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Differential Role of Galanin Receptors in the Regulation of Depression-Like Behavior and Monoamine/Stress-Related Genes at the Cell Body Level

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The present study on rat examined the role of galanin receptor subtypes in regulation of depression-like behavior as well as potential molecular mechanisms involved in the locus coeruleus (LC) and dorsal raphe (DR). The effect of intracerebroventricular (i.c.v.) infusion of galanin or galanin receptor GalR1- and GalR2-selective ligands was studied in the forced swim test, followed by quantitative *in situ* hybridization studies. Naive control, non-treated (swim control), saline- and fluoxetine-treated rats were used as controls in the behavioral and *in situ* hybridization studies. Subchronic treatment with fluoxetine reduced immobility and climbing time. Intracerebroventricular infusion of galanin, the GalR1 agonist M617 or the GalR2 antagonist M871 increased, while the GalR2(R3) agonist AR-M1896 decreased, immobility time compared to the aCSF-treated animals. Galanin also decreased the time of climbing. Galanin mRNA levels were upregulated by the combination of injection + swim stress in the saline- and the fluoxetine-treated groups in the LC, but not in the DR. Also tyrosine hydroxylase levels in the LC were increased following injection + swim stress in the saline- and fluoxetine-treated rats. Tryptophan hydroxylase 2 and serotonin transporter mRNAs were not significantly affected by any treatment. 5-HT_{1A} mRNA levels were downregulated following i.c.v. galanin, M617 or AR-M1896 infusion. These results indicate a differential role of galanin receptor subtypes in depression-like behavior in rodents: GalR1 subtype may mediate 'prodepressive' and GalR2 'antidepressant' effects of galanin. Galanin has a role in behavioral adaptation to stressful events involving changes of molecules important for noradrenaline and/or serotonin transmission.
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INTRODUCTION

Depression is among the most prevalent mood disorders involving genetic and environmental predisposing factors, particularly exposure to stressful events (see Holsboer, 2001; Nestler *et al*, 2002). Dysfunctions of brain noradrenaline (NA) and serotonin (5-hydroxytryptamine, 5-HT) neurotransmission as a consequence of uncontrollable (chronic) stress are implicated in depression (see Weiss *et al*, 1981; Heninger *et al*, 1996; Holsboer, 2001). In fact, the current pharmacotherapy of depression is also mainly based on use of compounds correcting dysfunctions in monoaminergic transmission, including selective serotonin reuptake inhibitors (SSRIs) (see Frazer, 1997; Millan, 2004). However, recent studies have also implicated several neuropeptides in the pathophysiology of depression and in the action of antidepressant drugs (see Heilig, 2004; Lu *et al*, 2005a, 2007; Nikisch *et al*, 2005; Ögren *et al*, 2006).

In this context, the neuropeptide galanin (Tatemoto *et al*, 1983) is of particular interest, since it coexists with NA in the locus coeruleus (LC) and with 5-HT in the dorsal raphe (DR) nucleus (Melander *et al*, 1986; Xu and Hökfelt, 1997; Xu *et al*, 1998b). Electrophysiological, behavioral, and neurochemical studies have shown that galanin exerts modulatory (mainly inhibitory) effects on both the noradrenergic and serotonergic systems (Seutin *et al*, 1989; Sevcik *et al*, 1993; Pieribone *et al*, 1995; Xu *et al*, 1998c; Razani *et al*, 2001; Kehr *et al*, 2002; see Ögren *et al*, 2006). Moreover, *in vivo* galanin can modulate 5-HT_{1A} pre- and postsynaptic receptor functions in an antagonistic manner (see Fuxe *et al*, 1998; Misane *et al*, 1998; Razani *et al*, 2001; Kehr *et al*, 2002; Ögren *et al*, 2006).

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The action of galanin is mediated via three G protein-coupled receptors, GalR1-GalR3 (see Branchek *et al*, 2000), which are expressed in the LC, DR, and their projection areas (Xu *et al*, 1998b, c; O'Donnell *et al*, 1999; Burazin *et al*, 2000; Larm *et al*, 2003; Hawes and Picciotto, 2004; Hawes *et al*, 2005; Swanson *et al*, 2005). Among these receptors, GalR1 and GalR3 mainly activate $G_{i/o}$ types of G proteins mediating inhibitory actions of galanin (Habert-Ortoli *et al*, 1994; Burgevin *et al*, 1995; Parker *et al*, 1995; see Branchek *et al*, 2000). In contrast, the GalR2 subtype can transmit either stimulatory effects of galanin, for example, on neurotransmitter release, acting via $G_{q/11}$ types of G proteins (Smith *et al*, 1997; Wang *et al*, 1997; Fathi *et al*, 1998), or it can inhibit neurotransmission via $G_{i/o}$ types (Fathi *et al*, 1998; Wang *et al*, 1998; see Branchek *et al*, 2000). Thus, the physiological effects of galanin are varied and complex.

A number of behavioral studies suggested a potential role of galanin in depression-like behavior in rodents (see Fuxe *et al*, 1998; Weiss *et al*, 1998, 2005; Yoshitake *et al*, 2004; Kuteeva *et al*, 2005, 2007; Lu *et al*, 2005a, 2007; Swanson *et al*, 2005; Barr *et al*, 2006; Karlsson and Holmes, 2006; Ögren *et al*, 2006). Bilateral infusion of galanin into the ventral tegmental area (VTA) (Weiss *et al*, 1998), or intracerebroventricular (i.c.v.) administration of galanin (Kuteeva *et al*, 2007), increased immobility time in the forced swim test (FST) in the rat, indicative of depression-like behavior. This increase was blocked by the non-specific galanin receptor antagonists M15 and M35, respectively (Weiss *et al*, 1998; Kuteeva *et al*, 2007). Moreover, infusion of the galanin antagonist M15 into the VTA, and i.c.v administration of the galanin antagonist M35, reduced immobility, suggesting an antidepressant-like profile of galanin antagonists (Weiss *et al*, 1998, 2005; Kuteeva *et al*, 2007).

Studies on the functional role of galanin receptor subtypes have been hampered by the lack of galanin receptor-selective agonists and antagonists (see Karlsson and Holmes, 2006; Ögren *et al*, 2006; Lu *et al*, 2007). Recently, both peptidergic and non-peptidergic compounds with relatively high selectivity for galanin receptor subtypes have been developed (Lundström *et al*, 2005; Swanson *et al*, 2005; Barr *et al*, 2006; Sollenberg *et al*, 2006), allowing studies on selective involvement of galanin receptor subtypes in regulation of physiological functions. For instance, newly developed non-peptide GalR3 antagonists, when given by systemic routes, were shown to have an antidepressant-like activity in behavioral models of anxiety and depression (Swanson *et al*, 2005; Barr *et al*, 2006).

The purpose of the present study was to assess the role of galanin receptor subtypes in depression-like behavior, using selective peptide ligands for the GalR1 and GalR2 receptors (Liu *et al*, 2001; Lundström *et al*, 2005; Sollenberg *et al*, 2006). As stress is involved in the pathogenesis of depression, the effects of forced swim on the endogenous galanin systems of the LC and DR was studied by *in situ* hybridization. In addition, the interaction between galanin receptor stimulation and exposure to stress was assessed by measuring the expression of neurochemical markers of relevance for depression in the cell bodies of the LC NA and DR 5-HT neurons.

MATERIALS AND METHODS

Animals

Adult male Sprague–Dawley rats ($n = 73$, 10–12 weeks old) (Scanbur, Sollentuna, Sweden), weighing 290–320 g at the time of surgery, were used in all experiments. The rats were housed four per cage in standard plastic cages (Type IV Makrolon®) in a colony room under standardized conditions (12 h light/dark cycle, lights on at 0700 h; temperature of $21 \pm 0.5^{\circ}$C; relative humidity 40–50%). Food and water were provided *ad libitum*. Rats, which underwent i.c.v. cannula implantation, were after the stereotaxic surgery housed two in each cage, separated by a transparent plastic wall (to avoid chewing of the chronic cannulae). All animals were treated according to guidelines approved by the local ethics committee (Stockholm Northern Ethics Board of Animal Experimentation, ethical number 267/05).

Stereotaxic Surgery

Rats were anesthetized with isoflurane (Apoteket, Stockholm, Sweden; induction 4.7%, maintenance 2.1–3.4% and airflow of 364–380 ml/min) using an anesthesia pump (AgnTho's, Lidingö, Sweden). The anesthetized rat was placed in a stereotaxic frame (David Kopff Instruments, Tujunga, CA, USA), and body temperature was maintained at 37°C using a temperature-controlled heating pad (CMA/105, CMA/Microdialysis, Stockholm, Sweden). A permanent steel guide cannula (26 GA; diameter 0.45 mm, Plastics One, Roanoke, VA, USA) was implanted into the right lateral ventricle at the following coordinates: 1.3 mm posterior to the bregma, 1.8 mm lateral to the mid-sagittal line and 3.7 mm ventral to the surface of the skull (infusion site 0.5 mm below the ventral coordinate). The guide cannula was secured to the skull using three microscrews and dental cement (Dentalon®, AgnTho's). Finally, a dummy cannula was inserted into the guide cannula.

After surgery, each animal received an injection of saline (1.5 ml subcutaneously in the neck) to compensate for the possible fluid loss during anesthesia, and one intramuscular injection of buprenorphin (Temgesic®, Shering-Plough AB, Stockholm, Sweden; 0.05 ml/kg) for postoperative pain relief. The animals were allowed to recover for 7 days in the colony room before the start of the experiment.

Compound and Peptides

The following compound and peptides were used in the present study. Fluoxetine hydrochloride (Sigma Aldrich, St Louis, MO, USA), rat galanin (Bachem, Budendorf, Switzerland), the GalR1 agonist M617 (galanin-(1–13)-Gln^{14}-bradykinin-(2–9)-amide) (Lundström *et al*, 2005), the GalR2(R3) agonist AR-M1896 (galanin-(2–11)-amide) (Liu *et al*, 2001), and the GalR2 antagonist M871 (galanin-(2–13)-Glu-His-$(Pro)_3$-$(Ala\text{-}Leu)_2$-Ala-amide) (Sollenberg *et al*, 2006).

Experimental Design

The present study was designed to assess the effects of exposure to swim stress, antidepressant treatment, and galaninergic system activation/blockade on depression-like

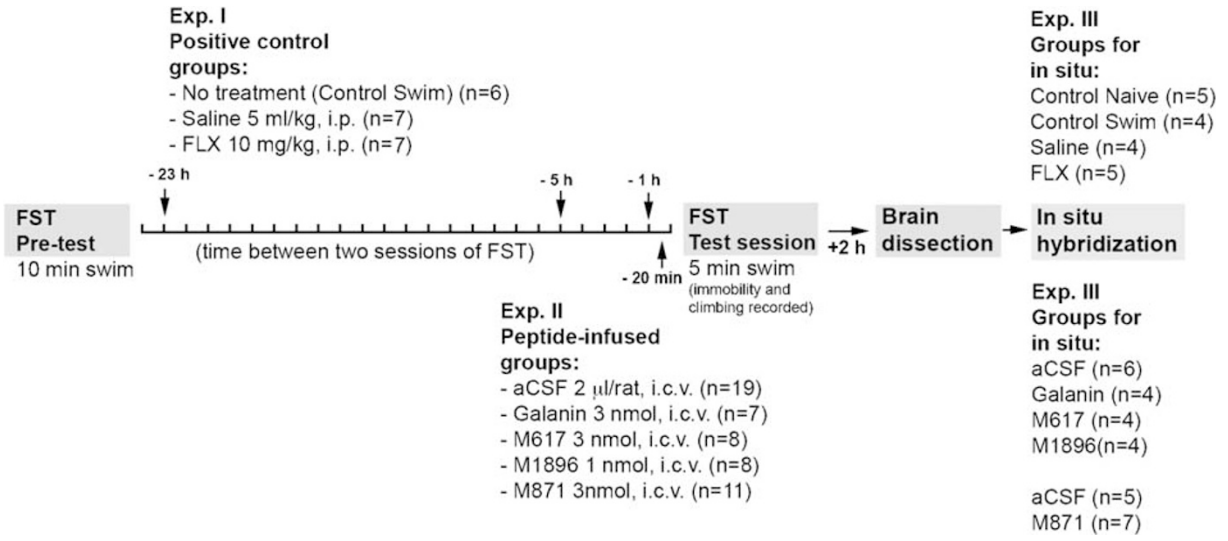

Figure 1 Schematic illustration of the experimental design. Depression-like behavior was assessed in the FST (experiments I and II), which consisted of two swimming sessions: a 10 min pre-test, 24 h later followed by a 5 min test when the immobility and climbing times were recorded. In the experiment I, rats received either no pharmacological treatment (only the FST, control swim), or three i.p. injections of saline (saline) or fluoxetine (FLX) 23, 5, and 1 h prior to the 5-min test (indicated by three arrows, above time scale). In the experiment II, rats received a single i.c.v. infusion of aCSF or peptide solution 20 min prior to the 5 min test (indicated by arrow, below time scale). Two hours after the FST animals were killed and brains were dissected out for *in situ* hybridization studies (experiment III). In the experiment III, also a group of rats, not exposed to the FST and not receiving any pharmacological treatment (control naive), was used for control. Doses of the compound and peptides and the number of animals per group are indicated. FLX- fluoxetine; aCSF- artificial cerebrospinal fluid; M617- the GalR1 agonist; M1896- the GalR2(R3) agonist AR-M1896; M871- the GalR2 antagonist.

behavior and on expression of various molecular markers in the LC/DR. For this purpose, rats receiving various pharmacological treatments and subjected to the FST were killed after the FST, and processed for *in situ* hybridization analysis. Thus, the same animals were used for behavioral studies and mRNA measurements. Naive rats, not receiving any pharmacological treatment and not subjected to the FST, were used as controls in the *in situ* experiments. The details of the experimental design are summarized in Figure 1.

The first experiment, consisting of three groups of rats, was performed to establish whether the stress of i.p. injection procedure could affect the results of the FST and of *in situ* hybridization. Therefore, a non-treated group (receiving only swim stress, 'control swim' group) was compared with the group receiving i.p. injections of saline 23, 5, and 1 h before the FST ('saline' group). As a positive control, a group of rats was treated with fluoxetine hydrochloride (10 mg/kg in 5 ml saline, i.p.) 23, 5, and 1 h before the FST (Detke and Lucki, 1996) ('fluoxetine' group). The fourth group of rats, which were not subjected to either swim stress or any injections ('naive control'), served as a non-stressed control for the *in situ* hybridization studies (see Figure 1).

In the second experiment, five groups of animals were given i.c.v. infusion of either artificial cerebrospinal fluid (aCSF, pH = 7.1), galanin or galanin receptor-selective ligands via the chronic cannula prior to the FST. A microinfusion pump (CMA/Microdialysis) and Hamilton syringe (25 µl) were used to infuse rat galanin (3 nmol per rat), the GalR1 agonist M617 (3 nmol), the GalR2(R3) agonist AR-M1896 (1 nmol) or the GalR2 antagonist M871 (3 nmol), or aCSF for the control group (see Figure 1 for the details). The dose of galanin was based on earlier *in vivo* results in the FST (Kuteeva *et al*, 2007), and the selection of doses for the galanin receptor-selective peptides was based on their relative *in vitro* affinity for the galanin receptors compared to galanin itself (Lundström *et al*, 2005; Sollenberg *et al*, 2006). The aCSF and peptide solutions (dissolved in aCSF) were freshly made and coded before the i.c.v. infusion. Each rat received a single infusion of 2 µl of aCSF or peptide solution during 1 min. The injection cannula was left inside the guide cannula for an additional 60 s after the injection to prevent back-flow. The animals were tested in the FST 20 min after the i.c.v. infusion.

Forced Swim Test

Depression-like behavior was assessed in the FST (Porsolt *et al*, 1977; Detke and Lucki, 1996). Animals were individually placed in a vertical glass cylinder (50 cm height, 20 cm in diameter) containing tap water ($25 \pm 0.5^{\circ}$C) to a height of 30 cm. Two swimming sessions were conducted: a 10 min pre-test, 24 h later followed by a 5 min test. The total durations of immobility and climbing behavior were recorded during the second, 5 min test. Immobility was defined as floating passively in an upright position in water, with only small movements necessary to keep the head above the water surface. Climbing was defined as vigorous forepaw movements directed toward the walls of the cylinder.

Histological Techniques

Two hours after the 5-min FST test session, animals were killed by decapitation. The brains were rapidly dissected out, immersed in ice-cold 0.9% NaCl and immediately frozen on dry ice.

For the histological control of cannula position in the right ventricle, sections were cut in a cryostat

(Microm, Heidelberg, Germany) at 20 μm thickness, thaw-mounted on gelatine-alume coated slides, stained with cresyl violet and viewed in a microscope.

For *in situ* hybridization, sections were cut in a cryostat (Microm) at 14 μm thickness and thaw-mounted on SuperFrost®Plus slides (VWR International AB, Stockholm, Sweden). Sections containing the LC were collected between −10.0 and −9.8 mm from bregma, and sections containing the DR/median raphe (MR) nuclei between −7.9 and −7.6 mm from bregma, according to the Stereotaxic Atlas of Rat Brain (Paxinos and Watson, 1998).

In Situ Hybridization

Oligonucleotides complementary to rat galanin nucleotides 152–199 (Vrontakis *et al*, 1987), tyrosine hydroxylase (TH) nucleotides 1441–1488 (Grima *et al*, 1985), tryptophan hydroxylase 2 (TPH2) nucleotides 489–536 (Walther *et al*, 2003), rat 5-HT_{1A} receptor nucleotides 762–809 and 1048–1095 (Albert *et al*, 1990) and 5-HT transporter (5-HTT) nucleotides 305–352 (Hoffman *et al*, 1991) were used to study mRNA expression levels in the LC (TH and galanin) and in the DR/MR (TPH2, galanin, $5HT_{1A}$, and 5-HTT). Oligonucleotide probes were labeled with ^{33}P (dATP) (NEN) at the 3′-end using terminal deoxynucleotidyltransferase (Amersham, Amersham, UK) and purified using ProbeQuant™ G-50 microcolumns (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Probes were added to the hybridization solution containing 50% of deionized formamide (Ambion Inc., Austin, TX, USA), 4 × standard saline citrate (SSC), 1 × Denhardt's solution and 50 mg/l denaturated salmon testis DNA (Sigma). Sections were air-dried overnight and hybridized with the oligonucleotide probes (0.5 ng per slide) for 16–24 h at 42°C. In case of the 5-HT_{1A} receptor, a mixture of two probes was used. After hybridization the sections were rinsed for 2 h (4 × 30 min) in 1 × SSC at 55–60°C followed by 1 h at room temperature. Sections were then rinsed in distilled water, rapidly dehydrated in ethanol and air-dried for at least 2 h. For the control of specificity, an excess (100 ×) of the unlabeled probe(s) was added to the hybridization solution. This procedure completely abolished the hybridization signals. The sections were exposed together with ^{14}C standards (Amersham) to a Kodak Biomax MR film (Kodak, Rochester, NY, USA). The films were developed in Kodak LX 24 and fixed in Kodak AL4, rinsed and air-dried.

In situ hybridization for the LC (TH and galanin) was performed in a single experiment for each probe and for the DR/MR for 5-HTT, while independent experiments were conducted for i.p.-injected/control and i.c.v.-infused groups for TPH2, galanin, and $5HT_{1A}$ receptor in the DR/MR. For the M871-treated group, a separate *in situ* hybridization experiment was performed for all the probes (see Figure 1, Exp. 3).

Image Analysis

Analysis of film autoradiograms was carried out by computerized microdensitometry using NIH Image 1.63 software (http:rsb.info.nih.gov/nih-image) after scanning films. ^{14}C standards (Amersham) coexposed with the slides were used to detect the semiquantified levels of radioactivity (nCi/g). Measurements were performed on four sections for the LC (left and right independently), and on 4–6 sections for the DR and MR, and the mean value was calculated for each animal. Since there exist significant anterior–posterior differences in the expression of various genes in the DR (Day *et al*, 2004), the analysis was performed on sections taken at identical levels of mid-rostral DR, the area projecting to the forebrain (Ungerstedt, 1971; Azmitia and Segal, 1978; Steinbusch, 1981). The DR nucleus was subdivided into lateral (lDR), dorsal (dDR), and ventral (vDR) subnuclei, which were each assessed individually.

Statistical Analysis

The results from behavioral and *in situ* hybridization experiments were analyzed using one-way analysis of variance (ANOVA) with treatment as between subject factor, followed by Fisher's protected significant difference test (Fisher's PLSD). The level of significance was set at $p < 0.05$. In case the values exceeded one SD from the mean, they were excluded from the further analysis.

RESULTS

Depression-Like Behavior in the FST

Rats were pre-exposed to water for 10 min, depression-like behavior was assessed 24 h later during a second, 5 min exposure to water.

In the first experiment, an overall ANOVA revealed a significant effect of treatment for immobility ($F2,17 = 3.98$, $p < 0.05$; Figure 2a). Subchronic treatment with the antidepressant drug fluoxetine (10 mg/kg) resulted in a significant decrease of immobility time as compared to both the control swim and the saline-injected groups ($p < 0.05$, Fisher's PLSD; Figure 2a). For climbing, only a tendency for statistical difference was detected using overall ANOVA ($F2,17 = 2.86$, $p = 0.08$; Figure 2b). However, since a clear reduction of immobility was observed in the fluoxetine-treated group, independent group comparisons were performed using the *t*-test. This analysis showed that the fluoxetine-treated group differed significantly from the control swim group ($t = 2.42$, $p < 0.05$; Figure 2b), but not from the saline-injected group ($t = 1.55$, $p = 0.14$).

In the second experiment, the galanin-, M617-, AR-M1896-, and M817-treated groups were run with concurrent independent aCSF-treated controls. ANOVA did not detect any significant difference between the aCSF-treated animals with respect to immobility or climbing time. The results were, therefore, pooled to create a single control group.

An overall ANOVA revealed a significant effect of treatment for the peptide-infused group for immobility ($F4,48 = 10.9$, $p < 0.01$; Figure 2c). *Post hoc* analysis using Fisher's PLSD tests showed that a single i.c.v. infusion of 3 nmol galanin significantly increased immobility time ($p < 0.01$; Figure 2c) compared to the aCSF-treated controls. Intracerebroventricular infusion of the GalR1 receptor agonist M617 (3 nmol) also increased immobility ($p < 0.001$; Figure 2c). In contrast, the GalR2(R3) agonist AR-M1896 (1 nmol) decreased immobility ($p < 0.05$ *vs* the aCSF-treated group, $p < 0.01$ *vs* the galanin-, M617-, and M871-treated groups; Figure 2c). Unlike the agonist

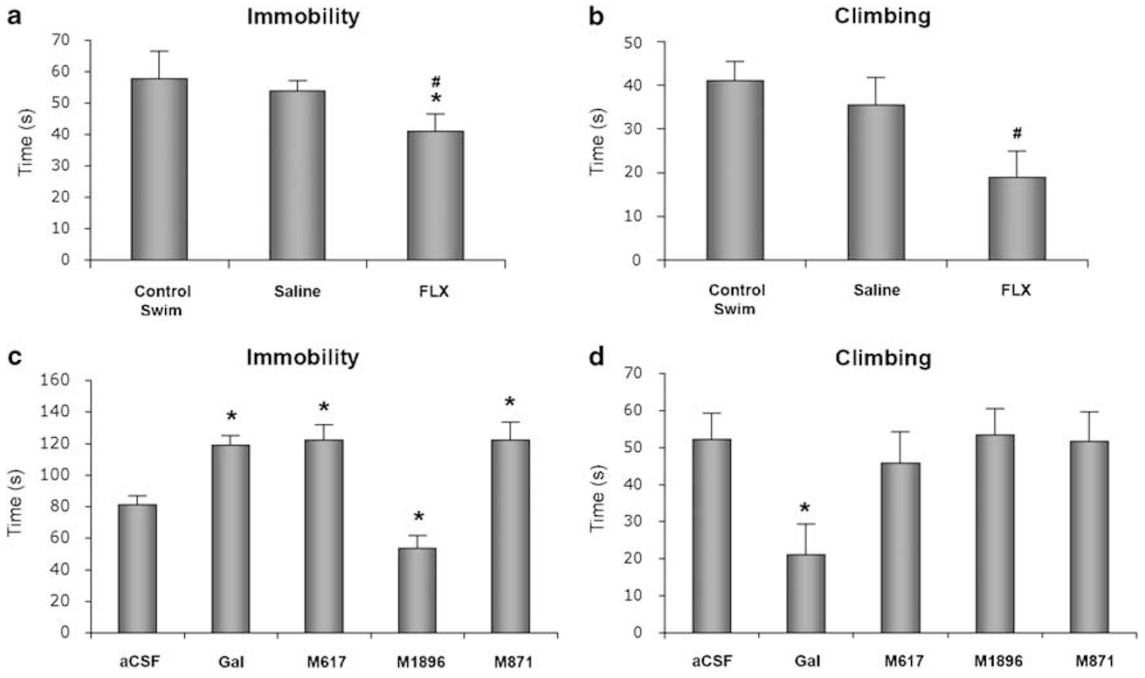

Figure 2 Time of immobility (a, c) and climbing (b, d) in the FST. In the experiment I (a, b), rats received either no pharmacological treatment (control swim, $n=6$), or were injected i.p. with saline (saline, $n=7$) or fluoxetine (FLX, $n=8$) 23, 5, and 1 h prior to the 5 min test. In the experiment II (c, d), rats received i.c.v. infusion of aCSF (aCSF, $n=19$), galanin (Gal, $n=7$), the GalR1 receptor agonist M617 (M617, $n=8$), the GalR2(R3) agonist AR-M1896 (M1896, $n=8$) or the GalR2 antagonist M871 (M871, $n=11$) 20 min prior to the 5 min test. Data presented as mean ± SEM. # on (a, b), significant difference from the control swim group; * on (a), significant difference from the saline group; * on (c, d), significant difference from the aCSF group; $p<0.05$–0.01, one-way ANOVA, Fisher's PLSD (a, c, and d) or *t*-test (b).

AR-M1896, the GalR2 antagonist M871 (3 nmol) increased immobility time ($p<0.001$; Figure 2c). An overall ANOVA did not detect a significant difference for climbing, ($F4.48=1.84$, $p=0.10$; Figure 2d). However, a closer examination of the data showed that i.c.v. galanin infusion clearly decreased the climbing behavior as compared to all other groups. Therefore, the aCSF control and the galanin groups were analyzed separately, showing that galanin markedly decreased climbing behavior ($F1,24=5.8$, $p<0.05$; Figure 2d).

In Situ Hybridization Studies

In the third experiment, semi-quantitative *in situ* hybridization studies were performed to measure expression of several neurochemical markers in NA, 5-HT and galanin systems of the LC and raphe nuclei in the naive control animals and after exposure to the FST.

Locus Coeruleus

In the LC, the levels of galanin and TH mRNA expression were assessed (see Table 1 for the summary of the experimental results).

Tyrosine hydroxylase. In situ hybridization showed a strong signal for TH mRNA in the LC (Figure 3a) in all groups studied. One-way ANOVA ($F3,14=4.79$, $p<0.05$) followed by Fisher's PLSD test showed that TH expression was significantly increased in the saline- (+94%, $p<0.01$) and fluoxetine-treated (+72%, $p<0.05$) groups compared to the naive, non-stressed control group (Figure 4a). Intracerebroventricular infusion of either galanin or the galanin receptor-selective ligands did not affect TH mRNA levels in the LC compared to the aCSF-treated group ($F3,13=0.79$, $p=0.5$, one-way ANOVA; Figure 4b). The GalR2 antagonist M871 had no effect on TH mRNA expression as compared to the aCSF-infused group ($F1,10=0.58$, $p=0.46$, data not shown).

Galanin. Galanin mRNA expression was detected in the majority of the LC cells (Figure 3b). One-way ANOVA indicated a significant difference for the i.p.-injected/control groups ($F3,14=3.81$, $p<0.05$, Figure 4c). Galanin mRNA levels were increased in both the saline- (+64%, $p<0.05$, Fisher's PLSD) and the fluoxetine- (+70%, $p<0.01$, Fisher's PLSD) treated groups, as compared to the control naive animals (Figure 4c). In the peptide-infused groups, only a tendency for the difference between the groups was observed ($F3,13=2.99$, $p=0.06$, one-way ANOVA, Figure 4d). No significant differences were detected between the M871- and the aCSF-treated groups ($F1,10=3.05$, $p=0.11$, one-way ANOVA, data not shown).

Dorsal and Median Raphe

In the raphe region, the levels of TPH2, galanin, $5HT_{1A}$ receptor or 5-HTT transcripts were assessed (see Table 1 for the summary of the experimental results).

Tryptophan hydroxylase 2. TPH2 mRNA expression was observed both in the DR and MR nuclei (Figure 3c). In the lDR and dDR TPH2 expression was not affected by any treatment (data not shown). The difference in TPH2 mRNA

Table 1 Summary of *In situ* Hybridization Studies in the Locus Coeruleus and in the Raphe Nuclei

	Locus coeruleus		Raphe nuclei			
Group	**Tyrosine hydroxylase**	**Galanin**	**Tryptophan hydroxylase 2**	**Galanin**	**5-HT$_{1A}$ receptor**	**5-HT transporter**
Control swim	NS	NS	NS	NS	NS	NS
Saline	↑ *vs* Naive	↑ *vs* Naive	NS	NS	NS	NS
Fluoxetine	↑ *vs* Naive	↑ *vs* Naive	NS	NS	NS	NS
Galanin	NS	NS	NS	NS	↓ *vs* aCSF (vDR)	NS
GalR1 agonist, M617	NS	NS	NS	NS	↓ *vs* aCSF (vDR).	NS
GalR2(R3) agonist, AR-M1896	NS	NS	NS	NS	↓ *vs* aCSF (vDR)	NS
GalR2 antagonist, M871	NS	NS	NS	NS	NS	NS

NS, not significant.
↑ indicates a significant increase in mRNA levels ($p<0.05$, one-way ANOVA, Fisher's PLSD), ↓ indicates a significant decrease in mRNA levels ($p<0.05$, one-way ANOVA, Fisher's PLSD).

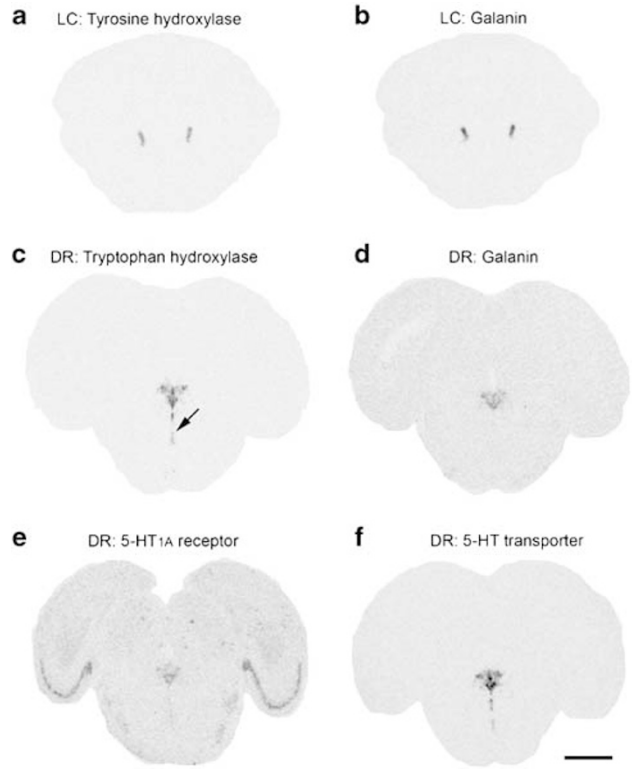

Figure 3 *In situ* hybridization autoradiographs of coronal sections of naive rat brain, showing TH (a) and galanin (b) transcripts in the LC, and TPH2 (c), galanin (d), the 5-HT$_{1A}$ receptor (e), and 5-HTT (f) transcripts in the DR and MR nuclei. Arrow indicates the MR nucleus (c), scale bar = 250 μm.

expression between the i.p.-injected/control groups did not reach significance in the vDR ($F3,14=2.7$; $p=0.07$, one-way ANOVA; not shown) and in the MR ($F3,14=2.71$, $p=0.08$, one-way ANOVA; not shown). In the peptide-infused groups, no differences in TPH2 mRNA levels were found in either vDR ($F3,14=0.77$, $p=0.52$, one-way ANOVA; data not shown) or MR ($F3,14=1.08$, $p=0.3$, one-way ANOVA; data not shown). No differences in TPH2 mRNA expression were found between the M871- and the aCSF-treated groups (vDR: $F1,10=0.13$, $p=72$; MR: $F1,10=0.28$, $p=0.60$, one-way ANOVA; data not shown).

Galanin. Galanin mRNA (Figure 3d) was detectable in the DR, but not in the MR. Galanin mRNA expression was not affected by swim stress or any pharmacological or peptidergic treatment in the lDR or dDR (data not shown). In the vDR, there was no effect of swim stress or antidepressant treatment ($F3,14=1.3$, $p=0.31$, one-way ANOVA, data not shown), while in the peptide-infused groups only a tendency was observed ($F3,14=2.55$, $p=0.08$, one-way ANOVA; Figure 5a). No effect of M617 or M871 treatment on galanin expression in the vDR was detected (M871: $F1,10=0.16$, $p=0.69$, data not shown).

5HT$_{1A}$ receptor. 5HT$_{1A}$ receptor mRNA (Figure 3e) was not affected by swim stress or antidepressant treatment in either the DR or the MR nuclei (data not shown). In the peptide-infused groups, a significant difference was found in the vDR ($F3,12=4.81$, $p<0.05$, one-way ANOVA). *Post hoc* analysis indicated that the galanin-, M617- or AR-M1896-infused groups all differed from the aCSF control group ($p<0.05$, Figure 5b). 5-HT$_{1A}$ receptor expression was not affected by M871 in either DR or MR (data not shown).

5-HT transporter. 5-HTT mRNA expression (Figure 3f) was not affected by swim/injection stress or any pharmacological treatment in any of the DR subnuclei or in the MR (data not shown).

DISCUSSION

The present study examined the effect of i.c.v. administration of galanin and galanin receptor-selective ligands on depression-like behavior in the rat. In addition, possible changes in several markers in the NA and 5-HT systems of the LC and the DR, both of which express galanin, were studied following FST, antidepressant treatment, and galanin-receptor activation/antagonism. The results indicate that the galanin receptors GalR1 and GalR2 play a differential role in regulation of depression-like behavior.

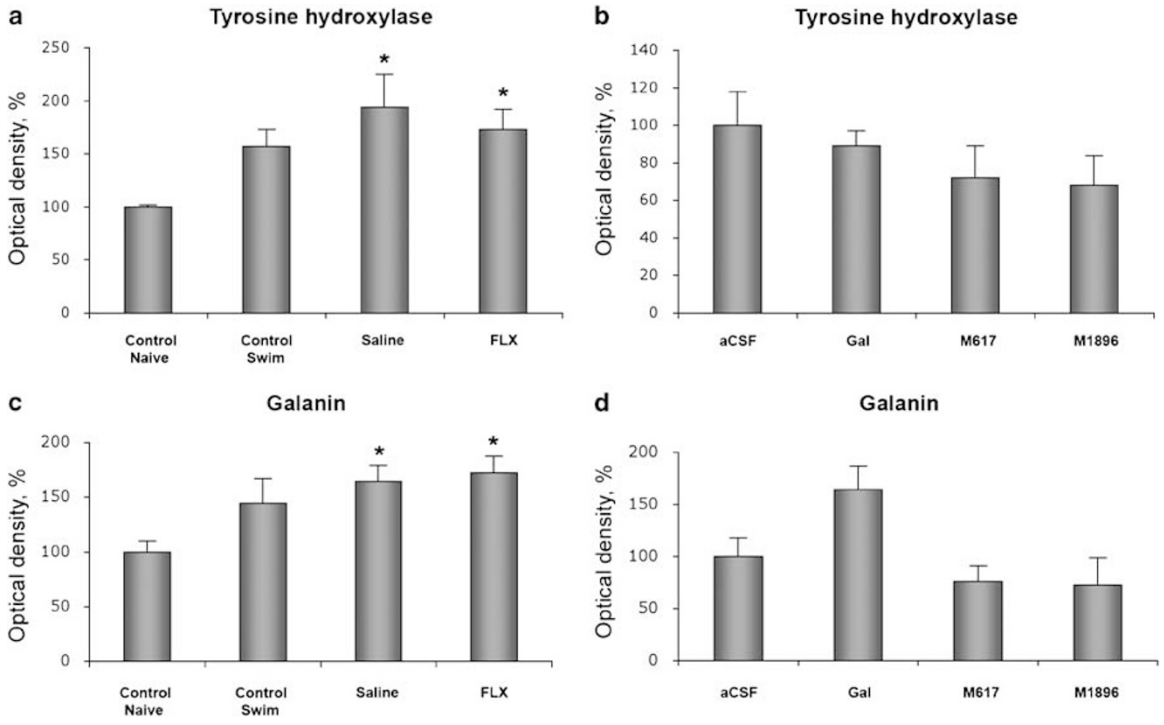

Figure 4 Semi-quantitative evaluation of TH (a, b) or galanin (c, d) mRNA levels (nCi/g, shown as % of optical density) in the LC of the control animals (control naive), after exposure to the FST (control swim), saline (saline) or fluoxetine (FLX) injections (a, c), or after aCSF, galanin, M617 or AR-M1896 infusion (b, d). Data presented as mean ± SEM. *, significant difference from the control naive group, $p < 0.05$–0.01, one-way ANOVA, Fisher's PLSD.

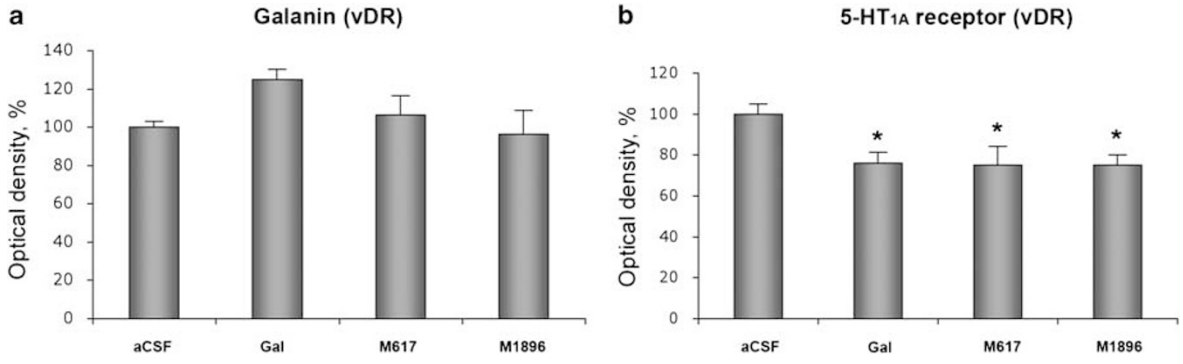

Figure 5 Semi-quantitative evaluation of galanin (a) or 5-HT_{1A} (b) mRNA levels (nCi/g, shown as % of optical density) in the ventral area of the DR after aCSF, galanin, M617 or AR-M1896 infusion. Data presented as mean ± SEM. *, significant difference from the aCSF-treated group, $p < 0.05$, one-way ANOVA, Fisher's PLSD.

In agreement, inescapable stress and activation of the galanin system modulate expression of molecules in monoamine circuits involved in coping with stress (see Weiss *et al*, 1981; Mongeau *et al*, 1997; Mann, 1999; Maier and Watkins, 2005).

Galanin Receptor Selectivity of the Peptidergic Ligands

It is important to note that receptor selectivity of peptidergic galanin receptor ligands is presently a matter of concern. *In vitro* studies have indicated that M617 exhibits a 25-fold subtype specificity for GalR1 *vs* GalR2 (Lundström *et al*, 2005), while M871 binds to the GalR2 with a 32-fold higher affinity than to GalR1 (Sollenberg *et al*, 2006). Neither M617 nor M871 were yet tested with respect to the GalR3 receptor subtype. Both *in vitro* and *in vivo* studies support the view that M617 acts as GalR1 agonist, and M871 is an antagonist at the GalR2 receptor (Lundström *et al*, 2005; Sollenberg *et al*, 2006).

Galanin (2–12), AR-M1896, was first described as a high affinity GalR2 selective agonist with a nanomolar affinity (Liu *et al*, 2001). However, it was subsequently shown that AR-M1896 can also act as a GalR3 ligand in transfected CHO and COS-7 cell lines, expressing GalR3 receptor, with a submicromolar affinity (Lu *et al*, 2005b, c). Thus, it is important to take into account the binding of this ligand also to the GalR3 receptor when interpreting functional results. However, the GalR3 expression is weak and restricted in the rat brain, compared to the GalR1 and the GalR2 subtypes (Wang *et al*, 1997; Smith *et al*, 1998; Waters

and Krause, 2000; see O'Donnell *et al*, 2003). In the DR, presence of the GalR3 receptor was indicated by electrophysiological studies, using selective GalR3 antagonists (Swanson *et al*, 2005). Thus, the GalR3 antagonist SNAP37889 partially blocked the inhibitory action of galanin on DR neuronal firing and 5-HT release, suggesting that the GalR3 receptor contributes to the inhibitory effects of galanin in the DR. In contrast, infusion of AR-M1896 into the DR increased 5-HT release in the hippocampus (Mazarati *et al*, 2005). Since the GalR2 subtype mediates excitatory actions of galanin on neurotransmitter release (see Branchek *et al*, 2000), it seems likely that AR-M1896 *in vivo* acts mainly as an agonist at the GalR2 receptor. In the discussion, we, therefore, consider AR-M1896 as a GalR2 receptor agonist, although a potential effect on GalR3 receptor cannot be excluded.

Depression-Like Behavior in Rats and the FST

Clinical depression is a complex disorder involving multiple symptoms including lowering of mood. In view of this complexity, it is difficult to develop valid animal models to study this disorder (Frazer and Morilak, 2005). However, one of the main predisposing factors in development of depression is exposure to traumatic and stressful events (see Kessler, 1997; Bilang-Bleuel *et al*, 2005), resulting in a dysregulated response of the hypothalamus–pituitary–adrenal (HPA) axis (see Holsboer, 2001). Moreover, depression is often viewed as inability to cope with stress (see Anisman and Zacharko, 1990; Kessler, 1997).

The FST was originally proposed as a model of stress-induced depression-like behavior (Porsolt *et al*, 1977). However, exposure to inescapable swimming may not be sufficiently strong to induce long-lasting depression-like symptoms in the normal rat. Nevertheless, the FST does represent an acute stressful event, since it is associated with a profound increase in adrenocorticotropin and corticosterone levels (Bilang-Bleuel *et al*, 2002; Rittenhouse *et al*, 2002), as well as with neurochemical and morphological changes in the hippocampus (Bilang-Bleuel *et al*, 2005) and prefrontal cortex (Izquierdo *et al*, 2006). Importantly, the immobility response in the FST can be prevented by glucocorticoid antagonists (Jefferys and Funder, 1987; Korte *et al*, 1996; Bilang-Bleuel *et al*, 2005) and various types of antidepressant treatments, including tricyclic antidepressants, monoamine oxidase inhibitors, SSRIs, and NA reuptake inhibitors (see Borsini and Meli, 1988).

Effects of Fluoxetine and Galanin Receptor Ligands on Depression-Like Behavior

Subchronic treatment with the SSRI fluoxetine decreased immobility time as compared to the non-treated (control swim) and the saline-injected control groups indicative of antidepressant-like effect, consistent with previous findings (Detke and Lucki, 1996; Lucki and O'Leary, 2004; Kuteeva *et al*, 2007). However, fluoxetine treatment also resulted in a significant reduction of climbing as compared to the control swim group. Also, in our earlier study, a similar effect of fluoxetine was detected (Kuteeva *et al*, 2007). The decrease in climbing behavior can be interpreted as a 'prodepressive' effect, indicating that fluoxetine does not display a consistent antidepressant profile in the FST. In fact, it has been difficult to detect 'antidepressant' effects of SSRIs in the FST (Porsolt *et al*, 1979), although later studies, using a modified FST with increased water levels, suggested a differential response of SSRIs and tricyclic antidepressants. While tricyclic antidepressants, such as desipramine, cause robust increase in climbing, SSRIs were shown to profoundly increase swimming behavior (Detke *et al*, 1995). Thus, increases in swimming behavior following fluoxetine treatment may explain the decrease in climbing observed in our studies.

Unlike fluoxetine, i.c.v. administration of galanin was found to increase immobility in the FST, suggesting 'prodepressive' properties of this neuropeptide (Kuteeva *et al*, 2007). Galanin also decreased the time of climbing, unlike the receptor-selective peptidergic ligands, suggesting that the native peptide can effectively evoke depression-like behavior. The observed increase in immobility and suppression of climbing are probably not simply related to a decrease in locomotor activity, since i.c.v. galanin only marginally reduces spontaneous locomotor activity in rat (Kehr *et al*, 2002).

Similarly to galanin, the GalR1 agonist M617 increased immobility time. In contrast, the GalR2(R3) agonist AR-M1896 decreased immobility, similar to fluoxetine, and, importantly, the GalR2 antagonist M871 increased immobility time in the FST. The latter finding supports the hypothesis that stimulation of the GalR2 receptor has an antidepressant-like effect, in agreement with the study by Lu *et al* (2005a). The results with the GalR2 antagonist indicate that there exists a tonic activation of the GalR2 receptor under *in vivo* conditions of the forced swim, which is sufficiently strong to have physiological consequences. This finding gives functional evidence for the view that galanin is released under stressful conditions (Lundberg and Hökfelt, 1986), stimulating the multiple receptor subtypes. Blockade of the GalR2 receptor attenuates the 'antidepressant' effect related to the GalR2 stimulation, leaving the 'prodepressive' activation of the GalR1/R3 receptors intact.

It is notable that the galanin antagonists M15 and M35 (Weiss *et al*, 1998, 2005; Kuteeva *et al*, 2007), as well as the GalR2(R3) agonist (present data), exert an antidepressant-like effect following a single administration, while fluoxetine requires at least a subchronic administration (three injections) to be effective in the FST. These findings suggest that the mechanisms of action of galaninergic ligands differ from that of SSRIs. Single-dose administration of fluoxetine prior to the test exposure in the FST failed to modulate the immobility response (Wardi *et al*, to be published). This indicates that the galaninergic system may act on mechanism(s) underlying stress-coping behavior, which are not directly affected by acute SSRI treatment.

Integrative Mechanism of Galanininergic Regulation of Depression-Like Behavior: Interaction with Monoaminergic Systems

It is generally assumed that dysfunctional monoaminergic transmission may underlie various symptoms of depression, including the susceptibility to stress (see Heninger *et al*, 1996; Mongeau *et al*, 1997; Holsboer, 2001; Morilak

and Frazer, 2004). Since galanin and the galanin receptors are expressed in both the NA and 5-HT systems, it is possible that the role of galanin in depression-like behavior may, at least partially, be related to modulation of brain NA and/or 5-HT functions at the cell body level and/or their projection areas.

Our previous studies have shown that galanin is a potent inhibitory modulator of the 5-HT system *in vivo* under basal (non-stressful) conditions. Thus, i.c.v. galanin caused a significant long-lasting reduction of the basal (Kehr *et al*, 2002) and SSRI-induced (Yoshitake *et al*, 2003) 5-HT release in the hippocampus, as measured by *in vivo* microdialysis. Moreover, galanin decreased TPH2 mRNA expression in the DR (Kehr *et al*, 2002), suggesting that galanin also inhibits 5-HT synthesis. In contrast to a profound inhibition of 5-HT release, i.c.v. galanin only marginally and transiently decreased basal and desipramine-induced NA release in the hippocampus (Yoshitake *et al*, 2003). However, under the present stressful conditions, the most significant effects following exposure to swim/injection stress were detected in the LC, as indicated by elevation of both TH and galanin mRNA levels. These results suggest that the action of galanin in the NA and 5-HT systems differ under basal and stressful conditions.

Locus coeruleus. The stressful event (forced swimming) combined with repeated i.p. injections resulted in increased activity of the LC NA system, evidenced by an increase of TH and galanin mRNA levels in the LC, in agreement with previous findings (Biguet *et al*, 1986; Berod *et al*, 1987; Richard *et al*, 1988; Austin *et al*, 1990; Holmes *et al*, 1995). Stress-induced hyperactivity of the LC neurons and increased release of NA have been proposed to contribute to development of human depression (Nestler *et al*, 1990; see Mongeau *et al*, 1997; Grant and Weiss, 2001; Morilak and Frazer, 2004). Importantly, chronic treatment with NA reuptake inhibitors was shown to reduce activation of the LC and exaggerated NA release in response to acute stress, but to increase synaptic levels of NA under basal conditions (Valentino and Curtis, 1991; Morilak and Frazer, 2004; Bondi *et al*, 2007).

The increase in TH and galanin mRNA levels observed following swim/injection stress indicates enhanced release and compensatory synthesis of both NA and the coexisting peptide (Schalling *et al*, 1989; Meister *et al*, 1990). Importantly, i.c.v. galanin did not reduce TH mRNA expression in the LC, when animals were exposed to the FST. This finding is critical, since it suggests that the effects of galanin differ under basal (Counts *et al*, 2002) and stressful conditions. Under basal conditions, galanin has been shown to reduce LC neuronal activity (Pieribone *et al*, 1995) and NA release (see above), probably via stimulation of the GalR1(R3) receptors (Ma *et al*, 2001). Under stressful conditions, galanin would probably increase the stress-reactivity of the LC NA system (Yoshitake *et al*, 2004; Kuteeva *et al*, 2005) and/or exert a direct post-synaptic effect in the LC projection areas, further contributing to depression-like behavioral changes (Weiss *et al*, 1998, 2005).

By acting in the VTA, galanin could inhibit the mesolimbic dopaminergic system (Tsuda *et al*, 1998; Ericson and Ahlenius, 1999), which may affect (impair) both motor activity and reward mechanisms, related to depression (see Weiss *et al*, 1998, 2005; Nestler and Carlezon, 2006). In line with this hypothesis, infusion of galanin into the VTA significantly increased immobility time in the FST, while infusion of the galanin antagonist M15 had an antidepressant-like effect (Weiss *et al*, 1998, 2005). Moreover, in an animal model of depression, in which prolonged decrease in spontaneous locomotor activity occurs after animals are exposed to a highly stressful event (inescapable electric shock), chronic micro-infusions of the galanin antagonist M15 into the VTA significantly accelerated recovery from the depression of the locomotor activity (Weiss *et al*, 2005).

Dorsal raphe. In the DR, neither TPH2 nor galanin mRNA transcript levels were significantly affected by the swim/injection stress, consistent with the view that the LC NA system is more sensitive to acute stress than the DR (Abercrombie and Jacobs, 1987; Wilkinson and Jacobs, 1988). However, galanin, as well as galanin-receptor selective ligands may affect depression-like behavior by directly influencing 5-HT neurotransmission. The available data indicate a profound inhibitory effect of galanin on 5-HT transmission under basal conditions, which is probably mediated by the GalR1/GalR3 receptors (Xu *et al*, 1998a, c; Kehr *et al*, 2002; Yoshitake *et al*, 2003; Mazarati *et al*, 2005; Swanson *et al*, 2005). The inhibitory effect of galanin on the DR 5-HT system after exposure to stress probably affects galanin receptor-mediated mechanism, since both the neurochemical and behavioral effects of i.c.v. galanin are blocked by the non-selective galanin antagonist M35 (Kehr *et al*, 2002; Kuteeva *et al*, 2007). Also the GalR3 selective antagonists exerted an antidepressant-like effect in several behavioral models relevant for anxiety- and depression-like behavior, including the FST (Swanson *et al*, 2005; Barr *et al*, 2006).

In the 5-HT neurons, however, the GalR2 receptor may be present and functional at the cell body level (Mazarati *et al*, 2005), in contrast to the LC (Ma *et al*, 2001). This receptor subtype could transmit an excitatory action of galanin, which is mediated via the phospholipase C pathway (see Branchek *et al*, 2000), as well as by inhibition of the G-protein coupled inwardly rectifying potassium (GIRK) channels (Kerekes *et al*, 2003). In agreement, activation of the GalR2 receptor in the DR by local infusion of AR-M1896 was shown to increase 5-HT release in the hippocampus (Mazarati *et al*, 2005), contrary to the profound reduction seen after the i.c.v. or local infusion of galanin into the DR (Kehr *et al*, 2002; Mazarati *et al*, 2005). The increase of serotonergic transmission following selective GalR2 receptor activation may underlie the antidepressant-like effect observed here after i.c.v. infusion of AR-M1896. Importantly, our results support recent data showing that repeated fluoxetine treatment upregulates GalR2 binding sites in the DR and thus un antidepressive role of the GalR2 (Lu *et al*, 2005a).

The galaninergic modulation of the 5-HT system could also be related to the interaction with the 5-HT_{1A} receptor. A small but significant decrease in the 5-HT_{1A} receptor expression was observed following galanin infusion, in

agreement with previous findings showing that galanin inhibits 5-HT_{1A} mRNA expression in the DR (Razani *et al*, 2000). Also the GalR1 agonist M617 and the GalR2(R3) agonist AR-M1896 downregulated the 5-HT_{1A} mRNA levels, suggesting that both receptor subtypes can contribute to this effect.

Downregulation of the 5-HT_{1A} receptor was proposed to underlie the increase in 5-HT transmission and therapeutic action of SSRIs (Artigas *et al*, 1996a; Le Poul *et al*, 2000). Moreover, coapplication of a 5-HT_{1A} receptor antagonist and SSRIs was shown to accelerate antidepressant responses in patients (see Artigas *et al*, 1996b; Blier *et al*, 1998). In view of these findings, antagonistic interaction of galanin and the 5-HT_{1A} receptor in the DR (see Ögren *et al*, 2006) may seem paradoxical, since galanin, in contrast to SSRIs, downregulates the 5-HT_{1A} autoreceptor (Razani *et al*, 2000), but at the same time reduces 5-HT transmission (see above). The present data indicate that the mechanisms underlying the impairment of coping mechanisms by galanin, although partially related to disturbances in 5-HT neurotransmission, cannot be directly related to changes in 5-HT_{1A} autoreceptor functions. In agreement, a recent paper has shown that the effects of chronic fluoxetine treatment in socially stressed rats is not associated with downregulation of the 5-HT_{1A} receptor function in the DR neurons (Cornelisse *et al*, 2007).

CONCLUSIONS

Mood disorders, such as depression, involve dysfunctions in brain NA and 5-HT systems, as well as impaired coping with traumatic events. The present results indicate that exposure to stress results in release of galanin, a neuropeptide expressed both in NA LC and 5-HT DR neurons, and stimulation of multiple galanin receptor subtypes. These receptors appear to play differential roles in stress coping mechanisms, involving changes in gene expression of molecules important for NA and/or 5-HT transmission in the LC and the DR.

In view of the present findings it is suggested that GalR1 and/or GalR3 receptor antagonists, or GalR2 receptor agonists may represent new therapeutic principles for the development of drugs for treatment of mood disorders.

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DISCLOSURE/CONFLICT OF INTERESTS

Drs E Kuteeva, T Wardi, L Lundström, U Sollenberg and Ü Langel do not have any conflicts of interest. Dr T Hökfelt served a consultant and received compensation from Lundbeck America in 2005. Dr SO Ögren has received research grants and owns shares from AstraZeneca.

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