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Short communication

Plasma vasopressin concentrations positively predict cerebrospinal fluid vasopressin concentrations in human neonates

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ABSTRACT

Central arginine vasopressin (AVP) plays a critical role in mammalian social behavior and has been hypothesized to be a biomarker of certain human neurodevelopmental disorders, including autism. However, opportunities to collect post-mortem brain tissue or cerebrospinal fluid (CSF) from children are extremely limited, and the use of less invasive peripheral assessments (e.g., blood, urine, or saliva) of AVP as a proxy for more invasive central measures has not been well validated. Further, almost nothing is known about AVP biology in very young infants. Therefore in the present study we concomitantly collected basal CSF and plasma samples from $N = 20$ neonates undergoing clinical sepsis evaluation (all were sepsis negative) and quantified AVP concentrations via well-validated enzyme-immunoassay methodology. Plasma AVP concentrations significantly and positively predicted CSF AVP concentrations ($r = 0.73$, $p = 0.0021$), and this relationship persisted when variance attributed to sex, gestational age, and sample collection time was controlled for in the statistical model ($r = 0.75$, $p = 0.0047$). These findings provide preliminary support for the use of basal plasma AVP measurement as a proxy for basal brain AVP activity in pediatric populations. Future studies are now required to determine the relationship between behavioral measures and AVP concentrations in both central and peripheral compartments in young infants and older children.

Introduction

The neuropeptide arginine vasopressin (AVP) has been linked to a wide variety of physiological processes (e.g., water balance and cardiac function) and more recently to complex psychological processes including human social cognition and behavior [15]. Several studies have outlined the potential for utilizing plasma AVP concentrations as a biomarker of disease status in neuropsychiatric disorders, including autism and schizophrenia [17,20]. Indeed, our group recently found that plasma AVP concentrations positively predict social cognition performance in children with autism, but not in siblings of probands or in matched neurotypical controls (D.S. Carson et al., manuscript submitted).

Plasma AVP concentrations as a biomarker of social functioning are most meaningful if they are associated with brain AVP activity. The relationship between central and peripheral nervous system AVP activity, however, is not well understood. AVP is primarily produced in the paraventricular and supraoptic nuclei of the hypothalamus. It is transported via axonal projections from hypothalamic magnocellular neurons to the posterior pituitary for storage and release into peripheral circulation where it regulates water balance, glucose, sodium, and potassium concentrations [19]. Importantly, AVP is also released centrally from the perikaryon of hypothalamic parvocellular neurons, as well as from their dendritic and axonal projections, and is delivered to a diverse range of brain regions via volume transmission. AVP is additionally released from neurons in the bed nucleus of the stria terminalis, medial amygdala, medial preoptic area, and suprachiasmatic nucleus [6]. Given that the extracellular fluid of the brain interconnects freely with

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the cerebrospinal fluid (CSF), it is generally accepted that the neuropeptide contents of CSF are a good, albeit non-specific, measure of their activity in the brain [11]. In adults, CSF circulates within the ventricular system of the brain and is thought to be reabsorbed in to the vascular system by entering the dural venous sinus via the arachnoid granulations. There is some evidence, particularly in neonates in which arachnoidal granulations are sparse, that suggests CSF largely flows along cranial nerves and spinal nerve roots where it then enters into lymphatic channels and subsequently in to circulating venous blood [27]. Further, differences in the anatomy of the blood–brain barrier (BBB) in neonates and adults, whereby the endothelial junctions of the brain's venous system are not as tightly formed during the early stages of life compared with adulthood, potentially allows for larger molecules (including neuropeptides) to flow more freely between the brain and body [22]. Thus, although large molecules such as proteins and neuropeptides are known to be sequestered by the BBB, the mechanisms of potential shared central and peripheral circulation of large molecules in neonates, or during certain disease states, may differ meaningfully from healthy adults [1].

There are some data from preclinical research that shows stimulation of AVP release from the neurohypophysis inhibits the release of AVP from the magnocellular perikaryon and dendrites, which in turn reduces further release from the neurohypophysis. These studies have largely measured concomitantly collected central microdialysates and peripheral blood samples following both psychological and physiological stressors in adult rodents [5,12]. Thus, how these findings inform our understanding of synchronized central and peripheral release patterns under basal conditions across the lifespan largely remains unclear. Further, previous studies investigating the relationship between CSF and plasma AVP concentrations in adult animals and humans have reported mixed findings [10,24,25], likely due to state specific factors (e.g., the stress of forced swimming) in animals, or disease and physiological imbalance (including osmolality and natremia status) inherent in the clinical indications that require invasive lumbar CSF sampling in humans. This collective information indicates that it is essential to consider all such variables in studies of AVP concentrations in both central and peripheral compartments and further highlights the importance of assessing neuropeptide activity during the early stages of life.

Given the growing interest in the role of AVP in neurodevelopmental disorders, there is an urgent need to clarify the relationship between basal CSF and plasma AVP concentrations in young humans. Due largely to the invasive nature of assessment, there has been only one prior pediatric study that investigated the relationship between AVP concentrations in concomitantly collected CSF and plasma samples. Bartrons et al. [2] provided evidence for a positive relationship between CSF and plasma AVP concentrations in neonatal humans with hypoxic-ischemic encephalopathy. Our research group was in a unique position to analyze basal AVP concentrations in concomitantly collected CSF and plasma samples from a small cohort of neonatal humans undergoing clinical sepsis evaluation. Only a small portion (< 5%) of these patients is generally found to be sepsis positive, which provides the rare opportunity to assess AVP concentrations in disease/syndrome free human newborns.

Methods

Participants

The study was approved by the Stanford University Institutional Review Board. Twenty human neonates (11 males, 9 females) undergoing clinically indicated sepsis evaluation for standard risk factors (e.g., maternal fever, prolonged rupture of membranes, infant respiratory distress) were recruited to the study. All subjects received <48 h of antibiotic treatment. All participants were found to be sepsis negative. Gestational age ranged from 31 to 40 weeks at birth ($M = 36.1$, $SD = 3.2$). Demographic information and maternal-infant medical condition necessitating neonatal CSF collection are presented in Table 1. Exclusion criteria consisted of known chromosomal anomalies and major malformations on the basis of historical evidence. A subset of these participants was included in a previously published study from our group [4].

Sample collection and processing procedures

Parental consent was obtained prior to initiation of the study procedures. Within 72 h of birth, and at the time of clinically indicated lumbar puncture, additional CSF (up to 0.5 mL) was obtained for research purposes using standard sterile procedures, and whole blood (up to 3 mL) was collected into chilled, aprotinin-treated EDTA vacutainer tubes from a central or arterial line, or via heel prick, if a line was not placed. CSF was immediately aliquoted and snap frozen on dry ice, and stored at −80 °C until the time of the assay. Blood samples were transported on wet ice, centrifuged at 4 °C for 10 min at 1300 × *g*, and then the plasma supernatant was aliquoted and snap frozen on dry ice, and stored at −80 °C until the samples were assayed in the Parker Laboratory.

Sample preparation and vasopressin quantification

CSF and plasma AVP concentrations were quantified using a commercially available enzyme immunoassay kit (Enzo Life Sciences, Inc., Farmingdale, NY). This kit has been validated for use in humans and is highly specific and selectively recognizes AVP and not related peptides (i.e., cross reactivity with oxytocin is <0.001%). Our laboratory has optimized procedures for determination of AVP concentrations in a variety of biological matrices (i.e., plasma, saliva, CSF) and in several species (i.e., adult, child, and infant humans as well as in juvenile and adult rhesus monkeys) using the methodology outlined here. Data obtained from an optimization experiment using neonatal CSF and plasma samples, run in duplicate, provided evidence that 300 μL of unextracted and hyperconcentrated CSF, and 400 μL of extracted and hyperconcentrated plasma, was sufficient for measuring AVP concentrations above the limit of detection. The minimum assay sensitivity where the standard curve is still linear is 3.39 pg/mL. A trained technician without knowledge of the experimental conditions performed sample preparation and AVP quantification. All sample preparation procedures were initiated by thawing samples in an ice bath. CSF samples were then mixed with an equal volume of ice-cold acetone, briefly vortexed, and centrifuged at 1 °C for 15 min at 4,000 × *g* prior to hyperconcentration (see below). Based on our optimization experiment, the small volumes of CSF available for research purposes, and evidence that there is minimal matrix interference in CSF, we followed the methods outlined in the previously published literature and did not perform extraction on these CSF samples [9,24]. However, there is a growing body of evidence that suggests extracting plasma samples is necessary in order to accurately determine concentrations of neuropeptides [14,23]. Thus, plasma samples were extracted using the solvent method recommended by the manufacturer. Briefly, equal volumes of 40:60 butanol:diisopropyl ether were added to samples prior to centrifugation at RT for 5 min at 8000 × *g*. The top organic layer was discarded and the aqueous solution transferred to a new mircocentrifuge tube. A 2:1 volume of ice cold acetone was then added to all samples prior to centrifugation at 4 °C for 20 min at 12,000 × *g*. Supernatent was then transferred to 15 mL Falcon tubes and a volume of 5:1 ice cold petroleum ether was added. Samples were

Table 1
Patient demographic and maternal-infant medical characteristics.

Patient number	CSF AVP (pg/mL)	Plasma AVP (pg/mL)	Sex	Gestational age (weeks)	Sample collection time	Indication for sepsis evaluation
1	1.5	2.2	Male	40.5	11:34	Chorioamnionitis
2	3.7	7.7	Male	40.2	18:50	Chorioamnionitis
3	2.4	2.1	Male	32.4	19:55	PPROM
4	3.9	2.9	Male	39.1	15:27	Chorioamnionitis
5	3.3	4.8	Male	36.4	12:48	Neonate respiratory distress
6	2.6	2.1	Female	31.7	20:13	Neonate respiratory distress, PPROM
7	2.8	2.4	Female	35.6	20:05	PPROM
8	1.8	3.1	Male	32.4	15:35	Neonate respiratory distress
9	5.4	8.5	Female	32.4	09:05	Neonate respiratory distress
10	2.6	3.9	Male	39.0	12:53	Chorioamnionitis
11	2.5	3.0	Male	36.6	10:34	Neonate respiratory distress
12	2.7	3.9	Male	37.0	17:24	Neonate respiratory distress
13	2.9	2.2	Female	33.5	13:25	Neonate respiratory distress
14	1.9	2.5	Female	32.6	14:10	Neonate respiratory distress
15	2.2	1.5	Male	40.2	16:09	Premature rupture of membranes

AVP, arginine vasopressin; CSF, cerebrospinal fluid; PPROM, preterm premature rupture of membranes.

briefly vortexed, centrifuged at 1 °C for 10 min at 3350 × *g*, and the top ether layer discarded. Following sample preparation, each CSF and plasma sample was transferred to a borosilicate tube where it was hyperconcentrated under a stream of nitrogen gas before reconstitution in Enzo Life Sciences assay buffer. Samples were assayed in singlicate due to limited volumes that could be safely obtained from newborns for research purposes. CSF and plasma samples were run on separate microplates, and all like samples were run together on a single microplate. All standards were run in triplicate and provided intra- and inter-assay Coefficients of Variation below 5%. Samples were read with a tunable microplate reader for 96-well format at 405 nm. The final AVP concentrations were then extrapolated from the pre-processing sample volumes. That is, for hyperconcentrated CSF with a starting volume of 300 μL we divided the values obtained from the assay by three to determine the final concentration of AVP in 100 μL of sample. This hyperconcentration method, which has been validated in our laboratory [26] and is used widely in this research field [23], provides the ability to assay small sample volumes that would otherwise be below the level of detection for the commercial EIA.

Statistical analyses

Study data were managed using REDCap [7] and analyzed using JMP V.10 (SAS Institute Inc., Cary, NC). Comparisons between AVP concentrations in CSF and plasma were determined using a paired student *t*-test. Comparisons between male and female AVP concentrations in both compartments were made using an unpaired student *t*-test. The relationship between plasma and CSF AVP concentrations was assessed using a General Linear Model, which included the following blocking factors: sex, gestational age, and sample collection time. These blocking factors were chosen as they were considered the most likely to contribute extraneous sources of variability. To correct for the blocking factors in the analysis, the regression line is partialed (controlled) for other variables in the analysis, and calculated at the mean value of those variables. Under the null hypothesis that a blocking factor has no effect, it follows from elementary mixed-model theory that the blocking factor variance will equal the error variance. As a result, including a non-primary blocking factor will subtract degrees of freedom and sum of squares (i.e., variability) from the residual such that the error variance estimate (the residual mean square) remains constant [16]. Four participants provided insufficient quantities of blood to conduct the assays and were removed from further analysis.

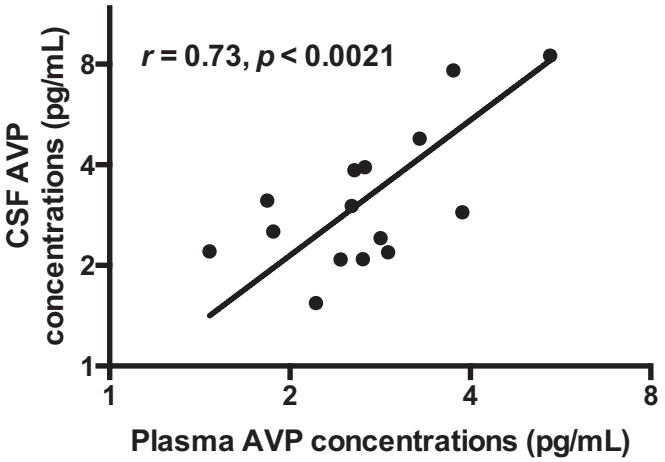

Fig. 1. Plasma arginine vasopressin (AVP) concentrations significantly and positively predict cerebrospinal fluid (CSF) AVP concentrations in neonatal humans.

Results

One of the CSF samples fell below the limit of detection, even after hyperconcentration, and was excluded from all analyses. Mean AVP concentrations were 2.77 pg/mL ($SD = 0.95$, $N = 15$) in CSF and 3.31 pg/mL ($SD = 2.15$, $N = 15$) in plasma. The data were square root transformed prior to conducting all inferential statistical analyses in order to obtain homogeneity of variance. CSF and plasma AVP concentrations did not differ significantly ($T_{13} = 1.81$, $p = 0.09$) and there were no sex differences noted for AVP concentrations in either CSF ($T_{13} = 0.83$, $p = 0.42$) or plasma ($T_{13} = 0.11$, $p = 0.91$). Plasma AVP concentrations significantly and positively predicted CSF AVP concentrations ($F_{1,13} = 14.73$, $r = 0.73$, $p = 0.0021$; Fig. 1). This strong positive relationship was maintained after controlling for the influence of the study blocking factors ($F_{1,10} = 13.09$, $r = 0.75$, $p = 0.0047$), and these blocking factors [sex ($F_{1,10} = 1.43$, $p = 0.26$), gestational age ($F_{1,10} = 0.14$, $p = 0.72$), and sample collection time ($F_{1,10} = 0.14$, $p = 0.72$)] each had no detectable effect.

Discussion

There has been an abundance of recent studies assessing the relationship between peripheral (i.e., blood, urine, saliva) AVP concentrations and psychological functioning in healthy and clinical populations [3,17,20]. Important relationships between peripheral AVP concentrations and a variety of behavioral and cognitive

processes have been documented. These studies have occurred, however, in the absence of compelling evidence that peripheral assessments of AVP are related to brain AVP activity. We addressed this important gap in knowledge in the present study by evaluating AVP concentrations in concomitantly collected CSF and plasma samples from $N = 15$ neonatal humans undergoing clinical evaluation for sepsis. We found that plasma AVP concentrations significantly and positively predicted CSF AVP concentrations ($r = 0.73$; $p = 0.0021$), and this strong positive relationship remained highly significant ($r = 0.75$; $p = 0.0047$) after controlling for variance attributed to sex, gestational age, and sample collection time. Importantly, none of these variables had a detectable effect on their own. Our findings from relatively healthy newborns are in agreement with similar findings from a study of newborns with hypoxic-ischemic encephalopathy [2]. These two complementary studies provide data on central and peripheral AVP activity in neonates ranging from relatively healthy to severely ill, with both studies yielding preliminary evidence supporting the use of basal plasma AVP concentrations as a proxy for basal brain AVP activity, at least in pediatric subjects.

Whether the predictive relationship between plasma and CSF AVP concentrations in the present study generalizes across the lifespan remains to be determined. The neonatal period represents a rapidly changing state of development when the barriers that separate the brain and body (i.e., BBB and blood-CSF barrier) may be more permeable, or more vulnerable to disruption (and therefore potentially more functionally related), than in adults (see [22] for review). Although Yang et al. [25] found a strong positive relationship between baseline CSF and plasma AVP concentrations in adult patients suffering from chronic headaches, several other studies have not found this relationship. For example, Kagerbauer et al. [10] found no relationship between pre-operative CSF and plasma AVP concentrations when analysing samples collected from adult patients undergoing spinal anesthesia for minor surgical procedures. This same research group found no evidence for a relationship between CSF and plasma AVP concentrations in adult patients with aneurysmal subarachnoid hemorrhage [13]. The reasons for these conflicting findings in adults are unknown, and the available data do not help differentiate whether the observed differences in the pediatric and adult cohorts are due to developmental differences, experimental considerations, or both. These findings nevertheless highlight the importance of rigorously addressing the extent to which developmental, state, trait, and disease characteristics influence the complex release patterns of AVP into both central and peripheral compartments [18] to better inform the validity of using proxy measures of brain activity in specific study populations. Importantly, recent evidence from our laboratory has confirmed the findings outlined here by showing a strong positive relationship between basal plasma and CSF AVP concentrations in young children and adults (D.S. Carson et al., manuscript submitted).

AVP concentrations in the present study also differed appreciably across individuals. Small increases in plasma AVP concentrations (i.e., 1–2 pg/mL) have been shown to strongly correlate with enhanced behavioral functioning in children with autism following transcutaneous electrical acupoint stimulation [28]. It therefore would have been interesting to determine whether neonatal patients who had higher or lower AVP concentrations were at increased risk to develop psychiatric or neurological disorders later in childhood. In fact, this was one of the main aims of our original study. Unfortunately, due to our small sample and high participant attrition rate, it was not possible to follow the development of these individuals as planned. Further, evidence suggests that it may be critical to control for other physiological variables, such as osmolality, that are known to impact plasma AVP concentrations before strong conclusions can be made about the relationship between AVP concentrations in the two compartments [8,19]. Due to the limited sample volumes available for research purposes and the limitations set by clinical protocols on what chemistry labs can be conducted on the available sample volumes from neonates, we were unfortunately unable to assess the impact of these other variables.

Biomarkers that facilitate early detection of disease are extremely valuable, as early interventions are crucial for maximally altering the developmental trajectories of individuals with disorders such as autism [21]. Recruitment from hospital nurseries allows for potentially very early identification of children at risk for neurodevelopmental disorders (e.g., premature infants), and future research should pursue this outcome-based approach in larger neonatal cohorts with the inclusion of a wider range of physiological and behavioral covariates. In conclusion, we provide evidence that basal plasma AVP concentrations positively predict basal CSF AVP concentrations in a small sample of neonatal humans. These findings provide support for claims that the measurement of AVP in plasma samples might be a valid tool for inferential assessment of brain AVP activity, at least in pediatric populations. Future studies are now needed to investigate the complex relationships between CSF and plasma AVP activity and various physiological and psychological states across the lifespan.

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Conflict of interest

All authors declare that they have no conflicts of interest.

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