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Research report

Increased CRF mRNA expression in the sexually dimorphic BNST of male but not female GAD67 mice and TMT predator odor stress effects upon spatial memory retrieval

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HIGHLIGHTS

- Male GAD67 with 50% decreased GAD67 protein in the brain show increased CRF mRNA in the BNST.
- A deficit in GABAergic transmission in male GAD67 mice could lead to a compensatory CRF increase.
- GAD67 mice show already increased working memory errors in a RAM task after control odor exposure.
- Wild types show increased working memory errors only after TMT predator odor exposure.

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ABSTRACT

The bed nucleus of the stria terminalis (BNST) is an important region for 2,5-dihydro-2,4,5-trimethylthiazoline (TMT) predator odor-induced stress responses in mice. It is sexually dimorphic and a region for corticotropin-releasing factor (CRF)-enhanced stress responses. Dense GABAergic and CRF input from the amygdala to the BNST gives point to relevant interactions between CRF and GABA activity in these brain regions. Hence, to investigate sexual dimorphism of stress-induced neuronal changes, we studied effects of acute TMT exposure on CRF mRNA expression in stress-related brain regions in male and female GAD67 mice and their wild-type littermates. In GAD67 mice, heterozygous knock-in of GFP in GABAergic neurons caused a 50% decrease of GAD67 protein level in the brain [91,99]. Results show higher CRF mRNA levels in the BNST of male but not female GAD67 mice after TMT and control odor exposure. While CRF neurons in the BNST are predominantly GABAergic and CRF enhances GABAergic transmission in the BNST [20,51], the deficit in GABAergic transmission in GAD67 mice could induce a compensatory CRF increase. Sexual dimorphism of the BNST with greater density of GABA-ir neurons in females could explain the differences in CRF mRNA levels between male and female GAD67 mice.

Effects of odor exposure were studied in a radial arm maze (RAM) task. Results show impaired retrieval of spatial memory after acute TMT exposure in both sexes and genotypes. However, only GAD67 mice show increased working memory errors after control odor exposure.

Our work elicits GAD67 mice as a model to further study interactions of GABA and CRF in the BNST for a better understanding of how sex-specific characteristics of the brain may contribute to differences in anxiety- and stress-related psychological disorders.

1. Introduction

The predator odor 2,5-dihydro-2,4,5-trimethylthiazoline (TMT), a synthetic component of fox feces, is commonly used to induce stress and unconditioned fear in rodents [10,31,38,46,61]. It is an ethologically relevant stressor that modulates learning and memory processes [37,47,62,63]. TMT activates a special pattern of limbic structures such as the amygdala, the bed nucleus of the

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stria terminalis (BNST) and the paraventricular nucleus (PVN) of the hypothalamus, as well as their downstream targets in the brainstem [24,31–33,46]. It is widely known that stress influences cognitive functions and additionally to the role of corticosterone in these mechanisms; there is evidence for an important contribution of corticotropin-releasing factor (CRF) in stress-induced memory changes [12,42,57,65,100]. CRF is a stress-related neuropeptide that coordinates the physiological and behavioral responses to stress, partly through activation of limbic structures related to fear and anxiety, like the amygdala and the BNST [1,51,55,75]. The BNST is a complex and heterogeneous structure critically involved in anxiety behavior and the control of the hypothalamus-pituitary-adrenal (HPA) axis [13,48,49,94]. Rodents exposed to the synthetic predator odor TMT show increased neuronal activation of the BNST indicated by the immediate-early-gene c-fos [3,24,46]. The BNST receives important projections from the amygdala, originating in neurons of the central nucleus of the amygdala (CeA), which co-express GABA and CRF [34,51,77,92]. The role of CRF in anxiety in the BNST network is well established [1,34,75,80,90,94]. CRF neurons in the BNST have a predominantly GABAergic phenotype and express GAD67 [19,20] and BNST neurons densely innervate the hypothalamic PVN [13,19,28,78], thus elevated CRF levels in the BNST are probably important for stress-induced modulation of the HPA axis [13,94]. During conditions of fear and anxiety, GABAergic and CRF input to the BNST seems to be critical for the function of the nucleus and for shaping BNST output [77].

Hence, we suggest that alterations of glutamic acid decarboxylase (GAD67), the enzyme responsible for the conversion of glutamate to GABA [8,29,39,86], whose expression was shown to be induced by acute stress in the BNST [26,76], may influence the complex interactions of large populations of GABA- and CRF-containing neurons located within the CeA, the BNST and the PVN, brain areas crucial for fear and anxiety [34].

Here, we investigated the effects of TMT exposure, as an ethologically relevant stressor, on CRF mRNA induction in stress-related brain regions of heterozygous GAD67-GFP knock-in mice (GAD67 mice) and their wild-type (WT) littermates of both sexes. In GAD67 mice, GFP is specifically expressed under the control of the endogenous GAD67 promotor [89,91]. GFP cDNA knock-in into the endogenous GAD67 gene caused consistent labeling of GABAergic neurons [50,89] and is associated with a decreased GAD67 protein level in the brain to half as compared to wildtype mice [91,99]. Therefore, the GAD67 mouse is a useful tool to investigate the effects of lower GAD67 expression, the most relevant enzyme for stress-induced GABA synthesis.

Because CRF has been shown to modulate learning and memory [64,73], we looked for effects upon retrieval of spatial memory in a radial arm maze (RAM) learning task. Hippocampus-dependent learning in the RAM was frequently used to measure spatial and non-spatial learning and memory [17,78]. Several studies have shown differences in learning abilities between different mice strains [17,78] and sex effects upon RAM performance [9,65]. Previously, we have shown that TMT stress has different actions on the strength of spatial memory formation depending on the timing with regard to memory phases. We showed that acute TMT stress before retrieval facilitates RAM performance in male GAD67 mice and their wildtype (WT) littermates, while repeated TMT stress during consolidation exerts no influence [47].

We and several other investigators showed TMT-induced neuronal activation of the BNST [24,31,32,46,52,97]. Most of these studies were performed in male rodents. Hence, with respect to the known sexual dimorphism of the BNST [15,25,41,70,81–84], in this study we examined TMT effects on spatial memory retrieval in male and female GAD67 mice compared to WT and looked for sex-specific differences in CRF mRNA expression peptide in the BNST and other brain regions important for stress- and anxiety-related behavioral and endocrine responses. Prior studies showed a significant decrease in hypothalamic CRF-like immunoreactivity levels 15 and 30 min after the start of restraint stress followed by a significant increase at 60 min [60]. Since CRF mRNA expression peaked at 60 min [74] and c-fos mRNA showed a peak 30 min after acute stress, different time peaks of expression in the brain disallowed detection of both. Hence, we focused on CRF mRNA detection in stress-related brain regions, because we and other groups already showed strong c-fos increase after TMT exposure in these brain regions.

2. Material and methods

2.1. Subjects

Twenty-four adult, heterozygous GAD67-GFP knock-in mice (GAD67 mice; 13 male, 11 female) and 24 WT littermates (C67BL/6J mice; 12 male, 12 female), 12–20 weeks of age, were used. GAD67 mice were heterozygous for insertion of the gene encoding green fluorescence protein (GFP) to the Gad 1 gene. In these mice, enhanced GFP is expressed in GABAergic neurons under the control of the endogenous GAD67 gene promoter [89,91]. GAD67 mice were bred by crossing heterozygous animals with C67BL/6J mice. Animals were analyzed by PCR analysis of tail DNA, using primers specific for the GFP sequence (TR GFP-8: CTG CTT GTC GGC CAT GAT ATA GAC G; TR-16: GGC ACA GCT CTC CCT TCT GTT TGC; TR-3: GCT CTC CTT CGC GTT CCG ACA G). Before the RAM experiment, mice were housed in transparent cages in groups of two to five with water and standard pellet food ad libitum and maintained on a 12 h:12 h light/dark cycle. All experiments were carried out according to the European Communities Council Directive (ECG) 86/609/EEC and approved by local authorities.

2.2. Odorants

2,5-dihydro-2,4,5 trimethylthiazoline (TMT) is a volatile component of the anal gland secretion of the red fox. The chemically derived substance was obtained from PheroTech, Delta, Canada. Diethylphthalate (DEP; Sigma-Aldrich, Taufkirchen, Germany) was used as control substance.

2.3. Spatial learning

2.3.1. Eight-arm radial arm maze

The maze consisted of a central platform and eight identically shaped arms (25 cm long, 6 cm wide and 6 cm high). The platform and the arms had a cover plate, allowing the operator to place, remove and observe the mice in the maze. Mice were not able to see whether there was still the reward or whether it was eaten already. The reward (a small food pellet) was placed behind a black non-transparent barrier (for details, see Ref. [47]).

Before training, mice were weighted, separated and food deprived until they reduced their weight to 80–90% of initial body weight (body weight reduction to an average of about 83% of initial weight). Access to water was warranted throughout the whole time.

Mice received two pretraining sessions (habituation) at 24 h interval to get familiarized with the test situation (pre 1 and pre 2 in Fig. 1). During pretraining, mice were allowed to freely explore the maze for 10 min with free access to food in each arm. After 2 days of habituation, training took place for 5 consecutive days with one trial per day. Final training after odor exposure took place on day 6. To prevent mice from using intramaze cues, the arms were rotated after each pretraining and training day. All arms were rewarded (spatial memory test). A trial was stopped when all