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Subtle sex differences in vasopressin mRNA expression in the embryonic mouse brain

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Abstract

Arginine vasopressin (AVP) is a neuropeptide which acts centrally to modulate numerous social behaviors. One receptor subtype through which these effects occur is the AVP 1a receptor (AVPR1A). The modulatory effects of Avp via the AVPR1A varies by species as well as sex, since both AVP and the AVPR1A tend to be expressed more prominently in males. Beyond these neuromodulatory effects there are also indications that the AVP system may play a role in early development to, in part, organize sex-specific neural circuitry that is important to sexually dimorphic social behaviors in adulthood. However, to date, AVP's role in early development is poorly understood, particularly with respect to its differential effect on males and females. In order to determine the timing and distribution of the AVP system in early brain development, we examined the brains of male and female C57BL/6J mice between embryonic day (E) 12.5 and postnatal day (P) 2 and quantified *Avp* and *Avpr1a* mRNA using qPCR and AVPR1A protein using receptor autoradiography. The mRNA for *Avp* was measurable in males and females starting at E14.5, with males producing more than females, while *Avpr1a* mRNA was found as early as E12.5, with no difference in expression between sexes. AVPR1A binding was observed in both sexes starting at E16.5, and while there were no observed sex differences, binding density and the number of neuroanatomical areas did increase over time. These data are significant as they provide the first whole-brain characterization of the vasopressin system in the embryonic mouse. Further, these findings are consistent with data from other species, that have documented a sex difference in the vasopressin system during early brain formation.

Keywords

embryonic development; receptor autoradiography; vasopressin; vasopressin 1a receptor

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Conflict of interest statement

The authors of the manuscript have no conflicts of interest to declare.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Introduction

Arginine vasopressin (AVP) is a nine amino acid neuropeptide whose sequence is conserved across mammalian species and whose actions are associated with the regulation of homeostatic functions in addition to the modulation of behavior [1]. AVP is synthesized mainly in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus where it is either released into the bloodstream via the posterior pituitary gland or throughout the brain via its central projections [2]. While there are three identified AVP receptors, the most prevalent in the brain and spinal cord is the AVP 1a receptor (AVPR1A) [1]. In adult rats and mice, the AVPR1A is commonly found in the somatosensory cortex (SC), olfactory tubercle (OT), nucleus accumbens, lateral septum (LS), hippocampus, amygdala, bed nucleus of the stria terminalis (BNST), hypothalamus, and ventral tegmental area (VTA) [3,4]. Its presence in these brain regions is known to be important to the modulation of a variety of social behaviors, including aggression, social memory, vocal communication, and social play [5–8].

While the vasopressin system is broadly distributed throughout the brain, the amount of AVP, as well as the specifics of its location and the density in AVPR1A expression, varies between sexes. Beyond the PVN and SON, AVP is also produced in several other brain regions, where it tends to be more abundant in males as compared to females. In male rats and mice, these regions include the suprachiasmatic nucleus, the BNST, the medial amygdala (MeA), the LS, and the medial preoptic area (MPOA) [9–13]. Similar to observations of AVP, AVPR1A binding is elevated in the SC, BNST, dentate gyrus, and hypothalamus of adult male rats compared to female rats[14]. Sex-specific differences in AVPR1A expression have also been observed in other rodent species [15,16], suggesting that sex differences in the vasopressin system may be evolutionarily conserved in mammals.

While males having more peptide and/or AVPR1A than females in specific brain regions does not necessitate sex differences in the function of this system, there is evidence to support the assertion that these sex-specific anatomical differences can have behavioral consequences [14]. That said, only a handful of studies have directly compared the vasopressin systems of males and females and its impact on behavior, and the data from these studies are mixed. For instance, in one study of *Avpr1a* knockout mice, it was reported that males, but not females, have decreases in anxiety-like behaviors [6]; another study in a different line of these mice did not find these same effects [17]. Peripheral administration of an AVP antagonist affects social memory in adult male rats, but not females [18,19], whereas central administration of an AVPR1A antagonist impairs social recognition in adult rats of both sexes, while having no effect in juveniles [20]. Though, in juveniles, central administration of an AVPR1A antagonist does have sex-specific effects on play behavior, i.e. it results in a decrease in males and an increase in females [8], possibly due to changes to AVPR1A expression around the time of puberty[21]. Taken together these data suggest that there are many neuroanatomical- and species-specific effects with respect to possible sex differences in AVP's modulation of behavior. Further, if the vasopressin system and its contributions to behavior are to be understood, there is clearly a need to consider how the age of an animal as well as their sex may interact.

If we look earlier in development, evidence suggests that the vasopressin system may have an important role in the developing brain — perhaps helping to organize sex-specific brain circuits. In a study published by Yamamoto and colleagues[22], postnatal administration of oxytocin (OXT) or an OXT antagonist to prairie voles on postnatal day (P)1, results in changes in AVP immunoreactivity only in males, but not females. During prenatal development, in rats, AVP protein and AVPR1-type receptors are found as early as embryonic day (E) 16 [23,24]. In mice, *Avp* mRNA is found in the hypothalamus as early as E13.5 [25,26], while AVPR1A binding is observed at P0 in the neocortex, as well as the septum, hippocampus, and VTA [27]. So far, the data suggest that both AVP and AVPR1A expression tend to increase over the course of development, and that AVPR1A expression tends to shift in terms of its region-specific expression [21,27,28]. Perhaps due to these types of dynamic changes in the system, there have been few studies that have made comparisons across time points. Even more scarce are studies that have compared males and females within the same study.

As little is known, or understood, about the developing vasopressin system, we sought to understand how this system emerges and changes during embryonic development in both males and females. This study differs from previous efforts to examine differences in the vasopressin system as it is the first to examine multiple embryonic timepoints, in addition to a postnatal time point, in both males and females. It is also the first whole-brain characterization of the AVPR1A distribution across embryonic mouse development. Given the lack of information regarding late development in embryonic mice, we chose to specifically focus on *Avp* and *Avpr1a* mRNA expression, as well as AVPR1A binding. These experiments align with some of our earlier work, which reported marked sex differences in the developing Oxt system, with females producing *Oxt* transcripts before males, and having greater expression of both *Oxt* and the *Oxt* receptor (OXTR) mRNA at all measured timepoints [29]. Based on the sequence similarities of the two peptides and documented receptor crosstalk [30], we hypothesized that AVPR1A expression would be initiated around the same time as the expression of the OXTR. We further hypothesized that we would see elevated levels of transcripts for *Avp* and *Avpr1a* in males; this hypothesis was based on the known sex differences in expression of the vasopressin system in adult rodents, as well as previous reports regarding OXT expression [22,29]. We are optimistic that the information gained by this study will help inform future studies on the possible organizational effects of the vasopressin system during fetal life and possible downstream behavioral effects.

Methods

Tissue collection

C57BL/6J dams between the ages of two and four months were mated with experienced C57BL/6J breeder males for up to two weeks until pregnant. Pregnancy was determined by presence of a sperm plug, considered E0.5. At the appropriate timepoint (E12.5, E14.5, E16.5, or E18.5) dams were euthanized via cervical dislocation and the embryos collected. Once removed, the embryos were decapitated and the heads fresh frozen on dry ice. P2 pups were also decapitated and whole heads fresh frozen. For control tissue, adult male C57BL/6J

brains were collected. At the time of euthanasia, a tail snip was taken from each embryo or pup to determine their sex using PCR for the SRY gene, as described previously [29]. Tissue was stored at −80°C until processed. All experiments were conducted in accordance with protocols approved by the Kent State University Institutional Animal Care and Use Committee.

Avp and *Avpr1a* qPCR

Snout, skin, and skull were dissected away in layers from whole heads using a scalpel before the brain was sliced in half. Single hemispheres were sonicated, and the RNA isolated using TRI Reagent® (Invitrogen, Carlsbad, CA, USA; n=5 males and n=5 females from n=5 dams per time point and one adult male control). RNA purity and quality was verified using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and only samples with a 260/280 of 1.8 and above and a 260/230 of 1.6 and above were used. Reverse transcription and multiplex qPCR steps were combined using the QuantiFast® Multiplex qPCR kit (Qiagen, Hilden, Germany) and the following PrimeTime® primer-probes (Integrated DNA technologies, Skokie, IL, USA): *Gapdh*(Mm.PT.39a.1), *Avp* (Mm.PT.58.7627488), *Avpr1a* (Mm.PT.58.43862564). Each sample was run in triplicate, with blank and no-template controls run on each plate. qPCR was performed using the Stratagene Mx3005P (Agilent Technologies, Santa Clara, CA, USA) as described previously [29].

AVPR1A receptor binding

Whole heads/brains (n=5 males and n=5 females from n=5 dams for each time point and one adult male used as a positive control) were cut coronally at 15μm at −20°C on a cryostat (Leica 1950; Leica Biosystems, Buffalo Grove, IL, USA) and mounted onto Superfrost™ Plus slides (Fisher Scientific, Hampton, NH, USA). Slides were stored at −80°C until receptor binding was performed, at which time tissues were labeled with an ^{125}I linear AVP receptor antagonist (Product ID: NEX310050UC; Perkin Elmer, Waltham, MA, USA), as described previously[31]. Briefly, slides were incubated in 0.1% paraformaldehyde for two minutes, then rinsed in 1X Tris buffer for 10 minutes. Slides were then incubated in tracer buffer (50 pM with ^{125}I linear vasopressin antagonist, 1X Tris buffer, 0.001% bovine serum albumin, and 0.0005% bacitracin) for one hour. Afterwards, slides were rinsed twice in 1X Tris buffer with 0.002% MgCl for five minutes each, followed by a third rinse with agitation for 35 minutes. Lastly, slides were dipped in ice cold distilled water and dried with a hair dryer using the “cool” setting. When slides were completely dry, they were laid with an ^{125}I microscale (American Radiolabeled Chemicals, St. Louis, MO, USA) against Kodak® BioMax® MR film (Fisher Scientific, Hampton, NH, USA) for one week. Film was developed using an SRX-101A (Konica, Newark, NJ, USA) film processor. Films were scanned at 1200 dpi (HP DeskJet 3830; HP Inc, Paolo Alto, CA, USA) and binding density across the whole brain was quantified in dpm/mg using NIH ImageJ software calibrated to the microscale. Neuroanatomical specificity of binding was determined by comparing the films to cresyl violet-stained sections of the same slides used for binding, as well as fetal brain atlases [32,33]. A 25 pixel by 25 pixel box was used for all areas measured. The box was placed at a pre-determined anatomical landmark and density measured on both left and right

side across three consecutive sections. Each measure was subtracted from the film background before being averaged for each animal.

Statistics

For qPCR: C_q values for *Avp* and *Avpr1a* were each subtracted from the reference gene (*Gapdh*) per well, then averaged for each sample. 2Δ-C_q values were calculated for each sample and normalized to the male P2 time point for both target genes. Only samples which amplified within 45 cycles were used. A two-way analysis of variance (ANOVA) was run with sex and embryonic day as the main factors, and a Tukey post-hoc analysis was performed where appropriate. All analyses were performed using SPSS® (IBM, Armonk, NY, USA). *For receptor binding:* One-way ANOVA tests were run at each timepoint with sex as the main factor.

Results

Avp and *Avpr1a* mRNA expression

In the expression of *Avp* mRNA, there was a main effect of embryonic day ($F_{4,40}$=11.169, p<0.001) as well as sex ($F_{1,40}$=5.261, p=0.027), but no significant interaction ($F_{4,40}$=2.428, p=0.064) (Fig. 1). Specifically, a Tukey multiple comparisons analysis showed expression was significantly higher on E16.5 and P2 compared to the other time points and females were found to have less *Avp* mRNA than males. In our qualitative assessments, we noted that transcripts were first detectable in males and females at E14.5, though expression was initially weak. Expression rose at E16.5, wherein levels of *Avp* transcripts were comparable to levels observed at P2. A sharp decline in expression was found at E18.5, where it was comparable to the E14.5 timepoint.

Unlike the expression of *Avp*, in measures of *Avpr1a* mRNA there was no main effect of embryonic day ($F_{4,40}$=2.315, p=0.074), or sex ($F_{1,40}$=3.253, p=0.079), nor any interaction ($F_{4,40}$=0.827, p=0.516) (Fig. 2). Though, it should be noted that while transcripts were detected in all male E12.5 samples, they were only detected in one female E12.5 sample. Both males and females expressed *Avpr1a* transcripts at E14.5, and expression remained consistent into E16.5. Qualitatively, mean expression was highest at P2 for both sexes. Despite these fluctuations in *Avp* and *Avpr1a* levels, *Gapdh* levels remained similar between sexes at each timepoint.

AVPR1A binding

No AVPR1A binding was detectable in males or females at E12.5 or E14.5, and there was no significant difference between males and females in any brain region at E16.5, E18.5, or P2. At E16.5, faint binding was detectable in both males and females in the nucleus of the vertical limb of the diagonal band of Broca (NVL), fornix (F), OT, indusium griseum (IG), and ventral hypothalamic area (Fig. 3). No area had binding stronger than 100 dpm/mg (Table 1.1). AVPR1A binding density increased at both E18.5 and P2, in addition to appearing in more areas at each timepoint. At E18.5, receptor binding was detectable again in the NVL and OT, with additional binding observed in the diagonal band of Broca (DBB), septohippocampal nucleus (SHN), medial septal nucleus (MSN), glial wedge (GW), medial

tuberal nucleus (MTN), and the arcuate hypothalamic nucleus (AHN) (Fig. 4). In all but the SHN, binding intensity was qualitatively noted above 200 dpm/mg (Table 1.2). At P2, AVPR1A binding was again observed in the NVL, SHN, and MSN, as well as in the nucleus of the horizontal limb of the diagonal band (HLDB), SC, ventral pallidum (VP), LS, cornu ammonis 1 of the hippocampus (CA1), hippocampal area (HA), caudate putamen (CP), and caudal tuberomammillary nucleus (CTMN) (Fig. 5). Qualitative observations identified that most areas had binding intensity above 200 dpm/mg at P2, with the exception of the SC and the VP of males and females, as well as the CTMN of males (Table 1.3).

Discussion

In this study we have evaluated the developmental time course of the male and female mouse embryonic vasopressin system using qPCR for *Avp* and *Avpr1a* mRNA and receptor autoradiography for AVPR1A quantification. Transcripts for *Avp* were detectable as early as E14.5 in both males and females, with males having higher *Avp* mRNA expression than females. *Avpr1a* transcripts were detected as early as E12.5 in males, and E14.5 in females, but there were no sex differences within or between timepoints. Previous studies found *Avp* transcripts a day earlier than *Avpr1a* transcripts [25,26], which may be the case here as well, though we did not sample on E13.5. Binding for the AVPR1A was not detectable until E16.5 in males and females, and expression increased at E18.5 and again at P2. The AVPR1A was observed throughout the brain, but no differences were evident across embryonic time points, between the sexes, or within brain regions. However, our qualitative observations that binding intensity increased across ages have also been noted in the juvenile rat[21], and patterns of transient AVPR1A expression follow neuronal migration timelines for brain development in those regions [34–36].

The delay in processing AVP from its early, neurophysin-associated form may explain its offset expression from its receptor, a feature also observed in the developing Oxt system [37]. However, while we observed a dissociation in the appearance of *Avp* as compared to the *Avpr1a* in both sexes, we previously found that prenatal transcripts for *Oxt* are found only in females [29]. Based on these data, as well as previous studies showing that perinatal OXT has sex-specific behavioral effects in voles [22], it seems that OXT and AVP have unique developmental time courses during embryonic development. We speculate that they may contribute to sex-specific brain development, though what the precise consequences of these differences are, as well as the mechanism of those differences, requires further inquiry.

It is well established that in adult rodents there are sex differences in the amount of AVP produced, as well as in the density of AVPR1A expression, in specific brain regions[10,14]. Our data suggest that while there are sex differences in the expression of *Avp*, there are not sex differences in *Avpr1a* or AVPR1A density or distribution. It is however important to consider that the absence of a sex difference in the *Avpr1a* may well be an artifact of our sampling method and sampling intervals, which utilized half-hemispheres of fetal brain collected every other day. As our goal was to determine when in development the AVPR1A was transcribed and translated in the fetal brain, this was the most reasonable approach. However, it will be important for future studies to seek more targeted avenues to determine if there are subtle, brain region-specific, sex differences in either *Avp* or *Avpr1a* expression

in the fetal brain. It may also be that sex-dependent differences in the vasopressin system might emerge briefly closer to the time of birth, as this is a time when the vasopressin system is known to begin to interact with the hypothalamic-pituitary-adrenal axis [38]. We also speculate that the high degree of variability in the receptor binding data could be the result of male-to-female sex ratios in utero[39,40], as testosterone is known to influence expression in the vasopressin system[13,41].

As documented in the juvenile and adult brain, we found AVPR1A binding in the LS, CP, SC, VP, hippocampus, and hypothalamus. Areas such as the MeA, BNST, MPOA, and VTA did not appear to have AVPR1A binding in any of the timepoints sampled, but it is possible that this could be related to length of film exposure. Nonetheless, the localization and intensity we see here in the AVPR1A binding is reinforced by other work completed in postnatal C57BL/6J mice [27]. Notably, all of the above areas where binding was observed are associated with the neural regulation of social behavior in adults [1,14]. Thus, these data confirm findings which suggest that AVP and the AVPR1A may have a role in the development of brain regions that are important to the neural regulation of social behavior. Perhaps contributing to the organization of the neural circuitry that is a part of the social behavior neural network.

The NVL, F, IG, DB, and GW are located at the midline and associated with the formation of the corpus callosum, which occurs with the fusion of the developing brain hemispheres at approximately E15 in mice. Glia arising from the midline initiate this process and help shape the path that neurons migrate across, eventually forming the corpus callosum [42]. mRNA for AVP has been found at E13.5 in areas expressing factors like PAX6, which affects differentiation of progenitor cells into neurons or glia and is also associated with appropriate formation of the corpus callosum [43,44]. Here, we show binding of the AVPR1A in many of these areas beginning at E16.5, and transcripts for *Avp* and the *Avpr1a* even earlier. This suggests that the vasopressin system may contribute to brain organization and connectivity by acting through neurons and/or glia. Further study is necessary to determine on which cell types the receptor is acting. Additionally, because mRNA does not always indicate protein levels or localization, determining and quantifying the presence of the AVP protein is also necessary. Nevertheless, given the conserved nature of AVP and the many roles it plays in the brain, it would be fitting that it would contribute to neurodevelopment through several avenues.

In summary, the data presented here describe the early global appearance of mRNA transcripts for *Avp* and the *Avpr1a*, as well as receptor density, in the fetal C57BL/6J mouse brain. Based on our data it is likely that the observed sex differences in the vasopressin system that are found across postnatal development and adulthood are initiated during embryonic development. It also seems that for the fetal AVP and OXT systems [29], E16.5 is a critical timepoint, as both the AVPR1A and the OXTR are first detectable at this time. However, how or if these systems interact at E16.5 to influence brain development is currently unknown. Direct comparison between systems is necessary to determine individual contributions at E16.5. Additionally, while we noted the presence of the AVPR1A in areas known to promote social behaviors, its expression varies between developmental stages. Further, it is unknown whether AVP is acting through the receptor at the times observed here

or if there is crosstalk from OXT, which could result in different outcomes [30,45,46]. Finally, the extent to which the vasopressin system influences neural organization is unclear, though given the extent of its evolutionary conservation and early presence in the central nervous system, better understanding its effect in mice may also shed light on its function in other species.

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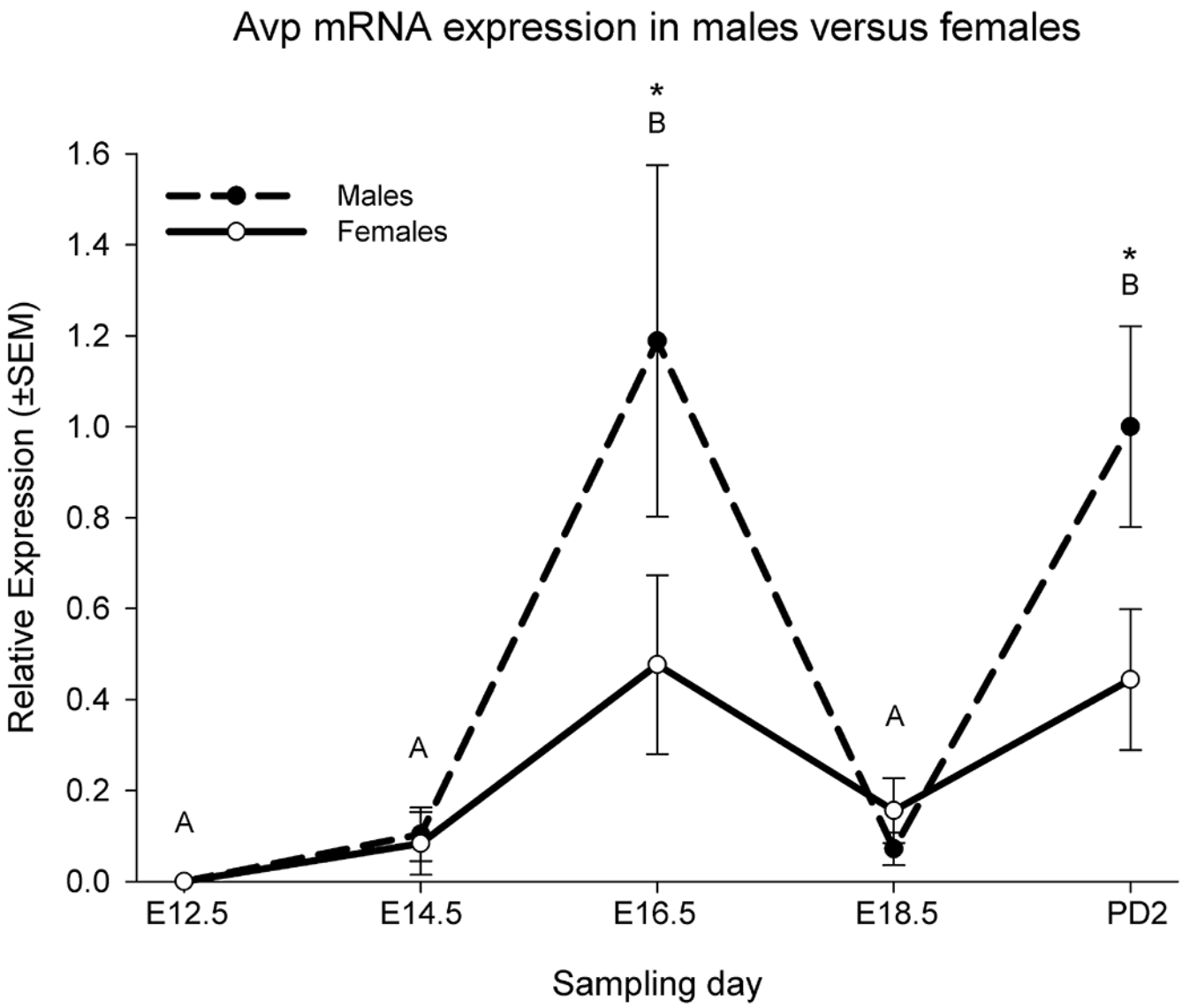

Figure 1:

Graph of arginine vasopressin (*Avp*) mRNA levels in the brains of male and female mice on embryonic day (E) 12.5 (males=5, females=5), E14.5 (males=5, females=5), E16.5 (males=5, females=5), E18.5 (males=5, females=5), and P2 (males=5, females=5). Values are expressed as a percentage relative to P2. There was a main effect of sampling day ($F_{4,40}$=11.169, p<0.001), and sex ($F_{1,40}$=5.261, p=0.027), but there was no significant interaction ($F_{4,40}$=2.428, p=.064). *Avp* was first detectable at E14.5 in both sexes. A Tukey HSD test showed E16.5 and P2 were significantly higher than the other time points, but not different from each other. * indicates p<0.005

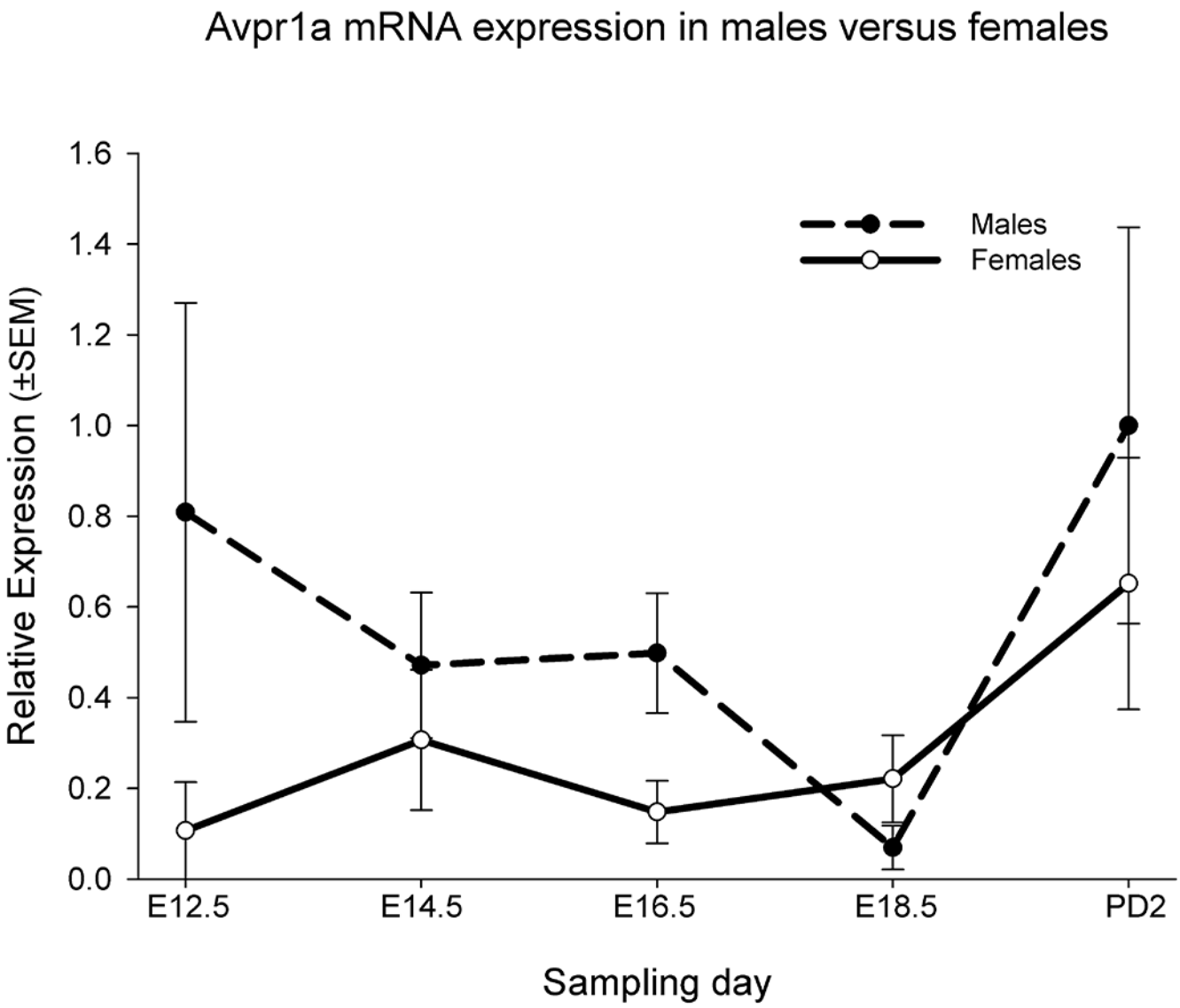

Figure 2:

Graph of arginine vasopressin 1a receptor (*Avpr1a*) mRNA levels in the brains of male and female mice on embryonic day (E) 12.5 (males=5, females=5), E14.5 (males=5, females=5), E16.5 (males=5, females=5), E18.5 (males=5, females=5), and P2 (males=5, females=5). Values are expressed as a percentage relative to P2. There was no effect of sampling day ($F_{4,40}$=2.315, p=0.074) or sex ($F_{1,40}$=3.253, p=0.079), nor was there any significant interaction ($F_{4,40}$=0.827, p=0.516). *Avpr1a* was detectable as early as E12.5 in both sexes.

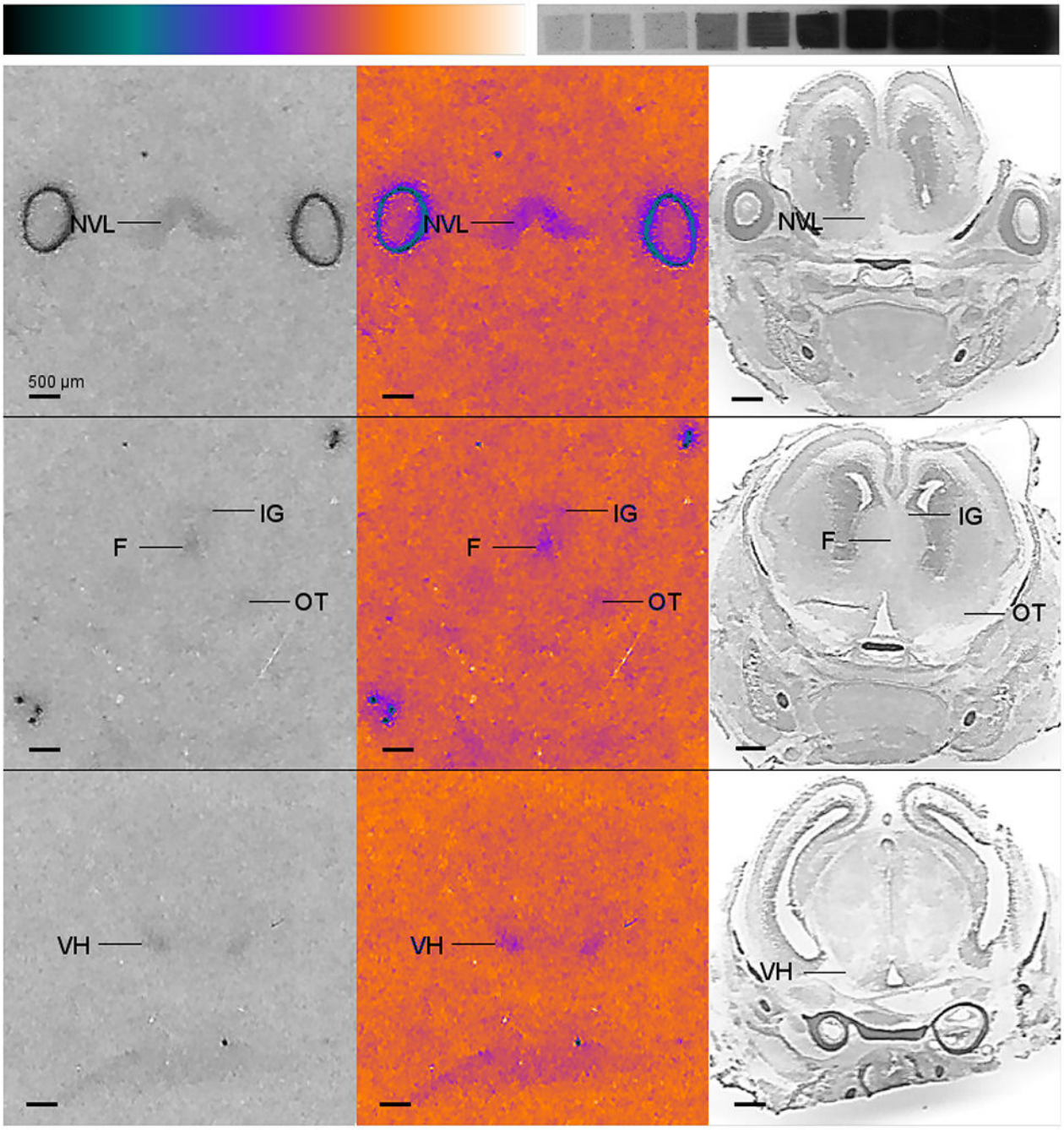

Figure 3:

Arginine vasopressin 1a receptor (AVPR1A) binding on embryonic day (E) 16.5 mice (left column). AVPR1A binding was performed using an ^{125}I-tagged linear AVP receptor antagonist on heads/brains collected at E16.5 (microscale top right), after which the same sections were cresyl-stained (right column). Representative images of the film autoradiograms were selected from both males and females as there were no observed sex differences in any area. Pseudocolor (middle column) was applied using ImageJ using the "Cool" LUT (scale top left). Scale bar = 500µm. NVL= nucleus of the vertical limb of the

diagonal band of Broca, F= Fornix, OT= olfactory tubercle, IG= indusium griseum, VH= ventral hypothalamus

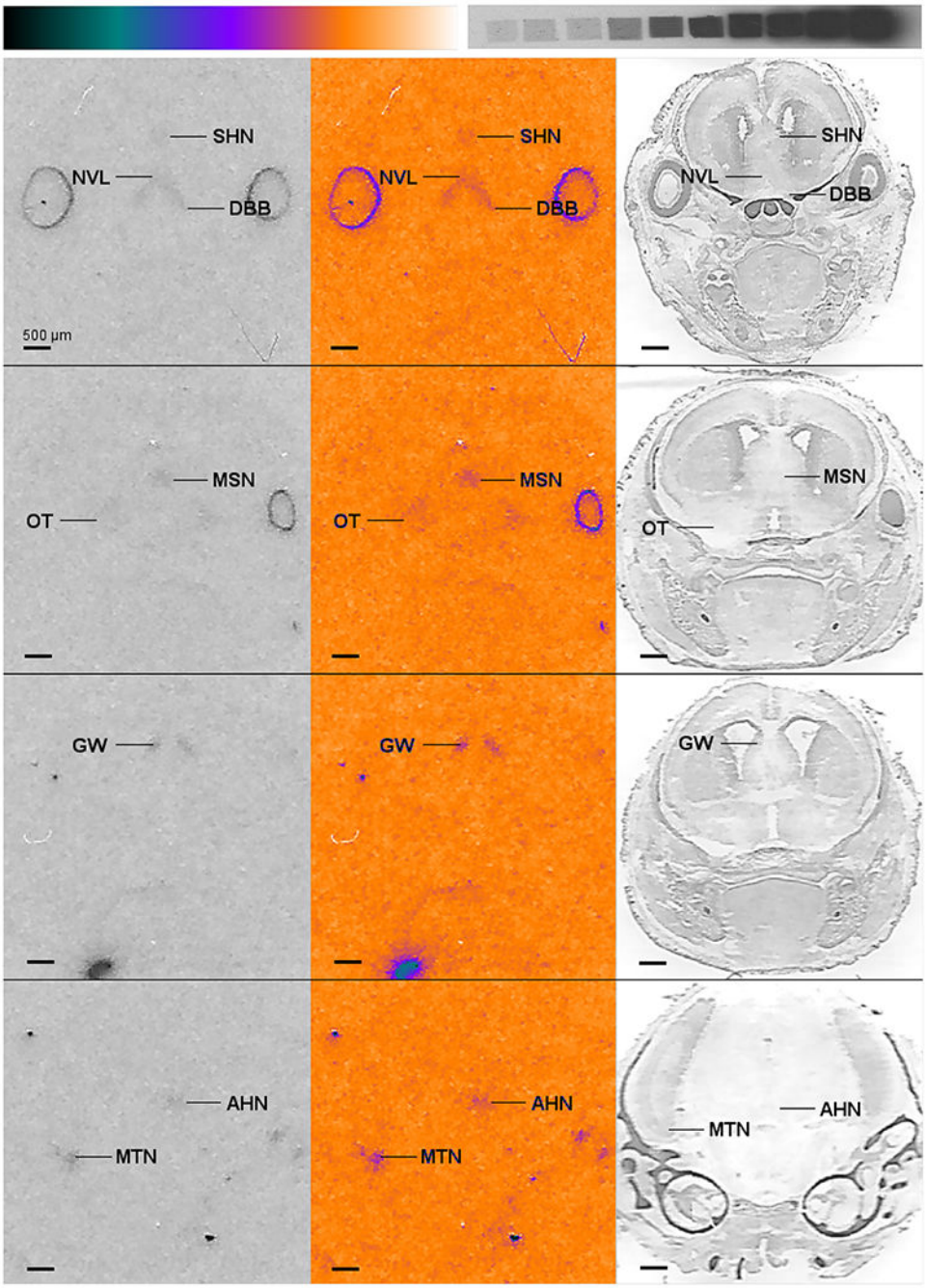

Figure 4:

Arginine vasopressin 1a receptor (AVPR1A) binding on embryonic day (E) 18.5 mice (left column). AVPR1A binding was performed using an ^{125}I-tagged linear AVP receptor antagonist on heads/brains collected at E18.5 (microscale top right), after which the same sections were cresyl-stained (right column). Representative images of the film autoradiograms were selected from both males and females as there were no observed sex differences in any area. Pseudocolor (middle column) was applied using ImageJ using the "Cool" LUT (scale top left). Scale bar = 500µm. NVL= nucleus of the vertical limb of the

diagonal band of Broca, DBB= diagonal band of Broca, SHN= septohippocampal nucleus, OT= olfactory tubercle, MSN= medial septal nucleus, GW= glial wedge, MTN= medial tuberal nucleus, AHN= anterior hypothalamic nucleus

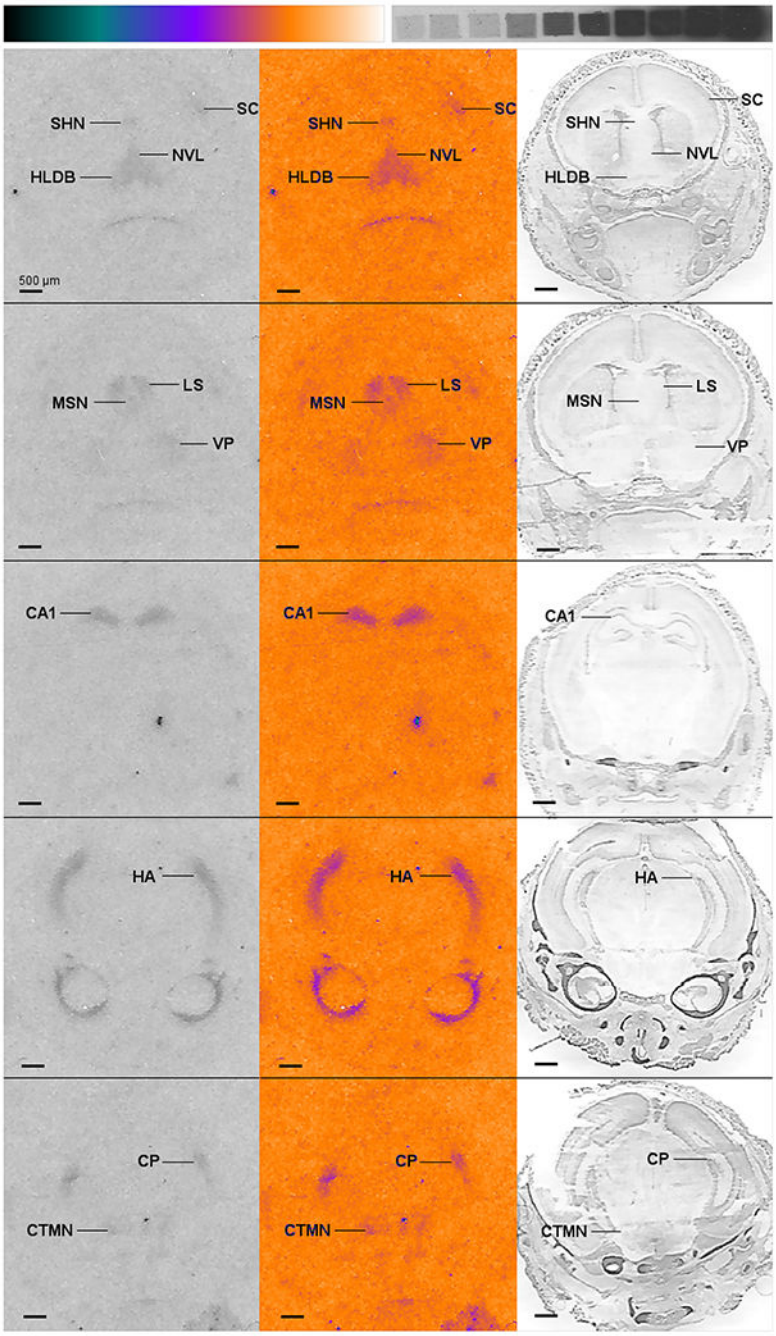

Figure 5:

Arginine vasopressin 1a receptor (AVPR1A) binding on postnatal day (P) 2 mice (left column). AVPR1A binding was performed using an ^{125}I-tagged linear AVP receptor antagonist on heads/brains collected at P2 (microscale top right), after which the same sections were cresyl-stained (right column). Representative images of the film autoradiograms were selected from both males and females as there were no observed sex differences in any area. Pseudocolor (middle column) was applied using ImageJ using the "Cool" LUT (scale top left). Scale bar = 500µm. NVL= nucleus of the vertical limb of the

diagonal band of Broca, HLDB= nucleus of the horizontal limb of the diagonal band, SC= somatosensory cortex, SHN= septohippocampal nucleus, MSN= medial septal nucleus, LS= lateral septum, VP= ventral pallidum, CA1= cornu ammonis 1 of the hippocampus, HA= hippocampal area, CP= caudate putamen, CTMN= caudal tuberomammillary nucleus

Table 1:

Table of statistical results comparing binding density (in dpm/mg) of the arginine vasopressin 1a receptor (AVPR1A) in the brains of male (grey band) and female (white band) mice following the application of an ^{125}I-tagged linear AVP receptor antagonist. No brain regions exhibited a significant difference between sexes at embryonic day (E) 16.5, E18.5, or postnatal day (P) 2.

1.1: Binding density (in dpm/mg) in male and female mice at E16.5			
Brain Regions at E16.5	**Mean dpm/mg ± SEM**	**F**	**p-value**
*NVL	♂ 27.983 ± 24.283	$F_{1,8}$=0.253	0.630
	♀ 52.822 ± 47.122		
F	27.946 ± 23.597	$F_{1,8}$=0.444	0.526
	9.926 ± 4.394		
+OT	3.066 ± 2.837	$F_{1,8}$=0.246	0.635
	1.365 ± 1.365		
IG	7.315 ± 5.886	$F_{1,8}$=0.107	0.754
	4.919 ± 3.476		
VH	7.988 ± 3.877	$F_{1,8}$=0.079	0.786
	9.743 ± 5.031		
1.2: Binding density (in dpm/mg) in male and female mice at E18.5			
Brain Regions at E18.5	**Mean dpm/mg ± SEM**	**F**	**p-value**
*NVL	♂ 465.768 ± 86.767	$F_{1,8}$=0.182	0.309
	♀ 341.529 ± 81.005		
DBB	257.686 ± 46.341	$F_{1,8}$=0.160	0.700
	296.467 ± 85.239		
+OT	199.973 ± 83.589	$F_{1,8}$=0.250	0.879
	181.483 ± 82.170		
°SHN	57.205 ± 30.665	$F_{1,8}$=0.387	0.551
	84.321 ± 31.011		
°MSN	578.886 ± 76.484	$F_{1,8}$=5.182	0.052
	344.610 ± 68.866		
GW	322.427 ± 101.484	$F_{1,8}$=0.516	0.493
	232.046 ± 74.511		
MTN	355.453 ± 95.665	$F_{1,8}$=0.690	0.430
	504.847 ± 152.353		
AHN	276.494 ± 34.855	$F_{1,8}$=0.068	0.811
	250.720 ± 118.334		
1.3: Binding density (in dpm/mg) in male and female mice at P2			
Brain Regions at P2	**Mean dpm/mg ± SEM**	**F**	**p-value**
*NVL	♂ 496.417 ± 107.854	$F_{1,8}$=0.001	0.980
	♀ 499.531 ± 54.161		
HLDB	391.521 ± 93.665	$F_{1,8}$=0.254	0.628

1.1: Binding density (in dpm/mg) in male and female mice at E16.5			
Brain Regions at E16.5	**Mean dpm/mg ± SEM**	**F**	**p-value**
	342.072 ± 28.935		
SC	57.365 ± 35.450	$F_{1,8}$=0.031	0.864
	65.749 ± 31.300		
°SHN	164.996 ± 65.538	$F_{1,8}$=0.253	0.628
	201.647 ± 29.685		
°MSN	213.580 ± 87.806	$F_{1,8}$=0.009	0.926
	204.525 ± 36.364		
LS	511.830 ± 90.628	$F_{1,8}$=0.350	0.567
	425.296 ± 114.745		
VP	66.051 ± 45.039	$F_{1,8}$=0.357	0.570
	116.726 ± 71.893		
CA1	688.263 ± 229.733	$F_{1,8}$=0.004	0.951
	667.633 ± 227.383		
HA	630.094 ± 212.698	$F_{1,8}$=0.078	0.788
	546.014 ± 214.136		
CP	399.034 ± 246.019	$F_{1,8}$=0.646	0.445
	715.444 ± 307.249		
CTMN	60.565 ± 40.039	$F_{1,8}$=0.936	0.362
	280.070 ± 223.271		

SEM = standard error of the mean, NVL= nucleus of the vertical limb of the diagonal band of Broca, F= Fornix, OT= olfactory tubercle, IG= indusium griseum, VH= ventral hypothalamus, DBB= diagonal band of Broca, SHN= septohippocampal nucleus, MSN= medial septal nucleus, GW= glial wedge, MTN= medial tuberal nucleus, AHN= anterior hypothalamic nucleus, HLDB= nucleus of the horizontal limb of the diagonal band, SC= somatosensory cortex, LS= lateral septum, VP= ventral pallidum, CA1= cornu ammonis 1 of the hippocampus, HA= hippocampal area, CP= caudate putamen, CTMN= caudal tuberomammillary nucleus.

* indicates area appears in all three sampling days,

+ indicates area appears in E16.5 and E18.5,

° indicates area appears in E18.5 and P2.