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Characterization of the distribution and ontogeny of galanin containing systems during the development of the brain

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Iowa State University, 1993

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Characterization of the distribution and ontogeny of galanin containing systems during the development of the brain

by

Joel Keith Elmquist

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Veterinary Anatomy
Major: Veterinary Anatomy

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Members of the Committee:

Signature was redacted for privacy.

Iowa State University
Ames, Iowa

1993

DEDICATION

To my wife, Kristie, and my parents, Bruce and Donna.

Their support made this work possible.

TABLE OF CONTENTS

GENERAL INTRODUCTION

Galanin (GAL) is a biologically active peptide of 29 amino acids in length originally isolated from porcine small intestine (Tatemoto et al., 1983). Galanin received its name from its N-terminal glycine and its C-terminal alanine. The amino acid sequence and structural data indicate that GAL is unrelated to any of the presently known peptide families and is the only member of its own family (Kaplan et al., 1991). Galanin has been detected throughout the mammalian central nervous system (CNS). Distribution of galanin-like immunoreactivity (GAL-IR) both in the central and peripheral nervous systems of several species has been reported (Melander et al., 1986a; Rokaeus 1987; Skofitsch and Jacobowitz 1985; Ch'ng et al., 1985; Kowall and Beal 1989; Kordower and Mufson, 1990; Jacobowitz and Skofitsch, 1991; Kordower et al, 1992).

In the adult rat, immunohistochemistry (IHC) has shown that GAL-IR is present in several cell populations in the adult brain. Nuclear groups containing GAL-IR (cell bodies) were seen in the telencephalon, thalamus, hypothalamus, pons, medulla, and the spinal cord. Galanin immunoreactive neuronal fibers were also found throughout the rat CNS. The highest concentrations of GAL-IR were seen in the preoptic area, hypothalamus, median eminence, and brainstem (Melander et al., 1986a; Skofitsch and Jacobowitz, 1985; Jacobowitz and Skofitsch 1991).

It has been previously shown that many neuropeptides are coexistent in neurons with other neurotransmitters. This has also been demonstrated in the case of GAL (Melander et al., 1991). For example, GAL has been shown to coexist in cholinergic neurons (choline

acetyltransferase positive cells) in the septum and basal forebrain (Melander et al., 1985). In the arcuate nucleus of the hypothalamus, GAL was colocalized in cells with tyrosine hydroxylase (TH; dopaminergic cells), growth hormone releasing hormone (GHRH), and glutamic acid decarboxylase (Melander et al., 1986b, Meister and Hokfelt, 1988). In the paraventricular and supraoptic nuclei of the hypothalamus, GAL has been colocalized with vasopressin (Melander et al., 1986b). In the brainstem, GAL has been shown to be present in serotoninergic (5-HT) positive cells (Melander et al., 1986b).

The distribution of GAL receptors (as defined by autoradiography) in the adult nervous system has also been documented (Melander et al., 1986c; Skofitsch et al., 1986; Skofitsch and Jacobowitz, 1991; Rosier et al., 1991; Mantyh et al., 1992; Hedlund et al., 1992). In the adult rat brain, GAL receptors were found in areas similar to that of the fiber terminals of GAL-IR. Specifically, moderate to dense levels of GAL binding sites were seen in the amygdaloid complex, septum, hypothalamus, and brainstem (Skofitsch and Jacobowitz, 1991).

The presence of GAL and its receptor in discrete areas in the mammalian brain and the coexistence of GAL with a number of neurotransmitters indicates that GAL may be involved in mediating a variety of functions. This is further substantiated by an increasing number of reports attributing physiological functions to GAL. Specifically, administration of GAL has been shown to inhibit insulin release (McDonald et al., 1985), increase release of growth hormone and prolactin (Murakami et al, 1989; Koshiyama et al., 1990), modify intestinal contractions (Ekbald et al., 1985), and increase food intake (Kyrouli et al., 1986 and 1990).

Recently, it has been shown that GAL containing systems undergo changes in the face of neuronal insult or injury (Vilar et al., 1991). For example it has been demonstrated that GAL synthesis is increased in hypothalamic magnocellular neurons after hypophysectomy (Vilar et al., 1990), in the dorsal raphe nucleus after decortication (Cortes et al., 1990), and in the corresponding dorsal root ganglia after sciatic nerve transection (Vilar et al., 1991). It has also been shown that intracerebroventricular colchicine administration causes an increase in GAL mRNA synthesis and causes GAL mRNA expression in brain regions which normally do not express the peptide (Cortes et al., 1990). Additional studies demonstrated that GAL mRNA was induced in both neuronal and glial cells following colchicine administration (Xu et al., 1992). Further, it has been demonstrated that GAL levels are increased in the basal forebrain of patients with Alzheimer's disease (Chan-Palay, 1988, Beal et al., 1990). These observations have led to speculations that GAL may be an important component of some neuronal systems in response to injury or insult (Vilar et al., 1991).

As has been shown with other regulatory peptides, molecular analysis has revealed that GAL is encoded as a precursor polypeptide, preprogalanin (Rokaeus and Brownstein 1986). The structure of preprogalanin demonstrates an amino-terminal signal sequence, the GAL peptide, and a 60 amino acid extension peptide. This peptide has been named galanin message associated peptide (GMAP) (Rokaeus and Brownstein, 1986, Kaplan et al., 1991). The distribution of GMAP-like immunoreactivity has been compared to that of GAL-IR in the rat (Hokfelt et al., 1992). The distribution was very comparable with a few exceptions. It was indicated that most tissues express both GAL and GMAP, although some expressed only one or the other of the peptides. This has led to the speculation that in some tissues

there may be differential posttranslational processing of preprogalanin (Hokfelt et al., 1992). The exact function and significance of GMAP in the nervous system is currently unclear.

Although the adult CNS distribution of GAL and its receptor have been described in several species, relatively little has been reported on the developmental aspects of GAL in the mammalian brain. The distribution of GAL-IR in the neonatal rat brain has been described (Sizer et al., 1990). This study found that GAL-IR in the rat brain was scant at birth and suggested that the ontogeny of GAL-IR is entirely a postnatal process in the rat. This is contrasted however, by findings reported by other labs. Specifically, GAL-IR has been detected in the dorsal horn of the spinal cord of the rat as early as embryonic day 12 (E12) and as early as 6 weeks of gestation in the human (Polak et al., 1991). Further, detectable levels (utilizing radioimmunoassay(RIA)) of GAL-IR are present in the brain and gastrointestinal (GI) tract of E15 rats (Gabriel et al., 1989). Northern blot analysis has been used to demonstrate the presence of GAL mRNA as early as E3 in the rat conceptus (Vrontakis et al. 1991). When the reports of early GAL expression are compared to the Sizer paper a possible discrepancy is seen. However, the discrepancy may be explained by the differences in the sensitivities of the techniques used. The report which concluded an entirely postnatal development of the GAL systems in the brain (Sizer et al., 1990) utilized indirect immunofluorescence. On the other hand, the previously mentioned studies used more sensitive approaches including avidin-biotin\nickel enhanced IHC, RIA, and northern blot analysis to detect GAL early in development (Polak et al., 1991, Gabriel et al., 1989, Vrontakis et al., 1991).

The investigation of the distribution of neuropeptides has focused largely on neurons.

Recently, however, several reports suggest that glial cells may also contain neuropeptides (Vilijn et al., 1988; Shinoda et al., 1989; Melner et al., 1990; Klein and Fricker, 1992;). As discussed earlier, GAL has been localized in glial cells as well. Specifically, it was demonstrated that after administration of colchicine or vinblastine, GAL mRNA and peptide are detectable in glial cells (presumed microglia). These results demonstrated that glial cells have the potential to synthesize GAL after administration of mitosis inhibitors (Xu et al., 1992). As discussed earlier, it has been shown that GAL containing systems undergo changes following neuronal insult or injury (Vilar et al., 1991). Thus, it appears that both neuronal and glial cell types have the ability to increase synthesis of GAL in changing cellular environments. Another period with a changing environment is that of CNS morphogenesis. However, the relationship of GAL in neurons to that of glial elements during development and the potential for GAL expression in glia during CNS development has not been reported.

The morphogenesis of the CNS is characterized by a series of complex processes including the interaction of neuronal and glial cells (reviewed in Silver et al., '93). One example involves radial glial cells which are believed to serve as physical scaffolds along which neurons can migrate (reviewed in Rakic, '88, '90). In addition, glial cells are thought to play integral roles in the guidance of axons during the formation of neuronal circuits. For example, glial elements are known to provide favorable environments through which growing axons migrate (Silver and Sidman, '80; Sakaguchi et al., '89; reviewed in Silver et al., '93). Conversely, it appears that glial structures can also deter the growth of axonal pathways (reviewed in Silver, '84; Silver et al., '93). Thus, glial subtypes have multiple functions

during formation of the CNS. Further, glial cells may be involved in the formation of GAL containing systems in the forming CNS.

The aforementioned studies indicate that GAL appears during the development of the brain. The early presence of GAL may suggest that it may be functioning early in the developing CNS in a similar fashion as it does in the adult CNS. On the other hand, the early appearance of GAL may indicate that it is involved in a distinct formative role. Along these lines, it has been hypothesized that GAL acts as a growth promoter in forming dorsal root ganglia cells (Polak et al., 1991). This information was used as the basis of my thesis research and led to the investigation of the distribution and possible developmental roles of GAL in the forming mammalian CNS. To this end we have employed the Brazilian opossum, *Monodelphis domestica,* as a developmental model.

The Brazilian opossum is a small pouchless marsupial which breeds well under laboratory conditions. The young are born in an extremely immature state well before CNS neurogenesis is completed (Jacobson 1984; Larsen and Jacobson 1986; Jacobson laboratory unpublished observations). *Monodelphis*, as most marsupials, has a minimal gestation period (14 days) and a protracted postnatal developmental period. The absence of a pouch makes the young very accessible for developmental studies and thereby circumvents technically difficult *in utero* surgical procedures that are a necessity in studies utilizing more conventional laboratory animals such as the rat. Therefore, *Monodelphis* is an excellent model to study development of the CNS and is currently being used by several laboratories (Rivkees et al., 1988; Schwanel-Fukuda et al., 1988; Saunders et al., 1989; Dore et al., 1990; Nicholls et al., 1990; Fox et al., 1991a and b; Treherne et al., 1992; Brunjes et al.,

1992; Wang et. al., 1992; Kuehl-Kovarik et al., 1992).

The experiments described in this dissertation are designed to characterize the anatomy of GAL containing systems during critical periods of neurogenesis and morphogenesis of the opossum brain. Specifically, the distribution of GAL-IR and GAL receptors were investigated during development of the opossum CNS. The distribution of glial cells during similar periods of brain morphogenesis was also investigated. Finally, the relationship of GAL-IR to the distribution of other hypothalamic neuropeptides and neurotransmitters will be presented.

Explanation of Dissertation Format

This dissertation includes four papers of which the first two have been published in peer reviewed journals (Paper I: *Developmental Brain Research* (1992) **67**:161-179; Paper II: *Molecular and Cellular Neurosciences* (1993) 4:354-365). The remaining two have been submitted for publication. The papers are preceded by a general introduction and followed by a general summary and discussion. The references of the general introduction, summary, and discussion follow the general discussion. All of the experimental work described herein was performed by myself in the laboratory of and under the guidance of Dr. Carol Jacobson.

PAPER I: GALANIN-LIKE IMMUNOREACTIVITY IN THE ADULT AND DEVELOPING BRAZILIAN OPOSSUM BRAIN

INTRODUCTION

Galanin is a biologically active peptide of 29 amino acids in length originally isolated from porcine small intestine[49]. Many biological effects have been shown by galanin administration *in vivo* and *in vitro*. These include inhibition of insulin release, increase of glucagon secretion, modification of intestinal contraction, alteration of luteinizing hormone release, increase in growth hormone release, and increase in food intake[7,9,25,26,31,36,41,49,50]. Distribution of galanin-like immunoreactive (GAL-IR) elements both in the central and peripheral nervous systems has been reported[3,33,34,47]. The distribution of GAL-IR in the central nervous system has been described in a number of mammals including the rat, pig, monkey and human[4,17,22,23,29,34,46,47]. In the rat, immunohistochemistry has shown that GAL-IR structures are present in several cell populations in the brain. Nuclear groups containing GAL-IR cells were seen in the telencephalon, thalamus, hypothalamus, pons, medulla, and the spinal cord. Galanin immunoreactive neuronal fibers were also found throughout the rat CNS[34,40,47].

Although the adult distribution of GAL-IR elements has been described in several species, relatively little has been reported on the developmental aspects of galanin in the mammalian brain[45]. Thus, we have investigated the distribution of GAL-IR containing structures in the Brazilian gray short-tailed opossum, *Monodelphis domestica*. The Brazilian opossum is a small pouchless marsupial which breeds well under laboratory conditions [24,51]. The young are born in an immature state before sexual differentiation and neurogenesis is completed[19,27]. The absence of a pouch makes the young very accessible for developmental studies. Therefore, *Monodelphis* is being used to study the development of the mammalian

CNS[6,8,11,13,35,39,42,43]. In this study we have employed immunohistochemistry to characterize the distribution of galanin-like immunoreactivity in the brain of the adult and developing Brazilian opossum.

MATERIALS AND METHODS

Animals

Adult and developing opossums were obtained from a colony maintained at Iowa State University and used in the studies described here. The initial animals of the colony were obtained from the Southwest Foundation for Research and Education (San Antonio, TX). The animals were maintained at a constant temperature (26°C) and on a 14:10 light-dark cycle. Food and water (Reproduction Fox Chow; Milk Specialties Products, Madison, WI) were available ad libitum. The animals used in the developmental portions of the study ranged in age from 1 day postnatal (PN) to 60 days PN. At least four animals were used at each of the timepoints in the study (1, 5, 7, 10, 16, 25, and 60 PN). Animals used for each timepoint came from a minimum of two different litters. A minimum of three males and three females were used at each age older than 5PN. At the ages of 1 and 5 PN the gender of the neonates cannot be determined grossly, and thus the animals were considered sexually undifferentiated.

Tissue Preparation

Five male (80-120g) and five female (50-100g) adult opossums (approximately 180 days of age) were examined in the first portion of the study. The animals were perfused transcardially as described previously[10-13]. Briefly, the animals were deeply anesthetized with ether, injected with 1000 units of heparinized saline in the left ventricle, and perfused

transcardially with 0.9% saline (until the perfusate was free of blood). Zamboni's fixative (1.8% paraformaldehyde, 7.5% saturated aqueous picric acid, sodium phosphate buffer; pH 7.5) was then perfused at a constant rate for 15 minutes. The brains were removed from the calvaria, postfixed for 24 hours in Zamboni's fixative, sunk in a 30% sucrose solution, and stored in a cryoprotectant solution at -15°C prior to sectioning.

After sinking each brain in buffered 30% sucrose to remove cryoprotectant, 30μm thick coronal sections approximating the transverse planes as described previously[10,12] were cut on a cryostat. Sections from one male and one female adult were collected directly onto slides and used for immunohistochemistry. The sections from the remaining adult animals were collected into cryoprotectant solution in tissue culture wells. One third of the sections from the wells were taken for immunohistochemistry.

The brains were collected from 1, 5, 10, and 16 PN opossums by cooling the animals in a -15°C freezer until anesthetized. The animals were then decapitated and the heads placed in Zamboni's fixative for 48 hours. After fixation, the heads were infiltrated with 30% sucrose overnight, and then cut into 20μm thick coronal sections on a cryostat (Reichert Instruments). The sections were thaw mounted onto slides and stored at 4°C until processed for immunohistochemistry.

Twenty five day old pups were anesthetized by cooling. These animals were perfused transcardially with 15 ml of Zamboni's fixative. The brains were isolated and postfixed in Zamboni's fixative for 48 hours. After postfixation, the brains were processed as described above for the developing animals.

Sixty day old animals were anesthetized and perfused as described above for the adult

opossums. After the brains were removed from the calvaria, they were postfixed for 48 hours in Zamboni's fixative, and processed as described above for the developing animals.

In addition, brains cut in the sagittal plane from developing animals (1 and 7 days PN) were taken and processed for immunohistochemistry as described above.

Immunohistochemistry

The protocol utilized for immunohistochemistry was a modification of that reported previously[11,13]. The slide mounted sections were rinsed with 50mM potassium phosphate buffered saline, incubated with a 0.3% H_2O_2 solution to remove endogenous peroxidase activity, exposed to normal goat serum as a blocking agent (Vector; 1:67) and then incubated in galanin primary antibody (made in rabbit, Cambridge Research Biochemicals; 1:5000) for 20 hours at room temperature. After adequate washing, the tissue sections were incubated in goat anti-rabbit IgG (Vector; 1:200) for 2 hours at room temperature, rinsed, and reacted with avidin-biotin complex (Vector Elite Kit; 1:50) at room temperature for an additional hour. After washing, the tissue sections were stained by exposing them to a substrate composed of 0.04% 3,3' diaminobenzidine tetrahydrochloride (DAB; Sigma), 2.5% nickel sulfate (Fisher Scientific) and 0.01% hydrogen peroxide, dissolved in 0.1 M sodium acetate. After staining for 6 minutes, the reaction was terminated by placing the slides into two successive rinses of 0.9% saline. The sections were then dehydrated in graded alcohols, cleared in xylene and coverslipped with permount mounting media and analyzed with a light microscope.

The unmounted adult tissue sections were immunohistochemically processed using a floating tissue technique described previously for adult tissue[10]. This allowed comparison of slide mounted tissue with floating tissue immunohistochemistry. The results obtained for the two procedures were comparable.

Negative controls were also run in parallel with the tissue to be stained. For this procedure, incubation of the tissue in primary antibody was replaced by incubation in normal goat serum. No specific staining of any of the tissue was observed.

Preabsorbtion controls consisted of coincubation of the tissue sections with galanin peptide (15 μM; Cambridge Research Biochemicals) and antibody. All staining was abolished in the developing animals exposed to the primary antisera along with the peptide. There was no specific staining observed in the adult preabsorbtion controls. However, in the paraventricular and supraoptic nuclei of the hypothalamus (Pa and SO) non-specific staining was seen in cell bodies. This staining was much lighter than that seen in sections incubated with galanin antibody alone.

To test the staining pattern observed using the galanin antibody obtained from Cambridge Research Biochemicals, adult and developing tissue was incubated with galanin antibody obtained from Peninsula Laboratories (1:5000). The staining pattern observed in both the tissue sections incubated with antibody and the preabsorbtion controls were similar for both antibodies.

Analysis of Tissue

Tissue sections at 60 μm intervals from areas immediately caudal to the olfactory bulbs to the caudal brainstem were analyzed from the adult animals. Consecutive sections at 20 μm intervals were examined from the developing animals. Sections were observed with a light microscope and regions containing GAL-IR elements were identified on maps of coronal sections of the opossum brain (Figs. 1-3) as described previously[10,11,12]. Identification of structures within and outside the developing brain was aided by the use of an atlas of the developing rat brain [37].

RESULTS

We have characterized the distribution of GAL-IR elements in the brain of the adult and developing opossum, *Monodelphis domestica*. Galanin-like immunoreactivity was seen in many distinct nuclear groups throughout the brain in cell bodies as well as in neuronal fibers both in the adult brain and in the developing brain.

GAL-IR Somata in the Adult Brain

Several nuclear groups from the rostral to caudal aspects of the adult opossum brain contained GAL-IR cell bodies (Fig. 1). Specifically, GAL-IR cell bodies were consistently seen in the medial preoptic area (MPA; Figs. 1b-c), periventricular hypothalamic nucleus (Pe; Figs. 1c-d), medial division of the bed nucleus of the stria terminalis (BSTM; Fig. 1c), supraoptic nucleus (SO; Figs. 1c and 4a), paraventricular hypothalamic nucleus (Pa; Figs. 1d and 4b), arcuate nucleus (Arc; Figs. 1e-f and 5a), medial amygdala (Fig. 1c), dorsomedial hypothalamic nucleus (DM; Figs. 1e and 5b), and the nucleus of the solitary tract (Sol; Figs. 1i-j and 6a). The GAL-IR cells seen in the Sol were in the caudal aspects of the nucleus with most cells located at or caudal to the obex (Figs. 1j and 6a). Although the subnuclear divisions of the opossum are not as distinct as those seen in the rat, GAL-IR cell bodies were seen in the dorsolateral portions of the Sol and would approximate the medial and dorsal medial subnuclei in the rat as described previously [16]. Although, the GAL-IR cells were not quantified the Pe, Arc, SO, and Sol contained the most dense collections of GAL-IR cell bodies.

GAL-IR Fibers in the Adult Brain

Galanin immunoreactive fibers were observed throughout the adult opossum brain extending from areas rostral to the preoptic area to the spinomedullary junction. The nuclear groups containing GAL-IR fibers are summarized in Fig. 1. Areas consistently stained included the lateral septal nucleus (LS; Figs. 1a-b), BST (Figs. 1a-c), preoptic area (Figs 1a-c), SO (Figs. 1c and 4a), Pa (Figs. 1d and 4b), Pe (Figs. 1c-d), Arc (Figs. 1e-f and 5a), DM (Figs. 1e and 5b), median eminence (ME; Figs. 1e and 5a), medial amygdala (Fig. 1c), lateral hypothalamus (LH; Figs. 1d-e and 5b), paraventricular thalamic nucleus (PVA, PV; Figs. 1c-d), central gray of the midbrain (CG; Figs. 1e-g), parabrachial nucleus (PB; Fig. 1h), spinal trigeminal nucleus (SpC and Sp5C; Figs. 1i-j), Sol (Figs. 1i-j and 6a), and the area postrema (AP; Fig. 6a). Fiber staining intensity ranged from light to moderate in the CG to very dense fibers in the Arc, ME, Pe, LS, MPA, and the dorsal vagal complex including the Sol.

As discussed earlier, cells in the Sol (Figs. 1j and 6a) were not seen very far rostral to the obex, but fibers were observed throughout the nucleus (Figs. 1i-j). Fibers were also seen in the area dorsal to the locus coeruleus (LC; Fig. 1h).

There were no apparent sex differences in the density or distribution of GAL-IR fibers and cell bodies in the brains of the adult animals analyzed.

GAL-IR Somata and Fibers in the 1 Day PN Brain

The brain of the neonatal opossum is very immature and specific nuclear groups are in the process of forming. Thus, when a specific nuclear group or area is discussed it will

be in the context of the presumptive or differentiating area or nuclear group. The brain of the 1 PN pup contained several areas with GAL-IR cells and fibers (Figs. 2a-j, 7a, and 8a-c). Cell bodies were observed in the rostral portions of the brain in the area of the lateral ventricles and adjacent to the forming third ventricle (presumptive medial preoptic area ; Figs. 2b-c, 7a, and 8a). A collection of immunoreactive cell bodies were also present in the area of the forming hypothalamus (hy) at the level of the pituitary (Figs. 2e-f, 7a, 8b, and 9a). Interestingly, a circular area of the forming diencephalon appeared to be devoid of immunoreactivity although it was encompassed by GAL-IR elements (Fig. 9a). This area is interpreted to be the forming VMH due to its location and the lack of immunoreactivity seen in this area at all ages examined including the adult. No GAL-IR cell bodies were seen caudal to the level of the pituitary gland. In the area of the forming olfactory bulbs, some very lightly staining GAL-IR cell bodies were seen as well as a low density of GAL-IR fibers (Fig. 2a).

Galanin-like immunoreactive fibers were seen throughout the extent of the developing 1 PN opossum brain (Fig. 2). Fibers were seen in the developing forebrain in the area between the lateral ventricles (septum; Figs. 2b and 7a), the preoptic area (Figs. 2b-c, 7a, and 8a), and areas lateral to the third ventricle, *ie* the forming hypothalamus. At the level of the forming pituitary gland, a dense collection of fibers were observed (Figs. 2e-f, 7a, 8b, and 9a). The areas stained includes the presumptive Arc, ME, DM, and LH. Fibers were also seen at this level coursing dorsally through the developing thalamus towards the cortex. The area of the presumptive VMH was devoid of staining and was encompassed by GAL-IR cells and fibers (Fig. 9A). In the forming midbrain, GAL-IR fibers were seen running

dorsoventrally (Fig. 8b). In the lateral aspects of the brainstem, GAL-IR fibers were observed in the region of the presumptive spinal trigeminal nucleus (Sp5C; Figs. 2h-j, 8c, and 10a). Fibers were also observed coursing from the vagal ganglia into the brainstem. These GAL-IR fibers were interpreted as afferent fibers to the forming Sol.

GAL-IR Somata and Fibers in the 5 PN Brain

By 5 days PN, the organization of the brain and the forming nuclear groups have become further differentiated, but have not taken on their adult appearance and are still considered to be presumptive. As was seen in the 1 PN pups, GAL-IR elements were observed throughout the developing brain. In the developing forebrain, GAL-IR somata were observed in the septum and preoptic area (Fig. 11a), as well as in the hypothalamus in the presumed Pa. As in the 1 PN pups, at the level of the pituitary, a dense collection of cell bodies was seen in the areas of the Arc and DM encircling an area devoid of staining, that is, the VMH (Fig. 9b). No GAL-IR somata were seen caudal to the level of the Arc and the pituitary gland.

The distribution and density of GAL-IR fibers at 5 PN is quite extensive. Fibers were observed in all areas described above that contained GAL-IR cell bodies. At the level of the forming pituitary, the Arc\ME area contained a very dense collection of GAL-IR fibers (Fig. 9b). In addition, GAL-IR fibers were observed in the posterior pituitary (Fig. 9b), although very little immunoreactivity was detected in the anterior pituitary. In the midbrain, GAL-IR fibers were seen running dorsoventrally as well as in the forming CG. In the brainstem, GAL-IR fibers were observed in axons arising from the vagal ganglia and

coursing to the Sol (Fig. 10b). In addition, fibers appearing to arise from the dorsal motor nucleus of the vagus (10) also contain galanin immunoreactivity. As in the 1 PN pup, the Sp5C contains GAL-IR fibers.

GAL-IR Somata and Fibers in the 10 PN Brain

At 10 days PN, areas containing GAL-IR somata included the medial septum, preoptic area, DM and Arc (Figs. 3a-f, 9c, and 11b). As was the case with the younger animals, an especially dense collection of cell bodies was observed in the Arc and DM. Once again the VMH was devoid of GAL-IR elements but was surrounded by positively stained structures (Fig. 9c). For the first time GAL-IR somata were observed in the Sol (Fig. 6b). As in the adult, the cells were located in the dorsolateral portion of the nucleus.

Galanin-like immunoreactive fibers were identified in regions throughout the brain. Areas observed include the septal nuclei, MPA, BST, Pa, SON, LH, Pe, Arc, ME, DM, CG, Sp5C, Sol, and 10 (Figs. 3a-j, 9c, and 11b). Fibers were also seen in the thalamus and amygdala. In the midbrain, fibers were observed in the presumed PB and LC. In the coronal sections of the brain where the forming tectum, cerebellum, and brainstem are present, GAL-IR fibers were believed to be running horizontally in the cerebellum. However, using the sagittal sections as guides these fibers were characterized as exiting the trigeminal ganglia and coursing to the brainstem.

GAL-IR Somata and Fibers in the 16 PN Brain

The patterns of GAL-IR elements observed at 16 days PN were not significantly different than those seen at 10 days of age. By this age most nuclear groups are discernable using the light microscope.

GAL-IR Somata and Fibers in the 25 PN Brain

By 25 days PN, the brain of the Brazilian opossum is fairly well differentiated and resembles the brain of the adult in shape and nuclear organization. The distribution of GAL-IR cells and fibers is also very similar to that seen in the adult. One difference at this age is the appearance of a small number of scattered GAL-IR somata in the hippocampus. These cells are not observed in the 10 and 16 days PN pups nor in 60 days PN or adult opossums. Thus, these cells seem to be transiently immunoreactive. In contrast to the adult, no GAL-IR cell bodies were observed in the SON although fibers were observed in the nucleus.

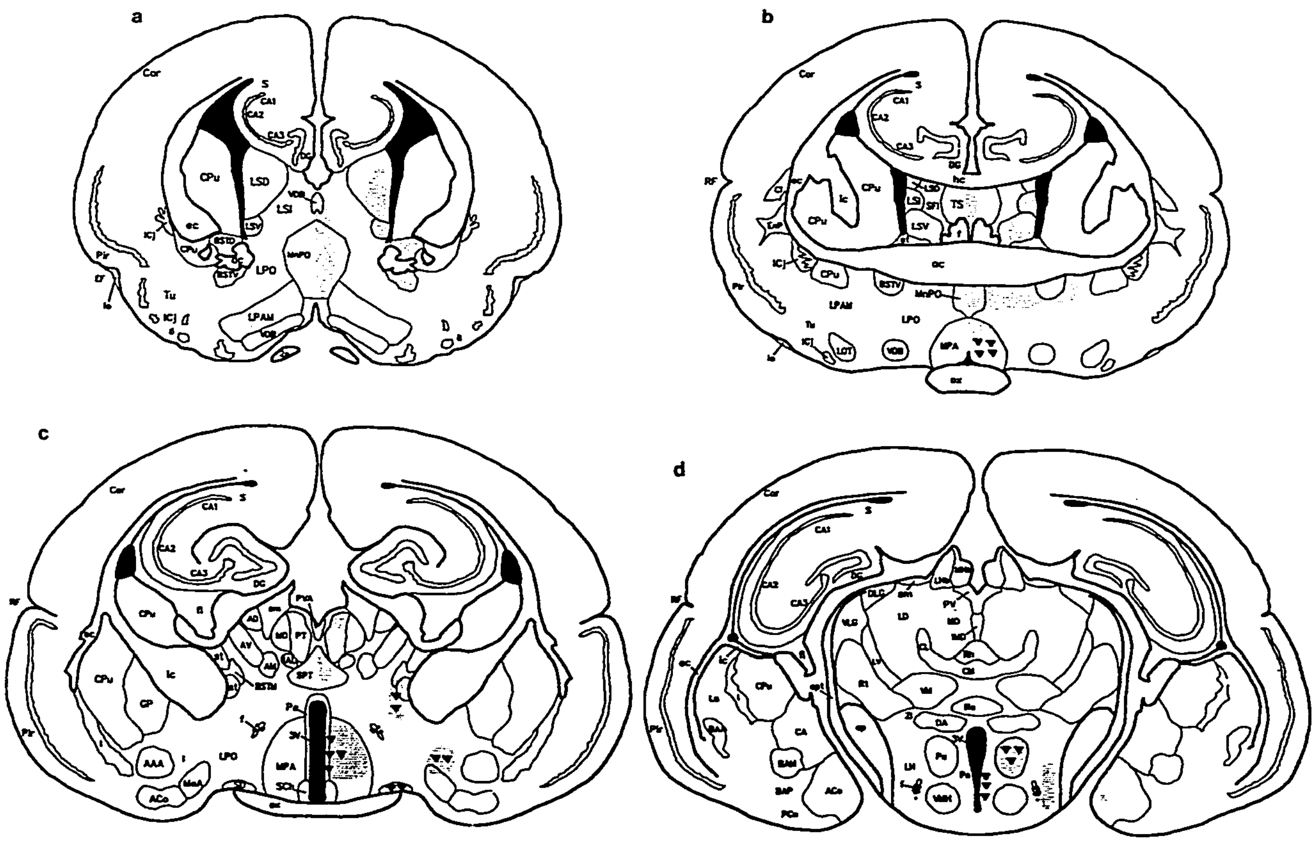
GAL-IR Somata and Fibers in the 60 PN Brain

The patterns of GAL-IR cells and fibers observed were very similar to those seen in the adult opossum brain with respect to location and density of immunoreactivity. As was seen in the 25 PN pups, there were no GAL-IR cell bodies in the SON but GAL-IR fibers were seen in the nucleus.

GAL-IR Elements Outside the Brain

As described earlier the brains of 1, 5, 10, and 16 days PN opossums were not removed from the calvaria prior to sectioning. Rather, the entire head was sectioned and processed for immunohistochemistry. This allowed for the visualization of GAL-IR elements outside the brain proper in the developing Brazilian opossum. Several structures were observed to contain galanin-like immunoreactivity including retinal ganglion cells and their axons forming the optic nerve, the trigeminal ganglion as well as many branches of the trigeminal nerve (Figs. 8b, 9a, and 12). In addition, the vagal and glossopharyngeal ganglia and the axons of these ganglia coursing into the brainstem were immunoreactive. Immunoreactive fibers were seen in the submucosal and myenteric layers of the esophagus in the 1 and 5 PN opossums. By days 10 and 16 PN a very dense collection of GAL-IR fibers as well as GAL-IR neurons were seen. The GAL-IR fibers were seen in the lamina, submucosa, and myenteric layers. The GAL-IR neurons seen in the days 10 and 16 PN esophagus were localized to the myenteric plexus. In addition, there were GAL-IR fibers just beneath the epithelium in the nasal cavity as well as in the larynx. Salivary glands (parotid) also contained GAL-IR fibers. The sphenopalatine and superior cervical ganglia contained GAL-IR elements. The immunoreactive elements described above were seen at all the ages examined (day 1, 5, 10 and 16 PN).

Figure 1: Drawings of selected coronal sections from rostral to caudal (a-j) of the adult Brazilian opossum brain showing regions with fibers (stippling) and cells (triangles) containing galanin-like immunoreactivity. See Table I for list of abbreviations.



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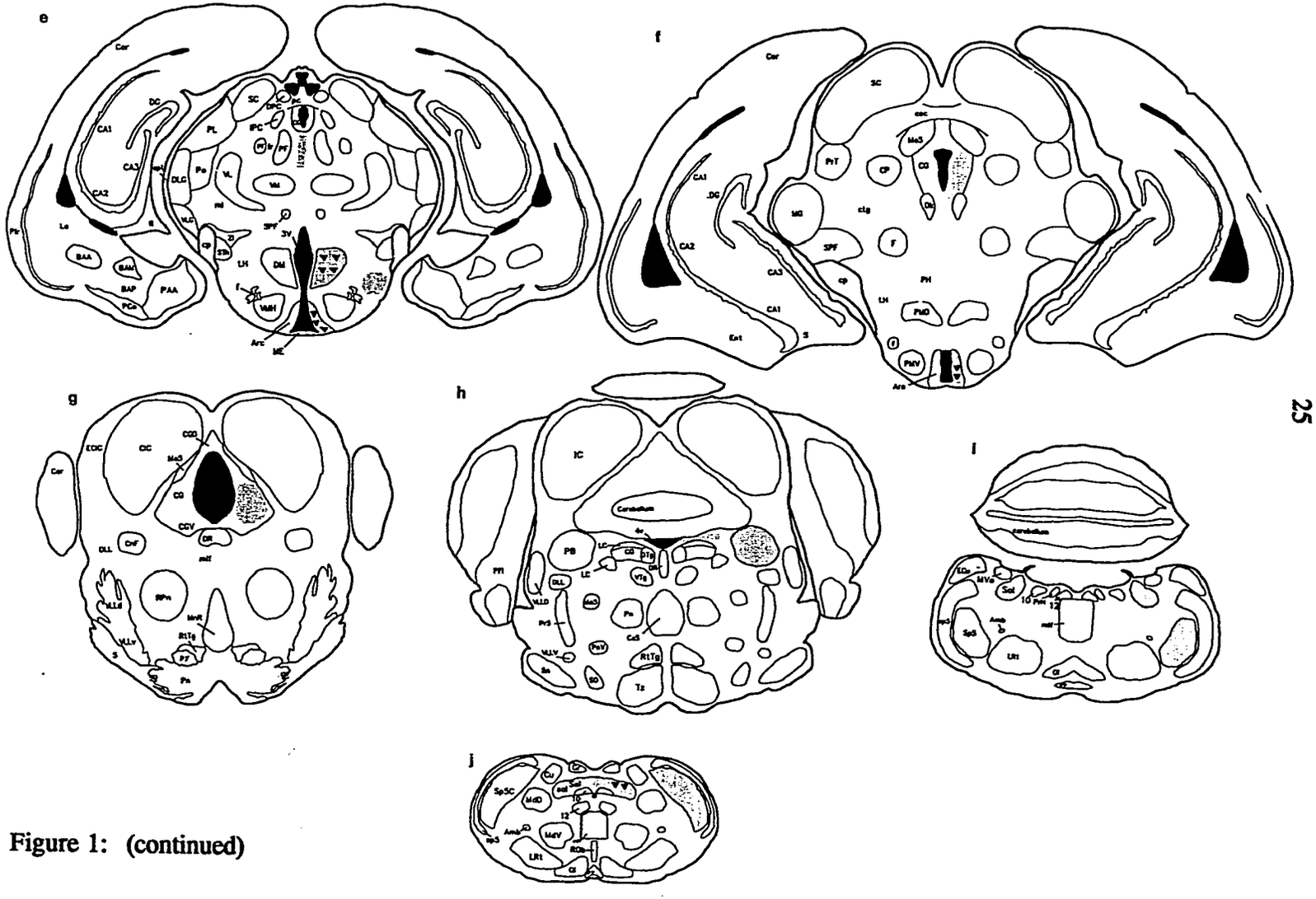

Figure 1: (continued)

Figure 2: Drawings of selected coronal sections from rostral to caudal (a-j) of the 1 day postnatal Brazilian opossum brain showing regions with fibers (stippling) and cells (triangles) containing galanin-like immunoreactivity. Drawings are produced at a similar scale factor as used in Figure 1.

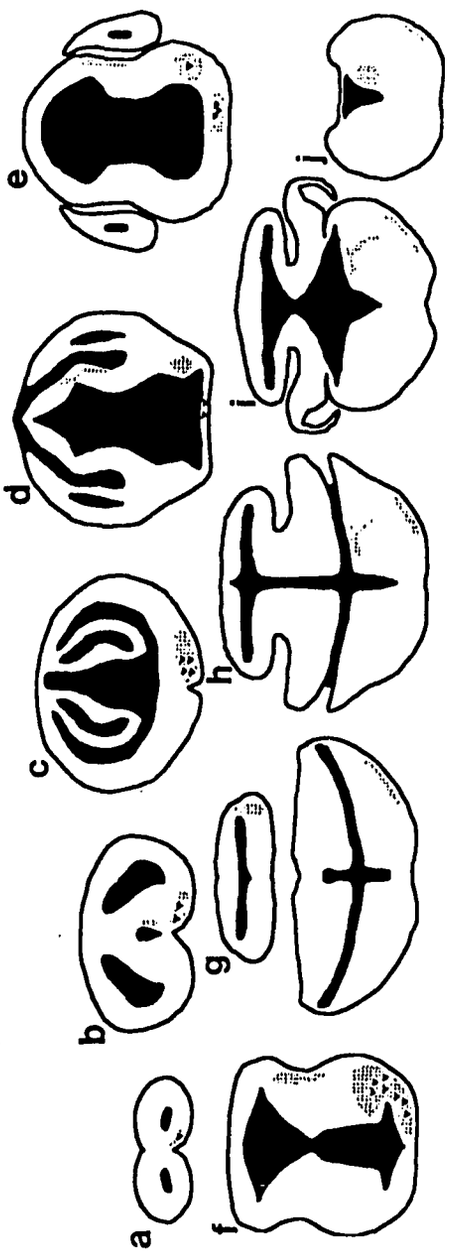
a
b
c
d
e
f
g
h
i
j

Figure 3: Drawings of selected coronal sections from rostral to caudal (a-j) of the 10 day postnatal Brazilian opossum brain showing regions with fibers (stippling) and cells (triangles) containing galanin-like immunoreactivity. Drawings are produced at a similar scale factor as used in Figure 1.

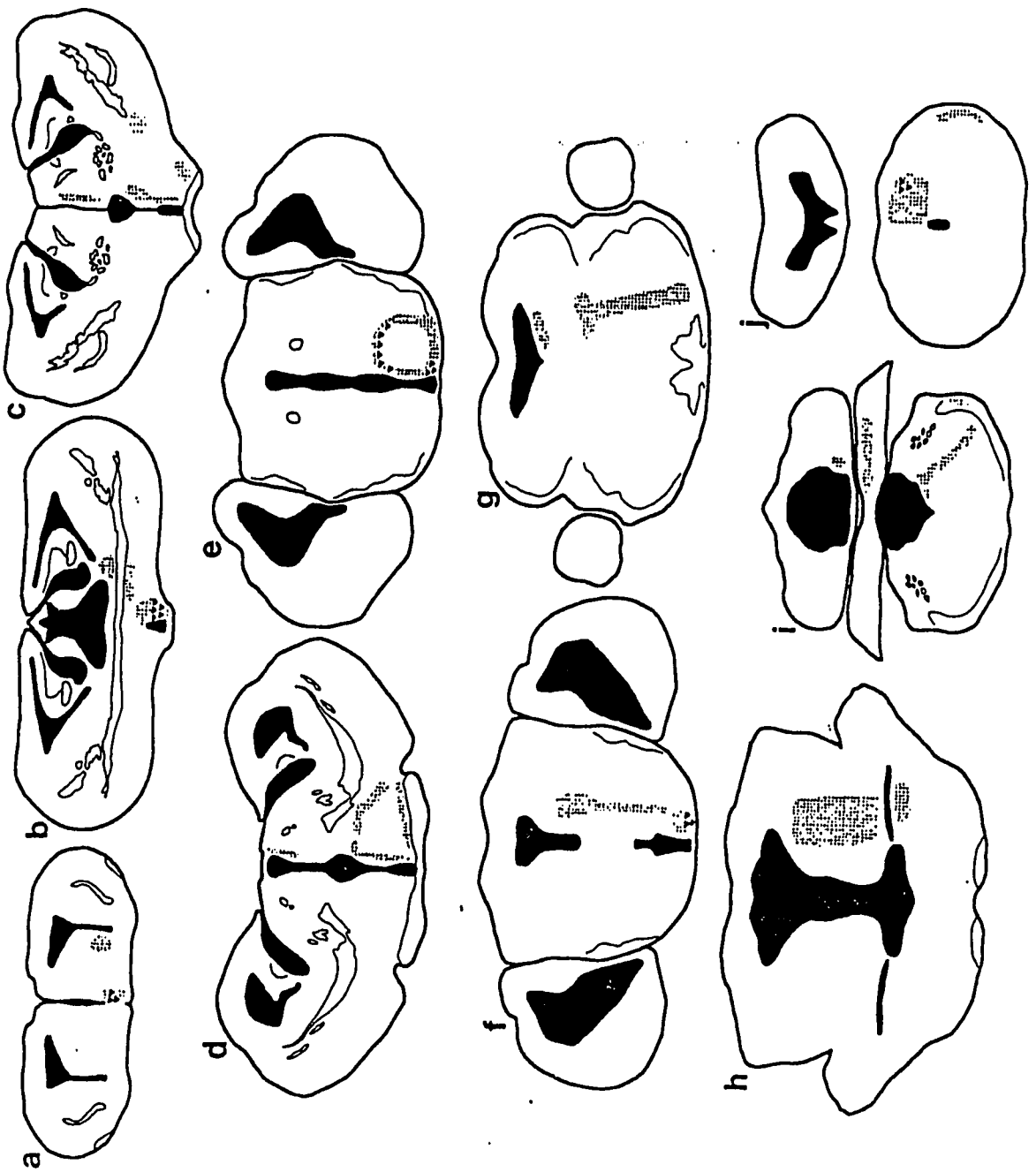
a
b
c
d
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Figure 4: Photomicrographs of galanin-like immunoreactive cells and fibers in (a) the supraoptic nucleus of the hypothalamus (SO), and (b) the paraventricular nucleus of the hypothalamus (Pa) of the Brazilian opossum. ox, optic chiasm; 3V, third ventricle. Bar = 200 μm.

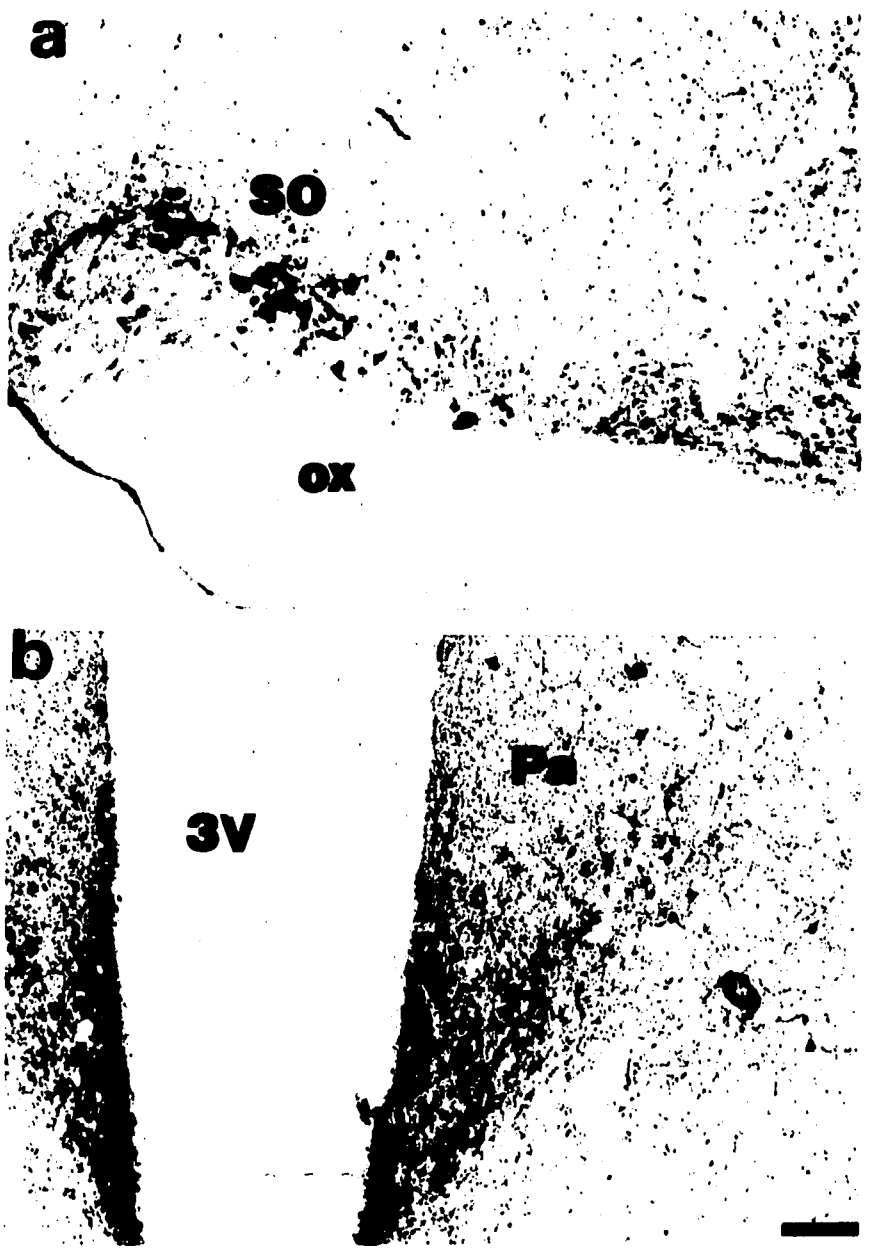
a
SO
ox
b
3V
Pa

Figure 5: Photomicrographs of (a) the median eminence and arcuate nucleus of the hypothalamus (Arc) and (b) the dorsal medial nucleus of the hypothalamus (DM) demonstrating galanin-like immunoreactive cells and fibers. The area on the left in a and b is the 3rd ventricle (3V). LH, lateral hypothalamus; Bars = 200 μm.


a
VMH
Arc
3V
b
DM
LH
VMH

Figure 6: Photomicrographs of the brainstem of the adult (a) and 10 day postnatal (b) Brazilian opossum demonstrating galanin-like immunoreactive cells and fibers. The cells shown in the adult are in the nucleus of the solitary tract (Sol) and those shown in b are in the presumed Sol. Galanin-like immunoreactive cells are first seen in the Sol at 10 days postnatal. cc, central canal; AP, area postrema; Bars = 200 μm.

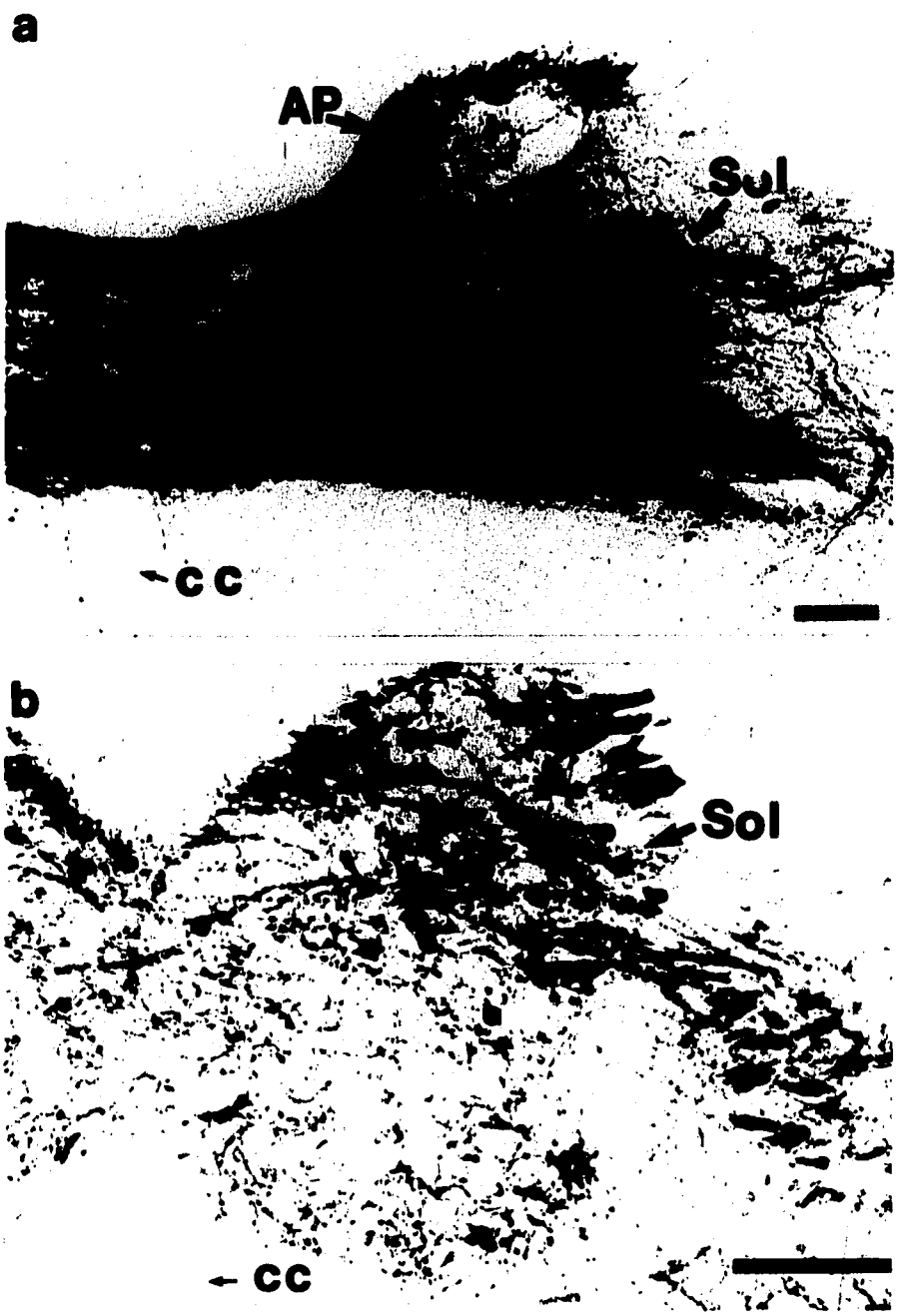
a
AP
Sol
cc
b
Sol
cc

Figure 7: Photomicrographs of sagittal sections of the Brazilian opossum brain at 1 day postnatal (a) and 7 days postnatal (b) demonstrating galanin-like immunoreactive elements. Rostral is to the left and caudal is to the right. Robust galanin-like immunoreactivity is present at 1 day postnatal but is confined to specific areas of the brain. bfb, basal forebrain; hy, hypothalamus; Bar = 800 μm.

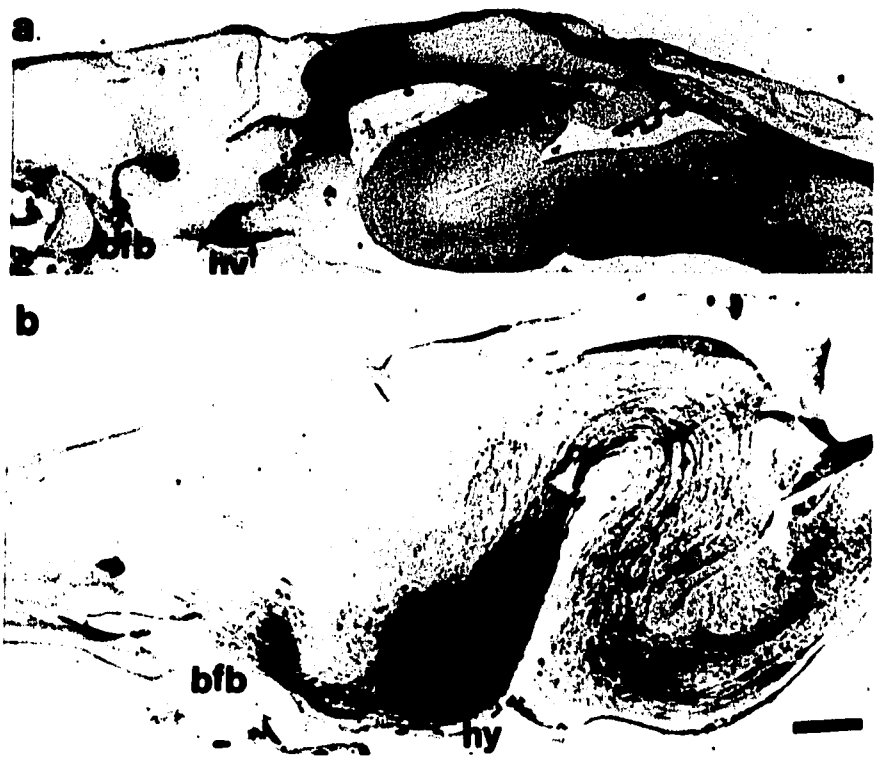
a
bfb
hy
b
bfb
hy

Figure 8: Photomicrographs of rostral to caudal (a-c) coronal sections of the head of the 1 day postnatal Brazilian opossum brain. a is a section through the developing preoptic area. b is at the level of the developing hypothalamus and pituitary gland. c is in the developing medulla. In b note the fibers coursing dorsally in the developing midbrain. In c note the fibers entering the brainstem laterally and coursing dorsomedially. These fibers are presumed to be of vagal origin. 5Gn, trigeminal ganglion. Bar = 800μm.

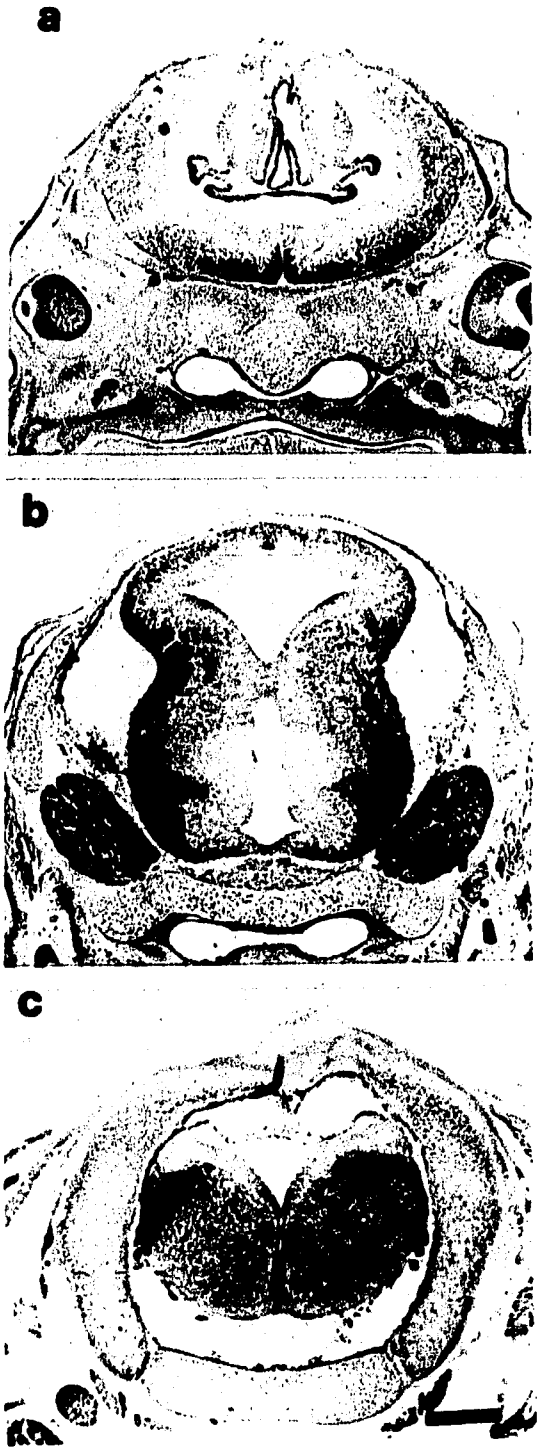
a
b
c

Figure 9: Photomicrographs of the developing hypothalamus and pituitary gland of the (a) 1, (b) 5, and (c) 10 days postnatal Brazilian opossum demonstrating galanin-like immunoreactivity. Note the graded appearance of the staining and the fact that the presumed ventromedial hypothalamic nucleus (VMH) contains relatively little galanin-like immunoreactivity at all three ages. APit, anterior pituitary; Arc, arcuate nucleus; e, ependymal lining of the third ventricle (3V); PPit, posterior pituitary; 5Gn, trigeminal ganglion; Bar = 200 μm.

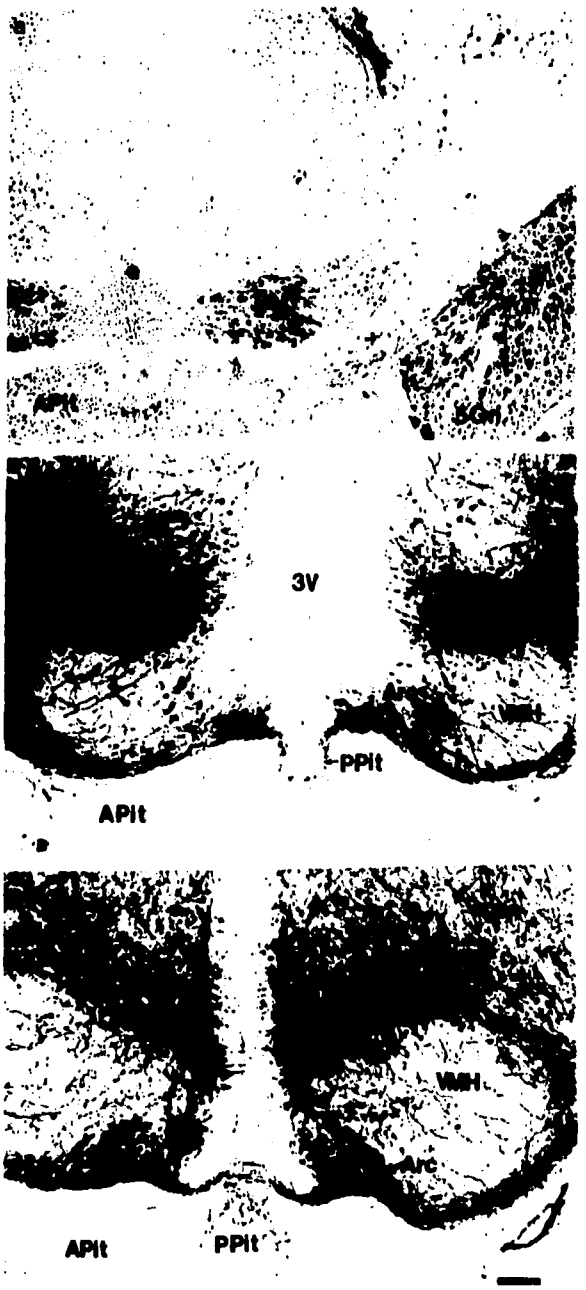
APlt
SCm
3V
PPlt
APlt
VMH
Arc
APlt
PPlt

Figure 10: Photomicrographs of the developing medulla in (a) 1 and (b) 5 days post natal opossum brain demonstrating galanin-like immunoreactivity. The immunoreactive fibers running dorsomedially were interpretted as vagal afferent fibers entering the presumed nucleus of the solitary tract (Sol). Note in a the fibers on the lateral rim of the brainstem which were interpreted as fibers in the developing nucleus of the spinal tract of the trigeminal (Sp5). Bar = 200 μm.

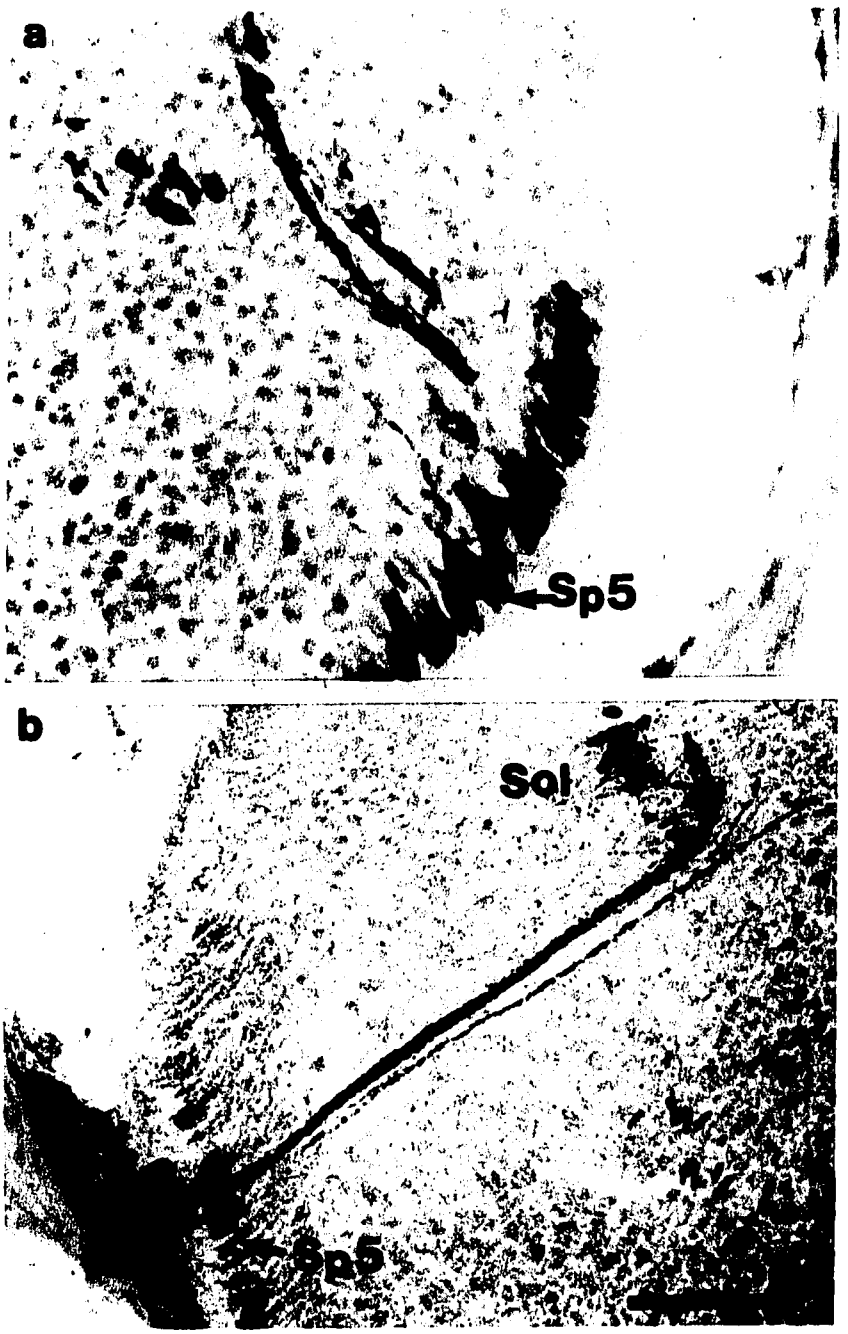
a
Sp5
b
Sol
Sp5

Figure 11: Photomicrographs of the developing preoptic area in (a) 5 and (b) 10 days postnatal opossum demonstrating galanin-like immunoreactive cells and fibers. 3V, third ventricle. Bar = 200 μm.

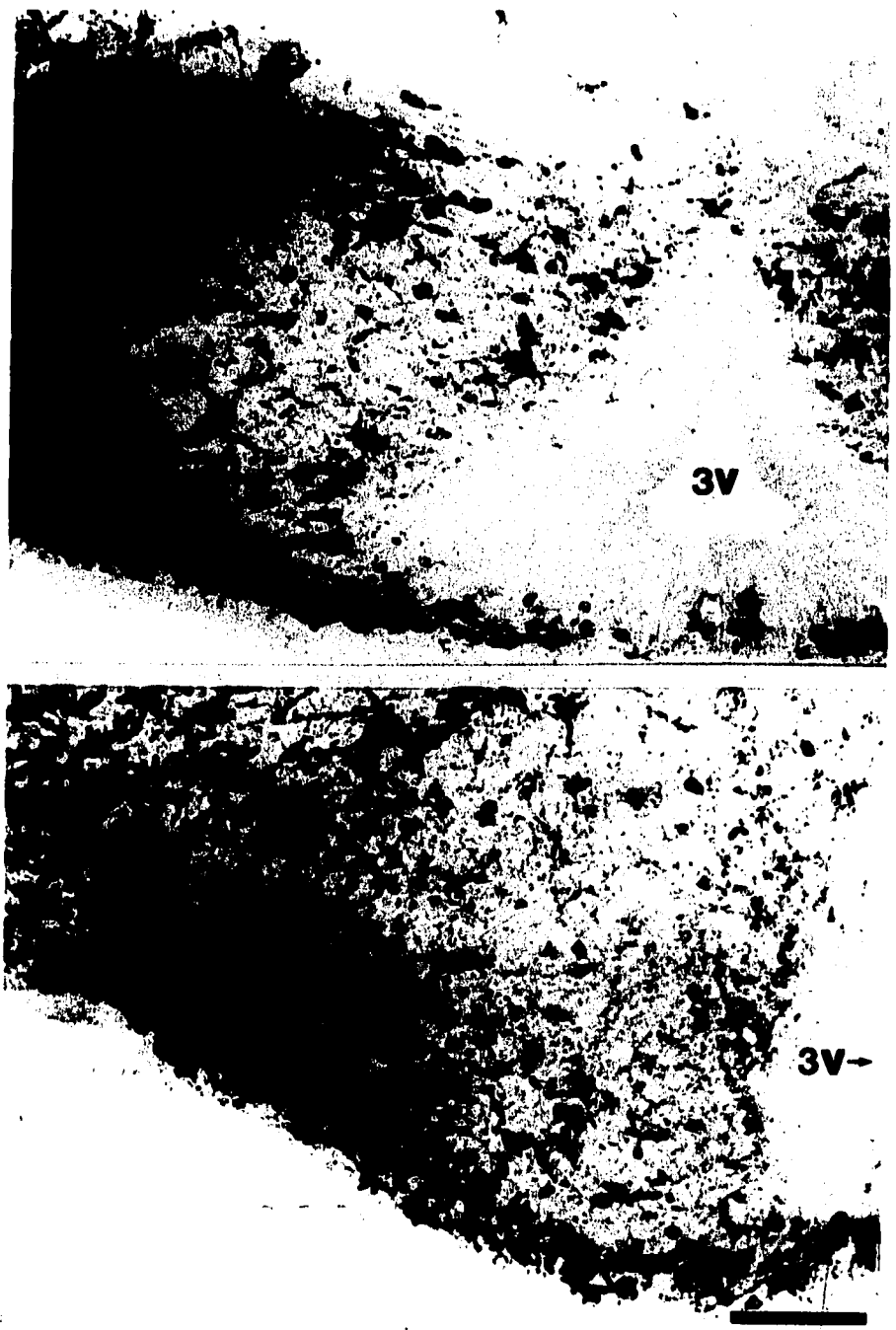
3V
3V

Figure 12: Photomicrograph of a sagittal section of a 1 day postnatal opossum demonstrating galanin-like immunoreactivity in cells and fibers in the trigeminal ganglion (5Gn). Note the dense collection of fibers seen entering the brainstem (bs) in the right portion of the photo. Bar = 200 μm.

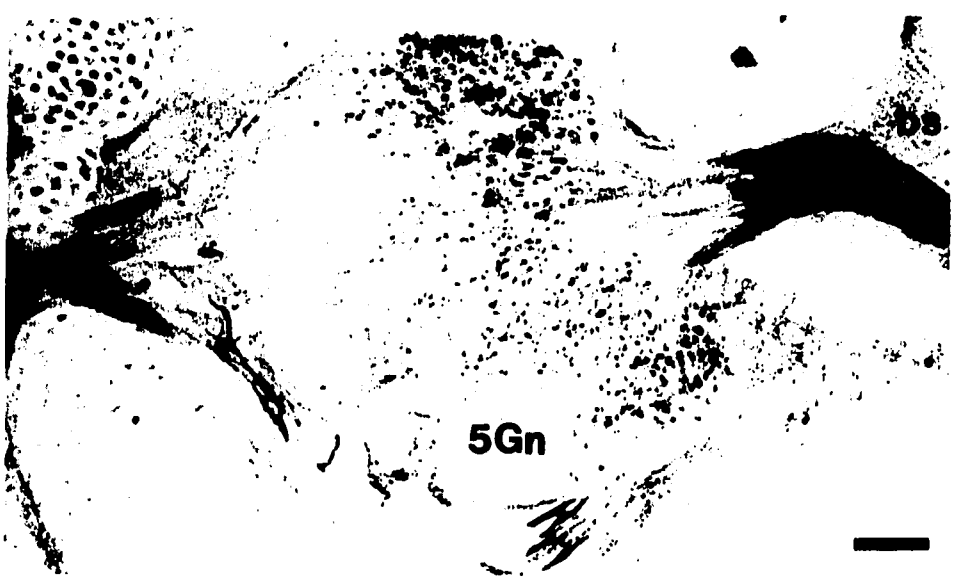
5Gn

TABLE 1: Summary of galanin immunoreactivity in the adult opossum brain

Nuclear groups/areas	Galanin immuno-reactive fibers	Galanin immuno-reactive cell bodies
AP	+	-
Arc	+	+
BSTM	+	+
BSTV	+	+
CG	+	-
DM	+	+
LC	+/-	-
LH	+	-
LSD	+	-
LSV	+	-
ME	+	-
Medial amygdala	+	+
MnPO	+	-
MPA	+	+
Pa	+	+
PB	+	-
Pe	+	+
PT/SPT	+	-
PV/PVA	+	-
SO	+	+
Sol	+	+
Sp5C	+	-
st	+	-
10	+	-

DISCUSSION

The adult distribution of GAL-IR fibers and cell bodies in *Monodelphis* is similar to that reported for other species including the rat and pig[4,34,46]. Although the biological effects of galanin in the opossum are not known, galanin has been shown to cause many responses in other mammals. Effects reported include; alteration of pituitary release of hormones such as GH and LH, inhibition of insulin release, and increase in feeding behavior[7,9,25,26,31,36,41,49,50]. The distribution of galanin in similar areas of the rat and opossum brain indicates that this peptide may play similar roles in the opossum.

The presence of GAL-IR cells and fibers in the Arc and GAL-IR fibers in the ME suggest that galanin may be a modulator of the hypothalamic pituitary axis affecting the release of hormones as has been shown in other mammals [20,36,41]. Further evidence for galanin's modulatory role includes our observation that GAL-IR fibers exist in the posterior pituitary as early as 5 days PN.

The opossums in this study were not colchicine treated, yet contained many areas of the brain with immunoreactive cell bodies. Previously, immunofluorescence histochemical studies in the rat, used animals that were pretreated with colchicine. Colchicine inhibits microtubular transport of cellular products and allows for the visualization of certain neuropeptides in neurons using immunohistochemistry. It has been reported that virtually no GAL-IR cells are seen in rats that are not first colchicine treated[34]. However, we performed immunohistochemistry on a non-colchicine treated adult rat and have been able to identify GAL-IR cell bodies in several nuclear groups of the rat brain. In particular, we see a dense collection of GAL-IR cell bodies in the locus coeruleus. These results imply that

our immunohistochemical technique is sensitive enough to visualize GAL-IR cell bodies and fibers in non-colchicine treated animals.

Besides the inherent difficulty of administering colchicine to the extremely small newborn opossum, it has been recently shown that colchicine treatment of rats may elevate the levels of galanin mRNA while decreasing levels of mRNA of other peptides such as cholecystokinin (CCK)[5]. It has also been theorized that the expression of galanin mRNA is induced in areas of the brain that do not normally express galanin. Furthermore, the authors point out that caution should be used in interpreting immunohistochemical data that employs the use of colchicine[5]. Although colchicine is a valuable tool in localizing immunoreactive cell bodies, the present results indicate that the immunohistochemical techniques employed in this study may be used to help circumvent the use of colchicine in histochemical studies for galanin distribution. We realize that our technique may not allow the visualization of all GAL-IR cell bodies present in the opossum brain and thus have initiated *in situ* hybridization studies to optimally localize galanin producing cells in the adult and developing brain of *Monodelphis*.

In general, the staining patterns observed for GAL-IR elements in the adult opossum brain were similar to that reported in other species. One area that appears to be somewhat different in *Monodelphis* as compared to the rat is the locus coeruleus (LC), an area that contains abundant GAL-IR elements in the rat. In the opossum, the LC was devoid of GAL-IR cells and contained relatively few GAL-IR fibers that were sometimes isolated to areas dorsal to the nucleus. One explanation for this finding is that galanin is present in cells and fibers in the opossum LC, but it is transported at a high enough rate that at any given time,

there are undetectable amounts of galanin immunoreactivity in the LC of the opossum. It could also be possible that the opossum may not have GAL-IR structures in the LC. Experiments utilizing *in situ* hybridization to detect galanin mRNA will help answer these questions.

The data from the adult portion of the study corresponds very nicely with what has been previously reported (especially for the rat). However, similar developmental results have not been reported. The distribution of embryonic GAL-IR elements in the gut and spinal cord have been described for the rat and human [15,21,28]. These studies showed that GAL-IR cells and fibers were present before birth. The ontogeny of galanin immunoreactivity in the brain has not been heavily investigated. A study employing radioimmunoassay (RIA) reported that a detectable level of galanin immunoreactivity was present in the brain at embryonic day 15 (E15) in the rat [14]. A fluorescent immunohistochemical study conducted in rats reported that GAL-IR elements were not present in the brain of 1 day PN rat pups, although fibers were seen in the superficial laminae of the dorsal spinal cord [45]. Thus, the authors concluded that the ontogeny of galanin in the rat brain is entirely postnatal. This would indicate that galanin is not present until neurogenesis and brain morphogenesis are completed in the rat [1]. Although all opossums in this study were postnatal, the large difference in relative maturity of a 1PN rat brain and a 1 PN opossum brain, coupled with the detection of galanin immunoreactivity using RIA at E15 in the rat, may refute this claim. A possible explanation for this discrepancy is that galanin may be found at higher levels in the rat brain during neurogenesis and morphogenesis and then these levels decline. Postnatally, galanin levels may then rise

to participate in physiological processes as discussed earlier.

The brain of a 1 day PN opossum is very immature and neurogenesis is still actively taking place. For example, neurogenesis of the suprachiasmatic nucleus (SCh) is not completed in *Monodelphis* until 8PN. It is not until 17PN that the SCh could be identified morphologically using light microscopy. On day 20 a clear day-night rhythm in metabolic activity was observed[39]. Further, neurogenesis of the medial preoptic area is not completed in *Monodelphis* until day 10 or 11PN. Morphogenesis follows neurogenesis by approximately 5-9 days in this region as well [19,27]. As discussed earlier GAL-IR cells and fibers are present in multiple sites in the developing brain as early as 1 day PN when brain differentiation is still very active. Thus, it appears that galanin is present at an important time in the development of the brain of *Monodelphis*.

Galanin-like immunoreactivity is very extensive in the newborn opossum. Although its distribution is region specific, many brain areas do not contain GAL-IR elements. One such area is the VMH, which contains relatively little galanin immunoreactivity at any of the ages examined. Conversely, regions of the brain which do contain galanin-like immunoreactivity in the early postnatal opossum continue to contain GAL-IR structures into adulthood. This data suggests that galanin may be playing a role in the formation of nuclear groups that will contain galanin in the adult Brazilian opossum brain.

Galanin-like immunoreactivity was also seen outside the brain proper in the heads of developing opossums. Galanin-like immunoreactivity was seen in the esophagus of the developing opossum. Galanin-like immunoreactive fibers were observed at 1, 5, 10, and 16 days PN while GAL-IR cell bodies were seen only at days 10 and 16 PN. The density of

GAL-IR fibers also increased with age from day 1 PN onward. The adult distribution of GAL-IR elements in the gastrointestinal tract of *Monodelphis* has not been reported. The patterns observed in the esophagus of the day 16 PN animals correspond very nicely with previous reports in other species including the rat and North American opossum [32,44]. It is possible that some of the GAL-IR fibers seen at 1 day PN are of vagal origin and are not entirely intrinsic. The evidence for this is that GAL-IR fibers are seen in the Sol and vagal ganglia at day 1 PN. Also galanin has been isolated in vagal sensory neurons which may suggest that galaninergic nerve terminals may relay sensory information from the gut [38]. Studies are currently underway investigating the role of galanin in the development of the innervation of the gastrointestinal tract in *Monodelphis*.

Other areas seen to contain GAL-IR elements included the retina and optic nerve of the eye, trigeminal ganglion and branches of the trigeminal nerve, the vagal ganglia, salivary glands, and the respiratory tract. Presence of GAL-IR fibers throughout the head of the developing opossum suggests that galanin may be playing a role in the formation or early functioning of the peripheral and autonomic nervous systems.

The presence of galanin receptors during the early developmental stages would provide more evidence that galanin is playing a role in the forming brain. The adult opossum contains galanin binding sites (Elmquist, Kao and Jacobson, unpublished) that corresponds well with what has been previously reported in the rat [48]. Thus, we have begun to investigate the presence and distribution of galanin receptors in the neonatal opossum.

The formation and differentiation of the brain involves significant plasticity of neurons. Another condition in which neuronal plasticity is present is after an injury to the

adult central nervous system [30]. Interestingly, it has been demonstrated that galanin levels are increased following insult. After transection of the sciatic nerve, both galanin-like immunoreactivity and galanin mRNA are significantly increased in the corresponding dorsal root ganglia [18,52]. Hypophysectomy increases both the peptide and mRNA in the Pa and Sol [53]. Also, as discussed earlier, colchicine administration has been shown to up regulate the synthesis of galanin [5]. Additionally, galanin immunoreactivity has been shown to be elevated in the basal forebrain in the brains of individuals with Alzheimer's disease [2]. This increase in galanin content in all situations is occurring in conditions in which neurons are responding to changing conditions and/or injury. This is quite interesting in that the developing brain in which neurogenesis and morphogenesis are occurring has changing environmental cues as well.

The ontogeny of CCK-IR elements of the Brazilian opossum brain has also been described [11]. The developmental presence of GAL-IR elements in the opossum brain differs from that for cholecystokinin. Unlike galanin, there is no detectable CCK-IR cells or fibers present at 1 day PN in the brain. CCK-IR fibers in the brainstem were not detected until 5 days PN. At 10 days PN, the only CCK-IR soma were seen in the presumptive Sol. Cholecystokinin-like immunoreactivity is not seen in the hypothalamus until 35 days PN. These findings are in stark contrast to those found for GAL-IR elements in this study. We have concluded that since CCK-IR appears relatively late during differentiation it is not needed for morphogenesis of nuclear groups which contain CCK in the adult. Galanin, on the other hand, is present during neurogenesis and morphogenesis of the opossum brain which may implicate it as a factor involved in these processes.

In summary, we have described the distribution of GAL-IR cells and fibers in the brain of the adult and developing Brazilian opossum. The presence of GAL-IR cells in specific areas of the adult brain indicate that galanin may be exerting physiological effects in the opossum similar to those reported for other species. Additionally, we have described the distribution of GAL-IR cells and fibers in the developing opossum brain. The presence of galanin-like immunoreactivity as early as 1 day PN, while neurogenesis is still actively occurring, indicates that galanin may be playing a role in the formation and differentiation of the Brazilian opossum brain.

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PAPER II: DEVELOPMENTAL PROFILE OF GALANIN BINDING SITES IN THE MAMMALIAN BRAIN

INTRODUCTION

Galanin (GAL) is a 29 amino acid peptide originally isolated from porcine intestine (1). Galanin has been detected throughout the gastrointestinal tract as well as the central nervous system (CNS) (reviewed in 2). A myriad of physiological functions have been attributed to GAL. Administration of GAL has been shown to cause inhibition of insulin release, increased release of growth hormone and prolactin, modification of intestinal contractions, and increased food intake (3, 4, 5, 6, 7, 8). The distribution of galanin-like immunoreactivity (GAL-IR) in the adult mammalian CNS has been described (2, 9, 10, 11, 12, 13, 14, 15, 16). The distribution of galanin binding sites in the adult nervous system has also been well documented (17, 18, 19, 20, 21).

Our laboratory recently described the distribution of GAL-IR in the brain of the adult and developing Brazilian opossum, *Monodelphis domestica* (16). The Brazilian opossum is a small pouchless marsupial which breeds well under laboratory conditions. The young are born in an immature state before sexual differentiation and neurogenesis is completed (22, 23). The absence of a pouch makes the young very accessible and thus circumvents the necessity of *in utero* manipulations for developmental studies. Therefore, *Monodelphis* is an excellent model to study development of the CNS and is currently being used by several laboratories (16, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33).

In the Brazilian opossum, GAL-IR was shown to be present in specific brain regions as early as 1 day of postnatal (PN) life and was robustly expressed by 5 PN in areas where neurogenesis is still actively taking place. The striking GAL expression seen during times

of CNS morphogenesis and neurogenesis led us to the hypothesis that GAL may play a role in the formation of the nervous system. To further substantiate a developmental role for GAL, its binding sites were elucidated in the brains of adult and developing Brazilian opossums.

MATERIALS AND METHODS

Animals

Adult and developing opossums were obtained from a colony maintained at Iowa State University. The initial animals of the colony were obtained from the Southwest Foundation for Research and Education (San Antonio, TX). The animals were maintained at a constant temperature (26°C) and on a 14:10 light-dark cycle. Food and water (Reproduction Fox Chow; Milk Specialties Products, Madison, WI) were available ad libitum. The animals used in the developmental portions of the study ranged in age from 1 PN to 60 PN. The day of birth was designated as day 1 PN. At least four animals each at 1 and 5 PN were used. At day 1 and 5 PN the gender of the neonates cannot be determined grossly, and thus the animals were considered sexually undifferentiated. At least four male animals were used at each of the following timepoints: 10, 25, 60 PN, and adult (greater than 180 PN). Animals used for each timepoint came from a minimum of two different litters. The animal housing and use of animals was in accordance with the guidelines of the Iowa State University Committee on Animal Care.

Tissue Preparation

Adult and 60 PN animals were decapitated and their brains were rapidly removed and immediately placed on cryostat chucks and placed in the cryostat at -20°C. The 1, 5, 10, and 25 PN animals were decapitated and the whole heads were mounted on chucks and frozen as described above. The brains were sectioned on a cryostat (Reichart Instruments) at 20 μm in the coronal plane and thaw mounted onto poly-L-lysine coated slides. The slides

were air dried and then refrigerated until processed for receptor autoradiography.

Autoradiography

The protocol for receptor autoradiography was similar to that reported for other neuropeptides in *Monodelphis* (32) and the rat (34, 35). The concentrations of labelled and unlabelled GAL were similar to that used in previous reports (20). Briefly, sections were preincubated for 30 minutes at room temperature in 50 mM Tris HCl, containing 130 mM NaCl, 4.7 mM KCl, 5 mM $MgCl_2$, and 1 mM EGTA (Tris saline buffer) plus 0.5% BSA (pH 6.5). Sections were subsequently incubated for 2.5 hours at room temperature in an incubation solution containing Tris saline buffer containing 0.025% bacitracin, 1 mM dithiothreitol, 4 μg/ml leupeptin, and 125 pM ^{125}I-GAL (New England Nuclear, specific activity 2200 Ci/mmol). Negative controls were generated by coincubation of the incubation solution containing ^{125}I-GAL with 1.5 μM unlabelled peptide. Following incubation, sections were washed six times (15 minutes each) in preincubation buffer at 4°C and then briefly immersed in ice cold water and dried. Labelled sections were exposed to LKB ^{3}H-Ultrofilm for 5-6 days. Films were hand-developed and the images of the brain sections were analyzed.

Brain sections were fixed by dipping the slides in Zamboni's fixative for 10 minutes or by exposure to paraformaldehyde fumes overnight. The sections were then stained with 1% neutral red, dehydrated through graded alcohols and coverslipped.

Analysis

Analysis of the films and slides was done using an atlas of the adult and developing opossum constructed in our laboratory (16, 25, 26). Atlases of the developing mouse and rat brain were also used (36, 37). The autoradiograms were analyzed using a light box and a hand held lens and also using a projecting scope (Bausch and Lomb). The sections were examined at intervals of 80 μm for the developing animals and at 160 μm for the adult animals.

The intensity of labelling in the forebrain of the 60 and 25 PN animals was quantified to assess the amount of variability of binding between animals of the same age associated with our procedures. A densitometric analysis employing a Zeiss SEM-IPS image analysis system (Zeiss-Kontron; IBAS version 2.00) was used. Briefly, the autoradiograms were transilluminated using a ChromaPro 45 lightbox and viewed with a Contax 60 mm macro lens. For analysis of tissue from the 60 and 25 PN animals, sections containing the anterior commissure at its widest diameter were scanned. The lateral septal nuclei, caudate putamen (CPu), bed nucleus of the stria terminalis ventralis (BSTV), medial preoptic area (MPA), cortex, and an area adjacent to the tissue section on the film (background) were identified in these sections and outlined with a digitizer tablet. Areas to be analysed were determined interactively as described and the mean grey value (0= black; 255= white) and standard deviation of the grey value of each area was determined. The mean grey value of each of the specific areas was divided by the grey value of the corresponding background reading in order to obtain a ratio corresponding the intensity of binding. The mean and standard error of the mean for each ratio was calculated in order to assess the amount of variability

present between animals and films. In addition, the intensity of binding in the anterior pituitary (APit) was quantified to assess differences in binding intensities between ages 5, 10, and 25 PN.

RESULTS

In this study, we have obtained data indicating that ^{125}I-GAL binding sites are present in the brain of the adult and developing opossum, *Monodelphis domestica*. Binding sites were observed throughout the brain of the adult opossum including the preoptic area, basal hypothalamus, and brainstem. Specific GAL binding sites were observed in the developing brain and in surrounding tissues on day 1 PN. An index of variability (SEM/mean for the area x 100) in the intensity of binding between animals and between films in the forebrain of the 60 and 25 PN animals was found to be less than 15%.

Three qualitative levels of densities of GAL binding sites (high, moderate, low) were assigned to specific brain regions based on the density of silver grains deposited on the autoradiograms. A comparison of sections of the same age exposed on the same film and between films was also made to aid in categorizing the density of binding sites. Co-incubation of the tissue with ^{125}I-GAL and unlabelled GAL abolished all specific binding in the brain (Fig. 1). However, binding was observed in the eyes (area of developing cornea and retinal pigment epithelium) of whole head sections of developing animals (Figs. 1, 4A, and 6A) that was not abolished by coincubation with unlabelled galanin. Thus, the binding seen in the eyes was regarded as nonspecific.

^{125}I-GAL Binding in the Adult and Day 60 PN Brain

Forebrain

The density of binding in the forebrain ranged from very high to low. A widespread

distribution of areas having high densities of binding was observed in nuclear groups including the nucleus accumbens, olfactory nuclei, the septal nuclei (Figs. 2A and 3A), the bed nucleus of the stria terminalis (BST; Figs. 2A and 3A), MPA (Figs. 2A-B and 3A-B), periventricular hypothalamic nucleus (Pe; Figs. 2B and 3B), supraoptic nucleus (SO; Figs. 2B and 3B), suprachiasmatic nucleus (SCh; Figs. 2B and 3B), paraventricular hypothalamic nucleus (Pa), dorsomedial hypothalamic nucleus (DM), lateral hypothalamic area (LH), arcuate nucleus (Arc), and the amygdaloid nuclei (Figs. 2B and 3B). Moderate binding was seen in the CPu (Figs. 2A-B and 3A-B), lateral preoptic area (LPO; Figs. 2B and 3B), nucleus of the vertical limb of the diagonal band (VDB; Figs. 2A and 3A), paraventricular thalamic nucleus (PV; Figs. 2B and 3B), the habenular nuclei, central grey (CG), posterior hypothalamic area (PH), premammillary nuclei, the external layer of the superior colliculus (SC) and in the inferior colliculus (IC; Figs. 2C and 3C). The patterns seen in the day 60 PN brains were comparable to that seen in the adult. One area of difference was the hippocampus (HI). Moderate densities of binding were noted in the 60 PN brain (Figs. 2A-B and 3A-B) while the adult contained only faint binding in the HI (not shown).

Hindbrain

The medulla contained very dense binding in the dorsal vagal complex (nucleus of the solitary tract (Sol) and dorsal motor nucleus of the vagus (10); Figs. 2D and 3D) and in the caudal spinal trigeminal nucleus (Sp5C; Figs. 2D and 3D). Moderate binding was seen in the parabrachial nucleus (PB; Figs. 2C and 3C), the motor trigeminal nucleus (Mo5; Figs. 2C and 3C), principal sensory trigeminal nucleus (Pr5; Figs. 2C and 3C), and the vagus

nerve (10n). Moderate binding was also seen in an area ventral to the fourth ventricle thought to include the locus coeruleus (LC), dorsal tegmental area (DTg), and the dorsal raphe (DR; Figs. 2C and 3C).

^{125}I-GAL Binding in the Developing Opossum Brain

The brain of the neonatal opossum is very immature and specific nuclear groups are in the process of forming. Thus, when a specific nuclear group or area is discussed it will be in the context of the presumptive or differentiating nucleus or area.

DAY 1 PN

The brain of the 1 PN animal contained low to moderate densities of binding sites. Moderate binding was seen in the medulla at the level of the vagus nerve and its ganglia. Moderate GAL binding was seen in the forming brainstem and midbrain at a coronal level in which the tectum is situated above the forming cerebellum and brainstem. In the forebrain low GAL binding was observed in the ventro-lateral portions of the olfactory bulbs, preoptic area, and hypothalamus. The area of the presumed septum also contained faint GAL binding. Moderate binding was observed in the APit. Little to no binding was seen in the posterior pituitary. Low densities of binding were seen in the presumed optic (2n) and trigeminal (5n) nerves.

Day 5 PN

By 5 days PN the brain of the opossum has become more differentiated but areas are still considered to be presumptive. Throughout the brain at 5 days PN GAL binding sites were observed (Figs. 4A-C, 5A-C, 8A, and 9A). High densities of binding were seen in the basal cortex/amygdala (Figs. 8A and 9A) and at different levels of the brainstem. At the level in which the tectum is situated above the brainstem there was a high density of binding in areas thought to include the forming raphe nuclei, dopaminergic nuclear complex (substantia nigra (SN) and ventral tegmental area(VTA)), pontine nuclei, and forming trigeminal nuclei (Figs. 4B and 5B). In the medulla the pattern became diffuse and nearly the entire medulla contained binding sites (Figs. 4C and 5C). The vagus nerve and its ganglia contained high binding as was seen in the 1 PN animal (Figs. 4C and 5C). The APit also contained high densities of GAL binding (Figs. 8A and 9A). Moderate binding was observed in the preoptic area/basal forebrain (Figs. 4A and 5A), ventrolateral hypothalamus, tectum, 2n, and the 5n (Fig. 4C).

DAY 10 PN

By day 10 PN the binding pattern seen in the brain has increased in density and locations. High densities of binding were seen in the forming septum (Figs. 6A and 7A), preoptic area (Figs. 6A and 7A), CPu (Figs. 6A and 7A), BST, amygdala (Figs. 6B, 7B, 8B, and 9B), hypothalamic nuclei (Figs. 6B, 7B, 8B, and 9B), dorsal vagal complex (Figs. 6D and 7D), the trigeminal nuclei including Sp5C, and the pontine reticular area (PRA; Figs. 6C-D and

7C-D). In addition, in the midbrain binding was noted in areas including the SN and VTA (Figs. 6C and 7C). Moderate binding was seen in the tectum (Figs. 6C and 7C), CG, and rostral brainstem including the PB and LC. Light binding was seen in the forming HI, entorhinal and piriform cortices (Figs. 6B, 7B, 8B, and 9B). As was seen in the 1 and 5 days PN animals the vagus and its ganglia contained high binding densities. The 2n (Fig. 6A), 5n, and the APit (Fig. 8B) also contained moderate to dense GAL binding sites.

Day 25 PN

By 25 days PN, the brain of *Monodelphis* is fairly well differentiated and resembles the brain of the adult in shape and nuclear organization. The distribution of GAL binding sites was also very similar to that seen in the adult with a few exceptions. Moderate binding was observed in the HI and entorhinal and piriform cortices. In contrast to the 1, 5, and 10 PN animals little binding was noted in the APit.

Anterior Pituitary Gland:

Since it appeared that the level of binding of ^{125}I-GAL in the APit decreased with age, image analysis was conducted to get a quantitative measure of the changes which occurred. Image analysis demonstrated that the ratios of binding in the APit to that of the corresponding background values of the 5, 10, and 25 PN animals were 0.407 ± 0.0330; $.529 \pm 0.032$; and 0.846 ± 0.057 (mean $\pm$ SEM) respectively. Specifically, in the 5 PN animals, the ratio of the highest mean grey values to that of the background values was less

than one half that of the highest APit grey value ratios at age 25 PN (indicating less binding in the 25 PN animals). The mean grey values of the 10 PN animals were comparable to those of the 5 PN animals.

Figure 1: Darkfield photograph of an autoradiogram of a coronal section of the head of a 3 day postnatal Brazilian opossum. This photograph demonstrates the lack of specific ^{125}I-galanin binding when the tissue was coincubated with 1.5 μM unlabelled galanin. Note that the binding in the brain was abolished, but was not abolished in the eyes by coincubation with unlabelled galanin and ^{125}I-galanin. Thus, the binding seen in the eyes was regarded as nonspecific.

Figure 2: Darkfield photographs of autoradiograms demonstrating the presence of ^{125}I-Galanin binding in coronal brain sections (rostral to caudal; A-D) of a 60 day postnatal Brazilian opossum. ^{125}I-Galanin binding is demonstrated by white areas in the image. See figure 3 for atlas drawings of similar levels (A-D). AAA, anterior amygdaloid area; ac, anterior commissure; CPu, caudate putamen; DR, dorsal raphe; DVC, dorsal vagal complex; IC, inferior colliculus; ic, internal capsule; ox, optic chiasm; POA, preoptic area; Sep, septal nuclei; bar = 2 mm.

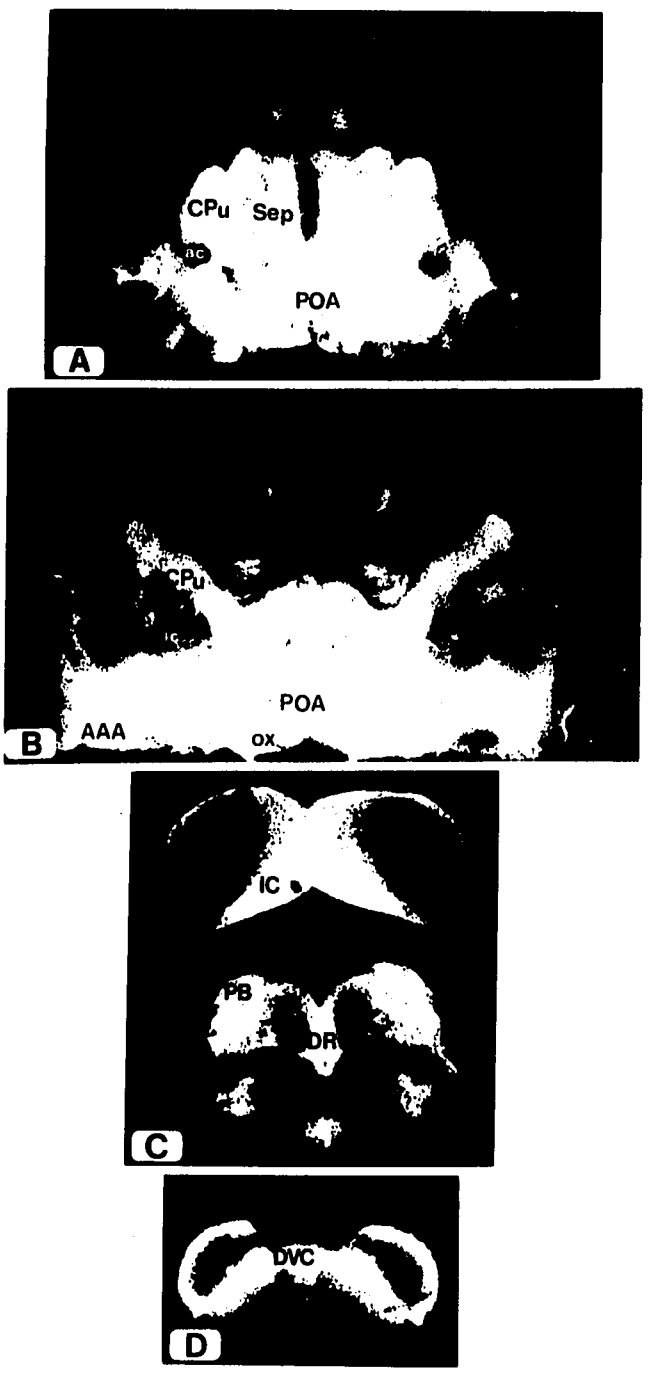

CPu
Sep
ac
POA
A
CPu
POA
AAA
ox
B
IC
PB
DR
C
DVC
D

Figure 3: Drawings of coronal sections (rostral to caudal; A-D) of the adult Brazilian opossum brain. The drawings are of similar levels to the sections shown in figure 2.

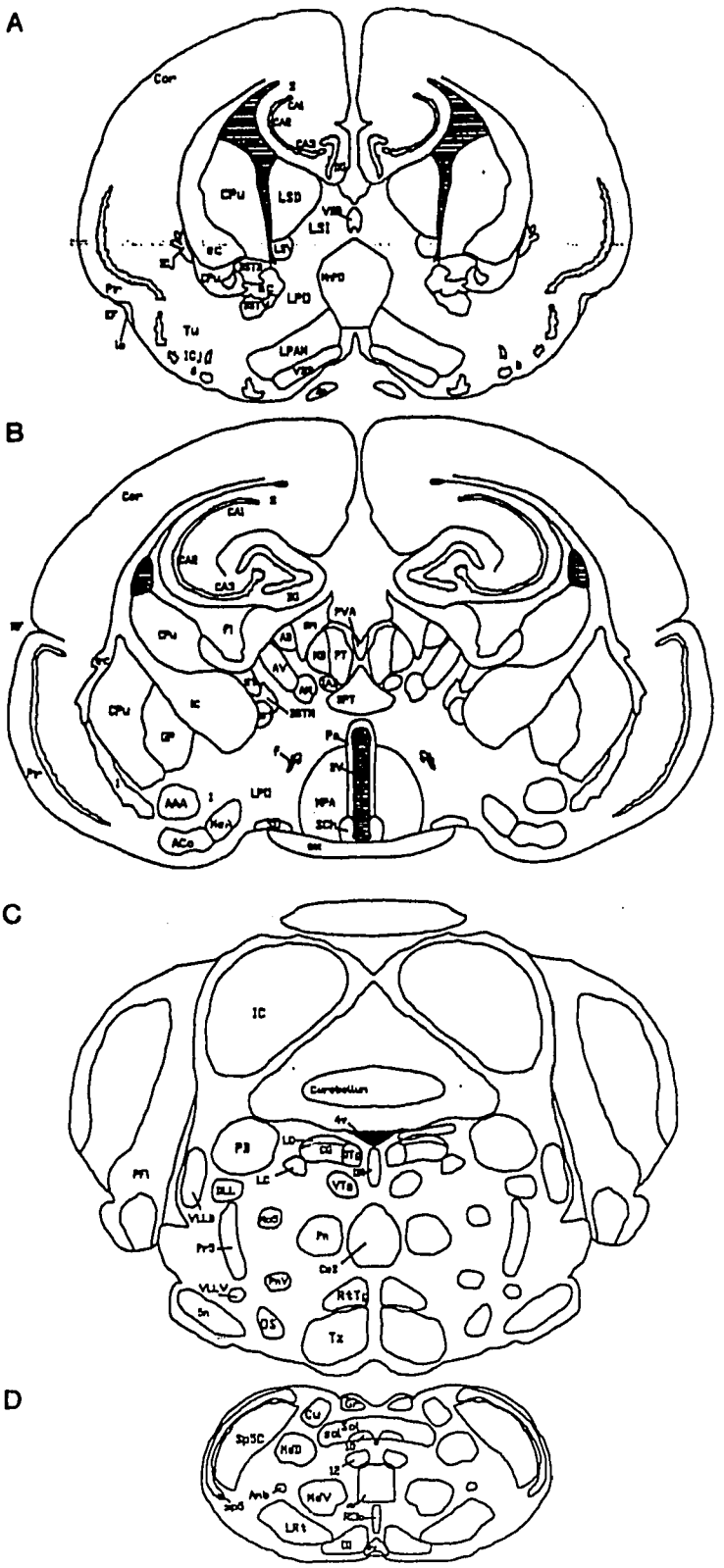

A
B
C
D
Cerebellum
IC
CPu
LSD
LSI
LPO
LPAH
Tu
AAA
ACo
PB
LC
VLL
Pn
Tz
Sp5C
Cu
Gr
12
Amb
LRt
CC

Figure 4: Darkfield photographs of autoradiograms demonstrating the presence of ^{125}I-Galanin binding in coronal sections (rostral to caudal; A-C) of the head of a 5 day postnatal Brazilian opossum. ^{125}I-Galanin binding is demonstrated by white areas in the image. See figure 5 for atlas drawings of similar levels (A-C). BFB, basal forebrain; PRA, pontine reticular area; TEC, tectum; 5n, trigeminal nerve; 10n, vagus nerve; 10 gn, vagal ganglia; bar = 1 mm.

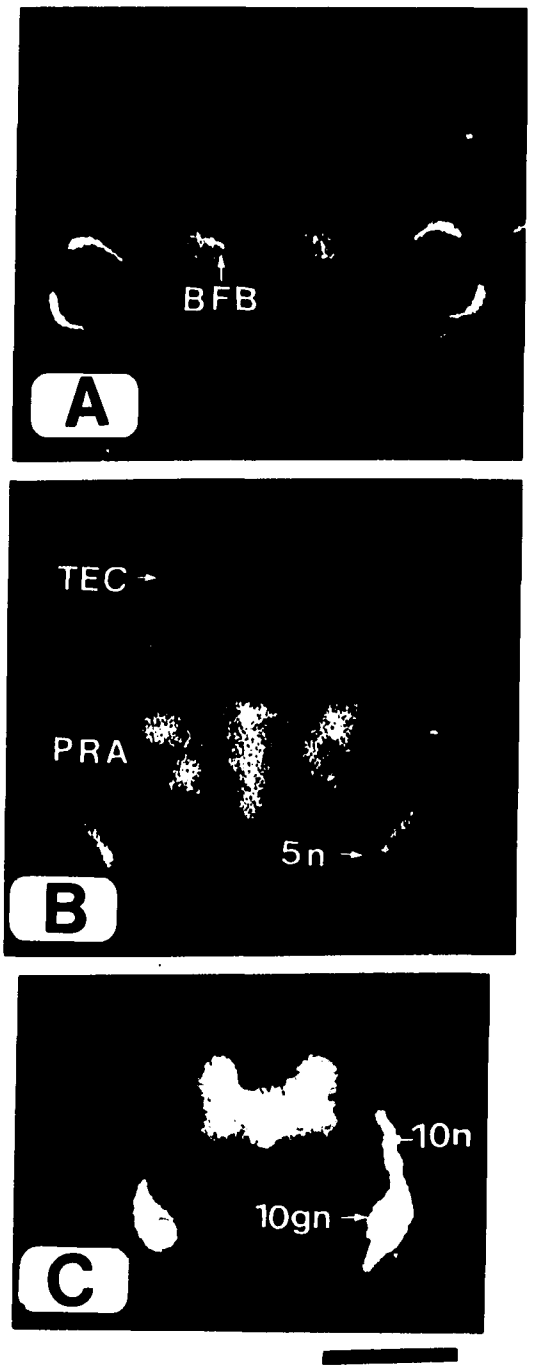

BFB
A
TEC
PRA
5n
B
10n
10gn
C

Figure 5: Drawings of coronal sections (rostral to caudal; A-C) of the 5 day postnatal Brazilian opossum brain. The drawings are of similar levels to the sections shown in figure 4. BFB, basal forebrain; DNC, dopaminergic nuclear complex; DVC, dorsal vagal complex; IOA, inferior olivary area; LV, lateral ventricle; MSA, medial septal area; Nu 5, trigeminal nuclei; PRA, pontine reticular area; PT, pyramidal tract; Sp5, Spinal trigeminal nucleus.

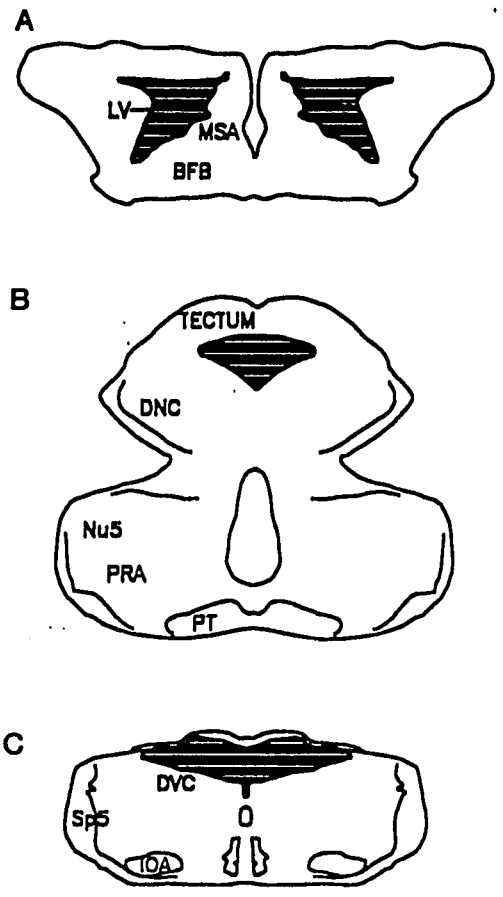

A
LV
MSA
BFB
B
TECTUM
DNC
Nu5
PRA
PT
C
DVC
Sp5
O
IOA

Figure 6: Darkfield photographs of autoradiograms demonstrating the presence of ^{125}I-Galanin binding in coronal sections (rostral to caudal; A-D) of the head of a 10 day postnatal Brazilian opossum. ^{125}I-Galanin binding is demonstrated by white areas in the image. See figure 7 for atlas drawings of similar levels (A-D). AAA, anterior amygdaloid area; BFB, basal forebrain; DVC, dorsal vagal complex; HI, hippocampal formation; LV, lateral ventricle; PRA, pontine reticular area; SNA, substantia nigral area; Sp5, Spinal trigeminal nucleus; 2n, optic nerve; 3v, third ventricle; bar = 1 mm.

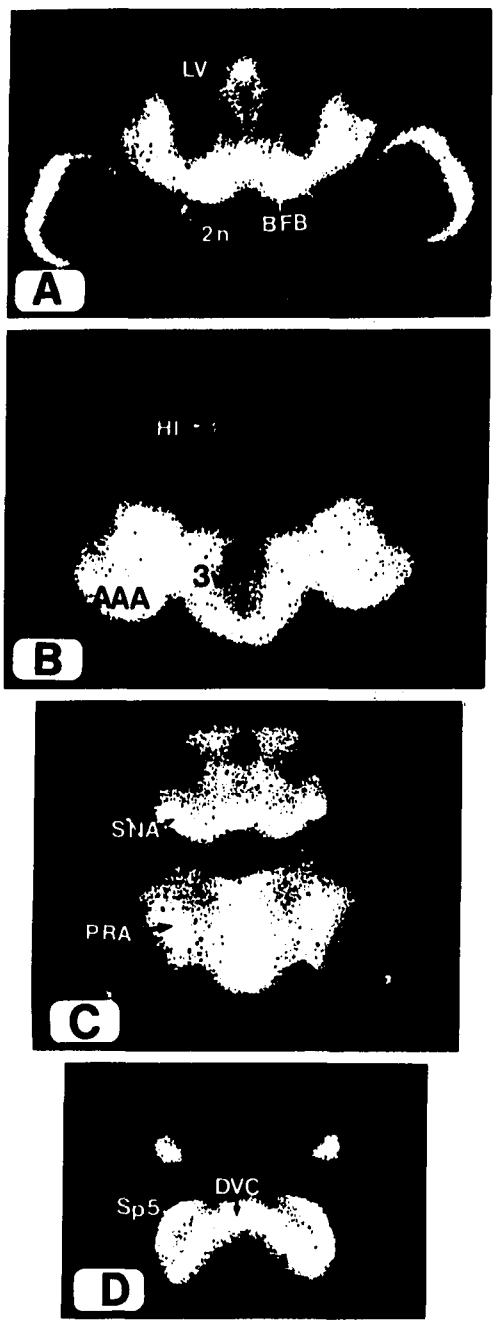
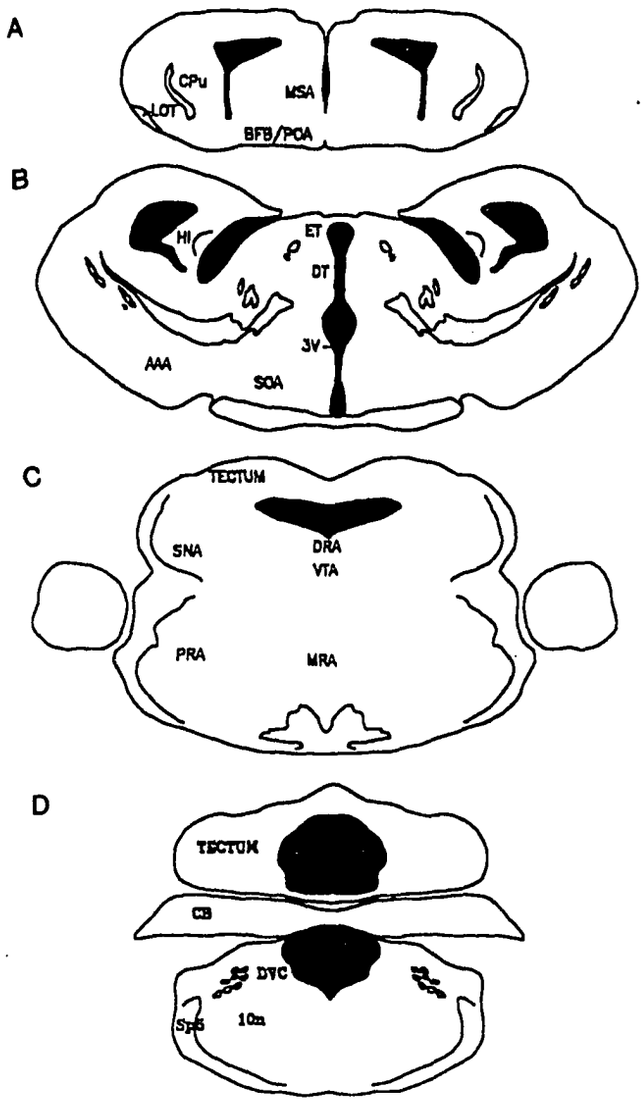

LV
2n
BFB
A
AAA
3
B
SNA
PRA
C
Sp5
DVC
D

Figure 7: Drawings of coronal sections (rostral to caudal; A-D) of the 10 day postnatal Brazilian opossum brain. The drawings are of similar levels to the sections shown in figure 6. AAA, anterior amygdaloid area; BFB/POA, basal forebrain/preoptic area; CPu, caudate putamen; CB, cerebellum; DRA, dorsal raphe area; DT, dorsal thalamus; DVC, dorsal vagal complex; ET, epithalamus; HI, hippocampal formation; LOT, lateral olfactory tract; MRA, median raphe area; medial septal area, MSA; PRA, pontine reticular area; SNA, substantia nigral area; SOA, supraoptic area; Sp5, Spinal trigeminal nucleus; VTA, ventral tegmental area; 3V, third ventricle; 10n, vagus nerve.


A
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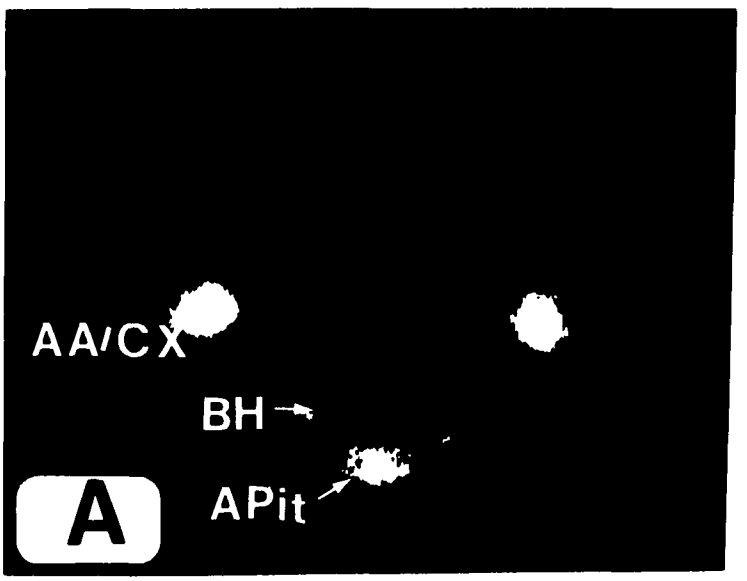
SNA
DRA
VTA
PRA
MRA
D
TECTUM
CB
DVC
SpV
10n

Figure 8: Darkfield photographs of autoradiograms demonstrating the presence of ^{125}I-Galanin binding in coronal sections of the head of a 5 day postnatal (A) and 10 day postnatal (B) Brazilian opossum. ^{125}I-Galanin binding is demonstrated by white areas in the image. See figure 9 for atlas drawings of similar levels (A-B). AA, amygdaloid area; AA/CX, amygdaloid area/cortical transition; APit, Anterior pituitary; BH, basal hypothalamus; bar = 1mm.


AA/CX
BH
APit
A

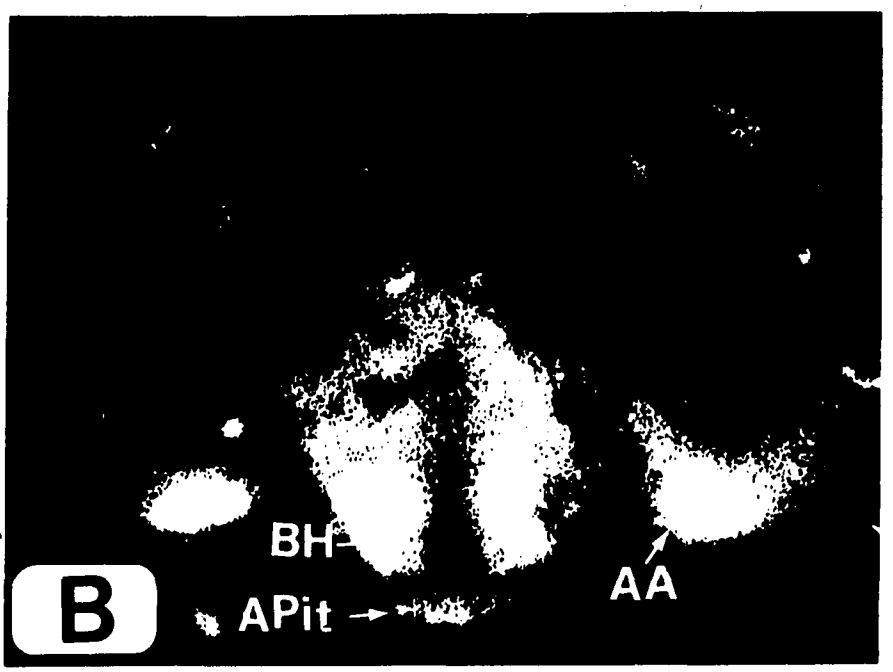
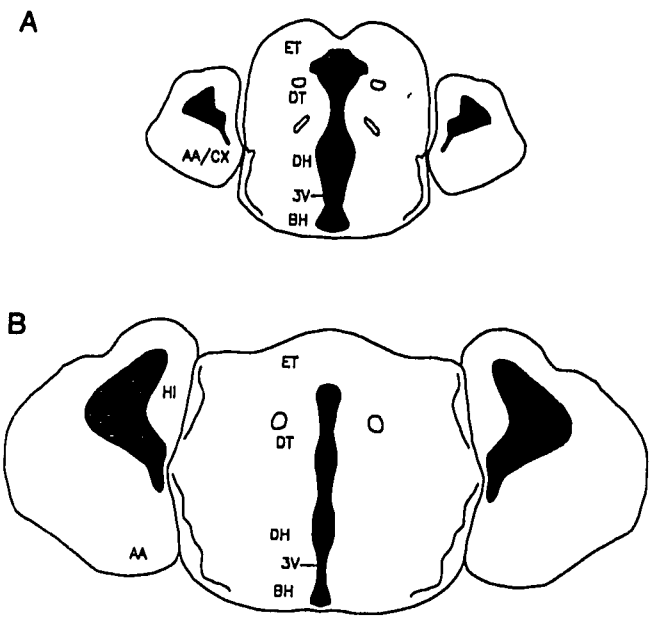

BH
APit
AA
B

Figure 9: Drawings of coronal sections of the brain of a 5 day postnatal (A) and of a 10 day postnatal (B) Brazilian opossum brain. The drawings are of similar levels to the sections shown in figure 8. AA, amygdaloid area; AA/CX, amygdaloid area/cortical transition; APit, Anterior pituitary; BH, basal hypothalamus; dorsal hypothalamus; DT, dorsal thalamus; ET, epithalamus; HI, hippocampal formation; 3V, third ventricle.


A
ET
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3V
BH

DISCUSSION

We have described the distribution of ^{125}I-GAL binding in the brain of the adult and developing opossum, *Monodelphis domestica*. In general, the pattern seen in the adult is very similar to that which has been reported in the rat (17, 18, 19). The distribution of GAL receptors as defined by receptor autoradiography also corresponds well to the distribution of GAL-IR in the adult opossum (16). The presence of GAL-IR and GAL binding sites in areas very similar to that of the rat and other species indicates that GAL may be playing similar physiological roles in the adult opossum.

Using a novel mammalian model for developmental studies, we have obtained results indicating that GAL receptors are present during periods of morphogenesis of the brain. Galanin receptors were detected in specific regions of the brain on day 1 PN and by 5 days PN dense binding was noted both in the forebrain and brainstem. These results are significant in that the brain of the Brazilian opossum is actively forming for a significant period of time following parturition. For example, neurogenesis of the suprachiasmatic nuclei is not completed in *Monodelphis* until 8PN. It is not until 17PN that the SCh can be identified morphologically using light microscopy. On day 20 PN a clear day-night rhythm in metabolic activity is observed (28). Further, neurogenesis of the medial preoptic area is not completed in *Monodelphis* until day 10 or 11PN. Morphogenesis follows neurogenesis by approximately 5-9 days in this region as well (22, 23). Experiments are currently under way utilizing bromodeoxyuridine as a marker for DNA synthesis (and neurogenesis). Preliminary results indicate that GAL-IR and GAL receptors are present at the time when DNA synthesis is ongoing in the neuroepithelium of the brain of the opossum (Jacobson lab,

unpublished). Characterization of the temporal relationship of GAL expression and its receptor to that of neurogenesis may help discern a developmental role for GAL.

Galanin is one of a number of peptides which are found in abundance both in the gastrointestinal (GI) tract and the CNS. These brain/gut peptides are thought to have multiple functions in adult mammals. Other examples of brain/gut peptides include cholecystokinin (CCK) and neuropeptide Y (NPY). Neither CCK nor NPY could be detected immunohistochemically in the brains of the opossum at 1 day PN (25, Jacobson lab unpublished). Specifically, CCK-like immunoreactivity (CCK-IR) was not detected in the brainstem until day 5 PN and in the hypothalamus until 15 PN. Further, CCK-IR in hypothalamic cell bodies were not detected until 35 days PN. These findings are in stark contrast to those for GAL in which GAL-IR and GAL receptors are detected as early as 1 day PN. Thus it appears that GAL may be functioning at a time when neurogenesis and morphogenesis are actively occurring. We anticipate that developmental roles of GAL may be distinct from its roles in the adult. Recently, reports have demonstrated effective GAL antagonists (38, 39). The application of these antagonists during early development may help to further elucidate a distinct developmental role for GAL in the forming CNS.

The aforementioned brain/gut peptides are known to affect mammalian feeding behavior. Specifically, CCK inhibits and GAL and NPY enhance food intake (reviewed in 40). Infusion of GAL into the paraventricular hypothalamic nucleus of adult rats causes an increase in feeding behavior (7, 8) and causes a preferential fat intake (41). Moreover, it has been recently reported that GAL levels are elevated in genetically obese rats that have high basal food intake levels (especially fat) and that the preferential fat intake can be

blocked by a GAL antagonist (42). Further, GAL injected into the brain has been shown to decrease insulin secretion (43). This data indicates that GAL may be involved in the regulation of energy stores and caloric balance in the adult animal. Galanin and its receptor are present during the early postnatal period in forming areas of the opossum brain (medulla and hypothalamus) that are thought to control feeding behavior. Further, CCK and NPY are not detectable immunohistochemically at day 1 PN. Thus, GAL may be involved in the early drive to assimilate nutrients for energy utilization and in control of the metabolic state of the newborn animal prior to the appearance of other regulatory systems.

As discussed earlier, GAL-IR and GAL receptors are present in multiple sites in the developing brain on day 1 PN when brain differentiation is still very active. The distribution of GAL binding sites in the developing brain correlated well with the GAL-IR at the same ages by day 5 PN (Figs. 4A-C, 5A-C, 6A, and 7A). In the 1 day PN animal a significant amount of GAL-IR was seen in the forming preoptic area, septum, hypothalamus, midbrain, and medulla (16). In contrast, the brainstem of the 1 PN animal was the only area in the brain that contained binding densities classified as moderate. The rest of the binding, although detectable, was considered low to faint. One possible explanation for the discrepancy between the detection of receptors and immunoreactivity is that our immunohistochemical technique may be more sensitive than our autoradiographic method. This is in contrast however, to our results for CCK. Specifically, CCK-IR is not detected until day 5 PN in the brainstem and day 15 PN in the hypothalamus, but CCK binding sites are detectable at day 1 PN in the brainstem and at 5 days PN in the hypothalamus (25, 32).

The detection of GAL-IR prior to GAL receptors may be caused by the presence of GAL in terminals of neurons in close association with the neuroepithelium. That is, GAL may induce the expression of GAL receptors in specific groups of neurons within or in neurons which have just left the neuroepithelium. This could be a means by which GAL may affect the development of a specific neuronal phenotype. Alternatively, it may be possible that this apparent lag in receptor detectability is due to the fact that with increasing age different cell types acquire GAL binding sites during the maturation of specific nuclear groups.

Galanin has been shown to be a regulator of pituitary function. Administration of GAL to rats increases GH secretion (44) and increases PRL secretion (5). Several studies support the hypothesis that GAL acts via the hypothalamus rather than directly on the APit to increase secretions (4, 44, 45). However, other reports indicate that GAL exerts mild effects directly on the APit (46, 47). Very low levels of binding have been reported in the adult rat APit using receptor autoradiography (19).

In the present study, transient GAL binding was detected in the APit. Moderate binding was seen at 1 PN. By days 5 and 10 PN the density of binding was high (Fig. 8A-B) but by 25 PN the binding in the APit was negligible. Image analysis demonstrated a more than two fold increase in the ratio of the mean grey values of the APit to that of the background values (reflecting less binding) of the 25 PN APit when compared to that of the 5 PN animals. The significance of transient APit binding during development is not known. One hypothesis is that GAL is regulating secretion in the APit prior to the maturation of other releasing factor pathways that are present in the adult. Presently, it is unknown how

neonatal opossums regulate growth and to what extent the APit functions in early life. Preliminary immunohistochemical studies in our lab indicate that ACTH and GH are present at days 4 and 9 PN. In contrast PRL-like immunoreactivity is not detectable at these ages (Jacobson lab unpublished). It appears that both corticotropes and somatotropes are present when GAL binding is noted in the APit. Thus, it is possible that GAL may directly regulate APit function in the very young animal. Then as the CNS matures, GAL may assume its adult type role. *In vitro* studies are currently underway investigating a possible age dependent direct action of GAL on the APit.

The formation and differentiation of the brain involves significant plasticity of neurons. Another condition in which neuronal plasticity is present is after an injury to the adult central nervous system (48). Interestingly, it has been demonstrated that galanin levels are increased following insult. After transection of the sciatic nerve, both galanin-like immunoreactivity and galanin mRNA are significantly increased in the corresponding dorsal root ganglia (49). Hypophysectomy increases both the peptide and mRNA in the Pa and Sol (50). Also, colchicine administration has been shown to up regulate the synthesis of galanin (51). Additionally, galanin immunoreactivity has been shown to be elevated in the basal forebrain of individuals with Alzheimer's disease (52, 53). This is quite interesting in that the developing brain in which neurogenesis and morphogenesis are occurring also has changing environmental cues. The robust GAL expression and presence of GAL receptors during CNS morphogenesis is further evidence for the hypothesis that GAL may be involved in the CNS response to changing conditions and/or injury.

In summary, we have described the distribution of GAL binding sites in the brains

of adult and developing opossums, *Monodelphis domestica.* The distribution of GAL receptors as revealed by autoradiography in the adult brain was very similar to reports in other species. Further we have described, for the first time, a profile of GAL receptors during early development of the mammalian brain. Binding was detected on day 1 PN when neurogenesis is actively taking place. In addition, a transient binding pattern was seen in the APit. The presence of GAL-IR and GAL receptors in similar regions is further evidence that GAL is functioning as a neurotransmitter/neuromodulator in the brain of the developing mammal. Future studies will determine the potential role of galanin in morphogenesis and early functioning of the CNS.

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PAPER III: DEVELOPMENTAL DISTRIBUTION OF GFAP AND VIMENTIN IN THE BRAZILIAN OPOSSUM BRAIN

INTRODUCTION

The development of the mammalian central nervous system (CNS) is extremely intricate, much of which occurs prenatally in placental mammals. This timecourse of developmental events often necessitates the use of *in utero* procedures to study critical events in the forming CNS. These factors make the use of marsupials an attractive alternative for *in vivo* studies of the developing CNS. The Brazilian opossum, *Monodelphis domestica,* is a small pouchless marsupial which breeds well under laboratory conditions. The young are born in an immature state well before neurogenesis is completed (Jacobson, '84). The absence of a pouch makes the young very accessible and circumvents the necessity of *in utero* manipulations for developmental studies. Therefore, *Monodelphis* is being used to study development of multiple CNS regions including the hypothalamus (Rivkees et al., '88; Schwanzel-Fukuda et al., '88; Fox et al., '91a,b; Elmquist et al., '92; Elmquist et al., '93; Keuhl-Kovarik et al., '93), brainstem (Wang et al., '92; Keuhl-Kovarik et al., '93), cerebellum (Dore et al., '90), olfactory bulbs (Brunjes et al., '92) and cerebral cortex (Saunders et al., '89).

The morphogenesis of the CNS is characterized by a series of complex processes including the interactions that occur between neurons and glial cells (reviewed in Silver et al., '93). In addition, studies have shown that disruption of neuron-glial cell interactions during critical periods of development can lead to structural and functional malformations of the brain (reviewed in Norenberg et al., '88). In the embryonic cerebral cortex and cerebellum intimate association between migrating neurons and radial glial cells have been observed (Rakic, '71, '72; Goldwitz and Mullen, '82). In these areas it is believed that

radial glial cells serve as physical scaffolds along which neurons can migrate (reviewed in Rakic, '88, '90). Radial glial cells are thought to be immature astrocytes which at later developmental stages differentiate into astrocytes (Voigt, '89). Glial cells are also thought to play integral roles in the guidance of axons and growth cones during the formation of neuronal circuits. For example glial elements provide favorable environments through which growing axons migrate (Silver and Sidman, '80; Sakaguchi et al., '89; reviewed in Silver et al., '93). Conversely, it appears that glial structures can also deter the growth of axonal pathways (reviewed in Silver, '84; Silver et al., '93). Thus, glial cells have multiple functions during formation of the CNS.

Numerous studies have utilized immunohistochemistry for the intermediate filament proteins glial fibrillary acidic protein (GFAP) and vimentin (VIM) to characterize glial elements of astrocytic lineage in the mammalian brain (see Voigt, '89; Hutchins and Casagrande, '90; Stichel et al., '91; Silver et al., '93). Of the two, GFAP is considered a major intermediate filament protein of astrocytes in the CNS of several mammalian species (see Voigt, 1989). Vimentin is expressed in cells of mesenchymal origin and is thought to exist in cells of the meninges, ependyma, and a few glial cells in the adult CNS (Dahl et al., '81; Bignami et al., '82; Hutchins and Casagrande, '89). Conversely, during development much more VIM is present, especially in radial glial cells (see Hutchins and Casagrande, 1989). As the CNS develops vimentin-like immunoreactivity (VIM-IR) becomes less apparent as cells mature into astrocytes (Pixley and de Vellis, '84; Hutchins and Casagrande, '89; Voigt, '89). Thus, VIM-IR and GFAP-like immunoreactivity (GFAP-IR) have been interpreted as markers of immature and mature astrocytes, respectively (Stichel et al., '91).

Several studies have investigated the distribution of GFAP-IR and VIM-IR during development of numerous mammalian species (Bignami and Dahl, '74; Voigt, '89; Hutchins and Casagrande, '89, '90; McDermott et al., '89; Oudega and Marani, '91; Stichel et al., '91; Silver et al., '93). Although the astroglial differentiation in the superior colliculus of the North American opossum (Barradas et al., '89) and the olfactory bulb of *Monodelphis* (Brunjes et al., '92) have been described, relatively little has been reported on the distribution of GFAP and VIM in the developing opossum brain. The characterization of the distribution of glial elements during morphogenesis of the brain of *Monodelphis* is of importance to both *in vivo* and *in vitro* studies investigating the development of the opossum brain. Therefore, we initiated studies to characterize the distribution of glial elements and their relationship to some previously characterized neuronal systems in the forming brain of *Monodelphis*. In these studies, we have used immunohistochemistry to visualize the distribution of VIM and GFAP during periods of neurogenesis and morphogenesis of the opossum brain.

MATERIALS AND METHODS

Animals

Adult and developing Brazilian opossums were obtained from a colony maintained at Iowa State University and used in the studies described. The initial animals of the colony were obtained from the Southwest Foundation for Research and Education (San Antonio, TX). The animals and procedures used were in accordance with the guidelines and approval of the Iowa State University committee on animal care. The animals were maintained at a constant temperature (26°C) and on a 14:10 light-dark cycle. Food and water (Reproduction Fox Chow; Milk Specialties Products, Madison, WI) were available ad libitum. At least four animals were used at each of the timepoints in the immunohistochemistry study (1, 5, 10, 15, 25). The day of birth was designated as 1 day postnatal (PN). Three adult males (greater than 180 PN) were used. Animals used for each timepoint came from a minimum of two different litters. At the ages of 1 and 5 PN the gender of the neonates cannot be determined grossly, and thus the animals were considered sexually undifferentiated. Only male animals were used at ages older than 5 PN.

Tissue Preparation

Adult opossums (greater than 180 days of age) were anesthetized by overetherization and perfused transcardially with Zamboni's fixative (1.8% paraformaldehyde, 7.5% saturated aqueous picric acid, sodium phosphate buffer; pH 7.5) as described previously (Elmquist et al., '92). The brains were removed from the calvaria, postfixed for 24 hours in Zamboni's

fixative, sunk in a 30% sucrose solution, and 40μm thick coronal sections were cut on a cryostat and placed into tissue culture wells containing cryoprotectant.

The brains were collected from 1, 5, 10, and 15 PN opossums by cooling the animals in a -15°C freezer until anesthetized. The animals were then decapitated and the heads placed in Zamboni's fixative for 48 hours. After fixation, the heads were infiltrated with 30% sucrose overnight, and then cut into 20μm thick coronal sections on a cryostat (Reichert Instruments). The sections were thaw mounted onto poly-lysine coated slides and stored at 4°C until processed for immunohistochemistry.

Twenty five day old pups were anesthetized by cooling. These animals were perfused transcardially with 15 ml of Zamboni's fixative. The heads were isolated and postfixed in Zamboni's fixative for 48 hours. After postfixation, the brains were processed as described above for the developing animals.

Immunohistochemistry

The protocol utilized for immunohistochemistry was a modification of that reported previously in our laboratory for the Brazilian opossum brain (Elmquist et al., '92; Pearson et al., '93). The slide mounted sections were rinsed with 50mM potassium phosphate buffered saline, incubated with a 0.3% H_2O_2 solution to remove endogenous peroxidase activity, exposed to normal horse serum as a blocking agent (Vector; 1:67) and then incubated in mouse monoclonal antibody against Vimentin (DAKO, 1:2500) or GFAP (ICN, 1:1000) for 20 hours at room temperature. After adequate washing, the tissue sections were incubated in horse anti-mouse IgG (Vector; 1:600) for 2 hours at room temperature, rinsed,

and reacted with avidin-biotin complex (Vector Elite Kit; 1:200) at room temperature for an additional hour. After washing, the tissue sections were stained by exposing them to a substrate composed of 0.04% 3,3' diaminobenzidine tetrahydrochloride (DAB; Sigma), 2.5% nickel sulfate (Fisher Scientific) and 0.01% hydrogen peroxide, dissolved in 0.1 M sodium acetate. After staining for 10-12 minutes, the reaction was terminated by placing the slides into two successive rinses of 0.9% saline. Sections were counterstained with neutral red (Fisher Scientific) and then dehydrated in graded alcohols, cleared in xylene and coverslipped with permount mounting media and analyzed with a light microscope. In each run negative controls were generated by omission of the primary antisera.

The adult tissue sections were immunohistochemically processed using a floating tissue technique described previously for adult tissue (Elmquist et al., '92).

Analysis of Tissue

Sections were observed with a Zeiss Axiophot microscope and regions containing VIM-IR and GFAP-IR were identified and recorded using maps of coronal sections of the opossum brain as described previously (Fox et al., '91a,b; Elmquist et al., '92). Identification of structures within and outside the developing brain was aided by the use of an atlas of the developing rat brain (Paxinos et al., '91).

RESULTS

Distribution of VIM-IR and GFAP-IR

General Observations

Immunohistochemistry demonstrated a reciprocal developmental relationship in the distribution of VIM and GFAP during maturation of the opossum brain. In particular, results GFAP-IR was sparse during the early stages of development and increased dramatically by 25 PN and in the adult brains. In the adults, GFAP-IR was present in astrocytes throughout the white and gray matter of the brain. In contrast, VIM-IR was seen as early as 1 PN and was still very robust at 15 PN. The VIM-IR was diminished by 25 PN as well in the adult brains where the VIM-IR was apparent only in a few specific regions. At all of the ages examined, VIM-IR was observed in the meninges, ependyma, and was also associated with blood vessels. In general, at the early developmental stages, the VIM-IR was seen mainly in radial glial elements with their processes spanning the width of the forming brain. These processes were uniformly spaced throughout the brain. In addition, dense bundles containing VIM-IR were seen in specific regions of the forming brain. Both antibodies (VIM and GFAP) labelled cells that were radial glial and/or astrocytic in appearance. However, the anti-VIM antibodies labelled more radial glial cells while more astrocytes were labelled with the anti-GFAP antibody. Additionally, VIM-IR was visualized in cells of the neuroepithelium of various ages. The immunoreactive cells were predominantly seen at the ventricular surface of the ne with few cells seen in the other layers. Omission of the primary antisera resulted in no specific staining at any of the ages examined.

1 PN

The brain of *Monodelphis* is extremely immature at the time of birth and grows and matures rapidly during the first month of postnatal life. Thus, when a specific nuclear group or area is discussed it will be in the context that particular area is still differentiating. Radial glial cells and fibers containing VIM-IR were seen in the entire rostral to caudal extent of the 1 PN brain. However, the VIM-IR was more abundant caudally in the forming medulla. Fine evenly spaced, radial cells were observed spanning the width from the pial surface to the ventricular zone (Fig. 1A). In the forebrain, rostral to the fusion of the cerebral hemispheres, dense VIM-IR cells and fibers were seen on the midline between and on the lateral aspects of the hemispheres (Fig. 2). Cells in the meninges and blood vessels also contained VIM-IR.

In addition to the evenly spaced immunoreactive radial glial elements, VIM-IR was observed in other structures as well. Specifically, dense VIM-IR was seen along the raphe of the brainstem (pons and medulla). Further, robust VIM-IR was observed in several cranial nerves including the olfactory (1), optic (2), trigeminal (5), vestibulocohclear (8), glossopharyngeal (9), and vagus (10) nerves. Labelling in these structures was seen outside the brain as well as in their presumed passages in the brain. The spinal tract of the trigeminal (Sp5) and the intramedullary portion of 10 were well delineated (Fig. 1A). Additionally, VIM-IR was seen in ganglia including those of cranial nerves 5, 8, 9, and 10. Vimentin-IR was also observed in the formative median eminence and neural lobes. No GFAP-IR was seen at 1 PN.

5 PN

As was the case at 1 PN, VIM-IR was seen throughout the rostral to caudal extent of the developing brain. From the medulla rostrally to the olfactory bulbs, VIM immunoreactive radial processes were evenly spaced with cell bodies located at the pial surface (Figs 1B). Vimentin-IR was also present in cells of the neuroepithelium.

Dense bundles of VIM-IR were present along the midline in the pontine and medullary raphe and the dorsal midline of the tectum. As seen in the 1 PN brains, cranial nerves 1, 2, 5, 8, 9, and 10 (including peripheral ganglia) contained robust VIM-IR (Fig. 3). Specifically, within the brain a well defined Sp5, 8, and 10 were observed (Fig. 1B). Dense VIM-IR was present in the median eminence (ME) and neural lobe of the forming pituitary gland.

In addition to the VIM-IR in the cranial nerves and in radial glial elements, VIM-IR was also seen in dense bundles of fibers in the forming forebrain. Specifically, collections of VIM-IR in fibers were observed in the internal capsule (Fig. 4) and in fibers running dorsoventrally in the diencephalon. Additionally, VIM-IR bundles were observed at the junction of the septum and cerebral cortex (corticoseptal junction, CSJ) and at the hypothalamic sulcus (HS) which extended from the ventricular to the external surface of the brain. No specific GFAP-IR was observed in the 5 PN brain.

10 PN

The pattern of VIM-IR in the 10 PN brain was similar to that observed for the 1 and 5 PN animals, although the overall distribution and intensity of immunoreactivity was

increased. As seen at the younger ages, immunoreactive radial glial cells and their processes were very prominent throughout the brain. Vimentin-IR was present in cells at the pial surface (Fig. 5A-B). The ME, neural lobe and cranial nerves maintained their robust VIM-IR as well (Figs. 1C and 6A). Vimentin-IR was still found in cells of the neuroepithelium.

In addition to the relatively even distribution of VIM-IR in radial glial cells and processes, collections of VIM-IR were observed in dense fiber bundles in multiple brain regions. For example, in the forming cerebral cortex, VIM-IR in fibers was observed running beneath the cortical plate extending ventrally into the internal and external capsules (Fig. 7). The fibers were seen mainly in the subplate zone with some extending into the subventricular zone as defined previously for *Monodelphis* (Saunders et al., '89). The anterior and hippocampal commissures (ac and hc, respectively) contained substantial VIM-IR in cells and fibers. Once again, dense VIM-IR was present along the midline of the tectum (Fig. 5A), pons, and medulla.

At the level of the CSJ and HS a tight band of VIM-IR fibers extended from the ventricular surface laterally to the pial surface (Fig. 8A-B). In addition, VIM-IR was seen in small cells surrounding the fiber bundles at the CSJ. Fibers and cells containing VIM-IR were seen in the stria medullaris. In the ventral hypothalamus, at the level of the optic chiasm, VIM-IR was seen in vertically aligned fibers on the midline.

By 10 PN GFAP-IR is detectable in the brain of the opossum. Cells containing GFAP-IR were observed near the pial surface of the ventral midbrain, and in the lateral portions of the pons and medulla (Fig. 9A). In addition, a few radial glial processes

contained GFAP-IR in the medulla.

15 PN

The 15 PN brain of Monodelphis still contained robust VIM-IR. The overall pattern was very similar to that seen at 10 PN with few exceptions. For example, immunoreactive radial glial cells and processes were very prominent in the forming cerebral cortex, tectum, cerebellum, hippocampus, and hypothalamus (Fig. 6B). As described in the 10 PN brain, a dense bundle of VIM-IR was seen beneath the cortical plate running parallel to the cortical surface that extended into the internal and external capsules. Vimentin-IR was once again seen along the midline of the tectum, pons, and medulla. Vimentin-IR in cells of astrocytic appearance were more prominent than at 10 PN especially in the cerebellum and fornix.

In addition to the areas containing VIM-IR described above, VIM-IR structures were observed at distinct anatomical locations. Specifically, VIM-IR fibers were seen at the CSJ and HS spanning the width of the brain. The point of attachment of the fibers was at sites of apparent ventricular kinks as described previously in the cat (Silver et al., '93). In addition VIM-IR was apparent surrounding the lateral aspects of the stria medullaris and in the ac.

By 15 PN, an increased distribution of GFAP-IR exists. Specifically, a few weakly positive radial glial cells and fibers were seen throughout the brain. Prominent GFAP-IR was observed on the midline of the tectum, in 2, and the medulla. Specifically, dense GFAP-IR was seen in the dorsal, ventral, and lateral portions of the pons and medulla (Fig. 9B). Although a few immunoreactive astrocytes were evident in the forebrain including the

preoptic area and lateral hypothalamus, a definite caudal to rostral expression of GFAP-IR was evident. In addition, an outside to inside pattern of GFAP-IR was also seen, with more immunoreactivity present in the periphery of the brain compared to more medial areas (Fig. 9B).

25 PN

The distribution of both VIM-IR and GFAP-IR was similar to that present at 15 PN. However, a general trend of decreasing VIM-IR and increasing GFAP-IR can be appreciated. For example, the VIM-IR observed in radial glial cells and processes was somewhat reduced throughout the 25 PN brain, although cells and fibers were still detectable. Prominent VIM-IR was still present in radial cells in the olfactory bulbs, hippocampus, and cerebellum. In addition, VIM-IR was seen in astrocytes in the ac, hc, and lateral pons and medulla including the dorsal cochlear nucleus, gracile and cuneate nuclei, and the area postrema. Vimentin-IR was still present in 2 and the midline raphe as well. The VIM-IR delineation of cranial nerves was less apparent than found at earlier developmental ages.

The labelling patterns observed in 25 PN brains further demonstrated the caudal to rostral and outside to inside appearance of GFAP-IR as discussed in the 15 PN brain. Specifically, the medulla of the 25 PN animals had an adult like appearance in which protoplasmic and fibrous astrocytes were visualized throughout the parenchyma (Fig. 10A). Rostrally, GFAP-IR was seen in astrocytes in the tectum, cerebral cortex, hc, ac, cerebellum, and internal and external capsules. In addition, GFAP-IR was seen prominently in the optic and trigeminal nerves.

Adult

The reciprocal developmental relationship of the relative levels of VIM-IR and GFAP-IR can be readily appreciated when adult brains were compared to those of younger ages. That is, the density and distribution of VIM-IR decreased markedly as compared to that seen at the younger ages. Conversely, the GFAP-IR increased dramatically. In the adult brain, VIM-IR is much less prominent as compared to that seen earlier in development. Radial glial cells and processes were no longer readily visualized. However, VIM-IR in radial fibers was observed spanning the molecular layer of the cerebellum (Fig. 11). In addition, certain areas of the brain maintained VIM-IR. Specifically, the meninges, blood vessels, and ependyma contained VIM-IR. Vimentin-IR in processes was seen extending laterally from the third ventricle into the hypothalamus (Fig 12A). The arcuate nucleus of the hypothalamus (Fig. 12B), ME, retrochiasmatic area, and the area postrema also contained substantial VIM-IR. Additionally, VIM-IR was observed in a few scattered protoplasmic and fibrous astrocytes throughout the brain.

The adult brains contained diffuse GFAP-IR in astrocytes at all rostral to caudal levels of the brain (Fig. 10B). The density and distribution of the GFAP-IR was very similar in appearance to previous reports of several mammalian species (Bignami and Dahl, '74; Onteniente et al., '83; Barradas et al., '89; Stichel et al., '91) and in the olfactory bulb of Monodelphis (Brunjes et al., '92).

Figure 1: A series of photomicrographs of coronal sections in the caudal medulla demonstrating vimentin-like immunoreactivity (VIM-IR) of a (A) 1 day postnatal (PN), (B) 5 PN, and (C) 10 PN opossum. Note the dense VIM-IR in the presumed vagus (10) and Spinal tract of the trigeminal (Sp5). Scale bars = 80 μm.

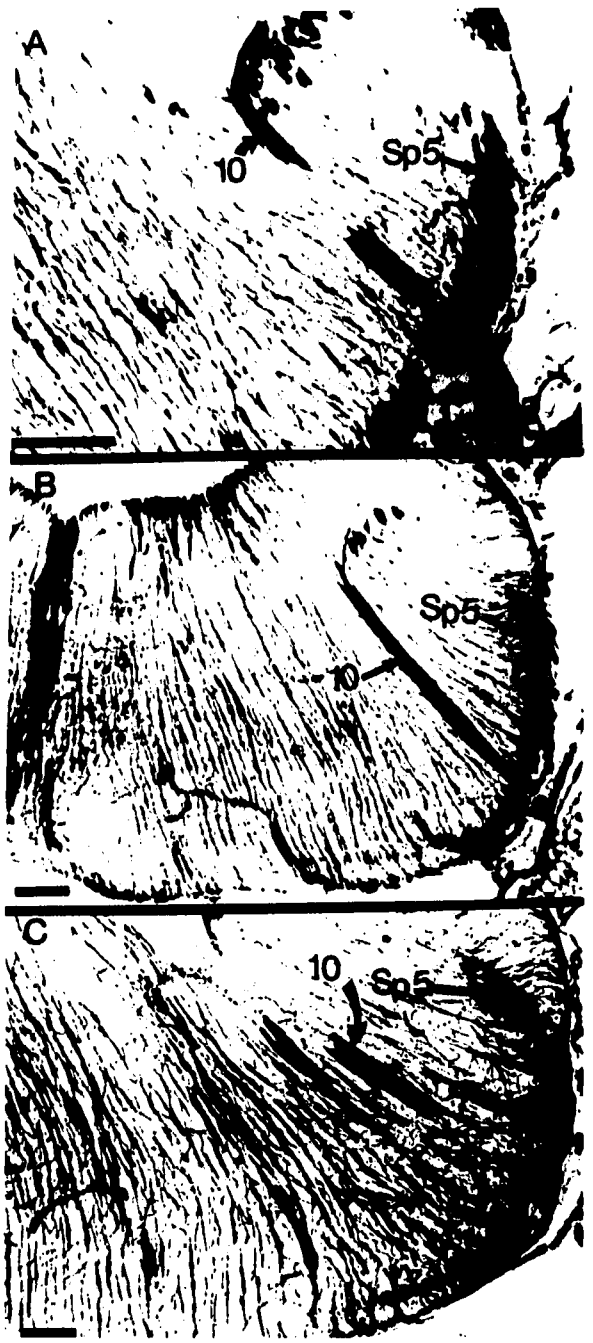

A
10
Sp5
B
Sp5
10
C
10
Sp5

Figure 2: A photomicrograph demonstrating vimentin-like immunoreactivity (VIM-IR) in a coronal section of the forming cerebral cortex of a 1 day postnatal opossum. Note the appearance of VIM-IR in radial fibers. Vimentin-IR is also seen outside the pial surface (arrow). Scale bar = 30 μm. LV, lateral ventricle.


LV

Figure 3: A photomicrograph of a coronal section demonstrating vimentin-like immunoreactivity in the trigeminal nerve (5) as it enters the trigeminal ganglion (5Gn) of a 5 day postnatal opossum. Scale bar = 30 μm.

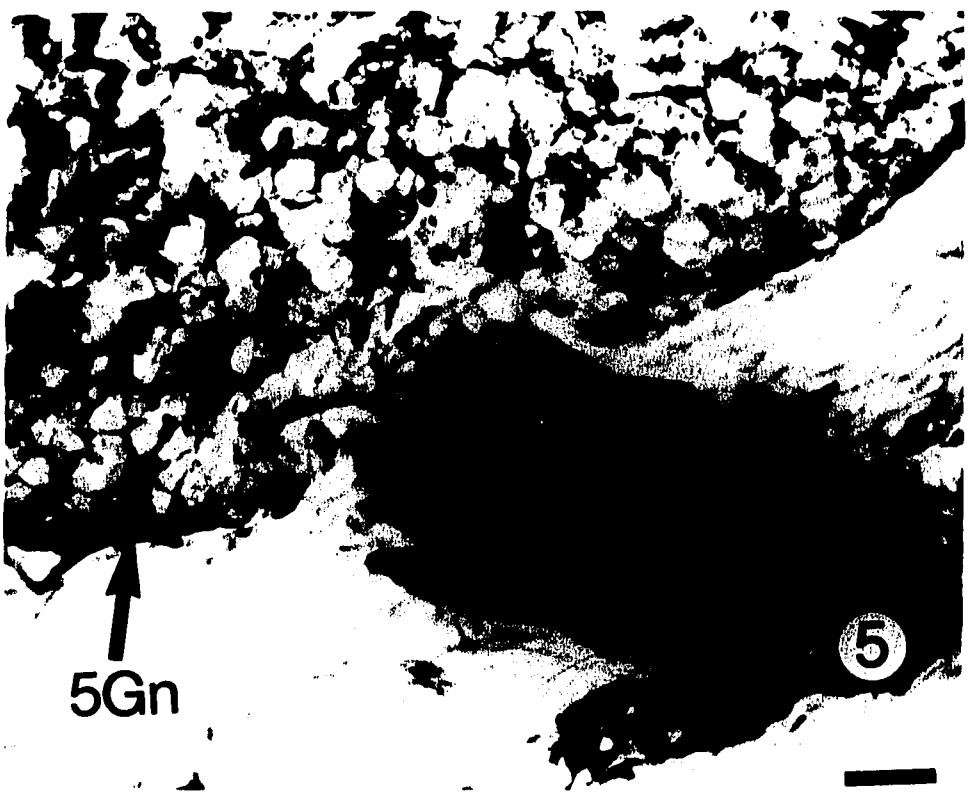

5Gn
5

Figure 4: A photomicrograph demonstrating dense vimentin-like immunoreactivity (VIM-IR) in the internal capsule (ic) of the 5 days postnatal opossum brain (coronal plane). VIM-IR is also seen in fine, radial fibers. Scale bar = 40 μm. Arrow denotes lateral ventricle.

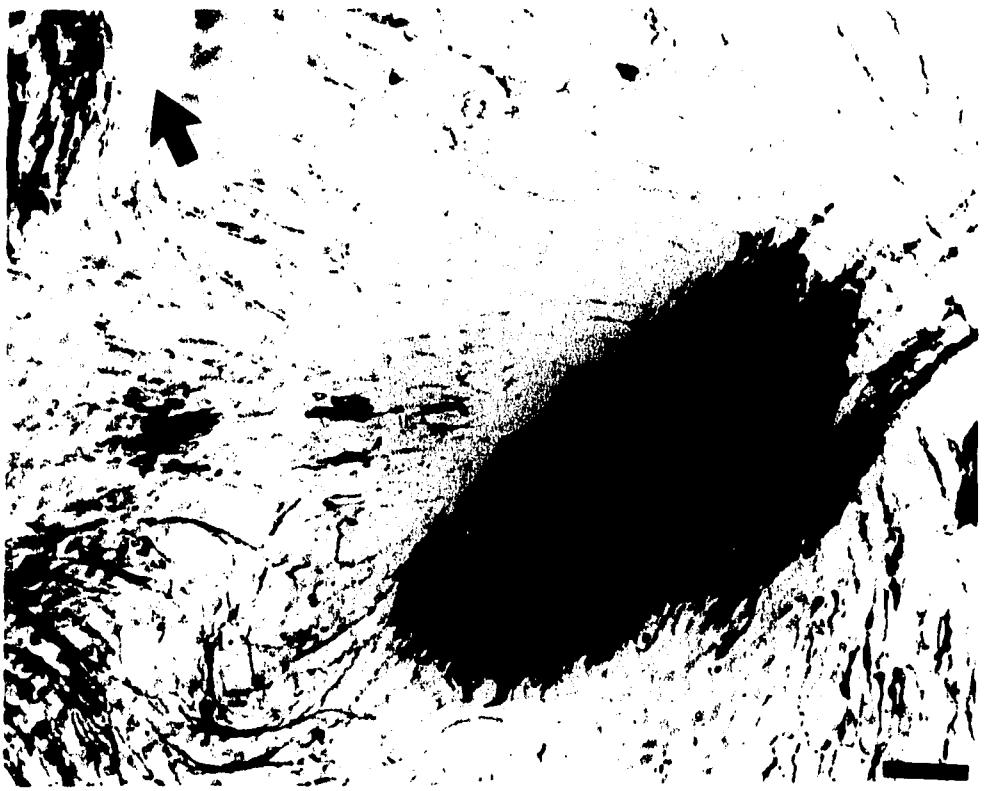


Figure 5: Photomicrographs demonstrating vimentin-like immunoreactivity (VIM-IR) in the tectum of a 10 day postnatal opossum brain (coronal plane). **A:** Note the presence of evenly spaced radial fibers containing VIM-IR. Dense VIM-IR is visible on the dorsal midline (arrow) extending from the pial surface to the cerebral aqueduct (aq). **B:** Photomicrograph demonstrating VIM-IR in cells at the pial surface and in radial fibers. Scale bars = 40 μm.

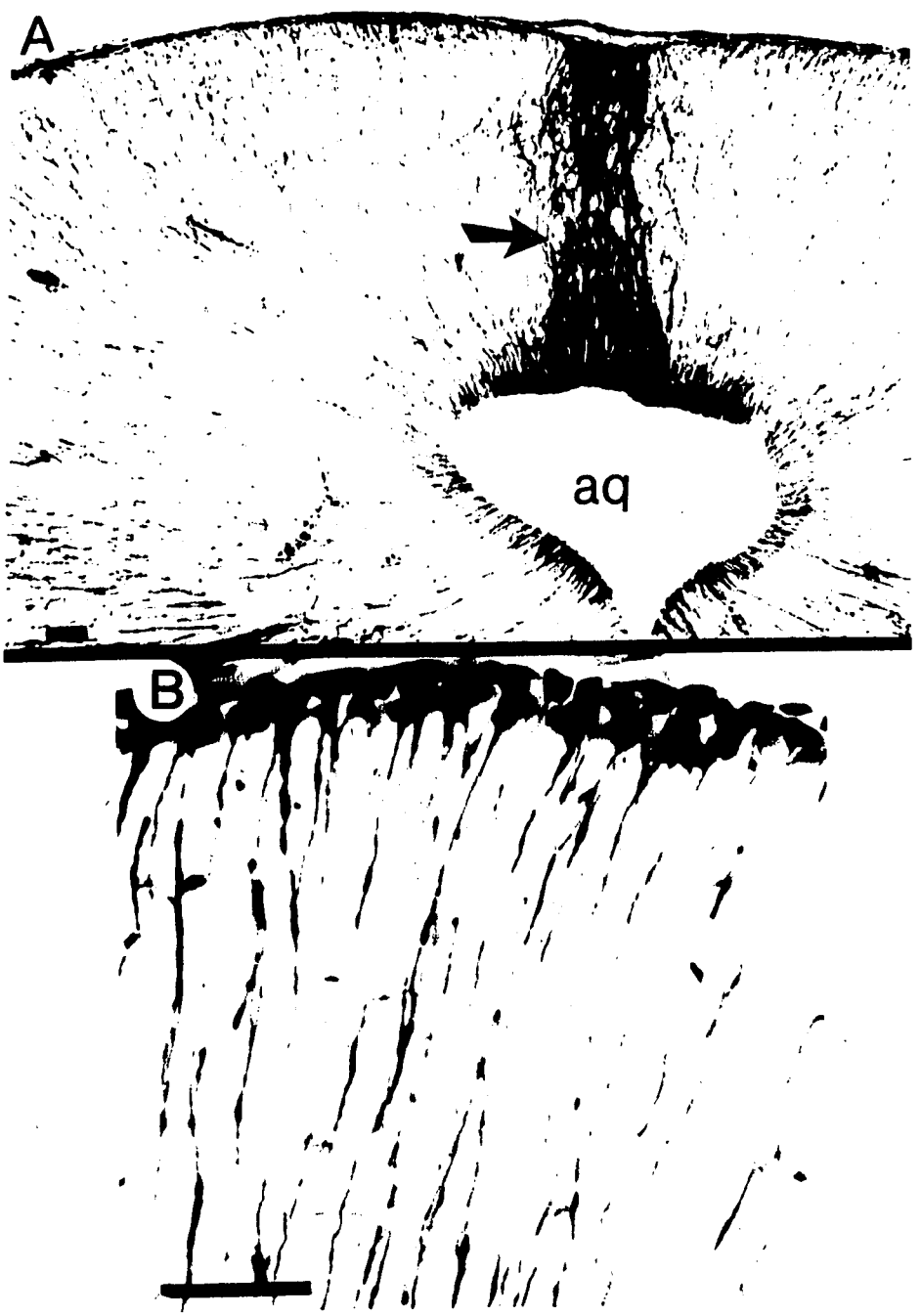

A
aq
B

Figure 6: Photomicrographs demonstrating vimentin-like immunoreactivity (VIM-IR) in the basal hypothalamus and neural lobe (NL; coronal planes) of (A) a 10 day postnatal (PN) and (B) 15 PN opossum. Note the dense VIM-IR in the area of the median eminence and NL. Scale bar = 80 μm. APit, anterior pituitary gland; 3v, third ventricle; VMH, ventromedial hypothalamic area.

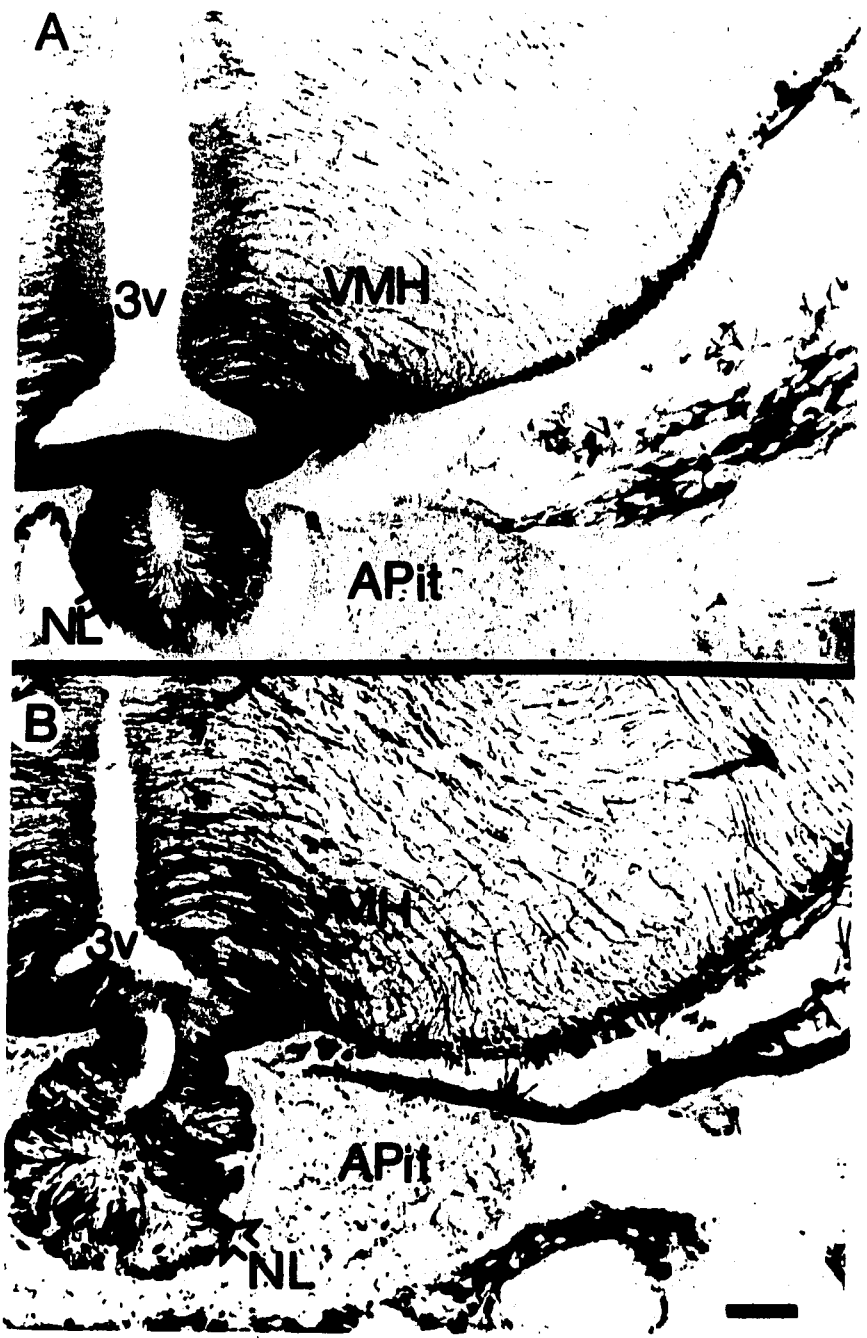

A
3v
VMH
APit
NL
B
VMH
3v
APit
NL

Figure 7: A photomicrograph demonstrating dense vimentin-like immunoreactivity (VIM-IR) in the forming cerebral cortex (coronal plane) of a 10 day postnatal opossum brain. Note the dense VIM-IR running beneath the cortical plate (asterisks). Also note the band of VIM-IR extending from the lateral ventricle (LV) to the corticoseptal junction (arrow). Scale bar = 80 μm


LV

Figure 8: Photomicrographs demonstrating vimentin-like immunoreactivity (VIM-IR) at (A) the corticoseptal junction and (B) the hypothalamic sulcus of a 10 days postnatal opossum brain (coronal planes). Closed arrows denote tight bands of VIM-IR extending from the lateral ventricle (LV) towards midline (A) and the third ventricle (3v) to the pial surface of the diencephalon (B). Note also in (B) VIM-IR running ventrally (open arrow). These fibers were interpreted as VIM-IR extending from the thalamus into the internal capsule. Scale bars = 40 μm.


A
LV
3
3

Figure 9: Photomicrographs demonstrating GFAP-like immunoreactivity (GFAP-IR) in the dorsolateral medulla (coronal planes) of **(A)** a 10 day postnatal (PN) and **(B)** 15 PN opossum brain. The GFAP-IR at these ages was seen predominantly in lateral aspects of the brainstem representative of the caudal to rostral and outside to inside pattern of appearance of GFAP-IR. Scale bars = 40 μm. cc, central canal; 4v, fourth ventricle.

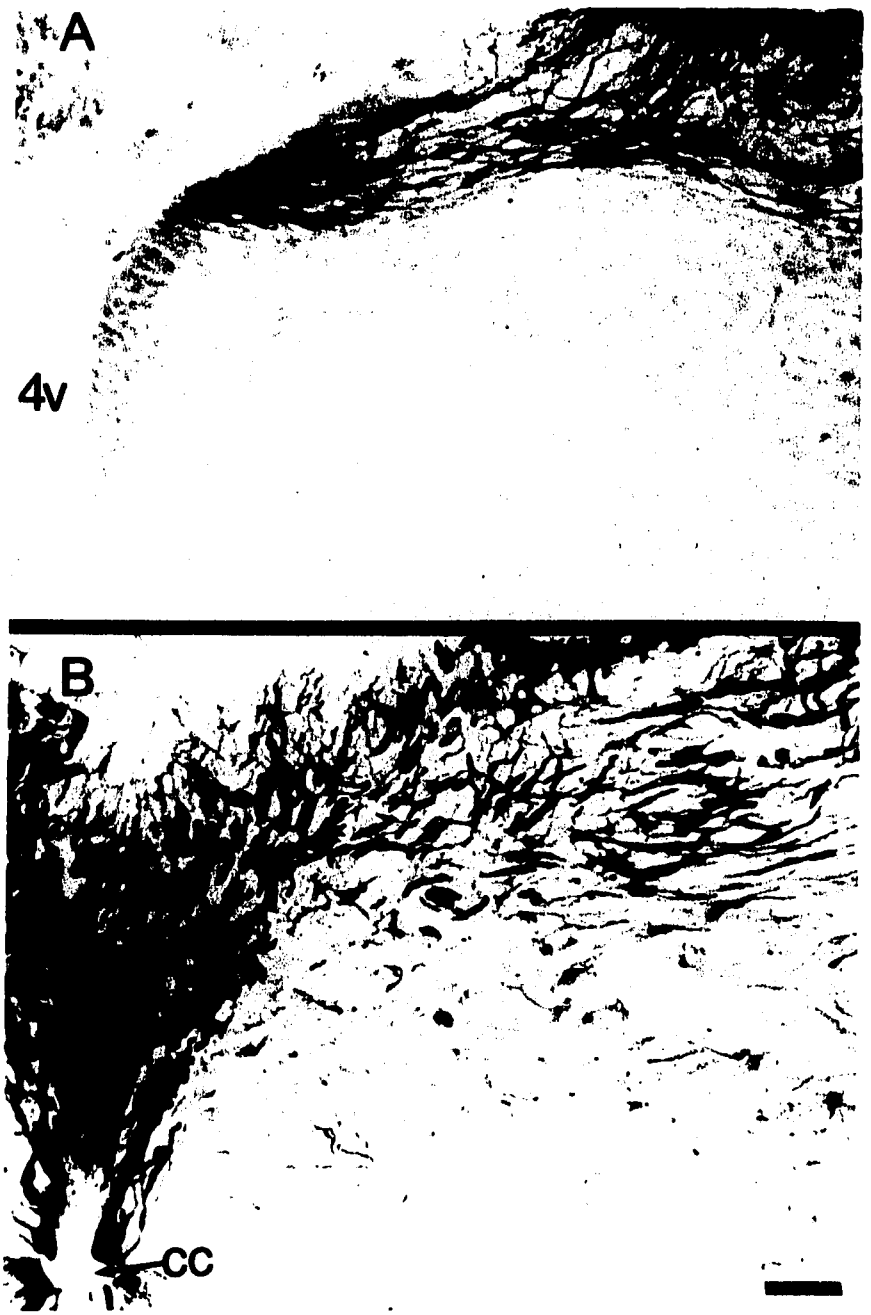

A
4v
B
CC

Figure 10: Photomicrographs demonstrating GFAP-like immunoreactivity (GFAP-IR) in astrocytes of (**A**) the ventrolateral medulla of a 25 days postnatal opossum and (**B**) the hippocampus of an adult brain (coronal planes). Scale bar = 40 μm.


A
B

Figure 11: A photomicrograph demonstrating vimentin-like immunoreactivity (VIM-IR) in radial fibers of the molecular layer of the cerebellum of an adult opossum (coronal plane). This is a region that maintains VIM-IR in radial cells in the adult brain. The asterisk denotes the purkinje cell layer. Scale bar = 40 μm.

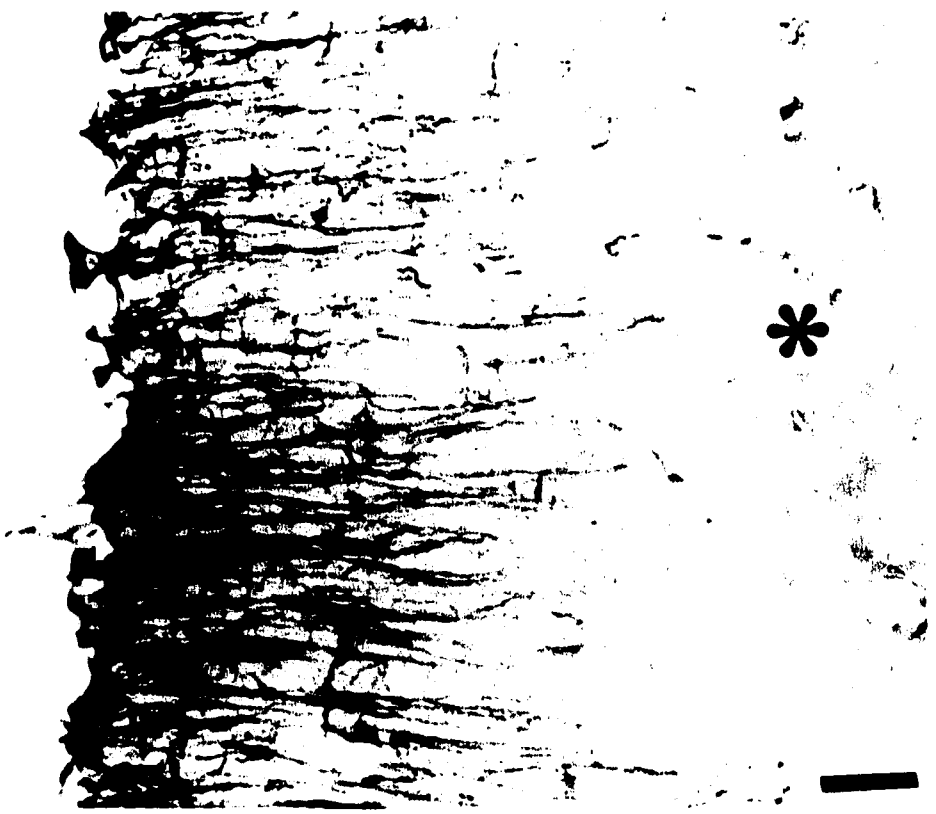
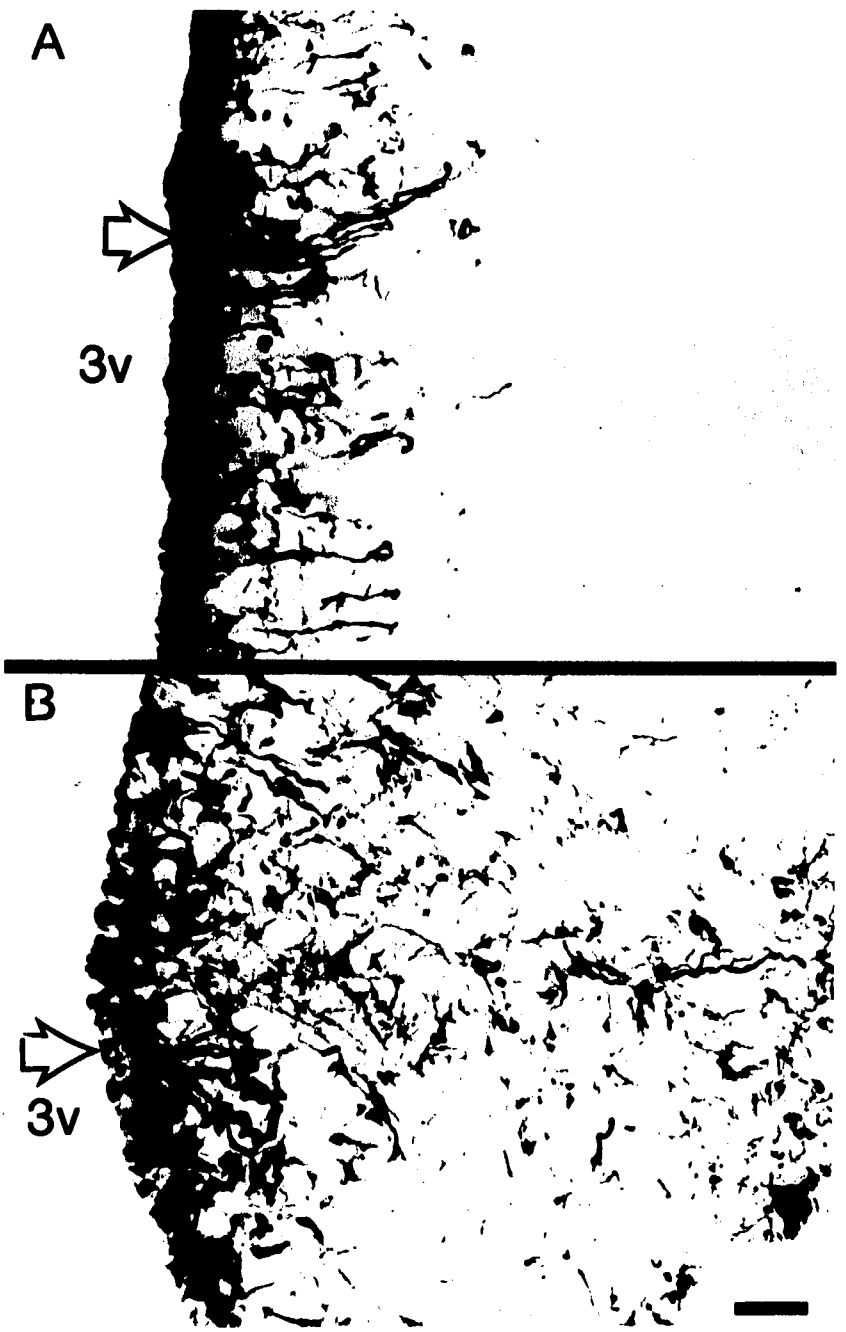


Figure 12: Photomicrographs demonstrating vimentin-like immunoreactivity (VIM-IR) in the adult opossum brain (coronal planes). (A) VIM-IR extending from the third ventricle (3v) into the dorsal hypothalamus. (B) VIM-IR in the arcuate nucleus of the hypothalamus of an adult. Note the VIM-IR in the ependymal cells (arrow) as well. Scale bar = 40 μm.


A
3v
B
3v

DISCUSSION

We have utilized immunohistochemistry to visualize the distribution of VIM and GFAP during periods of neurogenesis and morphogenesis of the opossum brain. Vimentin is an intermediate filament protein found in cells of mesenchymal origin, while GFAP is a major intermediate filament protein of astrocytes in the CNS. As discussed earlier, VIM-IR was observed in cells at the ventricular surface of the neuroepithelium of young opossums. It has been shown previously that some differentiating neurons in the spinal cord maintain VIM-IR (Bignami et al., '82). Thus, it is possible that the VIM-IR seen in the forming opossum brain is not present exclusively in differentiating glial populations. However, the majority of VIM-IR was seen in cells of radial glial appearance and thus we feel that the majority of VIM-IR is within cells of glial phenotypes.

It has been demonstrated previously that radial glial cells in the rat brain can mature into astrocytes as the forming brain ages (Voigt, '89). Additionally, immunohistochemistry using antibodies for VIM and GFAP has been used to identify immature and mature astrocytes, respectively (Stichel et al., '91). Thus, the relative abundance of GFAP and VIM may be an index of the relative maturity of the developing brain. The gradual decrease of VIM-IR and increase of GFAP-IR illustrates this point in the opossum brain. A caudal to rostral pattern of appearance of GFAP-IR was observed. This is consistent with observations that the medulla is more mature than the forebrain at early developmental ages of *Monodelphis*. For example neurogenesis of the medulla ends well before that of forebrain structures (Iqbal et al., '93; Jacobson lab, unpublished observations). Cholecystokinin (CCK) and galanin (GAL) receptors (^{125}I-binding sites) and CCK-like immunoreactivity also

have a caudal to rostral gradient (Kuehl-Kovarik et al., '93; Elmquist et al., '93; Fox et al., '91a). Further, axons (as defined by PHF-1 immunohistochemistry; Nelson et al., '93; Greenberg et al., '92) show a caudal to rostral gradient immunohistochemically as well (Jacobson lab, unpublished observations). Thus, the gradual decline of VIM-IR in radial glial elements and the increase in GFAP-IR in the brain of *Monodelphis* seems to mirror brain maturation.

In addition to the VIM-IR visualized in evenly spaced radial glial cells and processes, fascicles of VIM-IR were seen in other anatomical locations during development of the opossum brain. As discussed earlier, during development glial cells may provide favorable substrates that can guide neuronal migration and direct axonal growth to appropriate target regions (Silver and Sidman, '80; Sakaguchi et al., '89; Rakic, '91). In addition, glia may also provide boundaries for compartmentalization or impose barriers to deter the growth of axonal pathways (reviewed in Silver et al., '93). Potential examples of glial involvement in both functions in the brain of the opossum were visualized with VIM immunohistochemistry in this study. For example, dense VIM-IR fiber bundles were seen beneath the cortical plate (Fig. 7). These fibers ran parallel to the cortical surface and extended into the internal and external capsules. At 5 PN, the internal capsule contained dense VIM-IR (Fig. 4). Interestingly, immunohistochemistry for an axonal marker PHF-1 (Nelson et al., '93; Greenberg et al., '92), in similar regions demonstrates an apparent lag in appearance of PHF-like immunoreactivity as compared to VIM-IR. Thus, it is possible that the VIM containing structures may serve as guides for growth of incoming axons.

In the forming brainstem, very dense VIM-IR was seen in cranial nerves in the

periphery as well as in their presumed fiber tracks within the brain (Figs. 1A-C). These are potential examples of glial elements providing favorable substrates for axonal migration. We have previously shown that as early as 1 PN the neuropeptide galanin (GAL) can be localized immunohistochemically in areas including the presumed Sp5, 2, and 10 (Elmquist et al., '92). The immunohistochemical staining pattern of VIM and GAL in these anatomical locations is remarkably similar. Further, GAL binding sites are located in these structures as early as 1 PN as well (Elmquist et al., '93). The possibility that the GAL-IR and VIM-IR are located within the same cells and fibers cannot be ruled out. However, it is plausible that presumed axons containing neuropeptides such as GAL interact with VIM containing glial elements during neurogenesis and morphogenesis of the opossum brain.

Previously, it has been shown that specific populations of glial structures may be involved in partitioning the fetal brain (Silver et al., '93). Specifically, immunohistochemistry for GFAP revealed distinct bundles of GFAP-IR at several anatomical locations including the CSJ and HS. In addition, immunohistochemistry for RC1 (a marker for developing glial cells) in the mouse brain demonstrated intense immunoreactivity in glial elements at the CSJ. These immunoreactive structures were hypothesized to play roles in compartmentation of large portions of the forming CNS (Silver et al., '93). Interestingly, dense VIM-IR was present in strikingly similar regions of the formative opossum brain. This is especially apparent in the regions of the CSJ and HS. As discussed earlier, dense bundles of VIM-IR were seen in forebrain structures including the CSJ and HS (Figs. 8A-B). Thus, in addition to providing radial glial scaffolding and providing potential areas of axonal migration, it appears that glial elements may also be involved in compartmentation during

morphogenesis of the brain of *Monodelphis*. These findings coupled with the protracted postnatal period of neurogenesis indicate that *Monodelphis* is an extremely attractive model to investigate and manipulate the interactions of glial cells with neuronal systems during early development.

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PAPER IV: DEVELOPMENTAL RELATIONSHIP OF GALANIN TO OTHER NEUROENDOCRINE SYSTEMS

INTRODUCTION

Galanin (GAL) is a biologically active peptide of 29 amino acids in length originally isolated from porcine small intestine (Tatemoto et al., 1983). Distribution of galanin-like immunoreactivity (GAL-IR) in the central nervous system (CNS) of several mammalian species has been reported (Melander et al., 1986a, Skofitsch and Jacobowitz 1986, Ch'ng et al., 1985, Kowall and Beal 1989, Kordower and Mufson, 1990, Jacobowitz and Skofitsch, 1991, Kordower et al, 1992, Elmquist et al., 1992). The distribution of galanin binding sites in the adult nervous system has also been well documented (Melander et al., 1986c; Skofitsch et al., 1986; Mantyh et al., 1992; Hedlund et al; 1992).

In contrast to the well described adult distributions of GAL containing systems, less has been reported on the developmental distribution of GAL in the CNS. Our laboratory has previously shown that GAL is detectable with immunohistochemistry (IHC) during periods of neurogenesis and morphogenesis of the mammalian CNS (Elmquist et al., 1992). We have also shown that GAL binding sites are present during similar developmental periods (Elmquist et al., 1993). As a model for developmental studies we have used the Brazilian opossum, *Monodelphis domestica. Monodelphis* is a small pouchless marsupial which breeds well under laboratory conditions. The young are born in an immature state well before neurogenesis is completed (Jacobson, 1984; Jacobson laboratory unpublished observations). The absence of a pouch makes the young very accessible and circumvents the necessity of *in utero* manipulations for developmental studies. Therefore, *Monodelphis* is being used to study development of multiple CNS regions including the hypothalamus (Rivkees et al., 1988; Schwanzel-Fukuda et al., 1988; Fox et al., 1991a,b; Elmquist et al., 1992; Elmquist

et al., 1993;), brainstem (Wang et al., 1992; Keuhl-Kovarik et al., 1993), cerebellum (Dore et al., 1990), olfactory bulbs (Brunjes et al., 1992) and cerebral cortex (Saunders et al., 1989).

As discussed above, GAL and its receptor are present as early as 1 day postnatal (PN) in the opossum brain. Specifically, a striking level of GAL was detected in the forming hypothalamus. This was most pronounced in the regions of the forming median eminence (ME), and in the paraventricular (Pa), dorsomedial (DM), and arcuate (Arc) hypothalamic nuclei. Further, ^{125}I-GAL binding sites are present in these regions at similar ages. These findings have led us to hypothesize that GAL is involved in the formation and/or functioning of the hypothalamic-pituitary axis. Thus, the developmental distribution of other opossum hypothalamic neuropeptides or neurotransmitters (and their possible relationship to GAL) is of interest.

Our laboratory has demonstrated that arginine-vasopressin (AVP) is present in the opossum hypothalamus as early as 1 PN (Iqbal et al., 1993). These immunohistochemical studies indicated that in the formative hypothalamus, AVP is found in similar regions as GAL-IR. However, the distribution of other neuropeptides/neurotransmitters of the developing opossum hypothalamus have not been reported. We therefore have initiated immunohistochemical studies to compare the distribution of GAL-IR to several substances including, AVP, corticotropin-releasing hormone (CRH), tyrosine hydroxylase (TH), neuropeptide Y (NPY), and growth hormone releasing hormone (GHRH) during periods of

hypothalamic neurogenesis and morphogenesis. Additionally, we employed double label IHC to investigate the possible colocalization of GAL with AVP in the adult brain and during periods of hypothalamic neurogenesis and morphogenesis.

MATERIALS AND METHODS

Animals

Adult and developing opossums were obtained from a colony maintained at Iowa State University. The initial animals of the colony were obtained from the Southwest Foundation for Research and Education (San Antonio, TX). The animals and procedures used were in accordance with the guidelines and approval of the Iowa State University committee on animal care. The animals were maintained at a constant temperature (26°C) and on a 14:10 light-dark cycle. Food and water (Reproduction Fox Chow; Milk Specialties Products, Madison, WI) were available ad libitum. At least two male animals were used at each of the timepoints (1, 5, 10, 15 PN) for the single label IHC studies. The GAL/AVP double label study utilized three adults (two male and 1 female, greater than 180 days of age) and 3 males at 10 and 15 PN. At the ages of 1 and 5 PN the gender of the neonates cannot be determined grossly, and thus three animals were used at each age and were considered sexually undifferentiated.

Tissue Preparation

Adult opossums were perfused transcardially with Zamboni's fixative (1.8% paraformaldehyde, 7.5% saturated aqueous picric acid, sodium phosphate buffer; pH 7.5) as described previously (Elmquist et al., 1992). The brains were removed from the calvaria, postfixed for 24 hours in Zamboni's fixative, sunk in a 30% sucrose solution, and 40μm thick coronal sections were cut on a cryostat into tissue culture wells containing cryoprotectant.

The brains were collected from 1, 5, 10, and 15 PN opossums by cooling the animals in a -15°C freezer until anesthetized. The animals were then decapitated and the heads placed in Zamboni's fixative for 48 hours. After fixation, the heads were infiltrated with 30% sucrose overnight, and then cut into 20μm thick coronal sections on a cryostat (Reichert Instruments). The sections were thaw mounted onto slides and stored at 4°C until processed for immunohistochemistry.

Immunohistochemistry

The protocol utilized for single label IHC was a modification of that reported previously in our laboratory for the Brazilian opossum brain (Fox et al., 1991 a,b; Elmquist et al., 1992). For this study, the sections were incubated in primary antibody against AVP (Miles Scientific; 1:5000), CRH (gift of Drs. Akil and Watson; 1:5000), NPY (Incstar; 1:5000), TH (Incstar; 1:1000), or GHRH (Incstar; 1:5000) for 20 hours at room temperature. Secondary antibodies used were biotinylated horse anti-mouse IgG (TH) or goat anti-rabbit (CRH, NPY, AVP, GHRH) (Vector; 1:600). Visualization was accomplished with the avidin-biotin complex (Vector Elite Kit; 1:200). The chromogen was a substrate composed of 0.04% 3,3' diaminobenzidine tetrahydrochloride (DAB; Sigma), 2.5% nickel sulfate (Fisher Scientific) and 0.01% hydrogen peroxide, dissolved in 0.1 M sodium acetate. Sections were counterstained with neutral red (Fisher Scientific) and then dehydrated in graded alcohols, cleared in xylene and coverslipped with permount mounting media and analyzed with a light microscope. In each run negative controls were generated by omission of the primary antisera.

The double label technique consisted of slight modifications of the above protocol. Specifically, tissue was exposed to primary antisera against GAL (Peninsula, 1:500) and then exposed to biotinylated goat anti-rabbit secondary antibody (1:200) as described above. Tissue sections were then incubated with Texas red avidin D conjugate (Vector; 25 μg/ml) for one hour. After washing, sections were incubated in AVP primary antisera made in guinea pig for 20 hours (Peninsula labs; 1:500). Sections were then incubated in fluorescein conjugated goat anti-guinea pig antisera (Vector; 1:200) for two hours. Sections were coverslipped with glycergel (DAKO) and viewed with a Zeiss Axiophot fluorescence microscope.

Analysis of Tissue

Sections were observed with a Zeiss Axiophot microscope and regions containing immunoreactivity were identified and recorded using maps of coronal sections of the opossum brain as described previously (Fox et al., 1991a,b; Elmquist et al., 1992). The double labeled sections were visualized using a Zeiss Axiophot microscope equipped with a mercury light source. The Texas Red fluorescence was visualized with a rhodamine filter system centered at 546 nm and the fluorescein fluorescence with a FITC system centered at 450-490 nm. Potentially double labelled cells were detected by switching between the two filter systems. Photomicrographs were taken of immunohistochemical fields to compare patterns of GAL-IR and AVP-IR. In addition, double exposure photomicrographs were taken to aid in determination of double labelled elements.

RESULTS

Comparative Distributions

General Comments:

The brain of *Monodelphis* is extremely immature at the time of birth and grows and matures rapidly during the first month of postnatal life. Thus, when a specific nuclear group or area is discussed it will be in the context of that area is still forming. As discussed earlier, hypothalamic GAL-IR is robust during periods of morphogenesis and neurogenesis and its distribution was compared to the other substances examined. Immunohistochemistry revealed structures containing AVP-, CRH-, GHRH-, TH-, and NPY-like immunoreactivity (AVP-IR, CRH-IR, GHRH-IR, TH-IR, and NPY-IR) at various ages of the opossum brain. Of the substances investigated, only AVP contained hypothalamic distributions of immunoreactivity comparable to that of GAL. AVP-IR was seen in the forming hypothalamus in cells and fibers as early as 1 PN. Although TH-IR, GHRH-IR, and CRH-IR was detectable in cells as early as 1PN, little immunoreactivity was present in the GAL containing regions of the hypothalamus. A description of the hypothalamic distribution of AVP-IR, TH-IR, CRH-IR, NPY-IR, and GHRH-IR is presented below. Also, a brief description of other brain regions will be discussed. In addition, the relationship and possible coexistence of AVP-IR and GAL-IR in the forming opossum hypothalamus will be presented.

AVP-IR:

As early as 1 PN, robust AVP-IR in hypothalamic cell bodies and fibers was

observed. A detailed description of the developmental distribution and migration of AVP immunoreactive cells in the opossum hypothalamus has been presented previously (Iqbal et al., 1993). Briefly, the amount and intensity of staining in the forming magnocellular system of the hypothalamus increased with age. At all ages immunoreactive fibers extended from the presumed Pa and supraoptic (SO) nuclei to the retrochiasmatic area and into the neural lobe (Figs. 1B, 2B). The pattern of the AVP-IR overlapped with that of GAL-IR, especially in the regions of the lateral hypothalamus, retrochiasmatic area, and the caudal aspects of the Pa and SO (Figs. 1A-B, 2A-B).

TH-IR:

At 1 PN, TH-IR in cell bodies was seen only in the ventrolateral medulla. Immunoreactive fibers were seen at the base of the hypothalamus just outside the neuroepithelium and in the lateral diencephalon. By 5 PN cells containing TH-IR were visualized in the ventral tegmentum and in the lateral diencephalon (Fig. 3A). The amount of TH-IR increased by 10 and 15 PN outside the hypothalamus. Specifically, dense TH-IR in fibers was seen in the forming caudate putamen, internal capsule and zona incerta. Immunoreactive cells were seen in the olfactory bulbs, ventral tegmental area, substantia nigra (Fig. 3B), locus coruleus, and ventrolateral medulla. Within the hypothalamus, a few immunoreactive cells were seen in the Pa. However, little TH-IR was seen in the basal hypothalamus and median eminence (areas of dense GAL-IR) at any of the developmental ages (Fig. 1C).

CRH-IR:

Cells containing CRH-IR were seen as early as 1 PN outside the hypothalamus in areas including the forming cerebellum, tegmentum, and ventral medulla. A few immunoreactive fibers were present in the lateral diencephalon at 1 PN. In the region of the 5 PN hypothalamus, weakly stained cells were seen in the forming lateral preoptic area and in the dorsolateral hypothalamus. CRH-IR in fibers was seen in the forming ME, infundibulum and neural lobe. Outside the hypothalamus, immunoreactive cells and fibers were seen in the presumed ventral tegmental area, substantia nigra, inferior olivary complex, and cerebellum. Dense CRH-IR in fibers was seen in the internal capsule and in the presumed passage of the vagus nerve in the medulla. At 10 and 15 PN CRH-IR was present in the medial portions of the Pa, and in the ME and neural lobe (Fig. 1D). Nonhypothalamic regions containing CRH-IR at 10 and 15 PN included cells in the vertical limb of the diagonal band, median preoptic nucleus, anterior amygdaloid area, ventral to the fasciculus retroflexus, ventral pallidum, red nucleus, inferior olivary complex (Fig. 4A), and dorsal motor nucleus of the vagus. As seen in the 5 PN brains, the presumed vagus and nucleus of the solitary tract contained CRH-IR.

Analysis of adjacent sections processed for GAL and CRH IHC indicated a possible overlap of CRH-IR and GAL-IR in the ME (Fig. 1D). In addition, at later developmental ages (10 and 15 PN), GAL immunoreactive fibers and CRH cells may have similar distributions in the Pa. However, the vast majority of the GAL containing cells in the hypothalmus were seen in the ventral Pa and DM (ventral to cells containing CRH-IR).

GHRH-IR:

GHRH-IR was seen in cells and fibers as early as 1 and 5 PN in regions outside the hypothalamus including the basal forebrain, dorsal vagal complex, and in the tegmentum. At 5 PN, a few fibers were present in dorsal diencephalon. GHRH-IR in the hypothalamus was relatively sparse and no immunoreactivity was visualized in the region of the forming Arc and ME at all ages examined (1-15 PN; Fig. 1E). At 10 and 15 PN GHRH-IR in cells were seen in the basal forebrain (Fig. 4B), caudate putamen, amygdala, vagal complex, and trigeminal ganglion.

NPY-IR:

No specific NPY-IR was observed in the 1 and 5 PN brains. At 10 PN, weak NPY-IR was observed in the presumed dorsal vagal complex. The first hypothalamic NPY-IR was seen at 15 PN. Specifically, weak NPY-IR was seen in cells in the Arc. In addition, at 15 PN weak NPY-IR was seen in the olfactory bulbs, and in vagal and hypoglossal motor neurons.

GAL and AVP Double Label IHC

In the adult opossum brain, double label IHC revealed a coexistence of AVP-IR and GAL-IR in the Pa and SO. Specifically, several double labelled cells were visualized in the SO. Most of the cells were seen in the medial aspects of the nucleus. Fewer double labelled cells were seen in the Pa (Fig. 5A-B). Dense GAL-IR in fibers was seen in both the Pa and SO as well. Though several double labelled cells were visualized, the majority of the AVP

cells in the SO did not contain GAL. Both singly labeled GAL and AVP were neurons observed in the SO.

Double label IHC demonstrated developmental patterns of GAL-IR and AVP-IR that were coextensive but not colocalized. That is, no double labeled cell bodies or fibers could be easily distinguished. At all ages examined (1, 5, 10, and 15 PN) cells containing GAL-IR and AVP-IR were located in very close proximity to each other with immunoreactive fields overlapping (Figs. 1A-B, 2A-B, 5C-D). These areas of overlap were most prominent in the lateral hypothalamus in the area of the forming magnocellular system. Examples of double labelled elements could not be found, however. Double exposure photomicrographs demonstrated that AVP cells in the forming hypothalamus often appeared to be in contact with fibers containing GAL-IR. Conversely, apparent contacts of AVP-IR fibers with GAL cells were also visualized (Figs. 5C-D).

Figure 1: A series of photomicrographs of coronal sections demonstrating the comparative immunohistochemical distributions of (A) galanin (GAL), (B) arginine vasopressin (AVP), (C) tyrosine hydroxylase, (D) corticotropin releasing hormone (CRH), (E) growth hormone releasing hormone (GHRH), and (F) neuropeptide Y (NPY) in the 10 day postnatal opossum hypothalamus at the level of the anterior pituitary gland (asterisk). Note the robust GAL-like immunoreactivity in the hypothalamus and the lack of staining for NPY, TH, and GHRH. Scale bar = 80 μm. 3v, third ventricle.

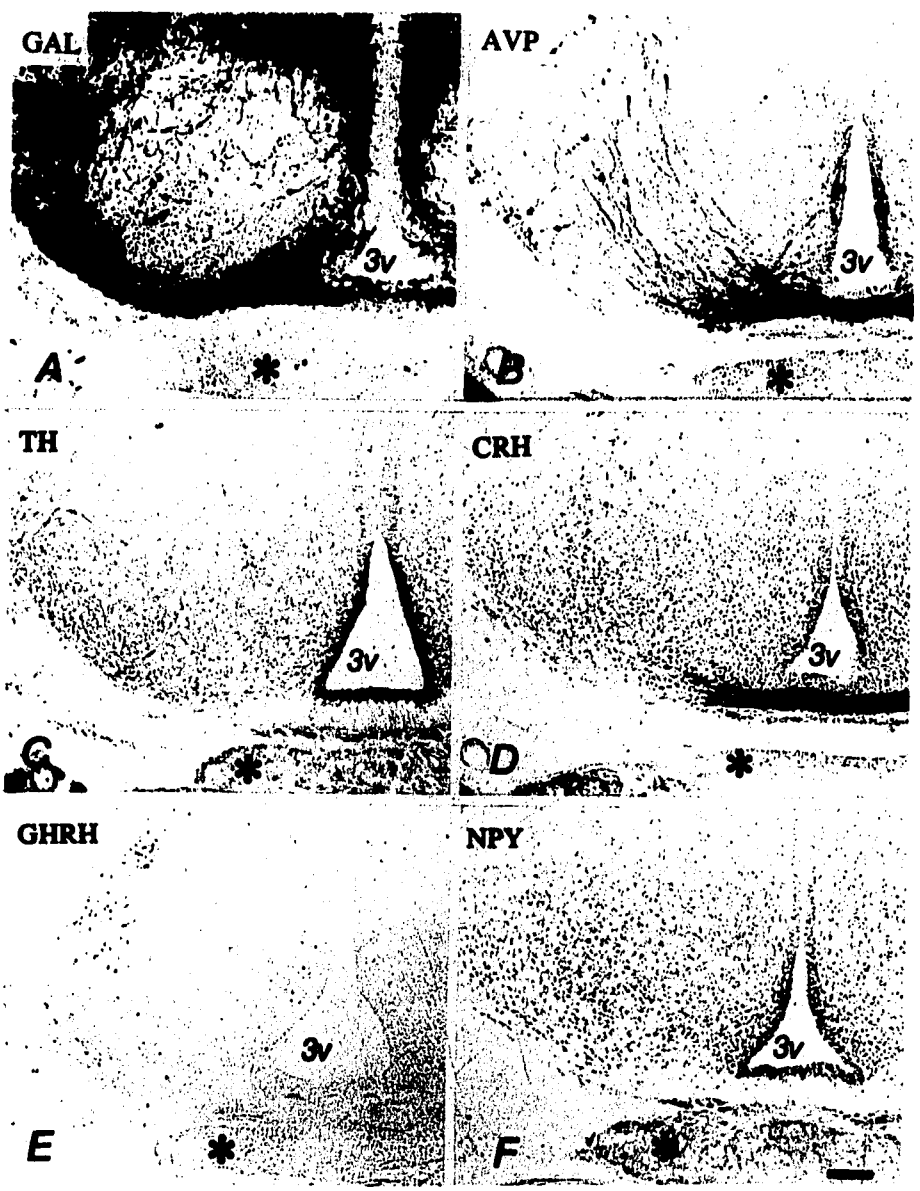

GAL
3v
A
AVP
3v
B
TH
3v
C
CRH
3v
D
GHRH
3v
E
NPY
3v
F

Figure 2: Photomicrographs of coronal sections of the 5 day postnatal opossum hypothalamus at the level of the anterior pituitary gland (APit) demonstrating the distributions of (A) galanin- and (B) arginine vasopressin-like immunoreactivities. Note the similar pattern of staining in the lateral hypothalamus. Scale bar = 80 μm. 3v, third ventricle.

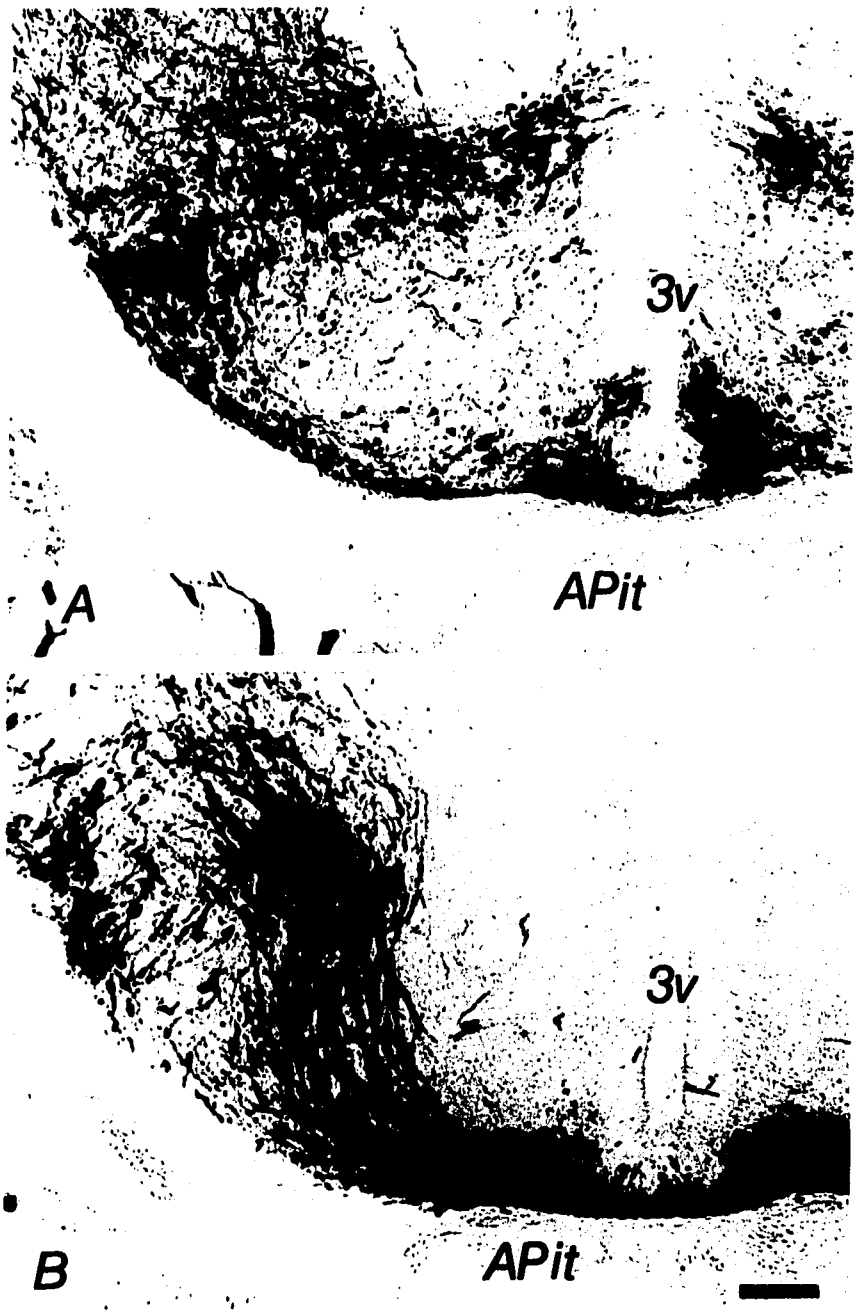

3v
A
APit
3v
B
APit

Figure 3: Photomicrographs of coronal sections of (A) 5 day postnatal (PN) and (B) 10 PN opossum midbrain demonstrating tyrosine hydroxylase-like immunoreactivity (TH-IR) in the ventrolateral portion of the mesencephalon. Scale bar = 40 μm.

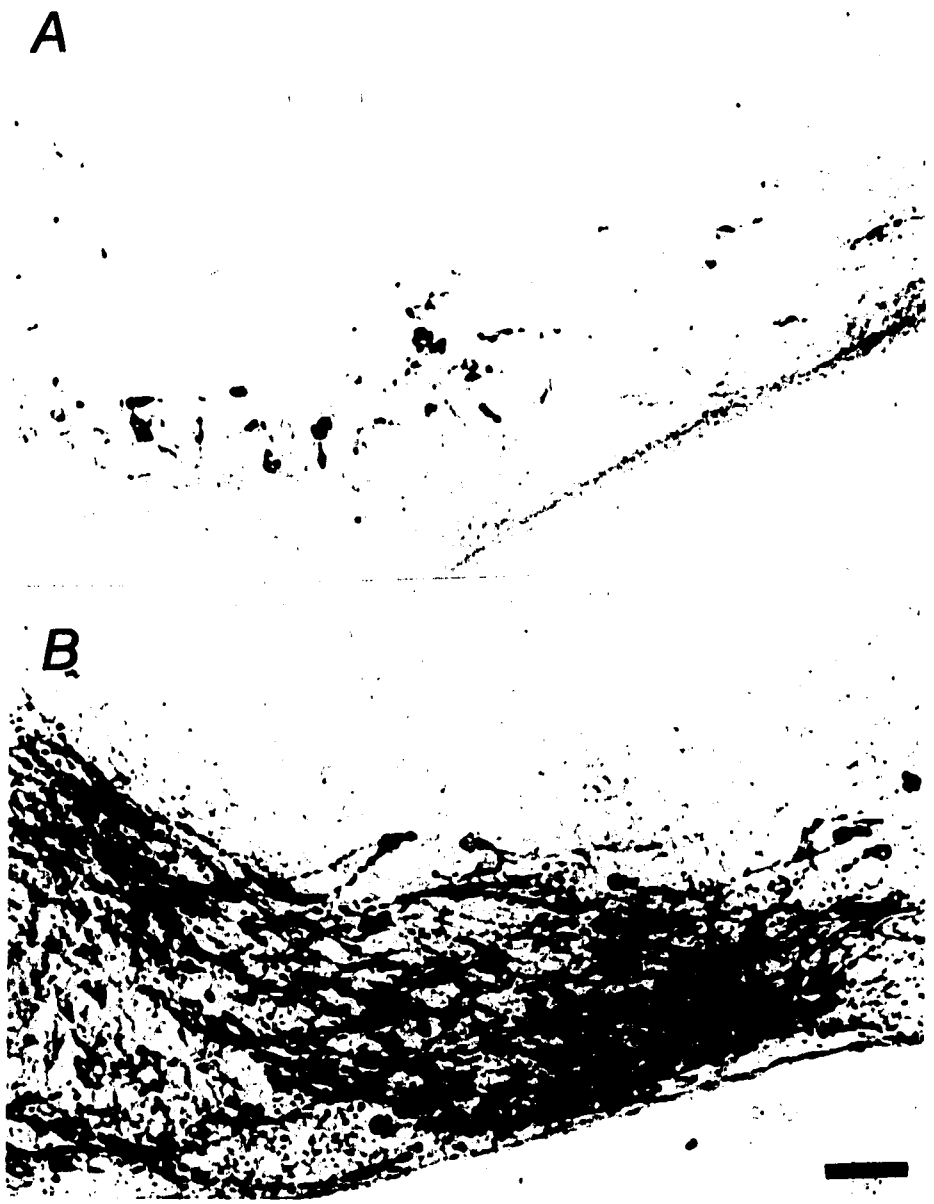

A
B

Figure 4: (A) A photomicrograph of a coronal section of a 15 day postnatal (PN) opossum brainstem demonstrating corticotropin releasing hormone-like immunoreactivity in the inferior olivary nuclear complex (arrow). (B) A photomicrograph of a coronal section of a 10 PN opossum brain demonstrating growth hormone releasing hormone-like immunoreactivity in the basal forebrain (ventral and lateral to the lateral cerebral ventricle). Scale bar = 80 μm.

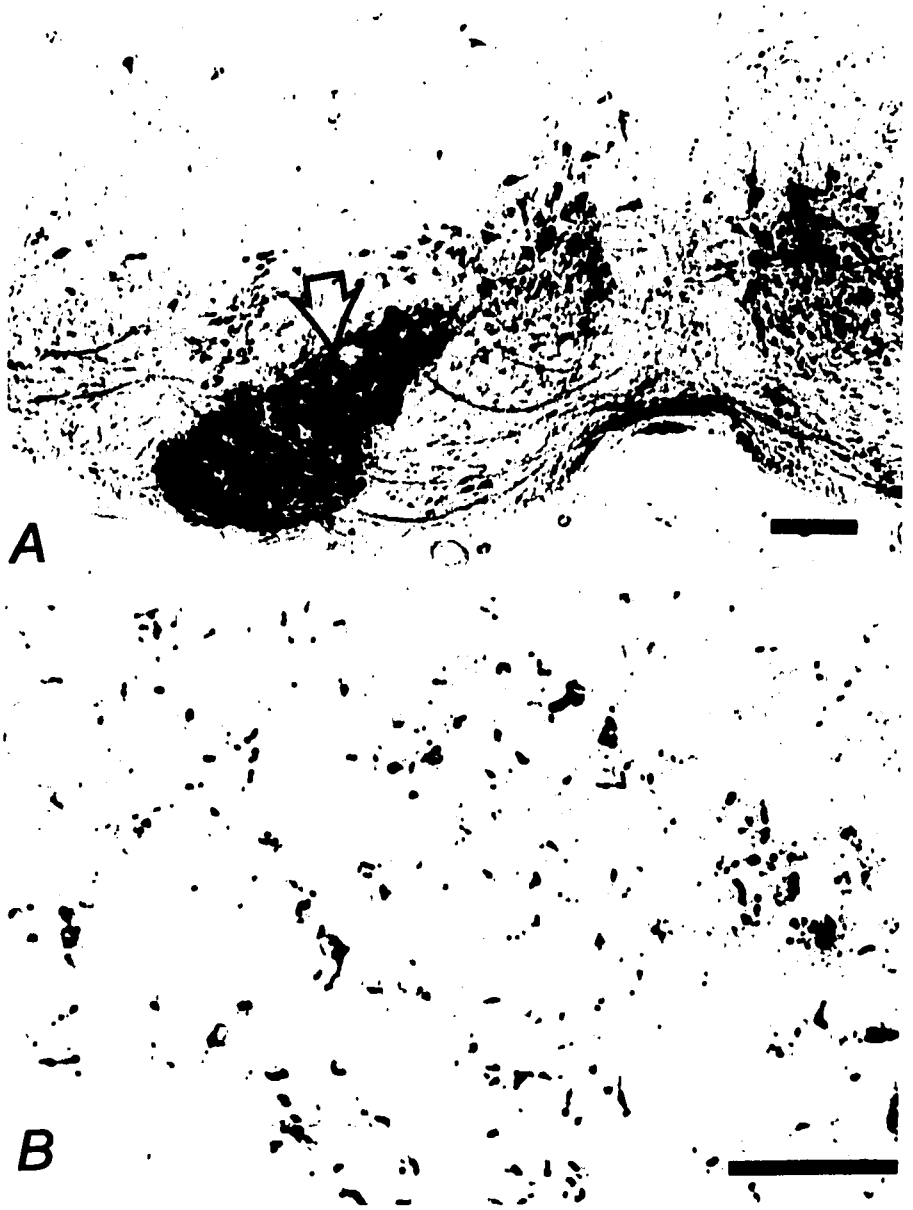
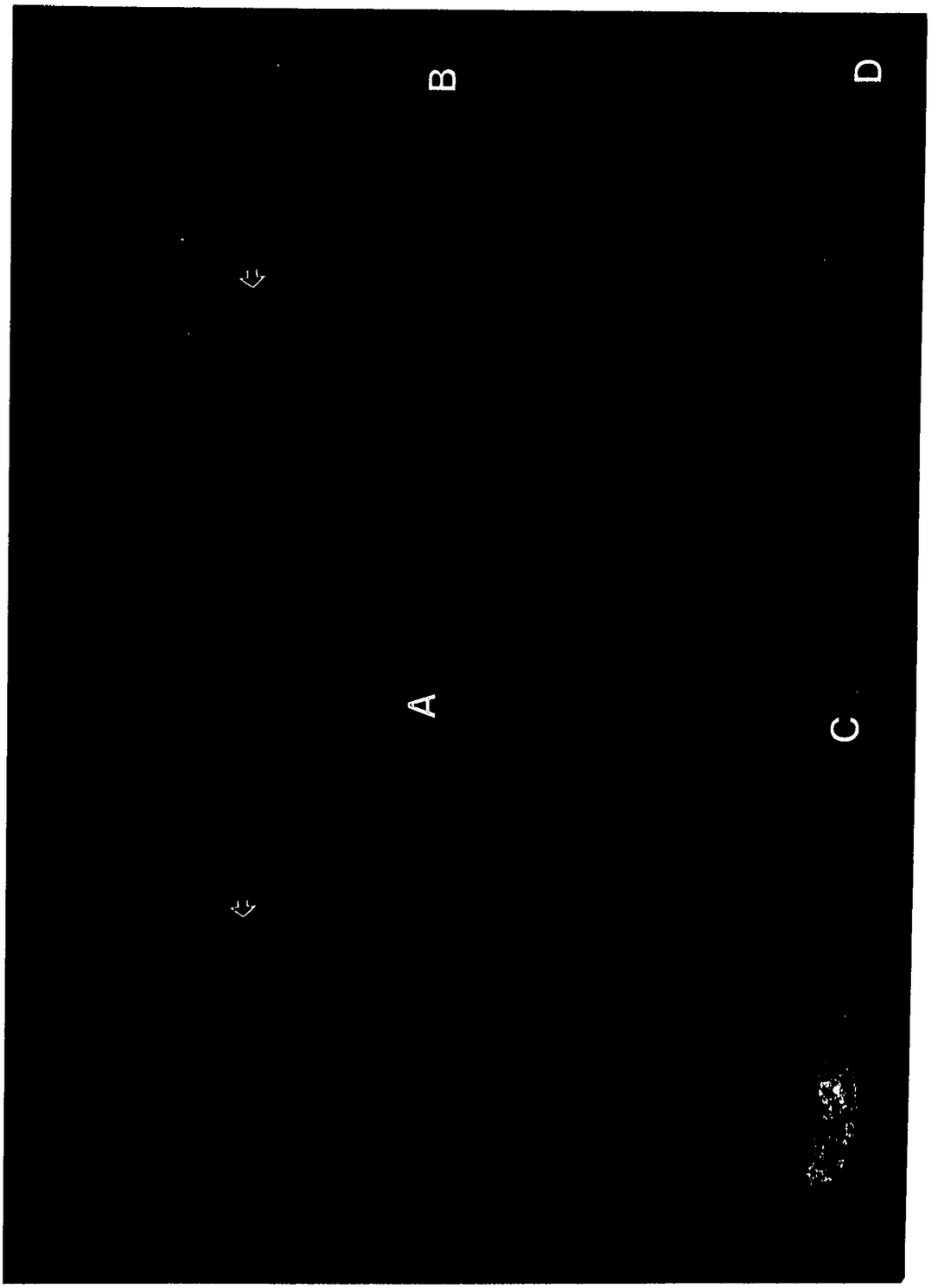

A
B

Figure 5: A series of color photomicrographs of coronal sections of the opossum hypothalamus demonstrating the presence of (A) galanin-like immunoreactivity (GAL-IR) in the adult paraventricular nucleus (Pa), and (B) arginine vasopressin-like immunoreactivity (AVP-IR) in the adult Pa. Note the colocalization of AVP-IR and GAL-IR in the Pa (arrow). (C and D) Double exposure color photomicrographs demonstrating cells and fibers containing GAL-IR and AVP-IR in close association in the 10 day postnatal opossum brain. Double labelled cells are not readily seen at this age, however.


B
D
A
C

DISCUSSION

Galanin is widely distributed neuropeptide of the mammalian nervous system. It has been demonstrated that GAL-IR is located in areas including the hypothalamus, basal forebrain, and medulla (Melander et al., 1986a, Skofitsch and Jacobowitz 1986, Kordower et al, 1992, Elmquist et al., 1992). Many physiological roles have been attributed to GAL including inhibition of insulin release, increase in growth hormone release, and increase in food intake (reviewed in Rokaeus, 1987).

Several neuropeptides/neurotransmitters have been shown to be coexistent with GAL in the adult mammalian brain (reviewed in Melander et al., 1991). For example, GAL has been shown to coexist in cholinergic neurons (choline acetyltransferase positive cells) in the septum and basal forebrain (Melander et al., 1985). In the Arc, GAL is colocalized with TH and GHRH (Melander et al., 1986b; Meister and Hokfelt, 1988). In magnocellular neurons of the Pa and SO of the hypothalamus GAL has been colocalized with AVP (Melander et al., 1986b) and cholecystokinin (CCK; Meister et al., 1990). Within the parvicellular Pa, GAL has been colocalized with CRH (Meister et al., 1990). In the brainstem, GAL has been shown to be present in seratoninergic (5-HT) positive cells (Melander et al., 1986b).

As discussed above the colocalization of GAL with other neuroactive substances in the adult brain has been well described. However, the developmental relationship of GAL to other neuropeptides/neurotransmitters has not been described. We therefore investigated the relationship of several neuropeptides (AVP, CRH, GHRH, and NPY) and the marker for catecholaminergic cells (TH) to that of GAL during periods of neurogenesis and

morphogenesis of the opossum hypothalamus. As discussed earlier, GAL-IR is robust throughout periods of hypothalamic development (Elmquist et al., 1992). Of the substances examined, only AVP was present in comparable levels and locations to that of GAL-IR. Although, CRH-IR, TH-IR, and GHRH-IR were detectable as early as 1 PN outside the hypothalamus, little immunoreactivity was seen in regions similar to that observed for GAL-IR.

Due to the apparent overlap of AVP-IR and GAL-IR during periods of hypothalamic development, we investigated the relationship and possible colocalization of GAL and AVP during these periods. Double label IHC for GAL and AVP indicated that GAL and AVP are colocalized in magnocellular neurons of the adult opossum Pa and SO. The brains of the developing opossums revealed a different pattern, however. As early as 1 PN, AVP and GAL containing cells were seen in the lateral hypothalamus. The amount and intensity of GAL-IR and AVP-IR increased with age in the forming SO and Pa. Unlike the adult hypothalamus, double labelled cells were not readily apparent. The possibility that some double labelled cells were not visualized can not be ruled out. However, the number of GAL and AVP cells and fibers was relatively large (especially at 10 and 15 PN) and it would seem logical that a few double labelled cells would be seen if both peptides were in the same cells. Photomicrographs demonstrated that GAL-IR and AVP-IR were coextensive but not colocalized. In particular, apparent contacts of GAL containing fibers with AVP cells were visualized.

The lack of double label is interesting for several reasons. Our results imply that AVP and GAL containing cells are distinct during periods of neurogenesis and

morphogenesis and at later stages (adult) cells contain both substances. Thus, it is plausible that GAL and AVP cells arise from distinct precursors and at later ages, a proportion of the magnocellular cells begin to synthesize and store both GAL and AVP. As discussed earlier GAL-IR is robust in the forming Arc, DM, Pa, and ME (Elmquist et al., 1992). GAL receptors (as defined by ^{125}I-binding) are present in similar regions throughout the postnatal development of the opossum hypothalamus (Elmquist et al., 1993). Further, a transient GAL binding pattern was seen in the forming anterior pituitary (APit). These results led us to the hypothesis that GAL is involved in formation and/or functioning of the APit. On the other hand, AVP is located in the forming neural lobe. Although GAL and AVP appear to not be colocalized during the development of the opossum hypothalamus, cells and fibers containing GAL-IR and AVP-IR are in close association with each other. Thus, GAL and AVP systems may interact to coordinate control of both anterior and posterior pituitary function during periods of hypothalamic morphogenesis and differentiation.

Galanin is one of a number of peptides which are found in abundance both in the gastrointestinal (GI) tract and the CNS. These brain/gut peptides are thought to have multiple functions in adult mammals (reviewed in Morely, 1990). Other examples of brain/gut peptides include CCK and NPY. Like GAL, CCK and NPY have been shown to be physiological effectors of endocrine and autonomic processes (for a review see McCann, 1991). As discussed earlier, NPY could not be detected immunohistochemically in the opossum hypothalamus at 1 and 5 PN. At 15 PN NPY-IR weakly stained cell bodies were observed in the Arc. Our laboratory has previously described the developmental distribution of CCK-like immunoreactivity (CCK-IR) in the opossum brain. Specifically, CCK-IR was

not detected in the hypothalamus until 15 PN. Further, CCK-IR in hypothalamic cell bodies were not detected until 35 days PN. As discussed earlier, GAL and CCK have been colocalized in the Pa of the adult rat (Meister et al., 1990). In addition, NPY is located in the Arc of the adult opossum (Jacobson laboratory, unpublished observations). Thus it appears that GAL-IR is present prior to immunoreactivity for other brain/gut peptides involved in the control of the adult hypothalamic-pituitary axis. This further demonstrates that GAL may be an important physiological regulator of hypothalamic function during times of neurogenesis and morphogenesis.

Galanin has been shown to be a regulator of pituitary function. Administration of GAL to rats increases GH secretion (Ottlecz et al., 1986; Murakami et al., 1989) and increases PRL secretion (Koshiyama et al., 1990). As discussed earlier, GAL-IR is robust in the hypothalamus including the Arc. Additionally, in the Arc, GAL has been colocalized with GHRH and dopamine (TH immunoreactive cells; Melander et al., 1986b; Meister and Hokfelt, 1988). Dopamine and GHRH are potent regulators of prolactin and growth hormone secretion, respectively (reviewed in Hokfelt et al., 1989). These adult distributions indicate that hypothalamic GAL may be involved in the control of the Apit secretions. Several studies support the hypothesis that in the adult GAL acts via the hypothalamus rather than directly on the Apit to increase secretions (Murakami et al., 1989; Ottlecz et al., 1986; Maiter et al., 1990). However, other reports indicate that GAL exerts mild effects directly on the Apit (Gabriel et al., 1988; Sato et al., 1991). Very low levels of binding have been reported in the adult rat Apit using receptor autoradiography (Skofitsch and Jacobowitz, 1991).

As discussed earlier, in the developing opossum GAL binding sites are localized transiently in the Apit (1, 5, and 10 PN; Elmquist et al., 1993). Thus, it is plausible that GAL may exert direct effects on the Apit during periods of hypothalamic neurogenesis. This is substantiated by results in the present study in that TH-IR and GHRH-IR are not localized to the area of the ME in the developmental ages examined. In the adult opossum TH-IR and GHRH-IR are present in the Arc and ME. In addition, GAL-IR is colocalized with TH-IR in the adult Arc (Jacobson lab, unpublished observations). Thus, it is possible that GAL may directly regulate Apit function in the very young animal. Then as the CNS matures, GAL may assume its adult type role and affect Apit function through hypothalamic mechanisms.

In summary, we have compared the developmental distribution of GAL-IR to that of other hypothalamic neuropeptides/neurotransmitters. Of the substances examined, only AVP was found to have similar densities and distributions of immunoreactivity. Additionally, we found that GAL-IR and AVP-IR are have similar distributions but could not be localized to the same cells. These results provide further evidence that GAL may be involved in the early functioning and/or formation of the hypothalamic-pituitary axis.

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SUMMARY

In the first study, the distribution of galanin-like immunoreactivity (GAL-IR) was characterized in the brain of the adult and developing Brazilian opossum (*Monodelphis domestica*). Nuclear groups containing GAL-IR in cell bodies and fibers were seen throughout the adult opossum brain. The distribution of GAL-IR was similar to that reported for other mammals. In the developing brain, GAL-IR was seen as early as 1 day postnatal (PN) in the developing hypothalamus and brainstem. By days 5 and 10 PN, there was a robust expression of GAL-IR in specific regions of the brain. Since neurogenesis and brain morphogenesis are actively occurring postnatally in the opossum, galanin (GAL) may be playing a role in the differentiation of specific regions of the brain.

In the second study we used autoradiography to investigate the distribution of GAL receptors in the forming Brazilian opossum brain. Binding sites for ^{125}I-GAL were detected as early as 1 PN in regions of the brain which were still undergoing neurogenesis. High levels of GAL binding were region specific and correlated with our previous results on GAL-IR during development. In addition, a transient binding pattern was seen in the anterior pituitary. In the adult brain, the pattern seen was very similar to that of reports in other species. These observations also indicate a potential role of GAL and its receptor during times of active neurogenesis and morphogenesis of the mammalian CNS.

In the third study, we utilized immunohistochemistry to characterize the developmental profiles of glial fibrillary acidic protein (GFAP) and vimentin (VIM) and their possible relationship to GAL in the brain of the Brazilian opossum. At 1 PN, VIM-like immunoreactivity (VIM-IR) was present throughout the brain. The density of VIM-IR was

maximal at 10 and 15 PN (especially in radial glial elements) and decreased slightly by 25 PN. In addition, in the forming brainstem, dense VIM-IR was seen in cranial nerves in the periphery as well as in their presumed fiber tracks within the brain in a very similar pattern to that observed for GAL-IR. In the adult brain, VIM-IR was markedly reduced compared to that of younger ages. In contrast, GFAP-like immunoreactivity (GFAP-IR) in the brain of *Monodelphis* increased dramatically with age. No GFAP-IR was observed in the 1 and 5 PN brains. The timecourse of appearance of GFAP-IR demonstrated a defmite caudal to rostral and outside to inside pattern. By 25 PN the pattern of GFAP-IR in the brainstem resembled that of the adult and more GFAP-IR was present in the forebrain than seen at younger ages. The adult distribution of GFAP-IR was very similar to that reported for other mammalian species. These results indicate that GFAP and VIM are reciprocally related during periods of morphogenesis and differentiation of the opossum brain. Further, these results demonstrate a potential interaction of GAL containing axons with glial cells during CNS morphogenesis.

In the fourth and final study, the distribution of GAL-IR in the developing hypothalamus was compared to several neuropeptides/neurotransmitters thought to be involved in controlling the hypothalamic-pituitary axis. The patterns of GAL-IR was compared to the immunohistochemical distributions of arginine vasopressin (AVP), corticotropin-releasing hormone (CRH), tyrosine hydroxylase (TH), neuropeptide Y (NPY), and growth hormone releasing hormone (GHRH). All of these substances were detected with immunohistochemistry in the developing opossum brain, but only AVP-like immunoreactivity (AVP-IR) was comparable to that of GAL-IR in the hypothalamus. Due

to the similar distributions of AVP-IR and GAL-IR, double label immunohistochemistry was employed to ascertain whether GAL and AVP were colocalized during periods of hypothalamic morphogenesis. Cells and fibers containing AVP-IR and GAL-IR were found to be located in very close association but double labelled cells were not readily visualized. In contrast, GAL and AVP were found to be colocalized in the adult opossum hypothalamus. The inability to detect GHRH and TH in the developing opossum hypothalamus is further evidence that GAL may be a regulator of the APit during hypothalamic development. Further, GAL may interact with AVP containing systems to coordinate anterior and posterior pituitary function.

DISCUSSION

The information presented in this dissertation extends previous work and further demonstrates the effectiveness of the Brazilian opossum as a model to investigate the morphogenesis of the mammalian CNS. Specifically, the anatomical distribution of galanin (GAL) containing systems was studied and described. The results of these studies indicate that GAL containing systems are present during periods of neurogenesis. In addition, the distributions of glial cells during periods of brain morphogenesis was investigated. The results described in this dissertation provides additional data to be used in future studies utilizing *Monodelphis*.

Typical of marsupials, the brain of *Monodelphis* is extremely immature at birth. Despite this relative immaturity GAL-like immunoreactivity (GAL-IR) was found in the CNS at 1 PN and was very robust by 5 PN. These findings may indicate that GAL is involved in morphogenesis of the opossum CNS. Along these lines, it has been previously hypothesized that GAL may act as a growth promoter in forming dorsal root ganglia (Polak et al., 1991). Evidence for a developmental role of GAL includes the detection of GAL-IR in the embryonic day 15 (E15) rat brain (Gabriel et al., 1989), the E12 rat spinal cord, and the human spinal cord at 6 weeks of gestation (Polak et al., 1991). Northern blot analysis has been used to demonstrate the presence of GAL mRNA as early as E3 in the rat conceptus (Vrontakis et al. 1991). Our immunohistochemical results described in Paper I appear to be in agreement with these reports in that GAL-IR is detectable in the opossum brain well before the cessation of neurogenesis (Jacobson laboratory unpublished observations). For example GAL-IR was observed in discrete regions such as the preoptic

area as early as 1 PN while neurogenesis of this region continues until 10-11 PN (Jacobson, 1984).

Preliminary results in our laboratory indicate that GAL is present at even earlier periods of development. Specifically, GAL-IR is detectable as early as E12 in the opossum spinal cord, dorsal root ganglia, medulla, and diencephalon (Jacobson laboratory, unpublished observations). In addition, GAL-IR was observed in presumed parasympathetic and sympathetic neuronal fibers in the thoracic and abdominal cavities at E12 (Elmquist et al., 1993). These findings indicate that GAL is essential in early autonomic processes. On the other hand, the aforementioned findings may indicate distinct morphogenic roles for GAL.

As discussed earlier, GAL has been implicated in the regulation of a variety of endocrine and autonomic processes including inhibition of insulin release (McDonald et al., 1985), increase in prolactin secretion (Koshiyama et al., 1990), and increase in food intake (Kyrkouli et al., 1986). Additionally, GAL has been localized in catecholaminergic neurons (Melander et al., 1986b). Interestingly, many of the physiological effects attributed to GAL are similar to those seen following α-adrenergic system stimulation in the CNS (Koshiyama et al., 1990). Specifically, it has been shown that α-receptor antagonism abolishes increases in prolactin release and food intake following GAL administration (Koshiyama et al., 1990; Kyrkouli et al., 1990). These findings suggest that many of the effects of GAL are mediated via interactions with noradrenergic systems within the nervous system. It has been previously demonstrated that catecholaminergic neurons (tyrosine hydroxylase immunopositive cells) are present at very early (E12.5) stages of CNS development in the

rat (Specht et al., 1981). As discussed in Paper IV, tyrosine hydroxylase immunopositive cells are present in the forming opossum CNS as well. Although GAL-IR appears to precede tyrosine hydroxylase-like immunoreactivity in the forming hypothalamus, the extent of interactions between galaninergic and catecholaminergic systems during development of other regions of the opossum CNS is currently unknown. However, it would be of interest to compare and contrast the ontogeny of the two systems during periods of brain morphogenesis.

Recently, it has been demonstrated that another neuropeptide, vasoactive intestinal peptide (VIP) acts as a growth factor in the mouse embryo. In particular, in cultured whole mouse embryos, VIP was found to increase somite number, embryonic volume, and DNA and protein content (Gressens et al., 1993). Although we have no direct evidence that GAL acts in a similar manner, GAL and its receptor are present during periods of neurogenesis. Further, GAL-IR is present during periods of embryogenesis (Elmquist et al., 1993). Effective GAL antagonists have been synthesized (Wiesenfield-Hallin et al., 1992 and Lindskog et al., 1992). Specifically, these antagonists have been shown to inhibit a variety of GAL mediated events including release of insulin, increase in feeding behavior, and increase in growth hormone release (Lindskog et al., 1992, Leibowitz et al., 1992; Gabriel et al., 1993). Utilization of these antagonists may prove helpful to determine novel developmental roles of GAL.

As discussed earlier, several reports indicate that in addition to neurons, glial cells may also contain neuropeptides (Vilijn et al., 1988; Shinoda et al., 1989; Melner et al., 1990; Klein and Fricker, 1992;). Following administration of colchicine, GAL can also be

detected in glial cells (presumed microglia; Xu et al., 1992). These results demonstrated that glial cells have the potential to synthesize GAL after administration of mitosis inhibitors. As discussed earlier, it has been shown that GAL containing systems undergo changes in the face of neuronal insult or injury (Vilar et al., 1991). Thus, it appears that both neuronal and glial cell types alter the synthesis of GAL in the face of changing environmental cues. Another plastic period for the CNS is during development (Mattson, 1989). In Paper III of this dissertation, the developmental distributions of vimentin (VIM) and glial fibrillary acidic protein (GFAP) in the forming opossum brain were presented. Previously, immunohistochemistry using antibodies for VIM and GFAP has been used to identify immature and mature astrocytes, respectively (Stichel et al., '91). Vimentin-like immunoreactivity (VIM-IR) was observed throughout the developing opossum brain. The majority of VIM-IR was observed in radial glial structures. For example, in the forming hypothalamus (an area containing robust GAL-IR), it was apparent that GAL and VIM were not in similar structures. Specifically, the VIM-IR was seen in fine radial processes whereas the GAL-IR was seen in neuronal cell bodies. However, VIM-IR was seen in dense bundles in other brain regions. In the forming brainstem, very dense VIM-IR was seen in cranial nerves in the periphery as well as in their presumed fiber tracks within the brain. As discussed previously (Paper I), GAL-IR can be localized immunohistochemically in similar areas including the presumed spinal tract of the trigeminal (Sp5), and the vagus nerve within the medulla. The immunohistochemical staining pattern of VIM and GAL in these anatomical locations was remarkably similar. Further, GAL binding sites are located in these structures as early as 1 PN as well (Elmquist et al., '93).

The aforementioned areas (vagal and trigeminal ganglia and Sp5) are involved in processing sensory information. As early as E12, GAL-IR can be located within forming sensory systems such as cranial nerve ganglia, dorsal root ganglia, and the dorsal horn of the spinal cord. Interestingly, VIM-IR is located within similar structures at E12. Although the possibility that the GAL-IR and VIM-IR are located within the same cells and fibers cannot be ruled out, it is plausible that GAL-IR is located within neuronal axons and the VIM-IR in glial processes. In addition, the exact localization of the GAL binding sites in the perinatal brain is unknown. Therefore it is possible that GAL released from presumed axons may bind at GAL receptors in both neurons and glia. If this is the case, presumed axons containing neuropeptides such as GAL may interact with VIM containing glial elements during formation of specific sensory systems of the opossum brain.

Galanin-like immunoreactivity is robust within the opossum brain throughout the perinatal period. The GAL-IR was especially prominent in the forming basal hypothalamus in the region of the median eminence (ME), and in the arcuate (Arc), dorsomedial (DM), and paraventricular (Pa) hypothalamic nuclei. These regions are known to be intimately involved in regulation of anterior pituitary (APit) function (reviewed in Hokfelt et al., 1989). As presented in Paper II, GAL binding sites are present in the basal hypothalamus and within the APit in the neonate. The presence of GAL and its receptor in this region of the hypothalamus indicates GAL may be involved in the regulation of the hypothalamic-pituitary axis in the developing opossum.

It has been previously demonstrated that GAL is a regulator of the adult APit. Administration of GAL to adult rats increases GH secretion (Ottlecz et al., 1986; Maiter et

al., 1990) and increases PRL secretion (Koshiyama et al., 1990). Several studies support the hypothesis that GAL acts via the hypothalamus rather than directly on the APit to increase secretions (Ottlecz et al., 1986; Murakami et al., 1989; Maiter et al., 1990). Further, central administration of a GAL antagonist inhibits the release of GH in rats (Gabriel et al., 1993). However, other reports indicate that GAL exerts mild effects directly on the APit (Gabriel et al., 1988; Sato et al., 1991). Very low levels of binding have been reported in the adult rat APit using receptor autoradiography (Skofitsch and Jacobowitz, 1991). As presented in Section II, transient GAL binding was detected in the APit. Moderate binding was seen at 1 PN. By days 5 and 10 PN the density of binding was high, but by 25 PN the binding in the APit was negligible. The significance of transient APit binding during development is not known. One hypothesis is that GAL is regulating secretion in the APit prior to the maturation of other releasing factor pathways that are present in the adult.

Dopamine and growth hormone releasing hormone (GHRH) released from the Arc affect the secretion of PRL and GH, respectively (reviewed in Hokfelt et al., 1989). Results presented in Paper IV indicated that GHRH and tyrosine hydroxylase (TH; marker for dopaminergic cells) are not detectable in the area of the Arc and ME at 1, 5, 10, and 15 PN. However, both substances are present in the adult opossum Arc and ME (Jacobson lab unpublished observations). Presently, it is unknown how neonatal opossums regulate growth and to what extent the APit functions in early life. However, preliminary results in our laboratory indicate that GH and ACTH are present at 4 PN. Prolactin-like immunoreactivity can be seen at 9 PN. These findings coupled with the fact that GAL binding sites are seen

transiently in the APit, indicate that during development, the opossum APit may be regulated by different releasing factor systems than those operative in the adult. Although we have no direct evidence for support of this, it is plausible that GAL is released from the forming basal hypothalamus and directly affects the APit. Then with brain maturation and the appearance of releasing hormone systems such as GHRH and dopamine, GAL assumes its adult like role and affects the APit at the level of the hypothalamus.

In Paper IV, the comparative distributions of arginine vasopressin (AVP) and GAL during periods of hypothalamic neurogenesis were examined. The results from this study indicated an apparent overlap of AVP-like immunoreactivity (AVP-IR) and GAL-IR in the formative hypothalamus. We therefore investigated the possible colocalization of GAL and AVP during these periods. Double label immunohistochemistry indicated that GAL and AVP are colocalized in magnocellular neurons of the adult opossum Pa and SO. In the brains of the developing opossums, double labelled cells were not readily apparent, however. Photomicrographs demonstrated that GAL-IR and AVP-IR were coextensive but not colocalized. In particular, apparent contacts of between GAL and AVP containing cells were visualized.

The lack of double label is interesting for several reasons. Our results imply that AVP and GAL containing cells are distinct during periods of neurogenesis and morphogenesis and at later stages (adult) cells contain both substances. Thus, it is plausible that GAL and AVP cells arise from distinct precursors and at later ages, a proportion of the magnocellular cells begin to synthesize and store both GAL and AVP. As discussed earlier GAL is a potential regulator of the APit during hypothalamic development. On the other

hand, AVP is located in the forming neural lobe. Although GAL and AVP appear to not be colocalized during the development of the opossum hypothalamus, cells and fibers containing GAL-IR and AVP-IR are in close association with each other. Thus, GAL and AVP systems may interact to coordinate control of both anterior and posterior pituitary function during periods of hypothalamic morphogenesis and differentiation.

In summary, we have characterized the developmental distributions of GAL containing systems during periods of neurogenesis and morphogenesis of the mammalian brain. Although the significance of the presence of GAL during these periods is unclear, the anatomical evidence provided in this dissertation indicates that GAL may be critical in the early regulation of autonomic and endocrine processes. Further, the extremely early appearance of GAL-IR and GAL binding sites may indicate that GAL is involved in the formation of specific CNS regions. Future studies utilizing *Monodelphis* may help discern the functional significance of elevated levels of GAL during periods of changing CNS environments such as morphogenesis of the mammalian nervous system.

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ACKNOWLEDGEMENTS

The work described in this dissertation was accomplished only with the help of several people. I'd like to thank fellow graduate students Dr. Charles Fox, Dr. Cathy Kuehl-Kovarik, Dr. Javed Iqbal, Paul Pearson, and Jay Swanson for technical assistance and thoughtful criticism. I wish to thank Aimee Kao, Lynne Ross, Cathy Martens, and Margie Carter for expert technical assistance. I also thank Dr. Carl Jacobson for his expertise in computers and in the use of Autocad.

Last, but certainly not least, I'd like to thank my mentor and my friend, Dr. Carol Jacobson. Her confidence and enthusiasm allowed me to attain my goals and introduced me to the field of neuroscience.

APPENDIX

ABBREVIATIONS USED IN PAPERS 1 AND 2

AA	amygdaloid area
AA/CX	amygdaloid area/cortical transition
AAA	anterior amygdaloid area
ac	anterior commissure
ACo	anterior cortical amygdaloid nucleus
AD	anterodorsal thalamic nucleus
AM	anteromedial thalamic nucleus
Amb	ambiguus nucleus
APit	anterior pituitary gland
Arc	arcuate nucleus
AV	anteroventral thalamic nucleus
BAA	basal amygdaloid nucleus, accessory division
BAM	basal amygdaloid nucleus, magnocellular division
BAP	basal amygdaloid nucleus, parvicellular division
BFB	basal forebrain
BH	basal hypothalamus
BST	bed nucleus of the stria terminalis
BSTD	bed nucleus of the stria terminalis, dorsal division
BSTM	bed nucleus of the stria terminalis, medial division
BSTV	bed nucleus of the stria terminalis, ventral division

CA	Ammon's cortex
CA1	CA1 field of Ammon's cortex
CA2	CA2 field of Ammon's cortex
CA3	CA3 field of Ammon's cortex
CB	cerebellum
CeS	central superior nucleus
CG	central grey
CGD	central grey, pars dorsalis
CGV	central grey, pars ventralis
CIC	central nucleus of the inferior colliculus
Cl	claustrum
CL	centrolateral thalamic nucleus
CM	central medial thalamic nucleus
CnF	cuneiform nucleus
Cor	cerebral cortex
cp	cerebral peduncle
CPu	caudate putamen (striatum)
Cu	cuneate nucleus
DA	dorsal hypothalamic area
DG	dentate gyrus
DH	dorsal hypothalamus
DLG	dorsal lateral geniculate nucleus

DLL	dorsal nucleus of the lateral lemniscus
DM	dorsomedial hypothalamic nucleus
DNC	dopaminergic nuclear complex
DPC	dorsal nucleus of the posterior
DR	dorsal raphe nucleus
DRA	dorsal raphe area
DT	dorsal thalamus
DTg	dorsal tegmental nucleus
DVC	dorsal vagal complex
ec	external capsule
ECIC	external cortex of the inferior colliculus
ECu	external cuneate nucleus
EF	endorhinal fissure
EnP	endopiriform nucleus
Ent	entorhinal cortex
ET	epithalamus
f	fornix
fi	fimbria of the hippocampus
Fl	flocculus
fr	fasciculus retroflexus
Gi	gigantocellular reticular nucleus
GIV	gigantocellular reticular nucleus, ventral

GP	globus pallidus
Gr	gracile nucleus
hc	hippocampal commissure
HI	hippocampus
I	intercalated nuclei of the amygdala
IAD	interanterodorsal thalamic nucleus
ic	internal capsule
IC	inferior colliculus
ICj	islands of Calleja
IMD	intermediodorsal thalamic nucleus
IO	inferior olive
IOA	inferior olivary area
IPC	intersitial nucleus of the posterior
La	lateral amygdaloid nucleus
LC	locus coeruleus
LD	laterodorsal thalamic nucleus
LH	lateral hypothalamic area
LHb	lateral habenular nucleus
lo	lateral olfactory tract
LOT	nucleus of the lateral olfactory tract
LPAM	lateral preoptic area, magnocellular
LPO	lateral preoptic area

LRt	lateral reticular nucleus
LSD	lateral septal nucleus, dorsal part
LSI	lateral septal nucleus, intermediate part
LSV	lateral septal nucleus, ventral part
LV	lateral ventricle
LVe	lateral vestibular nucleus
MD	mediodorsal thalamic nucleus
MdD	medullary reticular nucleus, dorsal part
MdV	medullary reticular nucleus, ventral part
ME	median eminence
Me5	mesencephalic trigeminal nucleus
MeA	medial amygdaloid nucleus, anterior part
MHb	medial habenular nucleus
ml	medial lemniscus
mlf	medial longitudinal fasciculus
MnPO	median preoptic nucleus
MnR	median raphe nucleus
Mo5	motor trigeminal nucleus
MPA	medial preoptic area
MRA	median raphe area
MSA	medial septal area
MVe	medial vestibular nucleus

Nu 5	trigeminal nuclei
OI	olivary nucleus, inferior
opt	optic tract
OS	olivary nucleus, superior
ox	optic chiasm
Pa	paraventricular hypothalamic nucleus
PAA	posterior amygdaloid area
PB	parabrachial nucleus
pc	posterior commissure
PC	oposterior cortical amygdaloid nucleus
PCRt	parvicellular reticular nucleus
Pe	periventricular hypothalamic nucleus
PF	parafascicular thalamic nucleus
PFl	paraflocculus
PH	posterior hypothalamic area
Pir	piriform cortex
PL	posteriolateral thalamic nucleus
Pn	pontine nuclei
PnC	pontine reticular nucleus, caudal part
PnV	pontine reticular nucleus, ventral part
Po	posterior thalamic nucleus
POA	preoptic area

Pr5	principal sensory trigeminal nucleus
PRA	pontine reticular area
PrH	prepositus hypoglossal nucleus
PT	paratenial thalamic nucleus
PV	paraventricular thalamic nucleus
PVA	paraventricular thalamic nucleus, anterior part
py	pyramidal tract
Re	reuniens thalamic nucleus
RF	rhinal fissure
Rh	rhomboid thalamic nucleus
RMg	raphe magnus nucleus
ROb	raphe obscurus nucleus
RPn	reticular pontine nucleus
Rt	reticular thalamic nucleus
RtTg	reticulotegmental pontine nucleus
S	subiculum
SC	superior colliculus
SCh	suprachiasmatic nucleus
Sep	septal nuclei
SFi	septofimbrial nucleus
sm	stria medullaris of the thalamus
SNA	substantia nigra area

SO	supraoptic nucleus
SOA	supraoptic area
sol	solitary tract
Sol	nucleus of the solitary tract
sp5	spinal trigeminal tract
Sp5	spinal trigeminal nucleus
Sp5C	spinal trigeminal nucleus, caudal part
Sp5O	spinal trigeminal nucleus, oral part
SPF	subparafascicular thalamic nucleus
SPT	subparatenial nucleus
st	stria terminalis
STh	subthalamic nucleus
TEC	tectum
TS	triangular septal nucleus
Tu	olfactory tubercle
Tz	nucleus of the trapezoid body
VC	ventral cochlear nucleus
VDB	nucleus of the vertical limb of the diagonal band
VL	ventrolateral thalamic nucleus
VLG	ventrolateral geniculate nucleus
VLLd	ventral nucleus of the lateral lemniscus,
VLLv	ventral nucleus of the lateral lemniscus,

VM	ventromedial thalamic nucleus
VMH	ventromedial hypothalamic nucleus
VTA	ventral tegmental area
VTg	ventral tegmental nucleus
ZI	zona inserta
10	dorsal motor nucleus of vagus
10gn	vagal ganglia
10n	vagus nerve
12	hypoglossal nucleus
2n	optic nerve
3v	third ventricle
4v	fourth ventricle
5n	trigeminal nerve