Neurogenesis and neuronal migration in hypothalamic vasopressinergic magnocellular nuclei of the adult rat

Running title: Hypothalamic AVP magnocells' adult neurogenesis

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Abstract: In restricted mammalian brain regions, cell proliferation and migration continue postnatally and throughout adulthood. Here we show, using immunohistochemical reaction (ir) against arginine vasopressin (AVP), neurophysin II, glial fibrillar acidic protein (GFAP), cell division and early neuronal markers Ki67 and doublecortin (DCX) respectively, and neuroanatomical analysis on the rat brain in serial sections of coronal, sagittal, horizontal and septo-temporal oblique orientations from adult rats, that neurons from hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei are dispersed along visible tangential routes to other subcortical regions, guided by AVP-ir cell chains and axon scaffolds. Using 5′-bromo-2′-deoxyuridine (BrdU) injection in adult rats followed by BrdU-ir, we observed numerous twin-nuclei within SON and PVN, with some double-labeled for AVP. Chronic water deprivation significantly increased the BrdU+ nuclei within both SON and PVN. Immunofluorescent reaction showed double labeling of AVP/DCX within SON and PVN. Interestingly, NeuN, a mature neuron marker, was largely absent in SON and PVN, but present in neurons within adjacent hypothalamic regions. These findings provide evidence that adult neurogenesis and migration occur in hypothalamic vasopressinergic nuclei and reveal tangential migration routes of AVP-ir neuronal chains and ascending axonal scaffolds in adult rats.

Key words: hypothalamic neurogenic niche, radial glial cells, GFAP, BrdU, BNST, DCX

1. Introduction

Adult neurogenesis is defined as the ability of specialized cells in the postnatal brain to produce new functional neurons. This phenomenon was first formally reported six decades ago using intraperitoneal injection of thymidine-H^3 in rats and cats, that shed some light on the possibility that new neurons may be formed in forebrain structures in both rodents and carnivores, that was not considered possible previously (Altman 1962, Altman 1963, Altman and Das 1966). Today it is well established that the subventricular zone of the lateral ventricles and the subgranular zone of the hippocampus (Doetsch and Alvarez-Buylla 1996, Lois, Garcia-Verdugo et al. 1996, Zhao, Deng et al. 2008, Ming and Song 2011), are prominent regions of neurogenesis to occur thought lifespan in human (Zhou, Su et al. 2022), as well as the caudate nucleus and nucleus accumbens *septi* in the rat (Das and Altman 1970). Adult

neurogenesis has also been described to occur in striatum of human (Ernst, Alkass et al. 2014) and in hippocampal fissure region in rats under pilocarpine insult (Zhang, Hernandez et al. 2014). In the hypothalamus of several rodent and primate species, neurogenesis in adult animals has been reported (Kokoeva, Yin et al. 2005, Kokoeva, Yin et al. 2007, Migaud, Batailler et al. 2010, Cheng 2013, Yoo and Blackshaw 2018, Sharif, Fitzsimons et al. 2021, Yao, Taub et al. 2021). This new development has received renewed attention and enthusiasm as it may serve as a potential mechanism for brain physiological adaptation across the lifespan, with possible roles in coping with homeostatic adversities. However, the actual sites and organization of the neurogenic niches in hypothalamus remain elusive.

Hypothalamus hosts unique magnocellular neurosecretory neurons (MNN) which synthesize the neurohormones arginine vasopressin (AVP) and its homologue oxytocin (OT) (Bargmann and Scharrer 1951, Hatton 1990). Three loci of AVP-MNNs have been described, i.e., supraoptic (SON), paraventricular (PVN), and accessory (AN) nuclei (Ugrumov 2002). Most, if not all, of the AVP-MNN located within the SON, PVN and AN emit main axons carrying AVP to the pituitary gland, via the hypothalamic-neurohypophysial tract (Falke 1991), which exert control over body water and electrolyte homeostasis, blood pressure, and hepatic glucose metabolism (Sladek and Armstrong 1987, Cunningham and Sawchenko 1991, Ugrumov 2002, Armstrong 2004).

AVP-MNN hormonal regulation of these peripheral functions has been gradually established over the past century. However, a novel understanding of AVP-MNN involvement in higher brain functions, such as memory processes, emotion and motivation, complementary to their peripherical physiological role, has emerged in recent years (for reviews see (Zhang and Eiden 2019, Zhang, Hernández et al. 2021)).

During the past fifteen years, our group has carried out a series of systems studies on the AVP-MNN ascending projection system in adult and old rats (12-18 months), summarized in (Zhang, Hernández et al. 2021). Connections between hypothalamic AVP-MNNs and other brain areas involved in stress coping and emotional/motivational behaviors have been demonstrated, through the employment of techniques such as juxtacellular labeling and tract-tracing methods combined with brain serial sectioning in cutting planes at coronal, sagittal, septo-temporal oblique and horizontal orientations. Histochemical reaction against neurobiotin, immunohistochemistry (IHC) against antigens of interest and systematic neuroanatomical analyses were performed (Zhang and Hernandez 2013, Hernandez, Vazquez-Juarez et al. 2015, Hernandez, Hernandez et al. 2016, Zhang, Hernandez et al. 2016, Campos-Lira, Kelly et al. 2018, Hernandez-Perez, Hernandez et al. 2019, Hernandez-Perez, Hernandez et al. 2022). *During these studies, a collateral observation was repeatedly made: AVP immunoreactive (AVP-ir) magnocellular neurons seemed to be dispersed from the above-mentioned loci to adjacent subcortical regions. Lateral hypothalamus (LH), zona incerta (ZI) and latero-posterior division of the bed nucleus of stria terminalis (BNSTlp) were observed as the main regions hosting these dispersed large AVP-ir neurons (DNs), but they were also seen in more distant subcortical regions, including entopeduncular nucleus (EP), nucleus basalis of Meynert (B), and postero-dorsal division of medial amygdala (MEApd).* The DNs were observed to be adjacent to blood vessels and AVP-ir fibers in scaffolds, some forming cords of AVP-ir cells ("cell chains" as referred to hereafter). These chains were visualized first with AVP IHC, with AVP-ir cell bodies within it. The phenomenon was also observed in healthy old rats (18 months). These

observations collectively suggested the occurrence of adult neuronal migration, raising the questions of the mechanism of their replenishment and potential generation via adult neurogenesis within the major AVP-MNN loci.

In the early 1990s, Swaab and colleagues reported that the hypothalamic vasopressin and oxytocin nuclei (VON) change in size and cell number at various postnatal developmental stages in the pig (van Eerdenburg, Poot et al. 1990, van Eerdenburg and Swaab 1991, van Eerdenburg and Swaab 1994). These changes were also observed upon alteration of gonadal-reproductive status (van Eerdenburg, Lugard-Kok et al. 1991), and suggested that the VON neurons undergo postnatal neurogenesis according to physiological demands (van Eerdenburg, Poot et al. 1990). However, these observations went largely overlooked.

To better characterize potential adult AVP neurogenesis in the context of AVP neuronal dispersion patterns from rat hypothalamic magnocellular loci, we conducted a systematic re-examination of the sets of permanently mounted rat brain sections with peroxidase immunohistochemistry against AVP and glial fibrillary acidic protein (GFAP), accumulated from our previous studies (*vide supra*). We examined the serial sections with *coronal, sagittal, horizontal and septo-temporal-oblique* orientations from male and female, juvenile to old (18 months) rats, and here report evidence *strongly suggesting* that AVP neurons could migrate following blood vessels, glial processes and axons of other AVP neurons. We challenged rats with chronic water deprivation every other day, and administered intraperitoneal 5-bromo-2'-deoxyuridine (BrdU), a synthetic nucleoside analogue with a chemical structure similar to thymidine, commonly used to assess cell proliferation in living tissues (Altman 1962, Altman and Das 1966, Lieber, Shaw et al. 1978) for two weeks. Immunohistochemical studies revealed BrdU-ir large round-shaped nuclei inside the AVP-magnocellular loci, with some of these paired as twin-BrdU-ir nuclei. It has been documented that preprovasopressin gene expression and subsequent vasopressin biosynthesis occur after a time lapse of 3 to 4 days following the birth of neurons destined to be AVP-MNNs (Ugrumov 2002). This may explain the low rate observation of double labeling with BrdU/AVP co-expressed, within the SON and PVN in the hypothalamus. However, a dividing AVP-ir cell with Ki67 labeled twin nuclei was observed. We also assessed the co-expression of AVP and neuroblast marker doublecortin (DCX) finding numerous DCX neurons within the SON and PVN and some of them co-expressed AVP. Our studies reveal AVP neurons in rat hypothalamic SON and PVN undergo low-rate neurogenesis during adult life and suggest that some of them disperse to adjacent subcortical regions guided by cell chains and axon-scaffolds visualized with IHC against GFAP and AVP.

2. Materials and methods

2.1 Animals

This study was performed using thirty-four Wistar rats (for the 2nd part of the study, *vide infra*). All animal procedures were approved by the local bioethical committee. Rats were housed in plexiglass cages (2-3 per cage) attached to an air recycler device, with controlled humidity, temperature (22-25°C), and

filtered air ventilation, under controlled illumination (12:12 h light/dark photocycles). Food and water were provided *ad libitum* unless indicated otherwise.

2.2 Experimental design

The study was divided in two parts. In the first part, systematic analysis and documentation of the patterns that some AVP neurons dispersed from SON and PVN were made by anatomical examination of permanently mounted *serial sections* samples with *coronal, sagittal, horizontal and septo-temporal oblique* orientations, which were accumulated from our previous studies. The ethical statements, animal experiment licenses and experimental details of those studies can be found elsewhere (Zhang and Hernandez 2013, Irles, Nava-Kopp et al. 2014, Hernandez, Vazquez-Juarez et al. 2015, Hernandez, Hernandez et al. 2016, Zhang, Hernandez et al. 2016, Campos-Lira, Kelly et al. 2018, Hernandez-Perez, Hernandez et al. 2019, Hernandez-Perez, Hernandez et al. 2022).

The second part concerned neurogenesis detection and demonstration using 5′-bromo-2′-deoxyuridine (BrdU) protocol (Zhang, Hernandez et al. 2014) and immunohistochemistry (IHC) against cell proliferation markers BrdU and Ki67, as well as neuronal precursor marker DCX, and their relationship with GFAP and AVP expression. This part was performed under the license FM-CIE-079-2020.

2.3 Chemicals

Chemicals were obtained from Sigma–Aldrich, St. Louis, MO, USA, if not indicated otherwise. Sources of primary antibodies and their dilutions are depicted in Table 1.

Table 1. Primary antibodies used and their dilutions

Antibody to	**Host**	**Dilution**	**Source**
[Arg8]-vasopressin	Rabbit	1:2000	Peninsula laboratories international, T4563
[Arg8]-vasopressin	Rabbit	1:2000	Prof. R. M. Buijs, UNAM, Mexico
Neurophysin II	Mouse	1:250	Prof. Harold Gainer, NINDS/NIH, Bethesda
Bromo-2-deoxyuridine (BrdU)	Rat	1:100	Accurate Scientific, NY OBT0030
Ki67	Rabbit	1:250	Biocare Medical, SP6
Glial fibrillary acidic protein (GFAP)	Rabbit	1:1000	Biocare Medical, 901-040-031511
Neuronal nuclear antigen (NeuN)	Mouse	1:2000	Millipore, MAB377
Doublecortin (DCX)	Goat	1:1000	Santa Cruz, SC-8066

2.4 Experimental treatment of water deprivation (WD) and BrdU injection:

Twenty-four rats (250+/-20g, 12 per each sex) were used in this experiment which consisted of two protocols. Rats were divided into two groups: controls, n=6 x 2 (male and female) and water deprivation WD, n=6 x 2 (sexes). WD subjects were restricted from water intake every other 24 hrs. For protocol A, n=4, intraperitoneal injection of the thymidine analog 5-bromo-2-deoxiurydine (BrDU, Sigma Aldrich) diluted in NaCl 0.9% (10 mg/ml) was given to each experimental subject for 14 days, with daily doses of

50 mg/kg body weight fractionated in 3 injections per day. Protocol B consisted in the same control and water deprivation treatment but without BrdU injection (n=2 per group, N=8), for Ki-67, GFAP, and DCX immunolabeling vs. AVP-ir.

2.5 Immunohistochemistry

Rats were deeply anaesthetized with an overdose of sodium pentobarbital (63mg/kg, Sedalpharma, Mexico) and perfused trans-aortically with 0.9% saline followed by cold fixative containing 4% of paraformaldehyde in 0.1 M sodium phosphate buffer (PB, pH 7.4) plus 15% v/v saturated picric acid for 15min (referred as Somogyi fixative (Somogyi and Takagi 1982)). Brains were immediately removed, blocked, then thoroughly rinsed with PB. Immunohistochemical reactions (IHC) were performed the same day of perfusion/fixation and sectioning (N. B. this timing scheme has given us a remarkably better AVP labelling) using the immunoperoxidase (for single antigen labeling) and immunofluorescence (multi-antigen labeling) methods. The IHC procedure of previous studies can be found elsewhere (Zhang and Hernandez 2013, Irles, Nava-Kopp et al. 2014, Hernandez, Vazquez-Juarez et al. 2015, Hernandez, Hernandez et al. 2016, Zhang, Hernandez et al. 2016, Campos-Lira, Kelly et al. 2018, Hernandez-Perez, Hernandez et al. 2019, Hernandez-Perez, Hernandez et al. 2022). Briefly, brains were sectioned using a Leica VT 1000S vibratome, at 70 µm thickness, soon after perfusion/fixation (15 min) and thoroughly rinsed (until the yellow color of picric acid was cleared). The following sectioning planes were used: sagittal (n = 3), coronal (n = 3), semi-horizontal (30° to the horizontal plane, n = 2) and septo-temporal (between coronal and sagittal planes, 45° to both planes, n = 2). Freshly-cut freely-floating sections (1 in 4) from different cutting planes were blocked with 20% normal donkey serum (NDS) in Tris-buffered (0.05 M, pH 7.4) saline (0.9%) plus 0.3% of Triton X-100 (TBST) for 1 h at room temperature and incubated with the primary antibodies listed in the table 1 in TBST plus 1% NDS over two nights at 4°C with gentle shaking. For immunoperoxidase reaction, conventional procedure were followed (detailed description can be found (Zhang and Hernandez 2013, Irles, Nava-Kopp et al. 2014, Hernandez, Vazquez-Juarez et al. 2015, Hernandez, Hernandez et al. 2016, Zhang, Hernandez et al. 2016, Campos-Lira, Kelly et al. 2018, Hernandez-Perez, Hernandez et al. 2019, Hernandez-Perez, Hernandez et al. 2022). For BrdU-IHC, sections were pretreated by incubating sections in 2N HCl for 30 minutes at 37°C for DNA denaturalization. After this step, sections were rinsed twice with 0.1 M borate buffer (pH 8.5) followed by a rinse with phosphate buffer (Zhang, Guadarrama et al. 2006).

2.6 Transmission electron microscopy

Technical details of electron microcopy photomicrograph displayed in the inset of figure 1 were reported elsewhere (Zhang and Hernandez 2013)

3. Results and specific discussion

3.1 *Vasopressin (AVP)-containing cells from hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei dispersed to other subcortical regions postnatally, forming chains of cells guided by axon scaffolds*

3.1.1 Re-examination of our previous finding on neonatal maternal separation effect on AVP system revealed an increased hypothalamic AVP neuronal population and more extended distribution as a function of a postnatal stress

In a previous study, we reported that early postnatal stress, resulted in enlarged AVP magnocellular nuclei, measured at both immunohistochemical and mRNA levels, as well as increased number of AVP-ir neurons in PVN and SON nuclei in rat adulthood (Fig. 1, modified from (Zhang, Hernandez et al. 2012) with permission granted). Interestingly, there was also an increase in the extension of distribution of AVP-ir neurons, outside the main magnocellular nuclei (i.e., the PVN and SON of the hypothalamus, in the rostro-caudal and medio-lateral dimensions (Fig. 1B and C).

In our previous report, we noted a remarkable lateral dispersion of some AVP-ir magnocelular neurons, classified according to the morphological criterion (Armstrong 2004), in the stressed group. For instance, in the Fig. 1, panels C and C', this phenomenon was indicated with yellow arrows. We previously interpreted this phenomenon as a result of reduced programmed cell death due to maternal separation induced upregulation of phospho-ERK 1/2 that promote the phospho-Bad pathway resulting in a reduced postnatal apoptosis (Irles, Nava-Kopp et al. 2014). However, re-analyzing those results together with other AVP-ir serial samples made for different experiments (*vide infra*) in not-stressed animals, we realized there could be another explanation besides the reduced neonatal apoptosis hypothesis, since it is consistently observed in adult rats, and an increase AVP-ir and cell dispersion can be also observed under adult osmotic challenges. Of course, this does not mean the signaling pathway we reported previously could be excluded but it surely opens a new viewpoint on this phenomenon.

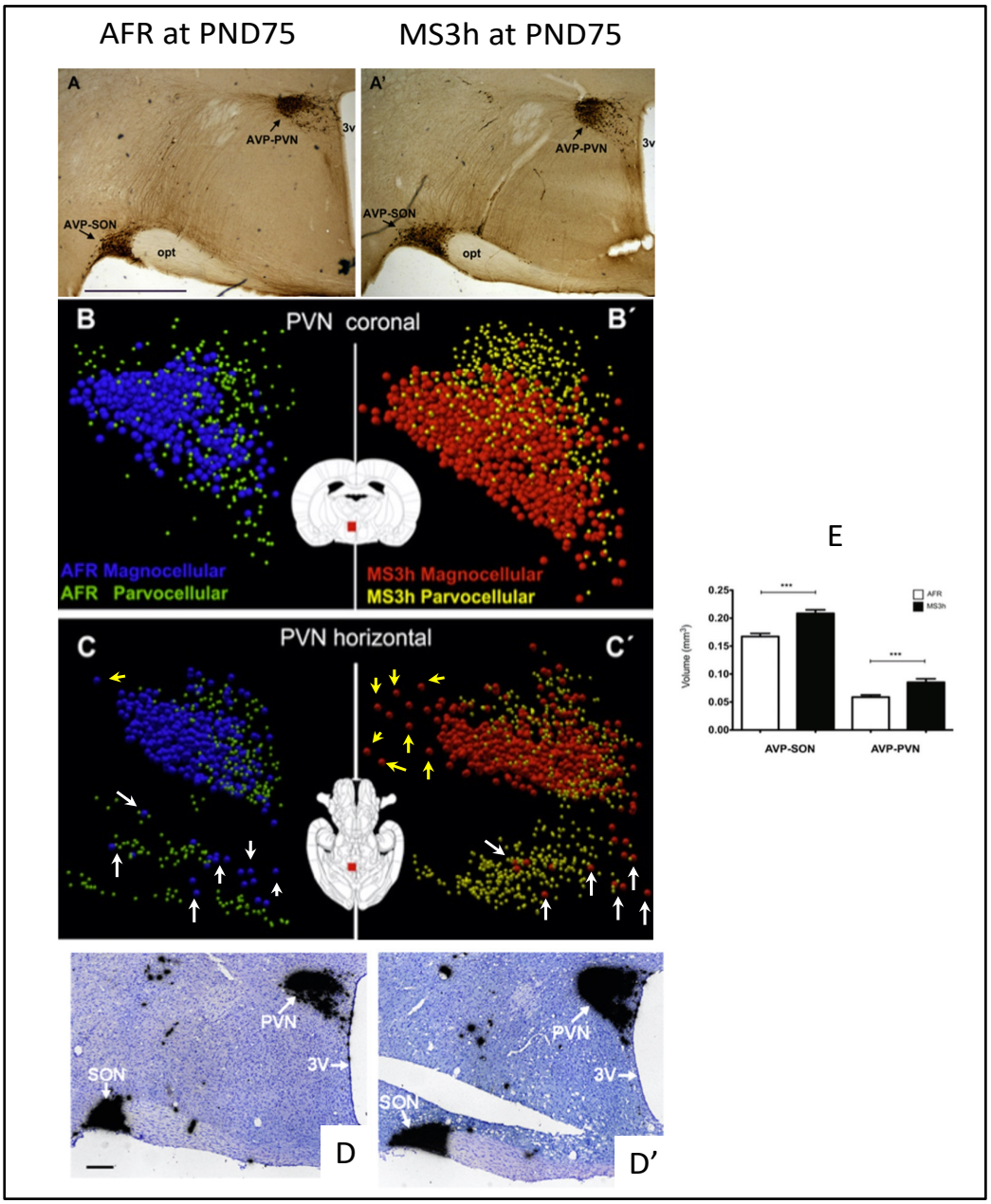


Fig. 1 **Neonatal maternal separation (3 hours daily, MS3h), ~~from postnatal day (PND1) through PND15,~~ resulted in enlarged AVP magnocellular nuclei**, measured at both immunohistochemical (panels A) and mRNA (panels D) levels. Bs and Cs: increased number of AVP-ir neurons in PVN and SON nuclei in rat adulthood (postnatal day 63). In order to visualize the directionality of the enlargement of the PVN, 3D digital reconstruction was made based on anatomical coordinates determined on digital photomicrographs (technical detail was described elsewhere (Zhang, Hernandez et al. 2012)). Magnocellular (MCN, control in blue vs MS3H in red) and parvocellular (control in green vs MS3H in yellow) neurons were determined according to their long axes were assigned according the morphological criterion, i.e., those with long axes more than 20µm (Armstrong 2004)) were assigned as MCN. The white arrows C and C' indicate the rostrally dispersed magnocellular neurons and the yellow arrows indicate the laterally dispersed magnocelular neurons, to lateral hypothalamus and zona incerta. Visible increases of the populations of both magnocells within the PVN and dispersed cells from the PVN can be noted. E: Histogram shows the mean ± SEM of the AVP+ volume extension measurements in SON and PVN regions. $p < 0.001$. 3v, third ventricle, opt, optic tract. Scale bar = 1 mm. Modified from (Zhang, Hernandez et al. 2012) with granted permission for the use of the material here.

3.1.2 Network of AVP-ir neuronal chains and tangential migration alongside the AVP-ir axon scaffolds in adult rats

In order to describe the morphology AVP-ir in the rat brain, we re-analysed serial sections sets with cutting planes of coronal, sagittal, horizontal, septo-temporal orientations, accumulated from our previous experiments. The immunohistochemical reactions (ir) were performed as in one of each three sections and we used a combination of two rabbit anti-vasopressin antibodies (gift from Prof. Ruud Buijs (Buijs, Pool et al. 1989) and Peninsula laboratories international, T4563, see Tab 1). This measure is implemented with the rationale that the neuropeptide changes its structure from its synthesis as a preprohormone to the releasing molecule as a nonapeptide inside a dense core vesicle, that one given antibody may only recognize one molecular form of the potential storage of the peptide. The usage of two antibodies against vasopressin apparently gave us an enhanced labeling of AVP system.

Selective planes which could best illustrate the phenomenon of adult neuronal tangential migrations occurring in AVP-ir populations are presented in figures 2 through figure 6 with detailed description in the figure legends. Sex and age of animals from which the series were obtained are also indicated in the legends.

Figure 2 shows examples from rat brain coronal section series with an emphasis on supraoptic nucleus (SON). AVP-ir cell-chains emerged from SON^{AVP} can be clearly seen (Fig. 2, panels As), which have been identified as neuronal precursors (indicated with red arrows). The cell-chains are neuronal precursors in migration ((Doetsch and Alvarez-Buylla 1996), Fig. 2A insets). Fig. 2 panels Bs show the AVP-ir axonal scaffolds intermingled with cell-chains (indicated with black arrows and red arrows respectively). AVP-ir cells seemed to "climb" into the AVP-ir scaffolds (Fig. 2, B3, black arrows). The proximity of AVP-ir axon with blood vessels taken from preoptic area of the rat hypothalamus is shown on Fig. 2, B4. Tight junctions (TJ) formed by processes of endothelium cells can be clearly seen in this electron photomicrograph, which indicates that the AVP-ir axons are separated from the lumen of blood vessels by brain-blood-barrier (BBB). AVP-ir neurons with tangential migrating phenotype were seen in entopeduncular nucleus (red arrows, Fig. 2 C and D) climbed in AVP-ir axon scaffolds (Fig. 2 C, black arrows). Through systematic microscopical examination and analysis of this series, we conclude that the AVP-ir neurons could take several routes to disperse from the SON (Fig. 2, E, green arrows).

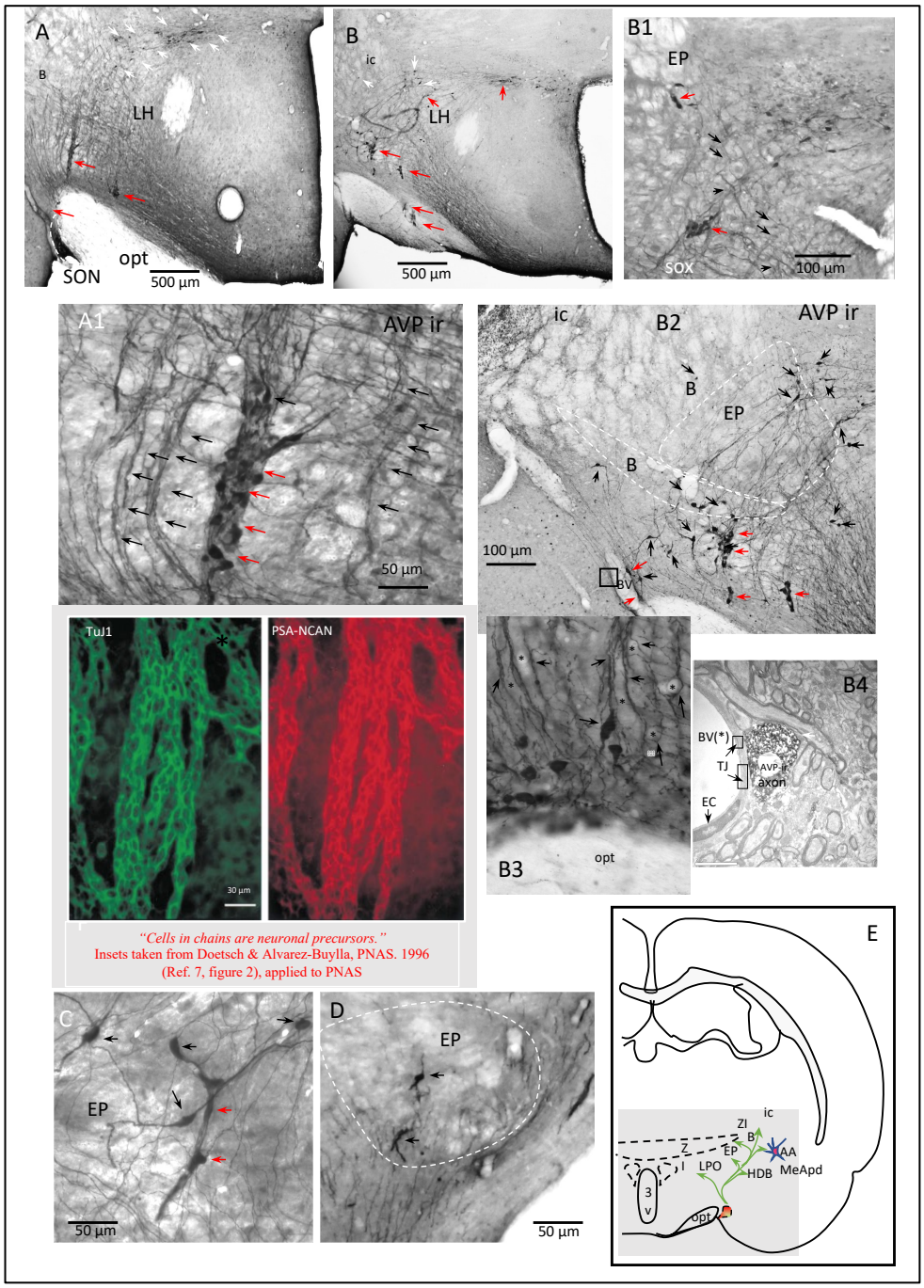


Fig. 2 **Network of AVP-ir cell chains and axon scaffolds in the adult rat hypothalamus (1/5) emerged from supraoptic nucleus (SON) - coronal view through serial sections from an adult male Wistar rat, age: 4 month.** A and A1: AVP-ir cell-chains (red arrows) and axonal scaffolds (indicate by black arrows) emerged from SON^{AVP}. The cell-chains have been previously characterized as neuronal precursors in migration ((Doetsch and Alvarez-Buylla 1996), fluorescent insets are examples taken from the same paper with granted permission for the use of the material here with copyright (1996) National Academy of Sciences, U.S.A. Panel B, B1 and B2 show the cell chains (red arrows) intermingled with AVP-ir axonal scaffolds (black arrows). B3: AVP-ir bipolar cells with tangential migration morphology climbed onto the AVP-ir axonal scaffolds. B4 shows the proximity of AVP-ir axon with blood vessels (bv) at electron microscopy level. Tight junctions (TJ) formed by processes of endothelium cells (EC) are indicated. The AVP-ir axons are separated from the lumen of blood vessels at ultra-structural level in the lateral preoptic of hypothalamus where the sample is taken. C and D: presence of AVP-ir cells with tangential migration morphology in entopeduncular nucleus (EP, C) and lateral hypothalamus (D). E: charting symbolizing the observed migration routes from coronal samples (green arrows).

Sagittal series of AVP immunostaining revealed massive ascending axonal routes toward the brain main conducting systems, i.e. fimbria and stria medullaris (Fig. 3). Numerous cell chains are discovered from this view (Fig. 3, A, B. C, E. F, red arrows). AVP-ir cells with tangentially migrating morphology (mainly bipolar) were found in adjacent regions to SON and PVN. Populational continuity between SON^{AVP} and $MeApd^{AVP}$ can be appreciated through serial section viewing (Fig. 3, from A through F).

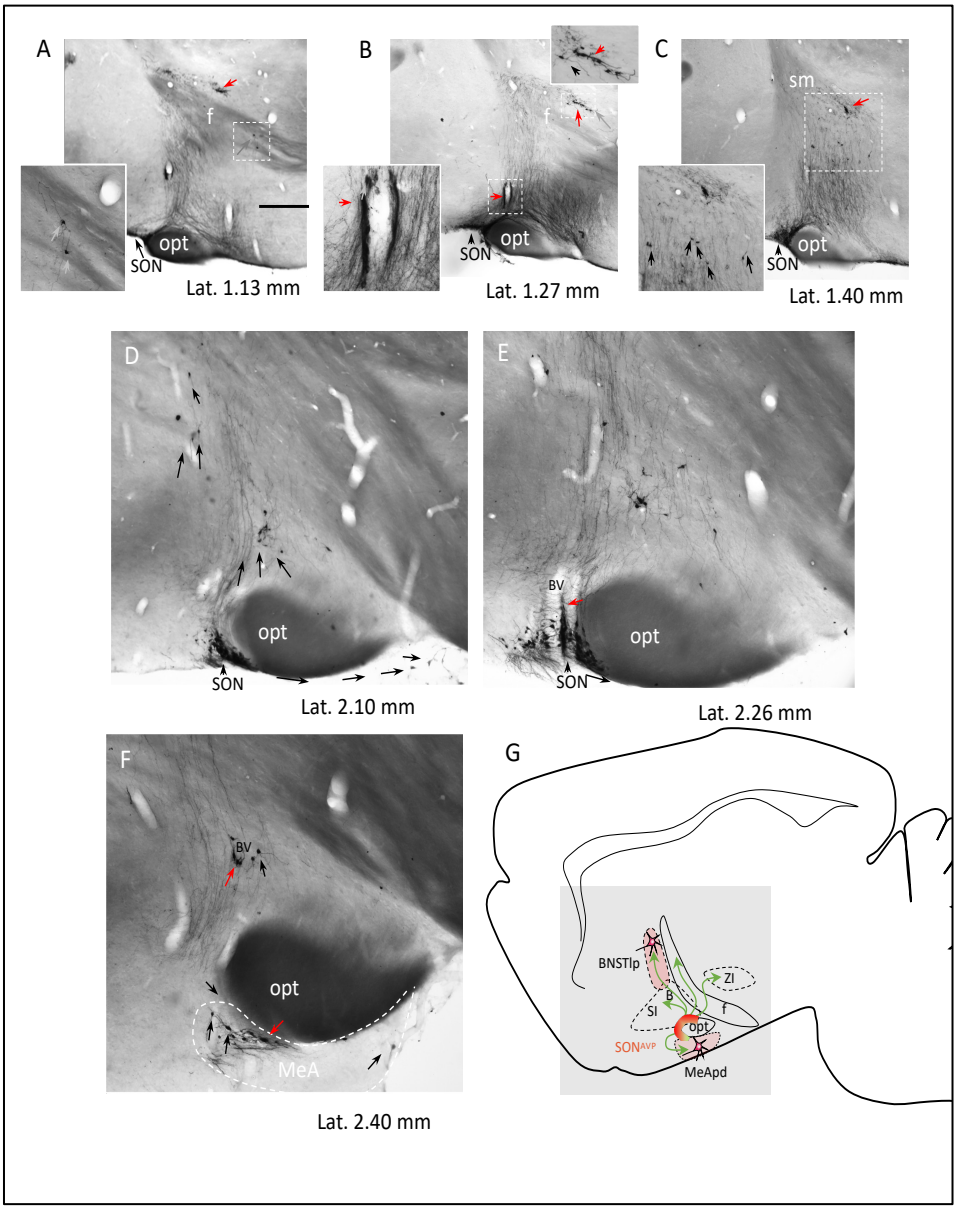


Fig. 3 **Network of AVP-ir cell chains and axon scaffolds in the adult rat hypothalamus (2/5) emerged from supraoptic nucleus (SON) - sagittal view through serial sections from an adult female Wistar rat of 4 month.** A-F: AVP immunostaining at representative medio-lateral levels. Ascending axonal stream can be clearly identified. AVP-ir cell chains are indicated by red arrows. Dispersed neurons with tangential migration morphology, climbing in the AVP-ir axonal scaffold are indicated by black arrows. D, E and F show the continuity and lateral migration path of AVP-ir cells from SON to the medial amygdala alongside the optical tract (opt). G. charting symbolizing the migration routes (green arrows) visualized from sagittal series analysis. Samples were osmicated for electron microscopy study, reason that the myelinated fibers are dark.

Horizontal sections series of AVP-ir allowed us to appreciate that many AVP-ir neurons disperse from the SON, alongside the optic tract, forming cell chains (Fig. 4, A, red arrows, note that the majority of the cell chains within the optic tract had a blood vessel within the chain). Other AVP-ir cells dispersed with tangential migration following AVP-ir axonal scaffolds (Fig. 4, A and A2, white arrows). Moreover,

it was surprising to observe that there are at least two populations of AVP neurons withing the region of hypothalamic bed nucleus of stria terminalis (BNST-Hy). One population is located in the rostral part, with small somata and weak AVP immunostaining and another population is posterior and at the boarder of the stria medullaris. The latter ones are strongly immunoreactive to AVP (Fig. 4, B1 and B2, white arrows). Cell chain alongside the stria medullaris is also observed (Fig. 4, C inset, red arrows). The migratory connection between the SON and the BNST-Hy, concluded from serial section analysis is synthesized in the diagram C of the Fig. 4. Vasopressin neuronal population's distribution and function in the BNST-Hy has been described with ample variations among reports. For example, vasopressin in the BNST has been linked to both anxiogenic (anxiety-promoting) and anxiolytic (anxiety-reducing) effects, depending on the circumstances (Wigger, Sanchez et al. 2004). A previous study of our group reported that one subpopulation of vasopressin neurons in the BNST-Hy co-expressed VGAT, a GABAergic neuronal marker, however, a substantial portion of the cells there did not express this marker which suggest the existence of subpopulations (Zhang, Hernandez et al. 2020).

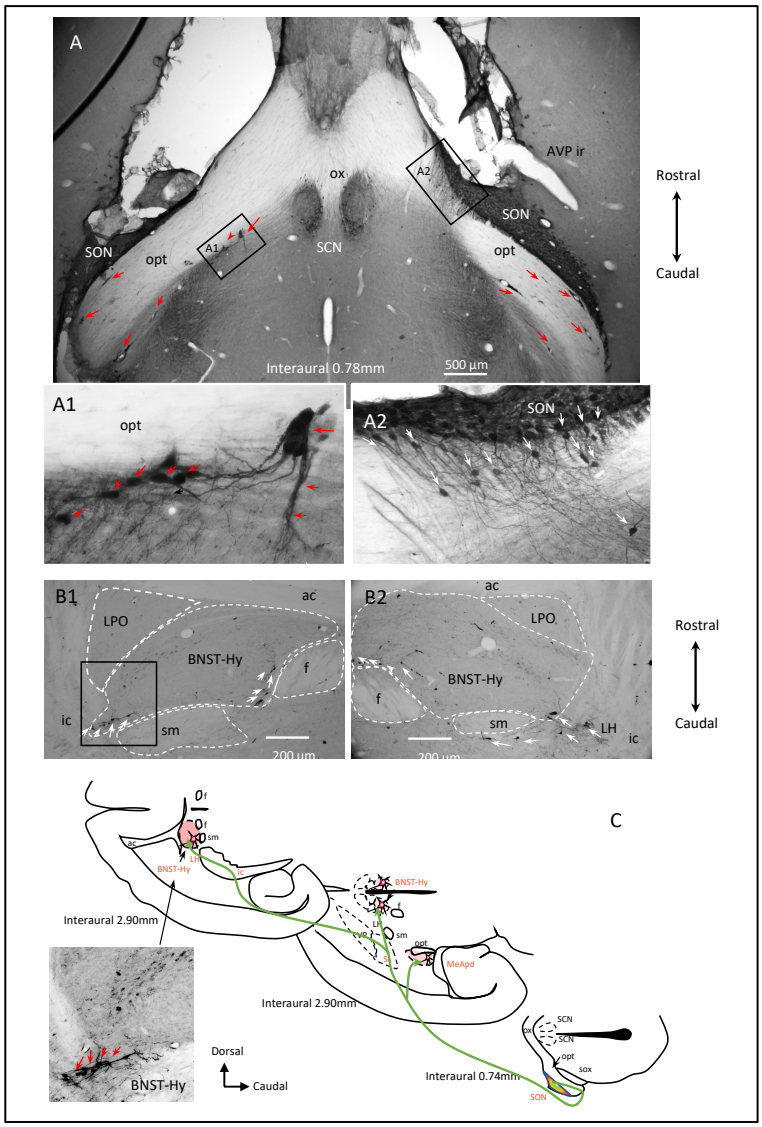


Fig. 4 **Network of AVP-ir cell chains and axon scaffolds in the adult rat hypothalamus (3/5) emerged from supraoptic nucleus (SON) - horizontal view through serial sections from an adult male Wistar rat of 4 month.** As: immunostaining at the interaural level 0.78mm, where SON and suprachiasmatic nucleus (SCN) vasopressinergic cells can be clear seen, however, with different features. While the SCN^{AVP} are well delineated, some neurons of SON^{AVP} dispersed tangentially following the axonal scaffolds (A1 and A2), mainly alongside the optic tract (opt). Numerous chains were indicated with red arrows. B1 and B2 show the tangentially migrated AVP-ir cells arriving the lateral portion of the bed nucleus of stria terminalis. C. charting symbolizing the migration routes (green arrows) from SON to medial amygdala posterodorsal division (MeApd) and bed nucleus of stria terminalis hypothalamic (BNST-Hy), passing through the sustantia innominata (SI), ventral pallidum (VP), lateral hypothalamus (LH) in 3 horizontal levels visualized by horizontal section analysis.

When the brains were sliced with a tilted angle of -30 º degree with reference of septo-temporal axis (Fig. 5, A, top right inset, red line), the immunostaining in serial sections provided a fresh view about the anatomical organization of the PVN^{AVP} in adult rat. Figure 5 shows examples from a male rat of 12 month old. An impressive AVP-ir cell chain, alongside a main blood vessel extended postero-laterally to zona incerta (ZI) (Fig. 5, A, red arrows). AVP-ir cells dispersed from the chain, moved tangentially reaching nucleus basalis of Meynert (B) and the magnocellular nucleus of lateral hypothalamus (MCLH) (Fig. 5, A, black and white arrows). The MCLH is a group of large cells located at the lateral border of the lateral hypothalamic area at the medial border of the internal capsule of the rat (Swanson 2004). It is one of twenty six regions, zones, and nuclei constituting the motor lateral hypothalamus. Functionally it belongs to the motor lateral hypothalamus of the subcortical motor system (Swanson 2004). This pathway is further described in the Fig. 6, with horizonal view at interaural level 2.20 mm, where AVP-ir axon scaffolds guided cell migration can be appreciated.

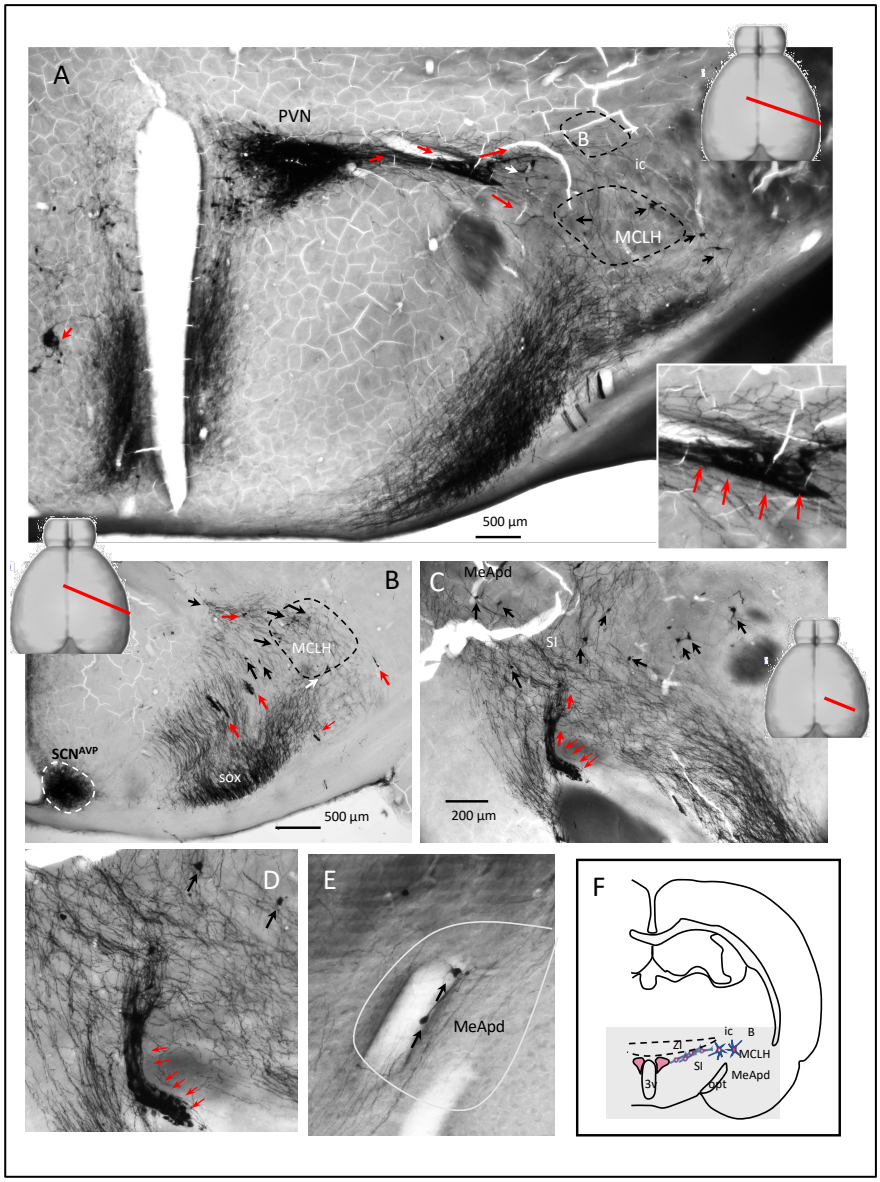


Fig. 5 **Network of AVP-ir cell chains and axon scaffolds in the adult rat hypothalamus (4/5) emerged from paraventricular (PVN) and supraoptic nucleus (SON) - septo-temporal oblique view (-30 º degree) through serial sections from an adult male Wistar rat of 12 month old.** A: AVP-ir of a septo-temporal oblique cutting plane passing through the center of PVN with -30 degrees of inclination toward temporo-caudal direction revealed thick AVP-ir cell chain emerged from PVN alongside a thick blood vessel which bifurcated to latero-dorsal (B: nucleus basalis of Meynert) and latero-ventral (MCLH: magnocellular nucleus of the lateral hypothalamus, MCLH) directions. Red arrows indicated cell chains and black arrows indicate the dispersed cells. B: more examples of dispersed cells intermingled with axonal scaffolds. Note that the suprachiasmatic nucleus (SCN^{AVP}) showed distinct feature that no cell chains or dispersed neurons were observed to emerge from there. C, D. and E: more examples of AVP-ir cell chains and dispersed neurons. F. charting synthesizing the observation of this figure. Samples were osmicated for electron microscopy study, reason that the myelinated fibers are dark.

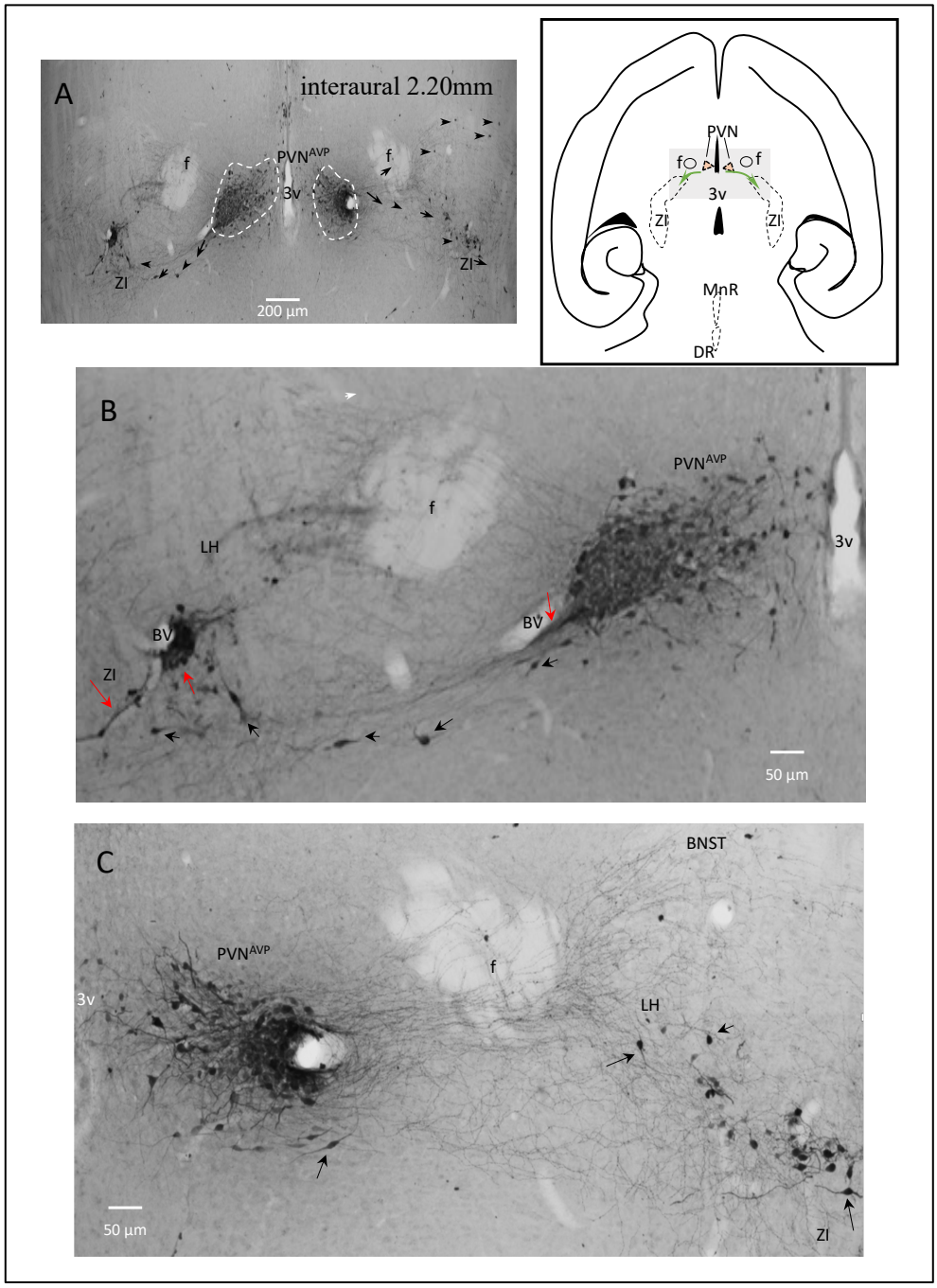


Fig. 6. **Network of AVP-ir cell chains and axon scaffolds in the adult rat hypothalamus emerged (5/5) from paraventricular nucleus (PVN) - horizonal view reveals migration route for direct connection between PVN and ZI APV-ir cells (sections from an adult male Wistar rat of 4 month).** A: AVP-ir cell-chains (red arrows) and axonal scaffolds (indicate by black arrows) emerged from $PVN^{AVP.}$ B and C are amplifications of each side of A.

3.2 Radial glial-like cells serving glial scaffolds for AVP-ir cell's tangential migration

Glial fibrillar acidic protein (GFAP) is the principal 8-9 nm intermediate filament in mature astrocytes and in the radial glial cells in the central nervous system (Eng, Vanderhaeghen et al. 1971, Barry, Pakan et al. 2014). It is known that astrocytes, through their interaction with neuroblasts and endothelial cells, play a key role in the genesis, migration, and integration of neurons in the adult brain (Gengatharan, Bammann et al. 2016, Bressan and Saghatelyan 2020). We used GFAP to explore the astrocyte distribution

in the hypothalamus. Surprisingly, we found a distinct pattern of immunostaining where long processes of many GFAP-ir fibers were aligned and seemed to serve for marking several routes (Fig. 7, A, A1 and A2, indicated with white arrows). The labeling density is remarkably heterogeneous and the observed routes coincided with the AVP-ir cells tangential migrations routes described in the Fig. 2-6. Those cells are apparently similar to the tanycytes around the third ventricle (3v) in the hypothalamus (Fig. 7, B) (Yoo and Blackshaw 2018) and those found in the adult dentate gyrus, subgranular zone (SGZ) of the hippocampal formation (Fig. 7, C) (Doetsch and Alvarez-Buylla 1996). These two types of glial cells are subtypes of the radial glial cells serving for adult neuronal migration (Barry, Pakan et al. 2014).

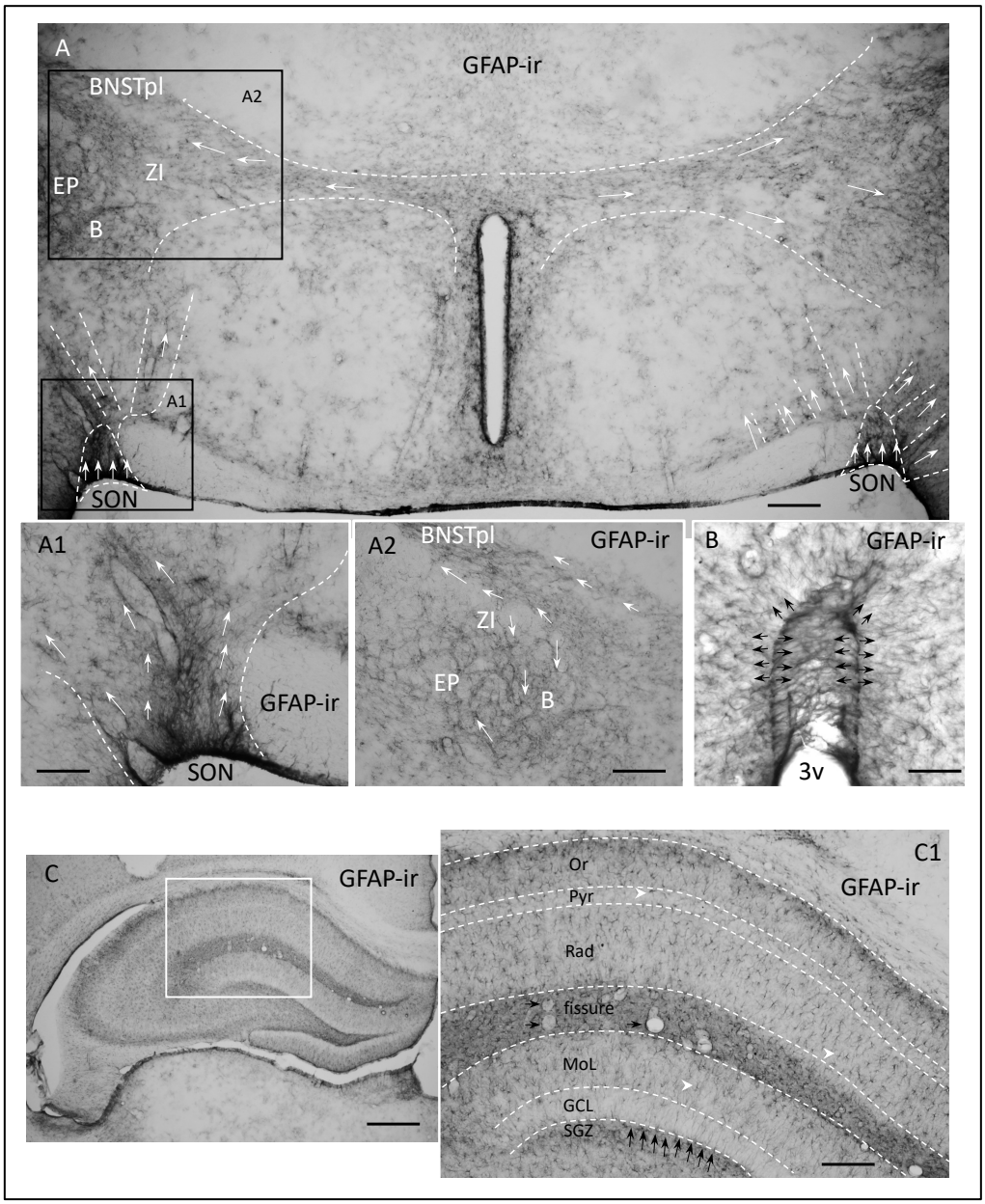


Fig. 7. Glial fibrillar acidic protein (GFAP) immunostaining reveals a distinct feature of the GFAP-ir morphology, similar to the radial glial cell and its heterogenous distribution resembling the migration routes observed with AVP IHC in the rat hypothalamus (A, resembling of tangential migration routes described above were indicated with white arrows) A1 and A2: example of distribution of the GFAP-ir cells. B. Tanycytes, a type of radial glial, in the periventricular region of the hypothalamus of the same reaction. C and inset, GFAP labeling in hippocampus. Note the similarity of the radial glial in the subgranular zone (SGZ), black arrows.

We further performed a double immunofluorescence reaction AVP/GFAP and found that all the cell chains with AVP-ir cells within them, are outlined by GFAP processes (Fig. 8 panels As and Bs). GFAP-ir long processes were seen intertwined with tangential-shaped AVP-ir cells and axons (Fig. 8, C2-C4, yellow arrows) serving as migration scaffolds for AVP-ir soma and axons (Fig.8 panels C).

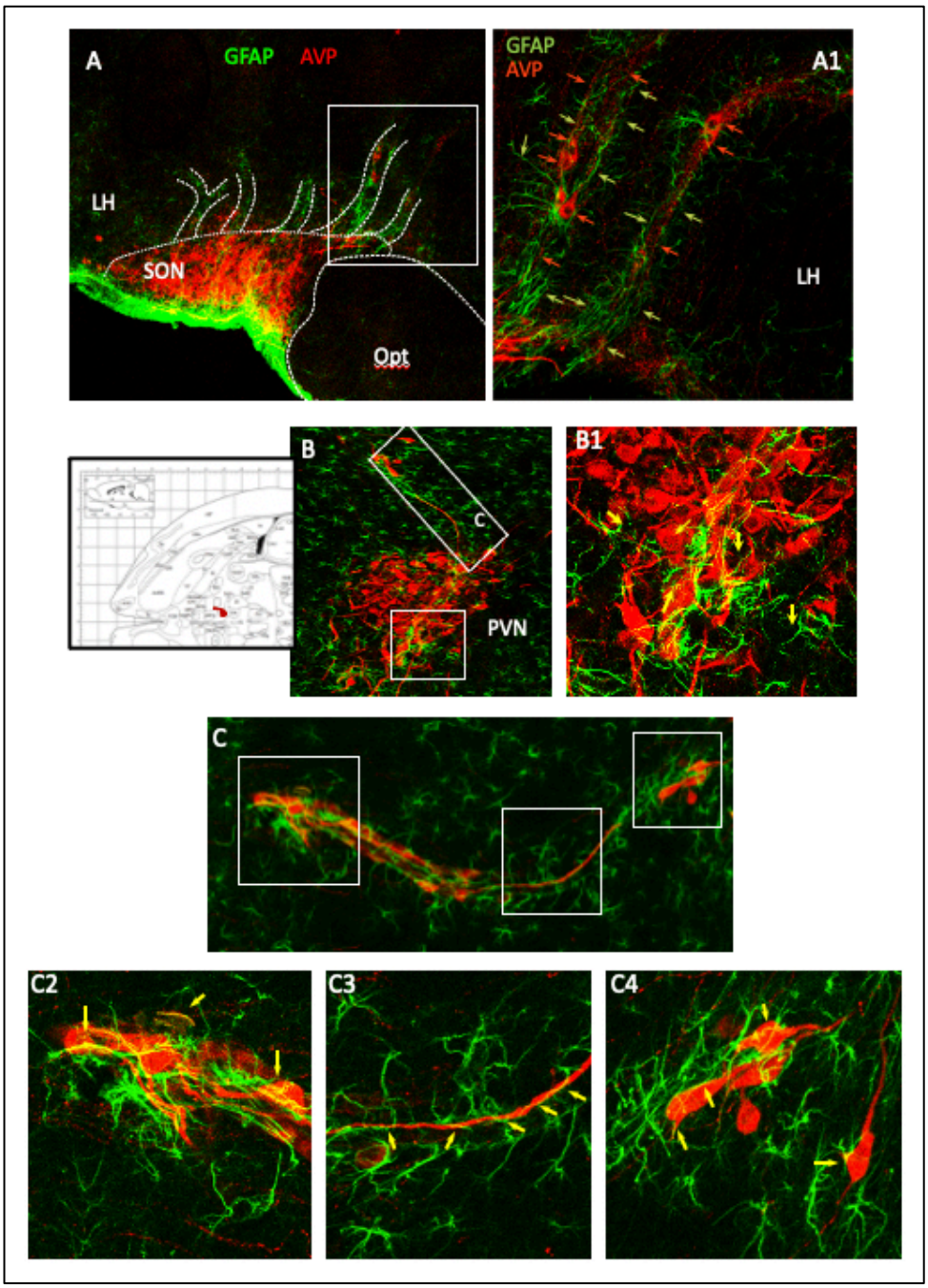
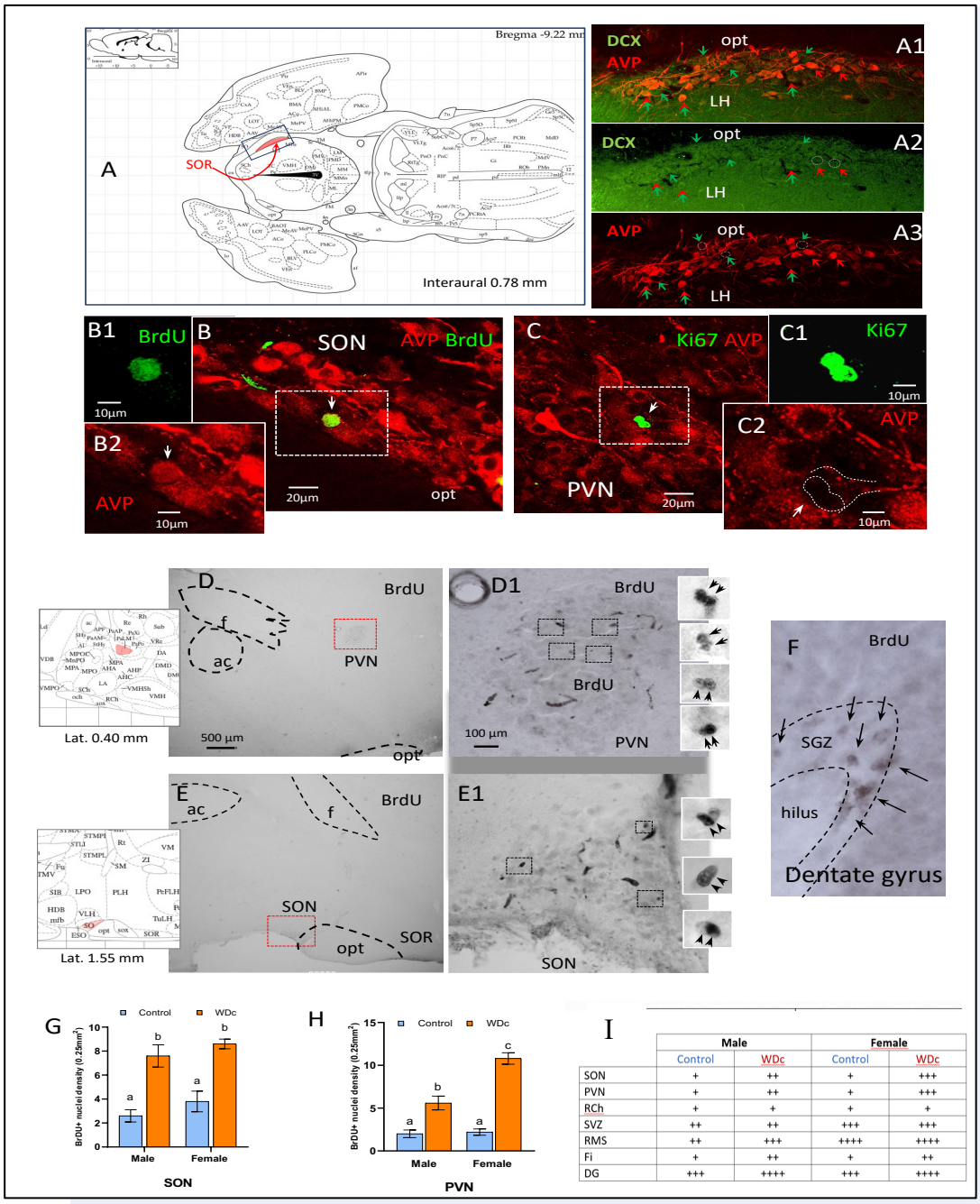


Fig. 8. Double immunofluorescence reaction against AVP/GFAP revealed the intimate relationship between the AVP-ir dispersion, AVP neuronal chains, and the GFAP fibers. A-C, GFAP-ir tunnel-like structures containing AVP-ir cells and axons. The phenomenon of AVP-ir axons "climbing" the GFAP-ir scaffolds is indicated with yellow arrows.. C2-C4 show the higher magnification of GFAP-ir long processes serving as migration scaffolds for AVP-ir soma and axons.

3.3 *Neurogenesis occurs within the adult SON and PVN and osmotic challenge significantly increases its rate, with some cells becoming AVP-ir cells.*



	Male		Female	
	Control	WDc	Control	WDc
SON	+	++	+	+++
PVN	+	++	+	+++
RCh	+	+	+	+
SVZ	++	++	+++	+++
RMS	++	+++	++++	++++
Fi	+	++	+	++
DG	+++	++++	+++	++++

Fig. 9. **Neurogenesis occurs within the adult SON and PVN, and osmotic challenge significantly increases its rate. Some cells become AVP-ir cells.** Panels As show abundant doublecortin (DCX)-expressing neurons in horizontal brain sections. Panels B show AVP/BrdU-ir neurons within the SON. Panels Cs show AVP/Ki67 double labeling in a large magnocellular neuron within PVN. Panels D and E show BrdU-ir nuclei within the PVN (D) and SON (E). Insets show examples of "twin" BrdU-ir nuclei observed within those regions. Panel F shows reference labeling of stem cells in the hippocampus subgranular zone of the same immunoreaction. Panels G and H: statistical comparison of BrdU-ir nuclei in SON (G) and PVN (H), control vs chronically water-deprived subjects. Panel I: whole brain semi-quantitative assessment of BrdU-ir, control vs WD. Serial sagittal brain sections were analyzed and an index of positive nuclei (BrdU) in each 40x (0.45 mm) field was assessed as follows: 1-4 nuclei (+); 5-9 nuclei (++); 10-15 nuclei (+++); more than 15 nuclei (++++). SON: hypothalamic supraoptic nucleus; PVN: hypothalamic paraventricular nucleus; RCh: retro-chiasmatic area; SVZ: subventricular cell zone; RMS: rostral migratory stream; Fi: Fimbria; DG: dentate gyrus.

Using doublecortin (DCX), an indicator of neurogenesis (Brown, Couillard-Despres et al. 2003), as well as a marker for neuronal migration (Gleeson, Lin et al. 1999, Filipovic, Santhosh Kumar et al. 2012), we observed abundant expression of DCX, yet heterogeneous within the SON magnocellular region (Fig. 9, A panels). Some neurons co-expressed DCX and AVP, which are labeled with bicolor arrows. Cells that were immunoreactive (ir) to only one of the two antigens are also highlighted with single-colored arrows, aiming to show the heterogeneity of this DCX expression with respect to AVP expression in the SON.

We also used 5-bromo-2´-deoxyuridine (BrdU) injection followed by immunostaining method to evaluate cell proliferation (Gratzner 1982) in both euhydrated and chronic water-deprived (every other day, during 15 days). Panels B show AVP/BrdU-ir neurons within the SON. We used another neuronal proliferation marker, Ki67 (Kee, Sivalingam et al. 2002), to test the neurogenesis potential of AVP-MNN loci. The AVP content in a dividing cell is shown (Fig. 9 C, white arrow). Figure 9's panels D and E show BrdU-ir nuclei within the PVN (D) and SON (E) with insets showing examples of "twin" BrdU-ir nuclei observed within those regions. Panel F shows reference labeling of stem cells in the hippocampus subgranular zone (SGZ) of the same immunoreaction.

With the observations mentioned above, we then asked if neurogenesis could be modified as an allostatic mechanism for stress adaptation. AVP-MNNs are able to respond to homeostatic challenges by increasing their metabolic activity generating physiological changes in dehydrated rats (Zhang, Hernandez et al. 2016, Zhang, Hernandez et al. 2018, Hernandez-Perez, Hernandez et al. 2022). A recent comprehensive transcriptomic study has reported change of 2247 RNA transcripts, including Ephrin receptor 6, a gene related to adult neurogenesis, in the SON of Wistar Han rats, after 3 days of water deprivation (Pauza, Mecawi et al. 2021). To test the hypothesis that neurogenesis of AVP-MNNs could be a mechanism for stress adaptation, we developed an experimental approach by repeatedly exposing adult rats to water deprivation (see materials and methods for detail) and injected daily three doses of the thymidine analogue BrdU for 15 days. The same procedure of dehydration without BrdU injection was performed in order to discriminate the possibility of BrdU interference with cell proliferation. Figure 9, panels G and H: show statistical comparisons of BrdU-ir nuclei in SON (G) and PVN (H), in control vs chronically water-deprived subjects. Two-way ANOVA of SON revealed an effect of dehydration ($F(1,16)=47.54$, $p<0.001$= as the source of variation. No effect of sex ($p>0.1$) nor interaction between factors ($p>0.5$). Multiple comparisons of groups revealed significant differences between male controls vs male WD animals ($p>0.0001$), between male control vs female WD ($p<0.0001$) and between female control vs female WD ($p<0.01$). In case of PVN, differences were noted between male control vs male WD ($p<0.01$), between male control vs female WD ($p<0.001$); between male WD and female control ($p<0.001$). In this case, an effect of sex was observed in WD animals as a difference between male WD vs female WD ($p<0.001$) was detected by statistical analysis. Female control vs female WD also had differences ($p<0.0001$) in the number of BrdU positive nuclei. Different letters indicate differences between experimental groups. Figure 9, panel I depicts a whole brain semi-qualitative assessment of BrdU-ir, control vs WD in different brain areas where neurogenesis has been reported and the effect of WD in the BrdU-ir neuron density. As observed in the table, WD increased the number of BrdU + nuclei

in SON and PVN but also increased the number of positive nuclei in other neurogenic niches, as the rostral migratory steam and subventricular zone.

3.4 *Absence of NeuN, a mature neuron marker, from SON and PVN in adult rat*

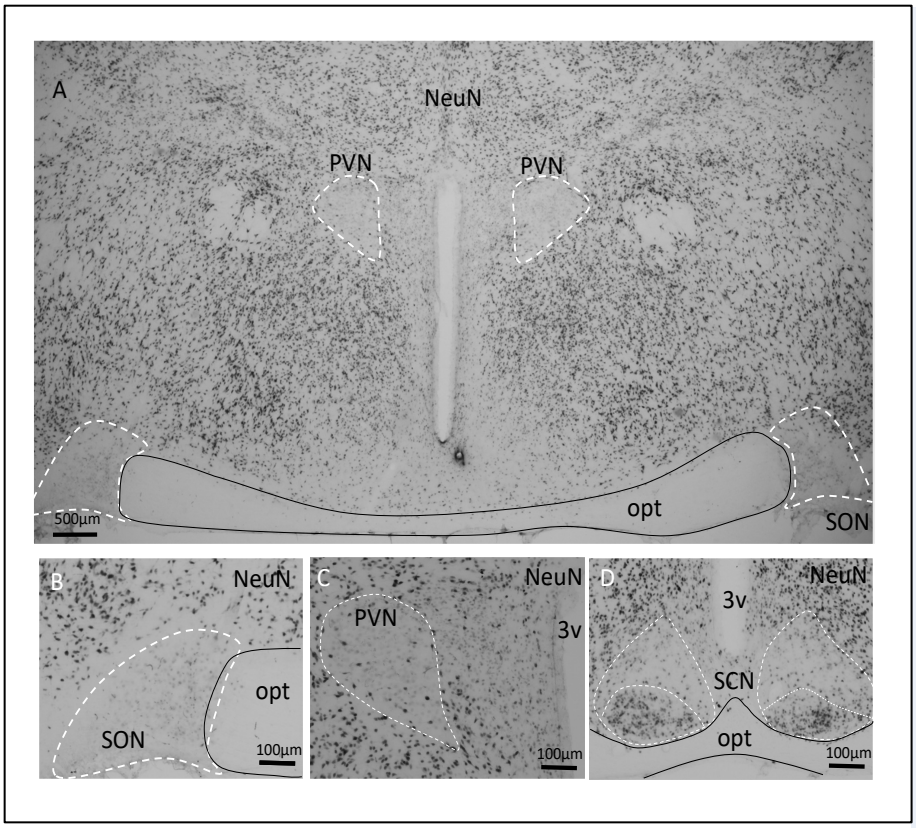


Fig. 10. **Absence of NeuN, a mature neuron marker, in the SON^{AVP} and PVN^{AVP}.** NeuN, a mature neuron marker, is widely expressed in the rat hypothalamus (A). However, both supraoptic (SON, A and B) and paraventricular (PVN, A and C) lack the NeuN expressing nuclei. In suprachiasmatic nucleus (D), the ventral subdivision, which is mainly occupied by vasoactive intestinal polypeptide VIP-expressing neurons, shows high expression of NeuN, and the dorsal part, which is mainly occupied by AVP-expressing neurons, lacks NeuN expression. opt: optical tract; 3v: third ventricle.

To evaluate if there were changes in the density of neurons in the rat hypothalamus after maternal separation (Irles, Nava-Kopp et al. 2014), we made immunostaining of the postmitotic neuron marker NeuN. Surprisingly, in the magnocellular hypothalamic PVN and SON nuclei, there was a general lack of NeuN expression (Fig. 10). In the suprachiasmatic nucleus, the ventral subdivision, which is mainly occupied by vasoactive intestinal polipeptide (VIP)-expressing neurons (Kawamoto, Nagano et al. 2003), showed a high expression of NeuN, while the dorsal part, mainly occupied by AVP-expressing neurons (Zhang, Hernandez et al. 2020), lacked NeuN expression. Although NeuN has been used for a long time as a reliable marker for mature neurons (Mullen, Buck et al. 1992), its identity as an epitope of the Rbfox3 alternative splicing regulator protein was elucidated only in recent years ago (Kim, Adelstein et al. 2009), and its possible functions on the neurobiology of the neurons have only recently been investigated in more detail (Duan, Zhang et al. 2016). Interestingly, it has been reported that some neurons of regions that undergo postnatal neurogenesis, such as the olfactory bulb, the dentate nucleus, and the cerebellum, lack NeuN/Rbfox3 expression. Moreover, It has been described as a reduction of NeuN immunoreactivity in pathophysiological circumstances, such as local injury, diseases, physical or chemical stress, in which

there is a high demand to the central nervous system to repair itself (Unal-Cevik, Kilinc, et al. 2004, Gusel'nikova and Korzhevskiy 2015, Duan, Zhang, (Unal-Cevik, Kilinc et al. 2004, Gusel'nikova and Korzhevskiy 2015, Duan, Zhang et al. 2016). In a recent *in vitro* study, it was shown that knocking out the PTBP2 protein induced alternative splicing of genes involved in synaptic transmission, signal transduction, and axon formation, but if NeuN/Rbfox3 was knockdown, this enhancement was blocked (Zhu, Fisher et al. 2023)..

4. General discussion and conclusion

In the present study, we provide evidence of the neurogesis and migration potential of AVP-MNNs in adult rats. The main findings of this study are three folds. First, through systemic neuroanatomical analysis, we conclude that an important population of AVP expressing neurons from the hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei migrate to adjacent subcortical regions, including the lateral hypothalamus, zona incerta, bed nucleus of stria terminalis hypothalamic lateral posterior division, the nucleus basalis of Meynert, the magnocellular nucleus of the lateral hypothalamus (MCLH) and entopeduncular nucleus, coinciding with visible streams of radial glial-like cells and processes. Tangentially migrated AVP+ cells were observed following AVP+ longitudinal tunnel-like bundles and matrices, that were outlined by GFPA+ radial glial-like fibers. Second, AVP-MNNs can undergo cell division that can be increased as a response to a repeated osmotic stressor. Third, the expression of the neuroblast marker doublecortin (DCX) lack of expression of NeuN in the MNN nuclei, suggested a transcriptomic identity MNN that is different to the adjacent hypothalamic nuclei.

The paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus are known for their role in the integration of hormonal, neuroendocrine and behavioral responses as well as to be central hubs for adaptation to stress (de Wied, Diamant et al. 1993, Zhang, Hernandez et al. 2016, Hernandez-Perez, Hernandez et al. 2019, Hernandez-Perez, Hernandez et al. 2022). Animal studies have shown that exposure to stressors during the perinatal period leads to the increased metabolic activity of Arginine-vasopressin magnocellular neurosecretory neurons (AVP-MNNs) leading to increased volume and to a robustness in their dendritic branching (Zhang, Hernandez et al. 2012).

The process that blood vessels guide the neuronal migration has been reported previously to occur with adult neuroblasts as scaffolds (Bovetti, Hsieh et al. 2007, Segarra, Kirchmaier et al. 2015). These neuronal precursors are guided by several factors originated in the vasculature that use tyrosine kinase-associated receptors to activate migration. Examples of this are the presence of vascular brain derived neurotrophic factor, BDNF which is trapped by glial following its production by vascular cells to control the stationary/migratory phase of neuroblasts along the rostral migratory stream (Grade, Weng et al. 2013) or the vascular endothelial growth factor (VEGF), that regulates glial processes to orientate and reorganize the vascular scaffolding as well as to act as a chemoattractant to neuronal progenitors (Bozoyan, Khlghatyan et al. 2012). This latter observations result of interest as there are studies showing that magnocellular neurons are able express VEFG (Alonso, Gallibert et al. 2008) and that vasopressin is able

to induce the production of VEGF via the stimulation of V1aR in endothelial cells (Tahara, Tsukada et al. 2011).

We are aware about the limitation of the first finding drawn based on our anatomical observation on serial sections from wild type animals and analysis. The conclusion solely based on these data shall be only intuitive. However, we have an ongoing collaboration with the Grinevich laboratory (Heidelberg University) who has created a line of transgenic rat with AVP-IRES2-Cre knock-in (Krabichler, Lefevre et al. 2023) (journal publication in preparation). In the supplemental information (SI) we report a case of an adult female rat with an injection AAV Cre-dependent virus vector targeted the supraoptic nucleus (SON). One month later, the rat was euthanized through anesthesia, perfusion and fixation. The authors of this study were provided the brain tissue and processed of IHC against mCherry. We found surprisingly many similar features of tangential migrating mCherry immunostained cells from this AVP-Cre rat's brain confirming our wild type rats using IHC against vasopressin (SI figure 1 vs Fig 2-6). It is worth noting that the injection was precise and specific. Two adjacent AVP containing structures, the suprachiasmatic nucleus (SCN) and the retro-supraoptic nucleus (SOR) are free of labeling (SI Fig. 1, G and H). The physiological meaning of this phenomenon, *i.e.,* adult AVP expressing MNN to disperse to other subcortical regions (*vide supra*) is mysterious, however, it raised important questions whether hypothalamic AVP system plays role(s) for adult brain health vs neurodegeneration. For instance, in a study using in situ hybridization in human tissues, Swaab and col. reported that the amount of AVP mRNA in hypothalamic suprachiasmatic nucleus of Alzheimer's disease (AD) patients showed 3-fold decrease vs control (Liu, Zhou et al. 2000). In another study, the authors report that patients with AD have decreased response to hyperosmolarity challenge and are prone to dehydration (Albert, Nakra et al. 1994). In a recent study (journal publication in preparation, for conference proceeding and presentations see (Duque, Greenwood et al. 2023, Mecawi 2023)) reported intriguing multi-omic data that described the spatial transcriptome, proteome, phosphoproteome, and lipidome data from the hypothalamic supraoptic nucleus that there are four groups with distinct genetic features within the SON, that are all related to AVP MCNs, offering new insight on possibilities of other AVP magnocelular groups within the SON, other than hypothalamo-neurohypophysial system and candidates to study the mechanisms involved in regulating those neurons under physiological and pathological contexts (Duque, Greenwood et al. 2023).

Regarding the neurogenesis in adult hypothalamus, one of the first studies that suggested that the hypothalamus could bear neurogenic potentials was published almost twenty years ago, where the authors showed the presence of cells with immunohistochemical and electrophysiological characteristics of immature neurons in tissue cultures derived from adult rat hypothalamus (Evans, Sumners et al. 2002) and a subsequent study showed that adult rat hypothalamus cultured neurogenic precursors had the capacity to generate neuropeptide-expressing neurons (Markakis, Palmer et al. 2004). Interestingly a new population of radial glia-like neural stem cell population has been recently reported in the postnatal hypothalamus, where Irx3 and Irx5 transcription factors modulate neurogenesis of postnatally generated leptin-sensing neurons (Son, Dou et al. 2021). A recent comprehensive transcriptomic study has reported change of 2247 RNA transcripts, including Ephrin receptor 6, a gene related to adult neurogenesis, in the SON of Wistar Hannover rats's SON AVP MCNs, after 3 days of water deprivation (Pauza, Mecawi et al. 2021). The possible role of neuropeptides in neurogenesis has been studied extensively in hippocampus,

for a review see (Zaben and Gray 2013). For instance, oxytocin has been shown to promote adult neurogenesis in rat hippocampus (Leuner, Caponiti et al. 2012, Lin, Chen et al. 2017) and vasopressin has shown to have a neuroprotective/antiapoptotic role in hippocampal neurons cultures (Chen and Aguilera 2010). However, to our knowledge, no further studies have evaluated the possible neurogenic potential of hypothalamic magnocellular neuropeptidergic neurons.

Furthermore, the presence of DCX in AVP immunopositive neurons and the absence of the mature neuron marker NeuN suggest that a significant portion of population are neuroblasts that they have the ability to migrate as DCX is a known marker of cell proliferation as well as migration (Gleeson, Lin et al. 1999, Filipovic, Santhosh Kumar et al. 2012). The lack of expression of NeuN in the magnocellular PVN and SON nuclei and segment of the suprachiasmatic nucleus that the majority of the neurons express vasopressin, suggested a transcriptomic identity that is different to the adjacent hypothalamic nuclei. It has been reported that some neuronal populations express low levels of NeuN (Kumar and Buckmaster 2007) recently identified as an epitope of Rbfox3, an alternative splicing regulator (Kim, Adelstein et al. 2009) (Kim, Adelstein et al. 2009). Interestingly, many of the regions identified as not expressing NeuN, are known to exhibit postnatal neurogenesis*, i.e.* the subventricular zone (Lois and Alvarez-Buylla 1994, Lois, Garcia-Verdugo et al. 1996), the hippocampal subgranular zone of dentate gyrus(Gage, Kempermann et al. 1998), the cerebellum (Ponti, Peretto et al. 2008), and the substantia nigra (Zhao, Momma et al. 2003). A number of several other studies have shown that in local pathophysiological situations (disease/lesions) there is a reduction in the expression of NeuN without implying cellular death, but loss of immunoreactivity (Duan, Zhang et al. 2016). For instance, after axotomy of facial nerve there is a reduction of NeuN immunoactivity in facial nucleus neurons (McPhail, McBride et al. 2004); after stroke, there is a reduction in neurons expressing NeuN in the penumbra and ischemic areas (Unal-Cevik, Kilinc et al. 2004), a re-localization of NeuN to the cytoplasm of HIV infected neurons has been reported (Lucas, Calvez et al. 2014). It has also been reported that a decreased expression of NeuN immunoactivity in hippocampal neurons is linked with exposure to neurotoxins (Collombet, Masqueliez et al. 2006) and a temporary loss of NeuN immunoreactivity in hippocampal neurons after radiation (Wu, Li et al. 2010). The above mentioned studies suggest that the non- or low- NeuN expression may be intimately linked with neuronal renewal mechanisms conserved to face brain homeostatic challenges and adaptation.

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