

miR-7b, a microRNA up-regulated in the hypothalamus after chronic hyperosmolar stimulation, inhibits Fos translation

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The transcription factor activator protein 1 (AP-1) is formed through the dimerization of immediate-early genes Fos and Jun family members. Activator protein 1 is known as a pivotal regulator of major biological events such as cell proliferation, differentiation, organogenesis, memory formation, and apoptosis. During a search for microRNAs (miRNAs; small, endogenous, noncoding RNAs that repress gene expression of target mRNAs in animals posttranscriptionally) that are differentially expressed in the mouse paraventricular and supraoptic nuclei after 10 days of drinking 2% saline, one candidate microRNA that is relatively highly expressed, mmu-miR-7b (miR-7b), was studied further because sequence analysis suggested a likely interaction with the 3' untranslated region of Fos mRNA. We show that miR-7b expression inhibits Fos translation *in vitro* and that it and its host gene are prominently expressed in the PVN and other brain areas, including the suprachiasmatic nucleus. No effect on Fos mRNA levels was observed. Normally, Fos is expressed at low to undetectable levels in cells, but it shows rapid induction and decay after acute stimuli. Various pathways have been identified through which Fos family proteins are degraded; our results indicate a significant additional mechanism by which Fos protein and activity may be regulated.

AP-1 | hyperosmolality | microarray | paraventricular nucleus | suprachiasmatic

The transcription factor activator protein 1 (AP-1) is formed through the dimerization of immediate-early genes Fos and Jun family members (1). AP-1 is known as a pivotal regulator of major biological events such as cell proliferation, differentiation, organogenesis, memory formation, and apoptosis (2–4). Expression of these immediate-early genes is also often studied as an indication of neuronal activity (5). For example, increased Fos expression accompanies acute and chronic hypersomolality (6, 7) and stress (8) in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus. These nuclei express arginine vasopressin (Avp) that is processed as it is transported to the posterior pituitary (9, 10). Avp is then released into the peripheral circulation to restore normal serum osmolality by reducing water secretion by the kidneys (11). Intraperitoneal injection of hypertonic saline also causes a rapid rise and fall in expression of Fos in these nuclei (12). Normally, Fos is expressed at low to undetectable levels in the parvocellular neurons of the PVN, but it is up-regulated within 30 min after acute stress, with a maximal protein induction between 1 and 3 h (8, 12). Although various pathways have been identified through which Fos family proteins are degraded (13), less is known about how their transcripts are regulated so tightly. The relative contributions of transcriptional and translational regulation and mRNA and protein degradation are still being investigated.

Potentially, regulation of Fos translation could occur through the binding to 3' untranslated regions (UTRs) of target mRNAs in animals of the small, endogenous, noncoding microRNAs

(miRNAs) that repress gene expression posttranscriptionally (14–16). miRNAs are believed to be expressed under the control of developmental or tissue-specific signaling (17–20) by which they regulate diverse biological functions, including neural differentiation (21, 22) and oncogenesis (23–25).

During a search for miRNAs that are differentially expressed in the mouse PVN and SON after 10 days of drinking 2% saline, one candidate miRNA that is relatively highly expressed, mmu-miR-7b (miR-7b), was studied further because sequence analysis suggested a likely interaction with the 3' UTR of Fos mRNA. Indeed, we found that miR-7b expression inhibits Fos translation *in vitro* and that it and its host gene are expressed prominently in the PVN and other brain areas, including the suprachiasmatic nucleus.

Results

Differential Expression of miRNAs in the PVN and SON of the Mouse After Saline Treatment. After 10 days of 2% saline ingestion, total RNA was isolated from PVN and SON micropunches (26). Avp expression was examined as a check for the efficacy of the treatment because its elevated expression after chronic hypertonic saline treatment is well documented (27). As expected, Avp transcripts showed a 2.6-fold increase under the hyperosmolar condition (Fig. 1 *Inset*). miRNA chip experiments were carried out three times (twice from one pair of RNA preparations and once from the other pair) with size-fractionated total RNA. The results are summarized in Fig. 1. Some mature miRNAs were overexpressed after hypertonic saline treatment with fold changes of >1.5. Examples include miR-9, miR-29c, miR-451, miR-137, and miR-7b. Interestingly, the levels of expression of a nearly equal number of miRNAs were depressed by this treatment (Fig. 1).

Interaction of miR-7b with the 3' UTR of the Fos mRNA. To find target genes of miRNAs, we analyzed candidates by using the bioinformatic tools at the miRBase target database (<http://microrna>.

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Abbreviations: AP-1, activator protein 1; AREs, adenylate- and uridylate-rich elements; Avp, arginine vasopressin; ISHH, *in situ* hybridization histochemistry; LNA, locked nucleic acid; miRNA, microRNA; PMA, phorbol 12-myristate 13-acetate; PVN, paraventricular nucleus; SON, supraoptic nucleus.

Data deposition: The microRNA data are deposited in the Array Express database, www.ebi.ac.uk/arrayexpress (accession no. E-MEXP-786).

See Commentary on page 15278.

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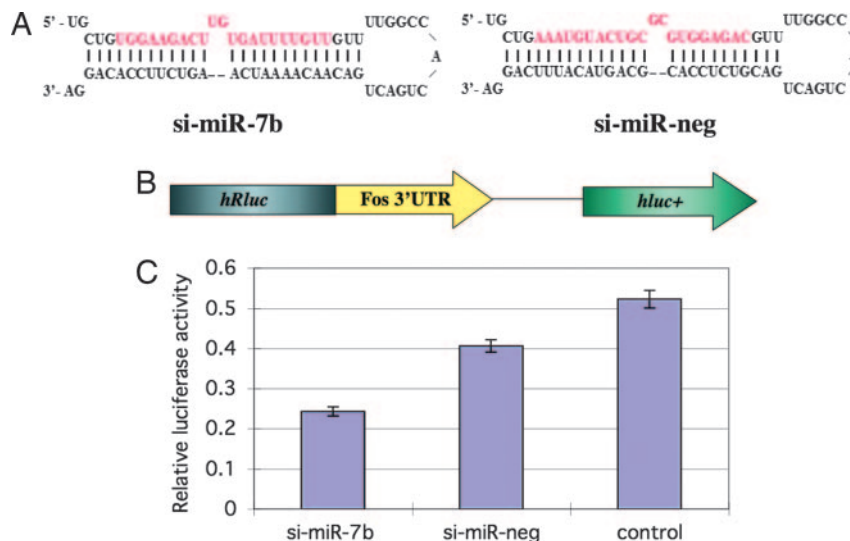


Fig. 3. Down-regulation of translation by miR-7b through the *Fos* 3' UTR. (A) Schematic of the si-miR-7b and si-miR-neg pre-miRNA sequences that were cloned into the expression vector as described in *Materials and Methods*. Targeting mature sequences are shown in red. (B) Construct map of the *Fos* 3' UTR used for the luciferase assay. The 3' UTR of *Fos* was cloned into the vector after the *Renilla* luciferase gene (*hRluc*) to form a fusion transcript. The luciferase activity would be decreased by binding of miRNAs to the 3' UTR of *Fos*. Firefly luciferase gene (*hLuc+*) is coexpressed in the vector as an internal control. (C) Luciferase activity of the *Fos* 3' UTR reporter gene in the absence or presence of the si-miR-7b and si-miR-neg expression vectors. 293T cells were cotransfected with both the reporter gene and si-miR-7b or si-miR-neg. *Renilla* luciferase data were normalized to firefly luciferase data. Data show the means from three independent transfections (error bars indicate standard deviations; $P < 0.001$ for each treatment compared with either of the other two).

that reduction of *Fos* levels by miR-7b occurs by translational suppression rather than by mRNA degradation.

Tissue-Specific Expression of the miR-7b-Containing Gene, *FICT*. *In situ* hybridization histochemistry (ISHH) using a conventional oligonucleotide probe targeting miR-7b failed to give any signal over background, presumably because of the short length and low GC content of the probe. However, at the coronal level used

to obtain the tissue punches, a locked nucleic acid (LNA) oligonucleotide probe revealed prominent expression in the PVN, suprachiasmatic nucleus, habenula, hippocampus, and deep layers of the neocortex (Fig. 5C). Expression in the SON appeared only slightly elevated. To confirm this distribution of the miRNA, we searched for a potential transcript containing miR-7b to use as a template for a more sensitive RNA probe. Intronic miRNAs are coexpressed with their host gene mRNA by sharing the same promoter (32). Mouse miR-7b is located on chromosome 17 after RIKEN cDNA A230051N06 (Ensembl gene A230051N06Rik; www.ensembl.org; Fig. 5A). Specific RT-PCR of hypothalamic total RNA showed that miR-7b is transcribed together with this gene (Fig. 5B). We therefore named the gene *FICT* (Fos-inhibiting-mir-7b-containing transcript). ISHH targeting bases 1–494 (GenBank accession no. AK038632) of the *FICT* gene cDNA confirmed the spatial localization of miR-7b, which we observed with the LNA oligonucleotide probe (Fig. 5D). It is likely that most neurons in these sections were labeled at a lower level because the sense probe showed a much lower in most, if not all, neuronal areas (Fig. 5E). We saw no obvious differences between sections from control and saline-treated animals.

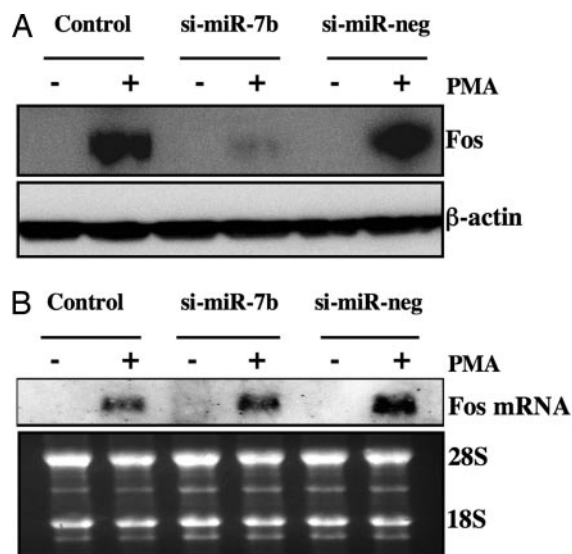


Fig. 4. PMA-induced Fos is inhibited by miR-7b. (A) Western blot of Fos from miRNA-transfected NIH 3T3 cells after PMA induction. Cells were transfected with si-miR-7b or si-miR-neg; after 48 h, PMA (40 ng/ml) was added. Cells were harvested 2 h later, and 30 μ g of whole-cell lysate was loaded into each lane. A β -actin antibody was used in a reprobings as a loading control. (B Upper) Northern blot of the *Fos* mRNA extracted after a 1-h treatment with PMA. Ten micrograms of total RNA was loaded into each lane. (B Lower) Ethidium bromide staining of the agarose gel (loading control).

Discussion

In the current study, we identified candidate miRNAs that are differentially expressed under hyperosmolar conditions in the hypothalamus in initial studies of hypothalamic gene regulation by miRNAs. Our findings suggest that the expression of approximately equal numbers of miRNAs increases and decreases after saline intake by mice. These regulated miRNAs may have crucial roles in regulating genes whose expression levels change in the PVN and SON with changes in serum osmolality (33–35). We focused on miR-7b because it has one of the highest levels of expression, and a previous report suggested that the miR-7 family is specific to the hypothalamus and pituitary in humans (36). We demonstrate that miR-7b down-regulates the expression of *Fos*, a marker for the activity of the neurons (6, 37). Various studies have shown that expression of *Fos* increases in

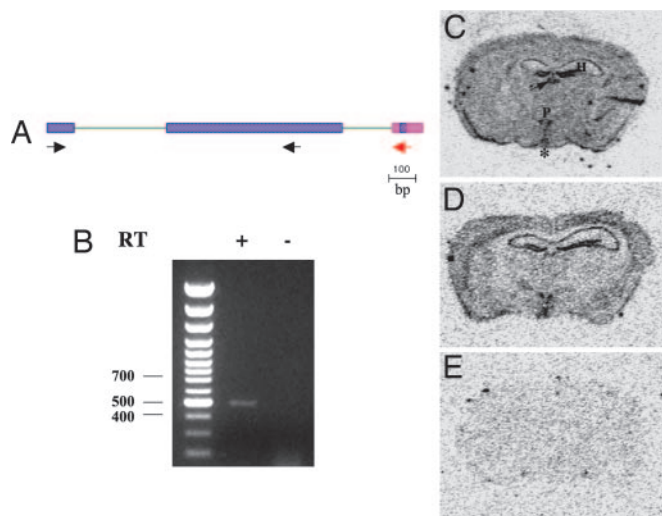


Fig. 5. ISHH of the gene *FICT* that contains *mmu-miR-7b*. (A) Schematic of the *FICT* gene and *miR-7b*. Reverse transcription was performed by using an antisense *miR-7b* primer (AS*mmu-miR-7b*, red arrow) for the first-strand synthesis. The *FICT* gene was subsequently amplified by using upstream primers (black arrows) on the cDNA. Blue and red boxes indicate exons and stem-loop sequences, respectively, of *miR-7b*. (B) RT-PCR confirmation of *miR-7b* location within the *FICT* primary transcript. A 495-bp product is produced (confirmed by sequencing). As a control, reverse transcriptase was omitted. ISHH is shown with an antisense oligonucleotide (LNA-*miR-7b*) probe (C) and antisense (D) and sense (E) riboprobes targeting *FICT* on sections through the mouse hypothalamus at a level containing the PVN (P above the paired nuclei), suprachiasmatic nucleus (above the asterisk), hippocampal formation (H), and habenula (arrow). Sections were from saline-treated mice, and exposures were to phosphorimaging plates for 1 month. The .tif files were transferred to Adobe Photoshop (Adobe Systems, San Jose, CA) and visualized with the automatic adjust levels.

response to hypernatremia in a number of brain regions (38, 39). Given the importance of AP-1 as a potent transcriptional activator, it is reasonable that various mechanisms would have evolved to regulate its activity. Our results suggest that after a transient perturbation (i.e., PMA treatment) leading to elevated Fos, inhibition of translation by *miR-7b* would provide a convenient method that would rapidly render ineffective any existing *Fos* transcripts until they are degraded. Under situations of prolonged stimulation of gene expression (e.g., chronic hyperosmolality), one might expect an equilibrium to be reached with *miR-7b* helping to restrain runaway gene expression.

Genes coexpressed with an miRNA have evolutionarily conserved target sites (36). This conservation of target sites is consistent with miRNAs acting as developmental switches or temporally regulating expression of gene sets (40). Given that *miR-7b* is colocalized with Fos in the PVN and SON and that the *Fos* gene has evolutionarily conserved target sites for *miR-7b* (Fig. 2), it is possible that *miR-7b* serves to regulate target genes temporally, including *Fos*, during osmotic stress, which could explain how Fos protein disappears rapidly after induction with acute hypertonic saline (12). Although we did not observe *Fos* transcript reduction (only reduced Fos protein levels) after *miR-7b* transfection, it is still possible that *miR-7b* binding to the *Fos* 3' UTR could affect mRNA stability if binding of one or more other miRNAs simultaneously is necessary for degradation (41). Interestingly, *Fos* harbors adenylate- and uridylylate-rich elements (AREs) that mediate mRNA degradation (42), and recent evidence suggests that miRNAs may participate in this process as well (43). Intriguingly, these AREs may also mediate de-repression of translation by miRNAs by binding the protein HuR that presumably displaces the miRNA-repressing complex (44). Therefore, further studies are needed to investigate whether other miRNAs and AREs are involved in Fos regulation.

Recently, microarray analyses have been used to profile gene expression in the hypothalamo-neurohypophyseal system (HNS) after hypoosmolar and hyperosmolar treatments (33–35). How those expression changes are controlled remains largely unknown. The inducible miRNAs identified in this study may be involved either directly by targeting those mRNAs or indirectly by affecting transcription factors such as Fos. ISHH localization of *miR-7b* within PVN in the hypothalamus also begs the question of whether other genes in addition to Fos are targets of *miR-7b*. Further studies will be important to learn which of the other miRNAs that we have identified by microarray have important roles in the HNS. Also, for example, the abundance of *miR-7b* in the suprachiasmatic nucleus, where Fos expression is prominently regulated (45, 46), indicates another place where *miR-7b* might play an important regulatory role.

The upstream region of *miR-7b* contains several putative AP-binding sites, so it will be interesting to learn whether AP-1 binds to these sites, forming a simple two-gene interacting system. Many miRNA genes are found in introns, and they are transcribed together with their host genes (47). However, it is possible that because of the slower turnover of miRNAs, their levels could differ from the those of the host genes (40). *miR-7b* shares its regulatory elements and primary transcript with its host gene *FICT*, and our ISHH data show that they share the same distribution, as would generally be expected.

In summary, microarray analysis can be used to study miRNA gene expression from selected brain regions after physiological manipulations. The miRNA *miR-7b* that we identified in this way may serve as a “molecular brake” to halt the regulation of other genes by Fos.

Materials and Methods

Animal and Tissue Isolation. Groups of 40 and 60 male mice (C57BL/6J), 5–6 weeks old, were purchased separately from The Jackson Laboratory (Bar Harbor, ME) for different tissue preparations. Half of each group was given 2% saline ad libitum as their only drinking fluid for 10 days to induce hyperosmolality. All procedures were carried out in accordance with National Institutes of Health guidelines on the care and use of animals with an animal study protocol approved by the National Institute of Mental Health Animal Care and Use Committee. All animals were killed by decapitation between 9:30 and 11:30 a.m., and the brains were removed quickly. PVN and SON tissues were isolated by using micropunches (26), combined and frozen on dry ice within 2 min, and stored at -80°C until processed further.

miRNA Microarrays. Twenty micrograms of total RNA was isolated from each of the four tissue preparations (two pairs of control and saline-treated animals) described above by using the mirVana miRNA isolation kit (Ambion, Foster City, CA). Small RNAs (<200 nt) were isolated from polyacrylamide gels. The RNAs were processed and used for microarray by LC Sciences (Houston, TX). Briefly, purified small RNAs were labeled with Cy3 or Cy5 fluorescent dyes (one dye for control, the other for saline-treated) and hybridized to dual-channel microarray $\mu\text{ParaFlo}$ microfluidics chips (LC Sciences). Each of the detection probes spotted a nucleotide sequence complementary to a specific miRNA sequence and a long nonnucleotide molecule spacer that extended the specific sequence away from the chip surface. The miRNA probe sequences used were from the miRBase Sequence database version 7.1 (Sanger Institute, Cambridge, U.K.; <http://microrna.sanger.ac.uk/sequences>). Each microarray chip contained six probe sets. Each probe spot measured was ≈ 100 pixels, and only those spots whose pixel intensities had a standard deviation of <0.001 were accepted. The data were then corrected by subtracting the background and normalizing to the statistical median of all detectable transcripts.

Microarray experiments were performed twice with one pair of RNA samples and once with the other pair. The data in Fig. 1 are the averages from two or three chips depending on whether at least one spot was acceptable from two or three chips, respectively.

Generation of DNA Constructs. The murine *Fos* 3' UTR (GenBank accession no. NM_010234 bases 1282–2092) was amplified by RT-PCR from adult mouse hypothalamus total RNA by using the primers 5'-GCAGTCAGAGAAGGCAAGGCAGCCG-GCA-3' and 5'-GCAGTCAGAGAAGGCAAGGCAGCCG-GCA-3'. The PCR product was cloned into pSICHECK (Promega, Madison, WI) downstream from the *Renilla* luciferase coding sequence (pSICHECK-Fos). si-miR-7b was cloned into the pcDNA6.2-GW/EmGFP-mir vector (Invitrogen, Carlsbad, CA) after annealing the oligonucleotides 5'-TGCTGTGGAA-GACTTGTGATTTGTTGTTTGGCCACTGACTGA-CAACAAAATCAAGTCTTCCA-3' and 5'-CCTGTGGAAG-ACCTGATTTGTTGTCAGTCAGTGGCCAAAACAACA-ATCACAAGTCTTCCAC-3' (Fig. 2). si-miR-neg, provided by Invitrogen, has an insert that can form a hairpin structure that is processed into mature miRNA, but it is predicted not to target any known vertebrate gene (according to Invitrogen). Transfection efficiency was checked by GFP expression under UV microscopy.

RT-PCR and Probes. The LNA oligonucleotide probe targeting mouse miR-7b was purchased from Exiqon (Vedbaek, Denmark), and it has been described (48). The LNA probe was labeled by using terminal deoxynucleotidyltransferase and ³⁵S-dATP. The *FICT* riboprobe targeting bases 1–494 (GenBank AK038632) was constructed by using the primers 5'-GGAAACGTCGGAAACTGGACAGC-3' and 5'-AAGTTG-GATTGAGGCTGGCTTCC-3' on first-strand cDNA generated from total hypothalamic RNA (3 μg). The RNA was initially treated with DNase (Promega) and then reverse-transcribed by using SuperScript III (Invitrogen) and a primer complementary to miR-7b (ASmmu-miR-7b; 5'-AACAAAAT-CACAAGTCTTCCA-3'). As a control, reverse transcriptase was omitted during the first-strand synthesis. The PCR product was purified and inserted into pGEM-TEasy vector (Promega). Antisense and sense riboprobes were labeled by using T7 and SP6 RNA polymerases, respectively, and ³⁵S-UTP.

Both the *Fos* probe, with the same primers used for pSICHECK-Fos, and the *Avp* probe, targeting bases 14–520 (GenBank, BC051997) and using the primers 5'-ATGCTCGCCAG-GATGCTC-3' and 5'-TCAGTAGACCCGGGGCTT-3', were prepared by RT-PCR with hypothalamic total RNA. Both probes were labeled with biotin by using the BioPrime DNA labeling system (Invitrogen).

Northern Blot Analyses. *Avp* mRNA was detected in 5 μg of total RNA isolated from the same tissue punches used to obtain the miRNA (see above) and resolved on a 0.8% denaturing-agarose gel. The RNA was transferred to Hybond N+ (Amersham, Piscataway, NJ) and hybridized with the *Avp* probe. *Fos* mRNA was detected in 10 μg of total RNA isolated from cultured NIH 3T3 cells by using TRIzol (Invitrogen). The RNA was resolved on a 1.2% denaturing agarose gel and transferred to a Hybond

N+ membrane. The *Fos* probe was applied to the membrane at 55°C. The signals were detected by using the chemiluminescent nucleic acid detection module (Pierce, Rockford, IL) according to the manufacturer's protocol.

ISHH. ISHH was performed as described by using either oligonucleotide or RNA (riboprobe) probes (49, 50). Briefly, mouse brains were removed and frozen on powdered dry ice. They were then stored at –80°C until 12-μm sections through the level of the PVN and SON were cut at –15°C and thaw-mounted onto Superfrost plus slides (VWR, Batavia, IL). They were stored again at –80°C until they were warmed to room temperature, then they were fixed in 4% formaldehyde in PBS, treated with acetic anhydride, and defatted through a series of alcohols. Radiolabeled oligonucleotides were applied to the sections for 20–24 h at 37°C before being washed in 16 mM sodium salts at 37°C. The riboprobes were incubated at 55°C before treatment with RNase and being washed in 16 mM sodium salts at 65°C. The sections were apposed to phosphorimaging plates for up to 4 weeks before scanning in a Cyclone system (PerkinElmer, Boston, MA).

Cell Culture, Transfection, and Stimulation. For the luciferase assay, 293T cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS. For *Fos* stimulation, NIH 3T3 cells were maintained in the same medium and treated with PMA (Sigma, St. Louis, MO) for 2 h (for Western blot analyses) or 1 h (for Northern blot analyses) before harvesting the cells. Forty-eight hours before treatment with PMA, cells were transfected with si-miR-7b or si-miR-neg by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Luciferase Assay. For the reporter assay, 293T cells were co-transfected in 24-well plates by using Lipofectamine 2000 with the appropriate miRNA expression vectors (2.4 μg) and pSICHECK-Fos (0.8 μg). Luciferase assays were performed 42 h later with the Dual-Luciferase reporter system (Promega) according to the manufacturer's instructions. All experiments were performed in triplicate.

Western Blot Analyses. Whole-cell protein extracts were prepared by using a 1× SDS loading buffer containing proteinase inhibitors (boiled for 5 min). Thirty micrograms of each protein extract was loaded onto an SDS/10% polyacrylamide gel and transferred to a PVDF membrane (Invitrogen). The membrane was incubated in 5% skim milk/0.1% TBS-Tween 20 at room temperature for 1 h followed by incubation with a *Fos* antibody (sc-52; Santa Cruz Biotechnology, Santa Cruz, CA) and then an anti-rabbit secondary antibody conjugated with horseradish peroxidase (Cell Signaling Technology, Danvers, MA). Signals were detected by using the Supersignal Western Pico chemiluminescent substrate (Pierce). Control for loading was checked by reprobing the membrane with a β-actin antibody (Cell Signaling Technology).

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