

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

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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.

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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 galanergic 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. 2A) and a significant decrease of both horizontal and vertical scores (Fig. 2B and C). In the FST, the CMS group exhibited a significant increase in immobility time (Fig. 2D) and a decrease in climbing time (Fig. 2E). All depression-like behaviors were significantly improved after escitalopram treatment (Fig. 2A-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. 3A-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. 3K-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. 3N).

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. 4A).

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. 4B 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. 4B and C).

In the FST, CMS groups showed a significant increase of immobility time (Fig. 4D) and a decrease of climbing time (Fig. 4E) 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. 4D and E).

Sucrose preference was decreased in the CMS group compared with controls (Fig. 4F). Such a decrease was not observed after infusion of Galr1 siRNA, whereas it remained decreased after scramble siRNA (Fig. 4F). 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. 5A). 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. 5A and 6A-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. 5B). Moreover, examining adjacent/semadjacent sections of naive rats (no double labeling), Galr1 mRNA showed a limited overlap with the tryptophan hydroxylase 2 (Tph2) transcript (Fig. 6A and D). However, glutamic acid decarboxylase (Gad) (Fig. 6B and E) and the vesicular glutamate transporter 2 (Vglut2) (Fig. 6C 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

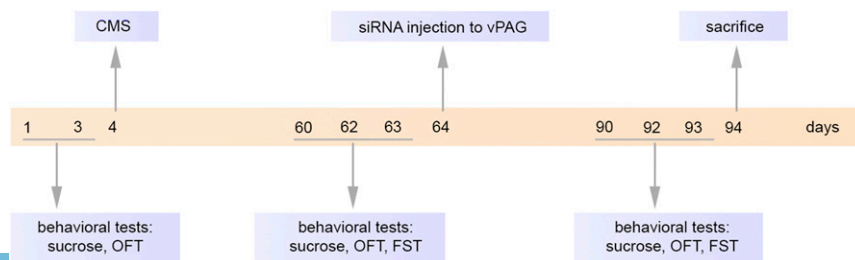


Fig. 1. Time schedule for the experiment. Note that the sucrose test is always followed by 1 d without testing. sucrose, sucrose intake test.

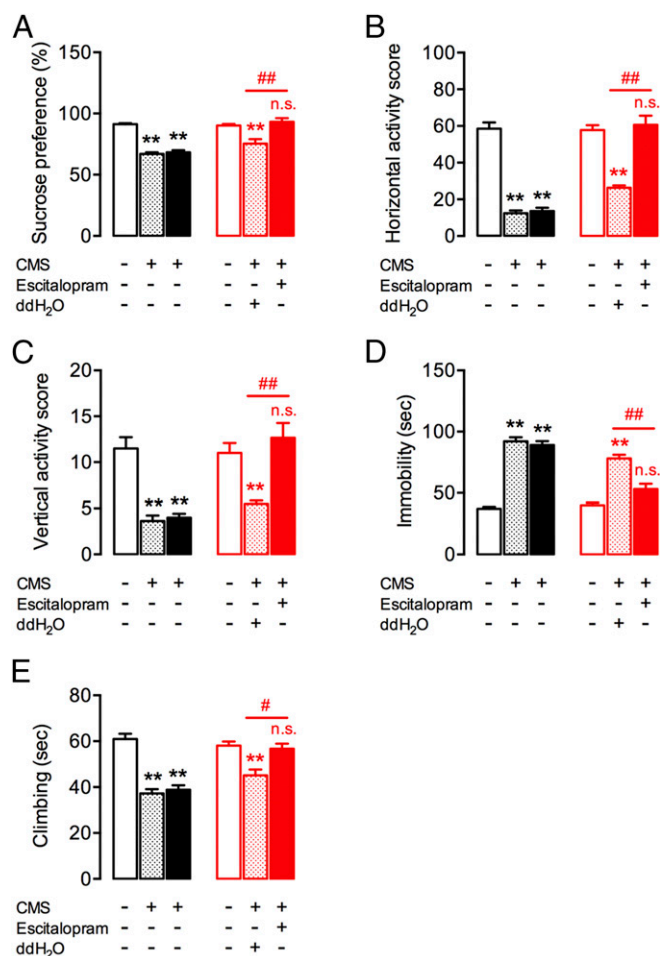


Fig. 2. Rats exposed to CMS show depression-like behaviors, and effects of escitalopram. (A–C) After the 8-wk stress schedule, the CMS group shows a significant decrease of sucrose intake and horizontal and vertical activity scores, which are rescued by escitalopram treatment [sucrose intake $F(2, 38) = 49.98, P < 0.0001$ 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 \pm 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 (ddH₂O). 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 venlafaxine 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

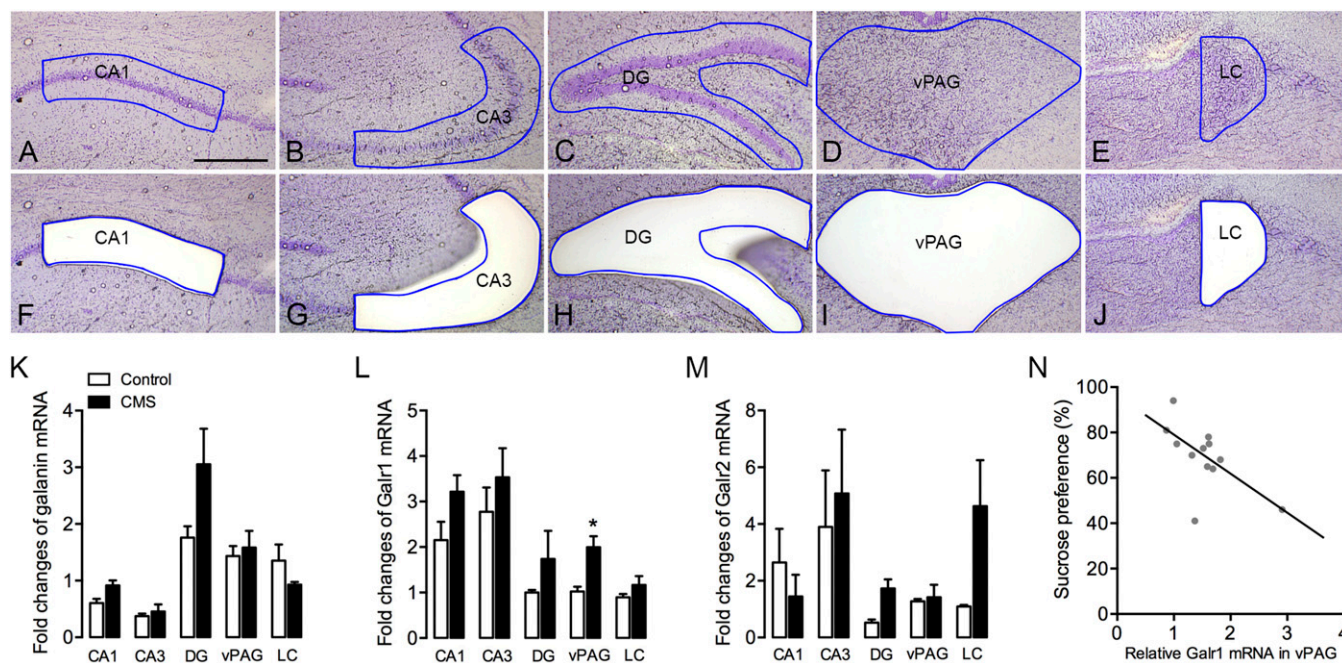


Fig. 3. Galr1 mRNA levels are increased in vPAG after CMS. Laser capture microdissection and transcript levels for galanin Galr1 and Galr2. In the section of the HPC, CA1 was first microdissected (A and F); then, CA3 (B and G) and, finally, DG (C and H) were microdissected. In the brainstem, the vPAG 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 \pm 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 μ m).

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 triphosphate 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-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 J) 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

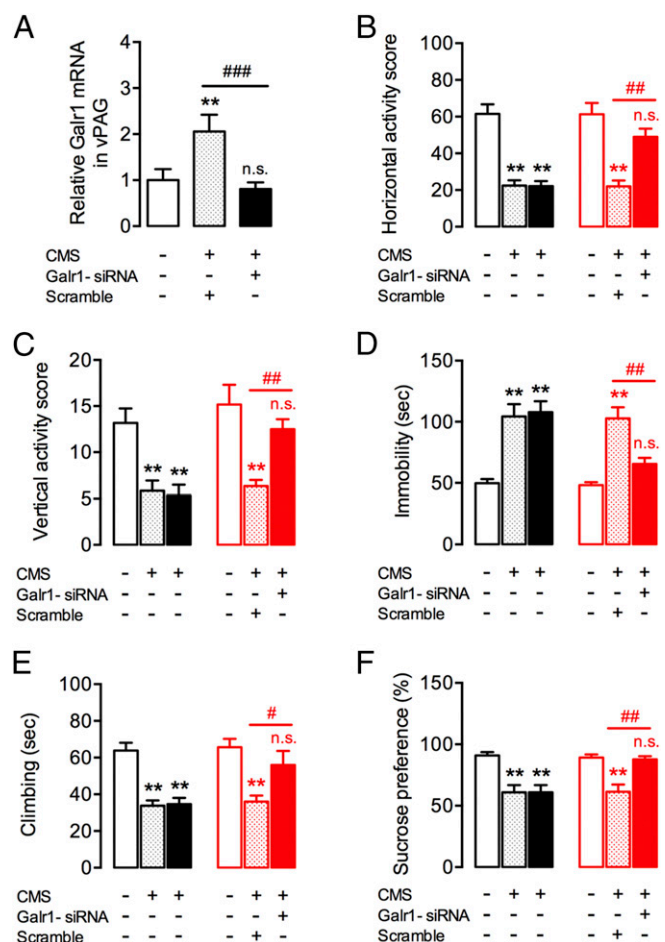


Fig. 4. Galr1-siRNA reduces Galr1 mRNA levels in vPAG and attenuates depressive-like behaviors. (A) Galr1 mRNA levels in the CMS-Galr1-scramble group are increased in the vPAG compared with control, and this increase is completely blocked after Galr1-siRNA treatment. $**P < 0.01$ compared with control 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 \pm 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

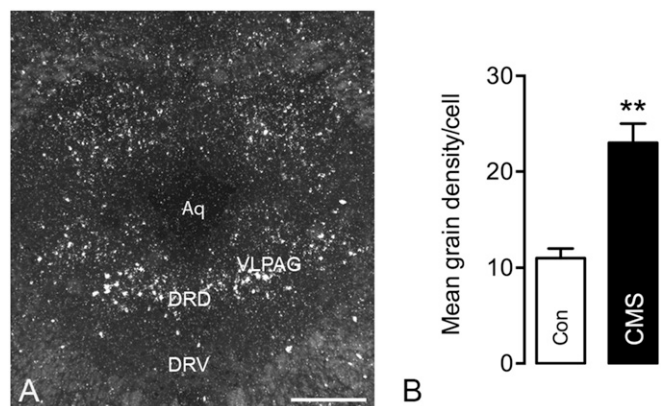


Fig. 5. Galr1 mRNA is present in the entire PAG and is increased after CMS. (A) Galr1 mRNA expression in a control rat. Note the presence of positive cells in the entire PAG with a stronger signal in some cells, especially in the VLPAG. Few cells are seen in the DRD, and hardly any cells are seen in 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 \pm SEM ($n = 6$). $**P < 0.01$ compared with control group (Student's *t* test). Aq, aqueduct; Con, control. (Scale bar: 500 μ m.)

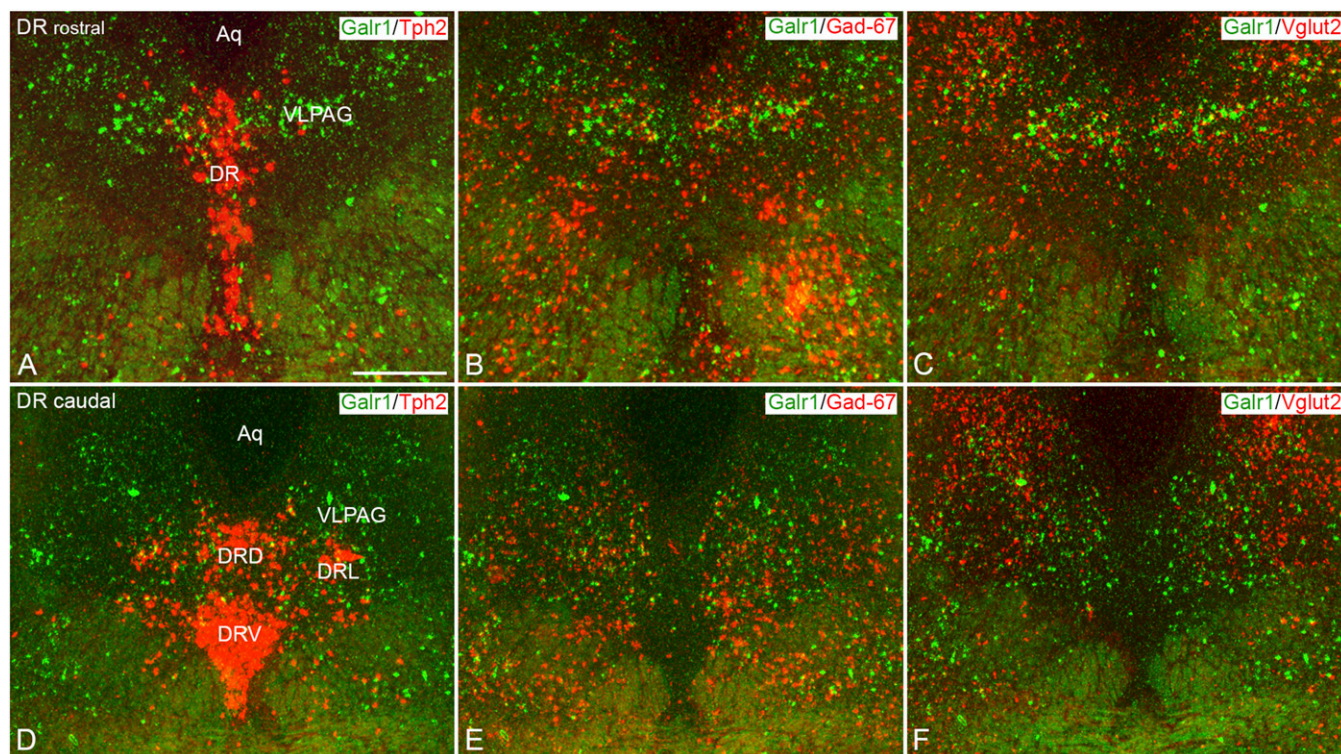


Fig. 6. Galr1 mRNA only partly overlaps with Tph2 mRNA in the vPAG gray, but it overlaps more extensively with Gad-67 and Vglut2 mRNAs. Adjacent slides were hybridized with probes for Galr1 (green) or for Tph2, Gad-67, or Vglut2 (all three red). The color-coded autoradiograms were then superimposed, 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 antidepressant 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 \times 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 (Rn99999916_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 μ m 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) para-

formaldehyde, and the brains were postfixed in the same fixative, kept in 10% (wt/vol) sucrose for 2 d, frozen, and sectioned at 20 μ m 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 $P < 0.05$.

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