The expression of cholecystokinin and vasopressin binding sites in the developing mammalian brain: a comparative study

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The expression of cholecystokinin and vasopressin binding sites
in the developing mammalian brain: A comparative study

by

Mary Cathleen Kuehl-Kovarik

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For the Graduate College

Iowa State University
Ames, Iowa

1995
DEDICATION

To my ever-supportive husband, Ken, and my parents,
Dave and Thelma,
for making all of this possible.

And to "the gang", Butch, Sabena, and Bartholomew -
for their unending love and patience.
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GENERAL INTRODUCTION

Mammalian neonates are born in various stages of development, depending upon the species requirements for postnatal survival. Some are born hairless and blind; others have the abilities to run and eat solid food. All eutherian neonates, however, share specific developmental patterns, including functional respiratory, excretory, and digestive systems at birth. In addition, eutherian mammals have, in general, completed neurogenesis by the time they are born. Thus, nuclei in the hypothalamus that control food intake and water balance, as well as brainstem respiratory centers, are developed and able to regulate those systems necessary to ensure neonatal survival. Marsupials, however, bear their young in an extremely undeveloped state [see Appendix 1]. Newborn marsupials do have specialized adaptations for external life, including well-developed forelimbs with claws, developed lungs, and a functional mesonephric kidney and digestive tract [Tyndale-Biscoe and Renfree, 1987]. However, the central nervous system in newborn marsupials is still actively undergoing neurogenesis. In the Brazilian opossum, Monodelphis domestica, neurons are produced in the forebrain well after birth [Iqbal et al., 1995; Larsen and Jacobson, 1986]. Thus, opossum neonates lack the central nervous system circuits that normally control food intake and water balance once the pup is born.

Neuronal proliferation in the central nervous system of the laboratory rat is almost exclusively a prenatal process [Bayer and Altman, 1995]. Neurogenesis in the hindbrain and midbrain is completed prior to forebrain development, as determined by [3H]thymidine autoradiography, although there is much overlap. Motor systems in the rat medulla develop between post fertilization day twelve and fourteen (PF 12-PF 14), sensory systems between PF 12 and PF 15, and reticular systems by PF 15 [Bayer and Altman, 1995]. Neurogenesis in the pons is completed by PF 15 as well. The mesencephalon is completely formed by PF 21, with neurogenesis in the tegmentum preceding that of the tectum [Bayer and Altman, 1995]. The thalamus forms between PF 14 and PF 19, the hypothalamus between PF 12 and PF 18 [Bayer and Altman, 1995]. Neurogenesis in the rhinencephalon and telencephalon, including the amygdala, the septum, the hippocampus, and the cortex, begins around PF 12
or PF 13 and continues until PF 20 [Bayer and Altman, 1995]. The striatum is completed by birth. Exceptions to the prenatal cessation of neurogenesis include the olfactory system, dentate granule cells in the hippocampus, and the interneurons (basket cells, stellate cells, and granule cells) of the cerebellum. Many of these neurons are born postnatally, and neurogenesis in these areas may continue until fifteen or twenty days of postnatal age [Bayer and Altman, 1995].

In contrast, a great deal of central nervous system development occurs postnatally in Monodelphis [Iqbal et al., 1995]. Although much less is known about the timing of neurogenesis in the Brazilian opossum, some major trends are evident. Notably, most forebrain structures do not complete neurogenesis until after the birth of the animal. The specific birthdates are known for several forebrain structures in the developing Brazilian opossum. Studies utilizing bromodeoxyuridine immunohistochemistry have established that the paraventricular nucleus and supraoptic nucleus of the hypothalamus complete neurogenesis by 7 days postnatal (7 PN) and 5 PN, respectively [Iqbal et al., 1995]. Neurogenesis is also extremely active in the septum and amygdala of the 7 PN opossum pup, and does not cease until 12 or 13 days of age [Swanson et al., 1994]. Experiments using [3H]thymidine have found neuronal birth in the suprachiasmatic nucleus until 7 PN [Rivkees et al., 1988], and active neurogenesis in the medial preoptic area until 10 or 11 PN [Larsen and Jacobson, 1986]. In addition, some neurogenesis is evident in the cerebral cortex until postnatal day 15 [J. Iqbal, personal communication]. Postnatal neurogenesis is also evident in the midbrain colliculi, and in the cerebellum [J. Iqbal, personal communication]. However, prenatal neurogenesis also appears to be a common feature in the developing Brazilian opossum brain. Very little postnatal neurogenesis is observed in the developing medulla [J. Iqbal, personal communication]. In addition, most motor nuclei in the pons and midbrain appear to have completed neurogenesis prenatally [J.Iqbal, personal communication]. Thus, motor control, and hindbrain development in general, appear to be important to the maintenance of external life in the newborn opossum.

It is obvious that the Brazilian opossum must regulate homeostatic processes prior to the development of forebrain control circuits, suggesting that this function may be taken over
by nuclei within the brain that are present by birth. Specific activities, such as nutrient intake and water balance, could, in some aspect, be controlled by brainstem motor nuclei, especially the trigeminal motor nucleus (MoS), the facial motor nucleus (FMN) and the hypoglossal nucleus. The MoS regulates jaw movement, while the FMN mediates those behaviors involved in facial movement or expression, behaviors which would include suckling, exploration, and defense. The hypoglossal nucleus, which controls tongue movement, would be involved in licking or suckling behaviors. Neurotransmitter systems involving one or more of these nuclei could potentially act as temporary controls of suckling behavior, and thus nutrient and fluid intake, until the hypothalamic circuits were functional.

If such a control mechanism exists, it may not be specific to metatheria. Maintaining homeostasis in mammalian neonates is a very different dilemma than homeostatic control in adult animals. Neonates are faced with a single, fairly constant source of food, although they appear to have no control over fluid versus nutrient intake. Thus, there may be neurotransmitter or receptor pathways that are specific to neonatal homeostasis. In support of this hypothesis, arginine vasopressin (AVP) binding sites have been demonstrated to be transiently expressed in the FMN of the neonatal rat [Tribollet et al., 1991]. Binding sites are present at birth and persist until around 30 PN, after which they disappear. No vasopressin binding sites are reported in the adult rat FMN. Vasopressin is a peptide intimately involved in water balance, and, therefore, vasopressin binding sites in the FMN could be mediating some aspect of water balance in the neonatal rat, and possibly the Brazilian opossum as well.

Multiple neuropeptides are involved in the regulation of food intake in the adult mammal. One of the most abundant and most intensively studied is cholecystokinin (CCK) [Beinfield et al., 1981; Crawley, 1985; Innis et al., 1979; Larsson and Rehfield, 1979]. Our laboratory has previously described the ontogeny of the CCK and AVP peptides in the developing Brazilian opossum brain [Fox et al., 1991; Iqbal and Jacobson, 1995], and they have been studied in the neonatal rat as well [Boer, 1987; Cho et al., 1983; Varro et al., 1983; Whitnall et al., 1985]. The purpose of the studies described in this dissertation was to focus on the expression of cholecystokinin and vasopressin binding sites in the neonatal
metatherian and eutherian brain, with attention focused on those brainstem nuclei that could potentially be involved in the regulation of nutrient or fluid intake. Vasopressin binding sites have already been described in the developing rat brain [Petracca et al., 1986; Tribollet et al., 1991], and this aspect of the project was not repeated.

**Literature Review**

Two peptide neurotransmitter systems will be explored in this dissertation. Cholecystokinin (CCK) has gained popularity among researchers as a potent modulator of food intake. Arginine vasopressin (AVP) has well-described effects on water balance.

**Cholecystokinin**

Cholecystokinin, a gut peptide, is now recognized as one of the most abundant peptides found in the mammalian central nervous system [Beinfield et al., 1981; Crawley, 1985; Innis et al., 1979; Larsson and Rehfield, 1979]. The localization and concentration of cholecystokinin within the brain and intestine has been studied extensively through the use of immunohistochemistry and radioimmunoassay [Beinfield et al., 1981; Crawley, 1985; Fallon and Seroogy, 1985; Gall, 1984; Larsson and Rehfield, 1979; Miceli et al., 1987; Vanderhaeghen et al., 1989]. Cholecystokinin receptors have also been localized by autoradiography in the gastrointestinal tract and central nervous system of several mammalian species, including the rat, mouse, hamster, guinea pig, primate, and human [Day et al., 1986; Gaudreau et al., 1985; Hyde and Peroutka, 1989; Miceli and Steiner, 1989; Niehoff, 1989; Pelaprat et al., 1988; Quirion et al., 1985; Van Dijk et al., 1984; Williams et al., 1986; Zarbin et al., 1983]. Cholecystokinin appears to have a role in many centrally controlled functions [for review, see Crawley and Corwin, 1994]. These include respiration [Hurle et al., 1988], thermoregulation [Shian and Lin, 1985], reproductive behavior [Bloch et al., 1987; Bloch et al., 1988], pituitary hormone secretion [Hashimoto and Kimura, 1986; Kimura et al., 1983; Vijayan et al., 1979], nociception [Baber et al., 1989; Faris et al., 1982; Faris et al., 1984; Wiertelak et al., 1992], anxiety [Costall et al., 1991; Hendrie et al., 1993; Singh et al., 1991], and memory and emotion [Crawley, 1985; Itoh et al., 1989]. By far the best-known role for CCK, however, is its action as a feeding satiety factor [see
Silver and Morley, 1991 for review].

Cholecystokinin was first described as a gut hormone, secreted by the duodenum, that stimulated gall bladder contraction and exocrine pancreatic secretion. Subsequent research demonstrated a role for CCK in the control of food intake [Baile and Della-Fera, 1985; Fried et al., 1991; Gregory et al., 1989; Moran et al., 1990; Morley et al., 1985]. In the adult mammal, a pathway of cholecystokinin immunoreactive cells and fibers is postulated to exist from the vagus and nucleus of the solitary tract, projecting through the parabrachial nucleus to feeding centers in the hypothalamus [Fulwiler and Saper, 1985; Herbert and Saper, 1990]. Less is known about the roles of CCK in feeding behavior other than direct satiety, although adult rats are reported to complete the full routine of post-feeding behavior after exogenous CCK administration [Antin et al., 1975]. Therefore, it seems likely that CCK has a central effect on motor systems involved in the control of feeding-related behaviors.

Cholecystokinin receptor binding is evident in rat gastric mucosa and muscle by PF 20 [Robinson et al., 1987], but appears to develop postnatally in rat brain [Hays et al., 1981; Pelaprat et al., 1988]. Cholecystokinin receptor localization in the developing mammalian brain has been reported only for the rat. In that study, binding sites were localized only to forebrain and midbrain regions [Pelaprat et al., 1988]. Thus, CCK binding sites in the developing brain have not been well studied, especially in hindbrain regions. Cholecystokinin in the developing mammalian hindbrain may play a role in the regulation of food intake. Therefore, knowledge of where and when CCK binding sites are expressed in the developing hindbrain of both the rat and the Brazilian opossum would be useful in elucidating potential mechanisms of such a relationship.

Arginine vasopressin

Arginine vasopressin plays a central role in water balance and blood pressure regulation [Tanaka et al., 1993; Zimmerman, 1983], an action mediated through magnocellular neurosecretory neurons located in the hypothalamus [Morris et al., 1987]. In addition, AVP appears to function in several other areas involving homeostasis, including central thermoregulation [Oloyami and Hart, 1992], circadian rhythmicity [Reghunandanan et al., 1990] and drinking and eating behavior [Burlet et al., 1992; Mangiapane et al., 1983].
Vasopressin has also been postulated to play a part in sexual and social behavior [Bluth and Dentzer, 1993; Wang et al., 1993; Winslow et al., 1993] and learning and memory processes [DeWied, 1980]. Along with these functional roles, AVP has been postulated to influence central nervous system development [Boer, 1985; Boer et al., 1980, 1993; Snijdewint et al., 1988].

In general, arginine vasopressin-like immunoreactivity (AVP-IR) appears prior to birth in the eutherian brain [Boer, 1987; Buijs et al., 1980; Whitnall et al., 1985]. Arginine vasopressin-like immunoreactivity is present prior to birth in the Brazilian opossum central nervous system [Iqbal and Jacobson, 1995] as well, a finding consistent with results in other species. Likewise, AVP binding sites have been detected early in development. Autoradiographical studies have demonstrated AVP binding as early as embryonic day 16 in the laboratory rat [Tribollet et al., 1991], as well as in the newborn rat [Petracca et al., 1986] and golden hamster [Delville et al., 1994]. Many of the earliest binding sites detected in the fetal rat are found in extrahypothalamic locations, including the ventral pontine reticular formation, the facial motor nucleus, the nucleus of the solitary tract, and the spinal trigeminal nucleus [Tribollet et al., 1991]. Binding in the anterior lobe of the pituitary, the suprachiasmatic nucleus, and the septal area is also evident prior to birth in the laboratory rat [Tribollet et al., 1991]. The significance of the early, and sometimes transient, expression of vasopressin binding sites remains to be investigated. Especially noteworthy is the transient expression of vasopressin binding sites in the facial motor nucleus of the developing rat [Tribollet et al., 1991]. The presence of AVP binding sites in the brain of the neonatal opossum has yet to be investigated.

Oromotor nuclei

Feeding and drinking behaviors involve the action of facial muscles, the jaw musculature, and the tongue. These muscles are controlled by cranial nerve nuclei in the brainstem known as oromotor nuclei [Travers, 1995]. Oromotor nuclei include the trigeminal motor nucleus (Mo5), the facial motor nucleus (FMN), and the hypoglossal nucleus (12) of the hindbrain. The trigeminal motor nucleus innervates the muscles of mastication, including the masseter, temporalis, and anterior digastric. Motoneurons in the
facial nucleus supply the innervation to various facial muscles, including the muscles of the mouth. Motoneurons located in the hypoglossal nucleus innervate the intrinsic and extrinsic tongue muscles.

There is little evidence for direct cortical input to brainstem motor nuclei in the adult. However, the situation in neonates is unknown at this time. In addition, forebrain nuclei influence the activity of oromotor nuclei through indirect routes, probably involving the reticular formation [Travers, 1995]. Thus, the relationship between forebrain structures and hindbrain motor nuclei is likely more complicated than originally thought.

The rat facial motor nucleus receives substantial input from nuclei of the midbrain, pons, and medulla [Travers, 1995]. Specific afferent projections include the lateral lemniscus, the red nucleus, the superior colliculus, the central gray, periocular nuclei, and the midbrain reticular formation. In the pons, the ventrolateral parabrachial nucleus, as well as specific regions of the reticular formation, contain cells that project to the FMN. Most projections from the medulla originate from the reticular formation, although projections from the spinal trigeminal complex, the medial vestibular nuclei, and the anterior nucleus of the solitary tract have been reported [Bystrzycka and Nail, 1985; Dom et al., 1975; Norgren, 1978; Travers, 1995].

Afferent projections reach the hypoglossal nucleus from cells in the midbrain, pons, and medulla; there appear to be no direct cortical projections [Travers, 1995]. Midbrain and pontine inputs project from the reticular formation, the medial parabrachial nucleus, and the principal sensory trigeminal nucleus [Travers, 1995]. Medullary projections originate from the reticular formation, the spinal trigeminal complex, and the nucleus of the solitary tract [Travers, 1995].

As for the other motor nuclei, there is little anatomical evidence for direct forebrain projections to the trigeminal motor nucleus. However, it appears that several forebrain structures, including the frontal cortex and central nucleus of the amygdala, have a direct influence on trigeminal motoneuron activity [Travers, 1995]. Rhythmic masticatory movements appear to be organized in the brainstem reticular formation [Travers, 1995]. Many projections to the Mo5 consist of pathways from sensory trigeminal nuclei, including
cells in the ipsilateral mesencephalic trigeminal nucleus, supratrigeminal, intertrigeminal and principal sensory trigeminal nuclei, and the spinal trigeminal complex. Pontine projections include adrenergic pathways [Travers, 1995]. Medullary projections are detected from the reticular formation and the raphe nuclei [Travers, 1995].

In the adult, pathways from the hypothalamus project to hindbrain regions that are involved in the control of homeostasis. These pathways are likely absent in neonatal marsupials, and possibly may be undeveloped in neonatal eutherians as well. Neonatal mammals must have unique mechanisms to maintain homeostasis and survive. In the case of marsupials, adaptations must be even more extreme, as newborn marsupials have not developed all of the organ systems necessary to ensure survival. The studies described in this dissertation are focused on two homeostatic mechanisms, nutrient intake and water balance, and trying to determine, through anatomical studies, how neonates regulate their internal environment at the level of the central nervous system. Thus, I am exploring how two neurotransmitter systems, cholecystokinin and vasopressin, may, through receptor expression, modulate the central nervous system control of nutrient and water intake.

Dissertation Organization

The body of this dissertation consists of three manuscripts. The first has been published in the Journal of Comparative Neurology [336: 40-52, (1993)]. The second has been accepted by Brain, Behavior, and Evolution, and the third has been submitted to the same journal. The papers are preceded by a general introduction and followed by a summary and general discussion. The literature cited in the introduction and discussion is presented after the general discussion section. In addition, a fourth manuscript describing the general biology and use of the Brazilian opossum has been included as Appendix 1. All of the research presented in this dissertation was performed by myself, under the guidance of Dr. Carol D. Jacobson.
CHAPTER ONE. LOCALIZATION OF CHOLECYSTOKININ BINDING SITES IN THE ADULT AND DEVELOPING BRAZILIAN OPOSSUM BRAIN

A paper published in the Journal of Comparative Neurology

M. Cathleen Kuehl-Kovarik, Lynne R. Ross, Joel K. Elmquist, and Carol D. Jacobson

Abstract

Cholecystokinin (CCK) is now recognized as one of the most abundant peptides in the mammalian central nervous system. We have previously used immunohistochemistry to localize CCK in the adult and developing Brazilian opossum brain. However, little is known about the distribution of CCK binding sites in the developing mammalian brain. Therefore, to further our knowledge of the sites of action for CCK during development, we initiated a series of studies to localize CCK binding sites in the adult and developing Brazilian opossum. This species was chosen since pups are born in a fetus-like state. Receptor autoradiography was performed on coronally sectioned brains of 1 to 60 day postnatal (PN) animals and adults with $^{125}$I-Bolton Hunter-CCK-8 as the radioligand. Binding is evident in the 1PN opossum brainstem, and is observed in the developing forebrain by 5PN. Region-specific binding increases during development, and binding in the 35PN brain resembles that of the adult pattern. Binding is evident prior to the detection of CCK-like immunoreactivity in many areas. The facial motor nucleus is identifiable and exhibits high levels of binding in Brazilian opossum pups of 10 to 35 days of age. However, binding is undetectable in the facial motor nucleus of 45 and 60PN pups. In general, the binding patterns for CCK in the adult opossum resemble those of other mammals, and likely mediate similar physiological functions. However, some cholecystokininergic pathways appear to be unique to neonatal mammals. Further studies will investigate the functional significance of CCK binding sites during development.
Introduction

Cholecystokinin (CCK), a well-known gastrointestinal peptide, is now recognized as one of the most abundant peptides in the central nervous system (Innis et al., 1979; Larsson and Rehfield, '79; Beinfeld et al., '81; Crawley, '85). Cholecystokinin appears to play a role in many centrally controlled functions, including respiration (Hurle et al., '88), thermoregulation (Shian and Lin, '85), reproductive behavior (Bloch et al., '87; Bloch et al., '88), pituitary hormone secretion (Vijayan et al., '79; Kimura et al., '83; Hashimoto and Kimura, '86), nociception (Faris et al., '82; Faris et al., '84; Baber et al., '89;), anxiety (Singh et al., '91), and memory and emotion (Crawley, '85). By far the best-known role for CCK, however, is its action as a feeding satiety factor. For a comprehensive review, see Silver and Morley ('91).

The localization and concentration of CCK within the adult brain has been studied extensively through the use of immunohistochemistry and radioimmunoassay (Larsson & Rehfield, '79; Vanderhaegen et al., '80; Vanderhaegen et al., '81; Beinfeld et al., '81; Gall, '84; Crawley, '85; Fallon & Seroogy, '85; Miceli et al., '87). Several studies have also examined the expression of CCK during development (Cho et al., '83; Varro et al., '83; Huang et al., '86; Scalise & Vigna, '88; Fox et al., '91a). Cholecystokinin immunoreactive cells and fibers appear by embryonic day 15 (ED15) in rat brain (Cho et al., '83), and CCK is present at birth in guinea pig cortex, brain stem, and hypothalamus (Huang et al., '86). Cholecystokinin immunoreactive fibers are present in the brainstem of the 5 day postnatal (5PN) Brazilian opossum, and cells are present in this region by 10PN (Fox et al., '91a).

With the use of autoradiography, cholecystokinin binding sites have also been localized in the central nervous system of many adult animal species (Gaudreau et al., '83; Zarbin et al., '83; Van Dijk et al., '84; Williams et al., '86; Sekiguchi and Moroji, '86; Miceli and Steiner, '89; Niehoff, '89), and humans (Gaudreau et al., '85; Vanderhaegen et al. '89). However, few studies have reported the existence and localization of CCK binding sites in the developing brain. Hays and coworkers ('81), using in vitro binding studies, found very low levels of CCK binding in 1 to 2 day old rats. Binding increased to a
maximum at 12 days. Localization studies in postnatal rats (Pelaprat et al., '88) have revealed receptor binding in the amygdaloid complex, endopiriform nucleus, and ventromedial nucleus of the hypothalamus on postnatal day 0 (P0), and in cortex, hippocampus, nucleus accumbens, striatum, dorsal thalamus, and mesencephalon by P10. However, localization of CCK binding sites in developing animals has not been reported for hindbrain regions.

In this study, we have used autoradiography to localize CCK binding sites in the brain of the Brazilian short-tailed opossum, *Monodelphis domestica*. *Monodelphis* 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. Initial studies have shown that neurogenesis in forebrain structures is active into the second week of postnatal life (Larsen and Jacobson, '86; Rivkees et al., '88). Thus the use of *Monodelphis* allows studies to be performed while neurogenesis is still occurring, but alleviates the necessity of performing cesarian sections in pregnant animals (Schwanzel-Fukuda et al., '88; Dore et al., '90; Fox et al., '91a; Brunjes et al., '92; Wang et al., '92). Previous studies in the adult opossum have demonstrated that neuropeptide systems and steroid receptor systems in the marsupial brain resemble that of the rodent (Fox et al., '91a,b; Elmquist et al., '92). Specifically, we have previously used immunohistochemistry to localize cholecystokinin cells and fibers in the adult and developing Brazilian opossum brain (Fox et al., '90; Fox et al., '91a). To further our knowledge of the sites of action for CCK, we have conducted a series of studies to localize CCK binding sites in these animals. The first study was designed to examine binding sites in adult opossums. Because CCK receptor localization has not been reported in the hindbrain of neonates, and as we have localized binding sites in regions of the adult brain not previously reported for other species, we subsequently localized CCK binding sites in developing *Monodelphis*. 
Materials and Methods

Animals

Adult and developing male and female Brazilian short-tailed opossums were obtained from a colony maintained at Iowa State University. The initial animals used to start the breeding colony were acquired from the Southwest Foundation for Research and Education in San Antonio, TX. The animals were housed in plastic cages, and maintained at 26°C on a 14:10 light-dark cycle. Water and food (Reproduction Fox Chow; Milk Specialties Products, Madison, WI) were provided ad libitum. For breeding, male and female animals were paired for 14 days. Animals were then separated and the female checked daily for the presence of pups. Day of birth was denoted 1PN. Young animals were housed with their mothers until 60PN. All animal housing and use of animals was in accordance with the guidelines and approval of the Iowa State University Committee on Animal Care.

Tissue collection

For the first study, three male and three female adult animals eight months of age were used. Animals were quickly decapitated and their brains were removed and frozen on dry ice. Frozen brains were stored at -20°C until used. Brains were cut into 20 μm thick coronal sections on a cryostat (Reichert Instruments) and thaw-mounted onto poly-L-lysine coated slides. Slides were stored at 4°C until processed for autoradiography.

Animals from 1 to 60PN were used in the second study. Animals of each age came from at least three different litters. Three 1PN pups were included. Four pups were collected at 5PN, an age when pups are still sexually undifferentiated upon visual inspection. Three male and three female pups were collected at 10, 15, 25, 35, 45, and 60PN. All pups were quickly decapitated. The heads of 1 to 25PN animals were frozen directly on dry ice. Brains were removed from 35, 45 and 60PN animals and similarly frozen. Tissue was sectioned as for the first study.

Autoradiography

The protocol for receptor autoradiography was a modification of the protocols reported by Herkenham and Pert ('82) and Niehoff ('89). Sections were preincubated for
30 minutes at room temperature in 50 mM Tris HCl, pH 7.4, containing 130 mM NaCl, 4.7 mM KCl, 5 mM MgCl₂, and 1 mM EGTA (Tris saline buffer) plus 0.5% BSA. Sections were subsequently incubated for 2.5 hours at room temperature in Tris saline buffer (pH 6.5) containing 0.025% bacitracin, 1 mM dithiothreitol, 4 μg/ml leupeptin, and 100 pM ¹²⁵I-Bolton Hunter-CCK-8 (New England Nuclear, 2200 Ci/mmol). Negative controls were generated by coincubation of ¹²⁵I labelled CCK solution with 1 μM unlabelled peptide. Following incubation, sections were washed in preincubation buffer at 4 °C, six times for 15 minutes each time, and dipped briefly in ice-cold water. Slides were air-dried overnight. Labelled sections were exposed to LKB ³H-Ultratfilm for four days (for neonates) or six days (for adults). Films were subsequently developed by hand.

Sections were fixed in paraformaldehyde fumes overnight. Fixed slides were dipped in NTB-2 photographic emulsion (Kodak), dried for four hours, and stored at 4 °C in a lightproof box for three weeks. Slides were then developed in Kodak Dektol developer, dipped in a glacial acetic acid bath, and fixed in Kodak Fixer. Tissue was washed for one hour in cold water, counterstained with 1% neutral red, dehydrated in alcohols, and coverslipped.

Analysis

Analysis was performed with the aid of a developing and adult Brazilian opossum atlas developed in our laboratory (see Fox et al., '91a; Elmquist et al., '92), supplemented by a developmental rat atlas (Paxinos et al., '91) and a developmental mouse atlas (Schambra et al., '92). Sections were analyzed at 160 μm intervals in adults, and 80 μm intervals in developing animals. Autoradiograms were viewed with a light-box and a hand-held lens. Emulsion-dipped slides were observed with a Zeiss microscope and the location and relative density of silver grains noted.

A standard curve of the amount of ¹²⁵I-labelled CCK bound to tissue sections has been created by using rat brain paste standards. Although we did not quantify our results in these studies, the density of labelling on the autoradiograms appears to be proportional to the amount of ¹²⁵I-CCK-8 bound.
Results

Coincubation of tissue with $^{125}$I-CCK-8 and unlabelled peptide resulted in the blocking of all labelling in the brain (Fig. 1). Therefore the labelling observed was assumed to be specific for CCK binding sites. Density of labelling, as determined by the density of silver grains on the LKB Ulrofilm or on the microscope sections, was denoted as high (heavy, dense), moderate, or low. A comparison of sections of the same age exposed on the same film and between films was also made to aid in categorizing the density of binding sites. Diffuse binding refers to a lack of specific nuclear borders in the binding pattern. Dramatic changes in binding levels within an area during development were noted.

*CCK binding sites in the adult opossum*

Cholecystokinin binding sites were observed throughout the adult Brazilian opossum brain (Table 1, Fig. 2). The highest levels of binding were observed in the cortex, hippocampus, ventromedial hypothalamic nucleus, interpeduncular nucleus, cerebellum, and nucleus of the solitary tract. Nissl stained tissue corresponding to the autoradiograms are shown in figure 2 to help identify the brain regions possessing the CCK binding sites.

**Rhinencephalon.** A high level of binding was evident in the olfactory bulb (OB), particularly in the external plexiform layer, and also in the piriform cortex (Pir). Moderate binding was observed in the region of the islands of Calleja (ICj).

**Telencephalon.** A high level of binding was observed throughout the neocortex (Cor, Fig. 2A, B). Moderate binding was evident in the basal ganglia. Binding was evenly distributed throughout the caudate putamen (CPu) and globus pallidus (GP). Dense binding was observed in the endopiriform nucleus (En). In the septal region, a high level of binding were observed in the nucleus accumbens (ACb). Low levels were evident in the ventral part of the lateral septal nucleus (LSV) and the medial division of the bed nucleus of the stria terminalis (BSTM). Within the hippocampus, the CA1, CA2, and CA3 regions of Ammon’s horn and the subiculum (S) and presubiculum (PrS) all displayed heavy binding. In caudal regions, binding was evident only in CA1 (Fig. 2A, B). Within the amygdala, moderate binding was observed in the basal amygdaloid nuclei (BAA, BAM) and the posterior cortical
amygdaloid nucleus (PCo). Light binding was evident in the anterior cortical amygdaloid nucleus (ACo) and the posterior amygdaloid area (PAA).

**Diencephalon.** A low level of binding was observed in midline thalamic regions (Fig. 2A, B). Although binding did not appear to be localized to discrete nuclei, parts of the paraventricular thalamic nucleus (PV), intermediodorsal thalamic nucleus (IMD), and rhomboid thalamic nucleus (Rh) were included in this area. Light binding was also observed in the reuniens thalamic nucleus (Re) and medial habenular nucleus (Mhb). A moderate amount of binding was localized to the subparafascicular thalamic nucleus (SPF).

As expected, the ventromedial hypothalamic nucleus (VMH) exhibited a very high level of binding (Fig. 2A, B). The paraventricular hypothalamic nucleus (Pa) and medial preoptic nucleus (MPA) had moderate levels of binding as well. Both the ventral (PMV) and dorsal (PMD) parts of the premammillary nucleus contained CCK binding sites. A higher level of binding was evident ventrally.

**Mesencephalon.** Dense binding was discretely localized to the lateral subnucleus of the interpeduncular nucleus (IPL, Fig. 2C, D). No other parts of the interpeduncular nucleus were labelled. Moderate binding was evident in the ventral tegmental area (VTA) and the compact part of the substantia nigra (SNC). Low levels of binding were observed in the central gray (CG) and dorsal raphe nucleus (DR). Binding was moderate in the mesencephalic trigeminal nucleus (Me5), and moderate to high binding was restricted to medial regions of the inferior colliculus (IC). High levels of labelling defined the ventral tegmental nucleus (VTg). Moderate binding was evident in the area of the dorsal tegmental nucleus (DTg).

**Metencephalon.** A moderately high level of binding was evident in the molecular layer throughout the cerebellum (Cb, Fig. 2E, F). Binding in other cerebellar layers was minimal.

A low level of binding was observed in the region of the parabrachial nucleus (PB). A moderate level of binding was observed in both the medial (MVe) and lateral (LVe) vestibular nuclei. A moderate level was also observed in the facial motor nucleus (FMN) and binding was denser in medial portions of this region.
Myencephalon. Binding was evident throughout the spinal trigeminal nucleus. Binding was low in the oral region (Sp5O), but increased caudally (Sp5C, Fig. 2E, F). Binding was extremely heavy in the nucleus of the solitary tract (Sol, Fig. 2E, F) and was also high in the area of the dorsal motor nucleus of the vagus (10) and the area postrema (AP). Low levels of binding were observed in the inferior olive (IO, Fig. 2E, F) and the hypoglossal nucleus (12, Fig. 2E, F).

CCK binding sites in the developing Brazilian opossum.

The distribution of cholecystokinin binding sites in discrete nuclei increases dramatically during the development of the central nervous system (Table 1). However, binding appeared to form a diffuse pattern during development. Although diffuse labelling could be due to our autoradiographic techniques, it is likely that, at early ages, cells of a specific phenotype are not well organized in discrete nuclear groups. Indeed, many nuclear groups in the brainstem cannot be recognized until 5PN, although neurogenesis of this region appears to be complete. Diffuse binding was confined to the developing brainstem of IPN opossum pups (Fig. 3). A high level of binding was also evident in the vagus nerve peripheral to the brain (10n, Fig. 3). No binding was observed in the forebrain at this age. Binding could be localized to discrete areas as early as 5PN. However, diffuse binding was evident in various regions until 35PN. Because differentiation is still active in some regions, many areas are difficult to identify or are considered presumptive in the younger animals.

CCK binding sites in the 5PN Brazilian opossum. Binding was low to absent in most of the forebrain of the 5PN opossum. Light homogeneous binding was present in the area of the presumptive hypothalamus. In the caudal telencephalic region, a well-defined area of labelling ran ventrocaudal, lateral to the third ventricle. A moderate level of binding was observed in a discrete midbrain region. Although nuclear groups are not evident histologically at this age, possible presumptive structures in this area include the red nucleus (R), the ventral tegmental area, and the interpeduncular nucleus (IP). Another discrete area, possibly the presumptive oculomotor (3) or trochlear (4) nucleus, labelled in the caudal midbrain. Labelling in the brainstem was generalized, but denser binding was evident in the areas of the FMN and the Sol. Heavy binding was observed in the trigeminal ganglion
CCK binding sites in the 10PN Brazilian opossum. Nissl stained tissue corresponding to the autoradiograms are shown in figure 4 to help identify the brain regions possessing the CCK binding sites. By ten days of age, low levels of binding were observed in the OB and Cor. Moderate to heavy levels of homogeneous binding was evident in regions of the amygdala (AA, Fig. 4A, B) and hypothalamus (Hyp, Fig. 4A, B). Diffuse labelling was also evident in the thalamus. The R/VTA/IP area was densely labelled (denoted as ventral mesencephalon, VMes, Fig. 4C, D). The labelled region in the caudal midbrain could now be identified as the presumptive 4 or Vtg. The trigeminal nerve (5n, Fig. 4C, D) labelled densely in the lateral midbrain, and the ventral nucleus of the lateral lemniscus (VLL, Fig. 4C, D) labelled also. In the brainstem, the FMN (Fig. 6C) was densely labelled. Binding in the caudal brainstem was diffuse, but could be localized to the Sol (Fig. 4E, F). The 5Gn and vagus nerve (Fig. 4E, F) were densely labelled.

CCK binding sites in the 15PN Brazilian opossum. Binding was evident throughout the 15PN brain. The OB was lightly labelled, and for the first time, binding was observed in the ACb, the CPU, and the En. Heavy binding was observed in the LSV. Binding was still considered low in the cerebral cortex and piriform cortex. There was dense binding in the S of the developing hippocampus. Binding was evident in the thalamus, in the region of the thalamic reticular nucleus (Rt), and light binding was observed in the midline thalamic region including the IMD, PV, and Rh. A dense level of binding was observed in the Re and the SPF. Within the hypothalamus, binding could be localized to the VMH, and was evident in the PMV as well. However, light binding was evident throughout the hypothalamic region which could not be localized to specific nuclei. Within the amygdala, binding appeared to be regionally specific in the ACo, the PAA and the basal amygdaloid nuclei. The ventral mesencephalic region still labelled densely, and there was some low level labelling rostrally that appeared to be VTA as well. There was discrete labelling in the 4/VTg region. Binding in the ventral periolivary nucleus (VPO) was observed for the first time, and moderate binding was evident at this age in the VLL. The presumptive molecular layer of the cerebellum was lightly labelled, but denser binding in the cerebellar nuclei (Cbn)
was evident. The FMN and the Sol/10 both exhibited dense binding, and the Sp5C displayed moderate binding. Heavy binding was still present in the 5Gn, 5n, and the vagus nerve.

**CCK binding sites in the 25PN Brazilian opossum.** Nissl stained tissue corresponding to the autoradiograms are shown in figure 5 to help identify the brain regions possessing the CCK binding sites. By this age, binding patterns were beginning to resemble the adult pattern, but diffuse labelling of regions was evident throughout the brain (Fig. 5). As observed in the 15PN pup, binding was evident in the OB and dense in the LSV. Binding was light in the Cor, Pir, CPu, and ACb. The subiculum and presubiculum of the hippocampus labelled densely, as did the CA1 (Fig. 5A, B), CA2, and CA3 regions of Ammon's horn. The Rt, Re, and midline thalamic nuclei (Fig. 5A, B) labelled as in the 15PN pup, and light binding was evident in the MHB. At this age, binding was evident in the MPA. A moderate level of binding was evident throughout the hypothalamus, but binding increased in the VMH (Fig. 5A, B) and PMV. Binding in the amygdala resembled that of the 15PN pup (Fig. 5A, B), as did labelling in the VPO and VLL (Fig. 5C, D). Low levels of binding were observed for the first time in the CG and the DR. Dense binding was now evident in the VTA (Fig. 5C, D), and lighter binding levels were observed in the IPL. No labelling was noted in the red nucleus, suggesting that binding in this region observed at an earlier age was localized to the presumptive VTA. Dense binding was localized to the VTg, and no labelling was observed in the 4 of 25PN or older animals. The inferior colliculus (IC) was lightly labelled by 25PN. The cerebellum and cerebellar nuclei labelled as in the 15PN animal, and the FMN and Sol/10 (Fig. 5E, F) still labelled densely. Heavy binding was also evident in the region of the AP by this age. Binding in the Sp5C (Fig. 5E, F) had increased in density, and binding was now evident in the hypoglossal nucleus (12, Fig. 5E, F). Binding was observed in the vagus nerve but was virtually absent in the 5Gn and 5n.

**CCK binding sites in the 35PN Brazilian opossum.** Many regions of the 35PN brain were not significantly different from the 25PN brain. At this age, the Cor and Pir had become densely labelled. Binding was evident for the first time in the ICj, the BSTM, and the GP of the forebrain. Within the thalamus, binding in the Rt had decreased significantly. Binding was now evident in the Pa. Dense binding was clearly evident in the IPL.
Moderate to heavy binding was expressed in the Me5 medial to the inferior colliculi. Also in the mesencephalon, binding was noted in the DTg, but was absent in the VPO and VLL. Binding was first noted in the PB, MVe and LVe, the Sp5O, and the IO at this age.

Because brains at this age and older were removed from the calvaria, binding peripheral to the brain could no longer be evaluated.

**CCK binding sites in the 45 and 60PN Brazilian opossum.** Binding patterns in the 45PN and 60PN brain resembled those of the adult Brazilian opossum, and binding was generally confined to discrete areas. By 45 days of age, binding in the Rt was completely absent. Binding had increased in the molecular layer of the cerebellar cortex but had greatly decreased in the cerebellar nuclei by 60PN, and also was evident in the SNC, as in the adult.

A significant difference in the brain of animals of these ages was observed in the brainstem. Although the facial motor nucleus exhibited binding sites in adults (Fig. 6A), and labelled heavily in younger animals (Fig. 6C), no CCK binding sites were evident in this region in animals of 45 or 60 (Fig. 6B) days of age.

**Summary and Trends**

The development of CCK binding sites in the Brazilian opossum central nervous system appears to progress from the caudal brain rostrally. Binding is observed in the brainstem of the IPN opossum pup, and binding is evident in the presumptive hypothalamus by 5PN. By 10PN, binding levels have increased and binding sites can be localized to specific nuclei. The 15PN opossum brain begins to resemble the adult brain morphologically and binding can be found in discrete nuclei throughout the brain, including the limbic system, basal ganglia, thalamus and hypothalamus, midbrain, cerebellum and brainstem. In animals of these ages, diffuse binding is evident throughout the brain but appears to decrease with maturation. By 25 days of age, binding patterns are beginning to resemble those of the adult opossum brain, and at 35PN, few changes are occurring. A significant finding in the 45 and 60PN opossum brain is the absence of binding in the FMN. Thus we see a robust, but transient expression of CCK binding sites in the facial motor nucleus of neonatal Brazilian opossums.
Discussion

The distribution of cholecystokinin receptors, as defined by \(^{125}\text{I}-\text{CCK}\) binding, in the adult Brazilian opossum brain generally resembles that found in other mammalian species (Zarbin et al., '83; Gaudreau et al., '83; Van Dijk et al., '84; Sekiguchi and Moroji, '86; Miceli and Steiner, '89; Niehoff, '89). However, few studies have examined CCK binding during development. The patterns of CCK binding in the developing Brazilian opossum forebrain are similar to those reported for the rat (Pelaprat et al., '88). Binding is evident at an early age in the limbic system and ventromedial hypothalamus, and is evident throughout the developing forebrain soon afterwards. However, receptor binding is observed in the 1PN opossum brain and is seen throughout the brain by 10PN, equivalent morphologically to a prenatal rat. Preliminary studies in our laboratory on embryonic day 18 rat tissue suggest that many more regions express CCK binding sites in developing opossums of a comparable morphological age (approximately 10 to 15 days postnatal). This may suggest that CCK binding sites are integral to postnatal physiology or behavior.

Cholecystokinin, pCCK mRNA, and CCK binding sites appear to develop postnatally, peak approximately 18 days postnatal, and then decline to adult levels in the rat forebrain (for review, see Micevych and Ulibarri, '92). Both whole-brain homogenate binding studies (Hays et al., '81) and CCK binding localization studies (Pelaprat et al., '88) support this trend. We do see decreased levels of CCK binding sites in specific brain areas, including the VTA, VLL, RT, VPO, and the FMN, with maturation. However, although we have not quantified our data, many areas in the developing Brazilian opossum brain obviously do not follow this pattern, including the cortex, molecular layer of the cerebellum, VMH, amygdala and caudate nucleus. Because the neonatal Brazilian opossum is still undergoing neurogenesis, and the developmental timecourse is protracted in relationship to rat development, different developmental patterns are to be expected.

Some differences do exist between patterns of CCK receptor binding observed in the adult Brazilian opossum brain and those observed for other species. Especially significant is the existence of CCK receptors in particular cranial nerve motor nuclei, including the facial and the hypoglossal. Also, binding confined to the molecular layer of the cerebellum
appears to be unique to this animal. Significant receptor binding in the Cb has been reported for the guinea pig, mouse, primate, and human (Zarbin et al., '83; Sekiguchi et al., '86; Niehoff, '89; Vanderhaeghen et al., '89; Hill et al., '90) but binding is consistently minimal in the rat (Gaudreau et al., '83; Van Dijk et al., '84; Sekiguchi et al., '86; Niehoff, '89). Receptors in the guinea pig and human are expressed at high levels in the granular layer and at lower levels in the molecular layer (Zarbin et al., '83; Sekiguchi et al., '86; Niehoff, '89; Vanderhaeghen et al., '89). Thus, even within the region there are species differences. Such differences could reflect true anatomical or physiological differences, or neurotransmitter variations within a physiological pathway. Thus, the significance of the variability in CCK receptor binding patterns is unclear.

Cholecystokinin immunoreactivity in the adult Brazilian opossum parallels binding patterns in many brain regions (Fox et al., '91a). However, striking differences do exist. Cholecystokinin-like immunoreactivity is absent in the VMH, IC, FMN, 12, and cerebellum of the Brazilian opossum. However, if the axonal terminals are small, or CCK transport is extremely rapid in these areas, the peptide may not be detected by our immunohistochemical methods. In support of this hypothesis, King and Bishop, using a different CCK antibody ('90; '92), report that cholecystokinin has been detected in the cerebellum of the North American opossum, Didelphis virginiana. Another possibility is that CCK is not being transmitted at a classical synapse in these brain regions (Kuhar, '85; Herkenham, '87).

Cholecystokinin-like immunoreactivity (CCK-IR), like CCK binding, progresses from caudal to rostral in the developing Brazilian opossum. Binding is observed in the forebrain in 5PN pups, and is as far rostral as the OB by 10PN. Pups are 10 days of age before CCK-IR cells and fibers are observed rostral to the Sol. Binding is apparent in the VMH by 15PN, but CCK expression in the hypothalamus does not appear to be well organized until 35PN. Thus, receptor binding is evident before the peptide is detected. CCK-IR cells and fibers are seen transiently in the CPu, and CCK-IR fibers are evident in the 12 at 10 and 15PN. Although neither of these regions appear to contain CCK in the adult opossum, CCK receptors are expressed in these regions in the adult.

The facial motor nucleus exhibits high levels of CCK binding sites in Brazilian
opossum pups of 10 to 35 days of age. Binding is evident in the brainstem of younger animals, but cannot be definitively localized to the FMN, so it cannot be accurately assessed. Binding in the FMN of pups appears to be homogeneous throughout the nucleus. Binding is undetectable in 45 and 60 day old opossums, but is once again evident in adult opossums, and appears to be concentrated ventromedially. The age at which binding sites are reexpressed in adults is unknown at this time.

The expression of CCK binding sites in the FMN of the adult appears, among the mammals studied, to be unique to the Brazilian opossum. However, the ubiquity of CCK binding sites in the facial motor nucleus in mammalian neonates is unknown and under investigation. Preliminary studies suggest a transient expression of CCK binding sites in the FMN of the neonatal rodent as well. Behaviors mediated by the facial motor nucleus are those involved in facial movement or expression. These behaviors would include nursing, exploratory behavior, as in feeding or reproduction, defense, and emotion. Because CCK binding is extremely high in neonatal, nursing pups, and disappears as the pups begin to consume solid food, it is possible that CCK receptors located within the facial motor nucleus could be mediating nursing behavior in neonatal mammals. Receptor expression in the FMN of adults is likely mediating an entirely different behavior, such as defense, exploration or reproduction. The significance of this finding to the Brazilian opossum or to all marsupials remains to be elucidated.

The Brazilian opossum provides a unique tool for studying CNS development. Although the brainstem appears to have completed neurogenesis by birth (Jacobson lab, unpublished), neurogenesis in forebrain structures is active through the first ten to fourteen days of neonatal life (Larsen and Jacobson, 1986; Rivkees et al., 1988). Thus, this mammal must adapt to ex utero life before neurogenesis is complete. We find it interesting that the cholecystokininergic system may mediate the motor aspects of nursing behavior in the neonatal opossum. Feeding is necessary for neonatal existence, but cannot be regulated through hypothalamic pathways in the neonatal Monodelphis as in the adult. Thus, other pathways must exist that modulate motor aspects of food intake in this neonate.

Cholecystokinin is best known as a satiety factor (Della-Fera and Baile, '80; Baile
and Della-Fera, '85; Morley et al., '85; Gregory et al., '89). Peripherally, CCK is known to decrease gastric emptying (Moran and McHugh, '89; Fried et al., '91). However, the role that central cholecystokinin plays in satiety is still controversial (Baile and Della-Fera, '85; Smith and Gibbs, '85). A pathway of cholecystokinin immunoreactive cells and fibers is postulated to exist from the vagus and nucleus of the solitary tract, projecting through the parabrachial nucleus to feeding centers in the hypothalamus (Norgren and Leonard, '73; Crawley and Kiss, '85; Fulwiler and Saper, '85; Herbert and Saper, '90). The high levels of CCK binding in the VMH and Sol, and the existence of CCK receptors in the parabrachial nucleus, support the use of the Brazilian opossum as a model for studying food intake modulation.

The expression of cholecystokinin in the medial preoptic area is known to be sexually dimorphic in the adult Brazilian opossum (Fox et al., '90) and rat (Frankfurt et al., '85; Micevych et al., '87; Micevych et al., '88). In addition, CCK immunoreactive perikarya are observed in the MPA of male Brazilian opossum pups from 25 to 35PN, while CCK-IR cells are not evident in the female opossum MPA at any time. However, no sex differences were observed in CCK receptor binding in the MPA at any age in this study.

In fact, no differences in CCK receptor expression between males and females were observed in any brain regions in Brazilian opossums. This finding is in agreement with Akesson and coworkers (Akesson et al., '87), who report no significant differences in receptor binding in the VMH of intact male and female rats. Exogenous estrogen administration in ovariectomized females will down-regulate CCK receptors in the VMH and PA, and female rats in estrus reportedly have significantly lower levels of CCK receptor binding in the VMH (Akesson et al., '87). However, as all of the adults in this study were intact, and most females were probably in diestrus since they were not housed with males (Fadem, '87), no influence of estrogen levels on receptor binding in the Brazilian opossum was apparent.

Two types of cholecystokinin receptors are now known to exist. An A-type or peripheral receptor is found in the gastrointestinal tract and pancreas (Jensen et al., '89), and in specific brain regions (Hill and Shaw, '88; Hill et al., '90; Hill and Woodruff, '90).
or brain type receptors are located throughout the brain (Hill and Woodruff, '90). As recent data has indicated specific functions for A and B receptor types in the rat mesolimbic pathway (Crawley, 1992), we feel that it is important to characterize CCK receptors in the brain of the adult and developing Brazilian opossum. Studies are currently underway, utilizing specific receptor antagonists, to accomplish this task.

In summary, we have described the distribution of cholecystokinin binding sites in the brain of the adult and developing Brazilian opossum. In general, the patterns of CCK receptor localization in the adult opossum resemble those of other mammals, and likely mediate similar physiological functions. However, some cholecystokininergic pathways may be unique to neonatal mammals. Further studies will investigate the physiological roles that neuropeptide systems may play before morphogenesis of the central nervous system is complete.

Acknowledgements

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Literature Cited


TABLE 1.  CCK Binding Sites in the Adult and Developing Brazilian Opossum Brain

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Note: '+' indicates presence, '-' indicates absence.
### Brainstem

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1. +, discrete receptor binding localized to a specific nucleus;
2. -, no receptor binding evident in the nucleus;
3. L, diffuse receptor binding evident throughout the area of the presumptive nucleus; however, binding is not discretely confined to the nucleus at this age.
Figure 1. Photographic images, generated from LKB ultrofilm, of adjacent coronal sections taken through the brainstem of a 15PN Brazilian opossum pup at the level of the facial motor nucleus (FMN). A. Tissue section incubated in 100 pm $^{125}$I-Bolton Hunter-CCK-8. Note the high level of binding in the FMN (arrow), and diffuse binding throughout the medulla at this level. B. Adjacent tissue section exposed to 100 pm $^{125}$I-Bolton Hunter-CCK-8 plus 1.0 $\mu$M unlabelled CCK-8. Coincubation with unlabelled peptide abolishes all binding in the brain, indicating that binding to brain tissue is specific. Nonspecific binding can be seen in hair follicles (arrow). Bar = 2mm.
Figure 2. Photomicrographs demonstrating the distribution of CCK binding sites in the adult Brazilian opossum brain. A, B. Coronal section through the diencephalon. High levels of binding are evident in the cortex (Cor), CA1 region of the hippocampus (CA1), and the ventromedial hypothalamic nucleus (VMH). C, D. Coronal section through the mesencephalon. Binding in the interpeduncular nucleus is restricted to lateral regions (IPL). E, F. Section through the medulla. High levels of binding are localized to the nucleus of the solitary tract (Sol). Binding is also evident in the spinal trigeminal nucleus, caudal part (Sp5C), the inferior olive (IO), the hypoglossal nucleus (12), and the molecular layer of the cerebellum (Cb). All sections are shown at the same magnification. Bar in E = 2mm.
Figure 3. A photographic image, generated from LKB Ultrofilm, of a coronal section through the brainstem of a 1PN Brazilian opossum. Binding is evident throughout the brainstem and is also observed in the vagus nerve (10n, arrow). Bar = 1mm.
Figure 4. Photomicrographs of coronal sections demonstrating the distribution of CCK binding sites in the 10PN Brazilian opossum brain. A, B. Section through the developing diencephalon. Diffuse binding is evident in the hypothalamus (Hyp) and amygdaloid areas (AA). C, D. Section through the mesencephalon. Asterisks are provided for orientation. At this age, high levels of binding are observed in the presumptive ventral mesencephalon (VMes). Binding is also evident in the ventrolateral lemniscus (VLL) and the trigeminal nerve (5n). E, F. Section through the developing brainstem. Although binding is diffuse, higher levels of I^{125} binding are evident in the nucleus of the solitary tract (Sol, note arrow in E). Dense binding is evident in the vagus nerve (10n). All sections are shown at the same magnification. Bar in E = 1mm.
Figure 5. Photomicrographs of coronal sections through the brain of a 25PN Brazilian opossum, demonstrating the distribution of CCK binding sites. A, B. Section through the diencephalon. Dense labelling is evident in the CA1 region of the hippocampus (CA1). Labelling is also evident throughout the hypothalamus, including the VMH, and extends dorsally into the reticular nucleus of the thalamus (Rt), midline thalamic nuclei, and medial habenular nucleus. Labelling in the amygdala (AA) is now localized to discrete areas. C, D. Section through the mesencephalon. Open arrow denotes cerebral aqueduct for orientation. Heavy labelling is observed in the ventral tegmental area (VTA). Labelling is also observed in the ventrolateral lemniscus (VLL). E, F. Section through the brainstem. Heavy labelling is evident in the nucleus of the solitary tract (Sol, note arrow in E), the caudal part of the spinal trigeminal nucleus (Sp5C), and the hypoglossal nucleus (12). All sections are shown at the same magnification. Bar in E = 1mm.
Figure 6. Photographic images, generated from LKB Ultrofilm, of coronal sections through the brainstem of the adult and developing Brazilian opossum at the level of the facial motor nucleus (FMN). Arrows indicate the location of the FMN. A. Section through the adult opossum brain. Moderate levels of binding are evident in the medial aspect of FMN. B. Section through a 60PN opossum brainstem. No binding is observed in the FMN at this age. C. Section through the brainstem of a 10PN Brazilian opossum. Heavy binding is evident throughout the facial motor nucleus. Bar in A, B = 2mm; bar in C = 1mm.
CHAPTER TWO. ONTOGENY OF CHOLECYSTOKININ BINDING SITES
IN THE LABORATORY RAT HINDBRAIN

A paper accepted for publication in Brain, Behavior, and Evolution

M. Cathleen Kuehl-Kovarik and Carol D. Jacobson

Abstract

Studies in our laboratory have revealed a robust, transient expression of cholecystokinin binding sites in the facial motor nucleus during development in the Brazilian opossum, Monodelphis domestica. To investigate the ubiquity of this phenomenon, we have performed receptor autoradiography on the hindbrains of embryonic and neonatal rat pups. Cholecystokinin binding sites are present at very low levels in the embryonic day 16 rat hindbrain, but binding sites are abundant prior to birth. The greatest increase in labelled nuclei occurs prior to 5 days of postnatal age. Binding levels are heavy in the nucleus of the solitary tract, medial vestibular nucleus, posterior dorsal tegmental nucleus, area postrema, and caudal spinal trigeminal nucleus by 30 days postnatal. Both A-type and B-type receptors are present in the neonatal brainstem, although most labelled areas appear to be B-type. A-type binding sites are present in the ventral cochlear nucleus, the nucleus of the solitary tract, the dorsal motor nucleus of the vagus, the area postrema, the spinal nucleus of the trigeminal, and the cuneate and gracile nuclei by 5 PN. As reported for the Brazilian opossum, cholecystokinin binding sites are expressed in the facial motor nucleus of neonatal rats, and are transient. In this study of the rat brainstem, a transient expression is also observed in the rubrospinal tract, parvo cellular reticular nucleus, raphe obscurus, cuneate and gracile nuclei, and the ventral median fissure of the spinal cord. As vasopressin binding sites and estrogen receptors have also been shown to be expressed transiently in the laboratory rat facial motor nucleus, the physiological and developmental significance of transient binding site expression remains to be elucidated.
Introduction

Cholecystokinin [CCK] is an abundant and well-characterized neuropeptide located throughout the mammalian central nervous system [Innis et al., 1979; Larsson and Rehfield, 1979; Beinfeld et al., 1981, 1982; Crawley, 1985; Fox et al., 1991]. Cholecystokinin appears to mediate a number of physiological and behavioral effects [for review, see Crawley and Corwin, 1994], including digestion and feeding [Baile and Della-Fera, 1985; Morley et al., 1985; Gregory et al., 1989; Moran and McHugh 1989; Fried et al., 1991], pituitary hormone secretion and reproductive behavior [Vijayan et al., 1979; Kimura et al., 1983; Hashimoto and Kimura, 1986; Bloch et al., 1987, 1988], dopamine release [Voight et al., 1986; Altar et al., 1988], analgesia [Faris et al., 1983, 1984; Dourish et al., 1990; Wiertelak et al., 1992], memory [Itoh et al., 1989], and anxiety [Costall et al., 1991; Singh et al., 1991; Hendrie et al., 1993].

The peptide is perhaps best known as a satiety factor, a role demonstrated in multiple species [for review, see Silver and Morley, 1991]. However, the effects of CCK on food intake in the neonatal mammal are unclear. Although Robinson and coworkers [Robinson, et al., 1985] report that CCK decreases food intake in 1 day old postnatal rats, Blass [Blass, et al., 1979] found that CCK does not appear to affect food intake in rat pups until 15 to 20 days of age. Thus, the age at which CCK begins to regulate food intake in the neonatal rat is controversial.

With receptor autoradiography, cholecystokinin binding sites have been localized in the central nervous system of a variety of species [Gaudreau et al., 1983; Zarbin et al., 1983; Van Dijk et al., 1984; Williams et al., 1986; Sekiguchi and Moroji, 1986; Miceli and Steiner, 1989; Niehoff, 1989]. However, little work has been done on receptor expression during neonatal development. The localization of binding sites during postnatal development has been reported for the rat forebrain [Hays et al., 1981; Pelaprat et al., 1988], but not for hindbrain regions. We have previously described the ontogeny of CCK binding sites in the developing brain of the Brazilian opossum, *Monodelphis domestica* [Kuehl-Kovarik et al., 1993]. The use of the Brazilian opossum allowed us to examine the developmental pattern
of CCK binding sites in the brain during ongoing neurogenesis and morphogenesis. In that study, we found an unexpected expression of CCK binding sites in the facial motor nucleus that was present at birth and robust by 10 days of postnatal life (10 PN), but was completely absent by 60 PN, the age at which the animals are weaned. Because the ontogeny of CCK binding in the mammalian hindbrain has not been described for other species, we were unsure if this phenomenon was unique to the marsupial, or a common theme in neonatal development.

In this study we have used autoradiography to localize CCK binding sites in the hindbrain of embryonic and neonatal rats. We have also employed CCK receptor antagonists to determine if both A and B receptor types are present in the neonate. We have determined the pattern of CCK binding in the facial motor nucleus during development, examined receptor expression in the embryonic versus postnatal animal, and compared binding patterns to that of other neonatal species studied in our laboratory. Whether CCK binding sites in the facial motor nucleus are playing a physiological or developmental role remains to be elucidated.

Materials and Methods

Embryonic and neonatal Sprague-Dawley rats were obtained from females (Harlan Sprague-Dawley, Indianapolis, IN) maintained at Iowa State University. The animals were housed individually in plastic cages, and maintained at 23 °C on a 14:10 light-dark cycle. Water and food (Teklad Rodent Diet; Harlan Sprague-Dawley) were provided ad libitum. For breeding, male and female animals were paired and the female was checked daily for the presence of sperm, defined as day 1 postfertilization. Animals were then separated and the female checked daily after three weeks for the birth of pups. Some females were sacrificed at 16, 18, or 20 days postfertilization to collect embryonic tissue. Day of birth, usually 23 days post coitum, was denoted 1 PN. Young animals were housed with their mothers until 21 days old. All animal housing and use of animals was in accordance with the guidelines and approval of the Iowa State University Committee on Animal Care.
Tissue collection

Animals of 1, 3, 5, 7, 10, 15, 20, 25, and 30 PN were included in this study, as well as tissue from animals of 16, 18 and 20 days postfertilization (PF). At least three animals were collected at each age, from a minimum of two different litters. All animals were decapitated and their brains were immediately removed and frozen on dry ice. Frozen brains were stored at -20°C until used. Brains were cut into 20 μm thick coronal sections on a cryostat (Reichert Instruments) and thaw-mounted onto poly-L-lysine coated slides, creating four duplicate sets of slides per brain. Every fourth section was processed for standard autoradiography. In addition, the three other sets of slides were used from 5 PN and 20 PN animals to investigate the effects of receptor antagonists and to generate negative controls. Slides were stored at 4°C until processed.

Autoradiography

The protocol for receptor autoradiography was a modification of the protocols reported by Herkenham and Pert [1982] and Niehoff [1989] and reported previously in our laboratory [Elmquist et al., 1993; Kuehl-Kovarik et al., 1993]. Sections were preincubated for 30 minutes at room temperature in 50 mM Tris HCl, pH 7.4, containing 130 mM NaCl, 4.7 mM KCl, 5 mM MgCl₂, and 1 mM EGTA (Tris saline buffer) plus 0.5% BSA. Sections were subsequently incubated for 2.5 hours at room temperature in Tris saline buffer (pH 6.5) containing 0.025% bacitracin, 1 mM dithiothreitol, 4 μg/ml leupeptin, and 100 pM ¹²⁵I-Bolton Hunter-CCK-8 (New England Nuclear, 2200 Ci/mmol). Negative controls were generated by coincubation of identical slides with ¹²⁵I labelled CCK solution and 1 μM nonradioactive CCK. Following incubation, sections were washed in preincubation buffer at 4°C, six times for 15 minutes each time, and dipped briefly in ice-cold water. Slides were air-dried overnight. Labelled sections were exposed to LKB ³H-Ulstrofilm for six days. Films were developed by hand. Subsequent to film development, slides were fixed in 4% paraformaldehyde for 20 minutes, followed by two 10 minute 0.9% NaCl washes. Sections were lightly counterstained with 1% neutral red (Fisher Scientific, Pittsburgh, PA), dehydrated in graded alcohols, cleared in xylene, and coverslipped with permount mounting media.
Tissue sections from 5 PN and 20 PN animals were incubated with the CCK receptor antagonist L-364,718 (10 nM), or L-365,260 (100 nM) added to the $^{125}$I labelled CCK solution. L-364,718 binds to A-type binding sites, effectively inhibiting CCK binding at these sites. Conversely, L-365,260 blocks CCK from binding to B-type sites. Antagonists were a generous gift from Dr. Roger Freidinger, representing the research laboratories of Merck, Sharp and Dohme, West Point, Pennsylvania. Prior to this experiment, both antagonists were diluted out to 1.0, 10, 50, and 100 nM and applied, with labelled CCK solution, to identical tissue sections to determine the most appropriate concentration. Concentrations used are in agreement with concentrations used by others [Hill et al., 1990]. Identical tissue sections were exposed to the $^{125}$I labelled CCK alone, or simultaneously with 1 $\mu$M nonradioactive CCK.

Analysis

Analysis was performed with the aid of a developmental rat atlas [Paxinos et al., 1991]. Sections were analyzed at 80 $\mu$m intervals for standard autoradiography, and 20 $\mu$m intervals for antagonists. Autoradiograms were viewed with a light-box and a hand-held lens. Counterstained slides were observed with a Zeiss light microscope.

A standard curve to determine the amount of $^{125}$I-labelled CCK bound to tissue sections was created using rat brain paste standards. The curve consisted of $^{125}$I-Bolton Hunter-CCK-8, diluted 1:5, 1:7.5, 1:10, 1:20, 1:50, and 1:500 in phosphate buffered saline. The diluted ligand was applied to mashed rat brain tissue that had been cut at 20 $\mu$m and mounted on slides. This curve was used to perform densitometric analyses employing a Zeiss SEM-IPS image analysis system [Zeiss-Kontron; IBAS version 2.00]. Autoradiograms were transilluminated using a ChromoPro 45 lightbox and viewed with a Contax 60mm macro lens. Images for analysis were captured with a Sony XC-77 camera. Densitometric analysis was applied to six tissue sections containing the nucleus of the solitary tract and the facial motor nucleus of animals at 5, 15, or 30 days of age on the same film to determine if and how binding density changes as the young rat matures. For all analyses, the area of interest was outlined with a digitizer tablet. The mean optical density for the nucleus of the solitary tract and the facial motor nucleus was determined from the measurements taken for
the 5, 15, and 30 PN animals. A ratio between the mean optical density of the 15 PN nucleus of the solitary tract and that of the 5 PN animal was obtained, and, similarly, a ratio was obtained between the mean optical density of the 30 PN nucleus of the solitary tract and that of the 5 PN tissue. The identical method was used to define the change in binding density over time in the facial motor nucleus. A t-test was performed to compare the change in binding density during development between the nucleus of the solitary tract and the facial motor nucleus.

**Results**

Coincubation of tissue with $^{125}$I-CCK-8 and unlabelled peptide resulted in the blocking of all labelling in the rat hindbrain. Therefore, the labelling observed was assumed to be specific for CCK binding sites. Cholecystokinin binding sites were very limited, and labelling was light, in the postfertilization day (PF) 16 rat. However, binding was robust and located throughout the hindbrain of rat pups after 16 PF but prior to birth. Changes in binding patterns observed from birth to weaning age in hindbrain regions were less dramatic.

**Analysis**

Binding in the nucleus of the solitary tract of the 15 PN and 30 PN animals is at least 85% of the binding in the comparable nucleus of the 5 PN animal (Table I). However, binding in the facial motor nucleus of the older animals is almost half that measured at 5 PN (Table I). Results from the quantitative analysis of the change in binding density over time reveal a significant decrease in binding over time in the facial motor nucleus as compared to the nucleus of the solitary tract, a result consistent in both the 15 PN vs. the 5 PN animal ($t= 15.77$, $p < 0.005$, Table I) and the 30 PN vs. the 5 PN animal ($t= 7.76$, $p < 0.005$, Table I). Thus, the decrease in binding seen in the facial motor nucleus is not a function of a general decrease in binding as the animal ages. An index of variability (SEM/mean X 100) [Elmquist et al., 1993] in the ratios generated between binding density at different ages was found to be less than 11%.
CCK binding sites in the developing rat hindbrain

Three qualitative levels of CCK binding site density were assigned to specific brain regions. Levels were based on the density of silver grains deposited on the autoradiograms as compared to brain paste standards ("heavy" defined as the darkest two brain standards, "moderate" the middle two, and "light" the lightest two).

**PF 16.** Binding sites in the embryonic day 16 rat were restricted to the caudal nucleus of the solitary tract and dorsal motor nucleus of the vagus, the raphe obscurus in the medulla, and the ventral median fissure of the rostral cervical spinal cord. Labelling was also observed in the vagus nerve exiting the central nervous system. Binding was very light in all regions at this age.

**PF 18.** At this age, distinct, moderate labelling was observed in the ventral laterallemniscus and median raphe nucleus. Heavy labelling was observed in the medial vestibular nucleus (MVe, fig. 1), the dorsal cochlear nucleus (DCo, fig. 1), and both the cuneate and gracile nuclei of the brainstem. Also, labelling in the caudal nucleus of the solitary tract, dorsal motor nucleus of the vagus, raphe obscurus, and ventral fissure of the spinal cord was heavier than that observed in the PF 16 rat.

**PF 20.** A few days prior to birth, many areas express CCK binding sites in the rat hindbrain (fig. 2). Rostrally, the ventral lateral lemniscus and the rubrospinal tract labelled moderately. Moderate labelling was observed in the locus coeruleus as well. Heavy labelling was observed in the median raphe nucleus (MnR, fig. 2A,B) and the dorsal cochlear nucleus. The medial subnucleus of the facial motor nucleus (fig. 2A,B), and the parvocellular reticular nucleus (PCRt, fig. 2A,B) dorsal to the facial motor nucleus exhibited moderate levels of binding. Heavy labelling was observed in the medial vestibular nucleus (MVe, fig. 2A,B) and the nucleus of the solitary tract (Sol, fig. 2C,D and E,F). Moderate levels were observed in the ventral cochlear nucleus, and the intermediate (IRt, fig. 2C,D) and medullary (MRt, fig. 2E,F) reticular zones. As in the PF 18 brain, heavy labelling was observed in the cuneate (Cu, fig. 2E,F) and gracile (Gr, fig. 2E,F) nuclei and the nucleus raphe obscurus (ROb, fig. 2E,F). The caudal nucleus of the trigeminal tract labelled very lightly.
1 PN. On day one of postnatal life, (1 PN), moderate labelling was observed for the first time in the dorsal and ventral parabrachial nuclei and the lateral cerebellar nucleus. Light labelling was seen in the interpolar spinal trigeminal nucleus. The rostral nucleus of the solitary tract was heavily labelled, and labelling in the caudal spinal nucleus of the trigeminal was heavier than that seen at previous ages. As brains were removed from the calvarium at this age, labelling in nerves exiting the brain could no longer be determined. In all other regions, labelling was similar to that seen in the PF 20 animal: heavy labelling was observed in the median raphe, the dorsal cochlear nucleus, the medial vestibular nucleus, the cuneate and gracile nuclei, and the nucleus raphe obscurus; moderate levels were evident in the ventral lateral lemniscus, rubrospinal tract, locus coeruleus, medial subnucleus of the facial motor nucleus, parvocellular reticular nucleus, ventral cochlear nucleus, and the intermediate and medullary reticular zones.

3 PN. After 1 PN, fewer changes were observed in overall binding patterns. Binding in the 3 PN brain resembled the 1 PN tissue in both pattern and intensity of binding. Binding was seen for the first time in the posterior dorsal tegmental nucleus and the area postrema, which labelled heavily. Light to moderate labelling was also observed for the first time in the intermediate subnucleus of the facial motor nucleus. The rubrospinal tract exhibited heavy labelling by this age, as did the parabrachial nuclei and caudal spinal nucleus of the trigeminal.

5 PN. Few changes were observed by this age. The intermediate cerebellar nucleus was labelled as well as the lateral. Both the lateral and medial subnuclei of the superior olive (SO, fig. 3A,D) labelled lightly for the first time, as did the mesencephalic trigeminal tract (fig. 3A,D). As in younger animals, moderate labelling was observed in the ventral lateral lemniscus. Labelling in the rubrospinal tract (rs; fig. 3A,D) the medial vestibular (MVe) and ventral cochlear (VCo, fig. 3A,D) nuclei, the nucleus of the solitary tract (Sol, fig. 3E,H), the dorsal motor nucleus of the vagus, the area postrema (AP, fig. 3I,L), the cuneate (Cu, fig. 3I,L) and gracile (Gr, fig. 3I,L) nuclei, and the caudal spinal trigeminal nucleus (Sp5C, fig. 3I,L) was still considered heavy. Moderate binding was seen in the lateral and medial (fig. 3A,D) parabrachial nuclei, the locus coeruleus, the medial and
intermediate subnuclei of the facial motor nucleus (FMN, fig. 3E,H; fig. 4), the parvocellular reticular nucleus (fig. 3E,H), the dorsal cochlear nucleus (fig. 3E,H), the cerebellar nuclei, the intermediate and medullary (fig. 3I,L) reticular nuclei, the interpolar spinal trigeminal nucleus, the raphe obscurus (ROb, fig. 3I,L), and the ventral median fissure of the spinal cord. Light labelling was still evident in the posterior dorsal tegmental nucleus (fig. 3A,D).

7 PN. At seven days of age, light labelling was seen for the first time in the reticulotegmental nucleus and the suprageniculate nucleus of the pons. The lateral and medial superior olive appeared to label more heavily than in the 5 PN animal. Labelling in the cerebellar nuclei, the mesencephalic trigeminal tract, the ventral lateral lemniscus, the rubrospinal tract, the posterior dorsal tegmental nucleus, the intermediate and medullary reticular nuclei, the nucleus of the solitary tract and dorsal motor nucleus of the vagus, the area postrema, the cuneate and gracile nuclei, and the caudal and interpolar spinal trigeminal nuclei remained comparable to that of the 5 PN animal. Binding was still evident in the intermediate subnucleus of the facial motor nucleus. However, labelling in the medial subnucleus of the facial motor nucleus, as well as the parvocellular reticular nucleus, raphe obscurus, and ventral median fissure of the spinal cord, was no longer observed.

10 PN. By this age, binding levels had increased in the posterior dorsal tegmental nucleus and suprageniculate nucleus. However, binding was lighter in the intermediate subnucleus of the facial motor nucleus and the rubrospinal tract. No changes in labelling were seen in the cerebellar nuclei, the mesencephalic trigeminal tract, the ventral lateral lemniscus, the parabrachial nuclei, the locus coeruleus, the reticulotegmental nucleus, the superior olive, the medial vestibular nucleus, the dorsal and ventral cochlear nuclei, the intermediate and medullary reticular nuclei, the nucleus of the solitary tract and the dorsal motor nucleus of the vagus, the area postrema, the cuneate and gracile nuclei, and the interpolar and caudal spinal trigeminal nucleus. No new areas of binding were observed.

12 PN. By 12 days of age, almost no changes were observed in binding patterns. A heavy level of binding was observed in the pyramidal tract for the first time. However, binding was no longer visible in the cuneate nucleus. Binding levels were greatly decreased.
in the cochlear nuclei, and no binding was observed in the intermediate subnucleus of the facial motor nucleus.

15 to 30 PN. At 15 PN, binding in the gracile nucleus was no longer visible. Binding levels were very low in the rubrospinal tract, which disappeared by 20 PN. No other changes were seen after 15 PN. Thus, by 30 days of age, moderate to heavy levels of binding were observed in the ventral lateral lemniscus, the parabrachial nuclei, the locus coeruleus, and the pyramidal tract of the rostral brainstem. Lighter levels of binding were noted in the mesencephalic tract of the trigeminal and the reticulotegmental nucleus. Further caudally, heavy labelling was evident in the medial vestibular nucleus and posterior dorsal tegmental nucleus, while more moderate levels were observed in the suprageniculate nucleus and cerebellar nuclei. Light binding was observed in the cochlear nuclei and the superior olive. In the caudal brainstem, the nucleus of the solitary tract, the dorsal motor nucleus of the vagus, and the area postrema displayed the heaviest labelling. Labelling was also heavy in the caudal part of the spinal trigeminal nucleus, while labelling in the interpolar part was more moderate. Moderate labelling was observed in the medullary reticular formation as well. Light labelling was evident in the intermediate reticular formation.

Characterization of CCK binding sites in the developing rat hindbrain.

The antagonists L-364,718, which inhibits CCK binding to A-type binding sites, and L-365,260, which blocks B-type sites, were used to classify CCK binding sites in the developing brainstem. Incubation of tissue sections from the 5 PN animals with $^{125}$I labelled CCK containing 100 nM L-365,260 blocked binding in most areas of the medulla (fig. 3C,G,K). However, binding in the lateral superior olive was unaffected (fig. 3C). Binding was still evident, but somewhat reduced, in the ventral cochlear nucleus (fig. 3C), the nucleus of the solitary tract (fig. 3G,K), the dorsal motor nucleus of the vagus, and the area postrema (fig. 3K), the spinal nucleus of the trigeminal (fig. 3K), the cuneate (fig. 3K) nucleus, and the gracile nucleus (fig. 3K). Thus, these areas are considered to express A-type receptors. The addition of 10 nM L-364,718 to the $^{125}$I labelled CCK solution caused a decrease in binding in a limited number of hindbrain areas (fig. 3B,F,J). Binding in the lateral superior olive (fig. 3B) was completely eliminated, and binding was decreased in the
ventral cochlear nucleus (fig. 3B,F), the nucleus of the solitary tract (fig. 3F,J), the dorsal motor nucleus of the vagus (fig. 3J), the area postrema (fig. 3J), the caudal spinal nucleus of the trigeminal (fig. 3J), the cuneate nucleus (fig. 3J), and the gracile nucleus (fig. 3J). Thus, the lateral superior olive appears to contain A-type receptors exclusively, while both A- and B-type binding sites are expressed in the ventral cochlear nucleus, the nucleus of the solitary tract, the dorsal motor nucleus of the vagus and the area postrema, the caudal spinal trigeminal nucleus, and the cuneate and gracile nuclei. All other labelled areas in the 5 PN hindbrain, including the facial motor nucleus, which were not blocked by L-364,718, are considered to express B-type CCK receptors exclusively (table 1).

At 20 PN, similar binding patterns were noted in the expression of A-type or B-type binding sites (table II). As in the 5 PN animal, the ventral cochlear nucleus, nucleus of the solitary tract, dorsal motor nucleus of the vagus, and area postrema, and the spinal trigeminal nucleus appear to express both A-type and B-type receptors. The cuneate and gracile nuclei are no longer labelled at this age. The lateral superior olive appears to express only A-type binding sites. All other labelled areas are considered to be exclusively B-type (table II).

Discussion

In general, CCK binding patterns in the developing rat hindbrain are as expected, based upon patterns seen in the developing Brazilian opossum [Kuehl-Kovarik, et al., 1993] and adult rat brain [Gaudreau et al., 1983; Zarbin et al., 1983; Van Dijk et al., 1984; Niehoff, 1989]. However, cholecystokinin binding sites appear to increase dramatically in the prenatal brain, and undergo few changes after birth. By fifteen days of postnatal age, the heaviest labelling is observed in the nucleus of the solitary tract and area postrema, caudal spinal trigeminal nucleus, and parabrachial nuclei. Heavy labelling is also observed in the posterior dorsal tegmental nucleus and medial vestibular nucleus. Many other places exhibited lower levels of binding. In the developing rat brain, binding sites are restricted to discrete nuclei or tracts, even in prenatal rat pups. This is in contrast to binding in the very young opossum [Kuehl-Kovarik, et al., 1993], where nuclear borders are less distinct,
especially in hindbrain areas. Binding sites also appear to be expressed transiently in selected areas in the neonatal rat.

Cholecystokinin binding patterns in the hindbrain are similar during the development of the neonatal opossum and the neonatal rat. In the neonatal opossum, CCK binding sites are present at birth in the nucleus of the solitary tract and area postrema, spinal trigeminal nucleus, medial vestibular nucleus, and parabrachial nuclei [Kuehl-Kovarik et al., 1993]. The corresponding early appearance of binding in these nuclei in the neonatal rat suggests an important role in prenatal or early postnatal physiology. Like the rat, CCK binding sites in the cerebellar nuclei of the opossum appear to be transiently expressed, disappearing sometime prior to adulthood. The most remarkable difference between binding patterns in the two mammals is the robust expression of CCK binding sites in the developing and adult opossum cerebellum [Kuehl-Kovarik et al., 1993; Madtes and King, 1994], while no binding sites are evident in the cerebellum of the neonatal rat. However, as very low levels of binding are reported in the adult rat cerebellum [Niehoff 1989], this is not unexpected.

Differences do exist between binding in the immature rat hindbrain and binding patterns reported for the adult rat. Specifically, even at thirty days of postnatal age, we observed binding in areas not reported for mature rats [Niehoff, 1989], such as the medial vestibular nucleus, the suprageniculate nucleus, and the cerebellar nuclei. Although it is doubtful that dramatic changes occur after 30 PN, CCK binding is influenced by the hormonal environment [Akesson and Micevich, 1986; Akesson et al., 1987]. Thus, hormones may be affecting CCK binding patterns between postnatal day 30 and adulthood.

However, a transient expression of cholecystokinin binding sites is observed in multiple hindbrain regions in the neonatal rat prior to postnatal day 30, including the rubrospinal tract, the facial motor nucleus, the parvocellular reticular formation, the raphe obscurus and the ventral median fissure of the spinal cord, the cuneate nucleus, and the gracile nucleus. The significance of this transient expression in multiple areas is unclear. The expression of transient binding sites during neonatal development has been reported for other neuropeptides [Tribollet et al., 1991; Yokosuka and Hayashi, 1992], indicating that binding sites may play a role in organizational events. This theory is supported by the fact
that many brain regions express CCK binding sites transiently. However, transiently expressed binding sites may also have a physiological or behavioral role in the neonate.

The transient expression of cholecystokinin binding sites in the neonatal rat facial motor nucleus is consistent with binding patterns in the neonatal opossum. In the rat, binding sites are evident as early as PF 20, and persist until 10 PN. Interestingly, the subnuclear arrangement appears to change, as binding sites are initially expressed medially, but only in the intermediate subnucleus just prior to their disappearance. In contrast, binding sites appear to be robustly expressed throughout the neonatal Brazilian opossum facial motor nucleus (subnuclear compartmentalization is very indistinct in the neonate of this species). Thus it is unclear whether binding sites are mediating similar functions in the two species. However, the transient expression of CCK binding sites in the facial motor nucleus of more than a single species of neonatal mammal suggests a significant role in neonatal physiology or behavior. Because facial muscles are intimately involved in suckling, and CCK is postulated as a regulator of food intake [Crawley and Corwin, 1994], it is possible that CCK binding sites in the facial motor nucleus of neonatal mammals may play a role in feeding.

Cholecystokinin binding sites in the facial motor nucleus in the neonatal rat disappear prior to weaning. In contrast, binding sites in the Brazilian opossum facial motor nucleus disappear around the time the animals are weaned. However, as brain development is greatly protracted in Monodelphis as compared to placental species, the discrepancy could simply reflect a differential timing of the maturation process. Also, cholecystokinin binding sites in the opossum facial motor nucleus appear prior to the completion of hypothalamic neurogenesis [Larsen and Jacobson, 1986; Rivkees et al., 1988; Iqbal et al., 1993], and prior to synaptic input into the nucleus [Swanson et al., 1993], but remain while afferent innervation is in progress. The timing of synaptic input into the neonatal rat facial motor nucleus is unknown, but is currently under investigation. Thus, the similar yet distinct expression of CCK binding sites in the Brazilian opossum may reflect not only a role in suckling (or some other) behavior shared by all mammals, but also the special needs of the neonatal marsupial.

Several studies report very little CCK binding in the prenatal rat brain [Hays et al.,
However, none of these studies examined hindbrain regions. In our study, cholecystokinin binding sites are present as early as PF 16, the earliest age examined, and are robustly expressed in many regions by PF 20. Few changes in binding patterns take place postnatally. The CCK peptide is also detected in the embryonic rat brain [Cho et al., 1983], implying a prenatal role for this neuropeptide system. Cholecystokin in binding site expression prior to birth may be related to brain development, the birth process, the changing hormonal milieu, or simply early up-regulation prior to postnatal functions. The temporal relationship between receptor expression and function remains to be more fully explored before this question can be answered.

Two types of cholecystokinin receptors are now known to exist, and their actions and pharmacology are the subject of intense research. The A-type or peripheral receptor is found in the gastrointestinal tract and pancreas [Jensen et al., 1989], and in specific brain regions [Hill and Shaw, 1988; Hill et al., 1990; Hill and Woodruff, 1990]. B- or brain type receptors are located throughout the brain [Hill and Woodruff, 1990]. As specific functions have been implicated for A and B receptor types in the rat mesolimbic pathway [Crawley, 1992], and the B-type now appears to play an important role in anxiety [Costall et al., 1991], we felt that it was important to characterize CCK binding sites during development. Both A- and B- type binding sites are present in the neonatal rat hindbrain, and in general, binding patterns resemble the adult pattern. The presence of A-type receptors in the lateral superior olive was an unexpected finding, and the significance is unknown at this time. We are currently utilizing our knowledge of CCK receptor types in behavioral studies using CCK receptor antagonists.

In summary, we have described the distribution of CCK binding sites in the developing rat hindbrain. In general, binding patterns during development were as expected, based on what is known about CCK binding in the adult rat and the neonatal Brazilian opossum. It is interesting to note that binding is transient in the facial motor nucleus of both species, suggesting a unique role in neonatal physiology or behavior. Further studies will investigate the physiological roles that neuropeptide systems may play in the mammalian neonate.
Acknowledgements

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References


Table I. Change in density of $^{125}$I-CCK-8 binding in selected hindbrain regions of postnatal rats during development

<table>
<thead>
<tr>
<th></th>
<th>15 PN/5 PN Mean</th>
<th>15 PN/5 PN SEM</th>
<th>30 PN/5 PN Mean</th>
<th>30 PN/5 PN SEM</th>
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</thead>
<tbody>
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<td>0.89&lt;sup&gt;1&lt;/sup&gt;</td>
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<td>0.85</td>
<td>0.09</td>
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<td>facial motor nucleus, medial</td>
<td>0.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.03</td>
<td>0.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.04</td>
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</table>

<sup>1</sup>, the ratio represents the mean optical density of a specified area measured at two different ages.  
<sup>a</sup>, binding over time for the facial motor nucleus is significantly decreased as compared to the change in binding over time in the nucleus of the solitary tract, p < 0.005.
Table II. Characterization of CCK binding sites in the 5 and 20 PN rat hindbrain

<table>
<thead>
<tr>
<th>Area</th>
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<th>20 PN</th>
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<td></td>
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<td>A</td>
<td>B</td>
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<tr>
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<td>X</td>
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<td>NP</td>
<td>NP</td>
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<tr>
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<td>X</td>
<td>X</td>
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<td>NP</td>
<td>NP</td>
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</tr>
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<td>facial motor nucleus, medial subnucleus</td>
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<td>NP</td>
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<td>raphe obscurus</td>
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<td>X</td>
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^X, CCK binding site characterized as either "A" or "B" based on antagonist studies using L-364,718 and L-365,260; NP, CCK binding sites not present at this age.
Figure 1. Images, taken from LKB Ultrofilm (A), and corresponding neutral red counterstained slides (B), of a coronal section through the hindbrain of a postfertilization day 18 rat. At this early embryonic age, CCK binding sites are evident in the medial vestibular nucleus (MVe) and dorsal cochlear nucleus (DCo) and in peripheral nerves outside the brain. Light labelling is also observed in the intermediate reticular nucleus and median raphe. Aq, cerebral aqueduct; Cb, cerebellum; 4V, fourth ventricle. Scale bar = 1 mm.
Figure 2. Images, taken from LKB Ultrafilm (A,C,E) and corresponding neutral red stained slides (B,D,F), of coronal sections through the hindbrain of a postfertilization day 20 rat, demonstrating the distribution of CCK binding sites. A,B: Section through the pons. Heavy labelling is evident in the median raphe (MnR) and medial vestibular nucleus (MVe). The dorsal cochlear nucleus is labelled moderately, and moderate levels of binding are evident in the medial subnucleus of the facial motor nucleus (arrow) and the parvocellular reticular nucleus (PCrt) dorsal to the facial. C,D: Section through the rostral medulla. Very heavy labelling is evident in the rostral nucleus of the solitary tract (Sol), while moderate levels are observed in the intermediate reticular formation (IRt) and vagus nerve outside the brain (arrow). E,F: Section through the caudal medulla. Heavy labelling is evident in the nucleus of the solitary tract (Sol) and dorsal motor nucleus of the vagus, cuneate (Cu), and gracile (Gr) nuclei, as well as the raphe obscurus (ROb). More moderate levels of binding are observed in the medullary reticular nucleus (MRt). All sections are shown at the same magnification. Aq, cerebral aqueduct; Cb, cerebellum. Scale bar = 1 mm.
Figure 3. Images, taken from LKB Ultrofilm (A-C; E-G; I-K) and corresponding neutral red stained slides (D,H,L), of coronal sections through the hindbrain of a 5 day postnatal rat, demonstrating the distribution and characterization of CCK binding sites. A - D: Identical sections through the rostral hindbrain. Figure A represents a tissue section treated with $^{125}$I-CCK-8 only. Heavy labelling is evident in the rubrospinal tract (rs) and the ventral cochlear nucleus (VCo), while a moderate level is seen in the medial parabrachial nucleus. Low levels are observed in the lateral and medial superior olive (SO), the mesencephalic trigeminal tract and the posterior dorsal tegmental nucleus. Figure B is the consecutive tissue section blocked with 10 nM L-364,718, an antagonist that inhibits CCK binding to A-type receptors. Note that labelling in the lateral superior olive is eliminated by this treatment, signifying that binding sites in this area are of the A-type. Figure C is the consecutive tissue section blocked with 100 nM L-365,260, an antagonist that inhibits CCK binding to b-type binding sites. Note that most binding is eliminated by this procedure. Binding is still evident in the lateral superior olive, and light binding is also observed in the VCo, indicating that these areas partially (VCo) or completely (lateral superior olive) express A-type binding sites. E - H: Consecutive sections through the caudal pons. Figure E represents a tissue section treated with $^{125}$I-CCK-8 only. Heavy labelling is evident in the nucleus of the solitary tract (Sol), while more moderate levels are observed in the facial motor nucleus (FMN), the parvocellular reticular nucleus, and the dorsal and ventral (VCo) cochlear nuclei. Figure F is the consecutive tissue section blocked with 10 nM L-364,718 (A-type blocker). Note that labelling in the VCo is reduced. Figure G is the consecutive tissue section blocked with 100 nM L-365,260 (B-type blocker). Note that labelling appears in the Sol and VCo, indicating that these areas partially express A-type binding sites. I - L: Consecutive tissue sections through the caudal medulla. Figure I represents a tissue section treated with $^{125}$I-CCK-8 only. Heavy labelling is observed in the nucleus of the solitary tract and dorsal motor nucleus of the vagus, area postrema (AP), the cuneate nucleus (Cu), the gracile nucleus (Gr), and the caudal spinal trigeminal nucleus (Sp5C). Moderate levels are observed in the medullary reticular nucleus and the raphe obscurus (ROb). Figure J is the consecutive tissue section blocked with 10 nM L-364,718 (A-type blocker). Note the greatly reduced labelling in the nucleus of the solitary tract, dorsal motor nucleus of the vagus, and area postrema. Labelling in the Sp5C is reduced as well. Figure K is the consecutive tissue section blocked with 100 nM L-365,260 (B-type blocker). Note the labelling in the nucleus of the solitary tract and dorsal motor nucleus of the vagus, AP, Cu, Gr, and Sp5C, indicating that these regions express A-type, as well as B-type binding sites. All sections are shown at the same magnification. IC, inferior colliculus; Cb, cerebellum; Mo5, trigeminal motor nucleus. Scale bar = 1.5 mm.
Figure 4. Higher magnification images of figure 3e and 3h, demonstrating binding in the facial motor nucleus of a 5 day postnatal rat. A. Higher magnification view of figure 3E, illustrating moderate levels of binding in the intermediate and medial subnuclei of the facial motor nucleus. B. Higher magnification view of figure 3H, the corresponding neutral red counterstained section. C. A schematic representation of the region presented in B. The location of the intermediate (7i) and medial (7m) subnuclei of the facial motor nucleus are indicated. P7, perifacial zone. Scale bar = 200 μm.
CHAPTER THREE. AUTORADIOGRAPHIC LOCALIZATION OF ARGININE VASOPRESSIN BINDING SITES IN THE ADULT AND DEVELOPING BRAZILIAN OPOSSUM BRAIN

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Abstract

We are currently utilizing the Brazilian short-tailed opossum, Monodelphis domestica, to study the development of the vasopressinergic system in relation to central nervous system neurogenesis and morphogenesis. Earlier studies have demonstrated that vasopressin-like immunoreactivity is present very early in the Brazilian opossum brain, suggesting a role for vasopressin in the developing mammalian central nervous system. In this study, we have utilized [3H]arginine vasopressin autoradiography to describe the distribution of vasopressin binding sites in the adult and developing Brazilian opossum brain, to further elucidate the role of the vasopressinergic system in CNS development. In general, vasopressin binding patterns in the adult opossum brain resemble those of other species. However, we found very few labelled areas in the neonatal opossum brain. At birth, only the ventral tegmental area and the nucleus of the solitary tract were labelled. Binding was not evident in the forebrain of the opossum until 25 days of postnatal age. The anterior pituitary was heavily labelled from birth onward, but binding in the brain itself remained at low levels until 35 days postnatal. Heavy binding was observed in only a few areas of the adult brain, including the dorsal part of the lateral septal nucleus, the suprachiasmatic nucleus, the dorsal and median raphe, the nucleus of the solitary tract, and the caudal part of the spinal trigeminal nucleus. Surprisingly, vasopressin binding sites in the opossum appear much later than the vasopressin peptide and, in many cases, after neurogenesis is complete. A more sensitive technique might indicate an earlier expression of binding sites. However, these findings
suggest that classical vasopressin binding sites are not playing a developmental role in the opossum, although the peptide is present.

**Introduction**

The localization and function of arginine-vasopressin (AVP) and AVP binding sites in the mammalian brain and periphery has been intensively studied, due to AVP's significant hormonal actions [see Morris et al., 1987 for review]. Arginine vasopressin plays a central role in water balance and blood pressure regulation [Zimmerman, 1983; Tanaka et al., 1993], an action mediated through magnocellular neurosecretory neurons located in the hypothalamus [Morris et al., 1987]. In addition, AVP appears to function in several other areas involving homeostasis, including central thermoregulation [Oloyami and Hart, 1992], circadian rhythmicity [Reghunandan et al., 1990] and drinking and eating behavior [Mangiapan et al., 1983; Buijs et al., 1992]. Vasopressin has also been postulated to play a part in sexual and social behavior [Bluth and Dentzer, 1993; Wang et al., 1993; Winslow et al., 1993] and learning and memory processes [DeWied, 1980]. Many of the actions of AVP are likely mediated through extrahypothalamic central nervous system pathways, which have been demonstrated to exist in multiple species [DeVries et al., 1981, 1985; Valiquette et al., 1985; Buijs, 1987; Dubois-Dauphin et al., 1990]. Along with these several functional roles, AVP has been postulated to influence central nervous system development [Boer et al., 1980, 1993; Buijs, 1987; Buijs et al., 1988]. Therefore, the ontogeny of the central vasopressin system has become an increasingly important area of study.

In general, arginine vasopressin-like immunoreactivity (AVP-IR) appears prior to birth in the mammalian brain [Buijs et al., 1980; Whitnall et al., 1985; Buijs, 1987]. Likewise, AVP binding sites have been detected early in development. Autoradiographical studies have demonstrated AVP binding as early as embryonic day 16 in the laboratory rat [Tribollet et al., 1991], as well as in the newborn rat [Petracca et al., 1986] and golden hamster [Delville et al., 1994]. Many of the earliest binding sites detected in the fetal rat are found in extrahypothalamic locations, including the ventral pontine reticular formation, the facial motor nucleus, the nucleus of the solitary tract, and the spinal trigeminal nucleus.
Tribollet et al., 1991]. Binding in the anterior lobe of the pituitary, the suprachiasmatic nucleus, and the septal area is also evident prior to birth in the laboratory rat [Tribollet et al., 1991].

One fascinating aspect of vasopressin binding site development in the neonatal rat is the apparently transient expression of functional AVP binding sites in multiple brain areas, most notably in the intermediate subnucleus of the facial motor nucleus (FMN) [Tribollet et al., 1991]. The transient expression of neurotransmitter binding sites in the facial motor nucleus during mammalian development appears to be a recurrent theme; immunohistochemical [Yokosuka and Hayashi, 1992] and in situ hybridization studies [R. Handa, personal communication] have demonstrated that estrogen receptors are also transiently expressed in the FMN during neonatal rat development. In addition, we have shown that cholecystokinin (CCK) binding sites are expressed transiently in the FMN of both the neonatal rat and the developing Brazilian opossum [Kuehl-Kovarik et al., 1993, Kuehl-Kovarik and Jacobson, 1995]. Thus, it appears that neurotransmitter binding sites in the facial motor nucleus may play a significant role, either organizational or physiological, during mammalian development.

Our laboratory has recently used immunohistochemistry to examine the developmental expression of AVP-like immunoreactivity in the Brazilian short-tailed opossum, Monodelphis domestica [Iqbal et al., 1993]. The offspring of this small, pouchless marsupial are born in an extremely immature state and have a protracted postnatal developmental period. Thus, Monodelphis has become a popular research animal for developmental studies [Fox et al., 1991; Brunjes et al., 1992; Elmquist et al., 1992; Treherne et al., 1992; Wang et al., 1992; Kuehl-Kovarik et al., 1993; Iqbal et al., 1995]. The use of the Brazilian opossum has allowed us to study the early development of the hypothalamic and extrahypothalamic vasopressinergic system in relation to CNS neurogenesis and morphogenesis. Vasopressin-like immunoreactivity is present prior to birth in the Brazilian opossum brain [Iqbal et al., 1993], suggesting a role for AVP in the development of the central nervous system.

In this study, we have utilized [3H]AVP autoradiography to describe the distribution of AVP binding sites in the adult and developing Brazilian opossum brain. The purpose of
this study was to 1) examine the role of the vasopressinergic system in CNS development; 2) compare the development of AVP binding sites in the opossum to that of higher mammals; and 3) determine if, and when, AVP binding sites are transiently expressed in the facial motor nucleus of the Brazilian opossum, in an ongoing attempt to determine the function of transient receptor expression in the facial motor nucleus of neonates.

Materials and Methods

Adult and developing male and female Brazilian short-tailed opossums were obtained from a colony maintained at Iowa State University. The initial animals used to start the breeding colony were acquired from the Southwest Foundation for Research and Education in San Antonio, TX. The animals were housed in plastic cages, and maintained at 26°C on a 14:10 light-dark cycle. Water and food (Reproduction Fox Chow; Milk Specialties Products, Madison, WI) were provided ad libitum. For breeding, male and female animals were paired for 14 days. Animals were then separated and the female checked daily for the presence of pups. The day of birth was considered postnatal day one (1 PN). Young animals were housed with their mothers until 60 PN. All animal housing and use of animals was in accordance with the guidelines and approval of the Iowa State University Committee on Animal Care.

Tissue collection

Animals of 1, 3, 5, 7, 10, 15, 25, 35, 45, and 60 PN were used for this study. Three to four animals studied at each age came from at least two different litters. Prior to 5 PN, animals were considered sexually undifferentiated. After that age, males were selected. However, females were also studied at 10, 25, and 60 PN to investigate any sex differences in binding. Three male and three female animals, ten months of age, were used to establish binding patterns in adults. Animals were decapitated and their heads (prior to 25 PN) or brains (after 25 PN) were quickly frozen at -20°C by immersion in isopentane. Frozen brains were stored at -80°C until used. Tissue was cut into 20 µm thick coronal sections on a cryostat (Reichert Instruments) and thaw-mounted onto poly-L-lysine coated slides. Slides were stored at -80°C until processed for autoradiography.
Autoradiography

The protocol for [\(^3\)H] receptor autoradiography followed that reported by Tribollet and coworkers [Tribollet et al., 1991]. Slides were dried at room temperature for 15 minutes, then lightly fixed for 5 minutes in 0.2% paraformaldehyde in phosphate buffered saline (pH 7.4). Slides were preincubated for 15 minutes in 50 mM Tris HCl, pH 7.4, then covered with an incubation medium consisting of 50 mM Tris HCl, 0.025% bacitracin, 5 mM MgCl₂, and 0.1% bovine serum albumin, containing 1.5 nM [\(^3\)H]AVP (New England Nuclear, 64.8 Ci/mmol) and 5 nM HO[Thr\(^4\),Gly\(^7\)]OT (Cambridge Research Biochemicals), a specific oxytocin agonist. The tissue was incubated in this solution for one hour at room temperature in a humid chamber. Adjacent tissue sections from a 15 PN and an adult animal were used to assess non-specific binding by the addition of 1 μM non-radioactive AVP (Sigma) to the incubation medium. Subsequent to the incubation, slides were bathed in ice-cold medium (without the peptides) for 15 minutes, quickly rinsed in ice-cold distilled water, and dried at room temperature overnight. Slides were then apposed to LKB [\(^3\)H]-Ultrofilm for three months at 4°C, along with [\(^3\)H] autoradiographic standards (Amersham). Prior to this study, test slides were exposed to film for various amounts of time to determine the appropriate time of exposure. Films were developed by hand, and slides were fixed in 4% paraformaldehyde for 20 minutes, lightly counterstained with 1% neutral red, and coverslipped for aid in analysis.

Analysis

Analysis was performed with the aid of a developing and adult Brazilian opossum atlas established in our laboratory [Fox et al., 1991; Elmquist et al., 1992], supplemented by a developmental rat atlas [Paxinos et al., 1991] and a developmental mouse atlas [Schambra et al., 1992]. Sections were analyzed at 120 μm intervals in adults, and 60 μm intervals in developing animals. Autoradiograms were viewed with a light-box and a handheld lens. Counterstained slides were observed with a Nikon light microscope.

Quantitative analysis was performed on a Macintosh IIci computer using the public domain NIH Image program (written by Wayne Rasband at the U.S. National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov). A
standard curve to determine the amount of $[^3]H$AVP (nCi/mg tissue) bound to tissue sections was created using $[^3]H$ autoradiographic standards, (Amersham). Images from an adult animal were captured with a Nikon Coolscan slide scanner. Selected areas, including the central amygdala, hippocampus, supraoptic nucleus, thalamus, cerebellum, and brainstem were outlined and measured for mean optical density, which was converted to $[^3]H$AVP binding using our standard curve. The mean and standard error were calculated for multiple measurements of each region, and binding was compared between test and negative control sections.

Results

The addition of HO[Thr$^4$,Gly$^7$]OT, an oxytocin agonist, to the incubation medium prevents AVP from binding to oxytocin receptors [Tribollet et al., 1991]. Thus, all $[^3]H$AVP labelling in this study was considered to indicate vasopressin binding sites only. No sex differences in AVP binding were observed in either the adult or developing Brazilian opossum brain. A summary of our results is presented in Table I.

Binding patterns in the adult opossum brain

Coincubation of tissue with $[^3]H$AVP and an excess of unlabelled peptide eliminated labelling in most areas of the adult opossum brain (Table II). Measurements of the thalamus and brainstem were taken to establish the amount of radioactivity present in tissue without binding sites (a "background" level of labelling). Measurements of the central amygdala and cerebellum show a dramatic increase in binding between the control and test sections. In contrast, measurements taken of the hippocampus show only a slight increase in binding; therefore, we considered this binding nonspecific. In addition, binding was not eliminated from the supraoptic nucleus of the hypothalamus, and binding in this area was considered nonspecific as well. All other radioactive areas were considered to reflect vasopressin binding sites.

Most areas of binding in the opossum brain were labelled at low levels compared to background. However, a few areas exhibited notably heavy labelling; these will be denoted as having high binding levels. Moderate binding refers to binding levels between these two
Rhinencephalon/telencephalon. High binding was evident in the choroid plexus of the lateral ventricles (ChP, fig. 1A-E), and in the choroid plexus of the fourth ventricle (fig. 1G), as well as over blood-filled areas outside the brain (fig. 1B, C). Low levels of labelling were evident in the anterior olfactory nucleus and the fundus striati (FStr, fig. 1A) of the rostral forebrain. The outer layers of the cortex (Cor, fig 1A-D) were moderately labelled. In the rostral lateral septum, the dorsal part was strongly labelled, and the ventral part (LSV, fig. 1A) was weakly labelled. In more caudal sections, the dark labelling was seen more ventrally so that at the level of the anterior commissure, the ventral part of the lateral septal nucleus (fig. 1B) was heavily labelled. A moderate binding level was observed in the area of the ventral pallidum (VP, fig. 1B) and the nuclei of the diagonal band (DB, fig. 1A) labelled darkly. A moderate level was observed in the ventral division (BSTV, fig. 1B) and medial division (BSTM, fig. 1C) of the bed nucleus of the stria terminalis. High binding was observed in the nucleus of the lateral olfactory tract (LOT, fig. 1C) and the central amygdaloid nucleus (Ce, fig. 1D). The subiculum (S, fig. 1E) portion of the hippocampus demonstrated heavy labelling. No labelling was observed in other areas of the hippocampus, amygdala, or basal ganglia.

Diencephalon. Within the thalamus, the reticular thalamic nucleus and ventromedial thalamic nucleus displayed low binding levels, while the posterior part of the paraventricular thalamic nucleus (PVP, fig. 1E) and subparafascicular thalamic nucleus (SPF, fig. 1E) exhibited moderate levels of binding. The suprachiasmatic nucleus (SCh, fig. 1D) had a high level of binding. The striohypothalamic nucleus (StHy, fig. 1D) exhibited a low level of binding, and a low level of binding was observed in the stigmoid hypothalamic nucleus as well. As stated above, the high level of binding observed in the supraoptic nucleus (SON, fig. 1D) was considered nonspecific, as binding was not eliminated by the addition of nonradioactive peptide.

Mesencephalon. The dorsal raphe (DR, fig. 1F) and median raphe exhibited high levels of binding. The only other labelled area in the midbrain, the lateral interpeduncular nucleus, was lightly labelled.
Metencephalon. The medial parabrachial nucleus labelled lightly. In addition, moderate labelling was consistently distributed throughout the molecular layer of the cerebellum (Cb, fig. 1F, G, H).

Myencephalon. The nucleus of the solitary tract (Sol, fig. 1H) exhibited a high level of binding. A low level of binding was observed in the spinal trigeminal nucleus, caudal part (Sp5C, fig. 1H), while moderate levels were observed in the inferior olive (IO, fig. 1H) and raphe obscurus (ROb, fig. 1H) of the caudal hindbrain.

Binding patterns in the developing opossum brain

Binding levels were consistently low in the developing brains. With the exception of the extremely high binding exhibited in the anterior pituitary, no moderate or high levels were observed until 35 PN. Coincubation of tissue with [³H]AVP and an excess of unlabelled peptide eliminated labelling in all areas of the 15 PN opossum brain.

1 - 5 PN. The brain of the newborn opossum is very immature. Neurogenesis is occurring, and many nuclear groups are still in the process of formation, especially in forebrain regions. Very little binding was evident during the first few days of postnatal age. However, light binding was observed in the ventral tegmental area of the midbrain at 3 PN and the presumptive nucleus of the solitary tract by 5 PN. Binding was also evident in the ventricles, apparently labelling the choroid plexus, and in areas surrounding the brain where blood vessels are present. A very high level of binding was observed in the anterior pituitary (APit, fig. 2).

7 - 10 PN. At this age, morphological changes are still occurring, but many nuclear groups have become identifiable. Binding was still noticeably absent from the forebrain. However, labelling was observed for the first time in the still-developing cerebellum (Cb, fig. 3), in what is presumed to be the forming molecular layer. Labelling was observed in the inferior olive as well. Binding levels did not noticeably change in the ventral tegmental area, nucleus of the solitary tract, or anterior pituitary. Binding was still evident in the choroid plexus.

15 PN. After two weeks of postnatal development, most nuclear groups in the opossum brain have formed and morphogenesis is almost complete. At this age, labelling
was evident in many areas of the midbrain and rostral brainstem, including the ventral tegmental nucleus, the principal sensory trigeminal nucleus, and the superior olive, as well as in the ventral tegmental area, as observed earlier. Although binding was now evident in the anterior olfactory nucleus, no labelling was observed in telencephalic or diencephalic forebrain areas. Binding in the cerebellum, inferior olive, and nucleus of the solitary tract, as well as in the anterior pituitary and the choroid plexus, remained unchanged.

**25 PN.** By this age, morphogenesis and neurogenesis are essentially complete, and the brain resembles that of the adult opossum. At this age, labelling was evident in multiple forebrain areas, including the ventral part of the lateral septal nucleus (LSV, fig. 4B), the area of the ventral pallidum (VP, fig. 4A) and nuclei of the diagonal band (DB, fig. 4B), and both the medial and ventral (BSTV, fig. 4B) divisions of the bed nucleus of the stria terminalis. Within the diencephalon, the suprachiasmatic nucleus (SCh, fig. 4C) exhibited moderate levels of binding. The subparafascicular thalamic nucleus exhibited a low level of binding. Binding was still evident in the anterior olfactory nucleus, anterior pituitary, and choroid plexus. In the midbrain and hindbrain regions, binding was observed for the first time in the dorsal tegmental nucleus (DTg, fig. 4D), the medial parabrachial nucleus (PB, fig. 4D), and the caudal part of the spinal trigeminal nucleus (Sp5C, fig. 4F). Binding levels remained low in the ventral tegmental area and ventral tegmental nucleus (VTg, fig. 4D), principal sensory trigeminal nucleus, superior olive (SO, fig. 4D), cerebellum, nucleus of the solitary tract (Sol, fig 4E), and inferior olive (IO, fig. 4E) as well.

**35 PN.** At 35 days of age and older, the brain was removed from the skull, so binding in the anterior pituitary could no longer be assessed. Binding was still evident in the choroid plexus (ChP, fig. 5A). Binding was evident for the first time in many forebrain areas. Light binding was evident in the outer layers of the cortex. Moderate binding was evident in the dorsal part of the lateral septal nucleus. Within the thalamus, the medial geniculate (MG, fig. 5B) demonstrated a moderate level of binding. The ventromedial thalamic nucleus was lightly labelled, as were the striohypothalamic nucleus (StHy, fig. 5A) and stigmoid hypothalamic nucleus. Light binding was observed in the central amygdaloid nucleus (Ce, fig. 5A) and the subiculum. Binding levels remained light in the anterior
olfactory nucleus, ventral pallidum and diagonal band, ventral lateral septum, and bed nucleus of the stria terminalis. Binding appeared to increase to moderate levels in the subparafascicular thalamic nucleus and binding in the suprachiasmatic nucleus (Sch, fig. 5A) was heavy. Further caudad, light binding was observed for the first time in the median raphe nucleus. However, binding was no longer evident in the ventral tegmental area and the superior olive. Binding in the nucleus of the solitary tract was heavy at this age. Light binding remained in the ventral and dorsal tegmental nuclei, principal sensory trigeminal nucleus, medial parabrachial nucleus, molecular layer of the cerebellum, inferior olive, and spinal trigeminal nucleus.

45 - 60 PN. Opossums of two months of age are ready to be weaned, but are not sexually mature. Therefore, this age group represents the "juvenile" opossum brain. Only a few changes were noted after 35 PN. Most notably, binding was no longer evident in the medial geniculate nucleus, the ventral tegmental and dorsal tegmental nuclei, and the principal trigeminal sensory nucleus. However, binding was noted for the first time in the fundus striati, the posterior part of the paraventricular thalamic nucleus, and the lateral subnucleus of the interpeduncular nucleus. Binding increased to a moderate level in the ventral pallidum and diagonal band. Binding remained moderate in the dorsal and ventral lateral septum and the subparafascicular thalamic nucleus, and heavy in the suprachiasmatic nucleus and the nucleus of the solitary tract. Binding remained light in the cortex, the anterior olfactory nucleus, the bed nucleus of the stria terminalis, the ventromedial thalamic nucleus, the striohypothalamic and stigmoid hypothalamic nuclei, the central amygdaloid nucleus, the subiculum, the median raphe nucleus, the medial parabrachial nucleus, the molecular layer of the cerebellum, the spinal trigeminal nucleus, and the inferior olive.

Discussion

In this study, we have described the distribution and development of arginine-vasopressin binding sites in the brain of the Brazilian opossum, Monodelphis domestica. The expression of vasopressin binding sites in the adult mammalian brain has been described for multiple species, including the laboratory rat [Phillips et al., 1988, 1990; Tribollet et al.,
1988; Gerstberger and Fahrenholz, 1989; Johnson et al., 1993; Kremarik et al., 1993],
golden hamster [Dubois-Dauphin et al., 1990; Szot et al., 1990], and laboratory guinea pig
[Tribollet et al., 1992]. In addition, the development of binding sites has been described in
the prenatal and postnatal rat [Petracca et al., 1986; Tribollet et al., 1991] and the postnatal
hamster [Delville et al., 1994]. Arginine vasopressin binding in the Brazilian opossum is
found in many regions reported for other species, yet differences do exist. Vasopressin
binding sites appear consistently in the lateral septum, suprachiasmatic nucleus, anteroventral
thalamus, fundus striati, central amygdaloid nucleus, and nucleus of the solitary tract of rats,
hamsters, and guinea pigs. In addition, binding has been reported in the bed nucleus of the
stria terminalis and the dentate gyrus of both rats and hamsters. Results from the adult
opossum agree well with most of these findings: binding is observed in the lateral septum,
bed nucleus of the stria terminalis, suprachiasmatic nucleus, fundus striati, central
amygdaloid nucleus, and nucleus of the solitary tract. However, binding is not observed in
the anteroventral thalamus or the dentate gyrus in Monodelphis. Binding in the opossum
seems to most closely resemble that of the laboratory rat. In addition to the aforementioned
areas, labelled regions in both species include the anterior olfactory nucleus, the stigmoid
hypothalamic nucleus, the interpeduncular nucleus, and the inferior olive. The adult
opossum appears to express AVP binding sites in novel regions as well. Labelling in the
areas such as the ventral pallidum, subparafascicular thalamic nucleus, and the parabrachial
nucleus appears to be unique to the opossum. Most notable is the labelling throughout the
molecular layer of the cerebellum.

Binding site expression during development is even more variable. The lateral
septum is labelled early in rat and hamster development [Tribollet et al., 1991; Delville et
al., 1994], and is one of the earliest forebrain areas to express binding sites in the opossum,
although no labelling in the forebrain is seen until 25 PN. The central amygdala is labelled
by birth in the rat and hamster, but is not labelled in the opossum until 35 PN, which would
correspond to a eutherian mammal of two weeks or older. Very different areas appear to
express binding sites transiently in different species. In the rat, binding in the dorsal raphe,
locus coeruleus, facial motor nucleus, hypoglossal nucleus, and spinal trigeminal nucleus
greatly decreases or disappears by adulthood [Tribollet et al., 1991]. In the hamster, binding in the bed nucleus of the stria terminalis is transient [Delville et al., 1994]. In contrast, transient binding in the opossum brain is mostly restricted to midbrain structures: the ventral tegmental area and tegmental nuclei, the principal sensory trigeminal nucleus, and the superior olive. Transient binding is noted in the medial geniculate nucleus as well. Binding was never noted in the locus coeruleus, facial motor nucleus, or hypoglossal nucleus in the opossum, although these are areas of transient expression in the rat.

The significance of these species differences, in particular during development, is unclear. Vasopressin may influence diverse pathways in various species, possibly due to differences in the rate of brain or body development. However, the technique used to detect binding sites must be taken into account. Localization with [³H]AVP is limited by low resolution and long exposure periods [Phillips et al., 1988]. In recent years, new vasopressin receptor ligands have become available that may change the detection of binding sites. Although some laboratories have shown improved detection with these methods [Phillips et al., 1988, 1990; Johnson et al., 1993], the variability within protocols is also reported to be greater than the variability between them [Gerstberger and Fahrenholz, 1989; Tribollet et al., 1992]. In addition, the vasopressin V₁A receptor has recently been cloned [Morel et al., 1992]; thus vasopressin receptor mRNA can now be detected by in situ hybridization. Detection of AVP receptor mRNA indicates a much more global distribution in the brain than previously thought [Ostrowski et al., 1992, 1994; Szot et al., 1994], but the relationship between AVP receptor mRNA and the actual expression of the protein requires further analysis. In the future, however, with improved methods of AVP binding site detection available, the importance of variable sites of action, as well as highly conserved pathways, may be more thoroughly investigated.

The presence of apparently specific AVP binding sites in the opossum cerebellum is an interesting phenomenon. Vasopressin binding is rarely reported in the cerebellum. When vasopressin binding sites are localized in the rat cerebellum, they are only displaced (if at all) by the addition of high concentrations of unlabelled AVP [Lawrence et al., 1988]. However, in homogenate studies, specific [³H]AVP binding is present in the cerebellum.
[Lawrence et al., 1988]. In addition, vasopressin V₁A receptor mRNA is abundant in rat cerebellum, specifically in the molecular and granular cell layers [Szot et al., 1994]. Perhaps binding sites in the rat cerebellum are unique in their affinity for AVP, whereas opossum cerebellar AVP binding sites resemble those found in other brain regions.

Vasopressin binding sites in the adult opossum appear to be expressed in many areas of AVP innervation [J. Iqbal, unpublished observation], both hypothalamic and extrahypothalamic, and, in some cases, in cell bodies as well [J. Iqbal, unpublished observation]. Vasopressin-containing fibers and binding sites are seen in the anterior olfactory nucleus, nuclei of the diagonal band, the ventral pallidum, the lateral septum, the bed nucleus of the stria terminalis, the subiculum, and the paraventricular thalamic nucleus in the forebrain. In mid- and hindbrain regions, binding sites and fibers appear in the interpeduncular nucleus, the dorsal and median raphe, the parabrachial nuclei, the inferior olivary nucleus, the nucleus of the solitary tract, and the spinal trigeminal nucleus. In addition, binding sites are abundantly expressed in the suprachiasmatic nucleus, a region of cellular localization of the vasopressin peptide (J. Iqbal, unpublished observation). Although sex differences are observed in vasopressin-like immunoreactivity in the lateral septum [J. Iqbal, unpublished observation] no differences in [³H]AVP binding were observed between male and female opossums of any age studied. Vasopressin binding in the adult golden hamster [Dubois-Dauphin et al., 1990] and vasopressin V₁A receptor mRNA [Szot et al., 1994] are also reportedly not sexually dimorphic.

The antidiuretic actions of vasopressin are well known, and AVP has been postulated to play a role in many other homeostatic mechanisms [Mangiapane et al., 1983; Morris et al., 1987; Raghunandanan et al., 1990; Burlet et al., 1992; Oloyomi and Hart, 1992]. In addition, AVP is currently being investigated as a factor in central nervous system development [Petracca et al., 1986]. This hypothesis is supported by the abnormal development of the Brattleboro rat. Genetically unable to synthesize AVP in the brain, these animals have stunted brain growth and several behavioral disturbances [Boer et al., 1980, 1993; Boer, 1985; Snijdewint et al., 1988]. The arginine vasopressin peptide is present very early in the developing brain of the normal rat and mouse [Buijs et al., 1980; Whitnall et al.,
In addition, AVP-like immunoreactivity is detectable in the mesencephalon of the developing opossum by twelve days postconception [Iqbal et al., 1993]. However, AVP binding site expression in the developing opossum brain does not appear to correlate with peptide expression. At birth, vasopressin binding sites are detected only in non-neuronal areas such as the choroid plexus and the anterior pituitary. Binding is first evident in the ventral tegmental area of the midbrain at 3 PN, almost a week after the peptide is detected immunohistochemically. Vasopressin-immunoreactive fibers are seen throughout the brain by embryonic day 13 and fibers are abundant in many forebrain areas by 1 PN, yet binding sites are not evident in the opossum forebrain until 25 PN. Neurogenesis is completed in the Brazilian opossum hindbrain prior to birth [J. Iqbal, unpublished observation], but continues in the forebrain for the first two weeks of life [Iqbal et al., 1995]. Thus, AVP peptide synthesis precedes the completion of neurogenesis, but binding site expression apparently does not. This could be a function of low sensitivity associated with the [$^3$H] binding technique. However, previous studies found no change [Tribollet et al., 1991], or an actual decrease [Szot et al., 1989] in binding affinity during development. Alternatively, AVP could be acting to influence cell growth or maturation through a non-receptor mediated mechanism. The fact that binding sites are, in many instances, detected in fiber-rich areas later in development, lends support to this hypothesis.

Vasopressin appears to have a high affinity for oxytocin receptors [Audiger and Barberis, 1985]. Therefore, previous studies that utilized [$^3$H] AVP to detect vasopressin binding sites often misidentified oxytocin binding sites as AVP receptors. The addition of the specific oxytocin antagonist HO[Thr$^4$,Gly$^7$]OT, used in this study, reportedly eliminates nonspecific binding to oxytocin binding sites [Tribollet et al., 1991]. Preliminary work in our laboratory supports this assumption. Tritiated AVP binding studies performed in the opossum without HO[Thr$^4$,Gly$^7$]OT resulted in binding in specific regions, such as the medial geniculate nucleus, where no binding was evident in the presence of this compound. In addition, negative controls were generated by the addition of nonradioactive AVP to the incubation medium. The only area of nonspecific binding detected in the controls was found in the supraoptic nucleus of the adult. Thus, the binding in all other brain areas in this study
can be considered to indicate the location of vasopressin binding sites. Although no
electrophysiology was performed in this study, previous studies performed in the rat
[Tribollet et al., 1991] have shown that AVP binding sites detected by the method we have
used are indeed functional. In addition, neonatal rats respond behaviorally to the central
exogenous administration of AVP [Winslow and Insel, 1993]. We assume, therefore, that
binding in this study reflects the localization of functional vasopressin binding sites.

Currently, three types of vasopressin receptor are known to exist [Jard et al., 1987].
In the rat, type-specific antagonists have been used to localize V₁a receptors to those brain
areas that express vasopressin binding sites [Phillips et al., 1988, 1990; Gerstberger and
Fahrenholz, 1989; Johnson et al., 1993]. Vasopressin V₁a binding sites are also present in
the liver, kidney, testes, and small blood vessels [Phillips et al., 1990]. Vasopressin V₂
binding sites are abundant in the cells of the renal collecting ducts, loops of Henle, and distal
convoluted tubules in the kidney [Phillips et al., 1990]. Interestingly, binding sites within
the anterior pituitary do not bind V₁ antagonists such as d(CH₂)₅SAVP [Jard et al., 1986;
Phillips et al., 1988, 1990], and are now characterized as V₁b receptors, distinct from V₁a
found throughout the central nervous system [Baertschi and Friedli, 1985; Jard et al., 1986].

Preliminary data demonstrates that vasopressin binding sites in the opossum brain resemble
those of other species. Binding studies with a tritiated V₁ receptor antagonist (New England
Nuclear; 45.1 Ci/mmol) in 10 and 25 PN opossums reveal identical patterns to binding with
[^H]AVP in all brain areas [unpublished observation]. However, no binding is seen in the
anterior pituitary with this antagonist. These results would indicate that, as in other
mammals, vasopressin binding sites in the Brazilian opossum brain are of the V₁ type, but
sites within the anterior pituitary are not. In addition, vasopressin receptor subtypes do not
appear to change during development. The V₁ receptor has been shown to act via
phosphatidylinositol hydrolysis [Shewey and Dorsa, 1988], while the V₂ binding site is
coupled to adenylate cyclase [Jard, 1983]. However, the physiological significance of
multiple vasopressin receptor subtypes is unclear at this point in time.

Although arginine vasopressin was used in this study to detect vasopressin binding
sites, another type of vasopressin receptor may exist in the Brazilian opossum. Lysine
vasopressin (LVP) has been reported to be an important neurotransmitter in other marsupials [Chauvet et al., 1983, 1984; Rouille et al., 1988], where it plays many of the same roles that AVP does in rats and other mammals. We have attempted to characterize the vasopressin peptide in the opossum using immunohistochemistry against both AVP and LVP. The LVP antibody we used was reportedly specific for LVP. However, the patterns of immunoreactivity seen for AVP and LVP were strikingly similar in the Brazilian opossum brain. Further, preabsorption with the LVP peptide did not block AVP-like immunostaining, but preabsorption with arginine vasopressin blocked the staining of both the AVP and LVP antibody. Thus, it is unclear at this time whether the vasopressin peptide in the opossum is arginine vasopressin, lysine vasopressin, or both. The basic similarities in binding patterns seen between the Brazilian opossum and other mammals would suggest that most, if not all, vasopressin binding sites in the Brazilian opossum brain will bind to arginine vasopressin. However, the affinity of vasopressin binding sites for LVP in the opossum is still an area of exploration.

The transient expression of binding sites appears to be a recurrent theme during neuronal development. In the Brazilian opossum, binding sites are detected transiently in multiple midbrain areas, including the ventral tegmental area, the dorsal and ventral tegmental nuclei, the principal sensory trigeminal nucleus, and the superior olive. In the rat, binding is transient in many hindbrain regions associated with cranial nerves, such as the facial motor nucleus, hypoglossal nucleus, and spinal trigeminal nucleus [Tribollet et al., 1991]. At the present time, the significance of these binding sites is unknown; however, they have been shown to be functional in the neonatal rat [Tribollet et al., 1991]. Transient receptor expression during development is particularly interesting in the facial motor nucleus. In the neonatal rat, not only vasopressin binding sites [Tribollet et al., 1991], but also estrogen receptors [Yokosuka and Hayashi, 1992; R. Handa, personal communication] and cholecystokinin binding sites [Kuehl-Kovarik and Jacobson, 1995] are expressed from birth through the first two to three weeks of life in the facial motor nucleus. In addition, cholecystokinin binding sites are expressed transiently, but robustly, in the facial motor nucleus of the developing Brazilian opossum until the age of weaning, between 45 and 60
PN [Kuehl-Kovarik et al., 1993]. Synaptogenesis also appears to be delayed in the Brazilian opossum facial nucleus [Swanson et al., 1993]. We believe that the transient expression of multiple receptor types in the facial nucleus is significant, either from a developmental or physiological aspect. It is possible that cholecystokinin binding sites in the developing opossum are performing a task executed by multiple receptor types in other mammalian species. It is interesting to note that binding sites for AVP are apparently not expressed in the facial motor nucleus of opossums of any age.

In summary, we have described the localization and development of arginine vasopressin binding sites in the Brazilian opossum, *Monodelphis domestica*. We see many similarities, and some differences, in binding patterns between *Monodelphis* and other mammalian species. Binding sites were not detected in the facial motor nucleus of the developing opossum, in contrast to the transient pattern observed in the neonatal rat. Therefore, the effects mediated by vasopressin in this region in rats may be mediated by an alternative peptide system in *Monodelphis*. Vasopressin binding sites, like the peptide, are found in many extrahypothalamic regions, suggesting a variety of homeostatic roles for the vasopressinergic system. However, binding sites are rarely detected prior to the completion of neurogenesis. Therefore, arginine vasopressin, acting on its classical binding site, is unlikely to play a major role in the development of the opossum central nervous system.

Acknowledgements

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References


vitro autoradiography using a radioiodinated \( V_1 \) receptor antagonist. Neuroscience, 27: 749-761.


Table I. Summary of the ontogeny of vasopressin binding sites in the Brazilian opossum brain.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Age (postnatal days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-5</td>
</tr>
<tr>
<td>Anterior olfactory tract</td>
<td>-1</td>
</tr>
<tr>
<td>Anterior pituitary</td>
<td>H2</td>
</tr>
<tr>
<td>Bed nucleus of the stria terminalis:</td>
<td></td>
</tr>
<tr>
<td>- medial division</td>
<td>-</td>
</tr>
<tr>
<td>- ventral division</td>
<td>-</td>
</tr>
<tr>
<td>Central amygdaloid nucleus</td>
<td>-</td>
</tr>
<tr>
<td>Cerebellum, molecular layer</td>
<td>-</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>-</td>
</tr>
<tr>
<td>Diagonal band, vertical limb</td>
<td>-</td>
</tr>
<tr>
<td>Dorsal raphe</td>
<td>-</td>
</tr>
<tr>
<td>Dorsal tegmental nucleus</td>
<td>-</td>
</tr>
<tr>
<td>Fundus striati</td>
<td>-</td>
</tr>
<tr>
<td>Inferior olive</td>
<td>-</td>
</tr>
<tr>
<td>Interpeduncular nucleus,</td>
<td></td>
</tr>
<tr>
<td>lateral subnucleus</td>
<td>-</td>
</tr>
<tr>
<td>Lateral olfactory tract</td>
<td>-</td>
</tr>
<tr>
<td>Lateral septal nucleus:</td>
<td></td>
</tr>
<tr>
<td>dorsal part</td>
<td>-</td>
</tr>
<tr>
<td>ventral part</td>
<td>-</td>
</tr>
<tr>
<td>Medial geniculate nucleus</td>
<td>-</td>
</tr>
<tr>
<td>Medial parabrachial nucleus</td>
<td>-</td>
</tr>
<tr>
<td>Median raphe</td>
<td>-</td>
</tr>
<tr>
<td>Brain Region (or Nucleus)</td>
<td>L</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
<td>---</td>
</tr>
<tr>
<td>Nucleus of the solitary tract</td>
<td></td>
</tr>
<tr>
<td>Paraventricular thalamic</td>
<td></td>
</tr>
<tr>
<td>nucleus, posterior</td>
<td></td>
</tr>
<tr>
<td>Principal sensory trigeminal</td>
<td></td>
</tr>
<tr>
<td>Raphe obscurus</td>
<td></td>
</tr>
<tr>
<td>Reticular thalamic nucleus</td>
<td></td>
</tr>
<tr>
<td>Spinal trigeminal nucleus, caudal part</td>
<td></td>
</tr>
<tr>
<td>Stigmoid hypothalamic nucleus</td>
<td></td>
</tr>
<tr>
<td>Striohypothalamic nucleus</td>
<td></td>
</tr>
<tr>
<td>Subiculum</td>
<td></td>
</tr>
<tr>
<td>Subparafascicular nucleus</td>
<td></td>
</tr>
<tr>
<td>Superior olive</td>
<td></td>
</tr>
<tr>
<td>Suprachiasmatic nucleus</td>
<td></td>
</tr>
<tr>
<td>Ventral pallidum</td>
<td></td>
</tr>
<tr>
<td>Ventral tegmental area</td>
<td>L</td>
</tr>
<tr>
<td>Ventral tegmental nucleus</td>
<td></td>
</tr>
<tr>
<td>Ventromedial thalamic nucleus</td>
<td></td>
</tr>
</tbody>
</table>

1. - indicates no binding in the region at the specified age.

2. Indicates level of binding based on quantitative measurements (established for adults) and relative levels determined for developing animals. L, low level of binding; M, moderate level of binding, more significant than low level but not remarkably high; H, notably high level of binding.

3. ? indicates that level of binding is unknown, as we remove the brain from the skull at this age.
Table II. A comparison of $[^3\text{H}]$AVP binding in test and control sections from selected regions of an adult Brazilian opossum brain.

<table>
<thead>
<tr>
<th>Region</th>
<th>$[^3\text{H}]$AVP (nCi/mg)</th>
<th>no. of measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test $^a$</td>
<td>Control $^a$</td>
</tr>
<tr>
<td>central amygdala</td>
<td>$2.13 \pm 0.26^b$</td>
<td>$0.47 \pm 0.04$</td>
</tr>
<tr>
<td>hippocampus</td>
<td>$0.90 \pm 0.07$</td>
<td>$0.56 \pm 0.03$</td>
</tr>
<tr>
<td>thalamus</td>
<td>$0.48 \pm 0.02$</td>
<td>$0.39 \pm 0.07$</td>
</tr>
<tr>
<td>cerebellum</td>
<td>$2.40 \pm 0.11$</td>
<td>$0.94 \pm 0.08$</td>
</tr>
<tr>
<td>brainstem</td>
<td>$0.62 \pm 0.07$</td>
<td>$0.59 \pm 0.03$</td>
</tr>
<tr>
<td>supraoptic nucleus</td>
<td>$2.30^c$</td>
<td>2.06</td>
</tr>
</tbody>
</table>

$^a$, test refers to tissue incubated in $[^3\text{H}]$AVP. Control tissue was incubated in radioactive AVP with an excess of unlabelled peptide to block all specific binding.

$^b$, mean and standard error representing $[^3\text{H}]$AVP binding in nCi/mg tissue.

$^c$, only one measurement was taken of the supraoptic nucleus.
Figure 1. Pseudocolor images, produced from LKB Ulrofilm, of [3H]AVP binding throughout the adult Brazilian opossum brain. Selected coronal sections are shown from rostral (A) to caudal (H). A: Section through the telencephalon, demonstrating high binding levels in the choroid plexus (ChP) of the lateral ventricles and the vertical limb of the diagonal band (DB), and moderate levels of binding in the outer layers of the cortex (Cor). Light binding can be noted in the ventral lateral septum (LSV) and the fundus striati (FStr). B: Section at the level of the anterior commissure (ac), demonstrating high levels of binding in the choroid plexus (ChP) and ventral part of the lateral septum (LSV), and moderate binding in the ventral pallidum (VP) and ventral part of the bed nucleus of the stria terminalis (BSTV). Moderate binding is noted in the cortex (Cor) as well. C: Section at the level of the preoptic area. High binding is observed in the nucleus of the lateral olfactory tract (LOT). Moderate binding is evident in the cortex (Cor) and the medial division of the bed nucleus of the stria terminalis (BSTM). D: Section through the diencephalon. High binding levels are observed in the suprachiasmatic nucleus (SCh), central amygdaloid nucleus (Ce) and suprachiasmatic nucleus (SON). However, labelling in the SON is considered nonspecific. Moderate binding is evident in the cortex (Cor), and low binding is observed in the striohypothalamic nucleus (StHy). E: Section through the caudal diencephalon. A high level of binding is noted in the subiculum (S). The cortex (Cor) demonstrates a moderate level of binding, and binding in the paraventricular (PVP) and subparafascicular (SPF) thalamic nuclei is present at moderate levels as well. F: Section through the mesencephalon. A high binding level is observed in the dorsal (DR) raphe nucleus. G: Section through the metencephalon. Moderate binding levels are observed in the molecular layer of the cerebellum (Cb). High binding is evident in the choroid plexus (ChP) of the fourth ventricle (4V). No other binding is observed in the brainstem at this level. H: Section through the caudal brainstem. High binding is observed in the nucleus of the solitary tract (Sol). The cerebellum (Cb) is labelled moderately. Low binding levels are noted in the caudal part of the spinal trigeminal nucleus (Sp5C), while moderate levels are observed in the inferior olive (10) and the raphe obscurus (ROb). Inset: pseudocolored standard bar, correlating color and binding intensity. Red, highest binding, above 3.0 nCi/mg. Yellow, moderate to high, around 2.0 nCi/mg. Green, moderate to low, around 1.0 nCi/mg. Blue to black, low binding, below 0.5 nCi/mg. ac, anterior commissure; HC, hippocampus; 4V, fourth ventricle. All images are at the same magnification. Scale bar = 2 mm.
Figure 2. Photomicrograph, produced from LKB Ultrofilm, of a coronal section from a 5 PN opossum forebrain, demonstrating [3H]AVP binding. Note the high level of binding in the anterior pituitary (APit). No other binding is noted in the forebrain at this age. 3v, third ventricle. Scale bar = 500 μm.

Figure 3. Photomicrograph, produced from LKB Ultrofilm, of a coronal section from a 7 PN opossum hindbrain, demonstrating [3H]AVP binding. Note the light binding in the cerebellum (Cb) at this age. No other binding is evident in the mesencephalon at this age. Teg, tegmentum; 4v, fourth ventricle. Scale bar = 750 μm.
Figure 4. Photomicrographs, produced from LKB Ultratfilm, of selected regions from a 25 PN opossum, demonstrating [³H]AVP binding. A: Section through the basal forebrain, demonstrating binding in the ventral pallidum (VP). B: Section slightly further caudal, demonstrating binding in the diagonal band (DB), ventral division of the bed nucleus of the stria terminalis (BSTV), and ventral lateral septum (LSV). C: Section through the ventral diencephalon, demonstrating binding in the suprachiasmatic nucleus (SCh). D: Section taken at the level of the mesencephalon, demonstrating binding in the dorsal (DTg) and ventral (VTg) tegmental nuclei, the parabrachial nucleus (PB), and the superior olive (SO). E: Section through the metencephalon, demonstrating binding in the nucleus of the solitary tract (Sol) and inferior olive (IO). Binding is also evident in the choroid plexus (ChP). F: Section through the caudal brainstem, demonstrating binding in the caudal part of the spinal trigeminal nucleus (Sp5C). ac, anterior commissure; 3V, third ventricle; 4V, fourth ventricle. Dotted line in A indicates base of brain. Scale bar in F applies to all figures. For A, B, C, E, and F, bar = 500 µm. Scale bar for D = 750 µm.
Figure 5. Photomicrographs, produced from LKB Ultrofilm, of selected regions from a 35 PN opossum, demonstrating $[^3H]$AVP binding. A: Section through the hypothalamus and amygdala. Note the low level of binding in the central amygdaloid nucleus (Ce), and also in the striohypothalamic nucleus (StHy). Heavier binding is demonstrated in the suprachiasmatic nucleus (SCh). Binding is also evident in the choroid plexus (ChP) of the lateral ventricle. B: Section through the midbrain. Note the binding in the medial geniculate nucleus (MG), a phenomenon only observed at this age. 3V, third ventricle; HC, hippocampus. Both photographs are at the same magnification. Scale bar = 500 $\mu$m.
GENERAL CONCLUSION

Summary

Chapter one examined the ontogeny of CCK binding sites in the Brazilian opossum brain. In general, the patterns of cholecystokinin binding site expression are as expected. Cholecystokinin binding sites in the adult opossum are found in similar regions as in other adult mammalian species, and binding in the developing opossum forebrain is comparable to that reported for the rat, although binding sites are noted earlier in development than was expected. Cholecystokinin binding sites are observed in the developing opossum hindbrain at birth, and are present in the forebrain as early as five days postnatal, well before the end of neurogenesis. In general, binding site expression appears to precede the appearance of the CCK peptide. In addition, there are many regions of apparent peptide-binding site mismatch, where binding sites are expressed but CCK immunoreactive fibers are not detected. A dramatic demonstration of this phenomenon is the consistent binding throughout the molecular layer of the cerebellum. Transient binding sites are observed in the developing reticular thalamic nucleus, ventral lateral lemniscus, ventral periolivary nucleus, cerebellar nuclei, and facial motor nucleus.

Cholecystokinin binding site expression in the rat and opossum brainstem also demonstrate similar overall patterns, as discussed in chapter two. The greatest development of CCK binding sites in the rat hindbrain occurs prenatally, in contrast to what has been reported for forebrain regions. These findings parallel the early expression of CCK binding sites in the developing Brazilian opossum brain. However, unlike binding patterns in the opossum, no CCK binding sites are observed in the developing rat cerebellum at any age. Many areas of the rat hindbrain appear to express CCK binding sites transiently during development. These include the rubrospinal tract, the facial motor nucleus (medial and intermediate subnuclei), the parvocellular reticular formation, the raphe obscurus, the cuneate nucleus, and the gracile nucleus.

Chapter three examined the expression of arginine vasopressin binding sites in the adult and developing Brazilian opossum brain. Vasopressin binding site expression in the
adult opossum parallels binding patterns in eutherian species in many areas, although a great deal of interspecies variability exists as well. Vasopressin binding sites are expressed much later in opossum brain development than was expected, based on developmental data in the normal and Brattleboro rat. In general, binding sites appear well after the completion of neurogenesis. In addition, AVP immunoreactivity is detected prior to binding site expression. Unlike the cholecystokinergic system, there appears to be very little peptide-binding site mismatch in the Brazilian opossum brain. One exception to this is in the molecular layer of the cerebellum, which appears to express vasopressin (as well as CCK) binding sites. In the developing Brazilian opossum, many midbrain regions appear to transiently express vasopressin binding sites, including the ventral tegmental area, the ventral and dorsal tegmental nuclei, the principal sensory trigeminal nucleus, and the superior olive.

Thus, the transient expression of binding sites is observed in both rat and opossum brain during development. Cholecystokinin binding sites are expressed transiently in both forebrain and hindbrain areas of the opossum. A transient expression of CCK binding sites is seen in the developing rat hindbrain as well, although commonly in different regions than observed in the opossum. However, CCK binding sites are expressed transiently in the facial motor nucleus of both species. Vasopressin binding sites are expressed transiently in some areas of the developing Brazilian opossum brain as well, mainly restricted to midbrain regions. No vasopressin binding sites are noted in the facial motor nucleus of the developing opossum.

No cholecystokinin or vasopressin binding sites are noted in the trigeminal motor nucleus of the rat or opossum at any age studied. Nor are CCK binding sites detected in the hypoglossal nucleus of the developing rat. However, cholecystokinin binding sites are expressed in the opossum hypoglossal as early as ten days postnatal, and these persist until adulthood. No vasopressin binding sites are present in the opossum hypoglossal nucleus, in contrast to what has been reported for the rat [Tribollet et al., 1991]. Thus, in these studies, the only constant characteristic in the oromotor nuclei between these two species during development is the transient expression of cholecystokinin binding sites in the facial motor nucleus.
Conclusions

A healthy mammal, whether neonatal or adult, basically consists of multiple organ systems working in synchrony to provide internal homeostasis. The regulation of homeostasis, as well as the behaviors necessary to ensure survival, is under the control of the nervous and endocrine systems. Within the nervous system, the hypothalamus influences multiple endocrine organs, and regulates many bodily functions, including temperature maintenance, fluid and nutrient balance, and sexual activity. Nuclei in the medulla are involved in the control of respiration, and the vagus controls heart rate, bronchial dilation, and gastrointestinal motility and secretion. Thus, the central nervous system can be considered the control center for the survival and maintenance of the organism.

The young of Monodelphis are born in an extremely immature state after a two week gestational period [see appendix 1]. These fetus-like animals have functional respiratory, excretory, and digestive systems to allow them to survive in the ex-utero environment [Tyndale-Biscoe and Renfree, 1987]. However, they are born in a greatly immature state of differentiation. Although the brainstem, in general, has completed neurogenesis, much forebrain neurogenesis is completed subsequent to the birth of the animal [Iqbal et al., 1995; Larsen and Jacobson, 1986]. Thus, Monodelphis young must control their internal environment and survive prior to the existence of functional forebrain nuclei.

Conversely, neonatal rats, like most mammals, are born with most organ systems intact. Neurogenesis is, for the most part, a prenatal event in Eutherian species [Bayer and Altman, 1995]. Even so, the developed brain of the baby rat would be expected to be organized for neonatal survival, and undergo many changes prior to adulthood. Therefore, transmitter systems specific for neonatal survival would be expected to be present in both eutherian and metatherian mammals. In addition, the specialized need of the baby marsupial would dictate differences in neuronal organization between the two subclasses.

Neurotransmitters constitute the classical basis of central nervous system function. Neurotransmitter systems allow cellular communication through the release of substances, manufactured by neurons (and perhaps glia) that diffuse across synapses and bind to
postsynaptic or intracellular binding sites. Signals within the central nervous system can be regulated by changes in neurotransmitter synthesis or release. However, modulation can also occur at the receptor level, with changes in binding site expression or binding affinity.

A transient expression of binding sites is observed in many areas of the developing rat and opossum brain during development. We have not yet proven that these binding sites are functional. However, electrophysiological studies in the neonatal rat have demonstrated that transient vasopressin binding sites in the facial motor nucleus (FMN) are indeed functional [Tribollet et al., 1991], supporting the assumption that other transiently-expressed sites are as well. In addition, receptors require considerable genetic and metabolic machinery for maintenance [Herkenham, 1987]. It is unlikely that an organism (or cell) would invest energy in manufacturing binding sites that were not functional, especially for a brief time in the animals life. The fact that multiple types of binding sites are expressed transiently during a specific period of development implies functional significance at that particular age. This transient expression is seen in neonatal life, when the central nervous system is undergoing many morphological changes. However, the neonate, as well, is changing physiologically and behaviorally as it matures. Thus, transient binding sites could be regulating developmental events at the level of the neuron or transmitter system, or globally altering the animal’s ability to cope with its environment.

Although CCK and AVP binding sites are expressed transiently in multiple brain regions, there are few common regions between the two mammals, nor do many regions express both types of binding sites. Interestingly, however, cholecystokinin binding sites are expressed for a short time in the facial motor nucleus of both species, and vasopressin binding sites are expressed in the rat FMN as well. Thus, the FMN appears to be a significant region for neurotransmitter modulation in the developing mammal.

The facial motor nucleus is one of a number of cranial motor nuclei known as the oromotor nuclei [Travers 1995]. These particular brainstem nuclei, which also include the trigeminal motor nucleus (Mo5) and the hypoglossal motor nucleus (12), regulate the activity of the facial and oral muscles, and thus play a role in feeding behaviors. The facial motor nucleus, therefore, could potentially play a significant part in the modulation of nutrient or
fluid intake in the neonate. Cholecystokinin binding sites are present in the opossum hypoglossal, and vasopressin receptors are expressed by neurons in the rat hypoglossal [Tribollet et al., 1991], suggesting that the hypoglossal nucleus may somehow be involved in the regulation of nutrient intake as well. The trigeminal motor nucleus is not involved in either of the receptor systems studied.

In addition to oromotor nuclei, other brainstem nuclei are intimately involved in the regulation of food intake in mammals. The dorsal vagal complex, consisting of the nucleus of the solitary tract and the vagal dorsal motor nucleus, is the source of input for the central control of gastric function, as well as the endpoint for vagal afferents [Finger, 1992]. Cholecystokinin binding sites are transported in the vagus [Moran and McHugh, 1992], and are expressed, possibly presynaptically, in the nucleus of the solitary tract [Moran and McHugh, 1992]. Cholecystokinin binding sites are first expressed in the dorsal vagal complex of the rat and opossum at or before birth. In addition, vasopressin binding sites have been reported to be present in the rat dorsal vagal complex by postfertilization day 20 (PF 20), and are observed in the opossum nucleus of the solitary tract by 5 PN. It is apparent that both the cholecystokininergic and vasopressinergic systems are playing a role in autonomic function early in neonatal life. However, binding site expression appears to remain high throughout the development and maturation of the animal. Therefore, the dorsal vagal complex appears to be a point of maintenance, rather than modulation, during mammalian development.

The facial motor nucleus

The facial motor nucleus has completed neurogenesis by birth in both metatherians and eutherians [Bayer and Altman 1995; J. Iqbal, personal communication], and expresses multiple binding site types, only during neonatal life, in multiple species. The FMN, therefore, is an excellent candidate for the regulation of some aspect of neonatal existence. In support of this hypothesis, we now know that estrogen receptors, as well, are transiently expressed in the facial motor nucleus of neonatal rats [Kuehl-Kovarik et al., 1995; Yokosuka and Hayashi, 1992]. There is considerable overlap in the timing of expression of these multiple receptor types. Cholecystokinin binding sites are expressed from PF 20 until 10
Vasopressin receptors have been demonstrated from PF 17 until 30 PN (Tribollet et al., 1991). Estrogen receptors are present from birth through 11 PN (Yokosuka and Hayashi, 1992) and disappear soon afterwards (Kuehl-Kovarik et al., 1995).

Within the neonatal rat FMN, there is a distinct subnuclear organization. Estrogen receptors are expressed only in the medial subnucleus (Yokosuka and Hayashi, 1992), while vasopressin binding sites are discretely localized to the intermediate subnucleus (Tribollet et al., 1991). Cholecystokinin binding sites are expressed in the medial subnucleus early in life, but later are expressed in the intermediate subnucleus before disappearing altogether. In the adult rat, the medial subnucleus controls posterior facial muscles, such as those involving ear movement (Travers, 1995), while the intermediate subnucleus innervates perioral and orbital musculature (Travers, 1995). However, this may not be the case in neonates. Cholecystokinin binding sites in the developing Brazilian opossum FMN show no nuclear compartmentalization.

We have attempted to characterize cholecystokinin binding sites in the facial motor nucleus of the neonatal rat and opossum with the use of antagonists. Two types of CCK binding sites have currently been distinguished. The A-type or peripheral receptor is found in the gastrointestinal tract and pancreas (Jensen et al., 1989), and in specific brain regions (Hill and Shaw, 1988; Hill et al., 1990; Hill and Woodruff, 1990). The B- or brain type receptor is abundant throughout the central nervous system (Hill and Woodruff, 1990). Specific functions have been implicated for both receptor types in the rat mesolimbic pathway (Crawley, 1992), and the B-type appears to play an important role in anxiety (Costall et al., 1991). Interestingly, CCK binding sites in the neonatal rat FMN appear to be of the B-type, as expected based on typical distribution patterns. However, CCK binding in the developing opossum FMN is strongly A (Kuehl-Kovarik et al., 1993), a type found in only a few discrete places in the mammalian brain. The significance of this is unknown, although the different receptor types do have different affinities for various forms of the CCK peptide. Thus, the cholecystokininergic system may be regulated at a different level in the neonatal opossum than in the young rat.
The control of homeostasis

It is generally assumed that the control of food intake in the neonatal rodent is mediated by the hypothalamus, as in the adult, although modifications within the central nervous system would be expected as the baby switches from a liquid diet to solid food. Cholecystokinin is a well-known satiety factor in the adult mammal [Silver and Morley 1991]. Multiple studies have been undertaken to examine the effect of CCK in neonates [Blass et al., 1979; Robinson et al., 1985; Weller et al., 1990], with varying results. Blass and coworkers [1979] found that CCK doesn't appear to affect food intake in rat pups until 15 to 20 days of age. However, Robinson and coworkers [1985] report a decreased intake as early as 1 PN with the administration of CCK. Weller and coworkers [1990] report that CCK has an effect in 9 to 12 day old pups. In addition, Rinaman and coworkers [1994] report that exogenous CCK does not induce intermediate-early gene expression in the hypothalamus of two day old rat pups, although it does in the hindbrain [Rinaman et al., 1994]. Thus, the role of CCK in the modulation of nutrient intake in the neonatal rat is still unclear, and even less is known about the site of peptide action in the neonate.

The control of nutrient intake in the young opossum is even more ambiguous. At birth, marsupials lack the forebrain circuits that normally function to control feeding behaviors. The neonatal marsupial is physically attached to the dam's nipple for a period of time. Therefore, the mother may, to some extent, regulate the amount of milk delivered to the pup. Yet there must be some input from the neonate, as nipples without pups do not release milk. In addition, pups are able to actively nurse an artificial "nipple". Brazilian opossum pups are not weaned until fifty to sixty days of age, and cholecystokinin binding sites are present in the FMN throughout the suckling period, disappearing when the babies are weaned. At this time, we have not yet been able to demonstrate an effect of exogenously administered CCK on food intake in two week old Brazilian opossum pups. However, a confounding factor is an apparent natural variability in milk consumption within a litter.

Like the regulation of food intake, the control of water balance in the mammalian neonate is not as straightforward as first assumed. Arginine vasopressin-like immunoreactivity has been detected in the supraoptic and paraventricular hypothalamic nuclei
well before birth in the rat and mouse [Buijs et al., 1980; Whitnall et al., 1985]. However, Dlouha and coworkers [1982] report that AVP does not play a role in water balance in the rat until fourteen days postnatal. They found that milk intake was not significantly different between homozygote and heterozygote Brattleboro rats for the first three weeks of postnatal life [Dlouha et al., 1982]. In addition, they found no differences in urine osmolarity, but they did detect a higher blood osmolarity in the homozygous Brattleboro rat pups. These results would indicate that vasopressin does not regulate water balance in neonates as it does in adults. However, the controls for this experiment were Brattleboro heterozygotes, rats with reduced, but not absent, levels of AVP. Thus, the controls could not necessarily be expected to exhibit "normal" osmoregulatory behavior [Valtin, 1982]. In addition, this experiment did not control for any mechanisms of compensation in the Brattleboro rats. From these results, it can only be concluded that AVP is not essential for osmoregulation in these neonates. However, the role that vasopressin plays in normal neonatal rats is unclear.

We have not yet determined the age that AVP is first released into the bloodstream of the neonatal Brazilian opossum. However, we do know that arginine vasopressin-like immunoreactive fibers are present in the median eminence as early as three days postnatal [Iqbal and Jacobson, 1995], and AVP-like immunoreactivity is found in the posterior pituitary by 5 PN [Iqbal and Jacobson, 1995], suggesting that AVP is released into the bloodstream at a young age. Whether AVP is necessary for water balance in the neonatal opossum is not known at this time, but as there are no vasopressin binding sites in the FMN of this species, we can assume that the FMN is not directly involved in this regulatory process.

A dramatic difference is seen between the eutherian and metatherian method of producing offspring. While eutherian mammals nurture their young through well-developed placental attachments, metatherian mammals support their offspring through lactation. A correlate of this difference is seen in milk composition. Eutherian milk changes very little during the course of lactation. In contrast, metatherian milk undergoes dramatic changes in consistency as the neonate grows [Tyndale-Biscoe and Renfree, 1987]. In the first half of
lactation, marsupial milk is low in total solids, lipids, and protein. During the second half, lipids and protein increase, and carbohydrates decrease. There is also evidence that changes in marsupial milk are regulated by the suckling offspring [Tyndale-Biscoe and Renfree, 1987]. Perhaps the cholecystokininergic system, regulated by CCK binding sites in the FMN, is acting in concert with changing milk composition to ensure that the opossum pup is getting the correct balance of liquid and nutrients, while, in rat pups, two different transmitter systems are necessary.

Mismatch

Although cholecystokinin binding sites are observed in both the neonatal Monodelphis and rat facial motor nucleus, and vasopressin binding sites are observed in the rat FMN as well, neither CCK nor AVP immunoreactive fibers are found in this region [Fox et al., 1991; Iqbal and Jacobson, 1995]. This phenomenon, known as mismatch, is common throughout the central nervous system [Herkenham, 1987]. Many reasons for peptide-binding site mismatch are apparent. These include technical factors, the possible distributions of neurotransmitter and receptors within a cell, and the biochemical properties of binding sites [Herkenham, 1987]. However, most of these factors appear to play a minor role in the mismatch dilemma. Current evidence suggests that binding sites are often nonsynaptically distributed, resulting in "parasynaptic" information flow [for review, see Herkenham, 1987]. In support of this evidence, we have found that immunoreactivity for synaptic vesicle proteins, classical markers of mature synapses, appear to be absent from the developing opossum FMN for the first two weeks of life [Swanson et al., 1993]. Transmitter action at a distance has been shown for multiple systems [Herkenham 1987]. In addition, there is now evidence that cerebrospinal fluid, which contains neuropeptides, may be moved between interstitial and ventricular compartments by bulk flow and active pumping mechanisms, rather than simple diffusion [Herkenham, 1987]. If this is the case in the neonatal brain, then the peptides could be available for their binding sites in the FMN. This would also place the regulation of the transmitter system at the receptor level.

Another interesting site of peptide-receptor mismatch is the cerebellum. Both cholecystokinin and vasopressin binding sites are found in the molecular layer of the adult
and developing opossum. However, neither CCK nor AVP immunoreactivity has been reported for this area, either in cells or fibers, except for the transient existence of AVP immunoreactive fibers in the 3 PN Brazilian opossum [Iqbal and Jacobson, 1995]. Therefore, volume transmission of these peptides likely occurs in the opossum cerebellum. In contrast to our findings in the opossum, neither binding site type has been found in the rat cerebellum, although CCK binding sites have been reported in cerebellar regions (granule cell layer or molecular layer) in other mammalian species, including humans [Niehoff, 1989; Sekiguchi et al., 1986; Vanderhaegen et al., 1989; Zarbin et al., 1983]. In addition, AVP receptor mRNA has been localized to the molecular and granule cell layers of the rat cerebellum [Szot et al., 1994], although binding sites have not been detected. The apparent species difference in this region could be due to lifestyle demands for balance and coordination, as the Brazilian opossum is arboreal.

Conclusion

In conclusion, we now know that cholecystokinin binding sites are transiently expressed in the facial motor nucleus of the Brazilian opossum and rat during early postnatal development. In addition, vasopressin binding sites and other binding sites, including estrogen receptors, are expressed transiently in the same time period in the rat. Because the demands on neonatal mammalian physiology and survival do not parallel that of the adult, and also due to the extreme fetus-like existence of the neonatal opossum, it is tempting to relate the transient modulation of these transmitter systems to the regulation of homeostasis. Although we have little data at this time to support this theory, it seems reasonable that transient receptor systems in the FMN may play a role in food intake and/or water regulation. Another logical possibility is that these receptor systems play a role in brain development itself. This argument could be supported by the facts that a) the cholecystokininergic system is present early in the forming brain; b) many receptor types are involved; and c) binding sites appear prior to synaptic input in the FMN. In addition, research on Brattleboro rats has shown that vasopressin plays an apparent role in brain development [Boer et al., 1980, 1993; Boer 1985; Snijdewint et al., 1988]. However, the reason that multiple receptor types are expressed in the FMN of the rat, versus one (so far)
in the opossum, is unclear, as more development is presumably occurring in the postnatal opossum than in the postnatal rat. Further studies need to be done to explore this theory. These include elucidating the timing of synaptogenesis in the developing rat FMN, and examining prenatal opossums for binding site expression, as well as exploring how manipulations of the system affect development.

We have shown that much remains to be learned about the facial motor nucleus during mammalian development, although it is considered a classical cranial nerve motor nucleus, with well-known afferents, efferents, and effects. The transient expression of multiple receptor types in at least two developing mammals, plus the apparent delayed synaptogenesis to this nucleus, leaves many questions to be answered. Future studies in our laboratory are planned to address many of these questions.
REFERENCES


APPENDIX 1. MONODELPHIS DOMESTICA, THE GRAY SHORT-TAILED OPOSSUM: A NOVEL MODEL FOR MAMMALIAN DEVELOPMENT

A paper accepted by Lab Animal

M.C. Kuehl-Kovarik, D.S. Sakaguchi, J. Iqbal, I. Sonea, and C.D. Jacobson

The Brazilian gray short-tailed opossum, Monodelphis domestica, is a small marsupial from South America. About the size of a hamster, the females weigh in at approximately 100 grams at puberty, while the males can be twice as large. Similar to other marsupials, Brazilian opossum pups are born in an extremely immature state after a short gestational period. Unlike many other marsupials, Brazilian opossums are pouchless. The pups are exposed on the mother's ventrum, allowing easy access for manipulation (Fig. 1A). In addition, Brazilian opossums are relatively docile when handled in the laboratory. Thus, the Brazilian opossum is an excellent experimental model for studies of mammalian development.

Biology, Care and Breeding

In our experience, Brazilian opossums have a lifespan of approximately three years in captivity. The young are born after fourteen to fifteen days of gestation and crawl up to the mother's nipples. Up to thirteen pups can be delivered; however, we have observed as few as two. Newborn pups, approximately one centimeter long, are essentially "living tubes". They have developed forelimbs, but their hindlimbs are still paddle-like. Many internal structures are still developing (Fig. 2). Neuronal birth and central nervous system development are in an extremely dynamic state for approximately two weeks after birth. Pups cannot be sexually characterized by gross observation for at least ten days postpartum, although histologically they can be differentiated earlier.¹
Postnatal development is greatly prolonged as compared to that of placental mammals. Pups remain physically attached to the female's nipples for two weeks, a period during which they undergo a dramatic increase in size, as well as morphological changes. After this time, the pups, which are beginning to grow fur, can come and go from the nipple, but they are absolutely reliant on the mother for survival. The pups' eyes do not open until they are one month of postnatal age (Fig. 1B). During the next month, the pups take on the physical and behavioral aspects of adult animals. The pups are weaned at approximately sixty days of age. However, we do not separate male and female siblings until they are about four months old, as puberty occurs at five to six months of age. Females remain anestrous until exposed to a unfamiliar male's pheromones.

Although they do have some special needs, adult Brazilian opossums are easily maintained at a research facility, and many breeding colonies have been established throughout North America and Europe. At our laboratory animal facility, males and females are housed singly in standard plastic rat cages on fine wood shavings. The animals are provided with plastic huts for nest building. The ambient temperature must be maintained at 26 °C, with a humidity of approximately 80%. Animals are kept on a 14:10 hour light:dark cycle. All animals are maintained on a diet of Reproduction Fox Chow (Milk Specialties Products, Madison, WI) provided ad libitum, and fresh fruit is provided three times a week. Nursing females are supplemented with bran cereal to increase their fiber intake. Water is available at all times.

Females in our colony are bred at five months of age or older. Following our standard breeding protocol, a female and male opossum are housed together for fourteen days. The female usually goes into estrus four to nine days after pairing, and will remain in estrus for less than 24 hours. After the two week pairing period, females are housed singly and checked daily for the presence of pups. If the female bears offspring, the day of birth is denoted as postnatal day 1 (1 PN). If the female does not have pups within a two week time period after separation from the male, she is rebred with a different male. Pups remain with the dams for sixty days. After they are weaned, the female is rested for one month, then rebred. Our litter success rate (# of litters/# of breedings) is at least 50%, and
is fairly consistent year-round. Females will occasionally consume their offspring, especially during the first two weeks after birth. However, this appears to be correlated more with small litter size than with manipulation. Even though we routinely disturb the females and their pups, we have consistently large numbers of juveniles reaching adulthood.

The Brazilian Opossum as a Developmental Model

Brazilian opossum pups are born in a state of immaturity approximately paralleling that of an embryonic day 13 rat. Thus, we have access to a fetus-like mammal while sparing the expense and manipulation of a caesarian section. In addition, we can utilize littermates at different time points, because the use of one pup does not imply the sacrifice of the entire litter. The Brazilian opossum can be used to study the early development of multiple organ systems. Our laboratories utilize *Monodelphis* to study aspects of central nervous system development.

Unlike many other organ systems, the proliferation of cells in the nervous system occurs for only a short period of time in an animal’s life. Once a neuron is born, it can no longer undergo mitosis to form new neurons. When neuronal proliferation (neurogenesis) ceases, the central nervous system has its complete complement of neurons. Considerable rearrangements within the nervous system will occur throughout the animal’s life. New synaptic connections are formed while others disappear, and many neurons will die as well, but, in general, new neurons will not be formed. The development of the central nervous system is a series of extremely complex events that must be highly regulated. Unfortunately for researchers, neurogenesis, including many of the organizational and regulatory events involved, occurs prenatally in placental animals. However, marsupials are born well before the end of neurogenesis. Immunohistochemically, we have been able to date the birth of neurons in the neonatal Brazilian opossum. Neuronal proliferation and subsequent differentiation in the Brazilian opossum brain is active for up to two weeks after birth (Fig. 3). Thus, the Brazilian opossum is a unique, accessible animal model for studying developmental events in the central nervous system.
Neuropeptide development

Many studies in our laboratory have focused on the development of peptide neurotransmitter systems in the forming brain that are involved in the regulation of feeding. We have established that, for many systems, the patterns of neuropeptide synthesis and corresponding receptor expression are similar between adult opossums and other, more standard laboratory mammals. Thus, we feel that the Brazilian opossum is an excellent model to study general mammalian development. We have found that neuropeptides associated with food intake regulation are present very early in the postnatal Brazilian opossum brain, where they may be playing a role in the regulation of feeding or other homeostatic functions. In addition, some neuropeptides are present in regions that are still undergoing neurogenesis, suggesting that these peptides may play a role in neuronal differentiation, migration, or synaptogenesis. Peptides involved in water balance regulation are present prior to birth, indicating an important role in early neonatal development.

Steroid receptor development

Another focus of study in our laboratory is the development of steroid hormone receptor systems in the brain. Steroid hormones play a key role in the organization of the embryonic central nervous system, ultimately determining whether the brain will be "male" or "female". In addition, steroids influence many cellular functions, including neuropeptide synthesis. We have examined the possible effect of steroids on the development of systems involved in food intake. We are currently investigating the developmental patterns of androgen receptor expression and concurrent hormonal influences on steroid receptor systems.

The Monodelphis visual system

The visual system has been particularly attractive for experimental analysis because it is a relatively accessible sensory system that can be naturally divided between the eye and brain, connected only by the cable-like optic nerve. The sensory retina, which lines the back of the eye, has a well defined, highly ordered anatomical organization. In addition, the retina is composed of only a few basic classes of cells. Ganglion cells, the only retinal neurons which establish connections with the brain, are among the first postmitotic cells to
be generated in the retina. In placental mammals, the vast majority of ganglion cells are produced during fetal development. In Monodelphis, however, although some ganglion cells are produced prenatally, the majority of retinal histogenesis occurs postnatally (Fig. 3). The postnatal neurogenesis of the retina in this species provides a unique opportunity to study the neuroanatomical development of this sensory organ.

The visual system also provides a dramatic example of the precise connectivity required within a functioning nervous system. How this precision in connectivity is established during development is an extremely important biological issue. During early development the presumptive eye differentiates into a highly organized structure. The ganglion cells elaborate axonal processes that will navigate a pathway from the eye to the primary visual centers in the brain. What are the molecular mechanisms used by the growing nerve processes in their journey to their final targets? The mechanisms which are involved in the precise patterning of the mammalian visual system have remained elusive, due largely to the fact that in placental mammals these initial events all occur prenatally. In Monodelphis however, the majority of the development of the visual system, from neurogenesis of the retina to the invasion of the primary visual centers by optic axons, all takes place after birth. Therefore, the visual system of the Brazilian opossum is proving to be a particularly useful experimental preparation for studying the development of the mammalian visual system, both in vivo and in vitro.

Unique concerns

Although it is an excellent model for general mammalian development, the Brazilian opossum faces a unique developmental dilemma. Many autonomic and homeostatic functions are regulated through a "control center" in the forebrain, the hypothalamus. However, the hypothalamus of the opossum is still forming at birth. Thus, the Brazilian opossum must have other regulatory circuits to survive. We are pursuing this issue, focusing on the regulation of food intake, and now have evidence that certain regions of the hindbrain may temporarily act as "control centers" until the forebrain can take over these critical regulatory functions. We are currently pursuing the question of how peptides may control the development of these novel hindbrain centers.
Further manipulations

The study of the newborn Brazilian opossum has provided numerous important new insights into many aspects of central nervous system development. However, we would like to study the animals even earlier in embryonic development. To this end, we are now able to establish the time of conception through observed matings. Our timed pregnancy protocol is a modification of protocols reported by other laboratories^4^, based on the fact that females will remain anestrous until exposed to a male. To induce a mating, a male and female opossum are paired for two to three days, then placed on either side of a perforated plexiglass partition for one to two days. The partition is removed in the late afternoon of the fourth day, and the animals are videotaped for any breeding activity (one to two hours). If mating has not occurred, animals can be separated and once again allowed contact at approximately twelve hour intervals. Mating should occur on the sixth to eighth day after pairing, and will usually take place within twenty minutes of removal of the partition. With this protocol, the exact time of mating can be established, and embryos of a very specific age can be collected (Fig. 4).

In addition, we have recently developed a protocol for diagnosing pregnancy by ultrasound. Once we have a confirmed mating on videotape, we ultrasound the female on gestational day twelve (day of breeding = G0). For the procedure, females are lightly anesthetized with isoflurane. If the female is pregnant, a thick-walled, fluid-filled uterus is evident in the ultrasound image^5^4. Combining the techniques of timed pregnancies and ultrasound, we are now able to manipulate the female at a specific gestational state and feel confident that we are affecting the fetuses. We are currently injecting pregnant females with a marker of cell birth at a known gestational age, allowing us to date the birth of neurons in the offspring even earlier than before.

Summary

The Brazilian opossum, *Monodelphis domestica*, is a unique model for studying mammalian development. Its relative ease of care and manipulation, combined with the production of fetus-like embryos at birth, makes the Brazilian opossum an excellent tool for
developmental research. This is especially important in the study of central nervous system development, as neurogenesis is a prenatal event in placental mammals. We have utilized the Brazilian opossum to study many aspects of neuropeptide and steroid hormone receptor development, and their actions in the forming brain. From our initial studies of the early postnatal development of the optic pathway it has become very clear that Monodelphis will provide a unique system to begin addressing the molecular basis of axonal pathfinding in the mammalian visual system. In addition, we are currently investigating how the neonatal opossum brain is organized to cope with its unique existence. We believe that, in the near future, many researchers will find Monodelphis a useful model for their developmental studies.

BIBLIOGRAPHY


FIGURE 1. Photographs of the Brazilian opossum, *Monodelphis domestica*. 1A. Newborn opossum pups attached to the mother’s nipples. Absence of the pouch makes these pups very accessible for developmental studies. 1B. A mother Brazilian opossum with one month old pups. At this age, the youngsters’ eyes have just opened.
FIGURE 2. Low-power photomicrograph of a sagittal section through a three day old Brazilian opossum body. Note the primordial gonad (G) and the developing kidney (K) and adrenal gland (A). Also evident are the lungs, liver, and gut.
FIGURE 3. High-power photomicrograph of a coronal section through the head of a twelve day postnatal Brazilian opossum pup. The dark staining indicates neurons that have just been born. Note the active neurogenesis in the brain (long arrow) and in the retina of the eye (short arrows).
FIGURE 4. Photograph of embryonic day thirteen Brazilian opossum embryos. Note the placental attachment at this developmental stage (arrow). This attachment apparently exists for only one day.
APPENDIX 2. LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tbody>
<tr>
<td>AA</td>
<td>amygdaloid area</td>
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<tr>
<td>ACb</td>
<td>nucleus accumbens</td>
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<td>ACo</td>
<td>anterior cortical amygdaloid nucleus</td>
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<td>Amb</td>
<td>ambigiuus nucleus</td>
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<td>AP</td>
<td>area postrema</td>
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<td>APt</td>
<td>anterior pituitary</td>
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<td>Aq</td>
<td>cerebral aqueduct</td>
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<td>AVP</td>
<td>arginine vasopressin</td>
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<td>BAA</td>
<td>basal amygdaloid nucleus, accessory division</td>
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<td>BAM</td>
<td>basal amygdaloid nucleus, magnocellular division</td>
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<tr>
<td>BAP</td>
<td>basal amygdaloid nucleus, parvocellular division</td>
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<tr>
<td>BSTM</td>
<td>bed nucleus of the stria terminalis, medial division</td>
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<tr>
<td>BSTV</td>
<td>bed nucleus of the stria terminalis, ventral division</td>
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<tr>
<td>CA</td>
<td>central amygdaloid nucleus</td>
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<tr>
<td>CA1</td>
<td>CA1 field of Ammon’s cortex</td>
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<td>CA2</td>
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<td>CA3</td>
<td>CA3 field of Ammon’s cortex</td>
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<tr>
<td>Cb</td>
<td>cerebellum</td>
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<td>CCK</td>
<td>cholecystokinin</td>
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<td>central grey</td>
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<td>choroid plexus</td>
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<td>central nervous system</td>
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<td>Cor</td>
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<td>Abbreviation</td>
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<tr>
<td>cp</td>
<td>cerebral peduncle</td>
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<td>central tegmental tract</td>
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<td>DB</td>
<td>nuclei of the diagonal band</td>
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<td>DCo</td>
<td>dorsal cochlear nucleus</td>
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<td>DG</td>
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<td>DLG</td>
<td>dorsal lateral geniculate nucleus</td>
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<td>DR</td>
<td>dorsal raphe nucleus</td>
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<td>ET</td>
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<td>fi</td>
<td>fimbria of the hippocampus</td>
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<td>FMN</td>
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<td>interpeduncular nucleus</td>
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<td>Description</td>
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<td>IPL</td>
<td>interpeduncular nucleus, lateral subnucleus</td>
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<td>IRt</td>
<td>intermediate reticular nucleus</td>
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<td>LRt</td>
<td>lateral reticular nucleus</td>
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<td>LSD</td>
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<tr>
<td>LSI</td>
<td>lateral septal nucleus, intermediate part</td>
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<tr>
<td>LSV</td>
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<td>LVe</td>
<td>lateral vestibular nucleus</td>
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<td>LVP</td>
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<td>posterior amygdaloid nucleus</td>
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<td>PB</td>
<td>parabrachial nucleus</td>
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PCo posterior cortical amygdaloid nucleus
PCRt parvocellular reticular nucleus
Pe periventricular hypothalamic nucleus
PF postfertilization day
Pir piriform cortex
PMD premammillary nucleus, dorsal part
PMV premammillary nucleus, ventral part
PN postnatal day
Pn pontine nuclei
PNRA pontine reticular area
PrH prepositus hypoglossal nucleus
PrS presubiculum
PV paraventricular thalamic nucleus
PVP paraventricular thalamic nucleus, posterior part
py pyramidal tract
P7 perifacial zone
R red nucleus
Re reuniens thalamic nucleus
RF rhinal fissure
Rh rhomboid thalamic nucleus
ROb raphe obscurus
rs rubrospinal tract
Rt reticular thalamic nucleus
S subiculum
SC superior colliculus
SCh suprachiasmatic nucleus
sm stria medullaris of the thalamus
SN substantia nigra
SNA substantia nigral area
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<td>supraventricular nucleus</td>
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