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Effects of intracerebroventricular galanin or a galanin receptor 2/3 agonist on the lesion induced by transient occlusion of the middle cerebral artery in female rats

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ABSTRACT

Several studies have shown that injury to the central and peripheral nervous system can increase expression of galanin, a 29 amino acid neuropeptide. Moreover, there is evidence that galanin, especially through its galanin receptor 2 (GalR2) receptor, plays a neuroprotective role in different injury models. However, direct studies of a possible neuroprotective effect of galanin in experimental stroke models are lacking. Galanin, a GalR2/3 agonist or artificial CSF was continuously infused intracerebroventricularly (i.c.v.) in naïve female rats after a 60 min transient and focal occlusion of the middle cerebral artery. The animals were sacrificed, and the ischemic lesion was visualized using 2,3,5-triphenyltetrazolium hydrochloride (TTC) staining. The lesion was 98% larger after i.c.v. administration of the GalR2/3 agonist (2.4 nmol/day) seven days after occlusion compared to artificial CSF ($p = 0.023$). No statistically significant differences were found after seven days in the groups treated with galanin in three different concentrations (0.24, 2.4 and 24 nmol/day; $p = 0.939$, 0.715 and 0.977, respectively). There was no difference in the size of the ischemic lesions measured after three days in the galanin-treated group (2.4 nmol/d) compared to artificial CSF ($p = 0.925$). The present results show, surprisingly, that a GalR2/3 agonist doubled the size of the ischemic lesion. Whether this effect primarily reflects the properties of the current model, species, gender and/or the mode of galanin administration, e.g. causing desensitization, or whether galanin indeed lacks neuroprotective effect of its own, remains to be corroborated.

1. Introduction

The 29/30 amino acid neuropeptide galanin (Tatemoto et al., 1983) is expressed both in the peripheral and central nervous systems (Rokaeus et al., 1984). Galanin exerts mainly inhibitory effects on classical neurotransmitters and may also serve other functions such as a growth/trophic factor (Holmes et al., 2000; Kerr et al., 2000; O'Meara et al., 2000; Zigmond, 2001).

It has been shown that several types of injury to central and peripheral neurons up regulate galanin and expression in discrete neuronal systems. Lesions/experimental manipulations so far studied include (1) dorsal root ganglion (DRG) neurons after peripheral axotomy (Hokfelt et al., 1987; Villar et al., 1989); (2) trigeminal ganglion neurons after damage of the vibrissae of rats (White et al., 1994); (3) medial septum-vertical diagonal band neurons after (i) electrocoagulation lesions of the ventral hippocampus or decortication (Cortes et al., 1990), (ii) transection of the septohippocampal pathway (Agoston et al., 1994) or (iii) tetrodotoxin injections into the vertical diagonal band (Agoston et al., 1994); (4) locus coeruleus neurons after olfactory bulbectomy (Holmes and Crawley, 1996); and (5) magnocellular hypothalamic neurons after hypophysectomy, a procedure that transects the axons of these neurons (Villar et al., 1994). The increase of galanin in nerve terminals in the basal forebrain of patients with Alzheimer's disease (AD) (Chan-Palay, 1988; Beal et al., 1990; Mufson et al., 1993) and in the hippocampal formation of AD mouse models (Diez et al., 2000, 2003) may, similarly and at least partly, be a result of neuronal damage.

Induction of galanin in DRG neurons by axotomy appears to be mediated by leukemia inhibitory factor (LIF) (Zigmond et al., 1996; Zigmond, 2001), a cytokine which influences neuronal differentiation and the expression of several neuropeptides. Thus, mice deficient in LIF do not show galanin over expression in the superior cervical ganglion after carotid nerve transection (Rao et al., 1993)

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or in DRGs after peripheral axotomy (Corness et al., 1996). The lesion-induced up regulation of galanin has been interpreted to indicate that the neuropeptide may serve as a survival/neurotrophic/reparative factor in the nervous system (Hobson et al., 2008).

The biological effects of galanin are mediated through three cloned G-protein-coupled receptor subtypes, GalR1, GalR2, and GalR3 (Habert-Ortoli et al., 1994; Branchek et al., 2000). The receptors have diverse signal transduction pathways exerting different intracellular effects, and their regional expression suggests involvement in a multitude of physiological functions. Galanin exerts inhibitory effects, mainly via GalR1 and −3 through $G_{i/o}$ type of G-proteins, whereas activation of GalR2 may typically result in stimulation of phospholipase *C* via $G_{q/11}$ G-proteins and protein kinase *C* (Habert-Ortoli et al., 1994; Branchek et al., 2000).

The protective/trophic effect of galanin is reported to be mainly mediated by GalR2, Akt and ERK signaling pathways (Mahoney et al., 2003; Elliott-Hunt et al., 2007). The galanin fragment Gal (2–11) (Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-NH2) (AR-MI896), an agonist with 500-fold selectivity for GalR2 over GalR1 (Liu et al., 2001), has in vitro showed neuroprotective properties by reducing the glutamate-induced excitotoxic response in hippocampal cells (Elliott-Hunt et al., 2004; Pirondi et al., 2005). The effect of exogenously administered galanin in an experimental traumatic brain injury model (central fluid percussion injury) in rat was studied by Liu et al. (Liu et al., 1994), where a single injection of galanin (1.0 or 10.0 μg) administered intracerebroventricularly (i.c.v.) prior to the trauma resulted in significantly less deficits compared to controls regarding motor skill and memory performance.

In a recent study on rat we showed a decrease in galanin concentrations in hippocampus in the ischemic hemisphere compared to the corresponding contralateral intact hemisphere three days after transient middle cerebral artery occlusion (MCAo) (Theodorsson and Theodorsson, 2005). Other studies have reported increases in galanin peptide and transcript after MCAo (Bond et al., 2002; Raghavendra Rao et al., 2002; Hwang et al., 2004; Lee et al., 2005; De Michele et al., 2006). Taken together, these data support that galanin is a strong reactant to damage of the nervous system. However, studies directly showing a neuroprotective property *per se* in an in vivo experimental stroke model are lacking.

The aim of this study was therefore to investigate whether or not exogenous administered galanin or a GalR2/3 agonist, continuously infused i.c.v., exerts neuroprotective effects in brain areas affected by the ischemic lesions occurring 60 min after a transient and focal occlusion of the middle cerebral artery (MCA) in naïve female rats.

2. Materials and methods

2.1. Animal model

The study was conducted in accordance with the National Committee for Animal Research in Sweden and Principles of Laboratory Animal Care (NIH publication No 86–23, revised 1985). The protocol was approved by the Local Ethics Committee for Animal Care and Use at Linköping University.

A total of 94 virgin female rats (Sprague–Dawley, B&K Universal, Sollentuna, Sweden) were used and housed at the Linköping University Animal Department for at least 1 week before the start of the experiments. The rats were kept two in each cage at constant room temperature (21 °C), with free access to water and standard rat chow (Lactamin, Vadstena, Sweden), and with 12-h light/dark (light on at 8.00 am) and sound (soft radio music) cycles before the experiments.

At the age of 12–15 weeks the rats (b.wt. 255–339 g) were randomly allocated to three treatment groups for i.c.v. administration of galanin, GalR2/3 agonist or artificial CSF (aCSF). The rats received an i.c.v. cannula, and the MCA was occluded for 60 min, and galanin, GalR2/3 agonist or aCSF was continuously administered. The rats were sacrificed by guillotine 3 and 7 days after the MCA occlusion.

An Alzet brain infusion kit including a 28 G stainless steel cannula and a height adjustment spacer (Alzet Infusion Kit II, 3–5 mm, Durect Corporations, Cupertino, CA) was used together with an Alzet osmotic pump, (volume 100 μL, releasing rate 0.5 μL/h, lasting 1 week) (Alzet Osmotic Pump 1007D, Durect Corporations). The kit was operated into the left ventricle at the stereotactic coordinates: bregma (B) −1.3 mm anterior/posterior, (B) −3.8 mm ventral to brain surface and + 1.8 mm lateral to midline and secured with three stainless screws and glue (Dental® plus, Heraeus Kutzer, Dormagen, Germany). The Alzet osmotic pump was inserted in the rat neck and connected to the brain infusion cannula. The osmotic pump was filled with rat galanin (SC 936, Neosystem, Strasbourg, France) dissolved in isotonic Ringer acetate solution (Braun) to a concentration of 8.4 μg/100μL, 84 μg/100μL or 840 μg/100μL, releasing a dose of 0.77 μg/day (0.24 nmol/day), 7.68 μg/day (2.4 nmol/day) or 76.8 μg/day (24 nmol/day). The GalR2/3 agonist (Gal 2–11, AR-M1896, Tocris Cookson Ltd., Bristol, UK) was dissolved as above to a concentration of 84 μg/100μL, releasing a dose of 7.68 μg/day (2.4 nmol/day) and processed as mentioned above.

Occlusion of the MCA was performed as described earlier (Theodorsson et al., 2005). In brief, anesthesia was induced by 4% isoflurane (Forene®, Abbott, Scandinavia AB, Kista, Sweden) in a mixture (30%/70%) of oxygen/nitrous oxide in an induction chamber. A soft endotracheal tube was inserted for controlled ventilation (Zoovent, CWC600AP, ULV Ltd., Newport U.K.) using 1–1.5% isoflurane in a mixture of oxygen/nitrous oxide (as above). The tidal volume and ventilation frequency were carefully regulated using on-site monitoring of blood gases and acid/base status (AVL, OPTI 1 Medical Nordic AB, Stockholm, Sweden) (Table 1). The rats were placed with their left side up on a thermostatic heating pad (Harvard Homeothermic Blanket system, Edenbridge, UK) to maintain the core/rectum temperature at 37.0 ± 0.5 °C. The left femoral artery was cannulated using a soft catheter Micro-renathane® tubing (MRE-025 Braintree Scientific, Inc., MA) primed with saline containing heparin (100 IU/mL, Lovens, Ballerup, Denmark) for registration of blood pressure and pulse [(AcqKnowledge software (BioPac system, Goleta, CA) and Blood Pressure Transducer (56360, Stoelting, IL)].

Using an operating microscope (Zeiss Opmi 6-H, West Germany), the left MCA was exposed transcranially (Tamura et al., 1981), removing part of the zygomatic bone but maintaining the temporal muscle and the facial and mandibular nerves. The MCA was occluded for 60 min with a microclip between the rhino-cortical branch and the lenticulostriate artery (Theodorsson et al., 2005).

After termination of the MCAo, the osmotic pump was inserted and the infusion started. The rats were kept in individual cages with free access to water and standard chow until termination of the experiment.

2.2. Measuring ischemic lesion

The ischemic lesion was measured after three and seven days. The rats were anesthetized by carbon dioxide and sacrificed by a rat guillotine. The brain was carefully dissected out and cooled in + 4 °C saline. Seven, 2 mm-thick coronary slices of the brain were cut with razor blades directed by a rat brain matrix (RBM-4000, ASI Instrument, Inc., Warren, MI) using B as position 0, and two slices, B + 2 and B + 4 mm anterior to B and four slices, posterior to B (B −2, −4, −6 and −8 mm, respectively).

The slices were freed from the dura mater and soaked for 10 min in a solution of 2% 2,3,5- triphenyltetrazolium hydrochloride (TTC)

Table 1
Blood gases, blood pressure and core temperature reported in the table were registered immediately prior to the 60 min MCA microclip occlusion.

	n	pO_2 kPa	pCO_2 kPa	pH	Temperature (°C)	Systolic blood pressure, mmHg
Galanin 2.4 nmol/d 3 days	15	14.5 ± 0.7	5.30 ± 0.12	7.48 ± 0.01	36.7 ± 0.1	116 ± 4
Placebo 3 days	15	14.6 ± 0.6	5.11 ± 0.11	7.49 ± 0.01	36.7 ± 0.1	115 ± 4
Galanin 0.24 nmol/d 7 days	6	14.3 ± 1.2	5.73 ± 0.28	7.47 ± 0.02	36.6 ± 0.1	109 ± 3
Galanin 2.4 nmol/d 7 days	15	14.9 ± 0.6	5.27 ± 0.09	7.49 ± 0.01	36.7 ± 0.1	114 ± 3
Galanin 24 nmol/d 7 days	5	13.2 ± 0.5	5.61 ± 0.05	7.47 ± 0.01	36.7 ± 0.1	114 ± 3
GalR2 agonist 2.4 nmol/d 7 days	11	13.5 ± 0.9	5.02 ± 0.26	7.46 ± 0.01	36.6 ± 0.2	108 ± 2
Artificial CSF 7 days	15	12.8 ± 0.6	5.00 ± 0.10	7.48 ± 0.01	36.8 ± 0.1	110 ± 3

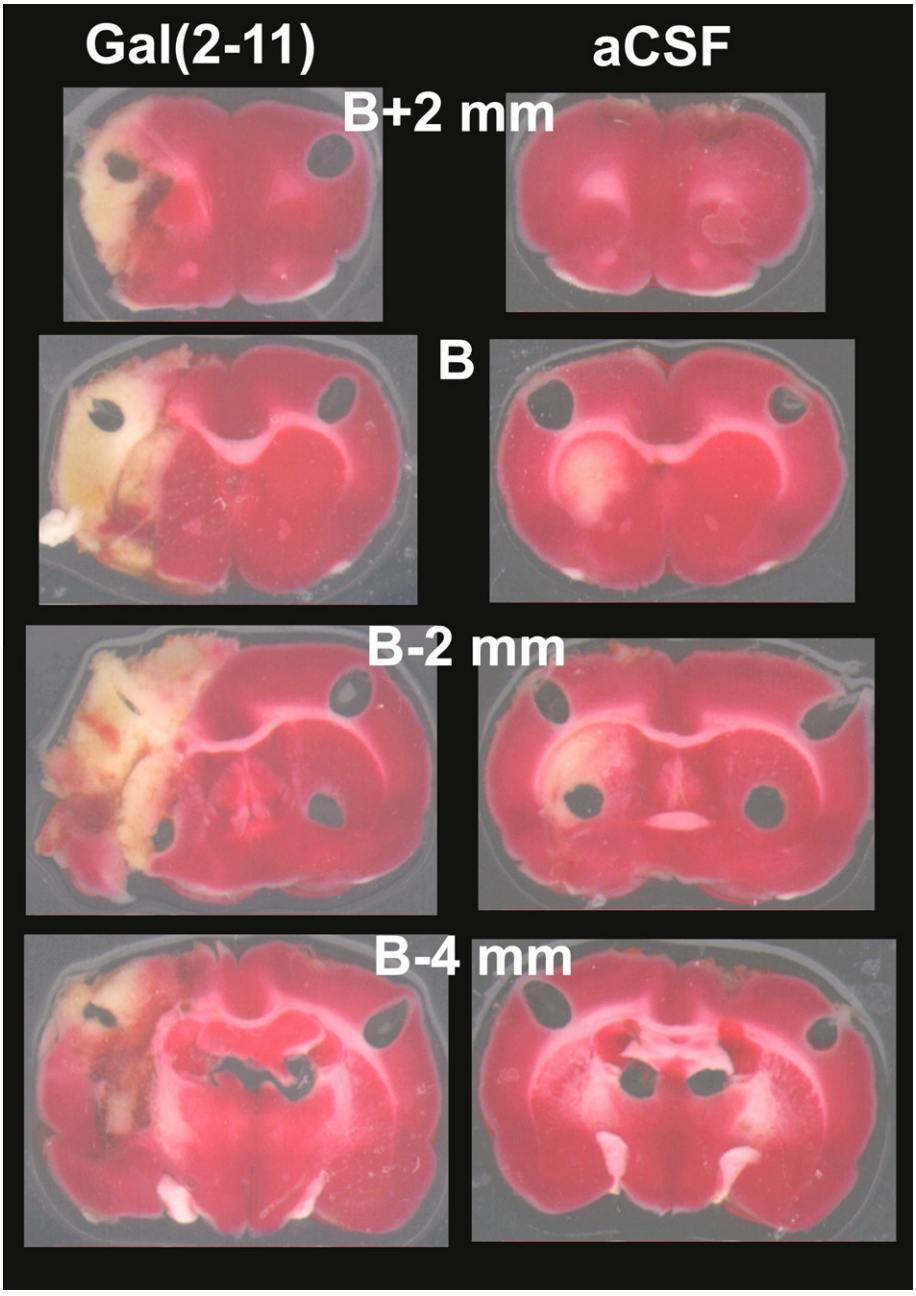

Fig. 1. Lesion areas visualized by TTC staining after i.c.v. administration of the GalR2/3 agonist (Gal 2–11) and artificial CSF. The TTC staining colors viable (well functioning mitochondria) tissue in red and ischemic tissue lesions in white. Punch biopsies allowing biochemical analysis have been taken.

in 0.1 mol/L PBS (pH 7.4) in a small Petri dish, maintained at 37 °C in a heater. Gentle stirring of the slices was used to ensure even exposure of the surfaces to staining. Excess TTC was then drained, and the slices were scanned (ScanJet 2c, Hewlett–Packard, Cupertino, CA).

The size of brain lesion was measured using SigmaScan Pro version 5 (SPSS, Inc). The image was divided into red, green and blue color spectra. The red spectrum was used to measure the total area of the slice and the green spectrum the area of the ischemic lesion. Before measurement, the intensity of the red spectrum was

maximized to sharpen the boundary between the slice itself and its surroundings. The intensity in the green spectrum was kept unchanged, but the outer boundaries of the ischemic area were marked in the "overlay draw mode" to demark the area from normal structures in the brain. An automatic threshold of 40% in the green spectrum was used in the function "fill mode" to automatically mark the ischemic area (Bederson et al., 1986; Goldlust et al., 1996; Theodorsson et al., 2005).

2.3. Study of recovery of galanin in Alzet pumps

Eight Alzet pumps of the type used in the experiments were filled with 100 μL of 1 μg/μL rat galanin (SC 936, Neosystem, Strasbourg, France) in isotonic Ringer acetate solution and covered with Parafilm®. Ten microliters of the same solution were aliquoted to each of 8 radioimmunoassay test tubes closed with stoppers. The Alzet pumps and test tubes were incubated at 37 °C for 7 days. The original 1 μg/μL galanin solution was stored in the refrigerator and used as calibrator for the galanin radioimmunoassay. After the incubation, the Alzet pumps were emptied using a Hamilton syringe, cut in half and incubated in 10 mL of radioimmunoassay buffer before analysis.

2.4. Radioimmunoassay of galanin

Rat galanin-like immunoreactivity (GAL-Li) was analyzed using antiserum RatGal4 raised against conjugated synthetic rat galanin (Theodorsson and Rugarn, 2000). HPLC-purified ^{125}I rat galanin was used as radioligand and rat galanin as standard. Immunoreactivity was measured using a Gamma counter (Wizard® 1470, Perkin Elmer, Turku, Finland). The detection limit of the assay was 5 pmol/L. Intra- and interassay coefficients of variation were 6% and 10%, respectively. The calibrator, Alzet pump galanin solutions and test tube solutions were all diluted to an expected concentration of 0.01 μg/μL and diluted 1:2 in 10 steps.

2.5. Statistical analysis

The mean and standard error of the mean were used as measures of central tendency and variation, respectively, throughout the study. Multivariate analysis of variance (ANOVA) and Tukey's post hoc test were used for significance testing (SYSTAT version 11, Systat Software Inc., Richmond, CA). P values less than 0.05 were considered significant.

3. Results

3.1. Size of ischemic lesion

The lesion area, involving the frontoparietal cortex and/or lateral striatum, was measured as percentage of the total area of each slice. Multivariate analyses of variance, factors = galanin (three concentrations)/GalR2/3 agonist/aCSF, time (three and seven days) and slice (7), revealed overall significant differences $p < 0.001$ for all three effects.

Multivariate analyses of variance in the seven-day groups, galanin (three concentrations), GalR2/3 agonist and aCSF showed overall significant differences ($p = 0.008$). Multiple comparisons with Tukey's post hoc tests revealed significantly larger ischemic lesions in the GalR2/3 agonist-treated group compared to aCSF group ($p = 0.023$, for all 7 slices) (Fig. 1). The mean lesion area as visualized with the TTC staining was larger in every slice in the GalR2/3 agonist-treated animals after seven days compared to the corresponding aCSF group. In the galanin group the lesions were largest at B (11.0 ± 2.7%) and B −2 mm (9.5 ± 2.5%), 111% and 98% larger than in the aCSF group, 5.2 ± 1.5% and 4.8 ± 1.1% respectively ($p = 0.023$, for all 7 slices) (Fig. 2).

In the galanin-treated groups, 0.24, 2.4 or 24 nmol/day were no differences found ($p = 0.939$, 0.715 and 0.977, for all 7 slices) (Fig. 2). The lesions were largest at B and B −2 mm, 7.2 ± 1.7% respectively 6.4 ± 1.2% in the galanin 0.24 nmol/day-treated group, 8.0 ± 1.9% respectively 5.9 ± 3.0% in the galanin 2.4 nmol/day-treated group, and 5.2 ± 2.0% respectively 8.1 ± 1.8% in the galanin 24 nmol/day-treated group (Fig. 2).

In the group analyzed after three days (only galanin 2.4 nmol/d) no differences were seen. The lesions were largest at B and B −2 mm, 16.2 ± 3.9% respectively 16.1 ± 2.9% in the galanin-treated group, and 15.1 ± 3.3% respectively 15.0 ± 2.7% in the aCSF group ($p = 0.925$, for all 7 slices), and in fact smaller at B + 2 mm and B + 4 mm in the galanin-treated group than in the aCSF group (Fig. 3).

3.2. Physiological measurements

Blood gases, blood pressure and rectal temperature were continuously monitored and kept within the physiological range during the operative procedures (Table 1).

3.3. Recovery tests of galanin

Recovery of galanin from Alzet pumps after 7 days at 37 °C for 7 days was 89 ± 2% and 93 ± 1% in the test tubes.

3.4. Mortality and exclusions

Five rats died unexpectedly within 30 min after intubation. This was also encountered in parallel in other experiments where in several animals from the same distributor died during a limited time period at Linköping University Animal Department. Two rats (belonging to the seven days GalR2/3 group) and one rat (belonging to the seven days aCSF group) were found dead after one and six days, respectively. Four rats were excluded from the study due to technical mishaps.

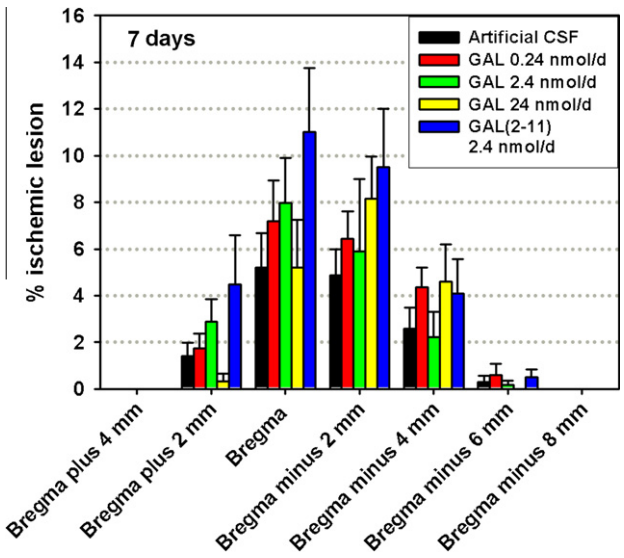

Fig. 2. Areas of ischemic lesions in i.c.v. coronal slices of the brain of naïve female rats treated with administered galanin in three different concentrations, a GalR2/3 agonist (Gal2–11) or artificial CSF (aCSF) during 7 days after a 60 min transient occlusion of the middle cerebral artery. The ischemic lesion as visualized with TTC staining was 98% larger after i.c.v. administration of the GalR2/3 agonist (2.4 nmol/d) compared to aCSF ($p = 0.023$). No differences were seen after galanin administration in the concentrations 0.24, 2.4 and 24 nmol/d ($p = 0.939$, 0.715 and 0.977).

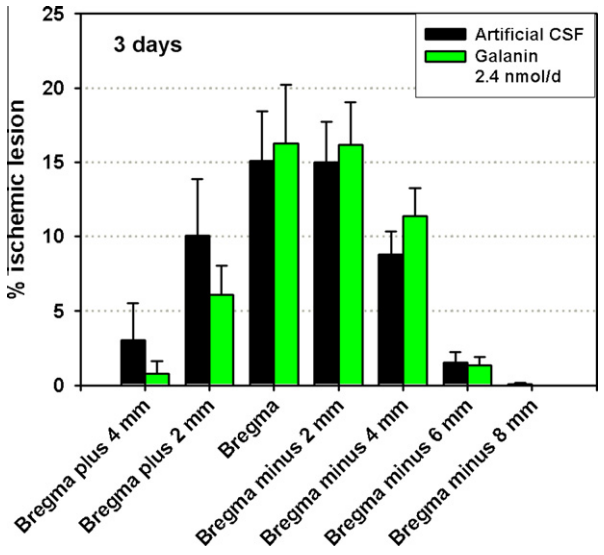

Fig. 3. Areas of ischemic lesion in coronal slices of the brain of naïve female rats treated with i.c.v. administered galanin (2.4 nmol/d) or artificial CSF (aCSF) during 3 days after 60 min transient occlusion of the middle cerebral artery. No significant effects were seen.

4. Discussion

The main result in this study on female rats is that the ischemic lesion, as visualized by TTC staining, surprisingly was 98% larger seven days after a 60 min transient MCAo and continuous i.c.v. administration of a GalR2/3 agonist, as compared to the aCSF group. There were no differences observed in the groups receiving a continuous infusion of three different concentrations of galanin after seven days and a single concentration after three days. These findings suggest that continuous stimulation of, presumably, GalR2 for a long period distinctly worsens the outcome after a transient MCAo. It also raises the possibility that concomitant activation of GalR1 (and perhaps GalR3) counteracts this effect. Galanin has in several CNS lesion models been shown to exert neuroprotective effects, primarily mediated via GalR2 (for references, see Introduction). However, the current study is – to our knowledge – the first direct investigation of the effects of centrally administered galanin in an experimental rat stroke model.

A possible mechanism for the lack of protective effects of galanin is degradation in the Alzet pumps, and this was therefore investigated. The recovery of rat galanin, stored at 37 °C in Alzet pumps and measured after 7 days was 89%, which lends no support to this hypothesis. Instead, the galanin infused i.c.v. in the present study was in the expected concentration range.

The galanin fragment Gal (2–11) acts as an agonist at GalR2 (Liu et al., 2001), that is the receptor which has been reported to play a central role in the reported neuroprotective properties of galanin (Mahoney et al., 2003; Elliott-Hunt et al., 2007). However, it was subsequently shown that this fragment also binds to GalR3 in transfected cell lines with a similar avidity as to GalR2 (Lu et al., 2005). Therefore a possible involvement of GalR3 remains to be analyzed. An effect via GalR3, however, appears less likely as the distribution of GalR3 mRNA is low in the rat brain and is mainly restricted to the hypothalamus (Wang et al., 1997; Smith et al., 1998; Waters and Krause, 2000; Mennicken et al., 2002). A further confounding fact is that GalR2 also binds the Gi/o type of G-proteins and thus could be inhibitory (Branchek et al., 2000). This is interesting but has, to our knowledge, not been tested in in vivo studies on GalR2.

A recent study has reported on a novel, GalR2 specific agonist M1145 (Runesson et al., 2009), which has more than a 90-fold higher affinity for GalR2 compared to GalR1, and a 76-fold higher affinity when compared with GalR3. Thus, M1145 represents a possibility to further explore a contribution of GalR3 to the increase in lesion size induced by Gal (2–11). Galanin, acting at all three receptor subtypes including GalR2, does not cause the same increase as the GalR2/3 agonist. It may therefore be speculated that concomitant activation of, in particular GalR1, could counteract the lesion induced via GalR2. If so, GalR1 in this particular model may counteract the development of the ischemia-induced lesion. This hypothesis could be tested by infusion of the GalR1 agonist M67 (Lundstrom et al., 2005).

The present study is based on continuous infusion of the peptides for three and seven days. It has been shown that application of galanin, but not Gal (2–11), blocks spontaneous firing of noradrenaline neurons (Seutin et al., 1989; Sevcik et al., 1993; Pieribone et al., 1995; Ma et al., 2001), which express both GalR1 and GalR2 (O'Donnell et al., 1999), and that a single application causes a strong desensitization, thus mediated via GalR1. To what extent this may occur for GalR1 (and/or GalR2/3) also in the forebrain in the present experimental paradigm remains to be established.

The distribution of galanin receptor protein in the rat brain is at present somewhat uncertain, since no reliable receptor-specific antibodies are available (Lu and Bartfai, 2009). Early receptor autoradiography has shown lack of binding to dorsal cortical areas and striatum of rat (Skofitsch et al., 1986; Melander et al., 1988), that is in those areas where the MCAo-induced lesion occurs. However, a study with ^{125}I-galanin (1–15-ol) showed strong binding in exactly these areas (Hedlund et al., 1992), perhaps reflecting GalR2 receptors, possibly presynaptic receptors on noradrenaline nerve terminals originating in the locus coeruleus. In situ hybridization shows in general low receptor transcript expression in dorsal cortex/hippocampal formation of rat, except for a clear signal from the granule cells in the dentate gyrus (Xu et al., 1998; O'Donnell et al., 1999).

Even if no studies on i.c.v. infusion of galanin have been made in rats subjected to transient cerebral ischemia, there are published reports related to that topic. Thus, Theodorsson and Theodorsson (2005) observed a decrease in galanin concentrations in hippocampus in the ischemic hemisphere compared to the corresponding contralateral intact hemisphere three days after transient MCAo. In rats a transient increase in galanin-positive cells have been reported in the dentate hilar region (Lee et al., 2005) and in non-pyramidal cells in the CA2/3 area (Hwang et al., 2004) of gerbils with a peak 12 h after insult, paralleled by an increase in protein. After four days galanin was expressed in microglia (Hwang et al., 2004). De Michele et al. (2006) detected both galanin accumulation in swollen axons 4 and 24 h after insult and galanin-immunoreative cell bodies after 72 h, all in the perinfarct zone. Finally, two gene expression studies on rats exposed to transient MCAo reported increases in galanin and GalR1 (Bond et al., 2002; Raghavendra Rao et al., 2002).

In conclusion, the present data show that continuous infusion of a GalR2/3 agonist does not result in neuroprotection in a rodent stroke model, but rather worsens the insult. Whether this surprising effect is related to the brain regions involved, this particular stroke model and/or the mode of drug administration, perhaps involving desensitization mechanisms, or whether galanin, via GalR2, to rats exposed to a transient occlusion of the MCA indeed lacks neuroprotective effect of its own, remains to be analyzed.

Conflict of interest

The authors have no conflict of interest to disclose. There is no duality of interests.

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