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

Running Head: *BAIAP3* is Associated with Anxiety and Benzodiazepine Abuse

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Abstract

Anxiety disorders and substance abuse, including benzodiazepine use disorder (BUD), frequently occur together. Treatment of anxiety disorders unfortunately still includes benzodiazepines, and patients with an existing co-morbid BUD or a genetic susceptibility for BUD may be put at risk of adverse treatment outcomes. The identification of genetic predictors for anxiety disorders, and especially for BUD, 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, 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. Using a phenotype-based genetic association study, we identify 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 find that male, but not female *Baiap3* KO mice develop tolerance to diazepam more quickly than control animals. Analysis of cultured *Baiap3* KO hypothalamus slices reveals an increase in basal network activity and an altered response to diazepam withdrawal. Thus, *Baiap3/BAIAP3* is gender-specifically associated with anxiety and BUD, and the analysis of *Baiap3/BAIAP3* related functions may help elucidate mechanisms underlying the development of both disorders.

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INTRODUCTION

Anxiety disorders have high lifetime prevalence rates (1) and exhibit a remarkable comorbidity with substance use disorders (SUD) (2-4). This association worsens treatment outcomes for both conditions (5), and represents a significant burden on individuals and society. Both anxiety disorders and SUD 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 would be 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 SUD (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 SUD (13-15). Although the interaction between anxiety disorders and SUD is likely bi-directional 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 employed 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). 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, that are involved in regulating autonomic functions, and that 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, using the previously described Göttingen Research Association for Schizophrenia (GRAS) database (31, 32). Employing this 2-pronged approach, we identify *Baiap3* as the first genetic risk marker for anxiety and benzodiazepine abuse in both mice and humans.

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MATERIALS AND METHODS.

Animals

All experiments were approved by the local Animal Care and Use Committee. The first 3 coding exons of the murine *Baiap3* gene were preplaced with a neomycin resistance cassette through homologous recombination in embryonic stem cells (129/ola) (Supplementary FigureS1A). *Baiap3* mutant mice of mixed 129/ola;C57Bl6/N background were backcrossed for 7 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 12h 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, Germany) was suspended in saline containing polysorbate80 for intraperitoneal (i.p.) injection, (II) the short-acting benzodiazepine midazolam (Ratiopharm GmBH, Germany) was added to 2% sucrose solution for oral administration. Antagonists used were: (I) flumazenil (Sigma-Aldrich Chemie GmBH, Germany), routinely applied in the clinic to counteract benzodiazepine overdoses, was dissolved in saline containing polysorbate80 and HCl, and (II) pentylenetetrazole (PTZ) (Sigma-Aldrich Chemie GmBH, Germany), a non-competitive GABA antagonist with epileptogenic properties was dissolved in saline for i.p. injection.

Phenotypical Characterization of *Baiap3* KO Mice

Behavioural characterization of naïve *Baiap3* KO mice and their WT littermates of both genders began at the age of 8 weeks and was performed in 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 figure legends.

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Elevated Plus-Maze

The mouse was placed in the central platform, facing an open arm of the plus-maze. Behavior was recorded over 5min by an overhead video camera. A PC 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 fear equivalent.

Open Field

Spontaneous activity in open field was tested in a grey Perspex arena (120cm in diameter, 25cm high), virtually divided into 3 zones: central, intermediate and peripheral. The mouse was placed in the center and the test started when the mouse reached the wall. Over 7min, the mouse was allowed to freely explore the open field. Behavior was recorded by a PC-linked overhead video camera and calculated using 'Viewer' software. Readouts were: velocity, distance traveled, time spent in each zone and initial latency to reach the wall.

Hole Board

The hole board apparatus (TSE, Bad Homburg, Germany) for measuring exploratory activity consisted of a 50cm×50cm×35cm transparent Perspex chamber with a non-transparent floor raised above the bottom of the chamber. The floor had 16 equally spaced holes, 2.4cm in diameter, fitted with a light barrier sensor (8mm below floor). Mice were allowed to explore the chamber for 5min 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 4-40 revolutions per minute over 5min. Each mouse was placed individually on a drum and the latency of falling from the drum was recorded using a stop-watch. To assess motor learning, the test was repeated 24h 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 2min, 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

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movement (excluding respiratory movements), was recorded by a video camera and a PC equipped with 'Video Freeze' software (MED Associates, St. Albans, Vermont, USA).

Pentylenetetrazole-Induced Seizures

Seizure activity was induced in wakeful mice using a single i.p. injection of PTZ (50 mg/kg body weight (b.w.)) (33). After injection of the compound, the mouse was placed in a small, clear home cage and closely observed for 30min. Latencies to focal (partial clonic), generalized (generalized clonic) and maximal (tonic-clonic) behavioral seizures were recorded. Furthermore, 4 phases in the continuum of behavioral response to i.p. PTZ injection were defined as follows: 1. 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). 2. Partial clonus (clonus seizure activity affecting face, head, and/or forelimb or forelimbs). 3. Generalized clonus (sudden loss of upright posture, whole body clonus involving all four limbs and tail, rearing and autonomic signs). 4. 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)x(1/PC latency) + (0.3)x(1/GC latency) + (0.5)x(1/TC latency)]x1000. 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 (5mg/kg b.w. i.p.) for 10 consecutive days. Rotarod test was performed 30min after each diazepam injection for 7 days, with baseline rotarod training performed for 2 days prior to starting injections. On day 11, diazepam withrawal was induced by flumazenil (15mg/kg b.w. i.p.), followed by injection of PTZ (50mg/kg b.w. i.p.) to induce withdrawal-related seizures. Seizure induction by PTZ (50 mg/kg b.w. i.p.) was also performed on drug-naïve mice.

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Midazolam Oral Self-Administration and Behavior Testing in the Addicted State

To induce benzodiazepine dependence as prerequisite for oral self-administration (document of addiction), group-housed mice received midazolam (Ratiopharm GmbH, Germany) in 2% sucrose (to reduce the bitter taste), instead of drinking water. Midazolam concentration was increased weekly, starting from 0.005mg/ml until the maximum concentration of 0.05mg/ml was reached after 10 weeks. 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 continued supply of midazolam (0.05mg/ml) for 2 weeks. For the preference test, every mouse had a choice of two bottles containing either midazolam (0.05mg/ml) in 2% sucrose or 2% sucrose alone for another 2 weeks. 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^{TM} system (Metris, Hoofddorp, Netherlands). The LABORAS^{TM} system 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^{TM} cage in the testing room for 2 consecutive nights (17.00-9.00). On the day of testing, Makrolon type 3 cages (840cm^2), filled with a 2cm layer of bedding used during the habituation phase, were placed on each triangular sensor platform (95x75x75cm). Food and sucrose solution with midazolam (addicted group) or 2% sucrose (control group) were provided *ad libitum*. Prior to each session, LABORAS^{TM} was calibrated using the calibration procedure and reference weights supplied by Metris. Movements during nighttime (18.00–9.00h) were recorded and distinguished as separate behavioral patterns by the LABORAS^{TM} software. Locomotion duration and scratching frequency during dark phase (20.00-8.00) was analyzed.

Statistical Analysis

Behavioral data were analyzed separately for males and females by Mann-Whitney *U* test and 2-way ANOVA including posthoc 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±s.e.m.

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Human Sample

Schizophrenic Patient Sample

The schizophrenic patient sample (N=1086) was recruited across 23 sites throughout Germany in the cross-sectional GRAS study and most comprehensively phenotyped (31, 32). The study has been 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 IV (DSM-IV) criteria for schizophrenia or schizoaffective disorder were included in the analyses regardless of their disease stage (acute, chronic, residual, or remitted). Almost all patients are of European Caucasian descent (Caucasian 94.7%; other ethnicities 1.9%; unknown 3.4%).

Healthy Control Sample

Voluntary blood donors (N=1142) 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 SUD (summarizing abuse and dependence based on the DSM-IV 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 (34) as well as chlorpromazine equivalents to estimate the relative dose of antipsychotic medication, and global assessment of functioning (GAF) scale (DSM-IV) as a measure of impairment of psychological, social and occupational functioning.

Target Variables

The dichotomous DSM-IV BUD diagnosis (summarizing abuse and dependence) as well as the quantitative anxiety composite score were our target variables. The anxiety composite score is based on the aggregation of 4 anxiety-related variables: (1) Brief Symptom Inventory (BSI) subscale anxiousness; (2) State-Trait Anxiety Inventory (STAI) subscale trait anxiety;

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(3) STAI subscale state anxiety; and (4) anxiety item of the PANSS general psychopathology subscale (Supplementary FigureS2).

DNA Extraction and Normalization

Genomic DNA was purified from whole blood using JETQUICK Blood and Cell Culture DNA Spin Kit (Genomed GmbH, Loehne, Germany) according to the manufacturer's protocol. DNA aliquots were stored at -80°C. For further analyses, DNA was normalized to 50ng/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 3 selected SNPs (rs11648169, rs2235632, rs1132358) of *BAIAP3* were analyzed using Simple Probes (TIB Molbiol, Berlin, Germany) and called using the LightCycler® 480 Genotyping Software implemented in the LightCycler® 480 system (Roche, Mannheim, Germany). The reaction mixture (10µl) was prepared with 20ng of DNA in 384 well plates following the standard protocol (Roche). In each run, 8 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=1082 (99.63%) were successfully genotyped for *BAIAP3* SNP1 (C/G) rs11648169, N=1086 (100%) for *BAIAP3* SNP2 (G/A) rs2235632, and N=1069 (98.43%) for *BAIAP3* SNP3 (C/T) rs1132358, and included in the analyses. Of the healthy control subjects, all N=1142 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 using SPSS for Windows version 17.0. Group differences in categorical and continuous variables were assessed using Chi^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 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 using Pearson's correlation coefficient and Cronbach's α (35). In the GRAS sample, the following items or scales were incomplete: BSI anxiousness 7.5% missing, STAI trait anxiety 20.2%, STAI state anxiety

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21.6%, and PANSS anxiety 3.2%. If all 4 anxiety variables were available, the mean was calculated for each respective subject as individual anxiety composite score. In case of missing data, a linear regression based multiple imputation model (10 iterations) of missing data was applied, if at least 3 out of the 4 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 schizophrenic subjects (36). 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 case of SNP rs1132358, C carriers (CC and TC) were aggregated and contrasted with TT individuals. SNP 11648169 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 immersion fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4). Brains were post-fixed for 1 hour, cryoprotected with 30% sucrose and frozen. For immunofluorescence analysis, free-floating brain sections of 40µm thickness or organotypic sections of 300µm thickness were incubated in primary antibodies for 72h followed by incubation with IgG-coupled Alexa Fluor 488, Alexa Fluor 555 and Alexa Flour 633 dyes (Invitrogen, Germany) for 24h. Rabbit and guinea pig antibodies to Baiap3 were raised to a purified fragment (amino acids 617-973) containing the munc homology domains (MHD)-1 and MHD-2 domains 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, 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^{TM}) and an ApoTome^{TM} fluorescence microscope (Axio Imager Z1, Zeiss), respectively.

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Hypothalamus Slice Culture

Organotypic hypothalamus slices of 300µm thickness from postnatal day (P)5 and P6 mice were prepared in Hank's balanced salt solution (24020-091, Invitrogen, Germany) with 20% glucose and 1mM kynurenic acid (Sigma-Aldrich, Germany) (pH 7.4), using a McIlwain Tissue Chopper. Slices were cultured in 6-well plates on confetti cut from 0.45µm filters (FHLC04700, Millipore, Germany) that were placed 0.4µm Millicell cell culture inserts (PICM03050, Millipore, Germany) for 5 days using a mixture of 41% Earle's Basal Medium Ealge (BME) (F0225, Biochrom, Germany), with 25% Earle's Balanced Salt Solution (1.8mM $CaCl_2$, 1mM NaH_2PO_4, 0.8mM $MgSO_4$, 116mM NaCl, 26.2mM $NaHCO_3$, 5.4mM KCl, 5mM Glucose), 20% heat inactivated horse serum, 10% H_2O, 25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Biochrom, Germany), 28mM Glucose, 1mM GlutaMAX™ (35050, Invitrogen, Germany), 1µg/ml Insulin, 88µg/ml ascorbic acid, 0.25% MEM Vitamine Solution (K0373 Biochrom, Germany) and 0.5% MEM Amino Acids (K0363, Biochrom, Germany). On day 5 *in vitro,* the cultures were switched to a medium with identical components but containing 5% horse serum, 55% BME, 2mM GlutaMAX™ and 10µM diazepam, diluted 1:6000 from a 60mM stock solution in dimethylsulfoxide (DMSO) or DMSO alone. The CO_2 concentration was 5% and medium changes were done on the day after culture and every 48h 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 30min recovery time, the extracellular recording solution contained 120mM NaCl, 26mM $NaHCO_3$, 1mM KH_2PO_4, 2mM KCl, 20mM glucose, 2mM $MgCl_2$, 2mM $CaCl_2$ and 250nM flumazenil. Cells were whole-cell voltage clamped at -70mV or -20mV or recorded in current clamp mode with an EPC 10 USB Double (HEKA, Germany) under control of the Patchmaster 2.52 program (HEKA, Germany). All analyses were performed using MiniAnalysis (Synaptosoft, USA). Recordings of miniature inhibitory postsynaptic currents (mIPSCs) were performed in presence of 1µm tetrodotoxin (Tocris, Germany) and 10µm 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX) (Tocris, Germany), with an intracellular solution containing 100mM KCl, 50mM K-gluconate, 10mM HEPES, 0.1mM EGTA, 0.3mM GTP, 4mM ATP and 0.2% Biocytin. Action potentials and sponanous inhibitory postsynaptic currents (IPSCs) were recorded with an intracellular solution

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containing 20mM KCl, 130mM K-Gluconate, 10mM HEPES, 0.1mM EGTA, 0.3mM GTP, 4mM ATP and 0.2% biocytin. Action potentials analyzed were from the first minute of a 2min recording, membrane potentials were measured after setting the current injection to 0pA at the end of the recording. IPSCs were recorded for 5min after switching the cell to a holding potential of -20mV and waiting for 1min. Statisticial analyses were performed using GraphPad Prism5.

Analysis of *BAIAP3* mRNA Levels in PBMCs

PBMCs from 121 patients were isolated 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 1µg RNA, a mixture of oligo dT, hexamer primers, dNTPS (10mM each) and SuperScriptIII (200U, Invitrogen) were used for transcription into cDNA (20µl reaction). The mixture was incubated for 10min at 25°C, 45min at 50°C, followed by 45min at 55°C. For the quantitative reverse transcriptase polymerase chain reaction (qRT)-PCR, a 1:10 dilution of the cDNA was used and 3 replicate experiments per sample were performed; 5µl Power SYBR mix (Applied Biosystems) and 1pmol of each primer were added. *BAIAP3* qRT-PCR primers used were: 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 10min; 45 cycles of 95°C for 15s, 60°C for 1min. Cycle threshold (CT) values of *BAIAP3* were standardized to CT values of GAPDH.

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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 8.7kb. We generated *Baiap3* KO mice by homologous recombination in embryonic stem cells, replacing the first 3 coding exons with a neomycin selection cassette (Supplementary FigureS1A). *Baiap3* KO mice are viable, fertile and indistinguishable from their wildtype (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 [http://mouse.brain-map.org/]. 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 (Figure1). 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 (Figure1). Baiap3 immunoreactivity appears punctate, but does not seem to localize to either glutamatergic or GABAergic pre- or post-synapses to a significant degree (Supplementary FigureS1C-G). Adult *Baiap3* KO mice lack any detectable expression of Baiap3 protein by immunofluorescence and Western blot analysis (Figure1B, Supplementary FigureS1B). 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 weeks and not present in adult animals (Supplementary FigureS1B).

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 genders to a battery of standard behavioral tests (Figure2A-L; Supplementary FigureS3A-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 (Figure2A,B).

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Female but not male KO mice also made fewer visits to the center (Figure2C,D), and spent significantly more time in the periphery (Figure2E,F). When placed in a novel chamber (fear-conditioning box), both male and female KO mice showed an increased novelty induced freezing response (Figure2K,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, i.e. elevated plus-maze and light/dark box, did not reveal any genotype differences (Figure2G-J). Furthermore, the distance traveled (motor activity) in open field and elevated plus-maze (Supplementary FigureS3A-D), exploratory behavior (hole board; Supplementary FigureS3E,F) motor learning and coordination (rotarod; Supplementary FigureS3G,H) and body weight (Supplementary FigureS3I,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 SUD. We selected 3 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 FigureS4A) 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, (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 3 SNPs revealed a similarly high degree of linkage disequilibrium between them in the GRAS sample (Supplementary FigureS4B) and in healthy controls (Supplementary FigureS4C). Case-control analysis of genotype frequencies of the 3 SNPs did not reveal any significant differences, indicating that the selected genetic variation in *BAIAP3* is not associated with schizophrenia risk (Supplementary FigureS4D). We subsequently used

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the PGAS approach (32), to analyze the 3 SNPs for association with specific phenotypic readouts relevant for anxiety disorders and SUD. For this, an anxiety composite score was constructed using 4 anxiety relevant variables (Supplementary FigureS2), which showed a significant association with only 2 of the 3 selected SNPs (as expected because of the high linkage disequilibrium between both markers and their similar MAFs) for women but not for men (Table1). 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 SUD 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 SUD. 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 (BUD) in men (Table1). Even though there was a similar tendency for women (BUD 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 due to the lower numbers of females as compared to males 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 none of these 2 SNPs was associated with disease-related or sociodemographic control variables (Table1).

For the purpose of an association analysis of the relevant *BAIAP3* genotypes (GG/AG/AA in rs2235632 and CC/TC/TT in rs1132358) with BUD, the GRAS sample delivers an ideal, nearly experimental setting: The distribution of these genotypes among benzodiazepine users versus non-users is highly comparable, allowing the identification of risk genotypes leading to BUD (Supplementary TableS1). Most importantly, the benzodiazepine dose was equal across all genotypes (Supplementary TableS1). Hence, the *BAIAP3* risk genotypes (AA for rs2235632, TT for rs1132358) appear to confer a specific genetic risk of developing BUD given equal dose and likelihood of exposure. Interestingly, neither alcohol nor cannabis abuse were found to be associated with the 2 SNPs, pointing to a specific benzodiazepine link with the selected *BAIAP3* genotypes (Table1).

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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 due to the lower numbers available for analysis (Supplementary FigureS5). 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

Based on the identification of human *BAIAP3* risk genotypes for benzodiazepine abuse in male patients, we tested *Baiap3* KO and WT littermates of both genders in experimental paradigms of chronic benzodiazepine administration to assess the development of tolerance, dependence, and withdrawal (Figure3A). The baseline performance of each mouse in the rotarod test was established on 2 consecutive days of rotarod training. No significant genotype-dependent differences were detected in baseline performance (Supplementary FigureS3G,H). Benzodiazepine dependence in *Baiap3* KO and WT mice of both genders was then induced with daily diazepam injections (5mg/kg i.p.) for 10 consecutive days. To monitor the development of tolerance to diazepam, motor performance on rotarod at 30min after each injection was evaluated over the first 7 days of diazepam treatment. Rapid development of tolerance to daily diazepam injections was apparent in both genders and genotypes by an increase of the latency of falling from rotarod over the course of 7 days (Figure3B,C). Here, male *Baiap3* KO mice performed significantly better than their WT littermates (Figure3B), whereas no such difference was detected for females (Figure3C). 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 diazepam withdrawal-related seizures, the susceptibility to pentylenetetrazole (PTZ)-induced seizures was first evaluated in diazepam-naïve mice. The seizure response of *Baiap3* KO mice of both genders to PTZ (50mg/kg i.p.) was higher than in WT animals, with the difference just failing to reach significance in males (Figure3D,E). To assess the effect of genotype on benzodiazepine withdrawal, the diazepam antagonist flumazenil (15mg/kg i.p.) was injected on day 11, after 10 days of daily diazepam treatment, immediately followed by PTZ injection (50mg/kg i.p.)

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to trigger withdrawal seizures (Figure3A). 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-naïve mice of both genotypes (Figure3F). In contrast, the genotype-dependent differences in diazepam-naïve females regarding seizure scores disappeared under conditions of diazepam withdrawal (Figure3G), 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 FigureS6). 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 FigureS6). These data indicate that the *Baiap3* genotype gender-specifically affects the development of tolerance, i.e. 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. Since *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*. Since 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 10µM 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 miniature inhibitory postsynaptic currents (mIPSCs) (Figure4A) 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-

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dependent effect under diazepam withdrawal conditions. Here, WT mIPSC amplitudes were 27% smaller (Figure4B) and rise times 13% longer than in KO neurons (Figure4C), 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 TableS2). Since 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 to vehicle treated WT slices (Figure4D). There was no significant difference in the resting membrane potentials (Figure4E), AP rise times, decay times and half-widths (Supplementary TableS2). Inhibitory postsynaptic currents (IPSCs) were recorded in the same cells at a holding potential of -20mV 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 to vehicle treated WT slices (Figure4F,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.

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DISCUSSION

In this study, we identify 2 human *BAIAP3* risk genotypes that are associated with anxiety in women and benzodiazepine use disorder (BUD) in men. We further 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) to 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 non-schizophrenic 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 (e.g. 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 BUD 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

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fulfill criteria for BUD. Neither SNP was associated with schizophrenia in our case-control analysis. Furthermore, no associations with SUD other than BUD 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 SUD (13-15), the impact of *BAIAP3* genotypes on anxiety disorders or BUD is likely to be small. However, the observed effects and their gender specificity (across 2 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 (37, 38).

Given the higher prevalence of both anxiety disorders and BUD in women (9, 39) 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 BUD 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 (40-42). Therefore, our use of 3 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 α2 subunit and muscle relaxation by α2, α3 and α5 subunits (43). 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

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increased seizure propensity observed in Baiap3 KO mice of both genders, 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 (44), and the amygdala, which also expresses Baiap3, is known to play a key role in epileptogenesis (45). Interestingly, the human *BAIAP3* gene is located on chromosome 16p13.3, which has been linked to electroencephalographic traits of idiopathic epilepsy syndromes (46, 47). We would thus argue that further investigation of *BAIAP3* as a candidate gene for epilepsy-related phenotypes is warranted. Since we found that Baiap3 did not co-localize with markers of GABAergic or glutamatergic pre- and post-synapses to a significant degree (Supplementary FigureS1C-G), the increased seizure propensity in *Baiap3* KO mice of both genders 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 much 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 (48-50), 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 (51, 52). Additional mechanisms, such as neuroendocrine responses to benzodiazepine treatment, may play a critical role in the development of BUD (53). Furthermore, since expression of *Baiap3* in both the VTA and in the nucleus accumbens is low (Allen Brain Atlas [http://mouse.brain-map.org/]), a direct effect 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. Since 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

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modulatory neurotransmitters or neuropeptides that influence the balance between GABAergic and glutamatergic neurotransmission. Baiap3-immuoreactivity appears punctate (Supplementary FigureS1C-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 post-synaptic 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 as 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 man, suggests that *Baiap3* KO mice will be a valuable tool in further elucidating the genetic, physiological and neuroanatomical underpinnings of anxiety disorders and BUD.

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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.

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Figure Legends

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 non-specific 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.

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 genders, whereas (C,D) visits to the center and (E,F) stay in the periphery revealed anxiety-like behavior only in females. (G,H) Elevated plus-maze and (I,J) light/dark box revealed no genotype-dependent differences in either gender. (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 2-way ANOVA (E-H), including Bonferroni testing, applied. Mean±s.e.m. presented.

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-naïve *Baiap3* KO mice display a higher PTZ-induced seizure

propensity as compared to 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), males, WT=7, KO=7; females, WT=8; KO=10. Mann-Whitney *U* test (D-G) and 2-way repeated measures ANOVA (B,C), including Bonferroni, testing applied. Mean±s.e.m. presented.

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. (B) Under diazepam withdrawal conditions, *Baiap3* WT mIPSC amplitudes were significantly smaller than in KO slices, and (C) WT mIPSC rise times were longer than in KO slices. (D) *Baiap3* WT slices showed an increase in AP frequency in response to diazepam withdrawal when compared to 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 to WT slices. (E) The resting membrane potential was not affected by experimental condition or *Baiap3* genotype. (F) IPSC amplitudes and (G) IPSC frequencies were increased in *Baiap3* WT slices under diazepam withdrawal compared to DMSO treated WT slices. Mann-Whitney *U* test for AP and IPSC frequencies; Student's *t*-test for all other parameters. Mean±s.e.m. presented.

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Phenotype comparison of GRAS patients sorted by BAIAP3 genotypes

Males (GRAS sample)	*BAIAP3* rs2235632 G carriers (GG/AG)	*BAIAP3* rs2235632 AA	*P* value (*F*/*T*/*Z*/χ^2 value) [a]	*BAIAP3* rs1132358 C carriers (CC/TC)	*BAIAP3* rs1132358 TT	*P* value (*F*/*T*/*Z*/χ^2 value) [a]
Target variables	**N=471-538** [b]	**N=155-187** [b]		**N=474-538** [b]	**N=152-175** [b]	
Benzo use disorder, No. (%)	11 (2.3)	9 (5.4)	**.047** (χ^2=3.93)	11 (2.3)	9 (5.7)	**.034** (χ^2=4.47)
Anxiety composite score, Mean±SD [c, d]	-0.06±0.74	-0.07±0.70	.499 (*F*=0.46)	-0.07±0.73	-0.05±0.72	.651 (*F*=0.21)
Sociodemographic						
Age (at examination), y, Mean±SD [range]	37.33±12.01 [18-78]	36.17±11.91 [17-75]	.225 (*Z*=-1.21)	37.28±11.96 [18-78]	36.34±12.06 [17-75]	.309 (*Z*=-1.02)
Education, y, Mean±SD [range] [e]	14.17±3.48 [8-28]	14.42±3.70 [8-27]	.680 (*Z*=-0.41)	14.21±3.48 [8-28]	14.32±3.76 [8-27]	.853 (*Z*=-0.19)
Unemployment, No. (%)	217 (44.7)	76 (44.7)	.990 (χ^2=0.00)	214 (44.2)	75 (46.6)	.601 (χ^2=0.27)
Substance use						
Alcohol use disorder according to DSM-IV, No. (%)	221 (42.3)	84 (45.7)	.435 (χ^2=0.61)	217 (41.6)	82 (47.4)	.180 (χ^2=1.80)
Cannabis use disorder according to DSM-IV, No. (%)	218 (41.8)	83 (45.1)	.430 (χ^2=0.62)	224 (42.9)	75 (43.4)	.919 (χ^2=0.01)
Clinical						
PANSS positive score, Mean±SD [range]	13.55±6.04 [7-36]	14.02±6.41 [7-38]	.427 (*Z*=-0.79)	13.53±6.03 [7-36]	14.23±6.50 [7-38]	.249 (*Z*=-1.15)
Chlorpromazine equivalents, Mean±SD [range]	707.90±694.69 [0-6324.29]	689.45±568.91 [0-3238.00]	.678 (*Z*=-0.42)	701.33±688.16 [0-6324.29]	712.11±592.75 [0-3238.00]	.424 (*Z*=-0.80)
GAF score, Mean±SD [range]	45.70±16.04 [10-90]	45.35±16.88 [5-90]	.862 (*Z*=-0.17)	45.75±16.05 [10-90]	44.90±16.89 [5-90]	.619 (*Z*=-0.50)
Females (GRAS sample)	**G carriers (GG/AG)**	**AA**	***P* value (*F*/*T*/*Z*/χ^2 value) [a]**	**C carriers (CC/TC)**	**TT**	***P* value (*F*/*T*/*Z*/χ^2 value) [a]**
Target variables	**N=223-269** [b]	**N=75-92** [b]		**N=229-273** [b]	**N=71-83** [b]	
Benzo use disorder, No. (%)	12 (4.7)	6 (7.0)	.406 (χ^2=0.69)	12 (4.6)	6 (7.7)	.281 (χ^2=1.16)
Anxiety composite score, Mean±SD [c, d]	-0.02±0.77	0.19±0.80	**.028** (*F*=4.91)	-0.02±0.78	0.21±0.77	**.017** (*F*=5.81)
Sociodemographic						
Age (at examination), y, Mean±SD [range]	42.36±12.92 [18-79]	44.86±12.52 [21-76]	.893 (*T*=1.61)	42.52±12.90 [18-79]	44.49±12.60 [20-76]	.221 (*T*=1.23)
Education, y, Mean±SD [range] [e]	14.56±3.95 [7-31]	14.12±3.54 [8-27]	.447 (*Z*=-0.76)	14.52±3.94 [7-31]	14.13±3.62 [8-27]	.454 (*Z*=-0.75)
Unemployment, No. (%)	76 (31.9)	20 (25.0)	.243 (χ^2=1.37)	74 (30.5)	20 (28.2)	.712 (χ^2=0.14)
Substance use						
Alcohol use disorder according to DSM-IV, No. (%)	59 (22.4)	14 (15.7)	.178 (χ^2=1.82)	60 (22.4)	13 (16.0)	.219 (χ^2=1.51)
Cannabis use disorder according to DSM-IV, No. (%)	30 (11.4)	8 (9.0)	.525 (χ^2=0.40)	29 (10.8)	9 (11.1)	.941 (χ^2=0.01)
Clinical						
PANSS positive score, Mean±SD [range]	13.84±6.66 [7-37]	14.41±6.38 [7-33]	.288 (*Z*=-1.06)	13.87±6.69 [7-37]	14.32±6.24 [7-32]	.366 (*Z*=-0.91)
Chlorpromazine equivalents, Mean±SD [range]	636.37±776.51 [0-7375.00]	704.59±762.50 [0-4370.00]	.612 (*Z*=-0.51)	634.40±771.10 [0-7375.00]	718.83±788.67 [0-4370.00]	.616 (*Z*=-0.50)
GAF score, Mean±SD [range]	46.34±19.42 [8-90]	44.22±17.59 [12-84]	.435 (*Z*=-0.78)	46.22±19.25 [8-90]	44.88±18.07 [12-84]	.645 (*Z*=-0.46)

[a] For statistical methods, Mann-Whitney *U* or *Chi*^{2} tests and for normally distributed variables *T*-tests were used. Bolded values, *P*<.05.
[b] Due to missing data, sample sizes vary.
[c] Results after multiple imputations (10).
[d] ANCOVA 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.

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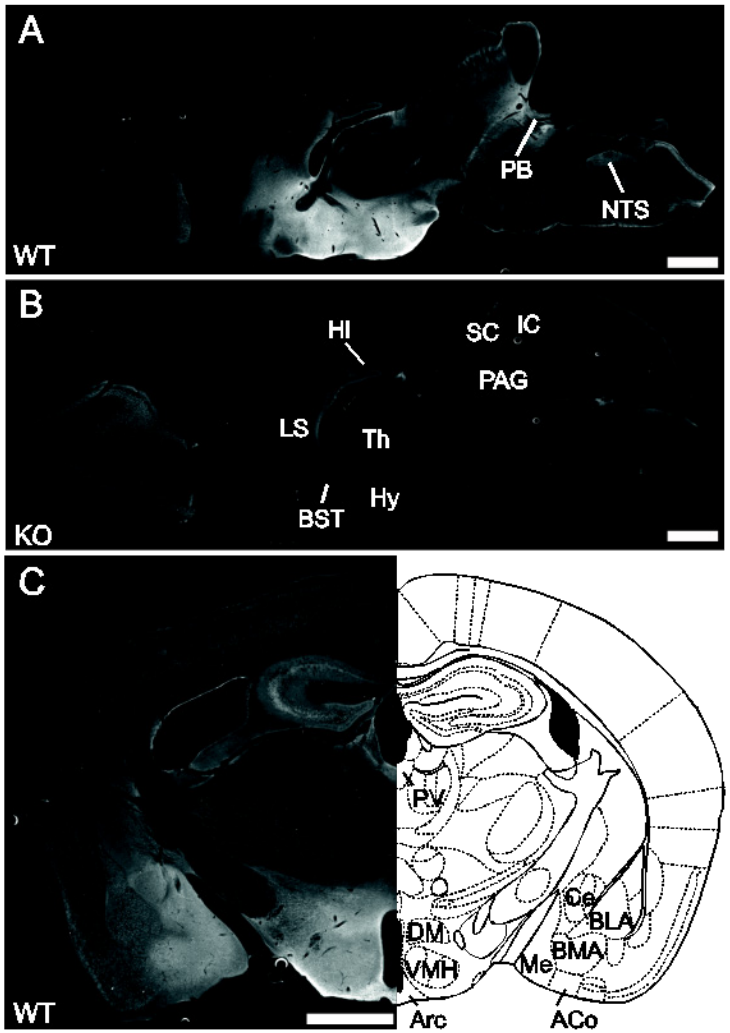
Wojcik et al Figure 1



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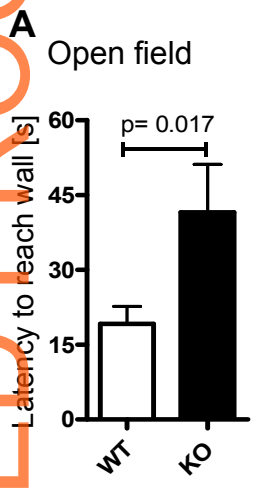

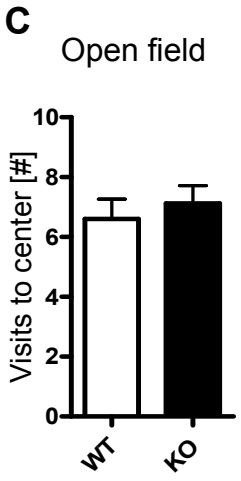

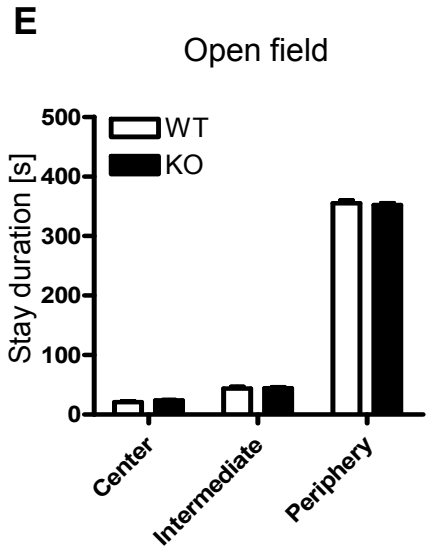

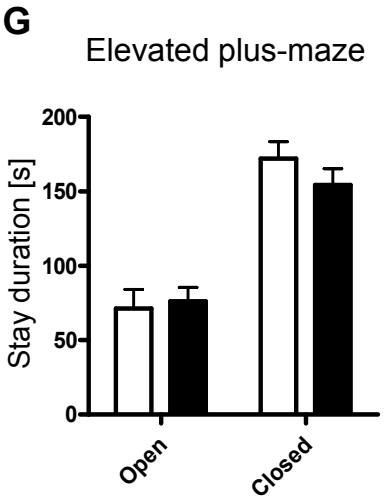

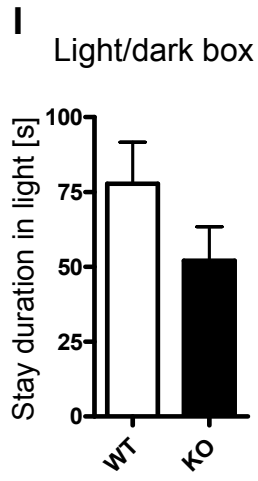

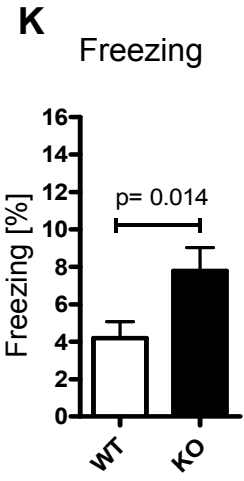

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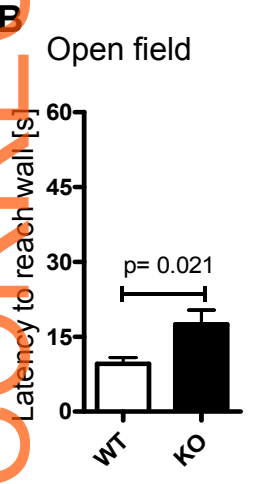

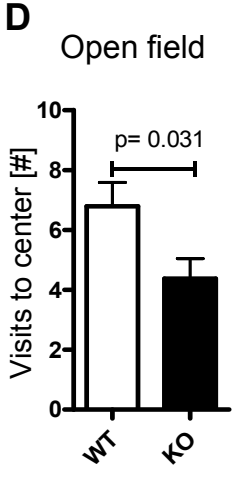

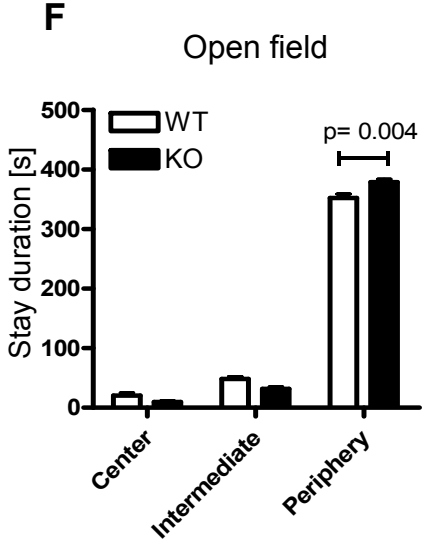

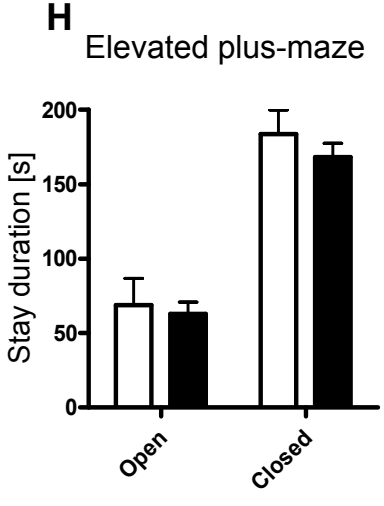

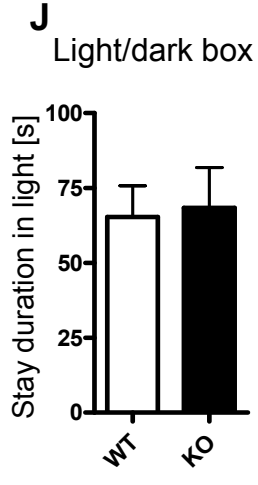

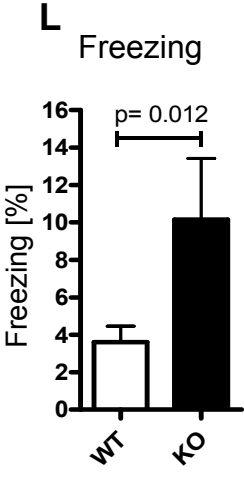

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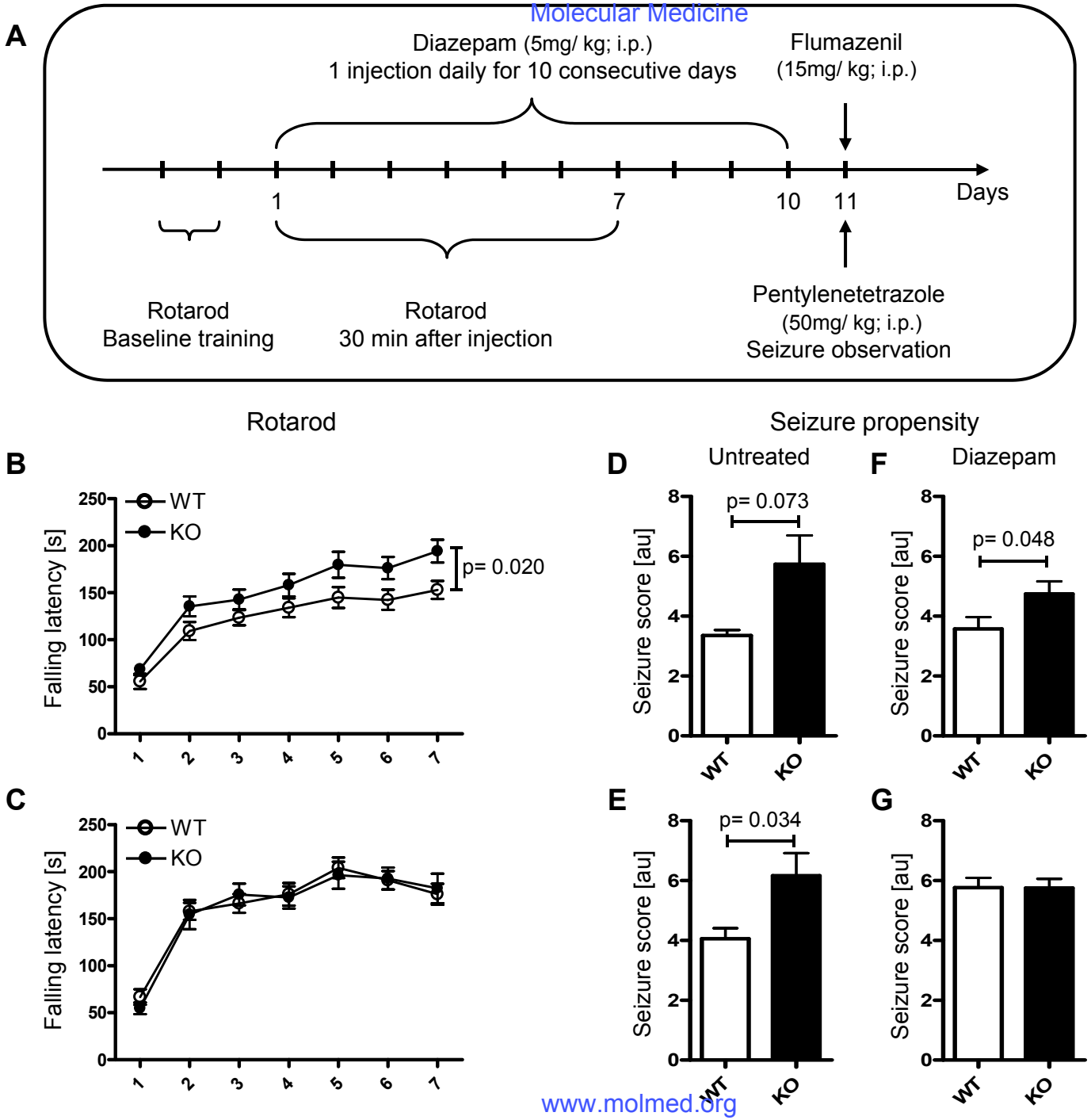

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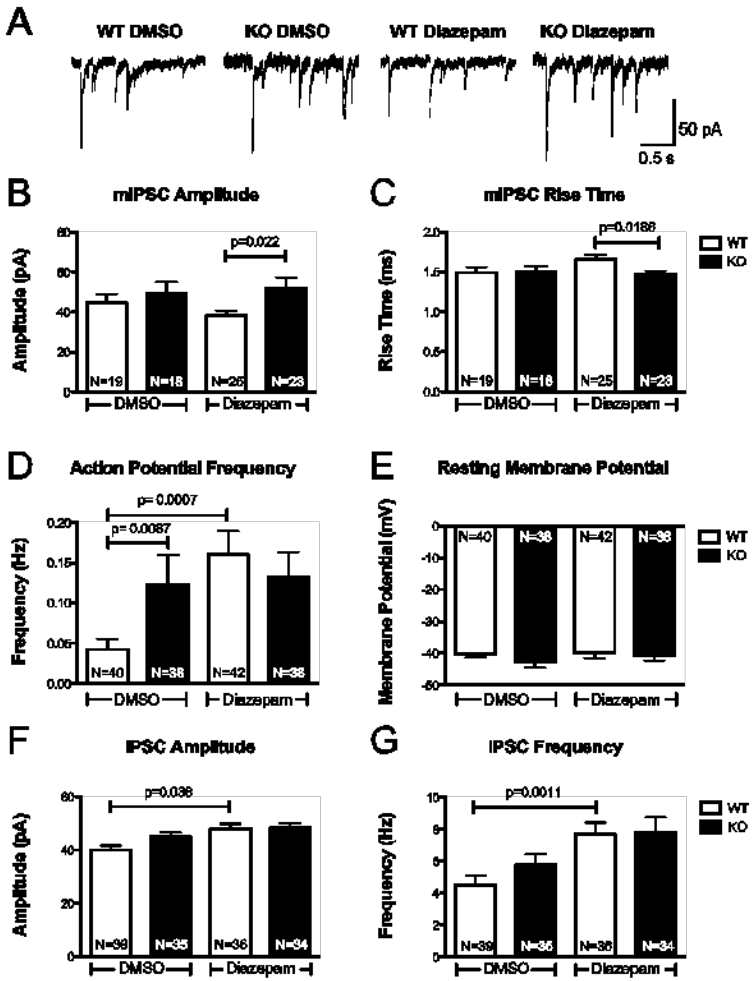
Wojcik et al Figure 4



Supplemental Data

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

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]

*Equal contribution

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CONTENTS:

Supplementary Table S1. Benzodiazepine use (%) and dose (lorazepam equivalents) in GRAS patients sorted by *BAIAP3* genotypes

	BAIAP3 rs2235632				*BAIAP3* rs1132358			
Males (GRAS sample)	**GG**	**AG**	**AA**	***p* value (χ^2 value) [a]**	**CC**	**TC**	**TT**	***p* value (χ^2 value) [a]**
	N=181	**N=351**	**N=187**		**N=184**	**N=347**	**N=175**	
Receiving benzodiazepines, No. (%) [b]	22 (12.2)	57 (16.2)	30 (16.0)	.427 (χ^2 =1.70)	20 (10.9)	57 (16.4)	30 (17.1)	.165 (χ^2 =3.60)
	N=21[d]	**N=52[d]**	**N=30**		**N=19[d]**	**N=52[d]**	**N=30**	
Benzodiazepine dose, mg, Mean±SD [b, c]	3.14±4.14	2.64±3.11	3.04±3.31	.833 (χ^2 =0.37)	2.95±4.31	2.67±3.11	3.00±3.32	.979 (χ^2 =0.04)
Females (GRAS sample)	**GG**	**AG**	**AA**	***p* value (χ^2 value) [a]**	**CC**	**TC**	**TT**	***p* value (χ^2 value) [a]**
	N=101	**N=167**	**N=92**		**N=105**	**N=167**	**N=83**	
Receiving benzodiazepines, No. (%) [b]	28 (27.7)	34 (20.4)	23 (25.0)	.363 (χ^2 =2.02)	30 (28.6)	34 (20.4)	20 (24.1)	.298 (χ^2 =2.42)
	N=27[d]	**N=33[d]**	**N=21[d]**		**N=29[d]**	**N=33[d]**	**N=18[d]**	
Benzodiazepine dose, mg, Mean±SD [b, c]	2.51±1.51	2.13±2.35	2.33±2.28	.227 (χ^2 =2.97)	2.44±1.49	2.08±2.37	2.52±2.40	.234 (χ^2 =2.90)

[a] For statistical methods *Chi*^{2} or Kruskal-Wallis test was used. Bolded values, $p<.05$.
[b] Refers to benzodiazepines as daily medical treatment.
[c] Calculation of lorazepam equivalents according to Bezchlibnyk-Butler, K. Z. & Jeffries, J. J. (Eds) (2003) *Clinical Handbook of Psychotropic Drugs (13th ed).* Cambridge (MA): Hogrefe & Huber.
[D] Discrepancies in N due to missing information on benzodiazepine compound or daily dose.

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Supplementary Table S2. The mIPSC frequencies and decay times and the AP rise times, decay times and half-width were not affected by experimental condition or *Baiap3* genotype.

	WT DMSO	KO DMSO	WT Diazepam	KO Diazepam
mIPSC Decay Time	20.27±4.35 ms	21.01±4.94 ms	21.40±4.22 ms	20.36±4.21 ms
mIPSC Frequency	3.99±3.26 Hz	5.59±5.78 Hz	4.43±3.74 Hz	3.61±2.80 Hz
AP Rise Time	0.97±0.36 ms	1.02±0.45 ms	1.05±0.37 ms	1.12±0.34 ms
AP Decay Time	1.17±0.17 ms	1.14±0.24 ms	1.14±0.24 ms	1.21±0.36 ms
AP Half-Width	1.22±0.19 ms	1.22±0.29 ms	1.20±0.26 ms	1.27±0.39 ms

Mann-Whitney *U* test for mIPSC frequencies and Student's *t*-test for all other parameters. Mean±SD presented.

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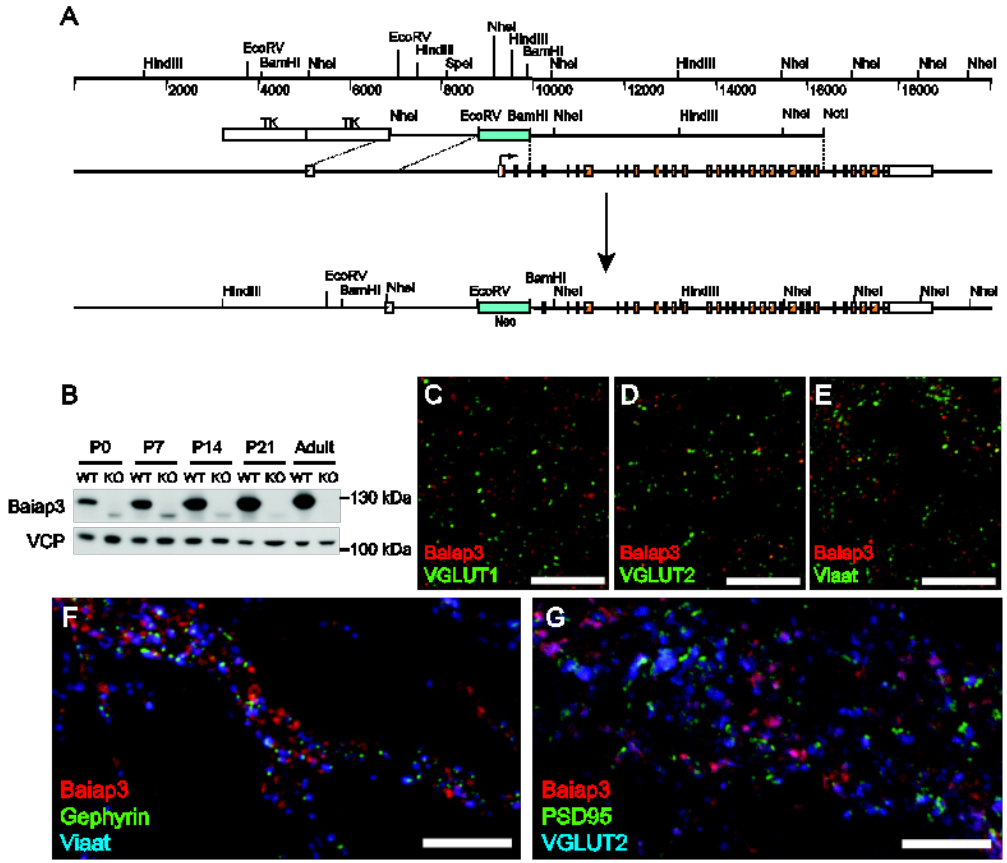

Supplementary Figure S1. Targeting strategy and expression of mouse *Baiap3*. (A) Targeting strategy for the mouse *Baiap3* gene. The first 3 coding exons were replaced with a neomycin selection cassette (Neo). The targeting vector further contained 2 thymidine kinase cassettes (TK) for negative selection. (B) Western blot analysis of the expression of *Baiap3*, in P0, P7, P14, P21 and adult *Baiap3* WT and KO animals. In *Baiap3* KO pups, we detected a truncated Baiap3-immunoreactive product that decreased after P7 and was not detectable in adult mice. An antibody to the valosin-containing-protein (VCP) ATPase was used as a loading control. (C-E) Immunostaining analyses in the hypothalamic medial preoptic area show that Baiap3 largely does not co-localize with markers of glutamatergic and GABAergic synapses. (C) Baiap3 (red) shows no co-localization with the vesicular glutamate tranporter (VGLUT)1 (green). (D) Baiap3 (red) shows only limited co-localization with VGLUT2 (green) (E) Baiap3 (red) shows only limited co-localization with the vesicular inhibitory amino acid transporter (Viaat) (green). (F) In organotypic hypothalamus slices Baiap3 (red) does not show significant co-localization with pre-synaptic (Viaat, blue) or post-synaptic (Gephyrin, green) markers of GABAergic synapses or (G) with pre-synaptic (VGLUT2, blue) or post-synaptic (postsynaptic-density-protein 95, green) markers of glutamatergic synapses. Scale bars equal 10µm.

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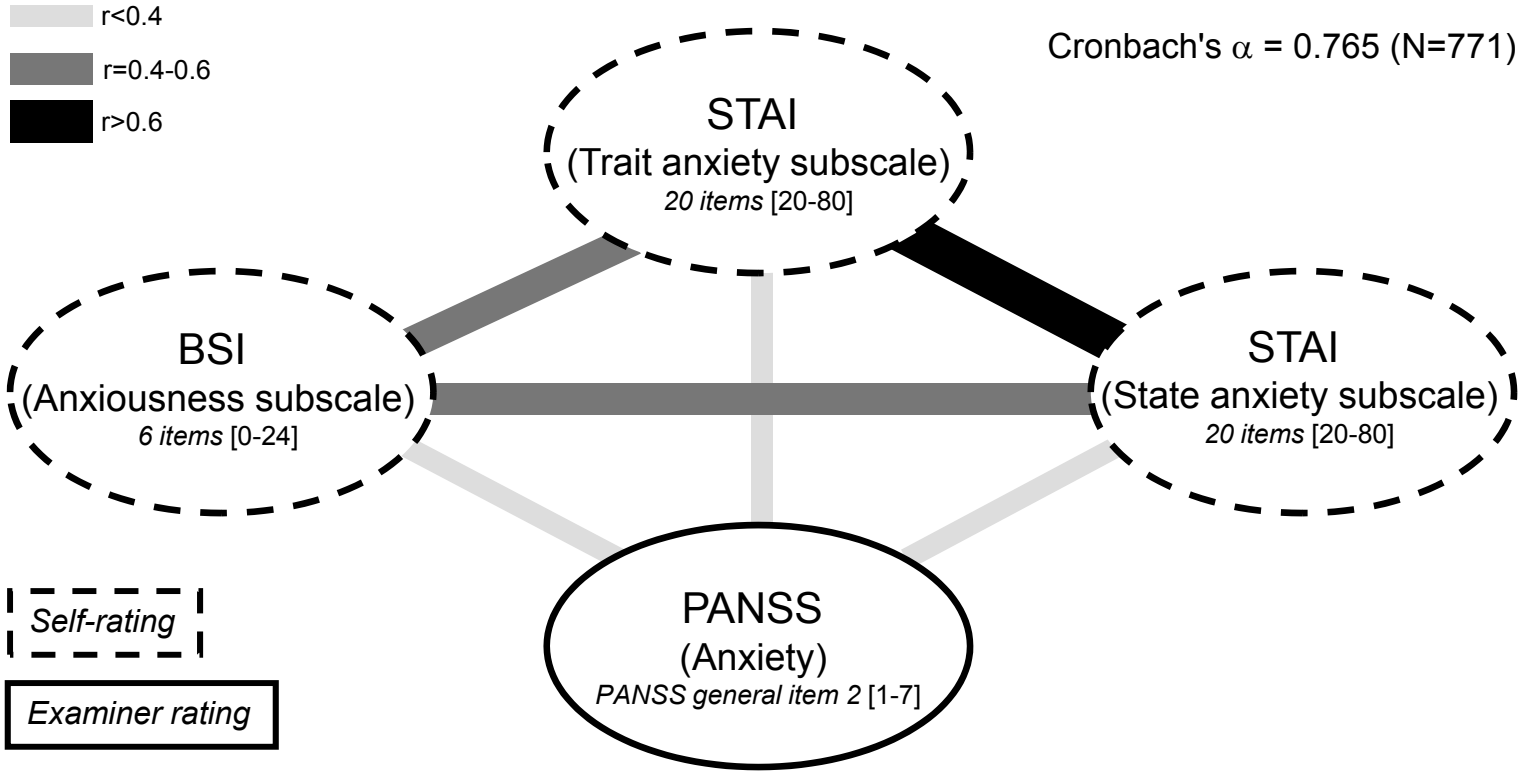

Supplementary Figure S2. Anxiety composite score. Shown are variables composing the anxiety composite score, their intercorrelations and internal consistency. Pearson's correlation coefficients and Cronbach's α given.

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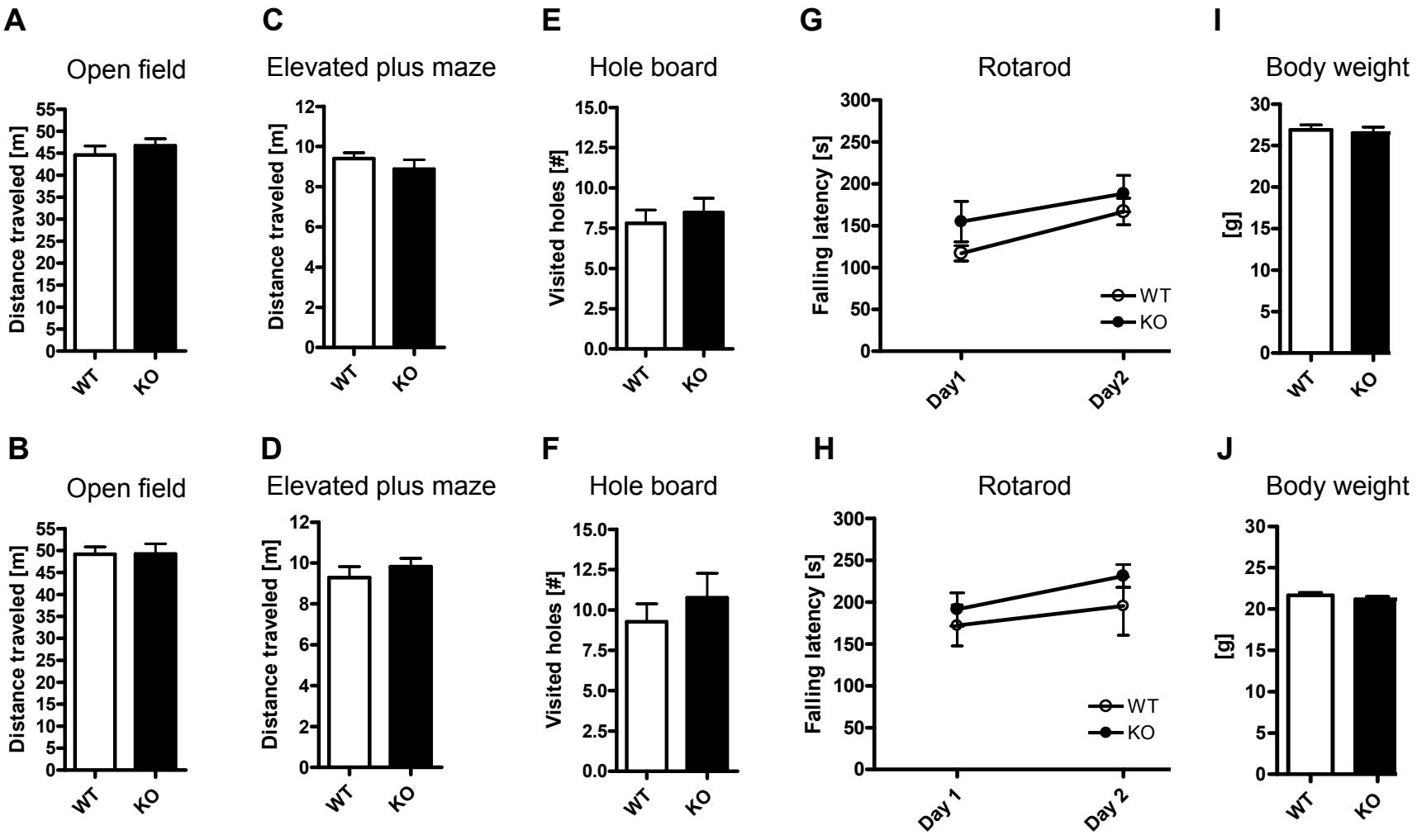

Supplementary Figure S3. *Baiap3* genotype does not affect activity level, exploratory behavior, motor function or body weight. (A,B) The distance traveled during a 7min session in the open field and (C,D) 5min session in the elevated plus-maze was comparable among genotypes and genders. (E,F) Exploratory behavior measured in the hole board, (G,H) motor coordination and learning, evaluated by rotarod, as well as (I,J) body weight were comparable for both genders between *Baiap3* KO and WT littermates. Numbers tested: males, WT=16-25, KO=16-25; females, WT=18-23, KO=10-28. Mann-Whitney *U* test (A-F, I,J) and 2-way repeated measures ANOVA (G,H), including Bonferroni testing, were applied. Mean±s.e.m. presented.

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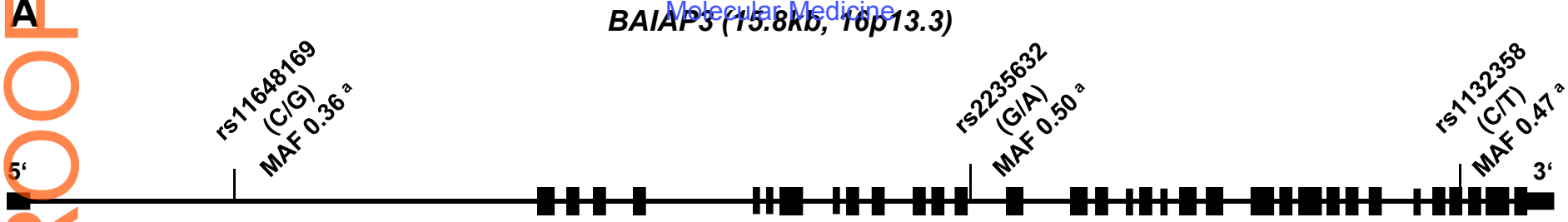
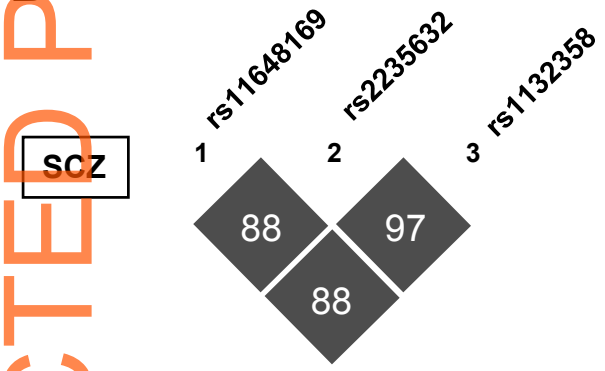


[a] MAF=minor allele frequency in European Caucasian population

B



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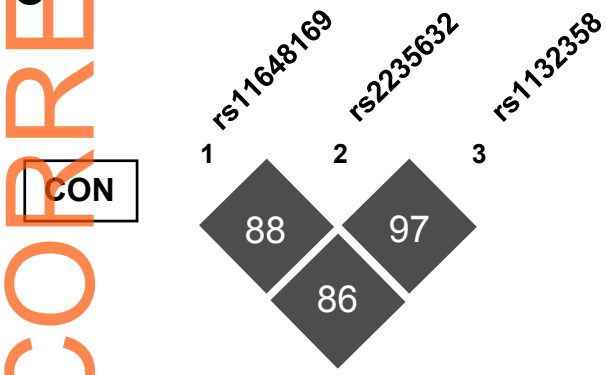

D

BAIAP3		Genotypic frequencies % (N numbers)			*p* value [a] (χ^2 value)	*p* value HWE [b]
		CC	**GC**	**GG**		
rs11648169	**SCZ**	43.2 (467)	44.2 (478)	12.7 (137)	0.792 (χ^2=0.47)	0.395
	CON	43.4 (495)	44.9 (513)	11.7 (134)		0.950
		GG	**AG**	**AA**		
rs2235632	**SCZ**	26.2 (284)	48.1 (523)	25.7 (279)	0.440 (χ^2=1.64)	0.225
	CON	26.6 (304)	50.0 (571)	23.4 (267)		0.971
		CC	**TC**	**TT**		
rs1132358	**SCZ**	27.3 (292)	48.6 (519)	24.1 (258)	0.178 (χ^2=3.46)	0.360
	CON	28.7 (328)	50.4 (576)	20.9 (238)		0.611

[a] Comparison of genotype distributions between GRAS sample (SCZ) and healthy controls (CON).
[b] Hardy-Weinberg-equilibrium.

Supplementary Figure S4. *BAIAP3* genotyping strategy and case-control analyses. (A) Location of the selected single nucleotide polymorphisms (SNPs) in the *BAIAP3* gene. Kb, kilobases. (B) Linkage disequilibrium map for N=1086 schizophrenic and schizoaffective patients and (C) Linkage disequilibrium map for N=1142 healthy blood donors indicating a high degree of linkage between the 3 selected SNPs in both groups. (D) Case-control comparisons reveal a similar distribution of the *BAIAP3* SNP genotypes for patients and healthy individuals, thus excluding the selected *BAIAP3* markers as risk factors for schizophrenia.

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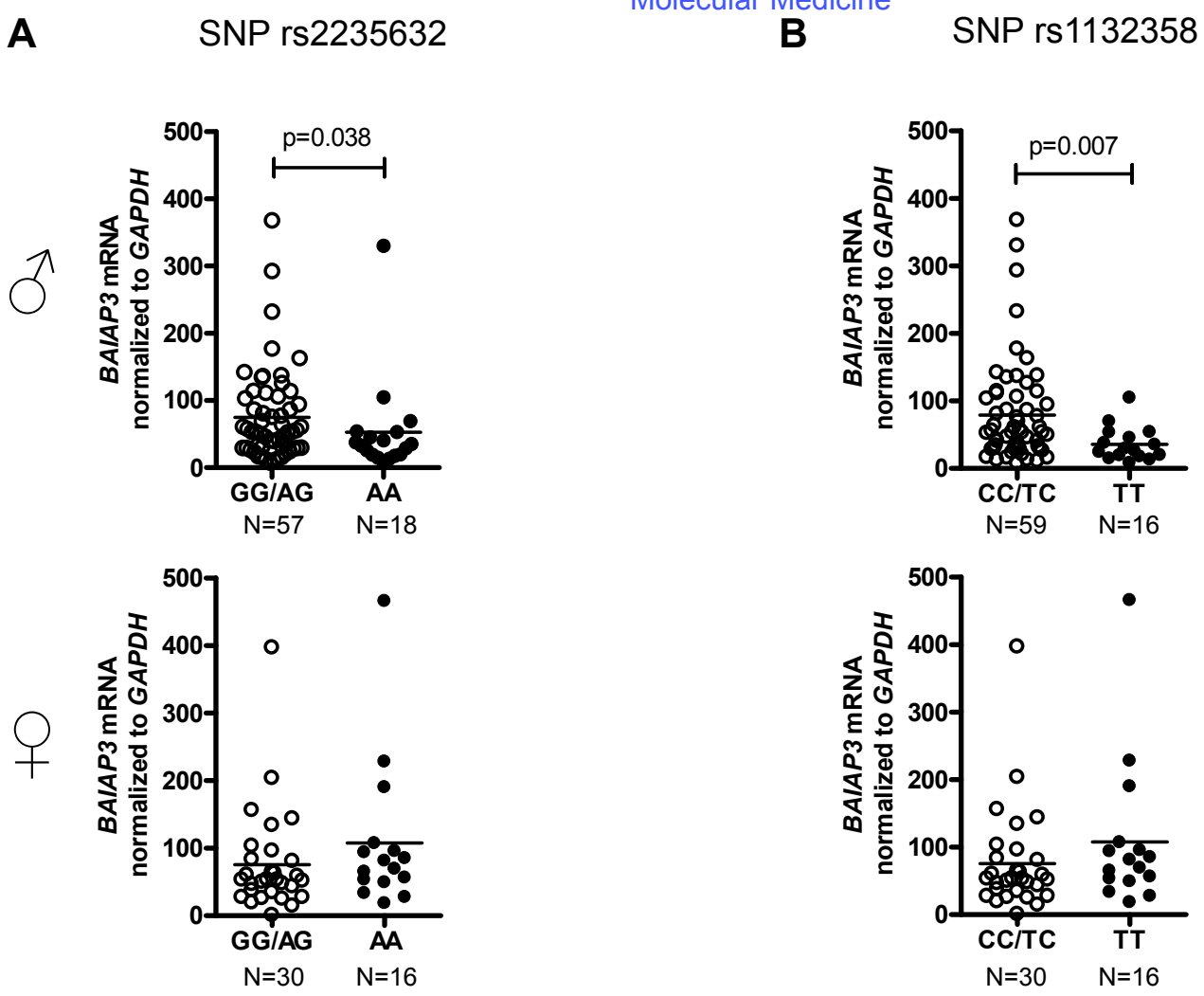

Supplementary Figure S5. *BAIAP3* mRNA expression in PBMCs. (A) SNP rs2235632: The risk genotype AA is associated with lower *BAIAP3* mRNA levels in male but not in female patients. (B) SNP rs1132358: The risk genotype TT is associated with lower *BAIAP3* mRNA levels in male but not in female patients. Mann-Whitney *U* test applied, due to non-normal data distribution (A,B). Mean±s.e.m. presented.

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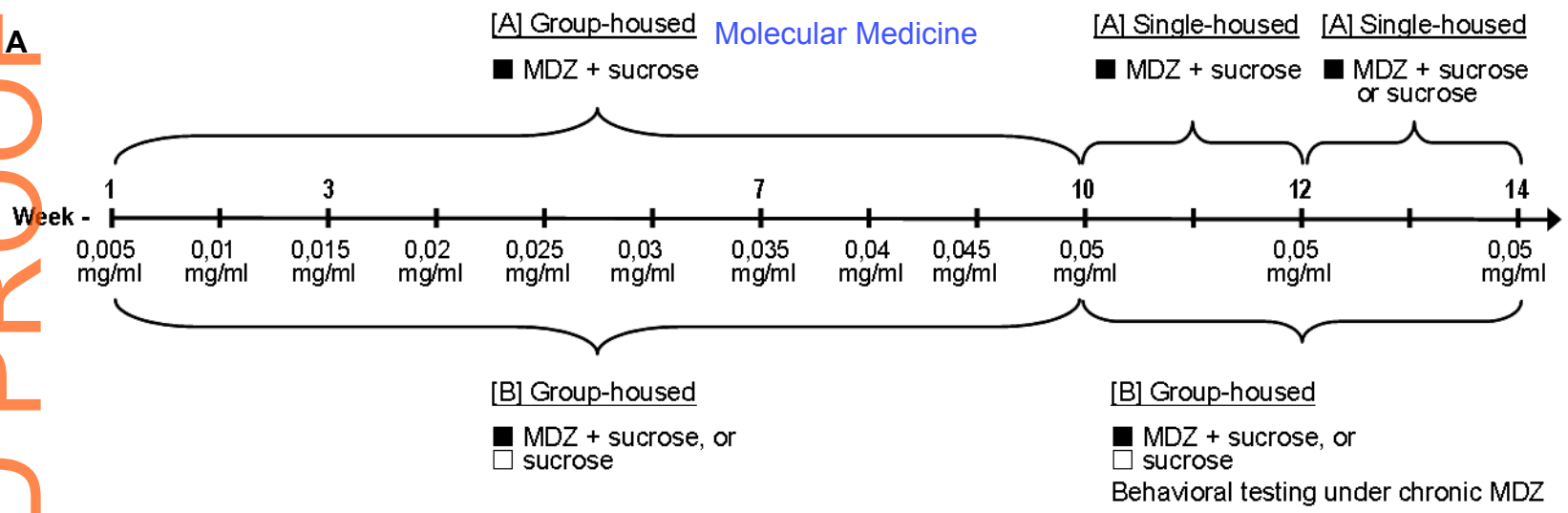

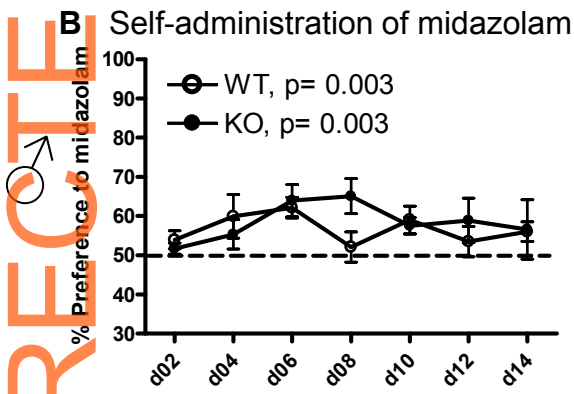

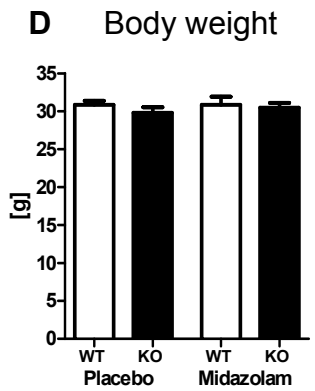

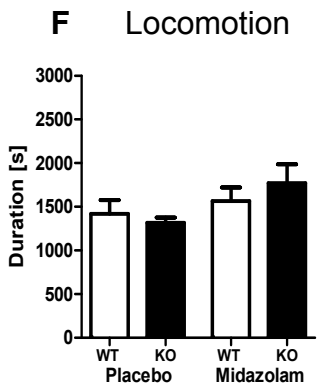

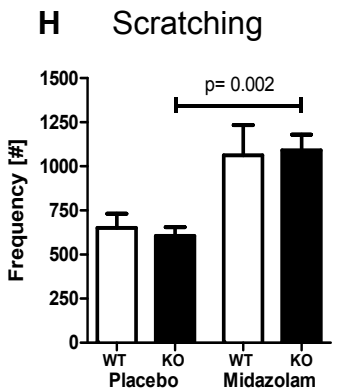

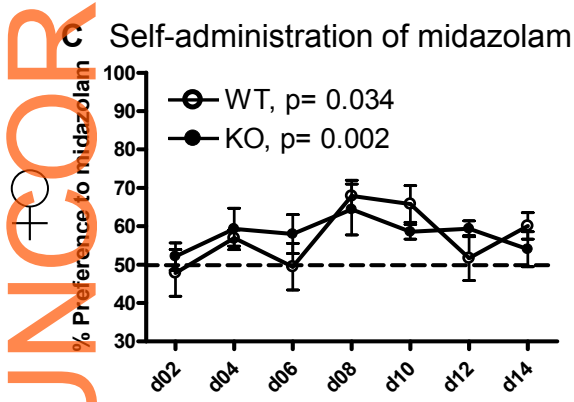

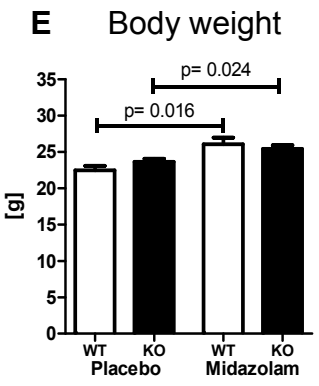

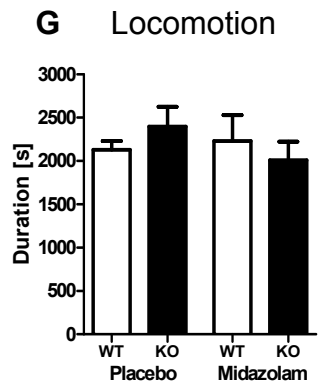

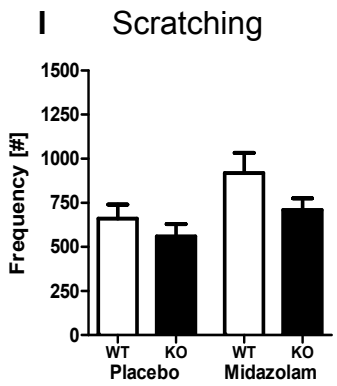

Figure S6

Supplementary Figure S6. High oral self-administration of midazolam (as readout of addiction), body weight, and basic behaviors in the chronic addicted state are not affected by *Baiap3* genotype. (A) Experimental design scheme. (B,C) *Baiap3* KO and WT mice of both genders displayed significant and comparable preference for midazolam over sucrose. (D,E) Chronic midazolam intake did not affect body weight of male, but increased that of female mice independently of genotype. (F,G) Locomotion duration in LABORAS^{TM} remained unaffected across genotypes and genders. (H,I) Scratching frequency in LABORAS^{TM} tended to be increased upon chronic high-dose midazolam across genotypes and genders. Numbers tested: males, WT= 6-8, KO=10-16; females, WT=6-10, KO=10-13, except for (B) and (C), males, WT=8, KO=4; females, WT=5; KO=4; 2-way repeated measures ANOVA (B,C) as well as 2-way ANOVA (D-I), including Bonferroni testing, where applicable. Mean±s.e.m. presented.

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