Injection of galanin into the dorsal hippocampus impairs emotional memory independent of 5-HT$_{1A}$ receptor activation

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A B S T R A C T

There is evidence that interaction between the neuropeptide galanin and the 5-HT$_{1A}$ receptor represents an integrative mechanism in the regulation of serotonergic neurotransmission. Thus, in rats intracerebroventricular (i.c.v.) galanin did not impair retention in the passive avoidance (PA) test 24 h after training, but attenuated the retention deficit caused by subcutaneous (s.c.) administration of the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT. This impairment has been linked to postsynaptic 5-HT$_{1A}$ receptor activation. To confirm these results in mice, galanin was infused i.c.v. (1 nmol/mouse) in C57BL/6/Bkl mice 30 min prior to training followed by s.c. injection (0.3 mg/kg) of 8-OH-DPAT or saline 15 min before PA training. In line with previous results, i.c.v. galanin significantly attenuated the PA impairment caused by 5-HT$_{1A}$ receptor activation in mice. To study if the galanin 5-HT$_{1A}$ receptor interaction involved the dorsal hippocampus, galanin (1 nmol/mouse) was directly infused into this brain region alone or in combination with s.c. 8-OH-DPAT. However, unlike i.c.v. galanin, galanin infusion into the dorsal hippocampus alone impaired PA retention and failed to attenuate the 8-OH-DPAT-mediated PA impairment. These results indicate that the ability of i.c.v. galanin to modify 5-HT$_{1A}$ receptor activation is not directly mediated via receptor interactions in the dorsal hippocampus. Instead, the galanin-mediated PA impairment suggests an important inhibitory role of galanin receptors in the dorsal hippocampus for acquisition (encoding) and/or consolidation of emotional memory. In addition, the interaction between galanin and 5-HT$_{1A}$ receptors probably involves a wide serotonergic network that is important for the integration of emotional and cognitive behaviors.

1. Introduction

Galanin is a 29 (30 in humans) amino acid neuropeptide [1] that is widely distributed in the brain of mammals, including rat [reviewed in 2] and mouse [3–5]. These studies show high to moderate levels of galanin in the ventral forebrain, amygdala, hypothalamus, thalamus, and brainstem, likely with a slightly lower abundance in the mouse than in the rat.

The biological actions of galanin are mediated by three different galanin receptor (GalR) subtypes, Gal$_1$R, Gal$_2$R and Gal$_3$R. The distribution of the transcript for the galanin receptors has been studied in both rat and mouse brain using various methods, including in situ hybridization, RT-PCR, cloning and pharmacological characterization [9].

The studies in rat present a wider distribution whereby Gal$_1$R > Gal$_2$R > Gal$_3$R, the latter at low levels in the central nervous system (CNS) but more abundant in the periphery [6,7,10]. The only comprehensive and very detailed study in mouse [5] also shows a broad expression of both Gal$_1$R and Gal$_2$R transcripts, but Gal$_3$R is not reported at all. Gal-R mRNA is found in many regions of the CNS, in the amygdala, thalamus, hypothalamus, dorsal raphe, whereas levels in the hippocampus are low. The Gal-R is also expressed in many areas, including the hippocampal formation, both in the CA1, CA3 and granule cell layers, and particularly high in the lower brain stem, including the dorsal raphe nucleus and locus coeruleus.

Central galanin is associated with a number of physiological functions, including effects on learning and memory via modulation of

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classical neurotransmitters [8,9,11]. Furthermore, galanin is implicated in both psychiatric and neurological disorders [11–14]. In this context it is of particular significance that brain galanin and its receptors co-distribute with classical neurotransmitters such as serotonin (5-HT) and noradrenaline (NA) [15,16].

Galanin is a potent in vivo inhibitory modulator of brain serotonergic neurotransmission [11] and of 5-HT1A Receptor-mediated actions at both the pre- and postsynaptic level. Galanin infused bilaterally into both lateral ventricles of rats reduces 5-HT release in the ventral hippocampus in microdialysis studies in the rat [17]. This effect was found to be galanin receptor-related, probably due to an inhibitory action on the firing activity of the ascending dorsal raphe nucleus neurons via direct stimulation of Gal1R on 5-HT neurons [18] or indirectly via GABAergic neurons [19].

From the so far known fourteen 5-HT receptor subtypes [20] the 5-HT1A receptor is involved in associative aversive learning as demonstrated in PA and fear conditioning in rodents [21,22] with a biphasic effect on passive avoidance learning in mice. At low doses (0.01–0.03 mg/kg) the 5-HT1A receptor agonist 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) facilitates PA learning in mice [21]. Compounds characterized as selective 5-HT1A receptor antagonists such as NAD-299 fail to block the facilitation caused by low doses, presumably activating 5-HT1A autoreceptors [21]. At higher doses (>0.2 mg/kg) 8-OH-DPAT causes a dose-dependent impairment of PA both in rats [reviewed in 23,24,25,26,27,28] and mice [21]. The PA impairment is replicated in fear conditioning and is attributed to activation of postsynaptic 5-HT1A receptors [29], including hippocampal receptor sites, based on local administration [22] as later confirmed in PA [30]. Pre-injection of the 5-HT1A receptor antagonists Rovalzotan (NAD-299) and WAY-100635, which have low affinity for the 5-HT1 receptor [31], completely blocks both the 8-OH-DPAT-mediated serotonin syndrome, the PA and conditioned fear retention impairment [21,22,24,25,29,30]. A more detailed discussion on the role of pre- and postsynaptic 5-HT1A receptors is provided elsewhere [23].

The galanin/5-HT1A receptor interaction was first identified in studies in which intracerebroventricular (i.c.v.) infusion of galanin was combined with systemic 8-OH-DPAT injection in rats. Thus, galanin significantly attenuated the PA retention deficit induced by systemic 8-OH-DPAT, while i.c.v. galanin by itself did not affect PA retention [26]. The hippocampus plays a major role in the fear conditioning impairment caused by infusion of 8-OH-DPAT, although other limbic brain regions may also contribute [22]. Further indication identifies a regulatory role of galanin in serotonergic modulation in brain areas associated with mood disorders and emotional memory [11].

Since 5-HT1A receptors in the dorsal hippocampus are implicated in cognitive and affective functions, it is important to better define the brain circuitry involved in the galanin/5-HT1A receptor interaction. So far, almost all studies on the effects of galanin in learning and memory have been carried out with i.c.v. galanin administration in rats [24]. Therefore, the major aim of the present study was to investigate, in mice, (i) the effects of galanin given i.c.v. or infused directly into the dorsal hippocampus on PA learning and (ii) the specific role of the dorsal hippocampus in the galanin/5-HT1A receptor interaction in fear memory modulation.

2. Material and method

2.1. Mice

A total number of 126 male C57BL/6/Bkl mice (Scanbur BK, Solentuna, Sweden) with a mean body weight of 25.6 ± 1.7 g (mean ± SD) was tested at the age of 11–12 weeks. They were divided into eight different groups of drug treatments (n = 7–13/group) with two different experimental conditions, as specified below. The animals were housed in groups of five in standard Macrolon® cages (Type 3: 42 × 26 × 20 cm) at a constant temperature of 21 ± 1°C and a relative humidity of 53 ± 3%.

The mice were maintained on a 12-h light/dark cycle with free access to water and standard rodent chow. The experiments were performed during the light phase of the cycle, with lights switched on at 7.00 a.m. The animal housing and all experimental procedures followed the provisions and recommendations of the Swedish animal protection legislation approved by the local animal committee in accordance with the European Council Directive (2010/63/EU). All mice were handled during four consecutive days before the behavioral experiments to familiarize the animals to the handling procedure and the experimenter.

2.2. Stereotaxic operations

Double guide cannulae (C235, Plastics One, Roanoke, VA) were implanted using a stereotaxic system (David Kopf Instruments, Tujunga, CA), to perform acute infusion into the lateral ventricles in four groups and into the dorsal hippocampus in the other four groups. The surgery was performed under aseptic conditions using 1.2% avertin anesthesia as previously described [22,30,32,33]. Avertin was injected intraperitoneally (i.p.) at a dose of 0.024 ml/g, i.e. 0.6 ml/25 g mouse. During surgery each animal was kept on a heat pad maintained at 37°C to prevent hypothermia. For surgery each mouse was fixed in the stereotaxic system with a customized mouse adapter without using ear bars. After removal of the scalp, holes (Ø 0.5 mm) were drilled through the skull ±1.0 mm bilaterally from bregma (target: lateral ventricles) or 1.6 mm caudally and ±1.0 mm bilaterally from bregma (target: dorsal hippocampus), according to the stereotaxic atlas of the mouse brain [34]. Each guide was fixed to the skull with dental cement (ESPE Durelon® carboxylate cement, Seefeld, Germany) as described [22,30,32,33]. Finally, a dummy cannula was inserted and fixed with a dust cap. For post-surgery analgesia, buprenorphine (Temgesic®) was injected to provide analgesia for approximately 12 h. The mice were closely monitored during recovery from surgery for 5 days before the PA experiments started.

2.3. Drugs and drug administration

Four groups of mice received i.c.v. infusion of artificial cerebrospinal fluid (aCSF) or galanin 30 min before training followed by s.c. injection of either saline or 8-OH-DPAT 15 min before training. The other four groups were infused with aCSF or galanin into the dorsal hippocampus 30 min before training, followed by s.c. injection of saline or 8-OH-DPAT 15 min before training. Concerning the timing of drug injection, we decided to use central injections before systemic because we aimed to extend earlier findings in the rat based on a reversal design [25]. Using a prevention design, we thereby could reconfirm findings in the rat in the present study in mice.

Galanin (porcine galanin; Bachem, Bubendorf, Switzerland; lot. no. 0559826) was dissolved in aCSF on the day of infusion. In previous rat studies [25,28,35] 3 nmol of galanin was infused into the rat brain. Since rats have an approximately 3-fold bigger ventricular volume than mice, a dose of 1 nmol/mouse (3.21 µg/mouse) was administered in the current study. Brain infusion occurred via a double injector connected with a polyethylene tubing to two 25 µl gas-tight Hamilton syringes and a microinjection pump (CMA/100, CMA/Microdialysis, Solna, Sweden). The injection system was filled with aCSF (300 mM/µM, pH 7.4) or galanin (2 nmol/µl) dissolved in aCSF. To minimize the stress of the infusion procedure the animals were briefly anesthetized (~90 s) using isoflurane (CuraMED Pharma, Karlsruhe, Germany) as described before [22,30,32,33] and as refinement according to the 3R principles in animal welfare. Infusion and injection without brief anesthesia is a procedure we found to affects various physiological functions resulting in tachycardia [36] and stress-induced hyperthermia stressor [37]. Dust cap and dummy cannula were removed and the injector was inserted before 0.25 µl of solution was infused into each hemisphere (flow rate: 0.333 µl/min). We explicitly discriminate infusion from injection to indicate a slower administration process of a very low volume. This is
necessary for precise brain injection followed by the PA training 30 min later. The injector remained in the guide cannula for another 10 s after the infusion to prevent back-flow. After the infusion the animal was returned to its transportation cage. Fifteen min after i.c.v. or dorso-hippocampal infusion (galanin or aCSF) saline or 8-OH-DPAT (Sigma, St. Louis, MO) was injected s.c. into the scruff of the neck under brief iso-flurane anesthesia. A dose of 0.3 mg/kg was chosen based on previous results on 8-OH-DPAT-mediated PA impairment in mice [21]. PA experiments were performed 15 min after the s.c. injection.

2.4. Passive avoidance experiments

Groups of mice were transferred from the housing location to the experimental room in new transportation cages and placed for accommodation in the experimental room 60 min before the actual experiments (infusion procedure or retention test) began. The PA experiments were performed using a shuttle box system (Ugo Basile, Comerio-Varese, Italy) consisting of two compartments (10 × 16 × 18 cm) connected by a sliding door (4 × 4 cm) and with a grid floor made of stainless steel. One compartment was dark with black walls, while the other had white walls and was illuminated by a light bulb. The light intensities in the light and the dark compartment were 330 lx and 3 lx, respectively. The entire PA retention latency under identical experimental conditions (data not shown). The latency to cross into the dark compartment (training latency) was recorded. After US exposure each mouse remained in the dark compartment for another 30 s to avoid an aversive association with the stimulus, US. The US intensity 0.5 mA was selected, since a 0.3 mA US was too low to elicit clear PA learning as indicated by the relatively low PA activity was visible in the initial 60-s period of exploration before the door to the dark compartment was opened. Combined administration of intracerebral galanin (or aCSF) and s.c. 8-OH-DPAT (or saline) had a post hoc testing with pair-wise comparisons between groups based on the Mann-Whitney U test Non-parametric data are presented as box plots with the ends of the box denoting the 25% and 75% interquartile range, the line in the box indicating the median, and the whiskers providing the upper and lower quartile ± 1.5 times the interquartile range, respectively (JMP, 5.0.1, SAS Institute, Cary, NC). An error probability of 0.1 > p > 0.05 was regarded as a trend. One mouse was excluded from the statistical analysis as its performance fulfilled the criteria of a statistical outlier determined by the Dixon outlier test [38].

3. Results

3.1. Training performance

Mice injected s.c. with 8-OH-DPAT (0.3 mg/kg) exhibited mild signs of the serotonin syndrome (flat body posture, head weaving) indicative of postsynaptic 5-HT1A receptor stimulation. A reduction of explorative activity was visible in the initial 60-s period of exploration before the door to the dark compartment was opened. Combined administration of intracerebral galanin (or aCSF) and s.c. 8-OH-DPAT (or saline) had a significant effect on the latency to enter the dark compartment during training (i.c.v.: F adorn = 17.10; p < 0.0001; Fig. 3A; dorsohippocampally (i.h.): F SAR = 13.50; p < 0.0001; Fig. 3Q). Post hoc analysis indicated that administration of aCSF + 8-OH-DPAT (0.3 mg/kg) significantly increased the latency to enter the dark compartment (~140–170 s) when compared with the latency of aCSF + saline-treated controls (~10–30 s; i.c.v.: p < 0.0001; i.h.: p < 0.0001; Fig. 3A, C). Galanin plus...
saline did not affect the training latencies when compared with aCSF plus saline controls, irrespective of galanin infusion site (i.c.v.: $p = 0.97$; i.h.: $p = 0.82$; Fig. 3A, C). In addition, the galanin infusion did not reduce the increased latency to enter the dark compartment produced by 8-OH-DPAT, when compared with the latency of aCSF plus 8-OH-DPAT-treated mice (i.c.v.: $p = 0.97$; i.h.: $p = 0.82$; Fig. 3A, C).
3.2. Retention performance after intracerebroventricular galanin infusion

The drug interventions had a significant effect on the retention latencies in the four groups (H = 25.75, p < 0.0001, Fig. 3B), 8-OH-DPAT (after aCSF) resulted in a strong STL impairment (U = 0.0, p = 0.0002 vs. aCSF + saline control group; Fig. 3B) in the PA retention test. i.c.v. galanin followed by s.c. saline did not alter PA retention latency compared to its control group (U = 35.0, p = 0.07 vs. aCSF + saline control group). i.c.v. galanin before s.c. 8-OH-DPAT modestly but significantly attenuated the impairment of PA retention by aCSF and 8-OH-DPAT (U = 16.0, p = 0.0102, Fig. 3B). However, the impairment of PA retention by galanin and 8-OH-DPAT (despite its ameliorating effect versus aCSF and 8-OH-DPAT) was still very strong compared to the galanin and saline group (U = 5.0, p = 0.0007; Fig. 3B). In conclusion, the impairment mediated by s.c. 8-OH-DPAT dominated the STL, and i.c.v. galanin had low beneficial effects only in combination with s.c. 8-OH-DPAT.

3.3. Retention performance after intrahippocampal galanin infusion

Again, the drug interventions had a significant effect on the retention latencies in the four groups (H = 16.62, p = 0.0008; Fig. 3D). As expected, aCSF plus 8-OH-DPAT resulted in a strong impairment of PA retention STL, when compared with the corresponding aCSF plus saline control groups (U = 12.0, p = 0.0003; Fig. 3D). In contrast to our expectation, galanin infused into the dorsal hippocampus (plus s.c. saline) impaired the PA retention latency when compared with that of the aCSF plus saline control group (U = 14.5, p = 0.02; Fig. 3D). The STL impairment by galanin (+ saline) was similar to that of 8-OH-DPAT following aCSF (U = 0.088, p = 0.088; Fig. 3D). Consequently, i.h. galanin failed to significantly attenuate the 8-OH-DPAT-mediated impairment of the PA retention (U = 20.0, p = 0.56; Fig. 3D). In conclusion, dorsohippocampal galanin resulted in a strong STL impairment unlike i.c.v. galanin.

4. Discussion

The present study is the first in mice to analyze the effects of galanin on cognition and the interaction between 5-HT1A and galanin receptors in PA learning after infusion into the dorsal hippocampus. Systemic 5-HT1A receptor activation with 8-OH-DPAT, a 5-HT1A receptor agonist, caused an impairment of the PA retention, which was attenuated by galanin when administered i.c.v. but not into the dorsal hippocampus. Whereas i.c.v. galanin by itself did not impair PA, dorsohippocampal galanin alone impaired PA retention. Hence, the results from this study indicate that galanin exerts its inhibitory action on 8-OH-DPAT-mediated PA impairment at other brain area(s) than the dorsal hippocampus.

4.1. Importance of strains of mice in PA learning and training performance

Pre-training activation of 5-HT1A receptors by systemic 8-OH-DPAT resulted in a significant delay to enter the dark compartment during training, a finding consistent with the reported decrease of exploratory tendencies in the four groups (H = 35.0, p < 0.0001, Fig. 3B), 8-OH-DPAT (after aCSF) resulted in a significant delay to enter the dark compartment compared to controls [21]. However, the impairment of PA retention by galanin and 8-OH-DPAT (despite its ameliorating effect versus aCSF and 8-OH-DPAT) was still very strong compared to the galanin and saline group (U = 5.0, p = 0.0007; Fig. 3B). In conclusion, the impairment mediated by s.c. 8-OH-DPAT dominated the STL, and i.c.v. galanin had low beneficial effects only in combination with s.c. 8-OH-DPAT.

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4.1. Importance of strains of mice in PA learning and training performance

Pre-training activation of 5-HT1A receptors by systemic 8-OH-DPAT resulted in a significant delay to enter the dark compartment during training, a finding consistent with the reported decrease of exploratory behavior already observed at 0.1 mg/kg in C57BL/6J mice [22]. C57BL/6/Bkl mice appeared to exhibit stronger signs of the serotonin syndrome (a set of behavioral symptoms related to postsynaptic 5-HT receptor activation with 8-OH-DPAT, a 5-HT receptor agonist, caused an impairment of the PA retention, which was attenuated by galanin when administered i.c.v. but not into the dorsal hippocampus. Whereas i.c.v. galanin by itself did not impair PA, dorsohippocampal galanin alone impaired PA retention. Hence, the results from this study indicate that galanin exerts its inhibitory action on 8-OH-DPAT-mediated PA impairment at other brain area(s) than the dorsal hippocampus.
galanin infused i.c.v. can activate galanin receptors in a wider neuronal network than when infused directly into the dorsal hippocampus. Immunohistochemical studies in rats suggest that i.c.v. galanin can modulate pre- and postsynaptic 5-HT_{1A} receptor transmission in vivo in discrete cell populations in brain regions such as the dorsal and ventral hippocampus, parts of the amygdala and also at the dorsal raphe nucleus [25]. There is also evidence that galanin when administered i.c.v. may be taken up in certain populations of nerve terminals in the periventricular zone for retrograde transport, suggesting that this peptide may also affect intracellular events [25].

It is notable that galanin, which is a large molecule, probably is internalized and concentrated in certain neurons which may take a long time [46], compared to the fast effect of 8-OH-DPAT which crosses the blood-brain barrier extremely rapidly with physiological effects reported in rats as fast as 3 min after drug treatment [47]. However, our attempt to identify a role of the hippocampus led to a very surprising outcome, by indicating substantial differences based on infusion in these two target areas (dorsal hippocampus versus lateral ventricles). This result challenges many claims of hippocampal involvement based on i.c.v. drug administration. Thus, as reported by us earlier, we cannot support the claims of substantial hippocampal involvement by i.c.v. galanin in PA learning, despite the fact that hippocampal neurons were labelled by fluorophore-galanin after i.c.v. administration [46]. One reason is that we cannot rule out the presence and detection of neuropeptide fragments.

Therefore, it seems unlikely that galanin infused i.c.v. attenuated the PA impairment by 8-OH-DPAT through a direct interaction with post-synaptic 5-HT_{1A} receptors in the dorsal hippocampus. The potential mechanism probably involves an indirect interaction through extra-hippocampal pathways, e.g. via the dorsal or median raphe nucleus [11]. In support, infusion of galanin i.c.v. into the lateral ventricles of the rat causes a dose-dependent and long-lasting reduction of the basal extracellular 5-HT levels in the ventral hippocampus, as measured by in vivo microdialysis [17]. This reduction was blocked by s.c. administration of the 5-HT_{1A} receptor antagonist NAD-299, indicating that galanin modulates 5-HT release via actions on 5-HT_{1A} receptor at the cellular level in the dorsal raphe region. Moreover, the non-selective galanin receptor antagonist M35 attenuates the inhibitory effect of galanin on hippocampal 5HT release when administered i.c.v. in mice [17]. However, galanin does not affect 5HT release when administrated directly into the ventricular hippocampus [17], suggesting that the action of galanin on hippocampal 5-HT release is not exerted on the nerve terminals of the 5-HT neurons, but on their cell bodies in the dorsal/median raphe nuclei. The development of selective subtype-specific galanin receptor agonists and antagonists is necessary for the further characterization of the action of galanin with respect to receptor subtype involved and the temporal effects on learning and memory.

5. Conclusion

In summary, in contrast to galanin administered i.c.v., dorsohippocampal infusion of this peptide impaired the PA retention in mice, and thereby galanin, not surprisingly, failed to attenuate the 8-OHDPAT-mediated PA impairment. No PA studies in rats and mice have reported a galanin-mediated impairment of fear learning through direct hippocampal interventions. However, galanin infusion into the dentate gyrus area of both the dorsal and the ventral hippocampus in rats impaired spatial learning in the water maze test [48] as confirmed by i.c. v. galanin [49]. The impairing effect of galanin in the PA test is consistent with impaired CA1 long-term potentiation by galanin after tetanic and theta burst stimulation [50]. However, a lower dose of galanin (1 nmol) in the ventral hippocampus caused a slight facilitation of spatial learning in rats whereas a higher dose (3 nmol) also impaired [35]. These results suggest dose-dependent differential role for galanin and its receptors in subregions of the hippocampal formation. In addition, there may be species-specific differences between mice and rats. So far, our knowledge of the type and distribution of galanin receptors in cortical and hippocampal regions is limited, especially in mice. GalR mRNA is widely distributed in the dorsal hippocampus of the mouse [5]. Therefore, it may be assumed that the impairment of PA learning was mediated by this receptor subtype. However, the involvement of other galanin receptor subtypes or interaction of galanin with other neurotransmitter system such as GABA [19], glutamate [51] and noradrenaline [15] cannot be excluded. Future experiments with GalR subtype-selective agonists and antagonists [52] are necessary to resolve the temporal involvement of specific galanin receptor subtypes in the hippocampus and the dorsal raphe region for PA learning.

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CRedit authorship contribution statement

Oliver Stiedl: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing - original draft, Writing - review & editing. Eugenia Kuteeva: Data curation, Formal analysis, Writing - review & editing. Tomas Hökfelt: Investigation, Writing - review & editing. Sven Ove Ogren: Conceptualization, Funding acquisition, Investigation, Project administration, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

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