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Identification and characterization of novel activity-dependent transcription factors in rat cortical neurons

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Abstract

Using gene chip analyses, we have identified novel neuronal activity-dependent genes. Application of 25 mM KCl to mature (14-day culture) rat cortical neurons resulted in more than 1.5-fold induction of 19 genes and reduction of 42 genes among 1200 neural genes. Changes in the overall gene expression profiles appeared to be related to the reduction of excitability and induction of cellular survival signals. Among the genes identified, three transcriptional modulators [encoding Cbp/p300-interacting transactivator with ED-rich tail 2 (CITED2), CCAAT/enhancer binding protein β (C/EBPβ) and neuronal orphan receptor-1, (NOR1)] were newly identified as activity-dependent transcription factors, and two of these (CITED2 and NOR1) were found to be influenced by electroconvulsive shock (ECS). NOR1 was induced in specific brain regions by behavioral activation, such as exposure to a novel environment. Because the brain regions that exhibited the induction of these newly identified neuronal activity-dependent transcriptional modulators were distinct from those showing the induction of previously identified activity-dependent genes such as c-*fos*, these genes might be useful markers for mapping neuronal activity *in vivo*.

Keywords: activity-dependent; microarray; neurons; neuronal orphan receptor-1; novel environment; transcription factor.
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Activity-dependent changes are required for the learning and memory of transient experiences. Many of these responses to neuronal activation are believed to be mediated by *de novo* synthesis of RNA and protein, because RNA or protein synthesis inhibitors repress a subset of these events in neurons (Walton *et al*. 1999; Yoneda *et al*. 2001). Although it is clear that long-term consolidation of the short-term changes in neurons requires gene expression, less is known about how neuronal activity induces gene expression and which gene product critically mediates activity-dependent neuronal plasticity.

Because transcription factors lead to alterations in downstream gene expression profiles, transcription factors that immediately respond following neuronal activation may play critical roles in the downstream alteration of neuronal gene expression. Supporting this idea, several activity-dependent transcription factors have been found to be critical for the neuronal plasticity. For instance, the expression of *zif268* (also known as Egr1) is induced by behavioral stimulations such as spatial learning or fear conditioning *in vivo* (Fordyce *et al*. 1994; Hall *et al*. 2001). The significant contribution of *zif268* to neuronal plasticity has been well demonstrated in *egr*1-deficient mice, which failed to exhibit long-term memory consolidation and reconsolidation (Bozon *et al*. 2003; Lee *et al*. 2004). Although the physiological significance to neuronal plasticity is less explored, the expression of activator protein (AP)-1 components such as Fos and Jun family molecules is highly up-regulated by neuronal activation and behavioral stimulations (Vann *et al*. 2000; Teather *et al*. 2005). Therefore, because of such dramatic and specific responses, the immediate-early class of transcription factors

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Woon Sun and Sang-Hyun Choi are co-first authors and contributed equally to this work. [Correction added after online publication (24 November 2006): The author's name is Woong Sun]

Abbreviations used: Amy, amygdala; CaMK-IV, calcium/calmodulin-dependant protein kinase-IV; C/EBP, CCAAT/enhancer binding protein β; Cbp, CREB-binding protein; CITED2, Cbp/p300-interacting transactivator with ED-rich tail 2; Con or CTL, control; Depol, depolarization; DIV, days *in* vitro; DTT, dithiothreitol; ECS, electroconvulsive shock; ED, Gln/Asp; FDR, false discovery rate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GO, gene ontology; HIF, hypoxia inducible factor; NE, novel environment; NOR-1, neuronal orphan receptor-1; SAM, significant analysis of microarrays; SS, somatosensory area; SSC, saline sodium citrate; TTX, tetrodotoxin.

(e.g. fos, Jun, zif268, arc) has been used to monitor the activation of specific neuronal circuits *in vivo*.

It is important to note that different types of neuronal activation induce different subsets of transcription factors in different brain regions, suggesting that different combinations of transcription factors are required for the plastic changes in specific neuronal circuits. In this respect, identification of novel activity-dependent transcription factors may significantly improve our understanding of the mechanisms involved in the transcriptional control of neuronal plasticity. To this end, we applied microarray technology to identify hitherto uncharacterized activity-dependent transcription factors, and evaluated the activity-dependent induction of expression of these genes in different brain regions using *in situ* hybridization histochemistry.

Materials and methods

Primary culture of cerebral cortex neurons

Cultures were prepared from the cerebral cortex or hippocampus of gestation day 17 Sprague–Dawley rat embryos. Embryonic cells were dissociated by trypsinization and trituration. The suspension of single cells was plated (2×10^6 cells per 60-mm culture dish) on to a plate coated with poly-D-lysine (Sigma, St Louis, MO, USA) and maintained in medium consisting of neurobasal medium, B27 supplement (Gibco BRL, Rockville, MD, USA), 0.5 mM L-glutamine and antibiotic–antimycotic (Gibco BRL). At 3 days *in vitro* (DIV), cytosine arabinoside was added to inhibit the proliferation of non-neuronal cells. At 14 DIV, the medium was replaced with depolarizing medium containing 25 mM KCl, and neurons were harvested after 4 h of KCl treatment. To inhibit spontaneous depolarization, some cultures were treated with 1 μM tetrodotoxin (TTX) (Tocris, Ballwin, MO, USA) at 13 DIV and harvested 24 h after treatment.

Microarrays

Samples preparation and labeling

Rat cortical neurons cultured for 14 days were treated with 25 mM KCl for 4 h. Total RNA was extracted using an RNA extraction kit (Qiagen, Valencia, CA, USA), and assessed for quantity and quality by UV spectrometry and RNA gel electrophoresis. RNA was labeled and hybridized to a GeneChip according to Standard Affymetrix Protocols (Affymetrix GeneChip Expression Analysis Technical Manual, revision 3, 2001). Affymetrix GeneChip RN U34 arrays were used in this study, and their characteristics are detailed at http://www.affymetrix.com/products/arrays/specific/rnu34.affx.

Each reaction involving a single GeneChip hybridization was started with 5 μg RNA, which was initially reverse transcribed using the Superscript Choice System (Life Technologies, Grand Island, NY, USA). *In vitro* transcription of double-stranded cDNA (0.5–1.0 μg) generated cRNA containing biotinylated UTP and CTP using a BIOarray RNA Transcript labeling kit (Enzo Diagnostics, Farmingdale, NY, USA). Labeled cRNA was purified on RNeasy affinity resin (Qiagen) and fragmented (50–100 bases) by incubation at 94°C for 35 min.

Hybridization procedures and parameters

The fragments of biotinylated cRNA were hybridized in 2-[*N*-morpholino]ethansulfonic acid buffer containing 0.5 mg/mL acetylated bovine serum albumin to the RN U34 GeneChip at 45°C for 16 h in a GeneChip hybridization oven under constant rotation (60 rpm), according to standard Affymetrix protocols. The RN U34 array allows monitoring of the relative abundance of more than 1200 mRNA transcripts relevant to the study of neurobiology, including genes for kinases, cell surface receptors, cytokines, growth factors and oncogenes. After hybridization, GeneChips were washed and stained with streptavidin–phycoerythrin using a GeneChip fluidics station.

Data measurement and specifications

An Affymetrix GeneChip scanner operated by GeneChip Operating Software version 1.3 (GCOS; Affymetrix) was used to generate original array images. The average difference of each probe set, which is a measure of the relative abundance of a transcript, and signals and detection calls such as present or absent were computed by GCOS. All hybridization intensities were corrected by a set value for a total intensity of 500, and the scaling factors were between 5.7 and 10.5. Significant analysis of microarrays (SAM) (Tusher *et al*. 2001) (http://www-stat.stanford.edu/~tibs/SAM/) was used to select differentially expressed transcripts. Only the transcripts called present for all four hybridizations were used for SAM. The delta value was set with false discovery rate (FDR) less than 10% and another cut-off of 1.5-fold was applied simultaneously.

Nomenclature, sequence and gene ontology (GO) information was obtained from NetAffx (http://www.affymetrix.com/analysis/netaffx), EntrezGene (http://www.ncbi.nlm.nih.gov/entrez/) and GeneOntology (http://www.geneontology.org). Over-representative GO biological processes were searched by EASE software (Hosack *et al*. 2003). Fisher's exact test was used to measure the gene enrichment of each GO functional cluster with respect to the total number of genes in whole genome and annotated within each functional cluster.

PCR

Total RNA (2 μg) was reverse transcribed, and an equal aliquot of the resulting RT product (50 ng) was amplified by PCR with specific primer sets under the following conditions [except for glyceraldehyde-3-phosphate dehydrogenase (GAPDH)]: an initial denaturation at 95°C for 1 min, followed by 25–35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and final extension of 72°C for 10 min. The number of PCR cycles for each cDNA amplification under the saturated value was decided empirically. Table 1 summarizes the primer sets used in this study. PCR products were electrophoresed on 2% agarose gels and visualized by staining with ethidium bromide.

Electroconvulsive shock (ECS)

ECS was applied to male Sprague–Dawley rats (200–230 g) via ear-clip electrodes connected to an Electroconvulsive therapy (ECT) apparatus (Medcraft Model B24-III ECT, NY, USA). The stimulation (140 V for 0.5 s) was sufficient to produce full tonic–clonic seizure with hind limb extension for 10–15 s in 70–80% of treated animals. Control animals were handled identically to experimental rats, but were not electrically stimulated. Animals

Table 1 Primers used in this study

Gene name	Primer sequences	PCR product size (bps)	Positions	Accession number
CITED 2	upper: ccgcccaatgtcatagacactgatttc lower: atttctttcagccgcgaggttaacc	202	871–1072	NM-053698
C/EPBβ	upper: cgggacttgatgcaatccggatc lower: aacatcaacagcaacaaccccgc	136	842–977	X60769
NOR-1	upper: tgagcacgtgcagcagttctac lower: gaaggcactgaagtcggtgc	230	1998–2227	NM_031628
GAPDH	upper: gtccacattgttgccatcaacgac lower: tttctcgtggttcacacccatcac	335	147–482	X02231

that did not exhibit a full tonic–clonic seizure were excluded from the experiment to minimize variability. All experimental procedures with animals were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Committee of Korea University College of Medicine.

Exposure to a novel environment (NE)

Male Sprague–Dawley rats were maintained in standard home cages for more than 1 week to allow accommodation. For exposure to a NE, rats were individually placed in a plexiglass open box (45 cm × 45 cm × 45 cm) for 5 min. During this period, animals exhibited typical exploratory behaviors such as sniffing, rearing and increased motor activity. Animals were then returned to the home cage, and killed 55 min later.

In situ hybridization

In situ hybridization was performed as described previously (Kim *et al*. 2004). cDNAs for the template of RNA probes were prepared by cloning PCR products into T-vector. Each plasmid was linearized, and antisense cRNA probe was *in vitro* transcribed with ^{35}S-labelled UTP. Tissue sections for hybridization were fixed in 4% paraformaldehyde for 5 min on glass slides, rinsed, and acetylated in 0.25% acetic anhydride (Sigma) in 0.1 M triethanolamine (pH 8.0) for 10 min, After rinses, the slides were then hybridized with ^{35}S-labeled probes (1.2×10^6 cpm/slide) overnight at 52°C. After hybridization, the slides were washed four times in 4 × saline sodium citrate (SSC) solution at room temperature (20–25°C), and then incubated with Rnase buffer [Rnase A (10 mg/mL in distilled water), 0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0] for 30 min at 37°C. After Rnase treatment, the sections were washed with 2 × SSC/10 mM dithiothreitol (DTT) twice for 5 min each, 1 × SSC/10 mM DTT for 10 min, 0.5 × SSC/10 mM DTT for 10 min, and 0.1 × SSC/10 mM DTT for 30 min at 62°C. Finally, the sections were dehydrated, air-dried and exposed to β-max Hyperfilm (Kodak, Rochester, NY, USA). The expression of c-fos, Cbp/p300-interacting transactivator with ED-rich tail 2 (CITED2), CCAAT/enhancer binding protein β (C/EBPβ) and neuronal orphan receptor-1 (NOR-1) was quantified by measuring the optical density of autoradiographic film images in the hippocampal CA1 and dentate gyrus, amygdala and somatosensory areas of the cortex. The relative intensity of the signals was quantified using the NIH image 1.61 program (http://rsb.info.nih.gov/nih-image/). Anatomical regions for the quantification of signals were determined from adjacent Nissl-stained sections.

Results

Identification of depolarization-related neuronal genes by microarray analysis

To identify the genes whose expression was induced by depolarization, we first compared gene expression profiles in control cortical neurons with those in cortical neurons after treatment with KCl (25 mM) for 4 h using Affymetrix Genechips specified for neurobiology. Transcripts not displaying a present call in any one array were filtered out and 506 of 1322 transcripts were analysed further. Scatter plots using these reliable 506 transcripts that demonstrated call present for all four hybridizations showed strong correlations between the replicates of the same treatments (control replicates, $r = 0.99$; KCl-treated replicates, $r = 0.99$), whereas a rather weaker correlation was noted between the averaged control and KCl-treated groups ($r = 0.95$; Fig. 1). In SAM, a delta value set at 0.94 with an FDR of 9.5% and a further cut-off of 1.5-fold were applied, and 76 transcripts (61 genes) were proven to be expressed differentially upon KCl treatment. The expression of 19 genes (among 25 transcripts) was up-regulated whereas that of 42 genes (among 51 transcripts) was down-regulated (Supplementary Tables S1 and S2).

We then classified these genes into GO categories based on their known biological functions: regulation of transcription (including transcription, regulation of nucleobase, nucleotide and nucleic acid metabolism), regulation of neuronal synaptic plasticity, synaptogenesis, G protein-coupled receptor protein signaling pathway, sensory perception, synaptic transmission, ion transport, cation transport, second messenger-mediated signaling and regulation of programmed cell death (including regulation of apoptosis, regulation of programmed cell death and apoptosis) (Table 2). The induced genes are listed in Table 2 and the reduced genes in Table 3 according to GO categories with $p < 0.01$ by Fisher's exact test. Our gene screening demonstrated induction of eight transcription factors by neuronal depolarization, whereas there was no transcription factor whose expression was down-regulated. On the other hand, the majority of genes in synaptic transmission or intracellular signal transduction cateories were down-regulated, suggesting that our experimental

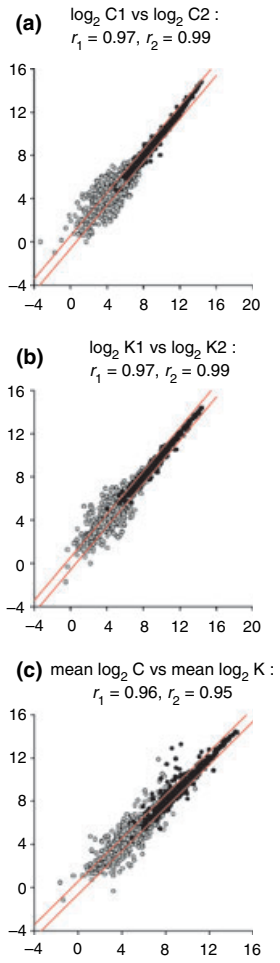

Fig. 1 Correlation plots of replicates of control (a, C1 vs. C2) and KCl-treated (b, K1 vs. K2). The averaged signal of KCl-treated groups was plotted against the averaged control signal (c, C vs. K). Logarithmic (log base 2) plots of normalized signals on *x* and *y* axes computed correlation coefficients. r_1 and r_2 were obtained from the plots using all 1322 transcripts and the 506 transcripts (black dots) selected by the present call filtering respectively. The signals of transcripts not displaying a present call in any one array are shown in gray. Reference lines represent differential expression thresholds (red line, 1.5-fold; blue line, 2.0-fold). [Correction added after online publication (24 November 2006): The preceeding sentence should read as: Reference line represent the expression threshold of 1.5 fold]

condition (prolonged depolarization) promoted compensatory suppression of neurotransmission to reduce possible damage as a result of prolonged neuronal activation. Taken together, the profile of overall gene expression appears to represent early transcriptional activation and defensive responses of neurons against prolonged neuronal depolarization.

Quantitative RT–PCR of transcription factor genes to validate microarray profiles

Among eight transcriptional factors identified, the activity-dependent induction of five genes (encoding c-fos, Nur77, JunB, JunD and zif268) has previously been reported (Sheng *et al*. 1993; Guzowski *et al*. 2001). However, three genes (encoding CITED2, C/EBPβ and NOR-1) had not been characterized earlier as their expression is activity dependent. Using semiquantitative PCR, we confirmed that the expression of C/EBPβ and NOR-1 was induced by 4 h after depolarization in both cultured cortical and hippocampal neurons (Fig. 2a). Conversely, addition of the sodium channel blocker TTX, which suppresses spontaneous depolarization substantially, reduced the expression of C/EBPβ and NOR-1 in both cultures (Fig. 2b). On the other hand, activity-dependent regulation of CITED2 expression was seen only in cerebral cortex neurons, and not in hippocampal neurons. Therefore, we concluded that the expression of these transcription factors is dependent on activity and neuronal type.

Expression of the activity-dependent genes following ECS *in vivo*

To assess whether the expression of these newly identified transcriptional factors is also influenced by neuronal stimulation *in vivo*, we administered ECS to rats and examined the expression of these genes by *in situ* hybridization histochemical analyses (Fig. 3a). Following ECS, c-fos induction was observed in several brain regions such as cerebral cortex, hippocampal formation and amygdala complex, consistent with earlier findings (Kim *et al*. 1994).

A low level of CITED2 mRNA expression was observed in several brain regions, including the thalamus, dentate gyrus and CA3 subregion of the hippocampal formation of normal brain. By 1 h following ECS, the induction of CITED2 mRNA was observed in the dentate gyrus, piriform cortex and somatosensory area of the cerebral cortex, and CITED2 expression returned rapidly to the basal level by 3 h. On the other hand, the expression level of C/EBPβ was not changed at any time point examined.

A relatively high basal level of NOR1 expression was observed in the hippocampus and amygdala. One hour after ECS, robust induction of NOR-1 expression was observed in the cerebral cortex, piriform cortex, amygdala, and hippocampal formation, including the dentate gyrus. Following transient induction, NOR-1 expression was reduced to below the basal level at 6–24 h after ECS. Quantitative results of the expression levels of the three genes in the dentate gyrus and amygdala are shown in Figs 3(b) and (c).

Expression of the activity-dependent genes following behavioral stimulation *in vivo*

We further examined whether physiological stimulation of the brain is sufficient to trigger the expression of these genes. For this purpose, we exposed the animals to a NE for 5 min. A NE is believed to induce several consequences in neuronal circuits involved in novelty seeking responses, fear/anxiety-

Table 2 Representative gene ontology categories (biological processes) and gene lists obtained by EASE analysis of genes upregulated by KCl treatment

ID	Genes	Folds
Regulation of transcription**		
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism**		
Transcription**		
X60769mRNA-at	CCAAT/enhancer binding protein (C/EBP), beta	2.96, 2.23
Rc-AA900476-g-at	Cbp/p300-interacting transactivator (CITED2)	3.06
D26307cds-at	Jun D proto-oncogene	2.06
X54686cds-at	Jun-B oncogene	7.96
AF023087-s-at	early growth response 1 (EGR1, zif268)	1.84
U17254-g-at	nuclear receptor subfamily 4, group A, member 1 (NR4A1, Nurr77)	9.89, 6.99
Rc-AI176710-at	nuclear receptor subfamily 4, group A, member 3 (NR4A3, NOR-1)	2.93, 2.07
X03347cds-g-at	c-Fos protein gene	19.91
Regulation of neuronal synaptic plasticity*		
Synaptogenesis*		
X04979-at	apolipoprotein E	2.42
D10938exon-s-at	brain derived neurotrophic factor (BDNF)	5.79

ID: Affymatrix probe set ID
Listed biological processes have Fisher Exact p-value less than 0.01(**) or 0.1(*).

related reactions and spatial information processes. This treatment is therefore expected to produce firing of multiple neuronal circuits within the cerebral cortex, amygdala and hippocampus.

Following this behavioral stimulation, however, changes in the expression of CITED2 and C/EBPβ were not observed (data not shown). However, we observed substantial induction of c-fos expression in a somatosensory area of cerebral cortex (Fig. 4). Induction of c-fos in this context has been reported previously (Handa *et al*. 1993), suggesting that our experimental model is valid. Compared with c-fos, the induction of NOR-1 was more profound and broad in many brain regions, including the cerebral cortex, CA1–3 subregions of the hippocampus and amygdaloid nuclei. Furthermore, although there was no induction of c-fos mRNA in amygdala, NOR-1 induction was robust in the CA1 region of the hippocampus and amygdala (Figs 4b–d). Such area-specific induction of NOR-1 was not observed after ECS, suggesting that this behavioral induction of NOR-1 expression is related to selective use of neural circuits in this context.

Discussion

Identification of novel activity-dependent genes in cultured neurons using microarrays

Using oligonucleotide microarray analyses, we identified novel activity-dependent genes including transcription factors. Our experimental procedure involving four-pair comparisons appeared efficiently to eliminate false-positive signals, because eight transcription factors that we screened as positive were either previously reported to be activity-dependent genes or verified in the present study. Overall changes in gene expression appear to represent the adaptive responses of neurons under conditions of prolonged neuronal depolarization.

Identification of novel activity-dependent transcription factors

In this study, we newly characterized three transcriptional modulators: CITED2, NOR-1 and C/EBPβ. Of these, the expression of CITED2 and NOR-1 mRNA was also found to be induced by ECS treatment *in vivo*. Conversely, all ECS-induced genes that we have recently identified (Sun *et al*. 2005) were induced by depolarization *in vitro*. These results suggest that early events occurring during *in vitro* cultured neuronal sustained depolarization and *in vivo* ECS treatments share many common features.

CITED2 was initially identified as melanocyte-specific gene related gene-1 (Shioda *et al*. 1997), and it was subsequently reported that the transcription of CITED2 is induced by cytokines in immune cells (Sun *et al*. 1998). An alternative splice form of CITED2 (p35srj) is a co-factor for hypoxia inducible factor (HIF)-1 activity (Bhattacharya *et al*. 1999), and HIF-1 is implicated in the upstream transcriptional control of CITED2 (Freeman *et al*. 2003). Therefore, the biological function of CITED2 appears to be related to inflammatory and neurodegenerative responses. In developing brain, CITED2 is strongly expressed in the restricted region of the brain (Dunwoodie *et al*. 1998) and genetic elimination of the *CITED2* gene is associated with late

Table 3 Representative gene ontology (biological processes) and gene lists obtained by EASE analysis of genes down-regulated by KCl treatment

ID	Genes	Folds
G-Protein coupled receptor protein signaling pathway**		
L22558_at	5-hydroxytryptamine (serotonin) receptor 7	0.66
S48813_s_at	adrenergic receptor kinase, beta 1	0.65
rc_AA964003_s_at	arrestin, beta 2	0.63
M16407_at	cholinergic receptor, muscarinic 3	0.56
M35077_s_at,S46131mRNA_s_at	dopamine receptor 1A	0.63, 0.61
L08490cds_at	gamma-aminobutyric acid A receptor, alpha 1	0.63
L08494cds_s_at	gamma-aminobutyric acid A receptor, alpha 5	0.58
M61099_at	glutamate receptor, metabotropic 1	0.61
D16817_g_at	glutamate receptor, metabotropic 7	0.62
rc_AI229237_at	opioid receptor-like	0.57
K02248cds_s_at,M25890_at	somatostatin	0.50, 0.34
Sensory perception**		
rc_AA955388_s_at	ATPase, Ca++ transporting, plasma membrane 2	0.55
rc_AA964003_s_at	arrestin, beta 2	0.63
M61099_at	glutamate receptor, metabotropic 1	0.61
D16817_g_at	glutamate receptor, metabotropic 7	0.62
AF021923_at	solute carrier family 24, member 2	0.58
Synaptic transmission**		
M16407_at	cholinergic receptor, muscarinic 3	0.56
L08490cds_at	gamma-aminobutyric acid A receptor, alpha 1	0.63
L08494cds_s_at	gamma-aminobutyric acid A receptor, alpha 5	0.58
X57573_at	glutamate decarboxylase 1	0.29
U11418_s_at	glutamate receptor, ionotropic, NMDA 1	0.66
M61099_at	glutamate receptor, metabotropic 1	0.61
Ion Transport**, Cation transport**		
rc_AA955388_s_at	ATPase, Ca++ transporting, plasma membrane 2	0.55
D90048exon_g_at	ATPase, Na+/K+ transporting, beta 2 polypeptide	0.63
D38101_s_at	calcium channel, voltage-dependent, L type, alpha 1D	0.62
L08490cds_at	GABA receptor, alpha 1	0.63
L08494cds_s_at	GABA receptor, alpha 5	0.58
U11418_s_at	glutamate receptor, ionotropic, NMDA 1	0.66
X98564cds_at	potassium channel, subfamily V, member 1	0.59
X62839mRNA_s_at	potassium voltage gated channel, Shaw-related subfamily, member 2	0.24
AF021923_at	solute carrier family 24, member 2	0.58
Second messenger mediated signaling*		
L22558_at	5-hydroxytryptamine (serotonin) receptor 7	0.65
D38101_s_at	calcium channel, voltage-dependent, L type, alpha 1D	0.62
M35077_s_at,S46131mRNA_s_at	dopamine receptor 1A	0.63, 0.61
D16817_g_at	glutamate receptor, metabotropic 7	0.62
Regulation of Programmed cell death, Regulation of Apoptosis, Apoptosis*		
U49729_at	Bcl2-associated X protein	0.65
U77933_at	caspase 2	0.65
M31837_at,rc_AI009405_s_at	insulin-like growth factor binding protein 3	0.56, 0.47

ID: Affymatrix probe set ID

Listed biological processes have Fisher Exact p-value less than 0.01 (**) or 0.1 (*).

embryonic lethality accompanied by enhanced apoptosis of neuroepithelial cells and exencephaly (Bamforth *et al*. 2001), suggesting a critical role of CITED2 in normal brain development including neural tube closure (Greene and Copp 2005). In a hypomorphic variation of CITED2 knockout mice, hippocampal development was impaired,

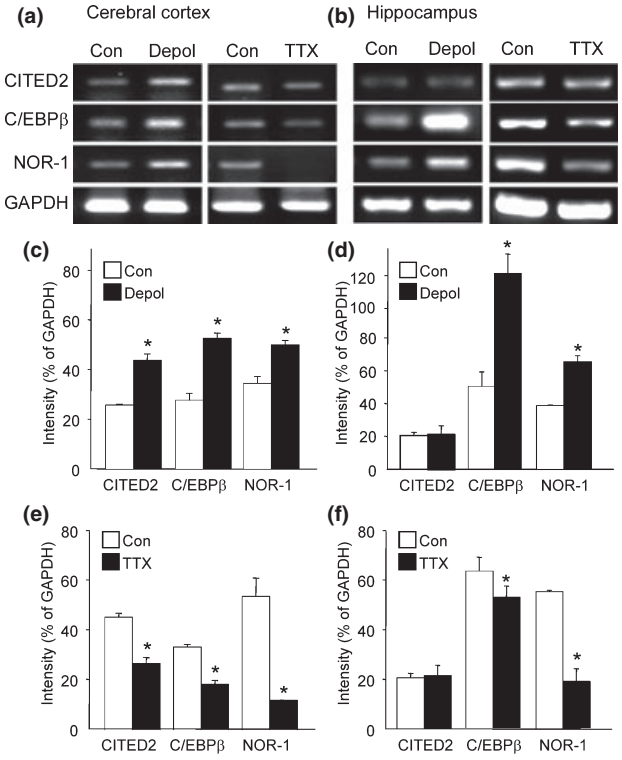

Fig. 2 RT–PCR confirmation of activity-dependent transcription factors *in vitro*. Comparison of gene expression levels in control neurons (Con) and those treated for 4 h with KCl (Depol), or control neurons (Con) and those treated for 24 h with TTX. Neurons from the cerebral cortex (a) or hippocampus (b) at 14 DIV were used. Amplication was carried out for 30 cycles for control versus depolarized groups, 35 cycles for control versus TTX groups, and 25 cycles for GAPDH. PCR cycle numbers were estimated empirically and the subsaturation condition of PCR amplification was verified by serial dilution of initial cDNA inputs (data not shown). (c–f) Quantitation of the PCR band intensities of control versus depolarized (c, d) or control versus TTX (e, f) cortical (c, e) or hippocampal (d, f) neurons. Values are mean ± SEM ($n = 4$). *$p < 0.01$ versus control (Student's *t*-test).

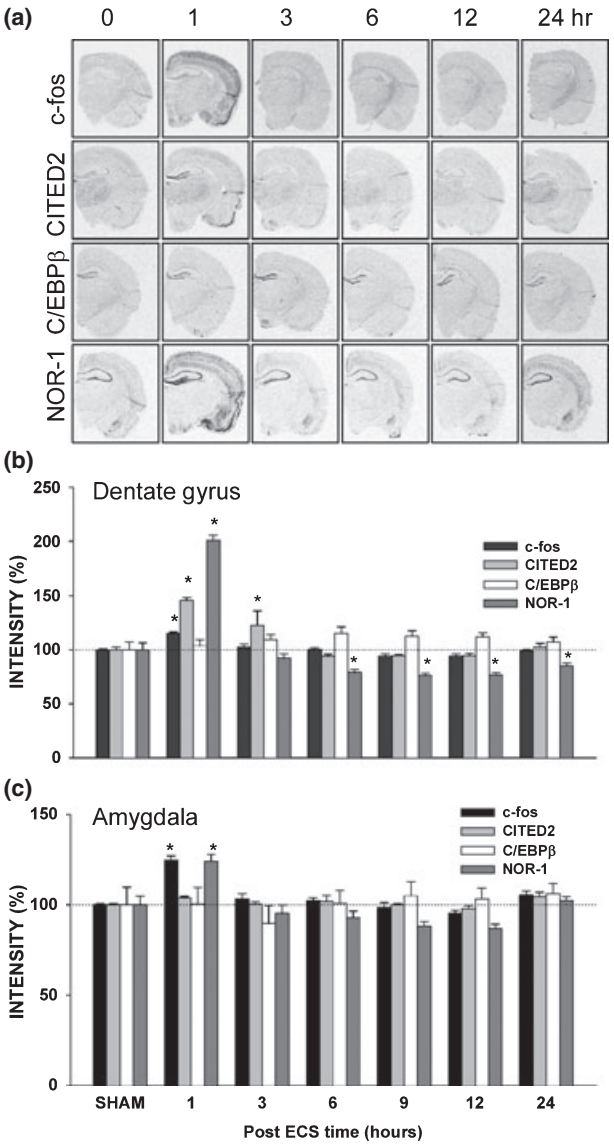

Fig. 3 (a) Expression of c-fos, CITED2, NOR-1 and c/EBPβ following sham treatment, and 1, 3, 6, 12 and 24 h after ECS. (b, c) Quantification of gene expression in (b) dentate gyrus and (c) amygdala. Values are mean ± SEM ($n > 4$). *$p < 0.05$ versus sham group (ANOVA followed by Dunnett's test).

and increased seizure susceptibility was noted (Bamforth *et al*. 2004). Recently, we observed that the expression of CITED2 was also increased following transient forebrain ischemia induced by four-vessel occlusion (Sun *et al*. 2006). This observation further supports the idea that CITED2 expression is related to the neuronal death evoked by aberrant neuronal activation/damage.

C/EBPβ (also known as NF-IL6, IL6-DBP, LAP, AGP/EBP or CRP2) is a transcription factor that was initially considered to be involved in hepatocyte-specific gene regulation, because it is expressed at a high level in liver cells (Descombes *et al*. 1990). Recently, however, it has become clear that C/EBPβ is also involved in adipogenesis, cell cycle control and programmed cell death in other tissues (McKnight 2001). As for the possible role of C/EBPβ in the brain, C/EBPβ mRNA has been shown to be expressed widely in adult mouse brain (Sterneck and Johnson 1998), and the expression of C/EBPβ to be induced in murine reactive astrocytes surrounding the senile plaques of Alzheimer's disease and by the pro-inflammatory cytokine tumor necrosis factor-α (Popovic *et al*. 1998). Furthermore, C/EBPβ promotes neuronal differentiation and neurite outgrowth through the phosphatidylinositol 3-kinase (PI-3-K) pathway (Cortes-Canteli *et al*. 2002). In *Aplysia* (Alberni *et al*. 1994) as well as in mammals (Yukawa *et al*. 1998), C/EBPβ plays an essential role in the consolidation of stable long-term synaptic plasticity. Taubenfeld and co-workers have recently shown

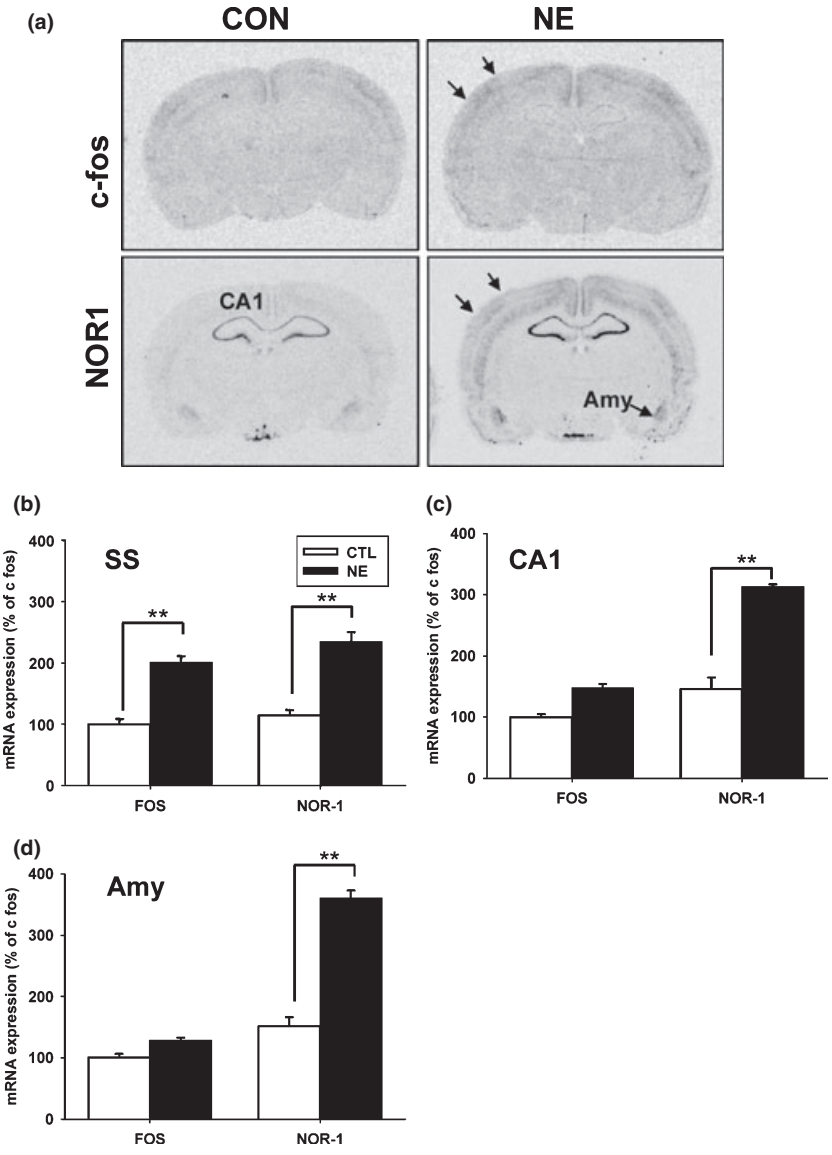

Fig. 4 (a) Changes in expression of c-fos and NOR-1 mRNA in rat brain 1 h after exposure to a NE. (b–d) Quantification of c-fos and NOR-1 mRNA levels in (b) the somatosensory area (SS) of the cortex [arrows in (a) indicate SS], CA1 region of the hippocampus and (c) amygdala (Amy). CTL, control' Values are mean ± SEM ($n = 6$). $^{**}p < 0.01$ (Student's *t*-test).

that consolidation of new memories requires cyclic AMP response element-binding protein-dependent C/EBPβ activation in the hippocampus (Taubenfeld *et al*. 2001). These results indicate that C/EBPβ could be a transcriptional factor involved in activity-dependent processes, such as learning and memory (Cortes-Canteli *et al*. 2004).

NOR-1 is a zinc finger-containing transcription factor with high degree of homology with Nurr1 and Nur77 (Mangelsdorf *et al*. 1995). NOR-1 was originally identified from cultured neurons undergoing apoptosis, suggesting a role in neuronal death (Ohkura *et al*. 1994). The transcription of NOR-1 is regulated by calcium/calmodulin dependant protein kinase-IV (CaMK-IV) signaling (Inuzuka *et al*. 2002). Considering that CaMK-IV activity is induced by increased intracellular free calcium level, activity-induced intracellular calcium may be able to induce NOR expression via CaMK-IV activation. In addition, other stimulators such as forskolin (protein kinase A activation), 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (protein kinase C activation) and nerve growth factor (trk activation) could induce NOR-1 activation (Bandoh *et al*. 1995). Although the endogenous ligand for NOR-1 has yet to be identified, a synthetic molecule that activates the transcriptional activity of NOR-1 has been developed (Hsu *et al*. 2004).

Induction of NOR-1 after behaviorally induced neuronal activation *in vivo*

The expression of NOR-1 was induced not only by ECS, but also by physiological stimulation of neuronal circuits elicited by a brief exposure of animals to a NE. We were not able to observe significant changes in the expression of CITED2 and C/EBPβ induced by a NE and it is not clear whether mild or physiological/behavioral stimulation of the brain is insufficient for the induction of these transcriptional factors or

whether they respond to other types of neuronal activity. We have examined whether other behavioral settings such as fear conditioning for hippocampal and amygdala-dependent learning have an effect, but failed to observe substantial changes in CITED2 and C/EBPβ mRNA levels (data not shown).

When the expression pattern of NOR-1 after exposure of animals to NE was compared with that after ECS treatment, NE appeared to induce NOR-1 in more specific brain regions than ECS. For instance, ECS-induced NOR-1 expression was observed in most brain regions, whereas NE-induced NOR-1 expression was more pronounced in the somatosensory area of the cerebral cortex. Similarly, the induction of NOR-1 was observed throughout the amygdala after ECS, whereas NOR-1 induction was prominent in the central nucleus of the amygdala, following exposure to NE. Finally, the induction of NOR-1 expression was observed predominantly in the dentate gyrus of the hippocampal formation after ECS, whereas NOR-1 induction was more profound in the CA1–3 region after exposure to a NE. Collectively, these results lead us to suggest that NOR-1 expression is dependent on neuronal activation within a specific context. On the other hand, the expression of c-fos was not observed in the amygdala under the same conditions. The expression of activity-dependent transcriptional factors appears to be dependent on neuronal type. Although *zif268*, another well characterized activity-dependent gene, has been used to monitor neuronal activity in the lateral amygdala (Malkani *et al*. 2004), its induction was less evident in the central amygdala. Thus, NOR-1 seems to be more suitable for visualizing the neuronal activity of the central amygdala *in vivo*.

In summary, we have identified three novel transcription factors (CITED2, C/EBPβ and NOR-1) whose expression is activity dependent *in vivo* and *in vitro*. NOR-1 especially is sensitive enough for monitoring behavioral activation of neuronal circuits with different regional specificity, compared with other widely used neuronal activation markers such as *zif268* and c-*fos*.

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Supplementary material

The following supplementary material is available for this article online:

Table S1 Genes specifically up-regulated after KCl-induced depolarization in rat cortical neurons

Table S2 Genes specifically down-regulated after KCl-induced depolarization in rat cortical neurons

This material is available as part of the online article from http://www.blackwell-synergy.com

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