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Berman, Joshua Aaron, Ph.D.

City University of New York, 1995

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Molecular and Pharmacological Characterization of Pituitary GABAergic Systems

by

Joshua Aaron Berman

A dissertation submitted to the Graduate Faculty in Biomedical Sciences
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy, The City University of New York

1995

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This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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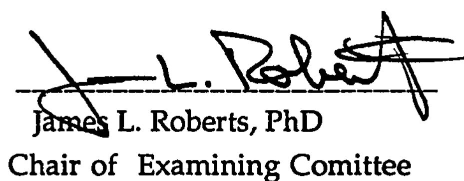

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Abstract

MOLECULAR AND PHARMACOLOGICAL CHARACTERIZATION OF PITUITARY GABAERGIC SYSTEMS

by

Joshua Aaron Berman

Adviser: Professor James L. Roberts

To better understand GABAergic regulation of pituitary cells, and study the function of different GABA-A receptors in physiologically well-characterized systems, I studied molecular composition and pharmacological characteristics of GABA-A receptors in the anterior and neurointermediate lobes of the rat pituitary. Of fourteen GABA-A receptor subunit mRNA species, I developed ribonuclease protection assays for eleven, including the generation of partial cDNA subclones for the β1, β2, β3, γ1, and δ subunits.

Anterior lobe expressed α1, β1, β2, β3 and γ2s mRNAs along with a small amount of γ1 mRNA; neurointermediate lobe, melanotrophs expressed α2, α3, β1, β3, β1, γ1 and γ2s mRNAs. The two sites displayed an absolute segregation of α1 versus α2 and α3 expression, and showed beta subunit heterogeneity. The neurointermediate lobe expressed as much γ1 as γ2s mRNA.

Pharmacological properties of pituitary GABA-A receptors were similar to those of brain GABA-A receptors in most respects, including a 2:1 ratio of muscimol to benzodiazepine sites. Quantities of receptor sites in the tissues studied were roughly proportional to the quantities of mRNA detected. As predicted by subunit composition anterior lobe receptors displayed pure Type I benzodiazepine pharmacology while neurointermediate lobe displayed pure Type II pharmacology, allowing clear correlation of these properties with alpha subunit composition in native receptors.

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Further, I was able to demonstrate synthesis of 3 alpha-hydroxydihydroprogesterone in the neurointermediate lobe, in what appear to be quantities sufficient to potentiate GABA actions at the GABA-A receptor.

Melanotrophs expressed subunit mRNAs sufficient in theory to direct synthesis of eighteen kinds of receptor with subpopulations possessing differential sensitivity to modulators of the GABA-A receptor. Taken together with the synthesis of a neurally active steroid in the neurointermediate lobe, this suggests a role for endogenous modulators of the GABA-A receptor in controlling actions of melanotrophs. Based on the findings described, I develop a hypothesis on the roles of GABA in melanotroph regulation in the concluding chapter, and present some data on the integration of signals through GABA-A and GABA-B receptors in melanotrophs.

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While science itself is an endeavor of great beauty, graduate training in the sciences would surely be a dreary undertaking if not for the scientists. In my five years in the Roberts lab, I have been privileged to work with one of the most colorful assortments of people I think anyone will ever meet anywhere, and I mean that in a good way; people can only become "characters" in an atmosphere where they care about one another, and take an interest in each other's unique qualities. And there is no shortage of unique qualities at the Fishberg Center. With that in mind, I would like to give my heartfelt thanks to all "Fishbergers" past and present for making my years here memorable ones.

Specifically, I'd like first to thank the postdocs past and present, Marc Glucksman, Andrea Gore, Nancy Levin, Ke Wen Dong, Moshe Jakubowski, Adrian Perrotti, and even Dominic Autelitano. You all helped me immensely, even at times when that might have been a difficult task.

For their collegial, scientific, and human companionship, I would like to thank my fellow graduate students and the technicians in the Roberts lab; in particular, Tracy "Leona" Yeo, Wei Dong Jin, Jim Lundblad (before he became a doc), Nomsa Bengani, Jaime Rodriguez, Wei and Linda Zhou, and non Robertsonians John Martignetti, Bill Probst, Mark Miller and Robert Wooley.

Most importantly, I want to thank my co-advisors, Dr. James L. Roberts and Dr. Dolan B. Pritchett. Dr. Roberts' incredible wealth of knowledge, his unique concern both for the well being of individual students, and for the integrity and well being of the enterprise of scientific education as a whole will serve as an inspiration to me for years to come. Dr. Pritchett, perhaps following the example of his advisor, has given unstintingly of his time, expertise and resources to a guest presence in his lab. In fact, I must say that both on a scientific level, and on a personal level, Dolan and his family have made me feel over the years that I have a "home away from home" in Philadelphia. It has truly been a privilege to come to know them.

Finally, I would like to acknowledge the contribution of my brother Jay Berman, my good friends Brian Ratner, Ed Portnoy, Roger Paz and other friends and family, along what has been a long road. I especially want to thank my mother and father, Sharon Berman and Dr. David Berman for their unselfish love and unwavering support, and most of all, for doing the hard work of discouraging their children from taking the path of least resistance.

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I dedicate this thesis to my mother and father, Sharon Berman and Dr. David Berman for their incalculable roles in its coming to fruition, and also to the memory of my uncle, Dr. Richard King, whose interest in my work over the years was a true source of encouragement, and who is deeply missed on all accounts.

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"What then is there?"...
"There is a thick mire."

- Isaac Bashevis Singer
Gimpel the Fool

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CHAPTER ONE:
BACKGROUND AND SIGNIFICANCE

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INTRODUCTION

Gamma-amino butyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system, and models of GABA actions on neuroendocrine cells have proposed GABA as an agent of tonic inhibition of peptide and hormone secretion (Racagni et al., 1979; Apud et al., 1981). Yet in several cases, investigators have found complex, biphasic effects of GABA on neuroendocrine cells, and in particular, excitation of peptide secretion by GABA-A agonists. To date, such phenomena have been described in melanotrophs (Tomiko et al., 1983; Demeneix et al., 1984), lactotrophs (Anderson, 1986), gonadotrophs (Virmani et al., 1990), and the GnRH neuron derived GT-1 cell line (Hales et al., 1992). The common pattern in all of these cases is the coexistence of GABA-A and GABA-B receptors in the same cell population, and the coupling of GABA-A receptors to a depolarizing chloride efflux. The emergence of this pattern merits a re-examination of the role of GABA in neuroendocrine systems, with special attention to recent discoveries

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about endogenous modulators of the GABA-A receptor, and about the molecular diversity of GABA-A receptor subunits.

Of great relevance to GABA actions on pituitary and other neuroendocrine cells has been the discovery of neurally active steroids which modulate GABA-A receptors, and in some cases also exert effects on voltage gated calcium channels. Neurally active steroids may be synthesized by the pituitary or in the hypothalamus from circulating adrenal or gonadal steroids, or synthesized *de novo* from cholesterol in a process which may be regulated by neurotransmitters (Baulieu, 1992). The neurally active steroids are of special interest to neuroendocrine investigators as they might constitute a source of fast acting feedback to the pituitary and hypothalamus for adrenal and gonadal steroids.

Also of great potential relevance to the nature of GABA actions on neuroendocrine cells is the recently discovered heterogeneity of GABA-A receptor subtypes(Olsen and Tobin, 1990). There are at least fourteen GABA-A receptor subunit genes, and we and other investigators have demonstrated the expression of multiple subunit types in individual neuroendocrine tissues (Valerio et al., 1992; Valerio et al., 1992; Berman, 1994). The various subunit types produce GABA-A receptors which vary in affinity for GABA and in their relative ability to interact with various modulators of GABAergic transmission such as neurally active steroids, beta-carbolines, and benzodiazepines.

To re-examine the role of GABA in regulation of neuroendocrine cells in light of these recent advances, I have sought to focus on a well defined population of cells, the melanotrophs of the neurointermediate lobe, and to use the lactotrophs of the anterior lobe as a point of comparison. The work presented in this thesis aims to answer the following questions: how diverse is

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the complement of GABA-A receptor mRNA expressed in a single population of neurosecretory cells? If there is receptor heterogeneity, how is it likely to be employed? What sorts of pharmacological properties can be linked to the expression of various GABA-A receptor subunit genes in pituitary cells? Are endogenous modulators of the GABA-A receptor likely to be important in the regulation of these cells through the GABA-A receptor sites observed?

Some of the studies described in the following chapters examine in detail, molecular and pharmacological characteristics of GABA-A receptors found in the pituitary. Others aim to probe the complex interactions of GABA and modulators of GABAergic transmission in the regulation of secretion of neuropeptides by melanotrophs.

I will begin by introducing general aspects of the biology of the pituitary intermediate lobe. I will then review the body of evidence concerning GABA effects on melanotrophs. This survey is followed by a review of the comparative pharmacology of GABA-A and GABA-B receptors. I will then discuss the various endogenous and exogenous pharmacological modulators of GABA receptor action and finally, review the molecular biology of the GABA-A receptor.

OVERVIEW OF MELANOTROPH PHYSIOLOGY

The studies I will present all aim to elucidate in one way or another, aspects of regulation of melanotrophs by GABA. I will begin, then, with a general overview of this population of neurosecretory cells. In this way, the

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components of the GABAergic system under study, namely receptor mRNA, receptor sites, and modulators, can be understood in terms of the unique physiological and anatomical characteristics of the cells on which they occur.

Anatomical Considerations:

The melanotroph is the major constituent cell of the intermediate lobe of the pituitary, a structure found in all mammals except cetaceans and humans, and in many amphibians, reptiles, and fish. The intermediate lobe, which develops from the epithelium of Rathke's pouch, is separated from the anterior pituitary by a pituicyte lined virtual space, the hypophyseal cleft. These pituicytes are rich in decarboxylases, monamine oxidases, and peptidases, thus direct neuropeptide and neurotransmitter communication between anterior and intermediate lobes is strictly limited (Howe and Thody, 1967).

Whereas the anterior lobe receives hypothalamic input via diffusion from the median eminence through a capillary plexus, the intermediate lobe is directly innervated by tuberohypophyseal projections from the arcuate nucleus and midbrain reticular formation, as well as numerous other projections (Bjorklund et al., 1973). Adrenergic, dopaminergic, serotonergic, cholinergic, and GABAergic fibers have been observed (Dahlstrom and Fuxe, 1966; de Bold et al., 1980; Oertel et al., 1982; Mezey, 1984; Schimchowitsch et al., 1991) with GABA and dopamine localized in the same endings. Figure 1-1 gives an overview of pituitary anatomy.

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The intermediate lobe is poorly vascularized, and the route by which IL peptides reach general circulation is poorly characterized; it is thought that a capillary plexus closely associated with the *pars nervosa* may play a role in the release of IL peptides. It is also possible that there are vascular routes for retrograde transport of secreted IL peptides to the hypothalamus (Howe, 1973). In addition, the melanotrophs are surrounded by a system of canaliculi. These narrow passages between cells may function both to conduct secreted products back to general circulation and to conduct neurotransmitters and neuro- modulators to melanotrophs in a non-synaptic manner (de Bold, 1980).

The melanotrophs, which are large, polygonal, strongly PAS positive cells, make up the vast majority of the cells of the intermediate lobe. Also found in the parenchyma of the IL, especially in the rostral zone, are small numbers of weakly PAS positive, smaller, angular corticotroph-like cells. In addition to the pituicytes lining the hypophyseal cleft, there are astrocyte-like glial cells dispersed throughout the IL parenchyma (Thody, 1980).

There is a single mention of the expression of the gap junction protein, connexion-43 (cx-43) in the intermediate lobe of the pituitary in a paper on the distribution of connexion immunoreactivity in endocrine tissues. (Meda et al., 1993) Unfortunately, the investigators did not include a photograph showing the intermediate lobe, thus it is not possible to say where the very small amount of immunoreactivity they reported was located or how it was distributed; quite possibly it was limited to the small glial or pituicyte population of the intermediate lobe. A contradictory result was obtained by another group, showing no expression of cx-43 in the intermediate lobe; this result was readily apparent in photographs of several immunostained pituitaries in which cx-43 immunoreactivity was clearly seen in the adjoining

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sections of neural and anterior lobe. (Yamamoto et al., 1993) To the best of my knowledge, there are no reports documenting electrical coupling of melanotrophs to one another; thus the weight of evidence indicates that melanotrophs do not form gap junctions with one another.

The Secretory Products of the Melanotroph and Their Actions:

The primary secretory products of the melanotroph are derived from the precursor molecule proopiomelanocortin (POMC); in contrast to the corticotroph where ACTH and βLPH are the major products, the melanotroph further cleaves these peptides to yield its major secretory products, alpha melanocyte stimulating hormone (αMSH) and β-endorphin (βEP). The βEP is secreted primarily in an N-acetylated, inactive form (Smyth et al., 1979).

Alpha-MSH has a broad range of actions, and it is regulated by numerous physiological stimuli. Its "classical' effects involve changes in skin color in amphibians and reptiles. The events leading to the identification and isolation of αMSH span a period of fifty years, starting with the observation that the removal of the pituitary in frogs resulted in a loss of skin coloration. Thus it was noted that the pituitary contained some kind of compound possessing melanophore stimulating activity. This discovery initiated a long quest to isolate the so-called "intermedins," a search in which melanophore stimulating activity of pituitary extracts was the primary bioassay. Alpha-MSH was finally isolated and sequenced in the 1950's as were ACTH and βMSH. All three were shown to share a core of seven amino acids to which the melanophore stimulating activity was attributed (Eberle, 1988).

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The relationship between these peptides was clarified somewhat when the corticotropin-like intermediate lobe peptide (CLIP) was isolated and shown to comprise those portions of ACTH not represented in αMSH. It was concluded that αMSH represented a cleavage product of ACTH (Scott et al., 1973). By the late 1970's, POMC was isolated and shown to be the precursor of ACTH, αMSH, CLIP, βMSH, βLPH and β-endorphin. Beta-LPH was shown to contain the sequences of βEP, γLPH, and βMSH was shown to derive from γLPH (Roberts and Herbert, 1977). Thus the origins of the various melanophore stimulating activities were defined.

Although the effects of IL derived αMSH are too numerous to list here, there are broad categories of effects worth mentioning. Targets for melanocyte stimulating hormone include the melanophore and sebaceous glands in the skin, the anterior pituitary, adipocytes, the adrenal cortex, the immune system, the heart, and the kidneys as well as numerous locations in the brain.

One subset of αMSH responses may be interpreted as participating in responses to combinations of physical and anxiogenic stresses. Alpha-MSH appears to act at a variety of sites to oppose the actions of IL-1β in the immune system, the general outcome of its effects being a suppression of inflammatory processes. At the adrenal cortex, αMSH may synergize with ACTH to increase adrenal output, and may act as a potent stimulus for aldosterone production (Baumann et al., 1986). In addition, αMSH increases heart rate. When injected systemically, αMSH is reported to enhance avoidance learning tasks, and to prevent their extinction (de Wied, 1964; de Wied and Bohus, 1966; Witter et al., 1975) while enhancing impulse conduction and locomotor activity (Strand and Cayer, 1975; de Wied and Jolles, 1982). This implies that αMSH secreted by the IL into systemic circulation may exert central effects, either by direct

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stimulation of brain MSH receptors or by some indirect route. Thus some actions of αMSH appear to complement and augment the functions of ACTH, namely, limiting potentially damaging responses to systemic injury, and preserving cardiac output; another "wing" of the response to αMSH secretion might be seen as preparing an animal to evade a perceived hazard and to remember the experience. Beta-endorphin secreted along with αMSH may also play a role in these responses. Its opiate activity could be unmasked either by βEP being desacetylated peripherally, or more likely, if the IL fails to acetylate βEP altogether during conditions of high stimulation. This particular scheme is admittedly speculative, but it is not difficult to imagine the utility of links and overlaps in responses to anxiogenic and physical stresses; in nature, anxiogenic stimuli are in fact those events which are likely to lead to physical injury, and the systems for avoiding and then responding to injury must be well integrated.

Another interesting subset of αMSH activities involves endocrine development in the fetus. It has long been known that the growth of the fetal adrenal gland is dependent on an intact pituitary. Fetal hypophysectomy induces adrenal atrophy, and in the early fetus this phenomenon can be prevented by injection of αMSH (Dupouy, 1982). It is interesting to note that while the IL is absent in adult humans, it is present in fetal life and in pregnancy (Silman et al., 1976; Vissers et al., 1989). Possible roles of IL MSH in pituitary and brain development are difficult to demonstrate as the presence of brain MSH represents a potential confounding factor in investigations of this topic.

Alpha-MSH also stimulates the activity of the preputial glands (Thody, 1980). The preputial glands produce odorants which act to attract sexual

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partners in rodents; thus secretion of αMSH may act to signal sexual receptivity in rats. The correlation of IL αMSH levels and secretion with the estrous cycle shows that IL output is greatest at proestrous when sexual receptivity, estrogen, and progesterone levels are highest. Furthermore, administration of either estrogen or progesterone to ovariectomized rats leads to an increase in plasma αMSH levels. Taken together, these observations lend credence to a steroid driven role for αMSH in sexual receptivity. (Thody et al., 1981; Celis, 1977) .

The variety of effects of the MSH peptides and the large number of target organs make for a somewhat confusing picture of overall regulation. It is probable that the actions of other circulating hormones along with varying affinities and number of MSH receptors allow a sort of "physiological separation" of the varied actions of MSH.

Regulation of Melanotrophs by Circulating Hormones and Neuronal Inputs

As is implied in the previous discussion, there is evidence that melanotrophs have receptors for estrogen; melanotrophs express mRNA encoding the estrogen receptor which is a member of the steroid receptor superfamily (Pelletier et al., 1988). As discussed above, the effect of estrogen is to increase melanotroph output. This observation of estrogen receptor mRNA, however, is not well matched at the protein level. While *in situ* hybridization with an estrogen receptor mRNA probe identified the intermediate lobe as a site of relatively high expression, another group using immunocytochemistry observed only a very weak estrogen receptor immunoreactivity. (Yamashita, 1989). It is possible that the IL estrogen receptor is modified or sequestered in

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some way which renders it less immunoreactive, or that it is translated only at a low level, but is present at levels sufficient for regulation of the cells by estrogen. Another possiblity is that the estrogen receptor mRNA observed in melanotrophs does not lead to functional receptors at all. In this case, estrogen might exert its effects on melanotrophs through some other mechanism such as an as yet undiscovered G-protein linked membrane receptor.

Receptors for glucocorticoids are probably also present on melanotrophs, but the feedback effects on the synthesis and secretion of POMC products and down regulation of CRF receptors seen in the corticotroph are largely absent (Autelitano, 1989). While glucocorticoid receptor mRNA is present in the IL (Sheppard et al., 1990), the receptor itself has been difficult to detect both by immunocytochemical means and by steroid autoradiography (McGimsey, 1991). This situation is somewhat reminiscent of that of the estrogen receptor. Denervation may bring back the glucocorticoid receptors (Antakly et al., 1985), a process thought to be under the regulation of dopamine; under these conditions, glucocorticoids appear to stimulate POMC mRNA (Levin, 1991). Recently, it was shown that puromycin treatment in IL primary cultures restored the inhibition of POMC gene expression by dexamethasone (Sheppard et al., 1993). The authors suggested that an endogenous protein is expressed in the IL which inhibits glucocorticoid receptor function. It has also been shown recently that cfos overexpression in corticotrophs can inhibit dexamethasone effects on POMC (Roberts, 1993). Taken together, all of these results suggest that glucocorticoid, and perhaps other steroid receptors in the IL are under the regulation of an

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endogenous protein which could be c-fos, and which may be dependent on intact innervation.

In discussing the effects of adrenal steroids on melanotrophs, it should be mentioned that the progesterone metabolite 3-alpha-hydroxydihydroprogesterone (3αOHDHP) and the glucocorticoid metabolite tetrahydrodeoxycorticosterone (THDOC) can exert direct effects on GABA-A receptors (Majewska et al., 1986; Sutanto and de, 1991). Furthermore, THDOC has been shown in humans to be under hypothalomo- pituitary axis control (Schambelan and Biglieri, 1972). Pregnenolone sulfate and other sulfated pregnenolone derivatives have also been shown to modulate GABA-A receptors by decreasing chloride flux. These steroids, as well as pregnenolone itself, can also modulate voltage gated calcium channels via G-proteins (Ffrench-Mullen et al., 1994). These compounds have been shown to be synthesized *de novo* by neural tissue (Baulieu, 1991) and their synthesis is regulated by agonists of the peripheral benzodiazepine receptor (Costa and Guidotti, 1991). Interestingly, the neurointermediate lobe is a major site for the expression of diazepam binding inhibiting peptide (DBI), an endogenous peripheral benzodiazepine agonist (Alho et al., 1988). The potential role of neurally active steroids in regulation of melanotrophs will be discussed in greater detail in later chapters.

In addition to circulating steroids, melanotrophs may respond to circulating catecholamines of sympathetic origin, and in particular to noradrenaline. Noradrenaline stimulates melanotroph output, presumably via receptors positively coupled to cAMP. Passive avoidance, or stresses such as foot shock or restraint, have been shown to increase βEP and αMSH levels; sympathectomy, medullectomy, and treatment with propanolol

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eliminated or reduced these effects (Berkenbosch et al., 1983; Berkenbosch et al., 1984; Kvetnansky et al., 1987). In addition to ciruculating noradrenaline, the noradrenergic projections from the reticular formation to the IL may also contribute to these phenomena (Bjorklund et al., 1973).

CRF is another positive regulator of melanotrophs, and like noradrenaline, it is probably positively coupled to cAMP. Although the source of CRF innervation to the melanotroph is unclear, some investigators have suggested that neural lobe magnocellular neurons provide CRF and vasopressin input to the IL. CRF stimulates αMSH and βEP release (Proulx et al., 1982). It has been further suggested that CRF effects on neural lobe oxytocin release are mediated through stimulation of IL peptide release (Bondy, 1989). Thus CRF outflow to melanotrophs occurs under a set of circumstances different from those in which CRF stimulates anterior lobe corticotrophs. The participation of melanotrophs in responses to systemic stresses is likely to be mediated through other pathways. Vasopressin stimulates αMSH release in response to hypertonic saline but does not increase the effects of CRF as it does in the anterior lobe (Lutz-Bucher, 1983).

As mentioned above, among the important inputs to the intermediate lobe are the tuberohypophyseal projections from the arcuate nucleus which have been demonstrated to contain both dopamine and GABA in the same terminals (Schimchowitsch et al., 1991). Dopamine in melanotrophs is negatively coupled to cAMP through the D2 dopamine receptor and has been repeatedly shown to have inhibitory effects on POMC synthesis and peptide secretion in melanotrophs (Loeffler et al., 1988; Taraskevich and Douglas, 1990). Further, dopamine appears to prevent some changes in melanotroph function and morphology when the IL is denervated by transplantation to the

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kidney capsule. Specifically, transplantation produces a population of cells which stain only lightly for αMSH and which lack secretory granules and have enlarged nuclei. These cells, taken to represent hypersecreting cells do not appear in animals treated with ergocryptine (Iturriza, 1989). In another study, a population of light and dark cells in intact pituitary was demonstrated by simple chemical staining with toluidine blue. The dark cells in this study were interpreted as cells more active in the synthesis of POMC peptides, and both the number of these cells and the levels of POMC mRNA by in situ hybridization were shown to rise with haloperidol treatment and to fall with bromocriptine treatment (Chronwall, 1988). These two studies are some what difficult to reconcile; different visualization methods and tissue preparations yielded what appear to be conflicting results. But it is clear that dopaminergic input to the IL plays an important role in regulating the output of POMC peptides, and that its influence on synthesis and secretion is primarily inhibitory. The restoration of dark cells by ergocryptine in the transplant study may reflect a difference in effects on synthesis versus release of αMSH; dopaminergic inhibition of synthesis of POMC may require other hypothalamic inputs which were not present in the transplant study.

The tuberohypophyseal GABAergic projections to the melanotroph serve both inhibitory and excitatory functions. Initial studies on the effects of GABA on melanotrophs showed that high concentrations of GABA (10^{-4}M) or low concentrations of GABA (10^{-6}M) in the presence of benzodiazepines cause a brief train of action potentials followed by a period of inhibition in isolated rat neurointermediate lobes (Taraskevich and Douglas, 1982). In addition, these GABA treatments were found to cause a spike of αMSH release followed by a period of release below basal levels observed in the cultures (Tomiko et

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al., 1983). The stimulatory effect of GABA was found to be dependent on the presence of calcium, and to be blocked by GABA-A antagonists; it is attributed to the opening of low-threshold voltage dependent calcium channels as the cell reaches the chloride equilibrium potential. The chloride currents through melanotroph GABA-A receptors are outwardly directed, presumably because the melanotrophs actively concentrate chloride ions. The inhibitory component of the response to GABA could not be eliminated with GABA-A antagonists, and has been attributed to the GABA-B receptor. In fact, in several studies, application of the specific GABA-B agonist baclofen to melanotrophs in culture resulted in decreases in both POMC mRNA synthesis and αMSH release (Demeneix et al., 1984; Demeneix et al., 1986; Loeffler et al., 1986). GABA-A receptors also seem to contribute to the inhibitory phase of GABA action on melanotrophs, however, as potentiators such as clonazepam have been shown to increase the degree of inhibition of αMSH release following the excitatory "spike". Eight hour treatment of IL cultures with specific GABA-A agonists results in reduction of POMC mRNA (Loeffler et al., 1986). The transition of GABA-A receptors from excitatory to inhibitory in these circumstances could be the result of changes in the cellular chloride gradient; indeed since the direction of the chloride gradient determines whether GABA-A receptors will be hyperpolarizing or depolarizing, it may be an important point of regulation in these cells.

In summary, melanotrophs are stimulated to synthesize POMC and release αMSH either directly or indirectly by estrogens, CRF, and noradrenaline. Dopamine exerts an inhibitory effect while GABA has a biphasic effect on release of POMC derived peptides and exerts an inhibitory effect on the synthesis of POMC mRNA.

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GABA RECEPTOR PHARMACOLOGY AND MOLECULAR BIOLOGY

A brief review of GABA receptor pharmacology and molecular biology will help in clarifying the effects of GABA on melanotrophs, and in establishing the rationale for the experiments described in the chapters which follow.

GABA-A and GABA-B receptors:

GABA, the major inhibitory neurotransmitter in the central nervous system, acts at two classes of receptors, GABA-A receptors and GABA-B receptors. Effects mediated by the GABA-B receptor are pertussis toxin sensitive, so the receptor is thought to be a one of the seven-membrane spanning family of receptors which are linked to G-proteins. GABA-B receptors hyperpolarize cells by indirectly opening potassium channels or oppose excitation by indirectly closing calcium channels (Bowery,1991). The GABA-A receptor, in contrast, is a ligand-gated chloride channel which hyperpolarizes cells in most instances by holding them at or near the chloride equilibrium potential. Affinities for GABA differ at the two classes of sites, with affinity and EC-50 values ranging from 100nM to 1μM for GABA-B receptors and from 1μM to 100μM for GABA-A receptors (Bowery, 1980; Wong, 1984).

The binding of GABA in some preparations of brain tissue has been observed to be cooperative. In addition, two molecules of GABA must bind to the receptor in order to open the chloride channel. The GABA-A receptor is

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structurally and genetically related to the nicotinic acetylcholine receptor which is also known to exhibit a cooperativity effect (Schofield et al., 1987). A commonly used GABA-A specific antagonist is bicuculline. Picrotoxin blocks the action of GABA by blocking the chloride channel. The comparative pharmacology of GABA-A and GABA-B receptors is summarized in a table shown in Figure 1-2.

The most striking feature of the GABA-A receptor is the wide variety of modulators of GABAergic transmission which act at distinct sites on the receptor. GABA and muscimol are the major agonists at the GABA receptor, but the actions of GABA can be affected by benzodiazepines, β-carbolines, ethanol, barbiturates, and neurosteroids.

The benzodiazepines (BZD), used clinically as anxiolytic drugs, potentiate GABAergic transmission by increasing the affinity of the GABA-A receptor for GABA and thereby increasing the frequency of channel openings in response to a given dose of GABA. β-carbolines act at the same site, but in almost every instance reduce the cellular response to GABA, and are therefore referred to as "inverse agonists" at the BZD site. Affinity of receptors for β-carbolines has been used to define two different types of GABA/BZD receptors. Type I BZD receptors are characterized by a high affinity for the β-carbolines and the compounds 2-oxaquazepam and CL 218 872. Type II receptors are less numerous and show 5-10 fold lower affinity for these compounds (Olsen et al., 1990) There is a growing body of evidence to suggest that β-carbolines and benzodiazepines occur endogenously in the brain. Of particular interest are the findings that levels of β-carboline compounds in the brain are doubled during anxiogenic stresses of the type that activate melanotrophs, and that β-carbolines are found in the arcuate

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nucleus (Shoemaker et al., 1978; Deitrich and Erwin, 1980). These findings must be balanced against the fact that thus far, no one has localized β-carbolines to synaptic vesicles and that the concentrations of these compounds in samples of whole brain tissue appear to be well below concentrations required to observe effects on GABAergic transmission (Deitrich and Erwin, 1980; Izquierdo, 1989). Related to the effects of benzodiazepines are the effects of ethanol on GABAergic transmission. Ethanol potentiates GABAergic transmission, increasing whole-cell chloride currents in response to GABA (Suzdak et al., 1986). Ro15-4513, a behavioral antagonist of ethanol induced ataxia, binds to the BZD site on the GABA receptor (Syapin et al., 1987). Furthermore, ethanol and benzodiazepines are clinically cross-tolerizing, thus providing further evidence for direct action at the same receptor.

Another important class of GABA modulators is the barbiturates. Barbiturates act at a site distinct from benzodiazepines, and in fact may potentiate benzodiazepine binding. The barbiturates potentiate GABAergic transmission, but they do so by increasing the duration rather than the frequency of channel opening (Olsen, 1982). Neurosteroids appear to act at or near the barbiturate site, but by a different mechanism; the neurosteroids increase the frequency of channel opening in response to GABA in a manner analogous to that of the benzodiazepines, as well as increasing the duration of channel opening. As mentioned previously, neurosteroids include progesterone derivatives, pregnenolone derivatives , and the glucocorticoid derivative tetrahydrodeoxycorticosterone (THDOC). Because THDOC is under HPA regulation (Schambelan and Biglieri 1972), it is possible that THDOC serves as a form of rapid and direct feedback from the adrenals to GABAergic circuits in the brain and pituitary, including the melanotroph.

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The pharmacological complexity of the GABA-A receptor is matched by complexity at the molecular level. The initial isolation and cloning of the subunits of the GABA-A receptor began with attempts to purify muscimol and flunitrazepam binding proteins from preparations of bovine cerebral cortex . SDS-polyacrylamide gel electrophoresis revealed a 48kD flunitrazepam binding subunit designated α and a 51kD muscimol binding subunit designated β. These subunits were observed to occur in the stoichiometry $\alpha 2\beta 2$ (Sieghart,1989). Sequence analysis of these isolated subunits led to the isolation of cDNA clones for the α and β subunits which were found to be 35% identical and to have 57% homology including conservative amino acid substitutions. The two genes were shown to be homologous to the nicotinic acetylcholine receptor (nAchr), particularly in hydrophobic putative membrane spanning regions. Based on this information a pentameric structure, analogous to that of the acetylcholine receptor was proposed, with each subunit containing an N-terminal extracellular loop, four membrane spanning regions, and a cytoplasmic loop between the third and fourth putative membrane spanning regions. Consistent with its role as an anion channel it was noted that the GABA-A receptor subunits have positively charged residues or neutral residues in locations analogous to those locations in the nAchr which have negative charges and are thought to line the pore of the nAchr cation channel (Schofield et al.,1987).

Long-standing pharmacological evidence for GABA-A/BZD receptor diversity, and the structural comparison to the multisubunit nAchr provided the impetus to search for additional GABA-A receptor subunits. Low stringency screening of cDNA libraries with an alpha subunit specific probe detected the $\alpha 2$, and $\alpha 3$ subunits which were subsequently found to have 70%

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to 80% identity with the previously isolated α1 subunit and differed in GABA affinity within a 30 fold range. Injected into oocytes with the β1 subunit, these subunits were shown to confer an inward chloride current in response to GABA that was blocked by bicuculline and picrotoxin, and potentiated by pentobarbital but not by benzodiazepines (Levitan et al., 1988). Subsequent studies isolated an α5 subunit which was shown to be most abundant in the hippocampus and to exhibit spontaneous channel opening when injected into oocytes with a β subunit (Khrestchatisky et al., 1989). Finally, an α6 subunit was shown to be located exclusively in the cerebellar granule cells and to bind the ethanol antagonist Ro15-4513 (Luddens et al., 1990).

Similarly, three variants of the β subunit were isolated in the rat using a probe based on a conserved domain in the second membrane spanning region. These subunits conferred varying sensitivities to GABA when coinjected into oocytes with the α1 subunit, and were all observed to possess a putative cAMP dependent phosphorylation site in the cytoplasmic loop (Ymer et al., 1989).

Robust responses to benzodiazepines in reconstituted receptors were not observed until the discovery of the γ2 subunit, 40% homologous to the α1 and β1 subunits (Pritchett et al., 1989). The γ2 subunit has been shown to occur in two forms; alternative splicing can insert a putative protein-kinase C phosphorylation site in the cytoplasmic loop (Kofuji et al., 1991). Another subunit type designated δ was shown to be present in locations where non-BZD binding GABA-A receptors were known to exist, and did not confer BZD responsiveness in reconstitution experiments (Shivers et al., 1989). Some benzodiazepine binding receptors have been shown to be immunoprecipitable with δ subunit binding antibodies, however (Mertens et al., 1993). This result

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may indicate that the behavior of the δ subunit is dependent on what other subunits are present, or that binding of benzodiazepines does not insure benzodiazepine responsiveness. Also in reconstitution studies in Cos cells and oocytes, the γ2 subunit in combination with apha and beta subunits was found to confer a response to β-carbolines (Pritchett et al., 1989). The presence of α1 subunits was found to specify Type I BZD pharmacology while α2 and α3 gave type II BZD pharmacology. A γ1 subunit, highly homologous to the γ2 subunit, has been isolated and has been shown to confer a benzodiazepine response, but with a reduced sensitivity to β-carbolines (Pritchett and Seeburg, 1990; Ymer et al., 1990). It is important to note that the properties observed in these reconstitution experiments may depend greatly on the specific subunit configurations used. The large number of subunits necessitates that in most experiments only one subunit is varied, so that the results obtained may not be reflective of subunit combinations observed in-vivo.

Figure 1-3 illustrates the proposed structure of GABA-A receptors in schematic form, and also summarizes the various classes of subunits and the pharmacological properties of GABA-A receptors for which each is thought to be responsible.

SUMMARY

As a prelude to describing studies on components of the GABAergic neurotransmitter system which regulates melanotrophs, I have reviewed the anatomy of the neurointermediate lobe, the synthesis and actions of its major secretory products, the various hormonal and neurotransmitter systems which

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regulate melanotrophs, and finally, the various kinds of GABA receptors and their characteristics.

This information provides the context in which to think about the central question of this thesis, namely, what kinds of GABA-A receptors and GABA-A receptor modulators are present in the pituitary, and particularly on melanotrophs, and how might they contribute to melanotroph function.

The following three chapters outline, respectively, the generation of GABA-A receptor subunit cDNA probes, studies on identification and quantitation of GABA-A receptor subunit mRNA in the pituitary, and finally, pharmacological characterization of GABA/benzodiazepine receptors in the pituitary. The next chapter describes experiments which investigate the capacity of neurointermediate lobe tissue to synthesize neurally active steroids which may modulate the GABA-A receptor.

In the concluding chapter I will attempt to draw these observations together into a hypothesis on how biphasic responses to GABA may be of use in regulating basal levels of activity in populations of neurosecretory cells. This discussion will focus in part on how the particulars of pituitary GABA systems described in this work may contribute to this type of regulation.

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1-1a

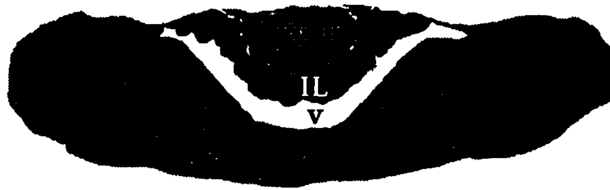

Figure 1-1a demonstrates the relation between the three main parts of the rat pituitary in a horizontal section. Anterior lobe, AL; intermediate lobe, IL; neural lobe, NL. A virtual space separating AL and IL is designated "v". This space typically appears as an artifact of fixation. Note the continuity of the neural lobe and intermediate lobe. In dissection of the pituitary, these tissues separate from the anterior lobe as a single unit designated as the neurointermediate lobe. The arrangement of the pituitary is such that a coronal section would look quite similar.

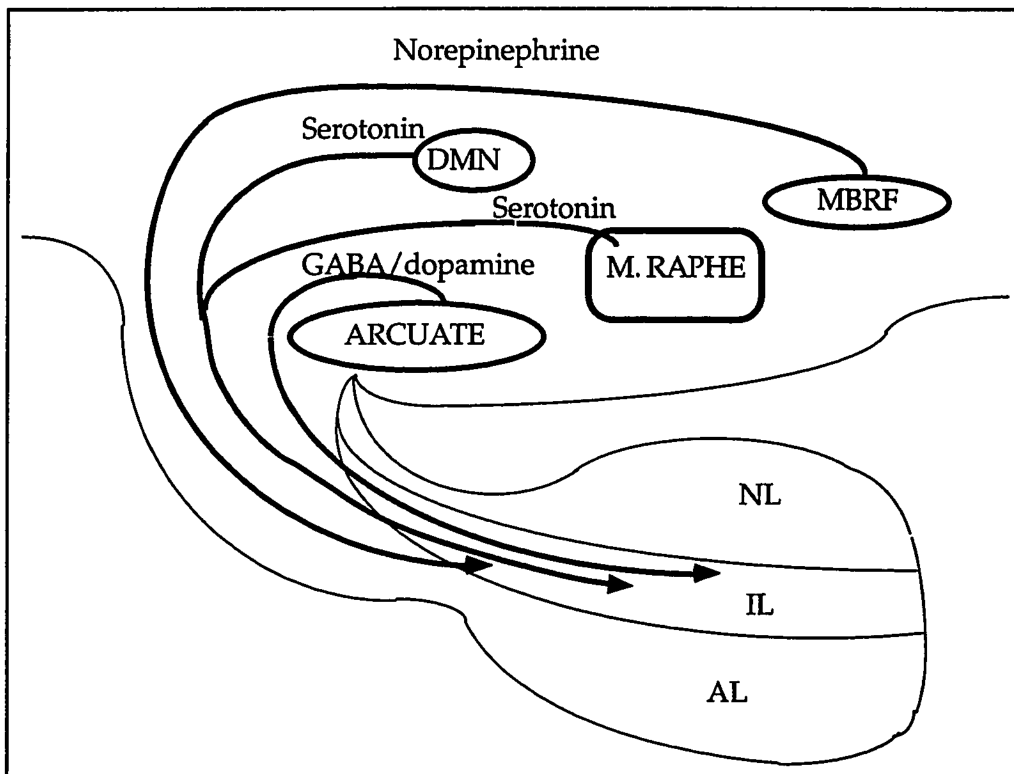

Figure 1-1b depicts major projections to the intermediate lobe. The view of the pituitary in this schematic is midsagital. DMN, dorsal medial nucleus of the hypothalamus; M. Raphe, median raphe nucleus; MBRF, midbrain reticular formation; arcuate, arcuate nucleus. Not shown are peptidergic and cholinergic inputs of unknown origin.

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1-2 Summary of GABA pharmacology

	GABA-A	GABA-B
Class	Ligand Gated Ion Channel	Seven Membrane Spanning
Relatives	nAchR, glycine receptor..	mAchR, dopamine receptor..
Action	Chloride channel, holds cells at ECl; usually hyperpolarizing	Opens K+ or closes Ca++ channels via a G-Protein; hyperpolarizing, inhibits secretory events
Agonists	GABA, Muscimol, Isoguvacine	GABA, Baclofen
Antagonists	Bicuculline,	Phaclofen, Saclofen, CGP35348
Potentiators	Benzodiazepines (frequency) Barbiturates (duration) Neurosteroids (both) Ethanol	
Negative Modulation	Beta-carbolines (inverse agonist at BZD site) Some neurosteroids (sulfated) Picrotoxin (Cl^- channel block)	Pertussis toxin

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1-3 CLASSES OF GABA-A RECEPTOR SUBUNITS

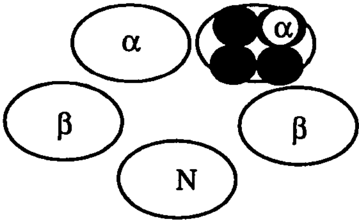
α: β-Carboline, Benzodiazepine, GABA, affinities

β: GABA affinity, required for efficient assembly, phosphorylation site on cytoplasmic loop related to cAMP regulation

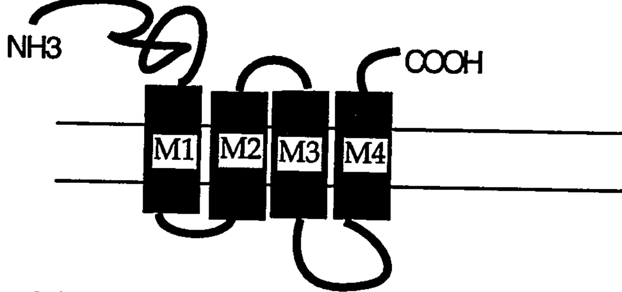
γ: Required for robust benzodiazepine effects, phosphorylation site on cytoplasmic loop in some subtypes

δ: Does not participate in benzodiazepine sensitive receptors

All subunits confer some picrotoxin, barbiturate, and neurosteroid responses but relative sensitivities may vary depending on receptor composition.



Schematic view from above of proposed GABA-A receptor pentameric structure highlighting four membrane spanning regions in one of the subunits. (After Olsen, R.W, and Tobin, A.J. *FASEB Journal* 4: pp. 1469-1480



Schematic view of proposed structure of a single GABA receptor subunit with four membrane spanning regions, cytoplasmic loop between M3 and M4, and N terminal ligand binding domain. (After Schofield et al., *Nature* 328: pp. 221-227, 1987)

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CHAPTER TWO:
GENERATION OF RIBOPROBES FOR RAT GABA-A RECEPTOR SUBUNIT mRNAs

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INTRODUCTION

Of 14 known vertebrate GABA-A receptor subunits (reviewed in the introductory chapter), we selected eleven for investigation in our studies of GABA-A receptor subunit composition in rat pituitary. The basis for this selection included known patterns of expression and abundance, and the apparent species specificity of some subunits. Specifically omitted from these studies were subunits thus far found only in avian species, and the ρ subunit whose expression has been observed mainly in the retina and at very low levels elsewhere (Cutting et al., 1991). Also omitted were the $\alpha 4$ and $\gamma 3$ subunits which similarly have been found to be expressed only at extremely low levels in specific neuronal populations (Wisden et al., 1992).

The cDNA probes for the $\gamma 2$ subunit and the five alpha subunits found in high abundance in rat brain were available to us from previous investigations conducted by our collaborator, Dr. Dolan B. Pritchett.

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We used the polymerase chain reaction (PCR) to generate a set of probes for the beta, gamma, and delta subunits, using published sequence information to design oligonucleotide primers. The general strategy was to cover regions of the message ranging from 300 to 600 bp in length, so as to produce probes suitable for use in ribonuclease protection assays. We also tried avoid highly conserved regions, thus producing a set of probes which might be used simultaneously, and which might be used in other applications such as Northern blots or *in situ* hybridization if the occasion arose. The oligonucleotide sequences employed are given in Figure 2-1. We screened the initial PCR products initially first by restriction digest where possible. In several cases, after subcloning into Bluescript, we also screened candidate cDNA segments by use in a ribonuclease protection assay. A PCR based method for efficiently screening riboprobes from several candidate cDNAs prior to large scale plasmid preparation is described below, and the rationale for the use of this screening step is given in the discussion. Finally, after plasmid preparation, we sequenced the cDNAs selected for use in our studies and compared them to the published sequences.

EXPERIMENTS AND RESULTS

Generation of partial cDNAs for rat β1 and β3 subunits by PCR: The first rounds of PCR from brain cDNA for the β1, β2, and β3 subclones produced primary PCR products of 284 bp, 584 bp and 633 bp respectively, which are shown in Figure 2-2. Restriction digest of the primary β3 PCR product with

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BamH1 and EcoR1 produced a 268 bp fragment thus demonstrating the internal BamH1 site at 301-307 bp (Ymer et al., 1989). This observation provided early confirmation of the identity of the β3 subclone. The β3 product was ligated into Bluescript SK and the β1 and β2 products were ligated into Bluescript KS. Transformation of TB-1 bacteria with the products of the ligation resulted in only about 15-20 transformants for β1, no transformants for β2, and more than 500 transformants for β3. Plasmids amplified from transformed colonies, when linearized outside of the insert in the Bluescript polylinker, directed the synthesis of antisense riboprobes of 330 bases for β1 and 319 bases for β3. When hybridized to 5 μg of cerebellar total RNA in a ribonuclease protection assay, these probes protected bands of 284 bases and 268 bases respectively, as shown in Figure 2-3. The sequences of 200-300 bp of these subclones in Bluescript KS and Bluescript SK appear in Figure 3-6; schematic maps appear in Figure 2-7.

Generation of partial cDNAs for rat β2, γ1 and δ subunits using a ribonuclease protection assay and PCR in combination: In all subsequent PCR reactions, I used oligonucleotides with restriction sites 6 bases downstream from the 5′ end, rather than at the extreme 5′ end. PCR from cerebellar cDNA for the γ1, δ, and β2 subunits produced primary PCR products of the predicted sizes as shown in figure 2-4a. The β2 PCR product is 584 bp, the γ1 band is 432 bp, and δ is 506 bp. These products were subcloned into Bluescript KS and I transformed TB-1 bacteria with the resulting plasmids. Using the T7 or T3 oligonucleotides along with the downstream oligonucleotides for the GABA-A receptor subunit sequences, I carried out PCR based screening of transformed TB-1 bacterial colonies with the following

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result: of colonies transformed with the β2 and γ1 containing plasmids, 4/5 contained inserts and of colonies transformed with δ containing plasmids, 1/2 contained inserts. The results of this screening appear in Figure 2-4b.

I selected two to four insert-containing subclones for each subunit and cut these with EcoRV. This enzyme cut all of the PCR products except for the β2 subclones. In the case of the β2 clones I decided to attempt riboprobe generation with one subclone in spite of its failure to cut with EcoRV. The linearized PCR-generated templates produced riboprobes which I ran on a 4.5% polyacrylamide denaturing gel. Size measurements were made by plotting the log of migration of the probes against their predicted sizes, and the migration of xylene cyanol and bromophenol blue against their theoretical migration values on a 4.5% denaturing gel. Regression values of greater than 0.97 were observed for the plot of the log of migration of both the probes and markers against the predicted probe sizes and the expected migration of the markers. The templates directed the transcription of riboprobes of the expected sizes in 3 of 4 cases for δ and in 1 of 2 for γ1. For β2, the candidate subclone produced a species of about 650 bases. This band is consistent with that which would result from a riboprobe transcribed from the uncut PCR product.

When hybridized to cerebellar total RNA in a ribonuclease protection assay, the riboprobes for γ1 and δ protected bands of the expected sizes as ascertained by the method described above as well as by visual comparison with Msp markers. The β2 riboprobe protected a band of 578 bases, which is the size of the full β2 insert uncut by EcoRV. These results are shown in Figure 2-5b. Based on these results I selected subclones β2-4, δ14, and γ1-5 for large scale plasmid preparation.

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Large scale plasmid preparation from colonies selected in the screens described above produced plasmids containing segments of the sequences for the β2, γ1, and δ subunits of the GABA-A receptor which, when subjected to dideoxy sequencing, were identical to published sequences for these subunits (Shivers et al., 1989; Ymer et al., 1989; Ymer et al., 1990). Figure 2-6 shows the sequences of the first 200 to 300 bp of the inserts in Bluescript KS. Figure 2-7a shows schematic maps of the plasmids with inserts. Plasmid maps for the alpha and γ2 subunit clones obtained from Dr. Dolan Pritchett appear in figure 2-7b.

DISCUSSION

Both screening methods described above resulted in the production of partial cDNAs for five GABA-A receptor subunit genes which were free of base substitutions which are sometimes encountered when the polymerase chain reaction is used. Because these partial cDNAs were isolated for use in generating antisense riboprobes for nuclease protection assays, total sequence identity at least from the 3' end of the probe to a useful internal restriction site was an absolute requirement. Thus it seemed useful to use the ribonuclease protection assay early in the screening of candidate subclones. A great pitfall exists in using the ribonuclease protection assay as a preliminary screen; it is that if the PCR band is of a certain size and comes from total brain RNA then it is already known that a riboprobe generated from that PCR product should protect some RNA species of that length. This issue arose in the production of

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the β1 and β2 subclones which were tried in ribonuclease protection assays without having been cut at an internal site. In fact, both subclones isolated were correct as shown by dideoxy sequencing. The initial failure of the β2 subclones to linearize with EcoRV was evidently not due to absence of the site, since plasmids derived from this clone contained the site in their sequence and were subsequently cut by the enzyme. As a functional template with the correct sequence was generated, this issue was not investigated further.

The ribonuclease protection assay screen was useful in excluding at least one delta subunit clone, δ5, which produced a band smaller than expected and probably had a PCR error in the insert. It appears, however, that the majority of clones with inserts functioned in ribonuclease protection assays suggesting that for these relatively small inserts, PCR errors do not occur frequently.

MATERIALS AND METHODS

RNA Isolation: I isolated total RNA from cerebellum and cerebral cortex of adult male Sprague Dawley rats. Tissues were retained at -70°C after dissection, and thawed in ice cold lysis buffer in diethyl pyrocarbonate (DEPC) treated glass homogenizers with teflon pistons. For tissue slices of about 50-100 mg, I used 0.5 ml of lysis buffer. The lysis buffer consisted of 10mM Tris-HCl, 1.5 mM $MgCl_2$, 0.5% NP-40, 2.5 mg/ml sodium deoxycholate, and 0.3M sucrose. To lyse the issue, I applied 10-20 strokes of the piston with the tube continuously on ice. The lysate was then layered over a cushion of ice

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cold buffer containing 0.4M sucrose, 10mM Tris-HCl, and 1.5mM $MgCl_2$ in microcentrifuge tubes, and spun for 15 minutes at about 1000XG in a microfuge at 4°C. I removed the supernatants containing cytoplasmic RNA to a microfuge tube containing 50 µl of 10X SET buffer (100mM Tris-HCl pH 7.5, 10mM EDTA, 10% SDS) and then added a 10µl aliquot of Proteinase K (10 mg/ml, Sigma) to each tube. Samples incubated in a 45°C water bath for two minutes, followed by brief vortexing, and then further incubation for up to one hour. After incubation, each tube received 5 µl of 5M NaCl to aid in phase separation. Phenol/chloroform extraction was followed by re-extraction with chloroform/isoamyl alcohol (24:1). I retained the aqueous phase and added 2.5 volumes of ethanol. After at least one hour at -20°C, the samples were spun at full speed in a microcentrifuge at 4°C for 20 minutes. I washed the resulting pellets twice with 1500 µl of 70% ethanol, dried them briefly under vacuum, and resuspended them in TE buffer, pH 8.0 (10mM Tris HCl, 1mM EDTA). Total RNA isolated in this manner generally displayed 280 nm/260 nm absorbance ratios of 2.0 or greater, and the yield of RNA was in the range of hundreds of micrograms per extraction as determined by UV absorbance at 260 nM. I ran samples of 0.5 to 1 µg of the total RNA on 1% agarose gels post-stained with ethidium bromide, and found the RNA to be intact as evidenced by the presence of 28S and 18S bands in a 2:1 ratio and the absence of detectable RNA below the tRNA band.

Production of cDNA: To produce cDNA for subsequent use in the polymerase chain reaction, I incubated aliquots of 10 µg of total RNA from rat cerebellum and cerebral cortex with 5 µg acetylated bovine serum albumin (Promega); 1µg oligo dT (Pharmacia); 0.5mM dATP, dTTP, dCTP, and dGTP

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(Pharmacia); 5 µl of 10X reverse transcriptase buffer (BRL); and 200 units of M-MLV reverse transcriptase (BRL) for one hour at 37°C. The total reaction volume was 50 µl.

Polymerase Chain Reaction: Each polymerase chain reaction consisted of a 2 µl aliquot of cDNA, 16.6mM NH_4SO_4, 67mM Tris-HCl pH 8.0, 2mM $MgCl_2$, 0.2 mg/ml bovine serum albumin, and 0.25mM dNTPs (Pharmacia). Oligonucleotides (synthesized by the DNA core facility, Brookdale Center for Molecular Biology, Mount Sinai School of Medicine) were directed toward the 5′ and internal regions of GABA-A receptor subunit genes. The oligonucleotides, were present at a concentration of 100 ng per reaction. Oligonucleotides contained 6 bases of filler sequence on the extreme 5' end, followed by BamH1, HindIII, EcoRI, or Xho1 sites, and 20-30 bases of the GABA-A receptor subunit sequences. The oligos used to generate β1 and β3 PCR products, and in our first attempt to subclone the β2 subunit sequences had restriction sites at the extreme 5′ end. The sequences for all of the oligonucleotides used are shown in Figure 2-1. Before use in PCR reactions, each oligonucleotide was dried overnight under vacuum, resuspended in 1 ml TE, extracted with ether, resuspended again in TE, and brought to the desired concentration of 10 µg/ml with TE after quantitation of the stock solution by UV absorbance at 260 nM. The reaction profile was 55°C annealing for one minute, 78°C elongation for two minutes, and 94°C for one minute, for 45 cycles. For each reaction, I used 2 units of Taq polymerase (Promega) or 0.5 units of Amplitaq (Cetus). Each reaction was topped with 50 µl of mineral oil to prevent volume loss due to evaporation. PCR products were initially

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analyzed on 1.5% agarose gels stained with ethidium bromide and run at 60V for approximately one hour.

Subcloning and Bacterial Transformation: To prepare the PCR products for subcloning, I removed the reaction product from under mineral oil, performed a phenol/chloroform extraction, and precipitated the products with two volumes of ethanol and 0.1 volumes of 0.3M sodium acetate. After two hours at -20°C, a fifteen minute spin at full speed in a 4°C microfuge, and two washes with 70% ethanol, I resuspended the PCR products in a total volume of 100 µl with 40 units each of BamH1 and EcoRI or HindIII or Xho1 (New England Biolabs), 10 µl of the appropriate 10X buffers , and 0.1 µg/µl bovine serum albumin. Following restriction digest, the PCR products ran for one to two hours on a 1.5% agarose gel at 60V. I used the GeneClean system (Bio 101) to remove PCR products from the gel.

Purified PCR products were resuspended in water at concentrations of about 100 ng/µl and combined with 100 ng of linearized Bluescript KS in the presence of 1 mM ATP, 400 units of T4 DNA ligase (Promega), and 2.5 µl 10X ligation buffer (0.5M Tris, pH 7.4; 0.1M $MgCl_2$; 0.1M dithiothreitol; 10mM spermidine; 1 mg/ml BSA) in a total volume of 25 µl. As positive and negative controls, I prepared additional tubes containing the ligation mix and either uncut Bluescript KS or linearized Bluescript with no insert. These mixtures incubated overnight at 16°C. I then added 5 µl of each ligation mix to 200 µl of competent TB-1 bacteria. Following one hour incubation on ice, and a two minute heat pulse at 42°C, I added each transformation mix to a 5 ml conical tube containing LB with glucose. This mix incubated for two hours at

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37°C with shaking, after which it was spread onto LB-Ampicillin plates and incubated overnight at 37°C.

PCR Screening for Inserts: For β2, γ1, and δ subunits, up to five bacterial colonies for each clone were picked with sterile pipette tips. After streaking a sample of each colony picked onto a master plate, I washed the pipette tip in 10μl of water and transferred this material to a PCR mix essentially similar to that described above. This reaction mix included oligonucleotides representing the Bluescript T7 or T3 polymerase promoter sequence upstream, and the GABA-A receptor subunit sequence used in the first round of PCR downstream. After 30 cycles of PCR, as described above, I ran 1/20 of the products of each reaction on a 1.5% agarose gel stained with ethidium bromide. I used EcoRV to digest about 1-2 μg of PCR products from each clone shown to have an insert and ran the digested PCR products on a 1.5% agarose gel.

Riboprobes from PCR products: Following recovery of the linear PCR templates using the GENECLEAN system, I resuspended the DNA from each candidate subclone in 10μl of TE. I added 1 μl of this material to 4 μl of transcription mix for a final composition of 0.25mM ATP, GTP, CTP, 7.5 μCi α ^{32}P UTP (NEN 800 Ci/mmol), 10mM DTT, 1x Stratagene transcription buffer, 40 units rRNAsin (Promega) and 5 units of T7 or T3 polymerase (Stratagene). These reactions incubated for 30-45 minutes at 37°C. To stop the reactions I added 95 μl of deionized DEPC treated water. At this point, I removed two samples of 1 μl from each to spot on DE81 paper to test for incorporation of labelled UTP; one sample representing total label was dried

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immediately and the other representing incorporated label was washed three times in 0.5M NaP_i prior to scintillation counting. Incorporations ranged from 10-50%. I then added, 9.5 µl of a mixture containing 10 units of DNAse I (Worthington), 200mM Tris-HCl, pH 8.0, 40mM $MgCl_2$, 5 units of rRNAsin, and 5-10 µg yeast total RNA to each riboprobe reaction. The reactions incubated for another 10 minutes at 37°C, after which I added 50 µl of 7.5 M NH_4Ac and two volumes of ethanol to precipitate the probes. After the probes precipitated for one hour at -20°C, I recovered them by centrifugation at full speed in a 4°C microcentrifuge. Two 70% ethanol washes removed free nucleotides. I resuspended the probes to a concentration of approximately 200 pg/µl in 80% formamide hybridization buffer, as calculated based on the incorporation measured and specific activity inferred. Under the conditions described, riboprobes are made with a specific activity in the range of 1.5×10^6 CPM/ng. I combined a portion of each riboprobe with a running buffer containing xylene cyanol and bromophenol blue, and ran the probes on a 4.5% denaturing polyacrylamide gel at 250 volts until the leading dye just reached the bottom of the gel. After drying the gel, I exposed it to XAR-5 film at -70°C for two to three hours with an intensifying screen, and developed the film in order to visualize the probes and determine their size.

Ribonuclease Protection Assay Screening: I produced riboprobes from two to five subclone candidates for each GABA-A receptor subunit. For each assay I added 5 µl of the probe to either 5 µg rat cerebellar total RNA in 5 µl TE or 5 µg yeast total RNA in 5 µl TE, and then added 20 µl of 80% formamide. After two minutes in an 80°C bath, these reactions incubated overnight at 45°C. After 12 -18 hours, I added 300 µl of a solution of 2 µg/ml Ribonuclease T1

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(Sigma) and 40 μg/ml Ribonuclease A (Sigma) in buffer (10mM Tris-HCl, 3mM EDTA, 300mM NaCl) to each reaction. Digestion proceeded for one hour at 30°C after which I added 20 μl of 10% SDS and 5μl of Proteinase K (10 mg/ml, Sigma). The samples incubated at 37°C for 30 minutes. After adding 12 μl of 5M NaCl and 5 μg yeast total RNA to each sample, I extracted the samples with phenol and chloroform. After ethanol precipitation of the samples, I resuspended each in 7 μl of running buffer and performed polyacrylamide gel electrophoresis as described above.

Plasmid Preparation: To produce milligram quantities of plasmid, I used 1.5 liter overnight cultures of 5 ml saturation cultures derived from the selected clones. Plasmids were isolated by alkaline lysis, followed by ethanol precipitation, lithium chloride precipitation, and isopropanol precipitation. Following treatment of the DNA with heat killed ribonuclease A, I performed an additional precipitation with 13% polyethylene glycol, followed by phenol chloroform extraction and isopropanol precipitation in the presence of 1.25M NH_4Ac. After resuspension in TE, I added 100 μg of proteinase K and 1/10 volume of 10X SET to each plasmid prep. After an additional phenol/chloroform extraction and ethanol precipitation, I resuspended the DNA in 250 μl of TE, added 140 μl of 7.5M NH_4Ac, and removed the sample to a darkened room. After adding 15 μl of a 10 mg/ml solution of ethidium bromide, I performed a phenol/chloroform extraction. This was followed by a chloroform extraction, a final ethanol precipitation, resuspension in TE to a concentration of about 1-5 μg/μl, and finally, storage at 4°C.

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Dideoxy Sequencing: To sequence segments of the plasmids starting in the Bluescript sequence and spanning 200-300bp of the insert, I used the Sequenase system. In brief, I added 2-5 μg of plasmid DNA in 6 μl to 1 μl of DMSO and 1 μl of T3 primer (20 ng/μl). This mixture was heated to 95°C for 3 minutes, and placed on dry ice for 5 minutes, thawed, and then quickly centrifuged. I then added 2 μl of 5X Sequenase annealing buffer. After the reactions sat for 20 minutes at room temperature, I added 6 μl of reaction buffer containing 1 μl of 0.1M DTT, ^{35}S-dATP, 2 μl of a 1:7 dilution of Sequenase, and 2 μl of a 1:5 dilution of Sequenase labeling mix. After an additional 5 minutes at room temperature. I added 3.5 μl of the reaction mix to four tubes each containing three deoxynucleotides and either dideoxy A ,G, C or T. After 5 minutes, 4 μl of formamide-containing "stop dye" was added and the reactions were stored at -20°C prior to electrophoresis on a 5% acrylamide gel. The gel was placed on filter paper, dried, and exposed to Kodak XAR-5 X-ray film overnight.

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2-1 Oligonucleotide Sequences

β1

Upstream sequence: GAATTCCTTCTCTCTTTTCCTGTGATGGTT
26-49, EcoR1 at 5' end, SENSE
Downstream: GGATCCAATGGGATTCCAGAATATGAA
290-310, BamH1 at 5' end, ANTISENSE

β2

Upstream sequence: gagact**AAGCTTATTTGGTCATTTCCCTTAATAATC**
34-57, HindIII at 5' end, SENSE
Downstream: gagact**GGATCCCTTTGTCACTCCCGTGACTGCA**
597-619, BamH1 at 5' end, ANTISENSE

β3

Upstream sequence: GAATTCCATCTTCTCGGCCCCGGTGCTGGTG
34-57, EcoR1 at 5' end, SENSE
Downstream: GGATCCATTCCTGGAGACCAGACGGTGCTC
644-667, BamH1 at 5'end, ANTISENSE

γ1

Upstream sequence: gagact**AAGCTTATGGGTTCTGGGAAAGTCTTCCTT**
1-24, HINDIII at 5' end, SENSE
Downstream:
gagact**GGATCCCTTCCATCACTCCATATCCGCAGCAGGCG**
496-524, BamH1 at 5'end, ANTISENSE

δ

Upstream sequence: gagact**CTCGAGATGGACGTTCTGGCTGGCTG**
1-20, Xho1 at 5' end, SENSE
Downstream: gagact**GGATCCATTCCTGGAGACCAGACGGTGCTC**
480-506, BamH1 at 5'end, ANTISENSE

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2-2a

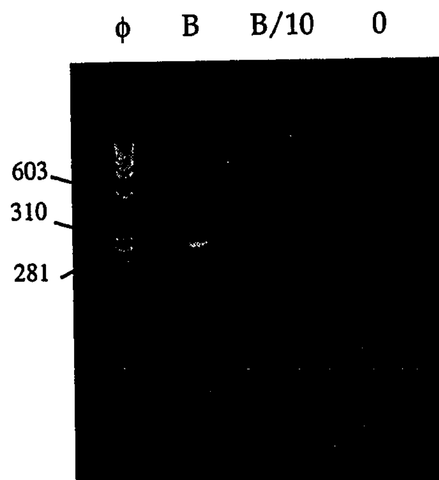

Figure 2-2a shows 284 base PCR product for the β1 subunit of the GABA-A receptor. B, cerebellar cDNA; B/10; tenfold dilution of cerebellar cDNA; 0, no DNA; ϕ, Hae II digest of ϕx-174 DNA used as marker. 1.5% agarose gel stained with ethidium bromide.

2-2b

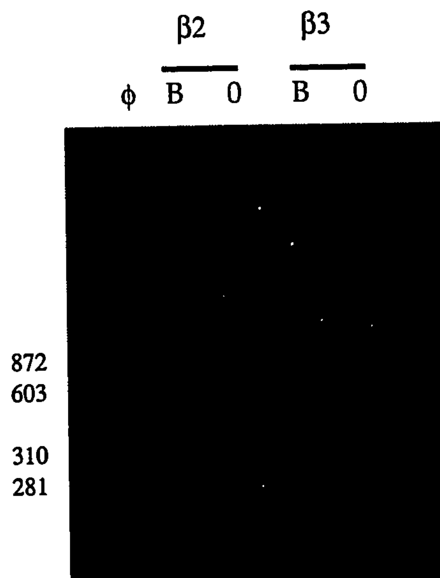

Figure 2-2b shows primary PCR products for the β2 and β3 subunits of the GABA-A receptor. B, cerebellar cDNA; B/10; tenfold dilution of cerebellar cDNA; 0, no DNA; ϕ, Hae II digest of ϕx-174 DNA used as marker. 1.5% agarose gel stained with ethidium bromide. For β2, the PCR product is 584 bases and for β3 it is 633 bases.

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2-3

β1

M P 10 5 1 0 0 B B

β3

M P 10 5 1 0 0 B B

Figure 2-3 shows ribonuclease protection assays for the β1 and β3 subunits with probes generated by linearized plasmid DNA bearing inserts putatively encoding segments of the β1 and β3 subunits of the GABA-A receptor. Protected bands (arrows) are at 284 and 268 bases respectively. M, Msp markers: P, undigested riboprobe: 10, 5, 1; 10pg, 5 pg, and 1pg of sense strand hybridized with riboprobe: 0=5 μg yeast total RNA hybridized with riboprobe: B, 1μg cerebellar total RNA hybridized with riboprobe. Assays were run on 4.5% acrylamide gels, dried, and exposed to XAR-5 film at -70°C with an intensifying screen for 48 hours.

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2-4a

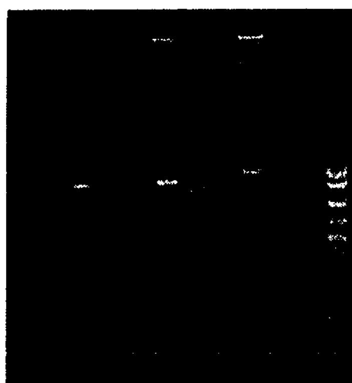

Figure 2-4a shows primary PCR products generated from brain cDNA by oligonucleotides directed at 5' and internal regions of mRNAs for the β2, γ1, and δ subunits of the GABA-A receptor run on a 1.5% agarose gel stained with ethidium bromide. M, Hae III digest of ϕx-174 markers; B, brain cDNA; 0, no DNA in PCR reaction. PCR products generated for β2, γ1, and δ subunits are 578 bp, 524 bp, and 506 bp respectively.

2-4b

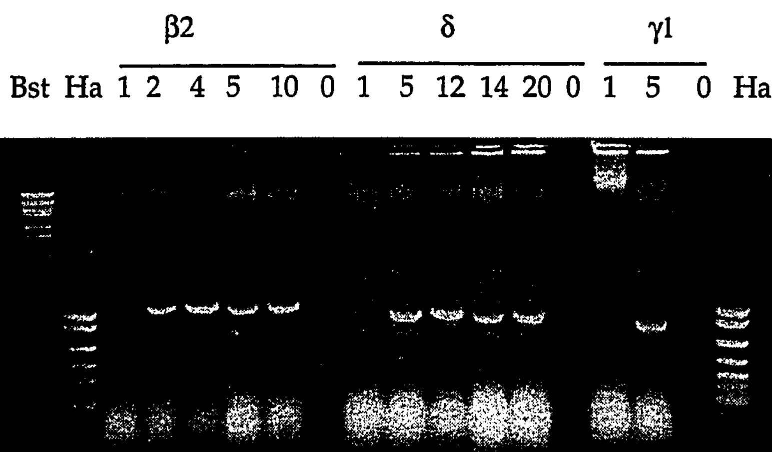

Figure 2-4b shows a PCR based screen for inserts encoding segments of GABA-A receptor subunit genes. T7 and upstream internal oligos were used in a 30 cycle reaction with material from colonies picked from agar plates. These PCR products were subsequently used to direct riboprobe transcription from the T7 promoter. Bst, BstE II marker; Ha, Hae III Φx 174 digest; 0, oligonucleotides and no DNA added; numbers at top denote colonies selected for screening.

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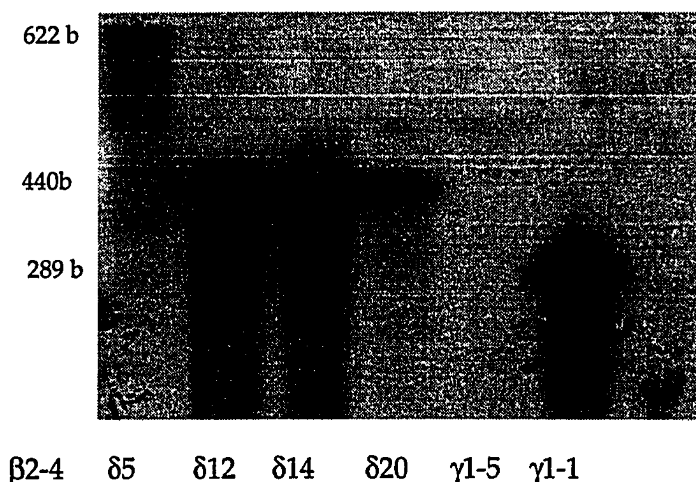
2-5a



2-5b

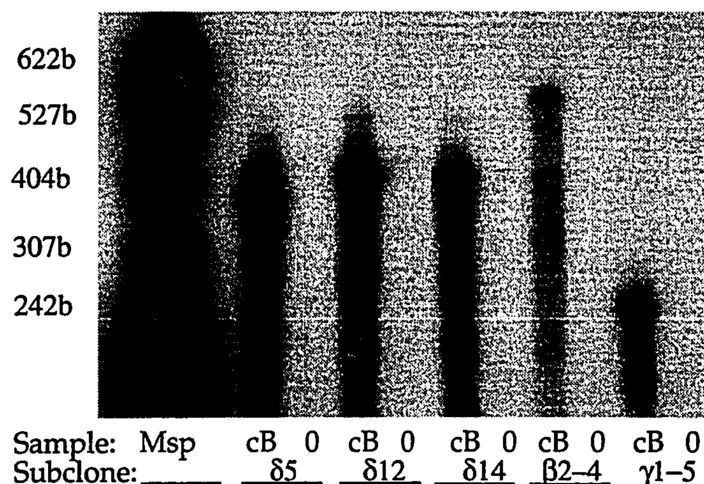


Figure 2-5a shows riboprobes transcribed from PCR products encoding the candidate cDNA clones for segments of the β2, δ and γ1 subunits of the GABA-A receptor. Msp; Msp-1 digest of pBR322 DNA used as a marker. Apparently unlinearized β2 template generated a 622 base probe. Three of four Eco RV linearized δ subclones screened generated probes of 440 bases as predicted. For γ1, one of two linearized subclones screened generated the predicted 289 base probe. The autoradiogram was a five hour exposure, with intensifying screen at -70°C on XAR 5 film.

Figure 2-5b shows ribonuclease protection assays conducted with the riboprobes generated from PCR products. Msp, Msp marker; cB, probes hybridized against 5 μg cerebellar total RNA; 0, probes hybridized against 5 μg yeast total RNA. All subclones tested appear to recognize bands of the predicted sizes (578 for β2, 244 for γ1, and 396 for δ) with the exception of δ5 which appears to protect a doublet with the major component smaller than the predicted size. The autoradiogram was an overnight exposure, with intensifying screen at -70°C on XAR 5 film.

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2-6

Figure 2-6 shows sequences obtained by dideoxy sequencing for segments of GABA-A receptor subunits confirming their identity. Portions of sequence that include PCR oligonucleotides are underlined. Segments of subclones that are Bluescript are in italics. Sequences are numbered so that ATG starts at +1. Predicted amino acid sequence is given to aid in correlation with published sequences. All sequences were found to be identical with published sequences (Shivers et al., 1989; Ymer et al., 1989; Ymer et al., 1990).

β1

Bluescript EcoR1 29
atcgataagcttgatatc<u>*gaa tt c* ctt ctc tct ttt cct gtg atg gtt</u> gcc atg gtt tgt
L L S F P V M V A M V C

tgt gca cac agc tcc aat gaa ccc agc aac atg tca tac gtg aaa gag aca gtg gac
C A H S S N E P S N M S Y V K E T V D

cga ctg ctc aaa gga tat gac att cgc ttg cgg cca gac ttt gga ggg ccc ccg gtg
R L L K G Y D I R L R P D F G G P P V

gac gtc ggg atg cgg atc gat gtc gcc agc ata gac atg gtc tcg gaa gtg aat
D V G M R I D V A S I D M V S E V N

263
atg gat tat aca ctc acc atg tat ttt
M D Y T L T M Y F

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2-6 (continued)

β2

HindIII 34
taag ctt att tgg tca ttt ccc tta ata atc gcc gct gtc tgt gct cag agt gtc aat
L I W S F P L I I A A V C A Q S V N

gac cct agt aat atg tcg ctg gtt aaa gag acg gtg gtg gac aga ctg ttg aaa ggc
D P S N M S L V K E T V V D R L L K G

tat gac att cgt ctg aga cca gat ttc gga ggt ccc cct gtg gca gta gga atg aac att
Y D I R L R P D F G G P P V A V G M N I

EcoRV (221) 275
gat atc gcc agc atc gat atg gtt tct gaa gtc aat atg gac tac acc ttg acc atg
D I A S I D M V S E V N M D Y T L T M

β3

82
ta aac gac ccc ggg aac atg tcc ttt gtg aag gag acg gtc gac aag ctg ttg aaa
N D P G N M S F V K E T V D K L L K

ggc tac gac att cgc ctg aga ccg gac ttc ggg ggt ccc cca gtc tgc gtg ggg atg
G Y D I R L R P D F G G P P V C V G M

aac atc gac atc gcc agc atc gac atg gtt tct gaa gtc aac atg gat tat acc tta
N I D I A S I D M V S E V N M D Y T L

298 BamH1 *Bluescript*
act atg tat ttc caa caa tat tgg aga gat aaa agg ctc ccc tac tct **gg***gatc cac ta*
T M Y F Q Q Y W R D K R L P Y S G

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2–6 (continued)

γ1

HindIII 4
gctt atg ggt tct ggg aaa gtc ttc ctt ttc tct cct tcc ctc ctg tgg agt caa act
M G S G K V F L F S P S L L W S Q T

aga gga gtg agg ttg ata ttc ttg tta cta acc ctg cat ctg gga aac tgc att gat
R G V R L I F L L L T L H L G N C I D

aaa gca gat gat gaa gat gat gaa gat tta act atg aac aaa aca tgg gtc ttg gca
K A D D E D D E D L T M N K T W V L A

EcoRV (187) 221
cct aaa att cat gaa gga **gat atc** aca cag att ctc aac tct tta ctt caa ggc
P K I H E G D I T Q I L N S L L Q G

δ

40
ctg ggc tgg ctg ctg ctg ccg ctc ctt ctg ctg tgc acg cag ccg cac cat ggc gcc
L G W L L L P L L L L C T Q P H H G A

EcoRV (110)
aga gca atg aat gac att ggg gac tac gtg ggc tcc aac ctg gag **ata tcc** tgg ctc
R A M N D I G D Y V G S N L E I S W L

ccc aac ctg gat gga cta atg gag ggc tac gcc cga aac ttc cga cca ggc att gga
P N L D G L M E G Y A R N F R P G I G

258
ggt cct cca gtg aat gtg gcg ctt gcc cta gag gtg gcc agc att gac
G P P V N V A L A L E V A S I D

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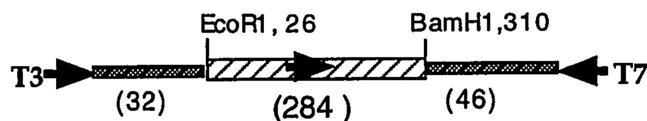
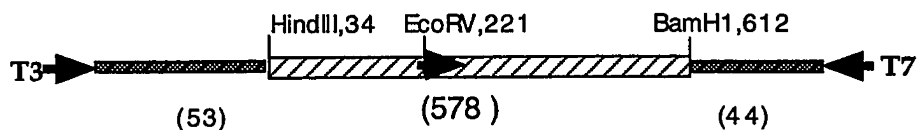
2-7a Maps of PCR generated GABA-A receptor subunit Subclones in Bluescript SK and KS

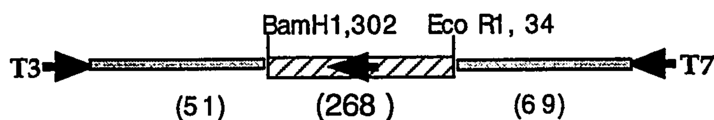
β1



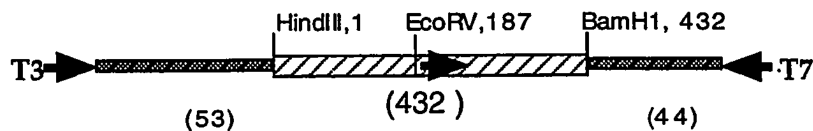
β2



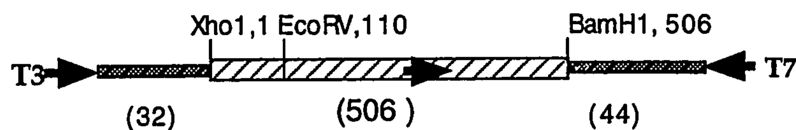
β3



γ1



δ



Numbers for restriction sites assume ATG=1
(see text for references)

GABA-A receptor subunit inserts

Bluescript KS +

Bluescript SK +

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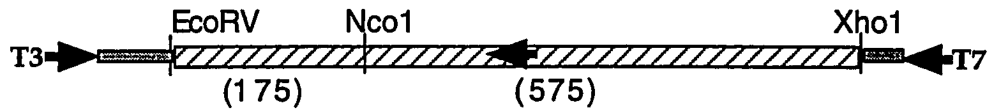
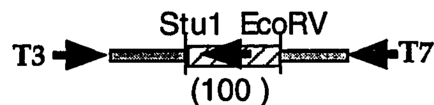
2-7b Maps of GABA-A receptor subunit Plasmids in Bluescript SK

α1



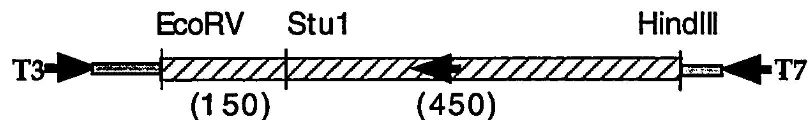
Linearized with Nco1, T3 probe covers 175 nucleotides

α2



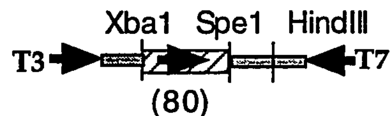
Linearized with EcoRV, T3 probe covers 100 nucleotides

α3



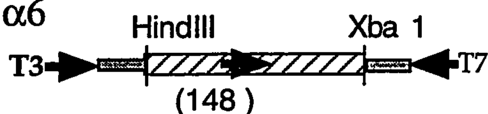
Linearized with Stu1, T3 probe covers 150 nucleotides

α5



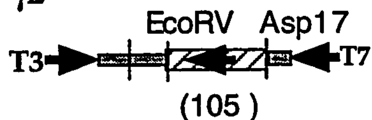
Linearized with Xba1, T7 probe covers 80 nucleotides

α6



Linearized with Xba1, T7 probe covers 148 nucleotides

γ2



Linearized with Asp 17, T3 probe covers 105 nucleotides of long form and 70 nucleotides of short form

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CHAPTER THREE:
MOLECULAR CHARACTERIZATION OF PITUITARY GABA-A RECEPTOR mRNAS IN THE RAT

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INTRODUCTION

The studies described in this chapter have two sets of reciprocal goals. One is to better understand GABAergic regulation of pituitary neurosecretory cells by means of a detailed investigation of the complement of mRNAs encoding GABA receptors. The second is to shed light on the uses and consequences of GABA receptor subunit heterogeneity, by examining that heterogeneity in a somewhat simplified preparation. In the brain, different types of GABA/benzodiazepine receptor bearing cells are impossible to separate from one another for biochemical studies, making it difficult to correlate pharmacological and physiological properties with subunit composition *in vivo*. In contrast, in the lactotrophs of the anterior lobe and the melanotrophs of the neurointermediate lobe, the pituitary contains functionally distinct, anatomically separable populations of cells expressing GABA/benzodiazepine receptors.

The problem here, then, is to establish to which, if any, of the known GABA-A receptor mRNAs are used in the pituitary. How many kinds of receptor mRNAs are present? Is the subunit composition simple or complex?

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Do the receptors seem to be encoded by a collection of subunit mRNAs similar to what might be observed in the brain?

The presence of GABA-A receptors in the pituitary has been inferred in the past based on the binding of GABAergic and benzodiazepine ligands to pituitary tissue, and on the actions of GABA ligands on neurosecretion (Locatelli et al., 1979; Taraskevich and Douglas, 1982; Anderson and Mitchell, 1984; Anderson and Mitchell, 1984; Anderson and Mitchell, 1985). The experiments described in this chapter establish in both the anterior and the neurointermediate lobes, the presence of mRNA encoding the GABA-A receptor subunits, the identity of the subunits encoded, and the relative amounts of the various species of mRNA.

At this point, it is useful to review some of the issues raised by GABA-A receptor subunit heterogeneity. As discussed in the introductory chapter, there are at least 14 different GABA-A receptor subunits. Hypotheses about how these are arranged into receptors have been guided by several considerations. The first is that a one to one stoichiometric relationship of alpha and beta subunits was identified in early investigations (Sieghart, 1989). Another is the observation that a gamma subunit must be present in order for GABA-A receptors to respond to benzodiazepines (Pritchett et al., 1989). Conversely, brain regions low in benzodiazepine binding have been observed to express the delta subunit (Shivers et al., 1989). Since the presumed structure of the GABA-A receptor is pentameric, based on analogy to the nicotinic acetylcholine receptor, a stoichiometry of $\alpha2\beta2N$ has been proposed.

If *in vivo,* functional receptors could result from all of the possible combinations of subunits, there would be several hundred different kinds of GABA-A receptors. Indeed, identifying what are "real" in-vivo configurations

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of the GABA-A receptor subunits has been one of the major objectives of investigators in this field. Approaches vary from attempts to reconstitute known functional properties of *in vivo* receptors, to attempts at co-localization of subunit genes, to more rigorous biochemical attempts to demonstrate subunit association by immunoprecipitation of linked subunits (Duggan et al., 1991; Duggan et al., 1992; Pollard et al., 1993).

Some indication of GABA-A receptor heterogeneity in the pituitary has been provided by early studies employing PCR. Expression of $\alpha1$, $\beta1$, $\beta3$, and $\gamma2s$ subunit mRNAs has been reported in the anterior lobe, using a polymerase chain reaction (PCR) based technique (Valerio et al., 1992). Another group has reported the presence of trace amounts of the $\gamma2l$ subunit mRNA, also using a PCR technique (Criswell, 1993).

The polymerase chain reaction is of great utility in detecting mRNA of low abundance, but as applied in the above studies without the use of standards for quantitation, it is of limited use in establishing relative quantities of various subunit mRNAs. This might lead to equation of major and very minor components of the population of receptor subunit mRNAs. Further, PCR would amplify signals in the anterior lobe which are the result of cross-contamination from the neurointermediate lobe and *vice-versa*. To obtain a more representative and complete view of GABA-A receptor composition in the pituitary, we have used ribonuclease protection assays to obtain quantitative measurements of the mRNA for eleven of the known $GABA_A$ receptor subunits in both the anterior lobe and the neurointermediate lobe.

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EXPERIMENTS AND RESULTS

Ribonuclease protection assays demonstrated a marked difference in alpha subunit gene expression in the anterior and neurointermediate lobes as seen in Figure 3-1; the anterior lobe contained only $\alpha 1$ mRNA while the neurointermediate lobe contained only $\alpha 2$ and $\alpha 3$ mRNAs. Messenger RNA for $\alpha 5$ and $\alpha 6$ were not found in either tissue as shown in Figure 3-2. The $\alpha 5$ mRNA, however, was found in hippocampal total RNA. Finally, the $\alpha 6$ mRNA was found in cerebellum but not in any other brain region, consistent with previous findings (Luddens et al., 1990).

Messenger RNA for all three beta subunits was detected in anterior lobe, as seen in Figure 3-3. In the neurointermediate lobe only $\beta 1$ and $\beta 3$ were detected at high levels, and $\beta 2$ was detected at a very low level. In contrast, all three subunits were detected in the cerebellum, with $\beta 2$ being by far the most abundant species. Both anterior and neurointermediate lobe expressed $\gamma 1$ and $\gamma 2$ subunit mRNAs, shown in Figure 3-4. In both tissues, the $\gamma 2$ subunit mRNA was detected only in the $\gamma 2s$ form. The $\gamma 2l$ form, however, was detected as the major species of $\gamma 2$ mRNA in both cerebellum and cerebral cortex. As shown in the right panel of Figure 3-4, mRNA for the δ subunit is not detectable in the pituitary. Consistent with previous findings (Shivers et al., 1989), however, the δ subunit was detected in our assays in cerebral cortex.

For all assays, quantitative analysis was performed by measuring the amount of radioactive antisense probe protected by known amounts of sense strand RNA. A typical standard curve appears in Figure 3-5, showing a linear signal over a range spanning from 150 fg to 5 pg of RNA. The detection limits

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of the ribonuclease protection assays were all in the range of .05 amol of mRNA per μg total RNA with the exception of the assays for β2, γ1, and δ mRNAs. These assays used larger probes and therefore gave a stronger signal resulting in sensitivities up to 0.025 amol/μg total RNA.

Quantitative analysis shows that the abundance of GABA-A receptor subunit mRNAs on the basis of amol/μg total RNA is generally greater in cerebellum than in either pituitary location. The exact basis for comparison is developed further in the discussion which follows. To compare levels of subunit mRNA expression between pituitary sites requires some analysis of cellular composition of the anterior pituitary which also appears in the discussion below.

The alpha and beta subunit mRNAs expressed in neurintermediate lobe, and the beta subunit mRNAs expressed in anterior lobe, are present in quantities which do not vary by much more than a twofold range. Notably, the γ1 and γ2 subunit mRNAs are expressed in the neurointermediate lobe in the same quantities. In the anterior lobe, however, the level of γ1 mRNA is forty times lower than the level of γ2 mRNA, and we cannot rule out the possibility that γ1 mRNA detected in the anterior lobe could come from slight contamination by neurointermediate lobe tissue. All quantitative measurements of GABA-A receptor subunit mRNAs are summarized in Figure 3-6. The overall subunit mRNA distribution is summarized schematically in Figure 3-7.

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DISCUSSION

Abundance of Receptor mRNAs

Individual $GABA_A$ receptor subunit genes are expressed in the pituitary with an abundance in the range of 1-15 amol mRNA per µg of total RNA and in the cerebellum in the range of 3-80 amol mRNA per µg of total RNA. (A typical mRNA species of 1.5kb found at a level of 10 amol/µg total RNA would occur in the range of 5pg/µg total RNA.) Assuming that there are 200,000 cells per intermediate lobe, and that the intermediate lobe yields on average about 4 µg of total RNA (Autelitano, 1990), we estimate that there are an average of about 200 copies of alpha subunit mRNA, 35 copies of beta subunit mRNA, and 80 copies of gamma subunit mRNA per cell. It has been estimated that the lactotrophs contribute about 10% of $2x10^6$ anterior pituitary cells in a male rat, (Costoff, 1973), and one can extract on average 30 µg of total RNA from the anterior lobe. (Autelitano, 1990) Using these parameters, we estimate that in the anterior lobe there are 300 copies of alpha subunit mRNA, 600 copies of beta subunit mRNA, and 250 copies of gamma subunit mRNA per cell expressing $GABA_A$ receptors.

In any sample of brain tissue, there are large numbers of cell types which differ in the kind of GABA-A receptors they express, or whether they express GABA-A receptors at all. This complexity precludes the possibility of estimating copy numbers per cell for the various mRNAs observed in our cerebellar samples. It is possible, however, to make some pharmacological predictions based on comparison of pg of message per µg of total RNA assayed in brain and pituitary. There can be a high degree of variation in which

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individual subunit mRNAs from the alpha, beta, or other classes of receptors are expressed in any given location. Therefore, comparisons between tissues of the expression of a particular subunit mRNA cannot be expected to predict the relative numbers of receptor sites in those locations. A better comparison can be made between the total mRNAs for a given class of subunits in the two tissues.

On the basis of copies per cell, it is apparent that the lactotrophs express GABA-A receptor mRNAs at a much higher level than do the intermediate lobe melanotrophs. The greatest difference is observed in the beta subunits for which there about about fifteen times as many copies per cell in the anterior lobe as in the intermediate lobe.

If the abundance of mRNA for a particular subunit class can be related to the number of receptor sites, then one would predict a higher density of receptor sites per cell in the anterior lobe. On the basis of pg per μg total RNA, the expression of GABA receptor mRNAs in cerebellum is fifteen to thirty times greater than in either pituitary tissue. Once again, one would predict a higher density of receptor sites in brain than in pituitary. The accuracy and potential significance of these predictions will be discussed in correlation with pharmacological data in the next chapter.

Heterogeneity and Tissue Specificity

Heterogeneity is exhibited with respect to gamma subunit expression in the pituitary. The intermediate lobe, in particular, expresses a significant amount of γ1 subunit message in addition to γ2. Indeed, it is one of small number of locations inside or outside the central nervous system where γ1

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subunit gene expression has been observed on par with that of γ2 (Wisden et al., 1992). The major pharmacological characteristics conferred on GABA-A receptors by the γ1 subunit are a heightened response to neurosteroids (Puia, 1994) and a blunted response to the benzodiazepine inverse agonists, β-carbolines (Ymer et al., 1990). In one study, transfecting cDNA constructs for the γ1 and α2 subunits actually reversed the effects of βCCM and DMCM, resulting in an *agonist* effect at the benzodiazepine site (Puia et al., 1991). These properties would imply that the intermediate lobe contains three separate populations of GABA-A receptors, roughly equal in abundance, which are inhibited, stimulated, or not affected by β-carbolines.

Also of relevance to β-carboline pharmacology is what is perhaps the most striking aspect of these results -the apparently absolute segregation of α1 versus α2 and α3 transcripts in the anterior and intermediate lobes. As stated in the introductory chapter, previous investigations have correlated the presence of these subunits with Type I and Type II benzodiazepine sites, respectively. The implications and presence or absence of Type I and Type II benzodiazepine sites will be discussed in relation to pharmacological studies described in the following chapter, and at that point I will return to the potential role of these compounds in melanotroph function.

Another potential consequence of differential expression of GABA-A receptor subunit genes in the pituitary might be in selectivity for the neurosteroid compounds which act on the GABA-A receptor. Given the roles of lactotrophs and melanotrophs in neuroendocrine regulation, the possibility that GABA-A receptors on these cells might respond differently to corticosteroid, progesterone, and pregnenolone derivatives is indeed an attractive one from a physiological standpoint. A small body of evidence exists

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that this may be the case. In one experiment, the neurosteroid 3 alpha-hydroxy dihydroprogesterone (3αOHDHP) enhanced flunitrazepam binding to cells transfected with α3 subunit cDNAs with more than twice the efficacy of enhancement of binding to cells transfected with α1 cDNAs (Lan et al., 1991). Another group demonstrated that the same steroid was twice as effective in enhancing chloride currents in oocytes injected with mRNAs encoding the α1 subunit as it was on oocytes injected with the mRNA encoding α2 or α3 subunits (Shingai et al., 1991). These apparently contradictory results point to the need to examine the effects of subunit composition and cellular context on neurosteroid response in native receptors. The pituitary, with its well separated population of cells with GABA receptors encoded by α1 versus α2 and α3 may be a good setting for such studies. Based on current information, one might expect that the presence of α1 subunit mRNA in the anterior lobe would result in a population of GABA-A receptors of uniform high sensitivity to 3αOHDHP. In contrast, the presence of γ1 subunits would be predicted to confer a high sensitivity to 3αOHDHP to a portion of the α2 and α3 containing receptors of the intermediate lobe, but those containing a γ2 subunit would have a lower responsiveness. It should be noted, however, that the relative sensitivities of the various subunits to other neurally active steroids such as pregnenolone sulfate, dehydroepiandrostane sulfate, or THDOC has not been examined to date. Thus the relative sensitivities of pituitary GABA-A receptor sites to steroids other than 3αOHDHP may be quite different from the predictions which are made here.

The relationship of alpha subunit composition to the efficacy and affinity of GABA is complex, and seems to depend greatly on the combinations of alpha and beta subunits present. Indeed, our finding of multiple beta

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subunit mRNAs in both anterior and intermediate lobes is probably as relevant as variations in the distribution of alpha subunit mRNAs to questions about GABA-A receptor efficacy and affinity. The relative abundance of β1 and β3 subunit mRNAs as compared to β2 mRNA is in contrast to the situation in most adult brain regions (Ymer et al., 1989; Wisden et al., 1992) and may mean that pituitary GABA-A receptors have somewhat higher affinities for GABA than receptors in the brain (Sigel et al., 1990). This may be a consequence of their form of innervation which is diffuse in the anterior pituitary and mixed in the intermediate lobe. Finding multiple beta subunit mRNAs in each location implies either that they encode receptors having multiple affinity sites for GABA on single cells, or that there is microheterogeneity with respect to GABA affinity within melanotroph and lactotroph populations.

The possibility of microheterogeneity within the intermediate lobe is supported by in-situ hybridization and histochemical studies which have demonstrated varying levels of proopiomelanocortin synthesis and storage in subpopulations of melanotrophs (Chronwall, 1988).

MATERIALS AND METHODS:

Animals and tissues: I obtained frozen (-70°C) pituitaries from intact male Sprague Dawley rats three months of age or older from Rockland Inc. Batches of 30 were thawed in phosphate buffered saline (1XPBS), pH 7.2 on ice. I used

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sharp forceps to separate neurointermediate and anterior lobes on an ice cold stage under a dissecting microscope. I then rinsed the tissues in buffer, and immediately froze them on aluminum foil over dry ice for storage at -70°C until further processing. Frozen tissues were used because of the large numbers of pituitaries required to carry out binding experiments and experiments in which low abundance mRNAs were detected. For some ribonuclease protection assays I used fresh tissues from male Sprague Dawley rats three months of age. In these instances, I separated neurointermediate and anterior lobes immediately following removal of the pituitary after sacrifice by decapitation. I transferred these to foil over dry ice and then stored the frozen pituitary lobes at -70°C.

RNA extraction: To extract cytoplasmic RNA I used the NP-40/sodium deoxycholate method as described in the previous chapter. I isolated total cytoplasmic RNA from cerebellum, cortex, hippocampus, and from several pools of 50-150 neurointermediate lobes, and 10-20 anterior lobes. I precipitated the RNA under ethanol, resuspended it at concentrations of 1-3 µg/µl, and stored it as 5 µl aliquots at -70°C until use. Aliquots from each pool of RNA were viewed on 1% agarose or agarose/acrylamide composite gels and found to be intact as indicated by the presence of 28S and 18S bands in a 2:1 ratio and no RNA visible below the tRNA band.

Ribonuclease protection assays: Riboprobes were synthesized as described in the previous chapter. I added aliquots of 5 µl of frozen total pituitary or brain RNA or 5 µl of sense strand standards to 20 µl of 80% formamide hybridization

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buffer and 5 μl containing 250 fg to 1 ng of high-specific activity probe (0.5 to 1.2 x 10^6 CPM/ng). I then heated the samples to 80°C for 1-2 minutes and let them hybridize overnight at 45°C. The following day, I added 300μl of a solution containing ribonuclease A (2μg/ml) (Sigma) and ribonuclease T1 (40μg/ml) (Sigma) in buffer (10mM Tris-HCl, 3mM EDTA, 300mM NaCl) to each sample and all samples were incubated for one hour at 30°C. I stopped the digestion by adding 20 μl of 10% sodium dodecyl sulfate and 10 μl of a 10mg/ml solution of proteinase K (Sigma). After 15 minutes at 37°C, I extracted the samples with phenol/chloroform and then added 30 μl of 7.5M NH_4OAc and 2.5 volumes of ethanol. Following 1-2 hours at -20°C, the samples were precipitated with a 15 minute spin at 15000xG on a microcentrifuge and washed twice with 70% ethanol. I dried the samples in a Speed Vac and resuspended them in 5 ml of loading buffer. Samples ran on 4.5% acrylamide gels at 180 V for two hours. The gels were vacuum dried and exposed to XAR-5 film overnight at -70°C with an intensifying screen. To quantify the protected bands, I placed the gels in Phosphorimager cassettes for 12 hours and scanned the cassettes on the Molecular Dynamics Phosphorimager system. Quantification of messenger RNA in samples for each subunit was based on comparison of detected units from protected bands to signals from standard curves using known quantities of sense strand RNAs for the appropriate probe.

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3-1

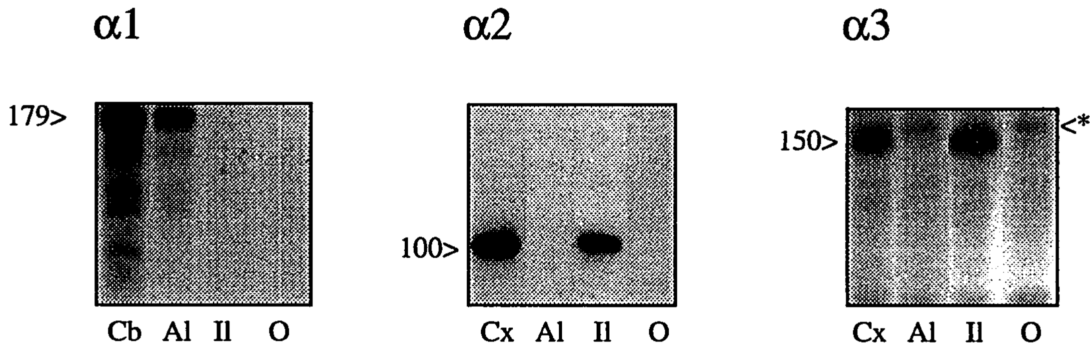

Figure 3-1 shows ribonuclease protection assays in various tissues for alpha subunits of the GABA-A receptor. Note the absence of α1 subunit in the intermediate lobe and absence of α2 and α3 subunits in anterior lobe. All assays used 5-10 μg total RNA. Symbol "<*" denotes a probe artifact. Cb, cerebellum; Cx, cerebral cortex; Al, anterior pituitary; Il, neurointermediate lobe; 0, yeast total RNA. 4.5% acrylamide gels were exposed for 12-24 hours to XAR-5 film with an intensifying screen at -70°C or were exposed to a phosphor storage screen for 6-12 hours and then scanned using a Molecular Dynamics Phosphorimage system. Autoradiograms and phosphorimage scans are reproduced here using a Microtek Scanner and Adobe Photoshop.

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3-2

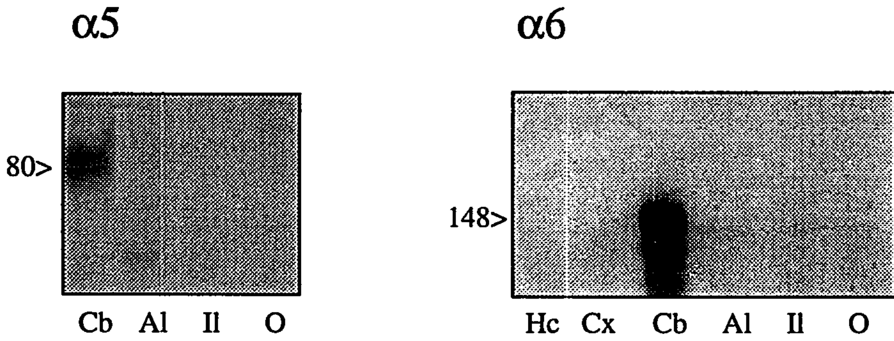

Figure 3-2 shows ribonuclease protection assays in various tissues for α5 and α6 subunits of the GABA-A receptor. Note the absence of both subunits in pituitary tissues, and the specific expression of α6 in cerebellum. All assays used 5-10 μg total RNA. Cb, cerebellum; Cx, cerebral cortex; Hc, hippocampus; Al, anterior pituitary; Il, neurointermediate lobe; 0, yeast total RNA. 4.5% acrylamide gels were exposed for 24-48 hours to XAR-5 film with an intensifying screen at -70°C or were exposed to a phosphor storage screen for 6-12 hours and then scanned using a Molecular Dynamics Phosphorimage system. Autoradiograms and phosphorimage scans are reproduced here using a Microtek Scanner and Adobe Photoshop.

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3-3

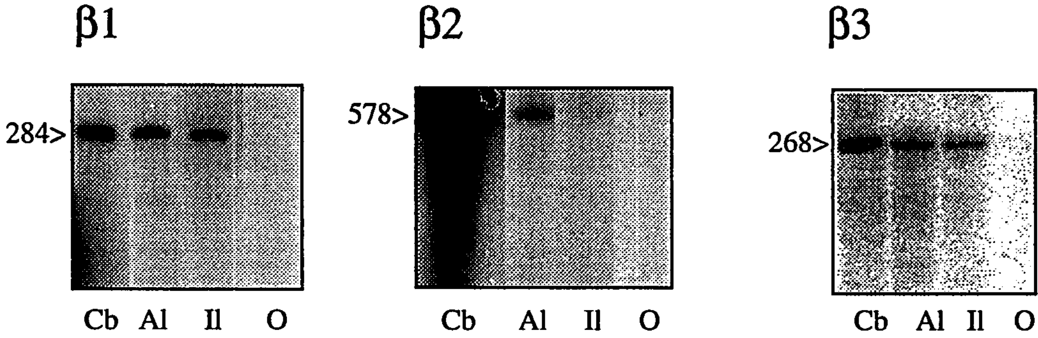

Figure 3-3 shows ribonuclease protection assays in various tissues for beta subunits of the GABA-A receptor. Beta subunits are expressed at roughly equal levels in all tissues except that β2 is by far the most abundant beta subunit in cerebellum and is nearly absent in the intermediate lobe. All assays used 5-10 μg total RNA. Cb, cerebellum; Al, anterior pituitary; Il, neurointermediate lobe; 0, yeast total RNA. 4.5% acrylamide gels were exposed for 12-24 hours to XAR-5 film with an intensifying screen at -70°C or were exposed to a phosphor storage screen for 6-12 hours and then scanned using a Molecular Dynamics Phosphorimage system. Autoradiograms and phosphorimage scans are reproduced here using a Microtek Scanner and Adobe Photoshop.

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3-4

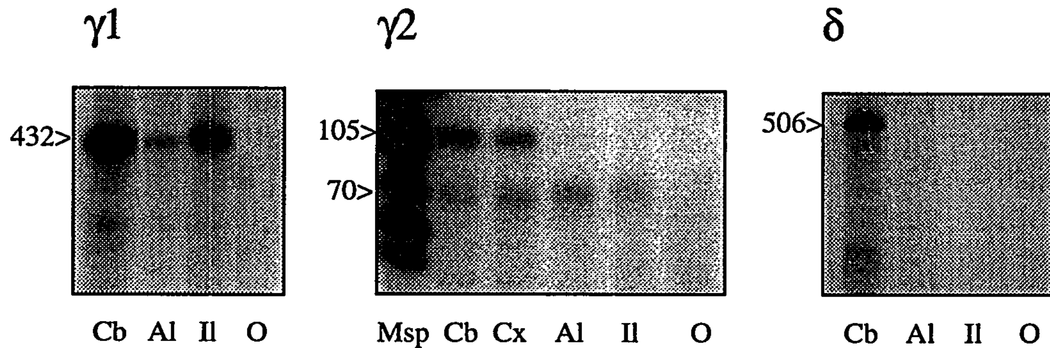

Figure 3-4 shows ribonuclease protection assays in various tissues for gamma and delta subunits of the GABA-A receptor. Note expression of the γ1 subunit in the intermediate lobe and absence of δ subunit in both pituitary tissues. Pituitary tissues are seen to express only the short form of the γ2 subunit while the long form predominates in cerebellum. All assays used 5-10 μg total RNA. Msp, Msp digest of pBR322 DNA. Cb, cerebellum; Al, anterior pituitary; Il, neurointermediate lobe; 0, yeast total RNA. 4.5% acrylamide gels were exposed for 12-24 hours to XAR-5 film with an intensifying screen at -70°C or were exposed to a phosphor storage screen for 6-12 hours and then scanned using a Molecular Dynamics Phosphorimage system. Autoradiograms and phosphorimage scans are reproduced here using a Microtek Scanner and Adobe Photoshop.

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3-5

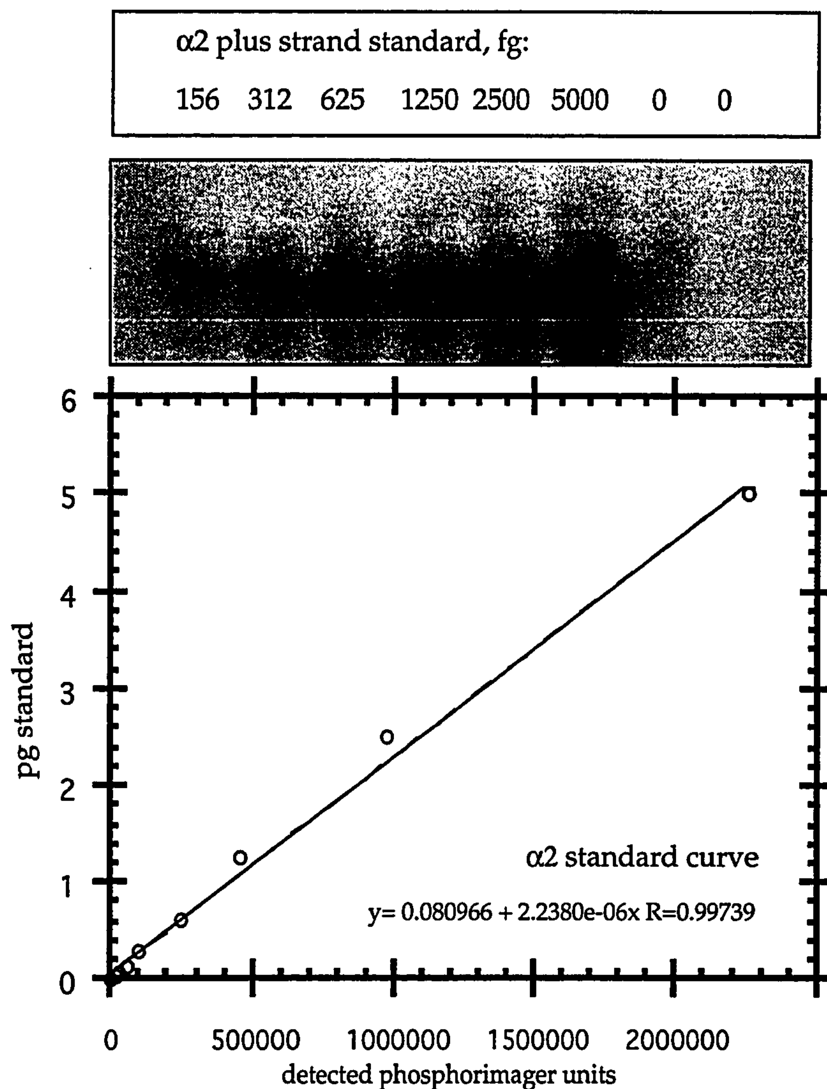

Figure 3-5 shows a typical standard curve obtained for a ribonuclease protection assay, in this case for the α2 subunit, using sense strands synthesized from the same templates used to synthesize antisense riboprobes. A regression coefficient > 0.99 and passage through the y axis at y=0 give evidence of linearity in a standard curve with values spanning from 156 fg to 5 pg of RNA. Inset above is the phosphorimage scan on which the standard curve was based.

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3-6 *Quantification of GABA-A receptor subunit messages in cerebellum and pituitary with estimates of message copies per cell in pituitary.*

Subunit	amol/μg total RNA			Estimated Copies/Cell	
	AP	IL	Cerebellum	AP	IL
α1	3.4±0.9	<0.05*	ND	305	--
α2	<0.05*	13.9±0.9	ND	--	165
α3	<0.05*	5.6±0.7	ND	--	65
β1	2.0±0.3	1.0±0.5	3.0	180	10
β2	0.9±0.7	0.3±0.1	80	80	3
β3	4.0±0.2	2.0±0.2	7.2	360	25
γ1	0.5±.05	3.6±0.2	13.0	45	40
γ2	2.3±0.7	3.7±0.7	40	200	40

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3-7

Distribution of GABA-A receptor subunit mRNAs in the rat pituitary.

AP	IL
$\alpha 1$	$\alpha 2 > \alpha 3$
$\beta 3 > \beta 1 > \beta 2$	$\beta 3 > \beta 1 >> \beta 2$
$\gamma 2s >> \gamma 1$	$\gamma 1 = \gamma 2s$

$\alpha 5$, $\alpha 6$ and δ mRNA s were not detected in pituitary tissue in assays with detection limits on the order of 0.05 amol/μg total RNA.

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CHAPTER FOUR:
PHARMACOLOGICAL CHARACTERIZATION OF PITUITARY GABA-A RECEPTORS IN THE RAT

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INTRODUCTION

The definition of the molecular composition of two populations of pituitary GABA-A receptors presents the opportunity to correlate observations on subunit mRNA composition and abundance with pharmacological observations. How well are the pharmacological properties of pituitary GABA-A receptors accounted for by the various subunit mRNAs we have observed in these tissues? Do observations on pharmacological properties conferred by specific subunits in oocytes and cell lines also hold true for native receptors encoded by groups of mRNA species which include these subunits? Do the quantitative relationships between the amounts of receptor subunit mRNAs expressed in specific tissues predict the relative numbers of receptor sites in these tissues as measured in binding assays?

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Previous investigators have reported binding sites for muscimol, GABA, and central type benzodiazepines in the pituitary, but have not attempted to determine the ratios of these sites in each tissue. One group conducted two separate studies, one of benzodiazepine binding using flunitrazepam, and one of muscimol and GABA binding. These investigators found high affinity sites for both classes of compounds, but in the neurointermediate lobe, observed more benzodiazepine sites (34 fmol/mg) than muscimol or GABA sites (9 fmol/mg). In the anterior lobe, they obtained the opposite result, finding more total muscimol binding (30 fmol/mg) than central type benzodiazepine binding (8 fmol/mg). Muscimol binding was distributed between high and low affinity sites of 1nM and 30 nM (Anderson and Mitchell, 1984; Anderson and Mitchell, 1984; Anderson and Mitchell, 1986). The significance of such a finding is difficult to interpret, particularly since it is the result of combining two separate studies. There are no known central type benzodiazepine sites which lack muscimol or GABA binding, and the investigators were careful to use competition with unlabeled peripheral benzodiazepine ligands to exclude these sites from their benzodiazepine binding totals. They observed a twentyfold excess of peripheral type, clonazepam insensitive flunitrazepam binding sites over central type benzodiazepine sites. It is possible that the large number of peripheral type sites affected the accuracy of central type measurements, and secondly, that some of the discrepancy arises from the fact that muscimol and benzodiazepine sites were measured in two separate studies, in two different batches of tissue. Since the number of binding sites was given on the basis of fmol/mg of membranes, variations in membrane preparation between batches could significantly effect the density of binding sites observed.

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A second group used autoradiography to investigate the distribution of peripheral and central benzodiazepine sites in the pituitary, and found that the neural lobe contains peripheral sites only while the anterior lobe and the intermediate lobe contain both types of sites (Brown, 1984). This study did not include any attempt at quantitation of binding sites.

To obtain quantitative information about the numbers and kinds of sites for GABAergic ligands, and to correlate this information with our molecular data, we carried out Scatchard and Hill analysis for muscimol and benzodiazepine sites from the same batches of neurointermediate lobe and anterior lobe membranes, derived from the same pools of pituitaries used in our molecular studies. We used a pure central benzodiazepine agonist, Ro15-1788 in order to circumvent problems caused by interference from the peripheral site. As a point of comparison, and to validate our measurements, we carried out parallel measurements in cerebellar membranes.

Based on subunit distribution we predicted that the anterior pituitary would have Type I benzodiazepine sites while the neurointermediate lobe would have Type II sites. To test this prediction, we used a competition assay which measures the ability of the compound CL 218 872 to compete for benzodiazepine sites with the benzodiazepine antagonist Ro15-1788. Ro15-1788 does not discriminate between Type I and Type II sites. In contrast, CL 218 872 is like the β -carbolines which define these two receptor classes in that it has an affinity tenfold higher at Type I sites than at Type II sites.

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EXPERIMENTS AND RESULTS

Neurointermediate lobe, anterior lobe, and cerebellum all displayed high affinity sites for muscimol and Ro15-1788 as seen in binding assays which appear in Figures 4-1, 4-2, and 4-3 respectively. For each tissue, the data are expressed as a simple binding curve, a Scatchard plot from which kd and Bmax were calculated, and a Hill plot, from which the Hill coefficient was calculated. Assays for Ro15-1788 appear in 4-1a, 4-2a, and 4-3a while assays for muscimol appear in 4-1b, 4-2b, and 4-3b.The sites were present in ratios of approximately two muscimol sites for every Ro15-1788 site, with Hill coefficients near unity, as is summarized in tabular form in Figure 4-4.

Affinity and Hill coefficients of pituitary sites were comparable to those observed in cerebellar membranes, but cerebellar membranes had a density of receptor sites more than 20 times greater than that seen in pituitary. The cerebellum seemed to have a lower affinity for muscimol by about threefold, but on close examination, the Scatchard plot shows that about 2/3 of the sites have an affinity comparable to that found in pituitary. The differences in affinity and relative numbers of sites, however, were not sufficient to result in a Hill coefficient less than unity.

CL 218 872 displaced Ro15-1788 from anterior lobe membranes with an inhibition coefficient (K_i) of 150 nM and from neurointermediate lobe membranes with a K_i of 1500 nM. This result indicates that the anterior lobe expresses a Type I benzodiazepine site and that the neurointermediate lobe expresses a Type II benzodiazepine site. The CL 218 872 competition assay is shown in Figure 4-5.

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DISCUSSION

Receptor number and affinity

We found that each intermediate lobe contained 50 mg membrane protein and each anterior lobe contained 300 mg; the concentration of Ro15-1788 sites was 60-70 fmol/mg membrane protein at both pituitary sites. Using the parameters for cell numbers established in the previous chapter, we therefore estimate that the intermediate lobe has 10,000 benzodiazepine receptor sites per cell. In the anterior lobe we estimate that each cell expressing the GABA/benzodiazepine receptor has 50,000 benzodiazepine sites. The larger number of receptor sites and higher mRNA levels on cells in the anterior pituitary may reflect the need to detect a weaker GABAergic signal supplied diffusely via the median eminence and portal blood as opposed to the direct innervation of the intermediate lobe. The 5:1 ratio of receptor sites in anterior lobe to sites in the intermediate lobe is not as large as the 15:1 ratio of copies of beta subunit message per cell between the two tissues. Similar amounts of alpha subunit mRNAs were expressed in both tissues, however, while the relative ratio of gamma subunit messages was only 3:1 -less than the ratio of receptor sites in the two tissues. One might predict that, although beta subunits seem to be required for efficient receptor assembly *in vitro,* (Pritchett et al., 1988) they may be translated more efficiently or subunits may be more stable than other subunits. In this way, the very small amount of beta subunit message in the neurointermediate lobe would be sufficient to produce a population of receptors proportionally greater than would be expected based on what is seen in the anterior lobe. On the basis of gamma subunit message or

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alpha subunit message alone, the number of sites in anterior lobe and neurointermediate lobe should be more similar, thus the lower copy numbers of beta subunit message probably do play some role in determining the lower number of sites.

High affinity muscimol sites were found to be present at twice the concentration of benzodiazepine sites in all tissues tested. The vast majority of GABA-A receptor sites in cerebellar tissue are benzodiazepine sensitive, and there is no reason to expect non-benzodiazepine binding sites in the anterior lobe, based on the receptor composition we observed. While the high affinity muscimol site is thought to represent a functionally desensitized state of the GABA-A receptor (Olsen and Venter, 1986), it still represents binding to the GABA-A receptor protein complex. Under the conditions of the equilibrium binding assay at which Bmax was measured, (specifically, exposure for one hour to high concentrations of ligand) all of the GABA-A receptors would exist in the high affinity desensitized state . Therefore, our results are consistent with a 2:1 ratio of muscimol sites to benzodiazepine sites on the GABA-A receptor complex.

In the neurointermediate lobe, however, a 2:1 ratio of muscimol to benzodiazepine sites implies that there is a possibility that muscimol sites were undercounted. Since the neural lobe has been shown to have no central type benzodiazepine binding, but to possess functional GABA-A receptors, muscimol sites in excess of twice the benzodiazepine sites would be predicted to occur. Further, $\gamma 1$ subunit containing receptors, both *in vitro* and immuno-precipitated from cerebellar tissues have been reported not to bind Ro15-1788; thus a portion of the intermediate lobe benzodiazepine sites should have been undetected in our assay, again leading to an apparent excess of muscimol sites.

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The *in vitro* studies, however, were conducted by pairing the $\gamma 1$ subunit with the $\alpha 1$ subunit. Furthermore, $\alpha 1$ is the most common alpha subunit in cerebellum. In the intermediate lobe, the $\gamma 1$ subunit is paired with $\alpha 2$ and $\alpha 3$, and this may restore its ability to bind Ro15-1788. The population of neural lobe receptors may be sufficiently small as not to contribute an excess of muscimol sites, or alternately, some aspect of the neurointermediate lobe may contribute to lability of muscimol sites making undermeasurement a possibility.

At present, we have not found a satisfactory way to assess the heterogeneity of affinities for GABA which we predict in pituitary based on the presence of multiple beta subunit types. One attempt at measuring the EC50 of GABA in potentiating diazepam binding failed, with poor signal to noise ratios presumably due to the extraordinarily high number of peripheral sites present in pituitary. This problem pertained even when the peripheral benzodiazepine site blocker PK11195 was used in the assay at high concentrations. Because the pituitary has greater than a twentyfold excess of peripheral sites, failure of PK11195 to block even a small number of these sites would result in high levels of diazepam binding at peripheral sites, and subsequently, poor signal -to-noise ratios in our assay. Even with a fairly low assay variability, differences as high as tenfold in affinity among subpopulations of GABA-A receptors can be difficult to detect in potentiation experiments. Because the affinity of GABA for the GABA-A receptor is greater than 100nM, direct ^{3}H GABA binding studies would also be somewhat difficult to perform; the high off-rate of the ligand necessitates the use of centrifugation assays rather than equilibrium binding assays. The small amounts of neurointermediate lobe tissue available for study in such assays

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would mitigate against obtaining very accurate or informative results, even with adequate blockade of high affinity GABA-B sites and GABA transporters. Perhaps the best approach to this question would be to measure efficacy of GABA in generating chloride currents in single cells or better still, in patch clamp preparations. In such an experiment, the EC50 values of a population of channels could be obtained, giving direct and accurate information about the number of different GABA affinities sites represented.

Tissue Specificity:

CL 218 872 competition demonstrated a clear separation of type I benzodiazepine sites on the anterior lobe and type II benzodiazepine sites on the neurointermediate lobe. In fact, the IC-50 values observed for CL 218 872 displacement of Ro15-1788 binding in the two tissues were exactly those observed in previous studies in which $\alpha 1$, or $\alpha 2$ and $\alpha 3$ transcripts were transfected into 293 cells (Pritchett et al., 1989). Since previous studies have shown an absence of neural lobe binding of Ro15-1788 (Brown, 1984) the results of CL 218 872 competition in the neurointermediate lobe can be taken to reflect only the sites encoded by the mRNAs expressed in the intermediate lobe. The mRNA for neural lobe sites would presumably be expressed in cell bodies in the hypothalamus. Our finding then, provides *in vivo* confirmation of the observation that $\alpha 1$ subunits participate in type I benzodiazepine receptors while $\alpha 2$ and $\alpha 3$ subunits participate in type II benzodiazepine receptors. That this is true in populations expressing multiple beta subunit mRNAs further demonstrates the special role of the alpha subunits in determining this property.

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Taken together our results demonstrate a higher affinity for β carbolines in the anterior lobe than in the neurointermediate lobe, and given the presence of the γ1, α2, and α3 subunits in the intermediate lobe, suggest that melanotrophs maintain a sub-population of GABA-A receptors which either do not respond to β-carbolines, or are stimulated by β-carbolines. The requirement of a higher affinity site on the anterior lobe is difficult to explain as there is no evidence of role for these compounds in lactotroph physiology. Indeed, other properties of the α1 receptor subunit may account for its exclusive use in the anterior lobe. α1 is the most widely expressed and abundant alpha subunit in most brain regions, and its expression may in fact be a sort of "default" pattern for cells expressing GABA-A receptors. A complex pharmacology for β-carbolines in the intermediate lobe makes more physiological sense, as melanotrophs are involved in responses to the types of stresses which have been implicated in β-carboline release in the brain (Izquierdo, 1989). It should be noted, however, that a physiological role for endogenous β-carbolines at GABA-A receptors has been demonstrated only circumstantially as extracellular concentrations in quantities sufficient to effect receptors have not been verified and localization to nerve terminals has not been reported to date.

MATERIALS AND METHODS

Binding studies: Binding assays were performed as previously described (Pritchett et al., 1989). I homogenized tissues for binding assays in 5-10 volumes of ice cold 10mM potassium phosphate buffer, pH 7.2, followed by

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centrifugation at 15,000xG for 15 minutes. I then resuspended the pellets and subjected them to centrifugation twice before storage at -70°C prior to binding assays. Scatchard analysis and analysis of CL 218 872 competition at benzodiazepine sites was performed by means of equilibrium binding assays. At the time of the assays, I resuspended the membranes in assay buffer (9mM potassium phosphate, pH 7.2, 100mM KCl). Each incubation contained 500 μl of membranes and 100 μl of ^{3}H-Ro15-1788, or ^{3}H-muscimol, in a 1 ml total reaction volume. For Scatchard analysis, incubations at several ligand concentrations were carried out in the presence or absence of 10μM clonazepam for Ro15-1788 or 200μM GABA for muscimol. Confirmation of observed maximum binding (Bmax) values was obtained by additional incubations with Ro15-1788 or muscimol blockers at the dissociation constant (K_d), and at five and ten times the K_d initially observed for these ligands. For CL 218 872 competition assays, I incubated membranes with 1.5 nM Ro15-1788 and CL 218 872 ranging from 0.2nM to 30 μM, or with no additional drugs, or with 10μM clonazepam. All incubations took place at 4°C for 2 hours. Membranes were collected by filtration on GF/C #32 fiberglass filters (Schleicher and Schuell) and washed twice with 5 ml of cold phosphate buffer. The amount of ligand bound was then determined by liquid scintillation counting. Membrane protein concentration was determined by Lowry assay.

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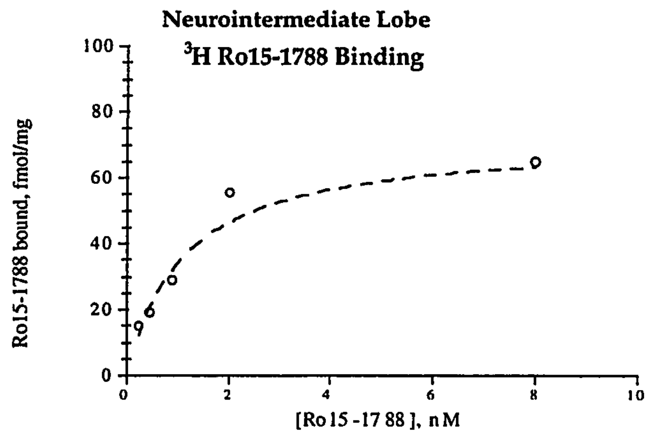
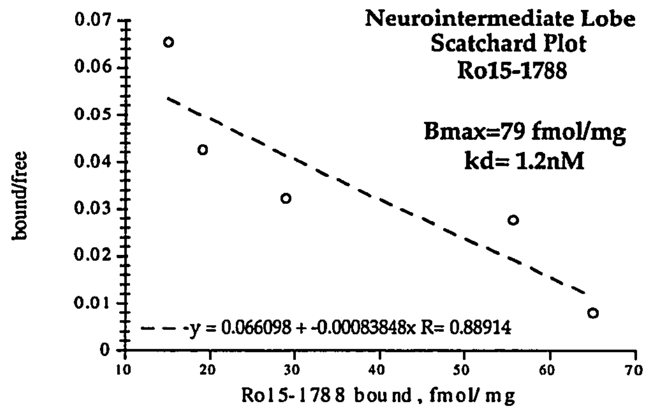
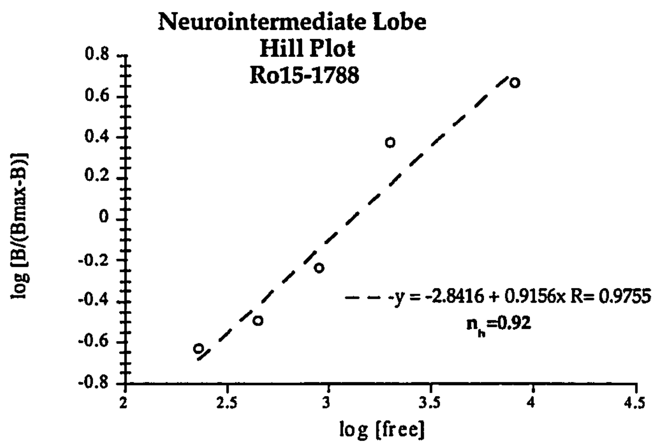
4-1a







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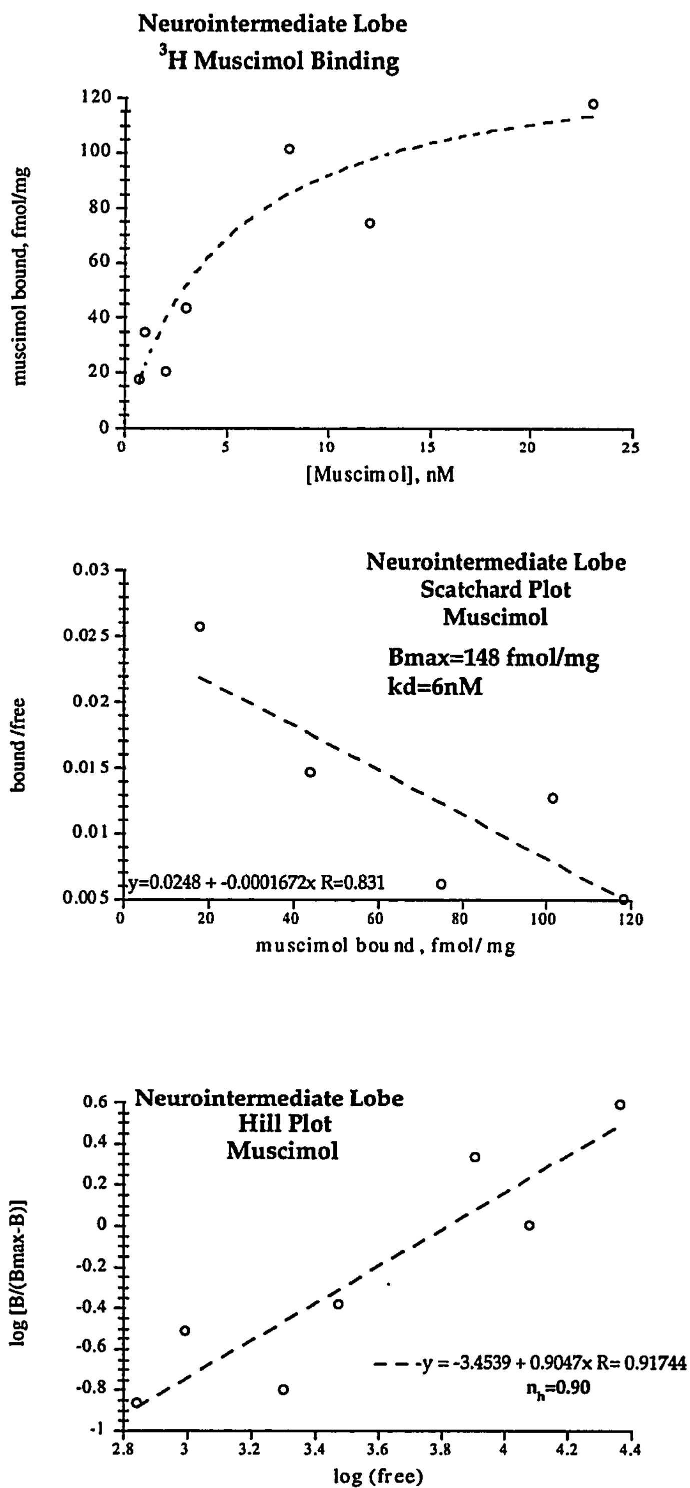

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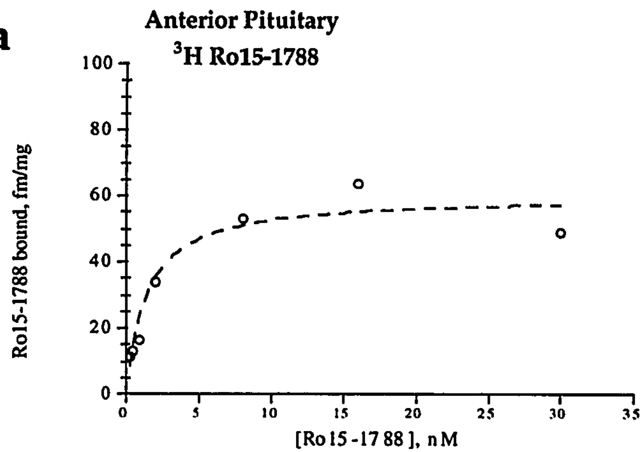
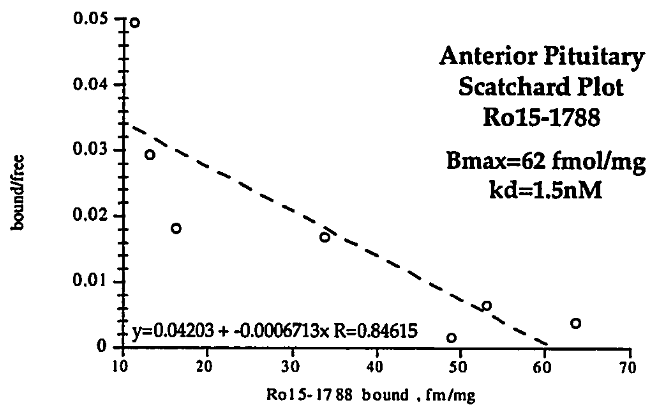
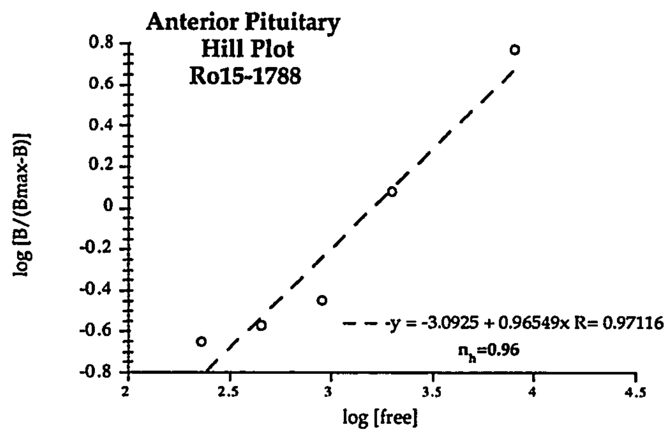
4-2a







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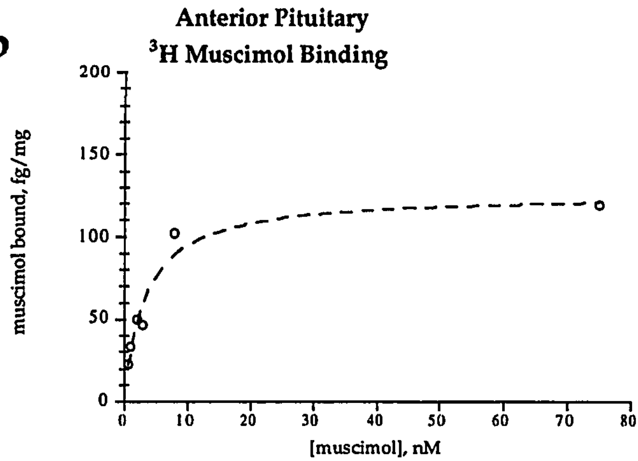

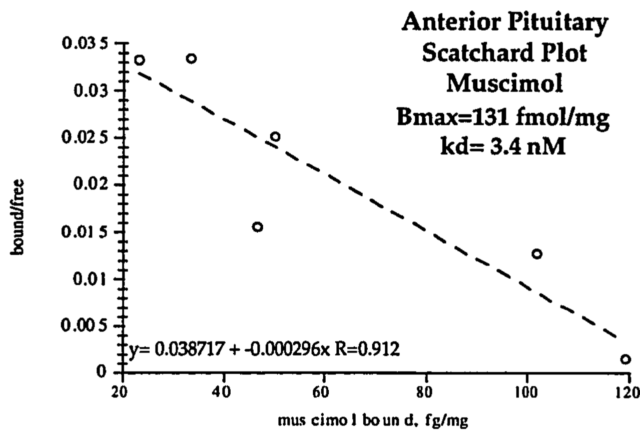

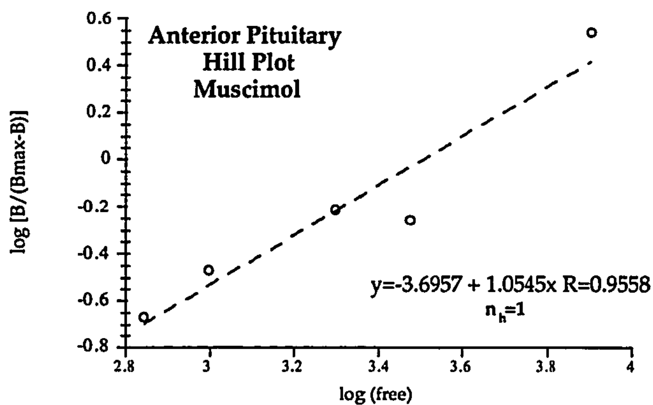

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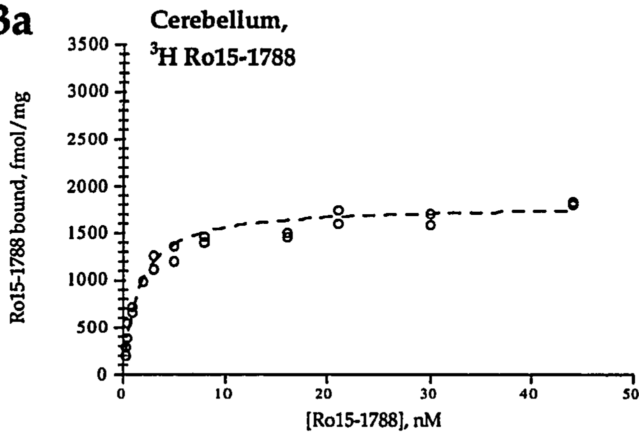
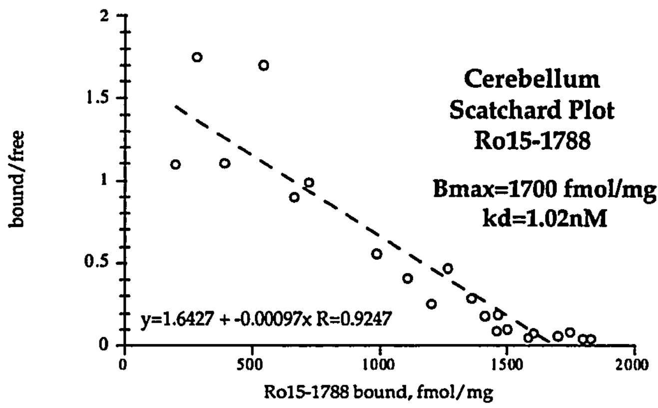
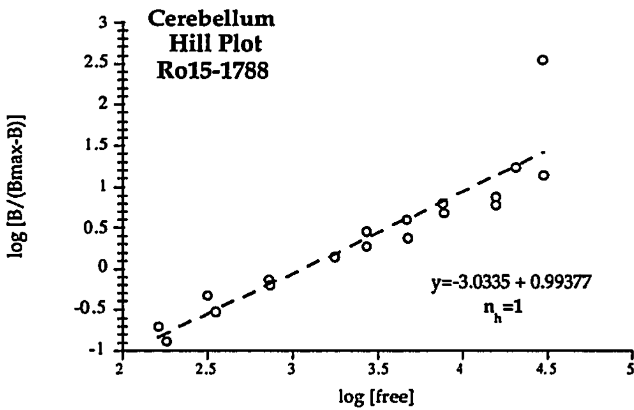
4-3a







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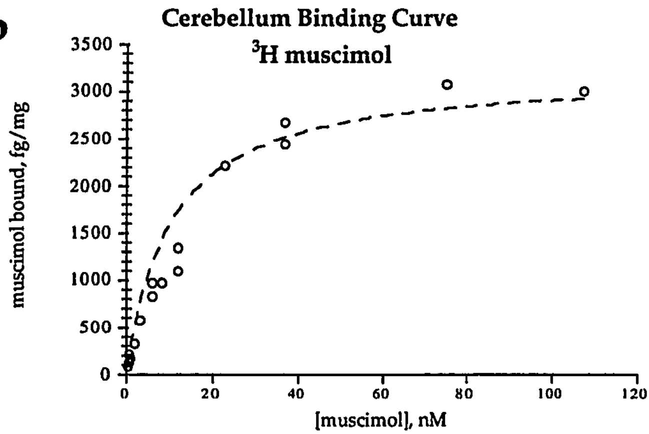
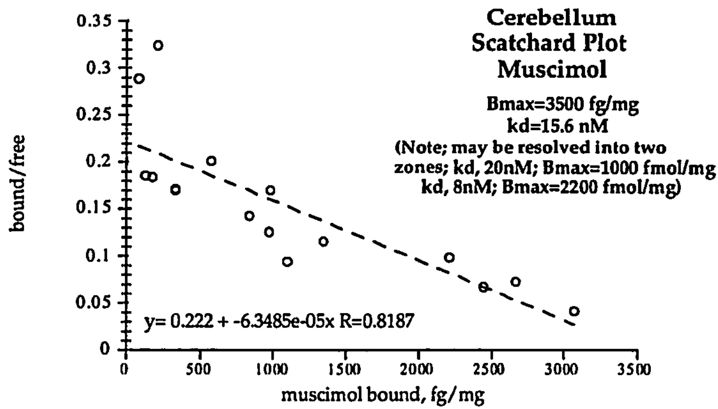
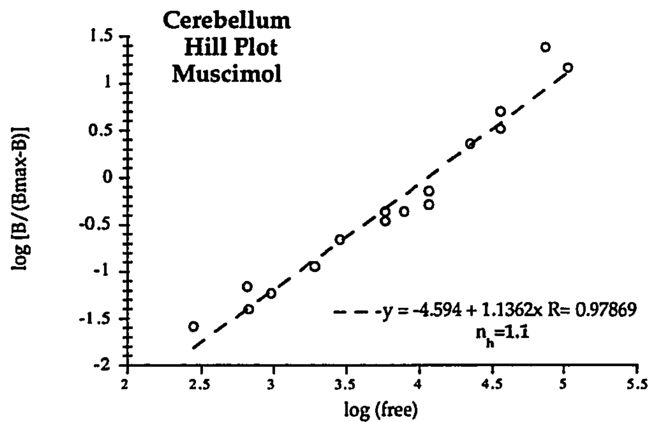
4-3b







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4-4 *Binding parameters of pituitary and cerebellar membranes for 3H RO15-1788 and 3H muscimol, and estimates of receptor sites per cell in pituitary.*

Tissue	Kd(nM)	Bmax(fmol/mg)	Hill#	*Receptors/cell
AP, RO15	1.5	62	.96	55,000
AP, muscimol	3.4	131	1.0	ND
IL, RO15	1.2	79	0.92	10,000
IL, muscimol	6.0	148	0.90	ND
Cb, RO15	1.0	1700	1.0	ND
Cb, muscimol	15.6	3500	1.1	ND

AP=anterior pituitary, IL=neurointermediate lobe, Cb=cerebellum, ND=not determined.
*(fmol/mg membranes)x(membranes/lobe)x[1/(cells/lobe)]x($6x10^{23}$ sites/mole): for intermediate lobe assume 200,000 cells, >90% melanotrophs; for anterior lobe assume $2x10^6$ cells, estimate 10% lactotrophs.

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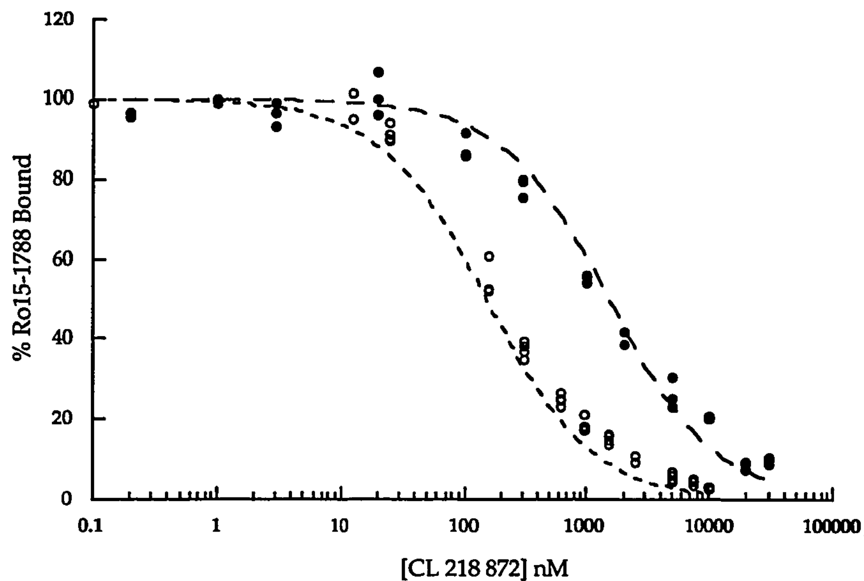
4-5

Cl 218 872 Competition Binding Curve



o Anterior Lobe

y = 100-(m0*100)/(m0+150)		
	Value	Error
Chisq	1082.8	NA
R	0.98853	NA

• Neurointermediate Lobe

y = 100-(m0*100)/(m0+1500)		
	Value	Error
Chisq	786.42	NA
R	0.99103	NA

Figure 4-5 shows CL 218 872 competition for Ro15-1788 binding sites in anterior lobe and neurointermediate lobe. Depotentiation curves generated using the values of 150nM as the k_i for anterior lobe and 1500 nM for the k_i for neuro-intermediate lobe fit the data with regression values near unity as determined using the general curve fitting application in Kaleidagraph software; curve fit parameters appear in boxes.

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CHAPTER FIVE:
SYNTHESIS OF A NEURALLY ACTIVE STEROID IN THE NEUROINTERMEDIATE LOBE OF THE RAT

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INTRODUCTION

The pattern of expression of GABA-A receptor subunit mRNAs in the pituitary raises the possibility that there is differential sensitivity to neurally active steroids both between anterior lobe and neurointermediate lobe, and between subpopulations of receptors within the neurointermediate lobe. While there are reports of synthesis of neurally active steroids by anterior pituitary, there are no such reports in the literature pertaining to the neurointermediate lobe. To address the likely physiological relevance of neurally active steroids to melanotroph function, we carried out studies to assess whether the neuro-intermediate lobe might be capable of synthesizing physiologically active concentrations of neurally active steroids.

Neurally active steroids are derived from several sources. One is the secretion of compounds into general circulation by classical steroidogenic

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tissues such as the adrenal gland. For example, in one study, tetrahydrodeoxycorticosterone (THDOC), was secreted in response to swim stress; this response was abolished by adrenalectomy. The resulting plasma levels of THDOC, however, were in the low nanomolar range, which is at least an order of magnitude below the EC50 for the actions of this compound at GABA-A receptors (Purdy et al., 1991).

Another source is *de novo* synthesis from cholesterol, a process which has been demonstrated to occur in neural tissue, particularly in glial cells. The preliminary steps of this pathway resemble those which take place in the adrenal, culminating in the synthesis of pregnenolone, dehydroepiandrostane, and their sulfated derivatives, and in some instances, progesterone and 3αhydroxy-dihydroprogesterone (3αOHDHP) (Baulieu, 1992). The initiation of this pathway by the cholesterol side chain cleavage enzyme, cytochrome $P450_{SCC}$, has been shown to be stimulated by agonists acting at peripheral benzodiazepine receptors (Costa and Guidotti, 1991) which are in great abundance in the neurointermediate lobe. The major steps of this pathway are shown in Figure 5-1.

A final source is from derivatization *in situ* of circulating adrenal and gonadal steroid precursors. A notable example of this pathway has actually been demonstrated in the anterior pituitary and hypothalamus, namely, the conversion of progesterone to 3αOHDHP via the intermediate 5α-dihydroprogesterone (5αDHP) in a series of two reductions in the A ring of the precursor steroid (Cheng and Karavolas, 1973). This series of reactions is also outlined in Figure 5-1. The enzymes responsible for the conversion of progesterone to 3αOHDHP in the anterior pituitary have been extensively studied (Robinson and Karavolas, 1973; Krause and Karavolas, 1980; Bertics

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and Karavolas, 1985; Campbell and Karavolas, 1990; Campbell and Karavolas, 1990). Progesterone is first reduced at the 5 position of the A ring to 5αDHP by progesterone 5α-reductase. This enzyme is localized in microsomes and has a Km of 117nM for progesterone.It requires NADPH as a cofactor. The second step, conversion to 3αOHDHP, is performed by one of two 5αDHP reductases. One is located in the cytosol, uses an NADPH cofactor and has a Km of 83nM for 5αDHP. The second is found in microsomes and has a Km of 230nM for 5αDHP, using NADH as a cofactor (Karavolas and Hodges, 1990).

The *de novo* synthetic pathway from cholesterol to pregnenolone in the brain can continue on to progesterone via the actions of Δ^{5}-3β-hydroxysteroid dehydrogenase-isomerase unless pregnenolone is shunted off into its sulfated derivative, or into dehydroepiandrostane and its sulfated derivative. This is an important point, because pregnenolone sulfate and 3αOHDHP have opposite effects on GABA-A receptors. Thus the point at which pregnenolone is converted to pregnenolone sulfate is likely to be an important point of physiological regulation of neurosteroid synthesis. That both circulating and *de novo* synthesized progesterone might serve as substrates for 3αOHDHP synthesis further emphasizes the potential physiological importance of the steroid A ring reduction pathway, and implies that it might be significant in contexts other than those involving the changes in gonadal and adrenal progesterone production.

It is, however, just such phenomena which point most directly to a role for 3αOHDHP in the neurointermediate lobe. Fluctuations of αMSH secretion and αMSH levels in the pituitary during the estrous cycle have been interpreted by some investigators to suggest a role for progesterone in peptide release from melanotrophs (Thody et al., 1981), and some studies have shown

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direct effects of progesterone on αMSH release (Celis, 1977). These observations along with the high potency of 3αOHDHP at GABA-A receptors and its synthesis from progesterone in the anterior pituitary led us to believe that this compound might be a likely candidate for a physiologically relevant neurally active steroid in the neurointermediate lobe. To investigate this possibility, we developed a simple chromatographic system for isolating and quantifying the major products of A ring reductions of ^3H progesterone, to determine whether these reactions occur in the neurointermediate lobe.

EXPERIMENTS AND RESULTS

Steroid Chromatography:

To develop a system for isolating and quantitating progesterone and its derivatives, we chromatographed unlabeled progesterone, 5αDHP, and 3αOHDHP on silica and C18 thin layer plates in several different solvent combinations. With silica we tested chloroform and ethyl acetate, chloroform with ethyl acetate and methanol, and toluene and acetone; with C18, we tried various ratios of methanol and water. On silica, toluene and acetone in a 7:3 ratio gave the best results. On C18, 80% methanol gave a satisfactory result, and reversed the order of progesterone and 5αDHP as compared to their order of migration on silica. Figures 5-2 and 5-3 show chromatograms on C18 and Silica respectively, along with the relative migrations of the various steroids

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with respect to the solvent front and with respect to deoxycorticosterone. (Further details concerning the chromatographic techniques employed appear in the legend to figures 5-2 and 5-3, and in the section on materials and methods at the end of this chapter.)

Synthesis of Progesterone Derivatives:

In the presence of 550nM total progesterone, crude homogenates of anterior lobe, neurointermediate lobe, and mediobasal hypothalamus, but not boiled anterior pituitary tissue converted ^{3}H progesterone to compounds comigrating in both chromatographic systems with 3αOHDHP and 5αDHP standards. Profiles of the distribution of radioactivity on the TLC plates appear for C18 in Figure 5-4 and for silica in Figure 5-5.

The percent of radioactivity recovered from fractions migrating with 3αOHDHP and 5αDHP for each tissue is given in tabular form for both chromatographic systems in Figure 5-6a. The amounts of the various steroids produced by each tissue are summarized in Figure 5-6b. Total conversion to A ring reduced forms was in the range of 30 fmol/min/mg for neurointermediate lobe, 10 fmol/min/mg for anterior lobe, and 5 fmol/min/mg for mediobasal hypothalamus. In both pituitary tissues, the A ring reduced steroids were almost equally distributed between the 3αOHDHP and 5αDHP fractions, while in mediobasal hypothalamus, about 75% of the converted material appeared as 5αDHP. These results were obtained consistently in both the C18 and silica chromatographic systems.

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DISCUSSION

The experiments presented here demonstrate the ability of the neurointermediate lobe to derivatize progesterone to a neurally active steroid. Identical percentages of total radiolabeled material migrating with cold 3αOHDHP were observed for each tissue in both chromatographic systems. The samples spotted to the C18 and silica plates were equal portions of the same reactions, to which equal amounts of carrier 3αOHDHP were added. Thus the 3αOHDHP after isolation in two independent chromatographic systems had the same specific activity. This observation can be taken as evidence confirming the identity of the radiolabeled steroid.

On a per mg basis, the neurointermediate lobe had the highest activity of all of the tissues tested in converting progesterone to its A ring reduced derivatives. Each incubation contained about 2 mg of tissue in the presence of 55 pmol of progesterone; 4% of this material was converted to 3αOHDHP in a period of one hour. This result implies that a single neurointermediate lobe has enough progesterone 5α-reductase and 5αDHP 3αhydroxysteroid oxido-reductase to produce about 20 fmol of 3αOHDHP from progesterone in one minute. If this amount of 3αOHDHP is distributed evenly throughout the volume of the neurointermediate lobe (less than 5 μl) then in one minute, a concentration of at least 5nM could be reached. The reaction rate given was observed in homogenate in which the neurointermediate lobe material was placed in over ten times its volume. *In vivo*, then, the effective enzyme concentration would be considerably higher, especially since the NADPH

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enzyme is not spread out over the whole cell, but rather concentrated in specific places. The *in vivo* reaction rate is likely to be much higher than what was observed in our homogenate. Further, it is highly unlikely that 3αOHDHP itelf is distributed over the entire volume of the cells of the neurointermediate lobe; the steroid is probably distributed over membranes and extracellular space in the neurointermediate lobe, a volume many times smaller than the total of 5 μl.

Taking these factors into account, it seems quite possible that the neurointermediate lobe is capable of synthesizing enough 3αOHDHP to reach local concentrations in the range of 100nM within a time frame on the order of minutes. At concentrations in this range, 3αOHDHP is reported to potentiate chloride current through the GABA-A receptor by more than twofold (Gee et al., 1988; Morrow, 1989). Furthermore,patch clamping studies have shown efficacy for 3αOHDHP at concentrations as low as 30nM in potentiation of chloride currents (Gee et al., 1988; Morrow, 1989; Lambert et al., 1990).

It should be noted in addition that effects of 5αDHP have been reported at classical nuclear progesterone receptors with an EC50 of 1nM (Rupprecht, 1993). In order for 5αDHP to be synthesized, however, progesterone must be present in the 100nM range. Since its EC50 at progesterone receptors is tenfold lower than that of 5αDHP, it would seem that potential effects of 5αDHP binding to progesterone receptors would be of little importance. Still, the possibility remains that even small amounts of binding of 5αDHP to progesterone receptors may have physiological consequences; such a steroid-receptor complex, for example, might be able to regulate elements which would not respond to a progesterone receptor bound to progesterone, or would respond differently. That the neurointermediate lobe synthesizes

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physiologically relevant concentrations of 3αOHDHP provides evidence that neurally active steroids may be involved in the regulation of melanotroph function. We are greatly intrigued by the high concentration of peripheral benzodiazepine sites in the neurointermediate lobe along with earlier findings of a high concentration of DBI in the pituitary, and our laboratory has undertaken studies of the *de novo* steroid synthetic pathway in this tissue. Of particular interest is whether pregnenolone, if synthesized, will be made into progesterone and ultimately 3αOHDHP, or diverted to pregnenolone sulfate and dehydroepiandrostane sulfate which are negative regulators of GABA-A receptors. The potential actions of all of these compounds in regulating melanotroph function will be discussed in the concluding chapter.

MATERIALS AND METHODS

Animals and tissues: I dissected anterior pituitary, neurointeremediate lobe, and mediobasal hypothalamus adult female Sprague Dawley rats, placing portions ranging from 6 mg neurointermediate lobe to 100 mg hypothalamic tissue, wet weight, in 300 µl of ice cold 100mM KPi, pH 7.2 in glass/teflon homogenizers on ice. After homogenizing the tissues, I transferred aliquots of 85 µl to glass tubes on ice. As a negative control, I placed a portion of anterior pituitary homogenate in an epindorf tube and kept it in an 80°C heat bath for 20 minutes to destroy any steroid converting activity. This "heat killed" material was then used in the same manner as the other tissue homogenates.

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Incubations: The reaction mix included ^{3}H progesterone, cold progesterone, NADPH, dithiothreitol (DTT), EDTA, and bovine serum albumin. To produce the mix, I placed 6 µl of ^{3}H progesterone in benzene (53.5 Ci/mmol, NEN) along with 5 µl of a stock of unalabeled progesterone (Sigma), at 1µM in methanol, and dried the steroids under a stream of helium in a borosilicate glass tube. Meanwhile, I combined 20 µl of a solution conaining 660 µg of NADPH/75 µl with 20 µl of 125mM DTT, 20 µl of 2.5mM EDTA, and 100 µl of 5% bovine serum albumin (Fraction V, Sigma) in another glass tube. To aid in keeping the steroid in solution, I heated this mixture in a 37°C water bath for about five minutes. I resuspended the steroids in 5 µl of methanol, washing the sides of the tube before transferring to the warmed reagent mix. To insure complete transfer of the steroids, I washed the tube a second time with methanol and transferred this material to the warm reagent mix as well. The incubations consisted of 85 µl of tissue homogenate and 15 µl of the final reagent mix in glass 12x75 tubes sealed with parafilm. Reactions incubated for one hour at 37°C. I performed the experiment on two separate occasions. In the second instance, two incubations were prepared for each type of homogenate. The results of both experiments were pooled for analysis.

Steroid Analysis: After incubation of the reactions, I added 5 µg of carrier 5αDHP, 3αOHDHP, and deoxycorticosterone to each sample. To remove steroids from the homogenate, I extracted each reaction mix twice with 200 µl of ethyl acetate, pooling the organic layers in a second glass tube. I removed the ethyl acetate by drying the samples under a helium stream, and resuspended the extracted steroids in 100 µl of methanol. Prior to chromatography, I added an additional 5 µg each 5αDHPand 3αOHDHP.

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I spotted 40μl of each sample onto the Linear K preabsorbent zones of silica and C18 glass backed with flourescent indicator and TLC plates (Whatman), and transferred the plates to closed glass chambers containing 100 ml of the appropriate solvents. For C18, I used 8 parts chromatography grade methanol (Fischer) to 2 parts distilled water. For silica, I used 7 parts toluene (Fischer) to 3 parts acetone (Sigma). Chromatograms ran until the solvent front was near the end of the plate. I sprayed the plates with a 1:1 mixture of ethanol and 50% sulfuric acid in order to visualize the steroid bands, and enhanced the image by brief exposure to iodine fumes. The sulfuric acid visualization method produces a specific color for each steroid, and it was noted that the carrier steroids retained their distinctive colors-golden brown for 3αOHDHP, darker brown for progesterone and 5αDHP, and purple-brown for deoxycorticosterone, throughout the extraction process. This indicates that the integrity of the carriers was not changed by the extraction or chromatographic process.

The visualized bands served to guide the division of the TLC plates into equal zones which followed the boundaries of the carrier bands. I scraped each zone into a scintillation vial and subjected it to scintillation counting in Ecoscint A. A preliminary experiment showed that the silica and C18 materials did seem to quench the scintillation signal, but the visualization process and presence of carrier steroids had no effect. Since the counts in each zone or fraction were normalized against the total counts recovered from the chromatogram, and the zones were all of equal size, this quenching would not change the result in terms of percent of steroid precursor converted to the compounds of interest.

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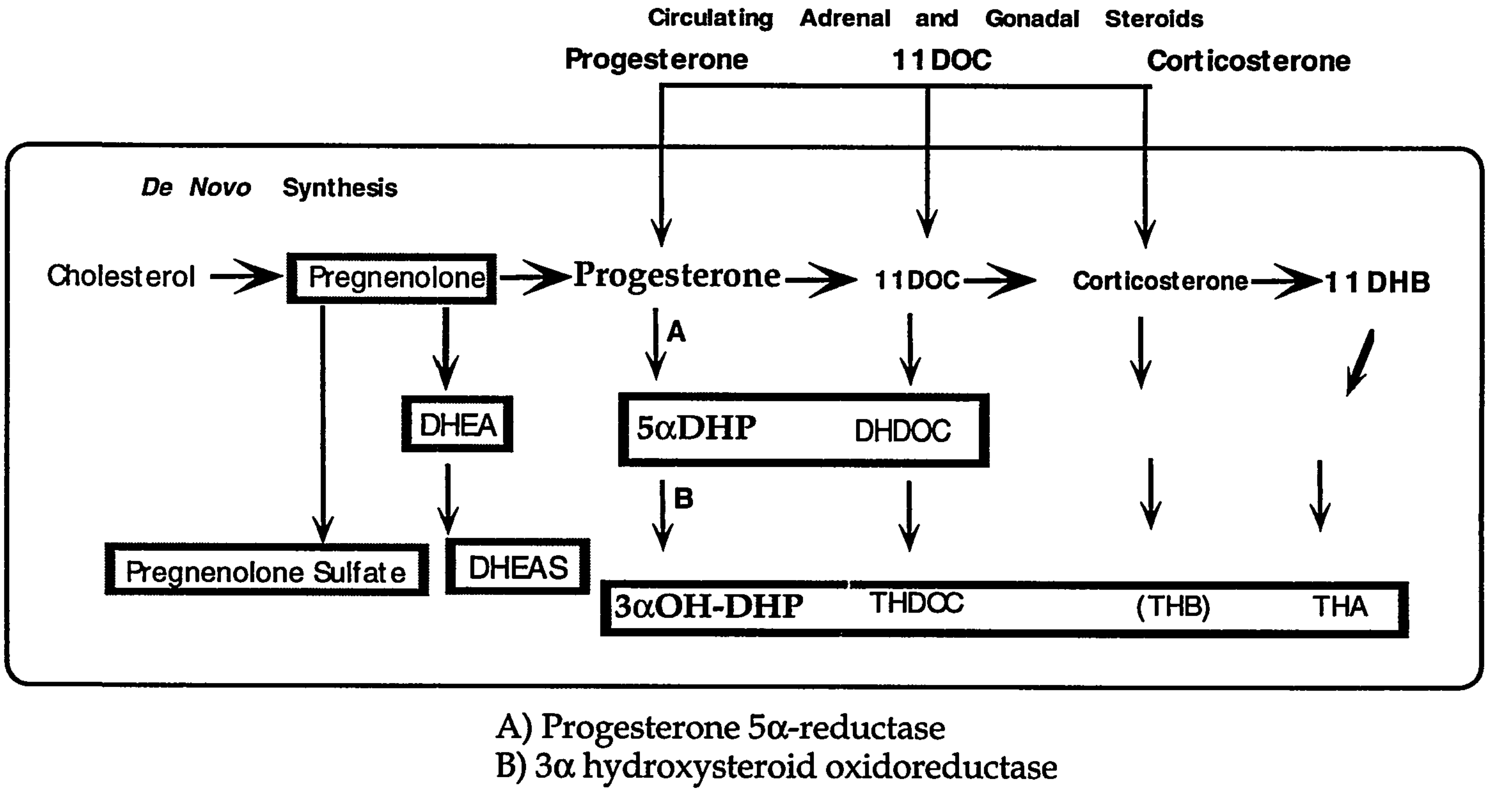
5-1 Schematic Illustration of steroid synthetic pathways and converging on synthetic pathway for 3αOHDHP



A) Progesterone 5α-reductase
B) 3α hydroxysteroid oxidoreductase

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5-2

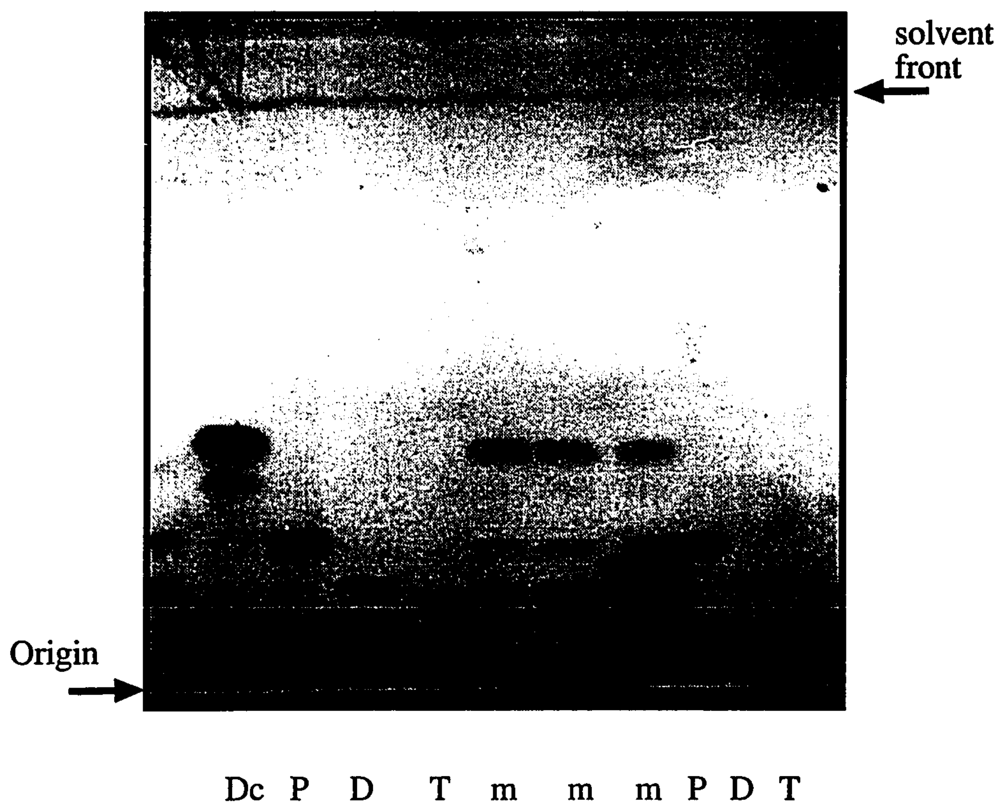

Figure 5-2 shows a C18 chromatogram of various steroids run in a mixture of 8 parts methanol to two parts water. P, progesterone; Dc, deoxycorticosterone; D, 5 alpha-dihydroprogesterone; T, 3 alpha-5 alpha-dihydroprogesterone; m, carrier mix of four steroids used in the experiments. Carrier mix contained approximately 4 µg of each steroid in 40 µl. Standards contained four times as much steroid. Visualization is achieved by spraying with a solution of 25% sulfuric acid in 50% ethanol with added development in an iodine chamber. The plate was placed over a UV table and photographed. Rf Values are as follows: progesterone, 0.25; 3αOHDHP, 0.16; 5αHDP, 0.18; and deoxycorticosterone, 0.4.

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5-3

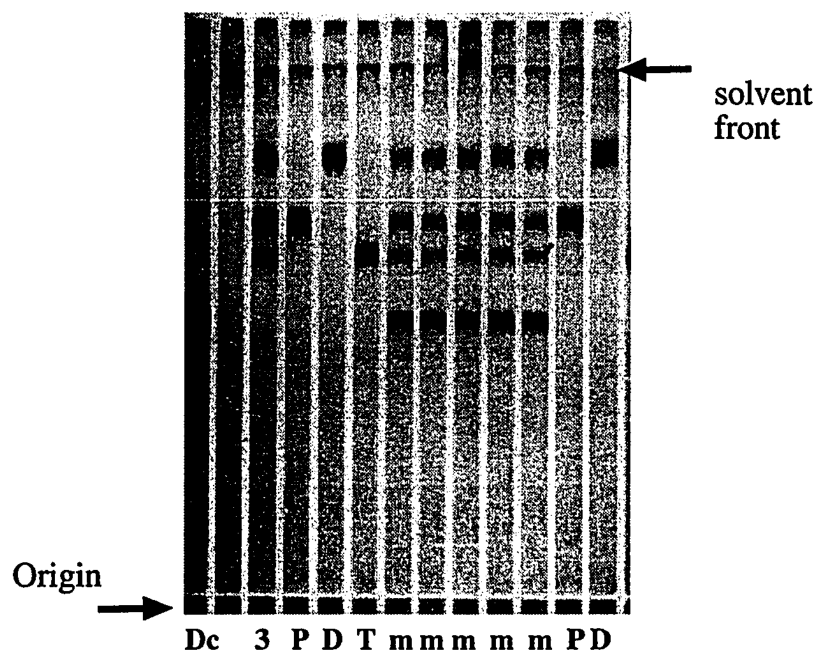

Figure 5-3 shows a silica chromatogram of various steroids run in a mixture of 7 parts toluene to three parts acetone.P, progesterone; Dc, deoxycorticosterone; D, 5 alpha-dihydroprogesterone; T, 3 alpha-5 alpha-dihydroprogesterone; m, carrier mix of the four steroids used in the experiments.Carrier mix contained approximately 4µg of each steroid in 40µl; Standards contained four times as much steroid. Visualization is by spraying with a solution of sulfuric acid in 50% ethanol with added development in an iodine chamber. The plate was placed over a UV table and photographed. Rf values are:progesterone, 0.7; 3αOHDHP, 0.64; 5αDHP, 0.83; deoxycorticosterone, 0.51

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5-4 C-18 chromatography of progesterone derived steroids

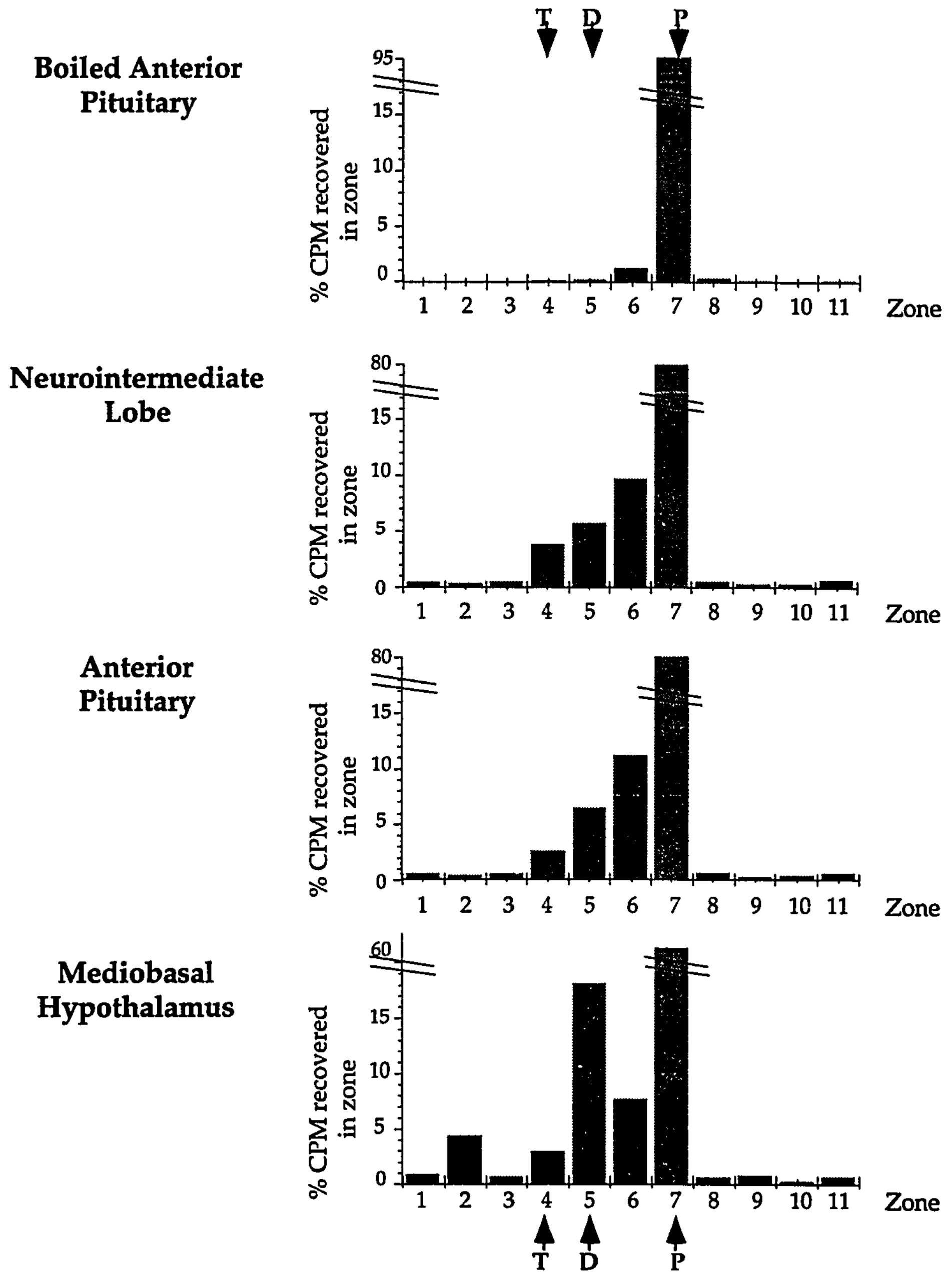

Figure 5-4 shows C-18 chromatography of ^{3}H progesterone and derivatives resulting from incubation with various tissues. T; 3αhydroxy-dihydroprogesterone; D, 5αdihydroprogesterone; P, progesterone.

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5-5 Silica chromatography of progesterone derived steroids

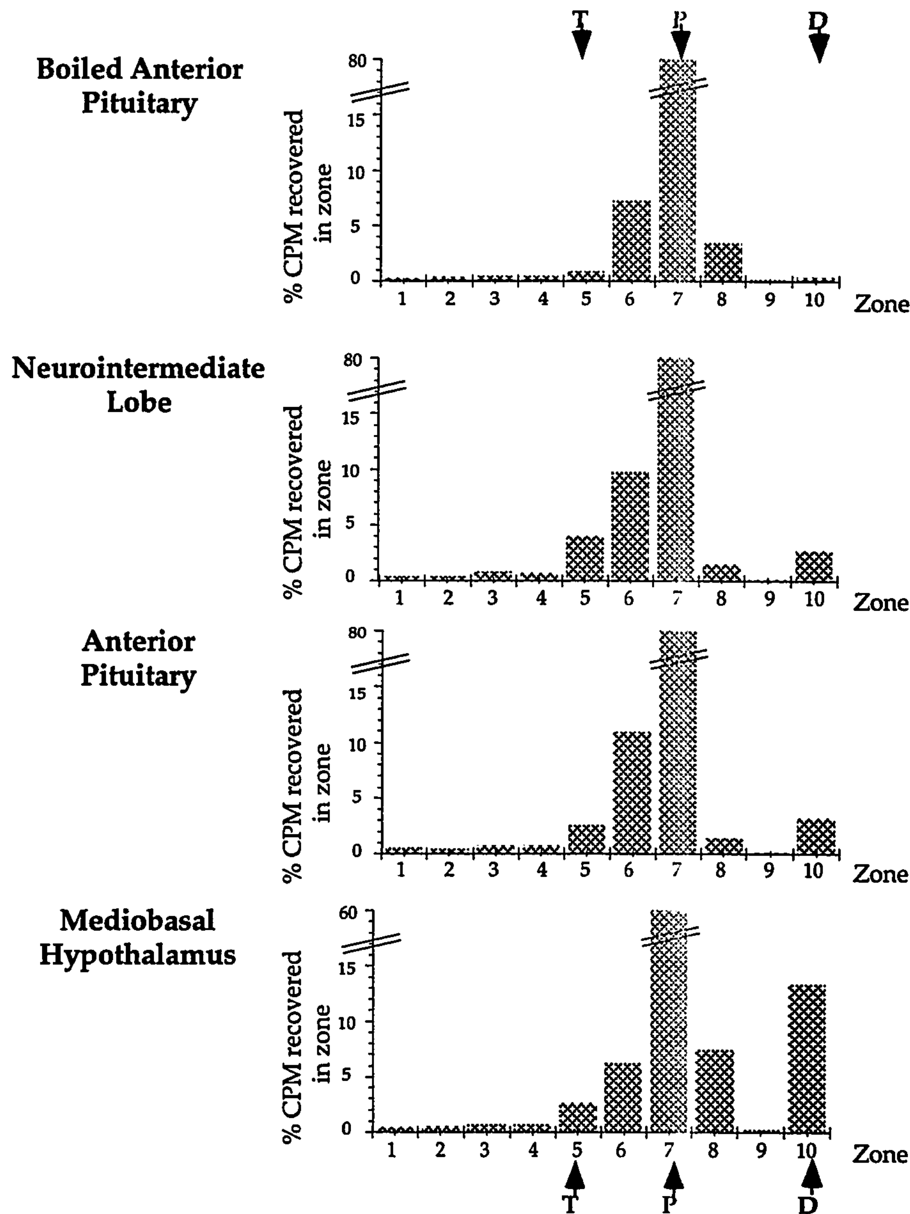

Figure 5-5 shows silica chromatography of ^3H progesterone and derivatives resulting from incubation with various tissues. T; 3αhydroxy-dihydroprogesterone; D, 5αdihydro-progesterone; P, progesterone.

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5-6a % of CPM recovered in Zones comigrating with standards

3αOHDHP

	C18	Silica
Nil	3.8	3.9
AP	2.5	2.5
MBH	2.9	2.6

5αDHP

	C18	Silica
Nil	5.6	2.7
AP	6.3	3.1
MBH	18.3	13.3

5-6b Conversion of ^{3}H progesterone to 3αOHDHP

	MBH	AP	NIL
% Conversion of ^{3}H prog	2.7	2.5	3.8
fmol made in 1 hour	8910	3080	3785
mg wet weight of tissue	30	2	6
Synthetic Rate fmol/mg/min	4.9	8.5	31.5

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CHAPTER SIX:
DISCUSSION AND CONCLUSIONS

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SUMMARY OF FINDINGS

The findings presented in these studies on pituitary GABA-A receptors present the overall picture of a population of receptors which, in their basic pharmacological properties, resemble the GABA-A receptors found in the brain; affinities for muscimol and benzodiazepine ligands, ratio of muscimol to benzodiazepine sites, and Hill numbers were similar or identical, for example, between brain and both pituitary sites. The major difference noted between pituitary and brain was a much lower density of receptor sites in both of the pituitary tissues.

Further, we found that GABA-A receptors in both anterior lobe and intermediate lobe are encoded by the same kinds of mRNA as GABA-A receptors in the brain. Messenger RNA for each of the major subunit classes is present in both locations, and the specific subunits represented account for such properties of the receptor as benzodiazepine binding, benzodiazepine responsiveness, which has been observed *in vivo* (Taraskevich and Douglas, 1985; Louiset et al., 1992), and the Type I versus Type II benzodiazepine pharmacology that we observed in our own studies.

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The lower levels of receptor sites detected in binding assays in pituitary as opposed to brain, and in neurointermediate lobe as opposed to anterior lobe was matched by the relative levels of receptor mRNAs at these locations, although the relationship was not a simple one. The results suggested that the relative levels of the alpha, beta, and gamma classes of subunit mRNAs all contributed to the final numbers of actual receptor sites present, rather than levels being solely determined by the least abundant subunit species. An alternate interpretation is that the impact of the relative level of mRNA for any one subunit class is modified by translational efficiency, subunit stability, and efficiency of assembly into receptor complexes at the protein level. The two interpretations given here are not mutually exclusive.

Although the number of species of subunit mRNAs found in the two pituitary tissues is not as high as would be found in many brain regions, there is still a surprising level of heterogeneity for what are fairly homogenous tissues. In the anterior pituitary, heterogeneity was basically limited to the population of beta subunit mRNAs, with β1, β2 and β3 mRNAs all present in similar amounts. In the neurointermediate lobe, a fairly complex situation exists, with significant quantities of mRNA from two different members of each subunit class expressed. Based on the level of heterogeneity observed at the mRNA level, we would predict that pituitary GABA-A receptors exhibit a wide range of sensitivities to GABA.

The distribution of GABA-A receptor subunit mRNAs in anterior pituitary and neurointermediate lobe also showed some important differences in tissue specificity. First, we observed the absolute segregation of α1 subunit mRNAs in the anterior lobe and α2 and α3 subunit mRNAs in the neurointermediate lobe and correlated this observation with binding properties

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consistent with pure Type I benzodiazepine sites in the anterior lobe and pure Type II sites in the neurointermediate lobe. This result is perhaps one of the clearest correlations of subunit mRNA composition and receptor properties in a native population of GABA-A receptors to date. A second notable difference between anterior and neurointermediate lobe was the presence of significant amounts of γ1 subunit mRNA in the neurointermediate lobe. The highly specific distribution of the various alpha and gamma subunit mRNAs in anterior and neurointermediate lobe might be predicted to result in varied responses to β-carboline and steroid modulators, pointing to possible physiological roles for these compounds in the pituitary.

In support of the importance of GABA-A receptor modulators in the neurointermediate lobe, we found that the neurointermediate lobe is highly active in synthesizing 3αOHDHP from progesterone. In fact, the neurointermediate lobe was the most active site for the synthesis of this neurally active steroid of all tissues tested and the amount of steroid synthesized is consistent with the presence of the compound at levels sufficient to activate GABA-A receptors.

HYPOTHESIS ON GABA REGULATION OF MELANOTROPHS

I will now attempt to consider the findings I have just detailed in light of of evidence on the effects of GABA in the neurointermediate lobe The goal of this discussion will be to develop a coherent hypothesis on how GABA regulates melanotroph function, and on how GABA receptor heterogeneity

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might contribute to that regulation. I will focus almost exclusively on the melanotrophs of the intermediate lobe, but some of the general concepts which emerge will be applicable to other neuroendocrine cell types such as GT1-7 cells or lactotrophs.

I will organize the discussion around answers to a series of questions about neurointermediate lobe GABA-A receptors, melanotroph heterogeneity, effects of GABA on melanotroph function, and potential effects of GABA-A receptor modulators on melanotroph function.

What kinds of GABA-A Receptors are likely to be found on melanotrophs?

In trying to predict what kinds of GABA-A receptors might be found on melanotrophs based on the expression of specific subunit mRNAs, a number of assumptions must be made. Chief among these is the assumption that the presence of a specific subunit mRNA can be correlated with the presence of the subunit protein. Where immunocytochemical studies can be correlated with *in situ* hybridization studies, this has been found qualitatively to be the case, e.g (Malherbe et al., 1990; Machu et al., 1993). Our own correlation of GABA-A subunit mRNA abundance with density of benzodiazepine receptor binding sites in brain, anterior lobe, and neurointermediate lobe also lends support to the correlation of subunit mRNA expression and subunit protein expression.

Another assumption is that one can extrapolate between different types of experiments. One group of relevant experiments is those in which subunit association is investigated by using immunoblotting to determine which subunits coprecipitate with other specifically immunoprecipiated subunits. Another group is those experiments in which subunits are expressed

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in oocytes or cell lines in various combinations, and pharmacological properties of the receptors are observed. A third group is results obtained *in vivo,* the most physiologically relevant, and yet also often the most difficult to interpret. Our own results with Type I and Type II benzodiazepine pharmacology confirm that in at least some respects, the extrapolation between transfection data and *in vivo* data is possible.

The present state of knowledge concerning many aspects of GABA-A receptor subunit asociation is murky. Similarly, while the properties of every individual GABA-A receptor subunit have been observed *in vitro* in combination with some subunit or subunits, every possible combination has not been studied. Thus for example, when it is observed that β1, β2,and β3 subunits can display different EC-50s in producing GABA evoked chloride currents in combination with an α1 and γ2 subunit, it is fair to say that these beta subunits might confer different GABA affinities and GABA efficacies to receptors containing other alpha and gamma subunits;one cannot, however, predict with any certainty their order of potency in the new contexts.

With the above considerations in mind, I will try to apply what is known about GABA-A receptor subunit association and GABA-A receptor subunit properties to the subunit mRNA distribution observed in neurointermediate lobe to convey the possible range of GABA-A receptors which could be synthesized from these mRNAs.

The first piece of information to take into account is the presence of both α2 and α3 subunit mRNAs in the same cell population. One group of investigators attempted in several different ways to determine whether different alpha subunits could associate in the same receptor complexes. They first performed immunoprecipitation of receptors isolated from brain

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membranes with antibodies to either one , two, or three different alpha subunits. They found that combining antibodies to two or three different subunits resulted in a higher percentage of total flunitrazepam binding sites being precipitated than with antibodies to any one subunit. Further, they found that the amounts precipitated by two or three antibodies were roughly additive. These results led them to conclude that the different alpha subunits were present mainly on non-overlapping sets of receptor complexes (Duggan and Stephenson, 1990). Then, in a second study, the same investigators performed immunoprecipitations and immunoaffinity purification sequentially with the various anti-alpha subunit antibodies. In this study, the key piece of information was the percentage of flunitrazepam binding retained in immunoaffinity columns following immunoprecipitation. Thus, for example, if all of the flunitrazepam binding of material first immunoprecipitated with an $\alpha2$ subunit antibody was retained with the $\alpha3$ subunit antibody, then one would conclude that $\alpha2$ was always associated with the $\alpha3$ subunit (but NOT necessarily the other way around). The results of this study supported the existence of both homo-oligomeric and hetero-oligomeric GABA-A receptor complexes with respect to alpha subunits. The authors concluded that hetero-oligomers exist, but are a minor component of the overall population of receptor complexes (Duggan et al., 1991), but their basic conclusion was mostly drawn with regard to the $\alpha1$ subunit. In a subsequent paper, they presented evidence that this result can be extended to interactions between the $\alpha2$ and $\alpha3$ subunits (Pollard et al., 1993).

Similar types of studies have not been performed for the beta subunits, largely because it was only recently that an antibody was developed that can distinguish between the $\beta2$ and $\beta3$ subunits (Machu et al., 1993). The ability of

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single types of beta subunit mRNA to generate responses to GABA measured by whole cell current recordings when transfected into cell lines in combination with alpha and gamma subunit mRNAs, suggests that receptors which are homo-oligomeric for the beta subunits can be efficiently assembled (Sigel et al., 1990). Still, the occurence of complexes hetero-oligomeric for beta subunits certainly cannot be ruled out.

In contrast, receptor complexes immunoprecipitated with anti-γ1 antibodies do not contain any γ2 immunoreactive material (Quirk, 1994), indicating that these subunits do not occur together in the same receptors.

In summary, it seems likely that most but not all GABA-A receptor complexes are homo-oligomeric with respect to alpha subunits, while all are homo-oligomeric with respect to the γ1 subunit, Both homo-oligomeric and hetero-oligomeric configurations are possible with regard to beta subunits, but there is not sufficient evidence to determine which arrangement is favored.

Applying these observations to the subunit mRNA distribution seen in melanotrophs, the range of possible combinations includes α2 or α3 or perhaps α2/α3 in combination with β1, β3 or β1/β3; all of these could be combined with either the γ1 or γ2 subunit. All told, there could be up to eighteen different kinds of GABA receptors found within the melanotroph population.

Some basic predictions can be made about the various components of the GABA-A receptor population on melanotrophs. The first is that a spectrum of different affinities and efficacies for GABA will be present. This prediction is based on evidence from several transfection studies in which substitutions of one alpha subunit for another or one beta subunit for another in combinations of alpha, beta, and gamma subunits, resulted in GABA EC50s in the induction

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of chloride currents varying by as much as tenfold (Ymer et al., 1989; Sigel et al., 1990; Verdoorn, 1994).

In at least one transfection study, the presence of γ1 subunit mRNA was associated with potentiation of GABA evoked chloride currents by 3αOHDHP more than twice what was observed with γ2 subunit mRNA present (Puia, 1994). Further, while GABA responses in cells transfected with mRNA encoding the γ2 subunit have been shown to be reduced by β–carbolines, responses in cells transfected with γ1 subunit mRNA either show no response or actually show potentiation by β–carbolines depending on whether α2 or α3 mRNAs are present.

Thus it is possible that within the population of melanotroph GABA-A receptors, there is a division between receptors which are highly sensitive to 3αOHDHP potentiation, but either unresponsive to or stimulated by β–carbolines and receptors which are inhibited by β–carbolines and have a smaller response to 3αOHDHP. The possible varieties of GABA-A receptor complexes in melanotrophs and their likely properties with respect to various modulators are illustrated in Figure 6-1.

To sum up, the question as to what kinds of GABA-A receptors are present on melanotrophs can probably be answered by saying that there are many kinds and that subpopulations among them vary in their sensitivity and even the nature of their responses to important modulators. At this point it will be useful to consider the possible heterogeneity of the cell population over which this complex set of receptors is distributed.

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Are all melanotrophs exactly the same?

The melanotrophs are part of a homogenous cell population in that they constitute more than 95% of the cells of the intermediate lobe and are all engaged in the synthesis of peptides derived from POMC. Immunocytochemical staining for αMSH or β-endorphin in the intermediate lobe appears fairly uniform, and virtually every cell is intensely stained. There is evidence at two levels, however, which would lead one to believe that all melanotrophs are not exactly alike.

One line of evidence is mainly histological, and was discussed in the introductory chapter. A group of investigators reported the presence of so-called light cells and dark cells as shown by toluidine blue staining in the intact intermediate lobe. Further, they noted an uneven distribution of POMC mRNA signal over individual cells in *in situ* hybridization studies with a ^{35}S labeled probe. These findings were taken by one group as evidence of heterogeneity within the cell population (Chronwall, 1988). Another group noted the appearence of light and dark immunostaining cells in intermediate lobes denervated and grafted onto the kidney capsule (Iturriza, 1989). In both cases, the presence of systemic dopaminergic agents abolished the heterogeneity that was observed. Thus both groups cited the innervation of melanotrophs by tuberohypophyseal projections as being of importance in setting up heterogeneity within the population.

This brings us to the second line of evidence relevant to intermediate lobe microheterogeneity, the nature of its innervation by tuberohypophyseal projections. The innervation of the intermediate lobe was extensively investigated as flourescence techniques for the visualization of catecholamines

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came into use. Applying these fluorescence techniques in combination with anatomical lesions and lesions by specific agents such as 6-hydroxydopamine, several groups of investigators demonstrated the presence of a fairly rich system of catecholaminergic and aminergic innervation of the intermediate lobe (Bjorklund et al., 1973; Fuxe et al., 1980). Fibers were shown to be most dense at the junction of the neural lobe and the intermediate lobe, with fibers penetrating and surrounding lobules of melanotrophs. Cells at the center of each lobule appeared to receive less innervation than those at the periphery. These fibers were identified as dopaminergic, serotonergic, and adrenergic based both on their susceptibility to lesioning agents and on the fluorescence wavelengths observed. Comparing these early results with later results using specific antibodies to serotonin (Mezey, 1984), or to tyrosine hydroxylase (TH) and glutamate decaroxylase (GAD) (Oertel et al., 1982), it seems apparent that the richest innervation in the rat intermediate lobe may actually be from the neurotransmitter which has received the least attention, serotonin. The immunoreactivity for TH and GAD has been co-localized to the same nerve endings (Oertel et al., 1982; Schimchowitsch et al., 1991), but is somewhat sparser than one might have been led to expect from the early fluorescence studies of catecholamine distribution. This disparity may well be due to the labeling of serotonin and other amines in the fluorescence technique. As in the earlier studies, the periphery of each lobule of melanotrophs appears more richly innervated than the interior, and some lobules appear to recieve much less innervation than others. At the electron microscope level, GAD immunoreactive varicosities were noted to make synapse-like contacts with melanotrophs, but also to terminate on some occasions without making such

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contact (Oertel et al., 1982). The distribution of TH and GAD immunoreactive fibers penetrating the intermediate lobe would seem to imply that some cells are better innervated than others, and that some tuberohypophyseal neurons communicate with their targets diffusely via the extracellular canaliculi mentioned in the introductory chapter, rather than at true synapses.

In summary, all melanotrophs are probably not exactly alike, and the supposition of previous investigators that innervation might make an important contribution to microheterogeneity was probably a good one. Differential innervation would imply that individual melanotrophs experience different concentrations of GABA and other neurotransmitters; this could in turn effect gene expression, peptide synthesis, and peptide release in the cells, leading to the observed microheterogeneity. One possible consequence of this situation is that the various GABA-A receptor subunit mRNAs we have detected may not all be expressed to the same extent in every cell. Thus it is possible that melanotrophs differ from one another both in the levels of GABA to which they are exposed and in their ability to respond to GABA through their highly varied population of GABA-A receptors. The significance of this arrangement might best be understood by remembering that GABA-A receptors are not the only GABA receptors on melanotrophs. There is substantial evidence for the negative regulation of melanotrophs through GABA-B receptors (Demeneix et al., 1984; Demeneix et al., 1986; Taraskevich and Douglas, 1990; Shibuya et al., 1991; Shibuya et al., 1992), and that is the topic to which I will now turn.

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Why have GABA-A and GABA-B receptors on the same cells?

That the effects of GABA on melanotrophs are exerted through both GABA-A and GABA-B receptors might have been inferred from the first data on GABA effects on melanotrophs. These early experiments showed a transient, calcium dependent stimulation of αMSH secretion by 10μM GABA, followed by a longer period of inhibition (Tomiko et al., 1983). The calcium dependent stimulation was thought to depend on a depolarizing chloride efflux which had been observed by the same group previously (Taraskevich and Douglas, 1982). At the time, the ability of GABA and muscimol to inhibit KCl induced peptide secretion was taken as evidence that the inhibitory phase of GABA actions could be fully attributed to the effects of GABA-A receptors. A few years later, another group of investigators used the specific GABA-B agonist baclofen in several studies to show that at least part of the inhibition of melanotrophs by GABA should be attributed to the actions of GABA-B receptors (Demeneix et al., 1984). One of these experiments showed that the pure GABA-A agonist isoguvacine potentiated the release of αMSH in response to the secretagogue barium while 10μM GABA in the presence of the pure GABA-A antagonist bicuculline inhibited peptide release. These results demonstrated clearly that short term inhibitory effects of GABA on melanotrophs occur through the GABA-B receptor. In more recent experiments it has been shown that GABA-B receptor activation reduces calcium levels in melanotrophs (Shibuya et al., 1992). Thus in this important respect the actions of the two receptors are exactly opposed to one another.

One important difference between GABA-A and GABA-B receptors is in their GABA sensitivity. Although assays of GABA *binding* typically give

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affinities for both sites around the 100nM range, assays of efficacy tend to show that at GABA-B receptors, GABA is effective at concentrations of 1μM or less, while efficacy at GABA-A receptors tends to begin at concentrations five or ten times higher (Bowery, 1980; Schwartz, 1986; Sigel et al., 1990; Wong et al., 1992).

In addition to being biphasic in time and dissectable by the use of pure GABA-A or GABA-B agonists or antagonists, the direction of the response of melanotrophs to GABA might then depend on the concentration of GABA present. We have begun to test this proposition in a perifusion system with dispersed primary cultures of melanotrophs. A preliminary result is shown in Figure 6-2. In this experiment, 10μM GABA stimulated melanotrophs to secrete β-endorphin in the same manner as did the pure GABA-A agonist isoguvacine. GABA applied at a lower concentration, 250nM, however, elicited an inhibitory effect similar to that of the pure GABA-B agonist baclofen. The concept of opposed populations of GABA receptors with differing actions on peptide secretion and differing affinities is depicted schematically in Figure 6-3.

One property conferred on melanotrophs by having both GABA-A and GABA-B receptors, then, is that melanotrophs have the capacity to respond in opposite ways to different amounts of the same neurotransmitter. It should also be noted that whenever GABA concentrations are sufficient to activate GABA-A receptors, GABA-B receptors will also be activated, holding the depolarization of melanotrophs in check. Thus there may be multiple benefits to having both GABA-A and GABA-B receptors on melanotrophs. In summary, the actions of GABA on melanotrophs may be viewed as being balanced between two sets of opposed receptors. This balance can be altered at

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several points, but the one on which I would like to focus now, is the potential role of modulators of the GABA-A receptor.

How could modulators of the GABA-A receptor alter melanotroph function?

Taken together, the properties of GABA-A receptor heterogeneity and differential innervation of individual melanotrophs define four potential groups of melanotrophs. One would be cells which are both maximally sensitive to GABA at GABA-A receptors and well innervated by GABA projections. A second group would be cells which are not maximally sensitive, but are still well innervated. A third group would be cells with maximal sensitivity at GABA-A receptors but poor innervation, and the final group would be cells both poorly innervated and not maximally sensitive at GABA-A receptors. Taking into account the higher potency of GABA at GABA-B receptors, one might predict that release of GABA from tuberohypohyseal projections would be likely to stimulate peptide release from the first group, inhibit release from the last group, and have varied effects on release from the two middle groups. The concept of GABA modulator induced shifts in the secretory behavior of melanotroph populations is illustrated schematically in Figure 6-4.

Positive modulators of the GABA-A receptor might act to push cells in the two middle groups to become stimulated in response to their GABAergic input, thus changing the overall "shape" of the population's GABA response. Positive modulators would certainly include 3αOHDHP and THDOC, and for some cells, might also include β-carbolines depending on the combination of γ and α subunits present. Conversely, negative modulators of GABA-A

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receptors would push cells toward an inhibitory response to GABA dominated by actions at GABA-B receptors. Such negative modulators would include pregnenolone sulfate and dehydroepiandrostane sulfate, as well as β-carbolines. It should be noted here that pregnenolone sulfate, dehydroepiandrostane sulfate, and pregnenolone have the ability to oppose the excitatory actions of GABA at voltage gated calcium channels as well as at the GABA-A receptor itself (Ffrench-Mullen, 1994). This convergence of negative regulatory actions of neurosteroids is illustrated schematically in Figure 6-5, as are the opposing effects of positive modulators. It is interesting to note that steroids expected to have negative effects are those generated in the *de novo* steroid synthetic pathway, probably subject to neurotransmitter regulation via the intermediate of the synthesis of DBI (Costa and Guidotti, 1991) while those predicted to have a positive effect at GABA-A receptors are likely to be derived from circulating steroids.

Concluding discussion:

In summary, at the level of the individual cell, I would propose that the combination of excitation through GABA-A receptors and inhibition through GABA-B receptors may set up a situation in which modulators of the GABA-A receptor may determine whether excitation or inhibition occurs in response to GABA. Applied to a large population of cells with complex GABA-A receptors and variable GABA inputs, the summation of the events in single cells could set a basal level of peptide secretion in response to tonic GABA input.

For the sake of clarity, the large numbers of other neurotransmitters acting on melanotrophs have been left out of this scheme. Two are of sufficient

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importance that they must now be mentioned, although a full examination of their actions is well beyond the scope of this discussion. One is dopamine, which inhibits peptide secretion from melanotrophs; dopamine, besides adding its own negative effect to that of GABA-B receptors, can be thought of as negatively modulating the excitatory pathway of GABA "from the inside" by indirectly inhibiting calcium channel function. Another is norepinephrine. Norepinephrine has been shown to be the major neurotransmitter responsible for the release of β-endorphin from the intermediate lobe during anxiogenic stresses. This effect is completely abolished by propanolol (Berkenbosch et al., 1984), ruling out a role for other neurotransmitters or modulators at least in the acute part of this response. It would seem then, that norepinephrine is able to take complete control of the system, overiding other signals and carrying out secretion of peptides in response to acute stimuli by itself.

When is the alteration of basal secretion via modulation of GABAergic pathways likely to be of importance in melanotrophs? Given our finding of synthesis of 3αOHDHP from progesterone by neurointermediate lobe, it is quite possible that the mechanisms proposed are involved in the increase in melanotroph output in response to progesterone seen during the estrous cycle. It is also possible that elevation of adrenal steroids such as corticosterone and progesterone during "physiological," stresses leads to the synthesis of neurally active steroids which would increase basal melanotroph function in the manner proposed. We have not investigated extensively the metabolism of corticosterone by neurointermediate lobe, but it is not impossible to see how corticosterone might be converted to compounds active at the GABA-A receptor. It should be noted that in these situations, changes in intermediate lobe function would occur well after activation of the anterior lobe

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corticotrophs, and after the activation of the adrenal gland. Conceivably, intermediate the lobe peptides might be involved in integrating the "aftermath" of physiological stresses.

The hypothesis proposed in this chapter is testable at many points. First, it should be possible to demonstrate the ability of the appropriate GABA modulators to alter dose dependent GABA inhibition or stimulation of peptide secretion from cultured melanotrophs. Our laboratory has undertaken these studies, and studies in GT1-7 cells which also have a biphasic response to GABA and have both GABA-A and GABA-B receptors. A second testable point is the heterogeneity of GABA-A receptor subunit mRNA distribution. This would be best adressed by *in situ* hybridization which can show whether individual GABA-A receptor subunit mRNAs found in the neurointermediate lobe are expressed to an equal extent in every melanotroph. As discussed in the chapter on pharmacology, the presence of GABA-A receptors with varied affinities for GABA would best be confirmed by patch clamping and whole cell recording studies of current responses to the application of GABA. The use of the *de novo* steroid synthesis pathway and the opportunities it may provide for neurotransmitter regulation leading to the synthesis of negative modulators is also an active topic of research in our laboratory. Finally, another testable point of the basic hypothesis given here is to assess the effects of administration of THDOC and 3αOHDHP *in vivo* on the secretion of intermediate lobe peptides.

Clearly, a project intended to take advantage of a homogenous population of cells in order to obtain a simplified preparation of a very complicated receptor has raised many more questions than it has answered. The surprising complexity of GABA-A receptors in melanotrophs, however, is

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well suited to a cell which wears many physiological "hats"; as discussed previously, melanotrophs are called upon to provide more than one type of peptide signal to participate in a staggering range of physiological events which take place in time frames ranging from minutes to months. Perhaps it would have been more surprising if the GABA receptors on melanotrophs were very simple. The components of the GABAergic system defined in the studies presented here point toward a system which might allow a large number of regulatory inputs to elicit subtle as well as dramatic responses, from subsets of melanotrophs as well as the whole population. I have tried here to develop a plausible hypothesis about how some of these components might be integrated to achieve these effects, and particularly, how the heterogeneity of GABA-A receptors can be used to this end.

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6-1

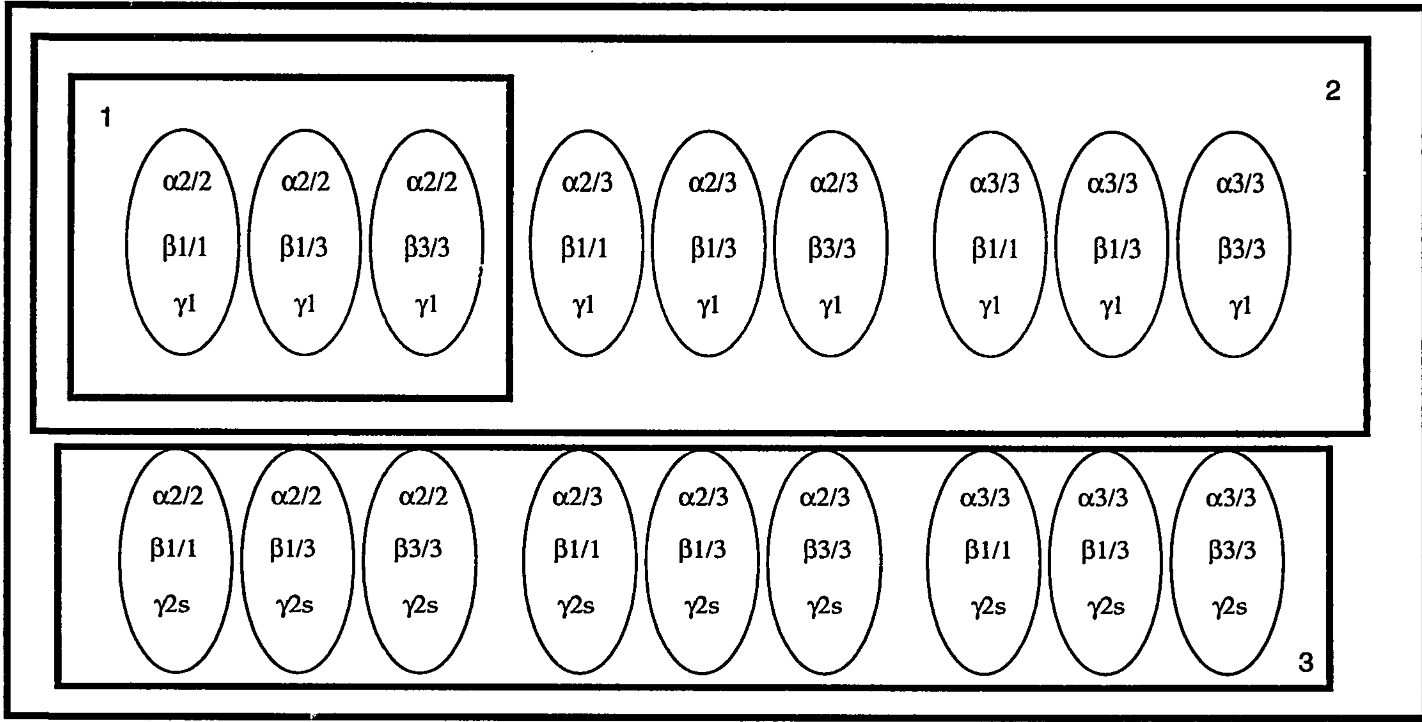

Figure 6-1 illustrates the varieties of GABA-A receptor complexes that might occur in melanotrophs. Group 2 would be characterized by higher sensitivity to neurally active steroids; group 1 might be stimulated rather than inhibited by beta-carbolines. Group 3 would be less sensitive than group 2 to neurally active steroids and would be negatively modulated by beta-carbolines. In addition, it is possible that each of the nine receptor complexes made with a given gamma subunit might have a different EC50 for GABA stimulation of chloride flux, one for each alpha and beta combination .

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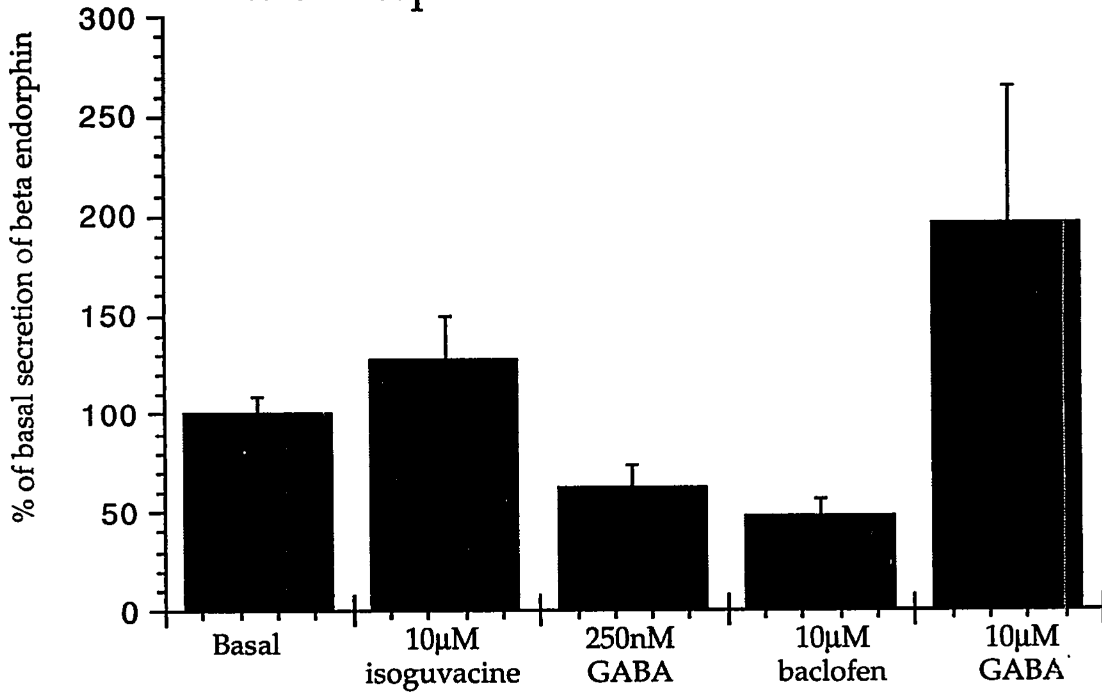

Figure 6-2 illustrates a perifusion experiment in which 250nM GABA inhibited beta-endorphin peptide secretion from perifused melanotrophs while 10μM GABA stimulated secretion. Each bar represents the average amount of beta endorphin secreted as compared to the basal level in five pooled fractions collected during the application of the drug indicated. Beta endorphin was quantified by radioimmunoassay. Standard error is shown.

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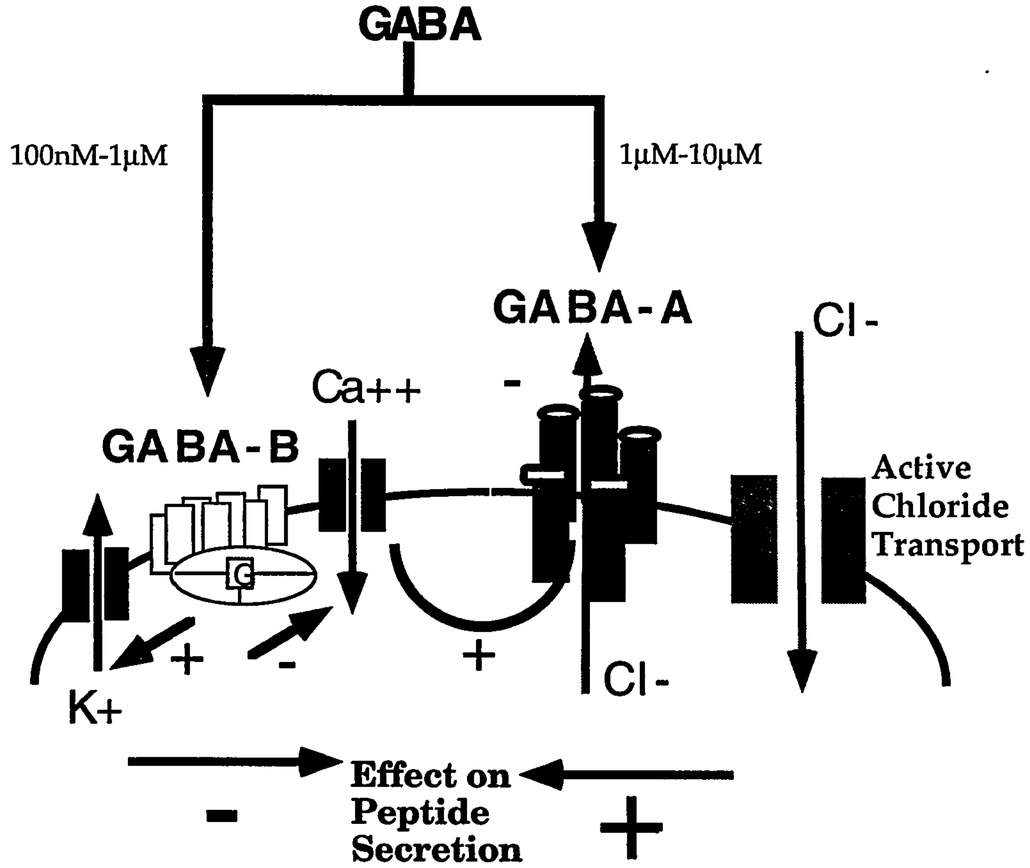
6-3 GABA acts on melanotrophs through opposing signal pathways



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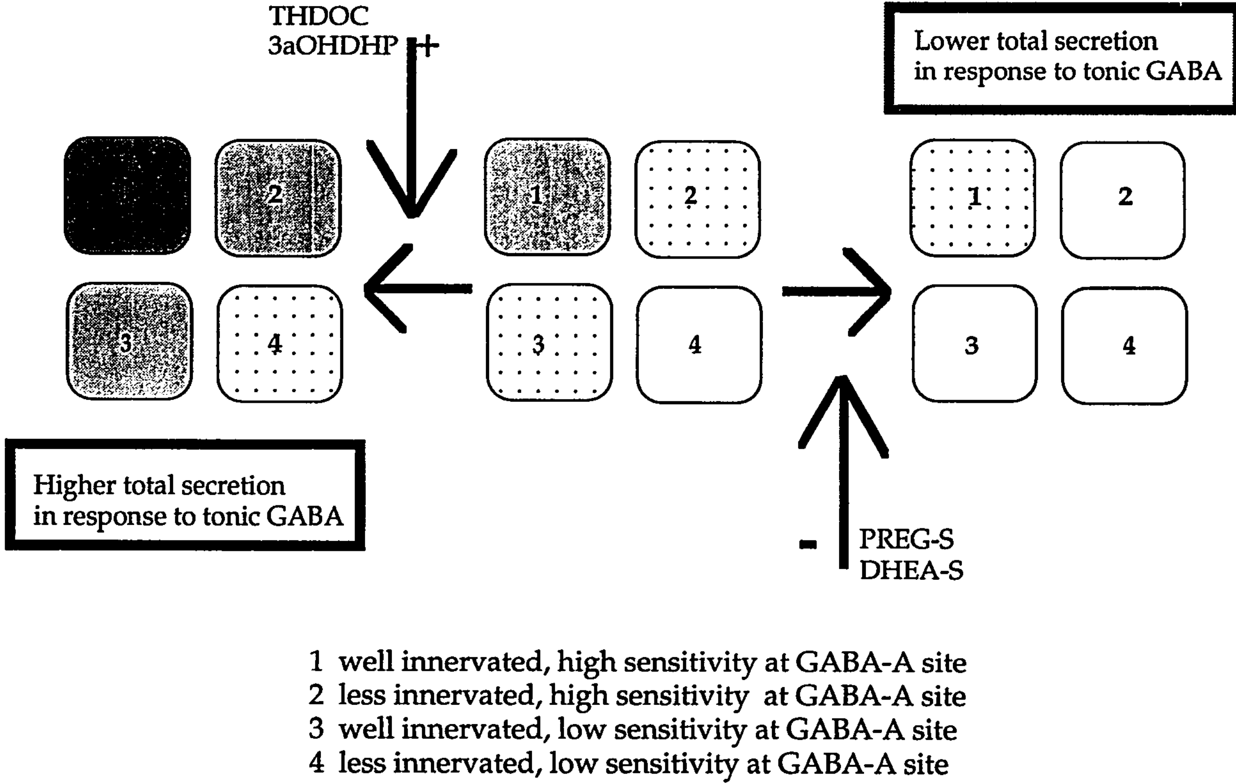
6-4 Schematic Illustration of regulation of basal secretion in a melanotroph population by positive and negative modulators of the GABA-A receptor



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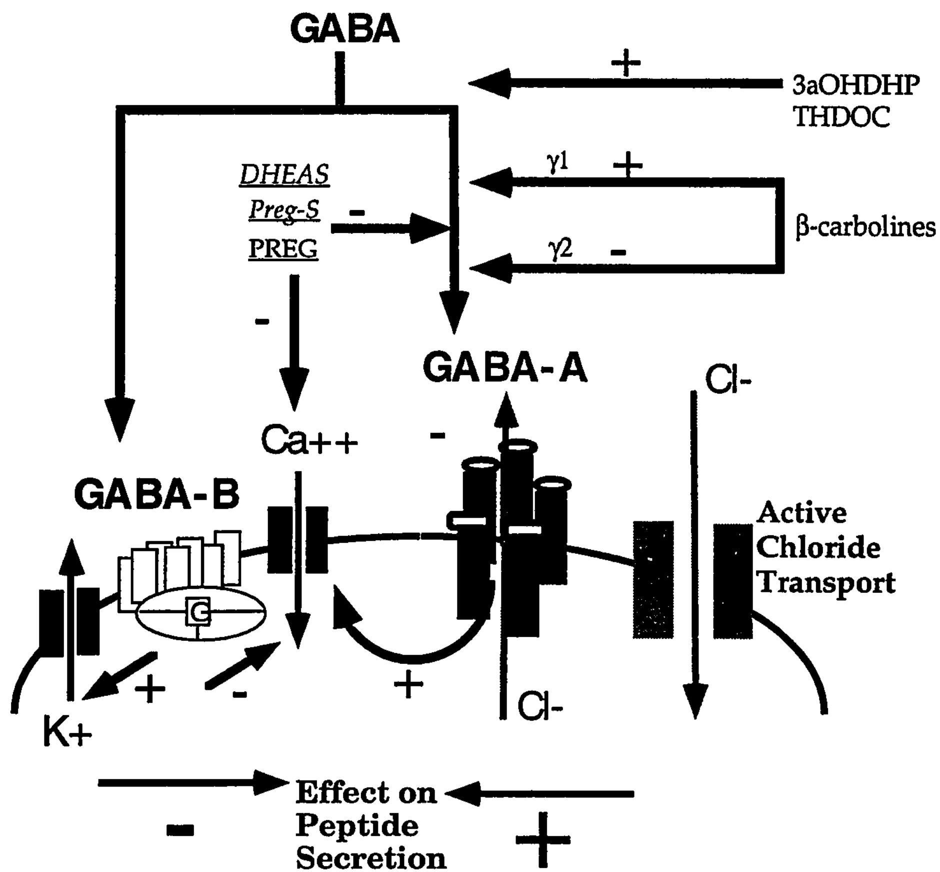
6-5

Converging of modulatory pathways on components of the GABA signalling system in melanotrophs, at the level of the individual cell.



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