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Dopamine D_2 Receptor Stimulation Alters G-Protein Expression In Rat Pituitary Intermediate Lobe Melanotropes

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Stimulation of dopamine D_2 receptors inhibits melanotrope pro-opiomelanocortin (POMC) biosynthesis and α-melanocyte-stimulating hormone (MSH) secretion. These effects are mediated by G-protein α_i- and α_o-subunits and are reversed by stimulating receptors linked to activation of $G\alpha_s$ protein. Melanotrope activity is increased by haloperidol, a D_2 receptor antagonist, and decreased by bromocriptine, a D_2 receptor agonist. Both the short and long isoforms of the D_2 receptor mRNA and protein increase following chronic haloperidol treatment. After chronic bromocriptine treatment the short isoform is downregulated, whereas the long isoform is upregulated *(1)*. Our hypothesis is that specific G protein α- subunits alter in pattern of expression similarly to the receptor isoforms. Using immunohistochemistry and *in situ* hybridization, this study examined changes in $G\alpha_i$, $G\alpha_o$, and $G\alpha_s$ protein and mRNA expression following chronic treatments with bromocriptine or haloperidol. $G\alpha_{i3}$ and $G\alpha_o$ immunoreactivities increased following bromocriptine treatment, whereas $G\alpha_s$ and $G\alpha_{i1/2}$ did not change. G_s immunoreactivity increased after haloperidol treatment, whereas $G\alpha_{i1/2}$, $G\alpha_{i3}$, and $G\alpha_o$ did not change. $G\alpha_i$ and $G\alpha_o$ mRNA increased following bromocriptine and decreased following haloperidol treatments, whereas the inverse results were observed with $G\alpha_s$ mRNA. These results suggest D_2 receptor activation can specifically increase $G\alpha_{i3}$ and $G\alpha_o$ expression, and D_2 receptor blockade increases $G\alpha_s$ expression.

Key Words: Gα-subunits; signal transduction; receptor regulation; *in situ* hybridization; immunohistochemistry.

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Introduction

There are two isoforms of the D_2 receptor, the short (D_{2short}) *(2)* and the long (D_{2long}) *(3–5)* generated by alternative mRNA splicing *(3–5)*. The two forms differ by a 29-amino acid insert in the third cytoplasmic loop, a site at which G proteins can bind *(6–10)*. Both D_2 receptor isoforms are abundant in melanotropes *(1,2,4,11)*, the predominant cell type in the pituitary intermediate lobe.

Affirming a key role for D_2 receptor regulation of melanotropes is that receptor stimulation reduces the number of cells entering the S-phase of the mitotic cycle *(12,13)*. Several other functions are also suppressed by D_2 receptor stimulation. Importantly, tissue content of α-MSH and β-endorphin, peptides derived from pro-opiomelanocortin (POMC), the major prohormone synthesized by melanotropes, are lowered via suppression of adenylyl cyclase *(14–17)* and α-MSH secretion is inhibited by D_2 receptor stimulation *(18,19)*. Melanotropes are electrically active and have spontaneous action potentials *(20)* the frequency of which is reduced *(18,21,22)* by G protein-mediated opening of K^+ channels upon D_2 receptor stimulation *(23)*. Furthermore, inositol phosphate production *(24)*, cytosolic Ca^{2+} levels *(24,25)*, and Ca^{2+} channel activity *(21,22,26–30)* are decreased. Specifically, the activities of L- *(25)* and P-type *(30)* Ca^{2+} channels are reduced. Following treatment with bromocriptine, a D_2 receptor agonist, the levels of both the D_{2short} *(1,31)* and the β_2/β_3 subunits of the $GABA_A$ receptor are decreased *(32)*. Thus, D_2 receptor stimulation seems to suppress a number of secretion-related activities in melanotropes.

$G\alpha_i$ and $G\alpha_o$-type G-proteins link D_2 receptors to several signaling pathways. In melanotropes, one or more $G\alpha_i$ subunits inhibit adenylyl cyclase activity *(33,34)*. This led us to the expectation that the mRNA and protein expressions of those G proteins constantly activated during chronic D_2 receptor stimulation would be upregulated in contrast to most other proteins, which are downregulated. We hypothesized that D_2 receptor agonist treatment would up-regulate $G\alpha_o$, $G\alpha_{i2}$ and/or $G\alpha_{i3}$ and downregulate $G\alpha_s$ and that D_2 receptor antagonist treatment would yield the

inverse results. We found that $G\alpha_o$ and $G\alpha_{i3}$ immunoreactivities increased, while the levels of $G\alpha_s$ and $G\alpha_{i1/2}$ were unchanged following chronic bromocriptine treatment, suggesting that $G\alpha_o$ and $G\alpha_{i3}$ are associated with the general downturn in melanotrope biosynthesis and channel activity upon receptor stimulation. Haloperidol treatment resulted in the predicted increase in $G\alpha_s$ immunoreactivity; again the levels of other Gα-subunits remained unchanged. These results allow a discussion of specific coupling of D_2 receptor isoforms to inhibitory activities in melanotropes and general requirements for maintenance of Gα subunit levels.

Results

Gα-Subunit In Situ *Hybridization*

To demonstrate tissue specificity of G-protein probe hybridizations, $G\alpha_i$, $G\alpha_o$ and $G\alpha_s$ mRNA autoradiographic grain counts were compared between the anterior, inter-mediate and neural lobes from vehicle treated rats. $G\alpha_i$ mRNA grain counts were greater in the anterior lobe (2.80 ± 0.33 grains/100 μm^2) than in the intermediate (1.63 ± 0.09 grains/100 μm^2) or neural (1.99 ± 0.31 grains/100 μm^2) lobes. $G\alpha_o$ mRNA was expressed almost equally in the anterior (5.51 ± 0.56 grains/100 μm^2) and neural (4.27 ± 0.68 grains/100 μm^2) lobes, but was lower in the intermediate lobe (3.11 ± 0.45 grains/100 μm^2). $G\alpha_s$ mRNA was higher in the anterior lobe (4.87 ± 0.25 grains/100 μm^2), but was equally expressed in the intermediate (3.38 ± 0.36 grains/100 μm^2) and neural (3.47 ± 0.76 grains/100 μm^2) lobes. Background grain counts in the cleft were low in all sections (less than 0.40 grains/100 μm^2).

Chronic haloperidol treatment significantly decreased (49%) the number of grains representing hybrids with $G\alpha_i$ mRNA per square micron of intermediate lobe, while chronic bromocriptine treatment significantly increased these grain counts (38%) (Fig. 1A and B). Anterior and neural lobe $G\alpha_i$ grain densities did not change following either treatment (data not shown). Chronic haloperidol treatment slightly, but not significantly, decreased (28%) and chronic bromocriptine treatment increased (27%) $G\alpha_o$ mRNA in the intermediate lobe (Fig. 1C and D). Concurrent changes in the anterior lobe were negligible following both treatments, and grain counts did not change at all in the neural lobe (data not shown). Chronic haloperidol treatment significantly increased (33%) and chronic bromocriptine treatment significantly decreased (27%) $G\alpha_s$ mRNA grain densities in the intermediate lobe (Fig. 1 E and F). Similar changes were detected in the anterior lobe following haloperidol and bromocriptine treatments. No changes in $G\alpha_s$ mRNA grain densities were observed in the neural lobe (data not shown).

Gα-Subunit Immunohistochemistry

Melanotropes

$G\alpha_{i1/2}$ immunoreactivity was located along the periphery of melanotropes, outlining the cells in a reticular pattern (Fig. 2A). The relative intensity of $G\alpha_{i1/2}$ staining did not change following either drug treatment (Table 1). $G\alpha_{i3}$ immunoreactivity was observed throughout the cytoplasm, leaving nuclei unstained (Fig. 3A). Bromocriptine treatment considerably increased $G\alpha_{i3}$ immunoreactivity in melanotropes, such that the intermediate lobe stood out brightly compared to the anterior and neural lobes (Fig. 3B). However, following chronic haloperidol treatment, no changes were detected compared to control.

$G\alpha_o$ immunoreactivity was observed in melanotrope cytoplasm (Fig. 4A). The intensity of the staining increased after bromocriptine treatment (Fig. 4B), but did not change following haloperidol treatment. $G\alpha_s$ immunoreactivity differed in intensity among individual melanotropes. Some melanotropes (15–20%) were strikingly bright (Fig. 5A). Following haloperidol treatment, this number rose to 40–50% (Fig. 5B), resulting in a brighter and sometimes a more uniform appearance of the immunoreactivity. Bromocriptine treatment did not alter immunoreactivity from control. These changes are summarized in Table 1.

Intermediate Lobe Glia, Pituicytes, and Anterior Lobe Cells

$G\alpha_{i1/2}$ immunoreactivity was also present in glial cells, demonstrated by colocalization studies with GFAP, an intermediate filament protein in a subpopulation of intermediate lobe glia, and vimentin, an intermediate filament protein found in all glia of the lobe *(35)* (Fig. 2A and B). $G\alpha_{i1/2}$ immunoreactivity was widespread in the anterior lobe, being colocalized with adrenocorticotrophic hormone (ACTH) in corticotropes, S-100 in folliculostellate cells, growth hormone (GH) in somatotropes, follicle-stimulating hormone (FSH) in gonadotropes and PRL in lactotropes. This distribution of $G\alpha_{i1/2}$ has been reported previously *(36)*. $G\alpha_{i1/2}$ immunoreactivity was present in pituicytes of the neural lobe, as demonstrated by colocalization with vimentin (Fig. 2A and B).

$G\alpha_{i3}$ was not present in intermediate lobe glia based on the morphology of cells expressing $G\alpha_{i3}$ and the lack of colocalization with vimentin. In the anterior lobe, $G\alpha_{i3}$ immunoreactive cells also contained ACTH, S-100, GH, or FSH, similar to previously described results *(36)*. While not detected in intermediate lobe glia, $G\alpha_{i3}$ was found in pituicytes as demonstrated by colocalization experiments with vimentin and S-100.

$G\alpha_o$ immunoreactivity was not present in intermediate lobe glia, but was widespread in endocrine cells of the anterior lobe and in pituicytes based on colocalization experiments. In the anterior lobe, $G\alpha_s$ was not detected with the antiserum from Santa Cruz, which recognizes amino acids 100–119 of the $G\alpha_s$. This was in contrast to a previous study using an antiserum recognizing the C-terminal region (amino acids 385-394) that detects $G\alpha_s$ in lactotropes, gonadotropes, and thyrotropes *(36)*. The region

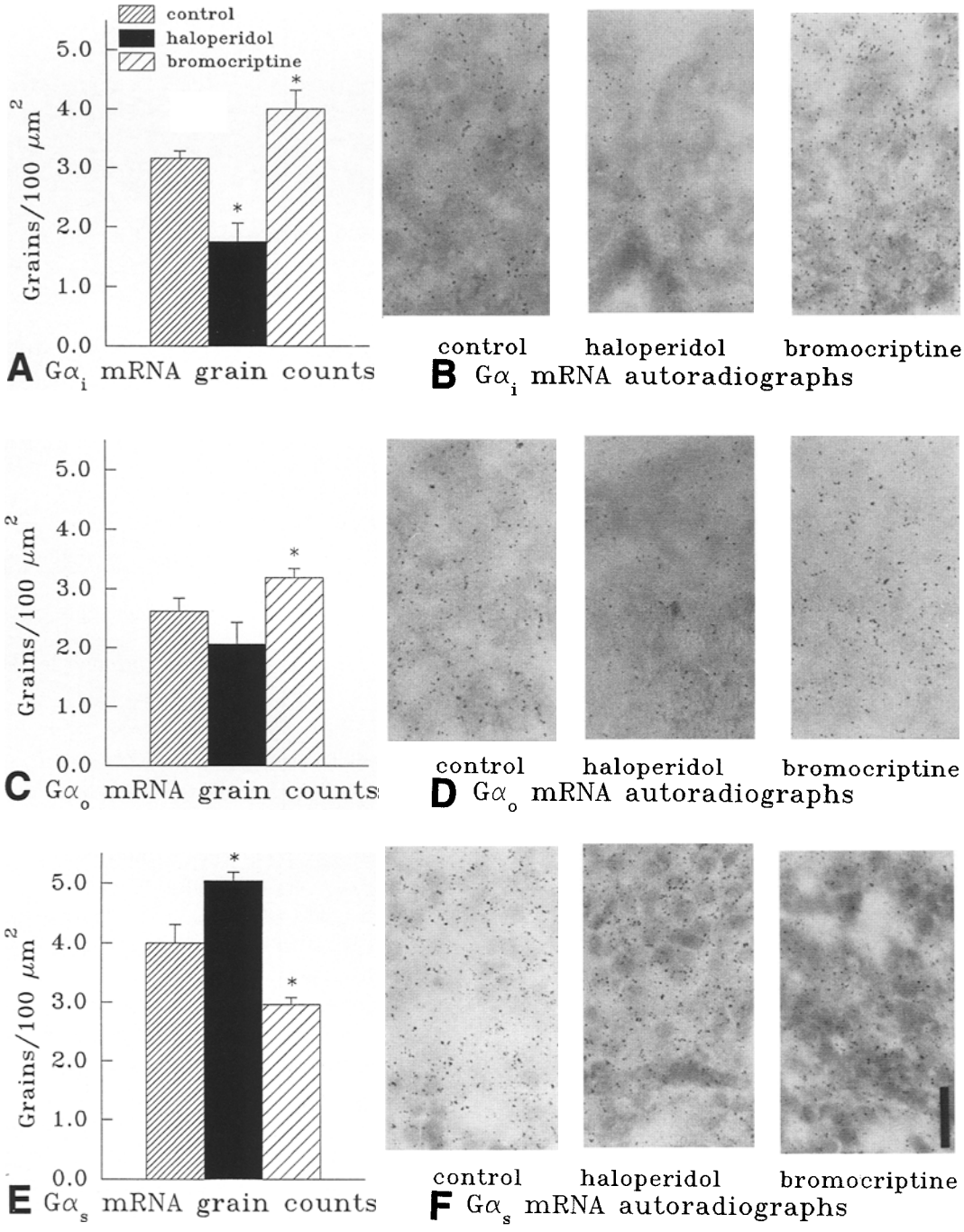

Fig. 1. *In situ* hybridization for $G\alpha_i$**(A,B)**, $G\alpha_o$ **(C,D)**, and $G\alpha_s$ **(E,F)** mRNAs. Bar graphs of mRNA grain counts (A,C,E) and autoradiographs (B,D,F) of rat pituitary intermediate lobes from control and chronically haloperidol or bromocriptine treated animals. Haloperidol treatment decreases grain densities of $G\alpha_i$ and $G\alpha_o$, but increases those of $G\alpha_s$. Bromocriptine treatment increases grain desities of $G\alpha_i$ and $G\alpha_o$, while decreasing those of $G\alpha_s$. *Indicates statistically significant difference ($P < 0.05$). In the autoradiographs (B,D,F) the plane of focus is on the silver grains. In the underlying tissue, only lightly counter-stained melanotrope nuclei are visible. Magnification bar = 25 μm.

consisting of amino acids 100–119 could be masked in the anterior lobe and not readily detected by our method, although why $G\alpha_s$ is detected with one antiserum and not the other only in the anterior lobe is unclear. $G\alpha_s$ was detected in varicosities and terminals of neural lobe axons as demonstrated by colocalization with neurofilament protein (Fig. 6).

Summary of Results

In situ hybridization showed differential expressions of $G\alpha_i$, $G\alpha_o$ and $G\alpha_s$ mRNAs in the lobes of pituitary glands of control animals. Likewise, immunohistochemistry using antisera for several Gα-subunits, demonstrated a lobe- and cell-specific distribution of the different subunits,

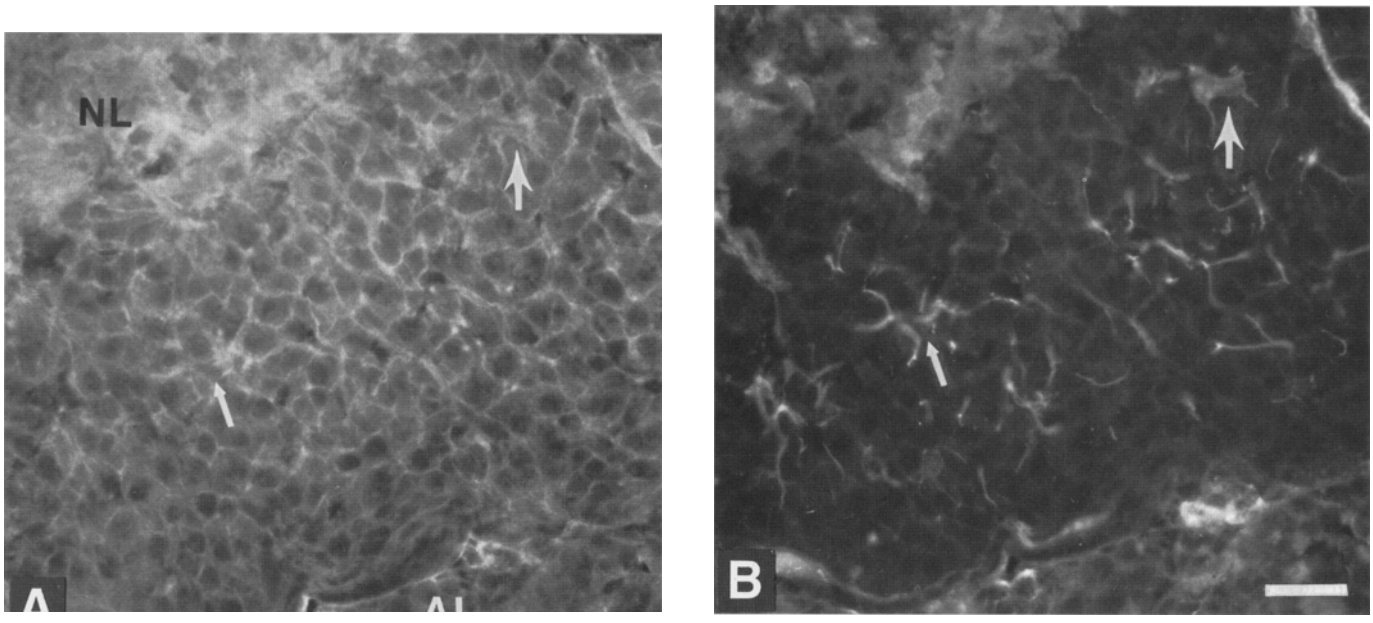

Fig. 2. Cellular localization of $G\alpha_{i1/2}$ immunoreactivity in rat pituitary intermediate lobe. $G\alpha_{i1/2}$ appears along the cell periphery of melanotropes, and in cells of the anterior (AL) and neural (NL) lobes **(A)**. Double label fluorescence immunohistochemistry for vimentin **(B)**, demonstrates that $G\alpha_{i1/2}$ is present in intermediate lobe glia (examples at arrows in A and B) and in pituicytes. Magnification bar = 25 μm.

Table 1
Gα Subunit Expression in Melanotropes

Gα-subunit	Control	Haloperidol	Bromocriptine
$G\alpha_{i1/2}$	++	++	++
$G\alpha_{i3}$	++	++	+++
$G\alpha_o$	++	++	+++
$G\alpha_s$	++	+++	++

Intensity (++ medium; +++ high intensity) of Gα-subunit immunoreactivity in melanotropes of rat pituitary intermediate lobe. Tissues from control and chronically haloperidol (D_2 receptor antagonist) or bromocriptine (D_2 receptor agonist) treated animals.

supporting specificity of the changes observed after drug treatment experiments. Importantly, in the experimental tissues, Gα-subunit mRNA level and the intensity of the corresponding immunoreactivity followed the same trends, indicating regulation at these levels. Although the $G\alpha_i$ oligonucle-otide probe did not distinguish between $G\alpha_{i1/2}$ and $G\alpha_{i3}$, it seems reasonable to assume that the elevation of $G\alpha_i$ mRNA after bromocriptine treatment corresponds to the marked increase in $G\alpha_{i3}$ immunoreactivity, since $G\alpha_{i1/2}$ did not change. G_o was likewise upregulated by the receptor agonist, while G_s was inversely regulated. Thus, these results support the hypothesis that repeated activation of a G-protein by chronic receptor stimulation results in an upregulation of the expression of the activated G-protein.

Discussion

Using *in situ* hybridization and immunohistochemistry on tissue sections, we find that D_2 receptor stimulation by an agonist increases $G\alpha_{i3}$ and decreases $G\alpha_s$ expressions, while a D_2 antagonist produces the opposite changes. These results agree with those from other cell types, such as the heart *(37,38)* and S49 mouse lymphoma cells *(39)*, where β-adrenergic receptor stimulation with isoproterenol increases $G\alpha_{i2}$ mRNA levels, while decreasing $G\alpha_s$ mRNA *(39)*. Taken together these results argue for an inverse regulation of $G\alpha_i$ and $G\alpha_s$.

$G\alpha_o$ expression significantly increases after chronic bromocriptine treatment in our experiments. $G\alpha_o$ is coupled to the inhibition of Ca^{2+} channel activity in several cell types. For example, acute $G\alpha_o$ activation by muscarinic and somatostatin receptors in GH_3 gonadotrope cells *(40)*, and α-adrenoceptor stimulation in cultured sympathetic neurons *(41)* inhibit Ca^{2+} channel activity. Additionally, acute D_2 receptor activation of $G\alpha_o$ inhibits Ca^{2+} channel activity in lactotropes *(42,43)* and melanotropes *(26,27)*, and may also be involved in the constant inhibition of Ca^{2+} channels in melanotropes seen with chronic application of a D_2 agonist *(25)*. Morris et al. *(44)* report that transfection of either D_{2long} or D_{2short} receptors into corticotrope-derived AtT20 cells results in functional coupling of both receptors to acute Ca^{2+} channel inhibition via $G\alpha_o$. Taken together, our results support the hypothesis that $G\alpha_o$ is the link to rapid and sustained inhibition of Ca^{2+} channels in melanotropes.

Generally, D_2 receptor stimulation downregulates activities in melanotropes. To accomplish this, those proteins required to effect downregulation hypothetically would themselves be upregulated. Both $G\alpha_{i2}$ and $G\alpha_{i3}$ have been implicated in inhibition of adenylyl cyclase. If both

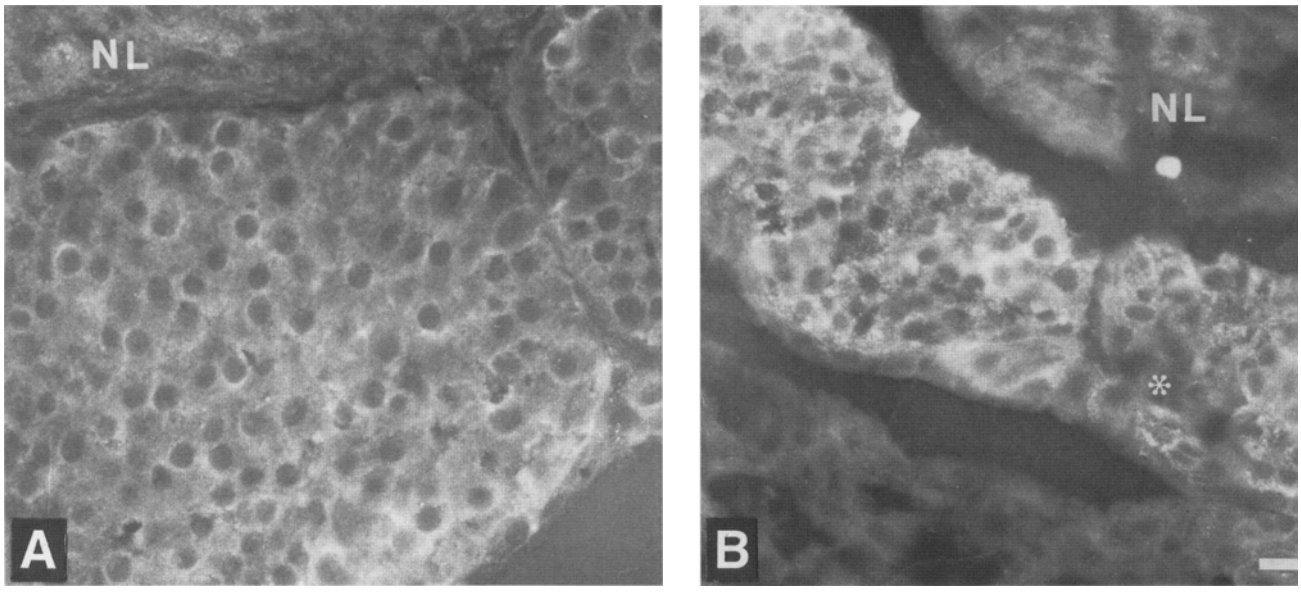

Fig. 3. $G\alpha_{i3}$ immunoreactive intensity increases in rat pituitary intermediate lobe melanotropes following bromocriptine treatment. Tissues from control **(A)** and treated **(B)** rats. The area in (B) showing background level of immunoreactive intensity (at asterisk) is occupied by a glial cell (data not shown). NL, Neural lobe; magnification bar = 25 μm.

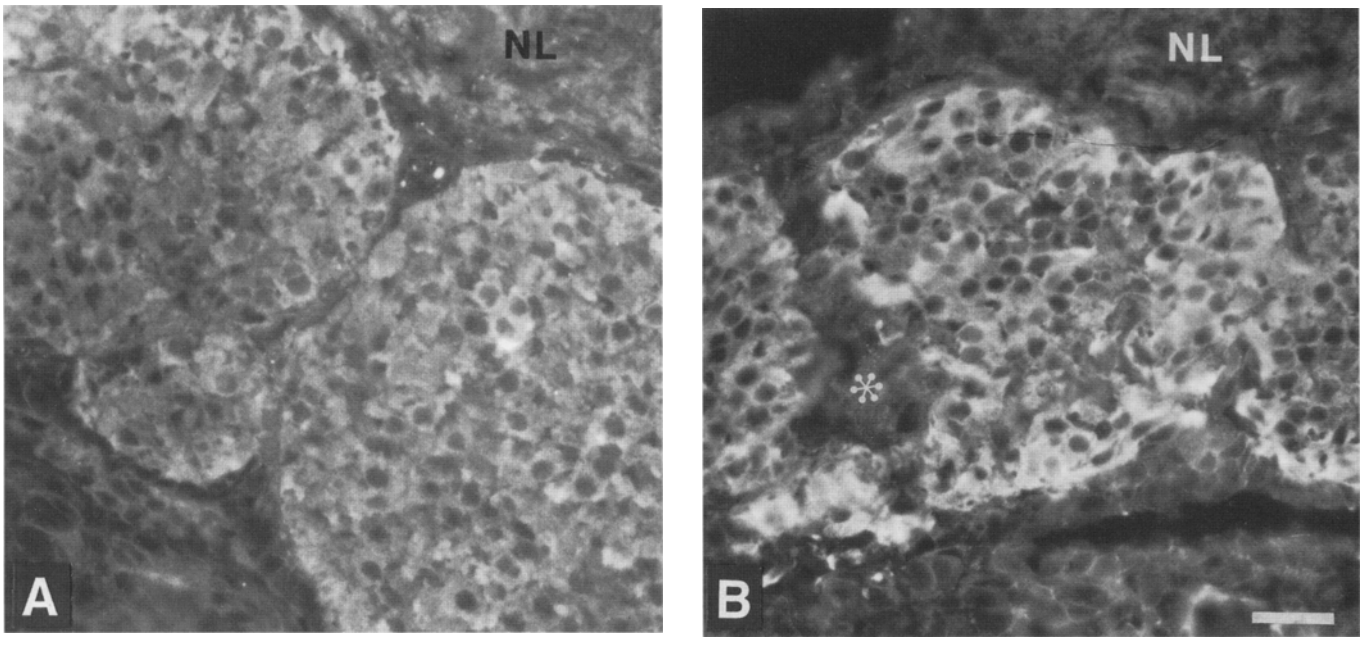

Fig. 4. $G\alpha_o$ immunoreactive intensity increases in rat pituitary intermediate lobe melanotropes following bromocriptine treatment. Tissues from control **(A)** and treated **(B)** rats. The area in (B), showing background level of immunoreactive intensity (at asterisk), is occupied by a glial cell (data not shown). NL, Neural lobe; magnification bar = 25 μm.

receptor isoforms were involved in the chronic suppression of melanotrope functions, increases in both of these $G\alpha_i$ proteins would have been expected. Since only $G\alpha_{i3}$ increased in our experiments, most likely only one of the two D_2 isoforms is involved in chronic inhibition. In a previous study, we demonstrated that chronic bromocriptine treatment somewhat increased D_{2long} whereas D_{2short} decreased significantly in melanotropes in pituitary tissue sections *(1)*. Likewise, in CHO cells, the isoforms are differentially regulated *(31)*. These findings imply that it is the D_{2long} form of the receptor which is involved in chronic downregulation and that this is signaled through $G\alpha_{i3}$.

Many experiments have aimed at resolving signal transduction pathways, linking specific receptors to G protein subtypes and then to specific cellular functions. Commonly, such studies are performed in cultured cells, whereas we

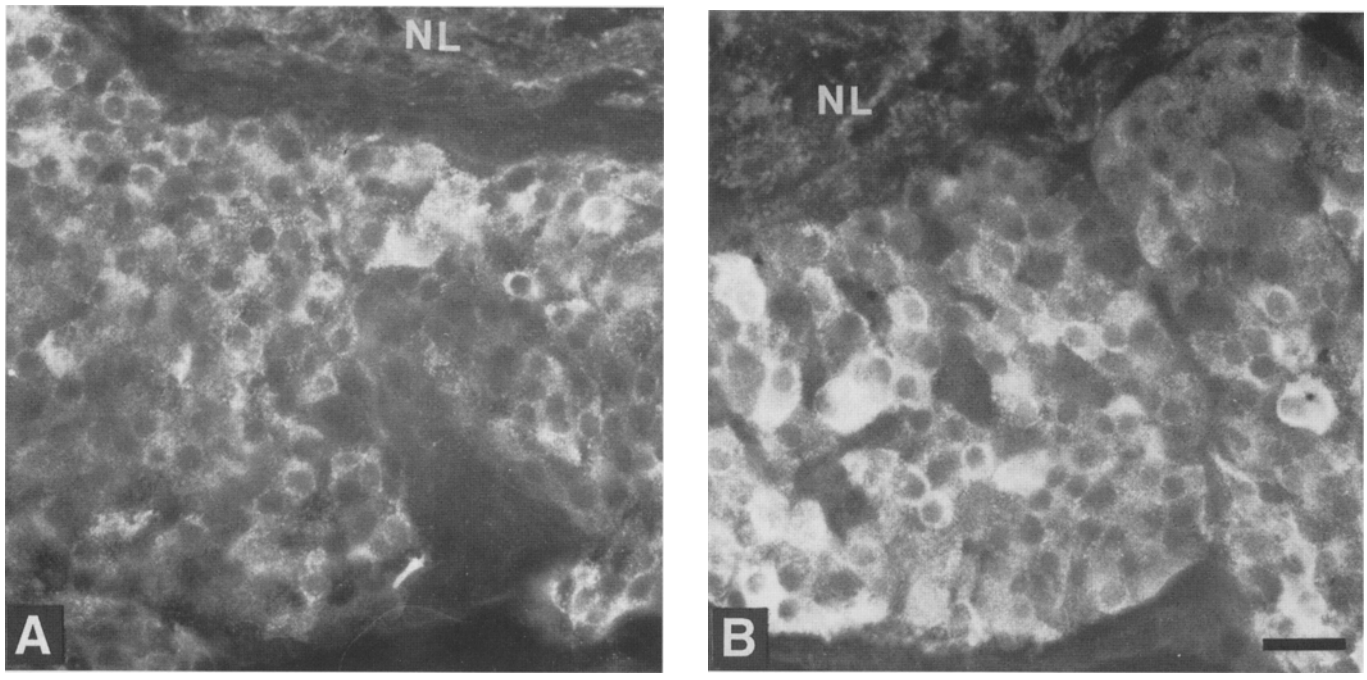

Fig. 5. $G\alpha_s$ immunoreactivity is heterogeneously expressed among melanotropes in the rat pituitary intermediate lobe (control tissue in [**A**]). Haloperidol treatment clearly increases the number of intensely stained melanotropes (**B**). NL, Neural lobe; bar = 25 μm.

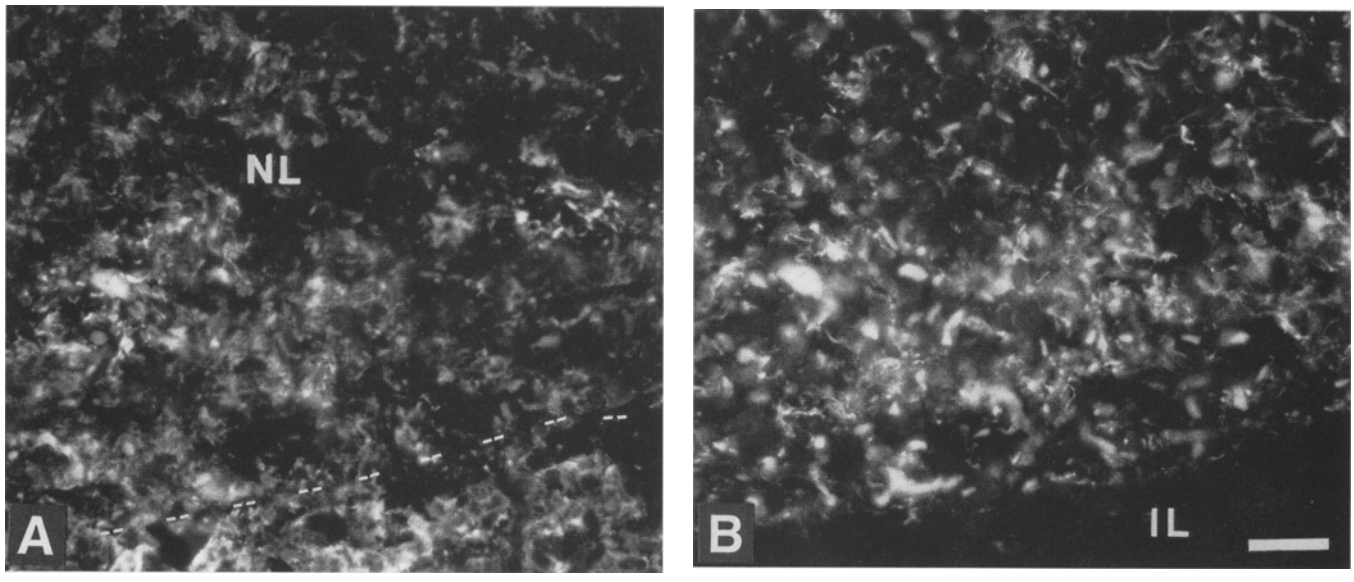

Fig. 6. $G\alpha_s$ (**A**) and neurofilament (**B**) double-label fluorescence immunohistochemistry in the neurointermediate lobe of the rat pituitary. The two proteins are colocalized in varicosities and terminals of neurosecretory axons of the neural lobe (NL). Note the presence of $G\alpha_s$, but lack of neurofilament protein in the intermediate lobe (IL). Magnification bar = 25 μm.

used tissue sections. D_2 receptor inhibition of adenylyl cyclase is well understood: the receptor activates one of the $G\alpha_i$-subunits which binds to the regulatory subunit of adenylyl cyclase and blocks its activity. Studies employing cell lines in which a specific D_2 isoform is heterologously overexpressed have generated conflicting results concerning which receptor isoform is linked to which $G\alpha_i$-subunit. In lactotrope-derived GH_4C_1 cells cotransfected with either D_{2long} or D_{2short} receptors, along with a large antisense oligonucleotide for either $G\alpha_{i2}$ or $G\alpha_o$ (which knocked down both $G\alpha_{o1}$ and $G\alpha_{o2}$), L-type Ca^{2+} channel activity is acutely inhibited by both D_{2long} and D_{2short} isoforms via $G\alpha_o$. D_{2long} also inhibited adenylyl cyclase through $G\alpha_{i2}$, while D_{2short} had little effect *(45)*. Likewise, in JEG-3 cells transfected with D_{2long}, $G\alpha_{i2}$ is required to effectively inhibit adenylyl cyclase *(46)*. Dif-

ferent results, instead suggesting coupling between D_{2long} and $G\alpha_{i3}$, were obtained by Senogles *(10)*. She constructed a series of $G\alpha_i$ mutants which are resistant to pertussis toxin and cotransfected one of these along with either D_{2long} or D_{2short} into GH_4C_1 cells. When naturally occurring G_i proteins were inhibited by pertussis toxin, the D_{2short} isoform signaled exclusively through $G\alpha_{i2}$, whereas D_{2long} coupled with $G\alpha_{i3}$ to inhibit adenylyl cyclase. Our results show that $G\alpha_{i3}$ expression is upregulated in melanotropes following chronic receptor stimulation, as is the D_{2long} isoform *(1)*. With the chronically stimulated receptor upregulated, constant activation of a G protein will ensue, likely requiring an upregulation of this G protein, our data suggest an association between the D_{2long} isoform and $G\alpha_{i3}$.

In contrast, during early development, the D_{2short} appears to be coregulated with $G\alpha_{i3}$ since the expression of both increase, while the D_{2long} and the $G\alpha_{i2}$ do not change as innervation of the intermediate lobe occurs *(47)*. Neurotransmitter control of the lobe begins during the first postnatal week *(48)*, at which time the D_{2short} is induced *(49)*. Thus, the $G\alpha_i$ proteins are similarly regulated upon receptor stimulation in the adult and during development, while the D_2 receptor isoforms are not, since D_{2short} increases upon the onset of innervation but decreases following bromocriptine treatment *(1)*. Taken together, these results suggest that other factors, like growth factors, in addition to dopamine could induce D_{2short} expression during development. Or, in developing rat melanotropes, the D_{2long} isoform may couple to both α-subunits, as the D_{2long} isoform is detected at approximately the same gestational day as $G\alpha_{i1/2}$ and $G\alpha_{i3}$. A similar kind of promiscuity was recently reported in that stimulation of transfected D_2 receptors can either increase or decrease intracellular Ca^{2+} levels by coupling through different Gα subunits to different second messenger pathways, depending on the cell line used *(50)*.

Although questions concerning exact mechanisms can only be fully resolved in cell culture systems, our results from a whole animal drug treatment paradigm complement such *in vitro* examinations. The findings of the present study demonstrate that, even if the whole animal in some respects represents a "black box," the correlation of in vivo results with those from in vitro experiments strengthen the physiological relevance of conclusions from in vitro studies.

Materials and Methods

Animals

Male Sprague-Dawley rats (150–175 gm; Sasco, Inc., Omaha, NE) were housed two to a cage with free access to food and water under a 12 h light–dark cycle. Rats were given a daily intraperitoneal injection for 14 d of either haloperidol (2 mg/kg; Sigma, St. Louis, MO), bromocriptine (2 mg/kg; 2-bromo-α-ergocriptine, Sigma) or vehicle (20 m*M* tartaric acid). Three animals per treatment group were utilized.

Rats were anesthetized and pituitaries were rapidly removed, immersed in 4% paraformaldehyde or periodate-lysine-paraformaldehyde (PLP) (0.01 *M* $NaIO_4$, 0.075 *M* lysine, 0.0375 *M* $NaPO_4$ buffer, pH 7.4; and 2% paraformaldehyde) at 4°C overnight, sunk in 25% sucrose at 4°C overnight, frozen on dry ice, embedded in OCT compound (Elkhart, IN) and stored at –70° C. Cryostat sections (5 μm) were cut serially and thawed onto subbed slides.

In situ *Hybridization Histochemistry*

The $G\alpha_s$, $G\alpha_i$, and $G\alpha_o$ oligonucleotides, characterized by Northern analysis, were purchased from DuPont NEN Products (Wilmington, DE). The sequences were: $G\alpha_i$ (GCC CGT GGT CTT CAC ACG GGT CCG CAG CAC ATC CTG CTG), corresponding to nucleotides 514–522, common to $G\alpha_{i1}$-, $G\alpha_{i2}$-, and $G\alpha_{i3}$-proteins; $G\alpha_o$ (GCC AGT TGT TTT GAC CCT GGT TCG GAG GAT GTC CTG CTC), corresponding to nucleotides 384–422; and $G\alpha_s$ (TCC AGA GGT CAG GAC ACG GCA GCG AAG CAG GTC CTG GTC), corresponding to nucleotides 580–618 *(51)*. The probes were 3'-end labeled with [^{35}S]-dATP (NEN) by incubation in terminal transferase at 37°C for 15 min. Probes were separated from unincorporated nucleotides using a NENSORB 20 column (NEN) primed with 0.1 *M* Tris-HCl and eluted from the column using 20% propanol.

Sections were rinsed in 2X SSC (0.3 *M* NaCl, 0.03 *M* sodium citrate), and placed in a humid chamber at room temperature (RT) for 1 h in hybridization buffer containing deionized formamide, 20X SSC, 50X Denhardt's solution, salmon sperm DNA (10 mg/mL), yeast tRNA (10 mg/mL) and 50% dextran sulfate. Slides incubated overnight at 37°C in 500 μl of the probe (1.5×10^6 cpm/500 μL hybridization buffer).

Slides were washed in 2X SSC (1 h, RT), 1X SSC (1 h, RT), 0.5X SSC (30 min, 37°C), and 0.5X SSC (30 min, RT), with all washes containing 0.5% β-mercaptoethanol. After a 40% ethanol wash, slides were air dried overnight, dipped in Kodak NTB-2 emulsion (diluted 1:1 with distilled water) and stored at 4°C for 6 wk. Autoradiographs were developed in Kodak D-19 (3 min, 17°C) and fixed with Kodak fixative. Slides were lightly counterstained with cresyl violet, dehydrated in graded alcohols, and coverslipped with Permount. Preparations to be compared were processed identically, keeping all parameters constant. Controls included omission of the labeled probe or incubation with unlabeled probe, resulting in only a few background autoradiographical grains. Specificity of G protein hybridization is discussed in the Results section.

Relative Quantitation of Autoradiographs

The autoradiographical silver grain density was calculated over the intermediate lobe and, to monitor hybridization

specificity, the neural and anterior lobes. The exposure time for the autoradiographs was timed to ensure that the ensuing grain density fell within the linear portion of the curve for radiation dose/number of grains. The data filter was set to count only objects that were within the size range of a single grain (0.5–4.0 μm^2) and of a gray level above background. The grain density of an area with no tissue present (e.g., pituitary cleft) was calculated and used as autoradiographical background, which was subtracted from grain density measurements. Three screen captures (representing 50% of the intermediate lobe cross section) per tissue section, from each of four sections per pituitary, with three animals per treatment group were evaluated. Two independent researchers quantitated coded slides. Data were statistically evaluated using two way analysis of variance (ANOVA) followed by the Bonferroni post-test for significance, with $p < 0.05$ considered significant. Values were expressed as mean ± standard error of the mean (SEM) for three animals per time point. The image analysis system consists of a Dage/MTI model 72 CCD camera mounted on the trinocular port of a Zeiss Axioplan microscope. The camera is connected to a 80486-based AT bus PC running MicroMeasure IL-4000 (Georgia Instruments, Inc., Roswell, GA).

Immunohistochemistry

Sections to be compared were prepared identically and in parallel using the same batch of antiserum. Slides were equilibrated for 5 min in phosphate-buffered saline (PBS) with 0.2% Triton X-100 (PBS-TX) before incubation in one of the following primary antisera overnight at 4°C: rabbit anti-$G\alpha_s$ (1:500, Santa Cruz), rabbit anti-$G\alpha_o$, rabbit anti-$G\alpha_{i1/2}$ (this designation indicates that this particular antiserum does not discriminate between $G\alpha_{i1}$ and $G\alpha_{i2}$), or rabbit anti-$G\alpha_{i3}$ (1:100; gifts from Dr. Allen Spiegel, NIH, Bethesda, MD), washed, incubated in secondary antibody ([fluorescein conjugated donkey antirabbit IgG (Jackson) or rhodamine conjugated goat antirabbit IgG (Cappel)], washed and cover slipped with polyvinyl glycerol (1:1 in PBS). Alternatively, before cover slipping, slides were used for double-label fluorescence immunohistochemistry to identify the cell types containing specific G proteins. For theses experiments slides were incubated in one of the following second primary antisera: mouse antivimentin (prediluted, Zymed Immunoresearch, South San Francisco, CA), a marker for folliculostellate cells, pituicytes, and intermediate lobe glia; mouse antineurofilament (1:50, Boehringer Mannheim, Indianapolis, IN), a marker for neural lobe axons; mouse anti-S-100 (1:300, Chemicon, Temecula, CA), a marker for folliculostellate cells, pituicytes, and intermediate lobe glia; monkey anti-GH (1:5000, gift from Dr. J. Schammel, University of South Alabama, Mobile, AL), a marker for somatotropes; guinea pig anti-FSH (1:100, Dr. J. Schammel, University of South Alabama, Mobile, AL), a marker for gonadotropes; and sheep anti-α-MSH (1:1000, gift from Dr. J. Tatro, Tufts Univ., Boston, MA), which also recognizes ACTH in tissue sections, was used as a marker for corticotropes and melanotropes. Slides were then washed three times for 5 min in PBS-TX, and incubated in a second secondary antiserum (rhodamine conjugated goat antimouse IgG (Jackson Immunochemicals, West Grove, PA), fluorescein conjugated goat antimonkey IgG (Jackson), fluorescein conjugated goat antiguinea pig IgG (Cappel) or rhodamine conjugated donkey antisheep IgG (Jackson) at 1:300 for 1.5 h.

Controls included omitting a primary antiserum or replacing it with the appropriate normal serum. No staining was obtained in either case. In double labeling experiments the secondary antisera did not cross react and switching the order of application of antisera did not change the outcome. The double label preparations served as further controls in that they demonstrated lobe and cell type specificity of Gα-subunit immunohistochemistry.

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