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ALTERED GABA TRANSMISSION IN A MOUSE MODEL OF INCREASED TRAIT ANXIETY

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Abstract—Anxiety disorders are the most prevalent central nervous system diseases imposing a high social burden to our society. Emotional processing is particularly controlled by GABA-ergic transmission in the amygdala. Using *in situ* hybridization and immunohistochemistry we now investigated changes in the expression of GABA synthesizing enzymes (GAD65 and GAD67), $GABA_A$ (α1–5, β1–3, γ1–2) and $GABA_B$ receptor subunits (GBBR1, GBBR2) in amygdaloid nuclei of high anxiety-related behavior (HAB) mice in comparison to mice selected for normal anxiety-related behavior (NAB). Levels of GAD65 and GAD67 mRNAs and protein, as well as those of GABA were increased in the amygdala of HAB mice. Relative to NAB controls, mRNA expression of the $GABA_A$ receptor subunits β1, β2 and γ2 was specifically increased in the basolateral amygdala of HAB mice while transcription of α5 and γ1 subunits was reduced in the central and medial amygdala. On the protein level, increases in β2 and γ2 subunit immunoreactivities were evident in the basolateral amygdala of HAB mice. No change in $GABA_B$ receptor expression was observed. These findings point towards an imbalanced GABA-ergic neurotransmission in the amygdala of HAB mice. On the other hand, FosB, a marker for neuronal activity, was increased in principal neurons of the basolateral amygdala in HAB mice, reflecting activation of excitatory neurons, possibly as a consequence of reduced GABA-ergic tonic inhibition through α5 and γ1 containing receptors. Ultimately these mechanisms may lead to the compensatory activation of GABA transmission, as indicated by the increased expression of GAD65/67 in HAB mice.

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Key words: $GABA_A$ receptor, glutamate decarboxylase, HAB, high anxiety-related behavior, amygdala, FosB.

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Abbreviations: BLA, basolateral amygdala; CEA, central amygdala; EPM, elevated plus maze; GAD65, glutamic acid decarboxylase of 65 kilo Dalton; GAD67, glutamic acid decarboxylase of 67 kilo Dalton; HAB, high anxiety-related behavior; LAB, low anxiety-related behavior; MEA, medial amygdala; NAB, normal anxiety-related behavior; ROD, relative optical density.

Anxiety disorders are the most prevalent disorders of the central nervous system imposing a high social burden to individuals as well as to the society (Andlin-Sobocki et al., 2005). While physiological anxiety is essential for the survival under changing environmental conditions, persistent generalized anxiety or exaggerated inappropriate fear are pathological manifestations that severely reduce quality of life (Belzung and Griebel, 2001; Norrholm and Ressler, 2009). Little is known, however, about the molecular mechanisms leading to anxiety-related conditions.

There is considerable evidence indicating a role of altered GABA-ergic transmission in human anxiety disorders (Millan, 2003). Thus, benzodiazepines acting through $GABA_A$ receptors are among the most widely prescribed anxiolytic drugs (Nemeroff, 2003). Changes in $GABA_A$ receptors were observed in limbic brain areas of panic disorder patients by [^{14}C]flumazenil positron emission tomography (Hasler et al., 2008) and single nucleotide polymorphisms in the glutamate decarboxylase65 (GAD65) gene were associated with increased susceptibility to anxiety disorders (Hettema et al., 2006).

GABA released from axon terminals can act on two classes of receptors, $GABA_A$ and $GABA_B$ receptors. $GABA_A$ receptors are ligand-gated chloride channels (Olsen and Sieghart, 2009) consisting of five subunits derived from more than 15 different genes. Most $GABA_A$ receptors, however, consist of two α-, two β- and one γ- or δ-subunit (Sieghart and Sperk, 2002). The subunit composition determines the physiological and pharmacological properties of individual receptor subtypes. In particular α2- and γ2-subunits of the $GABA_A$ receptor have been implicated in mediating the anxiolytic effects of benzodiazepines (Crestani et al., 1999; Low et al., 2000; Morris et al., 2006).

$GABA_B$ receptors are G-protein coupled, inhibitory receptors that are generally formed by heteromeric assembly of two receptor molecules, one $GABA_{B1}$ (GBBR1) with a $GABA_{B2}$ (GBBR2) subunit (Ulrich and Bettler, 2007). $GABA_B$ receptors may also play a role in the integration of anxiety, since mice deficient in $GABA_B$ receptors (GBBR1 or GBBR2) are more anxious in several behavioral paradigms (Mombereau et al., 2004, 2005).

The GABA-ergic system in the amygdala does not only modulate fear and anxiety under physiological conditions, but alterations in this system may also predispose individuals to pathological anxiety traits (Shen et al., 2010). The aim of our present study was therefore to elucidate the role of the GABA-erigc system in anxiety-related behavior in HAB and NAB mouse lines. These two mouse lines expressing either high (HAB) or normal (NAB) levels of inborn anxiety-related behavior were selected by breeding

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Table 1. Oligonucleotide sequence for *in situ* hybridization

mRNA	Access code	Oligonuceotide sequence
α1	NM_010250.3	5′ CCT GGC TAA GTT AGG GGT ATA GCT GGT TGC TGT AGG AGC ATA TGT 3′
α2	NM_008066.3	5′ CAT CGG GAG CAA CCT GAA CGG AGT CAG AAG CAT TGT AAG TCC 3′
α3	NM_008067.3	5′ GGC CAG ATT GAT AGG ATA GGT GGT ACC CAC TAT GTT GAA GGT GGT G 3′
α4	NM_010251.2	5′ GGA TGT TTC TGT GTG TTT CTC CTT CAG CAC AGG AGC AGC TGG 3′
α5	NM_176942.3	5′ TTG GGA TGT TTG GAG GAT GGG TCA GCT TTC CAG TTG T 3′
β1	NM_008069.4	5′ TGC CTG TCC AGC CCA CGC CCG AAG CCC TCG CGG CTG CTC AGT GG 3′
β2	NM_008070.3	5′ ACT GTT TGA AGA GGA ATC CAG TCC TTG CTT CTC ACG GAA GGC TG 3′
β3	NM_001038701.1	5′ CTG TCT CCC ATG TAC CGC CCA TGC CCT TCC TTG GGC ATG CTC TGT 3′
γ1	NM_010252.4	5′ GGG AAT GAG AGT GGA TCC AGC ATG GAG ACC TGG GGA 3′
γ2	NM_008073.2	5′ GGC AAT GCG AAT ATG TAT CCT CCC ATG TCT CCA GGC TCC TGT TCG GC 3′
ə	NM_008072.1	5′ GGC AAG GTC CAT GTC ACA GGC CAC TGT GGA GGT GAT GCG GAT GCT GTA T 3′
GAD65	NM_008078.2	5′ CTC CAT CAC ACG CTG GCA GGT CTG TTG CGT GCA G 3′
GAD67	NM_008077.3	5′ GCA GGT TCT TGG AGG ACT GCC TCT CCC TGA AGG CGC TCA C 3′
GBBR1	NM_019439.3	5′ AAG CCA CGG TAC CTG ATG CCA CCT TCC CAG GGC GGA TGT ATA ATC TGG C 3′
GBBR2	NM_001081141.1	5′ GAT GTA TGT GGT CTT CTC TGG TGT GTC TTG TAG CTG CAT GGT GAC TTC TTC C 3′

according to their anxiety-related behavior displayed on the elevated plus maze (EPM) (Kromer et al., 2005). The amygdala has a key role in the altered phenotypes of HAB mice, since mild emotional challenge of HAB mice results in differential activation of the amygdala and other brain areas (Muigg et al., 2009). We performed *in situ* hybridization and immunohistochemistry for GABA synthesizing enzymes, glutamate decarboxylase 65 (GAD65) and 67 (GAD67), as well as for $GABA_A$ (α1–5, β1–3, γ1–2) and for $GABA_B$ (GBBR1, GBBR2) receptor subunits in the amygdala under conditions of chronically increased trait-anxiety of HAB compared to NAB mice.

EXPERIMENTAL PROCEDURES

Animals

All procedures involving animals and animal care were conducted in accordance with international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996) and were approved by the Austrian Ministry of Science. All effort was taken to minimize the number of animals used and their suffering.

Experiments were carried out on adult male HAB (n=20) and NAB (n=23) mice at 12–15 weeks of age bred in the animal facilities of the Department of Pharmacology and Toxicology, University of Innsbruck, Austria. As previously described in more detail (Kromer et al., 2005; Muigg et al., 2009), the two lines were derived from a Swiss CD-1 outbred population selectively inbred for either high or normal anxiety-related behavior displayed on the EPM, with HAB mice spending less than 15% of the testing time on its open arms, compared with approximately 35% for NAB mice with no overlapping between the lines. The high anxiety phenotype of HAB mice was confirmed in the light/dark test and ultrasonic vocalization, previously (Kromer et al., 2005). In the current experiments the behavioral phenotype of each mouse was tested by an EPM test at 7 weeks of age. Animals were housed under standard laboratory conditions (12:12 h light/dark cycle with lights on at 7:00) with food and water available *ad libitum*. Mice were housed in groups of four to five animals per cage.

Histochemistry

Tissue preparation. In order to avoid detection of possible immediate changes of gene expression due to acute stress exposure of the mice during EPM testing, examination of the GABA-ergic system was performed 4 weeks after behavioral experiments. Mice were killed by carbon dioxide gas inhalation or by injecting an overdose of thiopental (Thiopental, Sandoz, Austria) for *in situ* hybridization and immunohistochemistry, respectively. Brains were either snap frozen (isopentane, −70 °C, 3 min) for *in situ* hybridization or perfused with 4% paraformaldehyde (PFA) for immunohistochemistry (Tasan et al., 2010). Using a cryostat, coronal sections of 20 μm and 40 μm were cut for *in situ* hybridization and immunohistochemistry, respectively. Every seventh section was Nissl stained and series of matching sections were selected for subsequent histochemistry.

In situ hybridization. *In situ* hybridization was performed as described previously in detail (Tsunashima et al., 1997; Tasan et al., 2010). Oligonucleotide probes targeting diverse markers of the GABA-ergic system are listed in Table 1. They were custom synthesized (Microsynth, Balgach, Switzerland, purified by HPLC). Oligonucleotides (2.5 pmol) were 3′ end-labeled by incubation with [^{35}S]α-dATP (50 μCi; 1300 Ci/mmol, Hartmann Analytic GmbH, Braunschweig, Germany) and terminal transferase (Roche Diagnostics, Basel, Switzerland), as described previously in detail (Tsunashima et al., 1997). Hybridization was performed in 50% formamide, 4× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.2), 500 μg/ml salmon sperm DNA, 250 μg/ml yeast tRNA, 1× Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 10% dextran sulfate, and 20 mM dithiothreitol (all from Sigma) at 42 °C for 18 h. The slides were washed at stringent conditions (50% formamide in 2× SSC, 42 °C) and briefly rinsed in water followed by 70% ethanol, and dried. Slides were exposed to BioMax MR films (Amersham Pharmacia Biotech, Buckinghamshire, UK) together with [^{14}C]-microscales for 7–14 days. For evaluation of the hybridization signal at the cellular level, some slides were dipped in Kodak NTB-2 photosensitive emulsion (Kodak, Rochester, NY, USA; diluted 1:1 with distilled water) at 42 °C, air dried over night, and then exposed for 4–6 weeks at 4 °C. The BioMax MR films and the dipped slides were developed with Kodak D19 developer. Sections were counterstained with Cresyl Violet, dehydrated, cleared in butyl acetate, and covered with a coverslip using Eukitt (Merck, Darmstadt, Germany).

Quantitative evaluation of *in situ* hybridization was done using digitized images of the autoradiographs (eight bit digitized picture, 256 gray values). Gray values were measured by the public domain program ImageJ 1.38x (NIH, USA; 255=white; 0=black) and converted to relative optical density (ROD). ROD values obtained from autoradiographic images were plotted against stan-

dard curves obtained from images of [^{14}C]-microscales exposed to the same film to insure that signal values are within the linear range of radioactivity, and then converted to percentage of control. Each value was obtained from the innermost 90% of the respective brain area. Adjacent background levels with no hybridization signal (internal capsule) were obtained separately for each brain section and side. The anatomical level was verified in sections counterstained with Cresyl Violet using a mouse brain atlas (Franklin and Paxinos, 2007).

Immunohistochemistry. Immunohistochemical analysis was performed on free-floating, PFA-fixed, 40 μm thick coronal sections using indirect peroxidase labeling, as described previously (Furtinger et al., 2001). The following antisera were used: polyclonal rabbit anti-FosB (1:2000 SC-48; Santa Cruz Biotechnology), polyclonal rabbit anti-GABA (1:2000 A2052, Sigma), monoclonal mouse anti-GAD67 (1:15,000 MAB 5406; Chemicon), monoclonal mouse anti-CaMKII (1:5000 MAB, clone 6G9, Millipore), polyclonal rabbit anti-β2 subunit (1:100, residues 351–404) and polyclonal rabbit anti-γ2 subunit (1:300, residues 319–366) (both gifts from W. Sieghart, Vienna). A mouse Ig blocking reagent (VECTOR® M.O.M. immundetection kit, Vector laboratories, Inc., Burlingame, USA) was used for monoclonal mouse antibodies (GAD67, CaMKII). In brief, free floating coronal sections were incubated in 10% normal goat serum (Biomedica, Vienna, Austria) in Tris–HCl buffered saline (TBS; 50 mM, pH 7.2) for 90 min, followed by incubation with primary antiserum. The resulting complex was visualized by incubation with horseradish peroxidase-coupled secondary antibody (1:250 P0448; Dako, Vienna, Austria) at room temperature for 150 min. Sections were further incubated in a solution containing 0.03% 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) and 0.005% H_2O_2 in TBS for 6 min. They were then mounted on slides, air-dried, dehydrated and coverslipped. After each incubation step (with the exception of preincubation with 10% normal goat serum), sections were washed three times for 5 min each with TBS. All buffers and antibody dilutions, except the buffer used for washing after peroxidase treatment and the diaminobenzidine reaction buffer, contained 0.4% Triton X-100. Normal goat serum (10%) was included in all buffers containing antibodies. In each experiment, sections without primary antibody were included as a control. No immunopositive elements were detected in these control sections.

In addition, dual labeling of FosB with either CaMKII as marker for pyramidal neurons or GAD67 as marker for non-pyramidal interneurons in the basolateral amygdala were performed to determine the type of FosB expressing neurons. Sections were incubated overnight at room temperature with rabbit anti-FosB antibody (1:500, SC-48; Santa Cruz Biotechnology) together with either a mouse monoclonal anti-CaMKII antibody (1:5000, Millipore, Billerica, MA, USA) or with a mouse monoclonal anti-GAD67 antibody (1:20,000, Chemicon, USA). Sections were rinsed in TBS-Triton (0.4%) and then incubated with a Vectastatin ABC-kit (Mouse IgG, Vector laboratories, Inc., Burlingame, USA) and donkey anti-rabbit Alexa Fluor 488 (1:500, Invitrogen, Eugene, USA) for 60 min at room temperature. After washing in TBS, sections were incubated in a tyramide amplification solution (1:100, TSA Plus Cyanine 3 System, PerkinElmer, MA, USA) for 2 min, mounted on slides and covered using Vectashield mounting medium (Vector laboratories, Inc., Burlingame, USA).

Number of FosB, GAD67 and GABA immunoreactivity positive cells was obtained for each marker bilaterally from two matched sections per animal (four NAB and four HAB mice) in the basolateral amygdala (BLA) at a magnification of 400 times in multiple separate fields and mean values were calculated for each mouse. Results are presented as number of immunoreactivity positive cells/mm^2 and expressed as mean±SEM.

Analysis of dual labeling immunofluorescence was done as described elsewhere (McDonald and Mascagni, 2010). In brief, two matched sections per animal (four NAB and four HAB mice) containing the BLA were processed for either FosB/CaMK or FosB/GAD67 dual localization. Identification of dual labeled cells was performed at 400 times magnification in multiple separate fields within the BLA in each section.

Behavioral testing

Elevated plus maze. This test was used as a widely accepted test for anxiety-like behavior as described in detail previously (Lister, 1987; Tschenett et al., 2003).

Statistical analysis

Data are presented as means±SEM. Data were analyzed for normal distribution and equal variances using GraphPad Prism software (Prism 5 for Macintosh, GraphPad Software Inc., San Diego, CA, USA). Statistical analysis of behavior was done by Student's *t*-test for data with normal distribution or Mann–Whitney test as a nonparametric test. ANOVA was used to analyze overall changes in percentage of mRNA ROD values in the CEA, BLA and MEA of HAB and NAB mice, with a Bonferroni *post hoc* test comparing HAB and NAB of the respective subregion. Linear regression and a Spearman correlation were used to investigate a correlation between mRNA expression and anxiety-related parameters as well as locomotor-related parameters on the EPM at 7 weeks of age.

RESULTS

HAB mice (n=20) showed a significantly reduced percentage of time spent in the open arms compared to NAB controls (n=23), (HAB: 5.2±0.89, NAB: 28.5±1.32, $P<0.0001$) and percentage of open arm entries (HAB: 21.1±2.11, NAB: 44.5±1.65, $P<0.0001$), indicating increased anxiety-related behavior and thus confirming the extremely anxious phenotype of each HAB mouse on the EPM. In addition, HAB mice exhibited reduced motor activity under stressful conditions as reflected by a decreased number of closed arm entries (HAB: 23.3±1.66, NAB: 33.9±1.53, $P<0.0001$) and reduced total distance traveled (HAB: 9.1±0.82 m, NAB: 14.3±0.46 m, $P<0.0001$) on the EPM during the 5 min testing period.

Histochemical results

Increased expression of GAD in the amygdala of HAB mice. As shown in Fig. 1A, the mRNAs encoding for the GABA synthesizing enzymes GAD67 and GAD65 (not shown) were particularly concentrated in the central amygdala (CEA) while their expression was lower both in the medial (MEA) and basolateral nucleus of the amygdala (BLA). In HAB mice, a pronounced up-regulation of mRNA abundance of the GABA synthesizing enzymes GAD65 and GAD67 was evident in the amygdala (Fig. 1A, B, Table 2), but not in other brain regions, including the granule cell layer of the dentate gyrus in the dorsal (% of control NAB: 100.0±7.00 and HAB: 107.0±6.15; P=0.47, Student's *t*-test, mean±SEM) (Fig. 1A, C) as well as in the ventral hippocampus (% of control NAB: 100.0±6.07 and HAB: 85.4±7.09; P=0.14, Student's *t*-test, mean±SEM). To investigate whether the increased mRNA expression translates into higher protein levels we performed immunohistochemistry for GAD67 and GABA (Fig. 2). Indeed immunoreactivity for GAD67 (Fig. 2A, C) as well as for GABA (Fig. 2B, C) was enhanced in the amygdala of HAB mice.

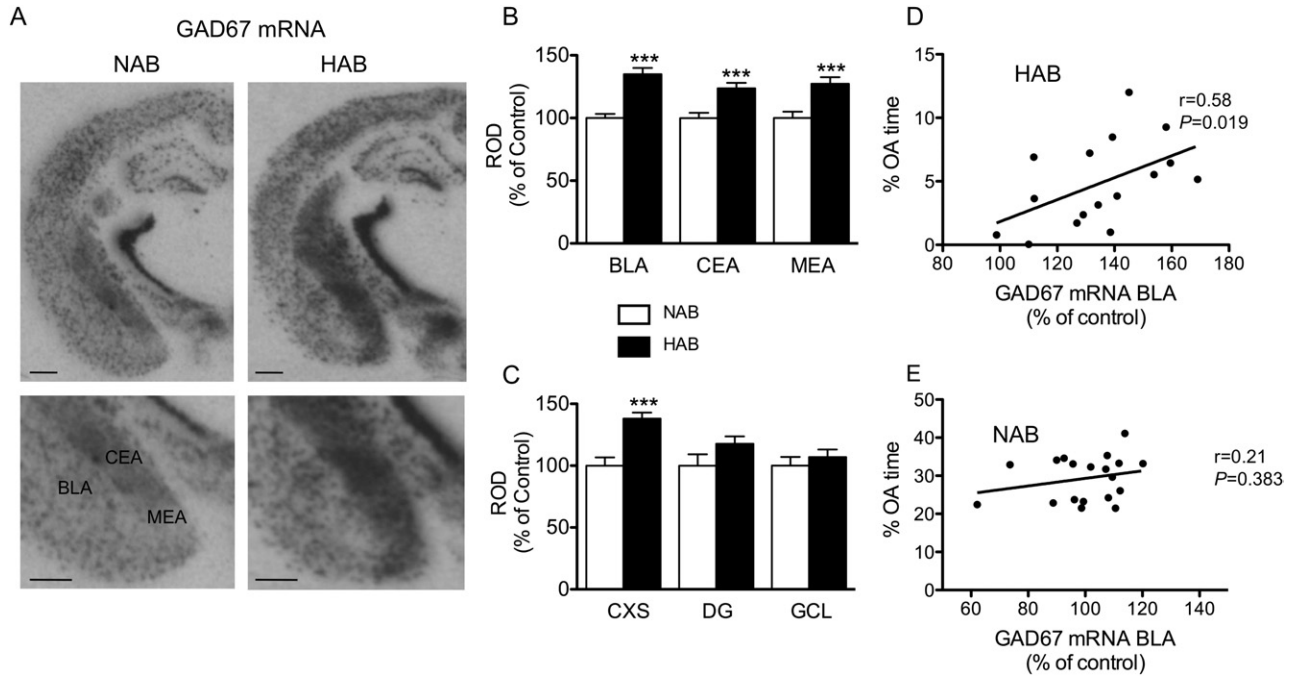

Fig. 1. Expression of GAD67 mRNA in HAB and NAB mice. (A) Film autoradiographs from *in situ* hybridization experiments displaying increased GAD67 mRNA in coronal sections of a HAB mouse brain compared to NAB controls, a higher magnification showing GAD67 mRNA expression in the amygdaloid complex of HAB mice, (B) increased expression of GAD67 mRNA in coronal brain sections of HAB mice, quantified in the BLA, CEA and MEA and (C) in the somatosensory cortex (CXS), hilus of the dentate gyrus (DG) and dentate granule cell layer (GCL), (D) correlation of ROD values of GAD67 mRNA with percentage open arm time in HAB mice and (E) NAB controls. Values in (A) and (B) are means±SEM, one-way ANOVA with Bonferroni *post hoc* test, *** $P<0.001$ (HAB $n=16$, NAB $n=19$); scale bars: 500 μm.

Since increased GAD67 mRNA expression may be caused by sustained neuronal activity due to chronically enhanced stress levels, we performed immunohistochemistry for ΔFosB, a marker of long-term adaptive changes in the brain. The number of FosB positive cells was significantly higher in the BLA of HAB mice than of NAB controls (Fig. 3A, B). In both, HAB and NAB mice, FosB labeling was co-localized with CaMKII labeling in the BLA (Fig. 3C), but was absent from GAD67 positive neurons (Fig. 3C), indicating chronic activation of principal neurons rather than of interneurons.

Expression of mRNA and immunoreactivity of $GABA_A$ and $GABA_B$ receptor subunits in the amygdala of NAB mice. Control NAB mice revealed a similar distribution of $GABA_A$ receptor subunit mRNAs in the amygdaloid nuclei as previously described for C57BL/6J mice (Heldt and Ressler, 2007a,b). High expression of $GABA_A$ receptor subunit $\alpha2$, $\alpha3$, $\beta3$ and $\gamma2$ mRNAs was observed in the BLA while $GABA_A$ receptor subunits $\alpha4$ and $\beta1$ were moderately and $\alpha1$, $\alpha5$, $\beta2$ and $\gamma1$ subunit mRNAs only weakly expressed. In the CEA, there was a predominant expression of $\alpha2$, $\alpha3$, $\beta3$ and $\gamma2$ mRNAs and a moderate expression of $\beta1$ and $\gamma1$ subunit mRNAs. The MEA was characterized by high expression of $GABA_A$ receptor subunit $\alpha2$, $\alpha3$, $\beta1$, $\beta3$, $\gamma1$ and $\gamma2$ mRNAs. Regarding $GABA_B$ receptors, GBBR1 subunit mRNA was equally high expressed in the BLA, CEA and MEA, whereas the mRNA for the GBBR 2 subunit was restricted mainly to the BLA and MEA.

Altered expression of $GABA_A$ receptor subunits in the amygdala of HAB mice. The expression profile of the different $GABA_A$ receptor subunit mRNAs in the amygdala of HAB mice was distinct from NAB controls. In HAB mice, there was a significant increase of $GABA_A$ receptor subunit $\beta1$ mRNA in all amygdala nuclei investigated (Table 2). In addition, the percentage of $\beta2$ mRNA was higher in the BLA, but not in the CEA or MEA of HAB mice (Fig. 4E–H, Table 2). Among the α-subunits only expression of $\alpha5$ mRNA was altered in the amygdala. Although basal levels of $\alpha5$ mRNA were already low throughout the amygdala of NAB controls, they were further reduced in the CEA and MEA of HAB mice (Table 2). Interestingly, in HAB mice the $\gamma1$ mRNA levels were reduced in all amygdaloid nuclei (Fig. 4A–D, Table 2), whereas those of $\gamma2$ mRNA were specifically increased in the BLA and CEA (Table 2). As shown in Fig. 5, $\beta2$ and $\gamma2$ subunit immunoreactivities were also clearly increased in the BLA of HAB mice.

No difference in the mRNA abundance of both $GABA_B$ receptor subunits GBBR1 and GBBR2 was detected in any of the amygdaloid nuclei of HAB mice compared to NAB controls.

Correlation of GAD expression in the amygdala with EPM behavior. To investigate whether increased expression of GADs may be associated with altered anxiety-like and/or locomotor-related behavior, we performed a correlational analysis of GAD67 and GAD65 mRNA levels with the behavioral data obtained from the EPM (percentage

Table 2. mRNA signals of amygdala nuclei in NAB and HAB mice

Subregions/ strains	$\alpha1$	$\alpha2$	$\alpha3$	$\alpha4$	$\alpha5$	$\beta1$	$\beta2$	$\beta3$	$\gamma1$	$\gamma2$	GAD67	GAD65	GBBR1	GBBR2
BLA														
NAB	100±3.9	100±3.2	100±2.3	100±3.2	100±4.2	100±5.1	100±4.6	100±3.1	100±6.4	100±3.0	100±3.3	100±3.8	100±7.9	100±2.7
HAB	95±3.7	109±4.7	106±2.5	105±4.8	102±7.5	120±6.0	129±5.9	96±4.8	74±7.2	115±4.4	135±5.0	126±5.1	106±3.3	94±5.9
P-value	0.340	0.134	0.081	0.387	0.783	**0.015**	**0.0005**	0.483	**0.009**	**0.007**	**<0.0001**	**0.0002**	0.491	0.357
CEA														
NAB	100±4.9	100±2.2	100±3.7	100±6.3	100±2.4	100±5.7	100±5.8	100±3.4	100±4.7	100±5.0	100±4.2	100±3.2	100±7.8	100±4.8
HAB	92±4.8	108±3.4	106±2.9	113±8.7	74±2.4	118±5.7	115±5.6	97±5.1	75±5.7	118±7.6	124±4.3	121±2.7	107±4.2	90±3.6
P-value	0.267	**0.047**	0.229	0.237	**<0.0001**	**0.032**	0.083	0.670	**0.002**	**0.046**	**0.0004**	**<0.0001**	0.433	0.139
MEA														
NAB	100±4.8	100±3.4	100±3.5	100±6.6	100±2.5	100±5.4	100±5.8	100±3.8	100±5.7	100±2.7	100±5.0	100±4.5	100±8.4	100±3.3
HAB	91±3.1	107±4.6	104±2.7	112±6.9	78±3.5	120±5.5	114±6.2	97±5.5	82±5.3	103±7.0	127±5.3	123±3.0	109±3.4	94±5.4
P-value	0.135	0.257	0.395	0.255	**<0.0001**	**0.013**	0.115	0.603	**0.024**	0.397	**0.0007**	**0.0003**	0.369	0.360

Data are mean±SEM of % of NAB. Numbers of animals: NAB, $n=19$; HAB, $n=16$.

open arm time and total distance traveled). Indeed, the expression of GAD67 mRNA in the BLA of HAB mice, was *positively* correlated with the percentage of open arm time ($r=0.58$, $P=0.019$; Fig. 1D), but not with the total distance traveled by the mice ($r=0.40$, $P=0.139$). In contrast, no correlation of GAD67 mRNA levels with percentage open arm time ($r=0.21$, $P=0.383$; Fig. 1E) or total distance traveled ($r=-0.005$, $P=0.983$) was observed in NAB mice. These findings imply that the up-regulation of the GAD67 may be a compensatory reaction to the high anxiety behavior phenotype of HAB mice. In contrast, when we included both groups, HAB and NAB mice, into the analysis there was a negative correlation of GAD65 and GAD67 mRNAs expression in all amygdaloid nuclei (BLA, CEA and MEA) with both, anxiety-like and locomotor-related behavior on the EPM (data not shown). These findings were not surprising, since they reflect largely the differences in the preselected phenotypes.

Altered $GABA_A$ receptor subunit mRNA expression correlated with anxiety- and motor activity-related behavior when both groups, HAB and NAB mice, were included. There was no correlation, however, at the level of the individual groups (not shown).

DISCUSSION

The present study identified prominent changes of the GABA system in the amygdala of HAB mice, an animal model of increased trait anxiety. These include markedly increased expression of the GABA synthesizing enzymes GAD65 and GAD67 together with increased expression of $GABA_A$ receptor subunit $\beta1$, $\beta2$, $\gamma2$ mRNAs, whereas subunit $\alpha5$ and $\gamma1$ mRNA levels were decreased.

In the BLA, most non-pyramidal neurons use GABA as a principal neurotransmitter and exert an inhibitory action on glutamatergic principal cells (Pare et al., 2003). This inhibition is predominantly generated through $GABA_A$ receptors that mediate either a phasic response when located within the synapse or maintain tonic inhibition at extrasynaptic sites (Semyanov et al., 2003, 2004). Spontaneous firing rates in the lateral and central amygdala are among the lowest in the brain, indicating potent tonic inhibition suppressing neuronal activity in the amygdala (Quirk and Gehlert, 2003). On the other hand, repeated stimulation of the BLA, leads to long-term synaptic facilitation and a behavioral state of chronic anxiety (Sajdyk and Shekhar, 2000).

GABA synthesizing enzymes (GAD65 and GAD67)

GAD65 and GAD67 are catalyzing GABA synthesis and their activity is tightly related to the tone of GABA neurons. They are abundantly expressed in the amygdala (Fig. 1A). In the BLA both enzymes are primarily expressed in interneurons (McDonald and Pearson, 1989) whereas in the CEA they are also contained in GABA-ergic projection neurons targeting forebrain and brain stem areas (LeDoux et al., 1988; Bourgeais et al., 2001). Impaired GABA-ergic transmission in GAD65 knockout mice results in increased anxiety-related behavior in the open field and elevated

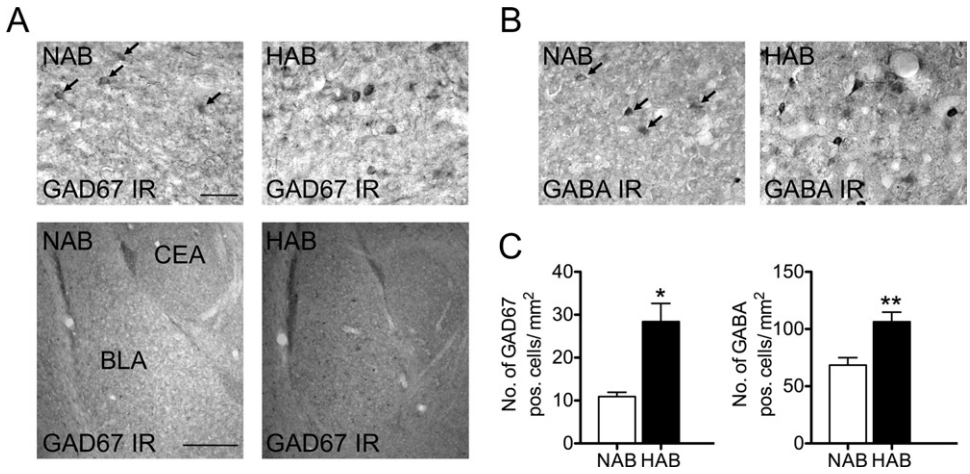

Fig. 2. GAD67 and GABA immunoreactivity (IR) in the amygdala of HAB and NAB mice. (A) Bright field photomicrographs of representative, matched sections demonstrating GAD67-IR labeling in the amygdala of HAB and NAB mice, higher magnification depicts increased number of GAD67-IR positive cells in the BLA of HAB mice compared to NAB controls, (B) bright field photomicrographs of GABA-IR labeling on representative, matched sections demonstrating an increased number of GABA-IR positive cells in the BLA of HAB mice, (C) quantification of GAD67-IR and GABA-IR positive cells in the BLA of HAB and NAB mice. Arrows in (A) and (B) depict GAD67-IR and GABA-IR positive cells in the BLA of NAB mice, respectively. Values in (C) are means±SEM, Mann–Whitney test, * $P<0.05$; ** $P<0.01$ (HAB $n=4$, NAB $n=4$), scale bars: low magnification 500 μm, high magnification 50 μm.

zero maze (Kash et al., 1999). Considerable evidence suggests that increased GABA-ergic transmission in the BLA is associated with reduced anxiety, while increasing the excitability of BLA projection neurons by inhibition of GABA transmission tends to be anxiogenic (Davis et al., 1994; Shekhar et al., 2003). In contrast, the high anxiety level in HAB mice is associated with an increased expression of GAD65 and GAD67. This increase in GABA synthesis may be part of a compensatory mechanism balancing the persistent over-stimulation of limbic brain areas, as indicated by the positive correlation of GAD mRNA expression with percentage open arm time. The increased expression of GADs may be driven by a sustained activation of the amygdala in HAB mice. On the other hand recent evidence suggests also excitatory actions of GABA in the basal amygdala (Woodruff et al., 2006), resulting in rapid activation of basolateral pyramidal neurons that may significantly contribute to increased anxiety-related behavior.

Activation of amygdala nuclei in response to stress has been well documented by increased expression of immediate early genes c-Fos and Zif-268 (Hoffman and Lyo, 2002; Singewald, 2007; Kovacs, 2008) and has also been

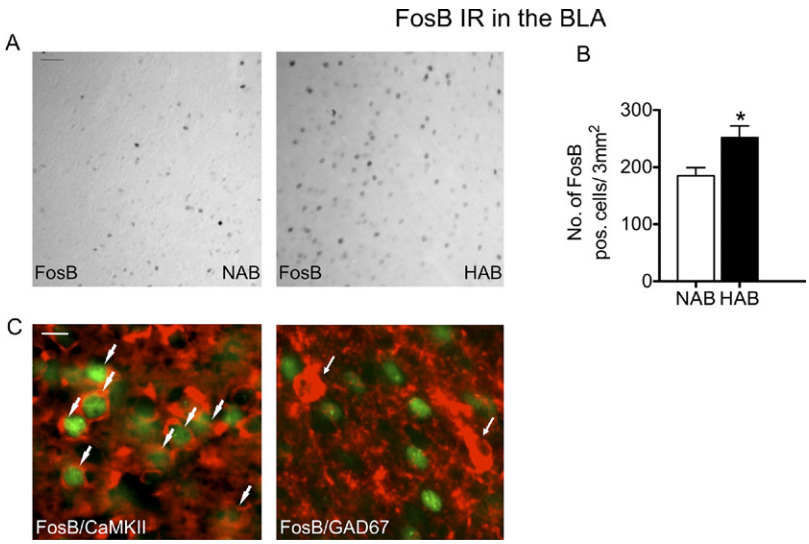

Fig. 3. FosB immunoreactivity in the BLA of HAB and NAB mice. (A) High magnification, bright field photomicrograph of representative, matched sections showing FosB positive cells in the BLA of NAB and HAB mice, (B) quantification demonstrating increased number of FosB positive cells in the BLA of HAB mice, (C) dual labeling immunofluorescence reveals colocalization of FosB (green) staining with (red) CaMKII but not with (red) GAD67-IR in the BLA, arrows in (C) show CaMKII positive and GAD67 positive cells, respectively. Values in (B) are means±SEM, Mann–Whitney test, * $P<0.05$ (HAB $n=4$, NAB $n=4$), scale bars: (A) 50 μm, (C) 10 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

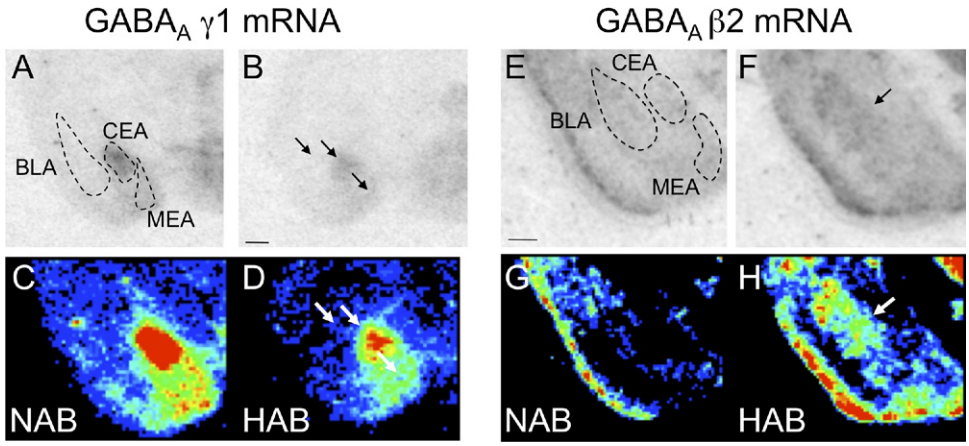

Fig. 4. $GABA_A$ receptor subunit mRNAs in the amygdala of HAB and NAB mice. (A+B) Film autoradiographs and (C+D) pseudocolor coded images of $GABA_A$ receptor $\gamma 1$ subunit mRNA in the amygdala of HAB (B+D) and NAB mice (A+C) are shown, arrows in (B) and (D) indicate reduced expression of $\gamma 1$ subunit mRNA in the amygdala of a HAB mouse, (E+F) film autoradiographs and (G+H) pseudocolor coded images of an *in situ* hybridization demonstrating increased $\beta 2$ mRNA in the BLA of HAB mice (F+H) compared to NAB controls (E+G), arrows in (F) and (H) indicate increased expression of $\beta 2$ mRNA in the BLA of a HAB mouse. Scale bars: (A–D) 500 μm; (E–H) 500 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

demonstrated in limbic areas of HAB mice (Muigg et al., 2009). Recently, Carta et al. (2008) reported increased expression of GAD67 mRNA in the CEA in response to stressful stimuli, reflecting long-term changes in neuronal activity (Carta et al., 2008). In the present study, the pronounced increases of GAD65 and GAD67 mRNAs in the amygdala of HAB mice may reflect chronic activation in amygdala nuclei due to consistently higher stress levels in these mice. This is supported by the increase in FosB-positive principal (glutamatergic) neurons in the BLA of HAB mice indicating chronic activation of the amygdala in the highly anxious HAB line already under baseline, non-stressful conditions.

This idea is further supported by a study in rats demonstrating that low exploratory activity on the EPM is associated with increased expression of GAD65/67 mRNA in the amygdala (Nelovkov et al., 2006). Notably, low exploratory behavior, besides reduced general motor activity may be related to increased anxiety, particularly when displayed in stressful situations like EPM exposure (Nelovkov et al., 2006). Our correlation analysis revealed lower anxiety in HAB mice correlating with high GAD67 mRNA levels (Fig. 1D). While this correlation provides informative evidence, further experiments, however, are needed to obtain a clear causal relationship between an altered GABA-ergic system and anxiety-related behavior in these mice.

$GABA_A$ receptor α subunits

Receptors containing the $\alpha 5$ subunit are often located extrasynaptically, predominantly mediating tonic inhibition (Scimemi et al., 2005; Zarnowska et al., 2009). Mice with reduced expression of $\alpha 5$ subunit in the hippocampus display facilitated trace fear conditioning, a special form of associative learning that requires hippocampal processing (Crestani et al., 2002). Although most of the investigations on $\alpha 5$ subunits have been performed in the hippocampus, a similar yet more anxiety-related role may be integrated in the amygdala. Since HAB mice displayed a decreased abundance of $\alpha 5$ subunit mRNAs in the CEA and MEA, a reduced inhibitory tonus in the amygdala of HAB mice may facilitate emotional activation and propagate a behavioral state of enhanced trait anxiety. Indeed, over-excitation after mild emotional challenge has been demonstrated recently in the amygdala of HAB compared to NAB mice (Muigg et al., 2009).

$GABA_A$ receptor β subunits

Given that β subunits contain part of the binding pocket for GABA, any change in their expression level is of physiological significance. *In situ* hybridization experiments indicate an increase of $\beta 1$ subunit mRNA in all amygdaloid nuclei investigated (BLA, CEA and MEA) and a selective increase of $\beta 2$ mRNA restricted to the BLA of HAB mice (Table 2). Recently, Heldt et al. (2007a) demonstrated an up-regulation of $\beta 2$ subunit in the amygdala after fear conditioning in mice after unpaired tone-shock exposure. They speculated that this reflected a state of hyperexcitability, similar to increased expression levels of β subunits seen in seizure-evoked animal models of epilepsy (Tsunashima et al., 1997; Pirker et al., 2003; Nishimura et al., 2005). Therefore also in HAB mice the selective increase in $\beta 2$ subunit mRNA may be due to over-excitation of the BLA as indicated by increased number of FosB positive neurons under baseline conditions.

$GABA_A$ receptor γ subunits

In HAB mice, we observed a reduction of the $\gamma 1$ subunit mRNA levels in all amygdaloid nuclei investigated (Table 2), whereas those of the $\gamma 2$ subunit were increased specifically in the BLA and CEA (Table 2). The mRNA levels for the δ subunit were below detection limit in the amygdala. The $\gamma 2$ subunit mediates postsynaptic clustering and is required for normal channel functioning

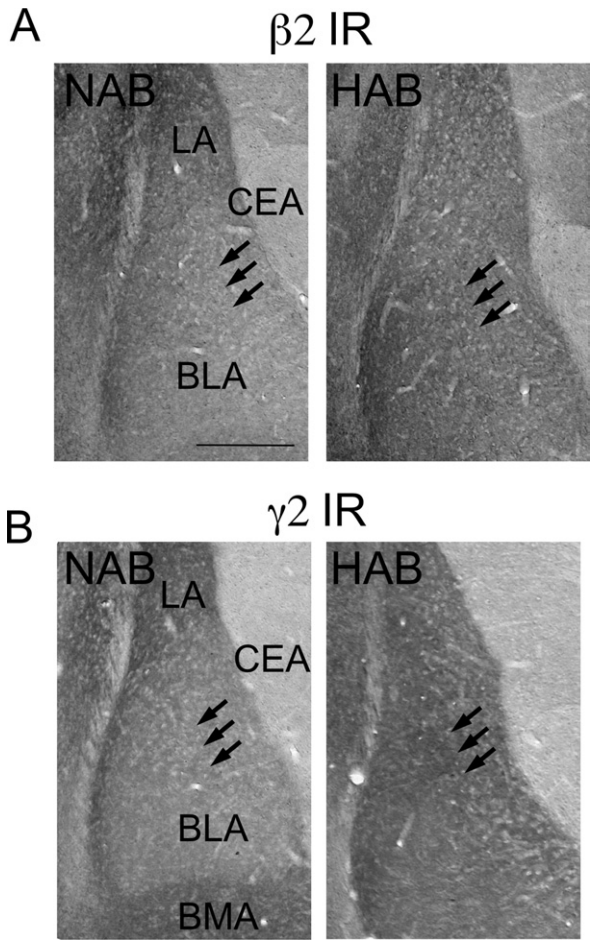

Fig. 5. Immunoreactivities for $GABA_A$ receptor $\beta 2$ and $\gamma 2$ subunits in the basolateral amygdala (BLA) of HAB and NAB mice. (A) Bright field photomicrographs of representative, matched sections demonstrating $\beta 2$ subunit-IR labeling and (B) $\gamma 2$ subunit-IR labeling in the BLA of HAB and NAB mice. Arrows indicate IR in the BLA. Note the increased labeling of the BLA compared to the lateral amygdala (LA) in HAB mice. BMA, basomedial amygdala. Scale bar: 500 μm.

(Crestani et al., 1999). Knockout mice, heterozygous for the $\gamma 2$ subunit, display increased anxiety-related behavior and reduced benzodiazepine binding sites. Although the total number of $GABA_A$ receptors is not altered, synaptic clustering is significantly reduced (Crestani et al., 1999). The increased expression of the $\gamma 2$ subunit mRNA in HAB mice may reflect an accelerated turnover of the $\gamma 2$ subunit containing $GABA_A$ receptors due to chronically high stress conditions and may indicate a compensatory mechanism counteracting increased amygdala activity. Following EPM exposure, Chacur et al. (1999) observed increased [^{3}H]-flunitrazepam but not [^{3}H]-muscimol binding specifically in the CEA and BLA, indicating a higher sensitivity of $GABA_A$ receptors to benzodiazepines although the total number of receptors may not be altered (Chacur et al., 1999).

Increased sensitivity to the anxiolytic effect of diazepam is generally observed in rodents with low explorative activity and changes in the subunit composition of $GABA_A$ receptors due to altered subunit expression may be an attractive explanation for this phenomenon (Liebsch et al., 1998; Bert et al., 2001; Nelovkov et al., 2006). In the present study, also HAB mice exhibit low explorative activity during EPM stress, presumably related to the high trait anxiety of these mice. Thus, the high sensitivity of HAB mice to the anxiolytic effect of benzodiazepines (Kromer et al., 2005) may, at least in part, be related to the higher number of $\gamma 2$ containing $GABA_A$ receptors, known to mediate the action of benzodiazepines in the amygdala.

Interestingly, whereas $\gamma 2$ subunits are increased in the amygdala of HAB mice, $\gamma 1$ subunits, particularly enriched in the CEA and MEA, are significantly reduced in these mice (Table 2). Esmaeili et al. (2009) recently demonstrated that GABA-ergic synapses formed by lateral inputs to the CEA, probably originating from intercalated cell masses, contain $\gamma 1$ subunits (Esmaeili et al., 2009). These connections are important for extinction of conditioned fear (Likhtik et al., 2008). Deficits in extinction learning have been described for HAB rats (Muigg et al., 2008) and mice (Yen et al., unpublished observation) and could be associated with decreased $\gamma 1$ subunit expression.

CONCLUSION

We demonstrated differential alterations of the GABA-ergic system in the amygdala of HAB mice, a model of pathological trait anxiety. Expression of GAD65 and GAD67 was highly up-regulated on mRNA as well as on protein level, suggesting enhanced GABA synthesis and release. Notably the increases in GAD65/67 and $\gamma 2$ subunit expression, but also that of subunits $\beta 1$ and $\beta 2$, point towards a facilitated GABA transmission, aiming to compensate the high-anxiety state of HAB mice. On the other hand, the reduced expression of the $\alpha 5$ subunit in the CEA and MEA may be part of the molecular mechanisms leading to the anxious phenotype of HAB mice. A reduced number of $\alpha 5$ subunit containing receptors may cause reduced tonic inhibition, favoring activation of the amygdala even under non-stressful conditions as illustrated by the increase in FosB expression. Both mechanisms may be causatively related to the compensatory increase in GAD65 and GAD67 expression.

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