MolecularMedicine

Genetic Markers of a Munc13 Protein Family Member, *BAIAP3*, Are Gender Specifically Associated with Anxiety and Benzodiazepine Abuse in Mice and Humans

Sonja M Wojcik,[1] Martesa Tantra,[2,3*] Beata Stepniak,[2*] Kwun-nok M Man,[1,3] Katja Müller-Ribbe,[2] Martin Begemann,[2] Anes Ju,[2] Sergi Papiol,[2,3] Anja Ronnenberg,[2] Artem Gurvich,[2] Yong Shin,[1,4] Iris Augustin,[1,5] Nils Brose,[1,3] and Hannelore Ehrenreich[2,3]*

[1]Max Planck Institute of Experimental Medicine, Department of Molecular Neurobiology, Göttingen, Germany; [2]Max Planck Institute of Experimental Medicine, Clinical Neuroscience, Göttingen, Germany; [3]DFG Center for Nanoscale Microscopy and Molecular Physiology of the Brain, Göttingen, Germany; [4]present address: BioElectronics, Institute of Microelectronics, Agency for Science, Technology and Research (A*STAR), Singapore, Singapore; and [5]present address: German Cancer Research Center, Department Signaling and Functional Genomics, Heidelberg, Germany

Anxiety disorders and substance abuse, including benzodiazepine use disorder, frequently occur together. Unfortunately, treatment of anxiety disorders still includes benzodiazepines, and patients with an existing comorbid benzodiazepine use disorder or a genetic susceptibility for benzodiazepine use disorder may be at risk of adverse treatment outcomes. The identification of genetic predictors for anxiety disorders, and especially for benzodiazepine use disorder, could aid the selection of the best treatment option and improve clinical outcomes. The brain-specific angiogenesis inhibitor I–associated protein 3 (Baiap3) is a member of the mammalian uncoordinated 13 (Munc13) protein family of synaptic regulators of neurotransmitter exocytosis, with a striking expression pattern in amygdalae, hypothalamus and periaqueductal gray. Deletion of *Baiap3* in mice leads to enhanced seizure propensity and increased anxiety, with the latter being more pronounced in female than in male animals. We hypothesized that genetic variation in human *BAIAP3* may also be associated with anxiety. By using a phenotype-based genetic association study, we identified two human *BAIAP3* single-nucleotide polymorphism risk genotypes (AA for rs2235632, TT for rs1132358) that show a significant association with anxiety in women and, surprisingly, with benzodiazepine abuse in men. Returning to mice, we found that male, but not female, *Baiap3* knockout (KO) mice develop tolerance to diazepam more quickly than control animals. Analysis of cultured *Baiap3* KO hypothalamus slices revealed an increase in basal network activity and an altered response to diazepam withdrawal. Thus, *Baiap3/BAIAP3* is gender specifically associated with anxiety and benzodiazepine use disorder, and the analysis of *Baiap3/BAIAP3*-related functions may help elucidate mechanisms underlying the development of both disorders.

Online address: http://www.molmed.org
doi: 10.2119/molmed.2013.00033

INTRODUCTION

Anxiety disorders have high lifetime prevalence rates (1) and exhibit a remarkable comorbidity with substance use disorders (2–4). This association worsens treatment outcomes for both conditions (5) and represents a significant burden on individuals and society. Both anxiety disorders and substance use disorders are complex disorders that arise from a combination of genetic influence and environmental factors. To improve upon established treatment options, which include pharmacological as well as cognitive-behavioral therapies (6,7), a more detailed picture of the etiology of these disorders is instrumental. Estimates of heritability from twin and family studies are in the range of 20–40% across the different anxiety disorders (8,9) and in the range of 40–70% for the major substance use disorders (10). Recent studies point to the involvement of a large number of genes with relatively small effect sizes for both anxiety disorder (11,12) and substance use disorder (13–15). Although the interaction between anxiety disorders and substance use disorders is likely bidirec-

*SMW, MT, and BS contributed equally to this study.

Address correspondence to *Sonja M Wojcik, Max Planck Institute of Experimental Medicine, Department of Molecular Neurobiology, Hermann-Rein-Str. 3, D-37075 Göttingen, Germany. Phone: +49-551-3899-722; Fax: +49-551-3899-715; E-mail: wojcik@em.mpg.de.*
Submitted April 12, 2013; Accepted for publication May 14, 2013; Epub (www.molmed.org) ahead of print May 14, 2013.

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tional and varies by the type of anxiety (16), genetically determined anxiousness personality traits may make the development of an addiction more likely (2,17–19). The recommended first-line pharmacological treatments of anxiety disorders are selective serotonin or serotonin/norepinephrine reuptake inhibitors and the calcium channel modulator pregabaline (6). However, primary care physicians often still prescribe benzodiazepines, which rank among the most frequently abused prescription medications (National Institute on Drug Abuse [http://www.nida.nih.gov]), to patients suffering from anxiety disorders (20). Identifying genetic risk markers would advance our understanding of the biology of anxiety and benzodiazepine abuse and would be a valuable step in improving treatment options for these complex diseases.

In addition to human family, twin and genome-wide association studies, animal models are used to study the genetic basis and neural circuitries of anxiety and addiction. For both animals and humans, anxiety is an adaptive defensive response to threatening stimuli necessary for the survival of the species, whereas anxiety disorders are an extreme and maladaptive manifestation of normal anxiety (21). Somatic anxiety symptoms are mediated by the release of specific neurotransmitters and neuropeptides. The selection of candidate genes that are being investigated in animal studies is still largely driven by hypotheses of the neural circuitries and neurotransmitter systems thought to be involved in mediating fear and anxiety (22). By using a candidate gene approach, we investigate the involvement of the brain-specific angiogenesis inhibitor I–associated protein 3 (Baiap3), which is highly expressed in brain regions involved in processing fear, such as the amygdalae, hypothalamus and periaqueductal gray, in behavioral phenotypes relevant for human psychiatric disorders.

Baiap3 is a member of the mammalian uncoordinated 13 (Munc13) family of synaptic regulators of neurotransmitter exocytosis (23–25). *Baiap3* has a unique and striking expression pattern (Allen Brain Atlas [http://mouse.brain-map.org]) in brain regions such as the central, medial and basomedial amygdaloid nuclei; the hypothalamus; and the periaqueductal gray. These areas are involved in regulating autonomic functions and are also critical in processing fearful stimuli and mediating anxiety-related behaviors (26,27). The cellular function of Baiap3 is currently unknown; however, all other Munc13 members are regulators of vesicle exocytosis in various cell types (28). In the brain, Munc13-1 and Munc13-2 are essential for membrane fusion of synaptic vesicles containing classical neurotransmitters, such as glutamate or γ-aminobutyric acid (GABA) (25). Munc13-4, a non-neuronal Munc13 isoform most closely related to Baiap3 at the sequence level, is involved in exocytosis in cells of the hematopoietic system (29,30).

To explore the function of Baiap3, we combined the behavioral analysis of *Baiap3* knockout (KO) mice with a phenotype-based genetic association study (PGAS) of the human *BAIAP3* gene by using the Göttingen Research Association for Schizophrenia (GRAS) database (31,32). Using this two-pronged approach, we identify *Baiap3/BAIAP3* as the first genetic risk marker for anxiety and benzodiazepine abuse in both mice and humans.

MATERIALS AND METHODS

Animals

Animal maintenance. All experiments were approved by the local Animal Care and Use Committee of Lower Saxony, Oldenburg, Germany. The first three coding exons of the murine *Baiap3* gene were preplaced with a neomycin resistance cassette through homologous recombination in embryonic stem cells (129/Ola) (Supplementary Figure S1A). *Baiap3* mutant mice of mixed 129/Ola;C57BL/6N background were backcrossed for seven more generations to C57BL/6N; all experiments were done with WT and KO littermates of the resulting generation 8. After weaning, mice were group-housed in standard plastic cages (n = 5 per cage) and maintained in a temperature-controlled environment (21 ± 2°C) on a 12-h light–dark cycle with food and water ad libitum, unless stated otherwise.

Drugs used in animal experiments. Two classical benzodiazepines, positive allosteric modulators of GABA type A receptors ($GABA_AR$) were used: (i) the long-acting benzodiazepine diazepam (ratiopharm GmbH, Ulm, Germany) was suspended in saline containing polysorbate 80 for intraperitoneal (IP) injection, and (ii) the short-acting benzodiazepine midazolam (ratiopharm) was added to 2% sucrose solution for oral administration. Antagonists used were as follows: (i) flumazenil (Sigma-Aldrich Chemie, Munich, Germany), routinely applied in the clinic to counteract benzodiazepine overdoses, was dissolved in saline containing polysorbate 80 and HCl; and (ii) pentylenetetrazole (PTZ) (Sigma-Aldrich Chemie), a noncompetitive GABA antagonist with epileptogenic properties, was dissolved in saline for IP injection.

Phenotypical characterization of *Baiap3* KO mice. Behavioral characterization of naive *Baiap3* KO mice and their WT littermates of both sexes began at the age of 8 wks and was performed in the following order: elevated plus-maze, open field, light–dark box, hole board, rotarod and exposure to a fear-conditioning chamber to assess novelty-induced freezing behavior. Mouse numbers of all individual experiments are given in the figure legends.

Elevated plus-maze. The mouse was placed in the central platform, facing an open arm of the plus-maze. Behavior was recorded over 5 min by an overhead video camera. A personal computer equipped with Viewer software (Biobserve, Bonn, Germany) was used to calculate the time each animal spent in open versus closed arms. The proportion of time spent in open arms (natural aversion) was used as a fear equivalent.

Open field. Spontaneous activity in open field was tested in a gray Perspex

arena (120 cm in diameter, 25 cm high), virtually divided into three zones: central, intermediate and peripheral. The mouse was placed in the center, and the test was started when the mouse reached the wall. Over 7 min, the mouse was allowed to freely explore the open field. Behavior was recorded by a personal computer–linked overhead video camera and calculated using Viewer software. Readouts were as follows: velocity, distance traveled, time spent in each zone and initial latency to reach the wall.

Hole board. The hole board apparatus (TSE Systems GmbH, Bad Homburg, Germany) for measuring exploratory activity consisted of a 50 cm × 50 cm × 35 cm transparent Perspex chamber with a nontransparent floor raised above the bottom of the chamber. The floor had 16 equally spaced holes, 2.4 cm in diameter, fitted with a light barrier sensor (8 mm below floor). Mice were allowed to explore the chamber for 5 min, and the number of explored holes (head dips) was recorded.

Rotarod. This test for motor function, balance and coordination consists of a rotating drum (Ugo Basile, Comerio, Varese, Italy), accelerated from 4 to 40 revolutions per minute over 5 min. Each mouse was placed individually on a drum and the latency of falling from the drum was recorded using a stopwatch. To assess motor learning, the test was repeated 24 h later.

Novelty-induced fear response. To assess novelty-induced fear response (indicated by freezing behavior), a chamber designed for training and testing of context fear conditioning was used. Mice were placed inside the chamber and allowed to explore the chamber freely for 2 min, during which time no additional stimulus was presented (equivalent to the assessment of baseline freezing of the fear-conditioning paradigm). Duration of freezing behavior, defined as the absolute lack of movement (excluding respiratory movements), was recorded by a video camera and a personal computer equipped with Video Freeze software (MED Associates, St. Albans, VT, USA).

Pentylenetetrazole-induced seizures. Seizure activity was induced in wakeful mice by using a single IP injection of PTZ (50 mg/kg body weight) (33). After injection of the compound, the mouse was placed in a small, clear home cage and closely observed for 30 min. Latencies to focal (partial clonic), generalized (generalized clonic) and maximal (tonic-clonic) behavioral seizures were recorded. Furthermore, four phases in the continuum of behavioral response to IP PTZ injection were defined as follows: (i) hypoactivity (progressive decrease in motor activity until the animal came to rest in a crouched or prone position with the abdomen in full contact with the cage bottom); (ii) partial clonus (clonus seizure activity affecting face, head and/or forelimb or forelimbs); (iii) generalized clonus (sudden loss of upright posture, whole body clonus involving all four limbs and tail, rearing and autonomic signs); and (iv) tonic-clonic (maximal) seizure (generalized seizure characterized by tonic hindlimb extension—also associated with death). Finally, latencies to partial clonus (PC), generalized clonus (GC) and tonic-clonic (TC) seizures were summed to assign a seizure score to each mouse, used as a quantitative trait measure for mapping according to the following equation: seizure score = [(0.2) × (1/PC latency) + (0.3) × (1/GC latency) + (0.5) × (1/TC latency)] × 1,000. The weighting factors (0.2, 0.3 and 0.5) in the equation were included as a means of incorporating a measure of the progressive nature of the PTZ-induced seizure phenotype into the severity rating because generalized clonus is regarded as a more significant event than partial clonus and tonic hind limb extension as the most severe component of the phenotype. Therefore, the seizure score reflects the degree of progression of the seizure phenotype in each mouse (33).

Diazepam dependence, tolerance and withdrawal. The mice received injections of diazepam (5 mg/kg body weight IP) for 10 consecutive days. Rotarod test was performed 30 min after each diazepam injection for 7 d, with baseline rotarod training performed for 2 d before starting injections. On d 11, diazepam withdrawal was induced by flumazenil (15 mg/kg body weight IP), followed by injection of PTZ (50 mg/kg body weight IP) to induce withdrawal-related seizures. Seizure induction by PTZ (50 mg/kg body weight IP) was also performed on drug-naive mice.

Midazolam oral self-administration and behavior testing in the addicted state. To induce benzodiazepine dependence as a prerequisite for oral self-administration (document of addiction), group-housed mice received midazolam (ratiopharm) in 2% sucrose (to reduce the bitter taste), instead of drinking water. Midazolam concentration was increased weekly, starting from 0.005 mg/mL until the maximum concentration of 0.05 mg/mL was reached after 10 wks. A respective control group received 2% sucrose only. One set of midazolam mice was then exposed to a midazolam preference test. For this purpose, mice were first switched to single housing with a continued supply of midazolam (0.05 mg/mL) for 2 wks. For the preference test, every mouse had a choice of two bottles containing either midazolam (0.05 mg/mL) in 2% sucrose or 2% sucrose alone for another 2 wks. The relative consumption of midazolam solution was calculated. The other set of mice (midazolam and control mice) stayed group-housed and underwent automated home cage observation using the LABORAS™ system (Metris, Hoofddorp, Netherlands). LABORAS is a fully automated system for continuous behavior recognition and tracking in small rodents. For habituation before testing, mice were temporarily put in single cages similar to the LABORAS cage in the testing room for 2 consecutive nights (1700 to 0900). On the day of testing, Makrolon type 3 cages (840 cm^2), filled with a 2-cm layer of bedding used during the habituation phase, were placed on each triangular sensor platform (95 cm × 75 cm × 75 cm). Food and sucrose solution with midazolam (addicted group) or 2% sucrose (control group) were provided *ad libitum*.

Before each session, LABORAS was calibrated by using the calibration procedure and reference weights supplied by Metris. Movements during nighttime (1800 to 0900) were recorded and distinguished as separate behavioral patterns by the LABORAS software. Locomotion duration and scratching frequency during the dark phase (2000 to 0800) was analyzed.

Statistical analysis. Behavioral data were analyzed separately for males and females by the Mann-Whitney *U* test and two-way analysis of variance (ANOVA), including post hoc Bonferroni testing, where applicable, using Prism4 (GraphPad Software, San Diego, CA, USA). Significance level was set to $p < 0.05$. All data are presented as mean ± standard error of the mean (SEM).

Human Sample

Schizophrenic patient sample. The schizophrenic patient sample (n = 1,086) was recruited across 23 sites throughout Germany in the cross-sectional GRAS study and most comprehensively phenotyped (31,32). The study was approved by the Ethics Committee of the Georg-August-University (Göttingen, Germany) and the review boards of participating centers and complies with the Declaration of Helsinki. Patients fulfilling *Diagnostic and Statistical Manual of Mental Disorders: DSM-IV-TR*, 4th edition, text revision (34) criteria for schizophrenia or schizoaffective disorder were included in the analyses regardless of their disease stage (acute, chronic, residual or remitted). Almost all patients were of European Caucasian descent (Caucasian 94.7%; other ethnicities 1.9%; unknown 3.4%).

Healthy control sample. Voluntary blood donors (n = 1,142) recruited following the national guidelines for blood donation were included for case control analysis (31,32). Also the majority of control subjects are of European Caucasian ethnicity (Caucasian 97.8%; other ethnicities 2%; unknown 0.2%).

Sociodemographic and clinical variables. Sociodemographic data (age, years of education, level of unemployment), information on substance use disorder (summarizing abuse and dependence based on the DSM-IV-TR criteria for alcohol and cannabis) and clinical variables describing disease severity were used to characterize the sample. Clinical variables included Positive and Negative Syndrome Scale (PANSS) positive scale as a measure of positive symptom severity (35) as well as chlorpromazine equivalents to estimate the relative dose of antipsychotic medication. The Global Assessment of Functioning (GAF) scale (DSM-IV-TR) was used as a measure of impairment of psychological, social and occupational functioning.

Target variables. The dichotomous DSM-IV-TR benzodiazepine use disorder diagnosis (summarizing abuse and dependence) and the quantitative anxiety composite score were our target variables. The anxiety composite score is based on the aggregation of four anxiety-related variables: (i) Brief Symptom Inventory (BSI) subscale anxiousness; (ii) State-Trait Anxiety Inventory (STAI) subscale trait anxiety; (iii) STAI subscale state anxiety; and (iv) anxiety item of the PANSS general psychopathology subscale (Supplementary Figure S2).

DNA extraction and normalization. Genomic DNA was purified from whole blood by using JETQUICK Blood and Cell Culture DNA Spin Kit (Genomed, Loehne, Germany) according to the manufacturer's protocol. DNA aliquots were stored at –80°C. For further analyses, DNA was normalized to 50 ng/μL with an automated robotic platform (Microlab Star, Hamilton, Bonaduz, Switzerland). Each sample was analyzed with a 0.8% agarose gel for quality control.

Genotyping. The three selected SNPs (rs11648169, rs2235632, rs1132358) of *BAIAP3* were analyzed by using Simple Probes (TIB Molbiol, Berlin, Germany) and genotyped using the LightCycler® 480 Genotyping Software implemented in the LightCycler 480 system (Roche, Mannheim, Germany). The reaction mixture (10 μL) was prepared with 20 ng DNA in 384-well plates following the standard protocol (Roche). In each run, eight positive controls (hgDNA, Bioline, Luckenwalde, Germany) and negative water blanks were included for quality and internal control purposes. Of the GRAS patients, a total of n = 1,082 (99.63%) were successfully genotyped for *BAIAP3* SNP1 (C/G) rs11648169, n = 1,086 (100%) for *BAIAP3* SNP2 (G/A) rs2235632 and n = 1,069 (98.43%) for *BAIAP3* SNP3 (C/T) rs1132358 and included in the analyses. Of the healthy control subjects, all n = 1,142 were successfully genotyped for SNP1, SNP2 and SNP3 of the *BAIAP3* gene.

Statistical analyses. For all analyses, statistical significance was set to 0.05. Statistical analyses of human data were performed by using SPSS for Windows, version 17.0. Group differences in categorical and continuous variables were assessed using χ^2 or Mann-Whitney *U* tests; in cases of normal distribution of the continuous variable, *t* tests were performed. Anxiety score composition was done using *z*-standardized mean subscale scores (BSI anxiousness, STAI trait anxiety, STAI state anxiety) or, in the case of PANSS anxiety, a *z*-standardized single item, organized such that higher values represent higher symptom severity. Intercorrelations and internal consistency of the anxiety composite score was calculated by using Pearson correlation coefficient and Cronbach α (36). In the GRAS sample, the following items or scales were incomplete: BSI anxiousness 7.5% missing, STAI trait anxiety 20.2%, STAI state anxiety 21.6% and PANSS anxiety 3.2%. If all four anxiety variables were available, the mean was calculated for each respective subject as an individual anxiety composite score. In the case of missing data, a linear regression-based multiple imputation model (10 iterations) of missing data was applied, if at least three out of the four variables per subject were available. For the 190 individuals with imputed values, the final anxiety composite score represents the mean of 10 imputed values for the missing item, increasing the availability of the anxiety score from n = 771 to n = 961 schizo-

phrenic subjects (37). Analysis of covariance (adjusted for age, PANSS positive subscale score and chlorpromazine equivalents) was used to analyze the effect of SNP genotypes on the standardized anxiety composite score. For the phenotype-genotype association analyses (including peripheral blood mononuclear cells [PBMCs]; see below) of the *BAIAP3* SNP rs2235632, G carriers (GG and AG) were aggregated and contrasted with individuals homozygous for the A allele, and in the case of SNP rs1132358, C carriers (CC and TC) were aggregated and contrasted with TT individuals. SNP rs11648169 was excluded from further analyses, since it yielded no statistically significant effects.

In Vitro Analyses

Immunofluorescence analysis. Brains were perfusion-fixed, and organotypic hypothalamus slices were immersion fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4). Brains were post-fixed for 1 h, cryoprotected with 30% sucrose and frozen. For immunofluorescence analysis, free-floating brain sections of 40 μm thickness or organotypic sections of 300 μm thickness were incubated in primary antibodies for 72 h followed by incubation with IgG-coupled Alexa Fluor 488, Alexa Fluor 555 and Alexa Flour 633 dyes (Invitrogen [Life Technologies, Darmstadt, Germany]) for 24 h. Rabbit and guinea pig antibodies to Baiap3 were raised to a purified fragment (amino acids 617–973) containing the munc homology domain (MHD)-1 and MHD-2 of mouse Baiap3. Commercial primary antibodies used were rabbit and guinea pig anti-vesicular glutamate transporter 1 (VGLUT1), rabbit and guinea pig anti-VGLUT2, rabbit and guinea pig anti-vesicular inhibitory amino acid transporter (Viaat), mouse anti-Gephyrin (mAB7a) (all from Synaptic Systems, Göttingen, Germany), and mouse anti-postsynaptic density protein 95 (PSD-95) (clone K28/48, NeuroMab). False color images of brain sections and organotypic slices were obtained with a fluorescence stereomicroscope (Leica FluoCombi III™) and an ApoTome™ fluorescence microscope (Axio Imager Z1; Zeiss), respectively.

Hypothalamus slice culture. Organotypic hypothalamus slices of 300-μm thickness from postnatal d 5 (P5) and P6 mice were prepared in Hanks balanced salt solution (24020-091; Invitrogen [Life Technologies]) with 20% glucose and 1 mmol/L kynurenic acid (Sigma-Aldrich, Germany) (pH 7.4), by using a McIlwain Tissue Chopper. Slices were cultured in six-well plates on confetti cut from 0.45-μm filters (FHLC04700; EMD Millipore [Millipore Ireland B.V., Tullagreen, Carrigtwohill County Cork, Ireland]) that were placed in 0.4-μm Millicell cell culture inserts (PICM03050; Millipore) for 5 d using a mixture of 41% Earle basal medium Eagle (BME) (F 0225; Biochrom, Berlin, Germany), with 25% Earle balanced salt solution (1.8 mmol/L $CaCl_2$, 1 mmol/L NaH_2PO_4, 0.8 mmol/L $MgSO_4$, 116 mmol/L NaCl, 26.2 mmol/L $NaHCO_3$, 5.4 mmol/L KCl, 5 mmol/L glucose), 20% heat-inactivated horse serum, 10% H_2O, 25 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Biochrom), 28 mmol/L glucose, 1 mmol/L GlutaMAX™ (35050; Invitrogen [Life Technologies]), 1 μg/mL insulin, 88 μg/mL ascorbic acid, 0.25% MEM Vitamine Solution (K0373; Biochrom) and 0.5% MEM Amino Acids (K0363; Biochrom). On d 5 *in vitro*, the cultures were switched to a medium with identical components but containing 5% horse serum, 55% BME and 2 mmol/L GlutaMAX. At the same time, diazepam was added to the medium from a 60 mmol/L stock solution in dimethylsulfoxide (DMSO) for a final concentration of 10 μmol/L. For control cultures, DMSO was added as a vehicle control at the same dilution of 1:6,000. The CO_2 concentration was 5%, and medium changes were done on the day after culture and every 48 h after that.

Electrophysiological analyses. Organotypic slices containing the ventromedial hypothalamus were transferred to the recording chamber between DIV10 and DIV17. Recordings were started after a 30-min recovery time, the extracellular recording solution contained 120 mmol/L NaCl, 26 mmol/L $NaHCO_3$, 1 mmol/L KH_2PO_4, 2 mmol/L KCl, 20 mmol/L glucose, 2 mmol/L $MgCl_2$, 2 mmol/L $CaCl_2$ and 250 nmol/L flumazenil. Cells were whole-cell voltage clamped at –70 or –20 mV or recorded in current clamp mode with an EPC 10 USB Double (HEKA, Lambrecht/Pfalz, Germany) under control of the Patchmaster 2.52 program (HEKA). All analyses were performed by using the Mini Analysis Program (Synaptosoft, Decatur, GA, USA). Recordings of miniature inhibitory postsynaptic currents (mIPSCs) were performed in the presence of 1 μmol/L tetrodotoxin (Tocris [R&D Systems, Wiesbaden-Nordenstadt, Germany]) and 10 μmol/L 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX) (Tocris [R&D Systems]), with an intracellular solution containing 100 mmol/L KCl, 50 mmol/L K-gluconate, 10 mmol/L HEPES, 0.1 mmol/L EGTA, 0.3 mmol/L GTP, 4 mmol/L ATP and 0.2% biocytin. Action potentials and spontaneous inhibitory postsynaptic currents (IPSCs) were recorded with an intracellular solution containing 20 mmol/L KCl, 130 mmol/L K-gluconate, 10 mmol/L HEPES, 0.1 mmol/L EGTA, 0.3 mmol/L GTP, 4 mmol/L ATP and 0.2% biocytin. Action potentials analyzed were from the first minute of a 2-min recording; membrane potentials were measured after setting the current injection to 0 pA at the end of the recording. IPSCs were recorded for 5 min after switching the cell to a holding potential of –20 mV and waiting for 1 min. Statistical analyses were performed using GraphPad Prism5.

Analysis of *BAIAP3* mRNA levels in PBMCs. PBMCs from 121 patients were isolated by using the standard Ficoll-Paque Plus isolation procedure (GE Healthcare, Munich, Germany). For RNA isolation, the miRNeasy Mini Kit (Qiagen, Hilden, Germany) was used. A total of 1 μg RNA, a mixture of oligo dT, hexamer primers, dNTPS (10 mmol/L each) and SuperScriptIII (200 U; Invitrogen [Life Technologies]) were used for transcription into cDNA (20-μL reaction). The mixture

was incubated for 10 min at 25°C and 45 min at 50°C, followed by 45 min at 55°C. For the quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), a 1:10 dilution of the cDNA was used and three replicate experiments per sample were performed: 5 μL Power SYBR mix (Applied Biosystems) and 1 pmol of each primer were added. *BAIAP3* qRT-PCR primers used were as follows: 5′-AGCTGGGCCCACCGCATCTCT-3′ with 5′-CTCGGCAGGCACGGAAAAGTAG-3′ and 5′-CTGACTTCAACAGCGACACC-3′ with 5′-TGCTGTAGCCAAATTCGTTGT-3′. The following cycling profile was run on the LightCycler480 system (Roche): preheating at 95°C for 10 min; 45 cycles of 95°C for 15 s, 60°C for 1 min. Cycle threshold values of *BAIAP3* were standardized to cycle threshold values of GAPDH.

All supplementary materials are available online at www.molmed.org.

RESULTS

Generation of *Baiap3* KO Mice

Baiap3 shares the basic domain structure of other Munc13 isoforms, with two munc-homology domains flanked by two C2 domains but lacks the N-termini contained in Munc13-1, -2 and -3 (23). The murine *Baiap3* gene contains 33 coding exons that span 8.7 kb. We generated *Baiap3* KO mice by homologous recombination in embryonic stem cells, replacing the first three coding exons with a neomycin selection cassette (Supplementary Figure S1A). *Baiap3* KO mice are viable, fertile and indistinguishable from their wild-type (WT) littermates in the home cage. In WT brain, the expression pattern of Baiap3 protein analyzed by immunofluorescence staining largely matches the distribution of *Baiap3* mRNA published in the Allen Brain Atlas. Baiap3 protein is prominently expressed throughout the hypothalamus and in the central, medial and basomedial amygdaloid nuclei, as well as in the paraventricular nucleus of the thalamus (Figure 1). Strong expression is further detected in the septum, bed nucleus of the stria terminalis, midbrain including the periaqueductal gray and inferior colliculus, and brain stem including the parabrachial nucleus and nucleus tractus solitarius (Figure 1). Baiap3 immunoreactivity appears punctate, but does not seem to localize to either glutamatergic or GABAergic pre- or postsynapses to a significant degree (Supplementary Figures S1C–G). Adult *Baiap3* KO mice lack any detectable expression of Baiap3 protein by immunofluorescence and Western blot analysis (Figure 1B, Supplementary Figure S1B). Western blot analysis of brains taken from newborn *Baiap3* KO animals revealed the presence of a weak band that most likely corresponds to Baiap3 protein expressed from a start codon present in coding exon 4; however, this putative truncated Baiap3 product is barely detectable by the age of 3 wks and not present in adult animals (Supplementary Figure S1B).

Novelty-Induced Anxiety in *Baiap3* KO Mice

The striking expression pattern of *Baiap3* in the amygdala and other brain regions involved in processing fear piqued our interest, and we chose to assess whether the genetic deletion of *Baiap3* led to any detectable behavioral alterations. We subjected *Baiap3* KO mice and WT littermates of both sexes to a battery of standard behavioral tests (Figures 2A–L; Supplementary Figures S3A–J). In the open field, both male and female *Baiap3* KO mice showed an increased latency to reach the wall upon release in the center zone (Figures 2A, B). Female but not male KO mice also made fewer visits to the center (Figures 2C, D) and spent significantly more time in the periphery (Figures 2E, F). When placed in a novel chamber (fear-conditioning box), both male and female KO mice showed an increased novelty-induced freezing response (Figures 2K, L). Taken together, these findings are indicative of a heightened novelty-induced anxiety level in *Baiap3* KO animals, with a more pronounced effect noted in females. In contrast, classical tests, measuring anxiety in the context of an inherent conflict between a protected and a more anxiogenic area, that is, elevated plus-maze and light–dark box, did not reveal any genotype differences (Figures 2G–J). Furthermore, the distance traveled (motor activity) in open field and elevated plus-maze (Supplementary Figures S3A–D), exploratory behavior (hole board; Supplementary Figures S3E, F), motor learning and coordination (rotarod; Supplementary Figures S3G, H) and body weight (Supplementary Figures S3I, J) were not affected by genotype.

BAIAP3 Is a Risk Marker for Anxiety in Women

To explore the possibility of an association of genetic variability in the human *BAIAP3* gene with specific biological readouts, we made use of the GRAS database of schizophrenic patients (31,32). Our hypotheses regarding Baiap3/BAIAP3 function were based on the anxiety phenotype observed in *Baiap3* KO mice and on the prominent expression of Baiap3 in brain regions involved in processing fearful stimuli as well as in substance use disorders. We selected three single-nucleotide polymorphisms (SNPs) in the *BAIAP3* gene: rs11648169 (C/G, intronic), rs2235632 (G/A, intronic) and rs1132358 (C/T, coding sequence, synonymous Asp1040Asp) (Supplementary Figure S4A) from public databases [http://www.ncbi.nlm.nih.gov/projects/SNP/; http://browser.1000genomes.org; http://hapmap.ncbi.nlm.nih.gov/]. The selection of SNPs was based on (i) a high minor allele frequency (MAF ≥ 0.36) distribution within the European Caucasian population (http://www.ncbi.nlm.nih.gov/SNP/), to increase the power to detect genetic effects, and (ii) the potential for functional consequences. The last criterion could only partially be fulfilled; the exonic SNP rs1132358 (C/T, Asp1040Asp, synonymous) might potentially affect mRNA structure or stability. All SNPs fulfilled Hardy-Weinberg equilibrium criteria, both in cases and in controls ($p > 0.05$). A construction of haplotype blocks of the three SNPs revealed a similarly

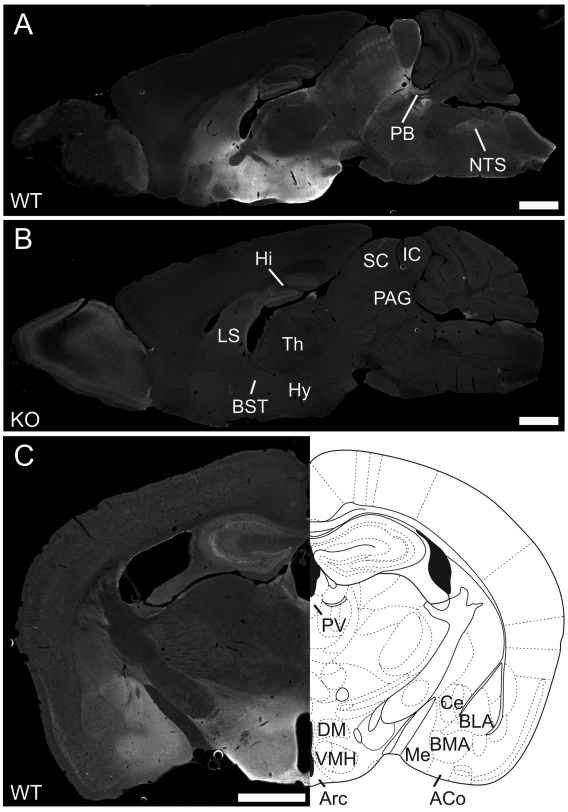

Figure 1. Immunofluorescence analysis of Baiap3 expression in mouse brain. (A) Sagittal brain section of adult *Baiap3* WT mouse stained with rabbit anti-Baiap3 antibody. (B) Sagittal brain section of adult *Baiap3* KO littermate showing the absence of Baiap3 immunoreactivity. Please note that the signal observed in the hippocampus of both WT and KO brain is a nonspecific background staining. (C) Coronal brain section of adult WT mouse stained for Baiap3 with a corresponding coronal diagram, adapted from the mouse Paxinos brain atlas (Bregma –1.46). PB, parabrachial nucleus; NTS, nucleus tractus solitarius; Hi, hippocampus; SC, superior colliculus; IC, inferior colliculus; PAG, periaqueductal gray; LS, lateral septum; Th, thalamus; Hy, hypothalamus; BST, bed nucleus of the stria terminalis; PV, paraventricular thalamic nucleus; DM, dorsomedial hypothalamic nucleus; VMH, ventromedial hypothalamic nucleus; Arc, arcuate nucleus; Ce, central amygdaloid nucleus; BLA, basolateral amygdaloid nucleus, anterior part; BMA, basomedial amygdaloid nucleus, anterior part; ME, medial amygdaloid nucleus; ACo, anterior cortical amygdaloid nucleus. Scale bars equal 1 mm.

high degree of linkage disequilibrium between them in the GRAS sample (Supplementary Figure S4B) and in healthy controls (Supplementary Figure S4C). Case control analysis of genotype frequencies of the three SNPs did not reveal any significant differences, indicating that the selected genetic variation in *BAIAP3* is not associated with schizophrenia risk (Supplementary Figure S4D). We subsequently used the PGAS approach (32) to analyze the three SNPs for association with specific phenotypic readouts relevant for anxiety disorders and substance use disorders. For this step, an anxiety composite score was constructed using four anxiety-relevant variables (Supplementary Figure S2), which showed a significant association with only two of the three selected SNPs (as expected because of the high linkage disequilibrium between both markers and their similar MAFs) for women but not for men (Table 1). SNP rs11648169 was excluded from further analyses, since it yielded no statistically significant effects.

BAIAP3 Is a Risk Gene for Benzodiazepine Abuse in Men

Because anxiety disorders and substance use disorders often occur together, and *BAIAP3* is expressed in brain regions relevant for emotionality and drug dependence, we also screened for a possible association between genetic variation in *BAIAP3* and substance use disorder. The same risk genotypes (AA for rs2235632, TT for rs1132358) that were associated with anxiety in women showed a statistically significant association with benzodiazepine use disorder in men (Table 1). Even though there was a similar tendency for women (benzodiazepine use disorder associated with 7.0%/7.7% in AA/TT genotypes versus 4.7%/4.6% in G/C carrier status), it did not reach statistical significance, perhaps because of the lower numbers of women than men in the GRAS sample. The genotype frequencies of rs2235632 and rs1132358 did not differ between men and women in the GRAS sample (rs2235632, GG/AG/AA: men 25.1%/49.1%/25.8%; women 28.3%/46.3%/25.5%; rs1132358, CC/TC/TT: men 26.1%/49.4%/24.5%; women 29.8%/46.9%/23.3%), and neither of these two SNPs was associated with disease-related or sociodemographic control variables (Table 1).

For the purpose of an association analysis of the relevant *BAIAP3* genotypes (GG/AG/AA in rs2235632 and CC/TC/TT in rs1132358) with benzodiazepine use disorder, the GRAS sample delivers an ideal, nearly experimental setting. The distribution of these genotypes among benzodiazepine users versus nonusers is highly comparable, allowing the identification of risk genotypes leading to benzodiazepine use disorder (Supplementary Table S1). Most importantly, the benzodiazepine dose was equal across all genotypes (Supplementary Table S1). Hence, the *BAIAP3* risk genotypes (AA for rs2235632, TT for rs1132358) appear to confer a specific genetic risk of developing benzodiazepine use disorder given equal dose and likelihood of exposure. Interestingly, neither alcohol nor cannabis abuse were found to

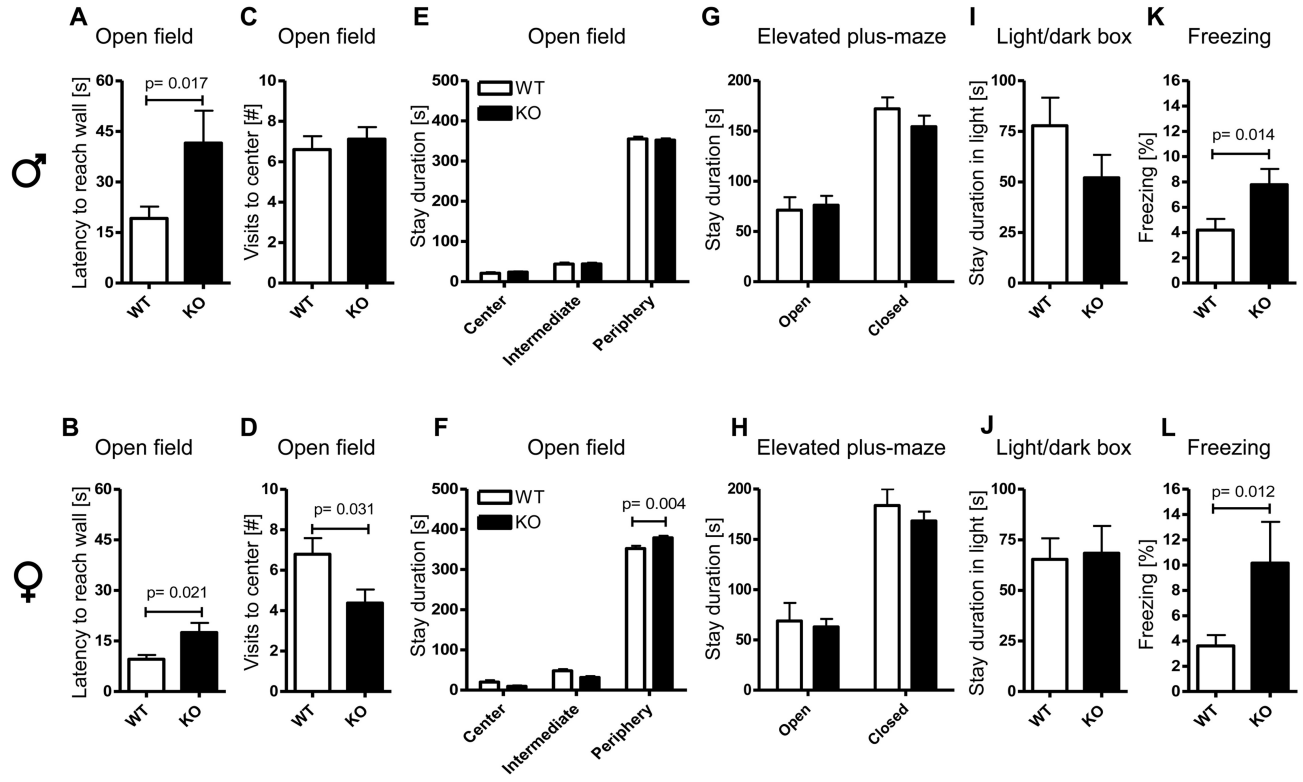

Figure 2. Anxiety phenotype in *Baiap3* KO mice. (A–F) Open field parameters. (A, B) The latency to reach the wall of the open field was significantly increased in *Baiap3* KO mice of both sexes, whereas visits to the center (C, D) and stay in the periphery (E, F) revealed anxiety-like behavior only in females. Elevated plus-maze (G, H) and light–dark box (I, J) revealed no genotype-dependent differences in either sex. (K, L) As readout of unspecific novelty-related anxiety, a higher freezing response was found in male as well as female *Baiap3* KO mice. Numbers tested: males, WT = 16–25, KO = 16–25; females, WT = 18–23, KO = 10–28. Mann-Whitney *U* test (A–D, I–L) and two-way ANOVA (E–H), including Bonferroni testing, were applied. Means ± SEM are presented.

be associated with the two SNPs, pointing to a specific benzodiazepine link with the selected *BAIAP3* genotypes (Table 1).

To determine whether the identified risk genotypes are associated with altered expression of *BAIAP3*, we analyzed the mRNA levels of *BAIAP3* in PBMCs obtained from 121 subjects by qRT-PCR. We found a statistically significant association of the *BAIAP3* risk genotypes (AA for rs2235632, TT for rs1132358) with lower *BAIAP3* mRNA levels in PBMCs of male individuals, which is at least partially comparable to a gene dose reduction or KO situation. This result is not found in women, possibly because of the lower numbers available for analysis (Supplementary Figure S5). However, these findings could also support the interpretation that the effects of *BAIAP3* risk alleles are gender specific.

Male *Baiap3* KO Mice Show Faster Development of Tolerance to Benzodiazepines

On the basis of the identification of human *BAIAP3* risk genotypes for benzodiazepine abuse in male patients, we tested *Baiap3* KO and WT littermates of both sexes in experimental paradigms of chronic benzodiazepine administration to assess the development of tolerance, dependence and withdrawal (Figure 3A). The baseline performance of each mouse in the rotarod test was established on two consecutive days of rotarod training. No significant genotype-dependent differences were detected in baseline performance (Supplementary Figures S3G, H). Benzodiazepine dependence in *Baiap3* KO and WT mice of both sexes was then induced with daily diazepam injections (5 mg/kg IP) for 10 consecutive days. To monitor the development of tolerance to diazepam, motor performance on rotarod at 30 min after each injection was evaluated over the first 7 d of diazepam treatment. Rapid development of tolerance to daily diazepam injections was apparent in both sexes and genotypes by an increase of the latency of falling from rotarod over the course of 7 d (Figures 3B, C). Here, male *Baiap3* KO mice performed significantly better than their WT littermates (Figure 3B), whereas no such difference was detected for females (Figure 3C). Thus, male *Baiap3* KO mice show faster development of tolerance to diazepam.

Baiap3 KO Mice Have an Increased Seizure Propensity

To evaluate whether *Baiap3* genotype would affect the propensity for di-

Table 1. Phenotype comparison of GRAS patients sorted by *BAIAP3* genotypes.

GRAS sample	*BAIAP3* rs2235632			*BAIAP3* rs1132358		
	G carriers (GG/AG)	AA	*p* (*F*/*T*/*Z*/χ^2 value)[a]	C carriers (CC/TC)	TT	*p* (*F*/*T*/*Z*/χ^2 value)[a]
Males	n = 471–538[b]	n = 155–187[b]		n = 474–538[b]	n = 152–175[b]	
Target variables						
Benzo use disorder (n (%))	11 (2.3)	9 (5.4)	**0.047** (χ^2 = 3.93)	11 (2.3)	9 (5.7)	**0.034** (χ^2 = 4.47)
Anxiety composite score (mean ± SD)[c,d]	−0.06 ± 0.74	−0.07 ± 0.70	0.499 (*F* = 0.46)	−0.07 ± 0.73	−0.05 ± 0.72	0.651 (*F* = 0.21)
Sociodemographic						
Age (at examination) (years) (mean ± SD (range))	37.33 ± 12.01 (18–78)	36.17 ± 11.91 (17–75)	0.225 (*Z* = −1.21)	37.28 ± 11.96 (18–78)	36.34 ± 12.06 (17–75)	0.309 (*Z* = −1.02)
Education (years) (mean ± SD (range))[e]	14.17 ± 3.48 (8–28)	14.42 ± 3.70 (8–27)	0.680 (*Z* = −0.41)	14.21 ± 3.48 (8–28)	14.32 ± 3.76 (8–27)	0.853 (*Z* = −0.19)
Unemployment (n (%))	217 (44.7)	76 (44.7)	0.990 (χ^2 = 0.00)	214 (44.2)	75 (46.6)	0.601 (χ^2 = 0.27)
Substance use						
Alcohol use disorder according to DSM-IV-TR (n (%))	221 (42.3)	84 (45.7)	0.435 (χ^2 = 0.61)	217 (41.6)	82 (47.4)	0.180 (χ^2 = 1.80)
Cannabis use disorder according to DSM-IV-TR (n (%))	218 (41.8)	83 (45.1)	0.430 (χ^2 = 0.62)	224 (42.9)	75 (43.4)	0.919 (χ^2 = 0.01)
Clinical						
PANSS positive score (mean ± SD (range))	13.55 ± 6.04 (7–36)	14.02 ± 6.41 (7–38)	0.427 (*Z* = −0.79)	13.53 ± 6.03 (7–36)	14.23 ± 6.50 (7–38)	0.249 (*Z* = −1.15)
Chlorpromazine equivalents (mean ± SD (range))	707.90 ± 694.69 (0–6,324.29)	689.45 ± 568.91 (0–3,238.00)	0.678 (*Z* = −0.42)	701.33 ± 688.16 (0–6,324.29)	712.11 ± 592.75 (0–3,238.00)	0.424 (*Z* = −0.80)
GAF score (mean ± SD (range))	45.70 ± 16.04 (10–90)	45.35 ± 16.88 (5–90)	0.862 (*Z* = −0.17)	45.75 ± 16.05 (10–90)	44.90 ± 16.89 (5–90)	0.619 (*Z* = −0.50)
Females	n = 223–269[b]	n = 75–92 [b]		n = 229–273[b]	n = 71–83[b]	
Target variables						
Benzo use disorder (n (%))	12 (4.7)	6 (7.0)	0.406 (χ^2 = 0.69)	12 (4.6)	6 (7.7)	0.281 (χ^2 = 1.16)
Anxiety composite score (mean ± SD)[c,d]	−0.02 ± 0.77	0.19 ± 0.80	**0.028** (*F* = 4.91)	−0.02 ± 0.78	0.21 ± 0.77	**0.017** (*F* = 5.81)
Sociodemographic						
Age (at examination) (years) (mean ± SD (range))	42.36 ± 12.92 (18–79)	44.86 ± 12.52 (21–76)	0.893 (*T* = 1.61)	42.52 ± 12.90 (18–79)	44.49 ± 12.60 (20–76)	0.221 (*T* = 1.23)
Education (years) (mean ± SD (range))[e]	14.56 ± 3.95 (7–31)	14.12 ± 3.54 (8–27)	0.447 (*Z* = −0.76)	14.52 ± 3.94 (7–31)	14.13 ± 3.62 (8–27)	0.454 (*Z* = −0.75)
Unemployment (n (%))	76 (31.9)	20 (25.0)	0.243 (χ^2 = 1.37)	74 (30.5)	20 (28.2)	0.712 (χ^2 = 0.14)
Substance use						
Alcohol use disorder according to DSM-IV-TR (n (%))	59 (22.4)	14 (15.7)	0.178 (χ^2 = 1.82)	60 (22.4)	13 (16.0)	0.219 (χ^2 = 1.51)
Cannabis use disorder according to DSM-IV-TR (n (%))	30 (11.4)	8 (9.0)	0.525 (χ^2 = 0.40)	29 (10.8)	9 (11.1)	0.941 (χ^2 = 0.01)
Clinical						
PANSS positive score (mean ± SD (range))	13.84 ± 6.66 (7–37)	14.41 ± 6.38 (7–33)	0.288 (*Z* = −1.06)	13.87 ± 6.69 (7–37)	14.32 ± 6.24 (7–32)	0.366 (*Z* = −0.91)
Chlorpromazine equivalents (mean ± SD (range))	636.37 ± 776.51 (0–7,375.00)	704.59 ± 762.50 (0–4,370.00)	0.612 (*Z* = −0.51)	634.40 ± 771.10 (0–7,375.00)	718.83 ± 788.67 (0–4,370.00)	0.616 (*Z* = −0.50)
GAF score (mean ± SD (range))	46.34 ± 19.42 (8–90)	44.22 ± 17.59 (12–84)	0.435 (*Z* = −0.78)	46.22 ± 19.25 (8–90)	44.88 ± 18.07 (12–84)	0.645 (*Z* = −0.46)

SD, standard deviation.

[a]For statistical methods, Mann-Whitney *U* or χ^2 tests and for normally distributed variables *t* tests were used. Bolded values: $p < 0.05$.

[b]Because of missing data, sample sizes vary.

[c]Results after multiple imputations (10).

[d]Analysis of covariance with age, positive symptoms (PANSS) and medication status (chlorpromazine equivalent) as covariates.

[e]Total years spent in education system; patients currently in school or educational training were excluded.

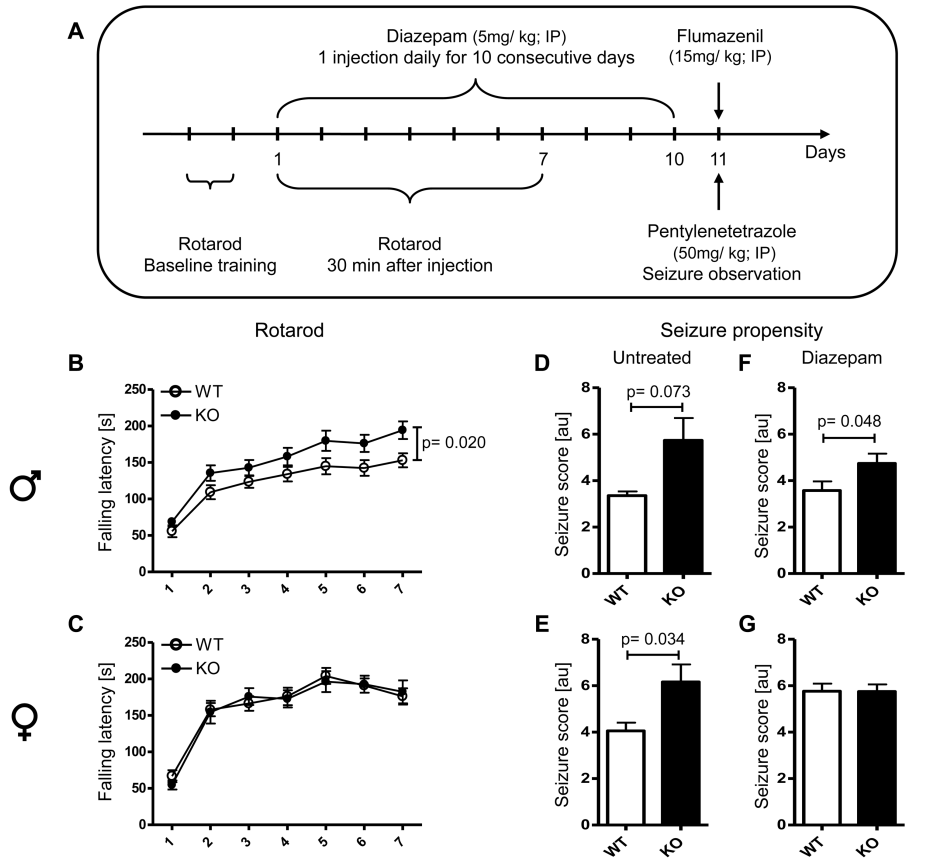

Figure 3. Diazepam tolerance and withdrawal in *Baiap3* KO and WT mice. (A) Experimental design scheme. (B) Male diazepam-treated *Baiap3* KO mice showed significantly faster improvement of performance on the rotarod, consistent with a more rapid development of tolerance to diazepam. (C) Rotarod performance of female mice was comparable between WT and KO. (D, E) Diazepam-naive *Baiap3* KO mice display a higher PTZ-induced seizure propensity compared with WT (significant in females, strong tendency in males). (F, G) Flumazenil-induced diazepam withdrawal does not further increase PTZ-induced seizure propensity in *Baiap3* KO mice. Seizure propensity of female mice became comparable between genotypes, pointing to a ceiling effect. Numbers tested: males, WT = 25, KO = 25; females, WT = 21, KO = 23, except for (D) and (E), where males, WT = 7, KO = 7; females, WT = 8; KO = 10. Mann-Whitney *U* test (D–G) and two-way repeated-measures ANOVA (B, C), including Bonferroni, testing applied. Means ± SEM are presented.

azepam withdrawal-related seizures, the susceptibility to PTZ-induced seizures was first evaluated in diazepam-naive mice. The seizure response of *Baiap3* KO mice of both sexes to PTZ (50 mg/kg IP) was higher than that in WT animals, with the difference just failing to reach significance in males (Figures 3D, E). To assess the effect of genotype on benzodiazepine withdrawal, the diazepam antagonist flumazenil (15 mg/kg IP) was injected on d 11, after 10 d of daily diazepam treatment, immediately followed by PTZ injection (50 mg/kg IP) to trigger withdrawal seizures (Figure 3A). Upon flumazenil-induced diazepam withdrawal, the response to PTZ in male *Baiap3* KO and WT mice did not differ appreciably from the one found in diazepam-naive mice of both genotypes (Figure 3F). In contrast, the genotype-dependent differences in diazepam-naive females regarding seizure scores disappeared under conditions of diazepam withdrawal (Figure 3G), which could be explained by a ceiling effect. Thus, female and male *Baiap3* KO mice are more seizure-prone than their WT littermates, and this propensity is not further increased by benzodiazepine withdrawal.

Drug Self-administration and Basic Behaviors Do Not Differ between *Baiap3* Genotypes upon Chronic Addiction

To assess whether *Baiap3* KO mice, once addicted, would also be more likely to orally self-administer benzodiazepines, we performed an experiment on chronic midazolam addiction, where self-application was assessed after forced long-term exposure to escalating doses of midazolam (Supplementary Figure S6). We detected no genotype or gender differences in the clear preference for midazolam. Moreover, no genotype effects on body weight or basic behavior in the chronically addicted state were noted (Supplementary Figure S6). These data indicate that the *Baiap3* genotype gender specifically affects the development of tolerance, that is, drug abuse at an early stage. In chronic addiction, genotype effects are no longer detectable.

Lack of Homeostatic Adaptation to Diazepam in *Baiap3* KO Hypothalamus Slices

One hypothesis regarding predisposition to the development of addiction at the cellular level is an altered response to the addiction-inducing substance and its withdrawal. Because *Baiap3* KO mice showed an increased seizure propensity and an altered development of tolerance to diazepam, we investigated whether lack of *Baiap3* leads to a measurably altered response to diazepam treatment and withdrawal in neurons *in vitro*. Because Baiap3 expression is highest in the hypothalamus, we cultured organotypic hypothalamus slices prepared from male P5/P6 *Baiap3* KO and WT animals in the presence of either 10 μmol/L diazepam or vehicle (DMSO) and recorded from neurons in the ventromedial hypothalamus in the presence of the diazepam antagonist flumazenil to

mimic diazepam withdrawal conditions *in vitro*. We hypothesized that diazepam treatment would lead to a homeostatic adaptation in the $GABA_AR$-mediated mIPSCs (Figure 4A) that would become apparent under diazepam withdrawal conditions. Although we observed no diazepam treatment–dependent differences that reached statistical significance, there was a significant genotype-dependent effect under diazepam withdrawal conditions. Here, WT mIPSC amplitudes were 27% smaller (Figure 4B) and rise times 13% longer than in KO neurons (Figure 4C), which is suggestive of a homeostatic adaptation to diazepam treatment in WT but not in KO slices. No significant differences in mIPSC decay times and frequencies were observed (Supplementary Table S2). Because the sudden withdrawal of diazepam should lead to an increase in overall network activity, we recorded action potential (AP) frequencies in ventromedial hypothalamus slices in the presence of flumazenil. Surprisingly, KO slices already showed significantly higher AP frequencies than WT slices under control conditions, with no further increase under diazepam withdrawal conditions. By contrast, in WT slices, we observed a significant increase in AP frequency under diazepam withdrawal conditions compared with vehicle-treated WT slices (Figure 4D). There was no significant difference in the resting membrane potentials (Figure 4E), AP rise times, decay times and half-widths (Supplementary Table S2). IPSCs were recorded in the same cells at a holding potential of –20 mV to be able to isolate spontaneous $GABA_AR$-mediated currents without drug application. In WT slices, we observed a significant effect of diazepam withdrawal, with an increase in IPSC amplitude and frequency compared with vehicle-treated WT slices (Figures 4F, G), which is in keeping with the overall higher firing rate and which was not apparent in KO slices. In summary, these data show that neurons in *Baiap3* KO hypothalamus slices have higher AP firing rates, likely consistent with the higher seizure propensity found *in vivo*, and that *Baiap3* KO slices show no obvious homeostatic adaptation to diazepam treatment and withdrawal.

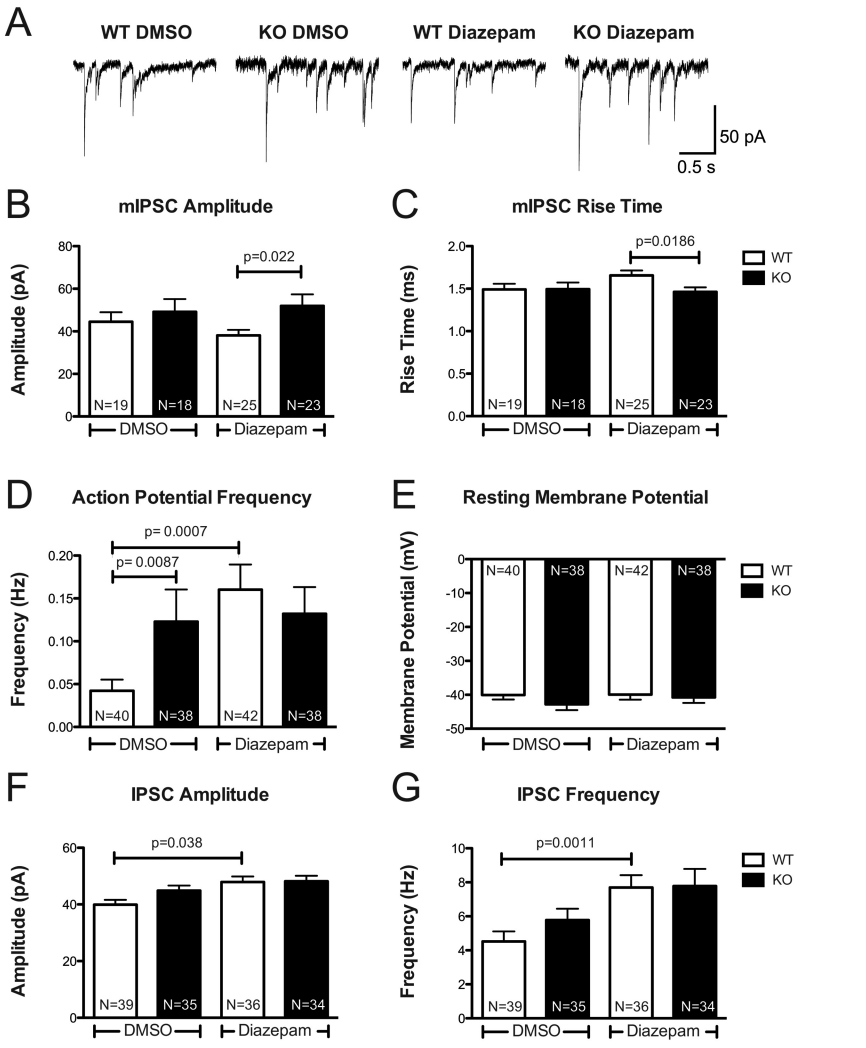

Figure 4. Increased basal network activity and lack of homeostatic adaptation to diazepam treatment in *Baiap3* KO hypothalamus slices. (A) Sample traces of mIPSC recordings from WT and KO hypothalamus slices that were cultured in the presence of diazepam or under vehicle control conditions with DMSO. Under diazepam withdrawal conditions, *Baiap3* WT mIPSC amplitudes were significantly smaller than in KO slices (B), and WT mIPSC rise times were longer than in KO slices (C). (D) *Baiap3* WT slices showed an increase in AP frequency in response to diazepam withdrawal when compared with DMSO-treated WT slices, whereas no such increase was apparent for *Baiap3* KO slices, which already showed an increased AP frequency under DMSO control conditions when compared with WT slices. (E) The resting membrane potential was not affected by experimental condition or *Baiap3* genotype. IPSC amplitudes (F) and IPSC frequencies (G) were increased in *Baiap3* WT slices under diazepam withdrawal compared with DMSO-treated WT slices. Mann-Whitney *U* test was used for AP and IPSC frequencies; Student *t* test was used for all other parameters. Means ± SEM are presented.

DISCUSSION

In this study, we identify two human *BAIAP3* risk genotypes that are associated with anxiety in women and benzodiazepine use disorder in men. We fur-

ther show that *Baiap3* deficiency in mice leads to (i) elevated seizure propensity; (ii) increased anxiety in both genders, with a more pronounced effect in females; and (iii) a faster development of tolerance to benzodiazepines in male mice. *In vitro* analysis of hypothalamic slices revealed an increase in neuronal baseline activity in the absence of *Baiap3*. Withdrawal from chronic benzodiazepine application *in vitro* results in a genotype-specific response pattern.

To the best of our knowledge, no other genetic risk marker that is associated with anxiety and benzodiazepine abuse has been reported to date. We are aware that, pending replication in nonschizophrenic individuals, we cannot be sure that our findings can be applied to the general population. In spite of this limitation, our findings suggest a role for *BAIAP3* and potential interaction partners in the development of anxiety and drug dependence.

Unfortunately, similar data from samples of equally well-phenotyped healthy individuals or even other disease groups are not available. This is particularly true with regard to benzodiazepine abuse, since short-term exposure is a primary goal of controlled and medically surveyed indications. Even looking at other rare situations of long-term exposure (for example, intractable epilepsies), a homogeneous sample comparable to the GRAS collection would be difficult to imagine. In the GRAS sample, there are no differences between *BAIAP3* genotypes regarding benzodiazepine exposure or daily dose (in case of exposure). This constellation allowed us to analyze the specific genetic risk of developing benzodiazepine use disorder in a setting close to an experimental condition.

Importantly, the present study was purely hypothesis-driven. Our hypotheses for performing a human phenotype-based genetic association study of *BAIAP3* were based on the anxiety phenotype we observed during basic behavioral characterization of *Baiap3* KO mice as well as on the distinctive Baiap3 expression pattern in brain, which includes regions relevant for addictive behaviors. We find that in humans, female carriers of the homozygous *BAIAP3* risk genotypes (AA for SNP rs2235632; TT for SNP rs1132358) are more likely to meet criteria for an anxiety disorder, whereas male carriers of the same risk genotypes are more likely to fulfill criteria for benzodiazepine use disorder. Neither SNP was associated with schizophrenia in our case control analysis. Furthermore, no associations with substance use disorder other than benzodiazepine use disorder were observed. In general, both genetic linkage and candidate gene studies suffer from lack of replicability (12). However, in our study, the parallel identification of a gender-specific association of *BAIAP3/Baiap3* with anxiety and an altered response to benzodiazepines in both mice and men, lends strong support to a causal link between *BAIAP3* and the observed phenotypes.

As for other genetic variations associated with anxiety disorders (11,12) or substance use disorders (13–15), the impact of *BAIAP3* genotypes on anxiety disorders or benzodiazepine use disorder is likely to be small. However, the observed effects and their gender specificity (across two species) are intriguing. While we currently have no mechanistic insight into this gender specificity, part of the explanation may lie in the fact that Baiap3 is expressed in sexually dimorphic brain regions such as the hypothalamus, amygdala and the bed nucleus of the stria terminalis (38,39).

Given the higher prevalence of both anxiety disorders and benzodiazepine use disorder in women (9,40) the present findings were surprising at first glance, but the similarity of gender differences in mice and humans underlines their significance, encouraging follow-up work on this gender effect. Admittedly, the gender effects in humans may ultimately turn out to be less prominent, since the total number of individuals with benzodiazepine abuse in the GRAS sample is low, resulting in moderate significance levels only for men. It cannot be excluded that, in a larger sample, an association of benzodiazepine use disorder with the genotypes investigated here might reach significance for women as well. Furthermore, research focusing on gender differences and social desirability in self-reported anxiety suggests an underreporting of fear and distress in men (41–43). Therefore, our use of three self-reported measures in the calculation of the anxiety composite score might partly explain the lack of association of the *BAIAP3* risk genotypes with anxiety in men. Nevertheless, gender differences in *BAIAP3/Baiap3* genotype-phenotype associations most likely exist and are worth pursuing.

Benzodiazepines are positive allosteric modulators of $GABA_AR$ and thus enhancers of inhibitory GABAergic neurotransmission. Their sedative, anti-convulsive and amnesic effects are largely mediated by the $GABA_AR\alpha1$ subunit, the anxiolytic effect by the $\alpha2$ subunit and muscle relaxation by $\alpha2$, $\alpha3$ and $\alpha5$ subunits (44). To date, no specific risk association of these obvious candidate genes has been identified. At present we have no evidence that would suggest that Baiap3 interacts with $GABA_AR$ subunits. However, the increased seizure propensity observed in *Baiap3* KO mice of both sexes, which is already apparent without prior diazepam treatment and withdrawal, is indicative of an altered balance of excitatory and inhibitory systems. Our comparison of neuronal firing rates in hypothalamus slices under baseline and diazepam withdrawal conditions uncovered an increase in basal network activity in the absence of Baiap3. This finding was unexpected, and although presently limited to the hypothalamus, is consistent with the increased seizure propensity observed *in vivo*. Even though we do not know whether the seizures observed in our PTZ-induction model originate in the subcortical regions that express Baiap3, subcortical epileptogenesis with origins in the hypothalamus is a feature seen in hypothalamic hamartomas (45), and the amygdala, which also expresses Baiap3, is known to play a key role in epileptogenesis (46).

Interestingly, the human *BAIAP3* gene is located on chromosome 16p13.3, which has been linked to electroencephalographic traits of idiopathic epilepsy syndromes (47,48). We would thus argue that further investigation of *BAIAP3* as a candidate gene for epilepsy-related phenotypes is warranted. Because we found that Baiap3 did not colocalize with markers of GABAergic or glutamatergic pre- and postsynapses to a significant degree (Supplementary Figures S1C–G), the increased seizure propensity in *Baiap3* KO mice of both sexes and the altered response to benzodiazepines in males is unlikely to be due to a direct effect of Baiap3 at GABAergic or glutamatergic synapses.

The neuronal circuitry underlying the addictive properties of benzodiazepines is less well understood than their molecular mechanism of action. Unlike many other addictive substances, benzodiazepines do not appear to increase dopamine levels in the nucleus accumbens (49–51), although electrophysiological studies suggest that benzodiazepines increase firing of dopaminergic neurons in the VTA through disinhibition of these neurons via inhibition of nearby inhibitory interneurons (52,53). Additional mechanisms, such as neuroendocrine responses to benzodiazepine treatment, may play a critical role in the development of benzodiazepine use disorder (54). Furthermore, because expression of *Baiap3* in both the VTA and in the nucleus accumbens is low (Allen Brain Atlas), a direct effect of Baiap3 on the mesolimbic dopamine pathway does not appear be the most likely explanation for the observed interaction between *Baiap3* genotypes and the response to benzodiazepines. Instead, our findings support the interpretation that the altered response to benzodiazepines could be a consequence of a local or global change in neuronal excitability. Because all other members of the Munc13 protein family have been shown to be regulators of SNARE-mediated exocytosis (25,29), Baiap3 may regulate the release of one or more modulatory neurotransmitters or neuropeptides that influence the balance between GABAergic and glutamatergic neurotransmission. Baiap3 immuoreactivity appears punctate (Supplementary Figures S1C–G) and may localize to peptidergic release sites, some of which may also contain VGLUT2 or Viaat. Although we presently cannot exclude the possibility that Baiap3 might have a postsynaptic function, given what is know about the function of all other members of the Munc13 protein family, we think that a pre-synaptic function is more likely. We can furthermore not exclude the possibility that alterations in the hypothalamic-pituitary-adrenal axis may play a role in the anxiety phenotype or the altered response to benzodiazepines seen in *Baiap3* KO mice. We are currently investigating whether Baiap3 is involved in regulating exocytosis of dense core vesicles and/or intracellular trafficking events that could influence neuropeptide release or extrasynaptic $GABA_A$Rs.

CONCLUSION

To conclude, *BAIAP3* had not previously been considered a candidate gene for either psychiatric disorders or epilepsy. Our study links *BAIAP3/Baiap3* genotypes to anxiety and an altered response to benzodiazepines in both mice and men and thus strongly argues for an involvement of *BAIAP3* in these neuropsychiatrically relevant phenotypes. The identification of human genetic variations that influence the risk for the development of pathological phenotypes as well as the response to pharmacological treatments may pave the way for more efficient treatments with fewer side effects. Rodent models are usually only imperfect representations of human psychiatric conditions; however, the simultaneous identification of *Baiap3* as a biomarker for anxiety and the response to benzodiazepines in mouse and humans suggests that *Baiap3* KO mice will be a valuable tool in further elucidating the genetic, physiological and neuroanatomical underpinnings of anxiety disorders and benzodiazepine use disorder.

ACKNOWLEDGMENTS

We are indebted to all patients for their participation in the GRAS study and all collaborating GRAS centers for their support. We are grateful to all colleagues who contributed to the GRAS data collection. We would also like to thank Astrid Zeuch, Astrid Ohle and the members of the DNA sequencing core facility for excellent technical assistance. This work was supported by the Max Planck Society, the Max-Planck-Förderstiftung, and the DFG Center for Nanoscale Microscopy and Molecular Physiology of the Brain.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES

1. Kessler RC, *et al.* (2005) Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. *Arch. Gen. Psychiatry.* 62:593–602.
2. Pasche S. (2012) Exploring the comorbidity of anxiety and substance use disorders. *Curr. Psychiatry Rep.* 14:176–81.
3. Swendsen J, *et al.* (2010) Mental disorders as risk factors for substance use, abuse and dependence: results from the 10-year follow-up of the National Comorbidity Survey. *Addiction.* 105:1117–28.
4. Conway KP, Compton W, Stinson FS, Grant BF. (2006) Lifetime comorbidity of DSM-IV mood and anxiety disorders and specific drug use disorders: results from the National Epidemiologic Survey on Alcohol and Related Conditions. *J. Clin. Psychiatry.* 67:247–57.
5. Myrick H, Brady K. (2003) Current review of the comorbidity of affective, anxiety, and substance use disorders. *Curr. Opin. Psychiatry.* 16:261–70.
6. Bandelow B, *et al.* (2012) Guidelines for the pharmacological treatment of anxiety disorders, obsessive-compulsive disorder and posttraumatic stress disorder in primary care. *Int. J. Psychiatry Clin. Pract.* 16:77–84.
7. Baker AL, Thornton LK, Hiles S, Hides L, Lubman DI. (2012) Psychological interventions for alcohol misuse among people with co-occurring depression or anxiety disorders: a systematic review. *J. Affect. Disord.* 139:217–29.
8. Hettema JM, Neale MC, Kendler KS. (2001) A review and meta-analysis of the genetic epidemiology of anxiety disorders. *Am. J. Psychiatry.* 158:1568–78.
9. Hettema JM, Prescott CA, Myers JM, Neale MC,

Kendler KS. (2005) The structure of genetic and environmental risk factors for anxiety disorders in men and women. *Arch. Gen. Psychiatry.* 62:182–9.
10. Ducci F, Goldman D. (2012) The genetic basis of addictive disorders. *Psychiatr. Clin. North Am.* 35:495–519.
11. Hovatta I, Barlow C. (2008) Molecular genetics of anxiety in mice and men. *Ann. Med.* 40:92–109.
12. Hamilton SP. (2009) Linkage and association studies of anxiety disorders. *Depress. Anxiety.* 26:976–83.
13. Wang JC, Kapoor M, Goate AM. (2012) The genetics of substance dependence. *Annu. Rev. Genomics Hum. Genet.* 13:241–61.
14. Gelernter J, Kranzler HR. (2010) Genetics of drug dependence. *Dialogues Clin. Neurosci.* 12:77–84.
15. Buckland PR. (2008) Will we ever find the genes for addiction? *Addiction.* 103:1768–76.
16. Marmorstein NR. (2012) Anxiety disorders and substance use disorders: different associations by anxiety disorder. *J. Anxiety Disord.* 26:88–94.
17. DeMartini KS, Carey KB. (2011) The role of anxiety sensitivity and drinking motives in predicting alcohol use: a critical review. *Clin. Psychol. Rev.* 31:169–77.
18. Zavos HM, Gregory AM, Eley TC. (2012) Longitudinal genetic analysis of anxiety sensitivity. *Dev. Psychol.* 48:204–12.
19. Kushner MG, Thuras P, Abrams K, Brekke M, Stritar L. (2001) Anxiety mediates the association between anxiety sensitivity and coping-related drinking motives in alcoholism treatment patients. *Addict. Behav.* 26:869–85.
20. Baldwin DS, Allgulander C, Bandelow B, Ferre F, Pallanti S. (2012) An international survey of reported prescribing practice in the treatment of patients with generalised anxiety disorder. *World J. Biol. Psychiatry.* 13:510–6.
21. Canteras NS, Resstel LB, Bertoglio LJ, Carobrez Ade P, Guimaraes FS. (2010) Neuroanatomy of anxiety. *Curr. Top. Behav. Neurosci.* 2:77–96.
22. Gratacos M, *et al.* (2007) Candidate genes for panic disorder: insight from human and mouse genetic studies. *Genes Brain Behav.* 6 (Suppl. 1):2–23.
23. Koch H, Hofmann K, Brose N. (2000) Definition of Munc13-homology-domains and characterization of a novel ubiquitously expressed Munc13 isoform. *Biochem. J.* 349:247–53.
24. Shiratsuchi T, *et al.* (1998) Cloning and characterization of BAP3 (BAI-associated protein 3), a C2 domain-containing protein that interacts with BAI1. *Biochem. Biophys. Res. Commun.* 251:158–65.
25. Varoqueaux F, *et al.* (2002) Total arrest of spontaneous and evoked synaptic transmission but normal synaptogenesis in the absence of Munc13-mediated vesicle priming. *Proc. Natl. Acad. Sci. U. S. A.* 99:9037–42.
26. Gorman JM, Kent JM, Sullivan GM, Coplan JD. (2000) Neuroanatomical hypothesis of panic disorder, revised. *Am. J. Psychiatry.* 157:493–505.
27. Gross CT, Canteras NS. (2012) The many paths to fear. *Nat. Rev. Neurosci.* 13:651–8.
28. Wojcik SM, Brose N. (2007) Regulation of membrane fusion in synaptic excitation-secretion coupling: speed and accuracy matter. *Neuron.* 55:11–24.
29. Feldmann J, *et al.* (2003) Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). *Cell.* 115:461–73.
30. Shirakawa R, *et al.* (2004) Munc13-4 is a GTP-Rab27-binding protein regulating dense core granule secretion in platelets. *J. Biol. Chem.* 279:10730–7.
31. Begemann M, *et al.* (2010) Modification of cognitive performance in schizophrenia by complexin 2 gene polymorphisms. *Arch. Gen. Psychiatry.* 67:879–88.
32. Ribbe K, *et al.* (2010) The cross-sectional GRAS sample: a comprehensive phenotypical data collection of schizophrenic patients. *BMC Psychiatry.* 10:91.
33. Ferraro TN, *et al.* (1999) Mapping loci for pentylenetetrazol-induced seizure susceptibility in mice. *J. Neurosci.* 19:6733–9.
34. American Psychiatric Association (APA). (2000) *Diagnostic and Statistical Manual of Mental Disorders: DSM-IV-TR.* 4th ed., text revision. Washington (DC): APA. 992 pp.
35. Kay SR, Fiszbein A, Opler LA. (1987) The positive and negative syndrome scale (PANSS) for schizophrenia. *Schizophr. Bull.* 13:261–76.
36. Cronbach LJ. (1951) Coefficient alpha and the internal structure of tests. *Psychometrika.* 16:297–334.
37. Rubin DB. (1987) *Multiple Imputation for Non-Response in Surveys.* New York: John Wiley & Sons.
38. Stefanova N, Ovtscharoff W. (2000) Sexual dimorphism of the bed nucleus of the stria terminalis and the amygdala. *Adv. Anat. Embryol. Cell Biol.* 158:III–X, 1–78.
39. Qureshi IA, Mehler MF. (2010) Genetic and epigenetic underpinnings of sex differences in the brain and in neurological and psychiatric disease susceptibility. *Prog. Brain Res.* 186:77–95.
40. Neutel CI. (2005) The epidemiology of long-term benzodiazepine use. *Int. Rev. Psychiatry.* 17:189–97.
41. Pierce KA, Kirkpatrick DR. (1992) Do men lie on fear surveys? *Behav. Res. Ther.* 30:415–8.
42. McLean CP, Anderson ER. (2009) Brave men and timid women? A review of the gender differences in fear and anxiety. *Clin. Psychol. Rev.* 29:496–505.
43. Stoyanova M, Hope DA. (2012) Gender, gender roles, and anxiety: perceived confirmability of self report, behavioral avoidance, and physiological reactivity. *J. Anxiety Disord.* 26:206–14.
44. Tan KR, Rudolph U, Luscher C. (2011) Hooked on benzodiazepines: GABAA receptor subtypes and addiction. *Trends Neurosci.* 34:188–97.
45. Fenoglio KA, *et al.* (2007) Hypothalamic hamartoma: basic mechanisms of intrinsic epileptogenesis. *Semin. Pediatr. Neurol.* 14:51–9.
46. Aroniadou-Anderjaska V, Fritsch B, Qashu F, Braga MF. (2008) Pathology and pathophysiology of the amygdala in epileptogenesis and epilepsy. *Epilepsy Res.* 78:102–16.
47. Pinto D, *et al.* (2005) Genome-wide linkage scan of epilepsy-related photoparoxysmal electroencephalographic response: evidence for linkage on chromosomes 7q32 and 16p13. *Hum. Mol. Genet.* 14:171–8.
48. de Kovel CG, *et al.* (2010) Whole-genome linkage scan for epilepsy-related photosensitivity: a mega-analysis. *Epilepsy Res.* 89:286–94.
49. Zetterstrom T, Fillenz M. (1990) Local administration of flurazepam has different effects on dopamine release in striatum and nucleus accumbens: a microdialysis study. *Neuropharmacology.* 29:129–34.
50. Invernizzi R, Pozzi L, Samanin R. (1991) Release of dopamine is reduced by diazepam more in the nucleus accumbens than in the caudate nucleus of conscious rats. *Neuropharmacology.* 30:575–8.
51. Finlay JM, Damsma G, Fibiger HC. (1992) Benzodiazepine-induced decreases in extracellular concentrations of dopamine in the nucleus accumbens after acute and repeated administration. *Psychopharmacology (Berl.).* 106:202–8.
52. Tan KR, *et al.* (2010) Neural bases for addictive properties of benzodiazepines. *Nature.* 463:769–74.
53. O'Brien DP, White FJ. (1987) Inhibition of non-dopamine cells in the ventral tegmental area by benzodiazepines: relationship to A10 dopamine cell activity. *Eur. J. Pharmacol.* 142:343–54.
54. Heberlein A, Bleich S, Kornhuber J, Hillemacher T. (2008) Neuroendocrine pathways in benzodiazepine dependence: new targets for research and therapy. *Hum. Psychopharmacol.* 23:171–81.