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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