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ECTOPIC GALANIN EXPRESSION AND NORMAL GALANIN RECEPTOR 2 AND GALANIN RECEPTOR 3 mRNA LEVELS IN THE FOREBRAIN OF GALANIN TRANSGENIC MICE

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Abstract—The functional interactions of the neuropeptide galanin (GAL) occur through its binding to three G protein-coupled receptor subtypes: galanin receptor (GALR) 1, GALR2 and GALR3. Previously, we demonstrated that GALR1 mRNA expression was increased in the CA1 region of the hippocampus and discrete hypothalamic nuclei in galanin transgenic (GAL-tg) mice. This observation suggested a compensatory adjustment in cognate receptors in the face of chronic GAL exposure. To evaluate the molecular alterations to GALR2 and GALR3 in the forebrain of GAL overexpressing mice, we performed complementary quantitative, real-time PCR (qPCR), *in situ* hybridization, and immunohistochemistry in select forebrain regions of GAL-tg mice to characterize the neuronal distribution and magnitude of GAL mRNA and peptide expression and the consequences of genetically manipulating the neuropeptide GAL on the expression of GALR2 and GALR3 receptors. We found that GAL-tg mice displayed dramatic increases in GAL mRNA and peptide in the frontal cortex, posterior cortex, hippocampus, septal diagonal band complex, amygdala, piriform cortex, and olfactory bulb. Moreover, there was evidence for ectopic neuronal GAL expression in forebrain limbic regions that mediate cognitive and affective behaviors, including the piriform and entorhinal cortex and amygdala. Interestingly, regional qPCR analysis failed to reveal any changes in GALR2 or GALR3 expression in the GAL-tg mice, suggesting that, contrary to GALR1, these receptor genes are not under ligand-mediated regulatory control. The GAL-tg mouse model may provide a useful tool for the investigation of GAL ligand-receptor relationships and their role in normal cognitive and affective functions as well as in the onset of neurological disease.

Key words: neuropeptides, galaninergic, galanin receptors, mouse, plasticity.

Galanin (GAL) is a 29 amino acid neuropeptide that was originally isolated from the porcine gut (Tatemoto et al., 1983). In mammals, GAL peptide is expressed at moderate to high concentrations in the cerebral cortex, striatum, hypothalamus, hippocampus, brainstem nuclei such as the nucleus accumbens, dorsal raphe and locus coeruleus, and dorsal horn of the spinal cord in mice (Perez et al., 2001), rats (Skofitsch and Jacobowitz, 1985; Melander et al., 1986), and primates (Kordower et al., 1992). GAL is involved in the regulation of cognition, feeding, and pain, and may contribute to the modulation of sexual behaviors, depression and anxiety (Bartfai et al., 1993; Weiss et al., 1998; Xu et al., 2000; Wrenn and Crawley, 2001; Wynick and Bacon, 2002; Counts et al., 2003). To accomplish these diverse biological functions, GAL gene expression is highly plastic. GAL is dramatically up-regulated in rat anterior pituitary by estrogen (Kaplan et al., 1988), in the rat basal forebrain by nerve growth factor (Planas et al., 1997) and following experimental injury to the rat central and peripheral nervous systems, including olfactory bulbectomy (Holmes and Crawley, 1996), hypophysectomy (Villar et al., 1994), neurochemical dorsal raphe lesions (Gabriel et al., 1995), immunotoxic basal forebrain lesions (Hartonian et al., 2002), potassium chloride (KCl)-mediated cortical spreading depression (Shen et al., 2003), and sciatic nerve transection (Villar et al., 1989).

GAL binds to at least three receptors termed galanin receptor (GALR) 1, GALR2 and GALR3, which have been cloned and characterized in rat, mouse and human (Branchek et al., 2000; Floren et al., 2000; Counts et al., 2003). All GALRs are membrane-bound, G protein-coupled receptors, but they differ with respect to their amino acid sequence, distribution, G protein coupling and the cellular signaling mechanism (Branchek et al., 2000; Floren et al., 2000; Counts et al., 2003). In general, GALR1 and GALR3 are strongly coupled to the inhibition of adenylyl cyclase, whereas GALR2 stimulates phospholipase C and inositol phosphate production or is weakly coupled to adenylyl cyclase inhibition (Smith et al., 1997, 1998; Wang et al., 1998).

Recent advances in cellular genetics have provided three GAL transgenic (tg) lines for the investigation of how the nervous system responds to a permanent excess of the neuropeptide GAL. In the first mutant mouse, the GAL gene was fused to a rat prolactin promoter (Cai et al.,

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Abbreviations: DBH, dopamine β-hydroxylase; DEPC, diethyl pyrocarbonate; GAL, galanin; GAL-ir, galanin immunoreactive; GALR, galanin receptor; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; ISH, *in situ* hybridization; NGS, normal goat serum; qPCR, quantitative real-time polymerase chain reaction; RIA, radioimmunoassay; SSC, standard saline citrate; TBS, Tris-buffered saline; tg, transgenic; UTP, uridine 5′-triphosphate; UW, University of Washington; UW GAL-tg, University of Washington galanin transgenic mouse; WT, wild type.

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1999). A second GAL-tg was generated by expressing GAL under the control of a human dopamine β-hydroxylase (DBH) promoter (University of Washington galanin transgenic mouse, UW GAL-tg) (Steiner et al., 2001). A third overexpresser was generated by linking the GAL gene to the platelet-derived growth factor B promoter (Kokaia et al., 2001). In a series of studies aimed at defining how the overexpression of GAL affects neuronal activity, we performed a quantitative *in situ* hybridization (ISH) analysis of GAL mRNA expression using the UW GAL-tg transgenic mice. These mutant mice express an approximately five-fold increase in GAL message in the locus coeruleus (Steiner et al., 2001) and a striking ectopic expression of GAL mRNA and peptide in the piriform cortex and hippocampal complex (Steiner et al., 2001; Hohmann et al., 2003). Radioimmunoassay (RIA) experiments revealed that GAL peptide was two-fold higher in the forebrain, 10-fold higher in the frontal cortex and four-fold higher in the hippocampus in the UW GAL-tg as compared with wild type (WT) mice (Steiner et al., 2001; Wrenn et al., 2002). Interestingly, GALR1 mRNA is increased in discrete brain regions in UW GAL-tg mice suggesting a compensatory adjustment in the expression of cognate receptors as a homeostatic response to the overexpression of this peptidergic neurotransmitter (Hohmann et al., 2003). Whether compensatory increases in GALR2 and GALR3 mRNA also occur in response to chronically increased GAL gene expression within brain remains to be determined. The present study used quantitative, real-time polymerase chain reaction (qPCR) to quantify GAL, GALR2 and GALR3 mRNA expression in forebrain regions of UW GAL-tg as compared with WT mice.

EXPERIMENTAL PROCEDURES

Subjects

Young adult C57BL/6J GAL-tg mice and WT littermates obtained from both The Jackson Laboratory (Bar Harbor, ME, USA) and the University of Washington (UW, Seattle, WA, USA) colony were used in this study. All animals were housed in a temperature controlled vivarium maintained at 20 °C on a 12-h light/dark cycle. Food and drinking water were available *ad libitum*. All procedures were approved by the Rush University Medical Center and UW Institutional Animal Care and Use Committees in accordance with the NIH Guide for the Care and Use of Laboratory Animals (publication no. 86-23, 1985). All efforts were made to minimize the number of animals used and to reduce their discomfort.

Tissue harvesting for qPCR

GAL-tg ($n=10$) and WT ($n=10$) mice obtained from The Jackson Laboratory were anesthetized with sodium pentobarbital (60 mg/kg i.p) and transcardially perfused with a cold 4% saline solution. Each brain was rapidly removed from the calvarium and placed into cold stainless steel mouse brain blocker (Stoelting Inc., Wood Dale, IL, USA). All brains were sectioned into 1 mm coronal slabs (rostral to caudal) and selected brain structures were dissected on a glass plate placed over wet ice under RNase free conditions. From the brain slabs samples were taken from the frontal cortex, posterior cortex, hippocampus, septal diagonal band complex, amygdala, piriform cortex and olfactory bulb. Dissections were based on fiduciary landmarks derived from the Franklin and Paxinos (1997) mouse atlas. All tissue samples from each mouse were placed in a plastic tube and immediately stored at −70 °C until processed.

qPCR

Total RNA was extracted from each tissue sample using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA samples were treated with 10 U RNase-free DNase I (Ambion, Austin, TX, USA) for 45 min at 37 °C to eliminate DNA contamination. Total RNA was re-extracted with Trizol and reverse transcribed into single-stranded cDNA for 90 min at 42 °C using 100 ng oligo-dT16 (Promega, Madison, WI, USA) and 400 U Superscript II reverse transcriptase (Invitrogen). For qPCR, primer pairs were generated using MacVector 7.1 software (Accelry, CA, USA) to amplify specific ~100 bp fragments from rat GAL, GALR2, GALR3 and glyceraldehyde 3-phosphate dehydrogenase (G3PDH), a housekeeping gene that served as an internal control for quantitative analysis. The primers sequences are listed in Table 1. SYBR Green Quantitative PCR Kit (Qiagen, Valencia, CA, USA) was used for qPCR. PCR reactions for each gene of interest were run in triplicate on an MJ Research DNA Engine Opticon 2 (Waltham, MA, USA) as follows: 95 °C×15 min, 40–50 cycles of 95 °C×15 s, 54 °C–57 °C×30 s, 72 °C×30 s. Melting curve analysis began at 65 °C and increased by 1 °C to 99 °C every 10 s. We performed qPCR on samples in the absence of reverse transcriptase in parallel experiments to a control for genomic DNA contamination. PCR products were verified by gel electrophoresis (1.5% agarose). Although we performed qPCR evaluation for GALR1 mRNA in forebrain regions of WT and GAL-tg mice, we were unable to reliably detect GALR1 message. This was most likely due to methodological difficulties in the construction of receptor-specific primers (data not shown).

A standard curve was established using cDNA from mouse brain total RNA. The standard curve was calculated by plotting the threshold cycle (Ct value) against the log nanograms of total RNA added to the reverse transcription reaction. Expression levels of GAL and GALRs were normalized to G3PDH levels. The Wilcoxon rank-sum test was used to compare differences between genotypes and mean expression levels. Significance was set at $P<0.01$. The sum of the normalized values+1 was log-transformed for graphical representation of the data.

GAL ISH

To determine the cellular localization of GAL mRNA, we hybridized tissue sections generated from a previous ISH study performed by our group using UW GAL-tg ($n=6$) and WT ($n=6$) mice.

Table 1. qPCR primer pairs

Target gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
GAL	TGGAGGAAAGGAGACCAGGAAG	GCCTCTTTAAGGTGCAAGAAACTG
GALR2	TGCTCTTCTGTACCTCTCACGTCTG	GCCCC AAGTTGGTTT TTATTGG
GALR3	CAGATTGCGAGAGTGGTGACATAG	GGATCTCAGGTAGTTCAAGGACTCC
G3PDH	CAGCAAGGACACTGAGCAAGAGAG	ATTCAAGAGAGTAGGGAGGGCTCC

Tissue was fixed in 4% paraformaldehyde, acetylated, delipidated in chloroform, rehydrated in 95% ethanol and air-dried. For riboprobe generation, a plasmid vector pGemT-Easy (Promega, Madison, WI, USA) containing a 493 bp cDNA corresponding to the entire coding region of preprogalanin (kindly provided by Dr. James Hyde, University of Kentucky) was linearized with S*ac*II and transcribed *in vitro* with SP6 RNA polymerase to generate a cRNA antisense probe complementary to mouse GAL mRNA. A sense probe was generated by linearizing the plasmid with P*st*I and transcribing with T7 RNA polymerase. *In vitro* transcription reactions included: 25 μl of 12.5 mCi/ml ^{35}S-labeled uridine 5′-triphosphate (UTP), which represents 25% of UTP, and 2 μl cold thio-labeled UTP in a final concentration of 50 mM α-thio-UTP; 2 μl mixture of 50 μM each of ATP, cytosine 5′-triphosphate, and guanosine 5′-triphosphate; 2 μl RNase block; 3 μl RNA polymerase; 2 μg linearized mouse GAL plasmid vector DNA; 2 μl 10× transcription buffer (Boehringer Mannheim, Indianapolis, IN, USA); and 10 μl diethyl pyrocarbonate (DEPC)-treated water. The transcription reaction was incubated at 37 °C for 1.5 h. The reaction mixture was then mixed with 1 μl DNase I for 15 min before the reaction was terminated with the addition of 4 μl EDTA and 1 μl tRNA. The probe mixture was brought to a final volume of 50 μl with DEPC-treated water, purified with a Quickspin G-50 Sephadex spin column (Boehringer Mannheim, Indianapolis, IN, USA) and run on a polyacrylamide gel to check for probe integrity. Tissue sections were then hybridized overnight at 60 °C with the ^{35}S-labeled riboprobe at a concentration of 0.25 μg/ml/kb. All slides were treated with RNase A and washed with standard saline citrate (SSC) at increasing stringencies, which included two washes in 0.1× SSC at 65 °C. Sections were then dehydrated in an ethanol series and air-dried. All slides were dipped in diluted NTB2 emulsion (Kodak, Rochester, NY, USA) and were exposed for 22 days before development with Kodak D-19 developer. Slides were counterstained with Cresyl Violet and examined under reflected dark-field microscopy for the presence of discrete clusters of silver grains in anatomically defined regions of the brain, from the frontal cortex to the midbrain. Specificity of GAL signal was determined by hybridization with sense riboprobes, which were negative for all sections examined (data not shown).

GAL immunohistochemistry

To more clearly delineate the topography and morphology of the neurons containing GAL mRNA as well as whether there was overexpression of GAL peptide, we immunohistochemically processed free-floating 40 μm thick sections from UW mice which were perfused with 4% paraformaldehyde prior to staining as previously described (Sobreviela et al., 1998; Perez et al., 2001). Briefly, following several rinses in Tris-buffered saline (TBS; pH 7.4) the sections were incubated for 20 min in TBS containing 0.1 M sodium periodate to inhibit endogenous peroxidase activity. Following rinses in TBS/0.25% Triton X-100, the sections were soaked in a blocking solution of TBS/0.25% Triton X-100/3% normal goat serum (NGS) for 1 h. Subsequently, sections were incubated in rabbit anti-GAL primary antibody (1:10,000; Peninsula Laboratories, Belmont, CA, USA) for 24 h at room temperature, with constant agitation in a medium containing TBS/0.25% Triton X-100/1% NGS. After washes in TBS/1% NGS, sections were incubated in biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA, USA) for 1 h. After several rinses, sections were incubated for 60 min in an avidin–biotin complex (1:500; "Elite Kit," Vector Laboratories, Burlingame, CA, USA). The tissue was washed in 0.2 M sodium acetate, 1.0 M imidazole buffer (pH 7.4) and then developed in acetate-imidazole buffer containing 2.5% nickel (II) sulfate, 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO, USA) and 0.0015% H_2O_2. Rinsing the sections with acetate–imidazole buffer terminated the histochemical reaction. Sections were mounted on gelatin-coated slides, dehydrated through graded alcohols (70%, 95%, 100%), cleared in xylene and cover slipped with Permount (Fisher Scientific, Pittsburg, PA, USA).

Immunohistochemical controls consisted of processing sections as described above except that 1) the primary antibody solvent was substituted for the primary antigen, and 2) an irrelevant IgG was substituted for the primary antibody. It should be noted that specificity of GAL immunoreactivity, as is the case for all peptides, is not absolute. Regardless of the fact that the control experiments eliminated specific staining, the potential for the an-

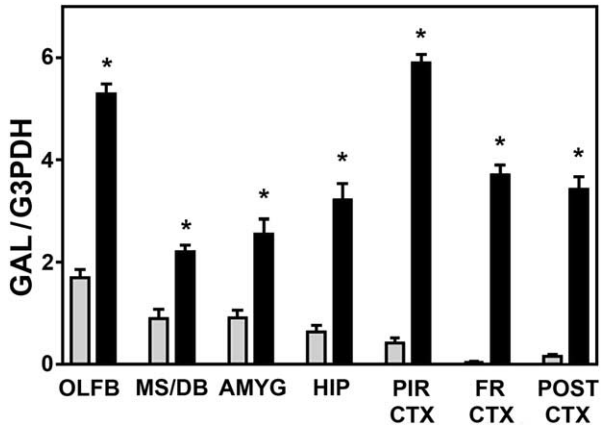

Fig. 1. Histogram showing GAL mRNA expression analyzed by qPCR in UW GAL-tg (black bars) and WT (gray bars) mice. * $P<0.001$ via Wilcoxon rank-sum test.

Abbreviations used in the figures

AMYG	amygdala
CA3	CA3 field of the hippocampus
Ce	central amygdaloid nucleus
cc	corpus callosum
DG	dentate gyrus
dhc	dorsal hippocampal commissure
ec	external capsule
EPl	external plexiform layer of the olfactory bulb
Fr CTX	frontal cortex
Gl	glomerular layer of the olfactory bulb
GO	granule cell layer of the olfactory bulb
Hip	hippocampus
I	layer I of the cerebral cortex
II	layer II of the cerebral cortex
III	layer III of the cerebral cortex
La	lateral amygdaloid nucleus
LEnt	lateral entorhinal cortex
lo	lateral olfactory tract
M	mitral cell layer of the olfactory bulb
MS/DB	medial septum/diagonal band of Broca
OLFB	olfactory bulb
opt	optic tract
Pir CTX	piriform cortex
PLCo	posterocortical amygdaloid nucleus
Post CTX	posterior cortex
PrS	presubiculum
RSG	retrosplenial granular cortex
V	layer V
VI	layer VI

tiserum to react with structurally related peptides could not be completely excluded. Thus, a degree of caution, which is inherent to immunohistochemical procedures, is warranted. In this regard, GAL immunoreactivity in this report refers to GAL-"like" immunoreactivity.

Tissue evaluation

In the present study, sections prepared for ISH were evaluated under both bright and dark field microscopy. GAL mRNA expression appeared as black or white grains over neurons under light and dark field filters, respectively. GAL immunoreactivity appeared as a dark blue precipitate in neurons and fibers. Photography was performed with a Nikon Microphot-FXA microscope coupled to a Nikon DXm1200 digital camera. Images were modified for brightness and contrast using Adobe Photoshop 7.0. The nomenclature used for the different regions of mouse brain was derived from Franklin and Paxinos (1997).

RESULTS

GAL mRNA in UW GAL-tg mice

qPCR analysis of GAL mRNA in select forebrain regions of WT mice revealed the highest expression in the olfactory bulb, followed by the septal diagonal band complex, amygdala, hippocampal formation, piriform cortex, posterior cortex and frontal cortex (Fig. 1). Relative GAL mRNA levels showed an apparent increase in all forebrain areas examined in GAL-tg mice compared with WT mice (Fig. 1).

GAL ISH

ISH experiments were performed to determine the cellular distribution of GAL mRNA up-regulation seen in the mutant mice. In WT mice, light to moderate numbers of GAL mRNA-containing cells were identified within the septal diagonal band complex, amygdala and within the ventral blade of the dentate gyrus of the hippocampus, but no labeling was seen in the piriform, entorhinal or neocortex (Fig. 2). In contrast, there was both an increase in GAL mRNA as well as evidence for ectopic GAL message expression in the forebrain of GAL-tg mice. Intense GAL mRNA labeling was seen in the inner layers of the piriform cortex (Fig. 2B) as compared with the virtual absence of label in WT mice (Fig. 2A). There was a marked increase in labeling within the amygdala (Fig. 2C, D) as well as an ectopic expression of the GAL mRNA in the cortical nucleus of the amygdala and the hippocampal–entorhinal cortex complex of GAL-tg mice compared with WT (Fig. 2E, F). In the latter region the labeling was confined mainly to the lateral entorhinal cortex in layers 2 and 5–6 (Fig. 2F). Ectopic expression of GAL mRNA was also seen in other areas of cortex particularly within the infragranular layers of the retrosplenial cortex in the mutant as compared with WT mice (Fig. 2G, H).

GAL immunohistochemistry

To determine whether those forebrain regions showing either increased or ectopic GAL gene expression exhibited similar changes in GAL peptide expression, we performed GAL immunohistochemistry. In WT mice a few scattered GAL-immunoreactive (-ir) neurons were seen in the main (glomerular layer) (Fig. 3A) and accessory olfactory bulbs, the septal diagonal band complex (not shown), hippocampus (CA3 fields and dentate gyrus) (Fig. 4A) and central amygdaloid nucleus (Fig. 4D). In GAL-tg mice, these structures displayed many more GAL-ir cells and fibers (Figs. 3B–D and 4B, C, F, G). In addition, we observed a striking ectopic expression of GAL-ir neurons in GAL-tg compared with WT mice in the piriform (Fig. 3, compare F, G to E), lateral entorhinal (Fig. 3, compare I, J to H) and retrosplenial granular (Fig. 3, compare L, M to K) cortex and presubiculum (Fig. 3, compare O, P to N). In general, the ectopic GAL-ir cells appeared round or oval (Fig. 3G, J). Ectopic GAL-ir cells were seen in the mitral and granule cell layers of the main olfactory bulb (Fig. 3B–D). In these layers an occasional neuron was found which displayed an extensive dendritic arbor (Fig. 3D). Ectopic round GAL-ir neurons were found mainly within the deep layer of the piriform cortex (Fig. 3F, G) and layers 2–6 of the entorhinal and retrosplenial granular cortex (Fig. 3I, J, L, M).

qPCR analysis of GALR2 mRNA levels

qPCR revealed varying GALR2 mRNA levels in the WT mouse forebrain (Fig. 5A). The highest levels of GALR2 mRNA expression were found in the olfactory bulb followed by the posterior cortex, septal diagonal band complex, amygdala, hippocampal formation, frontal cortex, and piriform cortex. There were no significant differences in GALR2 mRNA in UW GAL-tg mice in any of the forebrain regions examined.

qPCR analysis of GALR3 mRNA levels

The highest levels of GALR3 mRNA were found in the amygdala in WT mice, followed by the hippocampal formation, posterior cortex, olfactory bulb, piriform cortex, septal diagonal band complex, and frontal cortex (Fig. 5B). There were no significant differences in GALR3 mRNA expression in GAL-tg mice in any of the forebrain regions examined.

DISCUSSION

In the present study, corroborative evidence from qPCR, ISH and immunohistochemistry revealed that select forebrain regions of UW GAL-tg mice display dramatic increases in GAL mRNA and peptide, including ectopic neuronal expression, relative to WT control mice. However, qPCR analysis of these same regions did not reveal significant changes in GALR2 or GALR3 mRNA expression. While these data did not address potential effects of GAL overexpression on GALR protein, they suggest that baseline levels of these receptors may be sufficient for mediating GAL activity in the face of chronic increases in GAL concentration.

Previous ISH analysis of mRNA content in GAL-tg mice revealed a five-fold increase in GAL mRNA in the locus coeruleus and other brainstem nuclei known to express DBH, while ectopic GAL expression was seen in forebrain limbic regions such as the piriform and entorhinal cortex compared with WT mice (Steiner et al., 2001). The

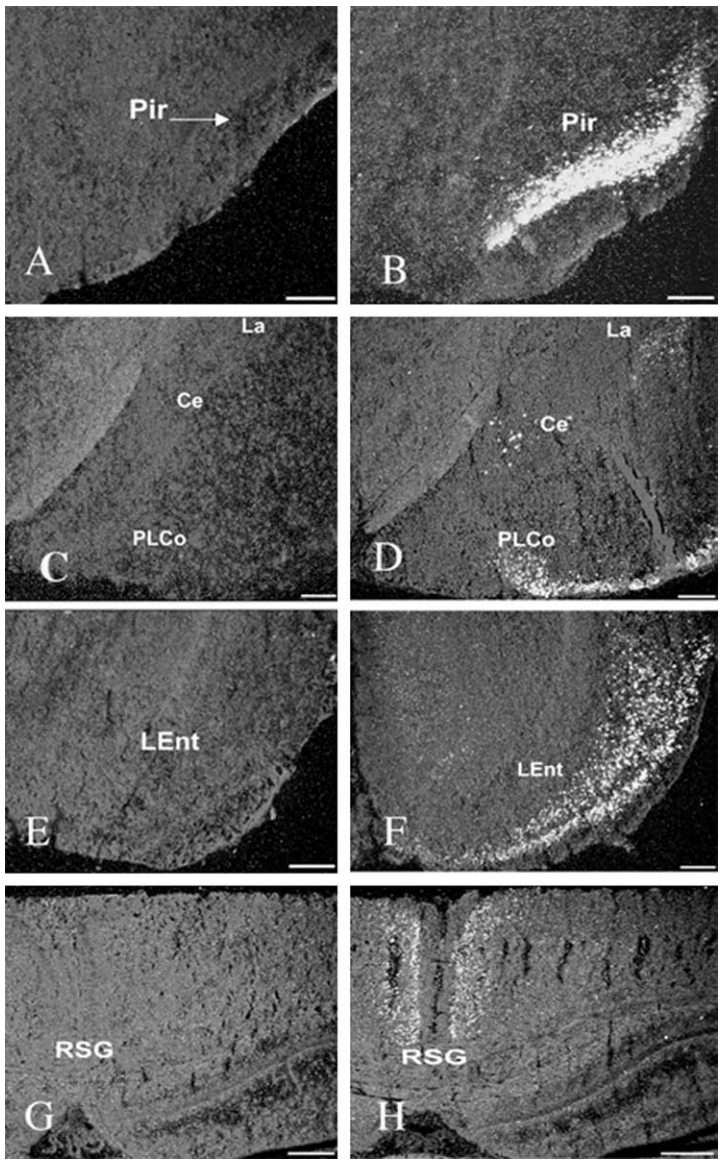

Fig. 2. Dark field photomicrographs showing the ectopic expression of GAL mRNA in the piriform cortex (B), amygdala (D), lateral entorhinal cortex (F) and the retrosplenial cortex (H) in GAL-tg mice compared with the absence of message in these areas in WT mice (A, C, E, G). Scale bar=250 μm.

present qPCR analysis extends these findings by showing a region-specific up-regulation of GAL mRNA in frontal cortex, posterior cortex, hippocampus, septal diagonal band complex, amygdala, piriform cortex and olfactory bulb of GAL-tg mice. The present ISH and immunohistochemical experiments also corroborate previous observations of ectopic GAL mRNA expression in the piriform and entorhinal cortex of GAL-tg mice (Steiner et al., 2001; Hohmann et al., 2003). In addition, we also provide evidence for ectopic GAL mRNA and peptide in neurons of the cortical nucleus of the amygdala, cortical areas such as the retrosplenial cortex and mitral cells of the olfactory bulb.

There are several possible mechanisms underlying the ectopic forebrain expression of GAL in UW GAL-tg mice. For instance, the 5.8 kb DBH promoter used to construct the GAL transgene has been shown to direct expression in neurons not traditionally associated with noradrenergic activity (Mercer et al., 1991; Hoyle et al., 1994). In particular, mice transgenic for a LacZ reporter under control of the DBH promoter display ectopic reporter expression in the piriform and entorhinal cortex as well as the amygdala (Mercer et al., 1991). Intriguingly, neuronal DBH transcription requires the activation of a cAMP response element in the upstream promoter region (Ishiguro et al., 1993; Kim et al., 1994; Afar et al., 1996), suggesting that cAMP-mediated transcription of the DBH-GAL transgene may contribute to ectopic GAL mRNA expression in forebrain neurons. Ectopic GAL expression in the mutant GAL-tg mice may also be due to well-documented GAL plasticity responses (Counts et al., 2003). GAL expression is highly plastic during embryogenesis (Anisimov et al., 2002), in the proliferative zone of the adult brain (Shen et al., 2003) and in response

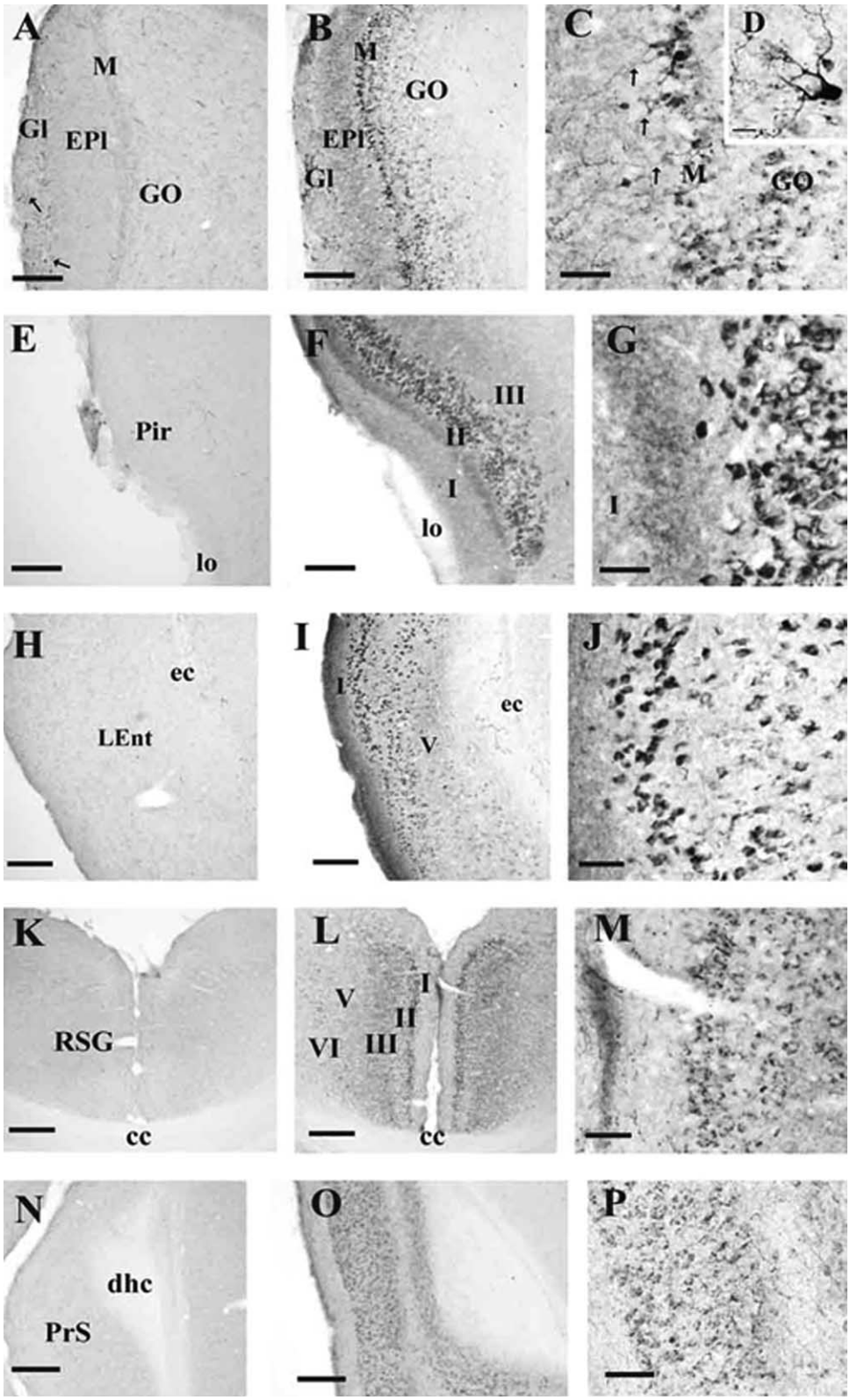

Fig. 3. Photomicrographs of GAL-ir in the forebrain of WT and GAL-tg mice. (A) Transverse section of the main olfactory bulb of a WT mouse showing GAL-ir neurons in the glomerular (arrows) layer as well as GAL-ir fibers in the internal plexiform and granule cell layers. Note the absence of the GAL-ir neurons in the mitral and granule cell layers. (B) Transverse section of an olfactory bulb of a GAL-tg showing ectopic GAL-ir neurons in the mitral and granule cell layers. Note the stronger GAL-ir in the external plexiform layer as compared with WT mice (A). (C) Detail of the ectopic GAL-ir neurons in the mitral and granule cell layers. Note the presence of GAL-ir processes in the external plexiform layer from neurons located within the mitral cell

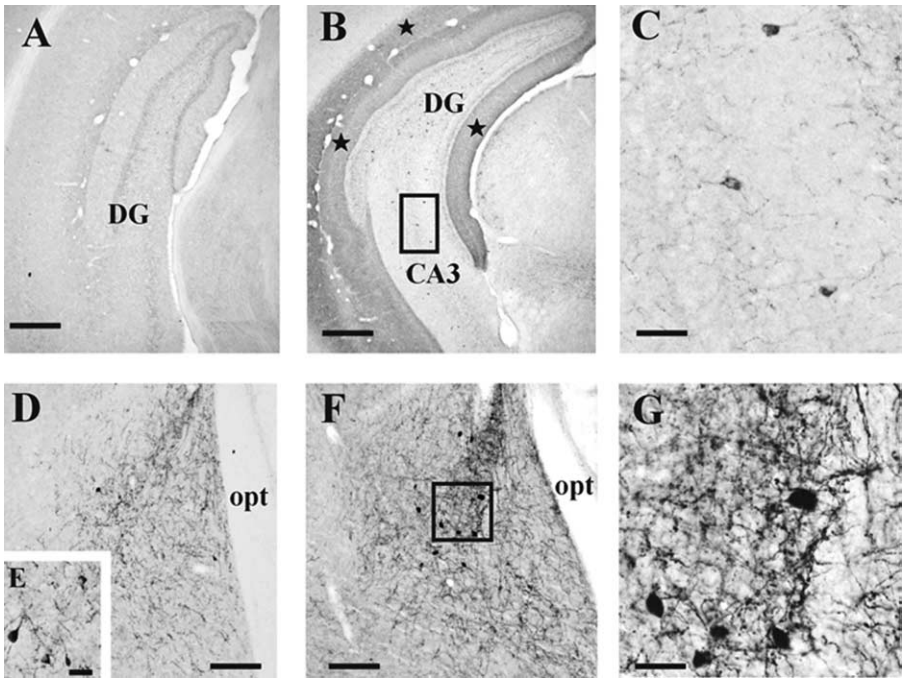

Fig. 4. Photomicrographs of GAL-ir in the hippocampus and amygdala in WT and GAL-tg mice. (A) Low magnification photo showing minor GAL staining in the hippocampus of a WT mouse. (B) Low power photomicrograph of the increased GAL immunostaining in the hippocampus of GAL-tg mice. Note the increase of GAL-ir in the molecular layer (stars). (C) Higher magnification image from the boxed area in B showing GAL-ir neurons in the CA3 region of the hippocampus in GAL-tg mice. (D) Low magnification photomicrograph showing GAL staining in the central amygdaloid nucleus in a WT mouse. (E) Inset shows a higher magnification image of the GAL-ir neurons in the central amygdaloid nucleus seen in WT mice. (F, G) Low (F) and high (G) power photomicrographs showing increased GAL-ir neurons and fibers in the central amygdaloid nucleus in a GAL-tg mouse. (G) is magnification of box in (F). Scale bars=200 μm in A, B, C=80 μm, D=250 μm, E=40 μm, F=200 μm and in G=70 μm.

to experimental brain trauma such as KCl-induced cortical spreading depression of the piriform and neocortices (Shen et al., 2003) or lesions to subcortical regions (Gabriel et al., 1995; Hartonian et al., 2002). Thus, physiological or pathological perturbations induced by the DBH-GAL transgene during development and adulthood could trigger GAL plasticity responses resulting in increased or ectopic forebrain expression. Such responses may reflect novel functions for this pleiotrophic peptide in the mammalian brain.

The present qPCR analysis of GALR2 and GALR3 mRNA expression in WT and GAL-tg mice revealed that GALR2 mRNA was expressed at highest levels in the olfactory bulb of both phenotypes, while moderate expression was seen in the posterior cortex, hippocampus, septal diagonal band complex, and amygdala. These results are similar to those obtained in the rat using semi-quantitative RT-PCR, RNase protection assays (Waters and Krause, 2000) or ISH (Kolakowski et al., 1998; O'Donnell et al., 1999). For example, RNase protection assays revealed moderate GALR2 expression in the cortex, hippocampus and amygdala in rat (Waters and Krause, 2000). In contrast, ISH demonstrated only moderate expression of GALR2 mRNA in the rat olfactory bulb relative to the hippocampus, whereas expression in the amygdala was relatively low (O'Donnell et al., 1999). qPCR analysis of GALR3 mRNA expression showed the highest levels in the amygdala with much lower expression in the cortex and hippocampus in both GAL-tg and WT mice. In contrast, an ISH study found only moderate GALR3 mRNA levels in the amygdala and no expression in the cortex or hippocampus (Mennicken et al., 2002), whereas RNase protection assays showed that GALR3 levels were not appreciably different in the amygdala compared with cortex and hippocampus in the rat (Waters and Krause, 2000). These differences between the mouse and rat are not attributable to the GAL transgene in the mouse since GALR2 and GALR3 mRNA levels were similar in the WT and GAL-tg mice. Rather, they are more likely due to either species-

layer (arrows). (D) GAL-ir neuron located in the mitral cell layer displaying an extensive dendritic arbor. (E) Piriform cortex of a WT mouse showing no GAL-ir neurons. (F) Piriform cortex of a GAL-tg mouse showing numerous ectopic GAL-ir neurons in layer II. (G) Detailed image of the GAL-ir neurons in layer II of the piriform cortex. (H) Lack of GAL-ir neurons in the lateral entorhinal cortex in WT mice. (I) In contrast, note the extensive ectopic expression of GAL-ir neurons in layers II–V of the lateral entorhinal cortex in GAL-tg mice. (J) Detail of GAL-ir neurons in layers II and III of the lateral entorhinal cortex. (K) Low magnification of the retrosplenial granular cortex showing lack of GAL-ir staining in WT mice. (L) In contrast, note the ectopic GAL-ir staining in the retrosplenial cortex in GAL-tg mice, mainly in layer II. (M) Higher magnification of the GAL-ir neurons in the retrosplenial granular cortex in a GAL-tg mouse. (N) Section of the presubiculum showing lack of GAL-ir staining in the WT mice. (O) By contrast, note the presence of numerous GAL-ir neurons in the presubiculum in a GAL-tg mouse. (P) Detail of the GAL-ir neurons of the presubiculum. Scale bars=150 μm in A, B, E, F, C=70 μm, D=20 μm, G=60 μm, H, I=180 μm, J=90 μm, K, L=200 μm, M=90 μm, N, O=170 μm and in P=100 μm.

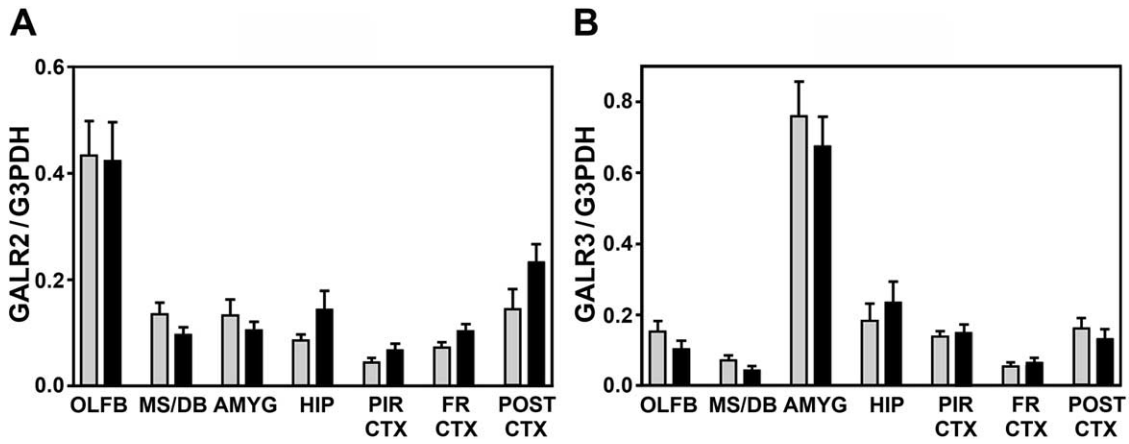

Fig. 5. Histograms showing (A) GALR2 and (B) GALR3 mRNA expression analyzed by qPCR in UW GAL-tg (black bars) and WT (gray bars) mice.

specific GALR expression in the forebrain or technical differences resulting from the assay employed.

Our results that GALR2 and GALR3 mRNA levels are unchanged in GAL-tg compared with WT mice contrast with ISH data showing an up-regulation of GALR1 mRNA in select nuclei of the hypothalamus and the CA1 region of the hippocampus in these tg mice (Hohmann et al., 2003). These regions receive afferent input from noradrenergic neurons of the locus coeruleus, which overexpress GAL in GAL-tg mice (Steiner et al., 2001). Thus, GALR1 gene expression in these target regions may be positively regulated by GAL as part of a homeostatic response to maintain normal galaninergic activity (Hohmann et al., 2003). It has been suggested that if GALR1 is coupled to inhibitory signaling mechanisms it may serve as an autoreceptor that restricts GAL neurotransmission in the face of chronic increases in GAL concentration (Hohmann et al., 2003). Alternatively, GALR1 increases in GAL-tg mice may reflect a post-synaptic mechanism by which target neurons regulate increased regional flux of GAL transmission. The stability of GALR2 and GALR3 mRNA levels in GAL-tg mice may indicate that the expression of these receptors is not regulated by the GAL ligand.

The discordance between GALR1 and GALR2/3 expression in GAL-tg mice might also be due to methodological differences inherent in qPCR and *in situ* techniques. For instance, we found a non-significant ~50–80% apparent increase in GALR2 and GALR3 mRNA in frozen samples of whole hippocampus by qPCR, whereas tissue based ISH detected ~50% increase in GALR1 in hippocampal CA1 neurons. Therefore, the application of qPCR to whole tissue pieces may lack the sensitivity to selectively detect subregion-specific changes in GALR expression within the forebrain regions examined. Future studies should employ GALR2 and GALR3-specific probes to determine whether ISH of select GAL-tg forebrain regions reveal discrete quantitative alterations in these specific receptor subtypes.

The functional significance of excess GAL in the face of GALR2/3 stability in the forebrain of the UW GAL-tg mice remains to be determined. Behavioral studies of the these mice show performance deficits on learning and memory tasks (Steiner et al., 2001; Wrenn et al., 2002), social and food preference tasks (Steiner et al., 2001; Wrenn et al., 2003), impaired long-term potentiation and evoked glutamate release in the hippocampus (Mazarati et al., 2000), reduced numbers of choline acetyltransferase-ir neurons in the basal forebrain (Steiner et al., 2001), and a selective reduction in ventral hippocampal acetylcholine release (Laplante et al., 2004) compared with WT mice. In addition, GAL overexpression appears to play a role in affective behaviors such as anxiety (Holmes et al., 2002; Kinney et al., 2002). Interestingly, the highest levels of GAL in the forebrain of these mice were found in the hippocampal complex, amygdala, olfactory and limbic cortical areas that mediate these cognitive and affective behaviors. These data support the interpretation that overexpressed GAL impairs cognitive and affective processes across a range of tasks, particularly on the more difficult components of learning and memory tasks related to limbic system neuronal function. Selective deficits in GAL-tg mice on learning and memory tasks are consistent with the pharmacological literature showing inhibitory actions of exogenously administered GAL in rats (Fisone et al., 1987; Dutar et al., 1989; McDonald et al., 1998; Wrenn and Crawley, 2001). While a role for GALR2 or GALR3 in these GAL-tg phenotypes has not been established, the present study indicates that overexpression of these receptors is not a prerequisite for the deleterious behavioral consequences of excessive GAL in this mutant.

In summary, we show that UW GAL-tg mice display substantial increases in neuronal GAL mRNA and peptide in the forebrain that are attributable in large part to an ectopic expression of GAL in limbic neuronal populations. The selective up-regulation of GAL may be related to DBH promoter-specific transgene regulation or to GAL-specific plasticity responses induced by the transgene during the development of these mice. The lack of evidence for changes in GALR2 or GALR3 expression in the face of chronic GAL activation suggests that, contrary to GALR1, these receptor genes are not under ligand-mediated regulatory control. This GAL mutant as well as others (Kokaia

et al., 2001; Elliott-Hunt et al., 2004) may serve as useful tools for determining GAL ligand-receptor relationships underlying cognitive decline in aging and neurological disorders such as Alzheimer's disease (Counts et al., 2003).

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