

Cellular correlates of anxiety in CA1 hippocampal pyramidal cells of 5-HT_{1A} receptor knockout mice

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Abstract

Rationale 5-HT_{1A} receptor knockout (1AKO) mice have a robust anxiety phenotype. Tissue-specific “rescue” strategies and electrophysiology have implicated a critical role for postsynaptic 5-HT_{1A} receptors, particularly in the CA1 region of the hippocampus.

Objectives In this study, we evaluated differences in membrane properties and synaptic activity in CA1 hippocampal pyramidal cells between 1AKOs and wild-type (WT) controls to better understand the cellular correlates of anxiety in this mouse model.

Methods Whole-cell patch-clamp recordings were conducted in CA1 pyramidal cells in hippocampal brain slices from 1AKOs and WTs that had previously been screened for anxiety with the elevated-plus maze. Spontaneous miniature inhibitory and excitatory postsynaptic currents (IPSCs and EPSCs) and stimulus-evoked eIPSCs and eEPSCs were recorded in addition to the effect of the benzodiazepine agonist diazepam or the inverse agonist FG 7142 on γ -aminobutyric acid (GABA)ergic eIPSCs.

Results Evoked EPSC amplitude was greater in 1AKOs than WTs. When subjects were pooled across genotypes, anxiety measures correlated with eEPSC amplitude, indicating enhanced postsynaptic glutamate synaptic activity under conditions of synaptic activation in anxious subjects. While GABA synaptic activity and sensitivity to diazepam were not affected by genotype or correlated with anxiety, sensitivity to the anxiogenic FG 7142 was smaller in anxious subjects.

Conclusions These data indicate enhanced postsynaptic glutamate receptor sensitivity and decreased GABAergic inhibition by a benzodiazepine inverse agonist in CA1 hippocampal neurons of anxious mice are produced by deletion of the 5-HT_{1A} receptor. These data provide new information about interactions between 5-HT, GABA, and glutamate systems during the expression of chronic anxiety.

Keywords Serotonin · 5-HT_{1A} receptor · Hippocampus · Anxiety · Elevated-plus maze · Electrophysiology

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Introduction

Dysfunction of γ -aminobutyric acid (GABA) and serotonin (5-hydroxytryptamine, 5-HT) systems have been implicated in anxiety disorders (for review, see Charney and Drevets 2002; Millan 2003; Sandford et al. 2000). Much of the evidence for the involvement of these neurochemical systems comes from the finding that certain GABAergic and serotonergic drugs are effective anxiolytics (Bourin and Hascoet 2001). Benzodiazepines, that have anti-anxiety properties, act at the GABA_A receptor. In addition, 5-HT has also been linked to anxiety by the observation that

activation of 5-HT_{1A} receptors with partial agonists such as buspirone or indirectly through serotonin-selective reuptake inhibitors are effective treatments for anxiety disorders (for review, see Lucki 1996; Ravindran and Stein 2010).

Animal studies have shown that mice with a genetic deletion of the 5-HT_{1A} receptor have a behavioral anxiety phenotype, making 5-HT_{1A} knockouts (1AKOs) a useful model to study anxiety disorders (Heisler et al. 1998; Parks et al. 1998; Ramboz et al. 1998). The behavioral phenotype of these mice also includes decreased hippocampal-dependent learning (Sarnyai et al. 2000) and an antidepressant-like response in tail-suspension and forced swim test models (Heisler et al. 1998; Mayorga et al. 2001; Ramboz et al. 1998). The 1AKO mouse anxiety model is further validated by the finding that 5-HT_{1A} receptors are downregulated in patients with panic disorder (Neumeister et al. 2004). Conditional tissue-specific rescue strategies have more recently implicated a critical role of postsynaptic 5-HT_{1A} receptors in the forebrain in anxiety-like behaviors. Specifically, Gross et al. (2002) found that restoration of postsynaptic 5-HT_{1A} receptors in the hippocampus but not somatodendritic 5-HT_{1A} autoreceptors in the raphe nuclei re-established normal anxiety-like behavior. In addition, Gross et al. (2002) found that a successful rescue of the anxiety phenotype in these mice was determined by receptor expression early in development, prior to postnatal day 21, rather than in adulthood. This critical period during which forebrain 5-HT_{1A} receptors must be expressed for normal maturation of emotion/anxiety circuits was later refined through pharmacological blockade of the receptor to postnatal days 13 through 34 (Lo and Gross 2008). 1AKO mice have also been shown to have an enhanced neuronal excitability in area CA1 of the hippocampus as well as magnified hippocampal theta oscillations, particularly in anxiety-provoking conditions (Gordon et al. 2005). These data indicate that deletion of the 5-HT_{1A} receptor may cause altered synaptic activity and signaling, specifically in the CA1 region of the hippocampus.

While there is a growing literature generally implicating the ventral rather than the dorsal hippocampus in anxiety (for a recent review, see Fanselow and Dong 2010), the majority of studies characterizing the specific role of the hippocampal 5-HT_{1A} receptor in anxiety, including local administration studies, have indicated a preferential role of the dorsal subdivision (reviewed in Menard and Treit 1999). Stimulation of 5-HT_{1A} receptors in dorsal hippocampus has primarily anxiolytic effects (Carli et al. 1993; Graeff et al. 1996; Jolas et al. 1995; Kataoka et al. 1991; Kostowski et al. 1989; Menard and Treit 1998; Schreiber and De 1993; Stefanski et al. 1993) and occasionally anxiogenic effects (Andrews et al. 1994; Dos et al. 2008; File et al. 1996) in animal models. By contrast, stimulation of 5-HT_{1A} receptors in ventral hippocampus are without

effect (File and Gonzalez 1996; Hogg et al. 1994). In addition, Gordon et al. (2005) examined neurophysiological correlates of anxiety in 1AKO mice and found the 1AKOs had elevated theta oscillations in dorsal hippocampus when animals were exposed to the elevated-plus maze (EPM) and further that the magnitude of theta oscillations correlated with behavioral anxiety measures. Similar studies from this group have more recently implicated ventral hippocampus as well in the anxiogenic phenotype of the 1AKO mice (Adhikari et al. 2010). It is clear that both subfields of the hippocampus may contribute to the anxiety phenotype of this mouse model, but the current study focuses first on the role of the dorsal hippocampus while reserving investigations of the ventral hippocampus for future studies.

The purpose of the present study was to further evaluate differences in membrane properties, GABA and glutamatergic synaptic activity, and responses to benzodiazepines at the single-cell level in CA1 pyramidal cells of the dorsal hippocampus. 1AKO mice and wild-type (WT) controls were pre-screened for anxiety in the EPM and later electrophysiology experiments performed using whole-cell patch-clamp techniques in hippocampal brain slices to better understand the cellular correlates of anxiety in this animal model.

Materials and methods

Subjects

Male homozygote 5-HT_{1A} receptor knockout and wild-type mice on a 129/Sv background (for details, see Phillips et al. 1999) were generated by homozygous mutant or wild-type breeding colonies at Temple University. These homozygote breeding colonies were derived from heterozygous breeding pairs generously provided by Dr. Mark Geyer at UCSD, San Diego, CA. While homozygous breeding colonies are at risk for genetic drift due to spontaneous gene mutations that develop over successive generations (Crawley 2007), random mating strategies were employed to minimize this confounding factor. As a test for phenotype changes due to genetic drift, behavioral data (see anxiety and locomotor measures described in the EPM section below) from the initial 1AKO litters were compared with data from the final 1AKO litters (and a similar comparison made in the WT group) and no statistical difference was obtained. Mice were 3–6 months of age at the time of study and were housed 4 per cage on a 12 h light schedule (lights on at 0,700 h) in a temperature-controlled (20°C) colony room. Mice were given access to standard mouse chow and water ad libitum. Animal protocols were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*.

Elevated-plus maze

Mice were pre-screened for anxiety in a 50-cm-high black Plexiglas EPM. Lighting conditions were adjusted using a single fluorescent lamp on the room periphery to achieve dim overall illumination but to maintain visibility on the video recordings. The EPM consisted of two open arms (28×7 cm), with a 0.5 cm lip on each open arm, and two enclosed arms (30×7×13.5 cm) that extended from a central platform (7×7 cm). Subjects were videotaped in the maze for 10 min. Time spent and numbers of entries into open and closed EPM arms and EPM center were measured in the first 5 min of EPM exposure. Time spent and the number of entries into open arms are considered anxiety measures; whereas, the number of total or closed arm entries are considered locomotor measures (File 2001; Hogg 1996).

Brain slice preparation

In order to ensure that exposure to the EPM did not influence electrophysiological measures, animals were returned to the animal colony for a minimum of 3 weeks following the EPM test. Mice were rapidly decapitated and the head placed in ice cold artificial cerebrospinal fluid (ACSF) in which sucrose (248 mM) was substituted for NaCl. The brain was rapidly removed, dorsal hippocampal slices (250 μm thick) were cut using a Leica microslicer (Leica, Allendale, NJ) or Vibratome 3000 Plus (Vibratome, St. Louis, MO) and placed in a holding vial containing ACSF at 35°C bubbled with 95% O₂/5% CO₂ for 1 h. Slices were then maintained in room temperature ACSF bubbled with 95% O₂/5%CO₂. The composition of the ACSF was (mM), NaCl 124, KCl 2.5, NaH₂PO₄ 2, CaCl₂ 2.5, MgSO₄ 2, Dextrose 10, and NaHCO₃ 26.

Electrophysiological recording

Slices were transferred to a recording chamber (Warner Instruments, Hamden, CT) and continuously perfused with ACSF at 1.5–2.0 ml/min at 32–34°C maintained by an in-line solution heater (TC-324, Warner Instruments). Only one cell was recorded per brain slice. CA1 pyramidal neurons were visualized using a Nikon E600 upright microscope fitted with a 40X water-immersion objective, differential interference contrast and infrared filter (Optical Apparatus, Ardmore, PA). The image from the microscope was enhanced using a CCD camera and displayed on a computer monitor. Whole-cell recording pipettes were fashioned on a P-97 micropipette puller (Sutter Instruments, Novato, CA) using borosilicate glass capillary tubing (1.2 mm OD, 0.69 mm ID; Warner Instruments). Recording pipettes (4–8 MΩ) were filled with an intracellular solution (in mM):

K-gluconate 70, KCl 70, NaCl 2, EGTA 4, HEPES 10, adenosine 5'-triphosphate magnesium salt (MgATP) 4, guanosine 5'-triphosphate sodium salt hydrate (NaGTP) 0.3, pH 7.3 for recording inhibitory postsynaptic currents (IPSCs) or (in mM): K-gluconate 120, KCl 10, MgCl₂ 1, HEPES 10, EGTA 0.02, MgATP 2, Na-phosphocreatinine 10, NaGTP 0.5, pH 7.3 for recording excitatory postsynaptic currents (EPSCs).

The majority of the cells recorded were pre-screened for the presence of strong spike frequency accommodation in response to a depolarizing current pulse which distinguishes pyramidal cells from interneurons (Freund and Buzsaki 1996; Lacaille et al. 1987). A visualized cell was approached with the electrode, a gigaohm seal established and the cell membrane ruptured to obtain a whole-cell recording using a Multiclamp 700B amplifier (Axon Instruments, Foster City, CA). Series resistance was monitored throughout the experiment. If the series resistance was unstable or exceeded four times the electrode resistance, the cell was discarded. Once the whole-cell recording was obtained, IPSCs and EPSCs were recorded in voltage-clamp mode ($V_m = -70$ mV). Signals were digitized by a Digidata 1320-series analog-to-digital converter and stored on-line using pClamp 9 software (Axon Instruments). Signals were filtered at 1 kHz and digitized at 10 kHz. The liquid junction potential was approximately 9–10 mV between the pipette solution and the ACSF and was not subtracted from the data obtained.

For stimulation experiments, bipolar tungsten stimulating electrodes (World Precision Instruments, Sarasota, FL) were placed ventral (100–200 μm distance) to the recorded cell in the Schaffer collateral pathway. For each cell, stimulus response curves were generated and a stimulus intensity that produced 50% of the maximum response was selected. Paired pulses (50 ms interval, every 20 s) of constant current were delivered with an Isoflex stimulus isolator (A.M.P.I., Jerusalem, Israel). The mean stimulus intensity was 0.62 ± 0.19 mA with a range of 0.02 to 2.5 mA for evoked IPSC (eIPSC) experiments and 0.45 ± 0.10 mA with a range of 0.02 to 3.0 mA for evoked EPSC (eEPSC) experiments.

GABAergic IPSCs were isolated by addition of the non-N-methyl-D-aspartate (NMDA) receptor antagonist 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX; 20 μM) and the NMDA receptor antagonist DL-2-amino-5-phosphonopentanoic acid (APV; 50 μM) and frequently confirmed by the addition of the GABA_A receptor antagonist bicuculline (20 μM) at the end of the experiment. Glutamatergic EPSCs were isolated by the addition of bicuculline (20 μM). Under these recording conditions (holding current = -70 mV with the presence of magnesium in the electrolyte), EPSCs should be mediated by non-NMDA receptors. This is further supported by the finding in

multiple cells and in multiple animals that addition of the α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/kainate receptor antagonist DNQX alone (20 μ M) at the end of an experiment completely suppressed EPSCs. The decay kinetics of miniature spontaneous EPSCs were rapid and fit by a single exponential that are characteristic of AMPA rather than slower kainate-mediated events (Cossart et al. 2002). Spontaneous inhibitory or excitatory postsynaptic currents (sIPSCs or sEPSCs) were recorded in the absence and miniature spontaneous IPSCs or EPSCs (mIPSCs or mEPSCs) in the presence of tetrodotoxin (1 μ M) to block action potential-mediated GABA or glutamate release.

Data analysis

MiniAnalysis software (Synaptosoft, Inc., Decatur, GA) was used to analyze spontaneous and miniature IPSC and EPSC events on the basis of amplitude, rate of rise, duration and area. Initially, noise analysis was conducted for each cell and amplitude detection thresholds set to exceed noise values (usually around 5 pA). Events were automatically selected, analyzed for double peaks, then visually inspected and confirmed. Event amplitude histograms were generated and compared with the noise histogram to ensure that they did not overlap. Synaptic activity was analyzed for frequency, amplitude and baseline holding current. Baseline synaptic activity data was compared between experimental groups by unpaired Student's *t*-test. To examine tonic inhibition by GABA or tonic excitation by glutamate, the effect of GABA or glutamate receptor blockade on resting membrane potential or holding current was compared between genotypes by two-way repeated measures ANOVA.

Evoked IPSC or EPSC amplitude was calculated by subtracting the peak current from the current obtained during a 5 ms window immediately preceding the stimulus artifact. Baseline eIPSC or eEPSC amplitude was averaged from at least 60 consecutive trials calculated over at least a 10 min period. Paired-pulse ratio (PPR) was calculated as the amplitude of the second eIPSC or eEPSC divided by the amplitude of the first eIPSC or eEPSC, respectively. The effects of diazepam or FG 7142 on eIPSCs were determined from the average of at least 60 consecutive trials 3–6 min following drug application when drug effect had reached steady state. The effect of these drugs on eIPSC amplitude or PPR was analyzed by comparing pre- and postdrug values by paired Student's *t*-test. Physiological characteristics and measures of synaptic activity were correlated with the percent of time in open arm index of anxiety measured during the EPM test by Pearson product-moment correlation. A probability of $p < 0.05$ was considered significant. Data are reported as mean \pm SEM.

Drugs

Drugs were bath applied and most chemicals for making the ACSF and electrolyte solution obtained from Sigma–Aldrich (St. Louis, MO). DNQX was purchased from Tocris (St. Louis, MO) and TTX was purchased from Calbiochem (San Diego, CA). FG 7142 was obtained from Sigma–Aldrich and diazepam (DZ) was generously provided by NIDA. Diazepam, FG 7142 and DNQX were dissolved in DMSO (final DMSO concentration in bath 0.015%). Diazepam has been shown to have a K_D of 6.9 nM in mouse brain membranes and FG 7142 inhibits [3H]-diazepam binding with an IC_{50} of 27.9 nM (Allan et al. 1992). We selected concentrations of diazepam (1 μ M) and FG 7142 (3 μ M) to fully saturate the receptor.

Results

Elevated-plus maze

Figure 1 shows the behavioral data from the EPM model. Figure 1a demonstrates that 1AKOs make significantly fewer open arm entries (as a percent of total arm entries; $p < 0.01$) and spend significantly less time in the open arms (as a percent of total time in the maze; $p < 0.05$) than WT controls. Both of these measures are indications of elevated anxiety in this animal model. Figure 1b shows that there is no effect of genotype on locomotor activity, as indicated by number of total arm entries.

Membrane characteristics and response to GABA_A and glutamate receptor blockade

There was no significant effect of genotype on either resting membrane potential (in current-clamp recordings) or holding current (in voltage-clamp recordings). Cells from WT mice had a resting membrane potential of -63.8 ± 1.2 mV ($N=27$). Cells from 1AKO mice had a resting membrane potential of -64.2 ± 1.1 mV ($N=35$). In a subset of cells, the effect of blockade of GABA_A receptors on resting membrane potential was examined and compared between genotypes. Cells from both WTs and 1AKOs showed a similar depolarization in response to bicuculline [from -66.1 ± 0.8 mV to -63.6 ± 0.8 mV in WTs ($N=18$) and from -68.0 ± 1.0 mV to -64.4 ± 0.9 mV in 1AKOs ($N=12$)]. There was a significant main effect of the drug ($F(1,28)=50.19$; $p < 0.001$) but no genotype \times drug interaction ($F(1,28)=1.79$; n.s.), indicating similar tonic inhibition by GABA in this preparation in the two groups. The effect of glutamate receptor blockade on holding current was also examined under voltage-clamp ($I=-70$ mV) conditions. A combination of APV and DNQX to block NMDA and non-

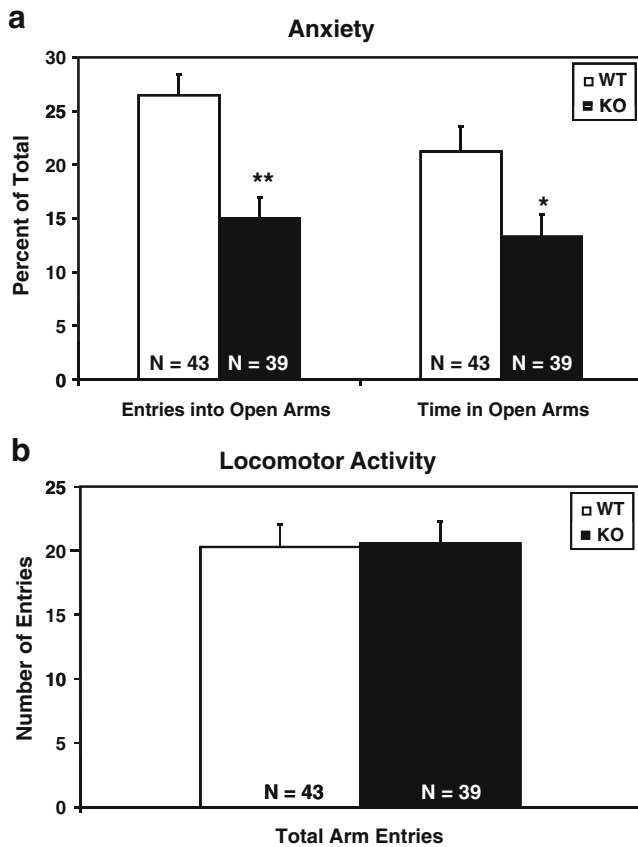


Fig. 1 1AKOs demonstrated an anxiety phenotype without alterations in locomotor activity when compared with WT controls in the elevated-plus maze (EPM) model. **a** Summary of the anxiety-like behavior of the 1AKOs in the EPM. 1AKOs had fewer open-arm entries (relative to total entries) and spent less time in the open arms of the EPM (relative to total time in EPM) than WT controls. **b** Total number of entries into both arms did not differ between groups. Data are represented by mean \pm SEM. * p <0.05; ** p <0.01 (statistically significant differences from WT controls by unpaired Student's t test)

NMDA receptors produced a small reduction of holding current in both groups [from -22.7 ± 3.5 pA to -31.0 ± 6.0 pA in WTs ($N=4$) and from -39.1 ± 6.2 pA to -41.5 ± 7.7 pA in 1AKOs ($N=16$)]. There was not a significant main effect of drug or a significant genotype \times drug interaction, indicating a mild tonic influence of glutamate that was similar in the two groups.

Glutamate synaptic activity

Fig. 2 compares glutamate synaptic activity characteristics in CA1 hippocampal pyramidal cells under spontaneous (Fig. 2a) and evoked release conditions (Fig. 2b) in WT and 1AKO mice. Figure 2a shows that there is no difference in basal sEPSC or mEPSC frequency or amplitude between the two groups. In addition, there is no difference in mEPSC kinetics (rise or decay time) between the two

groups (data not shown). In contrast, Fig. 2b shows that basal eEPSC amplitude is elevated in 1AKOs (p <0.05) when compared with WT controls. Since there is no difference in PPR between the two groups, these data indicate that 1AKOs have elevated postsynaptic AMPA receptor number or sensitivity, relative to controls. In addition, when the two groups are pooled, Fig. 3 shows a significant negative correlation between anxiety measures (percent of time in open arms of the EPM) and eEPSC1 amplitude ($R=-.491$, p <0.001, $N=61$) where eEPSC1 indicates the first EPSC evoked by the paired-pulse stimulus. There was also a similar negative correlation between this anxiety measure and eEPSC2 amplitude ($R=-.405$; p <0.01; data not shown) where eEPSC2 indicates the second EPSC evoked by the paired-pulse stimulus. Similar correlations were also observed when genotype was analyzed separately. There was a significant negative correlation between % time in open arms and eEPSC1 amplitude in the 1AKO group ($R=-.425$; p <0.05; $N=23$) and in the WT group ($R=-.482$; p <0.01; $N=38$). Nonetheless, these data indicate that anxious subjects, regardless of genotype, have elevations in postsynaptic AMPA receptor number or sensitivity.

GABA synaptic activity

Table 1 compares GABA synaptic activity characteristics in CA1 hippocampal pyramidal cells under spontaneous and evoked release conditions in WT and 1AKO mice. There is no difference in basal sIPSC or mIPSC frequency or amplitude or eIPSC amplitude or PPR between the two groups. In addition, there is no difference in mIPSC kinetics (rise or decay time) between the two groups (data not shown). These data indicate no effect of genotype on either spontaneous or evoked GABA synaptic activity.

Response to benzodiazepines

Figure 4 compares the effects of the anxiolytic benzodiazepine agonist, diazepam (Fig. 4a), and the anxiogenic partial inverse agonist at the benzodiazepine allosteric site on the GABA_A receptor, FG 7142 (Fig. 4b), on eIPSC activity in CA1 hippocampal pyramidal cells in WT and 1AKO mice. Diazepam significantly elevated amplitude of the first but not the second eIPSC (p <0.01 in WTs and p <0.05 in 1AKOs) and reduced PPR (p <0.01 in both groups). These data indicate that diazepam similarly augments evoked presynaptic GABA release in both genotypes. FG 7142 significantly reduced eIPSC amplitude (p <0.01 in both groups) without changing PPR. These data indicate that FG 7142 similarly inhibits evoked GABA synaptic activity in both groups. However, when data from both groups were pooled and

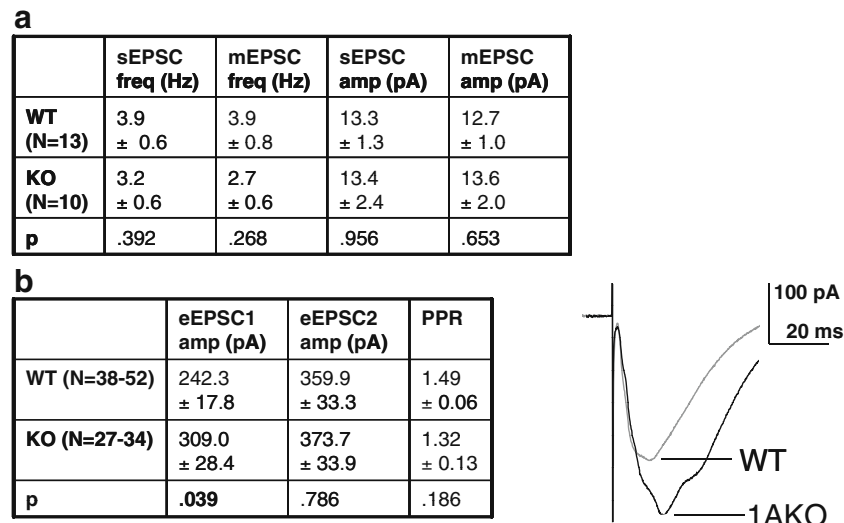


Fig. 2 CA1 hippocampal pyramidal cells recorded from 5-HT_{1A} receptor knockout mice (1AKOs) had greater postsynaptic glutamate receptor sensitivity under evoked release conditions. Figure 2a contains values for basal release conditions; 1AKOs did not significantly differ from WT controls in spontaneous or miniature spontaneous excitatory postsynaptic current (sEPSC or mEPSC) frequency or amplitude. Figure 2b contains the values for evoked potentials; 1AKOs had a higher evoked EPSC1 (eEPSC1) amplitude ($p=0.039$) but did not differ significantly from WT controls in

eEPSC2 amplitude or paired-pulse ratio (PPR). Next to Fig. 2b is an example of an averaged eEPSC1 from a WT (240 pA) and a 1AKO (328 pA). EPSCs were isolated by the addition of the GABA_A antagonist bicuculline (20 μ M). Spontaneous EPSCs were recorded in the absence and mEPSCs in the presence of tetrodotoxin (1 μ M). Evoked EPSCs were elicited by stimulation of the Schaffer collateral pathway and were 50% of the maximum response. Data are expressed as mean \pm standard error of the mean (SEM). Abbreviations: amp=amplitude, freq=frequency

correlated with anxiety measures from EPM performance (Fig. 5), there was a significant positive correlation between percent of time in open arms of the EPM and FG 7142 response ($R=.448$, $p<0.05$, $N=24$). When this correlation was examined in the separate genotypes, it was not significant in either due to the smaller N and consequent loss of statistical power (1AKOs: $R=.233$, n.s., $N=14$; WTs: $R=.556$, n.s., $N=10$). These data indicate that anxious subjects, regardless of genotype, show a decreased response to this anxiogenic benzodiazepine inverse agonist.

Physiological correlates of anxiety: a summary

Table 2 summarizes the correlation data of anxiety measures (percent of time in open arms) with physiological measures described above. Anxiety correlated with elevations in eEPSC amplitude and reduced eIPSC amplitude response to FG 7142. These findings indicate elevated postsynaptic AMPA receptors measured under conditions of synaptic activation and a desensitized response to this anxiogenic benzodiazepine inverse agonist in anxious subjects.

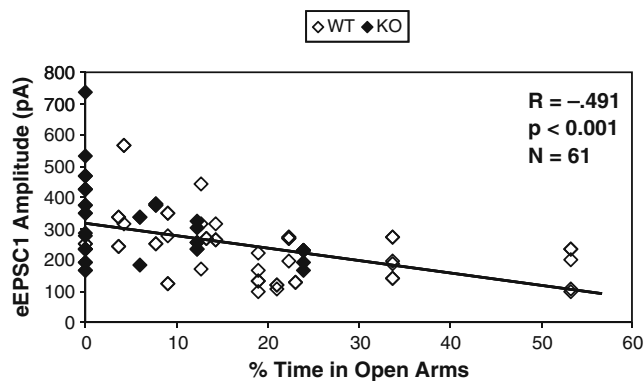


Fig. 3 Anxiety is correlated with increased postsynaptic glutamate receptor density or sensitivity in CA1 hippocampal pyramidal neurons. Regardless of genotype, there is a significant negative correlation between time spent exploring the open arms of the EPM and the glutamatergic eEPSC amplitude

Discussion

While behavioral characterization of the 5-HT_{1A} receptor knockout mouse describes a robust anxiety phenotype that persists on a variety of background strains, as measured by multiple anxiety models in multiple laboratories, an understanding of the physiological basis of the anxiety phenotype has been limited. The current study used a combination of behavioral measures, pharmacological challenges and hippocampal whole-cell recording techniques to uncover novel physiological correlates of anxiety in these subjects. Consistent with previous findings using the EPM (Bailey and Toth 2004; Ramboz et al. 1998; Sibille et al. 2000) and other anxiety models (Heisler et al. 1998; Parks et al. 1998), 1AKOs demonstrated elevated anxiety levels in the EPM model. Interestingly, many of the WT controls also exhibited

Table 1 Genotype had no effect on GABA synaptic activity under basal or evoked release conditions in CA1 pyramidal cells

	<i>sIPSC freq (Hz)</i>	<i>mIPSC freq (Hz)</i>	<i>sIPSC amp (pA)</i>	<i>mIPSC amp (pA)</i>
WT (<i>N</i> =14)	5.9±1.0	4.7±0.9	23.1±1.8	19.7±2.2
KO (<i>N</i> =19)	6.1±0.8	5.2±0.7	24.5±2.6	17.6±1.2
<i>p</i>	.876	.687	.680	.383
	<i>eIPSC1 amp (pA)</i>	<i>eIPSC2 amp (pA)</i>	<i>PPR</i>	
WT (<i>N</i> =33–46)	186.8±16.0	156.1±14.9	0.84±0.04	
KO (<i>N</i> =39–58)	206.7±19.6	154.2±13.4	0.78±0.04	
<i>p</i>	.446	.925	.356	

The data show that 1AKOs did not differ significantly from WT controls in spontaneous or miniature spontaneous inhibitory post synaptic current (sIPSC or mIPSC) frequency or amplitude. The data shows that under evoked release conditions, 1AKOs did not significantly differ from WT controls in eIPSC amplitude or PPR. IPSCs were isolated by the addition of the glutamate receptor antagonists 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX; 20 μ M) and DL-2-amino-5-phosphonopentanoic acid (APV; 50 μ M). Spontaneous IPSCs were recorded in the absence and mIPSCs in the presence of tetrodotoxin (1 μ M). Evoked IPSCs (eIPSCs) were elicited by stimulation of the Schaffer collateral pathway and were 50% of the maximum response. Data are expressed as mean \pm SEM. Abbreviations: amp = amplitude, freq = frequency

anxiety behavior that overlapped with that seen in the 1AKOs. As a result, for correlation analysis, subjects were pooled to provide a larger overall range of anxiety measurements so that physiological endpoints could be correlated with anxiety measures. Anxious subjects demonstrated elevated eEPSC amplitude, indicating elevated postsynaptic AMPA receptor sensitivity under conditions of synaptic activation. Anxious subjects also showed diminished GABAergic responses to the anxiogenic benzodiazepine inverse agonist FG 7142. These correlations indicate a link between anxiety and altered sensitivity of hippocam-

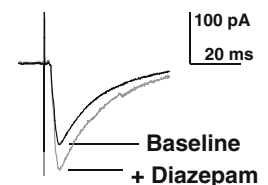
pal neurons to excitatory glutamatergic inputs as well as to anxiogenic, GABA-inhibiting benzodiazepines.

One general technical issue to consider in these experiments concerns the limitations of constitutive deletion of the 5-HT_{1A} receptor. Because the gene is constitutively deleted, it may result in developmental, physiological or behavioral compensatory processes which, rather than the targeted gene, could contribute to the observed phenotype (Gerlai 2001). These compensatory changes could also mask the functional consequence of the gene mutation, obscuring potential phenotypes in the animal (Crawley 1996). Our data demon-

Fig. 4 Genotype had no effect on sensitivity to the anxiolytic diazepam or the anxiogenic FG 7142. Figure 4a contains summary data demonstrating that diazepam (1 μ M) elevated eIPSC1 amplitude and reduced PPR in both genotypes. Next to Fig. 4a is an example of an averaged eIPSC from a WT at baseline (157 pA) and following diazepam application (207 pA). The data in Fig. 4b shows that FG 7142 (3 μ M) reduced eIPSC1 amplitude in both groups. Next to Fig. 4b is an example of an averaged eIPSC from a WT at baseline (180 pA) and following FG 7142 application (124 pA). Asterisks indicate a significant difference from predrug baseline by paired Student's *t*-test (* p <0.05, ** p <0.01). Data are expressed as mean \pm SEM. Abbreviation: amp=amplitude

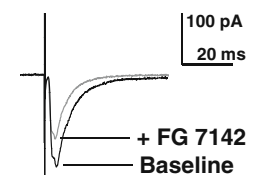
a Diazepam (1 μ M) Effects

	Base eIPSC1 (pA)	DZ eIPSC1 (pA)	Change in amp (pA)	Base PPR	DZ PPR	Change in PPR
WT (N=15)	177.3 \pm 18.8	234.4 \pm 25.9 **	57.1 \pm 18.9	0.85 \pm 0.08	0.70 \pm 0.06 **	-0.15 \pm 0.04
KO (N=13)	209.9 \pm 53.2	266.5 \pm 69.9 *	56.6 \pm 25.4	0.81 \pm 0.09	0.55 \pm 0.07 **	-0.25 \pm 0.08
<i>p</i>	.546	.653	.986	.686	.117	.219



b FG 7142 (3 μ M) Effects

	Base eIPSC1 (pA)	FG7142 eIPSC1 (pA)	Change in amp (pA)	Base PPR	FG 7142 PPR	Change in PPR
WT (N=10)	233.9 \pm 39.8	149.8 \pm 23.2 **	-84.1 \pm 21.6	0.86 \pm 0.07	0.93 \pm 0.10	0.07 \pm 0.06
KO (N=14)	205.9 \pm 14.1	154.0 \pm 12.6 **	-52.0 \pm 13.6	0.74 \pm 0.05	0.80 \pm 0.08	0.06 \pm 0.04
<i>p</i>	.462	.868	.199	.180	.312	.859



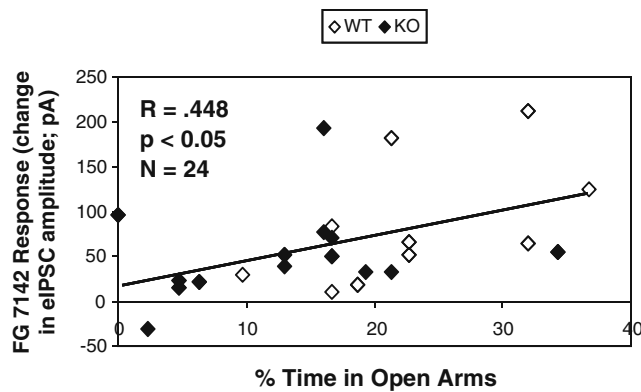


Fig. 5 Anxiety is correlated with a reduced response to the anxiogenic benzodiazepine inverse agonist FG 7142. Figure 5 illustrates a significant positive correlation between time spent exploring the open arms of the EPM and the magnitude of the change in GABAergic eIPSC amplitude in response to FG 7142 in CA1 hippocampal pyramidal cells

strate that in fact these compensatory responses do occur since we found a change in the glutamate neurotransmitter system which may be a physiological correlate underlying the anxiety phenotype. Another technical issue which is more specific to the current study concerns the potential influence of exposure to the behavioral model on the physiological endpoints examined. The EPM model is known to be stressful, resulting in elevated levels of stress hormones (Appenrodt et al. 1999; File et al. 1994; Pellow et al. 1985; Rodgers et al. 1999). There is some disagreement in the literature as to whether

exposure to the EPM, by itself, elevates anxiety levels. Some studies have shown that subjects tested in repeated EPM trials show stable measures of anxiety across trials (File et al. 1990; Lister 1987; Pellow et al. 1985) while others have shown progressive increases in anxiety with subsequent trials (Griebel et al. 1993; Rodgers et al. 1992; Rodgers and Shepherd 1993; Treit et al. 1993). Nonetheless, we chose to conduct the electrophysiology experiments at least 3 weeks following EPM testing in order to minimize any potentially confounding effects of the test itself on the electrophysiological measures, particularly in an area such as the hippocampus which is well-known for its responsiveness to stressors (McEwen et al. 1992; McEwen and Magarinos 1997). Our goal in this design was to capture two potential consequences of the 5-HT_{1A} receptor deletion: a behavioral measure of anxiety and changes in hippocampal physiology while minimizing the influence of one endpoint on the other.

In glutamate synaptic activity experiments, under conditions of spontaneous glutamate release, there were no genotypic differences or correlations with anxiety measures. By contrast, under conditions of synaptic activation, postsynaptic AMPA receptor number or sensitivity was significantly enhanced in 1AKOs and also significantly correlated with behavioral anxiety. One potential explanation of this discrepancy is that Schaeffer collateral stimulation to evoke glutamate release engages a different population and a greater number of synapses than those engaged during s/mEPSC recordings during which only synapses within the range of the patch

Table 2 Summary of correlations between anxiety-like behavior of WT and 1AKOs and electrophysiology differences in CA1 hippocampal pyramidal cells

Physiological correlates of anxiety in CA1 hippocampal neurons			
	Correlation coefficient (<i>R</i>)	<i>N</i>	Statistical significance
RMP (mV)	.140	62	n.s.
eEPSC1 amplitude (pA)	-.491	61	<i>p</i> <0.01
eEPSC2 amplitude (pA)	-.405	61	<i>p</i> <0.01
eEPSC PPR	.004	61	n.s.
eIPSC1 amplitude (pA)	.028	72	n.s.
eIPSC2 amplitude (pA)	.158	72	n.s.
eIPSC PPR	.175	72	n.s.
sEPSC frequency (Hz)	.054	23	n.s.
mEPSC frequency (Hz)	-.067	22	n.s.
sEPSC amplitude (pA)	.246	23	n.s.
mEPSC amplitude (pA)	.365	22	n.s.
sIPSC frequency (Hz)	.061	33	n.s.
mIPSC frequency (Hz)	.120	33	n.s.
sIPSC amplitude (pA)	-.097	33	n.s.
mIPSC amplitude (pA)	.320	33	<i>p</i> =0.07
DZ response (Δ eIPSC amp; pA)	-.076	28	n.s.
DZ response (Δ PPR)	.018	28	n.s.
FG response (Δ eIPSC amp; pA)	.448	24	<i>p</i> <0.05
FG response (Δ PPR)	.029	24	n.s.

Anxiety-like behavior was measured by percent of time spent in the open arms of the elevated-plus maze

RMP resting membrane potential, *amp* amplitude, *PPR* paired-pulse ratio, *DZ* diazepam, *FG* FG 7142

electrode contribute. Another potential factor is that behavioral differences between 1AKOs and WT controls are not readily apparent until subjects are challenged in animal models of anxiety, all of which involve stressful procedures to elicit the anxious behavior (i.e. active avoidance of open, exposed spaces (open-field test) or open arms (elevated-plus maze)). So, it is perhaps not surprising that this physiological hypersensitivity of hippocampal neurons to glutamatergic input is not observed in 1AKOs until the glutamate system is challenged by synaptic activation, as would be present during behavioral activation and stress conditions *in vivo*. This heightened glutamatergic sensitivity of hippocampal neurons is consistent with and may contribute to earlier findings in 1AKO mice of enhanced neuronal excitability in area CA1 of the hippocampus as well as magnified hippocampal theta oscillations that occur during anxiety-provoking conditions (Gordon et al. 2005). Since we observed greater sensitivity to glutamate in 1AKOs than WTs, it is possible that it represents a developmental response to the constitutive deletion of the 5-HT_{1A} receptor. It is unclear if the anxiety phenotype of 1AKOs is driven by the receptor deletion, *per se*, or potentially by secondary developmental responses in other systems such as the excitatory glutamatergic system as shown in the current study. Nonetheless, it is clear that greater excitatory drive to hippocampal neurons could contribute to anxiety as the hippocampus has long been associated with emotional behaviors, neural control of the hypothalamopituitary-adrenal (HPA) axis and stress psychopathology (Gray and McNaughton 2003; Jacobson and Sapolsky 1991; McEwen 2007).

Previous studies have also examined the sensitivity of 1AKO mice to benzodiazepines. Sibille, et al. (2000) initially reported that 1AKOs on a mixed Swiss-Webster x 129/Sv background demonstrated benzodiazepine-resistant anxiety coupled with reduced benzodiazepine receptor binding in cortex and amygdala, reduced GABA_A receptor binding in hippocampus, impaired GABA function in hippocampus and abnormal GABA_A receptor subunit expression in all three regions. Later reports showed that 1AKOs on a mixed Swiss-Webster x C57Bl6 background were similarly benzodiazepine-resistant (Bailey and Toth 2004) but that 1AKOs on the pure 129/Sv (Pattij et al. 2002) or C57/Bl6 backgrounds (Bailey and Toth 2004) had normal benzodiazepine sensitivity. We compared benzodiazepine responsivity in 1AKOs and WTs and found no effect of genotype on the response to either benzodiazepine agonists (increase in eIPSC) or inverse agonists (decrease in eIPSC), consistent with the normal benzodiazepine sensitivity reported for 1AKOs on a pure 129/Sv background (Pattij et al. 2002). However, when subjects were pooled and anxiety measures compared with benzodiazepine responsivity, we found a significant correlation between anxiety and diminished GABA synaptic

response to the inverse agonist. These data indicate that anxious behavior rather than genotype predict this decreased sensitivity to an anxiogenic benzodiazepine inverse agonist. Endogenous ligands for the benzodiazepine receptor have been proposed. For example, diazepam-binding inhibitor (DBI) is a neuropeptide that has been identified in both CNS and the periphery which acts an inverse agonist at benzodiazepine receptors (Costa and Guidotti 1991). The decreased sensitivity of the anxious subjects to the exogenous benzodiazepine inverse agonist could actually reflect a desensitization of the receptor system in these animals as a compensatory mechanism to high circulating levels of an endogenous inverse agonist such as DBI. In fact, DBI has anxiogenic behavioral effects when administered exogenously (Barbaccia et al. 1986; Costa and Guidotti 1991) and elevated expression of DBI has been reported in human depression with a strong anxiety component (Barbaccia et al. 1986). This elevation in endogenous inverse agonist could contribute to anxiety directly or indirectly via its effect on other anxiety-related neuropeptides. For example, inverse agonists modulating GABA_A receptor function have been shown to elevate levels of corticotropin-releasing factor (CRF) *in vitro* (Calogero et al. 1988) and *in vivo* (Skelton et al. 2000) and to enhance the behavioral effects of CRF (Britton et al. 1988). CRF is in turn responsible for initiating the hormonal, physiological and behavioral response to stress mediated by the HPA axis. In this manner, reduced sensitivity demonstrated by hippocampal neurons to an exogenously applied benzodiazepine inverse agonist maybe an adaptation to excessive circulating levels of an endogenous inverse agonist such as DBI which either directly or indirectly contributes to anxiety.

Previous studies have also examined physiological correlates of anxiety using different animal models. For example, Smith et al. showed decreased GABA_A receptor-mediated current in hippocampus and insensitivity to benzodiazepine agonists in a steroid withdrawal-induced anxiety model in rats (Smith et al. 1998). Cagetti et al. showed reduced benzodiazepine agonist sensitivity and reduced mIPSC decay in hippocampal neurons of rats during an ethanol withdrawal-induced anxiety model (Cagetti et al. 2003). Van Sickle et al. found increased AMPA but not NMDA receptor-mediated excitation of CA1 neurons in rats during benzodiazepine withdrawal-induced anxiety (Van Sickle et al. 2004). Maguire et al. showed reduced GABA_A receptor-mediated tonic inhibition of hippocampal neurons and elevated neuronal excitability during the estrus, as compared with diestrus, period of the ovarian cycle in female mice, a time of heightened anxiety (Maguire et al. 2005). All of these studies using different animal models are consistent with our findings and indicate a number of different mechanisms whereby heightened hippocampal neuron excitability may contribute to anxiety.

These experiments highlight at the single-cell level the potential importance of CA1 hippocampal pyramidal neurons and their GABA and glutamatergic synaptic inputs in the generation of anxiety. They identify altered sensitivity of hippocampal neurons to GABA modulators which ultimately may reflect underlying hyperactivity of an endogenous GABA-inhibiting system, an effect which could be linked to anxiety. In addition, these experiments identify enhanced glutamate synaptic drive to hippocampal neurons of anxious subjects. This mechanism would increase the excitability of hippocampal neurons, stimulating hippocampal output and would contribute to hippocampal-mediated behaviors that could include exaggerated emotional responses to environmental stressors and anxiety disorders.

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