Altered emotionality and neuronal excitability in mice lacking KCTD12, an auxiliary subunit of GABA_B receptors associated with mood disorders

F Cathomas1,2,5, M Stegen3,5, H Sigrist1, L Schmid1, E Seifritz2,4, M Gassmann3, B Bettler3 and CR Pryce1,4

INTRODUCTION

Gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the brain, is fundamental to brain function and implicated in the pathophysiology of several neuropsychiatric disorders. GABA activates G-protein-coupled GABA_A receptors comprising principal GABA_A1 and GABA_A2 subunits as well as auxiliary KCTD8, 12, 12b and 16 subunits. The KCTD12 gene has been associated with bipolar disorder, major depressive disorder and schizophrenia. Here we compare Kctd12 null mutant (Kctd12^−/−) and heterozygous (Kctd12^+/−) with wild-type (WT) littermate mice to determine whether lack of or reduced KCTD12 expression leads to phenotypes that, extrapolating to human, could constitute endophenotypes for neuropsychiatric disorders with which KCTD12 is associated. Kctd12^−/− mice exhibited increased fear learning but not increased memory of a discrete auditory-conditioned stimulus. Kctd12^+/− mice showed increased activity during the inactive (light) phase of the circadian cycle relative to WT and Kctd12^−/− mice. Electrophysiological recordings from hippocampal slices, a region of high Kctd12 expression, revealed an increased intrinsic excitability of pyramidal neurons in Kctd12^−/− and Kctd12^+/− mice. This is the first direct evidence for involvement of KCTD12 in determining phenotypes of emotionality, behavioral activity and neuronal excitability. This study provides empirical support for the polymorphism and expression evidence that KCTD12 confers risk for and is associated with neuropsychiatric disorders.

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ORIGINAL ARTICLE

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INTRODUCTION

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system, acting on ionotrophic GABA_A and metabotropic GABA_A receptors.1 GABA_A receptors control neuronal activity throughout the brain by regulating neurotransmitter release and gating of voltage-sensitive Ca^2+ and K_once-type K_ channels. The principal subunits GABAB^α1; GABAB^β1; and GABAB^β2 form fully functional heteromeric GABAB^α1β1,2 and GABAB^β1α1,2 receptors.2 GABAB^α1a and GABAB^α1b are subunit isoforms that determine pre- and postsynaptic receptor localization, respectively. Four cytosolic proteins of the KCTD family (named after their K+-channel tetramerization domain KCTD8, 12, 12b and 16) associate constitutively with GABA_B receptors as auxiliary receptor subunits.3,4 GABA_B receptors exhibit decreased expression in bipolar disorder, major depressive disorder (MDD) and schizophrenia,5 and have been proposed as promising treatment targets for these neuropsychiatric disorders as well as anxiety and addiction.6–9 In mouse, GABA_B^R1 and GABA_B^R2 knockout strains have been studied to investigate the role of GABA_B receptors in regulating various behavioral processes, including activity and innate and conditioned emotionality. Relative to wild-type (WT), GABA_B^R1−/− mice were mildly more active during the light phase and GABA_B^R1−/− mice were hyperactive during the dark phase.10 There was no clear evidence for phenotypes in GABA_B^R2−/− or GABA_B^R1b−/− mice in unconditioned anxiety tests including elevated plus maze and light–dark box.11 However, there were marked and specific phenotypes in paradigms of conditioned aversion and fear: In conditioned taste aversion, the characteristics of an otherwise rewarding stimulus (for example, saccharin solution) are paired with the malaise-inducing effects of injected lithium chloride such that the reward is now avoided: GABA_B^R1a−/− mice exhibited a deficit in acquiring conditioned taste aversion and GABA_B^R1b−/− mice exhibited a deficit in its extinction.12 Most relevant to the present study, there were also clear effects of GABA_B^α1a isoforms in a tone-electroshock fear-conditioning paradigm: GABA_B^R1a−/− mice exhibited normal fear expression to the conditioned tone but generalization of fear expression to a neutral tone, and GABA_B^R1b−/− mice exhibited low fear expression to the conditioned tone consistent with impaired acquisition or consolidation of fear conditioning.13 Therefore, GABA_A receptors appear to be particularly important in contributing to the learning and/or memory of conditioned aversive stimuli. A genome-wide association study of more than 1000 bipolar I disorder patients identified a polymorphism in the region of KCTD12 associated with increased disorder prevalence.14 In a microarray study comparing probands reporting chronic stress with matched controls, the former exhibited decreased KCTD12 expression in peripheral blood mononuclear cells (PBMCs).15 A
translational microarray study of the amygdala in MDD patients and in mice exposed to unpredictable chronic mild stress identified KCTD12/Kctd12 as one of about 30 genes with an upregulated expression in the amygdala in both MDD patients and unpredictable chronic mild stress mice relative to their control groups. Microarray studies showed reduced PBMC KCTD12 expression and increased postmortem hippocampal expression of KCTD12 in schizophrenia patients. In the adult mouse brain, Kctd12 is expressed highly in hippocampus and amygdala, with the KCTD12 protein localized primarily postsynaptically and associated almost exclusively with GABA B receptors. KCTD12 stabilizes GABA B receptors at the cell surface and regulates kinetic properties of the receptor responses. KCTD12 promotes fast desensitization of GABA B-activated K-currents by uncoupling activated G-protein βγ subunits from effector Kir3 channels. Activated G-protein βγ subunits from effector Kir3 channels. KCTD12 is not associated with GABA B receptors but will also regulate G-protein signaling of other G-protein coupled receptors and generate pleiotropic phenotypes.

In the present study, WT, Kctd12−/− and Kctd12+/− mice were studied to assess whether KCTD12 regulates phenotypes that could constitute risk factors (endophenotypes) for neuropsychiatric disorders with which KCTD12 downregulation is associated. As noted above, KCTD12 is associated with psychiatric disorders including bipolar disorder, which is highly (50%) comorbid with anxiety disorders. In the depressive phase of bipolar disorder, increased reward interest-pleasure are common, whereas in the manic phase, increased reward interest-pleasure is common and the day-night cycle is disrupted resulting in nocturnal hyperactivity. Infected by these psychopathologies, we investigated Kctd12−/− and Kctd12+/− mice in terms of fear learning and memory of an auditory-conditioned stimulus and of context, locomotion and innate anxiety, motivation for gustatory reward, and activity levels across the circadian cycle. We also investigated the electrophysiological properties of Kctd12-deficient neurons. Our results support a role for KCTD12 in the regulation of emotional learning-memory, activity during the inactive phase, and the intrinsic excitable excitability of neurons.

MATERIALS AND METHODS

Animals and housing

Male and female Kctd12−/− mice on a C57BL/6 × 129 mixed background (backcrossed to C57BL/6 for 6 generations) were paired to generate offspring of all 3 genotypes (WT, Kctd12−/− and Kctd12+/−). We conducted the present experiments with mice that were on the same genetic background as those used in previous electrophysiological studies. Mice were weaned at age 3–4 weeks and males were caged as littermate trios. They were maintained on a reversed 12:12 h light–dark cycle with light off at 0700 h in an individually ventilated cage system with temperature at 20–22 °C and humidity at 50–60%. Food (Complete pellet, Provimi, Klibad, Kaiseraugst, Switzerland) and water were both available ad libitum. Tissue probes for genotyping were obtained at age 5–7 weeks. There was no effect of genotype on body weight: WT 26.1 ± 0.7 g, Kctd12−/− 27.5 ± 0.8 g, Kctd12+/− 26.6 ± 0.5 g (P = 0.4). All the procedures were conducted under permits for animal experimentation issued by the Veterinary Office of Zurich or Basel-Stadt, Switzerland.

Genotyping

For genotyping, ear punches or toe clips were incubated overnight at 56 °C with shaking at 800 r.p.m. in 200 μl lys buffer containing 50 μl KCl, 10 μl Tris-HCl (pH 8.3), 2 μl MgCl2, 0.1 mg ml−1 gelatin, 0.45% NP-40, 0.45% Tween-20 and 0.15 mg ml−1 proteinase K (Roche, Rotkreuz, Switzerland, 03 115 879 001). Following inactivation of proteinase K for 10 min at 95 °C and centrifugation for 2 min at 9000 g, 2 μl of the supernatants were used for amplification of the 456 bp WT- and 396 bp knockout-specific amplicons in two separate PCRs with the following primers at 0.25 μM: WT, 5′-AAGAGGATGCGTGGGACAC-3′ (P2) and 5′-AGTGGGGTCAAGATGGATG-3′ (P3); KO, 5′-GATGCAGCCTCCTGGAAGAC-3′ (P1) and 5′-AGTGGGGTCAAGATGGATG-3′ (P3). RedTag DNA Polymerase (Sigma, Buchs, Switzerland, D4309-250/UN) was used according to instructions with the 10× reaction buffer containing 1.5 mM MgCl2. Brieﬂy, the apparatus consisted of a dark-walled Plexiglas arena (30 × 30 × 24 cm) that was placed on an electrified grid floor (the context). A black metal waste tray was placed under the grid floor. Infrared light-beam sensors allowed for measurement of movement in three dimensions. The aversive unconditioned stimulus (US) used was an electroshock of 2 × 0.2 mA. The discrete conditioned stimulus (CS) used was a tone of 5 kHz and 85 dB that was presented for 20 s with the final 2 s contiguous with the US. Four such arenas were used and each was placed in an attenuating chamber equipped with a ventilation fan and house lights (8 lux). Mice were tested in either a CS–US or Context–US fear-conditioning paradigm.

Behavioral tests

Each behavioral experiment was conducted with naive males aged 8–16 weeks, with mice derived from at least six different litters. Mice were handled on 3 days before the experiment, and all tests were conducted between 1100 and 1600 h.

Fear conditioning

The fear-conditioning experiment was conducted using a fully automated apparatus (MultiConditioning System, TSE Systems, Bad Homburg, Germany). Briefly, the apparatus consisted of a dark-walled Plexiglas arena (30 × 30 × 24 cm) that was placed on an electrified grid floor (the context). A black metal waste tray was placed under the grid floor. Infrared light-beam sensors allowed for measurement of movement in three dimensions. The aversive unconditioned stimulus (US) used was an electroshock of 2 × 0.2 mA. The discrete conditioned stimulus (CS) used was a tone of 5 kHz and 85 dB that was presented for 20 s with the final 2 s contiguous with the US. Four such arenas were used and each was placed in an attenuating chamber equipped with a ventilation fan and house lights (8 lux). Mice were tested in either a CS–US or Context–US fear-conditioning paradigm.

CS–US fear conditioning. This experiment was conducted on three consecutive days. Day 1, baseline mice were placed in the arena (context)
for 15 min without CS or US. Day 2, conditioning: following 2-min adaptation, mice were exposed to six CS-US pairings with an inter-trial interval (ITI) of 120 s. On day 1 and day 2, 24 mice per genotype were included. For the fear expression test on day 3, different protocols were used, with 12 mice per genotype allocated to each. Day 3, fear expression test in same context: after an adaptation time of 180 s, mice were tested for expression of CS fear using 15 × 30 s CS and 14 × 90 s ITI. Day 3, fear expression test in a novel context: mice were tested in a modified context with a dark Plexiglas wall containing a door (3.5 (W) × 10 (H) cm) placed across the center of the arena and a white instead of a black waste tray. The mice were given 180 s adaptation followed by 15 × 30 s CS and 14 × 90 s ITI.

Context-US fear conditioning. This experiment was conducted on two consecutive days, with 12 WT and 12 Kctd12−/− mice. Day 1, conditioning: mice were given 3 min adaptation followed by 6 × 2 s US and 5 × 138 s ITI. Day 2, fear expression test: in the same context, mice were tested for fear expression for 18 × 60 s.

In all the cases, freezing was defined as the complete absence of movement detection for at least 2 s. The output parameters were percent time spent freezing per CS or ITI. Mean freezing time per mouse was calculated for each two to three consecutive CSs or ITIs depending on experiment and phase.

Hot plate
The hot plate pain test was conducted using a programmable thermoelectric heating plate (Teca, Chicago, IL, USA). The temperature of the hot plate was set at 50 °C. The mouse was taken out of the home cage, placed inside a transparent Plexiglas chamber on the hot plate and the lid was closed. The latency (s) from the first contact with the hot plate until the first occurrence of either licking or lifting a forepaw or hindpaw or jumping, was scored. The maximum test duration was 60 s. Six mice per genotype were tested.

Open field
Mice, 10 per genotype, were placed in the experimental room at 24 h before testing. The open field was a black Plexiglas arena measuring 50 (L) × 50 (W) × 40 (H) cm. Lighting was set to 15 lux at the center. The open field was cleaned and wiped with 70% ethanol before each test. The test mouse was placed in the center of the arena and location and movement were monitored using a video-tracking system (VideoMot 2, Version 5.76, TSE Systems). The floor was divided virtually into center (30 × 30 cm) and periphery. The test duration was 30 min and data were analyzed in 5-min bins.

Sucrose preference
The sucrose preference test was conducted in the home cage. Mice, 10 per genotype, were caged singly at 3 days before the onset of the experiment. They were habituated to the test bottles (SwissPet, Delphin-Amazonia AG, Münchenstein, Switzerland) filled with water, for 3 h on the first day and 8 h on the second day. On the following 4 days at 0800–1600 h, mice were presented with two adjacent bottles, one contained 1% sucrose (Sigma-Aldrich, CH, St Louis, MO, USA) in water (W/V) and the other contained water only. The position of the two bottles was reversed daily to control for side preference. Consumption of sucrose solution and water was calculated by weighing the bottles at the beginning and end of the 8-h test. Sucrose preference (%) was calculated as 100 × (Δ weight of sucrose bottle/Δ weight of sucrose bottle + weight of water bottle).

Circadian activity
IntelliCage (NewBehavior, Zurich, Switzerland) is a system for continuous automated monitoring of the activity of individual mice in their home cage. Each mouse is fitted with a unique subcutaneous transponder that records its visits to the operant devices located in each corner of the cage, its operant nosepokes into a sensor (light-barrier) at the two doors per corner that open to allow access to drinking bottles, and the number of drinking bottles, measured via electrical contact of the tongue with the tip of the water bottle cap. The cage of 55 (W) × 38 (L) × 20 (H) cm was divided at the center to give two independent cages each containing two operant corner units. Four such divided IntelliCages were used with each placed in an attenuation chamber with a reversed 12:12 h dark–light cycle and ventilation. Two male littermates of the same genotype were placed in each cage-half with access to two corners. Ten mice per genotype were studied as follows: mice were habituated to the cage for 5 days with operant doors open, and then 5 days with operant doors closed and one nosepoke required to open a door for 10 s. Trained mice were then maintained in the IntelliCage for 20 days and were weighed daily. Output parameters were the total visits, nosepokes and licks. Data were divided into a 12-h dark phase and a 12-h light phase.

Electrophysiology
For electrophysiological experiments, 4–6-week-old males and females were used. Standard procedures were used to prepare 300 μm horizontal slices. CA1 pyramidal neurons were visualized by Dodt gradient contrast video microscopy (Luigs and Neumann, Ratingen, Germany) using an upright microscope (BX51WI, Olympus, Tokyo, Japan) with a × 60/0.9 objective. Slices were superfused continuously with ACSF containing (in mM): 125 NaCl, 25 NaHCO3, 2.5 KCl, 1.25 NaH2PO4, 2 CaCl2, 1 MgCl2 and 25 glucose (equilibrated with 95% O2-5% CO2). Patch pipettes were pulled from borosilicate glass (Hilgenberg, Malsfeld, Germany, 2 mm outer and 1 mm inner diameter) using a DMZ-universal puller (Zeitz, Martinsried, Germany) and filled with a solution containing (in mM): 140 K-glucuronate, 10 HEPES, 2 MgCl2, 2 Na2ATP. Pipette resistance was 5.6 ± 0.1 MΩ. The pipette capacitance was reduced electronically via the amplifier. Series resistance (14.8 ± 0.1 MΩ) was estimated in bridge mode and compensated. Seal resistance was 9.0 ± 0.6 GΩ. Recordings were accepted if cells had overshooting action potentials, a Vrest lower than –60 mV and R0 to Rseg ratio < 0.1 (P > 0.5). We used an Axoclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA) and signals filtered and digitalized at 40 and 10 kHz (current and voltage clamp, respectively) using a Digidata 1440A interface (Molecular Devices).

To isolate the cells synthetically, pharmacological voltage clamp experiments in CA1 pyramidal neurons were conducted in the presence of 20 μM 1,2,3,4- tetrahydro-7-nitro-2,3-dioxoquinoxaline-6-carbonitride disodium (CNOX), 100 μM picrotinox (PTX) and 0.5 μM tetrodotoxin (TTX), to block AMPA/kainate and GABAa receptors as well sodium channels, respectively. In some experiments, we additionally applied 5 μM CGP54626 to block GABAb receptors. Drugs were kept in H2O stocks at –20 °C (CNOX, TTX, BaCl2, CGP54626) or in DMSO stocks at –20 °C (PTX), diluted 1:1000 in glucose ACSF and bath applied. We obtained D-AP5, CNOX and TTX from Ascent Scientific (Weston-Super-Mare, UK), DTX from Alomone (Jerusalem, Israel), and all other substances from Sigma-Aldrich.

Data were analyzed using Clampfit 10.2 and IgorPro 6.2 (WaveMetrics, Portland, OR, USA) software. Liquid junction potential was ~10 mV and corrected offline accordingly. The resting membrane potential (Vrest) was calculated as the mean of a 10 s trace without current injection immediately after breakthrough. The current clamp input resistance (Rin) was the slope of the fit of the IV curve of two hyperpolarizing and two depolarizing current steps resulting in a voltage deflection of ~5 mV in current clamp. The voltage clamp input resistance was the slope of the fit of the IV from ±3 mV and ±6 mV voltage steps. Resting conductance was the reciprocal of voltage clamp Rin. The barium sensitive resting conductance was calculated via subtraction of resting conductance in ACSF and resting conductance in 200 μM Ba2++. The rheobase was defined as the smallest current inducing at least one action potential within the first 2 s of square current injection. Holding potential for voltage clamp experiments was –80 mV if not stated otherwise.

Statistical analysis
Behavioral data were analyzed using SPSS (version 19, SPSS, Chicago, IL, USA). Analysis of variance was used with a between-subject factor of genotype and, depending on behavioral test, a within-subject factor of trial. Post hoc testing was conducted using the least significant difference test. Electrophysiological data were analyzed using GraphPad Prism 5.01 (GraphPad, San Diego, CA, USA). In experiments where two sets of data were obtained from the same cell, results were compared using a Student’s paired t-test. When two sets of data were obtained from two unpaired groups, results were analyzed using Student’s t-test. When more than two sets of data were obtained from two unpaired groups, results were analyzed using repeated measures analysis of variance with Tukey’s test for post hoc analysis.

Statistical significance was set at P < 0.05. Data are presented as mean ± s.e.m. or mean ± s.d.
RESULTS
KCTD12 protein expression in Kctd12-deficient mice
To analyze the consequences of a lack of KCTD12 protein on behavior and neuronal function we generated Kctd12−/− mice (Figures 1a and b). Whole-brain lysates of Kctd12−/− mice lacked KCTD12 protein and had normal levels of GABAB1a, GABAB1b and GABAB2 protein (Figure 1c). Kctd12+/− mice had reduced KCTD12 protein levels, indicating that normally both Kctd12 alleles are transcribed. A compensatory upregulation of another KCTD auxiliary subunit (KCTD12b, KCTD8 or KCTD16) was not observed in whole-brain lysates of Kctd12+/− or Kctd12−/− mice (Figure 1c).

Quantitative analysis of KCTD12 protein in amygdala, ventral hippocampus and prefrontal cortex revealed that Kctd12+/− mice express ~30–40% of WT protein (Figure 1d). This was replicated with PBMCs, which provide a biomarker for KCTD12 protein expression in the brain. Kctd12−/− mice are therefore useful to analyze the consequences of a partial downregulation of KCTD12 protein in the brain.

Kctd12−/− mice exhibit altered auditory tone-electroshock fear conditioning and memory
Acquiring and consolidating the information that a discrete CS (for example, auditory tone) is associated with an aversive US (for example, electroshock), expressed in the form of fear-freezing behavior, are processes mediated primarily in the amygdala. 26 Such fear conditioning is increased in MDD patients 27 and in mouse models of stress-induced depression,28 and is disrupted in mice lacking GABAB1 subunits.13 On the basis of this evidence, and
given that Kctd12 is expressed highly in the amygdala, CS-US fear conditioning and expression were assessed in Kctd12-deficient mice.

On day 1, mice were placed in the novel arena (context) without electroshock to allow for exploration. Mice of each genotype were active and similarly so, as indicated by the low level of % time spent freezing (WT 7.3 ± 0.9%, Kctd12+/- 7.5 ± 1.1%, Kctd12−/− 7.8 ± 0.9% (P = 0.95)). On day 2, CS–US conditioning was conducted, and % time spent freezing during CS–US trials and inter-CS intervals was measured. For % time freezing during inter-CS intervals, there was a main effect of trial number (F(4,138) = 6.56, P < 0.001; Figure 2d), indicating acquisition of fear-freezing across the intervals between CS–US pairings. There was also a main effect of genotype (F(2,138) = 5.15, P < 0.008): Kctd12−/− mice exhibited increased fear-freezing in trials 3–4 compared with WT (P < 0.02) and Kctd12+/− (P < 0.001) and in trials 5–6 compared with WT and Kctd12+/− (both P < 0.001). For % time freezing during inter-CS intervals, there was a main effect of CS–US interval number (F(2,138) = 108.00, P < 0.001; Figure 2e), indicating acquisition of fear-freezing across the intervals between CS–US pairings. There was also a main effect of genotype (F(2,69) = 5.15, P < 0.008): Kctd12−/− mice exhibited increased fear-freezing in trials 3–4 compared with WT (P < 0.02) and Kctd12+/− (P < 0.01), and in trials 5–6 compared with WT (P < 0.02) and Kctd12+/− (P < 0.001). On day 3, the fear expression test was conducted. For the cohort tested in the same context as used for CS–US conditioning on day 2, there was a main effect of trial number (F(4,132) = 6.56, P < 0.001; Figure 2b) indicating a decrease in % time fear-freezing across trials, but no effect involving genotype (P > 0.43). For % time freezing during inter-CS intervals (Figure 2e), there was no effect of CS inter-trial interval number (P = 0.18) and no

Figure 2. Effects of Kctd12 genotype on fear-conditioned freezing. CS–US fear conditioning: (a–f) Twenty-four mice per genotype underwent CS–US fear conditioning in a specific context on day 2 and fear expression was then tested in either the same (N = 12, b and e) or a novel (N = 12, c and f) context on day 3. Freezing during the CS: (a) CS–US conditioning, (b) CS expression test in the same context, (c) CS expression test in a novel context. Freezing during inter-trial intervals: (d) CS–US conditioning, (e) expression test in the same context, (f) expression test in a novel context. Context–US fear conditioning (g and h): Twelve mice for each of WT and Kctd12−/− genotypes underwent context fear conditioning on day 1 and context expression test on day 2. Data are mean ± s.e.m. GT, genotype. *P < 0.05 for Kctd12−/− versus WT, ***P < 0.001 for Kctd12+/− versus WT, ###P < 0.001 for Kctd12+/− versus Kctd12−/−. CS, conditioned stimulus; KCTD, K+-channel tetramerization domain; US, unconditioned stimulus; WT, wild type.
Phenotypes of Kctd12 knockout mice
F Cathomas et al

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**Kctd12**−/− mice exhibit normal context–US fear conditioning and memory

Exposing mice to the aversive US in the arena in the absence of a CS exposed all genotypes to the conditioning effects of the CS when the US was presented. However, the observed effects of KCTD12 on CS fear conditioning did not extend to the context–US trials. This did not result in increased next-day fear expression to the CS, but the **Kctd12**−/− mice did exhibit increased fear expression during intervals between CS presentations when placed in a novel context.

**Kctd12**−/− mice exhibit normal pain sensitivity

**GABA** receptor agonists are involved in the control of pain sensitivity; for example, the agonist baclofen is antinociceptive and **GABA** receptor agonists exhibit hyperalgesia.15 Because altered pain sensitivity would impact on behavior in the fear-conditioning tests, where the US was a painful somatosensory stimulus, it was important to assess pain sensitivity in KCTD12-deficient mice. There was no genotype effect on reaction time (in seconds) in the hot plate test: WT 24.8 ± 5.3, **Kctd12**−/− 28.2 ± 2.5, **Kctd12**−/− 35.3 ± 4.6 (P = 0.037, N = 6 per genotype). Therefore, the absence of KCTD12 is not expected to impact on the sensitivity to the US in the fear-conditioning tests, and pain sensitivity differences do not explain the observed effects of KCTD12 on CS–US fear conditioning and expression.

**Kctd12**−/− mice exhibit normal locomotor activity and innate anxiety

The open field test was conducted to investigate for effects of KCTD12 (N = 10 per genotype) on general locomotor activity and innate anxiety in a novel, neutral environment. The total distance moved was used as an index of activity, and the distance moved in the more exposed center was used as a measure of anxiety, that is, less distance moved in center equates to greater anxiety. Acute intra-hippocampal injection of classical anxiolytic GABA drugs decreases anxiety in this test.16 For the total distance moved, there was a main effect of 5-min time intervals (F(5,135) = 17.78, P < 0.001), with mice exhibiting a consistent reduction in activity across the 30-min session, as expected for this test. There was no genotype main effect (P = 0.20) or time-interval interaction (P = 0.32). For the distance moved in center, there was no genotype effect (P = 0.20) and no time-interval interaction (P = 0.21). These findings indicate that the absence of KCTD12 impacts neither locomotor activity nor innate anxiety in a novel, neutral environment.

**Kctd12**−/− mice exhibit normal sucrose preference

The disruptions in interest-pleasure that are symptoms of MDD and bipolar disorder indicate psychopathology of reward processing. In rodents, reward sensitivity is typically studied using gustatory stimuli, with subjects drinking more of a sweet-tasting sucrose solution than water when a two-bottle choice test is conducted, suggesting a preference that is reduced in models of stress-induced depression.35 Reward processing is mediated by a circuit that includes GABA signaling between nucleus accumbens, ventral pallidum and ventral tegmental area, regions that exhibit low moderate **Kctd12** expression.19 In the present study, mice exhibited a sucrose preference and this was similar across genotypes: WT 77 ± 2%, **Kctd12**−/− 76 ± 1%, **Kctd12**−/− 79 ± 2% (P = 0.41, N = 10 per genotype). Therefore the absence of KCTD12 did not have an impact on gustatory reward processing.

**Kctd12**−/− mice exhibit increased behavioral activity during the light (inactive) phase

A distinctive circadian rhythm in terms of amount of activity during the dark and light phases is a biological norm. Both MDD and bipolar disorder are characterized by disrupted circadian rhythm including disturbed sleep or atypically high activity during the inactive period.23 The IntelliCage system does not provide data on sleep but does allow for continuous monitoring of activity in terms of mouse corner visits, operant nosepokes and water consumption, which in turn can be used to investigate phenotypes of absolute activity and drinking levels as well as their circadian distribution. Activity was greater during the 12-h dark period than the 12-h light period, and this was the case for each genotype on each measure (Figure 3). For example, for corner visits, there was a main effect of period (F(1,21) = 113.00, P < 0.001; Figure 3a versus d) in the absence of an effect of genotype. However, within the light period specifically, there were KCTD12-dependent phenotypes. For total number of corner visits, there was a main effect of genotype (F(2,21) = 4.71, P < 0.02); **Kctd12**−/− mice made more corner visits than did WT or **Kctd12**−/− mice (Figure 3a). There was also a main effect of genotype on total number of water licks during the light period (F(2,21) = 4.18, P < 0.03): **Kctd12**−/− mice made more licks than WT and **Kctd12**−/− mice (Figure 3c). There was no genotype effect on nosepokes during the light period (Figure 3b), and also no effects of genotype were observed during the dark period (Figures 3d and f). Therefore, downregulation but not complete ablation of KCTD12 resulted in increased behavioral activity during the inactive phase specifically.

KCTD12 activates a tonic K⁺ resting membrane conductance and reduces the intrinsic electrical excitability of hippocampal neurons

KCTD12 regulates kinetic properties of GABA receptor-activated Ca²⁺ and K⁺ currents.34 Downregulation of KCTD12 may therefore influence neuronal activity. We analyzed the electrophysiological properties of **Kctd12**−/− and **Kctd12**−/− CA1 pyramidal neurons. These neurons express uniformly high levels of KCTD12 transcripts and protein16,19 and are easily identifiable for electrophysiological recordings. Patch-clamp experiments using acute hippocampal slices revealed that the resting membrane potential (Vₘ(rest)) did not differ significantly between genotypes. However, the input resistance (Rₑ) recorded in current clamp was significantly higher.

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Translational Psychiatry (2015), 1 – 10
in Kctd12+/− and Kctd12−/− mice compared with WT mice (Table 1). The simplest explanation for these two observations is that KCTD12 deficiency reduces the number of open ion channels, and that the ion(s) carried by these channels have an equilibrium potential at or near the resting potential. Given that KCTD12 regulates the activity of Kir3-type K+ channels, the increase in input resistance likely relates to a decrease in open K+ channels. An increase in input resistance is expected to influence the intrinsic electrical excitability of neurons. Therefore, we next determined the rheobase current required to evoke action potentials (AP), which is a measure of the intrinsic excitability of neurons. When depolarizing neurons in current clamp from resting potentials (AP), which is a measure of the intrinsic excitability of neurons. Therefore, we next determined the rheobase current required to evoke action potentials (AP threshold), as well as the time from the stimulation to the peak of the first AP (AP delay), were significantly different between Kctd12−/− and WT mice (Table 1). Together, these data show that the intrinsic electrical excitability of CA1 pyramidal neurons is increased in mice with no or reduced KCTD12 expression.

The above results may be explained by a decrease in tonic K+ currents in Kctd12−/− and Kctd12+/− neurons. A decrease in tonic currents will decrease the resting membrane conductance (g_{rest}), which is measured in nano Siemens (nS) and is inversely related to the input resistance. Voltage-clamp recordings showed that g_{rest} was indeed significantly lower in Kctd12+/− than in WT neurons (Kctd12+/−: 5.1 ± 0.5 nS, n = 11; WT: 7.2 ± 0.5 nS, n = 12; P < 0.01). Surprisingly, however, inhibition of GABA_B receptors with the antagonist CGP54626 did not reduce the resting membrane conductance in WT neurons to that in Kctd12−/− neurons (WT: 6.7 ± 0.3 nS, n = 21, Kctd12−/−: 5.4 ± 0.4 nS, n = 15, P < 0.05). These results indicate that tonic GABA_B receptor activity is not responsible for the increase in resting conductance in the presence of KCTD12. We therefore addressed whether indeed K+ channels underlie the KCTD12-induced increase in resting conductance. Superfusion of slices with 200 μM Ba^{2+}, which inhibits Kir and K2P-type K+ channels, significantly reduced the resting membrane conductance of WT neurons to that of WT neurons.

Table 1. Passive membrane properties of CA1 pyramidal neurons recorded in acute hippocampal slices

| Genotype          | Membrane potential (mV) | Input resistance (MΩ) | Rheobase current (pA) | AP threshold (mV) | AP delay (ms) |
|-------------------|-------------------------|-----------------------|------------------------|-------------------|---------------|
| WT (n = 61)       | −69.4 ± 0.7             | 283 ± 13              | 49.5 ± 3.5             | −49 ± 0.6         | 471 ± 33      |
| Kctd12+/− (n = 16) | −67.1 ± 1.3             | 390 ± 30**            | 31.6 ± 5.2*            | −50 ± 0.6         | 303 ± 62      |
| Kctd12−/− (n = 55) | −68.9 ± 0.5             | 366 ± 17***           | 30.8 ± 2.4***          | −52 ± 0.5**       | 324 ± 34**   |

Abbreviations: AP, action potential; KCTD, K+-channel tetramerization domain; WT, wild type. Data are expressed as mean ± s.e.m. *p < 0.05, **p < 0.01, ***p < 0.001 (versus WT).
Kctd12−/− neurons (Figures 4b and d, P < 0.01). In line with this, the Ba2+-sensitive resting conductance determined from the values in Figure 4d (control minus Ba2+) was significantly larger in WT neurons compared with Kctd12−/− neurons (WT: 2.1 ± 0.2 nS, Kctd12−/−: 1.2 ± 0.1 nS, n = 16, P < 0.001). KCTD12 therefore induces a tonic Ba2+-sensitive K+ current in the absence of GABAB receptor activity.

DISCUSSION

Given the importance of GABAB receptors in emotional and cognitive processing, and the association of human KCTD12 with neuropsychiatric disorders, we looked for behavioral and electrophysiological phenotypes in Kctd12 knockout mice. Our study provides evidence for an involvement of KCTD12 in the regulation of behavioral phenotypes and the intrinsic electrical excitability of neurons. Specifically, homozygous ablation of Kctd12 led to changes in the fear learning and memory of a discrete auditory-conditioned stimulus. Heterozygous ablation of Kctd12 reduced protein expression in blood and brain to ~30–40%. This genotype led to hyperactivity during the inactive phase of the circadian cycle specifically. Kctd12+/− and Kctd12−/− mice exhibited reduced resting membrane conductances in CA1 pyramidal neurons that reduced the threshold for action potential
generation. Therefore, homozygous and heterozygous ablation of Kctd12 in mice lead to specific behavioral and neuronal phenotypes that, extrapolating to human, could constitute risk endophenotypes for the neuropsychiatric disorders associated with reduced KCTD12 transcription/translation.

In Kctd12−/− mice, the major finding was hyperactivity during the light phase of the circadian cycle. In GABA_A subunit-specific knockout mice, relative to WT mice, GABA_A10−/− mice were also mildly more active during the light phase, whereas GABA_A10−/− mice were hyperactive during the dark phase. The evidence for increased activity during the normally inactive phase in Kctd12−/− mice could indicate a deficit in the processes that typically suppress activity during this stage of the circadian cycle. There is indeed evidence for involvement of GABA_B receptors in circadian timing; for example, activation of GABA_B receptors in the suprachiasmatic nucleus leads to a shift in circadian phase. GABA_B receptors also regulate sleep, with the loss of GABA_B receptors delaying the onset of inactivity during the light phase. Postsynaptic GABA_B receptors are also important in terminating the persistent activity (UP) states in cortical neuron oscillations during slow-wave sleep. It is noteworthy that resting-state oscillations occur at a higher frequency and are proposed to contribute to the network abnormalities observed in bipolar disorder. It remains to be understood why a partial reduction in KCTD12 resulted in this phenotype whereas a complete loss of KCTD12 did not. As demonstrated, it was not the case that compensation occurred in expression of other KCTD subunits occurred in either Kctd12−/− or Kctd12+/− mice. Kctd12−/− mice, when tested for tone CS-electroshock US fear conditioning, exhibited phenotypes of suboptimal aversive learning and memory. They exhibited increased acquisition of fear-freezing to the CS and during the inter-CS intervals. However, on the following day, this was not recapitulated as increased expression of fear to the acquired CS. The Kctd12−/− mice did exhibit increased fear expression during the inter-CS intervals when placed in a novel context specifically. Therefore KCTD12 appears to exert inhibitory modulation of the amount of CS–US association learning that occurs. It is unusual for an increased acquisition of fear of a CS not to lead to increased fear expression to this same CS, suggesting that KCTD12 also contributes to consolidation of CS–US learning into memory. These data add to previous reports that GABA_B receptors are important regulators of learning about and/or forming memories of conditioned aversive stimuli. Increased aversive CS–US fear conditioning is also exhibited in MDD and KCTD12 expression was increased in amygdala in MDD. Together, the findings suggest that trait or state changes in KCTD12 levels can contribute to exaggerated focus on aversive stimuli and events, a common feature of mood disorder.

Regarding the neurocircuitry of fear conditioning, CS–US learning and memory occur primarily in the basal and lateral nuclei of the amygdala (BLA), and context-US learning and memory primarily in the hippocampus but dependent on bidirectional signaling with the BLA. GABA signaling is of major importance in the local inhibitory circuits that underlie adaptive CS–US conditioning, with GABA interneurons in the BLA integral to the feed-forward and feedback inhibitory circuits that control LTP output of the fear projection neurons. Both pre- and postsynaptic GABA_B receptors contribute to this. Studies in GABA_B10−/− mice have demonstrated that presynaptic GABA_B receptors inhibit postsynaptic LTP in the BLA and thereby prevent fear generalization. Mice lacking the postsynaptic GABA_B1 subunit exhibit impaired acquisition or consolidation of fear conditioning. Given that Kctd12 is highly expressed in mouse amygdala and hippocampus, and at the cellular level is expressed primarily postsynaptically, it is interesting that the fear-conditioning phenotypes of Kctd12−/− mice are markedly different from those of GABA_B10−/− mice. This is consistent with downregulation or lack of KCTD12 affecting subtle kinetic properties of postsynaptic GABA_B responses rather than preventing postsynaptic GABA_B responses altogether. It is also important to compare the positive evidence for the importance of GABA_B signaling to adaptive fear learning and memory as obtained with genetic mouse models, with the recent evidence for the lack of effects of GABA_A agonists and antagonists on mouse fear learning and memory, using either BALB/c or C57BL/6 strains: GABA_A positive modulators (GS39783, BHF177), the GABA_A agonist baclofen and a GABA_A antagonist (CGP52432), were all without effect on CS–US fear acquisition, recall or extinction. In rat, baclofen impaired CS extinction learning. Additional experiments are needed to reconcile these discrepancies.

Decreased KCTD12 is expected to affect the precise timing of GABA_B receptor influences on synaptic transmission and to result in excessive K^+_channel activity, which may cause intracellular K^+ depletion and thereby increase neuronal excitability. Here we show that decreased KCTD12 expression also increases neuronal excitability by reducing a tonic K^+ current. Surprisingly, the KCTD12-induced tonic current does not depend on GABA_B receptor activation. Since KCTD12 directly regulates the activity of heterotrimERIC G-protein subunits, it may induce tonic K^+ currents by activating the G-protein in the absence of receptor activity. Alternatively, KCTD12 may directly bind and activate effector K^+ channels. Lack of KCTD12 therefore increases neuronal excitability through synaptic and intrinsic mechanisms, which could be contributory to the suboptimal CS fear learning and memory observed in Kctd12−/− mice.

Given the human evidence for association of KCTD12 with bipolar disorder, MDD and schizophrenia, the current findings that Kctd12−/− and Kctd12+/− mice exhibit behavioral phenotypes of altered emotional and homeostatic behaviors and that these co-occur with increased excitability of hippocampal neurons, constitutes cause–effect evidence for the neurobehavioral importance of KCTD12. Of course, altered KCTD12 expression would be one specific factor in the complex gene–environment interactions that underlie the etiopathophysiology of these neuropsychiatric disorders. This study makes an important contribution to the animal model evidence that is essential to allow accurate assessment of GABA_B receptors as a potential target for the treatment of neuropsychiatric domains, both within and across some of the major disorders.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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**REFERENCES**

1. Padgett CL, Slesinger PA. GABA_B receptor coupling to G-proteins and ion channels. *Adv Pharmacol* 2010; 58: 123–147.
2. Gassmann M, Bettler B. Regulation of neuronal GABA_B receptor functions by subunit composition. *Nat Rev Neurosci* 2012; 13: 380–394.
3. Ivankova K, Turecek R, Fritzius T, Seddik R, Prezeau L, Comps-Agrar L et al. Up-regulation of GABA_B receptor signaling by constitutive assembly with the K^+ channel tetramerization domain-containing protein 12 (KCTD12). *J Biol Chem* 2013; 288: 24848–24856.
4. Schwenk J, Metz M, Zolles G, Turecek R, Fritzius T, Bildl W et al. Native GABA_B receptors are heteromultimers with a family of auxiliary subunits. *Nature* 2010; 465: 231–235.
5 Fatemi SH, Folsom TD, Thuras PD. Deficits in GABAergic receptor system in schizophrenia and mood disorders: a postmortem study. *Schizophr Res* 2011; 128: 37–43.

6 Mohler H. The GABA system in anxiety and depression and its therapeutic potential. *Neuropsychopharmacology* 2012; 62: 42–53.

7 Peters S, Slattery DA, Flor PJ, Neumann ID, Reber SO. Differential effects of baclofen and coxycodone on the increased ethanol consumption following following chronic psychosocial stress in mice. *Addict Biol* 2013; 18: 66–77.

8 Cryan JF, Slattery DA. GABA receptors and depression. Current status. *Adv Pharmacol* 2010; 58: 427–451.

9 Kumar K, Sharma S, Kumar P, Deshmukh R. Therapeutic potential of GABA B receptor ligands in drug addiction, anxiety, depression and other CNS disorders. *Pharmacol Biochem Behav* 2013; 110: 174–184.

10 Jacobson LH, Blettler B, Kaumann K, Cryan JF. GABA B receptor subunit isoforms exert a differential influence on baseline but not GABA B receptor agonist-induced changes in mice. *J Pharmacol Exp Ther* 2006; 319: 1317–1326.

11 Jacobson LH, Blettler B, Kaumann K, Cryan JF. Behavioral evaluation of mice deficient in GABA B receptor isoforms in tests of unconditoned anxiety. *Psychopharmacology* (Berl) 2007; 190: 541–553.

12 Jacobson LH, Kelly PH, Blettler B, Kaumann K, Cryan JF. GABA B receptor isoforms differentially mediate the acquisition and extinction of aversive taste memories. *J Neurosci* 2006; 26: 8800–8803.

13 Shaban H, Humeau Y, Herry C, Cassassus G, Shigemoto R, Ciocchi S et al. Generalization of amygdala LTP and conditioned fear in the absence of presynaptic inhibition. *Nat Neurosci* 2006; 9: 1028–1035.

14 Lee MT, Chen CH, Lee CS, Chen CC, Chong MY, Ouyang WC et al. Genome-wide association study of bipolar I disorder in the Han Chinese population. *Mol Psychiatry* 2011; 16: 548–556.

15 Miller GE, Chen E, Sze J, Marin T, Arevalo JM, Doll R et al. A functional genomic fingerprint of chronic stress in humans: blunted glucocorticoid and increased NF-kappaB signalling. *Biol Psychiatry* 2008; 64: 266–272.

16 Sible E, Wang Y, Joeyen-Waldorf J, Gailter C, Surget A, Oh S et al. A molecular signature of depression in the amygdala. *Am J Psychiatry* 2009; 166: 1011–1024.

17 Glatt SJ, Everall IP, Kremen WS, Deshmukh R, ad, Alpers H. GABA B receptor activation in the suprachiasmatic nucleus of diurnal and nocturnal rodents. *Brain Res Bull* 2004; 63: 531–545.

18 Vienne J, Blettler B, Franken P, Tafmi M. Differential effects of GABA B receptor subtypes, y-hydroxybutyric acid, and baclofen on EEG activity and sleep regulation. *J Neurosci* 2010; 30: 14194–14204.

19 Kim DJ, Bolbecker AR, Howell J, Rass O, Sporns O, Hetrick WP et al. Disturbed resting state EEG synchronization in bipolar disorder: a graph-theoretic analysis. *Neuroimage Clin* 2013; 2: 414–423.

20 Ongur D, Lundy M, Greenhouse I, Shinn AK, Menon V, Cohen BM et al. Default mode network abnormalities in bipolar disorder and schizophrenia. *Psychiatry Res* 2010; 183: 59–68.

21 Disner SG, Beesgar CG, Haigh EA, Beck AT. Neural mechanisms of the cognitive model of depression. *Nat Rev Neurosci* 2011; 12: 467–477.

22 Maren S. Neurobiology of Pavlovian fear conditioning. *Annu Rev Neurosci* 2001; 24: 897–931.

23 Maren S. Pavlovian fear conditioning as a behavioral assay for hippocampus and amygdala function: cautions and caveats. *Eur J Neurosci* 2008; 28: 1661–1666.

24 McGaugh JL. The amygdala modulates the consolidation of memories of emotionally arousing experiences. *Ann Rev Neurosci* 2004; 27: 1–28.

25 Phelps EA, LeDoux JE. Contributions of the amygdala to emotion processing: from animal models to human behavior. *Neuron* 2005; 48: 175–187.

26 Ehrlich I, Humeau Y, Grenier F, Ciocchi S, Herr C, Luthy A. Amygdala inhibitory circuits and the control of fear memory. *Neuron* 2009; 62: 757–771.

27 Sweeney FF, O’Leary OF, Cryan JF. GABA B receptor ligands do not modify conditioned fear responses in BALB/c mice. *Behav Brain Res* 2013; 256: 151–156.

28 Li X, Risborg VB, Cates-Gatto C, Kaczanowska K, Finn MG, Roberts AJ et al. Comparison of the effects of the GABA B receptor positive modulator BHF177 and the GABA B receptor agonist baclofen on anxiety-like behavior, learning, and memory in mice. *Neuropsychopharmacology* 2013; 70: 156–167.

29 Heaney CF, Bolton MM, Murtishaw AS, Sabbagh JH, Magcalas CM, Kinney JW. Baclofen administration alters fear extinction and GABAergic protein levels. *Neurobiol Learn Mem* 2012; 98: 261–271.

30 Beekhaver MP, Huguenard JR. Astrocytes as gatekeepers of GABA B receptor function. *J Neurosci* 2010; 30: 15262–15276.

31 Savitz J, Drevets WC. Bipolar and major depressive disorder: neuroimaging the developmental-degenerative divide. *Neurosci Biobehav Rev* 2009; 33: 699–771.

32 Malcangio M, Malmberg-Alieno P, Giotti A, Ghelardini C, Bartolini A. Desensitiza- tion of GABA B receptors and antagonists by CGP 33548, prevent bicuculline- and picrotoxin-induced anticonvulsions. *Neuropharmacology* 1992; 31: 783–791.

33 Schulzer V, Lusher C, Blanchet C, Kla N, Sarnis G, Klebs K et al. Eplepsy, hyper- algesia, impaired memory, and loss of pre- and postsynaptic GABA B responses in mice lacking GABA B. *Neuron* 2001; 31: 47–58.

34 Nazar M, Siemiatkowski M, Czlonkowska A, Sienkiewicz-Jarosz H, Plaznik A. The role of the hippocampus and 5-HT/GABA interaction in the central effects of benzodiazepine receptor ligands. *J Neurotransm* 1999; 106: 369–381.

35 Willner P, Valdivia VM, Clotia A, Van Orden M, Ades J, Zajac AM et al. Animal models of neuropyschiatric disorders. *Nat Neurosci* 2010; 13: 1161–1169.

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