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Neurotransmitter regulation of *c-fos* and vasopressin gene expression in the rat supraoptic nucleus

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Abstract

Acute increases in plasma osmotic pressure produced by intraperitoneal injection of hypertonic NaCl are sensed by osmoreceptors in the brain, which excite the magnocellular neurons (MCNs) in the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) in the hypothalamus inducing the secretion of vasopressin (VP) into the general circulation. Such systemic osmotic stimulation also causes rapid and transient increases in the gene expression of *c-fos* and VP in the MCNs. In this study we evaluated potential signals that might be responsible for initiating these gene expression changes during acute hyperosmotic stimulation. We use an *in vivo* paradigm in which we stereotactically deliver putative agonists and antagonists over the SON unilaterally, and use the contralateral SON in the same rat, exposed only to vehicle solutions, as the control SON. Quantitative real time-PCR was used to compare the levels of *c-fos* mRNA, and VP mRNA and VP heteronuclear (hn)RNA in the SON. We found that the ionotropic glutamate agonists (NMDA plus AMPA) caused an approximately 6-fold increase of *c-fos* gene expression in the SON, and some, but not all, G-coupled protein receptor agonists (e.g., phenylephrine, senktide, a NK-3-receptor agonist, and α -MSH) increased the *c-fos* gene expression in the SON from between 1.5 to 2-fold of the control SONs. However, none of these agonists were effective in increasing VP hnRNA as is seen with acute salt-loading. This indicates that the stimulus-transcription coupling mechanisms that underly the *c-fos* and VP transcription increases during acute osmotic stimulation differ significantly from one another.

Keywords

C-fos mRNA; Vasopressin heteronuclear RNA; Supraoptic nucleus; Quantitative real time PCR; Convection enhanced delivery; ALZET osmotic minipump; Hyperosmotic stimulation; Hypothalamus

Introduction

Neuronal activity causes long-lasting changes in gene expression in the central nervous system, which in turn can lead to profound developmental, homeostatic, and plasticity changes (Flavell and Greenberg, 2008). The earliest studies illustrating this phenomenon focused on changes in immediate-early gene expression, but more recent studies have elucidated other classes of genes with a variety of functions and kinetic responses that are regulated by neural activity

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(Greer and Greenberg, 2008; Hong et al., 2004; Braham et al., 2008). In this regard, it has been shown that five minutes of stimulation of NMDA-receptors in primary cultured cortical neurons can induce the expression of hundreds of specific genes with varying kinetics and which last from minutes to 24 hours post-stimulation (Hong et al., 2004). Genes that changed in expression include the neurotrophin, BDNF, various synapse-associated molecules such as Homer 1a, Arc, and microRNAs, and interestingly vasopressin. Most of these gene expression changes involve calcium-dependent signaling mechanisms, and activation of various transcription factor families (e.g., CREB, myosin enhancer factor 2), and epigenetic regulatory mechanisms, e.g., methyl CpG binding proteins (Flavell and Greenberg, 2008; Ooi and Wood, 2008).

The magnocellular neurons (MCNs) in the hypothalamo-neurohypophysial system (HNS) have long been known to respond to specific physiological stimuli with robust changes in gene expression (Burbach et al., 2001). In fact, the earliest demonstrations of activity-dependent regulation of *c-fos* expression as a marker of neuronal activity was made using the HNS (Sagar et al., 1988), and *c-fos* continues to be used as an activity marker in many functional studies on neuroendocrine systems (Sharp et al., 1991; Hoffman et al., 1993; Luckman et al., 1994; Hoffman and Lyo, 2002; Kovacs, 2008). Most studies in the HNS use systemic osmotic stimuli to induce changes in vasopressin (VP) and oxytocin (OT) gene expression (Burbach et al., 2001), and many other immediate-early genes, in addition to *c-fos*, have been shown to be robustly regulated by acute systemic hyperosmotic stimuli (Kawasaki et al., 2005). Hyperosmotic stimuli have also been shown to rapidly and transiently increase transcription of the VP gene in the HNS (Kawasaki et al., 2005; Herman et al., 1991; Arima et al., 1999; Yue et al., 2006 and 2008a) with kinetics similar to that of the immediate-early genes. This differs from the other major neuropeptide gene expressed in the HNS, the OT gene, which does not respond to acute osmotic stimuli with rapid changes in OT hnRNA in the MCNs (Yue et al., 2006 and 2008a), and time course studies have shown that there is a vast difference in the kinetics of stimulus-transcription coupling in the HNS between the VP and OT genes (Yue et al., 2008b), although the kinetics of their stimulus-secretion coupling mechanisms are virtually identical (Stricker and Verbalis, 1986).

In this paper we address the critical signals that might initiate the intracellular molecular cascades in the MCNs that lead to activity-induced increases in *c-fos* and VP-transcription. To date, most of our insights about osmotically-evoked signals to the HNS come from studies based on assays measuring the secretion of the peptides either centrally from the MCN dendrites (Ludwig, 1998; Ludwig and Pittman, 2003) or in the periphery from the neurohypophysis (Leng et al., 2001; Sladek and Kapoor, 2001; Sladek, 2004). These experiments indicate that there are potentially intrinsic (Bourque, 1998; 2008; Bourque and Olier, 1997) and a variety of afferent neurotransmitter signals to the MCNs (Sladek, 2004) that may be involved in the stimulus-secretion coupling. Some of these signals have also been shown to both evoke secretion of VP and OT, as well as VP and OT mRNA changes in an explant model of HNS (Yagil and Sladek, 1990; Sladek and Kapoor, 2001). Taken together, these studies suggest several signaling mechanisms that could be involved in the increases in *c-fos* and VP gene expression observed during hyperosmotic stimulation. These are: 1) that there is a direct effect of the hyperosmotic stimulus on osmoreceptors on the MCN membranes themselves that lead to depolarization and subsequent action potentials (Mason, 1980; Bourque, 2008) that could be the effective signals (Dudek and Fields, 2002; Zhao et al., 2005), 2) alternatively, the hyperosmotic stimuli are sensed by osmoreceptors in neurons in specific forebrain regions (e.g. lamina terminalis) which then send afferents that synapse on the MCNs and release glutamate which then activates ionotropic (e.g., NMDA, AMPA) receptors to the MCNs (Bourque, 1998; Richard and Bourque, 1995; Leng et al., 2001; McKinley et al., 1992; Sladek, 2004), and 3) that there are other osmotically evoked afferent inputs (e.g., catecholamines and

neuropeptides) that activate specific G-protein coupled receptors (GPCRs) on the MCNs (Sladek and Kapoor, 2001; Sladek, 2004).

In this study, we use novel delivery systems, i.e., convection enhanced delivery (CED) and ALZET osmotic minipumps, to deliver various candidate signaling molecules to the MCNs in the rat SON *in vivo*, in order to evaluate the above candidate mechanisms by studying the effects of various putative signalling molecules on *c-fos* and VP gene transcription by quantitative real-time PCR (qRT-PCR).

Materials and methods

Animals

Adult male Sprague-Dawley rats (250–350 g) obtained from CharlesRiver Laboratories (Wilmington, MA) were maintained under normal laboratory conditions (temperature: 21–23°C, 12 h light-dark cycle with light on at 6:00 am) with access to food and drinking water *ad libitum* prior to the experiments. They were caged individually following the experimental procedures, all of which were carried out in accordance with National Institutes of Health (NIH) guidelines on the care and use of animals and an animal study protocol approved by the NINDS Animal Care and Use Committee.

Hypertonic stimulation in rats

Rats were injected i.p. with 1 ml/100 g of either 1.5M or 2M NaCl and kept without access to water for 30 min, and then killed by decapitation.

Stereotaxic surgery

Rats were weighed and anesthetized with isoflurane, administered via a Stoelting gas anesthesia adaptor for the stereotaxic instrument (David Kopf Instruments, Tujunga, CA, USA). Application of pharmacological agents to the SON *in vivo* was by a CED (Szerlip et al., 2007) or an ALZET osmotic minipump method (Shahar et al., 2004; Scordalakes et al., 2008). Either bilateral intracerebral needles for the CED infusion or indwelling cannulae connected to osmotic minipump (ALZET osmotic minipump method) were directed over the SON (Shahar et al., 2004), using stereotaxic coordinates from the Paxinos and Watson (1986) atlas.

A schematic diagram of this paradigm is illustrated in Fig 1A which shows a coronal section of a rat brain in which experimental solutions are delivered unilaterally (EXP) and vehicle to the other control side (Vehicle) at a rate of 0.25 µl/min for 10 min. For the CED experiments, the 30 gauge syringe needles were inserted down to a level of 300 µM above the dorsal border of the SON, using the following stereotaxic coordinates: 1.1 mm posterior to bregma; 1.8 mm medial lateral on each side; and 8.8 mm dorsal to ventral.

For the ALZET osmotic minipump method, two 28 gauge sterile stainless steel cannulae (Plastics One, Inc., Roanoke, VA) were connected to ALZET osmotic minipumps (model 2001D, DURECT Corporation, Cupertino, CA, USA) via PE 60 tubes (Plastics One, Inc.) and fixed to the skull with sterile acrylic dental cement bonded to stainless steel screws (Plastics One, Inc), using the same stereotaxic coordinates as for the CED method. The pumps were filled with the solutions to be delivered at least 2–3 h prior to the surgery in a sterile environment and then activated by immersion in 0.9% saline at 37 °C. EXP solutions were delivered to one SON and vehicle to the contralateral SON for a period of 3.5 hours. The rate of flow from the ALZET osmotic minipump was 8 µl/hour (0.13 µl/min).

Following the above neurosurgical interventions, the skin incision was closed using simple interrupted sutures and Ketoprofen was administered (5 mg/kg, diluted in 0.9% NaCl) subdermally. Rats were sacrificed 30 min after agonist injection in the SON using the CED method. In the antagonist studies, where the solutions were delivered by ALZET osmotic minipump, rats were sacrificed 30 min after the i.p. injection of hypertonic 2M NaCl solution. For the qRT-PCR analysis, the rats were decapitated under isoflurane anesthesia, and their brains were quickly removed and placed into ice-cold Hanks' balanced salt solution. Coronal hypothalamic slices containing the SONs were made using a Jacobowitz brain slicer (Zivic Laboratories, Pittsburg, PA), and the 1mm slices, located approximately 0.8 to 1.8 mm in the Paxinos and Watson (1986) atlas, were placed on a piece of parafilm on a hard black surface to visualize the SONs. The SONs were immediately dissected out of the slices and placed in lysis/RNA stabilization buffer (STRATAGENE, La Jolla, CA) for subsequent purification of the RNA. For the immunohistochemical experiments, animals were anesthetized with isoflurane and immediately perfused transcardially with 100 ml of 0.1M phosphate-buffered saline (PBS) followed by 250 ml of fixative solution (4% paraformaldehyde in 0.1M PBS, pH 7.4). Brains were removed and post-fixed overnight in the same fixative solution, and subsequently were cryoprotected in 20% sucrose in 0.1 M PBS for 48 h at 4°C.

Experimental procedures

In order to evaluate the brain volumes perfused by solutions delivered by both CED and ALZET osmotic minipump methods, Fluoro-Gold (0.005%, Fluorochrome, LLC, Denver, CO) was used (main Fluoro-Gold filled space is shown as blue area, and the full extent of filled area is shown as dotted line in Figs. 1B and C).

The injections of various agonists, NMDA (Sigma Corp, St. Louis, MO, M3262), AMPA (Sigma A1455), bicuculline (Sigma B6889), veratridine (Sigma V5754), hyperosmotic mannitol (Sigma M4125), phenylephrine (Sigma P6126), senktide (Calbiochem 05-23-0613, Gibbstown, NJ), ATP (Sigma A2383), alpha-melanocyte-stimulating hormone (α -MSH) (Calbiochem 05-23-0751) and angiotensin II (Calbiochem 05-23-0101) were performed using the CED method. In these experiments, rats were sacrificed 30 min after injection for qRT-PCR assays. The antagonists, TTX (Sigma T5651) which inhibits sodium channels, CNQX (Sigma C127) and APV (Sigma A6553) which inhibit activation of AMPA and NMDA receptors was delivered using the ALZET osmotic minipump method. In both sets of experiments, the rats were injected with 2M NaCl i.p. 3.5h after surgery and sacrificed 30 min later for qRT-PCR assay.

Primers and qRT-PCR procedure

Total SON RNA was purified using an Absolutely RNA kit (Stratagene, La Jolla, CA), quantified on a spectrophotometer (NanoDrop, Wilmington, DE) and stored at -80 °C until use. The RNA used in the present study had a 260/280 nm ratio between 1.93 and 2.24, representing little if any contamination by proteins or other organic solutes. Three sets of primers were used for measuring the *c-fos* and VP transcription. The primer pairs for VP mRNA qRT-PCR were reported previously (Ponzio et al., 2007). The other two pairs of specific primers (Fig. 2A and Table 1) for *c-fos* mRNA and VP hnRNA, were designed with the help of genefisher software (Giegerich et al., 1996). These primer sets were used in separate reactions at concentrations of 0.6 μ M, and were included in a one-step qRT-PCR reaction (Qiagen, Valencia CA) along with 10 ng of the collected SON-enriched total RNA template. Procedures for qRT-PCR were done according to the manufacturer's protocol. The annealing temperatures were 60 °C for all primers for qRT-PCR reaction. Cycle threshold (Ct) values were measured and calculated by the real time PCR apparatus (StepOnePlus, Applied Biosystems, Foster City, CA). Ct values indicate a PCR cycle number at which the measured fluorescence of the indicator dye (SYBR green), corresponding to the quantity of amplified

products, is increasing in an exponential fashion above background. Each individual qRT-PCR reaction was run in duplicate and all animals in a given experiment (e.g., experimental and control samples) were run together. An average Ct value was calculated for each PCR primer pair, for each experimental condition. An additional reaction using the VP hnRNA primer pair was done in the absence of reverse transcriptases (–RT) to control for DNA contamination in the RNA template. Only templates with Ct differences >5 for the +RT versus –RT experiments, representing little if any DNA contamination, were included in the analysis.

Immunohistochemistry

The brains were cryoprotected and kept at –80°C until use. Coronal sections (16 µm) were cut on a cryostat (Reichert-Jung 2800; Frigocut, Heidelberg, Germany) and mounted onto poly-L-lysine coated slides (Fisher Scientific 12-550-19, Pittsburgh, PA). The sections were rinsed with 0.1M PBS for 5 min, followed by 0.3 % Triton-X in 0.1M PBS for 5 min, followed by 0.3 % gelatin (Sigma G6269) in 0.1M PBS as a blocking buffer for 10 min. Then, brain sections were incubated with Fos antibody (sc-52, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a dilution ratio of 1:2000 in 0.1M PBS containing 0.3 % gelatin. To identify VP and OT neurons in the double-label experiments with Fos immunostaining, the mouse monoclonal antibody PS41 against VP-neurophysin (NP), or PS45 mouse monoclonal antibody against both VP-NP and OT-NP (Ben-Barak et al., 1985; Whitnall et al., 1985), were used at dilution of 1:50 and incubated overnight at 4 °C. After incubation in primary antibodies, the slides were rinsed four times for 5 min in 0.1M PBS at room temperature. For Fos-IR, they were then incubated for 2 hr at room temperature in Alexa Flour® 594-conjugated goat anti-rabbit (Molecular Probes, Eugene, OR) secondary antibody (1:500 dilution in 0.1M PBS) to visualize the Fos-IR as red fluorescent nuclei. For the VP-IR and OT-IR the sections were incubated in Alexa Flour® 488-conjugated goat anti-mouse (Molecular Probes) secondary antibody (1:500) to yield cytoplasmic green fluorescence. After immunohistochemistry the sections were air dried in the dark and coverslipped using Prolong Gold Antifade reagent (Molecular Probes) as mounting medium.

Data analysis

Average Ct values ± SEMs were calculated for each experiment, and the unpaired Student's *t*-test was used for the statistical analysis of comparisons of Ct values for the *c-fos* mRNAs and VP hnRNAs between the experimental and control SONs, with statistical significance set at $P < 0.05$. Control reactions SON RNAs with VP hnRNA primers but without RT reactions were performed for each PCR run to monitor the assays for DNA contamination and primer artifacts. Relative quantification of *c-fos* mRNA and VP hnRNA changes were determined by the comparative cycle threshold (Ct) method using the formula, $2^{-\Delta\Delta C_t}$ (Pfaffl, 2001; Livak and Schmittgen, 2001; Schmittgen and Livak, 2008), and data were normalized to VP mRNA in each sample (Ponzio et al., 2007). The fold change in *c-fos* mRNA or VP hnRNA in each sample was calculated as follows: fold change or ratio = $\frac{(2.0)^{\Delta C_t \text{ between experimental side and control side of SONs (c-fos mRNA or VP hnRNA)}}}{(2.0)^{\Delta C_t \text{ experimental side and control side of SONs (VP mRNA)}}$ and is expressed as percent change from control.

Results

The experimental paradigm that we have used to evaluate the effects of specific pharmacological agents on the expression of the *c-fos* and VP genes in the SON, was to compare the changes of *c-fos* mRNA and VP hnRNA levels in SONs exposed to these agents versus SONs exposed to vehicle only. This paradigm is illustrated in Fig. 1A, and the spatial coverage of the delivered agent in and around the SON using the CED injection and ALZET osmotic minipump modes of delivery is shown in Figs. 1B and 1C, respectively. The measurements of

the changes in specific RNAs were determined by using one-step qRT-PCR (see Methods and Fig. 2). Validation of this treated versus contralateral (control) SON strategy, using the qRT-PCR assay is shown in Table 2 and Fig. 3. We tested the reliability of the qRT-PCR assay by comparing the Ct values in the left (Lt) and right (Rt) SON under control (n=3) and hyperosmotic stimulation (1.5M NaCl i.p., n=5) *in vivo*. The Ct values for *c-fos* mRNA, VP mRNA and VP hnRNA did not significantly differ between SONs sampled from Lt versus Rt SON, either in control or in osmotically stimulated rats (Table 2). This shows that in a given animal both SONs respond similarly to a systemic osmotic stimulus, and therefore the contralateral SON can serve as an appropriate control for the experimental SON in each individual animal (Table 2).

We also showed that the Ct values obtained from SONs in rats injected i.p. with isotonic (0.15M NaCl, n=5), versus in SONs of rats injected i.p. with hypertonic (2M NaCl, n=4) saline reflected the expectations predicted from other types of measurements described in the literature (Kawasaki et al., 2005; Yue et al., 2006). That is, the Ct values of *c-fos* mRNA and VP hnRNA were significantly decreased indicating an increase in transcription in the SONs of rats injected with 2M NaCl compared with the SONs of rats injected with 0.15M NaCl (Fig. 3A), and the increases of *c-fos* mRNA and VP hnRNA in the 2M NaCl-treated SONs were 326% and 201% respectively of that found in the 0.15M NaCl treated (control) SONs (Fig. 3B).

Evidence that the increased expression of *c-fos* mRNA and VP hnRNA after acute systemic hyperosmotic stimulation depends on sodium-dependent neural activity in the SON

TTX (0.5 μ M) and vehicle were perfused over experimental versus control SONs, respectively, by the ALZET osmotic minipump delivery method (n=5). The average percentage expression of *c-fos* mRNA and VP hnRNA was 64% and 41%, respectively, of that found in the control SON (Fig. 4A), indicating that the TTX-treatment decreased the hyperosmotic-induced increase in both of their expressions. Consistent with this, the average Ct values for *c-fos* mRNA and VP hnRNA in the SON 30 min after acute osmotic stimulus were significantly increased in the unilateral SON that was TTX-treated by ALZET osmotic minipump (Table 3). Also supporting this is the finding, using double immunostaining for Fos (Red) in the VP and oxytocin MCNs (Green) after i.p. injection of hypertonic 2M NaCl in rats, that the control SON, as expected, expressed Fos in the SON, whereas the TTX-treated side did not express Fos (Fig. 4B).

Effects of direct application of a depolarizing agent, Veratridine, and hyperosmotic solutions on the SON on *c-fos* and VP gene expression

Veratridine (100 μ M), which causes depolarization by opening voltage-dependent sodium channels, was applied directly to the experimental SON, and the vehicle control (0.1M PBS with 1% DMSO) was applied to the contralateral SON (n=4). The percentages of *c-fos* mRNA and VP hnRNA in the experimental SON were 390% and 93%, respectively, of that found in the control SON (Fig. 5A). Average Ct values for *c-fos* mRNA in the SON was significantly increased in the veratridine-treated SON, but not VP hnRNA in the SON (Table 3).

In order to evaluate the effect of direct hyperosmotic stimulation to the MCNs in the SON, hyperosmotic mannitol (494 mOsm/kg) and PBS (293 mOsm/kg) was applied directly to the experimental and contralateral SONs, respectively (n=6). The percentages of *c-fos* mRNA and VP hnRNA in the SONs of hyperosmotically stimulated rats were 97% and 138%, respectively, of that found in the control SONs (Fig. 5B). However, analysis of the average Ct values of the changes in *c-fos* mRNA and VP hnRNA in the SON were not significantly different (Table 3).

Effects of ionotropic-glutamate receptor agonists and antagonists on *c-fos* and VP gene expression in the SON

An excitatory cocktail (abbreviated NAB) consisting of ionotropic-glutamate receptor agonists, NMDA (100 μ M), AMPA (100 μ M) and the GABA_A receptor antagonist, bicuculline (500 μ M), was applied directly to the experimental SON, and PBS vehicle (control) was applied to the contralateral SON by CED (n=6). The percentages of *c-fos* mRNA and VP hnRNA expressed were 593% and 106%, respectively, of that found in the control SON (Fig. 6). Average Ct values for *c-fos* mRNA in the SON were significantly increased in the NAB-treated SON, but not for VP hnRNA in the SON (Table 3).

A cocktail of glutamate receptor antagonists, CNQX (>100 μ M) and APV (>100 μ M), was applied to the experimental SON, and the corresponding vehicle solution (control) was applied to the contralateral SON by ALZET osmotic minipump delivery (n=6). The percentages of *c-fos* mRNA and VP hnRNA expressed were 72% and 94%, respectively, of that found in the control SON (Fig. 7). The osmotically induced increases in Ct values for *c-fos* mRNA and VP hnRNA by i.p. injection of 2M NaCl (see Fig. 3) were not significantly inhibited by the direct application of the glutamate antagonists (Table 3).

Effects of direct application of selected GPCR agonists on *c-fos* and VP gene expression in the SON

Various GPCR agonists were applied directly to the experimental SONs, and saline vehicles were applied to the contralateral SONs (controls) by CED (each n=6). The percentages of *c-fos* mRNA and VP hnRNA for phenylephrine plus ATP-treated SONs were 204% and 98%, respectively, of that found in the control SON (Fig. 8A). The percentages of *c-fos* mRNA and VP hnRNA for the substance P analogue, senktide (NK-3 agonist), plus ATP-treated SONs were 153% and 84%, respectively, of that found in the control SON (Fig. 8B). ATP was added to the above agonists, since it is known to enhance their effectiveness in the HNS (Sladek, 2004). The percentages of *c-fos* mRNA and VP hnRNA for the α -MSH-treated SONs were 184% and 89%, respectively, of that found in the control SON (Fig. 8C). The percentages of *c-fos* mRNA and VP hnRNA for the angiotensin II-treated SONs were 118% and 87%, respectively, of that found in the control SON (Fig. 8D). Phenylephrine (100 μ M) plus ATP (1 mM), senktide (100 μ M) plus ATP (1 mM), and α -MSH (500 μ M) all significantly increased the Ct values of *c-fos* mRNA but not VP hnRNA in the SON (Table 3), whereas Angiotensin II (100 μ M) did not significantly increase the Ct values of either *c-fos* mRNA or VP hnRNA. The average Ct value $\pm$ SEMs for these experiments are shown in Table 3.

Test of possible synergistic effects of the combined actions of excitatory amino acids together with the selected GPCR agonists on *c-fos* and VP gene expression in the SON

A cocktail containing NAB, together with all the effective GPCRs (see Fig. 9), phenylephrine (100 μ M), senktide (100 μ M), α -MSH (410 μ M) and ATP (1 mM) was applied directly to the experimental SON, and the saline vehicle (control) was applied to the contralateral SON by CED (n=6). The percentages of *c-fos* mRNA and VP hnRNA were 384% and 64%, respectively, of that found in the control SON (Fig. 9). The average Ct values for *c-fos* mRNA in the SON was significantly increased in the agonist cocktail-treated SON, but not VP hnRNA in the SON (Table 3). These data indicate that there are neither additive nor synergistic effects of these simultaneous agonist activations on *c-fos* mRNA or on VP hnRNA expression.

Discussion

The HNS plays a critical role in the maintenance of fluid homeostasis in mammals largely through the regulated secretion of VP into the general circulation (Antunes-Rodriguez et al., 2004; Brownstein et al., 1980; Sladek, 1999; Hatton, 1990). In this homeostatic process,

specific osmosensitive neurons in the forebrain detect osmotic changes in plasma and through their axonal projections influence the MCNs in the SON and PVN to alter their firing rates and VP secretion appropriately (Bisset and Chowdry, 1988; Bourque, 2008; Bourque and Oliet, 1997; Richard and Bourque, 1995; McKinley et al., 1992; Leng et al., 2001). There are two distinct osmotically driven signaling mechanisms that are known to influence the neuronal activities of the MCNs, an intrinsic mechanism in which the changes in osmotic pressure are directly sensed by osmoreceptors on the MCNs themselves (Mason et al., 1980; Leng et al., 1982; Bourque, 1989; 1998; 2008), and synaptic inputs onto the MCNs that release a variety of neurotransmitters and neuropeptides which activate either rapid-acting, ionotropic receptors (e.g., glutamate receptors) or slower-acting, GPCRs (Burbach et al., 2001; Leng et al., 2001; Sladek and Kapoor, 2001; Sladek, 2004; Sabatier et al., 2003).

The increase in neural activity in the MCNs that occurs after acute hyperosmotic stimulation produces, in addition to VP secretion (Stricker and Verbalis, 1986), an increase in VP gene transcription (Arima et al., 1999; Herman et al., 1991; Murphy and Carter, 1990; Zingg et al., 1986; Yue et al., 2006; Burbach et al., 2001) as well as in many other genes in the MCNs (Mutsuga et al., 2005; Hindmarch et al., 2006). The immediate-early gene responses to osmotic stimulated neural activity in the MCNs have been extensively studied (Kawasaki et al., 2005). In other well-studied neuronal systems, increases in neuronal activity are often coupled to increased *c-fos* gene expression by calcium-dependent, phosphorylation mechanisms triggered directly by action potentials or indirectly by synaptic-driven neural activity (Deisseroth and Tsien, 2002; Fields et al., 2005; Flavel and Greenberg, 2008). However, the specific signals and signal-transduction mechanisms that mediate the robust *c-fos* and VP gene transcriptional responses to acute hyperosmotic stimuli in the HNS are still unknown.

In this report, we study whether signals to the HNS that are known to evoke secretion of VP, can also serve as effective stimuli to induce increase in *c-fos* and VP transcription when applied directly to the MCNs *in vivo*. We have focused on signals such as the direct application of hyperosmotic solutions or neurotransmitters that activate ionotropic receptors both of which depolarize the MCNs and lead to action potentials that might then initiate a calcium-dependent, signal-transduction cascade to increase gene expression described above. Another alternative is that the direct activation of GPCRs on the MCNs (e.g., neurokinins, α -MSH, etc.) could serve as key signals in the stimulus-transcription coupling cascade.

We approached these questions using a strategy in which we apply specific candidate stimulator reagents directly to one SON unilaterally by CED or ALZET osmotic minipump infusions, and control (vehicle) solutions directly to the contralateral SON in the same animal. The CED method is based on convective, bulk flow at rates ($>0.25 \mu\text{l}/\text{min}$) which are independent of the molecular weight, concentration or diffusibility of the infused reagent, and thus produces a wider distribution in the brain tissue than diffusion alone (Bobo et al., 1994; Morrison et al., 1999; Szerlip et al., 2007). The ALZET osmotic minipump method similarly relies on bulk flow (at a rate equal to $0.13 \mu\text{l}/\text{min}$) and can be used to infuse the SON with reagents over many days without any damage (Shahar et al., 2004). We found, in preliminary experiments using Fluorogold marker solutions, that these methods of delivery completely covered the entire SON with the infused reagent, and remained restricted in distribution to a maximum of $500 \mu\text{m}$ in diameter in the brain tissues, and therefore did not influence the contralateral SON which served as the control SON in each rat. We measured the levels of *c-fos* mRNA, VP mRNA and VP hnRNA in experimental and control SON punches from each rat using qRT-PCR, and determined the percentage changes in expression levels of the *c-fos* mRNA and VP hnRNA as a result of the experimental treatment, after normalization to the VP mRNA in each SON (see Methods). The rationale for using the VP mRNA as the normalizing factor is based on the fact that the VP mRNA does not significantly change after acute osmotic stimulation *in vivo*.

(Herman et al., 1991; Yue et al., 2006; Kawasaki et al., 2005) and therefore can correct for any variations in the SON punches.

We evaluated the efficacy of the above strategy by testing whether the known increases in *c-fos* mRNA and VP hnRNA observed after acute i.p. salt-loading could be measured by this approach. The data shown in Table 2 which compares the Ct values between the two sides of the SON under control (0.15M NaCl i.p.) conditions of salt loading, shows that there are no significant differences between the Ct values between two sides, thereby indicating that one SON can be used as the control and the contralateral one as the experimental SON in these qRT-PCR analyses. The changes in Ct values for the *c-fos* mRNA and VP hnRNA levels were as expected, significantly different after acute salt-loading, and the VP mRNA Ct values did not change under these conditions showing that the latter can be used for normalization (Fig 3A). The calculated % control changes for *c-fos* mRNA and VP hnRNA shown in Fig 3B by this qRT-PCR approach compared favorably with data from identical experiments but where the measurements were made by *in situ* hybridization (Kawasaki et al., 2005).

Using the above strategy, we addressed the following questions: 1) is the direct action of hyperosmotic solutions on the osmoreceptors on the MCNs themselves an effective stimulus to cause increased *c-fos* and VP gene expression? 2) Is the glutamate synaptic input to the MCNs, known to be effective signals for VP secretion by the MCNs (Leng et al., 2001; Sladek, 2004) also an effective stimulus for increased gene expression? And 3) are direct applications of agonists that activate GPCRs on the MCNs able to increase *c-fos* and VP gene expression in the MCNs?

Effects of direct osmotic stimulation and MCN action potentials on *c-fos* and VP gene expression in SON

Since activation of osmosensitive receptors on the MCNs is known to cause their depolarization and the generation of action potentials (Bourque, 2008; Ludwig et al., 1994; Mason, 1980; Kusano et al., 1999), we studied whether this stimulus would also increase either *c-fos* or VP gene expression. We found that the infusion of hypertonic solutions directly into the SON did not increase either *c-fos* or VP gene expression (Fig 5B), thereby eliminating this stimulus from consideration as a sufficient signal. This failure to stimulate *c-fos* expression in MCNs by hyperosmotic solutions is in agreement with previous reports *in vivo* using microdialysis delivery of the hyperosmotic stimulus (Ludwig et al., 1997) and also *in vitro* (House et al., 1998). It is of particular interest that the increase in action potentials in the MCNs in hyperosmotic solutions did not produce increased *c-fos*, suggesting that action potentials per se are not effective signals. This conclusion was previously drawn from experiments in which antidromic spikes from the pituitary invaded the MCN somas but did not evoke *c-fos* expression (Luckman et al., 1994). In addition, other experiments have shown that central injections of α -MSH could increase *c-fos* expression in OT MCNs in the SON, but inhibited spike activity, indicating an inverse correlation between these events (Sabatier et al., 2003; Sabatier, 2006).

The above data indicate that action potentials by themselves are not sufficient stimuli to evoke increased gene expression in the HNS, but do not discount the possibility that they might still play some role in the osmotic-stimulus evoked *c-fos* or VP gene increased expressions observed *in vivo*. Our experiments using the voltage-gated sodium channel opener, veratridine, which causes profound depolarization in neurons (Wang and Wang, 2003; Ulbricht, 1998), produced a four-fold increase in *c-fos* but not VP gene expression in the SON (Fig. 5A). However, we cannot attribute this change in *c-fos* expression to the depolarization in the MCNs alone, since we also observed in our immunohistochemical analyses after veratridine stimulation that in addition to the MCNs in the SON many other regions of brain (e.g., cortex) expressed Fos (data not shown). This raises the likely possibility that the veratridine also depolarized nearby afferent fibers which synapse on the MCNs and also in distal-brain regions. That action

potential activity in the SON, presumably in such afferents, are involved in the osmotically-stimulated *c-fos* and VP gene expression is supported by the experiments shown in Fig. 4. This experiment demonstrates that the increases in *c-fos* and VP hnRNA transcription in responses to acute hyperosmotic stimulation are blocked by application of TTX to the SON (Fig. 4). We reported previously that osmotically-evoked expression of Fos protein and VP hnRNA in MCNs in the experimental SON was inhibited by unilateral delivery of TTX via ALZET pumps, whereas the contralateral SON which received only control PBS underwent the expected increase in the expression of Fos immunoreactive neurons (Shahar et al., 2004). The results in Fig. 4A are consistent with this previous report and suggest that either pre- and/or post-synaptic action potentials are required to increase *c-fos* and VP gene transcription in the MCNs. Since as we show above, post-synaptic action potentials alone in the MCNs are insufficient to drive gene expression, we take this to mean that the effective signals must derive from presynaptic action potentials (Richard and Bourque, 1995; Bourque, 1998; McKinley et al., 1992), possibly through the secreted neurotransmitters.

Effects of ionotropic, glutamate agonists on *c-fos* and VP gene expression in SON

Previous studies have implicated the glutamate input to MCNs as being the primary signals for the release of VP *in vivo* (Van den Pol, 1990; Onaka and Yagi, 2001; Pak and Curren-Collazo, 2002; Meeker, 2002; Csaki et al., 2000; Leng et al., 2001; Bourque, 1998) and *in vitro* (Swenson et al., 1998; Morsette et al., 2001; Sladek, 2004). In addition, other experiments *in vitro* that employed organ-cultured explants of the HNS have shown that osmotic stimulation can increase VP mRNA (Yagil and Sladek, 1990) and that this VP mRNA increase in the explant can be blocked by general synaptic blockade using 15 mM MgSO₄, and also more specifically by inhibition of excitatory amino acid receptors by 200 mM Kynurenic acid (Sladek et al., 1995).

Given the above literature suggesting that excitatory amino acid inputs were the likely candidates to act as signals to couple neural activity to gene transcription in the MCNs during acute osmotic stimulation, we applied the ionotropic glutamate agonists, NMDA and AMPA, together with the GABA_A receptor antagonist, bicuculline (abbreviated NAB) to block the influence of any evoked GABA release which is known to inhibit the excitatory input *in vivo* (Leng et al., 2001), and examined its effects on *c-fos* and VP gene expression. The results of these experiments are illustrated in Fig. 6, which shows that there was a six-fold increase in *c-fos* mRNA, but no change at all in VP hnRNA. While the robust stimulation of *c-fos* gene expression was expected and reassured us that our paradigm was effective in selectively stimulating the SON unilaterally, the failure to alter VP transcription by this stimulus was surprising.

In a second, related experiment we tested whether ALZET osmotic minipump infusions of a mixture of the ionotropic glutamate antagonists, CNQX and APV, into the SON could inhibit the usual increases in *c-fos* and VP gene expression, when stimulated by an acute i.p. salt-loading (as seen in Fig. 3B). The results of this study, illustrated in Fig. 7, shows that these antagonists directed against both the NMDA- and AMPA-receptors did not block the acute, osmotically driven increase in VP hnRNA, and only reduced the increase of *c-fos* mRNA level by about 25%. However, the latter reduction was not statistically significant. This was the case even through the same antagonists, at even lower concentrations, did block *c-fos* gene expression induced by NMDA or AMPA-agonists in the SCN in *in vitro* (data not shown). Taken as a whole, these data suggest that activation of NMDA and AMPA receptors in the MCNs by itself is not sufficient to account for the increases in *c-fos* and VP transcription during acute osmotic stimulation *in vivo*.

Effects of selected G-protein coupled receptor agonists on c-fos and VP gene expression in the SON

In view of the failure of glutamate receptor agonist stimulation to produce an increase in VP gene expression, we then considered the possibility that other types of neurohumoral messengers that have been proposed to be VP secretagogues might be effective either alone or in combination with excitatory amino acid receptor agonists. There are several potential signaling candidates that are known to activate various GPCRs in the HNS and which can cause the secretion of VP (Sladek and Kapoor, 2001). Prominent among those agonists that can activate GPCRs in the SON are members of the tachykinin receptor family, such as substance P and neurokinin-3 receptor selective agonists, such as senktide. Neurokinin-3 receptors are found in the SON (Ding et al., 1999) and when selective inhibitors such as SB-22220 are injected intraventricularly they block VP release *in vivo* in response to osmotic stimulation (Haley and Flynn, 2007). Furthermore, intraventricular injection of tachykinin agonists causes increased c-fos expression in the SON and VP and OT secretion *in vivo* (Ding et al., 1999) and *in vitro* (Howe et al., 2004). It has also been shown that ATP acts synergistically with tachykinin receptor agonists to increase release of VP from HNS explants *in vitro*. Other GPCR-activating candidates in the SON are adrenergic agonists, such as phenylephrine which also synergizes with ATP (Kapoor and Sladek, 2006; Song et al., 2006; Song and Sladek, 2006), angiotensin II, and α -MSH, whose MC4R receptor is highly expressed in the SON and PVN (Mountjoy et al., 1994). None of these GPCR agonists evoked VP gene expression when infused into the SON, but direct infusion of phenylephrine plus ATP, senktide plus ATP, and α -MSH did significantly increase c-fos gene expression (Fig. 8). A combined cocktail containing the excitatory amino acids and bicuculline (NAB), and the three GPCR agonists that elicited a robust c-fos gene response, was infused into the SON by CED, and no synergistic effects on the gene expression of VP was found (Fig. 9). Therefore, it is possible that another combination of signals is necessary to activate VP gene expression.

Summary and Conclusions

In this study, we describe an experimental paradigm whereby we stereotactically infuse putative neurotransmitter/neuropeptides agonists and antagonists over one SON unilaterally and use the contralateral SON, into which only vehicle solutions were infused, as the control SON in the same rat. Quantitative comparisons of measurements of c-fos mRNA and VP hnRNAs in RNAs obtained from experimental and control SONs are made using qRT-PCR, and found to be highly reproducible. Using this paradigm, we have been able to show robust increases in c-fos mRNA in the SON after direct activation of ionotropic glutamate receptors, and various GPCRs (e.g., tachykinin, adrenergic, and α -MSH receptors), but not after applications of hypertonic solutions directly to the SON. In these experiments where the increased c-fos transcription in the SON occurs so robustly, and we can be sure that the MCNs are strongly activated, we were surprised to find that there was no concomitant increase in the transcription of the VP gene as occurs during acute systemic osmotic stimulation *in vivo*. It is possible that the difference between the fos and VP gene expression responses to the direct application of the NAB and peptide ligands might be due to the differential impact of potential depolarization block, accommodation, or receptor desensitization processes. However, irrespective of which mechanism underlies this difference in responses between these two genes, we can conclude from these data that the signal-transduction mechanisms that underlie the similar rapid kinetic responses of these two genes to acute hyperosmotic stimulation must be quite different. While the c-fos gene does not respond to increased action potential activity alone, it is promiscuously responsive to many neurohumoral signals, while the VP gene responds to none of these. These observations do not exclude the possibility that c-fos or any other immediate-early genes that increase in expression during acute hyperosmotic stimulation (Kawasaki et al, 2005) are involved in the regulation of VP gene expression, presumably acting through the functional AP1 element present in the rat VP gene (Yoshida et al, 2006). What specific signals are

necessary and sufficient to mimic the input to the MCNs during acute hyperosmotic stimulation to up regulate the expression of the VP gene remains unclear at present.

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Figure 1.

Convection enhanced delivery (CED) and ALZET osmotic minipump methods were used to stereotactically deliver various pharmacological agents to the supraoptic nucleus (SON) *in vivo*. (A) Illustrates a schematic diagram of this paradigm showing in a rat brain coronal section at the level of mid SON. The locations of the bilateral cannulae positioned approximately 300 μm over each SON. (B) Magnocellular neurons (MCNs) in the SON are shown by immunofluorescence in red using the PS45 antibody that cross-reacts with both vasopressin (VP) and oxytocin (OT)-NP. Fluoro-Gold (0.005%) was delivered by CED to one SON (right panel) and 0.9% saline was delivered to the contralateral SON (left panel). The Fluoro-Gold filling the SON and surrounding area is shown as blue fluorescence, and the dotted line shows the full extent of the dye spread. (C) MCNs in the SON are shown by single-label immunofluorescence in red using the PS45 antibody against VP and OT-NP. Fluoro-Gold (0.005%) was delivered by the ALZET minipump method to one SON and 0.9% saline was delivered to the contralateral SON (left panel). The extent of Fluoro-Gold filling the SON and surrounding area is shown as blue fluorescence, and the dotted line shows the extent of the dye spread.

OX, optic chiasm; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus. Scale bars = 100 μm

Figure 2.

Quantitative real time PCR (qRT-PCR) of *c-fos* mRNA, VP mRNA and VP heteronuclear (hn) RNA. (A) Locations of qRT-PCR primers in the RNAs are shown as arrows representing the positions of forward and reverse PCR primer binding sites (See Table 1 for sequences). In this single step assay, the reverse PCR primer also serves as the primer for reverse transcription (see Methods). (B) Representative fluorescence “growth curves” produced by quantitative real time (qRT)-PCR for *c-fos* mRNA, VP mRNA and VP hnRNA are shown for the SON 30 min after 0.15M NaCl intraperitoneal (i.p.) injection (control conditions).

Figure 3.

Changes in Ct values for *c-fos* mRNA, VP mRNA and VP hnRNA in rat SONs after acute osmotic stimulation as measured by the qRT-PCR assay. **(A)** Average $\pm$ SEM Cycle threshold (Ct) values from control SONs in rats injected with 0.15M NaCl (black bars) versus SONs in rats injected with 2M NaCl (stripped bars) are shown. **(B)** Calculated percent changes in *c-fos* mRNA and VP hnRNA in the SON 30 min after the injection of hyperosmotic 2M compared to rats injected with isotonic 0.15M NaCl injection. Statistically significant differences between Ct values in the 0.15M and 2M injected rats are shown by asterisks where $P < 0.05$.

Figure 4.

Inhibition of voltage-sensitive sodium channels in SON by TTX delivered by ALZET osmotic minipump blocks increased expression of *c-fos* mRNA and VP hnRNA in response to acute hyperosmotic stimulation by 2M NaCl i.p. injection. **(A)** The decreases in *c-fos* mRNA and VP hnRNA in the TTX-treated (3.5h) SON after acute osmotic stimulus (30 min) compared to their increases the control SON are illustrated as a percent of control SON values. The reductions of *c-fos* mRNA and VP hnRNA in the TTX-treated SON were 64% and 41% of the control SON, respectively. **(B)** Double immunostaining for Fos (in Red) and for both VP and OT-MCNs (PS45-ir, Green) after i.p. injection of hypertonic NaCl in rats. The PBS (Control) side **(B-I)** expressed Fos in the SON, but the TTX-treated side **(B-II)** did not express Fos, confirming the inhibition of Fos expression by ALZET osmotic minipump delivery of TTX. Scale bars=50 μ m

Figure 5.

Effects of direct depolarization and hyperosmotic stimulation on *c-fos* mRNA and VP hnRNA in the SON. **(A)** Veratridine (100 μ M) applied directly to the SON by CED increased the expression of *c-fos* fourfold but did not increase VP transcription in the SON. **(B)** Direct osmotic stimulation of the MCNs in SON by direct application of 494 mOsm/kg hyperosmotic mannitol to the SON did not significantly change the expression of either the *c-fos* or the VP gene in the SON.

Figure 6.

Direct application of an excitatory cocktail (NAB) consisting of ionotropic-glutamate receptor agonists (NAB), NMDA (100 μ M), AMPA (100 μ M) plus bicuculline (500 μ M), a GABA_A receptor antagonist to the SON by CED increased the expression of *c-fos* sixfold, but did not change VP gene expression in the SON.

Figure 7.

Ionotropic glutamate antagonists do not significantly block either *c-fos* mRNA or VP hnRNA expression in response to systemic hyperosmotic stimulation. The osmotically induced increases in *c-fos* and VP gene expression by i.p. injection of 2M NaCl (see Fig. 3) is not significantly inhibited by the direct application of glutamate antagonists, CNQX (>100 μ M) and APV (>100 μ M) by ALZET osmotic minipump delivery to the SON.

Figure 8.

Effects of the direct application of various G-protein coupled receptor (GPCR) agonists by CED on *c-fos* and VP gene expression in the SON. (A) Phenylephrine (100 μ M) plus ATP (1 mM), (B) Senktide (Substance P analogue) (100 μ M) plus ATP (1 mM), and (C) alpha-melanocyte stimulating hormone (α -MSH) (500 μ M) all increased the expression of *c-fos* mRNA but not VP hnRNA in the SON. (D) Angiotensin II did not increase the gene expression of either *c-fos* or VP.

Figure 9.

Test of possible synergistic effects by the combined actions of excitatory amino acids and selected GPCR agonists on *c-fos* and VP gene expression in the SON. Agonist cocktail containing NAB (NMDA (100 μ M), AMPA (100 μ M), bicuculline (500 μ M)), phenylephrine (100 μ M), senktide (100 μ M), α -MSH (410 μ M) and ATP (1 mM) was delivered by CED increased the expression of *c-fos* but not the VP gene in the SON.

Table 1qRT-PCR primers used to assay *c-fos* and vasopressin (VP) transcription

Primers	Sequence	Product	T _A [*]	T _M (°) [*]
<i>c-fos</i> mRNA primer pairs	5'-AGCATGGGCTCCCCTGTCA (forward) 5'-GAGACCAGAGTGGGCTGCA (reverse)	134 bp	60 °C, 30 s	83.4
VP mRNA primer pairs	5'-TGCCTGCTACTTCCAGAACTGC (forward) 5'-AGGGGAGACACTGTCTCAGCTC (reverse)	77 bp	60 °C, 30 s	80.7
VP hnRNA primer pairs	5'-GCCCTCACCTCTGCCTGCTA (forward) 5'-CCTGAACGGACCACAGTGGT (reverse)	100 bp	60 °C, 30 s	81.5

* T_A, annealing temperature and time; T_M, PCR product melting temperature.

Table 2

Comparisons of left (Lt) and right (Rt) SON Ct values (mRNA and hnRNA levels) under control and hyperosmotic stimulation *in vivo*

0.15 M NaCl i.p.	Ct (Lt) $\pm$ SEM	Ct (Rt) $\pm$ SEM	Lt vs Rt Ct difference	P value
<i>c-fos</i> mRNA	27.56 $\pm$ 0.31	27.55 $\pm$ 0.28	+0.01	0.97
VP mRNA	17.95 $\pm$ 0.41	17.99 $\pm$ 0.13	-0.04	0.90
VP hnRNA	29.25 $\pm$ 0.18	29.58 $\pm$ 0.58	-0.33	0.55
<i>1.5 M NaCl i.p.</i>	<i>Ct (Lt) $\pm$ SEM</i>	<i>Ct (Rt) $\pm$ SEM</i>	<i>Lt vs Rt Ct difference</i>	<i>P value</i>
<i>c-fos</i> mRNA	25.92 $\pm$ 0.44	25.61 $\pm$ 0.24	+0.31	0.23
VP mRNA	18.61 $\pm$ 0.55	18.26 $\pm$ 0.51	+0.35	0.67
VP hnRNA	28.91 $\pm$ 0.59	28.47 $\pm$ 0.46	+0.44	0.53

Average $\pm$ SEM Ct values for *c-fos* mRNA, VP mRNA and VP hnRNA were not significantly different in SONs sampled from left versus right side, neither in control (0.15 M NaCl intraperitoneal (i.p.) injection, n=3) nor in osmotically stimulated (1.5 M NaCl i.p., n=5) rats showing that the contralateral SON can serve as a control for the experimental SON in each animal, as shown in Fig. 1 paradigm.

Table 3

Comparison of Ct values between control and experimental sides after unilateral perturbation of SON by specific molecular activators

(A) *c-fos* mRNA

Exp. solution	Ct (Exp. side) ± SEM	Ct (Control side) ± SEM	n	P value
TTX	30.14 ± 0.28	29.23 ± 0.14	5	** 0.01
Veratridine	23.20 ± 0.36	25.17 ± 0.22	4	** < 0.01
Hyperosmotic mannitol	28.55 ± 0.68	28.31 ± 0.38	6	0.67
NMDA + AMPA + bicuculline(NAB)	22.79 ± 0.28	25.12 ± 0.17	6	** < 0.01
CNQX + APV	29.88 ± 0.26	29.39 ± 0.41	6	0.37
Phenylephrine + ATP	29.03 ± 0.29	29.99 ± 0.14	6	* 0.04
Senktide + ATP	29.12 ± 0.14	29.78 ± 0.14	6	** < 0.01
α-MSH	30.21 ± 0.13	31.10 ± 0.22	6	** < 0.01
Angiotensin II	29.31 ± 0.15	29.72 ± 0.17	6	0.07
Cocktail (NAB + phenylephrine + senktide + α-MSH)	27.99 ± 0.12	30.01 ± 0.25	6	** < 0.01

(B) VP mRNA

Exp. solution	Ct (Exp. side) ± SEM	Ct (Control side) ± SEM	n	P value
TTX	19.38 ± 0.32	19.25 ± 0.18	5	0.69
Veratridine	17.77 ± 0.42	17.86 ± 0.51	4	0.89
Hyperosmotic mannitol	18.83 ± 0.19	18.90 ± 0.21	6	0.80
NMDA + AMPA + bicuculline(NAB)	17.49 ± 0.44	17.41 ± 0.50	6	0.90
CNQX + APV	19.86 ± 0.20	19.96 ± 0.29	6	0.55
Phenylephrine + ATP	19.93 ± 0.19	19.95 ± 0.11	6	0.16
Senktide + ATP	19.75 ± 0.15	19.85 ± 0.12	6	0.32
α-MSH	20.11 ± 0.12	20.17 ± 0.098	6	0.96
Angiotensin II	19.29 ± 0.22	19.48 ± 0.22	6	0.21
Cocktail (NAB + phenylephrine + senktide + α-MSH)	19.66 ± 0.14	19.83 ± 0.16	6	0.56

(C) VP hnRNA

Exp. solution	Ct (Exp. side) ± SEM	Ct (Control side) ± SEM	n	P value
TTX	31.63 ± 0.57	30.18 ± 0.21	5	** < 0.01
Veratridine	29.65 ± 0.46	29.62 ± 0.57	4	0.96
Hyperosmotic mannitol	30.04 ± 0.38	30.42 ± 0.25	6	0.38
NMDA + AMPA + bicuculline(NAB)	29.37 ± 0.31	29.22 ± 0.39	6	0.75
CNQX + APV	30.17 ± 0.092	30.14 ± 0.24	6	0.89
Phenylephrine + ATP	30.85 ± 0.20	30.75 ± 0.18	6	0.46
Senktide + ATP	30.65 ± 0.17	30.43 ± 0.16	6	0.41
α-MSH	30.69 ± 0.085	30.53 ± 0.11	6	0.39
Angiotensin II	30.54 ± 0.15	30.51 ± 0.16	6	0.62
Cocktail (NAB + phenylephrine + senktide + α-MSH)	31.64 ± 0.24	31.16 ± 0.21	6	0.27

Average Ct values in each experiment for (A) *c-fos* mRNA, (B) VP mRNA and (C) VP hnRNA are shown. Statistically significant differences between the control and experimental SONs are shown as P-values, where

* $P < 0.05$,

** $P < 0.01$.