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Research article

Density of acetylcholine esterase (AchE) and tyrosine hydroxylase (TH) containing fibers in the amygdala of roman high- and low-avoidance rats

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HIGHLIGHTS

- Roman High Avoidance rats better in attention and two-way active avoidance task.
- Good active avoidance correlates with high cholinergic input to lateral amygdala.
- Roman Low Avoidance rats show enhanced stress reactivity and no escape response.
- Enhanced stress behavior correlates with high dopaminergic input to central amygdala.
- Dopamine may upregulate corticotropin-releasing hormone (CRH) in central amygdala.

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ABSTRACT

The cholinergic and dopaminergic innervation of the amygdala plays an important role in attention, emotional arousal, aversive forms of associative learning, conditioned responses, and stress responsivity. Roman High- (RHA) and Low-Avoidance (RLA) rats are an ideal model to study the potential impact of this innervation on behavioral responses, because they were selected bidirectionally for differences in their two-way active avoidance performance. RHA rats are known to quickly acquire two-way active avoidance and show indications of enhanced impulsive behavior, novelty seeking, and vulnerability to substance abuse, whereas RLA rats exhibit a passive coping style with high levels of immobility and enhanced stress responsivity. In the present study, the density of acetylcholine esterase (AchE)-positive cholinergic fibers and tyrosine hydroxylase immunoreactive (TH-ir) fibers were analyzed in various amygdala nuclei. In comparison to RLA rats, RHA rats displayed a significantly higher density of AchE-positive fibers in the lateral nucleus (La), the major sensory input area of the amygdala. In contrast, RLA rats showed a higher density of TH-ir fibers in the lateral division of the central nucleus (CeL), which modulates amygdala output and is known to contain more corticotropin-releasing hormone (CRH) positive neurons in RLA than in RHA rats. The findings suggest that a higher density of AchE-positive fibers in the La of RHA rats may facilitate attentional mechanisms and aversive forms of associative learning in RHA rats, whereas the increased density of TH-ir fibers in the CeL of RLA rats may be involved in the regulation of enhanced CRH expression and stress responsivity.

1. Introduction

The amygdala is increasingly recognized as an important player in a vast array of cognitive and emotional functions, which are mediated by at least two anatomically and functionally distinct divisions, namely the basolateral nuclear complex and the centromedial nuclei [1]. The basolateral complex that consists of the lateral (La), basal (Ba) and accessory basal (Ab) nuclei is richly

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interconnected with cortical areas and regarded as the main sensory input area for emotional learning, decision making, attention, and cognitive processes [16,18,28]. The main output station of the amygdala is the central nucleus (Ce), which by virtue of its widespread connections to the brainstem and hypothalamus coordinates autonomic, endocrine and behavioral responses to emotional stimuli including attention and arousal [16].

The sensory glutamatergic input from thalamic and cortical areas to amygdala nuclei and the output of the amygdala to other brain regions are modulated by cholinergic and monoaminergic systems [1,2,9,14,19,28]. The basal nucleus of Meynert provides the heaviest cholinergic innervation to the Ba, although the La and Ab also receive substantial input [30]. Whereas cholinergic transmission in the Ba is crucial for memory consolidation of emotionally arousing experiences [28,35], the role of cholinergic innervation in the La is less explored, although the La is critical for aversive forms of associative learning [16]. The dopaminergic innervation of the amygdala, originating mainly in the lateral portion of the substantia nigra, ventral tegmental area, dorsal raphe and periaqueductal grey [14,22], is particularly dense in the Ce and Ba [2,4], which is vital for regulating fear-related behaviors and stress-induced relapse in drug addiction [8,9,42].

The Swiss sublines of Roman High (RHA) and Low Avoidance (RLA) rats are an ideal model to study the impact of the cholinergic and dopaminergic innervation on an individual's cognitive abilities and emotional regulation. They were bidirectionally selected for more than a decade for two-way active avoidance learning [5,11], which is based on a form of cue fear-conditioning. In this test, a tone (becomes conditioned stimulus, CS), which is paired with shock, signals the delivery of shock (unconditioned stimulus, US). However, upon hearing the tone the animal can attempt to avoid the anticipated shock or it can escape from it when the tone-shock phase is initiated by running to the other compartment [7]. RHA rats quickly acquire the two-way active avoidance task, which requires learning, e.g., through activation of cholinergic attentional mechanisms, and show a higher responsiveness to rewarding stimuli [19,21]. In contrast, two-way active avoidance induces a passive coping style with a "no escape" response in RLA rats, which was related to their enhanced responsiveness to aversive stimuli, freezing tendency, stress responsivity, and elevated numbers of corticotropin-releasing hormone (CRH) positive neurons in the Ce [10,11,40,47]. Because CRH-positive Ce neurons receive a dense perisomatic dopaminergic innervation [13], which may upregulate CRH levels [17], we hypothesized that increased CRH levels in RLA rats may be induced by enhanced dopaminergic input, whereas an enhanced cholinergic input may contribute to the rapid acquisition of two-way active avoidance learning in RHA rats. The aim of the study was therefore to analyze the cholinergic and dopaminergic innervation in inbred Roman rat lines by focusing on amygdala nuclei involved in attentional mechanisms and conditioned fear responses.

2. Materials and methods

The Roman/Verh rat lines were psychogenetically selected for many decades by mating the animals based on their acquisition of two-way active avoidance behavior in the shuttle box [5]. The rats investigated in this study were derived from the inbred colony at the ETH Zurich/Switzerland [11] and continued at the University in Magdeburg starting in 1998. The animals were bred and kept under an artificial 12:12 h light-dark cycle with lights on at 6:00 a.m. (between 06:00–08:00 increase, and between 16:00–18:00 decrease of light intensities in continuous steps). Water and food were available ad libitum. Pups were weaned at the age of 4–5 weeks. All experiments were approved by the local council of animal care (AZ:28.14-42502/2-241 and −412, Uni MD), and comply with the European Communities Council Directive of November 24, 1986 (86/609/EEC).

Rats were transcardially perfused under sodium pentobarbital anesthesia (180 mg/kg i.p.) for 3 min with a saturated H_2S solution and for 15 min with a 4% glutaraldehyde solution made in 0.1 M phosphate buffer (PB). Following immersion fixation over night (in 20% sucrose with 2% glutaraldehyde), 4 series of 40 μm thick frozen sections were cut coronally. The first series was used for Nissl-staining (Cresyl violet). In the second series, acetylcholine esterase (AchE) positive fibers were visualized (RHA females = 6, males = 4, RLA: females = 4, males = 5) using a modified Karnovsky-Roots procedure [38]. Sections mounted on object slides were rinsed in 0.1 M maleate buffer (pH 8.0) and incubated for 2 h in 400 ml of a 0.1 M maleate buffer containing 0.29 mg promethazine, 46 mg maleic acid, 5.88 mg sodium citrate, 3 mg copper sulfate, 0.66 mg potassium ferricyanide, a drop of glacial acetic acid, and 8.5 mg acetylthiocholine iodide as substrate (pH 8.0). After rinsing sections with Tris buffer (pH 7.6) and dipping them into 0.5% cobalt chloride in 0.1 M Tris buffer for 10 min, staining was visualized in 0.1 M Tris buffer containing 0.05% DAB and 0.01% H_2O_2 (3 min). Tyrosine hydroxylase (TH) immunohistochemistry was performed in the third series by using free-floating sections (RHA females = 7, males = 4, RLA: females = 3, males = 6). Endogenous peroxidase activity was suppressed with 0.3% H_2O_2 in 0.1 M phosphate buffered saline (PBS) with a pH 7.4. Unspecific binding sites were blocked with 0.1 M PBS containing 5% normal horse serum (NHS) and 0.2% Triton X-100 for 10 min. Sections were incubated for 48 h at 4 °C using a monoclonal mouse anti-TH antibody at a concentration of 1:200 (Boehringer Mannheim, Germany) in 0.1 M PBS containing 1% NHS. Immunostaining was visualized with a biotinylated horse anti-mouse antibody (1.5 h) followed by incubation with avidin and biotinylated horseradish peroxidase macromolecular complex (ABC Kit, Vector Laboratories, Burlinggame, CA, USA) (1 h). Immunostaining was developed using diaminobenzidine tetrahydrochloride (DAB, 1 mg/1 ml), H_2O_2 (0.0075%) and heavy metal intensification (0.02% nickel ammonium sulfate, 0.024% and cobalt chloride). Sections were dehydrated, cleared in xylene, and coverslipped with Eukitt.

Densities of AchE-positive and TH-ir fibers were quantified in serial sections by taking digitized microphotographs with a 40X (49 μm × 33 μm) and 100X objective (oil immersion, 20 μm × 13 μm), respectively. A microscope (Leitz DM RXE) equipped with a CCD camera, computer and the software Kappa ImageBase Control 2.0 (Kappa Opto-electronics GmbH, Gleichen, Germany) was used to capture images. The images were taken under equal light conditions and microscope settings from the center of the La, Ba, accessory basal nucleus (Ab), and the medial (CeM), lateral (CeL) and lateral capsular (CeLc) divisions of the Ce. The average number of AchE images analyzed per amygdala nucleus were in the La 14.2 ± 0.7, Ba 22.5 ± 0.9, Ab 12.6 ± 0.7, CeM 13.6 ± 0.4, and CeLc 8.5 ± 0.7 (mean ± S.E.M.). For TH, the number of images analyzed was in the La 11.7 ± 0.7, Ba 15.7 ± 0.8, CeM 5.3 ± 0.4, CeL 5.5 ± 0.6, and CeLc 5.4 ± 0.6 (mean ± S.E.M.). Fiber densities were quantified by counting crossing points of fibers with lines superimposed on digitized images [26]. In each image, lines used to quantify crossing points covered a distance of 54 μm for the 40X objective and a distance of 22 μm for the 100X objective in approximately 70% of the image area. Numbers of crossing points were averaged for all images taken from an individual animal. The IBM SPSS software package was used for statistical analyses. Fiber densities were compared using a two-way ANOVA (line x sex) followed by the post hoc Tukey test. Data from both sexes were presented together as mean ± S.E.M. in the absence of sex differences. Two-tailed p-values < 0.05 were deemed to be significant.