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RESEARCH ARTICLE

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IMMUNOHISTOCHEMICAL IDENTIFICATION OF ARGININE VASOPRESSIN CONTAINING NEURONS AND FIBRES IN SOME HYPOTHALAMIC NUCLEI OF THE NEONATAL MOUSE

ABSTRACT:
Arginine vasopressin (AVP) is a neuropeptide synthesized in neurons of the mammalian brain and released via the posterior pituitary into the bloodstream where it influences the maintenance of fluid homeostasis and blood pressure. Cellular identification and distribution of AVP in the mouse hypothalamic nuclei before vision functioning has never been reported before. The distribution of AVP producing cells and their projections were examined in the hypothalamus and in the hypothalamic nuclei of the neonatal CD1 mouse using immunohistochemistry (immunoperoxidase and immunofluorescent labelling methods). Clusters of neurons labelled for AVP immunoreactivity (AVP-ir) were found in the hypothalamic nuclei as in the paraventricular (PVN), supraoptic (SON) and suprachiasmatic nuclei (SCN) of the hypothalamus. Scattered AVP producing cells were also found in the lateral hypothalamus, fornix, stria terminalis and preoptic area. Heavily stained AVP-ir fibres were found in the paraventriculo-supraoptico-neurohypophyseal tract while scattered AVP-ir fibres were found in the lateral hypothalamus and in the stria terminalis. The bed nucleus of the stria terminalis (BNST) contains more AVP-ir cells in the neonatals than in adult mice.

Key words:
Arginine vasopressin, immunohistochemistry, mice, hypothalamus, suprachiasmatic nucleus, paraventricular nucleus, preoptic nucleus.

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INTRODUCTION:

The hypothalamus is a collection of small nuclei in the diencephalon that lies just below the thalamus at the centre of the brain. It is said that the hypothalamus is the brain of the brain. It governs reproductive, homeostatic and circadian functions. The hypothalamus integrates autonomic and endocrine functions with behaviour. During the past decades, experimental evidence from numerous laboratories has led to the generally accepted view that neurons in the mammalian central nervous system, including the hypothalamus, synthesize a wide variety of neuropeptides that are used as messengers for intercellular communication. Arginine vasopressin (AVP) is one of these neuropeptides that is synthesized in magnocellular or parvocellular neurons (Wang et al., 1997, 2000). Magnocellular cell bodies, located mainly in the supraoptic and lateral paraventricular nuclei, synthesize the AVP released from the neural lobe of the hypophysis to the systemic circulation to regulate the extracellular fluid balance. On the other hand, parvocellular neurons are dispersed in a number of regions like the suprachiasmatic nucleus, the main cerebral circadian clock, and the medial PVN, and take part in the control of the corticotropic axis and multiple vegetative functions (Vacher et al., 2002).

AVP is also released into target areas within the central nervous system where it acts as a neurotransmitter or neuromodulator to regulate physiological and behavioural functions (Koolhaas et al., 1991; Johnson et al., 1993; Ferris, et al., 1995, 1997; Meredith and Marler, 2003; Tousson and Meissl, 2003; Vacher et al., 2003). Neural pathways for AVP differ markedly across mammalian species. For example, a dense cluster of AVP-ir fibres in the lateral septum was found in the rodents (Wang et al., 1996) but not in the monkey (Caffe et al., 1989) or human brain (Romijn et al., 1999). In voles, the distribution of brain AVP receptors shows a species-specific pattern associated with social organization and behaviour (Insel et al., 1994).

The aim of this study was to investigate, with the aid of a recently developed

immunoperoxidase and immunofluorescence techniques, cellular identification and distribution of arginine vasopressin immunoreactivity in the hypothalamic nuclei of mouse pups. Our goal was to systematically map the AVP system in the infant mice hypothalamic nuclei and compare results with those of previous studies in adult mouse and other rodents for further understanding of the evolution of the brain AVP system. A comprehensive picture of the central AVP system will provide important information for further studies of the functional significance of AVP. Together, these data provide a comprehensive picture of AVP pathways in the pup's brain, demonstrating differences from adult mouse in the distribution of cell bodies, and fibres.

MATERIALS AND METHODS:

The animals used in this study were male CD1 mouse (20 mouse of ages 6-8 days before vision), the mice were bred in the facilities of Max-Plank-Institute for Brain Research in Frankfurt, Germany under controlled conditions (12h:12h light – dark cycle, light on at 6:00 a.m) with about 60% humidity and 25°C temperature. Pups were taken from the animal house and killed by decapitation, the skulls were opened with fine scissors and the brains were quickly removed under sterile conditions into ice-cold artificial cerebrospinal fluid (124 mM NaCl, 5 mM KCl, 1.25 mM KH_2PO_4, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, 2.2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES). Brains were excised and fixed in 4% paraformaldhyde in phosphate buffered saline (0.1M PBS, pH 7.4) for 24 hours at 4°C and then cryoprotected in 30% sucrose in PBS at 4°C for 72 hours. Brains were sectioned with a freezing microtome at the level of the hypothalamus into 25 µm coronal sections and mounted on gelatine-coated slides and stored at -20°C prior to processing for ariginine vasopressin immunohistochemistry. Some sections were processed for the histological studies by cresyl violet (Nissl) staining (Adolph, 2002) and other sections were processed for the immunocytochemical studies.

Immunohistochemistry:

The hypothalamic brain sections were processed for AVP-ir immunoperoxidase or /and immunofluorescent labelling methods.

Immunoperoxidase labelling:

Sections were blocked for 1 hour (at room temperature) in a solution containing 10% normal goat serum (NGS) and 1% Triton- X-100 in PBS to block non- specific binding sites. These were then incubated with a rabbit polyclonal antibody direct against AVP (dilution 1:1000) in PBS containing 0.5% Triton- X-100 overnight at room temperature. Hypothalamic brain sections were then washed in PBS for 45 minutes (3X15 minutes) and then incubated for 12 hours in biotinylated goat anti-rabbit for AVP-ir with 0.5% Triton-X-100 at 4°C, rinsed in PBS, and subsequently incubated in ABC (Vector Labs) in PBS for 12 hours at 4°C and rinsed for 15 minutes (3X 5 minutes) in PBS. Treated sections were incubated in 0.5% 3, 3`-diamino-benzidine (DAB) in PBS with 0.03% H2O2 for 5 minutes. The reaction of DAB was stopped by rinsing for 10 minutes (2X 5 minutes) in PBS, then the treated sections were air-dried, dehydrated in a graded ethanol series (60-100%), delipidated in xylene, and cover-slipped with Mount-Quick (Daido Sangyo, Tokyo).

Immunofluorescence method:

Hypothalamic sections were blocked for 1 hour in a solution containing 10% NGS, 1% bovine serum albumin (BSA) and 0.5% Triton-X-100 in PBS. Sections were incubated in primary antibody diluent (3% NGS, 1% BSA and 0.5% Triton-X-100 in PBS) prior to incubation in rabbit anti-AVP (1:800) overnight at room temperature. After treatment with primary antibody, sections were rinsed in 0.1M PBS and then incubated for 1 hour (in dark room) in a goat anti-rabbit as secondary antibody (1:500). Then they were rinsed for 10 minutes in PBS and mounted and cover slipped with vectashield antifading mounting medium and examined under epifluorescence for AVP immunoreactivity. All fluorescent specimens were viewed by using a Leica TCS fluorescence microscope. Brightness and contrast of the images were adjusted using Adobe Photoshop software.

Abbreviations:

SCN, suprachiasmatic nucleus; PVN, paraventricular nucleus; SON, supra optic nucleus; 3V, 3rd ventricle; OC, optic chiasm; LH, lateral hypothalamus; AVP, arginine vasopressin; -ir, immunoreactive; AVP-ir, arginine vasopressin immunoreactivity; BNST, bed nucleus of stria terminalis; Fx, fornix; MPA, medial preoptic area; ARN, arcuate nucleus; DMH, dorsomedial hypothalamic nucleus; ST, stria terminalis; AH, anterior hypothalamus.

RESULTS:

General observations and hypothalamic nuclei cytoarchitecture:

The heterogeneity of cell types within most hypothalamic nuclei and areas has been emphasized with the consequent implications for heterogeneity of neuronal connections and functions. The hypothalamus can be seen in a coronal section of Nissl stained tissue (Figs. 1-8). The hypothalamus consists of a collection of nuclei and areas located at the base of the brain, ventral to the subthalamus. Along most of its length, the lateral border is formed by the fibres of the internal capsule. The preoptic region or anterior region of the hypothalamus is bounded by the basal forebrain.

The posterior hypothalamic region is continuous with the midbrain. The arcuate region is next to the posterior region. The hypothalamus is connected anatomically to both the forebrain and brain stem. The cell density is higher in the anterior region of hypothalamus, which contains numerous distinct nuclear structures while the lateral parts, in contrast, have fewer cells and contain more fibres. The important landmarks of the hypothalamic region are the optic chiasm (OC) and the median eminence.

The hypothalamus and the hypothalamic nuclei:

Hypothalamic nuclei refer to well-defined cell groups and can usually be localized by coronal sections of Nissl stained tissue. Hypothalamic areas are a more diffusely defined heterogeneous collection of cells and fibres that are not separated from each other by distinct boundaries. In the anterior division there are three important hypothalamic nuclei (SCN, PVN and SON), while in the arcuate (Fig. 8) and posterior regions there are other two hypothalamic nuclei (ARN and DMH). The majority of the hypothalamic nuclei are located medially. An important exception is the SON, which is both lateral and anterior (Figs. 5, 6).

The suprachiasmatic nucleus (SCN) is a dense accumulation of small neurons (7-9 µm in diameter) lying dorsal to the optic chiasm and lateral to the third ventricle (Figs. 3, 5, 9). The dorsal, lateral, rostral and caudal boundaries of SCN can be determined in Nissl stained material because of the greater packing density of cells in SCN relative to the surrounding other parts of the hypothalamus. Due to the small size and tight packing density of SCN cells (Fig. 2), it is difficult to ascertain the fine morphological characteristics of individual neurons in Nissl stained material. The PVN consists of paired nuclei located on either side of the midline just dorsal to SCN, and extending into the end of the 3rd ventricle (Figs.4, 9). PVN contains two types of cell clustered within the nucleus, the parvocellular cells (small cells) and the magnocellular cells (larger cells). The large, bipolar cells of the PVN, surrounding anterior hypothalamus and retrochiasmatic area are generally restricted to the periphery of the PVN.

The supraoptic nucleus (SON) is important because of its link to the posterior lobe of the pituitary. SON is a nucleus of magnocellular neurosecretory cells in the hypothalamus, their axons extend into posterior pituitary. It is a dense accumulation of large neurons (9 -10µm in diameter) lying lateral to the optic chiasm (Fig. 5). The dorsomedial hypothalamic nucleus (DMH) consists of paired nuclei located on either side of the 3rd ventricle just ventral to arcuate nucleus (Fig. 7). The arcuate nucleus (ARN) is a collection of neurons in the hypothalamus of the brain. The nucleus is located in the middle hypothalamus in the most ventral part of the third ventricle near the entrance of the infundibular recess (Fig. 8). Its small neurons (7-8µm in diameter) are in close contact with the ependyma.

AVP immunoreactivity neurons:

Numerous intensely labelled AVP-immuno-reactive neurons and fibres were distributed in a characteristic pattern throughout the hypothalamus coronal sections (Figs. 10-22). The septopreoptic - hypothalamic area contained several discrete clusters of AVP-ir structures (cells and fibres). Cell bodies and fibres containing AVP immunoreactivity were present within the PVN and in the SON (Figs. 12, 19). A dense cluster of AVP-ir neurons were found in a paraventricular position, lining the ependymal wall of the third ventricle, close to the pial surface of the preoptic area and in the PVN (Figs. 10-13). They were also seen more laterally in the lateral and dorsal thalamic areas. In addition, AVP-ir cells and fibres were observed in a discrete area located above and caudal to the anterior commissure (Figs. 18, 21), they have never been described in detail previously. Labelled structures were observed in the anterior preoptic nucleus, whereas scattered elements containing fluorescence were present in the very rostral portion of the SON (Figs. 19, 20, 22).

Moving caudally, a large number of labelled cells were observed within the boundaries of the dorsomedial hypothalamic nucleus (Fig.17). Labelling of vasopressinergic neurons outside the PVN and SON was associated mainly with the SCN and with scattered neurons in the sub-paraventricular zone (Figs. 12, 14). The distribution of AVP-ir neurons in the SCN delineated the middle region of the shell subdivision (Fig. 15). In rostral regions, AVP-IR cells were evenly distributed within the most shell portion of the SCN. In the middle and rostal regions, AVP-IR cells formed a cup around the dorsal half of the SCN. The highest concentration of vasopressin neurons was observed in the mediodorsal half of the central portion of the SCN. The immunoreactive somata were ovoid in shape (7-9 µm in diameter) and their processes possessed varicosities of different sizes (0.5-1.0 µm diameter). In the caudal third of the SCN, AVP-ir neurons were more homogeneously dispersed, and were morphologically similar to the cells in the rostral third of the nucleus. A moderate number of AVP producing cells was found in the dorsal anterior hypothalamus while a few AVP cells were also found in the fornix and in the pretectal nucleus of the hypothalamus (PT). Scattered AVP-ir cells were found in the bed nucleus of the stria terminalis (BNST) and in the area of lateral hypothalamus (Fig. 16). No AVP-ir cells were found in any aspects of the amygdala by either peroxidase or fluorescence technique. No AVP-ir structures could be detected in the ARN.

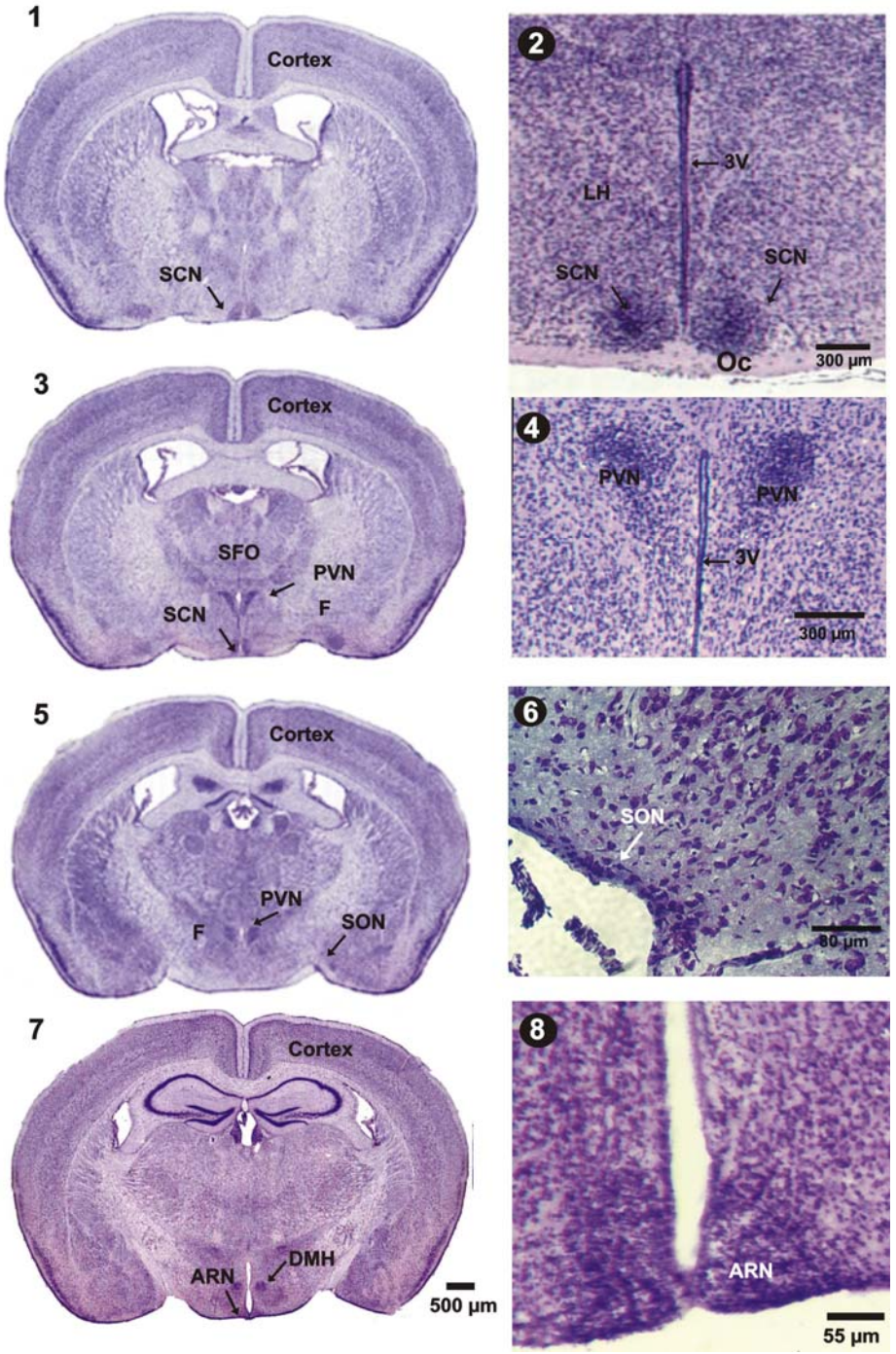
AVP immunoreactivity fibres:

Numerous intensely labelled AVP-ir fibres leave the SCN dorsally adjacent to the sub--paraventricular zone (Figs. 10 - 12), where many appear to terminate and to the ventral portion of PVN. Other AVP-ir fibres leave the SCN in a rostal direction along the midline to the preoptic area and other AVP-ir fibres extended from the SON to the PVN through the medial preoptic area (MPA). A lesser number of very fine fibres leave the lateral aspect of the SCN and can be followed for several hundred micrometres through the lateral hypothalamic area. Other AVP-ir fibres were observed in the SCN sometimes spanning the space of the third ventricle or extending ventrally into the area that had been occupied by the optic chiasm. Heavily stained AVP-ir fibres formed a paraventriculo-supraoptico-neurohypophyseal tract (Figs. 18, 19), as found in adult mouse. In addition, AVP-ir fibres were observed in the PVN and projected ventrolaterally to the preoptic area and dorsally to the PT with numerous fibres that extended to distribute in the rostral and medial portions of the PVN of the thalamus. AVP-ir fibres were also found between the SON and the lateral hypothalamus. Scattered AVP-ir fibres were found in the fornix and in the ST (Figs. 19, 21). Another small group of AVP +ve fibres observed caudally between the medial and lateral habenular nuclei. In the septum, a few AVP-ir fibres were found in the lateral area but these fibres did not form a dense plexus as found in adult mice.

DISCUSSION:

Cellular identification and distribution of AVP in the mouse hypothalamic nuclei before vision functioning has never been reported before. In the current study, we used immunohistochemistry to map the distribution of AVP immunoreactivity in the hypothalamus and hypothalamic nuclei of the neonatal CD1 mice. The distribution of AVP cells in the PVN, SON and SCN was similar to the pattern found in a variety of mammalian species such as rodents (Ibata et al., 1993; Romijn et al., 1997; Wang et al., 1997; Smale and Boverhof, 1999; Abrahamson and Moore, 2001; Moore et al., 2002; Vacher et al., 2003; Rao and Kanwa, 2004) and humans (Romijn et al., 1999); these support the assumption that hypothalamic AVP is highly conserved among mammalian species. Labelling for AVP by a two methods (Immunofluorescence and immunoperoxidase) confirmed the presence of AVP-ir elements in some of the hypothalamic nuclei of the neonatal CD1 mouse brain (SCN, SON, PVN, DMH and BNST) but failed to reveal any immunoreactive cell in the arcuate nucleus. This result, although surprising at first sight, is easily explained by the facts that the immunofluorescence staining is clearly less sensitive than the immunoperoxidase technique and the density of AVP immunoreactive material in the cells of the BNST/ is definitely lower than that in the magnocellular neurons. The same differential visualization has in fact already been reported when AVP-ir was detected with immunofluorescence labelling; no immunoreactive neurons were in this way detected in the BNST while they were quite abundant in the SON and PVN. Similar to other rodents, AVP-positive cells were found in hypothalamic nuclei as well as in the bed nucleus of the stria terminalis and the medial nucleus of the amygdala (Wang et al., 1996). Some species differences have been observed. For example, prairie voles have fewer AVP-positive cells in the DMH and BNST, but a higher density of AVP-ir fibres in the FX, than do non-monogamous voles (Wang 1995; Wang *et al*., 1996).

The present data indicate at least three differences in the pattern of AVP cells and fibres in the neonatal CD1 mice in comparison to adult mouse and / or in other rodents. First, AVP cells were not detected in any aspect of the amygdala in the neonatal CD1 mice brain. In adult, AVP mRNA-labelled or AVP-ir cells are found in the medial nucleus of the amygdala; and these cells project into the forebrain areas such as the lateral septum (Caffe and van Leeuwen, 1987; Wang *et al*., 1995, 1997; Moore *et al*., 2002). Although an early study reported AVP-ir cells in the medial amygdala in the macaque monkey (Caffe *et al*., 1989), the number of these cells was far less compared to rats, which may coincide with the absence of AVP cells in the human amygdala (Romijn et al., 1999). Secondly, a plexus of AVP-ir fibres was not found in the lateral septum in the neonatal CD1 mice. In contrast, the lateral septum in rat receives projections from AVP cells in the medial amygdala and the BNST (Barberis and Tribollet, 1997; Wang *et al.*, 1995, 2000). The third difference between the neonatal CD1 mice and adult rodants was noticed on the immunovisualization of AVP cells in the BNST. In adult CD1 mice, these cells usually cannot be reliably visualized by immunocytochemistry unless animals are treated with colchicine (Hermes *et al*., 1990; Wang et al., 2000). In the current study, the number of AVP-ir cells in the BST was similar to the number of AVP mRNA-labelled cells, suggesting that virtually all AVP cells in the BST were immunostained although the neonatal mouse was not treated with colchicine. In addition, the number of AVP-ir cells in the BST was not significantly increased by colchicine treatment in hamsters that possess large AVP cells in the BST (Ferris *et al.*, 1995).



Figs. 1-8: Coronal sections in the hypothalamus stained with cresyl violet showing the different hypothalamic nuclei in the CD1 mouse (ages 6-8 days).

Fig. 1: Photomicrograph showing the different hypothalamic areas. SCN can be clearly distinguished.

Fig. 2: High power micrograph stained with cresyl violet showing the bilateral SCN lying lateral to the third ventricle and dorsal to the optic chiasma.

Fig. 3: Photomicrograph showing two types of hypothalamic nuclei (SCN and PVN).

Fig. 4: High power micrograph showing the bilateral PVN.

Fig. 5: Photomicrograph showing two types of hypothalamic nuclei (PVN and SON).

Fig. 6: High power photomicrograph stained with cresyl violet showing SON.

Fig. 7: Photomicrograph showing two types of hypothalamic nuclei (DMH and ARN).

Fig. 8: High power photomicrograph stained with cresyl violet showing ARN.

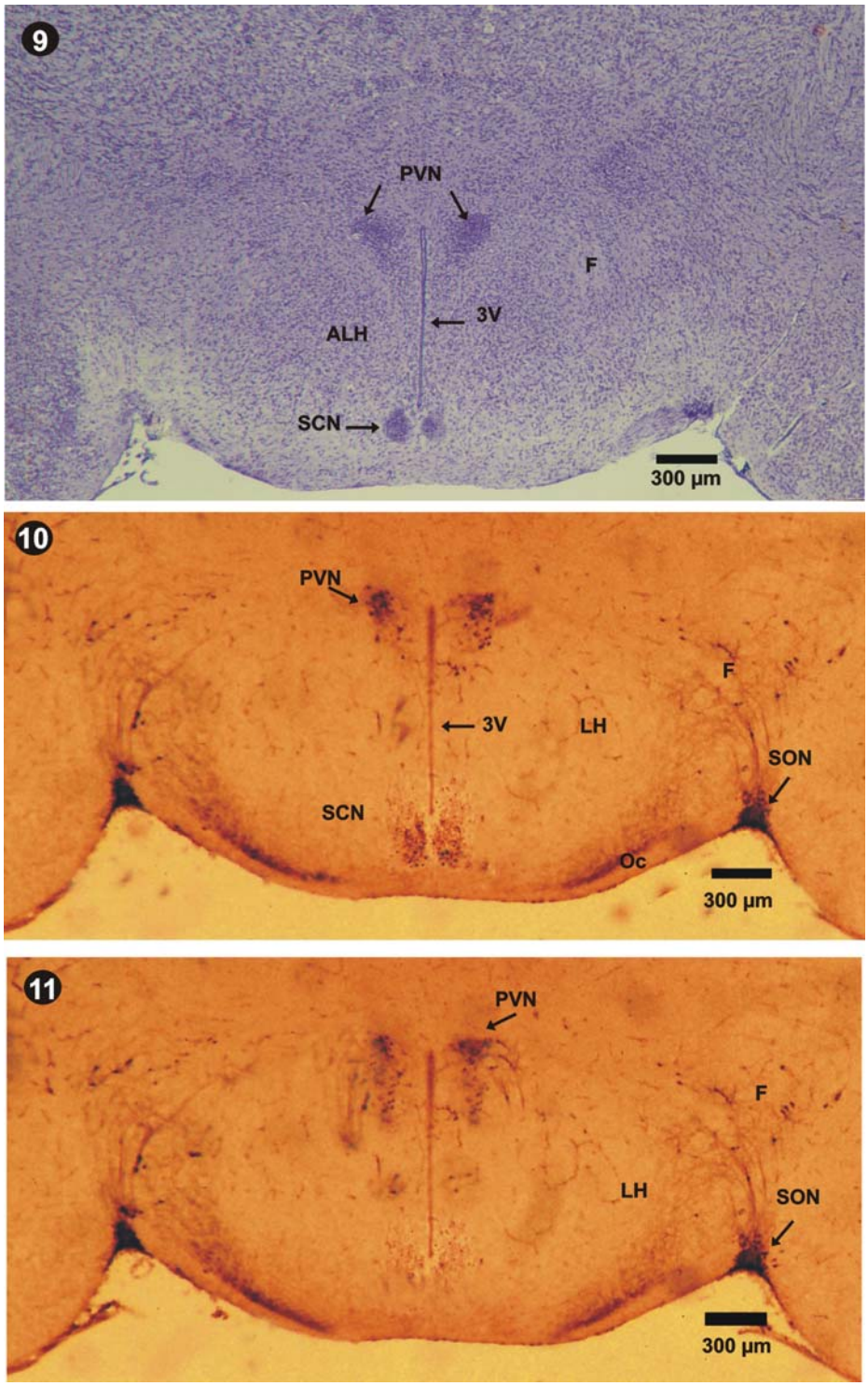


Fig. 9: Photomicrograph of coronal section in the hypothalamus stained with cresyl violet through the caudal SCN, the magnocellular neurons in the PVN can be seen above to SCN.

Figs.10-22: Photomicrographs AVP-ir (immunoperoxidase and immunofluorescence labelling) through in the hypothalamus coronal sections showing the different hypothalamic nuclei in the CD1 mouse (ages 6-8 days).

Figs. 10, 11: Low power micrographs of AVP-ir (immunoperoxidase labelling) through the caudal SCN. The magnocellular neurons in the PVN can be seen above to SCN. Thick AVP-ir axons from magnocellular neurons can be seen extending between the PVN and SON, and thin axons from paravecellular neurons can be seen dorsal to SCN, some of the latter extend from SCN into LH and sub- PVN zone.

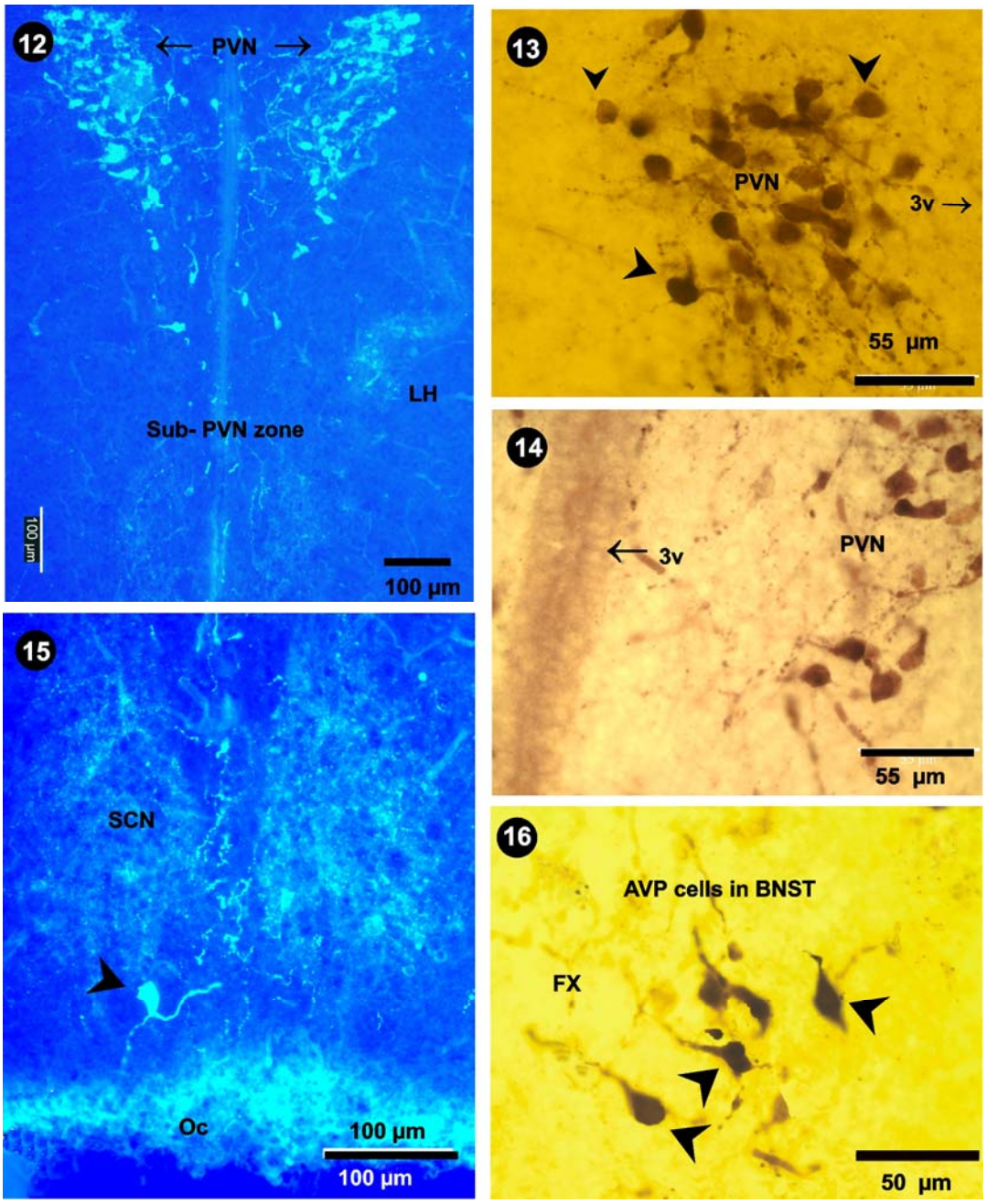


Fig. 12: Medium power micrograph of AVP-ir (immunofluorescence labelling) showing the bilateral PVN containing AVP immunoreactive cells and fibres. Many AVP-ir fibres in the sub-paraventricular zone can be clearly distinguished.

Figs. 13, 14: High power micrographs of AVP-ir (immunoperoxidase labelling) showing the AVP immunoreactive cells and axons in the PVN. Many processes close to the base of the third ventricle can be clearly distinguished.

Fig. 15: Medium power micrograph of AVP-ir (immunofluorescence labelling) showing the bilateral SCN containing AVP immunoreactive cells and fibres. Many AVP-ir can be clearly distinguished in the optic chiasm.

Fig. 16: High power micrograph (immunoperoxidase labelling) displaying AVP immunoreactive cells and fibres in the fornix and in the bed nucleus of stria terminalis.

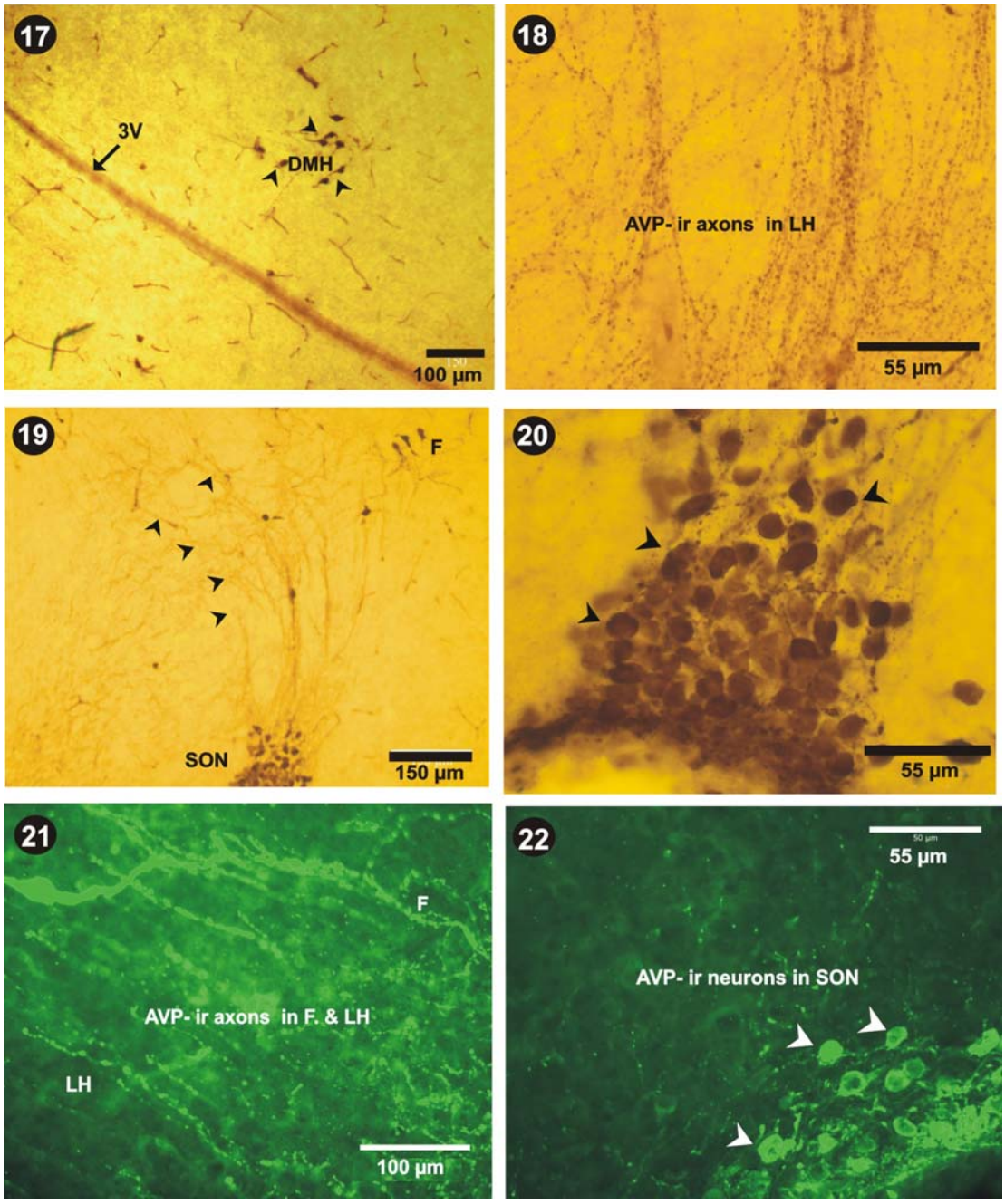


Fig. 17: Medium power micrograph of AVP-ir (immunoperoxidase labelling) showing positive AVP-ir cells and fibres in the dorsomedial hypothalamic nucleus.

Fig. 18: High power micrograph (immunoperoxidase labelling) displaying AVP immunoreactive fibres in the in the lateral hypothalamus.

Fig. 19: Low power micrograph of AVP-ir (immunoperoxidase labelling) showing positive AVP-ir cells and fibres in the SON.

Fig. 20: High power micrograph (immunoperoxidase labelling) displaying AVP immunoreactive cells in the SON.

Fig. 21: High power micrograph (immunofluorescence labelling) displaying AVP immunoreactive fibres in the in the fornix and in the lateral hypothalamus.

Fig. 22: High power micrograph (immunofluorescence labelling) displaying AVP immunoreactive cells and fibres in the SON.

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الكشف بالطرق الهستوكيميائيه المناعية عن الأرجنين القابض للأوعية الدموية في الخلايا والألياف العصبية لبعض الأنوية الموجودة في منطقة تحت المهاد لفئران حديثة الولادة

إيهاب مصطفى طوسون

قسم علم الحيوان ، كلية العلوم ، جامعة طنطا

يعتبر الأرجنين القابض للأوعية الدموية أحد أهم البيتيدات العصبيه التي تخلق داخل مخ الثدييات و التي تتدفق إلى تيار الدم عن طريق الفص الخلفي للغدة النخامية. تهدف الدراسة الحالية إلى الكشف بالطرق الهستوكيميائية المناعية عن الأرجنين القابض للأوعية الدموية في الخلايا والألياف العصبية في منطقة تحت المهاد للفئران حديثة الولادة. تم أخذ قطاعات في منطقة تحت المهاد من فئران ذات أعمار ما بين 3-5 أيام و تم تثبيتها بواسطة البارافورمالدهيد وتقطيعها بواسطة الكريوستات إلى قطاعات وقد أوضحت الدراسة الحالية أن الأرجنين القابض للأوعية الدموية يوجد في العديد من الأنوية الموجودة في منطقة تحت المهاد مثل النواة فوق الصليبية SCN و النواة فوق البصرية SON وكذلك النواة جانبية البطين PVN. كما أوضحت الدراسة وجود بعض الخلايا المبعثرة في منطقة تحت المهاد ذات قابلية للأرجنين القابض للأوعية الدموية مثل منطقة تحت المهاد الجانبي والفونكس ومنطقة ما قبل بصرية. وعلى مستوى الألياف العصبية وجد أن المنطقة التي تربط بين النواة جانبية البطين والنواة فوق البصرية غنية بالألياف ذات القابلية العالية للأرجنين القابض للأوعية الدموية بينما وجدت بعض الألياف المبعثرة في منطقة المهاد الجانبي والخط الطرفي. كما أوضحت الدراسة أن النواة السريرية للطرف الجانبي تحتوى على خلايا ذات قابلية عالية للأرجنين القابض للأوعية الدموية في الفئران الرضع عن الفئران البالغة.

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