Brief Communication

δ Subunit-containing $GABA_A$ receptor prevents overgeneralization of fear in adult mice

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The role of δ subunit-containing $GABA_A$ receptor ($GABA_A$(δ)R) in fear generalization is uncertain. Here, by using mice with or without genetic deletion of $GABA_A$(δ)R and using protocols in which the conditioned tone stimuli were cross presented with different nonconditioned stimuli, we observed that when the two tone stimuli were largely similar, both genotypes froze similarly to either of them. However, when they differed markedly, the knockout mice froze much more than their wild-type littermates to the nonconditioned but not conditioned stimuli. Thus, $GABA_A$(δ)R may prevent inappropriate fear generalization when the incoming stimuli differ clearly from the learned threat.

[Supplemental material is available for this article.]

Fear learning is a classical form of conditioning through which the organisms learn to associate certain objects or circumstances with threat (Maren 2001). In natural environments, one very seldom experiences the same form of stimulus exactly as they ever encountered. Thus, to help survival, the species ranging from the rodents to human are evolutionarily endowed with an ability to generalize fear to the stimuli resembling aspects of the threat they previously learned (Dunsmoor and Paz 2015; Onat and Büchel 2015). However, inappropriate or excessive generalization of fear to the harmless cues and context is maladaptive and characteristic of a spectrum of anxiety disorders such as panic disorder or post-traumatic stress disorder (PTSD) (Craske et al. 2009; Davis et al. 2010).

The A type of γ-aminobutyric acid (GABA) receptors ($GABA_A$Rs) are heteropentameric ligand-gated channels formed from limited combinations of subunits including α1-6, β1-3, γ1-3, δ, ϵ, π, ρ1-3, and θ subunits (Farrant and Nusser 2005). Among them, the δ subunit-containing $GABA_A$R ($GABA_A$(δ)R) is found to be exclusively located in the extrasynaptic membrane and exhibits several unique properties distinguishable from other members of $GABA_A$R family. For instance, they are highly sensitive to low level of GABA in the extracellular space and show far slower desensitization upon continuous GABA presence. As such, the $GABA_A$(δ)R is regarded as one of the primary mediators of tonic inhibition, one important form of GABAergic inhibition resulting from activation of peri- and extrasynaptic $GABA_A$Rs in neurons across brain regions including hippocampus, cerebellum, and cortex (Brickley and Mody 2012).

In the past decade, $GABA_A$(δ)R-mediated tonic inhibition has been demonstrated to regulate anxiety-related behaviors and seizure susceptibility (Maguire et al. 2005), learning, and memory (Shen et al. 2010). It is also shown to be engaged in regulating the expression and extinction of learned fear (Wiltgen et al. 2005). However, it remains unclear whether this receptor participates in regulating fear generalization. In the present study, we attempted to address this issue by comparing the generalization of learned fear in $GABA_A$(δ)R knockout (KO) mice and their wild-type (WT) littermates.

Adult male WT and KO mice with C57BL/6 J background were bred as previously described (Liu et al. 2016) and used at age of 60–70 d for all experiments. Auditory fear conditioning was performed as indicated in Figure 1A. On the first day, the mice were placed in a conditioning chamber (Med Associates) for 2 min of habituation to the apparatus, followed by five nonconditioned stimuli (CS−, 10 kHz, 80-dB tone; 20 sec) and five conditioned stimuli (CS+, 3 kHz, 80-dB tone; 20 sec) presented in random intervals (40–120 sec). On the second day, the animals were trained with 5 CS− and 5 CS+, with the CS+ being coterminated with a US (electrical shock; 0.5 mA, 0.5 sec). The CS− and CS+ were cross presented in random intervals (40–120 sec; Supplemental Fig. 1A). Twenty-four hours after the training, mice were placed in a different context for 3 min before testing their ability to generalize fear. 2 CS− and 2 CS+ were sequentially presented at an interval of 40 sec (Botta et al. 2015). The freezing of the mice was defined as a complete lack of movement except breathing and quantified by a video-based system (Freezeframe; Actimetrics) during the experiment.

We first determined whether $GABA_A$(δ)R was involved in fear generalization by using a training protocol in which the CS− differed from CS+ only in their frequency (10 versus 3 kHz, Fig. 1B, upper panel). Both the WT and KO mice were trained to learn association of fearful response with CS+ and differentiate them from CS−. Under this condition, there was no significant main effect of genotype and stimulus on the basal freezing during the habituation period (WT, $n = 10$; KO, $n = 10$; genotype: $F_{(1,36)} = 0.9930$, $P = 0.3257$; stimulus: $F_{(1,36)} = 1.060$, $P = 0.3101$,

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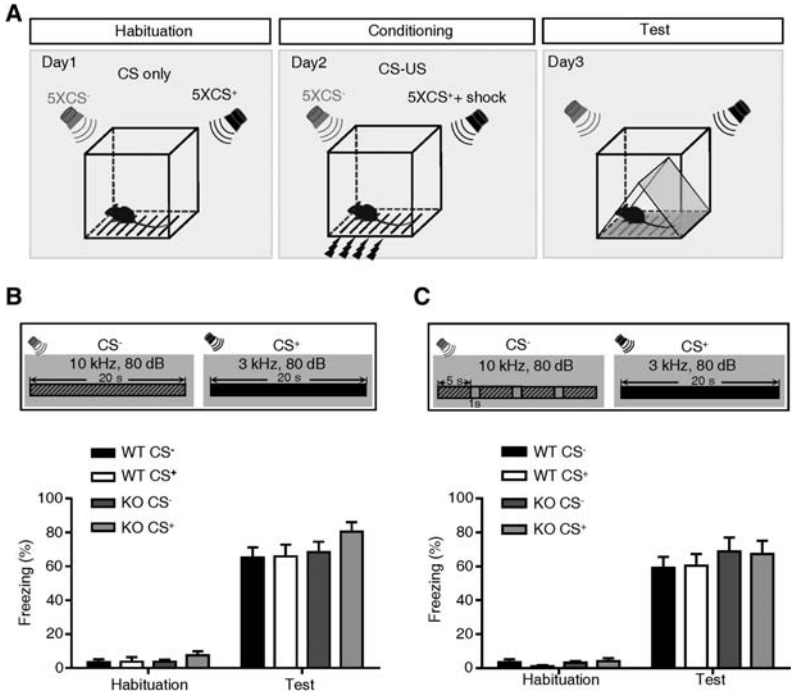

Figure 1. GABA$_A$(δ)R deletion has little effect on auditory fear generalization when CS− is presented for a continuum of 20 sec or separated into four sessions. (*A*) Schematic diagram showing the experimental design in the present study. (*B*) (*Upper* panel) Schematic diagram showing that CS− differs from CS+ only in the tone frequency (10 versus 3 kHz). (*Lower* panel) Freezing of the WT and KO mice to CS− and CS+ during habituation and test. (*C*) (*Upper* panel) Schematic diagram showing that CS− was presented in four 5-sec-long sessions and CS+ was presented continuously. (*Lower* panel) Freezing of the WT and KO mice to CS− and CS+ during habituation and test.

Although generalization of fear to events similar to the previously learned threat is adaptive for the survival of organisms, they also need to differentiate the harmless stimuli from those predictive of danger to prevent inappropriate expression of fear. To explore whether GABA$_A$(δ)R has a role in preventing inappropriate generalization of fear, we used a new form of CS− in which the tone was presented in short pips (0.5 sec) separated by a 1-sec no-tone interval (Fig. 2A). Neither genotype nor stimulus had significant main effect on the basal freezing of mice during the habituation period (WT, $n = 8$; KO, $n = 8$; genotype: $F_{(1,28)} = 3.448$, $P = 0.0739$; stimulus: $F_{(1,28)} = 1.7$, $P = 0.2029$, Fig. 2B). In addition, no significant between-genotype difference was observed on the freezing to CS+ during conditioning ($F_{(1,69)} = 0.6484$, $P = 0.4235$; Supplemental Fig. 1D). Twenty-four hours after training, an overall ANOVA with stimulus as a within-subjects factor and genotype as a between-subjects factor revealed significant main effect of stimulus ($F_{(1,28)} = 136.5$, $P < 0.0001$, Fig. 2B) but not genotype on the freezing of mice ($F_{(1,28)} = 1.963$, $P = 0.1730$, Fig. 2B). Moreover, a significant main effect of stimulus by genotype interaction was also observed ($F_{(1,28)} = 7.859$, $P = 0.0094$, Fig. 2B). Post hoc analysis demonstrated that this interaction was primarily driven by CS− because the different freezing between genotypes only existed when the CS− ($P = 0.0020$) but not CS+ ($P = 0.4115$) emerged (Fig. 2B). This indicates that GABA$_A$(δ)R deletion mainly affects the freezing of mice to CS− but not CS+. Thus, GABA$_A$(δ)R has a permissive role in preventing inappropriate expression of fear generalization.

Since overgeneralization of classically conditioned fear is characteristic of a spectrum of anxiety disorders (Lissek 2012; Fig. 1B, lower panel). Moreover, both genotypes showed similar response to CS+ during conditioning period ($F_{(1,88)} = 0.0960$, $P = 0.7574$; Supplemental Fig. 1B). Twenty-four hours after training, both genotypes showed robust freezing to either CS+ or CS−. Two-way ANOVA revealed that neither genotype nor stimulus had significant effect on the freezing of mice (genotype: $F_{(1,33)} = 2.037$, $P = 0.1629$; stimulus: $F_{(1,33)} = 1.066$, $P = 0.3095$; Fig. 1B, lower panel). On one hand, the similar and robust freezing of WT mice to CS+ and CS− indicates that they readily generalize the learned fear to CS−. On the other hand, the failure of GABA$_A$(δ)R KO to alter the mice's ability to discriminate between CS+ and CS− argues against an essential role of this receptor in generalization of learned fear.

To further examine the possible engagement of GABA$_A$(δ)R in fear generalization when CS− and CS+ also differed in the temporal pattern, we used another training protocol in which the CS− was altered from a continuum of 20 sec to four 5-sec-long sessions with each separated by a 1-sec no-tone interval (Fig. 1C, upper panel). Under this condition, both genotypes had similar basal response to CS+ and CS− during habituation (WT, $n = 7$; KO, $n = 7$; genotype: $F_{(1,24)} = 1.166$, $P = 0.2909$; stimulus: $F_{(1,24)} = 0.3417$, $P = 0.5643$, Fig. 1C), and indistinguishable freezing response to CS+ during conditioning ($F_{(1,60)} = 3.512$, $P = 0.0659$; Supplemental Fig. 1C). In addition, they showed strong freezing to either CS+ or CS− during the testing period and no significant effect of genotype and stimulus was found on the freezing of mice (genotype: $F_{(1,24)} = 0.084$, $P = 0.7743$; stimulus: $F_{(1,24)} = 0.288$, $P = 0.5972$; Fig. 1C, lower panel). Altogether, these findings indicate GABA$_A$(δ)R deletion is not sufficient to alter fear generalization under conditions when the learned fear is greatly generalized.

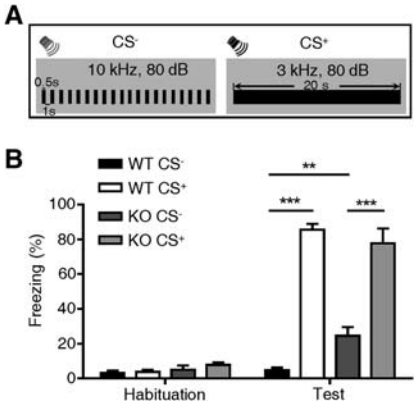

Figure 2. GABA$_A$(δ)R deletion results in inappropriate auditory fear generalization when CS− is presented in pips. (*A*) Schematic diagrams showing that CS− was given as pips tone (0.5 sec for 20 times, 1-sec interval) while CS+ was presented continuously. (*B*) Freezing of the WT and KO mice to CS− and CS+ during habituation and test. (**) $P < 0.01$, (***) $P < 0.001$.

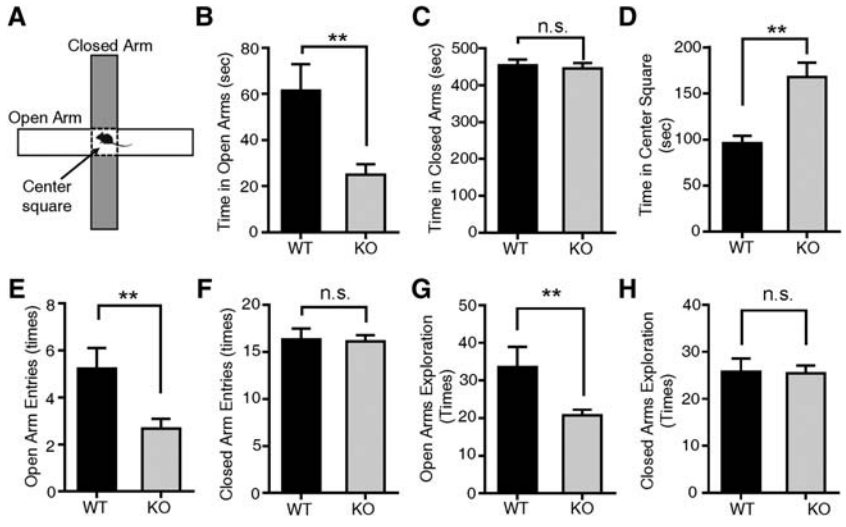

Figure 3. Effects of GABA$_A$(δ)R on anxiety-related behaviors. (*A*) Schematic diagram showing the elevated plus maze (EPM). (*B–D*) Comparison of the time spent in open arms (*B*), closed arms (*C*), and center square (*D*) between WT and KO mice. (*E,F*) Comparison of the number of open arm entries (*E*) and closed arm entries (*F*) between WT and KO mice. (*G,H*) Comparison of the exploration in open arms (*G*) and closed arms (*H*) between WT and KO mice. (**) $P < 0.01$. n.s., not significant.

Laufer et al. 2016), we next asked whether GABA$_A$(δ)R deletion would lead to aberrant anxiety-related behaviors. For this, we tested the anxiety-like behavior in KO and WT mice using the elevated plus maze (EPM). The EPM apparatus consisted of two opposing open and closed arms (Fig. 3A). Mice were given 1 h to habituate to the test room before the initiation of experiment. The animals were placed in the center square and faced to the open arm. The number of the animals' entry into either the closed or open arm, and the time they spent in each were recorded. Relative to their WT littermates, the KO mice spent less time in open arms (WT: $n = 9$; KO: $n = 12$; $P = 0.0037$; Fig. 3B) and had less entries to the open arms (WT: $n = 9$; KO: $n = 12$; $P = 0.0039$; Fig. 3E). However, both genotypes spent similar time in the closed arms (WT: $n = 9$; KO: $n = 12$; $P = 0.7087$; Fig. 3C) and had similar number of entries (WT: $n = 9$; KO: $n = 12$; $P = 0.8654$; Fig. 3F). On the other hand, the KO ones spent more time in center square (WT: $n = 9$; KO: $n = 12$; $P = 0.0029$; Fig. 3D), which was reconciled by reduced exploration times in open arms (WT: $n = 9$; KO: $n = 12$; $P = 0.0083$; Fig. 3G) and unaltered exploration times in closed arms (WT: $n = 9$; KO: $n = 12$; $P = 0.9140$; Fig. 3H). These data suggest that loss of GABA$_A$(δ)R results in more prominent anxiety-like behavior in mice.

Our findings provide the first piece of evidence highlighting the importance of GABA$_A$(δ)R in preventing excessive fear generalization. Indeed, recent studies have revealed that the GABA$_B$ receptor, which has similar subcellular localization as GABA$_A$(δ)R and is predominantly situated outside of synapses (Pan et al. 2009), plays a critical role in preventing the generalization of conditioned fear (Shaban et al. 2006; Botta et al. 2015). Due to the nature of their extrasynaptic location, both of them are activated by GABA diffused out of the synapses and show low level of desensitization in the continuous exposure of ambient GABA (Cope et al. 2005; Brickley and Mody 2012). More important, two early studies demonstrated that postsynaptic GABA$_B$ receptors enhanced the function of extrasynaptic GABA$_A$(δ) receptor-mediated tonic inhibition in dentate gyrus granule cells, thalamocortical cells and cerebellar granule cells (Connelly et al. 2013; Tao et al. 2013). It is thus likely that these two GABA receptors may participate in fear generalization through some overlapping mechanisms. Somewhat surprisingly, GABA$_A$(δ)R affects fear generalization only when the CS− are markedly different from the CS+ but not when they are similar. Thus, the role of GABA$_A$(δ)R in regulating fear generalization appears to be context specific.

GABA$_A$(δ)R has been observed to be extensively expressed in cerebellar granular cells (Jones et al. 1997; Nusser et al. 1999), dentate gyrus granule cells (Nusser and Mody 2002), thalamus relay nuclei, and neocortex (Cope et al. 2005; Botta et al. 2015). The expression of this receptor also varies drastically between the interneurons and projection neurons in these regions (Liu et al. 2016). Thus, the global deletion of GABA$_A$(δ)R in the current study raises a question regarding the specific brain region(s) in which this receptor acts to modulate fear generalization. Recent studies have consistently demonstrated that medial prefrontal cortex (mPFC), a region enriched with δ subunit (Maldonado-Avilés et al. 2009), is crucially involved in the generalization of learned fear. It is thus tempting to speculate that mPFC may be a plausible target for GABA$_A$(δ)R to prevent excessive generalization of fear.

While the present study has revealed a role of GABA$_A$(δ)R in regulating fear generalization, some potential interferences which may contribute to such GABA$_A$(δ)R effect should be considered. First, we exposed the animals to both CS+ and CS− during the habituation period, which may to some extent cause latent inhibition, a phenomenon referring to the fact that the prior nonreinforced exposure to CS impedes the conditioned response to CS when it is paired with US (Lubow and Moore 1959). Notably, the latent inhibition is thought to be mediated by trace conditioning effect in which CS and US are discontiguous and temporally segregated (Crestani et al. 2002). One recent study, however, reported that GABA$_A$(δ)R deletion unaltered trace conditioning in male mice (Wiltgen et al. 2005). Thus, the latent inhibition may play a minor, if there is some, role in overgeneralization subsequent to GABA$_A$(δ)R deletion. Second, in the three behavioral tests of the current study, the CS+ was presented with CS− with different temporal features, which might incur different pattern separation in mice and thus contribute to the different behavioral manifestations between WT and KO mice (Engin et al. 2015).

Our recent study has demonstrated that GABA$_A$(δ)R deletion results in decreased expression of learned fear in juvenile mice (30–40 d), which is associated with the loss of GABA$_A$(δ)R-mediated disinhibition in lateral amygdala (Liu et al. 2016). However, in the current study, no such influence was observed in adult mice. Thus, the role of this receptor in regulating fear learning may experience a shift during the transition from adolescence to adult. Several possible reasons may underlie this. First, the overall expression of GABA$_A$(δ)R in brain decreases during adolescent development (Shen et al. 2010), which may weaken its involvement in fear control. Second, several compensative mechanisms, for example, increased K^+ channel expression were observed to emerge upon the deletion of GABA$_A$(δ)R (Brickley et al. 2001). It is also likely that such mechanisms may become stronger during adolescent development, which may compensate the loss of function of GABA$_A$(δ)R.

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