Characterization of arginine vasopressin and oxytocin containing systems in the brain of the adult and developing Brazilian opossum, Monodelphis domestica

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Characterization of arginine vasopressin and oxytocin containing systems in the brain of the adult and developing Brazilian opossum, Monodelphis domestica

by

Javed Iqbal

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Ames, Iowa

1995
Characterization of arginine vasopressin and oxytocin containing systems in the brain of adult and developing Brazilian opossum, *Monodelphis domestica*

Javed Iqbal

Major Professor: Carol D. Jacobson
Iowa State University

We have characterized the development and distribution of arginine vasopressin- and oxytocin-like immunoreactivity (AVP-IR and OT-IR, respectively) in the Brazilian opossum (*Monodelphis domestica*) brain. Opossum pups are born after 14-15 days of gestation in an extremely immature state before morphogenesis and organogenesis is completed, which makes them an ideal model for developmental studies. Bromodeoxyuridine (BrdU) single and BrdU-AVP-OT double and/or triple label immunohistochemistry was employed to study postnatal neurogenesis of the hypothalamic paraventricular and supraoptic nuclei. Opossum pups of were injected with BrdU between days 1 and 11 of postnatal life (PN) and killed at day 60 PN. Results of BrdU single label immunohistochemistry revealed that neurogenesis in the supraoptic and paraventricular nuclei was occurring into the postnatal life and completed by day 5 and 7 PN, respectively. Double and triple label immunohistochemistry demonstrated that majority of AVP and OT secreting magnocellular neurons in the supraoptic and paraventricular nuclei are formed prenatally, whereas parvicellular neurons of the paraventricular nucleus are continue to form postnatally. The general distribution of AVP-IR
and OT-IR in the adult opossum brain resembled to that which had been reported for other species. In the developing opossum brain, AVP-IR was first detected at embryonic day 12 and 13 in the mesencephalon and diencephalon, respectively. In the supraoptic and paraventricular nuclei, AVP immunoreactive somata were observed on day 1 and 3 PN, whereas OT-IR in these nuclei appeared between day 3 and 5 PN. Few transiently expressing AVP immunoreactive cells and fibers were also seen in the dorsal thalamus, forming tegmental area, and cerebellum between day 1 and 3 PN. By day 5 and 7 PN, AVP-IR and OT-IR was observed in the median eminence, posterior pituitary, and in several forebrain areas. Somata and fibers containing AVP-IR and OT-IR increased in number and amount of immunoreactivity with the increasing age. The distribution patterns of AVP-IR and OT-IR became an adult-like by day 60 PN. A sex difference in the amount of AVP-IR in the lateral septum of the opossum brain was observed at day 60 PN, which persisted into the adulthood. These findings suggested that AVP and OT play some significant roles in postnatal development of the *Monodelphis*. 
DEDICATION

To my family and parents.

Without their support this piece of work would have not been possible.
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Arginine vasopressin (AVP) and oxytocin (OT) are two closely related neurohypophyseal neuropeptides, which are mainly synthesized and secreted by the neurosecretory neurons of the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei (Swaab et al., 1975a,b). Arginine vasopressin and oxytocin have also been localized in several other areas of the brain (De Vries et al., 1981; De Vries et al., 1985; Castel and Morris, 1988). In addition to their hormone-like functions in the periphery, AVP and OT may be acting as neurotransmitters and/or neuromodulators in centrally regulated functions such as neuroendocrine regulation of the anterior pituitary, autonomic and somatic functions, sexual and maternal behaviors, and learning and memory processes (Buijs, 1990; Richard et al., 1991; De Wied et al., 1993). Immunohistochemical studies have demonstrated that in addition to AVP and OT, a large number of other neuropeptides are also synthesized and secreted by the PVN and SON (Brownstein and Mezey, 1986; Hokfelt et al., 1989).

The SON and PVN are positioned in the anterior hypothalamus and are comprised of magnocellular and magnocellular and parvicellular groups of neurons, respectively. The magnocellular neurons project to the neurohypophysis and median eminence, and parvicellular neurons of the PVN project to the midbrain, brainstem, and spinal cord (Swanson and Kuypers, 1980; Swanson and Sawchenko, 1983). Both nuclei are functionally linked to each other because of homologous and complementary functions (Lazcano et al.,
In the rat, the anatomy of the SON and PVN has been studied and there exists an extensive literature on their cytoarchitecture (Felton and Cashner, 1979; Swanson and Sawchenko, 1983; Bruni and Permal, 1984), chemoarchitecture (Rhodes, 1981; Swanson and Sawchenko, 1983), and neuropeptide secretory activities (Browstein and Mezey, 1986; Hokfelt et al., 1989; Neumann et al., 1993).

In the majority of mammals, the SON consists of two groups of neurons which secrete AVP and OT separately. Although, the SON and PVN neurons produce both AVP and OT, it appears that the SON is predominantly vasopressinergic (Sofroniew et al., 1979). There also exists a topographical organization of AVP and OT secreting neurons which vary with the species (Sofroniew et al., 1979; Dierickx, 1980).

The PVN consists of three groups of neurons based on their morphology and efferent connections (Armstrong et al., 1980; Swanson and Kuyper, 1980; Swanson and Sawchenko, 1980; Swanson and Sawchenko, 1983). First, like the SON, the PVN contains a majority of magnocellular neurosecretory neurons which produce both AVP and OT, and whose axons project to the neurohypophysis, where they secrete their neurohormonal contents into the blood stream (Zimmermann, 1983). The second group of neurons are parvicellular neurosecretory cells which project to the median eminence and form the hypothalamo-adenohypophysial system, and participate in the control of release of the anterior pituitary hormones. The third group consists of neurons which are present in the medial and periventricular subdivision of the parvicellular neurons in the PVN which project to the brainstem and spinal cord (Armstrong et al., 1980; Luiten et al., 1985; Schwanzel-Fukuda
et al., 1984; Kiss, 1988; Kiss et al., 1991). Descending fibers from these neurons project to the dorsal motor nucleus of the vagus, nucleus of the solitary tract, intermediolateral column of the spinal cord (Swanson and Sawchenko, 1980), and sexually dimorphic nucleus of the bulbocavernosus muscle (Wagner and Clemens, 1993). It is these neurons and projections by which the PVN influences the centrally regulated autonomic functions. In addition to AVP and OT, more than 20 neuropeptides have been localized in the magnocellular and parvicellular neurons of the PVN including cholecystokinin (CCK), substance P (SP), enkephalin (ENK), corticotropin releasing factor (CRF), galanin, and neurotensin (Brownstein and Mezey, 1986; Hokfelt et al., 1989). Because of special secretory functions the neurons of the PVN and SON might differ in the timing of their birth, site of origin, and migration to their final destinations from the other neurons in the hypothalamus.

**Neurogenesis of the hypothalamic paraventricular and supraoptic nuclei**

Since the introduction of the autoradiographic techniques (Doniach and Pele, 1950), it has become possible to mark dividing cells and determine the approximate period of cellular proliferation. Various investigators have employed such techniques to study the developmental events in the mouse cerebellum (Uzman, 1960), cerebral cortex and hippocampus (Angevine and Sidman, 1962; Angevine, 1965), diencephalon (Angevine, 1970), and hypothalamus (Shmida and Nakamura, 1973). Similar studies on the time of neuronal origin, development of other brain regions, and nuclear groups have been conducted using tritiated thymidine autoradiography in the rat hypothalamus (Ifft, 1972; Altman and
Bayer, 1978) and preoptic area (Jacobson et al., 1981). Postnatal neurogenesis of the preoptic area (Larsen and Jacobson, 1986) and the suprachiasmatic nucleus (Rivkees et al., 1988) in the Brazilian opossum brain has also been studied using tritiated thymidine autoradiography.

In the rat and mouse brain, neurogenesis of the diencephalon is generally considered a prenatal phenomenon (Altman and Bayer, 1978; Krim and Slopper, 1980). Tritiated thymidine autoradiography has demonstrated that neurogenesis in the hypothalamic SON and PVN both in the rat and mouse is completed between day 12 and 14 postfertilization (Pf) (Ifft, 1972; Altman and Bayer, 1978; Shmida and Nakamura, 1973; Krim and Slopper, 1980). It has also been demonstrated that the neurons originate bilaterally from the germinal zone of the neuroepithelium of the third ventricle. Cells that form the SON complete their migration by day 15 Pf, and those forming the PVN remain in the periventricular area (Altman and Bayer, 1978). Although, Altman and Bayer (1978) in their autoradiographic studies had given a detailed account of the neurogenesis of the diencephalon, the origin and migration of neurosecretory neurons of the SON and PVN nuclei has not been fully investigated, specifically in relation to their neurochemical phenotypes.

Tritiated thymidine autoradiography has been a widely used technique for studying cell proliferation, migration, and differentiation. Recently, a thymidine analog bromodeoxyuridine (BrdU) has become an alternative to tritiated thymidine for the study of cell proliferation and birth dating (Miller and Nowakowski, 1988). Upon injection, BrdU is incorporated into the DNA during the synthesizing (S phase) phase of the cell cycle. If the cells do not divide many times subsequent to obtaining BrdU, the cells that have incorporated
BrdU can be detected by using monoclonal antibodies and standard immunohistochemical protocols (Gray, 1985). Bromodeoxyuridine immunohistochemistry has been proposed as a faster, sensitive, and valuable alternative tool to analyze the developmental events in the central nervous system (Soriano and Rio, 1991).

**Distribution of the neurohypophyseal neuropeptides**

The neuropeptides AVP and OT are synthesized and secreted mainly by the magnocellular neurons of the SON and the magnocellular and parvicellular neurons of the PVN (Swaab et al., 1975a,b; Sofroniew, 1985). Using immunohistochemical techniques, AVP localization has also been demonstrated in the suprachiasmatic (SCN) nucleus (Vandensande et al., 1975; Sofroniew and Weindel, 1980; De Vries et al., 1981). In addition, AVP-like immunoreactivity (AVP-IR) containing somata has also been observed in the bed nucleus of the stria terminalis, preoptic area, dorsomedial hypothalamus, and medial amygdala (Caffe and Van Leeuwen, 1983; Van Leeuwen and Caffe, 1983; De Vries et al., 1985; Riphagen, 1986; Wang et al., 1993). Upon neuronal stimulation AVP and OT are secreted into the circulation through the neural lobe of the hypophysis and act as peripheral hormones (Neumann et al., 1993). As has been demonstrated in the adult rat, these peptides also reach a number of other brain areas through an extra hypothalamic fiber system (Buijs, 1978; Buijs et al., 1978; Buijs, 1987; De Vries et al., 1985). The immunohistochemical demonstration of fibers containing these peptides that go to various brain regions suggests that AVP and OT may have neurotransmitter actions in the central nervous system (Boer et al., 1980; Voorn and Buijs, 1983; Valiquette et al., 1985; Buijs,
The distribution of AVP and OT binding sites in the adult and developing central nervous system as demonstrated by autoradiography has also been reported (Poulin and Pittman, 1988; Tribollet et al., 1989; Tribollet et al., 1991; Phillips et al., 1990; Dubois-Dauphin et al., 1990; Kremarik et al., 1993). In the rat brain, AVP and OT binding sites are found in areas similar to that for the distribution of these peptides. Arginine vasopressin binding sites are present in discrete regions in the telencephalon, diencephalon, metencephalon, and myencephalon. Whereas OT binding sites were present in specific brain regions including the olfactory nucleus, tenia tecta (hippocampus), bed nucleus of the stria terminalis, dorsomedial aspect of the caudate, amygdala, ventromedial hypothalamic nucleus, subiculum, and brainstem (nucleus of the solitary tract and dorsal motor nucleus of vagus).

It has also been demonstrated that many neuropeptides are colocalized in different brain regions/areas. For example, AVP is colocalized with CRF in the parvicellular and with OT in the magnocellular neurons of the PVN (Piekut and Joseph, 1991). Arginine vasopressin have also been reported to be colocalized in the PVN with galanin, CCK, ENK, and CRF (Hokfelt et al., 1989). The presence of AVP and OT and their binding sites in specific brain regions, and their colocalization with a number of other neuropeptides indicate that AVP and OT might be playing a role in mediating a variety of centrally regulated functions.
 Functional significance of neurohypophyseal neuropeptides

Arginine vasopressin exerts a variety of actions through membrane bound receptors, which are of two types. The first type, V1a vasopressin receptors are found in the liver, vasculature, adrenal, anterior pituitary, and brain, and the second type, V2 receptors are found in the kidney (Audigier and Barbereis, 1985; Phillips et al., 1990). By acting on V1a type receptors, AVP causes enhancement of phosphoinositide metabolism and release of intracellular calcium in the central nervous system (Stephens and Logan, 1986; Shewey and Dorsa, 1988; Briley et al. 1994). The V1a receptors have been shown to be widely distributed in the mammalian brain (Ostrowski et al., 1994; Szot et al., 1994). Recently, a third type of receptor, V1b has been shown to be expressed in the brain during development. It is hypothesized that AVP induces the formation of cyclic AMP in the developing brain through these receptors (Brinton and Brownson, 1993). By binding to V2 type receptors in the kidney, AVP functions as an antidiuretic hormone (Jard, 1983; Maning et al., 1987)

Physiological studies have shown that AVP also plays a role as a neurotransmitter and/or neuromodulator (Audigier and Barberies, 1985; Riphagen and Pittman, 1986; Buijs, 1987; Poulin et al., 1988; Raggenbass et al., 1989; Raggenbass et al., 1991). For instance, arginine vasopressin has been described to play a role in centrally regulated functions including control of blood pressure (Poulin et al., 1994), central thermoregulation (Riphagen and Pittman, 1986), antinociception (Oluyomi and Hart, 1992; Tanaka, 1993), circadian functions (Buijs, 1990; Reghunandanan, 1990; Felino et al., 1994) and water-salt balance (Zimmermann, 1983). On the basis of behavioral studies, it has been shown that AVP influences memory storage (De Wied, 1980; De Weid et al., 1993), drinking behavior
(Mangiapane et al., 1983), food intake (Burlet et al., 1992), and social and sexual behaviors (Bluth and Dentzer, 1993; Winslow et al., 1993). Electrophysiological studies have demonstrated that vasopressin can directly excite central motor neurons (Ma and Dun, 1985; Peters and Kreulen, 1985; Raggenbass et al., 1989; Liou and Albers, 1989; Sun and Guyenet, 1989), and increase the excitability of the facial motoneurons by generating a sodium dependent membrane current (Raggenbass et al., 1991; Tribollet et al., 1991).

In addition to the above mentioned functional roles of AVP, it has also been suggested that AVP is involved in the normal development of the brain. Brattleboro rats which are incapable of synthesizing AVP, have shown disturbances in their brain development, body growth, and avoidance behavior. Such alterations are suggestive of the importance of AVP in growth and central nervous system formation (Boer, 1980; Buijs, 1980; Buijs, 1990; Boer et al., 1993). Vasopressin along with other peptides is also involved in the growth and regulation of the adrenal cortex (Malendowicz, 1993).

The neuropeptide OT is synthesized in the hypothalamic PVN and SON, secreted into the circulation at the level of the neurohypophysis, and like AVP exerts a number of well established peripheral hormonal effects. Oxytocin is also present in extrahypothalamic fibers projecting to various structures in the brain, which indicate its functions as a neurotransmitter and/or neuromodulator as well (Sofroniew, 1985; Dreifuss et al., 1988). Other evidences for such a role includes the effects of oxytocin administration under experimental conditions. For example, small amounts of OT injection into the brain or the cerebral ventricles modulate neuroendocrine and autonomic functions (Freund-Mercier and Richard, 1984; Rogers and Herman, 1985; Benelli et al., 1994; Poulin et al., 1994). Electrical stimulation of the PVN
has been reported to influence gastric secretory functions via vagally mediated effects because of its direct connections with the dorsal motor nucleus of the vagus and nucleus of the solitary tract (Rogers and Nelson, 1984; Lawrance and Pittman, 1985; Rogers and Herman, 1987). A significant amount of data indicates that OT may be the neurotransmitter involved in the pathways from the PVN to the dorsal motor nucleus of the vagus and nucleus of the solitary tract (Renuad et al., 1987; Dreifuss et al., 1988; McCann and Rogers, 1990), and thus be a potential regulator of gastric activities (Verbalis, 1993). Many other centrally mediated functions in which OT is thought to play a role have also been suggested (reviewed in Richard et al., 1991; De Weid et al., 1993).

Development of neurohypophyseal neuropeptide systems

The ontogeny of the AVP and OT containing systems in the hypothalamic supraoptic and paraventricular nuclei have been studied in the rat. Vasopressin immunopositive cells appear in the fetal brain on day 16 and 18 Pf in the SON and PVN, respectively (Buijs et al., 1980; Whitnall et al., 1985). However, Hyodo and co-workers (1992) has shown that AVP-like immunoreactivity first appeared on day 14 Pf in the median eminence and on day 15 and 16 Pf in the SON and PVN, respectively. In the SCN, AVP immunopositive cells first appear on day 2 of postnatal life (De Vries et al., 1981). As compared to AVP, OT-like immunoreactivity in the rat brain was detectable after birth (Buijs et al., 1980, Whitnall et al., 1985; Alstein and Gainer, 1988), while OT precursors have been shown to be present from day 16 Pf onward (Laurent et al., 1989). Although the ontogeny of AVP and OT in the SON and PVN has been described in the rat and mouse, the development of AVP and
OT containing systems in the extrahypothalamic regions of the brain have not been thoroughly investigated.

Despite increasing evidence of functional significance of these neuropeptides in the adult central nervous system not much is known regarding their involvement in the processes which occur during prenatal and postnatal development of the mammalian brain. The early presence of neuropeptides and changes in their synthetic activity with increasing postnatal age suggest that AVP and OT may play a role in the development and formation of the mammalian brain.

Experimental model

To this end we utilized the Brazilian gray short-tailed opossum, *Monodelphis domestica*, as an experimental model to examine the development of AVP and OT containing systems in the mammalian brain. *Monodelphis* is a small marsupial which breeds well under laboratory conditions. Its young are born after 14 days of gestation in an extremely immature state, before sexual differentiation and neurogenesis are completed (Jacobson, 1984; Larsen and Jacobson, 1986). *Monodelphis*, as most marsupials, has a protracted postnatal developmental period. The absence of a pouch makes the opossum pups very accessible and thus provide an excellent experimental model for studying the developmental events in the mammalian central nervous system (Dore et al., 1990; Nicholls et al., 1990; Rivkees et al., 1988; Schwanzel-Fukuda et al., 1988; Treherne et al., 1992; Brunjes et al., 1992; Wang et al., 1992; Elmquist et al., 1994).
Dissertation organization

The present dissertation consists of three papers, of which the first has been published in Developmental Brain Research (1995) 85: 151-160. The second and third papers are prepared for submission to the Journal of Comparative Neurology. The papers are preceded by a general introduction and followed by a composite summary and general discussion. The literature cited in the general introduction and general discussion is listed after the general discussion section. All of the experimental and research work presented in this dissertation was carried out by myself in the laboratory and under the guidance of Dr. Carol D. Jacobson.
CHAPTER 1. POSTNATAL NEUROGENESIS OF THE HYPOTHALAMIC
PARAVENTRICULAR AND SUPRAOPTIC NUCLEI
IN THE BRAZILIAN OPOSSUM BRAIN

A paper published in Developmental Brain Research

J. Iqbal, J. K. Elmquist, L. R. Ross, M. A. Ackermann, and C. D. Jacobson

Abstract

We have used bromodeoxyuridine (BrdU) single and BrdU-arginine vasopressin-oxytocin (BrdU-AVP-OT) double and triple label immunohistochemistry to characterize postnatal neurogenesis of the supraoptic and paraventricular nuclei in the Brazilian opossum. Developing pups received a single injection of BrdU between days 1 and 11 postnatally. All brains were collected on day 60 of postnatal life (60 PN). Single label BrdU immunohistochemistry revealed that an injection at 1 PN resulted in heavy labelling in the hypothalamus including the area of the paraventricular nucleus, whereas only approximately one third of the cells in the supraoptic nucleus were labelled. Analysis of data indicated that neurogenesis of the supraoptic and paraventricular nuclei is completed by days 5 and 7 PN, respectively. Double and triple label immunohistochemistry demonstrated that following BrdU injection on day 1 or 2 PN, few of the AVP and OT secreting cells in the supraoptic nucleus were double labelled with either peptide and BrdU, and no double labelled cells were seen following BrdU injection on day 5 PN. Similarly, in the paraventricular nucleus most of the AVP and OT secreting magnocellular cells were not double labelled with either peptide and BrdU. Whereas several double labelled cells were observed in the parvicellular
part following BrdU injection on day 1 or 2 PN. No double labelled cells were present in any component of the paraventricular nucleus following injection on day 7 PN or later. These results indicate that the majority of the AVP and OT secreting magnocellular neurons are born prenatally and the OT and AVP parvicellular group of neurons are born during postnatal life. Our results also demonstrate that in contrast to that of eutherian rodents such as the rat and mouse, neurogenesis in the opossum hypothalamus continues into the postnatal period and provides a unique opportunity to study the neuroanatomical development of diverse regions such as the paraventricular nucleus.

Introduction

The paraventricular and supraoptic nuclei (PVN, SON respectively) are positioned in the anterior hypothalamus and constitute the hypothalamo-neurohypophyseal system. The PVN consists of magnocellular and parvicellular groups of neurons, whereas the SON is composed of magnocellular neurons. The magnocellular neurons of the PVN and SON project to the neurohypophysis and median eminence, while the parvicellular neurons of the PVN project to the brainstem and spinal cord and are involved in the control of autonomic homeostasis. Arginine vasopressin (AVP) and oxytocin (OT) are two closely related peptides synthesized and secreted by the PVN and SON. Immunohistochemical studies have demonstrated that many other neuropeptides including galanin, corticotrophin releasing factor, thyrotropin releasing factor, enkephalin, cholecystokinin, and neurotensin are also localized in the parvicellular neurons of the PVN and magnocellular neurons of the PVN and SON in the rat. Due to the presence of a wide variety of neuropeptides in the
neurons forming the PVN and SON, and the different functions of these neuronal populations, the time periods of neurogenesis and proliferation of these neurons in relation to their neurochemical phenotype, neurosecretions, and involvement in autonomic functions may differ as compared to that of other hypothalamic neurons.

Since the introduction of tritiated thymidine autoradiography, several investigators have studied neurogenesis of the central nervous system in multiple mammalian species including the rat and mouse. Neurogenesis of the diencephalon of rodents is generally considered a prenatal event. Tritiated thymidine autoradiography has demonstrated that neurogenesis in the hypothalamic SON and PVN, both in the rat and mouse, is completed between day 13 and 15 postfertilization, (eight to ten days prior to birth). Using tritiated thymidine autoradiography the migratory patterns of postmitotic cells have also been documented. Such studies have shown that neurons forming the PVN and SON originate from the germinal zone of the neuroepithelium of the third ventricle. The cells destined for the SON complete their lateral migration by day 15 postfertilization, while those forming the PVN remain in the periventricular area. Although previous autoradiographic studies have given a detailed account of the time of completion of neurogenesis of the diencephalon in the rat, the development of the PVN and SON, and specifically the possible existence of time differences in the formation of magnocellular and parvicellular neurons has not been fully investigated. Further, neurons that express a specific neurochemical phenotype and have a specific function may have different time periods of formation.

In this study, we have used the Brazilian gray short-tailed opossum as an
experimental model to study postnatal neurogenesis of the PVN and SON. The Brazilian gray short-tailed opossum, *Monodelphis domestica*, is a small pouchless marsupial. Brazilian opossum young are born after 14 days of gestation in an extremely immature and sexually undifferentiated state. The absence of the pouch in *Monodelphis* makes the fetus-like pups very accessible for developmental studies.

To study postnatal neurogenesis in the paraventricular and supraoptic nuclei, we have utilized bromodeoxyuridine (BrdU) immunohistochemistry. Bromodeoxyuridine is a thymidine analog which has recently become an alternative to tritiated thymidine for the study of developmental events in the central nervous system. Following exogenous administration, BrdU is incorporated into the DNA during the synthesizing phase (S phase) of the cell cycle. Like tritiated thymidine it remains in the cell permanently and the BrdU containing cells can then be detected by using an antibody directed against BrdU and standard immunohistochemical protocols. In order to determine: (1) if neurogenesis of the PVN and SON continues during the postnatal period, (2) if neurons of the PVN and SON complete neurogenesis at a different time point as compared to other neurons in the surrounding regions of the brain, (3) if AVP and OT secreting cells have the same birth dates as that of the surrounding neurons in the SON and PVN, and (4) if neurogenesis of magnocellular and parvicellular neurons is distinct, we have utilized BrdU single label, BrdU-AVP double label, and BrdU-OT-AVP triple label immunohistochemistry.
Materials and Methods

Animals

Developing Brazilian opossum pups were obtained from a colony maintained at Iowa State University. The animals were housed individually in plastic cages, maintained at a constant temperature (26°C) and a 14:10 h light-dark cycle. The animals were provided with water and food ad libitum (Reproduction Fox Chow; Milk Specialties Products, Madison, WI). For breeding purposes, male and female opossums were housed together and separated after a two week period. Subsequently the females were checked daily for the presence of pups. The day of birth of the pups was considered as day 1 of postnatal (PN) life. The animals and procedures used in this study were in accordance with those approved by the Iowa State University Committee on animal care.

BrdU injection and tissue collection

On each of the days between 1 and 11 PN, a different litter of pups was injected with BrdU. Individual pups were injected without separation from their mother. Each pup was given a subcutaneous injection along the dorsal midline of 20 μl of BrdU solution (Sigma; 1 mg/ml), dissolved in 0.9% saline. Preliminary results demonstrated that this method is quite effective in delivering BrdU throughout the brain. All animals in each litter were weighed, examined for presence of any abnormalities, and killed at 60 PN by etherization. At that time, the animals were perfused transcardially with 0.9% saline followed by Zamboni's fixative solution for 10 minutes as has been reported previously in the opossum. Brains were removed from the calvaria and postfixed for 48 hours at room temperature.
in the same fixative solution. Subsequently brains were sunk in 30% sucrose solution, and 20 μm thick coronal sections were cut on a cryostat (Reichert Instruments) at -20°C. Tissue sections were thaw mounted onto poly-L-lysine coated slides, kept on a slide warmer at 37°C overnight, and stored at 4°C until immunohistochemistry was conducted.

**Immunohistochemistry**

The protocol utilized for single label BrdU immunohistochemistry was a modification of that which we have been reported previously for BrdU and immunohistochemical detection of neuropeptides.

**Pretreatment and DNA denaturation**

The slide mounted tissue sections were rinsed with 50 mM potassium phosphate buffered saline (KPBS), and pretreated with trypsin (0.06% trypsin bovine type III; Sigma, and 0.06% calcium chloride dissolved in 300 ml of KPBS) for 30 minutes at 37°C. After washing for 10 minutes with KPBS, tissue sections were treated with 0.1N HCl (ice cold) for 10 minutes followed by incubation in 2N HCl at 37°C for 30 minutes. The tissue sections were then neutralized in basic KPBS (pH 8.5) for 10 minutes and followed by routine immunohistochemistry.

**Single label immunohistochemical staining**

Tissue sections were rinsed in KPBS, incubated with a 0.3% hydrogen peroxide solution to remove endogenous peroxidase activity, exposed to normal horse serum as blocking agent
and followed by an overnight incubation in BrdU antisera (1:200, Mouse monoclonal, Dako) at room temperature. After adequate washing, the tissue sections were incubated in horse anti-mouse IgG (Vector; 1:200) for 2 hours at room temperature, rinsed, and reacted with avidin-biotin complex (ABC; Vector Elite Kit, 1:50) at room temperature for an additional hour. After rinsing, the tissue sections were exposed 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 0.1M sodium acetate solution. After 6 minutes, the reaction was terminated by placing the tissue sections into 0.9% saline solution. The sections were then dehydrated in graded alcohols, cleared in xylene and coverslipped with permount mounting media.

**Double labelling immunohistochemistry**

The slide mounted sections were first processed for BrdU immunohistochemistry using ABC and DAB-nickel procedures to detect BrdU labelling as described above. Following termination of the DAB reaction the sections were rinsed in KPBS, incubated with 0.3% hydrogen peroxide solution to remove peroxidase activity due to the BrdU immunohistochemistry, blocked in normal goat serum (Vector; 1:67) for two hours at room temperature and followed by incubation with arginine vasopressin antisera (1:5000; rabbit polyclonal, Miles Scientific) for 20 hours at room temperature. After sufficient washing in KPBS, bound antibodies were visualized by incubating the tissue sections in biotinylated goat anti-rabbit IgG (Vector; 1:800) for two hours and avidin-biotin complex (ABC; Vector, 1:200) for one hour at room temperature. After rinsing, the tissue sections were reacted with
DAB substrate (without added nickel sulfate) that yielded a brown cytoplasmic reaction product. The reaction was terminated after 5 minutes by placing the tissue sections into 0.9% saline solution. The sections were then dehydrated in alcohol, cleared in xylene, and coverslipped with permount mounting media. Tissue sections were analyzed using a light microscope. Using this procedure we were able to visualize purple black (BrdU labelled nuclei) and brown (AVP labelled cytoplasm) reaction products simultaneously.

In addition to BrdU-AVP double labelling, we also processed slide mounted tissue sections from animals which were given BrdU injection at 1 and 2 PN for simultaneous BrdU-oxytocin-arginine vasopressin (BrdU-OT-AVP) labelling. The slide mounted tissue sections were first processed for BrdU immunohistochemistry using ABC and DAB-nickel procedures to detect BrdU labelling as described above. Following termination of the DAB reaction the sections were rinsed in KPBS, incubated with 0.3% hydrogen peroxide solution to remove peroxidase activity due to the BrdU immunohistochemistry, blocked in normal goat serum (Vector; 1:67) for 2 hours at room temperature and followed by incubation with oxytocin antisera (1:500; rabbit polyclonal, Peninsula Labs) for 20 hours at room temperature. After sufficient washing in KPBS, bound antibodies were visualized by incubating the tissue sections in biotinylated goat anti-rabbit IgG (Vector; 1:200) for 2 hours and Texas red avidin-D conjugate (Vector; 25 µg/ml) for one hour at room temperature. After rinsing, the sections were incubated in AVP primary antisera (generated in guinea pig) for 20 hours at room temperature (Peninsula Labs, 1:500). Sections were incubated in fluorescein conjugated goat anti-guinea pig antisera (Vector; 1:200) for two hours. The sections were rinsed and coverslipped with glycergel (DAKO) and viewed with a Ziess
Axiophot fluorescence microscope using appropriate filters cubes. Using this procedure we were able to visualize BrdU (purple black nuclei), OT (bright red), and AVP (bright green) labelling simultaneously.

Immunohistochemical control procedures

Negative controls were generated for every single, double and triple label runs by omitting the primary antisera and incubation of the tissue sections in the respective normal blocking serum. Tissue sections from animals which did not receive BrdU were processed concurrently and no specific nuclear staining was observed. Preabsorption controls were carried out by incubating the tissue sections in the OT and AVP primary antisera preabsorbed to OT and AVP peptides (15 μM; Sigma), respectively. All specific staining was abolished; whereas, preabsorption of OT primary antisera with AVP peptide or AVP primary antisera with oxytocin peptide (15 μM; Sigma) did not block the specific immunostaining.

Analysis of tissue

Following immunohistochemistry, tissue sections processed solely for BrdU localization, were coded so that all further analysis was performed without prior knowledge of the age and sex of the animals. For each day of injection, a minimum of four animals (2 males and 2 females) from each litter were analyzed using a Zeiss Axiophot microscope and an image analysis system. All sections that contained the PVN and SON were recorded for each animal using maps of coronal sections of the opossum brain and four representative sections lying within the full rostro-caudal extent of each nucleus were chosen. The field area
that contained the PVN or SON was delineated and the number of BrdU labelled cells identified by black staining nuclei was quantified using an IBAS-20 image analysis system as reported previously.16,39.

Tissue sections processed for BrdU-AVP double label immunohistochemistry were analyzed with a light microscope and tissue sections processed for BrdU-OT-AVP triple label immunohistochemistry were analyzed with a Ziess Axiophot microscope equipped with a mercury light source. Texas red fluorescence was visualized using a rhodamine filter system and fluorescein was visualized using FITC filter system. Double label and triple labelled tissue sections were observed by switching between the filter systems and bright field illumination. All observations were made by a single investigator.

Statistical analysis

Following quantitation of the single label immunohistochemistry the data was analyzed using two way analysis of variance (ANOVA) followed by the least significant difference test for the number of BrdU labelled cells in either the PVN or SON. In the analysis the independent variables were postnatal age (day of BrdU injection) and sex of the animal. Analysis of variance was also conducted on field area measurements in order to determine if the difference in the number of BrdU labelled cells resulting from the injection at various ages was not due to differential field area measurements used to quantify the total number of cells measured. The level of statistical significance was accepted at $p \leq 0.05$. 
Results

Our results demonstrated that the protocol we used to inject BrdU, effectively labelled the dividing cells in the brain of the opossum pups, and did not result in any abnormality in the growth of the animals. Immunohistochemical control procedures revealed that staining for BrdU, AVP, and OT were specific, and the labelling patterns of BrdU-AVP were comparable with two different AVP primary antiseras.

Qualitative analysis of BrdU labelling

Analysis of BrdU immunohistochemistry indicate that neurogenesis in the paraventricular is occurring at the earliest age studied (1 PN) and continues into the postnatal period. Injection of BrdU on day 1 PN resulted in a heavily labelled ependyma of the third ventricle and the hypothalamus including the PVN at 60 PN (the age of tissue collection). As seen in Figure 3, a high proportion of the cells in the PVN were BrdU labelled following injection on day 1 PN. Single label immunohistochemistry showed that the majority of the neurons in the magnocellular division of the PVN (as defined by Swanson and Kuypers\(^{51}\)) were not labelled regardless of the day of postnatal BrdU injection, whereas neurons in the parvicellular division (as defined by Swanson and Kuypers\(^{51}\)) had labelled nuclei following injection between day 1 and 5 PN (Fig. 1A, B and C). Further analysis indicated a progressive decrease in the number of BrdU labelled cells with increasing age of injection. None of the animals that received BrdU injection after day 7 PN had labelling in either the magnocellular or parvicellular divisions of the PVN (Fig. 1D). However, a few cells in the ependyma of the third ventricle and a few small cells with small darkly stained nuclei (glial-
like) in the parvicellular part of the paraventricular nucleus remained labelled following injection on later days. Although not quantified and included in this study, BrdU labelling was present in other areas of the brain including the dorsal and lateral hypothalamus following later day injections. As compared to the labelling in the PVN, in the SON approximately one third of the cells were BrdU labelled following injection on day 1 PN (Fig. 2A). There was no BrdU labelling in the SON after an injection on day 5 PN or later (Fig. 2B).

Quantitative analysis

The above findings were directly confirmed by quantifying the number of BrdU labelled cells in the PVN and SON from tissue sections processed for single label BrdU immunohistochemistry. Two-way ANOVA demonstrated that there was a significant (P < 0.05) effect of time at which BrdU was injected on the number of labelled cells both in the PVN and SON. As revealed by the ANOVA, the sex of the animal, and the field area measured for quantification of BrdU labelled cells in the PVN and SON had no effect on mean total number of BrdU labelled cells (data not shown).

Analysis of double and triple labelling

Double label (BrdU-AVP) and triple label (BrdU-OT-AVP) immunohistochemistry demonstrated several BrdU-AVP and BrdU-OT double labelled cells in the PVN. In the SON there were not many cells that appeared to be double labelled following injection on any day. Although not quantified, several double labelled cells were present in the PVN, specifically
in its parvicellular division, after a day 1 or 2 PN injection (Fig. 4A). The number of double labelled cells progressively decreased until injection on day 5 PN. No double label cells were found following injection on day 7 PN or later. In the SON, a small number of double label cells were found after injection on day 1 or 3 PN (Fig. 4B). No double labelled cells were observed subsequent to injection on day 5 PN or later.

**Discussion**

In this study we have defined the period of postnatal neurogenesis in the paraventricular and supraoptic hypothalamic nuclei in the Brazilian opossum brain. The results of the present study demonstrate that BrdU immunohistochemistry is an effective technique to study proliferation of neurons in the metatherian central nervous system. Our results also demonstrate that the injection of a BrdU solution subcutaneously to the developing opossum pup was quite effective in labelling dividing cells throughout the brain. Additionally, the dose of BrdU which we administered to the opossum pups did not appear to be toxic, as we did not observe any abnormality in body weight, brain morphology and/or other gross abnormalities in the development of other organ systems.

Bromodeoxyuridine has been used by various laboratories to study cell proliferation, migration and time of origin of neurons in the central nervous system, cytokinetic behavior in the cerebral ventricles, as well as determination of cell cycles in the fetal mouse brain\(^5\)\(^5\)\(^3\)\(^8\)\(^,\)\(^4\)\(^7\)\(^,\)\(^5\)\(^2\)\(^,\)\(^5\)\(^3\). Similarly, BrdU has been utilized to study the birth dates, and neurochemical phenotypes of different populations of neurons simultaneously using double label immunohistochemistry.\(^7\)\(^,\)\(^3\)\(^0\)\(^,\)\(^4\)\(^7\).
Previously, Miller and Nowakowski\textsuperscript{35} compared the labelling patterns after BrdU and tritiated thymidine injection given separately or together and have reported comparable numbers of BrdU and tritiated thymidine labelled cells in the rat brain. Analysis of tissue from animals injected with tritiated thymidine for a different study\textsuperscript{32} also revealed comparable timing and numbers of labelled cells following BrdU or tritiated thymidine both in the SON and PVN (Iqbal and Jacobson, unpublished results).

The results from the present study demonstrate that the neurons which comprise the PVN and SON continue to form during the postnatal period. Neurogenesis in the SON and PVN is completed by day 5 and 7 PN, respectively. Injection of BrdU after these ages resulted in no labelling of the neurons in these nuclei. However, following BrdU injection on day 8 PN, a few cells in the ependyma of the third ventricle and a few glial-like cells in the PVN were BrdU labelled. Triple label immunohistochemistry demonstrated that the majority of OT or AVP secreting magnocellular neurons in the SON and PVN never became BrdU labelled and thus were formed prenatally. Conversely, OT and AVP secreting neurons contained BrdU in the parvicellular division of the PVN and thus are formed during postnatal life. Recently, Raadsheer and coworkers\textsuperscript{40} have proposed methods to correct cell numbers based on cell size information. In the present study we did not take into account cell size when describing the total numbers of BrdU labelled neurons, thus it is possible that we might have under-estimated the number of labelled cells (especially small neurons) in the SON and PVN.

Immunohistochemical studies have demonstrated that in addition to OT and AVP there are many other neuropeptides synthesized in the PVN\textsuperscript{8,22,36}. Although several cells were
labelled with BrdU and AVP and/or OT, results indicate that many BrdU labelled cells in the PVN are not OT or AVP immunopositive. These results suggest that the BrdU single labelled cells may be those which synthesize other neuropeptides. Further double label studies are required to demonstrate the neurochemical phenotypes of the cells which are generated late in development.

*Monodelphis* pups are born in an extremely immature state after 14-15 days of gestation. Based on studies conducted in our laboratory and in others, we believe that the stage of neural development of the opossum pups seen at birth (1 PN) corresponds to that of the 13-14 day gestation rat. Further, 16 PN in the opossum resembles that of the 1 PN rat pup. Previously, various investigators have studied neurogenesis of the PVN and SON using tritiated thymidine autoradiography in the rat and mouse. In the rat, neurons which comprise the SON and PVN complete neurogenesis between day 13 and 15 postfertilization. Further, these nuclear groups are recognizable between day 16 and 18 postfertilization, respectively. Similar findings have been obtained for the mouse. However, these studies did not specifically define the magnocellular and parvicellular nature of the neurons and these studies did not allow for the identification of neurochemical phenotype of these cells.

In neurogenetic studies, it has been proposed that the large neurons in a given area of the brain are formed earlier than the small neurons located in the same region. In the mouse, magnocellular neurons in the PVN and SON form earlier than the small sized parvicellular neurons and neurons in the adjacent areas. Using tritiated thymidine autoradiography, neurogenesis of the anterior hypothalamus has been studied in the rhesus.
monkey\textsuperscript{56}. This study demonstrated that there are two waves of neurogenesis in the SON and PVN in the rhesus monkey. The first wave occurs between embryonic days 27 and 32 and the second wave is seen between embryonic days 38 and 45. Further, this study reported that labelled neurons in the first wave were present in the dorsal part of the PVN, whereas in the ventral part of the PVN labelled neurons were seen for the second wave. At present we do not know if there are two different waves of neuron formation in the opossum SON and PVN reported for the rhesus monkey\textsuperscript{56}. Despite the very immature state at which opossums are born, at present we still do not know the exact time difference between the initial formation of magnocellular and parvicellular neurons in these nuclear groups. To this end, further studies are in progress investigating prenatal labelling patterns.

In marked contrast to the rat and mouse in which development of the SON and PVN is completed \textit{in utero}, development of these nuclear groups in the Brazilian opossum starts during the prenatal period and continues into the second week of postnatal life. The difference in the timing of neurogenesis in the rat and opossum has been reported in earlier studies for other hypothalamic regions including the suprachiasmatic nucleus and medial preoptic area\textsuperscript{32,41}.

Arginine vasopressin and oxytocin are two well known peptides, synthesized and secreted by the SON and PVN. Immunohistochemical studies have demonstrated that vasopressin is present in the SON and PVN in the rat on day 16 and 18 postfertilization, respectively\textsuperscript{10}, which is preceded by the completion of neurogenesis of the neurosecretory cells by day 13 and 15 postfertilization, respectively\textsuperscript{2}. Recently, our laboratory has demonstrated that in the opossum brain arginine vasopressin-like immunoreactivity is present
in the forming diencephalon (presumably the SON) on embryonic day 13 and in the PVN by 3 PN, and oxytocin-like immunoreactivity is detectable in the SON at 3 PN and in the PVN at 5 PN, while neurogenesis in these nuclei is not completed until 5 and 7 PN, respectively\textsuperscript{24}. These findings provide evidence that in contrast to that seen in the rat and mouse, in the opossum neurogenesis continues even after neuropeptides are identifiable in neighboring cells. Secondly, these results suggest that a portion of the SON and PVN neurons are functional during the continued development of the hypothalamo-neurohypophyseal system in early postnatal life.

Studies using tract tracing and immunohistochemical techniques have demonstrated that neurons in the parvicellular division of the PVN project to the brainstem and spinal cord through which the PVN is involved in regulation of autonomic functions\textsuperscript{11,33,34,36,42,44,49,51}. The development of projections from the PVN neurons to the brainstem and spinal cord is not known at present for \textit{Monodelphis}. Further studies are needed to determine the relationship of birth dates of these neurons and the development of their axonal projections. Such information will aid in understanding the functional significance of neuropeptide containing cells during early postnatal life.

In summary, we have studied the development of the SON and PVN in the Brazilian opossum, and have demonstrated that neurogenesis continues into the second week of postnatal life. Our results indicate that a portion of the AVP and OT secreting magnocellular neurons become postmitotic prior to birth. We have also shown that magnocellular and parvicellular neurons of the paraventricular nucleus have distinct birth dates reflecting the functional differences of these cell groups in the adult brain.
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References


31
20 Gray, J.W., Monoclonal antibodies against bromodeoxyuridine, Cytometry, 6 (1985) 499.
23 Ifft, J.D., An autoradiographic study of the time of final division of neurons in the rat hypothalamic nuclei, J. Comp. Neurol., 144 (1972) 193-204.
32


35 Millar, M.W. and Nowakowski, R.S., Use of bromodeoxyuridine immunohistochemistry to examine the proliferation, migration, and time of origin of cells in the central nervous system, Brain Res., 457 (1988) 44-52.


50 Swanson, L.W. and Sawchenko, P.E., Paraventricular nucleus: a site for the integration of neuroendocrine and autonomic mechanisms, Neuroendocrinology, 31 (1980) 410-417


Figure 1. Photomicrographs of single label bromodeoxyuridine (BrdU) immunohistochemistry showing progressive decrease in the number of BrdU labelled cells in the paraventricular nucleus (PVN) after injection on either day 1 of postnatal life (1 PN) (A), 3 PN (B), 5 PN (C), or 7 PN (D). No BrdU labelling was seen in the PVN after injection on 7 PN (D). All tissue was collected at 60 PN. The dotted outline indicates the location of the PVN in each of the photographs. 3V, third ventricles.

Bar = 100 μm
Figure 2. Photomicrograph of single label bromodeoxyuridine (BrdU) immunohistochemistry. Injection on day 1 of postnatal life (1 PN) resulted in few labelled cells in the supraoptic nucleus (SON) (A). No BrdU labelling was observed in the SON after injection on 5 PN (B). All tissue was collected at 60 PN. The dotted outline indicates the location of the SON. OX, optic chiasm Bar = 100 μm
Figure 3. Number of bromodeoxyuridine (BrdU) labelled cells in the supraoptic (SON) and paraventricular (PVN) nuclei injected with BrdU between days 1 and 11 PN. The number of BrdU labelled cells decreased with increasing age. Neurogenesis in the SON and PVN is completed by 5 and 7 PN.
Number of BrdU labelled cells

Postnatal Age (Day of BrdU Injection)

- PVN
- SON
**Figure 4.** Photomicrograph of bromodeoxyuridine (BrdU, dark black nuclei), arginine vasopressin (green fluorescence), and oxytocin (red fluorescence) immunoreactive cells in the paraventricular (PVN) and supraoptic nuclei (SON) of an animal injected with BrdU at day 1 of postnatal life. The majority of arginine vasopressin (AVP) or oxytocin (OT) cells in the magnocellular part of the PVN are not double labelled (A). Few double labelled cells BrdU-AVP and BrdU-OT were seen in the supraoptic nucleus (B). Black arrows indicate BrdU-AVP and white arrows indicate BrdU-OT double labelled cells. Bar = 100 μm
CHAPTER 2. LOCALIZATION OF ARGinine VASOPRESSIN-LIKE IMMUNOREACTIVITY IN THE ADULT AND DEVELOPING BRAZILIAN OPOSSUM BRAIN

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Abstract

In this study, we have examined the distribution and development of arginine vasopressin-like immunoreactivity (AVP-IR) in the brain of the adult and developing Brazilian opossum, Monodelphis domestica. In the adult, cell bodies containing AVP-IR were found in the lateral preoptic area, medial preoptic area, bed nucleus of the stria terminalis, periventricular hypothalamic nucleus, suprachiasmatic nucleus, paraventricular hypothalamic nucleus, supraoptic nucleus, lateral hypothalamic area, the region surrounding the substantia nigra, and dorsal raphe nucleus. Immunoreactive fibers were found to be widely distributed in the forebrain, hypothalamus, thalamus, midbrain, and brainstem. The distribution of AVP-IR generally resembled that reported for other species including the rat, which suggests that AVP may have similar physiological roles in Monodelphis. In the developing opossum brain, AVP-IR was first seen in the mesencephalon and diencephalon between embryonic days 12 and 13. Subsequently, a distinct group of AVP immunoreactive cells was present in the forming supraoptic nucleus on day 1 of postnatal life (1 PN) and at 3 PN in the paraventricular nucleus. Between 1 and 3 PN, a few cells transiently expressed AVP-IR in
the forming thalamus and tegmental area. At this age a few immunoreactive fibers were also
detected in the forming cerebellum. These fibers were not seen at later ages in these areas.
By 5 PN, an increased expression of AVP-IR was seen in the forming supraoptic and
paraventricular hypothalamic nuclei, median eminence, and posterior pituitary. At day 7 PN,
immunoreactive cells and fibers were seen in several forebrain areas. The distribution pattern
of AVP-IR became adult-like by 60 PN. A sex difference in the amount of AVP-IR in the
lateral septum was also observed in the opossum brain at 60 PN. This difference persisted
in the adult brain. Due to the early presence of AVP-IR in the Monodelphis brain before
neurogenesis and morphogenesis is completed, we suggest that AVP may be involved in
developmental events in addition to its well defined physiological functions. Further studies
will define the significance of the early presence of AVP during the differentiation of the
central nervous system.

Introduction

The neuropeptide arginine vasopressin (AVP) is mainly synthesized and secreted by
the neurosecretory neurons of the supraoptic (SON) and paraventricular (PVN) nuclei in the
hypothalamus. These neurons project to the limbic structures, median eminence, posterior
pituitary, midbrain, brainstem, and spinal cord (Buijs, 1978; Buijs et al., 1978; Buijs et al.,
1980; Boer et al., 1980; Swanson and Kuypers, 1980; De Vries and Buijs, 1983). Immunocytochemical studies have shown that AVP is also secreted by the small neurons of
the suprachiasmatic nuclei (Vandesande et al., 1975; Sofroniew and Weindl, 1980; De Vries
et al., 1981). In addition, arginine vasopressin-like immunoreactivity (AVP-IR) in cells and
fibers has also been observed in other brain areas/regions (Caffe and Van Leeuwen, 1983; Van Leeuwen and Caffe, 1983; De Vries et al., 1985; Buijs, 1990). Upon neuronal stimulation, AVP is released from the neural lobe of the hypophysis into the circulation and functions as a potent antidiuretic and vasoconstrictor hormone. As has been demonstrated in the adult rat, AVP also reaches a number of other brain areas through an extrahypothalamic fiber system. Based on physiological and pharmacological studies, it has been shown that AVP also acts as a neurotransmitter and/or neuromodulator in the central nervous system (Audigier and Barderis, 1985; Riphagen and Pittman, 1986; Buijs, 1987; Dubois-Dauphin and Zakarian, 1987; Van Leeuwen, 1987; Freund-Mercier et al., 1988; Poulin et al., 1988; Brinton and McEwen, 1989; Tribollet et al., 1988; 1989).

Arginine vasopressin has been described to play a role in many centrally controlled functions which include blood pressure regulation (Goa et al., 1992; Tanaka et al., 1993), central thermoregulation and antinociception (Oluyomi and Hart, 1992), circadian rhythmicity (Reghunandanan et al., 1990), water and salt balance (Zimmerman, 1983), drinking behavior (Mangiapane et al., 1983), food intake (Burlet et al., 1992), sexual and social behavior (Wang et al., 1993; Bluth and Dentzer, 1993; Winslow et al., 1993), learning and memory processes (De Wied, 1980), and neuroendocrine regulation of the anterior pituitary (Swanson and Sawchenko, 1980; Familari et al., 1989; Shen et al., 1993). Electrophysiological studies have demonstrated that vasopressin can directly excite central motor neurons and increase the excitability of facial motoneurons by generating a sodium dependant membrane current (Raggenbass et al., 1989; Raggenbass et al., 1991; Tribollet et al., 1991).
In addition to the above mentioned functional roles, it has been suggested that AVP is also involved in brain development. Brattleboro rats which are incapable of synthesizing AVP have disturbances in their brain development, body growth, and resulting avoidance behavior patterns (Boer et al., 1980; Boer, 1985; Snijdewint et al., 1988; Boer et al., 1993). Such alterations are suggestive of the importance of this peptide in growth and development of the central nervous system.

Several studies have described the distribution of AVP and its binding sites in the rat, hamster, sheep, and pig. Immunohistochemical studies have demonstrated that in addition to the supraoptic, paraventricular, and suprachiasmatic nuclei, AVP containing cells are also present in the bed nuclei of the stria terminalis, preoptic area, medial amygdala, and locus coeruleus (Caffe and Van Leeuwen, 1983; De Vries et al., 1985). Arginine vasopressin-like immunoreactive fibers have been localized in many brain areas including the olfactory tubercle, frontal cortex, diagonal bands of Broca, ventral pallidum, septum, amygdala, hippocampal formation, habenular complex, mammillary nuclei, substantia nigra compacta, ventral tegmental area, interpeduncular nucleus, raphe nuclei, central gray, locus coeruleus, nucleus of the solitary tract, dorsal vagal complex, and the intermediolateral nucleus of the spinal cord (Buijs, 1978; Buijs et al., 1978; Boer et al., 1980; De Vries et al., 1985). Using autoradiography, binding sites for arginine vasopressin have been reported in similar areas of the rat brain (Dubois-Dauphin et al., 1990; Phillips et al., 1990; Tribollet et al., 1991; Kremarik et al., 1993).

The ontogeny of AVP secreting cells has been studied in the rat and mouse using various techniques. In the rat, AVP and AVP-neurophysin immunoreactivity appear in the
supraoptic nucleus at day 16 postfertilization (pf), and on day 18 pf in the paraventricular nucleus (Buijs et al., 1980; Whitnall et al., 1985). Using radioimmunoassay, AVP immunoreactivity was first detected between day 14 and 15 pf in the rat hypothalamic neurosecretory system (Buijs et al., 1980). Pro-AVP mRNA appears in the rat and mouse supraoptic nucleus on day 15 pf and in the paraventricular nucleus on day 18 pf (Laurent et al., 1989). In the suprachiasmatic nucleus of the rat, AVP immunopositive cells and fibers first appear on day 2 of postnatal life (De Vries et al., 1981). Although the distribution and ontogeny of AVP has been reported for the adult and fetal rat neurohypophyseal system, the development of the AVP containing extrahypothalamic systems in the mammalian brain has not been adequately described.

It is becoming increasingly evident that many neuropeptides such as AVP act as neurotransmitters and/or neuromodulators in the adult central nervous system, but relatively less is known regarding the functional significance of neuropeptides during prenatal and early postnatal development of the mammalian brain. The early presence of neuropeptides and changes in their synthetic rates during differentiation suggest their involvement in early developmental events which occur in the central nervous system.

In order for AVP to have a potential role in the development, maturation, and/or formation of the central nervous system, it should be present very early in the forming brain, before neurogenesis and morphogenesis is completed. To test the hypothesis that AVP is potentially involved in neuronal differentiation and maturation, we have characterized the distribution and ontogeny of AVP-IR using immunohistochemistry in the brain of the adult and developing Brazilian gray short-tailed opossum, *Monodelphis domestica*. The Brazilian
Opossum is a small pouchless marsupial which breeds well under laboratory conditions. Its young are born after 14 days of gestation in an extremely immature state, before neurogenesis and organogenesis is completed (Jacobson, 1984; Larsen and Jacobson, 1986; Saunders et al., 1989; Iqbal et al., 1994). Like most marsupials, Monodelphis has a protracted postnatal developmental period. Further, this animal does not have a pouch which allows easy access to developing pups and the ability to conduct developmental studies.

Materials and Methods

Animals

Adult and developing male and female Brazilian short-tailed opossums from a colony at Iowa State University were utilized in this study. The animals were housed in plastic cages, maintained on a 14:10 hours light dark cycle with a constant temperature (26 °C). The animals were provided water and food ad libitum (Reproduction Fox Chow; Milk Specialties Products, Madison, WI). In the first part of the study, the distribution of AVP-like immunoreactivity was investigated in four female and four male adult opossum brains. In the developmental part of the study, developing opossum pups of various ages from embryonic day 12 through day 60 of postnatal life were examined.

To obtain postnatal animals, an adult female and male animal were housed together for two weeks. Subsequently, the animals were separated, and the females checked daily for the birth of pups. The gestational period of the Brazilian opossum is approximately 14 days postfertilization. The day of birth of the pups was designated as day 1 of postnatal life (1 PN). To obtain embryonic opossums, four pairs of animals were videotaped during the dark
phase of the light dark cycle in order to identify the time of mating. To collect opossum embryos of known postfertilization age the day of mating was considered as embryonic day 0 (E 0). Since the gender of the embryos and pups cannot be identified grossly before 5 PN, the embryos and pups prior to day 5 PN were considered sexually undifferentiated. For the developmental part of the study, four embryos and four postnatal pups were investigated at the ages of E 11, 12, 13, and 1, 3, and 5 PN. At later ages, six animals (3 males and 3 females) from at least two different litters, were studied at each 7, 10, 15, 25, 35, 45, and 60 PN.

Tissue collection

The brains from the adult and developing animals were collected for immunohistochemistry as has been described previously (Fox et al., 1991a; Elmquist et al., 1992). To collect the embryos, the dam was killed by overetherization. Immediately the uteri were removed from the abdominal cavity, and opened in a petri dish containing 0.9% saline solution. The embryos were carefully transferred to Zamboni’s fixative for 48 hours. Subsequently, the embryos were sunk in 30% buffered-sucrose solution overnight, cut into 20 μm thick sagittal or coronal sections on a cryostat, thaw mounted onto poly-L-lysine coated slides, and stored at 4°C until immunohistochemistry was conducted.

Immunohistochemistry

The protocol utilized for immunohistochemistry was a modification of that which has been reported previously (Fox et al., 1991a; Elmquist et al., 1992). The slide mounted
sections were washed in 50 mM potassium phosphate-buffered saline (KPBS) solution followed by 30 minute incubation in 0.3% hydrogen peroxide solution. After blocking in normal goat serum (Vector; 1:67), the tissue sections were incubated in AVP primary antibody (made in rabbit; Miles Scientific, 1:5000) in a humidified chamber for 20 hours at room temperature. After adequate rinsing with KPBS, the tissue sections were incubated in goat anti-rabbit IgG (Vector; 1:800) for 2 hours, rinsed, and exposed to avidin-biotin complex (Vector Elite Kit; 1:200) for one hour in a humidified chamber at room temperature. After washing, the tissue sections were stained by reacting with 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 solution. After staining for 5-6 minutes, the tissue sections were washed in 0.9% saline solution two times to terminate the reaction. The sections were then dehydrated in an ascending series of alcohols, cleared in xylene and coverslipped with permount mounting media, and analyzed with the light microscope.

The unmounted adult tissue sections were processed for immunohistochemistry utilizing a free floating tissue technique reported previously (Fox et al., 1991a; Elmquist et al., 1992). The results obtained with the free floating tissue processing technique were comparable to those obtained for the mounted tissue sections.

**Immunohistochemical control procedures**

Both negative and preabsorption control procedures were run in parallel with the tissue to be immunostained. Negative controls were generated by incubation of the tissue in
the normal goat serum instead of primary antisera. No specific staining of the tissue was observed.

Preabsorption controls were carried out by co-incubating the tissue sections with arginine vasopressin peptide (15 μM; Sigma) and primary antibody. All staining was abolished in the tissue sections incubated in the primary antisera along with the peptide, whereas concurrent incubation of tissue sections in the primary antibody with either lysine-vasopressin or oxytocin (15 μM; Sigma) did not eliminate any of the specific immunostaining observed.

Analysis of tissue

Tissue sections at 60 μm intervals from areas caudal to the olfactory bulbs to the caudal brainstem from the adult animals and consecutive sections at 20 μm interval from the developing animals were analyzed. Sections were examined with a light microscope and structures containing AVP-IR were identified with the aid of maps of coronal sections of the opossum brains as have been described previously (Fox et al., 1991a; Elmquist et al., 1992) and an atlas of the developing rat brain (Paxinos et al., 1991). Brain structures containing AVP-IR in the embryos were identified with the aid of an atlas of mouse development (Kaufman, 1992) and an atlas of the prenatal mouse brain (Schambra et al., 1992).

Results

In this report we have described the distribution and ontogeny of hypothalamic and extrahypothalamic AVP-IR in the brain of the adult and developing opossum. Although in
the present study none of the animals were colchicine treated prior to use, AVP-IR was observed in cell bodies in several nuclear groups and in immunoreactive fibers in several regions throughout the brain of both adult and developing opossums.

**AVP immunoreactive somata in the adult opossum brain**

Arginine vasopressin immunoreactive cell bodies were detected in several nuclear groups and areas in the Brazilian opossum brain. Regions included the lateral preoptic area, medial preoptic area, bed nucleus of the stria terminalis, periventricular preoptic area, suprachiasmatic nucleus, paraventricular hypothalamic nucleus (Fig. 1A), supraoptic nucleus including its retrochiasmatic part (Fig. 1B), and scattered cells in the lateral hypothalamic area. Cells containing AVP-IR were also seen around the substantia nigra (Fig. 1C), and the dorsal raphe nucleus (Fig. 1D). Although we did not quantify the number of cells with AVP-IR in the opossum brain, the periventricular hypothalamic nucleus, paraventricular, supraoptic and suprachiasmatic nuclei each contained a dense collection of AVP immunopositive cells.

**AVP immunoreactive fibers in the adult opossum brain**

In the adult Brazilian opossum, AVP-IR in fibers was widely distributed throughout the brain, extending from the olfactory bulbs to the spinomedullary junction. For the purpose of description, the distribution of AVP immunopositive fibers is described as; low, if there was a few to single scattered immunoreactive fibers seen in the field area; dense, if there was an intense immunoreactivity and most of the field area was covered with immunostained
fibers; and moderate, if the immunoreactivity in the area was between dense and low.

**Rhinencephalon**

A low amount of immunoreactive fibers were observed in the limbic cortex, olfactory tubercle, and anterior olfactory nucleus. A moderate amount of immunoreactive fibers were present in the septohippocampal and medial septal nuclei. A dense collection of fibers was seen in the parietal cortex area 1.

**Telencephalon**

A low to moderate amount of immunoreactive fibers were present in the parietal cortex, presubiculum, and subiculum (Fig. 2A). The nuclei of the horizontal and vertical limbs of the diagonal band each contained a dense collection of immunoreactive fibers (Fig. 2B,C). In the lateral septum, the amount of immunoreactivity was different in male and female animals. Specifically, there was a greater amount of immunoreactivity observed in the male (Fig. 3A) as compared to that of the female (Fig. 3B). The lateral preoptic area including its magnocellular division, the medial preoptic area, and median preoptic nucleus each contained a moderate to dense amount of immunoreactive fibers. The bed nucleus of the stria terminalis contained a moderate number of immunoreactive fibers. A moderate amount of fibers was observed throughout the cortex running parallel to the external capsule, and a dense amount of immunoreactivity was present in the endopiriform nucleus. A low amount of immunopositive fibers were also seen in the islands of Calleja, as well as in the ventral pallidum. Among the limbic structures, the dentate gyrus contained moderate levels
of immunoreactive fibers and the CA1, CA2, and CA3 areas of Ammon's horn contained
a low amount of immunopositive fibers. A moderate to dense amount of immunoreactive
fibers was seen in the entorhinal cortex, anterior cortico-amygdaloid nucleus, anterior
amygdaloid area, and medial amygdala (Fig. 4A)

Diencephalon

A dense accumulation of AVP-IR in fibers was present in the paraventricular and
supraoptic hypothalamic nuclei. The dorsomedial hypothalamic nucleus contained a moderate
amount of immunoreactive fibers. Few immunoreactive fibers were also seen in the
ventromedial hypothalamic nucleus, whereas the lateral hypothalamic area contained a dense
amount of fibers. A dense collection of immunoreactive fibers was observed in the lateral
and medial habenular complex (Fig. 4B). A moderate to dense amount of immunoreactive
fibers was consistently seen in the various thalamic nuclei including the subparatenial,
mediodorsal, and paraventricular nuclei (Fig. 4B). A dense amount of AVP immunoreactive
fibers was consistently observed in regions including the dorsal premammillary nucleus (Fig.
5A), dorsal lateral and ventral lateral geniculate nuclei (Fig. 5B). No significant sex
difference in amount of immunoreactivity was seen for any of the diencephalic regions.

Mesencephalon

In the mesencephalon, a moderate amount of immunoreactive fibers were present in
the mammillotegmental tract, interpeduncular nucleus, periaqueductal gray, superior and
inferior colliculi, and ventral tegmental area.
Metencephalon

A low to moderate amount of immunoreactive fibers were present consistently in the pontine reticular formation, dorsal and median raphe (Fig. 1D), reticulotegmental pontine nuclei, parabrachial nuclei, locus coeruleus and the medial longitudinal fasciculus. Single immunoreactive fibers were seen in the facial motor nucleus.

Myelencephalon

A low to moderate amount of immunoreactive fibers were present in the lateral reticular nucleus, medial longitudinal fasciculus, inferior olivary nucleus, nucleus ambiguus, nucleus of the spinal tract of the trigeminal, nucleus of the solitary tract, and dorsal motor nucleus of vagus (Fig. 6).

AVP immunoreactive somata and fibers in the developing opossum brain

The opossum embryos are extremely immature and of small size at embryonic day 11 and undergo very rapid developmental changes between embryonic day 12 and 13. At this age the Brazilian opossum embryo resembles that of the developmental stage 33-34 Virginia opossum as described by McCrady (1938). At these stages of development, the brain of the opossum embryo morphologically resembles a neural tube. By birth, only the major divisions of the brain are recognizable. In the embryos and neonatal opossums used in this study, the brain is in the process of formation with neurogenesis and morphogenesis still actively occurring. Most of the nuclear groups and brain regions are differentiated enough to be identified at the age of 15 PN. Thus before this age, the specific area or nuclear group
discussed would be a presumptive or forming area, tract, or nuclear group.

*Embryonic Days 11-13*

As stated above, the brain of the embryo is extremely immature. Using immunohistochemistry no AVP-IR was detected at embryonic day 11. A few cells and fibers containing AVP-IR were detected in the mesencephalon for the first time at embryonic day 12 (Fig. 7A,B). By embryonic day 13, AVP-IR in cells was present in the diencephalon lateral to the third ventricle (Fig. 7C,D), and the number of immunoreactive cells detected in the mesencephalon increased in number and amount of immunoreactivity. Moderately immunostained fibers were seen in the diencephalon, mesencephalon, and brainstem.

*Day 1 of postnatal life*

The opossum brain at 1 PN is still very immature and is undergoing active morphological and developmental changes and different nuclear groups can not be distinguished.

Cells and fibers containing AVP-IR were visualized in the 1 PN opossum brain in several areas. The cell bodies were detected in the forming hypothalamus; presumably in the forming supraoptic nuclei (Fig. 8A,B). A collection of lightly stained cell bodies were seen in the forming thalamus and the midbrain tegmental area (Fig. 8C,D).

Fibers containing AVP-IR were observed in several areas in the 1 PN brain. In the forebrain, fibers were seen in the basal forebrain, hypothalamus, and median eminence (Fig. 8A,B). In the midbrain, immunoreactive fibers were present in the tegmentum, pons,
and in the medulla (Fig. 9A,B). Few scattered immunoreactive fibers were detected in the cerebellum at this age.

Day 3 of postnatal life

At 3 PN, although the brain has become more matured as compared to that seen at 1 PN, many brain regions and nuclear groups are still in the process of formation. As was observed in the 1 PN brain, AVP-IR was seen in many regions of the developing brain. The immunopositive cells in the supraoptic nuclei increased in number and staining intensity (Fig. 10A,B). Cells containing light immunostaining were detected lateral to the third ventricle, in the presumptive paraventricular nucleus and lateral hypothalamus (Fig. 10A,B). Few lightly stained cells were seen in the dorsal thalamus (Fig. 10C,D). The cells seen in the forming tegmental area in the 1 PN brain were not present at 3 PN.

Immunoreactive fibers increased in distribution and density in areas seen at 1 PN. A dense collection of fibers coursing towards the median eminence from the immunoreactive cells present in the forming supraoptic nucleus were seen (Fig. 10A,C). In addition, there was a moderate amount of immunoreactivity in the posterior pituitary (Fig. 10C).

Day 5 of postnatal life

At postnatal day 5, the brain is further organized and some of the brain regions and nuclear groups are becoming identifiable. By day 5 PN, there was a robust expression of AVP-IR in the supraoptic nucleus including its retrochiasmatic part, and cells containing AVP-IR in the paraventricular hypothalamic nucleus increased in number and
immunostaining intensity (Fig. 11A,B). Immunoreactive fibers of some cells were seen projecting into the ependyma of the third ventricle. Many immunoreactive cells were seen in the lateral hypothalamic area (Fig. 11B). These cells appeared to be in the process of lateral migration. The cells seen in the dorsal thalamus at 3 PN were not observed at this age. In addition, few lightly stained cells were seen in the lateral preoptic area and the bed nucleus of the stria terminalis. The distribution and density of immunoreactive fibers at 5 PN was increased as compared to that seen at 3 PN.

Fibers containing AVP-IR were detected in the forming lateral septum, lateral preoptic area, and vascular organ of the lamina terminalis.

Day 7 of postnatal life

At the age of 7 PN, many brain regions and nuclear groups are still forming and undergoing rapid changes. The distribution and quantity of AVP-IR in the supraoptic and paraventricular nuclei increased significantly by 7 PN (Fig. 11C,D). Since the supraoptic and paraventricular hypothalamic nuclei are not yet completely formed, many intensely immunostained cells present in the lateral hypothalamus still appeared to be in the process of migration (Fig. 11D). As compared to what was seen at 5 PN, the immunopositive cells in the lateral preoptic area and bed nucleus of the stria terminalis increased in number and intensity of immunostaining. Cells containing AVP-IR were also detected in the periventricular preoptic area at this age.

Immunoreactive fibers in the lateral hypothalamic area, median eminence, and posterior pituitary showed very intense immunostaining at 7 PN (Fig. 12). The fibers
containing AVP-IR also increased in numbers and were detected in additional structures at day 7 PN. Specifically fibers were observed in the magnocellular division of the lateral preoptic area, median preoptic nucleus, nuclei of the vertical and horizontal limbs of the diagonal bands, and ventral pallidum. Immunoreactive fibers were also observed in the pons, dorsal raphe magnus and central gray.

Day 10 of postnatal life

At 10 PN, the supraoptic and paraventricular nuclei are easily recognized and showed further increase in immunoreactivity both in the somata and fibers present. (Fig. 13A,B,C). Although, the subdivisions of the paraventricular nucleus are not clearly defined, it appeared from the size of the immunoreactive cells and the pattern of immunostaining that the majority of AVP immunoreactive cells were magnocellular neurons (Fig. 13B). Cells in the internuclear zone also increased in number and amount of immunostaining. A few scattered cells were observed in the forebrain areas including the medial preoptic area. An increased number of immunopositive cells were observed in the periventricular preoptic area at this age. A few cells and a dense collection of immunoreactive fibers were detected in the vascular organ of the lamina terminalis and a few immunoreactive fibers were also detected in the optic chiasm. A low to moderate amount of immunostained fibers were seen in the lateral preoptic area, diagonal bands of Broca, and islands of Calleja at this age. Fibers in the lateral hypothalamic area projecting to the median eminence and posterior pituitary showed a further increase in the intensity of immunostaining. Additional immunoreactive fibers seen projected to the arcuate nucleus. A moderate amount of immunoreactivity was
detected in many areas which included the premammillary nucleus, ventral tegmental area, median raphe nucleus, and dorsal tegmental nucleus. Few fibers were also seen in the raphe pallidal and in the pyramids in the medulla.

**Day 15-20 of postnatal life**

Between days 15 and 20 PN, most of the nuclear groups and areas in the opossum brain are well formed and clearly recognizable using the light microscope. The paraventricular nucleus has started assuming its characteristic adult shape and its subdivisions can be recognized. Based on the size and location of the immunostained cells it appeared that the magnocellular divisions contained more intensely stained cells whereas the parvicellular division contained less lightly stained cells. The pattern of AVP-IR in somata and fibers observed in other areas was not significantly different from that observed on 10 PN, except for a generalized increase in the intensity of immunostaining in the cells and fibers. At 20 PN, lightly immunostained cells were also seen in the suprachiasmatic nucleus.

**Day 25-35 of postnatal life**

The brain of the opossum at 25 PN is fairly well differentiated and resembles that of the adult in shape and nuclear organization. Immunostaining in the paraventricular and supraoptic hypothalamic nuclei begins to resemble that of the adult (Fig. 14 A,B,C). In the paraventricular nucleus, AVP immunoreactive cells were present in all of its subdivisions, however, most of the immunoreactive cells were present in the dorsal and medial magnocellular regions (Fig. 14B). At 25 PN, an intense AVP-IR appeared in the
suprachiasmatic nucleus (Fig. 15). A few labelled cells were also seen on the dorsal and ventral boundaries of the reticular part of the substantia nigra. A moderate amount of immunoreactive fibers were detected in the dentate gyrus and CA1, CA2, and CA3 areas of Ammon’s horn. Fibers were also seen in the cortex running parallel to the external capsule and in the medial amygdala at this age. A low density of immunoreactive fibers were also present in the paraventricular thalamic and intermediodorsal thalamic nuclei. In addition, fibers were seen in the subparatenial nucleus, habenular complex, and interpeduncular nucleus. Immunoreactive fibers in the medial septal area (Fig. 16A), vascular organ of the lamina terminalis (Fig. 16B), and lateral hypothalamic area showed further increase in immunostaining (Fig. 16C).

Days 45-60 of postnatal life

The distribution of AVP immunoreactive elements in the hypothalamic and extrahypothalamic brain regions at days between 45-60 PN were very similar to that seen in the adult opossum brain with respect to location and density of immunoreactivity (Fig. 17A,B,C, D). At 60 PN, a collection of small cells containing AVP-IR were observed in the dorsal raphe nucleus.

Although we did not quantify the immunoreactive fibers in the lateral septum, there was an apparent sex difference in the amount of immunoreactive fibers at 60 PN. The brain of male pups had a slightly greater number of immunoreactive fibers in the lateral septum (Fig. 3C) than that which was seen in the brain of the female pups of the same age (Fig. 3D). This difference was not apparent at 45 PN.
Discussion

In this study we have described the distribution and ontogeny of AVP-IR in the brain of the Brazilian opossum, *Monodelphis domestica*. In general, the distribution of AVP-IR in cell bodies and fibers of the adult opossum brain was similar to that which has been reported for other mammalian species including the rat (Vandesande et al., 1975a&b; Swaab et al., 1975; Sofroniew et al., 1979; Sofroniew and Weindl, 1980; Rhodes et al., 1981; De Vries et al., 1981; Caffe and Van Leeuwen, 1983; Van Leeuwen and Caffe, 1983; De Vries et al., 1985; Caffe et al., 1989; Dubois-Dauphin et al., 1990). In the opossum brain, cells having AVP-IR were detected in several areas including the lateral preoptic area, medial preoptic area, bed nucleus of the stria terminalis, periventricular preoptic area, paraventricular nucleus, supraoptic nucleus, lateral hypothalamic area, area around the substantia nigra, and dorsal raphe nucleus. Arginine vasopressin immunoreactive fibers were present throughout the brain extending from the olfactory bulbs to the spinomedullary junction in similar extra-hypothalamic areas as has been reported for the rat (Buijs, 1978; Buijs et al., 1978; De Vries et al., 1981; De Vries and Buijs, 1983; De Vries et al., 1985).

Arginine vasopressin containing fibers which project to the median eminence and arcuate nucleus from the parvicellular region of the paraventricular nucleus have been shown to be involved in the regulation of release of ACTH, endorphin, and prolactin from the anterior pituitary (Kjoer et al., 1992). Using immunohistochemical techniques, more than 20 neuropeptides have been localized in the paraventricular and supraoptic nuclei, and many are reported to be colocalized (Brownstein and Mazey, 1986; Hokfelt et al., 1989). Arginine vasopressin has been colocalized with galanin in the bed nucleus of the stria terminalis, and
medial amygdala of the rat (Miller et al., 1993; Planas et al., 1994). Such findings suggest that AVP might be acting synergistically with other peptides in the regulation of neuroendocrine functions in the brain.

The opossums used in this study were not treated with colchicine, yet AVP immunoreactive cell bodies were detected in many areas of the adult opossum brain. In general, the distribution resembled that reported for other species, particularly the rat. However, as compared to that of the rat, some differences exist in the localization of AVP cell bodies in the *Monodelphis* brain. In the rat, AVP-like immunoreactive cell bodies have also been localized in the medial amygdala and locus coeruleus (Caffe and Van Leeuwen, 1983; De Vries et al., 1985). In the opossum brain these areas were devoid of immunoreactive cell bodies and contained only a moderate amount of immunoreactive fibers. However, in the opossum immunopositive cell bodies were seen around the substantia nigra and in the nucleus of the dorsal raphe, areas not reported in other species. There could be several explanations for these findings. One possibility is that AVP secreting cells are present in the medial amygdala and locus coeruleus of the opossum, but the peptide is transported at a fast rate and therefore not detectable using our immunohistochemical protocols. Another explanation could be due to species differences, as we have detected cell bodies in areas which have not been reported even in the colchicine treated rat. This explanation is also supported by our previous findings that galanin containing cell bodies were not seen in the locus coeruleus of the opossum, whereas the rat contains an abundant number of galanin containing cell bodies in the locus coeruleus (Elmquist et al., 1992). The significance behind the species differences is not presently known.
Brazilian opossum pups are born after a 14 day gestational period. Unlike that of the rat, the opossum brain is extremely immature at birth. Based on previous studies conducted in our laboratory and in several others (Schwanzel-Fukuda et al. 1988; Saunders et al., 1989; Fadem and Harder, 1992; Elmquist et al., 1992; Elmquist et al., 1994; Iqbal et al., 1994), we believe that the stage of neural development of the opossum pups seen at birth corresponds to that of the 13-14 day of gestation rat. Further the 16 PN opossum pup resembles that of the 1 PN rat pup. In the rat, neurogenesis is considered a prenatal phenomenon; whereas, in the opossum brain neurogenesis and morphogenesis actively continues into the postnatal period (Larsen and Jacobson, 1986; Rivkees et al., 1988; Iqbal et al., 1994). In the rat and mouse, cells destined to form the supraoptic and paraventricular nuclei are formed between days 12 and 14 postfertilization (pf) and settle in the supraoptic and paraventricular nuclei between days 15 and 16 pf, respectively (Altman and Bayer, 1978). Arginine vasopressin-neurophysin and AVP-IR in the rat and mouse appear in the supraoptic nucleus on day 16 pf and in the paraventricular nucleus at day 18 pf, however, AVP precursors have been demonstrated using radioimmunoassay at day 14 pf in the mouse (Buijs et al., 1980; Boer et al., 1980; Whitnall et al., 1985; Laurent et al., 1989; Lazcano et al., 1990; Hyodo et al., 1992). In *Monodelphis*, AVP-IR is present in the mesencephalon and diencephalon as early as between E 12 and 13, and cell bodies containing AVP-IR are visible in the forming supraoptic and paraventricular nuclei at 1 and 3 PN, respectively.

In contrast to that of the rat and mouse, in the opossum brain neurogenesis in the supraoptic and paraventricular nuclei is completed by day 5 and 7 PN, respectively (Iqbal et al., 1994), and in other areas at even later times during the postnatal period (Larsen and
Jacobson, 1986; Rivkees et al., 1988; Swanson et al., 1994). Previously, we have reported that neurogenesis in the supraoptic and paraventricular nuclei is completed in postnatal life. The majority of the magnocellular neurons in the supraoptic and paraventricular nuclei are born prenatally whereas parvicellular neurons continue to be formed during the postnatal period of life (Iqbal et al., 1994). Although, the organization and cytoarchitectonic subdivisions of the paraventricular nucleus of the opossum have not been studied, based on the size and location of the cells and the early presence of AVP-IR in the supraoptic and paraventricular nucleus we believe that the magnocellular neurons become functional earlier than the parvicellular neurons.

Interestingly we also detected transiently expressed AVP immunoreactive fibers in the forming cerebellum at 1 PN, which were not detected after 5 PN. Cells containing AVP-IR in the forming thalamus and tegmentum detected at E 13 and 1 PN were not seen after day 3 PN. Presently, we do not have an explanation for the transient expression of AVP-IR in these areas. Further studies are required to know if these cells migrate to other areas of the brain, or synthesize AVP at a specific time period which then acts as an early, transient neurotransmitter or neuromodulator or die when the peptide is no longer required or when another peptide system becomes functional. The involvement of AVP in the early development of the central nervous system has been reported previously (Boer, 1985; Snijdewint et al., 1988; Boer et al., 1993; Brinton and Brownson, 1993). In Monodelphis, neurogenesis in the midbrain and brainstem is completed earlier than that of the hypothalamus and forebrain (Iqbal and Jacobson, unpublished results). Our findings of transient expression of AVP-IR in the forming tegmentum and cerebellum, and its presence
in the forebrain before neurogenesis is completed suggest that AVP may play a significant role in the development of the mammalian central nervous system.

As mentioned before, in the opossum brain AVP is detectable very early during embryonic life. The distribution of AVP immunoreactive elements increased in location and intensity of immunoreactivity with increasing age, and was detected in many extrahypothalamic areas including the olfactory tubercle, preoptic area, septal region, and vascular organ of the lamina terminalis during the first two weeks of postnatal life. These areas are involved in many centrally mediated functions such as drinking behavior, body fluid and cardiovascular regulation, thermoregulation, olfaction, and learning and memory (Tanaka et al., 1993; Bluth and Dentzer, 1993; Paredes et al., 1993; Kremarik et al., 1993; Larriva-Shad et al., 1993). The early presence of AVP in the young opossum brain further suggests that this peptide might have some neuromodulatory functions in the forming brain and have physiological significance in the neonatal opossum as well as in the adult animal.

Although at present it is not fully known when the hypophyseal portal circulation matures and becomes functional in the opossum; vascularization of the pars distalis of the adenohypophysis takes places in the developing opossum as early as 4 PN (Gasse and Meyer, 1993). In the present study, we detected AVP-IR in fibers projecting from the neurons in the forming supraoptic nucleus towards the median eminence at day 1 PN. Between day 3 and 5 PN robust immunoreactivity was present at the level of the median eminence and an intense amount of immunostaining was also seen in the posterior pituitary at 5 PN. Binding sites for AVP (Kuehl-Kovarik et al., 1994) and ACTH-like immunoreactivity is present between 5 and 10 PN in the anterior pituitary of developing opossum (Elmquist and
Jacobson, unpublished observations). These findings suggest that AVP might also be released into the circulation in the opossum, and be playing a neuromodulator-like role in the hypothalamo-hypophyseal axis during early postnatal life. One of the possible functions of AVP may include regulation of ACTH release from the anterior pituitary in order to acclimatize the pups to the stressful *ex utero* environment.

In extrahypothalamic areas, AVP containing somata have been identified in the bed nucleus of the stria terminalis and medial amygdala (Van Leeuwen and Caffe, 1983; De Vries et al., 1985; Wang et al., 1993; Miller et al., 1993; Planas et al., 1994). In our studies we could not detect any cell bodies in the medial amygdala but cell bodies were present in the bed nucleus of the stria terminalis and lateral preoptic area. A sex difference in the density of AVP immunoreactive fibers in the lateral septum and lateral habenula have been reported in the rat (De Vries et al., 1981; 1985). It has been reported that AVP expressing neurons in the bed nucleus of the stria terminalis provide a greater density of immunoreactive fibers to the lateral septum in the male as compared to that of the female (De Vries et al., 1981; 1985; Wang et al., 1993). De Vries and co-workers (1981) have demonstrated that the difference in the density of immunoreactive fibers in the rat appears at day 12 of postnatal life and persists into adult life. In the present study, we have also observed sex differences in the density of immunoreactive fibers in the lateral septum in the adult opossum brain. In contrast to the data for the rat, no significant difference in immunoreactivity was present in the lateral habenula of male and female opossums. Although we did not quantify the amount of immunoreactive fibers in the lateral septum of the developing opossum brain, the sex difference in the density of immunoreactivity became
apparent at day 60 PN. In the rat, puberty occurs between day 50 and 60 of postnatal life, whereas in the opossum puberty is not reached until day 145-150 of postnatal life. These findings indicate a delayed onset of puberty and sexually dimorphic behaviors in the opossum as compared to that in the rat.

In the rat, AVP-IR in the lateral septum and cells in the bed nucleus of the stria terminalis and medial amygdala have been reported to be influenced by the levels of the circulating gonadal steroid hormones (De Vries et al., 1981; 1985; Wang et al., 1993; De Vries et al., 1994). Recently, estrogen and androgen receptors have been localized in the AVP immunoreactive neurons in the bed nucleus of the stria terminalis (Axelson and Van Leeuwen, 1990; Zhou et al., 1994). Like AVP, other neuropeptides such as cholecystokinin (Frankfurt et al., 1985; Fox et al., 1990), and tachykinin (Akesson, 1993) in the mammalian brain are also reported to be sexually dimorphic and/or their level of immunoreactivity depends upon the levels of circulating steroid hormones. Previously, our laboratory has reported a sex difference in cholecystokinin-like immunoreactivity in the medial preoptic area of the opossum which becomes apparent between 25 and 35 PN. Estrogen receptor-like immunoreactivity appears in the medial preoptic area of the opossum brain at 15 PN (Fox et al., 1991 b&c) and suggests that estrogen may be involved in the establishment of the sex difference in cholecystokinin-like immunoreactivity. Currently, we have begun studies to investigate the ontogeny of androgen receptors in the opossum brain to understand the interactions of neuropeptides and gonadal steroid hormones in the developing brain.

In summary, in this report we have described the distribution and ontogeny of AVP-IR in the brain of the Brazilian opossum. Somata containing AVP-IR were seen in several
specific brain regions and nuclear groups and immunoreactive fibers were distributed throughout the opossum brain. The general distribution of AVP-IR in the opossum brain resembles that which has been reported for other species. These findings suggest that AVP may have similar physiological functions in the opossum as has been documented for other species. In addition, AVP-IR in somata and fibers in the developing opossum brain was detected as early as embryonic days 12 and 13. The early presence of AVP-IR in the embryonic opossum brain, while neurogenesis and morphogenesis is actively taking place suggests that AVP might have some significant physiological functions during the development of the central nervous system in the neonatal opossum.

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Literature cited


San Diego.


Immunocytochemical localization of luteinizing hormone-releasing hormone (LHRH) in the nervus terminalis of the adult and early neonatal gray short-tailed opossum (Monodelphis domestica). J. Comp. Neurol. 27:44-60.


Figure 1. A series of photomicrographs of coronal sections demonstrating AVP-IR in somata in the paraventricular hypothalamic nucleus (A), supraoptic nucleus (B), area around the substantia nigra (C), and in the dorsal raphe nucleus (D) in the brain of an adult opossum. Abbreviations: 3V, third ventricle; 4V, fourth ventricle; OX, optic chiasm; SN, substantia nigra; DR, dorsal raphe nucleus. The arrow in photomicrograph C points towards the midline. All photomicrographs are at the same magnification. Scale Bar = 100 μm.
Figure 2. Photomicrographs demonstrating AVP-IR in fibers in the subiculum (A) and in the medial septal area (B) in the brain of an adult opossum. C is a higher magnification photomicrograph of the area represented by the box in B. Abbreviations: LV, lateral ventricle; VDB, nucleus of the vertical limb of the diagonal band; HDB, nucleus of the horizontal limb of the diagonal band. Scale bar = 100 μm in A and C; 300 μm in B.
Figure 3. A series of photomicrographs of coronal sections demonstrating a sex difference in AVP immunoreactive fibers in the lateral septum of an adult male (A) and an adult female (B) opossum. The sex difference in AVP-IR in the lateral septum first becomes detectable in the male (C) and female (D) at 60 PN. Abbreviations: LV, lateral ventricle. All photomicrographs are at the same magnification. Scale bar = 100 μm.
Figure 4. Photomicrographs demonstrating AVP-IR in fibers in the medial amygdala (A), habenula and paraventricular thalamic nuclei (B) in the brain of an adult opossum. Abbreviations: 3V, third ventricle; Opt, optic tract; HB, habenular complex; PTA, paraventricular thalamic nucleus. Both photomicrographs are at the same magnification. Scale bar = 100 μm.
Figure 5. Photomicrographs of coronal sections from the brain of an adult opossum demonstrating the distribution of AVP-IR in fibers in the dorsal premammillary nucleus (A), and the dorsal and ventral lateral geniculate nucleus (B). Abbreviations: 3V, third ventricle; DLG, dorsal lateral geniculate nucleus; VPM, ventral premammillary nucleus. Both photomicrographs are at the same magnification. Scale bar = 100 μm.
Figure 6. A photomicrograph demonstrating AVP-IR in the nucleus of the solitary tract and dorsal motor nucleus of the vagus. Abbreviations: 4V, fourth ventricle; 10, dorsal motor nucleus of the vagus; 12, hypoglossal nucleus; cc, central canal. Scale bar = 100 μm.
Figure 7. Photomicrographs of sagittal sections demonstrating AVP-IR in opossum embryos. AVP-IR was first detected at embryonic day 12 in the mesencephalic flexure (box, A) and at embryonic day 13 AVP-IR was seen in the diencephalon as well (box, C). B and D are higher magnification photomicrographs of the areas indicated by the boxes in A and C. Arrows in B and D point to the AVP-IR in these areas, respectively. Note the immaturity of the opossum brain at embryonic days 12 and 13. Abbreviations: 4V, fourth ventricle; LV, lateral ventricle. The asterisk indicates the location of Rathke's pouch. Scale bar = 200 μm in A and C; 100 μm in B and D.
Figure 8. Photomicrographs of coronal sections through the 1 PN opossum brain demonstrating a distinct group of AVP immunoreactive cells in the developing hypothalamic supraoptic nucleus (arrow) and immunoreactive fibers projecting towards the median eminence (A). A few AVP immunoreactive cells were also seen in the forming tegmental area (box) in the 1 PN brain (C). B is a higher magnification photomicrograph of A, and D is a higher magnification view of the area indicated by the box in C. Abbreviations: 3V, third ventricle; 4V, fourth ventricle; ME, median eminence. Scale bar = 200 μm in A and C; 100 μm in B and D.
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Figure 10. Photomicrographs of coronal sections demonstrating an increase in the amount and intensity of AVP-IR in the somata and fibers in the supraoptic nucleus and median eminence at 3 PN (A). B is a higher magnification view of A, showing some lightly immunostained cells at 3 PN lateral to the third ventricle (arrow). At 3 PN, a few AVP-IR cells are also present in the dorsal thalamus (C, box) and moderate AVP-IR was present in the posterior pituitary (C). D is a higher magnification view of the area indicated by the box in C. Abbreviations: 3V, third ventricle; ME, median eminence. Scale bar = 200 μm in A and C; 100 μm in B and D.
Figure 11. Photomicrographs of coronal sections through 5 PN (A,B) and 7 PN (C,D) brains. B and D are higher magnification photomicrographs of A and C, respectively. Many AVP immunoreactive cells are present in the internuclear zone of the lateral hypothalamic area. These cells appear to be in the process of lateral migration. Abbreviations: 3V, third ventricle; PVN, paraventricular hypothalamic nucleus; SON, supraoptic nucleus. Scale bar = 200 μm in A and C; 100 μm in B and D.
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Figure 15. A photomicrograph of a coronal section demonstrating AVP immunoreactive cells in the suprachiasmatic nucleus as indicated by the arrow at 25 PN. Abbreviations: 3V, third ventricle; Sch, suprachiasmatic nucleus; OX, optic chiasm. Scale bar = 100 μm.
Figure 16. At 25 PN, AVP-IR in fibers was detectable in several areas of the brain. A series of photomicrographs of coronal sections demonstrating AVP-IR in fibers in the forebrain including the medial septal area (A) and vascular organ of the lamina terminalis (B). Dense AVP-IR is seen in the lateral hypothalamic area and median eminence (C). The dotted line in A indicates the midline. Abbreviations: 3V, third ventricle; OVLT, vascular organ of the lamina terminalis. All photomicrographs are at the same magnification. Scale bar = 100 μm.
Figure 17. A series of photomicrographs of coronal sections through the 45 PN brain demonstrating a dense amount of AVP-IR in the medial septal area (A), ventral pallidum (B), nuclei of the vertical and horizontal limbs of the diagonal bands (A,B), and stria terminals (C). Dense AVP-IR was seen in the interpeduncular nucleus (D). The dotted lines in A and B indicate the midline. The pattern of distribution of AVP-IR in fibers at 45 PN appeared very similar to that of the adult brain.

Abbreviation: IP, interpeduncular nucleus.

Scale bar = 100 μm.
CHAPTER 3. DISTRIBUTION AND ONTOGENY OF OXYTOCIN-LIKE IMMUNOREACTIVITY IN THE BRAIN OF ADULT AND DEVELOPING BRAZILIAN OPOSSUM, Monodelphis domestica

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Abstract

Many neuropeptides including oxytocin (OT) have been shown to function as neurotransmitters and/or neuromodulators in the adult central nervous system and are expressed during the perinatal or neonatal periods of brain development. Relatively little is known about oxytocin's role in early developmental events. In this study, we have used immunohistochemistry to describe the distribution of oxytocin in the adult and developing Brazilian opossum, Monodelphis domestica. As a marsupial, neurogenesis and morphogenesis in the Brazilian opossum brain continues into the second week of postnatal life and is therefore a good animal model for developmental studies. In the adult opossum brain, oxytocin-like immunoreactivity (OT-IR) was present in cells in the periventricular, paraventricular, supraoptic and dorsomedial hypothalamic nuclei. Scattered immunoreactive cells were also present in the medial preoptic, lateral preoptic and lateral hypothalamic areas. Oxytocin immunoreactive fibers were widely distributed in the opossum brain. The general distribution of OT-IR resembled that which has been reported for adults of other mammalian species. These findings suggest that OT may have similar physiological functions in the adult opossum. In the developing opossum brain, OT-IR was first detected in the forming median
eminence on day 1 of postnatal life (1 PN). Between 3 and 5 PN, OT-IR was present in the hypothalamic supraventricular and paraventricular nuclei and posterior pituitary. By 10 PN, OT-IR was seen in several areas of the brain. The early expression of OT-IR in the opossum brain suggests that OT may be involved in developmental processes. At 15 PN, OT-IR was seen in several brainstem nuclei including the nucleus of the solitary tract and dorsal motor nucleus of the vagus, which indicates that OT may be playing a role in autonomic regulation in the neonatal opossum.

Introduction

The neuropeptide oxytocin (OT) is principally synthesized and secreted by the neurosecretory cells of the paraventricular and supraventricular nuclei in the hypothalamus (Swaab et al., 1975; Swanson and Sawchenko, 1980; Swanson and Sawchenko, 1983). Oxytocin has a number of physiological functions in the periphery which include development and differentiation of mammary glands, milk ejection, uterine contraction, parturition, and prolactin release from the anterior pituitary (Brownstein et al., 1980; Antoni et al., 1988; Arey and Freeman, 1992; Sapino et al., 1993). Studies including immunohistochemical and tract tracing procedures have demonstrated that oxytocin-like immunoreactive fibers from the paraventricular and supraventricular nuclei project to a number of forebrain areas, the posterior pituitary, median eminence, brainstem, and spinal cord (Buijs, 1978; Buijs et al., 1978). These studies suggest that OT also acts as a neurotransmitter and/or neuromodulator (Buijs, 1983; Buijs, 1990; Csiffary et al., 1992; Ingram and Moos, 1992). Several physiological, pharmacological, and behavioral studies have demonstrated that OT is involved in many
centrally regulated functions (reviewed in Richard et al., 1991). For example, small amounts of OT injected into the cerebral ventricles can modulate neuroendocrine and autonomic functions (Antoni et al., 1988; Dreifuss et al., 1988; 1989; Roberts et al., 1993). Since the paraventricular nucleus has direct connections with the dorsal motor nucleus of the vagus and the nucleus of the solitary tract (Saper et al., 1976; Swanson and Sawchenko, 1980; Lawrence and Pittman, 1985), stimulation of the paraventricular nucleus has been reported to influence gastric secretory functions, as well as decrease gastric motility via vagally mediated effects (Luiten et al., 1985; Rogers and Nelson, 1984; Rogers and Herman, 1985; Rogers and Herman, 1987; McCann and Rogers, 1990; Siaud et al., 1990). Oxytocin has also been shown to inhibit food and salt intake in the rat (Arletti et al., 1990; Verbalis et al., 1993). A significant amount of data indicates that OT may be the neurotransmitter involved in the pathways from the paraventricular nucleus to the dorsal motor nucleus of the vagus and thus be a potential regulator of gastric activities (Charpak et al., 1984; Raggenbass et al., 1987; Tache et al., 1990).

Numerous studies have described the distribution of OT-like immunoreactivity (OT-IR) in a variety of species including the rat (Swaab et al., 1975; Castel and Morris, 1988; Hermes et al., 1988; Caffe et al., 1989; Dubois-Dauphin et al., 1989). Cells containing OT-IR are located in the paraventricular nucleus, supraoptic nucleus, periventricular hypothalamic nucleus, anterior commissural nucleus, and lateral preoptic and lateral hypothalamic areas. Oxytocin immunoreactive fibers have been detected in many extrahypothalamic regions, including the olfactory tubercle, lateral septum, bed nucleus of the stria terminalis, lateral preoptic area, nucleus of the solitary tract, dorsal motor vagal
nucleus, and intermediolateral nucleus and nucleus of the bulbous cavernous muscle in the spinal cord (Buijs, 1978; Buijs et al., 1978; Hermes et al., 1988; Dubois-Dauphin et al., 1989; Ingram and Moos, 1992; Wagner and Clemens, 1993). Using autoradiography, binding sites for OT have been reported in many brain areas including the components of the limbic system, various cortical structures, basal ganglia, hypothalamic mammillary nuclei, thalamic nuclei, brainstem, and spinal cord (Tribollet et al., 1988; 1989; Kremarik et al., 1993).

The ontogeny of the oxytocinergic neurons in the supraoptic and paraventricular nuclei have been reported in the rat using various techniques (Whitnall et al., 1985). In the rat, oxytocin immunopositive cells appear on embryonic day 20 (Buijs et al., 1980; Whitnall et al., 1985; Lazcano et al., 1990), however, its precursors have been demonstrated in the forming brain at embryonic day 16 (Whitnall et al., 1985). Tribollet and coworkers (1989) have reported OT binding sites in the fetal rat brain at embryonic day 14 in the forming dorsal motor nucleus of the vagus. They have also reported that OT binding sites are transiently expressed in many structures between days 16 and 35 of the postnatal period. On the basis of these findings, the authors have proposed that OT may have some neurotrophic functions during maturation of the brain.

Although the distribution of OT-IR has been documented for the adult brain of various mammalian species, developmental aspects of the extrahypothalamic OT containing systems have not been fully studied. Many neuropeptides including OT have been shown to function as neurotransmitters and/or neuromodulators in the adult central nervous system. Relatively little is known regarding the significance of neuropeptide expression during
In the present study, we have characterized the distribution of OT-IR in the brains of adult and developing Brazilian gray short-tailed opossums, *Monodelphis domestica*. The Brazilian opossum is a small pouchless marsupial which breeds well under laboratory conditions. Opossum young are born after 14 days of gestation in an extremely immature state, before sexual differentiation and neurogenesis is completed (Jacobson, 1984; Larsen and Jacobson, 1986; Saunders et al., 1989; Iqbal et al., 1994). *Monodelphis* similar to other marsupials, has a protracted postnatal developmental period. In addition, the absence of the pouch in *Monodelphis* makes the young a very accessible model for developmental studies (Schwanzel-Fukuda et al., 1988; Dore et al., 1990; Brunjes et al., 1992; Kuehl-Kovarik et al., 1993; Elmquist et al., 1994).

**Materials and Methods**

**Animals**

Adult and developing male and female Brazilian short-tailed opossums from a colony at Iowa State University were utilized in this study. The animals were housed in plastic cages, maintained on a 14:10 light hours dark cycle, and a constant temperature (26°C). The animals were provided water and food *ad libitum* (Reproduction Fox Chow; Milk Specialties Products, Madison, WI). In the first part of the study, the distribution of OT-IR was investigated in four female and four male adult opossum brains. In the developmental part of the study, the brains of opossum pups of various ages from 1 to 60 days of postnatal life were examined. For breeding purposes, female and male animals were housed together for
two weeks (14 days). The animals were then separated, and females were checked daily for the presence of pups. The day of birth of the pups was designated as day 1 of postnatal life (1 PN). The gender of the pups cannot be identified grossly before 5 PN, thus the pups were considered sexually undifferentiated. In the developmental part of the study, four animals were investigated at each of the ages of 1, 3, and 5 PN. Six animals (3 males and 3 females) from at least two different litters were used at each of the ages of 10, 15, 20, 25, 35, 45, and 60 PN.

*Tissue collection*

The adult animals were perfused with Zamboni’s fixative solution and brains were collected and processed for immunohistochemistry as has been described previously (Fox et al., 1991; Elmquist et al., 1992).

The brains from the developing opossum pups (1, 3, 5, 10, 15, and 20 PN) were collected by cooling the animals at -15 °C in the freezer until anesthetized. Following decapitation the heads were placed in Zamboni’s fixative solution for 48 hours at room temperature. After fixation, the heads were transferred into 30% buffered-sucrose solution overnight, and then cut into 20 μm thick coronal sections on a cryostat (Reichert Instruments). The section were thaw mounted onto poly-L-lysine (Sigma) coated slides and stored until immunohistochemistry was conducted.

The animals of 25, 35, and 45 days of postnatal age were anesthetized by cooling and were perfused transcendially with 15 ml of Zamboni’s fixative solution. The brains were removed and postfixed in the same fixative for 48 hours at room temperature. After
postfixation, the brains were processed as described for the developing animals. The brains from the 60 PN animals were collected as described for the adult animals and processed as for the developing animals. Additional brains of pups of 1 and 7 PN were cut in the sagittal plane and processed for immunohistochemistry as described above.

**Immunohistochemistry**

The protocol utilized for immunohistochemistry was a modification of that which has been reported previously (Fox et al., 1991; Elmquist et al., 1992). The slide mounted tissue sections were washed in buffer solution followed by incubation in $\text{H}_2\text{O}_2$ solution for 30 minutes. After blocking in normal goat serum (Vector; 1:67), the tissue sections were incubated in OT primary antibody (made in rabbit; Peninsula Laboratories, 1:3000) in a humidified chamber overnight at room temperature. After adequate rinsing, the tissue sections were incubated in goat anti-rabbit IgG (Vector; 1:800) for 2 hours, rinsed, and exposed to avidin-biotin complex (Vector Elite Kit; 1:200) for one hour in a humidified chamber at room temperature. After washing, the tissue sections were stained by reacting with 3,3’ diaminobenzidine tetrahydrochloride (DAB; sigma), with nickel sulfate and hydrogen peroxide. After staining for 5-6 minutes, the tissue sections were washed in 0.9% saline solution to terminate the reaction. The sections were then dehydrated, cleared, coverslipped, and analyzed with a light microscope.

The unmounted adult tissue sections were processed for immunohistochemistry utilizing a free floating tissue technique reported previously (Fox et al., 1991). The results obtained with the free floating tissue processing technique were comparable with those
obtained from the mounted tissue sections.

**Immunohistochemical control procedures**

Both negative and preabsorption control procedures were run in parallel with the tissue to be immunostained. Negative controls were generated by incubation of the tissue in the normal goat serum instead of in the primary antibody. No specific staining of the tissue was observed.

Preabsorption controls were carried out by co-incubating the tissue sections with oxytocin peptide (15 μM; Sigma) and primary antibody. All specific staining was abolished in the tissue sections except for a few fibers in the median eminence and posterior pituitary. Preabsorption of OT primary antisera with the arginine vasopressin peptide (15 μM; Sigma) did not eliminate any of the specific immunostaining.

**Analysis of tissue**

Tissue sections at 60 μm intervals from areas caudal to the olfactory bulbs to the caudal brainstem from the adult animals and consecutive sections at 20 μm intervals from the developing brains were analyzed. Sections were examined with the light microscope and structures containing OT-like immunoreactivity were identified with the aid of maps of coronal sections of the opossum brains (Fox et al., 1991; Elmquist et al., 1992) and an atlas of the developing rat brain (Paxinos et al., 1991)
Results

In the present study, we have investigated the distribution and ontogeny of OT-IR in adult and developing opossum brains. None of the animals in the present study were injected with colchicine, yet cell bodies containing OT-IR were detected in specific nuclear groups, and oxytocin immunoreactive fibers were seen in several brain regions.

Somata containing OT-IR in the adult opossum brain

Oxytocin-like immunoreactivity in cell bodies was observed in specific nuclear groups in the opossum brain including the periventricular, paraventricular (Fig. 1A), and supraoptic hypothalamic nuclei (Fig. 1B), medial preoptic area, lateral preoptic area, anterior and lateral hypothalamic areas (Fig. 1C), and dorsal medial hypothalamic nucleus. Although we did not quantify the number of immunoreactive cells in each region of the opossum brain, the periventricular, paraventricular, and supraoptic nuclei contained the dense collection of immunopositive cell bodies. No sex difference in the number of OT immunopositive cell bodies was observed.

Fibers containing OT-IR in the adult opossum brain

Oxytocin immunoreactive fibers were seen in several brain regions in the adult opossum brain. The distribution of OT immunoreactive fibers is discussed as: low, if there was a single to few scattered immunopositive fibers seen in the field area; high, if there was an intense immunoreactivity and most of the field area under view was covered with immunostained fibers; and moderate, if the immunoreactivity was intermediate between low
and high.

**Telencephalon**

In the telencephalon, single to low numbers of thin beaded OT immunoreactive fibers were observed in the anterior olfactory nucleus, olfactory tubercle, nuclei of the horizontal and vertical limbs of the diagonal band, ventral nucleus of the lateral septum (Fig. 2A), bed nucleus of the stria terminalis, lateral preoptic area; magnocellular division, median preoptic nucleus, and the optic chiasm. A moderate number of immunoreactive fibers were observed in the vascular organ of the lamina terminalis. Few immunoreactive fibers were seen in the medial amygdaloid nucleus (Fig. 2B).

**Diencephalon**

In the hypothalamus, a moderate to high number of OT immunoreactive fibers were consistently seen in the medial preoptic area, lateral preoptic area, anterior and lateral hypothalamic areas (Fig. 1B,C and Fig. 2C), periventricular, paraventricular and supraoptic nuclei (Fig. 1A and B), and median eminence. A low number of immunopositive fibers were present in the arcuate nucleus and ventral premammillary nucleus. Few fibers were observed in the paraventricular thalamic nucleus (Fig. 2D), habenular complex, and pineal gland.

**Mesencephalon and myelencephalon**

A moderate number of oxytocin immunoreactive fibers were detected in the dorsal and ventral central gray (Fig. 2E), ventral tegmental area, nucleus of the dorsal raphe (Fig.
2F), medial longitudinal fasciculus, pontine reticular formation, nucleus of raphe magnus, and the pyramids.

In the brainstem, a high number of OT immunoreactive fibers were detected in the dorsal motor nucleus of the vagus and nucleus of the solitary tract, and few fibers were also present in the inferior olivary nucleus.

OT-IR in somata and fibers in the developing opossum brain

In the neonatal opossum, neurogenesis and morphogenesis is actively taking place. Most of the nuclear groups and regions are not well differentiated until the beginning of the third week of postnatal life. Thus, for the purpose of discussion, during the early postnatal period, the nuclear groups or areas discussed are the presumptive regions or nuclear groups at this time.

Postnatal day 1

The brain of the opossum is very immature and undergoing active morphological and developmental changes at 1 PN. No OT immunoreactive cell bodies were observed at this age. There were a few immunoreactive fibers in the forming median eminence.

Postnatal day 3

Although the brain is further developed as compared to that at 1 PN, many regions and nuclear groups are still in the process of formation. At 3 PN, a distinct group of cells containing OT-IR was present in the forming supraoptic hypothalamic nucleus (Fig. 3). No
cell bodies were observed in other areas of the brain at this age.

A few immunoreactive fibers were seen in the vascular organ of the lamina terminalis. A moderate number of fibers coursing toward the median eminence along the pial surface were identifiable (Fig. 3). The posterior pituitary also contained low amounts of immunostaining at this age.

*Postnatal day 5*

By day 5 PN, in the supraoptic nucleus cells containing OT-IR increased in number and amount of immunostaining (Fig. 4A,B). Few lightly immunostained cells also appeared lateral to the third ventricle, presumably in the forming paraventricular hypothalamic nucleus (Fig. 4A,B). A few individual immunopositive cells were also detected in the forming medial preoptic area, lateral preoptic area, and periventricular hypothalamic nucleus.

Oxytocin immunoreactive fibers were detected in forming forebrain areas including the anterior olfactory nucleus, olfactory tubercle, medial septal area, nuclei of the vertical and horizontal limbs of the diagonal band (Fig. 5A), and the lateral preoptic and medial preoptic areas (Fig. 5B). Increased immunostaining was observed in the vascular organ of the lamina terminalis, supraoptic nucleus, median eminence, and posterior pituitary as compared to that observed at 3 PN.

*Postnatal days 7-10*

Between 7 and 10 PN, the opossum brain is further differentiated. The supraoptic and paraventricular nuclei are recognizable at the light microscopic level. There was an increased
number of OT immunoreactive cells in the supraoptic, paraventricular hypothalamic nuclei, and anterior periventricular preoptic area (Fig. 6A), and lateral preoptic and lateral hypothalamic areas. From the size of the cells that were OT immunoreactive in the paraventricular nucleus we believe these cells are the magnocellular neurosecretory neurons. A distinct group of immunoreactive cells were seen in the retrochiasmatic part of the supraoptic nucleus associated with the pial surface.

Fibers containing OT-IR were seen in additional areas including the medial septum and bed nuclei of the stria terminalis. A few fibers were present in the optic chiasm and along the ventral pial surface of the brain (Fig. 6A). The amount of staining and number of immunoreactive fibers in the periventricular, paraventricular and supraoptic nuclei, lateral preoptic and lateral hypothalamic areas, and median eminence was greater than that seen at 5 PN. A few immunoreactive fibers were also seen in the arcuate nucleus. The posterior pituitary contained very intense immunostaining at this age (Fig. 6B).

Postnatal days 15-20

Between days 15 and 20 of the postnatal period, the opossum brain is well differentiated and many nuclear groups and regions are easily recognizable using the light microscope. There was a further increase in the number of immunoreactive cells present in the periventricular (Fig. 7A), paraventricular and supraoptic hypothalamic nuclei (Fig. 7B), and lateral preoptic and lateral hypothalamic areas (Fig. 7A, B). In the supraoptic nucleus OT immunoreactive cells were mostly present dorsal to the optic chiasm and ventral to the optic tract in the retrochiasmatic area (Fig. 7C). The characteristic shape of the adult
paraventricular hypothalamic nucleus is definable between 15 and 20 PN. Most of the OT cells were observed in the medial magnocellular and periventricular parvicellular part of the paraventricular nucleus. Single scattered immunopositive cells were also detected in the medial preoptic area.

Oxytocin immunoreactive fibers in the lateral hypothalamic area (Fig. 7B), median eminence, and arcuate nucleus showed a further increase in number and immunostaining (Fig. 7C). Fibers containing OT-IR were detected in many additional areas. In the median preoptic nucleus thin immunoreactive fibers were detected for the first time at this age. A few immunoreactive fibers were seen in the dorsomedial hypothalamic nucleus, lateral habenula, pineal gland, and medial premammillary nucleus. Thick immunoreactive fibers were seen in the paraventricular and reticular thalamic nucleus, and a few immunoreactive fibers were observed along the optic tract. In the midbrain and brainstem, OT immunoreactive fibers were detected in many areas including the ventral tegmental area, central gray, pontine nucleus, pyramids, inferior olivary nucleus, nucleus of the solitary tract, and dorsal motor nucleus of the vagus (Fig. 9A).

Postnatal days 25-35

The opossum brain between days 25 and 35 of the postnatal period is well developed, and many nuclear groups and regions resemble those seen in the adult brain. No significant change was observed in the distribution of OT immunoreactive elements, except for a generalized slight increase in the amount of immunostaining as compared to that seen at 15-20 PN. In addition, occasionally a few single immunostained cells were detected in the
medial amygdala.

Postnatal days 45-60

The distribution pattern of OT immunoreactive somata and fibers, with regard to their number and density of immunostaining, both in the hypothalamic (Fig. 8A,B) and extrahypothalamic brain regions became very similar to that seen in the adult opossum brain (Fig. 9B). As in the adult, there were no sex differences found in OT-IR in the opossum brain.

Discussion

In the present study, we have described the distribution and ontogeny of OT-IR in the brain of the adult and developing gray short-tailed opossum, *Monodelphis domestica*. Although, the distribution and ontogeny of oxytocinergic neurons in the hypothalamus of various species including the rat has been reported, the development of the extrahypothalamic fiber systems has not been adequately studied. We have found that OT-IR was detected in various regions and nuclear groups both in the adult and in developing animals even though colchicine pretreatment was not used.

In the adult opossum brain, OT-IR in cell bodies was detected in the supraoptic, paraventricular and periventricular hypothalamic nuclei, the medial preoptic and lateral preoptic areas, lateral hypothalamic area, and dorsomedial hypothalamic nucleus. Oxytocin immunoreactive fibers were also seen widely distributed throughout the brain. Like OT, arginine vasopressin (AVP) is also mainly secreted by the paraventricular and supraoptic
nuclei. In the opossum brain, OT and AVP immunoreactivity is confined to separate populations of cells both in the supraoptic and paraventricular nuclei as has been shown for the rat (Mohr et al., 1988). However, there were a few cells in which OT was colocalized with AVP in the periventricular parvicellular region of the paraventricular and magnocellular cells of the supraoptic nucleus present dorsal to the optic chiasm (Iqbal and Jacobson, unpublished).

The general distribution of OT-IR in the opossum brain resembled that which has been reported for other mammalian species (Swaab et al., 1975; Sofroniew et al., 1979; Rhodes et al., 1981; Castel and Morris, 1988; Hermes et al., 1988; Caffe et al., 1989; Dubois-Dauphin et al., 1989; Ingram and Moos, 1992). In the present study, we did not quantify the number of OT immunoreactive cells in the opossum brain. Thus, it is possible that there may be some species differences in the actual number of OT immunoreactive cells. Secondly, our animals were not colchicine treated thus, we may not have been able to detect all oxytocinergic cells present in the adult brain.

Some differences in the distribution of OT-IR were found in the opossum as compared to that which has been reported for other species. In the rat and hamster, oxytocinergic cells in the paraventricular nucleus are intermingled with the vasopressinergic cells (Rhodes et al., 1981; Morin and Blanchard, 1993) whereas in the opossum most of the oxytocinergic cells are presumably located in the medial magnocellular, and dorsomedial and periventricular parvicellular divisions. In the supraoptic nucleus of the rat, oxytocinergic cells are reported to be present dorsal and lateral to the optic chiasm (Rhodes et al., 1981; Lazcano et al., 1990), in contrast to that, oxytocinergic cells in the opossum are present
dorsal and ventral to the optic chiasm a finding which resembled that seen for the guinea pig (Dubois-Dauphin et al., 1989). No oxytocinergic cells were found in the nucleus of the anterior commissure, median preoptic nucleus, and subchiasmatic area as has been reported for the guinea pig (Dubois-Dauphin et al., 1989). We have no justification to explain these differences in our results. Differences in staining patterns may be due to utilizing a new species or due to our inability to detect oxytocin cells in these areas because the animals used in this study were not colchicine treated. However, similar differences in the distribution of other neuropeptides in the opossum brain have been found previously (Elmquist et al., 1992; Iqbal and Jacobson, 1994).

Several studies have demonstrated that oxytocin in the adult is involved in many centrally regulated functions including milk ejection, reproductive and sexual behaviors, social behaviors, learning and memory processes, neuroendocrine regulation of the anterior pituitary, and control of food intake (reviewed in Richard et al., 1991; Argiolas and Gessa, 1991). Oxytocin-IR has been localized in several regions of the brain; for example, OT immunoreactive fibers are present in the bed nucleus of the stria terminalis (Van Leeuwen and Caffe, 1983; Ingram and Moos, 1992). Oxytocin has been reported to excite the neurons in the bed nucleus of the stria terminalis which facilitate milk ejection (Ingram et al., 1990). Oxytocin has also been shown to be involved in the regulation of release of ACTH and prolactin from the anterior pituitary (Antoni et al., 1988). Recently, it has been shown that OT immunoreactive fibers make synaptic contacts with the neurons containing endorphin in the arcuate (Csiffary et al., 1992) and noradrenaline (A1) cells in the brainstem (Buijs et al., 1990). The authors have suggested that OT might be involved in the neuromodulation of
endorphin secreting cells in the arcuate and A1 cells in the brainstem. Several investigations including tract tracing and immunohistochemical studies have demonstrated that neurons in the parvicellular part of the paraventricular nucleus project to various nuclear groups in the brainstem and spinal cord through which the paraventricular nucleus is involved in the control of many autonomic functions (Saper et al., 1976; Schwanzel-Fukuda et al., 1984; Dreifuss et al., 1988 & 1989; McCann and Rogers, 1990; Buijs, 1990). In the present study, we have detected OT-IR in similar areas of the opossum brain as has been reported for other mammals. These results suggest that OT may have similar physiological functions in the opossum as has been suggested for other species.

Brazilian opossum pups are born after a 14 day gestational period before neurogenesis and morphogenesis is completed. In contrast to the rat, the opossum brain is extremely immature at birth. Based on studies conducted in our laboratory and in several others (Schwanzel-Fukuda et al. 1988., Saunders et al., 1989., Fadem and Harder, 1992; Elmquist et al., 1992; Iqbal et al., 1994), we believe that the stage of neural development of the opossum pups seen at birth is comparable to that of the 13-14 day of gestation rat. Further the 16 PN opossum pup resembles that of the 1 PN rat pup. In the rat OT-IR is first detected between embryonic days 20-22, while arginine vasopressin is present on embryonic day 16 and 18 in the supraoptic and paraventricular nuclei, respectively (Buijs et al., 1980; Whitnall et al., 1985; Laurent et al., 1989). In agreement with these studies, in the opossum brain arginine vasopressin-like immunoreactivity in cell bodies and fibers was seen as early as embryonic days 12 and 13; whereas, OT-IR was first detected at later periods. In the opossum brain, OT-IR was first detected in the fibers in the median eminence at 1 PN, and
OT immunoreactive cell bodies were first observed at 3 PN in the forming supraoptic nucleus and later at 5 PN in the paraventricular hypothalamic nucleus. Several reasons for the delay in detection of OT-IR containing somata have been suggested. As has been suggested previously in the rat (Alstein and Gainer, 1988; Laurent et al., 1989), this delay could be due to a slow expression of the mRNA or post translational modification of the peptide as compared to that of AVP. The inability to see OT immunoreactive cell bodies prior to 3 PN in the supraoptic nucleus of the opossum may be due to the delayed migration of the OT secreting cells to the forming supraoptic nucleus, as opposed to those cells which express AVP from embryonic day 13 (Iqbal and Jacobson, 1994). This delay in expression/identification could also be due to low levels of the peptide which are transported at a fast rate into the axonal terminals. This explanation is plausible since we could detect OT-IR in fibers at 1 PN in the median eminence. Further studies using in situ hybridization might provide information regarding the delay in detection of OT-IR in cell bodies in the opossum brain.

In the developing opossum brain, OT-IR was detectable between 1 and 3 PN in the forming median eminence and supraoptic nucleus. Between days 5 and 10 PN, OT-IR was detectable in many brain regions including the paraventricular and periventricular hypothalamic nuclei, and lateral and medial preoptic areas. The distribution of OT immunoreactive cell bodies and fibers increased both in number and amount of immunoreactivity with increasing age. Neurogenesis in the supraoptic and paraventricular hypothalamic nuclei in the opossum is completed by 5 and 7 PN, respectively (Iqbal et al., 1994), whereas in other hypothalamic and extrahypothalamic regions/areas neurogenesis
continues into the second week of postnatal life (Rivkees et al., 1988; Larsen and Jacobson, 1986; Iqbal et al., 1994; Swanson et al., 1994). The findings of the present study suggest that in the opossum brain OT secreting cells become functional before neurogenesis is completed in areas where oxytocinergic cells or OT immunoreactive fibers will exist. Secondly, these results also raise the possibility that like several other neuropeptides, OT may also be involved in the normal development and functioning of the central nervous system or that OT may be acting as a neuromodulator and/or neurotransmitter during the early neonatal life of the opossum.

Many peptides are involved in the control of food intake including cholecystokinin. Cholecystokinin is known to inhibit food intake and gastric emptying and motility (Della-Fera and Baile, 1980; Baile and Della-Fera, 1985; Silver and Morley, 1991). Recently, several studies using c-fos immunohistochemistry have demonstrated that the peripheral administration of cholecystokinin activates the oxytocinergic neurons in the paraventricular nucleus and also increases OT plasma levels (Olson et al., 1992; Verbalis et al., 1991). These results have led the authors to suggest that OT is indirectly involved in the control of food intake. Previous studies in the adult rat have demonstrated that OT neurons in the paraventricular nucleus directly project to the dorsal motor vagal nucleus (Swanson and Sawchenko, 1980; Sawchenko and Swanson, 1982; Swanson and Sawchenko, 1983). Electrophysiological studies have demonstrated that stimulation of the paraventricular nucleus influences gastric motor and secretory function via vagally mediated mechanisms (Rogers and Nelson, 1984; Rogers and Herman, 1985; 1987). In vivo and in vitro studies have demonstrated that oxytocin can excite the dorsal motor vagal neurons (Tribollet et al., 1989;
McCann and Rogers, 1990). Tribollet and coworkers (’89) have also demonstrated functional OT binding sites in the dorsal motor nucleus of the vagus in the embryonic day 14 rat.

In other studies, we have shown that cholecystokinin-like immunoreactivity (CCK-IR) in fibers and cells first appears between 5 and 10 PN in the presumptive nucleus of the solitary tract and in the hypothalamus at 35 PN (Fox et al., 1991). In the present study, OT-IR was detected in the dorsal motor nucleus of the vagus and nucleus of the solitary tract at 15 PN, which almost parallels the appearance of CCK-IR in the brainstem. Conversely OT-IR in the paraventricular nucleus was detected between 5 and 7 PN, a time which is quite earlier than that for the appearance of CCK-IR in the hypothalamus. At present, in the opossum we do not know the time course for the development of the projections from the paraventricular nucleus to the brainstem, and/or from the brainstem to the hypothalamus. However, the early expression of OT-IR in the paraventricular nucleus suggests that OT may be the neurotransmitter involved in transmission of information or signals from the hypothalamus to the brainstem, and thus be involved in regulation of gastric and other autonomic functions. Further, the early presence of OT-IR in the paraventricular nucleus at 5 PN and oxytocinergic fibers between the hypothalamus and brainstem raises the possibility that in the young opossum, OT might be directly involved in the regulation of food intake. At a later age when the cholecystokinin pathways become functional, food intake regulation may be taken over by the cholecystokininergic pathways.

At present, we do not have an explanation for the delayed detection of OT-IR in the brainstem including the dorsal motor nucleus of the vagus and nucleus of the solitary tract as compared to that for the forebrain. One explanation could be that oxytocinergic
projections from the paraventricular nucleus to the vagus motor nucleus are not fully formed before day 15 PN. Secondly, the OT which may be present in descending fibers from the hypothalamus is quite low and was not detected prior to 15 PN. Another explanation could be that the OT from the paraventricular nucleus reaches its target through the cerebrospinal fluid. Interestingly between 5 and 7 PN, some of the neuronal processes of OT immunoreactive somata were seen in the ependymal layer of the third ventricle and were also associated with the pial surface in the reterochiasmatic part of the supraoptic nucleus. This latter explanation is supported by the findings of Tribollet and coworkers (1989) who have reported the presence of functional OT binding sites in the developing vagal motor nucleus at embryonic day 14 in the rat, whereas OT-IR in the rat brain was not detected until between 21 and 22 days of postfertilization (Whitnall et al., 1985). Further studies involving tract tracing, in situ hybridization, and autoradiography are required to determine if OT from the paraventricular nucleus is involved in the control of food intake in the neonatal opossum.

In summary, we have investigated the distribution and ontogeny of OT-IR in the adult and developing opossum, Monodelphis domestica. In the adult opossum brain, OT immunoreactive cells were localized in several specific nuclear groups and areas. Oxytocin immunoreactive fibers were seen in several hypothalamic and extrahypothalamic regions including the forebrain and brainstem. In addition, we have described the development of the hypothalamic and extrahypothalamic OT containing pathways. In the developing opossum brain, OT-IR in somata was present in the forming hypothalamus between 3 and 5 PN and in some brain areas OT-IR was seen even before neurogenesis of that area was completed. Although the exact functions of OT during development of the animal are not yet fully
understood, the widespread distribution of this peptide in the adult and its early presence in
the developing opossum brain suggest its involvement during the perinatal period.

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Literature cited

Alstein, M., and H. Gainer (1988) Differential biosynthesis and post translational processing
of vasopressin and oxytocin in rat brain during embryonic and postnatal development.
J. Neurosci. 8:3967-3977.

Antoni, F.A., K.J. Kovais, J. Dohanits, G.B. Makara, M.C. Holme, and M.F. Mazurek
20:729-736.

Arey, B.J., and M.E. Freeman (1992) Activity of oxytocinergic neurons in the
paraventricular nucleus mirrors the periodicity of the endogenous stimulatory rhythm

Rev. 15:217-231.


Figure 1. Localization of oxytocin-like immunoreactivity in the paraventricular hypothalamic nucleus (A), supraoptic nucleus (B), and lateral hypothalamic area (C) of an adult opossum brain. Abbreviations: 3V, third ventricle; OX, optic chiasm. All photomicrographs are at the same magnification. Scale bar = 100 μm.
Figure 2. A series of photomicrographs of coronal sections demonstrating the distribution of oxytocin-like immunoreactive fibers in the ventral division of the lateral septum (A), medial amygdala (B), lateral hypothalamic area (C), paraventricular thalamic nucleus (D), central gray (E), and dorsal raphe nucleus (F) of an adult opossum brain. Abbreviations: LV, lateral ventricle; 4V, fourth ventricle. All photomicrographs are at the same magnification.

Scale bar = 100 μm.
Figure 3. A photomicrograph of a coronal section demonstrating oxytocin-like immunoreactivity in cells in the forming supraoptic nucleus from the postnatal day 3 brain. Oxytocin immunoreactive fibers can be seen coursing towards the median eminence at this age. Abbreviations: 3V, third ventricle; ME, median eminence. Scale bar = 100 μm.
Figure 4. Photomicrographs of coronal sections through the postnatal day 5 brain demonstrating oxytocin-like immunoreactivity in the forming supraoptic hypothalamic nucleus. Some lightly immunostained cells are also seen in A and B lateral to the third ventricle, presumably in the forming paraventricular hypothalamic nucleus. In B, notice the immunoreactive cells and fibers in the forming lateral hypothalamic area and retrochiasmatic area. Abbreviation: 3V, third ventricle. Both photomicrographs are at the same magnification. Scale bar = 100 μm.
Figure 5. Photomicrographs of coronal sections demonstrating oxytocin-like immunoreactivity in fibers in the developing medial septal area and nuclei of the diagonal bands (A), and in the lateral preoptic area (B) of the postnatal day 5 brain. The black dashed line in A indicates the midline. Abbreviations: LV, lateral ventricle; 3V, third ventricle. Both photomicrographs are at the same magnification. Scale bar = 100 μm.
Figure 6. Localization of oxytocin-like immunoreactivity in the anterior periventricular preoptic area and lateral preoptic area of the postnatal day 7 opossum. Notice the immunoreactive fibers that are also present in the optic chiasm (A). An intense immunoreactivity can be seen in the median eminence and posterior pituitary (B). Abbreviations: 3V, third ventricle; LPO, lateral preoptic area; OX, optic chiasm; A Pit, anterior pituitary; P Pit, posterior pituitary. Both photomicrographs are at the same magnification. Scale bar = 100 μm.
Figure 7. Photomicrographs of coronal sections demonstrating the localization of cells and fibers containing oxytocin-like immunoreactivity in the anterior periventricular hypothalamic area and medial and lateral preoptic areas (A), paraventricular and supraoptic hypothalamic nuclei (B), and lateral hypothalamic area, median eminence, and arcuate nucleus (C) of the postnatal day 15 brain. A group of closely packed oxytocin immunoreactive cells can be seen in the retrochiasmatic area (C). Abbreviations: 3V, third ventricle; Arc, arcuate nucleus; LH, lateral hypothalamic area; LPO, lateral preoptic area; ME, median eminence; PVN, paraventricular hypothalamic nucleus; SON, supraoptic hypothalamic nucleus. All photomicrographs are at the same magnification. Scale bar = 100 μm.
Figure 8. Photomicrographs demonstrating oxytocin-like immunoreactivity in the paraventricular hypothalamic nucleus (A) supraoptic nucleus (B) and lateral hypothalamic area (A,B) of the postnatal day 45 brain. At this age, the distribution pattern of OT immunoreactive elements begins to resemble that of the adult brain. Note that most of the OT immunoreactive cells are present in the medial magnocellular and periventricular parvicellular part of the paraventricular nucleus. In the supraoptic nucleus oxytocin immunoreactive cells are present on the dorsal side of the optic chiasm. Both photomicrographs are at the same magnification. Scale bar = 100 μm.
Figure 9. Photomicrographs of OT immunoreactive fibers in the nucleus of the solitary tract and dorsal motor nucleus of the vagus in the brain of the postnatal days 15 (A) and 45 (B) opossum. Note a significant increase in the number of oxytocin immunoreactive fibers at the older age. Abbreviations: 4V, fourth ventricle; cc, central canal; 10, dorsal motor nucleus of the vagus; 12, hypoglossal nucleus; Sol, nucleus of the solitary tract. Both photomicrographs are at the same magnification. Scale bar = 100 μm.
SUMMARY

In the first study, bromodeoxyuridine (BrdU) single, BrdU-arginine vasopressin-oxytocin (BrdU-AVP-OT) double, and triple label immunohistochemistry was employed to characterize postnatal neurogenesis in the hypothalamic paraventricular and supraoptic nuclei in the Brazilian opossum brain. Analysis of the data revealed that neurogenesis of the supraoptic and paraventricular nuclei is completed by day 5 and 7 of postnatal life. Double and triple label immunohistochemistry demonstrated a few BrdU-AVP and/or BrdU-OT labelled cells in the supraoptic nucleus following injection on day 1 and 2 of postnatal life. No double labelled cells were seen after exposure on day 5 of postnatal life. In the paraventricular nucleus many double labelled cells were seen in the parvicellular part of the nucleus whereas the magnocellular part had few double labelled cells following exposure on day 1 and 2 of postnatal life. No double labelled cells were seen in the paraventricular nucleus after BrdU administration on day 7 of postnatal life. These results demonstrated that the majority of the magnocellular neurons in the SON and PVN must be born prenatally whereas parvicellular groups of neurons of the PVN continue to form during the postnatal period. Further, part of the magnocellular and parvicellular neurons acquire their neurochemical phenotype before neurogenesis is completed.

In the second study, the distribution and ontogeny of arginine vasopressin-like immunoreactivity (AVP-IR) was examined in the adult and developing Brazilian opossum brain. In the adult opossum, AVP-IR was found in several nuclear groups and AVP
immunoreactive fibers were widely distributed throughout the brain. The general distribution of AVP-IR in the adult opossum brain resembled that which had been reported in the other mammalian species. In the developing opossum brain, AVP-IR was detected in the mesencephalon and diencephalon on embryonic day 12 and 13, respectively. A distinct group of immunoreactive cells was seen in the forming supraoptic nucleus on day 1 of postnatal life, and in the forming paraventricular nucleus immunoreactive cells also appeared on day 3 of postnatal life. Between day 1 and 3 of postnatal life, a few AVP immunoreactive cells were observed in the forming dorsal thalamus and tegmental area, and a few AVP immunoreactive fibers were also seen in the developing cerebellum. The immunoreactive cells and fibers were not seen in these regions at later ages. Between day 5 and 7 of postnatal life, AVP-IR was observed in several of the brain areas. The distribution patterns of AVP-IR became adult-like by day 60 of postnatal life. A sex difference in the amount of AVP-IR was also observed in the lateral septum of the opossum brain at day 60 of postnatal life. This difference persisted in the adult brain. The early presence of AVP-IR in the *Monodelphis* brain suggest that, AVP may be involved in some developmental events in the central nervous system in addition to its well defined physiological functions in the periphery.

In the third study, the distribution and ontogeny of oxytocin-like immunoreactivity (OT-IR) was characterized in the brain of the adult and developing Brazilian opossum. In the adult opossum brain, OT-IR was present in cells in the periventricular, paraventricular, supraoptic, and dorsomedial hypothalamic nuclei. Scattered immunoreactive cells were also seen in the medial preoptic, lateral preoptic, and lateral hypothalamic areas. Oxytocin-like
immunoreactive fibers were also present in several areas of the brain. The general pattern of distribution of OT-IR in the adult opossum brain resembled that which had been reported for other mammalian species. In the developing opossum brain, OT immunoreactive fibers were seen in the median eminence as early as day 1 of postnatal life. Between day 3 and 5 of postnatal life, OT-IR was seen in the hypothalamic supraoptic and paraventricular nuclei. Between day 10 and 15 of postnatal life, OT-IR was seen in several of the brain areas including the brainstem. The early presence of OT-IR in the developing opossum brain suggests that OT might be involved in developmental and regulatory events during early postnatal development of the animal.
GENERAL DISCUSSION

The paraventricular and supraoptic nuclei are located in the anterior hypothalamus. The hypothalamic paraventricular nucleus consists of magnocellular and parvicellular group of secretory neurons, whereas the supraoptic nucleus is composed of magnocellular secretory neurons (Swanson and Kuypers, 1980). The magnocellular neurons of the PVN and SON project to the median eminence and posterior pituitary, which constitute the hypothalamo-neurohypophyseal system. The parvicellular neurons of the PVN also project to several brain regions including the forebrain, midbrain, brainstem, and spinal cord (Buijs, 1978; Buijs et al., 1978; Armstrong et al., 1980; Swanson and Kuypers, 1980). Arginine vasopressin and oxytocin are two well known neuropeptides secreted by these nuclei. Immunohistochemical studies have demonstrated that many other neuropeptides are also localized in these nuclei, and thus these nuclei are involved in many centrally mediated functions (Swanson and Sawchenko, 1980; Swanson et al., 1986; Hokfelt et al., 1989).

The information presented in this dissertation extends the previously reported work on the development of the hypothalamo-neurohypophyseal system. Specifically, the postnatal neurogenesis of the hypothalamic paraventricular and supraoptic nuclei, and distribution and ontogeny of arginine vasopressin- and oxytocin-like immunoreactivity containing systems were investigated and described in the brain of the adult and developing Brazilian opossum, *Monodelphis domestica*.

The Brazilian opossum, is a small marsupial which breeds well under laboratory
conditions (Kraus and Fadem, 1987; Tyndale and Renfree, 1987). Like other marsupials, *Monodelphis* pups are born after 14-15 days of gestation in an extremely immature state before neurogenesis and morphogenesis are completed (Jacobson, 1984; Larsen and Jacobson, 1986; Iqbal et al., 1994; Swanson et al., 1994). The day 1 postnatal opossum pup resembles the 13-14 day of gestation rat and reaches similar levels of neural development of the 1 PN rat by day 15-16 PN (Kraus and Fadem, 1987; Tyndale and Janssen, 1988; Saunders et al., 1989). Thus opossum pups provide an excellent model for *ex utero* developmental studies (Schwanzal-Fukuda et al., 1988; Dore et al., 1990; Brunjes et al., 1992; Kuehl-Kovarik et al., 1993; Elmquist et al., 1994; Iqbal et al., 1994).

In the first study, bromodeoxyuridine (BrdU) single label, double, and/or triple label immunohistochemistry (BrdU-AVP-OT) was employed to study postnatal neurogenesis in the PVN and SON. As had been demonstrated in the rat and mouse by earlier studies (Miller and Nowakowski, 1988; Nowakowski et al., 1989; Kono et al., 1991; Soriano and Rio, 1991; Beffo et al., 1992; Takahashi et al., 1992; Takahashi et al., 1993), our results demonstrated that BrdU immunohistochemistry is an effective method to study neurogenesis, cell proliferation, and the neurochemical phenotype of the dividing cells in the brain of a marsupial (Iqbal et al., 1994).

Results of the single label BrdU immunohistochemistry demonstrated that neurogenesis in the SON and PVN in the opossum brain continues into the postnatal period and is completed by day 5 and 7 PN, respectively. Bromodeoxyuridine injections after these ages resulted in there being no BrdU labelled cells in these nuclei except for few small glial-like cells in the parvicellular part of the PVN and ependyma of the third ventricle.
Double label and triple label immunohistochemistry showed that not many magnocellular AVP and OT secreting cells in the SON and PVN were double and/or triple labelled. However, in the parvicellular part of the PVN many double and/or triple labelled cells were observed after BrdU injections on day 1 and 2 PN. These results indicated that the majority of the magnocellular neurons both in the SON and PVN are formed prenatally, whereas parvicellular groups of neurons in the PVN continue to form during postnatal life. Interestingly, several BrdU labelled cells were not AVP and/or OT immunoreactive, which suggest that these cells may be those which synthesis and secrete other neuropeptides in these nuclei. Further studies using BrdU double label immunohistochemistry are required to identify the neurochemical phenotypes of the BrdU single labelled cells.

In the rat and mouse, neurogenesis is considered to be a prenatal phenomenon (Altman and Bayer, 1978; Karim and Slopper, 1980). Using tritiated thymidine autoradiography, neurogenesis in the SON and PVN have been investigated in the rat and mouse (Altman and Bayer, 1978; Karim and Slopper, 1980). Neurogenesis in the SON and PVN, both in the rat and mouse is completed between day 13 and 15 postfertilization, respectively. Immunohistochemical studies have shown that in the rat, AVP-IR in the SON and PVN appears between days 16 and 18 postfertilization, whereas OT-IR is detectable on days 19 and 21 postfertilization, respectively (Buijs et al., 1980; Whitnall et al., 1985). In the opossum brain, AVP-IR was detected in the diencephalon at embryonic day 13 (presumably the forming SON), the presence of AVP-IR in the SON was clearly demonstrable on day 1 PN, and AVP-IR in cells in the PVN also appeared on day 3 PN. These findings indicated that in marked contrast to that of the rat, in the opossum brain
neurogenesis continues into postnatal life and the neurochemical phenotype of some of the neurons can be identified before neurogenesis of other neurons is completed. These findings further suggest that a portion of the neuronal population in the SON and PVN might become functional very early during postnatal development of *Monodelphis*.

In the second study, we have characterized AVP immunoreactive systems in the opossum brain. The neuropeptide AVP is mainly synthesized in the hypothalamic PVN and SON. Immunohistochemical studies had shown that AVP immunoreactive cells are also localized in the bed nucleus of the stria terminalis, preoptic area, medial amygdala, anterior and lateral hypothalamic areas, and suprachiasmatic nucleus (Vandsande et al., 1975; Sofroniew and Wiendl, 1980; De Vries et al., 1981; Caffe and Van Leeuwen, 1983; Van Leeuwen and Caffe, 1983; De Vries et al., 1985) and AVP immunoreactive fibers are widely distributed in the extrahypothalamic areas of the brain (Buijs, 1978; Buijs et al., 1978; Buijs et al., 1980; De Vries et al., 1985).

In the adult rat and in other mammalian species, AVP has been shown to be a neuromodulator and/or neurotransmitter in a variety of centrally mediated functions. For example, it had been reported that AVP is involved in the regulation of blood pressure (Goa et al., 1992; Poulin et al., 1994), thermoregulation and antinociception (Oluyomi et al., 1992; Tanaka et al., 1993), circadian rhythmicity (Reghunandanan et al., 1990; Felino et al., 1994), drinking and food intake behaviors (Mangiapan et al., 1983; Burlet et al., 1992), sexual and social behaviors (Bluth and Dentzer, 1993; Winslow et al., 1993; Wang et al., 1994), and neuroendocrine regulation of the anterior pituitary gland (Familari et al., 1989; Shen et al., 1993). Electrophysiological studies have further demonstrated that AVP can
excite central motoneurons (Raggenbas et al., 1989; 1991; Tribollet et al., 1991), and cause release of intracellular calcium in the central nervous system (Stephens and Logan, 1986; Shewey and Dorsa, 1988; Briley et al., 1994).

The general distribution of AVP-IR in cells and in fibers both in the hypothalamic and extrahypothalamic areas of the adult opossum brain resembled that which had been reported for other mammalian species except for a few differences which may be attributed to the species difference. Differences in the localization of galanin, another neuropeptide, have been demonstrated previously for the opossum (Elmquist et al., 1992). However, the findings of the present study suggest that AVP may be playing similar functions in the Monodelphis brain and periphery as has been reported for the other species.

As discussed before, Brazilian opossum pups are born in a very immature state after 14-15 days of gestation before neurogenesis and morphogenesis is completed. At birth the opossum pups resemble the neuronal developmental stage of a 13-14 day gestation rat. In contrast to the rat, neurogenesis in the opossum brain continues into the second week of postnatal life (Larsen and Jacobson, 1986; Iqbal et al., 1994; Swanson et al., 1994). In the rat brain, AVP-IR appears at day 16 and 18 in the SON and PVN, respectively (Buijs et al., 1980; Whitnall et al., 1985). In the forming opossum brain, AVP-IR was detectable as early on embryonic day 12. By day 5 PN, a robust expression of AVP-IR was observed in the SON, PVN, median eminence, and posterior pituitary. A few AVP immunoreactive cells were observed in the forming dorsal thalamus and tegmental area, and a few immunoreactive fibers were also seen in the cerebellum between day 1 and 3 PN. This AVP-IR was not detected after day 3 of the postnatal period. Between day 7 and 10 PN, AVP-IR was
observed in several of the forebrain areas, including the olfactory tubercle, preoptic area, septal region, and vascular organ of the lamina terminalis.

The developmental role of AVP in the central nervous system has also been demonstrated in earlier studies using brattleboro rats, which are unable to synthesize AVP and have disturbances in their brain development, body growth and behavior (Boer et al., 1980; Boer, 1985; Snijdewint et al., 1988; Boer et al., 1993). Recently, it had also been shown that AVP increases the formation of cyclic AMP through V1b receptors which are expressed during development of the brain (Brinton and Brownson, 1993).

As mentioned earlier neurogenesis in the opossum forebrain is not completed until the second week of the postnatal period. However, in the midbrain and brainstem neurogenesis is completed earlier than that of the forebrain and hypothalamus (Iqbal and Jacobson, unpublished data). Our findings of the early detection of AVP-IR (as early as embryonic day 12), the transient expression in the dorsal thalamus, tegmental area, and cerebellum, and the later appearance of AVP-IR between day 7 and 10 PN in other brain areas in which neurogenesis is not completed, suggest that AVP might have some significant role during the development of the mammalian central nervous system. Alternatively, AVP might have some neuromodulatory roles and physiological significance during early postnatal development of the opossum.

Arginine vasopressin has been shown to be involved in the regulation and release of ACTH from the anterior pituitary gland in the adult rat (Kjoer et al., 1992). In the developing opossum pups, an intense AVP-IR was found to be present at the median eminence at day 3 PN and AVP-IR was also present in the posterior pituitary at day 5 PN.
In other studies in our laboratory, it had been shown that intense AVP binding sites are present in the anterior pituitary gland at day 5 PN (Kuehl-Kovarik et al., 1994), and ACTH-like immunoreactivity is also recognizable between day 4 and 5 PN (Elmquist and Jacobson, unpublished results). Vascularization of the anterior pituitary gland has also been demonstrated to be present at day 4 of the postnatal period (Gasse and Meyer, 1993). Taken together, these findings suggest that AVP may be involved in the release of ACTH from the anterior pituitary and may also be released into the circulation to regulate fluid homeostasis. Further studies would be required to understand the exact role of AVP during the early development of Monodelphis.

In the extrahypothalamic areas, AVP-IR in somata have been identified in the bed nucleus of the stria terminalis and medial amygdala (Van Leeuwen and Caffe, 1983; De Vries et al., 1985; Wang et al., 1993; Miller et al., 1993; Planas et al., 1994). In our studies, we could not detect any cell bodies in the medial amygdala but cell bodies were present in the bed nucleus of the stria terminalis and lateral preoptic area. A sex difference in the density of AVP immunoreactive fibers in the lateral septum and lateral habenula have been reported in the rat (De Vries et al., 1981; De Vries et al., 1985). It has been reported that AVP expressing neurons in the bed nucleus of the stria terminalis provide a greater density of immunoreactive fibers to the lateral septum in the male as compared to that of the female (De Vries et al., 1981; De Vries et al., 1985; Wang et al., 1993). De Vries and co-workers (1981) have also demonstrated that the difference in the density of immunoreactive fibers in the rat appears at day 12 of postnatal life and persists into adult life. In the present study, we have also observed a sex difference in the density of immunoreactive fibers in the
lateral septum in the adult opossum brain. In contrast to the data for the rat, no significant difference in immunoreactivity was present in the lateral habenula of male and female opossums. Although we did not quantify immunoreactive fibers in the lateral septum of the developing opossum brain, the sex difference in the density of immunoreactivity became apparent at day 60 PN.

Like AVP, other neuropeptides such as cholecystokinin (Frankfurt et al., 1985; Simerly and Swanson 1987; Mivevych et al., 1988; Fox et al., 1990), substance P (Malsbury and McKay, 1987; 1989), and tachykinin (Akesson, 1993) in the mammalian brain are also reported to be sexually dimorphic, and are involved in a number of sexual and social behaviors (Bloch et al., 1988; Bluthe and Dantzer, 1993; Winslow et al., 1993; Wang et al., 1994). Further, expression of these neuropeptides and their levels of immunoreactivity are influenced by the circulating levels of gonadal steroids (De Vries et al., 1981; De Vries et al., 1983; De Vries et al., 1985; Simerly and Swanson, 1987; Wang et al., 1993; De Vries et al., 1994). Recently, in the rat estrogen and androgen receptors have been localized in the AVP immunoreactive neurons in the bed nucleus of the stria terminalis (Axelson and Van Leeuwen, 1990; Zhou et al., 1994). In the developing opossum brain, we have shown that androgen receptor-like immunoreactive cells (AR-ir) were first detected in the ventral nucleus of the lateral septum at day 45 PN and in the bed nucleus of the stria terminalis and preoptic area at day 60 PN in the male (Iqbal et al., 1994b). No AR-ir cells were seen in these areas of the brain in females of the same age. These findings suggested that the sex difference in arginine vasopressin-like immunoreactivity in the
opossum brain appeared after androgen receptors were present and could potentially be involved in the regulation of arginine vasopressin expression.

Previously, a sex difference in cholecystokinin-like immunoreactivity in the medial preoptic area of the *Monodelphis* brain has also been reported (Fox et al., 1990). In the developing opossum brain, cholecystokinin-like immunoreactive fibers appear between day 25 and 35 PN in the medial preoptic area. The male animals contained a higher density of immunoreactive fibers than that of the female animals and this sex difference persisted into adulthood (Fox et al., 1991a). Data on the ontogeny of estrogen receptors in the opossum brain demonstrate that estrogen receptor containing cells in the medial preoptic area are present at day 15 PN (Fox et al., 1991b). In contrast to that, androgen receptor-like immunoreactivity in the medial preoptic area of the opossum does not start appearing until day 60 PN (Iqbal et al., 1994b), which substantiates the hypothesis that estrogen may be involved in the establishment of sex differences in cholecystokinin-like immunoreactivity in the medial preoptic area of the opossum brain. This concept is supported by the previous findings that castration of the adult male rat reduces cholecystokinin immunoreactivity (Simerly and Swanson, 1987; Micevych et al., 1988). As mentioned previously the appearance of a sex difference in the density of AVP-IR in the lateral septum of the opossum brain almost parallels the expression of androgen receptor-like immunoreactivity in the lateral septum and the bed nucleus of the stria terminalis. These findings suggest that unlike the sex difference in cholecystokinin-like immunoreactivity, a sex difference in the density of AVP-IR in the lateral septum of the opossum brain might be induced and regulated by androgens acting through their receptor.
Oxytocin is a closely related neuropeptide to AVP, synthesized by the hypothalamic paraventricular and supraoptic nuclei (Swanson and Sawchenko, 1983). Localization of OT-IR elements has also been reported in several of the extrahypothalamic areas of the brain and spinal cord (Castel and Morris, 1988; Hermes et al., 1988; Caffè et al., 1989; Dubois-Dauphin et al., 1989; Ingram and Moos, 1992; Wagner and Clemens, 1993). Oxytocin has well-known hormone-like functions in the periphery (Antoni et al., 1988; Arey and Freeman, 1992; Spino et al., 1993). Numerous physiological, pharmacological, and behavioral studies have demonstrated that OT is a neurotransmitter and/or neuromodulator in a variety of centrally mediated functions (Driefuss et al., 1988; Driefuss et al., 1989; Arletti et al., 1990; Buijs, 1990; Richard et al., 1991; Cisffary et al., 1992; Ingram and Moos, 1992; De Weid et al., 1993).

In the third study, we have examined the distribution and ontogeny of OT-IR in the *Monodelphis* brain. In the adult opossum brain, the localization and distribution of OT-IR generally resembled that which has been reported for other mammalian species. These results suggest that OT may be involved in regulation of similar physiological and central functions in the opossum as well.

As mentioned earlier, the opossum pups are born in an extremely immature state. Despite this immaturity, AVP-IR was observed as early as embryonic day 12. In contrast to that, OT-IR was first seen in the fibers in the median eminence on day 1 PN. Cells containing OT-IR was seen in the forming supraoptic nucleus at day 3 PN, and was later detected in the PVN at day 5 PN. Similar time differences in the appearance of AVP-IR and OT-IR in the rat brain have been reported (Buijs et al., 1980; Whitnall et al., 1985). In
the developing opossum brain, between days 5 and 7 PN, OT-IR was seen in several of the forebrain areas including the posterior pituitary. At days 10 and 15 PN, OT immunoreactive fibers were also seen in the brainstem specifically in the dorsal motor nucleus of the vagus and nucleus of the solitary tract.

Tract tracing and electrophysiological studies have demonstrated that the PVN projects to a number of midbrain and brainstem structures, which are involved in the regulation of a variety of autonomic functions including gastric activities (Saper et al., 1976; Swanson and Sawchenko, 1980; Schwanzel-Fukuda et al., 1984; Lawrence and Pittman, 1985; Arletti et al., 1990; McCann and Roger, 1990; Siaud et al., 1990; Tache et al., 1990; Moga and Saper, 1994). As discussed earlier, neurogenesis in the hypothalamic SON and PVN is completed by day 5 and 7 PN, respectively (Iqbal et al., 1994a), and continues even longer in other forebrain areas (Larsen and Jacobson, 1986; Rivkees et al., 1988; Swanson et al., 1994), whereas neurogenesis in the midbrain and brainstem is completed earlier than that for the forebrain (Iqbal and Jacobson, unpublished data). The early appearance of OT-IR in the forebrain areas and in the brainstem (dorsal motor nucleus of the vagus and nucleus of the solitary tract) suggests that OT systems become functional before neurogenesis is completed and might be involved in early developmental processes and regulation of autonomic functions.

Many neuropeptides like cholecystokinin are reported to be involved in the control of food intake (reviewed in Silver and Morley, 1991). Using c-fos immunohistochemistry and other techniques, it has been shown that peripheral administration of cholecystokinin and gastric distension activate the oxytocinergic neurons in the hypothalamic PVN (Renaud et al.,
These authors have proposed that OT may be involved in the regulation of gastric activities (Verbalis et al., 1993).

Previously, Fox and co-workers (1991a) have demonstrated that cholecystokinin-like immunoreactivity in the developing opossum brain is first detected in the presumptive solitary nucleus between days 5 and 10 PN, whereas it was not detected in the hypothalamus until day 35 PN. In contrast to that, OT-IR containing cells in the developing hypothalamus was detected as early as day 3 PN in the SON and day 5 PN in the PVN. In the nucleus of the solitary tract and dorsal motor nucleus of the vagus, OT-IR containing fibers started appearing at day 10 PN, which almost parallels the appearance of CCK-IR in these areas (Fox et al., 1991a). These findings suggest involvement of OT as a neurotransmitter between the hypothalamus and brainstem for the regulation of autonomic and gastric related activities in the neonatal opossum until other neuropeptide systems become fully functional. However, further studies would be required to prove this hypothesis.

In summary, we have characterized the postnatal development of the hypothalamic PVN and SON nuclei. In addition, we have examined the distribution and ontogeny of AVP-IR and OT-IR containing systems in the adult and developing Brazilian opossum brain. In the hypothalamic PVN and SON, neurogenesis is completed in postnatal life. The general distribution of AVP-IR and OT-IR resembled that which has been reported for other adult mammalian species. In the developing opossum brain, AVP-IR was detected as early as embryonic day 12 and OT immunoreactive fibers were present in the median eminence at day 1 PN. The significance of the early presence of these neuropeptides in the opossum brain before neurogenesis and morphogenesis is completed not fully understood at present.
However, the information presented in this dissertation suggest that AVP-IR and OT-IR containing systems might become functional very early during the postnatal development of *Monodelphis*, which raises the possibility of their involvement in developmental events, and/or autonomic and endocrine related activities. Further studies will help understand the significance of these peptides in the neonatal opossum.
REFERENCES


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