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NMDA receptor subunit expression in the supraoptic nucleus of adult rats: Dominance of NR2B and NR2D

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Abstract

The supraoptic nucleus (SON) of the hypothalamus contains magnocellular neurosecretory neurons (MNC) which synthesize and release the peptide hormones vasopressin and oxytocin. Glutamate is a prominent excitatory neurotransmitter in the SON and regulates MNC excitability. NMDA receptors (NMDAR), a type of ionotropic glutamate receptor, mediate synaptic plasticity of MNCs and are necessary for characteristic burst firing patterns which serve to maximize hormone release. NMDARs are di- or tri-heteromeric complexes of NR1 and NR2 subunits. Receptor properties depend on NR2 subunit composition and variable splicing of NR1. We investigated the expression profile of NR1 and NR2 subunits in the SON at the mRNA and protein levels, plus protein expression of NR1 splice variants in control and salt-loaded adult rats. There was robust mRNA expression of all subunits, with NR2D levels being the highest. At the protein level, NR1, NR2B and NR2D were robustly expressed, while NR2A was weakly expressed. NR2C protein was not detected with either of two antibodies. All four NR1 splice variant cassettes (N1, C1, C2, C2') were detected in the SON, though NR1 N1 expression was too low for accurate analysis. Three days of salt-loading did not alter mRNA, protein or splice variant expression of NMDAR subunits in the SON. Robust NR2D protein expression has not been previously shown in MNCs, and is uncommon in the adult brain. Though the functional significance of this unusual expression profile is unknown, it may contribute to important physiological characteristics of SON neurons, such as burst firing and resistance to excitotoxicity.

Keywords

hypothalamo-neurohypophyseal system; posterior pituitary; NR2B; NR2D; vasopressin; oxytocin

1. Introduction

The hypothalamo-neurohypophyseal system (HNS) consists of neurons in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus which produce arginine vasopressin (AVP), a peptide hormone necessary for regulation of fluid balance homeostasis, and oxytocin (OT), a closely related hormone that stimulates lactation and parturition. AVP and OT are produced in neurons referred to as magnocellular

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neurosecretory cells (MNCs) and are released into the blood stream via exocytosis from axon terminals in the neurohypophyseal lobe of the pituitary (Bourque et al., 1994; Swanson and Sawchenko, 1983). The HNS is tightly regulated by extracellular fluid osmolality and volume.

Glutamate is an important excitatory neurotransmitter in the SON (Meeker et al., 1993a; van den Pol et al., 1990) and plays an important role in many functions of the SON. These include: regulation of excitability (Bioulac et al., 1978; Hu and Bourque, 1991; Israel et al., 2010), synaptic transmission (Gribkoff and Dudek, 1988) and peptide hormone release (Meeker et al., 1999; Sladek et al., 1995; Swenson et al., 1998). The SON contains a high density of glutamatergic presynaptic terminals (Meeker et al., 1993b; Meeker et al., 1989), and SON MNCs express a variety of ionotropic and metabotropic glutamate receptors (Hattori et al., 1998; Meeker, 2002; Meeker et al., 1999; Morsette et al., 2001; van den Pol et al., 1990).

Functional N-methyl-D-aspartate receptors (NMDAR) are ubiquitously expressed on SON neurons (Al-Ghoul et al., 1997b; Currás-Collazo et al., 2000; Currás et al., 1998; Decavel and Curras, 1997), and are required for long-term potentiation (LTP) and depression (LTD) of synaptic responses in MNCs (Panatier et al., 2006), AVP release in response to osmotic stimuli (Swenson et al., 1998) and characteristic phasic burst firing of AVP MNCs, which is associated with enhanced hormone release (Hu and Bourque, 1992; Israel et al., 2010).

NMDARs are membrane-bound ionotropic glutamate receptors that function as ligand-gated, non-selective cation channels. They are characterized by voltage-dependent activity, high calcium permeability and slow activation/deactivation kinetics relative to other ionotropic glutamate receptors (Ascher and Nowak, 1988; Chaffey and Chazot, 2008). Membrane depolarization and ligand binding are both required for activation (Bliss and Collingridge, 1993). NMDARs are assembled as heterologous tetramers, composed of the obligatory NR1 subunit and modulatory NR2 subunits. Four NR2 subunits, each encoded by a separate gene, have been identified: NR2A, NR2B, NR2C, NR2D (Ishii et al., 1993; Kutsuwada et al., 1992; Monyer et al., 1994; Moriyoshi et al., 1991).

There is a single gene for the NR1 subunit, but alternative splicing creates eight different isoforms. These variants arise from the inclusion or exclusion of two insertional cassettes, one in the extracellular N-terminal domain (N1, exon 5) and one in the intracellular C-terminal domain (C1, exon 21). Plus, there are two alternate versions of the final exon (C2 or C2', exon 22 or exon 22'), such that each NR1 molecule contains either C2 or C2' (Cull-Candy and Leszkiewicz, 2004; Sugihara et al., 1992; Zukin and Bennett, 1995).

Receptors lacking the N1 cassette are partially inhibited (~50%) at physiological pH, potentiated by polyamines and inhibited by zinc ions, while those containing N1 are insensitive to these modulators (Traynelis et al., 1998; Traynelis et al., 1995). The C1 cassette contains phosphorylation sites for protein kinase A and protein kinase C (Tingley et al., 1997; Tingley et al., 1993), an endoplasmic reticulum retention signal (Scott et al., 2001; Xia et al., 2001) and binding sites for various intracellular proteins [calmodulin (Ehlers et al., 1996); neurofilaments (Ehlers et al., 1998); yotiao (Lin et al., 1998)]. The C2' cassette contains a PDZ binding domain which interacts with post synaptic density (PSD)-95. In hippocampal neurons, C2' containing receptors have been shown to have higher surface expression than C2 containing receptors. However, insertion of C2 containing receptors into the plasma membrane has been shown in response to LTP induction (Grosshans et al., 2002; Standley et al., 2000). The expression profile of NR1 splice variants has not been previously investigated in the SON.

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The NR2 subunit composition also determines various biophysical and pharmacological properties of NMDARs (Cull-Candy and Leszkiewicz, 2004). Receptors containing NR2A display a faster deactivation time (100ms) than those composed of NR2B or NR2C subunits (250ms), and the deactivation time of NR2D containing receptors (~4sec) is more than an order of magnitude slower than that for any other NR2 subtype (Monyer et al., 1994; Vicini et al., 1998). Plus, NR2C or 2D containing receptors respond more rapidly to fast depolarizations than NR2A or 2B containing receptors (Clarke and Johnson, 2006) and are less sensitive to blockade by magnesium ions (Momiyama et al., 1996), allowing for stronger activity at relatively negative membrane potentials.

Expression of NR2 subunits in the central nervous system (CNS) is regionally and temporally diverse. The NR2A subunit does not expresses until after birth and its highest expression levels are seen in the hippocampus and cortex (Portera-Cailliau et al., 1996; Wenzel et al., 1997). The NR2B subunit, on the other hand, is highly expressed before birth and remains high throughout development and adulthood, except in the cerebellum, where it is detectible only until post-natal day 22 (Wang et al., 1995). NR2B is predominantly expressed in forebrain areas, with particularly high expression in the hippocampus, thalamus and cerebral cortex, but there is also significant expression in the olfactory bulb and motor neurons of the spinal cord (Laurie et al., 1997; Loftis and Janowsky, 2003). NR2C replaces NR2B in the cerebellum in adults, and is expressed almost exclusively in cerebellar Purkinje and granule cells, though low levels are present in the olfactory bulb, thalamus and vestibular nuclei (Llansola et al., 2005; Wenzel et al., 1997). NR2D is prominently expressed throughout the brain in late embryonic and post-natal development, but is dramatically reduced in most brain regions after the first two to three weeks of life (Brothwell et al., 2008; Chaffey and Chazot, 2008; Dunah et al., 1996; Liu and Wong-Riley, 2010; Wenzel et al., 1996). Nonetheless, NR2D persists into adulthood in a few regions of the CNS [striatum (Bloomfield et al., 2007); substantia nigra (Jones and Gibb, 2005); dorsal root ganglia (Marvizón et al., 2002); globus pallidus, thalamus and superior colliculus (Wenzel et al., 1996)].

The existence of NR3 subunits has recently been reported [NR3A, 1995 (Ciabarra et al., 1995; Sucher et al., 1995); NR3B, 2001 (Matsuda et al., 2002; Nishi et al., 2001)], but there is no evidence to suggest that they would be expressed in the SON of adult rats. Although the NR3A subunit is widely expressed during development, expression declines with age to very low levels in the adult (Ciabarra et al., 1995; Sucher et al., 1995; Wong et al., 2002). NR3B, on the other hand, is expressed throughout the lifetime, but its expression is restricted to spinal cord neurons (Chatterton et al., 2002; Matsuda et al., 2002).

The full expression profile of NMDAR subunits in the SON has not been characterized, but in addition to ubiquitous NR1 expression in MNC neurons, NR2B has been shown to be expressed in the SON of embryonic, early post-natal and adult rats (Currás-Collazo and Dao, 1999; Currás and Dao, 1998) and is localized to neurons, not astrocytes (Currás-Collazo et al., 2000). Ifenprodil (an antagonist at NR2B-containing receptors) has been shown to reduce the amplitude of evoked excitatory postsynaptic currents (EPSCs) by 80% when AMPA receptors were blocked (Panatier et al., 2006). Although this suggests that NR2B is a dominant NR2 subtype in the SON, *in situ* hybridization has demonstrated mRNA expression of all four NR2 subunits in both OT and AVP neurons in the SON (Al-Ghoul et al., 1997b), but neither protein expression of NR2A, NR2C and NR2D subunits nor the expression of NR1 splice variants has previously been investigated.

Previous studies conducted in rats have shown reduced NR2B expression (Currás-Collazo and Dao, 1999) and either an increase (Decavel and Curras, 1997) or no change (Currás-Collazo and Dao, 1999) in NR1 expression after 7–10 days of salt-loading. We investigated

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the effects of a more moderate osmotic stimulus, since three days of salt-loading has been shown to be sufficient to activate the HNS [increased hematocrit, plasma osmolality, and plasma AVP (Somponpun and Sladek, 2003)], and prolonged salt-loading is likely to activate stress-related systems and pathways in addition to osmotically induced responses. The goal of this study was to establish a complete expression profile of NMDAR subunits in the SON of adult rats and investigate whether a moderate osmotic stimulus would alter this expression profile.

2. Results

Rats were salt-loaded by replacing their water with 2% sodium chloride for three days before sacrifice. To confirm that this osmotic stimulus activated the HNS, trunk blood was collected immediately after depcapitation for measurement of plasma osmolality, which was slightly but significantly increased (Fig. 1a). This change was sufficient to evoke a two-fold increase in AVP mRNA levels (Fig. 1b). Significantly increased nNOS mRNA expression (Fig. 1c) and decreased ERβ mRNA expression (Fig. 1d) were also observed. Since these effects have previously been described in the SON in response to dehydration (Somponpun and Sladek, 2003; Ueta et al., 1995), this further demonstrates that the salt-loading protocol activated the HNS in these rats. These results also confirm that expected up- and down-regulation of target mRNA can be detected by our quantitative real-time reverse-transcriptase PCR (qRT-PCR) protocol.

Next, qRT-PCR was used to assess mRNA expression of NR1 and NR2 subunits in the SON of control and salt-loaded rats (Fig. 2). All five subunits were robustly expressed at fairly equivalent levels, though NR2D was the most abundant with about double the amount of mRNA compared to the other subunits. Salt-loading did not significantly alter the mRNA expression of any of the subunits, though trends for an increase in NR1 and a decrease in NR2B are consistent with changes observed in previous studies using more prolonged salt-loading.

In order to test whether protein was also present for all five subunits in the SON, microdissected tissue was analyzed using SDS-PAGE and immunoblotting. As with the rats used for mRNA analysis, trunk blood was collected to assess plasma osmolality, which was significantly elevated in salt-loaded rats (Fig. 3a). Hematocrit levels were also elevated in salt-loaded rats (Fig. 3b). In addition, nNOS protein levels were increased in these rats (Fig. 3c), as would be expected from the literature (Ryu et al., 2008; Song et al., 2005; Ueta et al., 1995) and from our mRNA data. The nNOS data confirms that a 40% increase in band density can be detected using our quantification method.

Gels were run with SON protein homogenates, alternating lanes between tissue from control and salt-loaded rats (n=4 or 6). Protein bands corresponding to NR1, NR2A, NR2B and NR2D were detectible (Fig. 4a), but three days of salt-loading did not alter the level of any of the subunits (Fig. 4d). NR1 protein was prominent in SON tissue, but less plentiful than in the hippocampus (Fig. 4a). NR2A protein was detectible in SON tissue, but much less abundant than in either the cerebellum or hippocampus. NR2B protein was robustly expressed, though also at lower levels than in the hippocampus. Consistent with the literature, NR2B expression was minimal in the cerebellum (Fig. 4a). NR2C protein was not detected in the SON with either of two antisera, though they both detected NR2C in the cerebellum (Fig. 4b). The Phosophosolutions antibody (Fig. 4b, top) cross reacts with NR2A and NR2B, and these ~180kD bands are apparent in the SON, hippocampus and cerebellum, but the ~140kD NR2C band is only present in the cerebellum. The Santa Cruz antibody (Fig. 4b, bottom) cross reacts with NR2D, which shows up as a double band at ~150 and ~180kD, but as with the first antibody, the ~140kD band is only present in the cerebellum. The fact

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that neither of two NR2C antisera raised in different species, against peptides at opposite ends of the protein detect a ~140kD band in the SON in either control or salt-loaded rats is strong evidence that this subunit is not expressed at the protein level in the SON. This is somewhat surprising since robust expression of NR2C mRNA expression was found in the SON by us (Fig. 2) and others (Al-Ghoul et al., 1997b).

Surprisingly, NR2D was robustly expressed in the adult SON. Though NR2D protein levels in the SON were lower than in post-natal day 4 (P4) midbrain, they were notably higher than in micropunched caudate, one of the brain regions known to express NR2D in the adult rat brain. On the other hand, robust NR2D protein expression in the SON is consistent with the high levels of mRNA expression observed using both qRT-PCR (Fig. 2) and *in situ* hybridization (Al-Ghoul et al., 1997b). As with the cross-reaction with NR2D of the goat polyclonal NR2C antibody, the NR2D-specific antibody detects a double band at ~150kD (close to the predicted size of 143kD) and ~180kD. These bands were detected both by the NR2D antibody and by NR2C antibody that crossreacts with NR2D, as well as a polyclonal NR2D antibody raised in rabbit (Santa Cruz, H-119; data not shown).

Alternative splicing of the NR1 subunit is another aspect of NMDAR expression that could contribute to changes in MNC activity during dehydration, since different variants are known to confer distinct properties and function to the receptor. Immunoblots similar to those used for NMDAR subunit analysis (n=4 or 6) were used to probe for the NR1 splice variant cassettes (Fig. 5a). The N1 cassette (exon 5) was barely detectible in the SON, and band density was not enough above background for accurate analysis in either control or salt-loaded rats. On the other hand, the C1 cassette (exon 21) and both of the alternate C-termini of NR1, C2 and C2' (exon 22 or 22'), were present (Fig. 5a). As with the NR2 subunits, no differences in protein levels were detected between control and salt-loaded rats for any of the cassettes (Fig. 5b).

3. Discussion

In this study, we investigated the expression profile of NMDAR subunits in the SON of control and moderately salt-loaded rats both at the mRNA and protein levels. At the mRNA level, NR1 and all four NR2 subunits were strongly expressed, but at the protein level, only NR1, NR2B and NR2D showed strong immunoreactivity. The expression profile of NR1 splice variants was also assessed using specific antibodies raised against four sections of alternative splicing. Surprisingly, no changes in mRNA or protein expression were detected in response to salt-loading for any of the subunits or splice variants analyzed.

mRNA expression for NR1 and NR2A-D subunits was quantified using qRT-PCR, and all five subunits were robustly expressed. NR2D levels were almost double those of the other four subunits, which were all expressed in similar amounts. This contradicts previous *in situ* hybridization evidence that the amount of NR1 mRNA was equal to the combined sum of mRNA amounts for the four NR2 subunits (Al-Ghoul et al., 1997a). However, comparing quantities of mRNA from different genes using *in situ* hybridization is limited by the semi-quantitative nature of this detection method and by the impact of slight variations in parameters such as tissue fixation, probe affinity/activity or exposure on mRNA detection.

Protein expression of the same five subunits, as well as four alternatively spliced regions of NR1, was qualitatively analyzed using SDS-PAGE gel electrophoresis and immunoblotting. NR1 and NR2B were strongly expressed, as has been previously shown. NR2A was detectible, but consistent with previous reports, was expressed only at low levels. We demonstrate here for the first time that, at the protein level, NR2C expression is absent in the SON. More surprisingly, NR2D protein was found to be robustly expressed in the SON of

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adult rats. This subunit is widely expressed in the CNS during late embryonic and early post-natal development, but expression persists into adulthood in only a few regions of the brain, and generally at low levels (Brothwell et al., 2008; Chaffey and Chazot, 2008; Dunah et al., 1996; Liu and Wong-Riley, 2010; Wenzel et al., 1996). Overall, our data suggest that NR2B and NR2D are the dominant NR2 subtypes in SON NMDARs.

There is no literature on NR2D protein in the SON, but NR2D mRNA has been localized to both AVP and OT immunoreactive MNCs (Al-Ghoul et al., 1997b). NR2B protein expression in the SON has been shown to be exclusively neuronal (Currás-Collazo et al., 2000), and NR2B is thought to be the primary NR2 subunit involved in characteristic burst firing and synaptic plasticity of SON MNCs. In these cells, ifenprodil (an antagonist at NR2B-containing receptors) blocks synaptic plasticity [LTP and LTD (Panatier et al., 2006b)], and reduces the amplitude of evoked excitatory postsynaptic currents (EPSCs) by 80% when AMPA receptors were blocked (Panatier et al., 2006). This suggest that another NR2 subtype is also involved, possibly NR2D, since triheteromeric receptors (NR1/NR2B/NR2D) are also sensitive to ifenprodil, though less so than diheteromeric NR1/NR2B receptors (Brothwell et al., 2008).

The functional implications of these triheteromeric receptors are not fully understood, but there is evidence that they participate in synaptic transmission during development in both the cerebellum (Brickley et al., 2003) and substantia nigra (Brothwell et al., 2008; Jones and Gibb, 2005). Interestingly, burst firing is also observed in these regions [substantia nigra (Grace and Bunney, 1983); cerebellum (Aizenman and Linden, 1999)]. The predominance of the NR2D subunit in the SON could explain why MNCs are surprisingly resistant to various neurotoxic insults, particularly NMDA-mediated excitotoxicity (Hu et al., 1992). NR2D containing receptors (either NR1/NR2D diheteromeric, or NR1/NR2B/NR2D triheteromeric) have much lower conductance than receptors containing only NR1, NR2A and/or NR2B (Brickley et al., 2003). It is possible that the predominance of the NR2D subunit is protective against excitotoxic insult by limiting cation influx.

Expression of NR1 splice variants has not been previously analyzed in the SON. We found that expression of the N1 cassette was barely above the limit of detection, and expression was too low to allow for analysis. In heterologous expression systems, receptors composed of NR1 subunits lacking the N1 cassette are partially inhibited (~50%) at physiological pH, potentiated by polyamines and inhibited by zinc ions (Traynelis et al., 1998; Traynelis et al., 1995). Conversely, the C-terminal insertional cassette, C1, as well as both alternative C-termini were expressed.

Contrary to previous reports, no significant differences were found between control and salt-loaded animals (three days of 2% sodium chloride drinking solution). The most likely explanation for this is that three days of salt-loading is a milder, less stressful, stimulus than the 7–10 days of salt-loading which has been shown to increase NR1 immunoreactivity (Decavel and Curras, 1997) and decrease NR2B protein (Currás-Collazo and Dao, 1999). The observed difference between these two stimuli suggests that changes in NMDAR expression may be involved in the response and adaptation to a prolonged osmotic challenge, but that these changes are not required to stimulate AVP synthesis and release. Three days of salt-loading increases AVP mRNA synthesis (Fig. 1b) and plasma AVP concentration (Somponpun and Sladek, 2003) in the absence of detectible changes in NMDAR expression. Unfortunately, the relative amounts of protein of the five subunits expressed in the SON cannot be established from the immunoblots used in these studies, since differences in band density may reflect uncontrolled variables, such as antibody affinity.

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In summary, we investigated the expression profile of NR1 and all four NR2 subunits in the SON at both the mRNA and protein levels, as well as protein expression of NR1 splice variants in control and salt-loaded adult rats. The results showed robust mRNA expression of all five subunits with NR2D mRNA levels almost double those of the other subunits. At the protein level, NR1, NR2A, NR2B and NR2D were expressed at detectible levels, though NR2A expression was quite low. NR2C protein was not detected with either of two antibodies. Furthermore, all four NR1 splice variant cassettes (N1, C1, C2, C2') were detected in the SON, though NR1 N1 expression was too low for accurate analysis. Three days of salt-loading had no effect on mRNA, protein or splice variant expression of NMDAR subunits in the SON. Robust NR2D protein expression has not been previously shown in MNCs, and is uncommon in the adult brain. Though the functional significance of this unusual expression profile is unknown, it may contribute to important physiological characteristics of SON neurons, such as their burst firing patterns and resistance to excitotoxicity.

4. Experimental Procedures

The amount of mRNA of NR1 and each NR2 subunit was quantified in control and salt-loaded animals using quantitative real-time reverse-transcriptase PCR (qRT-PCR). SDS polyacrylamide gel electrophoresis (PAGE) combined with immunoblotting was used to assess which NR2 subunits and NR1 splice variants are expressed in the SON and whether these levels were affected by three days of salt-loading.

4.1. Animals

Adult male Sprague-Dawley rats (280–400g, Charles River) were housed at an ambient temperature of 21°C with a 12:12 hour light-dark cycle and were allowed to acclimate for at least two days after arriving in our facility. Food was provided ad libidum, as was water, except during salt-loading. To salt-load animals, 2% sodium chloride in tap water was provided ad libidum for three days (72h). All protocols were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Colorado Denver.

4.2. Tissue collection

Trunk blood was collected in heparinized micro-hematocrit capillary tubes immediately after depcapitation. The tubes were promptly centrifuged in a Marathon 13K/H centrifuge (Fisher Scientific) and hematocrit was measured as percent of blood volume. The tubes were then carefully broken to isolate and collect the plasma. Plasma osmolality was analyzed in triplicate on a vapor pressure osmometer (Vapro).

To collect SON tissue, brains were promptly removed from the skulls of decapitated rats, and the SON was bilaterally microdissected from the bottom of the brain using the optic chiasm as a landmark. SON tissue from both sides of each brain was pooled for each rat. For protein analysis, tissue was homogenized in 35µl 1% STE lysis solution (1% SDS, 10mM Tris-HCL, 1mM EDTA) using a pellet pestle motor; protein concentrations were measured using a CB-X Protein Assay kit (G Biosciences). For RNA analysis, SON tissue was placed in RNAlater immediately after microdissection. The tissue was later transferred into 50µl of Qiazol lysis reagent (Qiagen), and homogenized using a pellet pestle motor. Total RNA extraction was performed according the manufacturer's protocol, and further purified using the RNeasy kit clean-up protocol (Qiagen). Purified 12 total RNA was resuspended in 30µl of RNAse-free water, and stored at −80°C until it was used for qRT-PCR.

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4.3. Quantitative real-time reverse-transcriptase PCR (qRT-PCR)

The mRNA was quantified using real-time reverse-transcription polymerase chain reaction (qRT-PCR) on an ABI Prism 7900 Sequence detector at the UCCC RT-PCR core facility. Total RNA concentrations were determined using A260/280. 1μg of RNA was reverse transcribed in a 50μl reaction mixture with 125U of Moloney Murine leukemia virus reverse transcriptase (Life Technologies, INC.), 20U RNAsin ribonuclease inhibitor (Promega Corporation), 4mM deoxyribonucleoside triphosphates, 5mM $MgCl_2$, 1X PCR Buffer II and 0.5μg of random hexanucleotide primers (the last four from Applied Biosystems). The reaction mixture was sequentially incubated at 65°C for 5 min., 42°C for 60 min., and the reverse transcription reaction was stopped by heating to 95°C for 5 min. The reaction mixture was then cooled to 4°C before the next cycle.

Table 1 contains location and sequence information for the primers used. Amplification reactions were carried out using optimal concentrations of primers and probe with 10μl cDNA. The standard curves for amplification of each mRNA were established using 10-fold serial dilutions of SON tissue pooled from unmanipulated rats. Cycle threshold was then plotted against the log of the quantity of cDNA used in each reaction. Standard curve correlation coefficients and cycle thresholds in the linear range were: AVP, r = 0.9998 (Ct 17–38); ERβ, r = 0.9987 (Ct 25–38); NR1, r = 0.9994 (Ct 20–36); NR2A, r = 0.9995 (Ct 24–38); NR2B, r = 0.9974 (Ct 21–36); NR2C r = 0.9940 (Ct 21–37); NR2D, r = 0.9992 (Ct 23–38). The standard curves were subsequently used to calculate the relative amounts of target mRNA in test samples, which were normalized to 18s rRNA in the same samples (PE ABI, P/N 4308310), and the results were reported as pg ERβ mRNA/ng 18s rRNA.

4.4. Immunoblotting

Proteins in SON tissue homogenates were electrophoresed on SDS-PAGE gels and blots were probed with the subunit or splice variant specific antibodies. Before being loaded onto the gel for SDS-PAGE, samples were mixed 1:1 with Laemmli sample buffer containing 5% β-mercaptoethanol (Biorad), incubated for 5 minutes in a 92°C drybath and centrifuged (10 minutes, 10,000rpm/9400g, 4°C). Samples containing 5–40μg of protein were then loaded onto gels (7.5% or 4–12% gradient) along with multiple lanes of dual standard protein standard (Biorad) as a reference for protein size. The gels were run at 75V for 15 minutes, followed by 100V for 1 hour. Afterwards, gels were equilibrated in semi-dry transfer buffer for 10 minutes before being transferred to nitrocellulose (10V for 10 minutes plus 14V for 30 minutes).

Immunodetection was optimized with the Snap-id (Millipore), which provides vacuum assisted application of antibody, blocking and rinse solutions. Blots were loaded into a blot holder and rinsed with 1% Tween-20 (PBS-T). PBS-T was used for all rinse steps and blocking protocols, as well as for antibody dilution. Blots were incubated for 2 minutes with 30ml blocking solution (0.5% non-fat dry milk, 2% BSA or 2% normal rat serum). Next, 3ml of primary antibody were applied for 10 minutes, pulled through by applying vacuum, then reapplied for 5 minutes and pulled through again. Blots were then rinsed (4 × 30ml) with PBS-T, and 3ml of secondary antibody (horse radish peroxidase conjugated goat anti-rabbit) were applied for 10 minutes before being pulled through. The blots were then rinsed (4 × 30ml) and removed from the blot holder, then rinsed at least 5 minutes in PBS before being put in imaging solution (5–8 minutes in SuperSignal West Fempto Maximum Sensitivity Substrate). Blots were imaged promptly and band density was analyzed using the gel analyzer plugin on ImageJ (NIH) and normalized to GAPDH (1:15,000; Santa Cruz, FL-335, rabbit polyclonal) to control for variations in protein loading.

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Table 2 contains NMDAR antibody details. Antibody specificity has previously been established: NR1 (Luo et al., 1997), NR2A (Snell et al., 1996); NR2B (http://neuromab.ucdavis.edu/datasheet/N59_20.pdf); NR2D (Lindahl and Keifer, 2004; Salter and Fern, 2005). The calculated size of NR2D is 143kD, but all of the antisera which recognized NR2D detected two bands at ~150 and ~180kD [sc-1266, Fig. 4a; sc-1471, Fig. 4b; Santa Cruz H-119 (polyclonal NR2D antibody raised in rabbit), data not shown]. Though it is possible that the ~180kD band is non-specific, it is more likely to be a glycosylated form of NR2D. This band has also been reported in rat brain homogenate using a mouse monoclonal antibody that showed no cross reactivity with any other NR2 subunits, and enzymatic deglycoslyation of the protein homogenate resolved the two bands into a single band migrating at a lower molecular weight (Laurie et al., 1997). The splice variant antibodies are specific and do not cross react with other NR1 isoforms (Coultrap et al., 2005). Both of the NR2C antibodies are known to cross-react with other subunits; the rabbit polyclonal NR2C antibody also recognizes NR2A and NR2B, while the goat polyclonal NR2C antibody also recognizes NR2D. In both cases, however, the NR2C band was identified based on the smaller molecular weight of NR2C.

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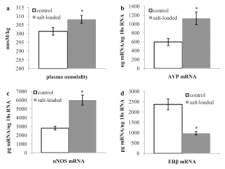

Figure 1. Confirmation of dehydration in salt-loaded rats used for NMDAR mRNA analysis

(a) Plasma osmolality, (b) AVP mRNA and (c) nNOS mRNA in the SON were all significantly increased, while (d) ERβ mRNA was significantly decreased. *$p<0.05$

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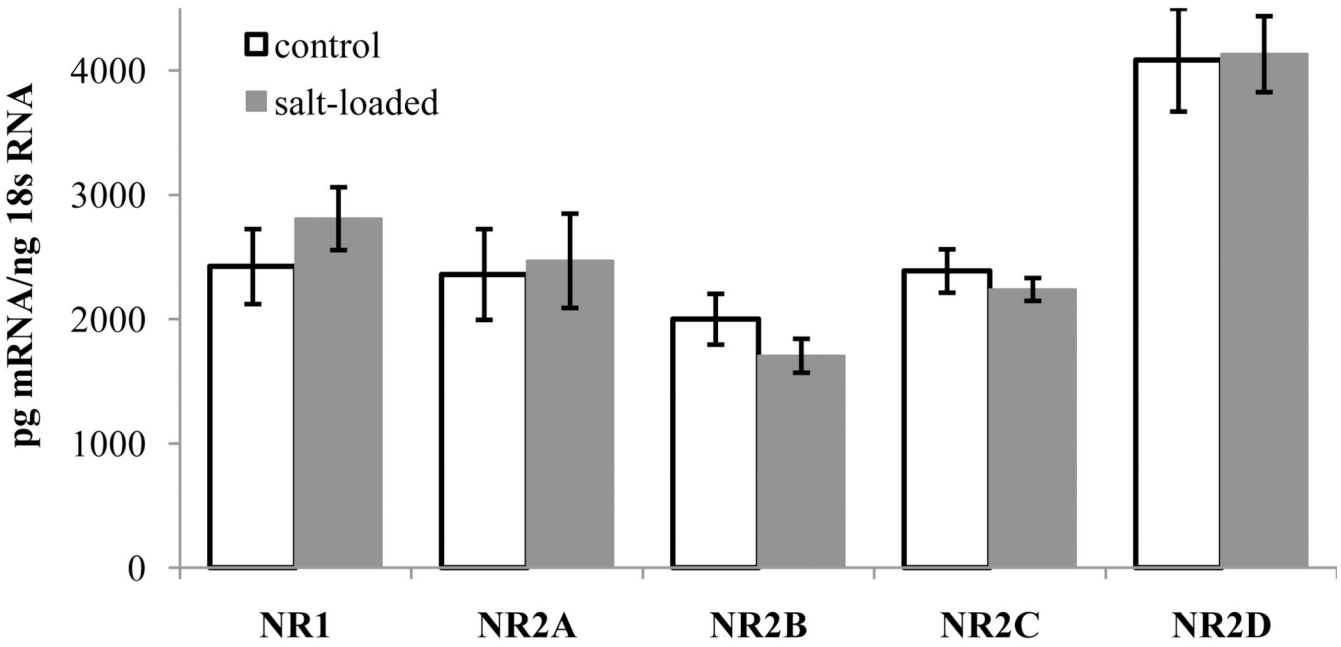

Figure 2. mRNA expression of NR1 and NR2A-D in the SON of control and salt-loaded rats

All five NMDAR subunits are transcribed in the SON. Of the NR2 subunits, NR2B is the least abundant, while NR2D is the most abundant. NMDAR subunit mRNA levels were not altered by three days of salt-loading. n=8 (NR1, NR2D); n=5 (NR2A-C).

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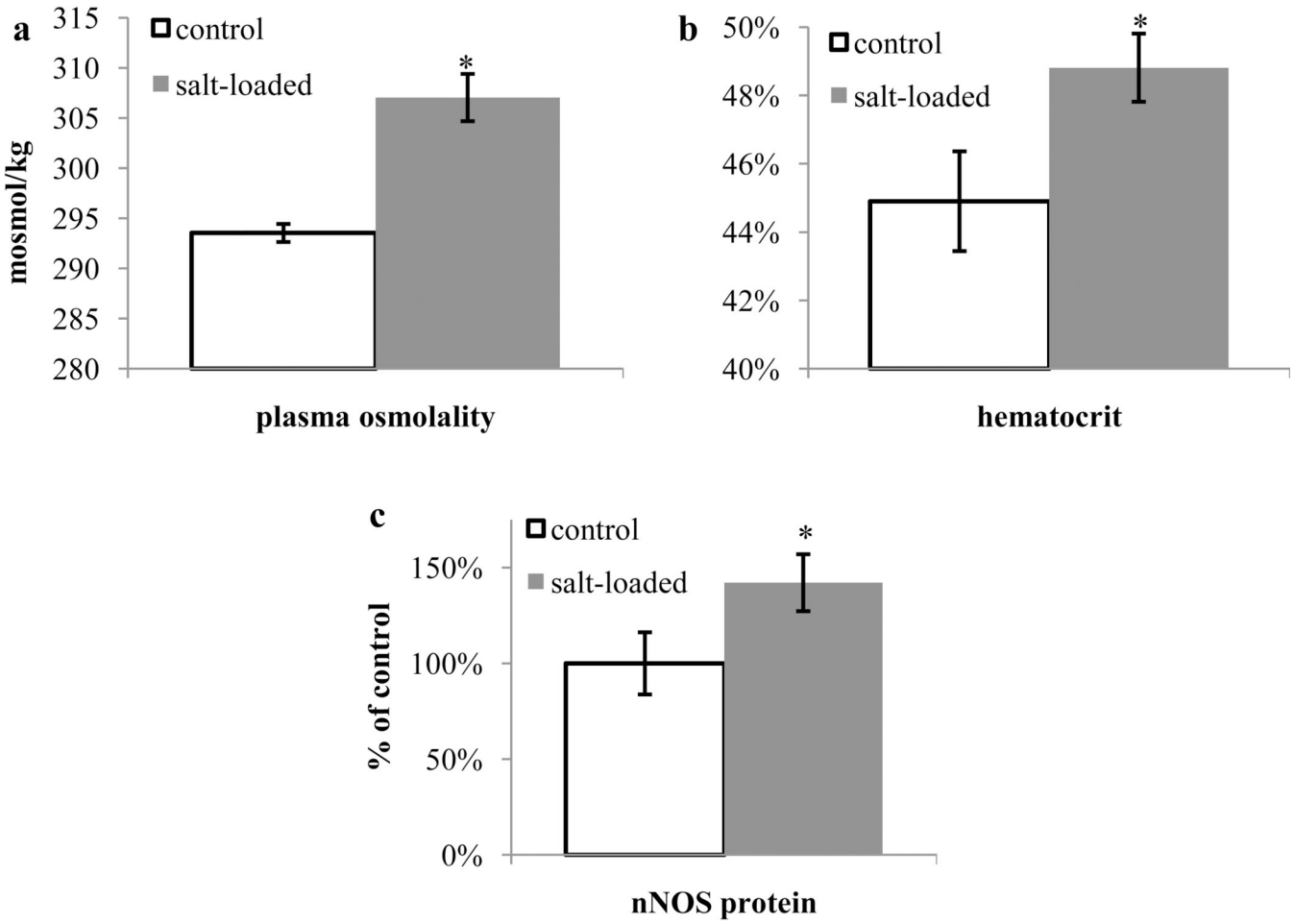

Figure 3. Confirmation of dehydration in salt-loaded rats used for NMDAR protein analysis

(a) Plasma osmolality and (b) hematocrit were significantly increased in salt-loaded rats relative to controls. (c) nNOS protein expression was significantly increased, showing that a 40% increase was detectible. *p<0.05

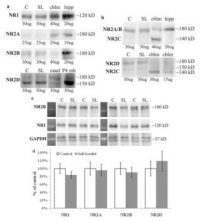

Figure 4. Protein expression of NR1, NR2A, NR2B and NR2D in the SON of control and salt-loaded rats

(a) Representative lanes with positive controls showing NR1, NR2A, NR2B and NR2D expression in the SON. (b) NR2C is not expressed in SON. Top: The Phosphosolutions antibody crossreacts with NR2A and NR2B. Bottom: The Santa Cruz antibody crossreacts with NR2D. (c) Full blots of NR1 and NR2B showing detectible differences between samples. (d) Protein expression of NMDAR subunits is not altered by three days of salt-loading. Density measurements were normalized to GAPDH as a loading control and are presented as percent of the average of the controls for the group. (n=6, except n=4 for NR1). C, control; SL, salt-loaded; cblm, cerebellum; hipp, hippocampus; caud, caudate; P4 mb, midbrain from postnatal day 4. μg amounts refer to total protein loaded.

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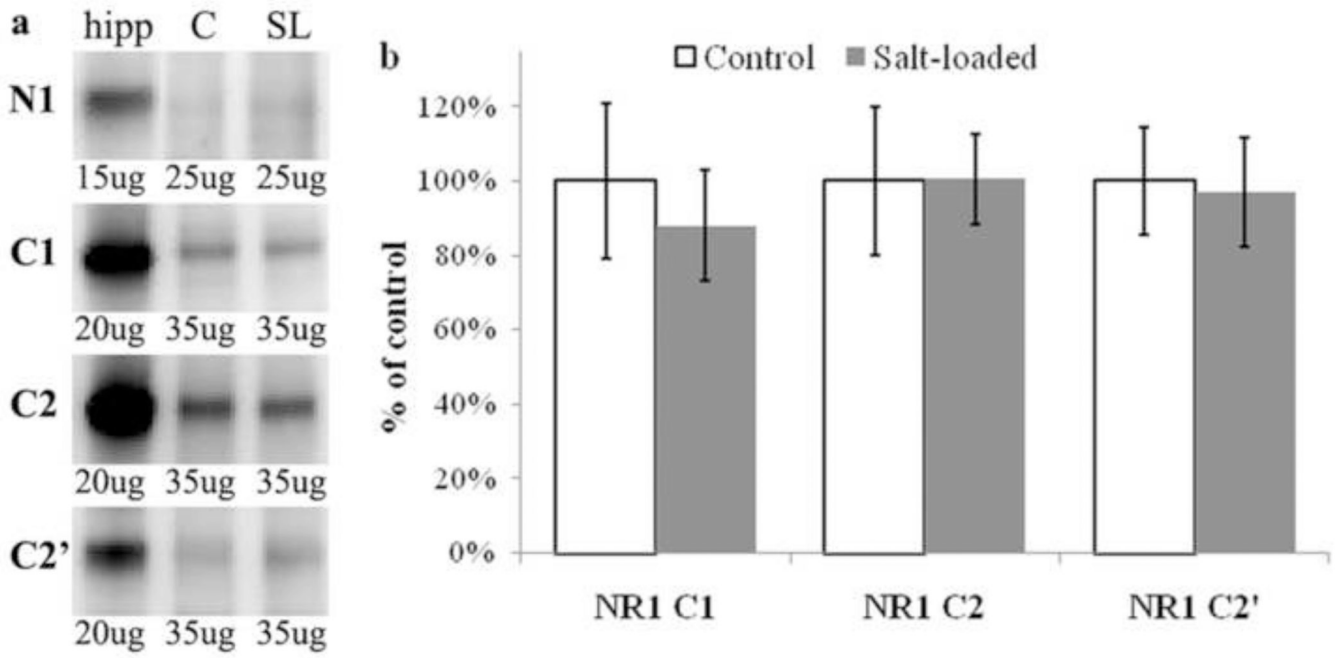

Figure 5. Protein expression of NR1 splice variants in the SON of control and salt-loaded rats

Protein expression of NR1 splice variants was not altered by three days of salt-loading. Density measurements were normalized to GAPDH as a loading control and are presented as percent of the average of the controls for the group. (n=6, except n=4 for C1). C, control; SL, salt-loaded; hipp, hippocampus. μg amounts refer to total protein loaded.

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Table 1

a NMDAR subunit specific primers for RT-PCR. (Applied Biosystems)

	TaqMan Assay ID	exons	amlicon location
AVP	Rn00566449_m1	1–2	154–219
NR1	Rn01436038_m1	3–4	823–890
NR2A	Rn00561341_m1	4–5	1343–1410
NR2C	Rn00561359_m1	12–13	2757–2813

b NMDAR subunit specific primers for RT-PCR. Custom designed primers

	Forward primer	Reverse primer	Exon	Amplicon location
NR2B	CGCAGCGTGAGCCTGAA	CTCAAACATATGGGCGTAGGG	13	4308–4373
NR2D	GCTCAGCGACCGGAAGTTC	GACCCATTGGGCACCGT	9–10	2806–2880

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Table 2

NMDAR antibodies used

	Species	Clonality	Company	Catalog #	Antigen	Concentration
NR1	mouse	monoclonal	Phosphosolutions	1508-NR1 (R1JHL)	aa 1–564 rat NR1	1:800 0.375 ug/ml
NR2A	rabbit	polyclonal	Phosphosolutions	1500-NR2A	C-terminus rat NR2A	1:300 0.667 ug/ml
NR2B	mouse	monoclonal	UC Davis/NIH NeuroMab Facility	75–097 (N59/20)	aa 20–271 rat NR2B	1:200 5.0 ug/ml
NR2C	rabbit	polyclonal	Phosphosolutions	1502-NR2C	N-terminus rat NR2C	1:450 0.444 ug/ml
NR2C	goat	polyclonal	Santa Cruz	sc-1471	C-terminus mouse NR2C	1:125 1.60 ug/ml
NR2D	goat	polyclonal	Santa Cruz	sc-1266	C-terminus mouse NR2D	1:80 2.50 ug/ml
NR1 N1	rabbit	polyclonal	Phosphosolutions	1504-N1	aa 191–211 (exon 5) rat NR1	1:450 1.11 ug/ml
NR1 C1	rabbit	polyclonal	Phosphosolutions	1505-C1	aa 864–900 (exon 21) rat NR1	1:500 1.0 ug/ml
NR1 C2	rabbit	polyclonal	Phosphosolutions	1506-C2	aa 901–938 (exon 22) rat NR1	1:500 1.0 ug/ml
NR1 C2'	rabbit	polyclonal	Phosphosolutions	1507-C2'	aa 901'–922' (exon 22') rat NR1	1:400 1.25 ug/ml