Depression-like behavior in rat: Involvement of galanin receptor subtype 1 in the ventral periaqueductal gray

Peng Wang[a,1], Hui Li[a], Swapnali Barde[b], Ming-Dong Zhang[b,c], Jing Sun[a], Tong Wang[a], Pan Zhang[a], Hanjiang Luo[a], Yongjun Wang[d], Yutao Yang[a], Chuanyue Wang[d], Per Svenningsson[e], Elvar Theodorsson[f], Tomas G. M. Hökfelt[b,2], and Zhi-Qing David Xu[a,2]

[a]Department of Neurobiology, Beijing Key Laboratory of Neural Regeneration and Repair, Beijing Laboratory of Brain Disorders (Ministry of Science and Technology), Beijing Institute for Brain Disorders, Capital Medical University, Beijing 100069, China; [b]Department of Neuroscience, Karolinska Institutet, SE-17177 Stockholm, Sweden; [c]Division of Molecular Neurobiology, Department of Biochemistry and Biophysics, Karolinska Institutet, SE-17177 Stockholm, Sweden; [d]Anding Hospital, Capital Medical University, Beijing 100088, China; [e]Center for Molecular Medicine, Department of Neurology and Clinical Neuroscience, Karolinska Institutet and Karolinska University Hospital, SE-17176 Stockholm, Sweden; and [f]Department of Clinical Chemistry and Department of Clinical and Experimental Medicine, Linkoping University, SE-58183 Linkoping, Sweden

Contributed by Tomas G. M. Hökfelt, June 16, 2016 (sent for review July 10, 2015; reviewed by Francesc Artigas, Marina R. Picciotto, Trevor Sharp, Rita J. Valentino, and Barry Waterhouse)

The neuropeptide galanin coexists in rat brain with serotonin in the dorsal raphe nucleus and with noradrenaline in the locus coeruleus (LC), and it has been suggested to be involved in depression. We studied rats exposed to chronic mild stress (CMS), a rodent model of depression. As expected, these rats showed several endophenotypes relevant to depression-like behavior compared with controls. All these endophenotypes were normalized after administration of a selective serotonin reuptake inhibitor. The transcripts for galanin and two of its receptors, galanin receptor 1 (GALR1) and GALR2, were analyzed with quantitative real-time PCR using laser capture microdissection in the following brain regions: the hippocampal formation, LC, and ventral periaqueductal gray (vPAG). Only Galr1 mRNA levels were significantly increased, and only in the latter region. After knocking down Galr1 in the vPAG with an siRNA technique, all parameters of the depressive behavioral phenotype were similar to controls. Thus, the depression-like behavior in rats exposed to CMS is likely related to an elevated expression of Galr1 in the vPAG, suggesting that a GALR1 antagonist could have antidepressant effects.

dorsal raphe | neuropeptide | siRNA | stress | transmitter coexistence

The indoleamine hypothesis of depression was formulated some five decades ago (1, 2), in parallel with the catecholamine hypothesis (3, 4), and has been supported by the success of selective serotonin reuptake inhibitors (SSRIs) for treatment of major depression (MD) (5). However, in a considerable number of cases, SSRIs are associated with side effects or lack of efficacy. For example, after treatment with SSRIs, only one-third of patients obtain full remission of symptoms (6). Therefore, there is an ongoing search for new medications targeting mood disorders, such as MD and anxiety. Here, neuropeptides and their receptors, the most diverse family of neurotransmitters in the brain (7), have been extensively explored (5, 8–17), including galanin and its three receptors.

Galanin is a 29-aa (30-aa in humans) neuropeptide (18) with a wide distribution in the rat brain (19, 20), where it is coexpressed with noradrenaline/norepinephrine (NA) in locus coeruleus (LC) neurons and with 5-hydroxytryptamine (5-HT) in dorsal raphe nucleus (DR) neurons (21–25). There are three galanin receptors, GALR1–GALR3, which all belong to the family of seven transmembrane-spanning, G protein-coupled receptors (26–29). They are found in many areas of the rat brain, as first shown with autoradiographic ligand-binding methodology (30, 31), and later with in situ hybridization (ISH)/quantitative real-time PCR (qPCR) (32–36). The galanin system has been associated with numerous physiological and pathophysiological functions (29), including depression- and anxiety-like behaviors.

There is early evidence from studies on the rat that central administration of galanin influences mood-related behavior in a region-specific way (37, 38), and also that galanin is prodepressive/anxiogenic, indicating a possible antidepressive/anxiolytic/antistress effect of galanin antagonists (23, 39–51). It has been suggested that this prodepressive/anxiogenic effect is mediated via the inhibitory GALR1 and that, in addition, galanin can have an antidepressive/anxiolytic action via GALR2 (23, 43, 46, 52–54). A recent clinical, genetic candidate study has reported an association of galanin and its three receptors with major depressive disorder (55).

In the present study, we have further explored the role of the galanin system in a rat model of depression based on exposure to chronic mild stress (CMS) (56, 57). The behavior was evaluated in the open field test (OFT), in the forced swim test (FST), and by monitoring sucrose consumption, as well as after treatment with the SSRI escitalopram. We used qPCR to monitor transcript levels of galanin, Galr1 and Galr2, in various laser-microdissected brain regions considered to be involved in depression-like behavior, including the hippocampal formation (HPC), the ventral periaqueductal gray (vPAG), and the LC. Because the results indicated an involvement of Galr1 in the vPAG, we analyzed the effect of intracerebral, parenchymal, lentivirus-mediated injection of Galr1 siRNA into the vPAG of CMS-exposed rats to determine whether or not there was a causal link between Galr1

Significance

The pathophysiology of depression remains unclear, but accumulated evidence implicates disturbances in monoaminergic transmission in the brain. Several studies suggest that members of the diverse family of neuropeptides may also be involved. In the rat, the neuropeptide galanin is coexpressed with noradrenaline and serotonin, and modulates the signaling of these neurotransmitters. Here, we explored a possible role of galanin and its receptors in a rat model of depression based on chronic mild stress using quantitative real-time PCR combined with viral-mediated delivery of galanin receptor 1 (Galr1) siRNA. Our results indicate involvement of the GALR1 receptor subtype in the ventral periaqueductal gray in depression-like behavior, possibly representing a novel target for antidepressant therapy.

Author contributions: P.W., T.G.M.H., and Z.-Q.D.X. designed research; P.W., H. Li, S.B., M.-D.Z., J.S., T.W., P.Z., H. Luo, Y.W., and E.T. performed research; P.W., H. Li, S.B., M.-D.Z., J.S., P.Z., H. Luo, Y.W., Y.Y., C.W., T.G.M.H., and Z.-Q.D.X. analyzed data; and P.S., T.G.H., and Z.-Q.D.X. wrote the paper.

Reviewers: F.A., Institut d'Investigacions Biomediques de Barcelona; M.R.P., Yale University School of Medicine; T.S., University of Oxford; R.J.V., Children's Hospital of Philadelphia; and B.W., Drexel University College of Medicine.

The authors declare no conflict of interest.

[1]Present address: Department of Anatomy and Histoembryology, Logistics University of People's Armed Police Force, Tianjin 300309, China.

[2]To whom correspondence may be addressed. Email: zhiqingx@ccmu.edu.cn or tomas.hokfelt@ki.se.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1609198113/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1609198113

expression in the vPAG and depression-like behaviors. We also compared the distribution of Galr1 in the vPAG with a number of transmitter-related markers using riboprobe ISH, and phenotyped the galaninergic input to the vPAG with immunohistochemistry. Some of these results were presented at the 2011 Society for Neuroscience meeting (58).

Results

CMS Rats Show Depression-Like Behaviors. Behavioral tests were carried out on three groups of rats (n = 6–8) according to the schedule shown in Fig. 1. To obtain baseline values, the rats were exposed to both the sucrose test and the OFT before CMS, and there was no difference in sucrose intake or in locomotor activity, as measured by horizontal and vertical activity scores. After the 8-wk stress schedule, the CMS group showed decreased sucrose preference (Fig. 2*A*) and a significant decrease of both horizontal and vertical scores (Fig. 2 *B* and *C*). In the FST, the CMS group exhibited a significant increase in immobility time (Fig. 2*D*) and a decrease in climbing time (Fig. 2*E*). All depression-like behaviors were significantly improved after escitalopram treatment (Fig. 2 *A*–*E*). The body weight of the CMS group was significantly lower than controls from the second week after exposure and onward (Fig. S1).

Galr1 Transcript Levels Show a Region-Selective Increase. We examined galanin, Galr1, and Galr2 mRNA levels in the cornu ammonis 1 (CA1), CA3, dentate gyrus (DG), vPAG, and LC following laser capture microdissection (Fig. 3 *A*–*J*) from rats of CMS and control groups using qPCR. The only selective change observed was an increase in Galr1 mRNA levels in vPAG (Fig. 3 *K*–*M*). Moreover, correlation analysis revealed a significant negative correlation between Galr1 mRNA levels in this region and sucrose preference in CMS rats (r = –0.619, P = 0.032; Fig. 3*N*).

Galr1-siRNA Reduces Galr1 mRNA Levels in vPAG. To determine whether increases in Galr1 levels in vPAG could be causally related to the change in behavior following CMS, we generated a lentiviral vector expressing an siRNA targeting Galr1. To evaluate the efficiency of this lentiviral siRNA construct to suppress Galr1 expression, qPCR and Western blotting analysis were performed in HEK293A cells after cotransfection of the lentiviral siRNA constructs with a myc-tagged Galr1 plasmid. The knockdown efficiency of the siRNA at the level of mRNA and protein of Myc-Galr1 was about 80% and 90%, respectively (Fig. S2). No significant effects were observed after administration of scrambled probes.

Rats were divided into three groups: CMS-Galr1-siRNA, CMS-Galr1-scramble-siRNA, and control. Control rats were left undisturbed in their home cages throughout the 8 wk, except for general handling once daily (5 min each day). CMS rats exposed to stressors randomly for 8 wk were divided into two groups, one group injected with lentivirus-based Galr1 siRNA into vPAG and the other group injected with scrambled siRNA into vPAG (Fig. S3). In vivo experiments combining laser microdissection and RT-qPCR showed that Galr1 mRNA levels in the CMS-Galr1-scramble group were increased (twofold) in the vPAG compared with the control group and that this increase was completely blocked by Galr1-siRNA treatment (Fig. 4*A*).

Galr1 Knockdown Attenuates Depressive-Like Behaviors. Before siRNA infusion, CMS groups showed a significant decrease of horizontal and vertical activity scores in the OFT compared with the control group (Fig. 4 *B* and *C*). After siRNA injection, these scores had recovered for the CMS-Galr1-siRNA group compared with controls, whereas CMS-Galr1-scramble-siRNA infusion had no effect (Fig. 4 *B* and *C*).

In the FST, CMS groups showed a significant increase of immobility time (Fig. 4*D*) and a decrease of climbing time (Fig. 4*E*) compared with controls. After siRNA injection, the immobility and climbing time for the CMS-Galr1-siRNA group were similar to the control group, but the scramble siRNA had no effect (Fig. 4 *D* and *E*).

Sucrose preference was decreased in the CMS group compared with controls (Fig. 4*F*). Such a decrease was not observed after infusion of Galr1 siRNA, whereas it remained decreased after scramble siRNA (Fig. 4*F*). In a control experiment, it also remained decreased in the CMS group after infusion of Galr1 siRNA into the ventral tegmental area (VTA) (Fig. S4).

Relation of Galr1 to Other Neurotransmitters. A Galr1 signal was detected in the entire vPAG, both in the ventral and dorsal aspects (Fig. 5*A*). Ventrally, a bilateral column of neurons with a strong signal could be discerned in the lateral parts of the DR (DRL) extending medially into the dorsal part of the DR (DRD) and laterally into the ventrolateral PAG (VLPAG), essentially avoiding the midline, however, and with only few cells in the ventral part of the DR (DRV) (Figs. 5*A* and 6 *A*–*C*). Monitoring the ISH signal, a significant increase of Galr1 mRNA levels was found in the vPAG in the CMS group compared with controls (Fig. 5*B*). Moreover, examining adjacent/semiadjacent sections of naive rats (no double labeling), Galr1 mRNA showed a limited overlap with the tryptophan hydroxylase 2 (Tph2) transcript (Fig. 6 *A* and *D*). However, glutamic acid decarboxylase (Gad) (Fig. 6 *B* and *E*) and the vesicular glutamate transporter 2 (Vglut2) (Fig. 6 *C* and *F*) mRNAs were both more abundant and "intermingled" with the Galr1 transcript.

Galanin and NA Innervation of the vPAG. To explore the possible origin of the galanin innervation of the vPAG, and thus the source of galanin potentially acting on GALR1 in the vPAG, we double-stained sections with antiserum against galanin and dopamine β-hydroxylase (DBH; the enzyme synthesizing NA and adrenaline/epinephrine) and triple-stained the sections with serotonin antibodies. At the levels studied, both galanin$^+$ and DBH$^+$ nerve terminals extended from the medial region of the serotonergic DR to the VLPAG, and, except for the midline areas, all these regions harbor Galr1$^+$ neurons. However, there were clearly varying densities, with the galanin$^+$ terminals being most abundant in the region around the aqueduct and in the DRL, extending into the

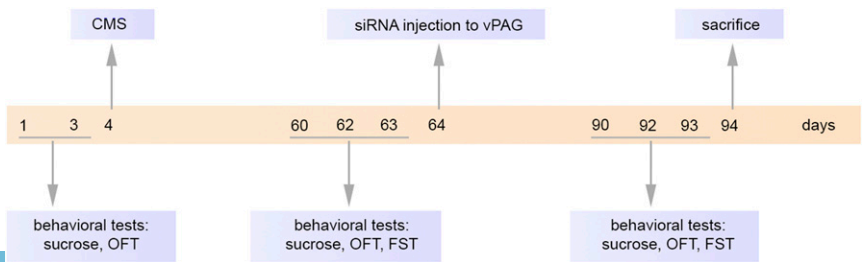

Fig. 1. Time schedule for the experiment. Note that the sucrose test is always followed by 1 d without testing. sucrose, sucrose intake test.

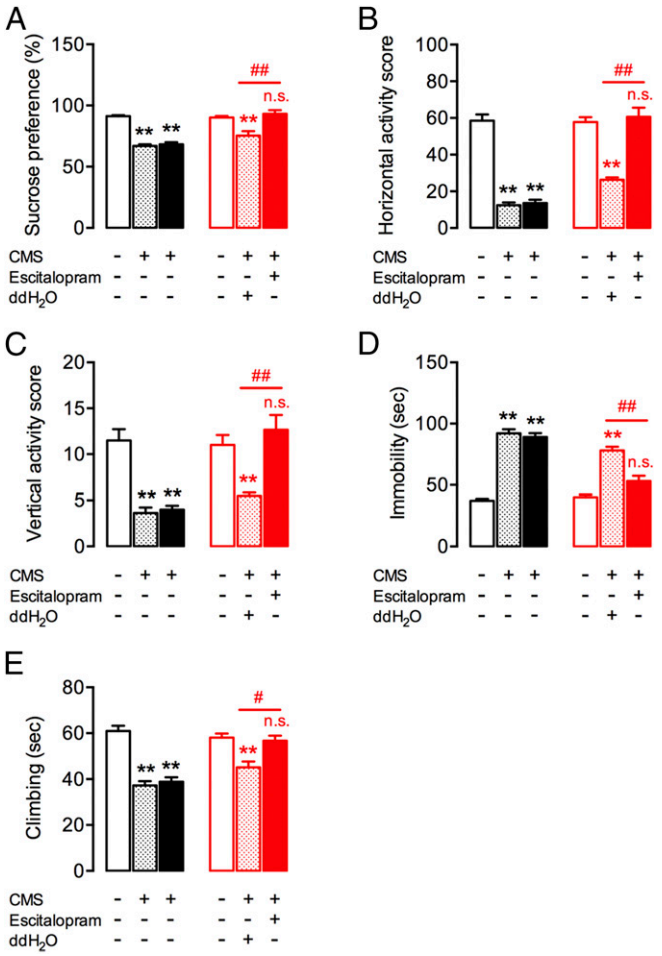

Fig. 2. Rats exposed to CMS show depression-like behaviors, and effects of escitalopram. (*A–C*) After the 8-wk stress schedule, the CMS group shows a significant decrease of sucrose intake and horizontal and vertical activity scores, which are rescued by escitalopram treatment [sucrose intake $F(2, 38) = 49.98$, $P < 0.0001$ and $F(1, 38) = 43.63$, $P < 0.0001$; horizontal activity $F(2, 38) = 98.77$, $P < 0.0001$ and $F(1, 38) = 77.78$, $P < 0.0001$; vertical activity $F(2, 38) = 24.09$, $P < 0.0001$ and $F(1, 38) = 17.64$, $P = 0.0002$]. (*D* and *E*) Eight-week CMS procedure results in a significant increase of immobility time and decrease of climbing time, and these changes are not seen after escitalopram treatment [immobility time $F(2, 38) = 141.7$, $P < 0.0001$ and $F(1, 38) = 44.33$, $P < 0.0001$; climbing time $F(2, 38) = 40.80$, $P < 0.0001$ and $F(1, 38) = 19.72$, $P < 0.0001$]. There is a significant CMS treatment interaction for the variances in *A–E*. Data are represented as mean ± SEM ($n = 6–8$ in each group). Two-way ANOVA, followed by the Bonferroni multiple comparisons test, is applied for the analysis. $^{**}P < 0.01$ vs. control group; $\#P < 0.05$, $\#\#P < 0.01$ vs. vehicle group (ddH_2O). n.s., no significant difference between the control group and CMS group treated with escitalopram.

VLPAG. In contrast, the highest density of DBH^+ fibers was observed in the VLPAG, presumably at least partly representing adrenaline terminals (59) (Fig. S5 *A–D*). Thus, the high-density "patches" did not overlap. We carefully examined several regions (boxes in Fig. S5 *B* and *D*) for coexistence: 5-HT cell body-rich regions (DRD) (Fig. S5 *E–H*), as well as more lateral areas with few 5-HT cell bodies (Fig. S5 *I–L*) and additional lateral areas without 5-HT cell bodies (Fig. S5 *M–P*) (both VLPAG). However, in all three regions, fibers harboring both galanin and DBH were rare, but some "cases" with coexistence were in fact encountered (Fig. S5 *Q–S*). It should, however, be emphasized that we have only carefully analyzed the illustrated levels, and coexistence may be more common in other subregions of the DR complex. In contrast, in cortical areas included in the same sections, almost all $galanin^+$ terminals were also DBH^+ (Fig. S5 *T–V*). Of note, no $galanin^+$ cell bodies were observed, because the rats had not been pretreated with the axonal transport-inhibiting molecule colchicine.

Discussion

The main results of the present study, based on an established rat model of depression (CMS), are as follows. First, Galr1 mRNA is significantly and selectively up-regulated in neurons in the vPAG. This up-regulation was shown with qPCR and confirmed by ISH. Notably, we found Galr1 $mRNA^+$ neurons distributed in the entire PAG (dorsal PAG and vPAG) and at all rostrocaudal levels, expanding and complementing previous mapping studies (32, 34). Interestingly, up-regulation of Galr1 selectively in the vPAG has also been observed in a rat traumatic (mild blast) brain injury model (60), further supporting Galr1 expression in vPAG being mood-related, perhaps to posttraumatic stress disorder in the latter case (61). Second, the depression-like symptoms were counteracted by Galr1 knockdown via microinjection of lentivirus-based Galr1 siRNA, supporting involvement of this receptor in the depression-like behavior in the CMS model. In a previous study using the same depression model, we observed, among other things, decreased levels of galanin mRNA in the dorsomedial hypothalamic nucleus and the lateral hypothalamic area vs. no change in the DR or LC (62); the latter is in agreement with the present study. Third, the 5-HT reuptake inhibitor escitalopram counteracted the behavioral effects induced by CMS, and therefore the question arises of whether this action is mediated via the galanin system. Interestingly, it has been shown that chronic antidepressant treatment increases galanin mRNA levels (23, 63) and GALR2-binding sites (23) in the DR, whereas no effect was observed on GALR1 expression in this nucleus (63). In a recent study, the selective NA reuptake inhibitor venlaflaxine failed to alter levels of galanin system transcripts in normal rats (64). It would be of interest to analyze whether escitalopram would influence GALR1 expression in the CMS model.

Taken together, these results suggest that activation of GALR1 in the rat vPAG has a prodepressive effect. However, studies on mice with genetically deleted Galr1 (Galr1-KO) showed no effect in the tail suspension test, pharmacological manipulations did not alter the depression profile (65), and such mice showed increased anxiety-like behavior in the elevated plus-maze (66). It should be noted that the 5-HT neurons in the mouse DR, in contrast to the rat DR, do not express galanin (67–70) and that the KO mice, of course, lack this receptor throughout life and in all parts of the brain (body).

Functional Implications. Our results suggest up-regulation of Galr1 mRNA levels in a so far unidentified population of neurons, some of which seem to form a bilateral horizontal band in the vPAG, only partly overlapping with the 5-HT neurons, and essentially localized in the DRL and VLPAG. As discussed below, they are thus, in any case, only partly 5-HT neurons but could represent GABA (GABA/5-HT) and/or glutamate neurons.

Evidence for involvement of GABA in mood disorders has been summarized (71–75), and $GABA_A$ receptor subunits are expressed in rat vPAG (76). Glutamate receptor antagonists have been shown to have antidepressant activity in animal experiments (77–79) and in treatment-resistant MD (72, 80–82), and glutamate receptors are expressed in rodent vPAG (83).

Functionally, in the rat brain, GALR1 is mainly associated with inhibition, often being postsynaptic (84), and it is possible that galanin also has this effect on $GALR1^+$ neurons in the vPAG. Thus, depending on the principle phenotype of these neurons, activation of GALR1 could, overall, result in inhibition (glutamate) or disinhibition (GABA). If present in interneurons innervating 5-HT neurons, the effect in the former case would be prodepressive and the effect in the latter case would be antidepressive. The outcome of the latter alternative is similar to the proposal by

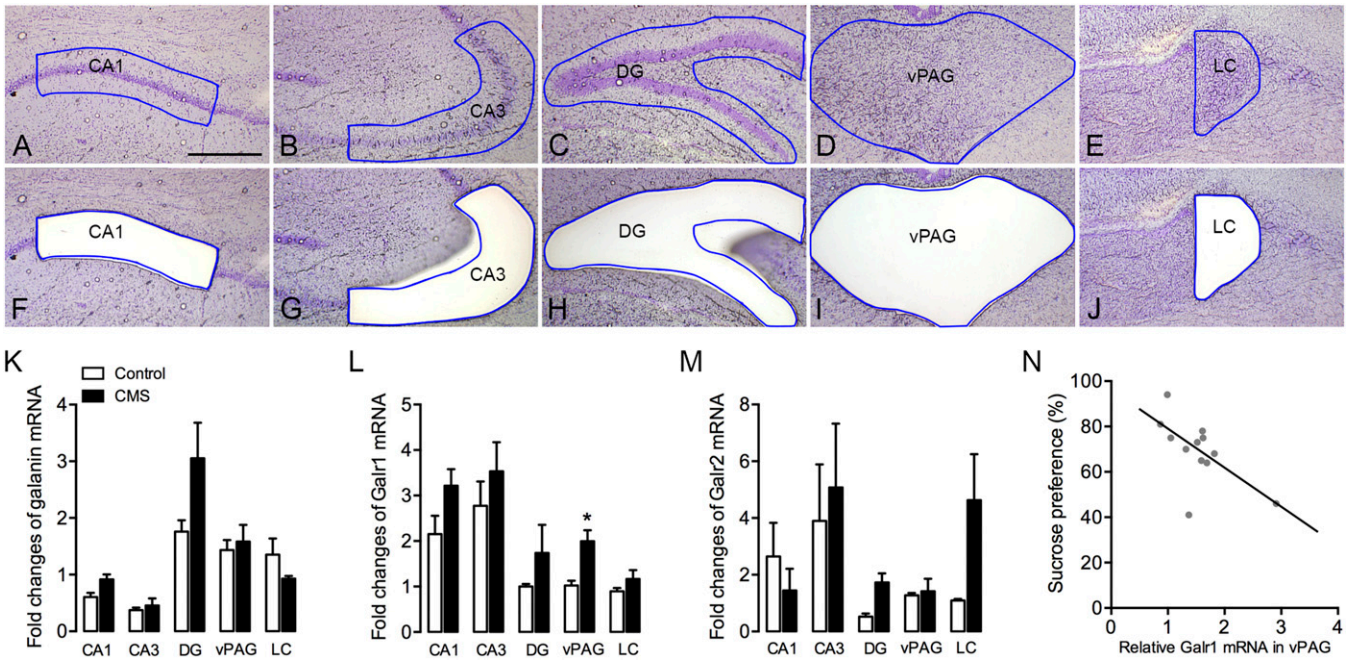

Fig. 3. Galr1 mRNA levels are increased in vPAG after CMS. Laser capture microdissection and transcript levels for galanin Galr1 and Galr2. In the section of the HPC, CA1 was first microdissected (*A* and *F*); then, CA3 (*B* and *G*) and, finally, DG (*C* and *H*) were microdissected. In the brainstem, the vPAG was first microdissected (*D*, *I*), followed by the LC (*E* and *J*). The quantitative analysis reveals region-specific significant increases of transcript levels only for Galr1 in vPAG (*L*, $P = 0.007$), but not for galanin (*K*, $P = 0.243$) or Galr2 (*M*, $P = 0.092$). Multivariate ANOVA (MANOVA) is applied for statistical analysis. Different brain regions are treated as repeated measurements of the same outcome variable. Regions are a within-subject factor, and group is a between-subject factor. For Galr1, interaction between region and group is not statistically significant ($P = 0.918$). $^*P < 0.05$, and data are expressed as mean fold change ± SEM ($n = 6$). (*N*) Galr1 mRNA expression in vPAG is correlated with the sucrose preference in the CMS rats ($P < 0.05$, $r = -0.6193$, $n = 12$). (Scale bar: 500 mm.)

Sharkey et al. (85) that GALR1 in the DR is a presynaptic, inhibitory receptor on GABA neurons innervating 5-HT neurons. This scenario also results in decreased GABA release, and thus disinhibition and increased firing of 5-HT neurons.

Interestingly, Galr1 transcript is also present in many cells in the human PAG but apparently not in 5-HT neurons (86). Previously, it has been proposed that a GALR3 antagonist is a candidate for treatment of depression acting on NA neurons in LC and 5-HT neurons in DR (86–88).

In other studies, it has been reported that activating GALR2 in the vPAG, via coupling to G_o/G_i causes increased inositol trisphosphate synthesis, Ca^{2+} mobilization, and depolarization (23, 43, 46, 53, 54). If postsynaptic on 5-HT neurons, then activation of the GALR2 receptors should cause increased firing and release of serotonin (i.e., an antidepressive effect). Taken together, a key question for understanding the role of galanin receptors in the rat vPAG in depression-like behavior is the phenotype of neurons expressing this receptor in the rat brain and the circuitry involved and, by extension, also in the human brain.

Distribution of Galanin Receptors in the vPAG. Two major ISH studies (32, 34) have shown a Galr1 mRNA signal in the PAG, essentially excluding the midline, however, as also reported by Xu et al. (89) and in the present study. There is a weak Galr2 mRNA signal in the midline area/cells of the DR (32, 34), which is supported by the data of others (23, 63) as well as present qPCR data, and speculatively expressed in a population of 5-HT neurons. These $GALR2^+$ neurons could express $VGLUT3^+$ (90–93), although there are also $VGLUT3^+$, $5\text{-}HT^-$ neurons in that area (91). Eberwine and Bartfai (94) and Bartfai et al. (95) have reported that a single warm-sensitive neuron in the hypothalamus may contain several hundred receptor transcripts, however. Many of these receptor transcripts are functional, even if histochemical approaches can, at most, detect some 5–10% of these receptors. Thus, negative results should be interpreted with caution.

More recent studies demonstrating galanin receptor heteromers have introduced a further degree of complexity in interpreting galaninergic mechanisms in the brain (96). In addition to GALR1 and 5-HT1A receptor heterodimers (97), an involvement of the galanin (1–15) fragment in mood regulation via GALR1 and GALR2 heterocomplexes in the dorsal HPC and DR has been reported (98). Interestingly, galanin (1–15) causes a dose-dependent hyperpolarization of hippocampal CA3 neurons (99), and, after iodination, it binds to, among other things, the dorsal HPC as shown in autoradiographic studies (100).

Possible Phenotype of $Galr1\ mRNA^+$ Neurons and Circuitries. The vPAG area microdissected in the present study (Fig. 3 *D* and *I*) harbors a large number of 5-HT neurons in the rat (101–103), and distinct rostrocaudal differences in function exist (104). In the rat, many of the 5-HT neurons express galanin (20, 22–24). However, a large number of nonserotonin cells exist in the rodent DR, with different electrophysiological characteristics (105–107) and neurotransmitter phenotypes, including GABA-, glutamate-, and peptidergic neurons, constituting some 50–70% of all DR neurons (70, 108–114).

Our attempts to phenotype the $Galr1^+$ neurons in the vPAG by double-ISH have failed. As an "emergency" approach, we have studied the distribution of the transcripts for Galr1, Gad, Vglut2, and Tph2 on adjacent sections, with the latter two having a wide distribution in the vPAG (refs. 101–106 for rat and ref. 114 for mouse). The results indicate only partial overlap between Galr1 and Tph2, suggesting that Galr1 is not present, and up-regulated, in the main bulk of 5-HT neurons. This view is supported by an in vivo single-cell transcriptomic study on the mouse, where no evidence for galanin receptor mRNA in the lateral wings was obtained (115). There are several other possibilities, including

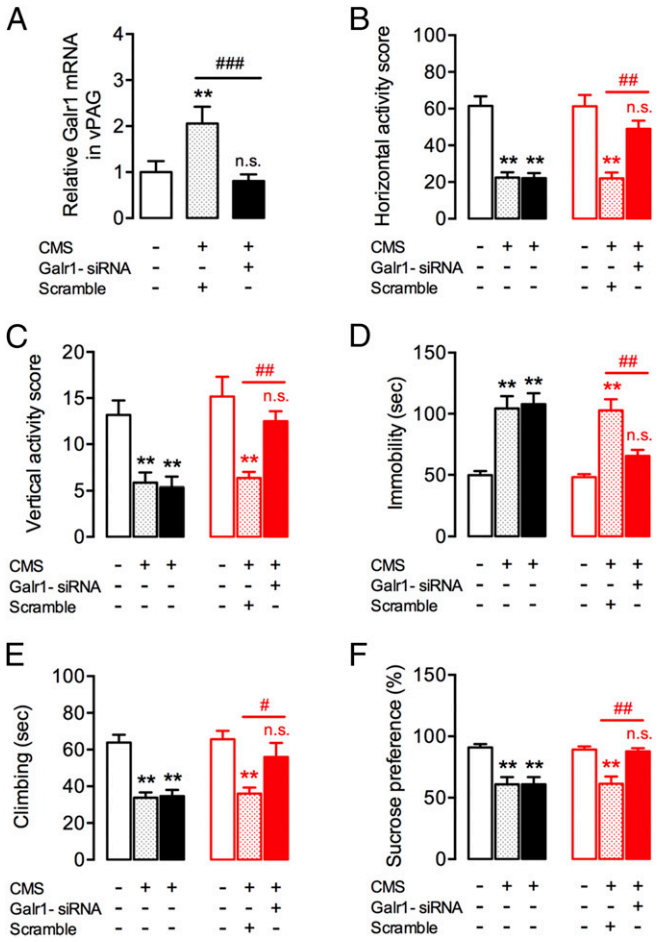

Fig. 4. Galr1-siRNA reduces Galr1 mRNA levels in vPAG and attenuates depressive-like behaviors. (*A*) Galr1 mRNA levels in the CMS-Galr1-scramble group are increased in the vPAG compared with control, and this increase is completely blocked after Galr1-siRNA treatment. $^{**}P < 0.01$ compared with control group; $^{###}P < 0.001$. n.s., no significant difference between the control group and CMS group treated with Galr1-siRNA (one-way ANOVA, $n = 6$). Decreased horizontal activity score [*B*; $F(2, 30) = 43.22$, $P < 0.0001$ and $F(1, 30) = 6.219$, $P = 0.0184$] and vertical activity score [*C*; $F(2, 30) = 17.88$, $P < 0.0001$ and $F(1, 30) = 8.278$, $P = 0.0073$] are reversed after Galr1-siRNA treatment, but not for the scramble groups. Increased immobility [*D*; $F(2, 30) = 30.55$, $P < 0.0001$ and $F(1, 30) = 6.751$, $P = 0.0144$] and decreased climbing time [*E*; $F(2, 30) = 21.44$, $P < 0.0001$ and $F(1, 30) = 5.00$, $P = 0.0329$] are reversed after Galr1-siRNA treatment. (*F*) Decreased sucrose intake is reversed after Galr1-siRNA treatment [$F(2, 30) = 19.82$, $P < 0.0001$ and $F(1, 30) = 5.360$, $P = 0.0276$]. There is a significant CMS treatment interaction for the variances in *B*, *D*, and *F*. Data in *B*–*F* are expressed as mean change ± SEM ($n = 6$). Two-way ANOVA, followed by the Bonferroni multiple comparisons test, is applied for the analysis. $^{**}P < 0.01$ compared with the basal values within each group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared between Galr1-siRNA and scramble groups.

expression of Galr1 in, for example, subpopulations of GABA and/or glutamate neurons. These neurons may be ascending and/or local.

GALR1 and GABAergic Neurons. GALR1 in the vPAG could be expressed in GABA interneurons, which are known to inhibit 5-HT neurons directly (116–127). Moreover, some 5-HT neurons in the rat and mouse lateral wings are GABA/GAD$^+$ (70, 117, 128–131), and thus probably corelease GABA and 5-HT. More recent studies report that of the ascending 5-HT neurons (132–134), 16% are GABAergic and project to the medial prefrontal cortex (135), as also suggested by electrophysiological studies (136). These GAD$^+$ 5-HT neurons have distinct electrophysiological characteristics and may, interestingly, be involved in stress-related responses and disorders (130, 137–139). Shikanai et al. (130) have shown that lateral, but not medial, 5-HT/GAD67 neurons are particularly sensitive to innocuous stressors. If these neurons express GALR1, they could, hypothetically, be involved in depression-like behavior.

GALR1 and Glutamatergic Neurons. Several glutamatergic cell groups are present in the vPAG (90–93, 140, 141), including VGLUT3 projection neurons in the midline area, many of which represent 5-HT neurons, clearly separated from the GABA (GAD67) neurons (91). The VGLUT3$^+$ neurons are likely not GALR1$^+$ in view of their mainly midline localization. There are also widespread local VGLUT2$^+$ neurons potentially coexpressing GALR1. In fact, there is evidence for glutamatergic interneurons innervating 5-HT neurons (127, 142).

With regard to descending systems, a glutamatergic projection from the forebrain (medial prefrontal cortex/hippocampus) to the vPAG/DR is well established (121, 143–150). In agreement, VGLUT1$^+$ and VGLUT2$^+$ boutons synapse both on TPH$^+$ and TPH$^-$ neurons (151). In the retrograde tracing study by Lee et al. (147), inspection of the two injection sites (figures 1 B and C of ref. 147) suggest that they probably include the area harboring the Galr1 mRNA$^+$ neurons.

Origin of Galanin in the vPAG. The exact source(s) of endogenously released galanin activating the up-regulated GALR1 receptors in vPAG remains to be established. There is a galanin terminal network of varying density in the entire rat vPAG (89), and some of these fibers could originate from galanin$^+$ 5-HT neurons because the 5-HT neurons send richly branching axon collaterals within the DR (104, 127, 152); there are also VGLUT3$^+$ 5-HT collaterals and at least some may contain galanin (140). Nevertheless, the galanin input to the GALR1$^+$ neurons is probably mainly from nonserotonergic neurons. Notably, galanin may also be available via volume transmission (153).

A large proportion (~80%) of the noradrenergic LC neurons can synthesize galanin (154, 155), and noradrenergic boutons originating in the LC may therefore release galanin in the vPAG. This finding is particularly relevant in relation to depression, because galanin expression is up-regulated in LC neurons after

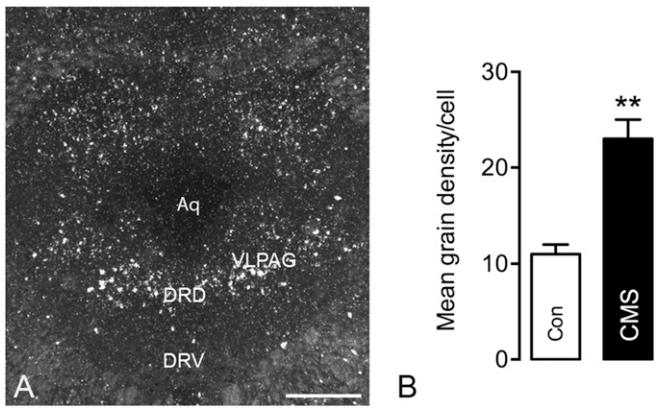

Fig. 5. Galr1 mRNA is present in the entire PAG and is increased after CMS. (*A*) Galr1 mRNA expression in a control rat. Note the presence of positive cells in the entire PAG with a stronger signal in some cells, especially in the VLPAG. Few cells are seen in the DRD, and hardly any cells are seen in the DRV. (*B*) ImageJ analysis of mean grain density in the vPAG shows a significant increase of Galr1 mRNA levels after CMS. Data are expressed as mean grain density ± SEM ($n = 6$). $^{**}P < 0.01$ compared with control group (Student's *t* test). Aq, aqueduct; Con, control. (Scale bar: 500 μm.)

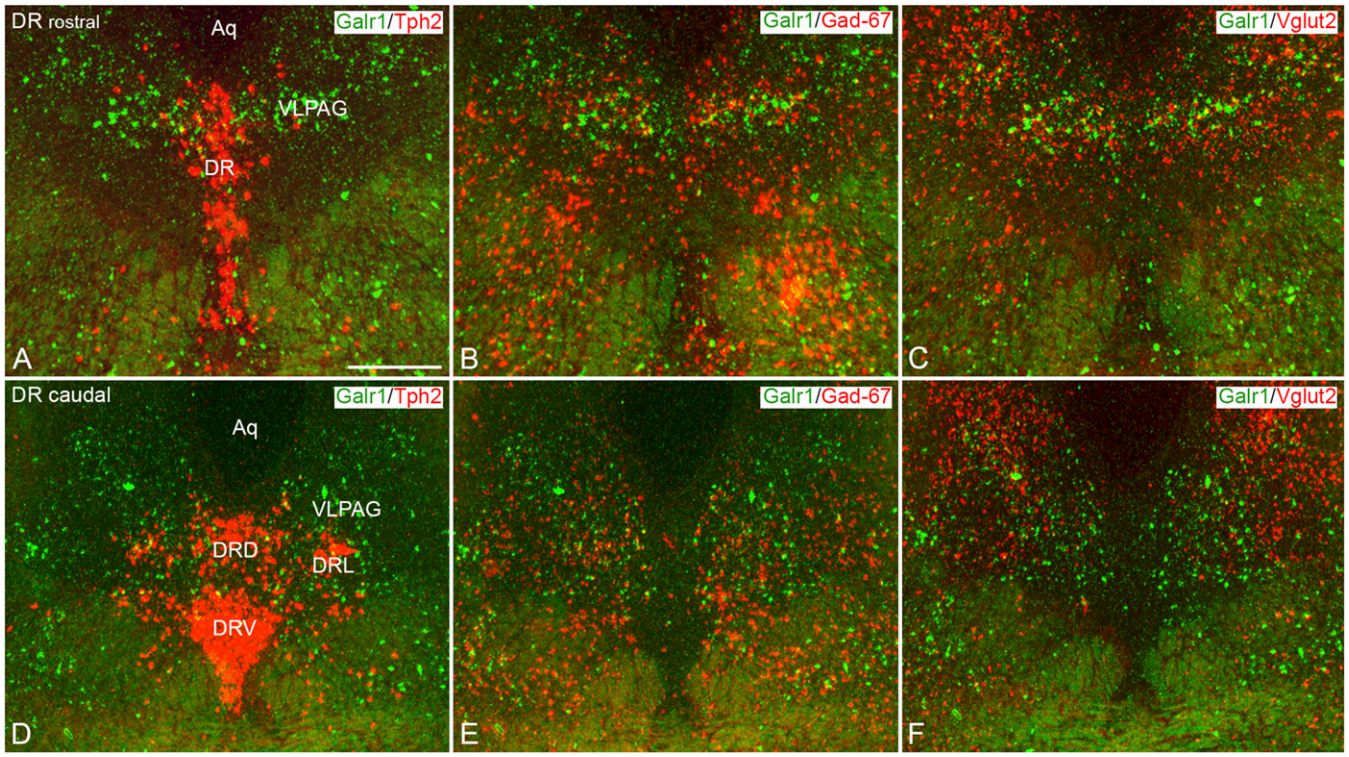

Fig. 6. Galr1 mRNA only partly overlaps with Tph2 mRNA in the vPAG gray, but it overlaps more extensively with Gad-67 and Vglut2 mRNAs. Adjacent slides were hybridized with probes for Galr1 (green) or for Tph2, Gad-67, or Vglut2 (all three red). The color-coded autoradiograms were then superimposed, showing Galr1 plus Tph2 (*A* and *D*), Galr1 plus Gad-67 (*B* and *E*), or Galr1 plus Vglut2 (*C* and *F*). Note only partial Galr1-Tph2 overlap vs. intermingling of Galr1 with Gad-67 and Vglut2. These figures do not show coexistence, only overlap, because adjacent sections have been compared. (Scale bar: 500 μm.)

stress (156–159). In fact, the 5-HT neurons receive a fairly dense noradrenergic innervation that exerts a tonic excitation via α_1-adrenoreceptors (160). However, the contribution of this innervation from LC seems to be minor (143, 161–164), and more caudally located catecholamine groups may be more important (164). Our results, based on double/triple labeling, are in agreement with this view, showing that the vast majority of galanin nerve terminals lack DBH at the levels analyzed in any case. This finding is in contrast to dorsal cortical regions (shown in the same sections), where virtually all galanin fibers are noradrenergic, as also previously shown (89). This finding is in agreement with studies on the amygdala, where stress activation of the LC system induces galanin release and an anxiolytic effect, although this galanin is not released from noradrenergic fibers originating from LC but, presumably, from local galanin neurons (42).

Concluding Remarks

The present results, based on the rat CMS depression model and a battery of tests, provide evidence for involvement of GALR1 in the vPAG in depression-like behavior. Thus, a GALR1 antagonist acting in the vPAG may have antidepressant actions, similar to the proposal that such antagonists, in the rat, may also mediate antidepression in the LC. However, a more reliable understanding of the role of galanin receptors in the rat vPAG in depression-like behavior requires identification of the phenotype of neurons expressing the galanin receptor and the circuitry involved.

Materials and Methods

Animals and the CMS Model. Male Sprague–Dawley rats were habituated, including handling daily for 1 wk. The study was approved by the Animal Care Committee at Capital Medical University. The CMS procedure, according Willner et al. (57), included exposure to one of the eight daily stressors randomly for 8 wk. Control rats were left undisturbed in their home cages except for handling once daily (*SI Materials and Methods*).

Behavioral Tests. As shown in Fig. 1, before starting exposure to CMS, the rats were subjected to the sucrose intake test as described (57), as well as to the OFT. The latter was based on an apparatus consisting of a black square arena (75 × 75 cm) with a 40-cm-high opaque black wall. The number of horizontal and vertical (rearings) activities during a 5-min period was counted. The behavioral tests were videotaped and quantified with a video tracking system program (EthoVision; Noldus). The FST was carried out on rats as described by Porsolt and colleagues (165). Briefly, rats were placed in the water for a 5-min session, and the immobility and climbing time of rats were recorded in seconds. The behavioral measurements were carried out 8 wk after the start of CMS, and the rats were exposed to the three tests on consecutive days, with the exception of the second day being free of tests (Fig. 1 and *SI Materials and Methods*).

Tissue Preparation and Staining. Rats were euthanized 1 d after having been exposed to the last of the three behavioral tests following the 8-wk period of CMS (Fig. 1), and the brains were removed, placed on dry ice, sectioned in a cryostat at a thickness of 40 μm, mounted on polyethylene naphthalate membrane glass slides, air-dried, exposed to ethanol, stained with cresyl violet, dehydrated, and air-dried before laser capture microdissection (*SI Materials and Methods*).

Laser Capture Microdissection and RNA Isolation. Laser microdissection of the CA1, CA3, DG of the HPC, and LC was performed. The dissected region in the PAG, including the 5-HT neurons (here termed vPAG), encompasses the entire DR (DRD, DRV, and DRL parts) plus the ventral aspects of the VLPAG (166). In this report, we use the term vPAG, unless specifically addressing the 5-HT neuron subnuclei. We thus follow the Paxinos–Watson nomenclature. The dissection was performed using a system equipped with a UV laser (Leica) and an isothiocyanate-containing lysis buffer. In the HPC slice, the CA1 was first microdissected (Fig. 3 *A* and *F*), followed by the CA3 (Fig. 3 *B* and *G*) and the DG (Fig. 3 *C* and *H*). In the midbrain slice, the vPAG was first microdissected (Fig. 3 *D* and *I*), followed by the LC (Fig. 3 *E* and *J*). Total RNA from these samples was extracted using the RNeasy Micro Kit (Qiagen).

qPCR. Total RNA was reverse-transcribed using random primers and SuperScript III reverse transcriptase. All qPCR reactions were performed by use of the 7300 ABI qPCR system. TaqMan gene expression assays for rat galanin (Rn00583681_ml), Galr1 (Rn02132426_sl), Galr2 (Rn01773918_mL), and Gapdh (Rn999999916_sl) as an endogenous control were obtained from Life Technologies (*SI Materials and Methods*).

Vector Construction and Knockdown by siRNA. The rat anti-Galr1 siRNA sequences started at nucleotide 888. For scramble siRNA control, a mismatch siRNA was designed, which contained the same, but scrambled, nucleotide composition. The green fluorescent protein-reporting lentivirus encoding the siRNA to Galr1 and scramble were custom-constructed into commercial pLenti B/X plasmid and injected into the vPAG and VTA of CMS rats. The final behavioral measurements were carried out 4 wk after the siRNA injections on consecutive days, with the exception of the second day being free of tests (Fig. 1 and *SI Materials and Methods*).

Western Blotting. The knockdown effect of siRNA on Galr1 protein levels was monitored using myc-Galr1–transfected HEK293A cells and Western immunoblotting as described (*SI Materials and Methods*).

RNA Probe Synthesis and ISH. RNA probes specific to rat Galr1, Tph2, Gad67, and VGlut2 were prepared from rat hypothalamus cDNA (*SI Materials and Methods*). Sections for the vPAG ($n = 3$) were prepared. Five sets of serial sections (P1–P5, 20 μm thick) were prepared in a cryostat. P2 and P4 were hybridized with the Galr1 antisense probe, and P1, P3, and P5 were hybridized with antisense probes to Tph2, Gad67, and VGlut2, respectively. Fixation, prehybridization, and hybridization were performed as previously described (*SI Materials and Methods*). Sections were placed against a film and/or dipped in an autoradiographic emulsion, developed, and mounted with glycerol/PBS medium (*SI Materials and Methods*).

Immunohistochemistry and Imaging. Adult male Sprague–Dawley rats (Charles River Laboratories) were perfused transcardially with 4% (wt/vol) paraformaldehyde, and the brains were postfixed in the same fixative, kept in 10% (wt/vol) sucrose for 2 d, frozen, and sectioned at 20 μm in a cryostat both coronally and sagittally. Sections were incubated with rabbit anti-galanin antibodies, followed by visualization using a TSA Plus Kit (PerkinElmer). Triple labeling was carried out with mouse anti-DBH and rabbit anti–5-HT antibodies (*SI Materials and Methods*). Images were acquired on a confocal laser-scanning microscope (LSM700; Zeiss).

Microscopic Analysis. Sections were analyzed using a Nikon Eclipse E600 microscope equipped with a bright- and dark-field condenser and epipolarization with side entrance illumination (Nikon). Sections were scanned using a Nikon LS-2000 film scanner (Nikon). Scanned and digital images were imported into Adobe Photoshop CS6 and optimized for brightness and contrast.

Statistical Analysis. Two-way ANOVA and the post hoc Bonferroni multiple comparisons test were applied for behavioral tests. Multivariate ANOVA was applied for qPCR results analysis. The siRNA knockdown efficiency was analyzed by one-way ANOVA, and the effects of Galr1 knockdown on CMS were analyzed by two-way ANOVA, followed by the post hoc Bonferroni multiple comparisons post hoc test. The statistical significance level was set at $P < 0.05$.

ACKNOWLEDGMENTS. We thank Prof. Staffan Cullheim [Karolinska Institutet (KI)] for critical support and Professor Kathryn Commons (Childrens Hospital, Harvard University) for valuable advice. Laser-scanning microscopy was made available by the center for Live Imaging of Cells at the KI, an imaging core facility supported by the Knut and Alice Wallenberg Foundation. This study was supported by the National Natural Sciences Foundation of China (Grants 30870815 and 31171032), Beijing Natural Science Foundation (Key Project 7091002), National Basic Research Program of China 973 Program (Grant 2010CB912003), Beijing Talent Project (Grant PHR20100510), Swedish Research Council (Grant 4X-2887), Seed Grant PXM2014_014226_000006 of the International Alliance of Translational Neuroscience, and Beijing Brain Project (Grant Z161100000216142), as well as by funds from the KI.

1. Coppen AJ (1968) Depressed states and indolealkylamines. *Adv Pharmacol* 6(Pt B): 283–291.
2. Maes M, Meltzer H (1995) The serotonin hypothesis of major depression. *Psychopharmacology: The Fourth Generation of Progress*, eds Bloom F, Kupfer D (Raven, New York), pp 933–944.
3. Schildkraut JJ (1965) The catecholamine hypothesis of affective disorders: A review of supporting evidence. *Am J Psychiatry* 122(5):509–522.
4. Schatzberg AF, Schildkraut JJ (1995) Recent studies on norepinephrine systems in mood disorders. *Psychopharmacology: The Fourth generation of progress*, eds Bloom F, Kupfer D (Raven, New York), pp 911–920.
5. Millan MJ (2006) Multi-target strategies for the improved treatment of depressive states: Conceptual foundations and neuronal substrates, drug discovery and therapeutic application. *Pharmacol Ther* 110(2):135–370.
6. Trivedi MH, et al.; STAR*D Study Team (2006) Evaluation of outcomes with citalopram for depression using measurement-based care in STAR*D: Implications for clinical practice. *Am J Psychiatry* 163(1):28–40.
7. Burbach JP (2010) Neuropeptides from concept to online database. *Eur J Pharmacol* 626(1):27–48. Available at www.neuropeptides.nl. Accessed May 10, 2016.
8. Griebel G, Holsboer F (2012) Neuropeptide receptor ligands as drugs for psychiatric diseases: the end of the beginning? *Nat Rev Drug Discov* 11(6):462–478.
9. Hökfelt T, Bartfai T, Bloom F (2003) Neuropeptides: Opportunities for drug discovery. *Lancet Neurol* 2(8):463–472.
10. Holmes A, Heilig M, Rupniak NM, Steckler T, Griebel G (2003) Neuropeptide systems as novel therapeutic targets for depression and anxiety disorders. *Trends Pharmacol Sci* 24(11):580–588.
11. Nemeroff CB, Vale WW (2005) The neurobiology of depression: Inroads to treatment and new drug discovery. *J Clin Psychiatry* 66(Suppl 7):5–13.
12. Steckler T (2008) Peptide receptor ligands to treat anxiety disorders. *Handbook of Anxiety and Fear*, eds Blanchard RJ, Blanchard DC, Griebel G, Nutt D (Elsevier, Amsterdam), pp 157–217.
13. Griebel G, Holmes A (2013) 50 years of hurdles and hope in anxiolytic drug discovery. *Nat Rev Drug Discov* 12(9):667–687.
14. Maubach KA, Rupniak NM, Kramer MS, Hill RG (1999) Novel strategies for pharmacotherapy of depression. *Curr Opin Chem Biol* 3(4):481–488.
15. Sajdyk TJ, Shekhar A, Gehlert DR (2004) Interactions between NPY and CRF in the amygdala to regulate emotionality. *Neuropeptides* 38(4):225–234.
16. Wu G, et al. (2011) Central functions of neuropeptide Y in mood and anxiety disorders. *Expert Opin Ther Targets* 15(11):1317–1331.
17. Valentino RJ, Commons KG (2005) Peptides that fine-tune the serotonin system. *Neuropeptides* 39(1):1–8.
18. Tatemoto K, Rökaeus A, Jörnvall H, McDonald TJ, Mutt V (1983) Galanin - a novel biologically active peptide from porcine intestine. *FEBS Lett* 164(1):124–128.
19. Skofitsch G, Jacobowitz DM (1985) Immunohistochemical mapping of galanin-like neurons in the rat central nervous system. *Peptides* 6(3):509–546.
20. Melander T, et al. (1986) Coexistence of galanin-like immunoreactivity with catecholamines, 5-hydroxytryptamine, GABA and neuropeptides in the rat CNS. *J Neurosci* 6(12):3640–3654.
21. Melander T, Hökfelt T, Rökaeus A (1986) Distribution of galaninlike immunoreactivity in the rat central nervous system. *J Comp Neurol* 248(4):475–517.
22. Fuxe K, et al. (1990) Galanin/5-HT receptor interactions. A new integrative mechanism in the control of 5-HT neurotransmission in the CNS. *Serotonin from Cell Biology to Pharmacology and Therapeutics*, eds Paoletti R, Vanhoutte PM (Kluwer, Dordrecht, The Netherlands), pp 169–185.
23. Lu X, et al. (2005) A role for galanin in antidepressant actions with a focus on the dorsal raphe nucleus. *Proc Natl Acad Sci USA* 102(3):874–879.
24. Xu ZQ, Hökfelt T (1997) Expression of galanin and nitric oxide synthase in subpopulations of serotonin neurons of the rat dorsal raphe nucleus. *J Chem Neuroanat* 13(3):169–187.
25. Priestley JV, Wotherspoon G, Savery D, Averill S, Rattray M (1993) A combined in situ hybridization and immunofluorescence procedure allowing visualisation of peptide mRNA and serotonin in single sections. *J Neurosci Methods* 48(1-2):99–110.
26. Branchek TA, Smith KE, Gerald C, Walker MW (2000) Galanin receptor subtypes. *Trends Pharmacol Sci* 21(3):109–117.
27. Habert-Ortoli E, Amiranoff B, Loquet I, Laburthe M, Mayaux JF (1994) Molecular cloning of a functional human galanin receptor. *Proc Natl Acad Sci USA* 91(21): 9780–9783.
28. Iismaa TP, Shine J (1999) Galanin and galanin receptors. *Results Probl Cell Differ* 26: 257–291.
29. Lang R, et al. (2015) Physiology, signaling, and pharmacology of galanin peptides and receptors: Three decades of emerging diversity. *Pharmacol Rev* 67(1):118–175.
30. Melander T, et al. (1988) Autoradiographic quantitation and anatomical mapping of 125I-galanin binding sites in the rat central nervous system. *J Chem Neuroanat* 1(4): 213–233.
31. Skofitsch G, Sills MA, Jacobowitz DM (1986) Autoradiographic distribution of 125I-galanin binding sites in the rat central nervous system. *Peptides* 7(6):1029–1042.
32. Burazin TC, Larm JA, Ryan MC, Gundlach AL (2000) Galanin-R1 and -R2 receptor mRNA expression during the development of rat brain suggests differential subtype involvement in synaptic transmission and plasticity. *Eur J Neurosci* 12(8):2901–2917.
33. Mennicken F, Hoffert C, Pelletier M, Ahmad S, O'Donnell D (2002) Restricted distribution of galanin receptor 3 (GalR3) mRNA in the adult rat central nervous system. *J Chem Neuroanat* 24(4):257–268.
34. O'Donnell D, Ahmad S, Wahlestedt C, Walker P (1999) Expression of the novel galanin receptor subtype GALR2 in the adult rat CNS: Distinct distribution from GALR1. *J Comp Neurol* 409(3):469–481.
35. Waters SM, Krause JE (2000) Distribution of galanin-1, -2 and -3 receptor messenger RNAs in central and peripheral rat tissues. *Neuroscience* 95(1):265–271.

36. O'Donnell D, et al. (2003) Localization of galanin receptor subtypes in the rat CNS. *Handbook of Chemical Neuroanatomy. Peptide Receptors, Part II*, eds Quirion R, Björklund A, Hökfelt T (Elsevier, Amsterdam), Vol 20, pp 195–244.
37. Bing O, Möller C, Engel JA, Söderpalm B, Heilig M (1993) Anxiolytic-like action of centrally administered galanin. *Neurosci Lett* 164(1-2):17–20.
38. Möller C, Sommer W, Thorsell A, Heilig M (1999) Anxiogenic-like action of galanin after intra-amygdala administration in the rat. *Neuropsychopharmacology* 21(4): 507–512.
39. Fuxe K, et al. (1991) Galanin/5-HT interactions in the rat central nervous system. Relevance for depression. *Galanin. A New Multifunctional Peptide in the Neuro-Endocrine System*, eds Hokfelt T, Bartfai T, Jacobowitz D, Ottoson D (Cambridge Univ Press, Cambridge, UK), pp 221–236.
40. Fuxe K, et al. (1998) Galanin modulates 5-hydroxytryptamine functions. Focus on galanin and galanin fragment/5-hydroxytryptamine1A receptor interactions in the brain. *Ann N Y Acad Sci* 863:274–290.
41. Weiss JM, Bonsall RW, Demetrikopoulos MK, Emery MS, West CH (1998) Galanin: A significant role in depression? *Ann N Y Acad Sci* 863:364–382.
42. Barrera G, et al. (2005) One for all or one for one: Does co-transmission unify the concept of a brain galanin "system" or clarify any consistent role in anxiety? *Neuropeptides* 39(3): 289–292.
43. Lu X, Sharkey L, Bartfai T (2007) The brain galanin receptors: Targets for novel antidepressant drugs. *CNS Neurol Disord Drug Targets* 6(3):183–192.
44. Ogren SO, Kuteeva E, Hökfelt T, Kehr J (2006) Galanin receptor antagonists: A potential novel pharmacological treatment for mood disorders. *CNS Drugs* 20(8): 633–654.
45. Weiss JM, et al. (2005) Testing the hypothesis that locus coeruleus hyperactivity produces depression-related changes via galanin. *Neuropeptides* 39(3):281–287.
46. Kuteeva E, et al. (2008) Differential role of galanin receptors in the regulation of depression-like behavior and monoamine/stress-related genes at the cell body level. *Neuropsychopharmacol* 33(11):2573–2585.
47. Sciolino NR, Dishman RK, Holmes PV (2012) Voluntary exercise offers anxiolytic potential and amplifies galanin gene expression in the locus coeruleus of the rat. *Behav Brain Res* 233(1):191–200.
48. Holmes A, Picciotto MR (2006) Galanin: A novel therapeutic target for depression, anxiety disorders and drug addiction? *CNS Neurol Disord Drug Targets* 5(2):225–232.
49. Khoshbouei H, Cecchi M, Dove S, Javors M, Morilak DA (2002) Behavioral reactivity to stress: Amplification of stress-induced noradrenergic activation elicits a galanin-mediated anxiolytic effect in central amygdala. *Pharmacol Biochem Behav* 71(3): 407–417.
50. Karlsson RM, Holmes A (2006) Galanin as a modulator of anxiety and depression and a therapeutic target for affective disease. *Amino Acids* 31(3):231–239.
51. Picciotto MR, Brabant C, Einstein EB, Kamens HM, Neugebauer NM (2010) Effects of galanin on monoaminergic systems and HPA axis: Potential mechanisms underlying the effects of galanin on addiction- and stress-related behaviors. *Brain Res* 1314: 206–218.
52. Kuteeva E, Hökfelt T, Wardi T, Ögren SO (2010) Galanin, galanin receptor subtypes and depression-like behaviour. *EXS* 102:163–181.
53. Saar I, et al. (2013) Novel systemically active galanin receptor 2 ligands in depression-like behavior. *J Neurochem* 127(1):114–123.
54. Saar I, Runesson J, Järv J, Kurrikoff K, Langel U (2013) Novel galanin receptor subtype specific ligand in depression like behavior. *Neurochem Res* 38(2):398–404.
55. Juhasz G, et al. (2014) Brain galanin system genes interact with life stresses in depression-related phenotypes. *Proc Natl Acad Sci USA* 111(16):E1666–E1673.
56. Moreau JL, Jenck F, Martin JR, Mortas P, Haefely WE (1992) Antidepressant treatment prevents chronic unpredictable mild stress-induced anhedonia as assessed by ventral tegmentum self-stimulation behavior in rats. *Eur Neuropsychopharmacol* 2(1):43–49.
57. Willner P, Towell A, Sampson D, Sophokleous S, Muscat R (1987) Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. *Psychopharmacology (Berl)* 93(3):358–364.
58. Wang P, Sun JHL, Hökfelt TG, Xu Z-Q (2011) Expression of galanin and its receptors in serotonergic and noradrenergic systems in the rat chronic mild stress depression model (Society for Neuroscience, Washington, DC), p S-15586 (abstr).
59. Hökfelt T, Johansson O, Goldstein M (1984) Central catecholamine neurons as revealed by immunohistochemistry with special reference to adrenaline neurons. *Classical Transmitters in the CNS. Part 1*, eds Björklund A, Hökfelt T (Elsevier, Amsterdam), pp 157–266.
60. Kawa L, et al. (2016) Expression of galanin and its receptors are perturbed in a rodent model of mild, blast-induced traumatic brain injury. *Exp Neurol* 279:159–167.
61. Hoge CW, et al. (2008) Mild traumatic brain injury in U.S. soldiers returning from Iraq. *N Engl J Med* 358(5):453–463.
62. Sergeyev V, et al. (2005) Neuropeptide expression in rats exposed to chronic mild stresses. *Psychopharmacology (Berl)* 178(2-3):115–124.
63. Rovin ML, et al. (2012) Influence of chronic administration of antidepressant drugs on mRNA for galanin, galanin receptors, and tyrosine hydroxylase in catecholaminergic and serotonergic cell-body regions in rat brain. *Neuropeptides* 46(2): 81–91.
64. Petschner P, et al. (2016) Chronic venlafaxine treatment fails to alter the levels of galanin system transcripts in normal rats. *Neuropeptides* 57:65–70.
65. Holmes A, et al. (2005) Phenotypic assessment of galanin overexpressing and galanin receptor R1 knockout mice in the tail suspension test for depression-related behavior. *Psychopharmacology (Berl)* 178(2-3):276–285.
66. Holmes A, et al. (2003) Galanin GAL-R1 receptor null mutant mice display increased anxiety-like behavior specific to the elevated plus-maze. *Neuropsychopharmacology* 28(6):1031–1044.
67. Cheung CC, Hohmann JG, Clifton DK, Steiner RA (2001) Distribution of galanin messenger RNA-expressing cells in murine brain and their regulation by leptin in regions of the hypothalamus. *Neuroscience* 103(2):423–432.
68. Larm JA, Shen PJ, Gundlach AL (2003) Differential galanin receptor-1 and galanin expression by 5-HT neurons in dorsal raphé nucleus of rat and mouse: Evidence for species-dependent modulation of serotonin transmission. *Eur J Neurosci* 17(3): 481–493.
69. Pérez SE, Wynick D, Steiner RA, Mufson EJ (2001) Distribution of galaninergic immunoreactivity in the brain of the mouse. *J Comp Neurol* 434(2):158–185.
70. Fu W, et al. (2010) Chemical neuroanatomy of the dorsal raphe nucleus and adjacent structures of the mouse brain. *J Comp Neurol* 518(17):3464–3494.
71. Kalueff AV, Nutt DJ (2007) Role of GABA in anxiety and depression. *Depress Anxiety* 24(7):495–517.
72. Kendell SF, Krystal JH, Sanacora G (2005) GABA and glutamate systems as therapeutic targets in depression and mood disorders. *Expert Opin Ther Targets* 9(1): 153–168.
73. Lloyd KG, Zivkovic B, Scatton B, Morselli PL, Bartholini G (1989) The gabaergic hypothesis of depression. *Prog Neuropsychopharmacol Biol Psychiatry* 13(3-4):341–351.
74. Möhler H (2012) The GABA system in anxiety and depression and its therapeutic potential. *Neuropharmacology* 62(1):42–53.
75. Taylor M, Bhagwagar Z, Cowen PJ, Sharp T (2003) GABA and mood disorders. *Psychol Med* 33(3):387–393.
76. Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G (2000) GABA(A) receptors: Immunocytochemical distribution of 13 subunits in the adult rat brain. *Neuroscience* 101(4):815–850.
77. Paul IA, Skolnick P (2003) Glutamate and depression: Clinical and preclinical studies. *Ann N Y Acad Sci* 1003:250–272.
78. Trullas R, Skolnick P (1990) Functional antagonists at the NMDA receptor complex exhibit antidepressant actions. *Eur J Pharmacol* 185(1):1–10.
79. Yilmaz A, Schulz D, Aksoy A, Canbeyli R (2002) Prolonged effect of an anesthetic dose of ketamine on behavioral despair. *Pharmacol Biochem Behav* 71(1-2):341–344.
80. Machado-Vieira R, Salvadore G, Diazgranados N, Zarate CA, Jr (2009) Ketamine and the next generation of antidepressants with a rapid onset of action. *Pharmacol Ther* 123(2):143–150.
81. Pittenger C, Sanacora G, Krystal JH (2007) The NMDA receptor as a therapeutic target in major depressive disorder. *CNS Neurol Disord Drug Targets* 6(2):101–115.
82. Zarate CA, Jr, et al. (2004) An open-label trial of riluzole in patients with treatment-resistant major depression. *Am J Psychiatry* 161(1):171–174.
83. Templin JS, Bang SJ, Soiza-Reilly M, Berde CB, Commons KG (2012) Patterned expression of ion channel genes in mouse dorsal raphe nucleus determined with the Allen Mouse Brain Atlas. *Brain Res* 1457:1–12.
84. Xu ZQ, Zheng K, Hökfelt T (2005) Electrophysiological studies on galanin effects in brain–progress during the last six years. *Neuropeptides* 39(3):269–275.
85. Sharkey LM, Madamba SG, Siggins GR, Bartfai T (2008) Galanin alters GABAergic neurotransmission in the dorsal raphe nucleus. *Neurochem Res* 33(2):285–291.
86. Le Maître E, Barde SS, Palkovits M, Diaz-Heijtz R, Hökfelt TG (2013) Distinct features of neurotransmitter systems in the human brain with focus on the galanin system in locus coeruleus and dorsal raphe. *Proc Natl Acad Sci USA* 110(6):E536–E545.
87. Barr AM, et al. (2006) A novel, systemically active, selective galanin receptor type-3 ligand exhibits antidepressant-like activity in preclinical tests. *Neurosci Lett* 405(1-2): 111–115.
88. Swanson CJ, et al. (2005) Anxiolytic- and antidepressant-like profiles of the galanin-3 receptor (Gal3) antagonists SNAP 37889 and SNAP 398299. *Proc Natl Acad Sci USA* 102(48):17489–17494.
89. Xu ZQ, Zhang X, Pieribone VA, Grillner S, Hökfelt T (1998) Galanin-5-hydroxytryptamine interactions: Electrophysiological, immunohistochemical and in situ hybridization studies on rat dorsal raphe neurons with a note on galanin R1 and R2 receptors. *Neuroscience* 87(1):79–94.
90. Gras C, et al. (2002) A third vesicular glutamate transporter expressed by cholinergic and serotoninergic neurons. *J Neurosci* 22(13):5442–5451.
91. Hioki H, et al. (2010) Vesicular glutamate transporter 3-expressing nonserotonergic projection neurons constitute a subregion in the rat midbrain raphe nuclei. *J Comp Neurol* 518(5):668–686.
92. Schäfer MK, Varoqui H, Defamie N, Weihe E, Erickson JD (2002) Molecular cloning and functional identification of mouse vesicular glutamate transporter 3 and its expression in subsets of novel excitatory neurons. *J Biol Chem* 277(52):50734–50748.
93. Shutoh F, Ina A, Yoshida S, Konno J, Hisano S (2008) Two distinct subtypes of serotonergic fibers classified by co-expression with vesicular glutamate transporter 3 in rat forebrain. *Neurosci Lett* 432(2):132–136.
94. Eberwine J, Bartfai T (2011) Single cell transcriptomics of hypothalamic warm sensitive neurons that control core body temperature and fever response Signaling asymmetry and an extension of chemical neuroanatomy. *Pharmacol Ther* 129(3): 241–259.
95. Bartfai T, Buckley PT, Eberwine J (2012) Drug targets: Single-cell transcriptomics hastens unbiased discovery. *Trends Pharmacol Sci* 33(1):9–16.
96. Fuxe K, et al. (2012) On the existence and function of galanin receptor heteromers in the central nervous system. *Front Endocrinol (Lausanne)* 3:127.
97. Borroto-Escuela DO, et al. (2010) Galanin receptor-1 modulates 5-hydroxtryptamine-1A signaling via heterodimerization. *Biochem Biophys Res Commun* 393(4):767–772.
98. Millon C, et al. (2015) A role for galanin N-terminal fragment (1-15) in anxiety- and depression-related behaviors in rats. *Intl J Neuropsychopharmacol* 18(3):1–13.
99. Xu ZQ, Ma X, Soomets U, Langel U, Hökfelt T (1999) Electrophysiological evidence for a hyperpolarizing, galanin (1-15)-selective receptor on hippocampal CA3 pyramidal neurons. *Proc Natl Acad Sci USA* 96(25):14583–14587.

100. Hedlund PB, Yanaihara N, Fuxe K (1992) Evidence for specific N-terminal galanin fragment binding sites in the rat brain. *Eur J Pharmacol* 224(2-3):203–205.
101. Jacobs BL, Azmitia EC (1992) Structure and function of the brain serotonin system. *Physiol Rev* 72(1):165–229.
102. Dahlström A, Fuxe K (1964) Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons. *Acta Physiol Scand* 62(Suppl 232):1–55.
103. Steinbusch HW (1981) Distribution of serotonin-immunoreactivity in the central nervous system of the rat-cell bodies and terminals. *Neuroscience* 6(4):557–618.
104. Commons KG (2015) Two major network domains in the dorsal raphe nucleus. *J Comp Neurol* 523(10):1488–1504.
105. Kirby LG, Pernar L, Valentino RJ, Beck SG (2003) Distinguishing characteristics of serotonin and non-serotonin-containing cells in the dorsal raphe nucleus: Electrophysiological and immunohistochemical studies. *Neuroscience* 116(3):669–683.
106. Marinelli S, et al. (2004) Serotonergic and nonserotonergic dorsal raphe neurons are pharmacologically and electrophysiologically heterogeneous. *J Neurophysiol* 92(6): 3532–3537.
107. Calizo LH, et al. (2011) Raphe serotonin neurons are not homogenous: Electrophysiological, morphological and neurochemical evidence. *Neuropharmacology* 61(3):524–543.
108. Brown RE, et al. (2008) Characterization of GABAergic neurons in rapid-eye-movement sleep controlling regions of the brainstem reticular formation in GAD67-green fluorescent protein knock-in mice. *Eur J Neurosci* 27(2):352–363.
109. Descarries L, Watkins KC, Garcia S, Beaudet A (1982) The serotonin neurons in nucleus raphe dorsalis of adult rat: A light and electron microscope radioautographic study. *J Comp Neurol* 207(3):239–254.
110. Moore RY (1981) The anatomy of central serotonin systems in the rat brain. *Serotonin Neurotransmission and Behavior*, eds Jacobs BL, Gelperin A (MIT Press, Cambridge, MA), pp 35–71.
111. Ottersen OP, Storm-Mathisen J (1984) Glutamate- and GABA-containing neurons in the mouse and rat brain, as demonstrated with a new immunocytochemical technique. *J Comp Neurol* 229(3):374–392.
112. Smith GS, et al. (1994) Distribution of messenger RNAs encoding enkephalin, substance P, somatostatin, galanin, vasoactive intestinal polypeptide, neuropeptide Y, and calcitonin gene-related peptide in the midbrain periaqueductal grey in the rat. *J Comp Neurol* 350(1):23–40.
113. Soiza-Reilly M, Commons KG (2011) Glutamatergic drive of the dorsal raphe nucleus. *J Chem Neuroanat* 41(4):247–255.
114. Lein ES, et al. (2007) Genome-wide atlas of gene expression in the adult mouse brain. *Nature* 445(7124):168–176.
115. Spaethling JM, et al. (2014) Serotonergic neuron regulation informed by in vivo single-cell transcriptomics. *FASEB J* 28(2):771–780.
116. Barbaresi P, Manfrini E (1988) Glutamate decarboxylase-immunoreactive neurons and terminals in the periaqueductal gray of the rat. *Neuroscience* 27(1):183–191.
117. Belin MF, et al. (1979) GABA-accumulating neurons in the nucleus raphe dorsalis and periaqueductal gray in the rat: A biochemical and radioautographic study. *Brain Res* 170(2):279–297.
118. Ford B, Holmes CJ, Mainville L, Jones BE (1995) GABAergic neurons in the rat pontomesencephalic tegmentum: Codistribution with cholinergic and other tegmental neurons projecting to the posterior lateral hypothalamus. *J Comp Neurol* 363(2): 177–196.
119. Gervasoni D, et al. (2000) Role and origin of the GABAergic innervation of dorsal raphe serotonergic neurons. *J Neurosci* 20(11):4217–4225.
120. Harandi M, et al. (1987) gamma-Aminobutyric acid and 5-hydroxytryptamine interrelationship in the rat nucleus raphe dorsalis: Combination of radioautographic and immunocytochemical techniques at light and electron microscopy levels. *Neuroscience* 21(1):237–251.
121. Jankowski MP, Sesack SR (2004) Prefrontal cortical projections to the rat dorsal raphe nucleus: Ultrastructural features and associations with serotonin and gamma-aminobutyric acid neurons. *J Comp Neurol* 468(4):518–529.
122. Mâgoul R, Onteniente B, Oblin A, Calas A (1986) Inter- and intracellular relationship of substance P-containing neurons with serotonin and GABA in the dorsal raphe nucleus: Combination of autoradiographic and immunocytochemical techniques. *J Histochem Cytochem* 34(6):735–742.
123. Sharp T, Boothman L, Raley J, Quérée P (2007) Important messages in the 'post': Recent discoveries in 5-HT neurone feedback control. *Trends Pharmacol Sci* 28(12): 629–636.
124. Sharp T, Cowen PJ (2011) 5-HT and depression: Is the glass half-full? *Curr Opin Pharmacol* 11(1):45–51.
125. Wang QP, Ochiai H, Nakai Y (1992) GABAergic innervation of serotonergic neurons in the dorsal raphe nucleus of the rat studied by electron microscopy double immunostaining. *Brain Res Bull* 29(6):943–948.
126. Varga V, Székely AD, Csillag A, Sharp T, Hajós M (2001) Evidence for a role of GABA interneurones in the cortical modulation of midbrain 5-hydroxytryptamine neurones. *Neuroscience* 106(4):783–792.
127. Weissbourd B, et al. (2014) Presynaptic partners of dorsal raphe serotonergic and GABAergic neurons. *Neuron* 83(3):645–662.
128. Belin MF, et al. (1983) Immunohistochemical evidence for the presence of gamma-aminobutyric acid and serotonin in one nerve cell. A study on the raphe nuclei of the rat using antibodies to glutamate decarboxylase and serotonin. *Brain Res* 275(2): 329–339.
129. Nanopoulos D, Belin MF, Maitre M, Vincendon G, Pujol JF (1982) Immunocytochemical evidence for the existence of GABAergic neurons in the nucleus raphe dorsalis. Possible existence of neurons containing serotonin and GABA. *Brain Res* 232(2):375–389.
130. Shikanai H, et al. (2012) Distinct neurochemical and functional properties of GAD67-containing 5-HT neurons in the rat dorsal raphe nucleus. *J Neurosci* 32(41): 14415–14426.
131. Stamp JA, Semba K (1995) Extent of colocalization of serotonin and GABA in the neurons of the rat raphe nuclei. *Brain Res* 677(1):39–49.
132. Abrams JK, Johnson PL, Hollis JH, Lowry CA (2004) Anatomic and functional topography of the dorsal raphe nucleus. *Ann N Y Acad Sci* 1018:46–57.
133. Chandler DJ, Lamperski CS, Waterhouse BD (2013) Identification and distribution of projections from monoaminergic and cholinergic nuclei to functionally differentiated subregions of prefrontal cortex. *Brain Res* 1522:38–58.
134. Celada P, Puig MV, Artigas F (2013) Serotonin modulation of cortical neurons and networks. *Front Integr Nuerosci* 7:25.
135. Bang SJ, Commons KG (2012) Forebrain GABAergic projections from the dorsal raphe nucleus identified by using GAD67-GFP knock-in mice. *J Comp Neurol* 520(18): 4157–4167.
136. Puig MV, Artigas F, Celada P (2005) Modulation of the activity of pyramidal neurons in rat prefrontal cortex by raphe stimulation in vivo: Involvement of serotonin and GABA. *Cereb Cortex* 15(1):1–14.
137. Johnson P, Lowry C, Truitt W, Shekhar A (2008) Disruption of GABAergic tone in the dorsomedial hypothalamus attenuates responses in a subset of serotonergic neurons in the dorsal raphe nucleus following lactate-induced panic. *J Psychopharmacol* 22(6):642–652.
138. Johnson PL, Lightman SL, Lowry CA (2004) A functional subset of serotonergic neurons in the rat ventrolateral periaqueductal gray implicated in the inhibition of sympathoexcitation and panic. *Ann N Y Acad Sci* 1018:58–64.
139. Crawford LK, Craige CP, Beck SG (2010) Increased intrinsic excitability of lateral wing serotonin neurons of the dorsal raphe: A mechanism for selective activation in stress circuits. *J Neurophysiol* 103(5):2652–2663.
140. Commons KG (2009) Locally collateralizing glutamate neurons in the dorsal raphe nucleus responsive to substance P contain vesicular glutamate transporter 3 (VGLUT3). *J Chem Neuroanat* 38(4):273–281.
141. Jackson J, Bland BH, Antle MC (2009) Nonserotonergic projection neurons in the midbrain raphe nuclei contain the vesicular glutamate transporter VGLUT3. *Synapse* 63(1):31–41.
142. Jolas T, Aghajanian GK (1997) Opioids suppress spontaneous and NMDA-induced inhibitory postsynaptic currents in the dorsal raphe nucleus of the rat in vitro. *Brain Res* 755(2):229–245.
143. Aghajanian GK, Wang RY (1977) Habenular and other midbrain raphe afferents demonstrated by a modified retrograde tracing technique. *Brain Res* 122(2): 229–242.
144. Celada P, Puig MV, Casanovas JM, Guillazo G, Artigas F (2001) Control of dorsal raphe serotonergic neurons by the medial prefrontal cortex: Involvement of serotonin-1A, GABA(A), and glutamate receptors. *J Neurosci* 21(24):9917–9929.
145. Gabbott PL, Warner TA, Jays PR, Salway P, Busby SJ (2005) Prefrontal cortex in the rat: Projections to subcortical autonomic, motor, and limbic centers. *J Comp Neurol* 492(2):145–177.
146. Hajós M, Richards CD, Székely AD, Sharp T (1998) An electrophysiological and neuroanatomical study of the medial prefrontal cortical projection to the midbrain raphe nuclei in the rat. *Neuroscience* 87(1):95–108.
147. Lee HS, Kim MA, Valentino RJ, Waterhouse BD (2003) Glutamatergic afferent projections to the dorsal raphe nucleus of the rat. *Brain Res* 963(1-2):57–71.
148. Peyron C, Petit JM, Rampon C, Jouvet M, Luppi PH (1998) Forebrain afferents to the rat dorsal raphe nucleus demonstrated by retrograde and anterograde tracing methods. *Neuroscience* 82(2):443–468.
149. Sesack SR, Deutch AY, Roth RH, Bunney BS (1989) Topographical organization of the efferent projections of the medial prefrontal cortex in the rat: An anterograde tract-tracing study with Phaseolus vulgaris leucoagglutinin. *J Comp Neurol* 290(2): 213–242.
150. Pollak Dorocic I, et al. (2014) A whole-brain atlas of inputs to serotonergic neurons of the dorsal and median raphe nuclei. *Neuron* 83(3):663–678.
151. Commons KG, Beck SG, Bey VW (2005) Two populations of glutamatergic axons in the rat dorsal raphe nucleus defined by the vesicular glutamate transporters 1 and 2. *Eur J Neurosci* 21(6):1577–1586.
152. Li YQ, Li H, Kaneko T, Mizuno N (2001) Morphological features and electrophysiological properties of serotonergic and non-serotonergic projection neurons in the dorsal raphe nucleus. An intracellular recording and labeling study in rat brain slices. *Brain Res* 900(1):110–118.
153. Fuxe K, et al. (2010) The discovery of central monoamine neurons gave volume transmission to the wired brain. *Prog Neurobiol* 90(2):82–100.
154. Holets VR, Hökfelt T, Rökaeus A, Terenius L, Goldstein M (1988) Locus coeruleus neurons in the rat containing neuropeptide Y, tyrosine hydroxylase or galanin and their efferent projections to the spinal cord, cerebral cortex and hypothalamus. *Neuroscience* 24(3):893–906.
155. Xu ZQ, Shi TJ, Hökfelt T (1998) Galanin/GMAP- and NPY-like immunoreactivities in locus coeruleus and noradrenergic nerve terminals in the hippocampal formation and cortex with notes on the galanin-R1 and -R2 receptors. *J Comp Neurol* 392(2): 227–251.
156. Sweerts BW, Jarrott B, Lawrence AJ (1999) Expression of preprogalanin mRNA following acute and chronic restraint stress in brains of normotensive and hypertensive rats. *Brain Res Mol Brain Res* 69(1):113–123.
157. Holmes PV, Blanchard DC, Blanchard RJ, Brady LS, Crawley JN (1995) Chronic social stress increases levels of preprogalanin mRNA in the rat locus coeruleus. *Pharmacol Biochem Behav* 50(4):655–660.

158. Sciolino NR, Holmes PV (2012) Exercise offers anxiolytic potential: A role for stress and brain noradrenergic-galaninergic mechanisms. *Neurosci Biobehav Rev* 36(9): 1965–1984.
159. Gundlach AL, Rutherfurd SD, Louis WJ (1990) Increase in galanin and neuropeptide Y mRNA in locus coeruleus following acute reserpine treatment. *Eur J Pharmacol* 184(1):163–167.
160. Baraban JM, Aghajanian GK (1981) Noradrenergic innervation of serotonergic neurons in the dorsal raphe: Demonstration by electron microscopic autoradiography. *Brain Res* 204(1):1–11.
161. Fritschy JM, Grzanna R (1990) Distribution of locus coeruleus axons within the rat brainstem demonstrated by Phaseolus vulgaris leucoagglutinin anterograde tracing in combination with dopamine-beta-hydroxylase immunofluorescence. *J Comp Neurol* 293(4):616–631.
162. Kim MA, Lee HS, Lee BY, Waterhouse BD (2004) Reciprocal connections between subdivisions of the dorsal raphe and the nuclear core of the locus coeruleus in the rat. *Brain Res* 1026(1):56–67.
163. O'Leary OF, Bechtholt AJ, Crowley JJ, Valentino RJ, Lucki I (2007) The role of noradrenergic tone in the dorsal raphe nucleus of the mouse in the acute behavioral effects of antidepressant drugs. *Eur Neuropsychopharmacol* 17(3):215–226.
164. Peyron C, Luppi PH, Fort P, Rampon C, Jouvet M (1996) Lower brainstem catecholamine afferents to the rat dorsal raphe nucleus. *J Comp Neurol* 364(3):402–413.
165. Porsolt RD, Le Pichon M, Jalfre M (1977) Depression: A new animal model sensitive to antidepressant treatments. *Nature* 266(5604):730–732.
166. Paxinos G, Watson C (2007) *The Rat Brain in Stereotaxic Coordinates* (Academic/Elsevier, Amsterdam), 6th Ed.
167. Paxinos G, Watson C (2005) *The Rat Brain in Stereotaxic Coordinates* (Academic, Sydney), 4th Ed.
168. Shi TJ, et al. (2012) Secretagogin is expressed in sensory CGRP neurons and in spinal cord of mouse and complements other calcium-binding proteins, with a note on rat and human. *Mol Pain* 8:80.
169. Theodorsson E, Rugarn O (2000) Radioimmunoassay for rat galanin: Immunochemical and chromatographic characterization of immunoreactivity in tissue extracts. *Scand J Clin Lab Invest* 60(5):411–418.
170. Steinbusch HW, Verhofstad AA, Joosten HW (1982) Antibodies to serotonin for neuroimmunocytochemical studies. *J Histochem Cytochem* 30(8):756–759.
171. Hökfelt T, Johansson O, Goldstein M (1984) Central catecholamine neurons as revealed by immunohistochemistry with special reference to adrenaline neurons. *Classical Transmitters in the CNS, Part 1: Handbook of Chemical Neuroanatomy*, eds Björklund A, Hökfelt T (Elsevier, Amsterdam), Vol 2, pp 157–266.