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Ontogeny of Basal and Regulated Proopiomelanocortin-Derived Peptide Secretion in Lobe Cells: Melanotrophs Exhibit Transient Glucocorticoid Responses during Development

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Proopiomelanocortin (POMC)-producing cells comprise nearly 100% of the adult rat intermediate lobe (IL) hormone-producing cells. Secretion by these cells in the adult is primarily under negative regulation by dopamine. Although the POMC-derived peptide α-MSH has been detected in plasma of fetal rats, the secretory capability of fetal melanotrophs has not yet been examined directly. Here we have used the reverse hemolytic plaque assay to assess, at the single-cell level, basal and regulated release by melanotrophs from fetal and early postnatal ages. Basal secretion was detected at the earliest age examined [Embryonic Day 17.5 (e17.5)], but CRH (10^{-8} *M*) stimulated secretion was not observed until e19.5. As development proceded, CRH increased both individual plaque sizes and the percentage of melanotrophs stimulated to secrete. An unexpected, transient inhibition of CRH stimulated release from melanotrophs by dexamethasone (DEX, 10^{-6} *M*) was observed from e19.5–p2 (postnatal Day 2). By p3, however, DEX no longer inhibited melanotroph secretion while inhibition of CRH-stimulated release from p3 corticotrophs was readily detected. The dopamine agonist ergocryptine (ERG, 10^{-6} *M*) inhibited basal secretion from melanotrophs, but not corticotrophs, at all ages examined. Taken together, these results indicate that melanotrophs undergo a maturation process in which they are initially nonresponsive to CRH, next possess functional CRH and steroid receptors, and finally, undergo functional uncoupling of steroid receptors which characterizes the adult IL. The loss of steroid-mediated inhibition of stimulated secretion parallels the arrival of catecholaminergic input into the IL. In contrast, the early response of melanotrophs to dopaminergic agonists, which can be detected 1 week prior to arrival of catecholaminergic fibers into the neurointermediate lobe, appears to be an intrinsic feature of these cells that is never present in corticotrophs.

INTRODUCTION

In the adult rat, the intermediate lobe (IL) of the pituitary gland is a quite homogeneous tissue comprised principally of proopiomelanocortin (POMC)-derived peptide producing cells (Howe, 1973; Chronwall *et al.*, 1987). Both the IL and anterior lobe (AL) develop from Rathke's pouch, which has been shown in the chick (Couly and LeDourain, 1985) and amphibian (Eagleson *et al.*, 1986) to arise from the anterior ventral neural ridge. Although the AL and IL share a common origin, the main products of POMC precursor processing differ between adult IL and AL POMC cells (Eipper and Mains, 1980). In addition, there are several major differences in the regulation of synthesis and secretion of POMC-derived peptides in each lobe. For example, although both adult AL corticotrophs and IL melanotrophs have CRH receptors and respond to CRH incubation with increased secretion of POMC-derived peptides, CRH receptors are less abundant in the IL (DeSouza *et al.*, 1984) and a 10-fold higher CRH concentration is needed to stimulate secretion from melanotrophs compared to corticotrophs (Meunier *et al.*, 1982; Vale *et al.*, 1983). In addition, adult melanotrophs, but not corticotrophs, are under inhibitory regulation by hypothalamic dopamine (Munemura *et al.*, 1980; Vermes *et al.*, 1980). Finally, in contrast to the adult corticotrophs,

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the melanotrophs lack functional glucocorticoid receptors (GR) (Schachter *et al.,* 1982; Eberwine and Roberts, 1984; Seger *et al.,* 1988) and thus glucocorticoids do not inhibit CRH-stimulated POMC secretion from these cells. However, if the IL is subject to denervation (Seger *et al.,* 1988) or the IL cells are cultured (Eberwine *et al.,* 1987), GR receptor function can be detected. Incubation with dopamine or dopamine agonists inhibits the appearance of GR in cultured IL cells, as detected by [^{3}H]DEX (dexamethasone) binding and GR immunocytochemistry (Antakly *et al.,* 1987), which suggested to us that the status of GR presence/function in the developing IL might vary as innervation procedes.

While immunocytochemical (Dupouy and Dubois, 1975; Chatelain *et al.,* 1979; Watanabe and Daikoku, 1979; Schwartzberg and Nakane, 1982; Kachaturian *et al.,* 1983; Pintar and Lugo, 1987; Lugo *et al.,* 1989) as well as *in situ* hybridization studies (Pintar and Lugo, 1987; Lugo *et al.,* 1989) have shown the presence of POMC cells in the fetal IL, studies on the secretory capabilities of these cells during development is limited. Only a few studies have been carried out in the human (Gibbs *et al.,* 1982), sheep (Newman *et al.,* 1987), and rat fetus (Davis *et al.,* 1984) as well as the neonatal rat (Davis *et al.,* 1984; Sato and Mains, 1986) and mouse (Schmitt *et al.,* 1981) and no study thus far has assessed directly, at the single-cell level, the secretory capabilities of fetal melanotrophs and the development of responsiveness to regulators of secretion. Furthermore, with the exception of the studies in the human fetus (Gibbs *et al.,* 1982), all other fetal studies have been carried out *in vivo*. Since sites other than the pituitary gland synthesize POMC mRNA and secrete POMC peptides, the origin of circulating peptides cannot be accurately determined in these studies (see Lugo and Pintar, 1996, for references). Moreover, the effect of *in vivo* physiologic conditions in modulating responses of individual cells or groups of cells cannot be evaluated.

As shown in the accompanying paper (Lugo and Pintar, 1996), the reverse hemolytic plaque assay, which was initially developed to study individual antigen-secreting cells (Molinaro *et al.,* 1981), can be utilized informatively to study the ontogeny of the secretory response of corticotrophs. Here, we have utilized this assay to study the ontogeny of basal and regulated secretion from individual melanotrophs from isolated neurointermediate lobes (NIL). These studies were carried out beginning at Embryonic Days 17–17.5 (e17–e17.5) since it is at this age that the percentage of POMC-derived peptide-containing cells in the IL becomes significant (Pintar and Lugo, 1987; Lugo *et al.,* 1989) and dissection of the pituitary into AL and NIL can be performed reliably and consistently. The secretory responses of immunocytochemically identified fetal (e17.5, e19.5, and e21) and neonatal (Postnatal Days 2, 3, and 5; p2, p3, and p5) melanotrophs to CRH and dopamine were investigated. Furthermore, since evidence has suggested that adult melanotrophs can increase the level of GR if deprived of neural input *in vivo* or *in vitro* and that this increase can be inhibited by incubation with dopamine or dopamine agonists *in vitro* (Antakly *et al.,* 1987), a major focus of this study was to determine whether the melanotroph response to glucocorticoids differed at ages prior to and after arrival of dopaminergic input into the IL.

MATERIALS AND METHODS

In general, methods used for this study were identical to those described in the accompanying paper (Lugo and Pintar, 1996). For specific descriptions of methods used to couple ovine red blood cells (oRBC) to staphylococcal protein A and to determine the efficiency of coupling, and for general aspects of the reverse hemolytic plaque assay, the basal secretion protocol, immunocytochemistry, and data analysis, see appropriate sections in the accompanying paper (Lugo and Pintar, 1996). The sections below include specific methods used to isolate or investigate NIL-derived cells.

Neurointermediate Lobe Cell Dispersion

Sources of NIL cells were embryos (e17.5, e19.5, and e21) isolated from decapitated timed-pregnant mothers and neonates (p2, p3, and p5). Isolated fetuses and neonates were decapitated, the pituitary glands removed, and AL and NIL separated. Isolated NIL from 10 to 18 animals (one to two litters) were pooled and dispersed as described in the accompanying paper (*Pituitary Cell Dispersion,* under Materials and Methods; Lugo and Pintar, 1996).

Studies on Stimulation of POMC-Derived Peptide Secretion from Melanotrophs

Studies examining stimulated secretion were performed identically to those on basal secretion but with the addition of the secretagogue CRH (10^{-7} *M*) together with the antiserum (Smith *et al.,* 1986). Initial experiments were conducted utilizing different CRH concentrations in order to determine the CRH concentration that resulted in the largest increase in melanotroph plaque area as compared to basal levels. The highest concentration used, CRH 10^{-7} *M,* produced the largest plaques and was used in all subsequent experiments.

Inhibition of POMC-Derived Peptide Release from Melanotrophs

1. The dopamine agonist 2-bromo-α-ergocryptine (ERG; 10^{-6} *M*) was used to investigate the inhibition of basal release of POMC-derived peptides from NIL cells of a variety of ages (e17.5, e19.5, e21, p2, p3, and p5). In these experiments, ERG (10^{-6} *M*) incubation began 45 min prior to addition of β-endorphin antiserum and continued throughout the duration of the experiment. ERG was used instead of dopamine since it is more stable than the latter (Antakly *et al.,* 1987); in addition, 1 m*M* ascorbic acid was included in all ERG-containing solutions in order to minimize oxida-

tion (Loeffler *et al.*, 1988). Initial experiments determined that 10^{-6} *M* ERG was the lowest ERG concentration that inhibited basal release in e21 NIL cultures, and this concentration was used in all subsequent experiments. Complement addition and subsequent treatment of the chambers was as described (Lugo and Pintar, 1996).

2. Glucocorticoid inhibition of CRH (10^{-7} *M*) stimulation was tested in isolated NIL from e19.5 to p5. In these experiments, incubation with DEX (10^{-6} *M*) began 45 min prior to addition of CRH (10^{-7} *M*) and β-endorphin antiserum; incubation with DEX continued throughout the duration of the experiment. Initial experiments performed with isolated AL cells determined that 10^{-6} *M* DEX produced the greatest inhibition of the CRH stimulatory response (Lugo and Pintar, 1996). Additional experiments have shown that inhibitory effects of DEX on CRH-stimulated transcription occur even at 10^{-8} DEX (Scott and Pintar, 1993). Subsequent treatment of chambers was as described (Lugo and Pintar, 1996).

3. ERG (10^{-6} *M*) inhibition of CRH (10^{-7} *M*) stimulation was tested at two ages (e19.5 and e21). These experiments were carried out as above but ERG (10^{-6} *M*) was substituted for DEX (10^{-6} *M*).

RESULTS

Melanotrophs from isolated NIL were capable of basal secretion beginning at the earliest stages examined (embryonic day 17.5, e17.5; Fig. 1). At this age, release of POMC-related peptides could not be enhanced even in the presence of a high CRH concentration (10^{-7} *M*); thus plaque sizes obtained upon incubation with CRH (10^{-7} *M*) were not significantly different ($P \leqslant 0.05$) than those obtained in the absence of CRH (Fig. 1). A stimulatory response of melanotrophs to CRH (10^{-8} *M*) was first observed at e19.5. At this age, plaque areas obtained upon incubation with CRH (10^{-7} *M*) (Figs. 1 and 2A) were significantly ($P \leqslant 0.05$) larger than control values (Figs. 1 and 2B). The response to CRH was dose-dependent and larger plaques were obtained with larger CRH concentrations at the ages tested, e19.5 (Fig. 3) and p3. The stimulatory effect of CRH on plaque size persisted in all subsequent ages studied (e21 and p2, p3, and p5 (Fig. 1). The melanotroph response to CRH incubation was heterogeneous. Some cells responded to CRH robustly, producing very large plaques, others responded moderately, while still others did not show an increase in plaque size above control values (see for example e21, Fig. 4). Plaque sizes under basal conditions were smaller and more homogeneous (Fig. 4). Incubation with CRH (10^{-7} *M*) also had a stimulatory effect on the percentage of melanotrophs that formed plaques (Table 1). This effect chronologically paralleled the CRH effect on increased plaque size. At e17.5, when incubation with CRH (10^{-7} *M*) had no effect on plaque size, the percentage of β-endorphin immunostained cells that produced plaques in its presence or absence was virtually identical (48 and 47%, respectively, Table 1). At e19.5 and in all older ages studied, incubation with CRH (10^{-7} *M*) produced an increase in plaque size (Fig. 1) paralleled with an increase in the percentage of plaque-producing cells over control values (Table 1). At all ages studied, there was a subset of immunostained cells that did not form plaques even upon CRH stimulation.

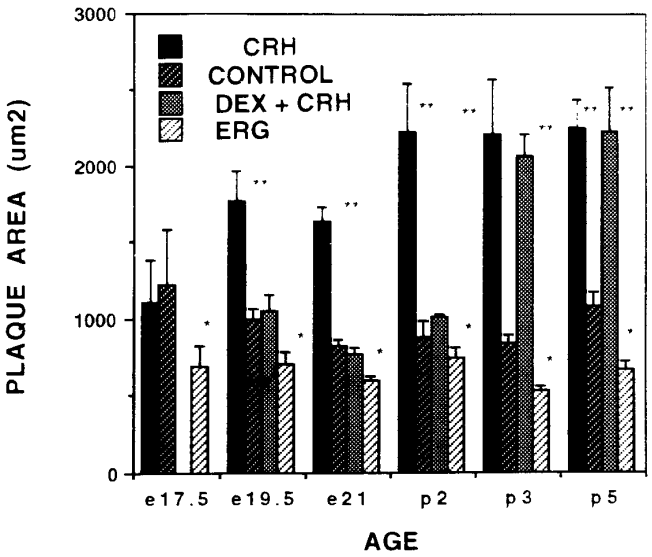

FIG. 1. Summary of the ontogeny of basal and regulated release from melanotrophs. At e17.5 mean plaque areas were unchanged in the presence or absence of CRH, but decreased in the presence of ERG to values below basal ($P \leqslant 0.05$). Statistically significant ($P \leqslant 0.05$) increases in plaque size following incubation with CRH were observed beginning at e19.5 and continued throughout all other older ages examined. Inhibition of CRH stimulation by the synthetic glucocorticoid DEX was only observed between the ages e19.5 and p2. DEX inhibited CRH-stimulated β-endorphin secretion to levels statistically ($P \leqslant 0.05$) similar to control. Beginning at p3, DEX no longer inhibited CRH stimulation of β-endorphin release from intermediate lobe cells. Each bar represents the mean $\pm$ SEM of three experiments (25 plaque areas/experiment). *Significantly different from other treatments within age. **Significantly different than control.

Melanotroph secretion was inhibited by the dopamine agonist ERG (10^{-6} *M*) beginning at the earliest age examined (e17.5), with plaque areas obtained after 45 min preincubation with ERG significantly ($P \leqslant 0.05$) smaller than control (basal) plaque areas (Fig. 1). This effect persisted throughout all ages examined (e19.5, e21, p2, p3, p5; Fig. 1).

The ability of ERG (10^{-6} *M*) to inhibit CRH-stimulated POMC peptide release was studied in two age groups, e19.5 and e21. Preincubation with ERG (10^{-6} *M;* 45 min) abolished the increase in plaque size normally obtained after incubation with CRH (10^{-7} *M*) alone; thus the values obtained upon CRH (10^{-7} *M*) addition following 45 min preincubation with ERG (10^{-6} *M*) were not significantly ($P \leqslant 0.05$) different from control values (see for example, e21, Fig. 5).

Glucocorticoid effects on CRH-stimulated POMC peptide release were examined beginning at e19.5, the age when

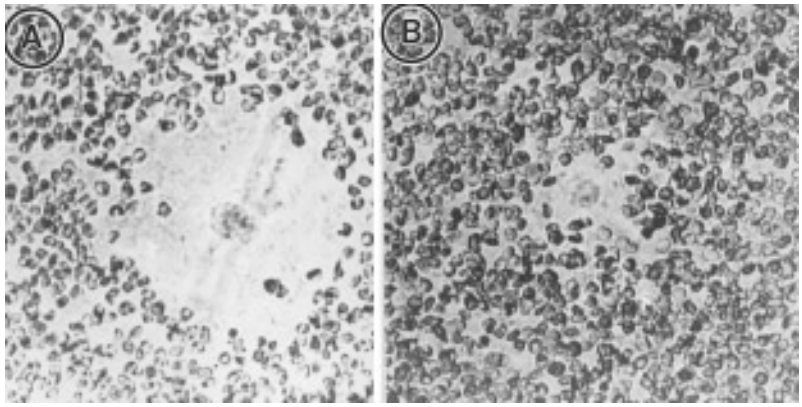

FIG. 2. Response of melanotrophs to secretory regulators at e21. Plaque areas are larger in the presence (A) versus the absence (B) of CRH.

CRH (10^{-7} *M*) enhancement of secretion was first detected. At this age, a 45-min preincubation with DEX (10^{-6} *M*) abolished the increase in plaque area observed upon incubation with CRH (10^{-7} *M*) alone (Fig. 1). The plaque areas obtained after addition of CRH (10^{-7} *M*) following a 45-min preincubation with DEX (10^{-6} *M*) were not significantly ($P \leqslant 0.05$) different from those obtained upon basal conditions (Fig. 1). Moreover, the plaque size distribution with both treatments was nearly identical (see for example e21 responses, Fig. 4). This inhibitory effect of DEX (10^{-6} *M*) persisted through Postnatal Day 2 (Fig. 1). Beginning at p3, the inhibitory effect of DEX on CRH (10^{-7} *M*)-stimulated release from melanotrophs was no longer present (Fig. 1). This lack of inhibition by DEX (10^{-6} *M*) was also observed in the oldest postnatal age examined, p5 (Fig. 1).

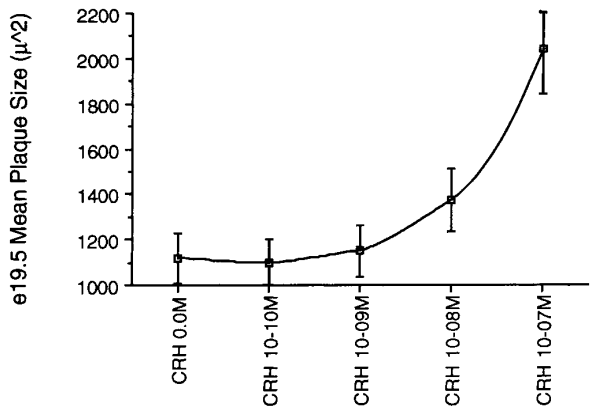

FIG. 3. CRH dose–response curves for melanotrophs. CRH dose–response curves were performed for melanotrophs at e19.5 and p3 from isolated NIL. Represented here are the mean values obtained at e19.5. Statistically significant ($P \leqslant 0.05$) increases in plaque areas above basal levels are observed with CRH concentrations equal to 10^{-8} *M* and higher. Each value represents the mean ± SEM of 25 plaques.

Cells were immunostained with β-endorphin antiserum following the plaque assay in order to determine the percentage of melanotrophs in the total NIL cell population at different ages as well as the possible effect of incubation with CRH (10^{-7} *M*) upon the percentage of plaque-producing melanotrophs. The percentage of melanotrophs in the total NIL cell population increased steadily throughout prenatal life and remained virtually unchanged throughout all postnatal ages studied (Table 1). At e17.5 melanotrophs comprised 32% of the NIL cell population, at e21 their percentage had increased to 47 and by p2 they comprised 57% of the NIL population (Table 1). As mentioned previously, while CRH incubation significantly ($P < 0.05$) increased the percentage of melanotrophs that formed plaques beginning at e19.5 (Table 1), there was a subpopulation of melanotrophs that did not form plaques even upon incubation with a high (10^{-7} *M*) CRH concentration.

DISCUSSION

While POMC mRNA-containing cells in the IL could be detected as early as e15, significant POMC-derived peptide immunoreactivity was not detected prior to e17 (Pintar and Lugo, 1987; Lugo *et al.*, 1989). We, therefore, began our studies of the ontogeny of basal and regulated secretion from individual melanotrophs at e17–e17.5. In addition, at this age we could separate the NIL from the AL reliably.

At e17.5 melanotrophs are capable of secreting POMC-derived peptides but do not respond to CRH even at high concentration (10^{-7} *M*). This initial lack of responsiveness to CRH by melanotrophs mimics the behavior of their young (e13.5) AL counterparts (Lugo and Pintar, 1995). Lack of responsiveness to CRH may be due to absence of CRH receptors, failure of coupling of CRH receptors to adenylate cyclase, or lack of function of protein kinase A substrates involved in CRH activation of secretion. Studies of the ontogeny of the recently cloned rat CRH receptor (Perrin *et*

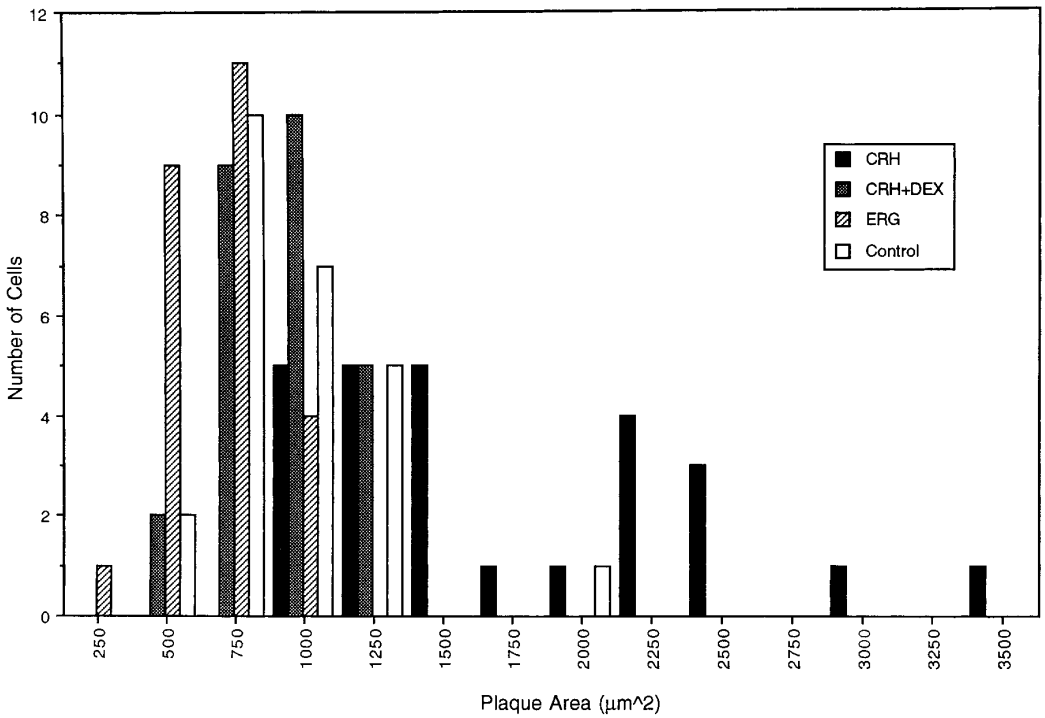

FIG. 4. Plaque area distributions of e21 melanotrophs following no treatment, CRH treatment, ERG treatment, or CRH–DEX treatment (45-min preincubation with DEX). Melanotrophs respond heterogenously to CRH stimulation, while plaque sizes obtained upon CRH–DEX treatment are not significantly ($P \leqslant 0.05$) different from basal values, have a similar size distribution, and are more homogeneous. Plaque sizes obtained upon ERG treatment are significantly ($P \leqslant 0.05$) smaller than basal values.

al., 1993) in addition to direct activation of the cAMP pathway by 8-Br-cAMP or forskolin in e17 melanotrophs are necessary in order to distinguish between these possibilities.

CRH (10^{-8} *M*) stimulation of secretion was first observed at e19.5. This response was similar to that observed in the adult IL (Meunier *et al.*, 1982; Vale *et al.*, 1983) and continued throughout all other older ages studied. As in the adult (Meunier *et al.*, 1982; Vale *et al.*, 1983; Loeffler *et al.*, 1988), the stimulatory effect of CRH could be abolished by preincubation with the dopamine agonist ERG. Thus far, CRH effects on fetal or neonatal melanotroph secretion have only been studied in superfused human NIL (fetus; Gibbs *et al.*, 1982) and in cultured rat pituitaries (neonate; Sato and Mains, 1986). Gibbs *et al.* (1982) did not observe a stimulatory effect of CRH on NIL from fetuses 19 to 23 weeks of gestation. In the neonatal rat, Sato and Mains (1986) showed approximately a twofold stimulation over the IL basal secretory rate upon CRH (10^{-7} *M*) exposure which was lower than that observed in the AL. Our results not only confirm those of Sato and Mains (1986) but extend their observations to prenatal ages. We demonstrated that while the fetal AL is responsive to CRH concentrations as low as 10^{-10} *M* (Lugo and Pintar, 1996), plaque sizes from IL cells did not increase significantly above control values at CRH concentrations lower than 10^{-8} *M*. Consistent with the observations of Sato and Mains (1986) in neonatal NIL cultures, the response of prenatal NIL cells does not reach a plateau even at high (10^{-7} *M*) CRH concentrations. Although the reason for this observation remains uncertain, it must be noted that the population assayed here contains at all times cells at different stages of differentiation which have not achieved adult steady-state values of POMC mRNA/cell (Lugo *et al.*, 1989) or POMC peptide content/cell (Sato and Mains, 1986). While the ontogeny of CRH receptors has not been studied thus far, it is likely that their number and functional status also change as development proceeds. Changes in any or all of these parameters may be responsible for the inability of differentiating melanotrophs to achieve plateau levels in response to CRH. Furthermore, the present studies demonstrate that CRH stimulates hormone release from fetal/neonatal melanotrophs via two mechanisms: (1) increase in the hormonal release from individual melanotrophs (larger plaque areas); (2) increase in the percentage of melanotrophs that form plaques.

Hotta *et al.* (1988), in their superfusion studies of whole embryonic rat pituitary glands, showed increases in α-MSH secretion at e17 upon incubation with CRH (10^{-11}–10^{-10} *M*) and inferred that this secretion was of IL origin. The present studies show that e17 melanotrophs do not respond to CRH even at concentrations as high as 10^{-7} *M* and a response to CRH was not detected prior to e19.5. Further-

TABLE 1
Effect of CRH on Melanotroph Plaque Formation

Age	Percentage of immunostained cells with plaques		Percentage of immunostained cells/ total NIL cells
	Control	CRH	
e17.5	47	48	32
e19.5	44	56[a]	38
e21	40	55[a]	47
p2	43	61[a]	57
p5	48	65[a]	56

Note. The percentage of melanotrophs was calculated from the number of immunolabeled cells following β-endorphin immunocytochemistry and the total number of cells on slides previously subjected to reverse hemolytic plaque assays. The percentage of plaque-forming melanotrophs was also determined. $n = 1000$ cells for percent plaque-forming cells/treatment; $n = 2000$ for percent of melanotrophs from total number of cells.

[a] Statistically different (<0.05) compared to age-matched control cells using paired t test.

more, fetal melanotrophs (unlike corticotrophs, Lugo and Pintar, 1996; see above) exhibited no response to CRH at concentrations lower than 10^{-8} *M*. Moreover, biochemical studies of peptide forms during fetal life have demonstrated that α-MSH is not detected in the fetal IL until e19 (Allen *et al.,* 1984). In addition, the presence of α-MSH-like peptides has been demonstrated in the fetal and neonatal AL by immunocytochemistry (Kachaturian *et al.,* 1983; Sato and Mains, 1985) and demonstrated to be des-acetyl-α-MSH by peptide analysis (Pintar *et al.,* 1986; Pintar and Lugo, 1987). Taken together, the aforementioned results indicate that Hotta *et al.*'s (1988) observations likely reflect des-acetyl-α-MSH secretion originating from corticotrophs rather than melanotrophs.

At all ages studied, there was a subpopulation of β-endorphin immunostained melanotrophs that did not form plaques even upon CRH (10^{-7} *M*) incubation. Since no study at the single cell level exists in the adult IL, it is not known whether every melanotroph releases POMC-derived peptides under basal or stimulated conditions. It is nonetheless known, that normally, the rat IL contains what has been described as dark and light melanotrophs. This difference in tinctorial properties has been interpreted to reflect differences in cell activity (Chronwall *et al.,* 1987, 1988). For example, a single injection of haloperidol (a dopamine antagonist) to intact animals increases the proportion of dark (active) melanotrophs and the number of silver grains over the cells after *in-situ* hybridization autoradiography with POMC-specific probes. Similar treatment with ergocryptine (a dopamine agonist) has opposite effects (Chronwall *et al.,* 1987). Perhaps the differences in tinctorial properties of these cells may also reflect differences in the secretory state of individual melanotrophs. In addition, melanotrophs have been shown to possess CRH (De Souza *et al.,* 1984) as well as β-adrenergic receptors (Cote *et al.,* 1980; Schimchowitsch and Pelletier, 1988) and to respond to CRH (Meunier *et al.,* 1982; Vale *et al.,* 1983) and β-adrenergic receptor agonists (Przewlocki *et al.,* 1979; Cote *et al.,* 1980; Pettibone and Mueller, 1982) by increasing POMC-derived peptide secretion. Thus, a phenomenon similar to that observed by Childs and Burke (1987) on AL corticotrophs in which maximal stimulation of secretion by these cells required the action of multiple secretagogues, might also exist for IL melanotrophs.

The ontogeny of the rat dopamine D2 receptor has been investigated both, by ligand binding assays (Sales *et al.,* 1989) as well as *in-situ* hybridization histochemistry (Rene *et al.,* 1994). In agreement with our data, both D2 receptor mRNA as well as D2 receptors have been detected in the IL as early as e17 which roughly coincides with the time when we first examined and detected a response to the dopamine agonist ERG. Thus far, only a few studies have investigated the responses of neonatal rodent IL to dopamine and shown that melanotrophs are responsive to dopamine from the time of birth (Schmitt *et al.,* 1981; Davis *et al.,* 1984; Sato and Mains, 1986). Our studies extend these results to the fetal period and demonstrate that these receptors are appropriately coupled to inhibit secretion many days prior to arrival of the dopaminergic input into the IL (p2, Davis *et al.,* 1984). Thus, the development of functional dopamine receptors on melanotrophs appears to be independent of hypothalamic dopaminergic influence, although influence from other areas such as neural lobe or basal lamina components can not be ruled out. For example, *in-vitro* studies of the rabbit pituitary showed that normal IL morphology in this species is dependent on contact between adenohypo-

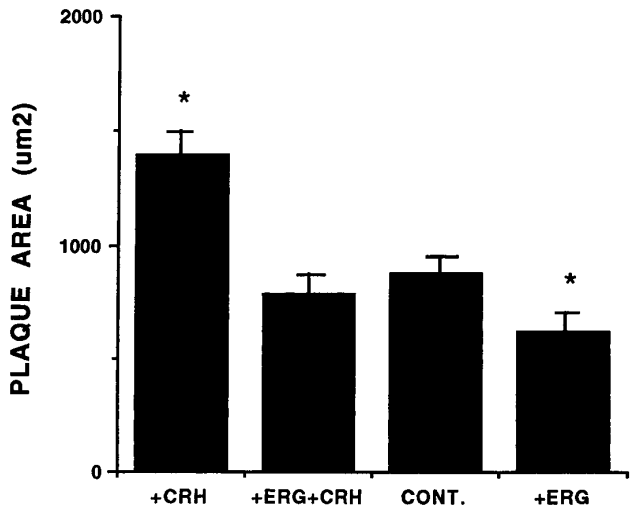

FIG. 5. ERG inhibition of basal and CRH-stimulated β-endorphin secretion from e21 melanotrophs. A 45-min preincubation with ERG produced inhibition of basal as well as CRH-stimulated release as determined by significant ($P \leq 0.05$) decreases in mean plaque areas. Each bar represents the mean ± SEM of 25 values. *Statistically different from all other treatments.

physial cells and the neural lobe (Gaillard, 1937). In addition, in species such as whales where the neural lobe never comes in direct contact with Rathke's pouch, the IL is absent (Wislocki and Geiling, 1936).

Dopamine inhibition of POMC-derived peptide secretion was present at e17.5 when responsiveness to CRH was undetectable even at high (10^{-7}) CRH concentration. Since both CRH and dopamine have been shown to exert their influence on melanotrophs via a cAMP-dependent mechanism (Meunier *et al.*, 1982; Cote *et al.*, 1986), the simplest explanation for the temporal discrepancy observed in these responses would be an absence of functional CRH receptors prior to e19.5. Thus far, no study has directly assessed the ontogeny of CRH receptors in the rat pituitary (see above). On the other hand, Loeffler *et al.* (1988) have shown that the dopamine effect also has a cAMP-independent component. Direct stimulation of the cAMP pathway by 8-Br-cAMP or forskolin does not completely abolish the D2 receptor-mediated inhibition of POMC peptide secretion or POMC mRNA synthesis in serum-free adult rat melanotroph cultures. Moreover, pretreatment with pertussis toxin almost completely abolishes the bromocryptine effect on POMC transcription and secretion, suggesting that the dopaminergic inhibition is mediated at least in part by guanosine triphosphate binding proteins (GTP-BP). These proteins have been shown to have a role as signal transducers in the noradrenaline and γ-aminobutyric acid-induced inhibition of voltage-dependent calcium channels in primary cultures of embryonic chick dorsal root ganglia neurons (Holz *et al.*, 1986). These data, together with the demonstration that dopamine blocks voltage-dependent calcium channels in some cells of the rat IL (Douglas and Taraskevich, 1982), suggest that the cAMP-independent component of the dopamine inhibition can be effected via GTP-BP-mediated inhibition of voltage-sensitive calcium channels. If the cAMP-dependent and independent mechanisms mature at different times during melanotroph differentiation, this would explain the difference observed here between the age when dopamine agonists first inhibit POMC-derived peptide release (e17.5) and the time when CRH first stimulates POMC release (e19.5).

Our studies clearly demonstrate that functional glucocorticoid responses can be transiently elicited during melanotroph ontogeny. Glucocorticoid inhibition of CRH stimulation was present between e19.5 and p2. After p2, preincubation with glucocorticoids had no effect on CRH stimulation of release. Studies by Scott and Pintar (1993) have shown that 30 min preincubation with DEX or the type II GR agonist RU 28362 (as low as 10^{-8} *M*) prior to CRH addition can inhibit CRH-stimulated increases in POMC hnRNA in short-term cultures of NIL from p1 but not p10 neonatal rats. Thus, both secretory and transcriptional effects are affected over the same time course and, taken together, demonstrate that a transient glucocorticoid response characterizes late fetal and early postnatal IL POMC cells. Although the most direct explanation for these results is that GR expression itself is being developmentally regulated, no data here addresses this point directly. Thus far, studies of GR gene expression during rat pituitary ontogeny utilizing *in-situ* hybridization histochemistry techniques (Cintra *et al.*, 1993; Rene *et al.*, 1994) have been inconclusive. For example, Cintra *et al.* (1993) reported no information about hybridization signal over the IL, while Rene *et al.* (1994) failed to detect signal over the presumptive IL after e15. Failure to detect signal over the IL by conventional *in-situ* hybridization techniques may reflect very low levels of GR mRNA that may require more sensitive methods such as PCR *in-situ* hybridization for optimal detection. Nevertheless, in our experiments, the loss of response to glucocorticoids coincides with the arrival of dopaminergic input into the IL (Davis *et al.*, 1984). This is particularly interesting because although the adult IL has been shown to have immunodetectable levels of GR (Bertini *et al.*, 1989), these cells are not responsive to glucocorticoids under normal conditions (Eberwine and Roberts, 1984). However, when hypothalamic input is removed by denervation (Seger *et al.*, 1988) or incubation of NIL cells *in vitro* for various days (Eberwine *et al.*, 1987), IL cells become responsive to glucocorticoids. Moreover, IL cells that have been maintained in culture for 6 days show high levels of GR which decrease substantially 48 hr after incubation with dopamine or its agonist bromocryptine (Antakly *et al.*, 1987). In contrast, dopamine agonists have no effect on GR levels in AL cells under identical conditions (Antakly *et al.*, 1987). Together these data suggest that dopaminergic input to the IL may somehow affect the ability of melanotrophs to respond to glucocorticoids regardless the presence of GR in these cells. Although the exact mechanism underlying this phenomenon is at present unknown, studies by Sheppard *et al.* (1993) show that pretreatment of DEX-insensitive IL-derived cells in culture with the protein synthesis inhibitor puromycin prior to DEX addition, leads to DEX inhibition of CRH-stimulated transcription. This result suggests that a rapidly metabolized protein(s) may be mediating the inhibition of function of the GR in adult melanotrophs, and, in this regard, it is interesting that negative regulation of the mouse proliferin gene by glucocorticoids depends not only on GR but also on fos/jun components of the AP1 transcription complex (Diamond *et al.*, 1990).

In summary, the fetal IL is capable of basal release as well as adult-like responses to CRH and dopamine agonist prior to birth. The latter responses can be elicited many days prior to arrival of dopaminergic input into the IL. Thus, development of functional dopamine receptors appears to be independent of hypothalamic input. Finally, melanotrophs respond transiently to glucocorticoids demonstrating the presence of functional GR in melanotrophs during IL ontogeny. This response is lost after the time of arrival of dopaminergic input into the IL. Thus, it appears that dopamine inhibition of GR expression/function is a normal event in melanotroph differentiation.

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