Osmotic and glutamate receptor regulation of c-Jun NH₂-terminal protein kinase in neuroendocrine cells

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Meeker, Rick, and Alda Fernandes. Osmotic and glutamate receptor regulation of c-Jun NH₂-terminal protein kinase in neuroendocrine cells. Am J Physiol Endocrinol Metab 279: E475–E486, 2000.—Expression of a c-Jun NH₂-terminal protein kinase (JNK), also known as stress-activated protein kinase (SAPK) in rodents, has been implicated in the ability of cells to respond to a variety of stressors. In nonmammalian cells, JNK participates in the regulation of cell volume in response to hyperosmotic stress. To explore the possibility that JNK may participate in the transduction of osmotic information in mammals, we evaluated the expression of JNK immunoreactivity in neuroendocrine cells of the supraoptic nucleus. Low basal expression of JNK-2 (SAPK-α) and JNK-3 (SAPK-β) was seen in vivo and in vitro. During water deprivation, JNK-2 increased in the supraoptic nucleus but not in the cortex. Osmotic or glutamate receptor stimulation in vitro also resulted in an increase in JNK-2 that was tetrodotoxin (TTX) insensitive and paralleled by increased nuclear phospho-c-Jun immunoreactivity. A TTX-sensitive increase in JNK-3 was seen in smaller neurons. Thus different JNK pathways may mediate individual cellular responses to osmotic stress, with JNK-2 linked to osmotic and glutamate receptor stimulation in magnocellular neuroendocrine cells.

stress-activated protein kinase; vasopressin; oxytocin; rat; brain

Expression of a c-Jun NH₂-terminal protein kinase (JNK), also known as stress-activated protein kinase (SAPK), has been implicated in the ability of cells to respond to a variety of stressors (6, 8, 11, 17, 26). For example, the role of JNK in the signal transduction cascade that leads to apoptosis has been extensively investigated (12, 16). However, evidence also suggests that JNK may have a number of physiological roles unrelated to cell death (4, 8). One physiological role of JNK that appears to have been highly conserved in the evolution from single cell to higher organisms is the regulation of cell volume in response to a hyperosmotic stress. In single-cell organisms, expression of the JNK analog HOG-1 has been shown to be crucial for survival of cells in hypertonic medium (8). Yeast cell mutants lacking this gene fail to grow in response to osmotic stress induced by raising the NaCl concentration to 0.9 M (8). The human gene, JNK-1, was shown to rescue these cells, indicating that the protein has highly conserved physiological properties (8).

In multicellular organisms, physiological systems have evolved to control the extracellular ionic environment, thereby minimizing the impact of unfavorable external environments. However, the high level of functional conservation of the JNK protein suggests that higher organisms could utilize this stress response to signal homeostatic mechanisms that regulate extracellular osmolality. Hypothalamic magnocellular neuroendocrine neurons, which specialize in the detection and response to changes in extracellular osmolality (2), may take advantage of this conserved regulatory response for the control of body fluid balance. These cells respond to increases in extracellular osmolality by virtue of intrinsic osmoreceptors (23) and extrinsic excitatory synaptic inputs originating from remote osmosensitive cells (9). The intrinsic sensors are thought to be mechanoreceptors responding to changes in cell volume (23), whereas the extrinsic inputs are thought to be largely due to the activation of postsynaptic glutamate receptors (9, 14). In particular, the characteristic bursting patterns that facilitate vasopressin secretion appear to be highly dependent on the activation of N-methyl-D-aspartic acid (NMDA) glutamate receptors (15). However, the signal transduction processes that follow osmotic and NMDA receptor stimulation and regulate cell activation and vasopressin synthesis are still poorly understood. The transcription factors c-fos and c-jun are both increased in response to osmotic stimulation (13, 27) and have been excellent candidates for linking cellular activity to transcriptional regulation. However, experiments designed to correlate c-Fos protein levels with transcriptional regulation are complicated by the divergent temporal patterns for each of these responses and the complex role of phosphorylation (1), a key step in transcription factor activation. Because JNK is a major enzyme for the phosphorylation of c-Jun (6, 17) and is widely expressed in rat brain (4), it could serve as an essential regulatory step for the control of transcriptionally active c-Fos/c-Jun heterodimers. To begin to explore the possibility that JNK could be an important step in the intracellular transduction of osmotic information, we
evaluated the expression of JNK immunoreactivity in the neuroendocrine cells of the supraoptic nucleus (SON) of the hypothalamus. The following studies illustrate that magnocellular neuroendocrine cells express low but significant basal expression of both α- and β-isoforms of the enzyme (SAPK-α/JNK-2 and SAPK-β/JNK-3) in vivo and in vitro and that this expression increases with osmotic or glutamate receptor stimulation.

MATERIALS AND METHODS

Animals. For the analysis of the effects of water deprivation on JNK immunoreactivity, Long-Evans rats in each experiment were split into two equal groups. One-half of the rats were deprived of water for 44 h starting at 1400, and the other one-half were fed water ad libitum. This deprivation interval produces a maximal increase in vasopressin mRNA (18) and therefore would be expected to correspond to an active period of transcriptional activity. At the end of the deprivation interval, the rats were rapidly and deeply anesthetized in their home cage with an isotonic preparation of pentobarbital sodium (60 mg/kg of a 40 mg/ml solution) designed to minimize environmental and osmotic stress. Each rat brain was then perfused with a balanced salt solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Sections were cut at a thickness of 30 μm on a vibratome, and alternate sections were stained for SAPK-α/JNK-2 and SAPK-β/JNK-3 (the isoforms predominantly expressed in adult rat brain).

JNK immunohistochemistry. The tissue was rinsed three times in 0.01 M PBS, pH 7.4, and incubated in 0.6% H2O2 for 15 min. After three rinses in PBS (5 min each), the tissue was incubated for 1 h in PBS containing 3% normal goat serum (NGS). The tissue was then incubated with rabbit anti-rat SAPK-α/JNK-2 (1:1,000, Upstate Biotechnology, Lake Placid, NY), SAPK-β/JNK-3 (1:2,000 Upstate Biotechnology), or mouse anti-phospho-c-Jun (1:2,000, Santa Cruz, Santa Cruz, CA) for 24 h at 4°C. These concentrations were chosen to give a moderate basal signal with low background on the basis of initial studies that examined dilutions ranging from 1:500 to 1:5,000. This allowed analysis of increases or decreases in enzyme immunoreactivity, particularly in the in vitro studies. On the next day, the tissue was rinsed three times in PBS and incubated for 1 h in biotinylated goat anti-rabbit (anti-mouse for the phospho-c-Jun antibody) IgG (1:200) in PBS with 3% NGS. The secondary antibody was washed from the tissue and the antigen was visualized using ABC reagent, with diaminobenzidine (0.5 mg/ml) as substrate, and 0.01% H2O2.

Primary cultures of magnocellular neuroendocrine cells. Punch cultures were prepared from fetuses harvested at embryonic day 16–17 from pregnant female Long-Evans rats under deep isoflurane anesthesia. After removal from the uterus, the fetuses were transferred to a 60-mm dish containing fresh HEPES-buffered Hanks’ balanced salt solution (HBSS, pH 7.4) at room temperature. The brain was dissected from each fetus, placed into fresh sterile HBSS, and washed three times. The brains were then transferred to complete culture medium (MEM) with glucose and sodium bicarbonate (GIBCO/BRL, Grand Island, NY) + 10% fetal bovine serum + 20 μg/ml gentamicin. Punches through the region of the SON were taken from the whole brain under a dissecting microscope, with the bifurcation of the middle cerebral artery from the circle of Willis as a marker for the general location of the SON. A blunt 23-gauge needle with sharpened edges connected to a 1-ml syringe was used to extract the tissue. The brains were kept totally immersed in culture medium throughout the punching procedure. The core of tissue (“punch”) was placed onto 25-mm round glass coverslips previously cleaned and coated with 0.1 mg/ml poly-D-lysine. The punches often fragmented into smaller pieces, providing several small foci of cell growth on the coverslip. Each dish was placed in a humidified incubator maintained at 36°C and 5% CO2, with just enough medium to wet the tissue. Viable punches attached loosely to the coverslip within 1–2 h. Additional medium was added to cover and feed the tissue after 2–4 h. Cells were fed every 2–3 days with 50% medium exchange. Neurons migrated from the punch, resulting in a field of neurons from the region of the SON that could easily be stained and individually analyzed. Coverslips with well-developed magnocellular neurons were used after 12–22 days in culture.

Data analysis. The density of regional staining in the SON and single cell staining in cultured neurons was analyzed semiquantitatively with a Bioquant Image Analysis System (R & M Biometrics). Controls and experimental conditions were always run at the same time under identical conditions. Mean gray level values were calculated for the entire SON at a final magnification of 424× or for single cellular perikarya at a magnification of 1,220×. The gray level value was converted to optical density (OD), and the ODs were averaged across all of the supraoptic nuclei or cells in each condition.

RESULTS

JNK expression in vivo. Both JNK-2 (SAPK-α) and JNK-3 (SAPK-β) showed weak-to-moderate basal levels of immunoreactivity throughout the brain. In the hypothalamus, JNK-2 showed weak or moderate levels of staining, localized principally to the SON (Fig. 1A). Cells scattered through the ventral anterior hypothalamus, suprachiasmatic nucleus, and periventricular regions were also lightly stained (not shown). In the dorsal frontoparietal cortex (Fig. 1C), stained cells were localized to the pyramidal cells in layer 5 (arrow), with lighter staining in layers 2 and 3. Moderate staining was also seen in the piriform cortex (arrow) and in cells scattered through the amygdala (Fig. 1E, arrowhead).

Immunoreactivity for JNK-3 in the hypothalamus was lighter than for JNK-2 but had a similar distribution. Very light staining was generally seen in neuroendocrine cells in the SON (Fig. 1B, arrowheads), periventricular regions, and the suprachiasmatic nucleus (not shown). In the cortex and amygdala, staining was seen for both JNK-2 (Fig. 1, C and E) and JNK-3 (Fig. 1, D and F), although staining was generally more robust for JNK-3. Individual cells were stained throughout the pyramidal cells in layers 2, 3, and 5 of the dorsal frontoparietal cortex (Fig. 1D, arrows) and within the piriform cortex (Fig. 1F, arrow) and amygdala (Fig. 1F, arrowhead). Other regions with light to moderate staining (not shown) included the hippocampus and paraventricular nucleus.

To test the ability of water deprivation to induce the expression of JNK in the SON, rats were deprived of water for 44 h, and the immunoreactivity for JNK-2 and JNK-3 in the SON was assessed under carefully matched conditions. Water deprivation resulted in a
significant 40% increase ($t = -2.82$, $P = 0.030$, $n = 7$ pairs) in the staining intensity of JNK-2 and a larger but more variable 80% increase ($t = -2.08$, $P = 0.076$, $n = 8$ pairs) in the staining of JNK-3 in the SON (Fig. 2). The increase in JNK-2 staining in response to water deprivation (Fig. 3B) was due to more extensive and intense cellular immunoreactivity relative to controls (Fig. 3A). Although increased nuclear staining for JNK-3 was apparent in a few cases (Fig. 3D), it was not a consistent finding. Controls stained in the absence of
primary antibody were devoid of immunoreactivity. In addition to the neuronal stain, an increase in JNK-2 immunoreactivity could be seen associated with the ventral glial layer at the base of the SON (small arrows).

Neurons in other regions of the brain, such as the cortex, also exhibited small increases in immunoreactivity, indicating that increased JNK expression is not exclusively associated with neuroendocrine cells. However, the increase was small and restricted to JNK-3 expression. An example of JNK-2 and JNK-3 staining in the dorsal frontoparietal cortex at the level of the SON is illustrated in Fig. 4 for both control and water-deprived rats. Moderate staining of large pyramidal cells was seen for JNK-2 (Fig. 4A) but did not change significantly after water deprivation (Fig. 4B). Quantification of the changes in JNK-2 staining intensity within the pyramidal cells of layers 3–5 demonstrated a 4.6% decrease in mean OD [0.359 ± 0.024 in controls vs. 0.342 ± 0.022 in water deprived; t = 2.00, n = 7 pairs, not significant (NS)]. Scattered staining of pyramidal cells was also evident for JNK-3 (Fig. 4C), including robust nuclear staining in layers 2, 3, and 5 of the cortex. After water deprivation, a small but consistent 10.6% increase in JNK-3 staining was seen (0.237 ± 0.029 in controls vs. 0.262 ± 0.028 in water deprived; t = 2.677, n = 8 pairs, P = 0.05), which was largely due to an increase in nuclear staining (Fig. 4D).

JNK expression in vitro in response to osmotic and glutamate receptor stimulation. To better understand the stimuli that might drive the expression of JNK in the neuroendocrine cells, we examined the ability of...
extracellular hyperosmolality and NMDA or metabotropic glutamate receptor stimulation to directly increase cellular JNK immunoreactivity in vitro. Cultures were analyzed after 1 or 24 h of exposure to evaluate acute and chronic JNK expression. During exposure to 30 mosmol/kg excess NaCl, cell size decreased acutely within 2 min of exposure and then recovered to normal over a period of 20 min (Fig. 5).

Examples of the results of a 1-h exposure to 30 mosmol/kg excess NaCl in the presence or absence of NMDA (50 μM) or to the metabotropic receptor agonist 1S,3R ACPD (100 μM) are illustrated in Fig. 6. Stimulation with 30 mosmol/kg excess NaCl resulted in an increase in staining intensity within the perikarya (arrows) and processes (arrowheads) for both JNK-2 (Fig. 6B) and JNK-3 (Fig. 6D) relative to untreated controls (Fig. 6, A and C). Stimulation of the cells with 100 μM 1S,3R ACPD had little effect on JNK-2 expression (Fig. 6E) and produced only a slight increase in JNK-3 (Fig. 6G) relative to unstimulated controls (Fig. 6, A and C). Combined stimulation with 1S,3R ACPD and 30 mosmol/kg excess NaCl resulted in a level of immunoreactivity that was greater than osmotic stimulation alone for JNK-2 (Fig. 6F) but not for JNK-3 (Fig. 6H). Stimulation with 50 μM NMDA alone increased the expression of JNK-2 (Fig. 6I) but had a negligible effect on JNK-3 (Fig. 6K). Combined osmotic and NMDA receptor stimulation resulted in strong expression of JNK-2 (Fig. 6J) but only a slight increase in JNK-3 (Fig. 6L). Staining in the absence of the primary antibody was negligible in each case (Fig. 6, M and N for JNK-2 and JNK-3, respectively).

A summary of the mean neuronal JNK-2 and JNK-3 expression across all cultures and conditions is provided in Fig. 7. Osmotic stimulation produced a rapid average increase in JNK-2 immunoreactivity of 33% ($t = -8.61, P < 0.002$) relative to isosmotic controls. This osmotically induced increase was enhanced when combined with 1S,3R ACPD (+64%, $t = -10.8, P < 0.002$) but not with NMDA. 1S,3R ACPD in the absence of osmotic stimulation resulted in a small increase in JNK-2 expression (+12%, $t = 3.96, P < 0.002$), whereas NMDA induced a 36% increase in JNK-2 ($t = 9.96, P < 0.002$).

A slightly different pattern was evident 24 h after stimulation. The effects of osmotic and NMDA stimulation were reduced (+13%, $t = -1.84, P = 0.069$ ($P = 0.018$ by Mann-Whitney) and +16.5%, NS, respectively), whereas the effect of 1S,3R ACPD alone was increased (+40.6%, $t = 7.50, P < 0.002$). The combination of osmotic and NMDA or ACPD stimulation was significantly greater than the untreated control condition or osmotic stimulation alone (+28–29%, $t$ values = 5.28–6.22, $P$ values < 0.002).

Fig. 5. Decrease and recovery of neuronal size relative to the pretreatment cell size after exposure to 30 mosmol/kg excess NaCl. A significant decrease in cell size was observed within 2 min of stimulation ($t = -4.05, P < 0.01, 13$ cells), followed by recovery to baseline by 20 min.

Fig. 4. Example of JNK-2 (A) and JNK-3 (C) staining in the pyramidal cells of the dorsal cortex in normal rats and in the cortex of rats deprived of water for 44 h (B, D). Cortical cells showed equivalent or less staining for JNK-2 after water deprivation (B), whereas increased JNK-3 staining appeared in pyramidal cell nuclei and cell bodies (D). Calibration bar (lower right) = 50 μm for all.
Fig. 6. Examples of immunoreactivity for JNK-2 (left 2 columns) and JNK-3 (right 2 columns) in cultured neuroendocrine cells exposed to vehicle (A, C), 30 mosmol/kg excess NaCl (B, D), 100 μM 1S,3R ACPD (E, G), 100 μM 1S,3R ACPD + 30 mosmol/kg excess NaCl (F, H), 50 μM N-methyl-D-aspartic acid (NMDA; I, K), or 50 μM NMDA + 30 mosmol/kg excess NaCl (J, L). In all conditions, staining was seen in neuronal perikarya (arrows) and in proximal dendritic processes (arrowheads) of magnocellular and other smaller neurons. Untreated controls (A, C) generally showed a light-to-medium staining intensity. Moderate increases in mean staining intensities were seen for cultures treated with hypertonic medium (B, D). Nuclear staining for JNK-3 was seen in occasional cells (open arrows), similar to in vivo observations. Stimulation with 1S,3R ACPD (E, G) had a negligible effect on JNK-2 expression and increased JNK-3 only slightly. Combination of osmotic stimulation with 1S,3R ACPD resulted in staining that was greater than osmotic stimulation alone for JNK-2 (F) but not for JNK-3 (H). NMDA stimulation produced strong staining for JNK-2 (I) but did not significantly increase JNK-3 (K). Combined NMDA and osmotic stimulation resulted in robust JNK-2 immunoreactivity and a small increase in JNK-3. Some cells showed nuclear staining for JNK-3 when stimulated with NMDA plus hyperosmotic medium (L, open arrow), but this was seen in controls and was not a consistent observation across all cultures. No staining was detected in the absence of primary antibodies for JNK-2 (M) or JNK-3 (N). Calibration bar (lower right) = 50 μm for all.
Increases in JNK-3 were also evident after osmotic and glutamate receptor stimulation, but with a different pattern from that for JNK-2. Acute osmotic stimulation resulted in a 13% increase in JNK-3 immunoreactivity (\( t = 5.2, P = 0.008 \)). 1S,3R ACPD increased JNK-3 by 20.2% (\( t = 5.16, P < 0.002 \)), whereas NMDA was ineffective (+3.3%, NS). Because the measurements included both the cell body and the nucleus, the increases could have been due to an increase in the number of cells with nuclear staining. However, a breakdown of the relative number of neurons with an immunoreactive nucleus showed similar numbers for osmotically stimulated (53 ± 7%) and control rats (44 ± 10%), indicating that, at best, this could only partially account for the increases seen. Combined osmotic and glutamate receptor stimulation failed to have a significant effect on JNK-3 expression and showed slight decreases relative to drug alone.

After 24 h of treatment, the osmotic effect was increased slightly (+24.8%, \( t = -2.38, P = 0.019 \)). Combined stimulation with 1S,3R ACPD and high osmolality gave results similar to osmotic stimulation alone (+20.1%, \( t = -3.03, P < 0.003 \)) with no significant direct contribution from 1S,3R ACPD. NMDA had a small but nonsignificant effect at 24 h (+18.7%) but in combination with osmotic stimulation induced a 47.3% increase in JNK-3 relative to controls (\( t = -3.43, P = 0.019 \)).

Because cells were selected at random from the cultures and therefore do not all represent magnocellular neurons, we segregated the data by cell size to evaluate whether the large magnocellular neurons responded differently from other neurons in the culture. The criterion for a magnocellular neuron was set at an area of 300 \( \mu m^2 \). Although few differences in response profiles were observed in large vs. small cells after 1 h, different patterns were apparent after 24 h (not shown). At this time, osmotically induced increases in JNK-2 (mean cellular OD) were restricted almost exclusively to large cells (15.0 ± 5.9%), with small cells showing only a 1.0 ± 2.6% increase (\( t = -2.18, large vs. small \)

Fig. 7. Summary of changes in neuronal staining for JNK-2 and JNK-3 in cultured neuroendocrine cells treated with control medium (C) or hyperosmotic medium (Osm) in the presence and absence of 100 \( \mu M \) 1S,3R ACPD (ACP) or 50 \( \mu M \) NMDA. Cultures were fixed with 4% paraformaldehyde 1 h or 24 h after stimulation and were stained with antibodies to JNK-2 (SAPK-\( \alpha \)) or JNK-3 (SAPK-\( \beta \)). Optical density readings were measured from 50 individual neurons sampled at random from 3–4 cultures. a, \( P < 0.05 \) relative to untreated controls; b, \( P < 0.05 \) relative to osmotic or drug stimulation alone.

Fig. 8. Failure of the NMDA receptor antagonist aminophosphonovaleic acid (APV; 100 \( \mu M \)) to block the osmotically induced increase in JNK-2 staining in cultured neuroendocrine cells. Optical density readings were taken from 63–102 individual neurons sampled from 3–4 cultures. Osmotic stimulation resulted in a 68.2% increase in staining intensity in cells exposed to high osmolality and a 91.3% increase in cells exposed to high osmolality in the presence of 100 \( \mu M \) APV. The increase in the osmotic + APV group relative to the osmotic alone group was small (13.7%) but indicated that JNK-2 may be expressed in response to the loss of basal activity at the NMDA receptor.

Fig. 7. Summary of changes in neuronal staining for JNK-2 and JNK-3 in cultured neuroendocrine cells treated with control medium (C) or hyperosmotic medium (Osm) in the presence and absence of 100 \( \mu M \) 1S,3R ACPD (ACP) or 50 \( \mu M \) NMDA. Cultures were fixed with 4% paraformaldehyde 1 h or 24 h after stimulation and were stained with antibodies to JNK-2 (SAPK-\( \alpha \)) or JNK-3 (SAPK-\( \beta \)). Optical density readings were measured from 50 individual neurons sampled at random from 3–4 cultures. a, \( P < 0.05 \) relative to untreated controls; b, \( P < 0.05 \) relative to osmotic or drug stimulation alone.
cells, $P = 0.034$). On the other hand, osmotically induced increases in JNK-3 tended to be exclusive to small cells, although results were variable and not significant (large cells, $-0.3 \pm 11.8\%$; small cells, $+30.8 \pm 9.4\%$, $t = 1.57$ large vs. small cells, $P = 0.122$). A similar trend was seen for NMDA stimulation, but it did not reach statistical significance.

The similarity between the effects of NMDA and hyperosmotic stimulation on JNK-2 immunoreactivity raised the question of whether the osmotically induced increase might be due to indirect activation or facilitation of endogenous NMDA receptors. To test this possibility, cultures were stimulated with 30 mosmol/kg excess NaCl, or with 30 mosmol/kg excess NaCl in the presence of the NMDA receptor antagonist aminophosphonovaleric acid (APV, 100 μM). The results, illustrated in Fig. 8, show that under these conditions, APV failed to block the increase in JNK-2 immunoreactivity associated with osmotic stimulation, suggesting the presence of two independent pathways for JNK up-regulation. In contrast, the increase in response to NMDA (0.246 $\pm$ 0.019) was blocked by APV in these cultures (OD $= 0.087 \pm 0.015$, $t = 5.24$, $P = 0.0001$, not shown).

Because the NMDA antagonist did not block the osmotically induced increase in JNK, we evaluated the general contribution of synaptic stimulation by incubating the cells in 1 μM tetrodotoxin (TTX) during stimulation. Osmotically stimulated cultures had many more heavily stained neurons (Fig. 9B) relative to controls (Fig. 9A). The increase was particularly apparent for the largest neurons, which were robustly stained after osmotic stimulation (Fig. 9B, inset, lower right). A similar increase in immunoreactivity was seen in neurons challenged osmotically in the presence of TTX (Fig. 9D). Neurons exposed to TTX alone showed some increase in immunoreactivity that was evident in small neurons (Fig. 9C, small arrows) but not large neurons (Fig. 9C, large arrow). A population analysis of all neurons, illustrated in Fig. 10, shows the shift in the frequency distribution from low to high density with osmotic stimulation ($\chi^2 = 40.8$, $P < 0.0001$). The greatest shift was seen for combined stimulation with 30 mosmol/kg excess NaCl and TTX ($\chi^2 = 46.1$, $P < 0.0001$), indicating that the osmotic stimulation effectively increased JNK-2 expression in the absence of synaptic activity. However, TTX alone also produced a significant increase (albeit smaller) in

Fig. 9. JNK-2 immunoreactivity in cultured magnocellular neurons after exposure to hyperosmotic medium in the absence or presence of 1 μM tetrodotoxin (TTX). Fifteen minutes after transfer of one-half of the cultures to TTX-containing medium, cells were osmotically challenged. After 12 h, cultures were fixed and stained for JNK-2 or JNK-3. The density of JNK-2 immunoreactivity was measured in neurons sampled from each of 4 conditions: control ($n = 186$), +30 mosmol/kg NaCl ($n = 116$), TTX alone ($n = 229$), and +30 mosmol/kg NaCl in the presence of 1 μM TTX ($n = 225$). Neurons from control cultures (A) showed a wide range of JNK-2 immunoreactivity, although most cells had light-to-medium staining. The lightest staining tended to be in the largest neurons (A, arrows). After exposure to hyperosmotic medium (+30 mosmol/kg), JNK-2 immunoreactivity increased in most cells and processes (B) and was particularly high in the largest neurons (inset, lower right). Treatment with TTX alone (C) tended to increase immunoreactivity in small cells (arrows). Some large cells also showed increased immunoreactivity, although most were still lightly stained (arrowhead). Osmotic stimulation in the presence of TTX (D) resulted in robust immunoreactivity similar to that seen in the absence of TTX (B). Calibration bar (lower right) = 50 μm for all.
JNK-2 immunoreactivity ($\chi^2 = 26.2, P < 0.0001$). A more detailed analysis of the responses based on separation of the cells into large ($>300 \, \mu\text{m}^2$) or small ($<300 \, \mu\text{m}^2$) subpopulations, summarized in Table 1, revealed that only the large neurons retained osmotic responsiveness in the presence of TTX on the basis of increases in JNK-2 immunoreactivity ($\chi^2 = 12.5, P = 0.014$), whereas small neurons failed to show a significant increase due to osmotic stimulation in the presence of TTX ($\chi^2 = 5.96, P = 0.202$).

Analysis of the density of JNK-3 immunoreactivity in neurons under the same four conditions revealed a similar pattern of results. Staining of neurons was generally lighter than JNK-2, with moderate immunoreactivity seen in occasional cells (not shown). Population analysis of all neurons (Fig. 11) illustrated the shift in distribution to higher densities associated with osmotic stimulation. Osmotic stimulation resulted in increased neuronal JNK-3 immunoreactivity relative to controls ($\chi^2 = 20.6, P < 0.0001$). TTX alone resulted in a distribution almost identical to controls ($\chi^2 = 1.06, P = 0.7879$). Osmotic stimulation in the presence of TTX was similar to controls and TTX alone ($\chi^2 = 1.14, P = 0.767$), indicating a full reversal of the osmotic effect. A separate analysis of small and large subsets of neurons in Table 1 revealed that osmotic stimulation increased JNK-3 staining in small neurons ($P$ values < 0.0001) but not large neurons. This effect of osmotic stimulation in small neurons was completely reversed by the presence of TTX.

The increase in JNK induced by osmotic and glutamate receptor stimulation would be predicted to give rise to phosphorylation of c-Jun. Cultures treated with hyperosmotic medium and or NMDA for 24 h were evaluated for phospho-c-Jun immunoreactivity in the nuclei of large cells. The results, summarized in Fig. 12, illustrate that under control conditions 14% of the large cells had immunoreactive nuclei. This value increased significantly to 30% ($P = 0.0038, t$-test) in the presence of 30 mosmol/kg excess NaCl and 22% ($P = 0.0531, t$-test) after stimulation with NMDA. The combination of NMDA and osmotic stimulation gave an increase of 36% ($P = 0.0169, t$-test).

### DISCUSSION

It is well established that the phosphorylation of the transcription factor c-Jun by JNK is a key event in the cellular response to stress (6, 10, 17). In addition, the activation of JNK may be an early step in the molecular cascade leading to apoptosis (7, 12, 16, 31). However, in contrast to the apoptotic cascade, our results demonstrate that, in mammalian cells, increased expression of JNK may have a protective physiological role under conditions of osmotic stress. In addition, it is clear that JNK is expressed in a variety of neurons under nonpathological conditions. The role of this basal JNK is poorly understood, although it seems likely that the cellular stress response has been adapted to various functional outputs in different cells. In lower or-

![Fig. 10. Population analysis of neurons stained for JNK-2 showing shift of frequency distribution to right (higher densities) in cultures treated with TTX alone or osmotic stimulation. All $P$ values reflect results of a $\chi^2$ analysis relative to controls.](image-url)

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Values represent optical density (OD, means ± SE) for immunoreactive neurons in each c-Jun NH$_2$-terminal protein kinase (JNK) group. The average size of large neurons was $398.0 ± 16.9 \, \mu\text{m}^2$ and that of small neurons was $193.4 ± 7.0 \, \mu\text{m}^2$. Statistical probability ($P$) values are given for comparisons with vehicle or tetrodotoxin (TTX) controls; by $t$-test relative to *controls; †controls receiving TTX only.
organisms, the jnk gene homolog hog-1 is part of the signal transduction pathway that protects cells from lethal dehydration in hypertonic environments (8). This pathway is linked to osmoreceptors, which are in turn coupled to the activity of MAP kinases (24). Expression of the human JNK-1 protein in yeast mutants lacking the hog-1 gene restored the ability to grow in hyperosmotic media (8). Thus this protein kinase is structurally and functionally conserved from yeast to mammals, suggesting that the stress response has been adapted to the more complex functions of specialized cells in higher organisms.

Our experiments showed that neuroendocrine cells of the SON and paraventricular nucleus exhibit JNK immunoreactivity, which increased in response to water deprivation. The most consistent increase in JNK expression in the magnocellular neuroendocrine cells by osmotic or NMDA receptor stimulation in vivo and in vitro was seen for the JNK-2 isoform. In contrast, JNK-3 was more likely to increase in the cortex in response to water deprivation or in smaller hypothalamic neurons osmotically challenged in vitro. These opposite response patterns suggested that the two JNK isoforms may be differentially regulated in different cells. These results also indicate that many types of neurons can respond to physiological increases in extracellular osmolality. The widespread nature of the response is consistent with previous reports of abundant JNK mRNA in rat cortex, hippocampus, and other regions throughout the brain (5). Functional and cellular specificity for neuroendocrine regulation may be dictated, at least in part, by the preferential expression of the JNK-2 isoform in response to osmotic and glutamate receptor stimulation, particularly over sustained periods of time. Little information is available to indicate how this difference might translate to differences in the JNK signal transduction pathway within the neuroendocrine cells.

Increases in nuclear phospho-c-Jun parallel the upregulation of JNK, but the significance of this effect on vasopressin expression is presently unclear, because the vasopressin gene does not have an activating protein 1 (AP-1) binding site in the promoter (21), a preferred target of the Fos-Jun complex. Other targets of phospho-c-Jun or the phosphorylation of other transcription factors such as ATF-2 (10), which bind to cAMP-regulating elements, may be involved in the signal transduction cascade associated with hyperosmotic stimulation.

Increases in JNK expression were induced in cultured neurons in vitro as little as 1 h, indicating that this response within the SON as well as in other brain regions can emerge rapidly with changes in extracellular osmolality. This timing is similar to the in vivo increases in the transcription factors c-Fos and c-Jun (13, 25) associated with hyperosmolality and hypovolemia. Message and protein levels for c-Fos increase rapidly after stimulation, beginning at ~15 min and reaching a peak by 1 h (13). Protein levels may persist for several hours (13) but must be phosphorylated and dimerized to be active at the AP-1 site on the target gene (1). The increased expression of JNK within 1 h of osmotic stimulation would be expected to facilitate the phosphorylation of its preferred substrate, c-Jun (6), and facilitate signal transduction. Thus JNK is well poised to provide a key step in the functional activation of the Fos-Jun complex in magnocellular and other neurons. This possibility is supported by the increase in nuclear phospho-c-Jun, which we observed in vitro after osmotic stimulation.

Role of glutamate receptors in JNK expression. The osmotic activation of neuroendocrine cells is under the control of both intrinsic and extrinsic stimuli. Increases in extracellular osmolality can activate intrinsic osmoreceptors that induce nonselective cationic currents (2, 23). In addition, a wide variety of synaptic

Fig. 11. Population analysis of neurons stained for JNK-3 showing shift of frequency distribution to right (higher densities) in cultures treated with hyperosmotic medium in the absence (n = 109) or presence of TTX (n = 188). TTX alone (n = 126) failed to affect JNK-3 staining relative to controls (n = 174) and fully reversed the effects of osmotic stimulation. All P values reflect results of a χ² analysis relative to controls.
inputs are thought to provide the extrinsic driving force for activation of the neuroendocrine cells (3). Much of the excitatory activity from extrinsic sources is provided by glutamate (9, 14, 20, 22, 29, 30). NMDA receptors, in particular, are responsible for the induction of bursting activity associated with hormone release (15). Either osmotic or glutamate receptor stimulation could potentially mediate the JNK response under physiological conditions. Indeed, in our cultures, both NMDA and osmotic stimulation increased JNK-2. The metabotropic glutamate receptor agonist 1S,3R ACPD also increased JNK-2 as well as JNK-3. These observations are consistent with other studies that have shown increases in JNK activity with osmotic (8, 11, 26) or glutamate receptor stimulation (28). In rat pituitary cells, ACPD increased JNK-2 as well as JNK-3. These data support the possibility of a direct osmotic signal transduction pathway in mammalian cells.

Role of synaptic stimulation in JNK-2 and JNK-3 expression. Differences in the expression of JNK-2 and JNK-3 continued to emerge when cultures were stimulated in the presence of 1 μM TTX to block release of endogenous transmitters. A significant osmotically induced increase in JNK-2 expression was maintained in large cells in the presence of TTX, whereas the osmotic expression of JNK-3 was completely suppressed by TTX. Thus osmotic stimulation of JNK-3 expression appeared to be dependent on synaptic stimulation, whereas JNK-2 expression was at least partially independent of synaptic activity. The relative contribution of synaptic vs. nonsynaptic stimulation of JNK-2 is difficult to extrapolate from these experiments. JNK-2 immunoreactivity in the presence of TTX was 80% of osmotic stimulation alone. However, TTX alone increased JNK-2 staining, suggesting that JNK expression may also be sensitive to the loss of synaptic activity. Thus nonsynaptic stimulation may account for as much as 80% or as little as 42% of the osmotic response, depending on which baseline is the appropriate comparison. Nevertheless, the presence of a significant nonsynaptic increase in JNK-2 restricted to large cells supports the possibility of a direct osmotic signal transduction pathway in magnocellular neuroendocrine cells. This pathway may constitute a relatively unique link between synaptic and osmotic activation of neuroendocrine cells and the coordinate regulation of transcriptional activity.

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