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Effects of footshocks on anxiety-like behavior and mRNA levels of precursor peptides for corticotropin releasing factor and opioids in the forebrain of the rat

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ABSTRACT

Corticotropin releasing factor (CRF) and dynorphin are neuropeptides that are associated with the negative emotional states. Experimental evidence indicates that dynorphin neurons located in the nucleus accumbens and CRF neurons in the bed nucleus of the stria terminalis (BST) and the central nucleus of the amygdala (CeA) mediate anxiety-like behaviors immediately after the stressful experience (24–48 h). The present study was done to evaluate if changes in the levels of the mRNA for these peptides in the striatum, BST, and CeA were associated with the long-lasting avoidance of novelty, a measure of an anxiety-like state, in a subset of rats exposed to unpredictable and moderately intense footshocks (5 × 2 s of 1.5 mA). Shocked rats with enhanced fear to a novel tone 24 h after the footshocks (high responders; HR) displayed long-lasting avoidance in the elevated T-maze whereas shocked rats with low levels of acute fear (low responders; LR) had low levels of avoidance similar to nonshocked rats. An increase in the level of proCRF mRNA was detected in the CeA of the HR compared to LR and nonshocked rats but not in other areas of the brain sampled. In contrast, prodynorphin and proenkephalin mRNA levels in the striatum, BST and CeA were not different between HR, LR and nonshocked rats. This study provides evidence that CRF neurons in the CeA may play a role in the anxiety-like state produced in a subset of rats exposed to footshocks.

1. Introduction

The extended amygdala is an anatomical macrostructure that extends along the anterior commissure and includes parts of the bed nucleus of the stria terminalis (BST) and the centromedial amygdala (Alheid, 2003; Alheid et al., 1999; de Olmos and Heimer, 1999; Heimer and Alheid, 1991). While not included in the original description of the extended amygdala, some researchers also include the shell of the nucleus accumbens because of some similarities between this part of the striatum and the rest of the extended amygdala (Heimer and Alheid, 1991; Koob, 2003). Experimental evidence from a variety of sources indicates that some parts of the extended amygdala are involved in the behavioral reaction to stress and in negative emotional states such as dysphoria, fear and anxiety (Davis et al., 2010; Koob, 2003). The lateral portion of the BST and the lateral subnucleus of the central nucleus of the amygdala (CeA) contain neurons that produce corticotropin releasing factor (CRF) (Cassell et al., 1999), a peptide shown to mediate the behavioral effects of stress (Heinrichs and Koob, 2004). For example, microinjections of CRF into the amygdala or the BST produced anxiety-like effects (Lee and Davis, 1997; Lee et al., 2008; Sahuque et al., 2006; Tazi et al., 1987) whereas microinjection of CRF antagonists into the CeA and BST blocked the negative emotional behaviors associated with a variety of aversive conditions (Erb et al., 2001; Heinrichs et al., 1992; Rassnick et al., 1993; Swiergiel et al., 1993). The opioid peptide dynorphin is another stress-related peptide found in high concentration in the extended amygdala and the shell of the nucleus accumbens (Cassell et al., 1986, 1999; Furuta et al., 2002; Poulin et al., 2009). A number of observations implicate dynorphin in the nucleus accumbens in the behavioral responses to stress (Knoll and Carlezon, 2010). For example, microinjections of dynorphin in the nucleus accumbens produce conditioned place aversion (Bals-Kubik et al., 1993) and exposure of rodents to stress leads to a dynorphin mediated dysphoria through mechanisms localized in the nucleus accumbens (Bruchas et al., 2007). Stress-induced behavioral depression was also prevented by inhibition of dynorphin synthesis and by microinjections of a kappa opioid antagonist in the nucleus accumbens (Newton et al., 2002). Other studies indicate that decreases in enkephalin activity in the amygdala contribute to anxiety-like behavior following acute and chronic stress and that the activity of these enkephalin neurons promote individual resilience to stressful situations (Berube et al., 2013, 2014; Hebb et al., 2004).

Exposure of rodents to unpredictable and moderately intense footshocks produces long-lasting generalized anxiety-states

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(Bruijnzeel et al., 2001a, 2001b; Chen et al., 2012; Louvart et al., 2005; Mikics et al., 2008a, 2008b; van Dijken et al., 1992) and shock-induced changes in brain peptide activity represent a mechanism for this effect (Chen et al., 2014). The purpose of the present study was to determine if the CRF and dynorphin systems become enhanced in a subgroup of rats that show a high level of anxiety-like behavior following footshock exposure. This was done by quantifying the levels of the precursor genes for CRF (proCRF), dynorphin (proDYN), and enkephalin (proENK) in subregions of the extended amygdala and striatum in shocked rats with anxiety-like behavior (high responders, HR) and no anxiety (low responders, LR) (Chen et al., 2012).

2. Materials and methods

2.1. Animals

An initial total of 40 male Sprague–Dawley rats of 6 weeks of age weighing 130–150 g (University of Manitoba, Manitoba, Canada) were pair-housed in plastic cages in a colony room on a 12-hour light–dark cycle (6:00–18:00) with controlled temperature (20–24 °C) and humidity (40–70%). Animals had free access to food and water. The rats were handled every other day during a 12-day adaptation period before being used for the experiments. All experimental procedures were approved by Research Ethics Review Board of University of Manitoba and in compliance with the Canadian Council on Animal Care.

2.2. Footshock procedure and grouping of subjects

Rats were transferred one at a time to a room which was exclusively dedicated to footshock delivery. After a 2 min acclimation period in the footshock chamber (MED Associates, Vermont, USA), rats received inescapable footshocks (5 × 2 s of 1.5 mA shocks presented randomly over 3 min with an intershock period of 10–50 s). The rats were kept in the chamber for another 60 s before they were returned to their home cages. Nonshocked rats were placed in the chamber for the same amount of time but no shocks were delivered. The shock chamber was cleaned using 10% alcohol and bedding under the grid floor was changed after each rat.

Rats were subdivided to HR and LR groups based on a previously described procedure (Chen et al., 2012). Briefly, fear to a tone in a different chamber (Plexiglas, length 65 cm × width 40 cm × height 50 cm; illuminated at 3–5 lx) was assessed 24 h after the footshock episode. The duration of this test was 6 min and comprised of 3 min of background noise (45–50 dB) followed by a 3 min presentation of a novel auditory tone of 9 kHz (75 dB). The chamber was cleaned with 0.5% liquinox after exposure of the chamber to each rat. Immobility (lack of body movement except breathing) during the novel tone was scored for each rat and the percentage of immobility (immobility duration/3 min × 100) was used to divide the shocked rats into LR (immobility < 40%) and HR (immobility > 60%), which respectively show low and high levels of avoidance (Chen et al., 2012).

2.3. Behavioral tests

Fear to the shock context was assessed by measuring immobility to the shock chamber for 5 min at 2 days after the footshock episode. The shock chamber was cleaned with 10% alcohol and bedding under the grid floor was changed between rats. Immobility to the shock context was scored and the percentage of immobility (immobility duration/5 min × 100) was calculated for each rat.

The elevated T-maze (ETM) was used at 10–11 days after the shock exposure to assess avoidance and escape. The ETM was made of black Plexiglas and consisted of three arms elevated 50 cm from the floor. One closed arm (length 50 cm × width 10 cm × height 30 cm) was perpendicular to the two open arms (length 50 cm × width 10 cm × height 0.5 cm) and the test was done in a dimly lit room (3–5 lx). The test involved placing a rat at the distal end of the closed arm facing the open arms and measuring the latency for the four paws of the rats to cross to the open area (baseline anxiety). The same procedure was repeated twice (avoidance learning 1 & 2). In addition, two escape trials (escape 1 & 2) were done following the avoidance trials. The escape trials involved placing of the rat at the distal end of one open arm and measuring the latency for the four paws of the rats to enter the closed arm. All the trials were terminated after 5 min and there was a 30 s interval between each trial involving returning the rat back to its home cage. The ETM was cleaned with 0.5% liquinox after each trial.

2.4. Tissue preparation

Fourteen days after the footshock exposure, the rats were anesthetized using chloral hydrate (600 mg/kg, i.p.) and perfused with 150 ml ice-cold 0.1 M phosphate buffered saline (PBS) before removal of the brain. Brains were placed in a matrix with 1 mm divisions to cut sections of the brain from which brain samples were collected. For microdissection, the coronal sections of the brain were placed on a plastic petri dish, and according to an anatomical atlas (Paxinos and Watson, 2005), areas of interest were visualized and dissected out using a dissecting microscope. Samples for the nucleus accumbens (core and shell) as well as the medial and lateral caudate–putamen were dissected from sections at the level of the striatum (1.0 to 2.0 mm anterior to bregma; Fig. 1B). Coronal sections were taken to dissect out the BST (0.0 to 1.0 posterior to bregma; Fig. 1B) and the amygdala samples (1.8 to 2.8 posterior to bregma; Fig. 1B). Amygdala samples contained all of the CeA and relatively small portions of the basolateral and medial amygdala. All procedures were done on ice with all surfaces in contact with the brain tissue cleaned with RNaseZap (Life Technologies Inc., ON, Canada); tissues were collected in RNase-free microtubes containing RNAlater solution (Life Technologies Inc., ON, Canada) and stored at −80 °C for later analysis.

2.5. Real-time PCR (RT-PCR)

Total RNA was extracted from individual homogenized samples using the protocol in the QIAzol® handbook (Qiagen, ON, Canada) and purified using RNeasy Mini Kit (Qiagen, ON, Canada). The concentration of purified RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Delaware, USA). The first-strand cDNA synthesis was performed using qScript™ cDNA SuperMix (Quanta BioSciences, Maryland, USA). Quantitative PCR was performed on the genes of interest in triplicate using an Eco™ Real-Time PCR system (Illumina, California, USA). All PCR reactions were carried out in a PCR MasterMix consisting of 5.1 μl of RNase-free water, 0.9 μl of each primer mix (FWD + REV; 20 μM), 2.0 μl of cDNA, and 2.0 μl of MBI EVOlution EvaGreen® qPCR master mix (MBI Lab Equipment, PQ, Canada). An amplification and detection procedure was carried out which consists of denaturation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 15 s, plus 72 °C for 15 s, followed by melt curve stage of 95 °C for 15 s, 55 °C for 15 s and 95 °C for 15 s. All primers were obtained from Integrated DNA Technologies and validated as previously described (Zhang et al., 2012). The housekeeping gene Hprt1 was used as the internal quantitative control. The primers used were: Hprt-1 forward, 5′-CTC ATG GAC TGA TTA TGG ACA GGA C-3′ and reverse, 5′-GCA GGT CAG CAA AGA ACT TAT AGC C-3′; proCRF forward, 5′-CAT CTC TCT GGA TCT CAC CT-3′ and reverse, 5′-TTG CTG AGC TAA CTG CTC TG-3′; proDYN forward, 5′-TGT CCT TGT GTT CCC TGT G-3′ and reverse, 5′-CAC TCC AGG GAG CAA ATC AG-3′; proENK forward, 5′-AAC TTC CTG GCA TGC ACA CT-3′, and reverse, 5′-CTC ATC CTG TTT GCT GCT GTC-3′.

2.6. Data analyses

All the behavioral tests were recorded with a video camera for subsequent analysis by two experimenters blind to the condition. A one-way