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Galanin Receptors in Human Basal Forebrain Differ from Receptors in the Hypothalamus: Characterization Using [^{125}I]Galanin (Porcine) and [^{125}I]Galantide

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ABSTRACT

Galanin, a 29-amino acid peptide, is uniquely distributed in human basal forebrain and may play a role in cholinergic cell dysfunction in Alzheimer's disease. We report a detailed evaluation of galanin receptors in human basal forebrain (67 ± 12 years) and hypothalamus (67 ± 15 years) with radioligand binding techniques. The binding of [^{125}I]galanin (porcine) (agonist) or [^{125}I]galantide [GAL (1–3)-substance P (5–11)-NH_2] (putative antagonist) saturated in 2 hr, and only 15% to 30% of either radioligand was removed in the presence of unlabeled peptide. [^{125}I]Galanin or [^{125}I]galantide binding in basal forebrain revealed similar B_{max} values, with [^{125}I]galanin having a higher affinity for the galanin receptor. In contrast, [^{125}I]galanin showed a lower affinity and labeled 42% more receptors than [^{125}I]galantide in the hypothalamus. Differences were noted in competition studies of galanin and galanin chimeric peptides (M15, M35, M40 and C7) between [^{125}I]galanin and [^{125}I]galantide binding and in both regions. M35, M40 and C7 showed high affinity for galanin receptors in the hypothalamus with Hill coefficients close to unity, whereas in the basal forebrain these peptides competed differently. 5′-Guanylylimidodiphosphate reduced the specific binding of either radioligand in both regions. Based on the derived data, both radioligands irreversibly bind with high affinity and act as agonists at galanin receptors in human basal forebrain and hypothalamus. Galanin and galanin chimeric peptides compete differently for galanin receptors depending on the radioligand and region tested, suggesting subtype differences.

GAL is a 29-amino acid peptide isolated from the porcine small intestine (Tatemoto *et al.*, 1983). This peptide is cleaved from preprogalanin, a 123-amino acid precursor molecule, to form a biologically active peptide (Rokaeus and Brownstein, 1986). The first 13 amino acid residues of GAL are homologous throughout the species identified, with residue differences occurring in the COOH-terminal portion of the sequence (Tatemoto *et al.*, 1983). The amino acid sequence of GAL has 90% homology among the species examined but does not share any significant homology with other neuroactive peptides, suggesting that GAL is a member of a new family of neuropeptides (Evans and Shine, 1991). GAL protein and mRNA are widely distributed throughout the mammalian CNS (Kordower *et al.*, 1990, 1992; Melander and Staines, 1986). For the most part, the distribution of GAL within the CNS correlates with the location of GAL binding sites as shown with *in vitro* receptor autoradiography (Fisone *et al.*, 1987; Kohler *et al.*, 1989; Melander *et al.*, 1988; Skofitsch *et al.*, 1986). Although the exact physiological role of GAL is not clear, various studies suggest that GAL exhibits a wide range of biological responses (Hokfelt *et al.*, 1991), including inhibition of glucose-induced insulin release (Amiranoff *et al.*, 1988; Praz *et al.*, 1983) and inhibition of adenylate cyclase, and is reported to be coupled to G_i proteins (Chen *et al.*, 1992). A recent report indicated that GAL activates at least three distinct G proteins: α_{i1}, α_{i2} and α_{i3} (Gillison and Sharp, 1994).

Conflicting reports regarding the functionality of GAL and GAL chimeric peptides in different species have impeded understanding of the role of GAL pharmacology (Bartfai *et al.*, 1991; Gregerson *et al.*, 1993; Gu *et al.*, 1993; Takahashi *et al.*, 1994) and physiology (Dunning *et al.*, 1986, Gilbey *et al.*, 1989; Holst *et al.*, 1993; McDonald *et al.*, 1994; Miralles *et al.*, 1990) in humans. These differences may be species sequence

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ABBREVIATIONS: GAL, galanin; GLT, galantide or M15, GAL (1–13)-substance P (5–11); AD, Alzheimer's disease; K_{obs}, slope of the line of association; CNS, central nervous system; GppNHp, 5′-guanylylimidodiphosphate; M35, GAL (1–13)-bradykinin (2–9)-NH_2; M40, GAL (1–13)-Ala-Leu-Ala-amide; C7, GAL (1–13)-spantide; hGAL, galanin (human); rGAL, galanin (rat); pGAL, galanin (pig); fGAL, galanin (fragment 1–16); BF, basal forebrain; HYP, hypothalamus.

dependent, and availability of the GAL receptor cDNA (Habert-Ortoli *et al.*, 1994) may resolve several of these issues. The primary sequence of hGAL peptide was first reported by Bersani *et al.* (1991) and shortly thereafter by Evans and Shine (1991) and differs from other known species sequences by having an additional serine residue and a nonamidated carboxyl terminus. The additional amino acid and nonamidated carboxyl terminus may indicate that the hGAL receptor differs from receptors in other species. Interestingly, comparative GAL immunohistochemical (Benzing *et al.*, 1993; Kordower *et al.*, 1990, 1992; Walker *et al.*, 1989) studies show a dramatic species difference among monkeys, great apes and humans in the expression of GAL within the subfields (*i.e.*, septal diagonal band complex and nucleus basalis) of the BF. For example, in monkeys, GAL colocalizes with the BF magnocellular cholinergic neurons, whereas in apes and humans, this peptide is found mainly in a separate population of small, local circuit interneurons (Benzing *et al.*, 1993; Kordower *et al.*, 1990, 1992). This dramatic species difference in the neuronal phenotypic organization of galaninergic systems within the BF suggests possible unique characteristics in GAL pharmacology within the human brain.

Numerous biochemical, neurobehavioral and clinical pathological studies have indicated that GAL may play a key role in cognitive function associated with the BF hippocampal neuronal system. GAL has been shown to inhibit acetylcholine production within the hippocampus (Fisone *et al.*, 1987) and to depress neural systems underlying working memory (Crawley, 1993) in rats. Recent immunohistochemical (Cortes *et al.*, 1990) and *in situ* hybridization studies show a dramatic difference between humans and primate species in the neuronal phenotypic distribution of GAL within the BF (Kordower *et al.*, 1990, 1992; Melander *et al.*, 1985; Walker *et al.*, 1991). This region is intimately involved in memory function and undergoes extensive neuronal degeneration in AD (Mufson *et al.*, 1989; Whitehouse *et al.*, 1982). We and others have demonstrated that galaninergic fibers hyperinnervate remaining cholinergic BF neurons in AD (Chan-Palay, 1988a; Mufson *et al.*, 1993). These findings have led to the suggestion that galaninergic systems play a role in cholinergic cell dysfunction in this disease (Chan-Palay, 1988b; Mufson *et al.*, 1993). Taken altogether, these observations suggest that GAL may represent a potential pharmacological strategy for AD-dependent BF dysfunction. It is important, therefore, to define GAL receptor pharmacology in normal human BF.

The purpose of the present study was to biochemically characterize GAL receptors in the human BF and to compare the pharmacology with HYP GAL receptors of the same individuals with the use of an agonist, [^{125}I] pGAL, or a putative antagonist, [^{125}I] GLT.

Materials and Methods

Brain tissue preparation. Human brains of seven men (average age, 56.6 years; range, 27–77 years) and five women (average age, 64.2 years; range, 27–88 years) without neurological or psychiatric illness were obtained at autopsy (Table 1). The average postmortem delay was 14.1 hr (range, 5–25 hr). After each brain was removed from the calvaria, it was sliced coronally into 1-cm-thick slabs with a calibrated Lucite brain slice apparatus. The slabs were then hemisected, and the right hemisphere was immersion fixed in 4% paraformaldehyde solution (pH 7.4; Fisher Scientific, Pittsburgh, PA) as described previously (Mufson *et al.*, 1993). Samples from the left hemisphere containing the anteromedial and anterolateral subfields of the nucleus basalis (n = 9) (Mufson *et al.*, 1989) or the medial HYP (n = 5) were dissected, snap frozen in liquid nitrogen, and stored at −80°C until processed for receptor binding assays. Sections from each brain containing the amygdala, hippocampal complex and temporal cortex were paraffin embedded for neuropathological examination of Alzheimer's-like degeneration (*i.e.*, neuritic plaques and neurofibrillary tangles) with Alz-50 antibody immunohistochemistry, thioflavin-*S* and Bielchowsky silver stains according to previously described protocols (Mufson *et al.*, 1988, 1989, 1993). Neuropathological evaluation revealed virtually no pathological degeneration of the Alzheimer's type.

Peptides. GAL (rGAL, hGAL, pGAL and fGAL) and GLT were obtained from Bachem (Torrance, CA) or Peninsula Laboratories (Belmont, CA). Chimeric GAL peptides M35, M40 and C7 were purchased from T. Bartfai and U. Langel, (Stockholm University, Stockholm, Sweden). [^{125}I]pGAL and [^{125}I]GLT were iodinated by DuPont-NEN (Boston, MA) with chloramine T and lactoperoxidase methods, respectively. The GLT used for custom labeling was obtained from Peninsula Laboratories. Cholecystokinin (26–33) amide, substance P (5–11) NH_2, bradykinin (2–9) and spantide II were purchased from Bachem or Peninsula Laboratories. All stock solutions of peptides (100 μM) were in 1% (v:v) dilute acetic acid at 100 μM, and 50-μl aliquots were stored at −80°C until use. Stock solutions of peptides were thawed and serially diluted in GAL buffer (50 mM HEPES, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.025% bacitracin and 0.025% NaN_3, pH 7.4) for assay.

Receptor preparation. Human BF or HYP (20–25 mg/ml wet wt.) were weighed and placed in 50-ml centrifuge tubes. The tissue

TABLE 1
Donor demographics

Donor	Age	Sex	Brain weight	Postmortem delay	Tissue sample
	years		*g*	*hr*	
1	88	F	1000	5	BF, HYP
2	64	F	1200	10.5	BF
3	77	M	1310	19.5	BF
4	56	M	1310	15	BF
5	63	M	1275	13	BF, HYP
6	59	M	1430	19	BF
7	27	M	1440	11	BF
8	29	F	1390	25	BF
9	71	F	1150	19	BF
10	51	M	1260	11.5	HYP
11	69	F	1200	11.5	HYP
12	79	M	1220	11	HYP

was homogenized in 35 ml of GAL buffer (50 mM HEPES, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.025% bacitracin, 0.025% NaN_3 and 0.1% bovine serum albumin, pH 7.4) and centrifuged for 12 min at 15,000 rpm (J2–21M [J-20], Beckman Instruments, Inc., Palo Alto, CA), and the supernatant was removed. The pellet was resuspended in 35 ml of stock buffer and recentrifuged for 12 min at 15,000 rpm. The supernatant was removed, and the pellet was resuspended in a volume that contained ~0.47 ± 0.09 mg/ml protein or 78 ± 0.4 μg/200 μl reaction volume. Protein determination was performed with the Pierce BCA protein assay (Smith *et al.*, 1985). Each experiment was done with a specific donor sample (Table 1); pooled samples were not used for this study because we wanted to collect information on individual samples.

Radioligand binding protocol. Membrane homogenate (100 μl) was added to each well of a 96-well microtiter plate followed by the addition of 50 μl of binding buffer or unlabeled peptide. Nonspecific binding was determined in the presence of 1 μM hGAL or GLT. Reactions were initiated by the addition of 50 μl of [^{125}I]GAL or [^{125}I]GLT in buffer, for a final reaction volume of 200 μl. The membranes were incubated (room temperature) on orbital shaker for 2 hr (25°C). Standard glass fiber filters (No. 32, Schleicher and Schuell, Keene, NH) were presoaked for 1 hr in 2% polyethyleneimine before filtration to reduce nonspecific binding. The membranes were harvested on a 96-well microtiter vacuum harvester (TomTec Mac II, Orange, CT), and the unbound radioactivity was removed by rinsing with five cycles (3.5 ml vol) of 50 mM Tris·HCl wash buffer (4°C, pH 7.2). Bound radioactivity was determined with a Packard (Meriden, CT) 10-channel Cobra gamma counter.

Kinetic experiments were carried out according to the methods of Bylund and Yamamura (1990). Briefly, an aliquot (100 μl) of membrane homogenate was added to each well of a 96-well microtiter plate. Total and nonspecific binding was determined for each time point. The reaction was initiated by the addition of radioligand at the set time for association experiments. For dissociation experiments, membranes and radioligand were preincubated for 2 hr, and at given time points, 1 μM GAL, μM GLT or 100 μM GppNHp (Sigma Chemical Co., St. Louis, MO) was added. Membranes were harvested as described above.

For membrane wash experiments, centrifuge tubes (50 ml) containing 6 ml of human BF (25 mg/ml wet wt.) membrane homogenates were incubated with hGAL (IC_{50} × 100 = 1 nM) to saturation 2 hr at room temperature. After incubation, each tube was vortexed; a 10-μl sample was taken for protein determination; and triplicate 100-μl aliquots were taken for each set of total and nonspecific samples. The volumes of the centrifuge tubes were restored to 6 ml with membrane buffer and centrifuged at 14,000 rpm for 10 min; the supernatant was removed, and the membrane was resuspended in 4.8 ml buffer. This procedure was repeated, with 100-μl aliquots removed from the centrifuge tubes and the volume restored to 6 ml. This wash procedure was repeated 3 times. After the last wash procedure, the final group of 100-μl aliquots were taken, and the binding assay was performed as indicated.

For acid treatment experiments, BF homogenates were incubated for 2 hr with either radioligand. The addition of 1 ml acid solution (0.2 M acetic acid plus 0.2 M NaCl; 4°C) was added to the membranes and incubated for 15 min (4°C). Control samples were run in parallel and received 1 ml binding buffer (4°C). The reaction was centrifuged for 5 min at 12,000 rpm, the supernatant was removed, and the wash protocol outlined above was followed to remove all unbound radioligand.

For experiments in which the effects on radioligand binding of the nonhydrolyzable GTP analogue GppNHp (Sigma Chemical Co.) were measured, a stock solution (10 mM) of GppNHp was made in binding buffer before assay. Radioligand binding assays were performed as described with the exception that 10 μl GppNHp (final concentration, 100 μM) and 40 μl binding buffer were added in place of 50 μl buffer.

Statistical analysis. EBDA analysis programs for competition and saturation studies (Mcpherson, 1985) were used. Due to the availability of age-matched human tissue from each brain area investigated, all experiments were run 2 or 3 times with different samples of BF or HYP. Values expressed are the mean ± S.E.M. of those experiments. Statistical analysis was done on data from competition studies using the SAS statistical package (Cary, NC). A nonparametric three-factor factorial followed by the nonparametric least-significant difference was used. Statistically significant differences were determined with a Student's paired *t* test, where a level of $P < .05$ was considered significant.

Results

The binding of [^{125}I]GAL or [^{125}I]GLT to GAL receptors was irreversible. Kinetic experiments with [^{125}I]GAL or [^{125}I]GLT (fig. 1, top) showed K_{obs} rates of 0.026 and 0.042 min^{-1}, respectively, with BF homogenates. For dissociation experiments, GAL or GLT (1 μM) or GppNHp (100 μM) was added at 2 hr, and ~90% of either radioligand remained bound after 1 hr. Kinetic experiments with [^{125}I]pGAL or [^{125}I]GLT (fig. 1, bottom) showed K_{obs} rates of 0.017 and 0.018 min^{-1}, respectively, with HYP homogenates. The same method as was used with BF was used with HYP for dissociation experiments, and approximately 75% to 80% of either radioligand remained bound after 1 hr. Dissociation studies were continued to 4.5 hr with both radioligands in both regions, and no change was noted (data not represented). Additional approaches were attempted to dissociate [^{125}I]pGAL or [^{125}I]GLT from the GAL receptor. BF membranes were incubated with [^{125}I]pGAL for 2 hr and then washed 4 times with binding buffer. A standard binding assay was performed on these membranes, and the bound to unbound radioligand ratios remained the same in comparison to unwashed control membrane values (data not illustrated). Exposure of acetic acid to BF membranes preincubated with

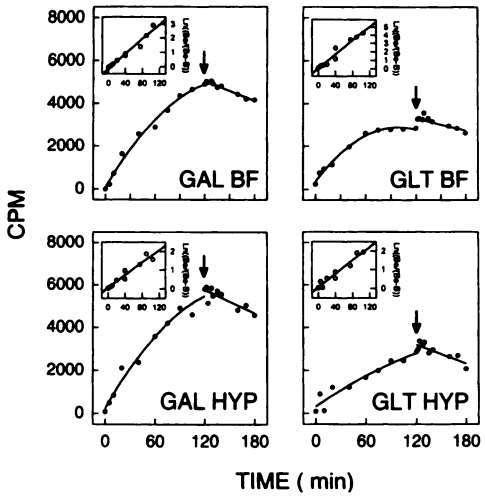

Fig. 1. Kinetics of [^{125}I]pGAL and [^{125}I]GLT binding in human BF and HYP. Top, 50 pM [^{125}I]pGAL or [^{125}I]GLT association binding in the BF. Insets, Pseudo-first-order association plots ([^{125}I]pGAL – K_{obs} = 0.026 min^{-1} and line correlation of .96; [^{125}I]GLT – K_{obs} = 0.042 min^{-1} and line correlation of .95). Bottom, 40 pM [^{125}I]pGAL or 50 pM [^{125}I]GLT association binding in the HYP. Insets, Pseudo-first-order association plots ([^{125}I]pGAL – K_{obs} = 0.017 min^{-1} and line correlation of .93; [^{125}I]GLT – K_{obs} = 0.018 min^{-1} and line correlation of .94). Data are mean values of triplicate determinations from one experiment that is representative of two other cases (table 1, BF 1, 4 and 6; HYP, 1, 5 and 12). Arrows, Addition of 1 μM GAL or GLT at 120 min.

either radioligand was done to determine whether the binding of the radioligands changed the conformation of the GAL receptor. The addition of acetic acid to BF membranes abolished the specific binding of either radioligand. To determine whether the acetic acid treatment destroyed the receptor, the BF membranes were washed and used again in a binding assay with [^{125}I]pGAL, and the specific binding was restored to control values (data not illustrated).

[^{125}I]pGAL and [^{125}I]GLT appeared to label the same receptor population in basal forebrain. Due to the irreversible binding of either radioligand, K_D and B_{max} values are relative values based on assumed saturation (2 hr) rather than steady state measurements. Rosenthal analysis with [^{125}I]pGAL or [^{125}I]GLT in BF homogenates of aged-matched control subjects (table 1) (67 ± 12 years old) revealed K_D values of 89 ± 23 and 193 ± 39 pM and B_{max} values of 35 ± 8.3 and 31 ± 0.7 fmol/mg protein, respectively (fig. 2, top). Maximal binding densities for GAL receptors were similar for both radioligands, and [^{125}I]pGAL showed the highest affinity for the receptor population labeled. Crude homogenates from two younger (28 ± 1.4 years old) BF samples (table 1, BF 7 and 8) were also evaluated with Rosenthal analysis and revealed similar K_D and B_{max} values (data not represented).

[^{125}I]pGAL labeled more receptors in the HYP than [^{125}I]GLT. In comparison, Rosenthal analysis with [^{125}I]pGAL or [^{125}I]GLT in human HYP of aged-matched control subjects (67 ± 15 years old) revealed K_D values of 264 ± 7 and 197 ± 11 pM and B_{max} values of 125 ± 9 and 72 ± 10 fmol/mg protein, respectively (fig. 2, bottom). [^{125}I]pGAL had a lower affinity value for the GAL receptor and labeled 42% more receptors in this region than [^{125}I]GLT.

GppNHp reduced the binding of [^{125}I]pGAL or [^{125}I]GLT to GAL receptors. The addition of GppNHp (100 μM) reduced the specific binding of 40 to 45 pM [^{125}I]pGAL or [^{125}I]GLT in BF by 87% and 100%, respectively (fig. 3). In the HYP, the specific binding of [^{125}I]pGAL or [^{125}I]GLT was reduced by 74% and 100%, respectively.

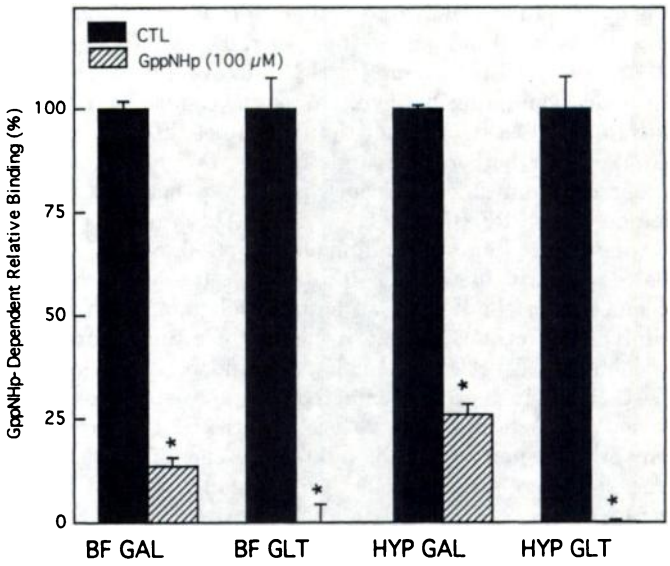

Fig. 3. Effect of 100 μM GppNHp on [^{125}I]pGAL or [^{125}I]GLT binding in human BF and HYP. Data illustrated as percent relative specific binding of six determinations from three experiments (table 1, BF 3, 5 and 9; HYP, 10, 11 and 12) with different membrane preparations (*P < .05).

Characterization of [^{125}I]pGAL or [^{125}I]GLT in human tissue with neuropeptides. The specific binding of 40 to 45 pM [^{125}I]pGAL or [^{125}I]GLT in the BF was 86 ± 6% and 70 ± 3%, respectively, in competition experiments with GAL or GAL chimeric peptides. The specific binding of 40 to 45 pM [^{125}I]pGAL or [^{125}I]GLT in the HYP was 75 ± 6% and 64 ± 10%, respectively, in competition experiments.

To determine specificity of the GAL receptor, four neuropeptides were selected for testing in the two areas examined. Substance P, spantide II, bradykinin (2–9) and corticotropin-releasing factor were competed at 0.01, 0.1 and 1 μM in the presence of [^{125}I]pGAL or [^{125}I]GLT. At the concentrations tested, substance P (1 μM) inhibited [^{125}I]pGAL bind-

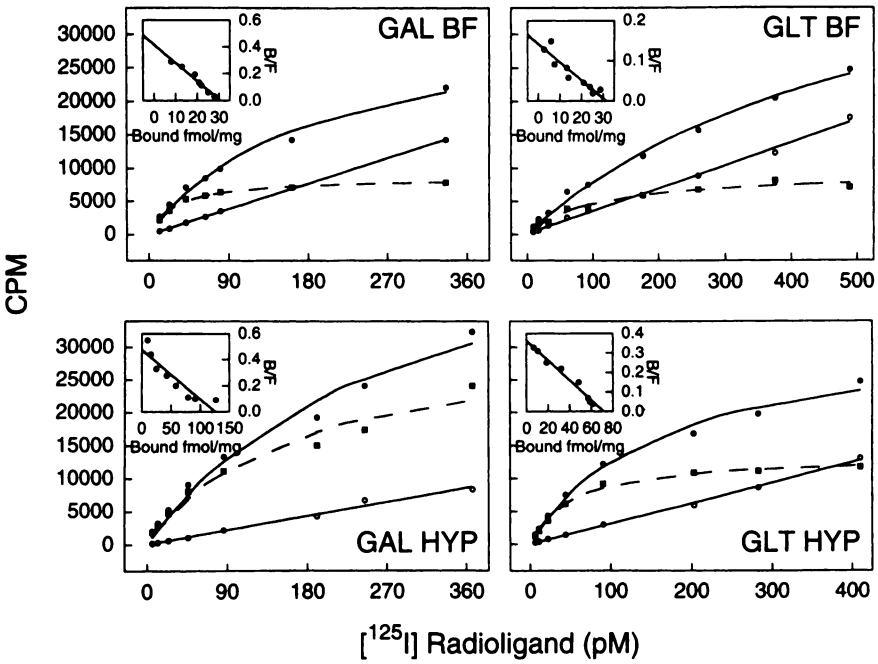

Fig. 2. Saturation of [^{125}I]pGAL and [^{125}I]GLT binding in human BF and HYP (case 5). Varying concentrations of radioligand were incubated with membrane suspensions for 2 hr at room temperature. Total (●), nonspecific (○), and specific (■) binding were estimated as indicated in "Materials and methods." Top, [^{125}I]pGAL or [^{125}I]GLT saturation binding in the BF. Insets, Scatchard transformations generated by EBDA analysis ([^{125}I]pGAL – K_D = 74 pM, B_{max} = 31 fmol/mg protein, R = .97; [^{125}I]GLT – K_D = 221 pM, B_{max} = 32 fmol/mg protein, R = .87). Bottom, [^{125}I]pGAL or [^{125}I]GLT saturation binding in the HYP. Insets, Saturation transformations generated by EBDA analysis ([^{125}I]pGAL – K_D = 264 pM, B_{max} = 125 fmol/mg protein, R = .83; [^{125}I]GLT – K_D = 197 pM, B_{max} = 71.8 fmol/mg protein, R = .98). Data are mean values of triplicate determinations from one experiment that is representative of three other cases (table 1, BF 1, 2, 5 and 6; HYP, 1, 5, 10 and 12).

ing by 26% in the BF, whereas none of these other neuropeptides inhibited the binding of either radioligand.

Because [^{125}I]pGAL and [^{125}I]GLT irreversibly bind to the receptor population defined, values for competition studies are reported as IC_{50} rather than K_i values. hGAL competed similarly for both radioligands in the HYP but showed an order of magnitude difference in affinity between radioligands in the BF (fig. 4). GAL and GAL chimeric peptides were competed for the binding of [^{125}I]pGAL or [^{125}I]GLT in both tissues to determine IC_{50} values. (table 2). Peptides competed for the binding of both radioligands with similar rank order in the HYP, but in the BF the affinities and rank order differed between radioligands and regions. GAL and GAL chimeric peptides competed and were statistically different for the binding of [^{125}I]pGAL in the BF compared with any other region or radioligand tested. The affinities of M35 and M40 were similar for both radioligands in the HYP but could not be determined in the BF with [^{125}I]GLT. Also, routine competitions showed M35 (1 pM) inhibited ~80% of [^{125}I]pGAL or [^{125}I]GLT binding in the BF. This finding was unusual because competitions with M35 for hippocampus (pig or rat) or RINm5F cells using [^{125}I]pGAL competed for the binding over a normal range of activity (data not shown). Kinetics or other neuropeptide receptor interference was considered a pausable explanation for this observation. Because M35 is the bradykinin chimeric peptide, interaction with other neuropeptides was investigated. BF membrane homogenates were preincubated for 2 hr with 1 μM fGAL, spantide or bradykinin (2–9) before competition of M35 for [^{125}I]pGAL binding. If [^{125}I]pGAL is labeling additional receptors such as substance P or bradykinin, the preincubation step should block these receptors and M35 should compete normally for [^{125}I]pGAL binding. No significant effect on M35 inhibition of [^{125}I]pGAL binding was noted in the BF in these experiments, eliminating the possibility of substance P or bradykinin interactions. Because both radioligands bind irreversibly, the kinetics of M35 was also considered a possible explanation for our reported findings. If the on rate of M35 was faster, then the order of addition of either M35 or radioligand would affect the competition. Either M35 or [^{125}I]pGAL was added to BF homogenates and incubated for 1 hr before the addition of either [^{125}I]pGAL or M35, respectively, to initiate the reaction. In these studies, no significant effect on M35 competition for [^{125}I]pGAL binding was noted, eliminating the possibility of a kinetic difference between M35 and the radioligand.

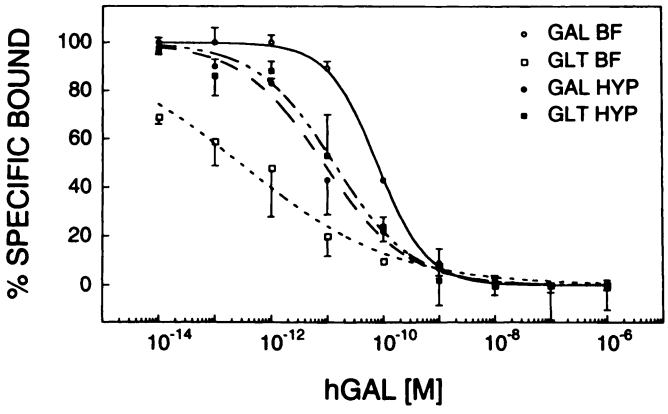

Fig. 4. Competition hGAL using either [^{125}I]pGAL or [^{125}I]GLT in the human BF or HYP. Graph is representative of one experiment ran in triplicate. Values represent the mean and S.D. of percent specific bound at each concentration tested. Competitions were repeated at least 3 times on different tissue homogenates (table 1, BF 1, 5 and 9; HYP, 1, 10, 11 and 12).

Discussion

The present findings indicate that [^{125}I]pGAL and [^{125}I]GLT are slowly reversible high-affinity agonists that differentiate GAL receptor subtypes in the BF and HYP. The ability to generate a large number of brain samples has limited the scope of the present study, and therefore certain questions regarding receptor subtypes, radioligand specificity and GAL chimeric peptide interactions require further investigation.

The results of the kinetic experiments showed that both [^{125}I]pGAL and [^{125}I]GLT irreversibly bind to the GAL receptor after association, even in the presence of >1000-fold excess of unlabeled peptide. This was an unexpected finding that may be due to a high-affinity state of the GAL receptor. Experiments were designed to address the irreversibility or slowly reversible action of [^{125}I]pGAL or [^{125}I]GLT binding for GAL receptors based on prior information. Several researchers reported that GAL elicits its biological effects by interacting with membrane-bound receptors that are associated with G_i proteins (Amiranoff *et al.*, 1988; Lagny-Pourmir *et al.*, 1989). If both [^{125}I]pGAL and [^{125}I]GLT are agonists that bind to high-affinity GAL receptors, forming a stable ternary complex (De Lean *et al.*, 1980), then the presence of GppNHp should dissociate the G protein from the receptor and reduce agonist binding. Routine dissociation experiments were done in the presence of GppNHp, and <20% of bound radioligand was removed within the first hour. Several additional approaches were undertaken to try to dissociate the radioligands from the GAL receptor. BF membranes were repeatedly washed after preincubation with [^{125}I]pGAL, and the amount of bound radioligand was measured. Readily reversible ligands can be removed with this wash technique (Deecher *et al.*, 1991, 1992). No reduction in specific binding could be measured. Treatment of BF membranes with acetic acid after preincubation with [^{125}I]pGAL removed all bound radioligand. These membranes were washed and reused in binding experiments, and the specific binding was restored.

These results indicate that [^{125}I]pGAL and [^{125}I]GLT bind very tightly to the receptor but that their interaction does not change the conformation of the GAL receptor. Data on the irreversibility or slowly reversible kinetics of [^{125}I]pGAL and [^{125}I]GLT binding have been shown in other reports, although not clearly identified. Servin *et al.* (1987) performed dissociation kinetics in the presence of 100 nM GAL with [^{125}I]pGAL in rat brain and reported a dissociation constant of $K_{-1} = 0.039\ min^{-1}$, even though after 1 and 2 hr, 50% and 25% of the specific binding remained, respectively. Other reports have noted similar [^{125}I]pGAL kinetics in agreement to those reported in the present study. Sharp *et al.*, (1989), with RINm5F cells, reported that the kinetics were partially reversible in the presence of 800 pM [^{125}I]pGAL, with 40% of the ligand still bound after 2 hr. Lagny-Pourmir *et al.* (1989) demonstrated that only 30% of [^{125}I]pGAL binding was dissociated after 2 hr in the presence of 50 nM GAL but that

TABLE 2

Competition of GAL and GAL chimeric peptides using [^{125}I]GAL or GLT in BF or HYP provides evidence to suggest the existence of different GAL receptor subtypes in the human brain

Results are expressed as mean ± S.E.M. of the IC_{50} (pM) corresponding to three independent experiments. Values for IC_{50} were estimated using the algorithm EBDA. Slopes of each fitting are given in parentheses. Statistical analysis was performed using the SAS statistical package (Cary, NC). A nonparametric three-factor factorial followed by the nonparametric least-significant difference was used.

Competitor	[^{125}I]GAL BF	[^{125}I]GLT BF	[^{125}I]GAL HYP	[^{125}I]GLT HYP
pGAL	4.0 ± 3.2 (0.44)	1.1 ± 1.0 (0.30)	7.2 ± 4.0 (0.70)	3.3 ± 0.3 (1.00)
rGAL	71.4 ± 3.4 (1.00)[a]	1.3 ± (0.50)	3.0 ± 1.7 (0.50)	1.6 ± 1.0 (0.50)
hGAL	100.0 ± 4.5 (1.10)[a]	3.2 ± 3.0 (0.30)	8.5 ± 2.0 (0.50)	14.0 ± 0.5 (0.70)
fGAL	560.0 ± 57.0 (0.80)[b]	268.0 ± 66.0 (0.50)	116.0 ± 52.0 (0.60)	140.0 ± 5.0 (0.60)
GLT	162.0 ± 12.0 (0.50)[a]	575.0 ± 35.0 (1.20)	416.0 ± 33.0 (0.50)	470.0 ± 30.0 (1.10)
M35	<1[e]	<1[e]	25.0 ± 1.8 (0.80)[d]	47.0 ± 11.0 (0.98)[d]
M40	146.0 ± 15.0 (0.60)[a]	<1[a,e]	35.0 ± 3.6 (0.70)	28.0 ± 1.7 (0.85)
C7	85.0 ± 5.0 (0.40)	<1[a,e]	250.0 ± 11.0 (1.20)[a]	87.0 ± 85.0 (0.20)

[a] $P < .05$ *vs.* all groups within the same competitor.
[b] $P < .05$ *vs.* HYP using both radioligands.
[c] $P < .05$ *vs.* BF/[^{125}I]GAL.
[d] $P < .05$ *vs.* BF using both radioligands.
[e] Values represent the results of six independent determinations in which the minimal dose of competitor added exhibited maximal inhibition.
BF/[^{125}I]GAL ranking of competitors: M35 < pGAL < rGAL = C7 = hGAL < M40 = GLT < fGAL.
BF/[^{125}I]GLT ranking of competitors: M35 = M40 = C7 < pGAL = rGAL = hGAL < fGAL = GLT.
HYP/[^{125}I]GAL ranking of competitors: rGAL = pGAL = hGAL < M35 = M40 = fGAL < C7 = GLT.
HYP/[^{125}I]GLT ranking of competitors: rGAL = pGAL = hGAL < M40 = M35 = C7 = fGAL < GLT.

>50% of the specific binding of [^{125}I]pGAL was removed within 5 min in the presence of 10 μM GppNHp. It is possible that the high-affinity state of the GAL receptor population is responsible for the irreversible portion of the binding. Previous investigators may be reporting the reversible binding portion of the low-affinity state of the receptor or studying a different subtype of the GAL receptor that is region and species specific.

Although our findings of irreversible kinetics are unusual, another neuropeptide, endothelin (Hocher *et al.*, 1992; Watakabe *et al.*, 1992; Wu-Wong *et al.*, 1993), has similar kinetic characteristics. Severne *et al.* (1987) proposed a theory regarding high-affinity agonists, termed "tight agonist binding." This theory states that when agonists bind, a stable receptor ligand complex is formed that locks the agonist in the binding pocket for as long as the receptor is functionally associated with their perspective G proteins. This tight agonist binding occurs in only a portion of the receptor population (high affinity) and is commonly characterized by slowly reversible kinetics. This theory fits well with the interaction of [^{125}I]pGAL and [^{125}I]GLT binding in the BF and HYP, with the exception of GppNHp not fully dissociating the radioligands in our kinetic experiments. It is probable that the tight agonist binding forms a very stable receptor G protein complex (ternary complex) that is not affected by the presence of GppNHp after saturation. If GppNHp were added before saturation, then binding of either [^{125}I]pGAL or [^{125}I]GLT was reversed. Preincubation of BF or HYP membranes with GppNHp reduced the specific binding of both radioligands, indicating that a major portion of the binding is associated with G proteins and that both radioligands are agonist. However, a portion of the specific binding of [^{125}I]pGAL was not GppNHp sensitive and therefore is not associated with G proteins.

Based on our observations, we propose two affinity states for the GAL receptor population that was identified with [^{125}I]pGAL labeling: a high-affinity and a low-affinity state. Gu *et al.* (1994) reported evidence of high- and low-affinity states of the GAL receptor in guinea pig stomach smooth muscle cells, which is in agreement with our findings in the human brain. It is probable, based on the tight agonist binding theory, that the 25% of the binding in our studies that showed reversible kinetics is associated with the low-affinity state of the GAL receptor population. The tight agonist binding theory fits well with our data with [^{125}I]pGAL labeling but not with [^{125}I]GLT labeling because all of the binding was GppNHp dependent. In contrast, a portion of the [^{125}I]pGAL binding was GppNHp insensitive, yet both radioligands showed similar proportional reversible kinetics, which is indicative of low-affinity binding. Our findings in conjunction with this theory suggest that both radioligands act as agonists because both ligands are GppNHp sensitive.

Rosenthal analysis on data taken at assumed saturation from binding experiments in the BF and HYP reveals that both radioligands bind with high affinity to GAL receptors and that the greatest number of binding sites were found in the HYP. A recent article characterizing GAL receptors in human HYP with [^{125}I]pGAL reported slowly reversible kinetics and similar binding affinities, which is in agreement with our study, but reported 4-fold greater binding density of the GAL receptor (Lorinet *et al.*, 1994). This finding is currently unexplainable because the tissue samples in both studies were taken from approximately the same age group.

In the BF, [^{125}I]pGAL and [^{125}I]GLT label the same number of sites, with [^{125}I]pGAL having the highest affinity for the receptor population in this tissue. Although these data may suggest that both radioligands label the same receptor population in the BF, the competition studies indicate otherwise. Also, our data demonstrate that [^{125}I]pGAL labels 50% more sites than [^{125}I]GLT in the HYP, suggesting an additional receptor population or affinity state of the GAL receptor that is not recognized by [^{125}I]GLT.

Studies have shown that the first 13 amino acids in the GAL sequence are essential for binding to the GAL receptor (Amiranoff *et al.*, 1989; Fisone *et al.*, 1989). These first 13 amino acids are 100% homologous between species in the GAL peptides (Tatemoto *et al.*, 1983) and the GAL chimeric peptides (Langel *et al.*, 1992) tested in this study. The affinities of the GAL peptides were determined with [^{125}I]pGAL or [^{125}I]GLT to discern any differences in binding interactions of either radioligand in the BF or HYP. hGAL competed similarly for both radioligands in the HYP but in the BF

showed a statistically significant lower affinity for [^{125}I]pGAL binding. Comparison of IC_{50} values from competition studies in the HYP shows that all peptides compete similarly in rank order for [^{125}I]pGAL and [^{125}I]GLT binding. In contrast, all peptides, with the exception of pGAL and C7, competed differently for [^{125}I]pGAL in the BF. The varying affinities noted in the competition studies and the results from the saturation experiments suggest at least two receptor subtypes depicted by the choice of radioligand and region tested.

The results from the competition studies of the GAL chimeric peptides M35, M40 and C7 were unusual in the BF homogenates. Due to lack of human tissue in these regions, additional studies on only one GAL chimeric peptide could be investigated. M35, the bradykinin chimera, inhibited at least 80% of the binding of either radioligand at low concentrations (1 pM) in the BF. To ensure that this was a substantiated finding, receptor preparations from human BF and HYP, pig and rat hippocampus and RINm5F were performed in parallel experiments in the presence of competing concentrations of M35 for [^{125}I]pGAL binding. The IC_{50} values were determined in the picomolar range, with the exception of the human BF homogenates. These observations suggest that the present findings are accurate. Kinetics or other neuropeptide interference was believed to be a possible explanation for this finding. In experiments to address neuropeptide specificity, several neuropeptides were competed for the binding of [^{125}I]pGAL or [^{125}I]GLT. None of the peptides tested inhibited the binding of either radioligand except for substance P (1 μM), which inhibited [^{125}I]pGAL binding by 26%. BF receptor preparations were also preincubated with fGAL, spantide or bradykinin (2–9) before routine binding experiments competing M35 were run. These experiments were done to determine whether the complete inhibition of [^{125}I]pGAL binding by M35 was due to other receptor interaction. These experiments showed only a 10% reduction on the inhibitory effect of M35 for [^{125}I]pGAL binding, indicating that M35 is primarily interacting with GAL receptors labeled by [^{125}I]pGAL.

The other possible explanation for the complete inhibition of [^{125}I]pGAL or [^{125}I]GLT binding by M35 in the BF is the fact that these ligands bind irreversibly. To address this issue, order of addition of competitor or radioligand was considered. Either M35 or radioligand was added directly to the BF homogenates and preincubated before the start of the binding assay. In these studies, complete inhibition of [^{125}I]pGAL binding was still noted regardless of order of addition. Therefore, kinetics or other neuropeptide receptor interference does not appear to be the reason for the complete inhibition of binding. It is possible that M35 is causing a conformational change of the receptor, inhibiting the binding of the radioligands. Further studies are required to fully determine the effect of M35 on the GAL receptor described in the human tissue.

In conclusion, we characterized GAL receptors in human BF and HYP using two radioligands, [^{125}I]pGAL and [^{125}I]GLT. No apparent age-related differences in GAL receptor pharmacology were noted in BF. Both radioligands, *in vitro*, are high-affinity agonists and irreversibly bind to GAL receptors in the BF and HYP, which is in line with the theory of tight agonist binding. Our study suggests that GAL subtype differences and high- and low-affinity states exist for the GAL receptor depending on the radioligand or brain region selected. At the time of the present study, [^{125}I]hGAL was not available, and further characterization must be done with the specific human peptide to compare and contrast receptor differences between radioligands and regions tested.

Based on several reports, GAL may be a potential pharmacological treatment strategy for BF dysfunction in AD. Thus, defining GAL receptor pharmacology in normal brain tissue will be useful when characterizing the receptor pharmacology of the human BF from patients with AD.

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