Published in final edited form as:

Prog Brain Res. 2008 ; 170: 43–51. doi:10.1016/S0079-6123(08)00404-4.

Experimental approaches for the study of oxytocin and vasopressin gene expression in the central nervous system

Elka M. Scordalakes^{1,2}, Chunmei Yue¹, and Harold Gainer^{1,*}

¹Laboratory of Neurochemistry, National Institute of Neurological Disorder and Stroke, National Institutes of Health, Bethesda, MD, USA

²Department of Pharmacology, University of Texas Health Science Center of San Antonio, San Antonio, TX, USA

Abstract

Intron-specific probes measure heteronuclear RNA (hnRNA) levels and thus approximate the transcription rates of genes, in part because of the rapid turnover of this intermediate form of RNA in the cell nucleus. Previously, we used oxytocin (*Oxt*)- and vasopressin (*Avp*)- intron-specific riboprobes to measure changes in *Oxt* and *Avp* hnRNA levels in the supraoptic nucleus (SON) by quantitative in situ hybridization (ISH) after various classical physiological perturbations, including acute and chronic salt loading, and lactation. In the present experiments, we used a novel experimental model to study the neurotransmitter regulation of *Oxt* and *Avp* gene expression in the rat SON in vivo. Bilateral cannulae connected via tubing to Alzet osmotic mini-pumps were positioned over the SON. In every experiment, one SON was infused with PBS and served as the control SON in each animal, and the contralateral SON received infusions of various neurotransmitter agonists and antagonists. Using this approach, we found that *Avp* but not *Oxt* gene expression increased after acute (2–5 h) combined excitatory amino acid agonist and GABA antagonist treatment, similar to what we found after an acute hyperosmotic stimulus. Since both OXT and AVP are known to be comparably and robustly secreted in response to acute osmotic stimuli in vivo and glutamate agonists in vitro, our results indicate a dissociation between OXT secretion and *Oxt* gene transcription in vivo.

Keywords

oxytocin; vasopressin; gene expression; glutamate; heteronuclear RNA; alzet osmotic mini-pumps; osmotic regulation

Introduction

Studies of neuropeptide gene expression in the central nervous system (CNS) are usually performed by in situ hybridization (ISH) using exon-specific probes and measure the steady-state levels of mRNA, which reflect both gene transcription and mRNA degradation processes in the neuron. In contrast, measurements using intron-specific probes measure pre-

*Corresponding author. Tel.: +301 496 6719; Fax: +301 496 1339; gainerh@ninds.nih.gov.

mRNA or heteronuclear RNA (hnRNA) levels in the neuron which, because of the rapid turnover of the primary transcript and intermediate forms of RNA in the cell nucleus, are believed to primarily reflect the transcription rate of the gene. About 20 years ago, Roberts and coworkers (Freneau et al., 1986, 1989) introduced the approach of using intron sequence-specific probes and ISH procedures to detect the pro-opiomelanocortin gene primary transcript in individual neurons of the hypothalamus. After an earlier report of using an intron-specific oligonucleotide probe for ISH (Young et al., 1986), a highly effective intron-specific vasopressin (*Avp*) riboprobe was developed (Herman et al., 1991) and widely used for studies of the regulation of *Avp* gene expression in the hypothalamo-neurohypophyseal system (HNS) in vivo and in vitro. There were also efforts made to develop intron-specific riboprobes for studying oxytocin (*Oxt*) gene expression (Brooks et al., 1993). However, use of this intron 1-based probe was found to produce variable results (Rivest and Laflamme, 1995; Yue et al., 2006). Therefore, we developed and validated a new *Oxt* intron-specific riboprobe, and used this new probe, together with other well-established intron- and exon-specific *Oxt* and *Avp* probes to reevaluate *Oxt* and *Avp* gene expression in the hypothalamus under various classical physiological conditions (Yue et al., 2006). In these experiments, we found that while there was, as expected, a large increase in *Avp* hnRNA after acute salt loading, there was surprisingly no change in the *Oxt* hnRNA (Yue et al., 2006). This suggested that acute hyperosmotic stimuli produce increased *Avp* but not *Oxt* gene transcription. These observations were subsequently confirmed using quantitative real-time PCR (Yue et al., personal communication). Since both neuropeptides are robustly and equivalently secreted from the neurohypophysis following acute salt-loading (Stricker and Verbalis, 1986), it had always been assumed that the gene expression responses of the OXT and AVP magnocellular neuron (MCN) phenotypes to osmotic perturbations would also be equivalent. However, our previous studies (Yue et al., 2006) suggested that there is a significant difference in the excitation–transcription coupling mechanisms that regulate these two neuropeptide genes in the rat MCN. Given the above finding that the *Oxt* and *Avp* MCNs' gene expression responses after acute osmotic stimuli are so dramatically different, in contrast to their similar evoked secretory responses, we sought to determine whether direct application of the presumed neurotransmitter signals for secretion (Sladek, 2000, 2004; Burbach et al., 2001) onto the SONs would also produce different transcriptional responses in the OXT and AVP MCN's.

It is well established that acute or chronic increases in plasma osmotic pressure are sensed by osmoreceptors in the lamina terminalis, which in turn activate the MCNs in the HNS to secrete OXT and AVP into the systemic circulation (McKinley et al., 2004). Various lesion and inhibitor experiments have pointed to synaptic inputs to the MCNs as critical mediators of this secretory response to hyperosmotic stimuli (Sladek and Johnson, 1983; Oldfield et al., 1994; Richard and Bourque, 1995; Sladek et al., 1995; Bourque and Richard, 2001). The apparent close coupling of both acute and chronic osmotic stimuli to OXT and AVP secretion from the posterior pituitary (Stricker and Verbalis, 1986; Shoji et al., 1994; Leng et al., 2001; Ventura et al., 2005) has been attributed primarily to the action of excitatory amino acids (i.e., glutamate) and the modulation of GABA inputs on the MCNs in the HNS (Sladek and Armstrong, 1987; Richard and Bourque, 1992, 1995; Sladek et al., 1998; Swenson et al., 1998; Leng et al., 2001).

Changes in *Oxt* and *Avp* gene expression in the HNS have similarly been interpreted as being closely coupled to the same osmotically evoked synaptic inputs during chronic osmotic stimulation (Lightman and Young, 1987; Sladek, 2000; Burbach et al., 2001; Ueta et al., 2002), and in the case of *Avp* gene expression, also after acute hyperosmotic stimulation (Herman et al., 1991; Arima et al., 1999; Ueta et al., 2002). Evidence in favour of this hypothesis that synaptic input and glutamate agonists (i.e., NMDA and AMPA receptor agonists) and GABA antagonist can cause increases in *Oxt* and *Avp* mRNA in the SON comes largely from in vitro studies by Celia Sladek and her colleagues (Yagil and Sladek, 1990; Sladek et al., 1995, 1998; Swenson et al., 1998; Morsette et al., 2001).

In this paper, we further examine the hypothesis that *Oxt* and *Avp* transcription in the SON is activated by glutamate agonists (and/or GABA antagonists, e.g., bicuculline), by using a novel in vivo experimental system and measurements of *Oxt* and *Avp* hnRNA. For this purpose, bilateral cannulae were implanted over the left and right SONs of male rats. The left SON was infused with a control solution (PBS) and its hnRNA levels were compared to those of the right SON, which received an experimental cocktail consisting of NMDA, AMPA and bicuculline in PBS delivered via ALZET osmotic mini pumps for less than 5 h. We determined the changes in *Oxt* and *Avp* gene transcription by using intron-specific riboprobes to measure *Oxt* (Yue et al., 2006) and *Avp* (Herman et al., 1991) hnRNA levels. Thus, hnRNA levels in the left (control) SONs are compared to those in the right SON in each animal, so that each experimental animal serves as its own control.

Materials and methods

Animals

Adult male Sprague–Dawley rats (250–350 g body weight) were obtained from Taconic Farms (Germantown, NY, USA) and group housed until the time of surgery and then subsequently separated into individual cages. The animals were kept under temperature- (22±1°C) and light-controlled conditions (12-h light–dark cycle, with lights on at 06:00 am). Regular rat chow and water were provided *ad libitum*. All experiments were conducted in accordance with the guidelines set forth by the NIH Animal Care and Use Committee.

Stereotaxic surgery

The surgical procedures were similar to those described elsewhere (Shahar et al., 2004). Briefly, male rats were weighed and anaesthetized with isoflurane, administered via a Stoelting gas anaesthesia adaptor for the stereotaxic instrument. Once anaesthetized, the rat was placed in the stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Using the stereotaxic coordinates from the Paxinos and Watson (1986) atlas, bilateral intracerebral cannulae connected to osmotic mini pumps were directed over the SON (see Fig. 1A). A small (1.5 mm diameter), parasagittal hole was made on each side of the skull (1.8 mm lateral to the sagittal suture and 1.30 mm caudal to the bregma) using dental burr. Two sterile stainless steel cannulae (28 gauge) 8.80 mm long, were inserted down to a level of 0.3 mm up to the dorsal border of the SON, using the following stereotaxic coordinates: 1.30 mm posterior to bregma; 1.80 mm medial lateral on each side; 8.80 mm dorsal ventral. Each cannula (Plastics One, Inc. 6591 Merriman Road, S.W. Roanoke, VA 24018) was attached

via sterile PE50 tubing (Plastics One, Inc.) to a sterile model 2002, ALZET osmotic mini-pump (DURECT Corporation, Cupertino, CA, USA), and fixed to the skull with sterile acrylic dental cement (Plastics One, Inc.) bonded to sterile stainless steel screws (Plastics One, Inc.) inserted in the skull. The two ALZET osmotic mini-pumps, connected to the PE50 tubes, were placed subcutaneously on the dorsal aspect of the rat, in between the two scapulae through subcutaneous tunnels which were formed using haemostatic forceps, starting from the mid-sagittal skin incision. One pump (for the left SON) always contained the vehicle solution (sterile PBS), while the other pump (for the right SON) contained either PBS or the experimental drug. All solutions were filtered through a 0.22 μ M Millipore filter. The pumps were filled at least 12 h prior to the surgery in a sterile environment and placed in the incubator (37°C) while immersed in 0.9% saline for the purpose of osmotic activation.

Following the above neurosurgical interventions, the sagittal skin incision was closed with surgical stainless steel clips and Ketoprofen was administered (5 mg/kg, diluted in 0.9% NaCl). Animals were killed within 2–5 h or 2 days of surgery. Prior to being killed, animals were anesthetized with isoflurane and immediately perfused transcardially with ice-cold 50 ml PBS followed by 250 ml of fixative solution (4% paraformaldehyde, 0.19% picric acid in 0.1 M phosphate buffer, pH 7.4). Brains were removed and post-fixed overnight in the same fixative solution (diluted 1:4 with PBS). Next, each brain was slowly agitated in a 30% sucrose solution (30% sucrose in 0.9% normal saline) for four nights changing the solution at least twice.

Experimental procedures

Control animals consisted of males that had either undergone stereotaxic surgery and infused bilaterally with PBS ($n = 2$) or males that had simply been killed and perfused without any surgical manipulation ($n = 3$). Of the males that had bilateral cannulae, one was killed 3 h after surgery, and the other was killed 2 days after surgery. The experimental male rats (NAB) received varying doses of NMDA (Sigma, St. Louis, MO, M-3262), AMPA (Sigma A1455) and bicuculline (Sigma B6889) infused over the right SON and PBS over the left SON for 2–5 h. Three animals had 100 μ M NMDA and AMPA and either 50 μ M, 500 μ M or 2.5 mM bicuculline. Two males received a cocktail of 500 μ M each of NMDA, AMPA and bicuculline (NAB). There are no significant differences in hnRNA responses between these NAB cocktails containing different doses, and these data are averaged. Three male rats received 3 μ M TTX (Sigma Corp) over the right SON and PBS over the left SON for 2 days. These rats also received an osmotic stress (1.5 M NaCl i.p., 1 ml/100 kg) 2 h prior to the cardiac perfusions.

Riboprobes specific for *Oxt* and *Avp*

Probes used in these experiments have been previously described (Yue et al., 2006). Briefly, DNA sequences in intron 2 (In2) of the rat *Oxt* gene were used to design the PCR primers for the *Oxt* intronic probe using DNASIS-Mac v3.5 software (Hitachi Software Engineering Co., Ltd. Tokyo, Japan). The BLAST results showed that these primers are specific for the *Oxt* gene. The primers used to obtain *Oxt* hnRNA probe are: 5'-ATGAAGCTT-GTGAGCAGGAGGGGCCTA-3' (sense), and 5'-ATGCTGCAGCTGCAAGAGAAATGGGTC-AGT-3' (antisense). These produced an 84 bp

fragment of In2. PCR was carried out in a 50 µl reaction volume, containing 0.8 µg rat genomic DNA, 1 × PCR buffer (20mM Tris-HCl, pH 8.4, 50mM KCl), 200µM each of dNTPs, 200 nM each of the primers and 2.5 U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). PCR was performed on a GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems, Foster City, CA) and consisted of an initial 2 min, 94°C denaturation, followed by 30 cycles of denaturing (94°C, 30 s), annealing (60°C, 30 s) and extension (72°C, 45 s), followed by a final extension of 7 min at 72°C. The PCR products were loaded on the 1.5% agarose gel and purified by the MinElute Gel Extraction Kit (Qiagen, Valencia, CA). PCR fragments were subcloned into pBlueScript II SK (+) (Stratagene, La Jolla, CA). Modified T7 (GCGCGTAATACGACTCACTATAGGG) and T3 (CGCGCAATTAACCCTCACTAAAGG) primers were used to PCR amplify the fragments and the resulting PCR products were used as templates for the synthesis of riboprobes. T7 and T3 RNA polymerases were used to obtain both sense and antisense labelled probes, respectively.

The rat *Avp* intronic riboprobe (Herman et al., 1991) was a 735 bp fragment of intron 1 of rat *Avp* gene subcloned into pGEM-3 vector (Promega, Madison, WI), and was kindly provided by Dr. Thomas Sherman (Georgetown University, Washington, DC). The latter was subcloned into pGEM-3 vectors (Mutsuga et al., 2004). Modified T7 (CATACGATTTAGGTGACACTATAG) primers were used to PCR amplify fragments to make templates that were used to synthesize the riboprobes. The T7 RNA polymerase was used to synthesize the antisense of hn*Avp* riboprobe.

Labelling of the *Avp* intronic riboprobes used 40 ng of the template and 50 µCi of [α -³⁵S]-uridine 5'-triphosphate (UTP) (Perkin Elmer Life Sciences, Inc., Boston, MA), 10 mM DTT and a MAXIscript in vitro transcription kit (Ambion, Inc., Austin, TX). *Oxt* In2 labelling was performed as described above using 40 ng of the PCR product, 50 µCi of [α -³⁵S]-UTP and 50 µCi of [α -³⁵S]-CTP.

In situ hybridization for the detection of *Avp* and *Oxt* heteronuclear RNA

The ISH protocol used in this study has been previously published (Young et al., 1986; Mutsuga et al., 2004). Briefly, serial 10 µm brain sections were cut on a cryostat and placed onto poly-l-lysine coated slides (Fisher Scientific Company, Newark, DE), dried on a slide warmer for 10–30 min at 37 °C, and then stored at –80 °C. Before hybridization with riboprobes, the sections were rinsed once and washed twice for 5 min in PBS, put into 0.1 M triethanolamine-HCl (pH 8.0) containing 0.25% acetic anhydride for 10 min at room temperature, rinsed with 2 × SSC buffer and transferred through graded ethanols (75–100%) and then air-dried. Hybridization was carried out in 80 µl of hybridization solution (20 mM Tris-Cl pH 7.4, 1 mM EDTA pH 8.0, 300 mM NaCl, 50% formamide, 10% dextran sulphate, 1 × Denhardt's solution, 100 µg/ml salmon sperm DNA, 250 µg/ml yeast total RNA, 250 µg/ml yeast tRNA, 0.0625% SDS, 0.0625% sodium thiosulphate) containing 10⁶ cpm denatured S³⁵-labelled riboprobe. After overnight hybridization at 55°C, the sections were washed 4 times in 4 × SSC, incubated with TNE buffer [10 mM Tris-Cl pH 8.0, 0.5 M NaCl, 0.25 mM EDTA pH8.0] containing 20 µg/ml ribonuclease A for 30 min at 37°C, and then washed twice in 2 × SSC, once in 1 × and 0.5 × SSC at room temperature, and twice in 0.1 ×

SSC at 65°C. The sections were rinsed in graded ethanol solutions and then air-dried. Finally, the sections were apposed to a low-energy storage phosphor screen (Amersham Biosciences, Piscataway, NJ) for 7–20 days, and developed using a phosphor imager (Storm 860, Amersham Biosciences).

Quantitative analysis of ISH

To evaluate the levels of hnRNA in the SONs, the average densities and unit areas from two representative sections in the central region of the nuclei recorded on the phosphor imager were measured using the Image Quant software version 5.2 (Amersham Biosciences).

Statistical analyses

The ratios of *Avp* and *Oxt* hnRNA of the right SONs, divided by the left SONs were statistically analyzed. Statistical significance of differences between groups was calculated by an unpaired *t*-test followed by Fisher's protected least significant differences (PLSD) post hoc test using the Statview 5.0 (SAS Institute, Inc., Cary, NC) program. Differences between groups were considered statistically significant when *p*-values were less than 0.05. Results are expressed as percent control (mean±SEM).

Results

The experimental paradigm used in these experiments is depicted in Fig. 1A, in which ALZET osmotic mini-pumps attached to pre-positioned cannulae located over each SON in male rats were used to infuse control (PBS only) solutions over the left SON and either control or experimental solutions over the right SON. In this way, each animal's left SON serves as a control for the experimental right SON, and the hnRNA measurements on the experimental side are expressed as a percentage of the values measured on the control side. Representative ISH results for various treatments are shown in Figs. 1B, C. Figure 1B illustrates the control experiments, where both SONs are treated equally. In the experiment shown in Fig. 1B the same PBS control solution was infused over each SON for 3 h, and ISH for *Avp* hnRNA was conducted. As can be seen in Fig. 1B, there is little difference in *Avp* hnRNA levels between the two sides of the SON. The quantitative data is shown in Fig. 2. For the *Avp* hnRNA, the average ratio of the right SON over the left SON in the five control animals is 1.10 ± 0.14 , and is expressed as a percentage of the PBS control (left side) in Fig. 2A, indicating that there was no intrinsic difference between the left versus the right SON in the absence of drug treatment. A similar result was obtained for the *Oxt* hnRNA measurements in these control experiments, which are illustrated in Fig. 2B (PBS), where the ratio between the two sides was 1.19 ± 0.08 .

Figure 1C illustrates an experiment in which the excitatory amino acid and GABA antagonist cocktail of NAB was infused over the right SON for 2.5 h. There was an increase of *Avp* hnRNA in the NAB-treated SON (right SON) as compared to the PBS-control infused SON, indicating that the NAB was a very effective stimulus to increase *Avp* hnRNA expression in the stimulated SON. This increase was comparable to the increase in *Avp* hnRNA that we observed in the SON 2 h after an i.p. injection of 1.5 M NaCl was given to the rat (illustrated in the left side of SON in Fig. 1D, see also Yue et al., 2006). That this

increase in *Avp* hnRNA in response to hyperosmotic stimulation is likely to be due to the osmotically evoked neural input to the SON, is supported by the observation that this increase is blocked by the infusion of 3 μ M TTX over the right SON (Fig. 1D, right SON).

Quantitative analyses of the effects of NAB infusion over the right SON on *Avp* and *Oxt* hnRNA levels is shown in Fig. 2A, B, respectively. The average ratio of *Avp* hnRNA levels of the NAB-stimulated SON over the PBS-control SON is 3.13 ± 0.84 ($n = 5$), which represents a threefold increase in *Avp* hnRNA in the NAB-treated SON over the control SON (Fig. 2A, $p < 0.04$). In contrast, however, there was no change in *Oxt* hnRNA in the NAB-treated SONs as compared to the PBS-control SON, where the ratio between the control and treated SONs was 0.99 ± 0.19 ($n = 5$). This did not significantly differ from the control animals (Fig. 2B, $p = 0.42$). Thus, these data are consistent with the view that the *Avp* hnRNA is regulated by excitatory amino acid input and GABA modulation, but that under the same conditions *Oxt* hnRNA is not.

Discussion

A substantial literature exists that links hyperosmotically evoked excitation in the HNS with the regulated secretion of OXT and AVP into the general circulation. Studies conducted by Leng et al. (2001) show that intravenous infusions of hypertonic saline solution resulted in a linear increase in activity of both AVP and OXT cells. These authors generated a computational model which predicts that both glutamate and GABA are simultaneously released onto the SON to mediate this linear increase in activity. They tested this hypothesis in part, by administering bicuculline to the SON during an intravenous hyperosmotic infusion. Under these conditions, there was a robust increase in the slope of OXT neural activity. These data suggest that both glutamatergic and GABAergic inputs are both activated under osmotic stress, and that GABA is modulating the glutamate excitatory input which induces MCNs to release OXT and AVP into the bloodstream. Other studies have shown in vivo and in vitro that glutamate agonists and/or GABA antagonists produce increased secretion of OXT and AVP from the posterior pituitary (see Sladek, 2004, for a review). Analogous to this excitation–secretion coupling scenario, a similar view exists for excitation–transcription coupling in the HNS (Sladek, 2000; Burbach et al., 2001).

Studies in vitro using a SON–pituitary explant system have shown that increases in *Avp* mRNA and AVP secretion are tightly correlated (Yagil and Sladek, 1990). In this system, endogenous GABA tonically inhibits AVP secretion, and addition of GABA antagonists stimulated release in a dose-dependent fashion (Sladek and Armstrong, 1987). Kynurenic acid, a general excitatory amino acid antagonist, blocked increases in *Avp* mRNA and AVP secretion in response to osmotic stress (Sladek et al., 1995), and subsequent experiments suggest that both AMPA receptors (Sladek et al., 1998) and NMDA receptors (Swenson et al., 1998) mediate the AVP release. These data suggest that during osmotic stimulation, the actions of the neurotransmitters glutamate and GABA are causally involved in the increase in *Oxt* and *Avp* gene expression in the SON.

To test this hypothesis in vivo, we adapted our previously described approach of bilateral infusion of experimental solutions over the SON using ALZET osmotic mini-pumps (Shahar

et al., 2004). In the latter study, we showed that osmotically evoked c-fos expression in the MCNs in the experimental SON was completely inhibited by unilateral delivery of TTX, whereas the contralateral SON that was infused by only control PBS solution underwent the expected increase in c-fos expression (see Fig. 2, in Shahar et al., 2004). In this regard, it is interesting to note that osmotically stimulated OXT and AVP release is also blocked by application of TTX to the HNS (Ludwig et al., 1995) and that the expected increased in *Avp* hnRNA under these conditions is also inhibited (see Fig. 1D in this paper). Using this bilateral infusion approach (see Fig. 1A), we set out to determine whether direct application of glutamate agonists and GABA antagonists to the SON could evoke increases in *Oxt* and *Avp* hnRNA, as measured by *Oxt*- and *Avp*-intron-specific riboprobes.

The results of these experiments, show that *Avp* hnRNA increased after <5 h infusions of a drug cocktail comprised of a mixture of NMDA, AMPA and bicuculline (see NAB in Figs. 1C and 2A), and this increase is comparable to that found with an acute osmotic stimulus (see left SON in Fig. 1D, and Yue et al., 2006). We found in pilot experiments that applications of AMPA, NMDA or bicuculline individually are not very effective in increasing *Avp* hnRNA (data not shown), in comparison to the NAB cocktail result shown in Figs. 1C and 2A. These data for *Avp* transcription are consistent with the hypothesis of closely linked excitation–transcription coupling. In contrast, however, are the data for *Oxt* transcription (Fig. 2B) in the SON, which exhibits no change in *Oxt* hnRNA under the same conditions as when *Avp* hnRNA increases robustly. The latter data do not support the hypothesis with respect to *Oxt* gene transcription, and indicates that *Oxt* transcription does not change in response to acute osmotic stimulation, in contrast to *Avp* gene transcription which does (Yue et al., 2006). Since it is known that a chronic osmotic stimulus activates transcription from both the *Oxt* and *Avp* genes, these data suggest a profound difference in the signal-transduction mechanisms of excitation–transcription coupling must exist between the *Oxt* and *Avp* genes in the SON during acute osmotic or neurotransmitter stimulation.

Acknowledgments

We thank Dr. Tal Shahar for his help in establishing the surgical technique in our laboratory, and Dr. Noriko Mutsuga for her technical assistance in ISH. This research was supported by the Intramural Research Program of the NIH, NINDS.

Abbreviations

AMPA	alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AVP	vasopressin
CNS	central nervous system
CON	control
GABA	gamma-aminobutyric acid
hnRNA	heteronuclear RNA
HNS	hypothalamo-neurohypophysial system

ISH	in situ hybridization
MCN	magnocellular neuron
NAB	NMDA, AMPA and bicuculline
NMDA	<i>N</i> -methyl-d-aspartic acid
OXT	oxytocin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
SON	supraoptic nucleus
TTX	tetrodotoxin

References

- Arima H, Kondo K, Kakiya S, Nagasaki H, Yokoi H, Yambe Y, Murase T, Iwasaki Y, Oiso Y. Rapid and sensitive vasopressin heteronuclear RNA responses to changes in plasma osmolality. *J Neuroendocrinol.* 1999; 11:337–341. [PubMed: 10320560]
- Bourque CW, Richard D. Axonal projections from the organum vasculosum lamina terminalis to the supraoptic nucleus: functional analysis and presynaptic modulation. *Clin Exp Pharmacol Physiol.* 2001; 28:570–574. [PubMed: 11458884]
- Brooks PJ, Kaplitt MG, Kleopoulos SP, Funabashi T, Mobbs CV, Pfaff DW. Detection of messenger RNA and low-abundance heteronuclear RNA with single-stranded DNA probes produced by amplified primer extension labeling. *J Histochem Cytochem.* 1993; 41:1761–1766. [PubMed: 8245424]
- Burbach JP, Luckman SM, Murphy D, Gainer H. Gene regulation in the magnocellular hypothalamo-neurohypophysial system. *Physiol Rev.* 2001; 81:1197–1267. [PubMed: 11427695]
- Fremeau RT Jr, Autelitano DJ, Blum M, Wilcox J, Roberts JL. Intervening sequence-specific in situ hybridization: detection of the pro-opiomelanocortin gene primary transcript in individual neurons. *Brain Res Mol Brain Res.* 1989; 6:197–201. [PubMed: 2615595]
- Fremeau RT Jr, Lundblad JR, Pritchett DB, Wilcox JN, Roberts JL. Regulation of pro-opiomelanocortin gene transcription in individual cell nuclei. *Science.* 1986; 234:1265–1269. [PubMed: 3775385]
- Herman JP, Schafer MK, Watson SJ, Sherman TG. In situ hybridization analysis of arginine vasopressin gene transcription using intron-specific probes. *Mol Endocrinol.* 1991; 5:1447–1456. [PubMed: 1775130]
- Leng G, Brown CH, Bull PM, Brown D, Scullion S, Currie J, Blackburn-Munro RE, Feng J, Onaka T, Verbalis JG, Russell JA, Ludwig M. Responses of magnocellular neurons to osmotic stimulation involves coactivation of excitatory and inhibitory input: an experimental and theoretical analysis. *J Neurosci.* 2001; 21:6967–6977. [PubMed: 11517284]
- Lightman SL, Young WS III. Vasopressin, oxytocin, dynorphin, enkephalin and corticotrophin-releasing factor mRNA stimulation in the rat. *J Physiol.* 1987; 394:23–39. [PubMed: 2895179]
- Ludwig M, Callahan MF, Morris M. Effects of tetrodotoxin on osmotically stimulated central and peripheral vasopressin and oxytocin release. *Neuroendocrinology.* 1995; 62:619–627. [PubMed: 8751288]
- McKinley MJ, Mathai ML, McAllen RM, McClear RC, Miselis RR, Pennington GL, Vivas L, Wade JD, Oldfield BJ. Vasopressin secretion: osmotic and hormonal regulation by the lamina terminalis. *J Neuroendocrinol.* 2004; 16:340–347. [PubMed: 15089972]

- Morsette DJ, Sidorowicz H, Sladek CD. Role of non-NMDA receptors in vasopressin and oxytocin release from rat hypothalamo-neurohypophysial explants. *Am J Physiol Regul Integr Comp Physiol.* 2001; 280:R313–R322. [PubMed: 11208557]
- Mutsaers N, Shahar T, Verbalis JG, Brownstein MJ, Xiang CC, Bonner RF, Gainer H. Selective gene expression in magnocellular neurons in rat supraoptic nucleus. *J Neurosci.* 2004; 24:7174–7185. [PubMed: 15306651]
- Oldfield BJ, Badoer E, Hards DK, McKinley MJ. Fos production in retrogradely labelled neurons of the lamina terminalis following intravenous infusion of either hypertonic saline or angiotensin II. *Neuroscience.* 1994; 60:255–262. [PubMed: 8052417]
- Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinate.* 2. Academic; Orlando, FL: 1986.
- Richard D, Bourque CW. Synaptic activation of rat supraoptic neurons by osmotic stimulation of the organum vasculosum lamina terminalis. *Neuroendocrinology.* 1992; 55:609–611. [PubMed: 1584342]
- Richard D, Bourque CW. Synaptic control of rat supraoptic neurones during osmotic stimulation of the organum vasculosum lamina terminalis in vitro. *J Physiol.* 1995; 489(Pt 2):567–577. [PubMed: 8847648]
- Rivest S, Laflamme N. Neuronal activity and neuropeptide gene transcription in the brains of immune-challenged rats. *J Neuroendocrinol.* 1995; 7:501–525. [PubMed: 7496392]
- Shahar T, House SB, Gainer H. Neural activity protects hypothalamic magnocellular neurons against axotomy-induced programmed cell death. *J Neurosci.* 2004; 24:6553–6562. [PubMed: 15269267]
- Shoji M, Kimura T, Kawarabayashi Y, Ota K, Inoue M, Yamamoto T, Sato K, Ohta M, Funyu T, Sonoyama T, Abe K. Effects of acute salt loading on vasopressin mRNA level in the rat brain. *Am J Physiol.* 1994; 266:R1591–R1595. [PubMed: 8203636]
- Sladek, CD. Antidiuretic hormone: synthesis and release. In: Fray, JCS., editor. *Handbook of Physiology, Section 7, The Endocrine System Volume III, Endocrine Regulation of Water and Electrolyte Balance.* Oxford University Press; Oxford: 2000. p. 436–495.
- Sladek CD. Vasopressin response to osmotic and hemodynamic stress: neurotransmitter involvement. *Stress.* 2004; 7:85–90. [PubMed: 15512851]
- Sladek CD, Armstrong WE. Gamma-aminobutyric acid antagonists stimulate vasopressin release from organ-cultured hypothalamo-neurohypophyseal explants. *Endocrinology.* 1987; 120:1576–1580. [PubMed: 2435537]
- Sladek CD, Badre SE, Morsette DJ, Sidorowicz HE. Role of non-NMDA receptors in osmotic and glutamate stimulation of vasopressin release: effect of rapid receptor desensitization. *J Neuroendocrinol.* 1998; 10:897–903. [PubMed: 9870746]
- Sladek CD, Fisher KY, Sidorowicz HE, Mathiasen JR. Osmotic stimulation of vasopressin mRNA content in the supraoptic nucleus requires synaptic activation. *Am J Physiol.* 1995; 268:R1034–R1039. [PubMed: 7733386]
- Sladek CD, Johnson AK. Effect of anteroventral third ventricle lesions on vasopressin release by organ-cultured hypothalamo-neurohypophyseal explants. *Neuroendocrinology.* 1983; 37:78–84. [PubMed: 6888661]
- Stricker EM, Verbalis JG. Interaction of osmotic and volume stimuli in regulation of neurohypophyseal secretion in rats. *Am J Physiol.* 1986; 250:R267–R275. [PubMed: 3946641]
- Swenson KL, Badre SE, Morsette DJ, Sladek CD. *N*-methyl-D-aspartic acid stimulation of vasopressin release: role in osmotic regulation and modulation by gonadal steroids. *J Neuroendocrinol.* 1998; 10:679–685. [PubMed: 9744485]
- Ueta Y, Levy A, Lightman SL. Gene expression in the supraoptic nucleus. *Microsc Res Tech.* 2002; 56:158–163. [PubMed: 11810718]
- Ventura RR, Giusti-Paiva A, Gomes DA, Elias LL, Antunes-Rodrigues J. Neuronal nitric oxide synthase inhibition differentially affects oxytocin and vasopressin secretion in salt loaded rats. *Neurosci Lett.* 2005; 379:75–80. [PubMed: 15823420]
- Yagil C, Sladek CD. Effect of extended exposure to hypertonicity on vasopressin messenger ribonucleic acid content in hypothalamo-neurohypophyseal explants. *Endocrinology.* 1990; 127:1428–1435. [PubMed: 2387259]

- Young WS III, Mezey E, Siegel RE. Vasopressin and oxytocin mRNAs in adrenalectomized and Brattleboro rats: analysis by quantitative in situ hybridization histochemistry. *Brain Res.* 1986; 387:231–241. [PubMed: 3828759]
- Yue C, Mutsuga N, Scordalakes EM, Gainer H. Studies of oxytocin and vasopressin gene expression in the rat hypothalamus using exon- and intron-specific probes. *Am J Physiol Regul Integr Comp Physiol.* 2006; 290:R1233–R1241. [PubMed: 16357095]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 1.

Vasopressin gene expression and excitatory amino acid stimulation in the SON. (A) Schematic diagram of in vivo experimental system is illustrated. Stereotaxic surgery was performed on adult male rats and bilateral cannulae were directed over the SON. Cannulae were connected via PE50 tubing to Alzet mini-pumps containing one of three different solutions: an excitatory cocktail of NMDA, AMPA and bicuculline (NAB); TTX or PBS. In all experiments, the left SON of each male served as an internal control and received vehicle infusion (PBS), while the right SON was infused with either PBS (control solution) or an experimental solution NAB or TTX. Representative photomicrographs of *Avp* hnRNA ISH results are shown in (B–C). (B) PBS was infused over both SONs for 3 h. (C) A cocktail of NAB was infused over the right SON for 2.5 h. (D) TTX was infused over the right SON for 2 days, and 1.5 M NaCl was injected i.p. to increase *Avp* hnRNA expression acutely for 2 h. Note that the left uninhibited SON showed enhanced *Avp* hnRNA comparable to the NAB stimulated SON in (C), whereas the TTX inhibited SON (right side) did not (see text).

Abbreviations: PBS, phosphate buffered saline (vehicle); EXP, experimental drug; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus; OX, optic chiasm; LV, lateral ventricle; 3V, third ventricle; f, fornix; ic, internal capsule; cc, corpus callosum.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 2.

Quantitative determinations of vasopressin (A) and oxytocin (B) hnRNA levels in control (unstimulated) SONs and SONs stimulated by infusions of the excitatory amino acid cocktail (NAB). The data are expressed as percentage changes in the NAB-treated (right side) SON as compared to the PBS-control (left) SON (see Fig. 1C). The control bars in the graph represent the mean control data from five animals in which both sides of the SON were equally treated (e.g., by PBS infusion only, Fig. 1B). The bars labelled NAB are from animals in which the right SON infused with PBS ($n = 5$). *Equals significant difference ($p < 0.05$) in hnRNA level between PBS and NAB stimulated SONs.