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TRACE AND CONTEXTUAL FEAR CONDITIONING IS ENHANCED IN MICE LACKING THE $\alpha 4$ SUBUNIT OF THE GABA_A RECEPTOR

M.D. Moore¹, J. Cushman², D. Chandra³, G.E. Homanics³, R.W. Olsen¹, and M.S. Fanselow²

¹Department of Molecular and Medical Pharmacology, UCLA, Los Angeles, CA

²Psychology Department, UCLA, Los Angeles, CA

³Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, PA

Abstract

The GABA_A $\alpha 4$ subunit is highly expressed in the dentate gyrus region of the hippocampus at predominantly extra-synaptic locations where, along with the GABA_A δ subunit, it forms GABA_A receptors that mediate a tonic inhibitory current. The present study was designed to test hippocampus-dependent and hippocampus-independent learning and memory in GABA_A $\alpha 4$ subunit-deficient mice using trace and delay fear conditioning, respectively. Mice were of a mixed C57Bl/6J X 129S1/X1 genetic background from $\alpha 4$ heterozygous breeding pairs. The $\alpha 4$ -knockout mice showed enhanced trace and contextual fear conditioning consistent with an enhancement of hippocampus-dependent learning and memory. These enhancements were sex-dependent, similar to previous studies in GABA_A δ knockout mice, but differences were present in both males and females. The convergent findings between $\alpha 4$ and δ -knockout mice suggests that tonic inhibition mediated by $\alpha 4\beta\delta$ GABA_A receptors negatively modulates learning and memory processes and provides further evidence that tonic inhibition makes important functional contributions to learning and behavior.

Keywords

GABA_R; trace fear conditioning; delay fear conditioning; $\alpha 4$; δ ; tonic inhibition; neurosteroids; sex difference; hippocampus

INTRODUCTION

The γ -aminobutyric acid A receptor (GABA_AR) functions as the major mediator of both phasic and tonic inhibitory neurotransmission in the brain. It is composed of five subunits selected from 19 gene products that are currently known to play a role in the mammalian CNS ($\alpha 1$ – 6 , $\beta 1$ – 3 , $\gamma 1$ – 3 , δ , ϵ , π , θ , and $\rho 1$ – 3) (Olsen and Sieghart, 2008). Receptors containing $\alpha 4\beta\delta$ are predominantly expressed extrasynaptically where they mediate a tonic inhibitory current (Glykys et al., 2008) that is sensitive to modulation by low doses of neurosteroids and ethanol (Brown et al., 2002; Smith et al., 2007; Wallner et al., 2003). The

$\alpha 4$ subunit is primarily expressed in the molecular layer of the dentate gyrus, thalamus, striatum, nucleus accumbens, tuberculum olfactorium, and superficial layers of the neocortex, while being notably absent from the cerebellum (Cestari et al., 1998; Chandra et al., 2006; Pirker et al., 2000). Although typically expressed together, the $\alpha 4$ and δ subunits can partner with other subunits. δ can combine with $\alpha 6$ in the cerebellar granule cells and $\alpha 1$ in inhibitory interneurons in the molecular layer of the dentate gyrus (Glykys et al., 2007; Pirker et al., 2000; Sperk et al., 1997), whereas $\alpha 4$ can combine with $\gamma 2$ to form sub-synaptic GABA_AR's (Benke et al., 1997, Sur et al., 1999).

Electrophysiological studies have found that GABA_AR $\alpha 4$ KO mice show greatly reduced tonic inhibitory currents and slower mIPSC kinetics in dentate gyrus granule cells and thalamic relay neurons (Chandra et al., 2006; Liang et al., 2008). GABA_AR-mediated inhibition plays a critical role in modulating the dynamics of the neural circuitry thought to underlie learning and memory processes, influencing both information processing and synaptic plasticity (Buzsaki, 1997; Crabtree and Isaac, 2002; Paulsen and Moser, 1998; Read et al., 1994). The goal of the present study was to determine the learning and memory phenotype of GABA_AR $\alpha 4$ KO mice utilizing Pavlovian tone fear conditioning.

In tone fear conditioning an initially neutral tone conditional stimulus (CS) is paired with an aversive shock unconditional stimulus (US). In delay conditioning, the shock is temporally contiguous with the tone whereas in trace conditioning the shock onset follows a “trace” interval of 20 seconds after tone offset. Delay tone conditioning results from strengthening of the thalamic and cortical inputs which convey auditory information to sites of CS and US convergence within the amygdala (Boatman and Kim, 2006). Trace conditioning, however, requires the additional contribution of the hippocampus in order to support formation of the CS-US association (Chowdhury et al., 2005; McEchron et al., 1998; Misane et al., 2005; Quinn et al., 2005; Quinn et al., 2002; Tseng et al., 2004). Trace and delay fear conditioning, therefore, allow for the assessment of hippocampus-dependent and hippocampus-independent learning, respectively. A previous study found that GABA_AR δ KO mice exhibit normal delay fear conditioning but enhanced trace and contextual fear conditioning, with this behavioral effect limited to female mice (Wiltgen et al., 2005). This indicated that loss of the δ -subunit produces a specific sex-dependent enhancement in hippocampus-dependent learning and memory. This sex difference was hypothesized to be due to fluctuating neurosteroid levels in the female mice and the action of these endogenous compounds on δ -containing GABA_ARs, specifically in the dentate gyrus (Wiltgen et al., 2005). The present study, utilizing identical experimental procedures, assessed trace and delay fear conditioning in male and female GABA_AR $\alpha 4$ KO mice.

MATERIALS AND METHODS

All mice were produced in Pittsburgh (Chandra et al., 2006), shipped to UCLA and allowed a 4–6 week acclimation time. Mice ranged from 4–7 months of age at the time of study. They were of a mixed C57Bl/6J X 129S1/X1 genetic background from $\alpha 4$ heterozygous breeding pairs. The GABA_AR $\alpha 4$ KO mice were compared to their WT littermates. Mice were genotyped as described earlier (Chandra et al., 2006). All were housed in the Psychology Department vivarium at UCLA on a 12 h light/12 h dark cycle in groups of 3–5

and had free access to food and water. Experiments were performed on both female and male mice during the light phase of the cycle ($n = 8-10$ per group, for a total of 74 mice).

For a more detailed description of the methods see Wiltgen et al. 2005. As outlined in Figure 1, training took place in four identical chambers (Context A) (28 X 21 X 21 cm; Lafayette Instrument Co.) in a brightly lit room. The floor of each chamber consisted of a shock grid wired to a shock generator and scrambler (Med-Associates Inc.) to deliver foot shock. On the training day (Day 1) $\alpha 4$ KO or WT mice underwent either trace or delay fear conditioning. For both procedures, mice were placed in Context A and left to explore for 3 min before tone onset (20 sec, 75 dB, 2800 Hz). For delay conditioning, termination of tone was contiguous with foot shock (2 sec, 0.5 mA). For trace conditioning, tone and foot shock were separated by a 20-sec trace interval. Both groups received 5 tone-shock trials. In order to keep the overall session length the same, the trace-conditioned group received a 200-sec ITI (inter-trial interval, defined as the time between CS onset and the next CS onset) and the delay-conditioned group received a 220-sec ITI. After the last shock, animals were left for 2 min and then returned to their home cages.

For the tone test (Day 2), 24 hours after training, the animals were placed in a separate room with 4 novel and structurally distinct chambers (Context B). The tone was delivered in the same way as in Context A during training, but with shock omitted. On Day 3, 24 hours after the tone test, animals were returned to the holding room for 30 min before being placed back in Context A for an 8 min context test.

An observer blind to the genotype of the animal scored the presence or absence of freezing as a measure of conditional fear. For the tone test, freezing scores were grouped into 3 different bins: baseline (BL), tone, and post-tone or “trace” interval (the 20 sec immediately following tone termination). For the BL bin, freezing was scored every 8 sec. For both tone and trace bins, freezing was scored every 2 sec. Freezing data from the five tone presentations were averaged together. During the context test on Day 3, freezing was scored every 8 seconds. All freezing scores were transformed into percent freezing by dividing the total number of freezing by the total number of observations and multiplying by 100 (% Freezing = $\text{Freeze}_{\text{TOT}} / \text{Observations}_{\text{TOT}} \times 100$). The data was analyzed using the general linear model statistical package SPSS (SPSS Inc) with α set at 0.05.

RESULTS

The results indicate that GABA_AR $\alpha 4$ subunit deletion tended to enhance Pavlovian fear conditioning but the precise effects depend on the sex of the animal and the type of conditioning. Data from both trace and delay conditioned animals were combined and a 3-way ANOVA, with the factors genotype (WT vs. KO), training (trace vs. delay), and sex (male vs. female), was performed for baseline, tone, trace, and context freezing. Data for the tone test for delay conditioned animals are shown in Figure 2, data for the traced conditioned animals are shown in Figure 3, and the results of the context test are shown in Figure 4. Female mice froze significantly more than male mice during both the tone and post-tone intervals, as well as the context test, regardless of training or genotype ($F_{(1,73)} = 7.909, p = 0.006$; $F_{(1,73)} = 7.023, p = 0.010$, and $F_{(1,74)} = 6.498, p = 0.013$ respectively). As

expected, there was an overall enhancement in delay conditioned relative to trace conditioned mice during the tone presentation ($F_{(1,73)} = 20.828, p < 0.001$). GABA_AR $\alpha 4$ KO mice showed a significant enhancement of freezing during the tone ($F_{(1,73)} = 11.616, p = 0.001$), trace ($F_{(1,73)} = 4.515, p = 0.037$), and context ($F_{(1,74)} = 5.437, p = 0.023$) tests. Trace conditioned GABA_AR $\alpha 4$ KO mice froze significantly more during the tone ($F_{(1,36)} = 7.994, p = 0.008$), but delay conditioned GABA_AR $\alpha 4$ KO mice did not differ ($F_{(1,37)} = 3.233, p = 0.081$). Context freezing showed an overall enhancement in GABA_AR $\alpha 4$ KO mice, however, this enhancement depended on both sex and the type of training as indicated by a significant genotype x training x sex interaction ($F_{(1,74)} = 6.556, p = 0.013$). As shown in Figure 4, GABA_AR $\alpha 4$ KO males showed enhanced context freezing in the delay conditioned ($F_{(1,17)} = 7.25, p = 0.017$) but not the trace conditioned mice ($F_{(1,17)} = 0.129, p = 0.725$), whereas in GABA_AR $\alpha 4$ KO females the opposite pattern was observed, i.e. the significant enhancement was seen in trace ($F_{(1,17)} = 7.25, p = 0.017$), but not delay conditioned mice ($F_{(1,17)} = 0.129, p = 0.725$). Freezing during the baseline period showed no overall effect of genotype, sex, or training. There was, however, a genotype x training x sex interaction ($F_{(1,73)} = 4.739, p = 0.033$). This interaction was driven by a significant decrease in freezing specifically in the trace conditioned female GABA_AR $\alpha 4$ KO mice ($F_{(1,20)} = 9.086, p = 0.007$).

DISCUSSION

The results from this study demonstrate that a global loss of the GABA_AR $\alpha 4$ subunit enhances both trace and contextual fear conditioning in a sex-dependent manner. These findings add to the growing body of literature suggesting that GABA_AR-mediated inhibition plays a critical role in regulating learning and memory processes (Crestani et al., 2002; Vicini and Ortinski, 2004; Wiltgen et al., 2005). The sex interactions are consistent with a similar study in GABA_AR δ KO animals (Wiltgen et al., 2005), providing further evidence that neurosteroid sensitive $\alpha 4\beta\delta$ -containing GABA_AR's play a sex-dependent role in regulating learning and memory.

GABA_AR $\alpha 4$ KO mice showed increased freezing to a trace conditioned tone and during the post-tone trace interval. This suggests that the loss of tonic inhibition and alteration of mIPSC kinetics in dentate gyrus granule cells (Liang et al., 2008) results in an enhancement of hippocampus-dependent learning and memory. The trend towards an overall enhancement in tone conditioning suggests the possibility that the $\alpha 4$ -subunit in regions supporting auditory conditioning in general, such as the thalamus, may also contribute to the behavioral differences. A general increase in fear expression in GABA_AR $\alpha 4$ KO mice is unlikely given the overall pattern of results in this experiment, such as equivalent or even reduced baseline levels of freezing, as well as previous experiments, which have found no differences in anxiety or locomotor activity in the elevated plus maze or open field tests (Chandra et al., 2006).

The enhancement of freezing during the context test in GABA_AR $\alpha 4$ KO mice provides further support for the role of the GABA_AR $\alpha 4$ subunit in hippocampus-dependent learning and memory (Kim and Fanselow, 1992; Phillips and LeDoux, 1992). This enhancement showed a complex interaction with sex and the type of training. In GABA_AR $\alpha 4$ KO males,

the enhancement was limited to delay conditioned animals, whereas in females it was found specifically in the trace conditioned animals. One possible explanation for the complexity of this interaction is that the enhanced hippocampal function resulting from $\alpha 4$ -subunit deletion interacts with underlying sex differences in the mechanisms of associative competition. Discrete conditional stimuli, such as a tone, compete with other stimuli, such as the multimodal contextual CS, for association with the US (Kamin, 1968; Mackintosh, 1976; Rescorla and Wagner, 1972). The mechanisms of this associative competition are still controversial, however, in fear conditioning the conditional analgesia produced as part of the fear response is believed to play a critical role (Fanselow, 1998; McNally et al., 2004; Young and Fanselow, 1992). Females have greatly reduced conditional analgesia relative to males (Stock et al., 2001) suggesting that the rules by which the tone competes with the context in trace vs. delay fear conditioning might differ between males and females. These different rules of associative competition could interact with the loss of the $\alpha 4$ -subunit to produce the observed genotype by training by sex interaction in context fear.

Sex differences in the performance of learning and memory tasks have been widely reported in both rodents and humans (Cahill, 2003; Lambrey and Berthoz, 2007; Maren et al., 1994; Rizk-Jackson et al., 2006; Wiltgen et al., 2001). The overall enhanced freezing in females relative to males in the present study is consistent with previous findings (Wiltgen et al., 2005) and may reflect a general increase in the expression of defensive behavior in female rodents (Blanchard et al., 1990; Shors et al., 2000). Although the underlying mechanisms of sex differences in learning and memory are not well understood (Cahill, 2006), the overall phenotype of GABA_AR $\alpha 4$ KO mice in this study likely involves the differential contribution of neurosteroids in females relative to males.

Neurosteroids, namely the progesterone metabolite allopregnanolone (ALLO) and the deoxycorticosterone metabolite tetrahydrodeoxycorticosterone (THDOC), can act as high affinity GABA_AR modulators, especially at $\alpha 4\beta\delta$ -containing extrasynaptic receptors (Belelli and Lambert, 2005; Brown et al., 2002; Smith et al., 2007). The expression level of these neurosteroid-sensitive GABA_AR subunits in the hippocampus, periaqueductal gray, and amygdala have been shown to fluctuate in response to changes in ovarian hormone levels, estrous cycle phase, and stress (Corpechot et al., 1997; Gulinello and Smith, 2003; Lovick, 2006; Maguire and Mody, 2007). In addition, neurosteroids have been shown to modulate anxiety, learning and memory processes, and synaptic plasticity (Bitran et al., 1999; Engel and Grant, 2001; Johansson et al., 2002; Mukai et al., 2006; Robel et al., 1995). Loss of neurosteroid-sensitive $\alpha 4\beta\delta$ -containing receptors in GABA_AR $\alpha 4$ KO mice could therefore interact with sex and estrus cycle-dependent differences in neurosteroid levels to produce different behavioral effects in males compared to females as seen in this study.

The majority of the present results are in line with previous findings in GABA_AR δ KO mice; in fact, the effects in the female trace-conditioned groups are essentially identical (Wiltgen et al. 2005). Our findings, however, suggest a role for the GABA_AR $\alpha 4$ subunit in learning and memory in both sexes, a marked contrast to the findings in the GABA_AR δ KO mice in which enhanced trace and context fear was restricted to female mice. Although the GABA_AR δ and $\alpha 4$ subunits have regional overlap in the brain (Pirker et al., 2000; Sperk et al., 1997) and loss of one finds a concomitant down-regulation of the other, their functional

contributions are not identical, particularly in relation to synaptic inhibition (Korpi et al., 2002; Liang et al., 2008; Peng et al., 2002; Spigelman et al., 2002). Electrophysiological recordings from the dentate gyrus of GABA_A α 4 KO mice reveal slower synaptic mIPSC kinetics compared to WT mice (Liang et al., 2008), whereas GABA_A δ KO mice actually have faster synaptic mIPSC kinetics (Mihalek et al., 1999), despite similar reductions in tonic inhibitory currents in both genotypes. These differences are likely due to the faster kinetics of α 4 β 2 GABA_AR's (Smith and Gong et al, 2005), which are thought to show a compensatory increase in δ -subunit KO mice but are totally absent in α 4-subunit KO mice (Peng et al, 2002). Additionally, the expression of α 1 β 6 GABA_AR's in inhibitory interneurons is intact in α 4 KO mice, whereas it is absent in δ KO mice (Glykys et al, 2007). Disinhibition mediated by these α 1 β 6 GABA_AR's and/or the divergent effects on synaptic inhibition could underlie the greater prevalence of the learning enhancement in α 4-subunit KO mice.

The convergent findings of reduced tonic inhibition and enhanced hippocampus-dependent learning in both α 4 and δ KO mice strongly argues that tonic inhibition mediated by α 4 β 6 GABA_AR's negatively modulate learning and memory processes. At the cellular level, the overall reduction in neuronal excitability produced by tonic inhibition may limit the activity dependent synaptic modifications that are necessary for stabilization of the memory trace, whereas, at the systems level, tonic inhibition may regulate the speed and accuracy of information encoding and retrieval (Buzsaki, 1997; Crabtree and Isaac, 2002; Paulsen and Moser, 1998; Read et al., 1994). Future research will be required to determine the validity of these potential mechanisms and to unravel the prominent sex differences observed in this study.

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Figure 1. Experimental Design

(A) Outline of three-day procedure. (B) Diagram depicting temporal relationship between tone and shock in delay vs. trace conditioning.

Figure 2. GABA_AR α 4 KO mice show no differences in delay tone fear conditioning
Mean ($\pm$ SEM) percent freezing of α 4 KO and WT mice during the BL, tone, and trace intervals of the tone test for delay conditioned animals.

Figure 3. GABA_A α 4 KO mice exhibit an enhancement of trace tone fear conditioning
Mean ($\pm$ SEM) percent freezing of α 4 KO and WT mice during the BL, tone, and trace intervals of the tone test for trace conditioned animals.

Figure 4. The enhancement of context fear in GABA_A α 4 KO mice depends on both sex and type of tone conditioning

(A) Mean ($\pm$ SEM) percent freezing of male α 4 KO and WT mice during the context test.

(B) Mean ($\pm$ SEM) percent freezing of female α 4 KO and WT mice during the context test.