GAL_3 *receptor* KO mice exhibit an anxiety-like phenotype

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Edited by Leslie Lars Iversen, University of Oxford, Oxford, United Kingdom, and approved April 1, 2014 (received for review October 8, 2013)

The neuropeptide galanin (GAL) is widely distributed in the central and peripheral nervous systems. It is a modulator of various physiological and pathological processes, and it mediates its effects via three G protein-coupled receptors ($GAL_{1–3}$ receptors). A role for GAL as a modulator of mood and anxiety was suggested, because GAL and its receptors are highly expressed in limbic brain structures of rodents. In recent years, numerous studies of animal models have suggested an involvement of GAL and GAL_1 and GAL_2 receptors in anxiety- and depression-related behavior. However, to date, there is sparse literature implicating GAL_3 receptors in behavioral functions. Therefore, we studied the behavior of *GAL_3 receptor*-deficient (*GAL_3*-KO) mice to elucidate whether GAL_3 receptors are involved in mediating behavior-associated actions of GAL. The *GAL_3*-KO mouse line exhibited normal breeding and physical development. In addition to behavioral tests, phenotypic characterization included analysis of hematology, amino acid profiles, metabolism, and sudomotor function. In contrast to WT littermates, male *GAL_3*-KO mice exhibited an anxiety-like phenotype in the elevated plus maze, open field, and light/dark box tests, and they were less socially affiliated than WT animals to a stranger mouse in a social interaction test. In conclusion, our data suggest involvement of GAL_3 receptors in GAL-mediated effects on mood, anxiety, and behavior, making it a possible target for alternative treatment strategies for mood disorders.

galanin receptor | serotonin | Gcat variant 2

Thirty years ago, Tatemoto et al. (1) isolated the neuropeptide galanin (GAL), a 29-aa (30-aa in humans) peptide, from porcine intestine. The peptide is highly conserved throughout evolution and found in many other species. GAL is widely distributed in the CNS and peripheral nervous system, and it has a variety of biological and physiological functions, ranging from energy homeostasis, reproduction, and feeding to cognition and learning (2). In the murine brain, GAL mRNA is extensively expressed in the hypothalamic and brainstem areas. The highest expression levels were observed in the preoptic, periventricular, and dorsomedial hypothalamic nuclei; bed nucleus of the stria terminalis (BNST); medial and lateral amygdala; locus coeruleus; and nucleus of the solitary tract (3). Furthermore, GAL coexists with the serotonin and norepinephrine systems in the rodent brain and acts as an inhibitory neuromodulator of norepinephrine, serotonin, dopamine, glutamate, and acetylcholine function (4). The expression pattern and neuromodulatory functions of GAL suggest a role for this neuropeptide in mood disorders like anxiety and depression. Accordingly, administration of GAL via the intracerebroventricular (i.c.v.) route or into the dopaminergic ventral tegmental area induced depression-like behavior in the rat forced swim test (FST) (5, 6). Several studies in GAL-overexpressing transgenic mice reported an increased depression-like behavior in the FST (7, 8) but found no differences in anxiety-related behavior under baseline conditions (7, 9). Holmes et al. (9) suggested, however, that GAL might have protective effects during periods of elevated stress, because GAL overexpression counteracted anxiogenic effects evoked by noradrenergic stimulation.

To date, three GAL receptors ($GAL_{1–3}$ receptors) have been identified, and they are all members of the G protein-coupled receptor superfamily. The GAL receptor subtypes have substantial differences in their functional coupling and signaling activities, contributing to the diverse effects of GAL (2). In the CNS, GAL_1 and GAL_2 receptors are detected in the BNST, amygdala, hippocampus, hypothalamus, dorsal raphe nucleus, locus coeruleus, dorsal root ganglia, and thalamus. *GAL_1 receptor* is additionally expressed in the brainstem (medulla oblongata and lateral parabrachial nucleus) and in the dorsal horn of the spinal cord, and *GAL_2 receptor* expression is further found in the cerebral cortex, cerebellum, and spinal cord. Expression of *GAL_3 receptor* in the CNS is more limited, with mRNA being preferably detected in the hypothalamus (10, 11). This differential localization of the three GAL receptors in the brain, as determined by in situ hybridization, suggests that different functions of GAL might be mediated by individual receptor subtypes. Evidence from animal models indicates that all three GAL receptor subtypes are involved in functional processes related to anxiety and depression. Stimulation of the GAL_1 receptor with selective ligands results in a depression-like

Significance

In the modern world, stress-related diseases, including depression and anxiety disorders, are rapidly increasing. Neuropeptides are important modulators of these diseases. The neuropeptide galanin (GAL) has already been implicated in anxiety- and depression-related behaviors, but the relevant receptor subtypes remain to be elucidated. In the present work, we are the first, to our knowledge, to examine the role of the GAL_3 receptor in anxiety- and depression-related behaviors in *GAL_3 receptor*-deficient mice. We provide evidence that this receptor subtype is involved in stress-related diseases, and we propose this receptor as a target for alternative treatment strategies for mood disorders.

Author contributions: R.L., G.S., P.H., and B.K. designed research; S.M.B., A.F., F.L., B.S.H., M.D., F.R., A.A.L., J.J.V., X.N., and G.S. performed research; J.A.M. and R.L. contributed new reagents/analytic tools; S.M.B., A.F., M.D., J.A.M., J.J.V., G.S., P.H., and B.K. analyzed data; and S.M.B., A.F., X.N., G.S., P.H., and B.K. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1318066111/-/DCSupplemental.

phenotype (12), whereas KO of this receptor in mice elicits increased anxiety-like behavior in the elevated plus maze (EPM) test but not in the light/dark (L/D) exploration, emergence, or open field (OF) tests (13). Consistent with an antidepressant-like effect of GAL_2 receptor signaling (14), Lu et al. (15) observed a depressive-like phenotype in *GAL_2 receptor*-deficient (*GAL_2*-KO) mice but found no GAL_2 receptor-mediated effects on anxiety-related behavior. GAL_3 receptor stimulation was suggested to induce a depression-like profile because decreased immobility and increased swimming in the rat FST were observed after treatment with the nonpeptidergic GAL_3 receptor-selective antagonist 1-phenyl-3-[[3-(trifluoromethyl)phenyl]imino]-1*H*-indol-2-one (SNAP 37889). Furthermore, SNAP 37889 induced anxiolytic-like behavior in the social interaction test (SIT) (16). In fact, GAL_3 receptor-selective antagonists like SNAP 37889 or SNAP 398299 and others (16, 17) have been and are still being developed to treat depressive disorders and/or anxiety. However, to our knowledge, any involvement of GAL_3 receptors in anxiety- or depression-related behavior was not previously verified in *GAL_3 receptor*-deficient (*GAL_3*-KO) animals. Therefore, we investigated behavior in a novel *GAL_3*-KO mouse line to elucidate whether the GAL_3 receptor is involved in mediating behavior-associated actions of GAL. Furthermore, for phenotypic characterization, analysis of *GAL_3*-KO animals included hematology, metabolism, and sudomotor function measurements.

Results

Phenotypic Analysis of *GAL_3*-KO Mice. *GAL_3*-KO mice were produced by targeting both coding exons of the *GAL_3 receptor* by homologous recombination (https://beta.infrafrontier.eu/sites/infrafrontier.eu/files/upload/public/lexicon/combined_lexicon_data/LEXKO-230-treeFrame.html) (Fig. S1*A*) and then backcrossing the mutant on a C57BL/6 background for at least seven generations. *GAL_3*-KO mice were viable and fertile, reproduced normally, and could not be distinguished from their non-homozygous siblings in appearance and general behavior. To investigate possible changes in brain development, we performed immunohistochemistry for neuronal nuclei (NeuN; a marker for adult neurons), calcium/calmodulin kinase II (CamKII), and GABA in the basolateral amygdala and hippocampus (areas involved in the integration of emotions) and in the hypothalamus, the area with the highest *GAL_3 receptor* expression (Fig. S2). We counted NeuN- and GABA-immunoreactive neurons in the basolateral amygdala (250-μm area) at a magnification of 200×. There were 496 ± 27 and 486 ± 7 ($P = 0.74$) NeuN-positive neurons and 95 ± 10 and 98 ± 10 ($P = 0.83$) GABA-immunoreactive neurons per 250 μm^2 in *GAL_3*-KO and WT mice, respectively ($n = 3$ per group). Thus, neither the density of total (NeuN-positive) neurons nor that of GABA-immunoreactive neurons was significantly altered in the basolateral amygdala by the *GAL_3*-KO. Also, no difference in GABA, CamKII, and NeuN immunoreactivities in the hippocampus or the hypothalamus was observed by visual inspection (Fig. S2). Therefore, there is no indication that the *GAL_3*-KO results in a developmental phenotype.

For phenotypic characterization of *GAL_3*-KO animals, clinical hematology was performed to evaluate the status of blood cell components, including erythrocytes, leukocytes, and thrombocytes. Hematology parameters showed no significant difference between *GAL_3*-KO and WT mice (Fig. S3). The European Mouse Mutant Archive (EMMA) network reported increased cholesterol and triglyceride levels of male homozygous *GAL_3*-KO mice compared with sex-matched WT littermates (https://beta.infrafrontier.eu/sites/infrafrontier.eu/files/upload/public/lexicon/combined_lexicon_data/LEXKO-230-treeFrame.html). In our *GAL_3*-KO mice, which were backcrossed on the C57BL/6 background, we observed a similar trend of higher cholesterol and triglyceride levels compared with age-matched WT animals (Fig. S4). However, cholesterol and triglyceride levels of our mice were significantly lower than those reported by the EMMA network (https://beta.infrafrontier.eu/sites/infrafrontier.eu/files/upload/public/lexicon/combined_lexicon_data/LEXKO-230-treeFrame.html), but they are in the range of baseline levels reported for C57BL/6 mice by the EuroPhenome database (www.europhenome.org). Detailed genomic analysis revealed that the first exon of the GAL_3 receptor gene shares a 367-bp overlap with the last exon of an alternative splice variant of *glycine C-acetyltransferase* (2-amino-3-ketobutyrate–CoA ligase; Gcat) *variant 2* (Fig. S1 *A* and *B*). Murine Gcat variant 1 (UniProtKB O88986) (18) was reported to catalyze the reaction between L-2-amino-3-oxobutanoic acid and CoA to form glycine and acetyl-CoA during degradation of L-threonine to glycine. Compared with *Gcat variant 1*, splice *variant 2* uses an alternate 3′ exon and differs in the 3′ coding and 3′ UTR, resulting in a protein with a shorter and distinct C terminus with unclear relevance (Fig. S1*A*). Accordingly, no catalytic activities are known for Gcat variant 2. To elucidate if Gcat variant 2 could play a substantial role in amino acid metabolism, we compared the expression of these two variants in murine liver, spleen, lung, kidney, testis, and brain, and we found an average 2,300-fold lower expression of the alternative splice *variant 2* compared with *variant 1* (Fig. S5). Due to the overlap of the GAL_3 receptor gene with *Gcat variant 2* and the involvement of Gcat variant 1 in the degradation of L-threonine to glycine, we assessed if the KO of *Gcat variant 2* in *GAL_3*-KO animals has any influence on the amino acid profiles in various tissues. Amino acid levels in murine sera, brain, and liver tissues did not differ significantly between WT and transgenic animals (Fig. S6).

Behavioral Phenotype of *GAL_3*-KO Mice. We compared the behavior of male *GAL_3 receptor* mutant mice and WT animals using a behavioral test battery to evaluate anxiety and depression.

EPM test. Anxiety-related behavior of WT and *GAL_3*-KO mice was assessed with the EPM test, in which the time spent on the open arms and the number of entries into the open arms were taken as established indices of anxiety, because they are inversely related to the level of anxiety in the subject animals (19). The time spent on the open arm of the maze was expressed as a percentage of the total time spent on any arm during the 5-min test session. Consistent with a more anxious phenotype, *GAL_3*-KO mice spent significantly less time on the open arms of the maze (3.0 ± 0.7% vs. 10.0 ± 1.7%) (Fig. 1*A*) and, conversely, more time on the closed arms of the maze (77.9 ± 2.5% vs. 52.7 ± 7.7%) compared with WT mice (Fig. 1*B*). To assess locomotor activity in the EPM test, the total distance traveled on the open and closed arms and the total number of entries into any arm during the 5-min test session were analyzed. In contrast to the parameters of anxiety, neither the total distance traveled nor the total number of entries into any arm differed between WT and KO mice (Fig. 1 *E* and *F*).

L/D box test. The L/D box test was performed to examine anxiety-related behavior of WT and *GAL_3*-KO animals. The distance traveled and time spent in the light compartment were evaluated as parameters of anxiety and expressed as a percentage of the total distance traveled and the total time spent in the box during the 10-min test session, respectively. Furthermore, delay in entering the light compartment for the first time after having

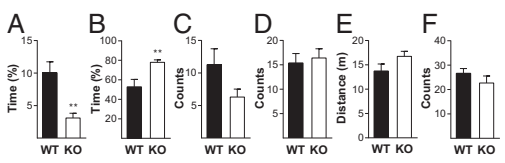

Fig. 1. Anxiety-like behavior in *GAL_3*-KO mice in the EPM test. Graphs show the time spent on the open (*A*) and closed (*B*) arms, number of entries into the open (*C*) and closed (*D*) arms, total distance traveled (*E*), and total number of entries into any arm (*F*) measured in male WT and *GAL_3*-KO mice. Time spent on the open and closed arms is expressed as a percentage of the 5-min test duration. Values represent mean ± SEM ($n = 8$–10). $^{**}P < 0.01$ vs. WT mice.

entered the dark compartment and transitions between the dark and light compartments were monitored as further indices of a more anxious phenotype. The distance traveled in both compartments was analyzed to study differences in general motor activity. Whereas the GAL_3-KO mice showed a trend toward an increased latency to enter the light compartment and a trend toward decreased transitions between the two compartments, the time spent (13.7 ± 2.5% vs. 30.8 ± 4.4%) and the distance traveled (18.5 ± 2.8% vs. 32.5 ± 2.4%) in the light compartment of the box were significantly decreased compared with those in WT animals (Fig. 2 *A–D*), which is consistent with a more anxious phenotype. In contrast, locomotion expressed as the total distance traveled did not differ between genotypes (Fig. 2*E*).

OF test. The OF test was used to examine the locomotor/exploratory, as well as anxiety-related, behavior of GAL_3-KO mice. The time spent in the central area, the number of entries into the central area, and the number of fecal boli shed were considered to be indices of anxiety. GAL_3-KO mice spent significantly less time in the central area of the field (16.3 ± 2.3% vs. 28.0 ± 3.7%) and visited the central area significantly less often (17.7 ± 2.2 vs. 27.8 ± 3.1) than WT mice (Fig. 3 *A* and *B*). In addition, the number of fecal boli shed during the test was significantly higher in the KO mice (3.4 ± 0.7 vs. 0.6 ± 0.3) (Fig. 3*D*). The total traveling distance was also significantly reduced in the GAL_3-KO mice compared with the WT mice (17.5 ± 3.6 m vs. 22.7 ± 5.5 m) (Fig. 3*C*).

SIT. As an estimation of social affiliation, the SIT was conducted. WT mice spent significantly more time in the compartment containing the stranger mouse (Fig. 4*A*), as well as in the immediate vicinity of the stranger mouse (Fig. 4*C*), compared with the time spent in or in the immediate vicinity of the empty compartment. WT mice also had significantly more visits to the compartment containing the stranger mouse and to the area surrounding the stranger mouse (Fig. 4 *B* and *D*) compared with the empty compartment. In contrast, GAL_3-KO mice did not display a significant preference for the compartment containing the stranger mouse, neither in terms of time spent in the compartment (Fig. 4*A*) or in the immediate vicinity (Fig. 4*C*) nor in terms of visits to the compartment or to the immediate vicinity of the stranger mouse (Fig. 4 *B* and *D*). When parameters were compared between GAL_3-KO and WT mice, no significant differences were observed.

Tail suspension test. Considered to mirror despair/depression-like behavior, the time of immobility was analyzed in the tail suspension test (TST), along with the time spent curling and swinging. Three animals of each genotype were disregarded from the analysis because of tail climbing behavior. In the TST, GAL_3-KO mice showed a trend toward a shorter period of immobility compared with WT animals ($P = 0.052$), suggesting a decreased depression-like phenotype of the GAL_3-KO mice (Fig. 5*A*). The time spent curling and swinging did not differ between GAL_3-KO and WT animals (Fig. 5 *B* and *C*).

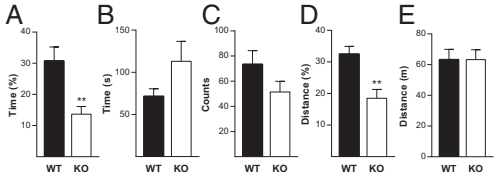

Fig. 2. Anxiety-like behavior in GAL_3-KO mice in the L/D box test. Graphs show the time spent in the light compartment (*A*) and latency to enter the light compartment (*B*), number of transitions between the light and dark boxes (*C*), and distance traveled in the light compartment (*D*) and in the entire box (*E*), comparing male WT and GAL_3-KO animals. Distance traveled and time spent in the light compartment are expressed as a percentage of the total traveling distance and the total 10-min test duration, respectively. Values represent mean ± SEM (n = 8–10). $^{**}P < 0.01$ vs. WT mice.

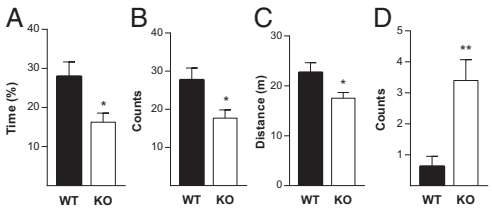

Fig. 3. Anxiety-like behavior in GAL_3-KO mice in the OF test. Graphs show the time spent in the central area (*A*), number of entries into the central area (*B*), total distance traveled (*C*), and number of fecal boli shed (*D*) measured in male WT and GAL_3-KO mice. Time spent in the central area is expressed as a percentage of the total 5-min test duration. Values represent mean ± SEM (n = 8–10). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. WT mice.

FST. The FST was performed to measure depression-like behavior by means of the time spent immobile, swimming, or climbing. No genotype-related differences in the duration of immobility, climbing, and swimming were detected (Fig. 5 *D–F*), which shows that depression-like behavior in the FST did not vary with genotype.

Analyses of Molecular and Compensatory Mechanisms of Behavioral Phenotype. The central neurotransmitter serotonin [5-hydroxytryptamine (5-HT)] is implicated in a variety of behavioral disorders, including depression and anxiety (20, 21). Because we observed an anxiety-like phenotype in the GAL_3-KO mice, we wanted to elucidate whether the 5-HT system is altered in GAL_3-KO animals. Therefore, we investigated expression levels of several members of the 5-HT system in GAL_3-KO and WT animals in microdissected brain regions (hypothalamus, thalamus, hippocampus, medial prefrontal cortex, striatum, and amygdala). Expression analysis included *tryptophan hydroxylase isoform 2* (22), which catalyzes the first and rate-limiting step of 5-HT biosynthesis; the *5-HT$_{1A}$*, *5-HT$_{1B}$*, *5-HT$_{2A}$*, and *5-HT$_{2C}$ receptors*, which are all implicated in emotional behaviors (23); and the 5-HT transporter *SLC6A4*. We observed similar expression levels of all examined 5-HT–related genes in WT and GAL_3-KO animals (Fig. S7), indicating that changes of the 5-HT system are not causing the observed behavioral phenotype. However, it could also be possible that compensatory mechanisms of the GAL system in the GAL_3-KO animals are associated with the observed anxiety-like phenotype. Therefore, we also analyzed expression levels of *GAL* and the GAL receptors in microdissected brain regions of GAL_3-KO and WT mice. Because expression of the GAL system did not differ with genotype (Fig. S7), compensatory mechanisms of the GAL system can be excluded.

Sudomotor Function. Recently, it was reported that GAL and GAL_3 receptor are involved in eccrine sweat gland secretion (24, 25). Because we found GAL_3 receptor to be involved in anxiety-related behavior, and because sweating is influenced by anxiety, we investigated the role of GAL_3 receptor in sudomotor function. After partial immobilization of mice, basal sweating at room temperature was intense at 10 min but declined significantly over time to ∼50% of initial values without discernible differences between WT and GAL_3-KO animals (Fig. 6). The cholinergic response was similar in WT and GAL_3-KO mice, where the maximal number of sweat glands reactive to pilocarpine was observed 10 min after pilocarpine injection, and it remained unchanged over time (Fig. 6). Thermal stimulation activated a lower number of sweat glands than pilocarpine, but there was no difference in the number of active sweat glands between GAL_3-KO mice and WT mice during heat exposure (Fig. 6). Immobilization activated ∼90% of the sweat glands reactive to pilocarpine, and stress-induced sweating was also similar in both GAL_3-KO mice and WT mice at any of the times tested (Fig. 6).

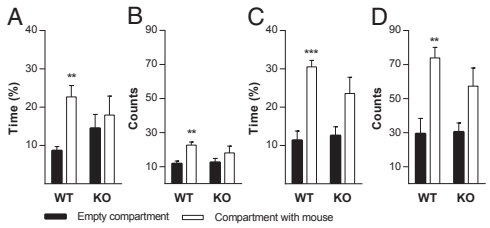

Fig. 4. Social affiliation in *GAL$_3$*-KO mice in the SIT. Graphs show the time spent in the chamber with the stranger mouse and in the empty chamber (*A*), number of entries into each chamber (*B*), time spent in the immediate vicinity of the grid enclosures (*C*), and number of entries into the immediate vicinity of the grid enclosures (*D*) measured in male WT and *GAL$_3$*-KO mice. Times spent in each chamber and in the immediate vicinity of each chamber are expressed as percentages of the total 5-min test duration. Values represent mean ± SEM (n = 8–9). $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. empty compartment.

Discussion

Accumulating data of behavioral studies in rodent models suggest that the neuropeptide GAL is involved in the pathophysiology of depression and anxiety disorders. Therefore, it is proposed as a therapeutic target. However, the roles of the GAL receptors in mediating depression- and anxiety-associated actions of GAL remain unclear. Although in vivo animal studies point to involvement of $GAL_{1–3}$ receptors in anxiety- or depression-related behavior, *GAL$_3$ receptor*-deficient animals have not yet been investigated in behavioral studies. In the present work, we studied the behavior and physiological phenotype of a novel *GAL$_3$ receptor*-deficient mouse line.

Mutant animals with targeted deletion of the GAL_3 receptor gene on the C57BL/6 background did not differ from WT animals in general health, reproductive performance, and hematology. We have to take into account that the *GAL$_3$*-KO animals also lack the C-terminal part of Gcat variant 2. Because of this double KO, we analyzed the expression of both Gcat splice variants in various tissues of WT animals and observed *variant 2* to be expressed an average of 2,300-fold less than *Gcat variant 1*. Because no functions or catalytic activities are known for variant 2, and because of its low expression profile, we do not believe that disruption of *Gcat variant 2* interferes with our results in any way. This assumption is supported by amino acid profiles of serum, liver, and whole brain, where no differences were observed between *GAL$_3$*-KO and WT mice. Thus, we are confident that an alteration of Gcat variant 2 mRNA does not influence the behavior of *GAL$_3$*-KO mice. Nevertheless, we cannot exclude the possibility that *Gcat variant 2* is more highly expressed in behavior-related neurons, which might then contribute to the observed phenotype.

Our behavioral studies show that, relative to WT animals, *GAL$_3$*-KO mice exhibit increased anxiety-like behavior. GAL itself has been reported to induce anxiolytic, anxiogenic, or no responses depending on several factors, such as the site of administration, behavioral paradigm used, and species tested (26). Anxiogenic-like actions of GAL were induced in the punished drinking test following microinjections of the peptide into the rat amygdala (27). Furthermore, whereas administration of the GAL antagonist M40 into the lateral BNST had no effect on the baseline behavior of rats, M40 attenuated the anxiogenic-like effects of immobilization stress, suggesting that endogenous GAL released in the lateral BNST facilitates acute behavioral reactivity to stress (28). Furthermore, the release of GAL in the central amygdala was increased when the noradrenergic response to stress was amplified by administration of the α2-adrenoreceptor antagonist yohimbine. This effect led to attenuation of the anxiety-like behavioral response to acute stress, which could be blocked by the GAL antagonist M40 (29). In GAL-overexpressing transgenic mice, the proanxiety effects of yohimbine were absent, whereas the behavioral phenotype in the EPM, L/D box, and OF tests was unaltered under basal conditions. These observations led to the conclusion that GAL exerts its anxiolytic actions exclusively under stressful conditions, implicating high noradrenergic activation (9). Also, i.c.v. administration of GAL caused an anxiolytic-like action in the highly stressful Vogel punished drinking test in rats (30), whereas i.c.v. administration of GAL in mice failed to evoke anxiolytic-like effects in the EPM or L/D box tests (31).

Studies in *GAL$_1$ receptor*-deficient (*GAL$_1$*-KO) mice showed increased anxiety-like behavior in the EPM test, whereas anxiety-related behavior under the less stressful test conditions of the L/D box, emergence, and OF tests was normal (13). These observations support GAL's anxiolytic actions, which preferentially take place under stressful conditions, with GAL acting at the GAL_1 receptor subtype. Moreover, *GAL$_2$*-KO mice displayed an anxiogenic-like phenotype that was again specific to the EPM test, reminiscent of that seen in *GAL$_1$*-KO mice (32).

Involvement of the GAL_3 receptor in anxiety has so far been studied with the help of the GAL_3 receptor antagonists SNAP 37889 and SNAP 398299 (16). In contrast to our findings in the KO mice, pharmacological antagonism of the GAL_3 receptor produced an anxiolytic effect, as seen in the Vogel conflict test and by an increased social interaction time in rats. The discrepant results between the genetic and pharmacological analyses of GAL_3 receptor action in anxiety may be due to several factors, including limited selectivity of the antagonists for the GAL_3 receptor and restricted access to the cerebral GAL_3 receptor involved in anxiety control. Furthermore, nonspecific effects of SNAP 37889 cannot be excluded. In the mouse brain, GAL_3 receptor mRNA and protein are prominently expressed in the periaqueductal gray, thalamus, hypothalamus, amygdala, hippocampal formation, and prefrontal cortex (10, 11), which are brain areas that play an important role in emotional regulation and stress sensitivity. It is likely that GAL modulates stress and anxiety through GAL_3 receptors located in the hypothalamus, either by directly modulating neuronal circuitries involving the amygdala, hippocampus, raphe nucleus, and locus coeruleus or by interfering with the hypothalamic–pituitary–adrenal axis (33). In addition, the effect of gene deletion during embryogenesis may be compensated for during brain development, which can result in an adult phenotype different from that of acute pharmacological blockade.

Despite their anxiogenic-like phenotype, *GAL$_3$*-KO mice displayed a trend toward decreased immobility time in the TST, which we interpret as a reduction in depression-like behavior. This observation is in line with evidence for a prodepressive effect of GAL (34, 35). In accordance with these observations, mice overexpressing GAL were shown to display increased immobility in the FST, indicative of enhanced depression-like behavior (7).

However, similar to the complex effects of GAL on anxiety, recent studies indicate an antidepressant-like effect of GAL as well (34). Thus, i.p. administration of the GAL agonist galmic, which displays low affinity for the GAL_1 receptor, and of the nonselective agonist galnon, which also acts via non-GAL receptors, induced a decrease in immobility time in the FST in rats (36, 37). There is also evidence for an antidepressant-like effect of GAL in men, as measured by the Hamilton Depression Rating Scale, and a suppression of rapid eye movement sleep after i.v. administration of GAL (38).

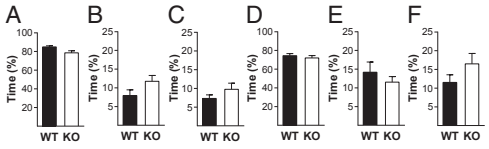

Fig. 5. Depression-related behavior in *GAL$_3$*-KO mice in the TST and FST. Graphs show the time of immobility (*A*), curling (*B*), and swinging (*C*) in the TST and the time of immobility (*D*), climbing (*E*), and swimming (*F*) in the FST in male *GAL$_3$*-KO animals compared with WT mice. Times are expressed as percentages of the 6-min test duration. Values represent mean ± SEM (n = 5–7).

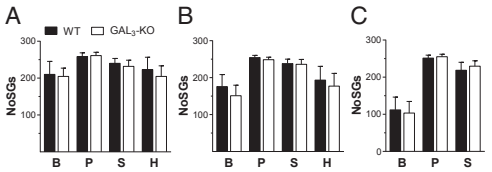

Fig. 6. Sudomotor function. Total number of secreting sweat glands (NoSGs) in the hind paw of WT and *GAL$_3$*-KO mice at room temperature [basal (B)] and following pilocarpine (P) injection, thermal stimulation [heat (H)], and immobilization [stress (S)] measured 10 (*A*), 15 (*B*), and 30 (*C*) min after the onset of tests. Values represent mean ± SEM (n = 9–10).

These contradictory results may be attributed to the differential actions of the three GAL receptor subtypes (34). Whereas stimulation of the GAL_2 receptor seems to be responsible for antidepressant-like effects (15, 39, 40), the inhibitory receptor subtypes GAL_1 (41) and GAL_3 seem to contribute to the prodepressive effects of GAL (42). Thus, in addition to its anxiolytic effect, the GAL_3 receptor antagonist SNAP 37889 exerts an antidepressant-like activity in rats (16). A different GAL_3 receptor antagonist, 3-(3,4-dichlorophenylimino)-1-(6-methoxypyridin-3-yl)indolin-2-one, was also able to exert antidepressant-like effects in rats and mice (17). An overlap of the behavioral phenotype, as seen for GAL_1 and GAL_3 receptors, could also be an indication of heterodimerization of GAL receptor subtypes. Indeed, heterodimerization of GAL_1 (43) and GAL_2 (44) receptors has been observed. A recent study suggested heterodimerization of GAL_1 and 5-HT_{1A} receptors (45), because several studies presented evidence for interactions of GAL with 5-HT_{1A} receptor functions (46, 47), leading us to propose GAL receptor heterodimerization in the context of behavior.

States like anxiety are closely connected to sudomotor function, because the main function of sweat glands is secreting sweat for thermoregulatory and emotional responses. *GAL* has been shown to be expressed in the human eccrine sweat gland cell line NCL-SG3, along with *GAL$_2$* and *GAL$_3$ receptors*. Furthermore, GAL has functions in eccrine sweat gland physiology, because application of GAL to NCL-SG3 cells increased short-circuit (Isc) currents. Application of SNAP 37889 inhibited the effect of GAL on Isc currents, indicating that the observed GAL effects are mediated via GAL_3 receptors (24). Based on these results, we hypothesized that sudomotor function differs between *GAL$_3$*-KO and WT mice. Similar to human sweating from palms and soles, sweating in the footpad of mice is predominantly emotional. The restraint required to perform the sweat imprint technique causes activation of almost all sweat glands in most mouse strains, as also shown in the present results. Although basal emotional sweating is initially intense, it declines quickly, in contrast to the more sustained response observed after complete immobilization at room temperature. Pilocarpine is a cholinergic agonist that directly stimulates sweat secretion by binding to sweat gland muscarinic M3 receptors (48). The number of pilocarpine-reactive sweat glands indicates a normal cholinergic response of sweat glands in *GAL$_3$*-KO mice. In addition, physiological stimulation by heating or stress produced similar sudomotor responses in *GAL$_3$*-KO and WT mice, without significant differences observed in the temporal evolution of the number of secreting sweat glands. These findings are consistent with the normal sudomotor response observed after cholinergic stimulation in *GAL*-KO mice (25), as well as after thermal stimulation in GAL-overexpressing mice, thus supporting the suggestion that GAL receptors may not be present in sudomotor sympathetic fibers or in sweat glands in the mouse (49). However, we recently reported that GAL seems to play a role in central thermoregulatory pathways or in regions implicated in stress responses (25). The present results suggest that the GAL_3 receptor subtype is not involved in either thermoregulatory or stress-induced sudomotor responses.

The primary findings of the present study are that *GAL$_3$*-KO mice show an anxiogenic-like phenotype compared with WT mice and that the GAL_3 receptor does not play a role in sweat gland function.

Materials and Methods

Behavioral Testing. Male mice were housed in groups of four or five per cage at the Institute of Experimental and Clinical Pharmacology, Medical University of Graz, under controlled temperature (21 °C) and a 12 h light/dark cycle (0530/1730 hours), and they were allowed to adapt to the test room at least 1 d before every experiment. The same cohort of animals was used in the behavioral test battery with the following test sequence: EPM test, TST, L/D box test, OF test, SIT, and FST. Experiments were performed over a total period of 7 wk. At the beginning of the behavioral studies, the mice were 12–14 wk old.

EPM test. The animals were placed in the center of a maze with four arms arranged in the shape of a plus (19). The maze consisted of a central quadrangle (5 × 5 cm), two opposing open arms (30 cm long and 5 cm wide), and two opposing closed arms of the same size but equipped with 15-cm high walls at their sides and the far end. The device was made of opaque gray plastic and elevated to 55 cm above the floor. The light intensity was 20, 30, and 5 Lux at the central quadrangle, on the open arms, and in the closed arms, respectively. At the beginning of each trial, the animals were placed on the central quadrangle facing an open arm. The movements of the animals during a 5-min test period were tracked by a video camera above the center of the maze and recorded with VideoMot2 software (TSE Systems). This software was used to evaluate the animal tracks and to determine the number of entries into the open and closed arms, the time spent on the open and closed arms, and the total distance traveled in the open and closed arms during the test session. Entry into an arm was defined as the instance when the center of the body of the mouse crossed the border to the arm. Locomotion was quantified by measuring the total distance traveled in the open and closed arms and the total number of entries into any arm during the 5-min test session. Anxiety-related behavior was deduced from the time spent on the open arms and the number of entries into the open arms.

L/D box test. The L/D box consisted of a cage [37 × 21 × 20.5 cm (length × width × height)] divided into two sections of equal size by a partition containing a door (4.5 × 6 cm) (TSE Systems) (50).The light compartment consisted of transparent walls and was brightly illuminated (300–400 Lux, 18.5 × 21 cm), whereas the dark compartment (1 Lux, 18.5 × 21 cm) was composed of black acrylic walls. Animals were placed in the light compartment facing the opening to the dark compartment, and locomotion and exploration of the animals were tracked by two external IR frames recording the light beam interruptions (counts) during a 10-min test period. Activity and time spent in the light compartment were taken as indicators of anxiety-like behavior.

OF test. The OF test apparatus consisted of a box (50 × 50 × 30 cm) that was made of opaque gray plastic and illuminated by 35 Lux at floor level (51). The ground area of the box was divided into a 36 × 36-cm central area and the surrounding border zone. Mice were individually placed in the center of the OF, and their behavior during a 5-min test period was tracked by a video camera positioned above the center of the OF and recorded with VideoMot2 software. This software was used to evaluate the time spent in the central area, the number of entries into the central area, and the total distance traveled in the OF.

SIT. The social interaction box consisted of a three-chambered apparatus [20 × 40 × 22 cm (length × width × height) for each of the compartments] that was made of a transparent Perspex cage, a special nonreflective gray-colored floor, and two grid enclosures (Ugo Basile) (52). For habituation, mice were individually placed in the central compartment, whereas entrance to the other compartments was blocked by two sliding doors [5 × 8 cm (width × height)]. In the consecutive test sessions, a control mouse was placed into one of the grid enclosures. The doors between the compartments were opened, allowing free access to all three chambers. Mouse behavior was tracked during a 5-min test period evaluating the time spent in the two compartments with grid enclosures, with one being empty and the other containing the stranger mouse, as well as the time spent in the immediate vicinity (5 cm) of the grid enclosures and number of entries into each.

TST. Each mouse was suspended by its tail with 2-cm wide strapping tape (Leukotape classic; BSN Medical S.A.S.) to a lever mounted at the top of a box [39 × 39 × 39 cm (length × width × height)]. Each trial took 6 min and was carried out at a light intensity of 15 Lux. The duration of immobility, swinging, and curling (53) was scored by a trained observer.

FST. Mice were individually placed in glass beakers (inner diameter of 13.5 cm, height of 19 cm, 2-L capacity) containing tap water at 25 °C. The water depth was 14 cm, which prevented the mice from touching the bottom of the beaker

with their paws or tail. Mice were tested for 6 min, and the time of immobility, swimming, and climbing was scored by a trained observer. Mice were considered immobile when floating passively in the water, performing only those movements required to keep their heads above the water level (54).

Additional Materials and Methods. Additional information on the ethics statement, experimental animals and housing, generation of GAL_3-KO mice, genotyping (Table S1 and S2), immunohistochemistry, expression analysis (Table S1 and S2), measurements of blood parameters, amino acid profiles, functional studies, and data analysis and statistics is provided in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Sabine Ebner, Karin Tomicek-Gründl, Sepideh Aminzadeh Gohari, Anneliese Bukovac, Marta Morell, Mónica Espejo, Xavier Belda, and Antonio Armario for their technical help and Sabine Schmidhuber for introduction and backcrossing of GAL_3-KO mice. We thank the European Commission (EC) Seventh Framework Programme (FP7) Capacities Specific Program-funded EMMA service project for providing B6;129S5-Galr3^{tm1Lex}/Orl mice. We also acknowledge the measurement of blood parameters by the Department of Laboratory Medicine, Paracelsus Medical University Salzburg. The study was supported by Centro de Investigación Biomédica en Red Enfermedades Neurodegenerativas (CIBERNED) funds, the Austrian Research Promotion Agency (822782/THERAPEP), EC FP7 (AIRGAL 274684), and the Paracelsus Medical University Salzburg (PMU-FFF E-10/11/059-LAN).

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