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UNCONDITIONED STIMULUS PATHWAYS TO THE AMYGDALA: EFFECTS OF LESIONS OF THE POSTERIOR INTRALAMINAR THALAMUS ON FOOT-SHOCK-INDUCED c-Fos EXPRESSION IN THE SUBDIVISIONS OF THE LATERAL AMYGDALA

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Abstract—The lateral nucleus of the amygdala (LA) is a site of convergence for auditory (conditioned stimulus) and foot-shock (unconditioned stimulus) inputs during fear conditioning. The auditory pathways to LA are well characterized, but less is known about the pathways through which foot shock is transmitted. Anatomical tracing and physiological recording studies suggest that the posterior intralaminar thalamic nucleus, which projects to LA, receives both auditory and somatosensory inputs. In the present study we examined the expression of the immediate-early gene c-*fos* in the LA in rats in response to foot-shock stimulation. We then determined the effects of posterior intralaminar thalamic lesions on foot-shock-induced c-Fos expression in the LA. Foot-shock stimulation led to an increase in the density of c-Fos-positive cells in all LA subnuclei in comparison to controls exposed to the conditioning box but not shocked. However, some differences among the dorsolateral, ventrolateral and ventromedial subnuclei were observed. The ventrolateral subnucleus showed a homogeneous activation throughout its antero-posterior extension. In contrast, only the rostral aspect of the ventromedial subnucleus and the central aspect of the dorsolateral subnucleus showed a significant increment in c-Fos expression. The density of c-Fos-labeled cells in all LA subnuclei was also increased in animals placed in the box in comparison to untreated animals. Unilateral electrolytic lesions of the posterior intralaminar thalamic nucleus and the medial division of the medial geniculate body reduced foot-shock-induced c-Fos activation in the LA ipsilateral to the lesion. The number of c-Fos labeled cells on the lesioned side was reduced to the levels observed in the animals exposed only to the box. These results indicate that the LA is involved in processing information about the foot-shock unconditioned stimulus and receives this kind of somatosensory information from the posterior intralaminar thalamic nucleus and the medial division of the medial geniculate body.

Key words: emotional learning, fear conditioning, pain, freezing, electrolytic lesions, somatosensory thalamus.

In recent years classic fear conditioning has become a leading model for studying the neural mechanisms of learning and memory in mammals (LeDoux, 2000; Maren, 2005). In this behavioral paradigm, a neutral (innocuous) conditioned stimulus (CS), such as a tone, is presented in association with an aversive unconditioned stimulus (US), such as a mild foot shock. After a few such paired presentations, the animal begins to respond defensively to the neutral stimulus. Considerable evidence suggests the lateral nucleus of the amygdala (LA) is a site of auditory CS and somatosensory US convergence (Romanski et al., 1993; Bordi and LeDoux, 1994b) and is crucial for the formation of the association between the CS and US (Blair et al., 2001; Fanselow and LeDoux, 1999; LeDoux, 2000; Maren, 2001, 2005; but see Cahill et al., 1999). While the CS pathways to LA have been characterized in detail (LeDoux et al., 1990a, 1991; Bordi and LeDoux, 1994a), less is known about the origin of the US pathways to LA (see Shi and Davis, 1999; Lanuza et al., 2004).

Anatomical tracing and physiological recording studies suggest that the posterior intralaminar thalamic nucleus (PIN) and the medial division of the medial geniculate body (MGm) receive both auditory and somatosensory inputs (LeDoux et al., 1987; Bordi and LeDoux, 1994b) and project to LA (LeDoux et al., 1990b). While many cells in LA respond to both auditory CS-like and somatosensory US-like stimulation (Romanski et al., 1993), most cells in PIN process one or the other modality rather than both (Bordi and LeDoux, 1994a,b). Moreover, this thalamo-amygdaloid pathway is enriched in stathmin, a protein involved in the expression of learned and innate fear (Shumyatsky et al., 2005). The MGm/PIN complex is thus a likely candidate to provide the LA with information about a foot-shock US.

The expression of the immediate-early gene c-*fos* is widely used as a marker of cellular activity in the brain (Sagar et al., 1988). In the present study we therefore examined whether foot shock stimulation would increase c-Fos expression in different LA subnuclei and whether lesions of MGm/PIN would prevent the foot-shock-induced c-Fos expression. Previous studies on foot-shock-induced c-Fos expression in the amygdala gave rise to contradictory results (see Knapska et al., 2007). Some studies reported increased c-Fos expression in the LA after foot shock (Schettino and Otto, 2001; Holahan and White,

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Abbreviations: ANOVA, analysis of variance; c-Fos-li, c-Fos-like immunoreactive; CS, conditioned stimulus; DL, dorsolateral subnucleus of the lateral nucleus of the amygdala; LA, lateral nucleus of the amygdala; MGm, medial division of the medial geniculate body; PIN, posterior intralaminar thalamic nucleus; US, unconditioned stimulus; VL, ventrolateral subnucleus of the lateral nucleus of the amygdala; VM, ventromedial subnucleus of the lateral nucleus of the amygdala.

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2004; Knapska et al., 2006), whereas others found no foot-shock-induced increases in the LA (Pezzone et al., 1992; Smith et al., 1992; Rosen et al., 1998) or in the LA analyzed together with the basal nucleus (Milanovic et al., 1998; Radulovic et al., 1998). Recent anatomical data suggest that the LA is heterogeneous, and that the different subnuclei, and different anteroposterior divisions, may have different roles in fear conditioning (Pitkänen et al., 1997; Doron and LeDoux, 1999). This raises the possibility that foot shock might induce differential c-Fos expression in subdivisions of LA. Due to the small size of these subdivisions, previous studies may have either been successful or failed to notice increases in c-Fos expression depending on the particular area of the LA chosen to count the c-Fos-labeled nuclei.

We therefore analyzed in detail foot-shock-induced c-Fos expression in each of the subdivisions of LA throughout the anteroposterior extension of the nucleus. To determine the role of the MGm/PIN complex in relaying US information to the LA, we examined the effects of unilateral electrolytic lesions of this thalamic complex on foot-shock-induced c-Fos activation in the LA ipsilateral to the lesioned thalamus, using the contralateral LA as a control.

EXPERIMENTAL PROCEDURES

Subjects

All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and were approved by the New York University Animal Care and Use Committee. The number of animals used was the minimum judged necessary to obtain significant results, and appropriate measures were taken to minimize their suffering, especially during surgery. Studies were performed using adult male Sprague–Dawley rats (Hilltop Laboratories, Scottsdale, PA, USA) which weighted 275–300 g on arrival at the laboratory. All rats were housed individually in clear plastic cages, with food and water available *ad libitum*, and kept on a 12-h light/dark cycle with lights on at 7:00 am. All experiments were conducted during the light phase of the cycle. Rats were allowed to acclimate to the vivarium for at least 5 days before the start of the experiments.

Experimental design

Four experimental groups were used. A control, No-treatment group of rats ($n=6$) was carried out to determine the basal c-Fos expression in response to the conditions under which the animals were housed. These rats were killed after living in the animal facility for 10 days without any specific experimental stimulation. Their brains were then processed for immunocytochemical detection of the c-Fos protein. To assess c-Fos expression induced by the surgery procedure and the conditioning apparatus, six rats (Box group) received sham surgery and 4 days later were placed in the conditioning box for 30 min. They were killed 90 min after removal from the box. Immunocytochemical detection of c-Fos was then carried out. To characterize the foot-shock-induced c-Fos expression in the lateral amygdala, a group of nine animals (Foot-shock group) received sham surgery and, 5 days later, 10 foot shocks during a 30 min stay in the conditioning box. Ninety minutes later they were killed for c-Fos immunocytochemistry. Finally, to study the possible role of the posterior intralaminar thalamic complex in relaying foot-shock information to the amygdala, 14 rats received unilateral electrolytic lesions of the MGm/PIN complex, and, after 5 days of recovery, received 10 foot shocks in the conditioning box. Ninety minutes later they were killed for c-Fos immunocytochemistry. Eight of the 14 rats had acceptable thalamic lesions (Lesioned group), and were included in the cell counting procedure.

Surgery

All surgical procedures were performed under anesthesia with sodium pentobarbital (45 mg/kg i.p.) complemented with atropine sulfate (0.4 mg/kg). Animals were placed in a stereotaxic frame and unilateral electrolytic lesions of the posterior intralaminar thalamic nuclei were performed using coordinates based on the atlas of Paxinos and Watson (1998) and a previous study (Shi and Davis, 1999). Electrolytic lesions were made passing constant (positive) current of 0.8 mA for 10 s through stainless steel electrodes (0.25 mm in diameter) insulated except for 500 μm of the tip. Since the MGm/PIN complex is a long rostrocaudal structure, three lesions were made at anteroposterior (AP) coordinates -4.8 mm, -5.3 mm, -5.8 mm respectively. All lesions were placed at 2.6 mm from the midline and 6.5 below bregma. Sham electrolytic lesions were made by using the same procedure except that the dorsoventral coordinate was -4.0 mm, and no current was passed. Lesions and sham operations were performed counterbalanced in the left and right hemispheres.

Apparatus and stimulation procedure

Behavioral tests took place in a rodent chamber (Coulbourn Instruments, Lehigh Valley, PA, USA, model E10-10) housed in a sound attenuating cubicle. Each animal on the Foot-shock and Lesioned groups received 10 foot shocks (1 mA, 0.5 s each) during 30 min delivered through the grid floor of the chamber (shocker model E13-08, Coulbourn Instruments). The average intertrial interval was 160 s. Freezing behavior, defined as immobility except for respiratory-related movements, was used as an index of fear. Freezing was timed for each animal during the 30 s following the delivery of the first five shocks (post-shock freezing).

c-Fos immunocytochemistry

Ninety minutes after the end of stimulation, each animal was deeply anesthetized with an i.p. injection (75 mg/kg) of sodium pentobarbital and perfused transcardially with saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Following perfusion, the brains were removed and immersed in fixative for 4–6 h at 4 °C. Subsequently, each brain was immersed in a solution of 30% sucrose in phosphate buffer at 4 °C until it sank. Serial, coronal 40 μm-thick sections were then cut from each brain with a freezing microtome and collected into six matching series. Alternate series were processed for c-Fos immunocytochemistry, for Nissl staining (with 1% acidic Toluidine Blue), or for acetylcholinesterase histochemistry (following the procedure given in Paxinos and Watson, 1998). The Nissl and acetylcholinesterase preparations facilitated the accurate localization of the cytoarchitectonic boundaries of the amygdaloid nuclei (especially of the subdivisions of the LA), as well as the extent of the lesion sites in the thalamus.

The tissue sections were processed for immunocytochemistry using the avidin–biotin complex method. Endogenous peroxidase activity in the tissue sections was suppressed by incubation in a 1% H_2O_2 solution for 30 min. The sections were then incubated for 24 h at 4 °C in anti-c-Fos IgG raised in rabbit (cat. #: sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:20,000 in Tris-buffered saline containing 0.3% Triton X-100 (Sigma, St. Louis, MO, USA) and 2.5% normal goat serum (Vector Laboratories, Burlingame, CA, USA). The sections were then incubated in biotinylated goat anti-rabbit IgG (Vector Laboratories.) diluted 1:200 in Tris buffered saline containing 0.3% Triton X-100 for 2 h