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Neuroscience Letters 369 (2004) 191–196

Neuroscience Letters

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# Regulation of neuronal nitric oxide synthase mRNA expression in the rat magnocellular neurosecretory system

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Received 19 January 2004; received in revised form 25 June 2004; accepted 16 July 2004

**Abstract**

We examined the activation of nNOS mRNA expression within the supraoptic and paraventricular nuclei (SON and PVN) of the hypothalamus. In salt-loaded rats nNOS mRNA expression was significantly increased in both nuclei. In rats given i.p. injections of 1.5 M NaCl (4 ml/kg), a small but significant increase in nNOS mRNA expression in the SON and PVN was found 6 h after injection; no change was detected 2 or 4 h after injection. In rats in which hyponatraemia had been induced experimentally, nNOS mRNA was downregulated in the SON, and expression levels were not increased within 4 h after intense acute osmotic stimuli. Finally, neurons of the SON were antidromically-activated by neural stalk stimulation for 2 h. No increase of nNOS mRNA expression in the SON was observed 2 h after stimulation. Thus, increased electrical activity is not directly coupled to rapidly increased expression of nNOS mRNA, and hence acute increases in nNOS mRNA expression are unlikely to play a role in short-term adaptation of the magnocellular system to osmotic stimulation.
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*Keywords:* In situ hybridization; Hypothalamus; Oxytocin; Osmotic stimulation; Neurohypophysial stalk stimulation

Neuronal NOS (nNOS) is highly expressed throughout the magnocellular neurosecretory system [2]. This expression is physiologically regulated during circumstances in which the secretion of oxytocin and vasopressin is altered [3,6,20]. In particular, nNOS mRNA expression in the magnocellular neurons of the supraoptic and paraventricular nuclei (SON and PVN) is up-regulated after chronic salt loading [5,21,23] and dehydration [19]. These stimuli also increase nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase activity (a histochemical indicator of NOS activity) in the SON [14] and the posterior pituitary [15]. Changes in NOS expression have also been reported through the course of pregnancy [9,12,17].

NO is produced in an activity-dependent manner in magnocellular neurones, possibly in response to increases in intracellular calcium concentration following calcium entry through voltage-gated calcium channels. NO production results in negative-feedback inhibition of the electrical activity of magnocellular neurons [17], reflecting both direct and indirect inhibition via the stimulated release of GABA [18]. The up-regulation of this system during chronic hyperosmotic stimulation would seem to enhance restraint of secretion, possibly helping to conserve stores of oxytocin and vasopressin during periods of sustained high demand.

However, during parturition, nNOS mRNA expression in the PVN and SON [17] is about 20% higher in rats killed 2 h after the start of parturition than in rats killed before parturition has begun. Since oxytocin secretion only increases very close to the onset of parturition in rats, the apparent ability of this system to show rapid upregulation suggested that acute changes in NOS expression might also influence neuronal responses to short-term osmotic stimulation. Vasopressin cells in particular show a marked evolution of firing pattern during the hours after osmotic activation, displaying

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doi:10.1016/j.neulet.2004.07.045

phasic discharge activity that becomes progressively more distinct, conceivably reflecting enhanced activity-dependent mechanisms [7]. In the present study, we examined whether acute strong osmotic stimulation or antidromic activation results in a rapid change in nNOS mRNA expression in the SON and PVN.

Female Sprague–Dawley rats (250–300 g, Bantin & Kingman, UK) were housed under standard conditions (12-h light:12-h light cycle, ambient temperature 20 ± 1 °C) with free access to food and tap water, except where stated otherwise.

In situ hybridization was performed as previously described [17] using three different 45-mer antisense oligonucleotide probes for nNOS complementary to bases 223–267 (5′-non-coding region), 4714–4758 (3′-non-coding region) and 1662–1706 of the rat nNOS sequence [2] and were labelled at the 3′ end with [α-$^{35}$S]deoxy-ATP (NEN, Boston, MA) using terminal deoxynucleotidyltransferase (Amersham Pharmacia Biotech, Little Chalfont, UK). Specific radioactivity of the labelled probes was between 367 and 1457 Ci/mmol. Coronal brain sections (15 μm) were cut on a cryostat through the SON, PVN, amygdala and subfornical organ, according to a rat brain stereotaxic atlas [13] and thaw-mounted onto gelatin-coated glass slides under RNAse-free conditions. Slides were then stored in desiccated slide boxes at −70 °C. For in situ hybridization, sections were fixed in 4% paraformaldehyde in 0.1 M PB for 30 min, washed in 0.1 M PBS, followed by acetylation in 0.1 M PB containing 0.1 M triethanolamine and 0.25% acetic anhydride (v/v) for 10 min. The sections were then dehydrated through a graded ethanol series, delipidated in 100% chloroform for 5 min and partially rehydrated in 100 and 95% ethanol before being air-dried. Slides were covered with hybridization buffer containing the mixed $^{35}$S-labelled nNOS oligonucleotide probes at 2500 dpm/μl and left to hybridize for 17 h at 37 °C in a humidifying chamber. Following incubation, excess hybridization solution was drained and the slides were briefly rinsed three times in 1× SSC at room temperature and then washed three times for 30 min in 1× SSC at 55 °C followed by 1 h in 1× SSC at room temperature. They were then air-dried and dipped in 300 mM ammonium acetate, 70% ethanol and air-dried again. Slides were exposed to Hyperfilm-βmax autoradiography film (Amersham Pharmacia Biotech, Little Chalfont, UK) for 3 weeks at 4 °C together with slides containing $^{35}$S brain paste standards. The films were developed (Kodak D-19, Sigma, Poole, UK) and fixed (Hypam Rapid Fixer, Ilford, Knutsford, Cheshire, UK).

The sections collected for the hyponatraemia study were subsequently dipped in liquid autoradiographic emulsion (Type G.5, Ilford, Knutsford, Cheshire, UK) and exposed for 12 weeks at 4 °C. Slides were developed (Kodak D-19, Sigma, Poole, UK), fixed (Hypam Rapid Fixer, Ilford, Knutsford, Cheshire, UK), counterstained (haematoxylin and eosin), dehydrated through an alcohol series, cleared in xylene and coverslipped.

The amount of nNOS mRNA was assessed by measuring silver grain density on the autoradiographic films over the SON, PVN, amygdala and subfornical organ under a light microscope (10× objective), quantified (NIH Image analysis system, version 1.58) and corrected for tissue background. A logarithmic relationship of radioactivity against grain density was plotted from standards and acceptable tissue grain density values lay on the linear part of this curve. For each identified brain area, the mean grain density determined from four sections of each rat was used to calculate the group mean grain density. All data are reported as mean ± S.E.M. Data were analysed statistically using a *t*-test (SigmaStat) or one-way repeated measures ANOVA followed by Student–Newman–Keuls test as appropriate. $P < 0.05$ was considered statistically significant. In the emulsion-dipped and counterstained sections, the percentage of positive cells was calculated from the total number of cells visible (25× objective) and the number of cells positive for nNOS mRNA per SON. A positive cell was defined as possessing more overlying silver grains than 3 S.D. above the mean background (taken from five non-magnocellular cells in a region lateral to the SON).

Control rats ($n = 8$) had access to food and tap water ad libitum, while salt-loaded-rats ($n = 8$) received access to food and 2% NaCl drinking solution. After 4 days of salt loading, rats were decapitated. Brains were removed, frozen on crushed dry ice and stored at −70 °C.

Rats were given intraperitoneal (i.p.) hypertonic saline (4 ml/kg 1.5 M NaCl; $n = 8$) or isotonic saline ($n = 8$) at around 10:00 h, the water bottle was removed and they were decapitated 2, 4 or 6 h later and the brains treated as above.

Hyponatraemia was induced as previously described [21,22]; in brief, rats were given 40 ml/day (70 kcal/day) of a nutritionally balanced liquid diet (AIN-76, Bioserv, Frenchtown, NJ, USA). After 2 days on the liquid diet, osmotic minipumps (Alzet model 2002, Alza, Palo Alto, CA, USA) containing 1-desamino-[8-D-arginine]-vasopressin (DDAVP; Rorer Pharmaceuticals, Fort Washington, PA, USA) were implanted subcutaneously, under halothane anaesthesia, to deliver DDAVP at 5 ng/h. On the day of osmotic minipump implantation, the rats were given a more dilute preparation of the liquid diet (70 kcal in 60 ml), but thereafter resumed the more concentrated formula (70 kcal in 40 ml). Rats were denied access to drinking water following surgery and for the remainder of the drug infusion period. Seven days after the induction of hypoosmolality, rats were injected i.p. (4 ml/kg) with isotonic (0.15 M), "low" hypertonic (1.5 M) or "high" hypertonic (3 M) NaCl. Four hours after injection, rats were decapitated and the brains were removed. Trunk blood was collected for measurement of plasma [$Na^+$] using a flame photometer.

Rats were anaesthetised with sodium pentobarbitone (Sagatal, 50 mg/kg, i.p., Rhône Mérieux, Hertfordshire, UK) and a femoral artery and vein cannulated. In control rats the skull was exposed only. In sham-operated rats the pituitary stalk was approached dorsally in the midline (3 mm caudal

to bregma) with a concentric stimulating electrode (SNEX-100X, Clarke Electromedical Instruments, UK), but no current was applied. In the stalk-stimulated rats the stimulating electrode was lowered into the pituitary stalk as described above and trains of biphasic pulses (20 Hz, 10 s on 10 s off, 1 mA peak to peak) were delivered for 2 h. After stimulation, the rats were left a further 2 h before the brains were removed. Blood samples were taken from the left femoral artery via a heparinised polythene cannula 5 and 10 min before and then 15 and 20 min after the start of stalk stimulation to verify correct electrode placement. The plasma was separated by centrifugation and stored at −20 °C until oxytocin assay. The remaining blood cells were resuspended in isotonic saline and returned to the rat via the femoral vein cannula. Plasma oxytocin concentrations were measured before and after stalk stimulation by specific radioimmunoassay [4]; all samples were measured in a single assay to avoid interassay variance.

Data are shown as means ± S.E.M. Groups were analysed by ANOVA, using non-parametric tests where normality tests failed, and subsequent post hoc pairwise tests, using SigmaStat software.

In all brains, strong hybridization signals for nNOS mRNA were apparent in the SON and magnocellular part of the PVN (mPVN), apparently reflecting expression in many magnocellular neurons, and this was confirmed by microscopic analysis of emulsion-dipped sections (not shown). From the film images, as expected, a significant increase in nNOS mRNA signal was apparent in the SON (54%) and the mPVN (61%) after 4 days of saline drinking ($P < 0.05$ and $<0.005$ compared to controls, $t$-test, respectively, Fig. 1). There was no significant change in nNOS mRNA expression in the other areas measured (amygdala and subfornical organ; Fig. 1).

In rats exposed to i.p injection of 1.5 M NaCl, there was intense nNOS mRNA hybridization with significant upregulation in the SON (21%) and the mPVN (16%) after 6 h ($P < 0.05$, compared to isotonic saline administration, $t$-test, Fig. 2A). There was no significant change in nNOS gene expression in the SON at earlier times, and no significant change in the amygdala and subfornical organ at 2, 4 or 6 h after i.p. hypertonic saline injection (data not shown).

Thus, while we could confirm previous reports that chronic osmotic stimulation results in an upregulation of nNOS mRNA expression in the SON, the response to acute hyperosmotic stimulation appeared to be slow in relation to other changes. In particular, no change was detected over the time scale in which evolution of discharge patterning of vasopressin cells has been described, from initial fast continuous firing to phasic firing, indicating that changes in the level of nNOS expression probably do not contribute to this evolution of discharge patterning.

The lack of a rapid increase in nNOS mRNA expression was surprising in light of earlier findings of an abrupt upregulation at parturition. One possibility seemed to be that, since late pregnant rats are mildly hyponatraemic, basal expression of nNOS mRNA is low and changes may be more readily detectable. We, therefore, studied rats in which hyponatraemia

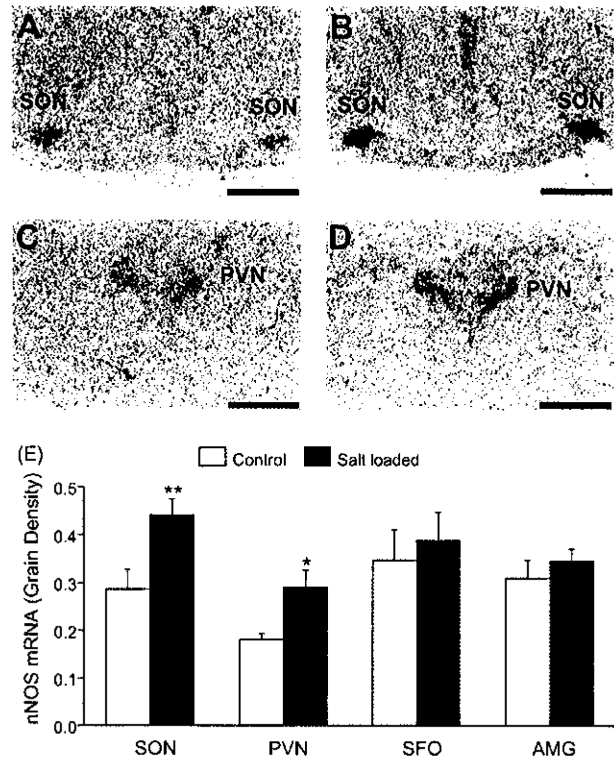


Fig. 1. (A–D) Example film autoradiographs of sections hybridized with $^{35}$S oligonucleotide probes against rat nNOS mRNA. Hybridization signal in the SON of 4 days salt-loaded rats (B) was greater than in control rats (A). The PVN of salt-loaded rats (D) also showed more signal than control rats (C). (E) nNOS mRNA expression measured as film grain density over the SON, PVN, subfornical organ and amygdala. Values are means ± S.E.M. ($^{*}P < 0.05$, $^{**}P < 0.005$, $t$-test, respectively, $n = 8$ each group). Scale bars, 1 mm.

had been induced chronically. Rats were given injections of 0.9 M NaCl (controls), 1.5 M NaCl or 3 M NaCl, and killed 4 h later. Control hyponatraemic rats had a plasma [$Na^+$] of 127 ± 1 mM ($n = 7$) compared to 140 ± 1 mM in control normonatraemic rats. In hyponatraemic rats, plasma [$Na^+$] was 130 ± 1 mM in rats given 1.5 M NaCl ($n = 7$) and 136 ± 1 mM in rats given 3 M NaCl ($n = 8$), indicating partial normalization of plasma [$Na^+$] at the highest dose of hypertonic saline.

In control (normonatraemic) rats, as found above, there was no significant change in nNOS mRNA expression 4 h after i.p. injection of 1.5 M NaCl, but there was a significant increase in expression in rats given the stronger stimulus (3 M NaCl) (one-way ANOVA on ranks $P < 0.01$; difference between control and hypertonic saline groups is significant on post hoc test (Dunn's method) at $P < 0.05$), as estimated either by the proportion of SON cells densely hybridized (Fig. 3), or by the silver grain density over the whole SON (data not shown).

In all groups of hyponatraemic rats, the proportion of cells densely hybridized was lower than in all groups of normonatraemic rats, and two-way ANOVA showed that this was a sig-

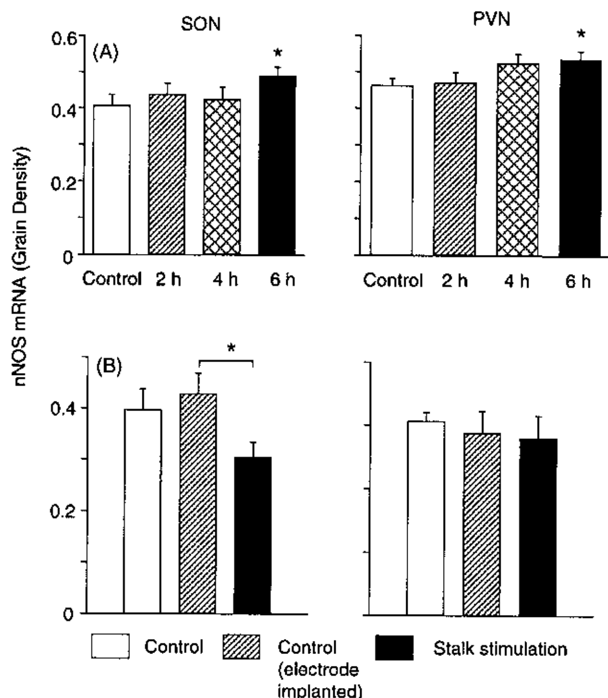


Fig. 2. nNOS mRNA expression measured as film grain density over the SON and PVN. (A) Significant increases in nNOS mRNA expression were observed in both the PVN and SON 6 h after hypertonic saline administration (4 ml/kg, 1.5 M NaCl, i.p.) compared to the control group (* $P < 0.05$, $t$-test, $n = 8$ each group). (B) A significant decrease of nNOS mRNA expression was observed in the SON after pituitary stalk antidromic stimulation ($n = 7$) (* $P < 0.05$, $t$-test compared to the control electrode implanted group, $n = 8$). There was no significant change in nNOS mRNA expression in the PVN. Values are means ± S.E.M.

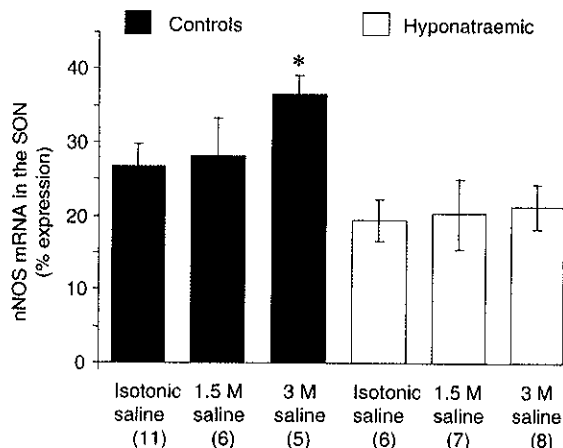


Fig. 3. nNOS mRNA expression in the SON measured as percentage of neurones expressing dense signal. Significant increases in nNOS mRNA expression were observed 4 h after injection of 3 M NaCl but not after injection of 1.5 M NaCl in normonatraemic rats. Expression was lower in hyponatraemic rats and did not increase significantly following acute stimulation. The bars show means (±S.E.) and the numbers of rats in each group are given in parentheses.

nificant difference between hyponatraemic rats and normonatraemic rats ($P < 0.05$). However, there were no significant differences among the three groups of hyponatraemic rats. Thus, in normonatraemic rats, osmotic stimulation results in a relatively slowly developing increase in nNOS mRNA expression in the magnocellular system; and we could detect no response within 4 h of an intense acute stimulus in hyponatraemic rats (Fig. 3).

We went on to test whether a more intense acute stimulus would increase nNOS mRNA expression more quickly. We used 2 h electrical stimulation of the neural stalk, at a frequency and intensity close to the maximal sustainable activity in magnocellular neurons, resulting in a large and prolonged secretion of both oxytocin and vasopressin; mRNA expression was measured 2 h after the end of stimulation. Basal oxytocin concentrations were not significantly different between groups, and in control and sham-operated rats, the plasma concentration of oxytocin did not change significantly. Stalk stimulation caused a very large increase in the plasma oxytocin concentration (4682%, $P < 0.05$, $n = 8$), but resulted, unexpectedly, in a significant decrease in nNOS mRNA expression in the SON (29%, $P < 0.05$ compared to sham-operated rats, Fig. 2B). The hybridization signal in the mPVN, amygdala and subfornical organ of stalk-stimulated rats were not significantly different from those in the control rats.

Although the nNOS gene is of the constitutive type, nNOS mRNA expression in the SON and PVN is increased by chronic osmotic stimulation as found here and in previous studies [5,19,21]; here we also report down-regulation of expression in chronic hyponatraemic conditions. However, although large changes in expression level accompany these chronic states of osmotic imbalance, expression levels of nNOS mRNA seem to change relatively slowly in response to acute osmotic stimuli. Here we show that 4 h after acute i.p. injection of 3 M NaCl or 6 h after acute i.p. injection of 1.5 M NaCl, we could detect a small but significant increase in nNOS mRNA expression in the SON. However, this response is slow in comparison to the increases in plasma osmolality and plasma vasopressin and oxytocin concentrations, which peak 30–60 min after injection [10], and increases in transcription of the vasopressin gene in the SON are detectable within 10 min of an acute osmotic stimulus [1], and certainly too slow to influence changes in oxytocin or vasopressin secretion in the acute phase of an osmotic response.

The lack of increased expression of NOS mRNA in hyponatraemic rats is consistent with the attenuated electrical and secretory response of oxytocin and vasopressin neurons to osmotic challenge in hyponatraemia [11]. In hyponatraemic rats, oxytocin and vasopressin cells respond progressively to increases in plasma [$Na^+$] by increasing their firing rate, but the slope of the response is much attenuated compared to the responses seen in normonatraemic rats [8], possibly because of down-regulation of expression of the stretch-sensitive membrane receptors that mediate osmosensitivity.

Thus, changes in NOS mRNA expression appear to reflect the extent of electrical activation of oxytocin cells, but as we also show in this study, spike activity per se is not sufficient to increase expression on NOS mRNA.

In cortical neurons, raised intracellular concentrations of calcium following influx through voltage-gated channels regulate the expression of nNOS gene through an alternate promoter region [16]. If this mechanism applies in magnocellular neurons, then electrical stimulation of the neural stalk should be effective in inducing changes in gene expression in magnocellular neurons, since stalk stimulation evokes action potentials that are conducted antidromically to the cell bodies. The action potentials at the cell bodies should increase intracellular calcium concentrations through voltage-gated channels similar to physiological activation of these neurones (see [7]). However, no increase in nNOS mRNA expression was observed 2 h after the end of 2 h of intense electrical stimulation; indeed there was a paradoxical decrease in expression in the SON. This was unexpected, and speculation on its possible physiological relevance is premature. However, we can conclude that calcium influx following antidromic activation in magnocellular neurones is not effective in increasing the expression of nNOS mRNA within 2–4 h.

Thus, changes in expression of nNOS mRNA that accompany chronic hyponatraemia or hypernatraemia seem likely to be specific adaptations to sustained changes in secretory demand. However, rapid up-regulation of nNOS mRNA expression probably plays no part in the evolution of patterning of neuronal activity that occurs in magnocellular neurons during the first few hours of activation after hyperosmotic stimulation. In previous studies we have shown that NO has a negative-feedback inhibitory action on the electrical activity of both oxytocin cells and vasopressin cells [17]; it may be that this role is particularly important during chronic activation of secretion, in order to conserve pituitary stores of hormone; when NO activity is blocked, dehydration leads to extreme depletion of pituitary hormone content [5].

## Acknowledgements

This work was supported in part by grants from the Wellcome Trust, the BBSRC, and by a Royal Thai Government scholarship to R.S.

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