these subnuclei is still unclear.

Connectivity is the most important factor dictating the function of a brain area. Among the subnuclei of the amygdala, the connections of the CeA to cardiovascular-related nuclei in the brain have received the most attention. Neurons in the CeA send projections to brainstem cardiovascular-related areas, including the nucleus tractus solitarius (NTS), dorsal motor nucleus of the vagus (DMV), and ventrolateral medulla (VLM) (Hopkins and Holstege, 1978; Price and Amaral, 1981; Veening et al., 1984; Danielsen et al., 1989; Takayama et al., 1990; Wallace et al., 1992; Pickel et al., 1995; Saha et al., 2000). By contrast, few studies have examined the possible connections among the Me and BLA with cardiovascular-related nuclei. Therefore, to address differentiation of the cardiovascular functions of the amygdala, it is crucial to examine the cardiovascular-related projection patterns of the 3 subnuclei of the amygdala.

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In this study, we used the anterograde tracer, biotinylated dextran (BD), to examine the cardiovascular-related projection pattern of the Me, CeA, and BLA of rats. The most essential cardiovascular-related brain structures (for example, cardiac-parasympathetic preganglionic nuclei and brain areas that project densely and directly to sympathetic preganglionic nuclei) were examined for amygdaloidal projection terminal boutons. In the medulla, the DMV, NTS, VLM, and nucleus ambiguus (NA) were examined, and the parabrachial nucleus (PB) and periaqueductal gray (PAG) were examined in the brainstem. In addition, the paraventricular hypothalamic nucleus (PVH), lateral hypothalamic area (LH), dorsomedial hypothalamic nucleus (DM), posterior hypothalamus (PH), and ventromedial hypothalamus nucleus (VMH) in the hypothalamus (Loewy and Spyer, 1990) were examined.

This study therefore addresses the very basic functional connections of the amygdala. Cardiovascular-related projection patterns of the BLA, Me and CeA were compared and contrasted. We found extensive medullary, brainstem and hypothalamic CeA connections, but the BLA and Me might go through the hypothalamus primarily to influence cardiovascular functions.

2. Materials and methods

Experiments were performed on adult male Wistar rats weighing 230 to 550 g ($n = 12$) in accordance with the guidelines provided in the *Codes for Experimental Use of Animals* of the Council of Agriculture of Taiwan, and based on the *Animal Protection Law* of Taiwan. All experimental protocols were approved by the Institutional Animal Care and Use Committee of National Taiwan University. Rats were obtained from the Animal Center, National Taiwan University Hospital (Taipei, Taiwan).

The rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). During surgery, the depth of anesthesia was verified by periodically testing for the absence of a pinch reflex. Supplementary doses were provided as necessary. The head of each rat was fixed on a stereotaxic apparatus in prone position, and its core temperature was maintained at 37.5 °C with a feedback-controlled heating pad. Small holes were drilled in the skull to expose the cortex overlying the amygdala (Paxinos and Watson, 2005). Tracer injections were administered through a glass microelectrode (o.d. 0.75 mm) filled with BD (MW 10,000; Molecular Probes, Eugene, OR, 10% in artificial cerebrospinal fluid, pH 7.4). A BD solution was injected into the right side of the amygdala with a pneumatic pressure pump. One injection was made in each animal. The injection volume was monitored by measuring the displacement (h) of the meniscus of the liquid surface under an operating microscope equipped with a reticle in the eyepiece, and was calculated as $\pi r^2 h$. A volume of 6 to 27 nL was individually injected into each subnucleus of the amygdala (CeA: 2.5 mm posterior to, 4.4 mm lateral to, and 6.5 mm below the bregma; Me: 2.5 mm posterior to, 3 mm lateral to, and 7.8 mm below the bregma; and BLA: 2.5 mm posterior to, 5 mm lateral to, and 8.2 mm below the bregma). To reduce backflow after the injection, the micropipette was left in the injection site for 30 min before withdrawal. The wound was sutured, and the animal was allowed to recover in its home cage.

After a recovery period of 10 days, the rats were anesthetized with sodium pentobarbital and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in a 0.1 M phosphate buffer at pH 7.4. The brain was removed and stored in a 30% sucrose buffer solution at 4 °C. The brain was then serially cut into 50-μm frozen sections in the coronal plane. The sections were rinsed 3 times in phosphate-buffered saline (PBS), and sequentially reacted with avidin-labeled peroxidase (ABC kit, PK-6100, Vector Laboratories, Burlingame, CA), and hydrogen peroxide/diaminobenzidine (DAB), combined with Ni and Co at room temperature. The development time of the DAB products was 10 min. After 3 rinses in PBS, the sections were mounted onto gelatin-coated slides and allowed to air-dry. Slides were rinsed in water, dehydrated in increasing concentrations of ethanol, cleared in xylene, and capped with a coverslip and Permount.

A 6A Zeiss microscope (Axioplan 2, Göttingen, Germany) equipped with a Q Imaging (Micro Publisher 3.3 RTV, Surrey, Canada) digital camera was used to capture bright-field BD images. Labeled boutons were digitally acquired using a Zeiss Axio Imager A1 microscope with DIC optics. The labeled profiles were also examined from line drawings made with a camera lucida attachment. To analyze the terminal densities in the projection areas, the DAB-labeled terminals were counted under high magnification. To analyze terminal densities in the projection areas, DAB-labeled terminals were counted under high magnification (100× objective, oil). The most densely labeled section in each target nucleus was selected from each case for analysis. The counted areas measured 200 μm in diameter. To analyze the sizes of axon fiber and terminal bouton diameters under high magnification (60× objective, oil), 3 sections were randomly selected in the hypothalamus and medulla, 1 to 3 images were acquired for each section. The diameters of the terminal boutons and the width of axons from each subnucleus in the amygdala of each individual animal were measured. The data were expressed as mean ± standard error of the mean (SEM). The one way analysis of variance was used to test the differences between the boutons and fibers. Holm–Sidak's multiple comparison was used to test the differences between the each 2 nuclei.

3. Results

After 10 days of anterograde transport, the BD-labeled axon terminals and terminal boutons were found to be densely distributed throughout the hypothalamus. By contrast, the terminals in the medulla were found only in the CeA- and Me-injected rats. A summary of terminal bouton densities is provided in Tables 1 and 2. Only rats with injection sites completely restricted to the CeA ($n = 3$), Me ($n = 4$), and BLA ($n = 5$) were analyzed.

The injection levels of each animal are summarized in Table 1. In the CeA group, the respective injection sites of Animals 0330, 0707, and 0512 were 1.56 mm, 1.8 mm, and 2.64 mm posterior to the bregma. The spread of injection of Animal 0707 was larger than that of the others. In the Me group, the injection sites in Animals 0804 and 0102 were both at 1.56 mm posterior to the bregma. The injection sites in Animal 0704, 1124 were 2.16 mm and 2.64 mm posterior to the bregma. The injection site was at 2.52 mm posterior to the bregma in Animal 0920 of the BLA group, and this injection was on the lateral side of the BLA. The injection site of Animal 0320 was at 2.92 mm posterior to the bregma and in the dorsal part of the nucleus. The injection site of Animal 0910 was at 2.92 mm posterior to the bregma and in the inferior part of the nucleus. The spread of injection of this animal was minimal. The injection levels in Animals 1011 and 1018 were both at 3.48 mm posterior to the bregma. The injection site of Animal 1018 was in the medial part of the nucleus, and on the lateral side for Animal 1011. The injection sites are listed in Fig. 1.

Table 1
Levels of unilateral tracer injection into subnuclei of amygdala in each animal.

Injection level (posterior to bregma)	CeA	ME	BLA
1.56 mm	0330	0804, 0102	–
1.80 mm	0707	–	–
2.16 mm	–	0704	–
2.52 mm	–	–	0920
2.64 mm	0512	1124	–
2.92 mm	–	–	0320, 0910
3.48 mm	–	–	1011,1018

CeA, central nucleus. Me, medial nucleus. BLA, basolateral nucleus. "–": no injection at that level.