

# Genetic Program of Neuronal Differentiation and Growth Induced by Specific Activation of NMDA Receptors

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**Abstract** Glutamate and its receptors are expressed very early during development and may play important roles in neurogenesis, synapse formation and brain wiring. The levels of glutamate and activity of its receptors can be influenced by exogenous factors, leading to neurodevelopmental disorders. To investigate the role of NMDA receptors on gene regulation in a neuronal model, we used primary neuronal cultures developed from embryonic rat cerebri in serum-free medium. Using

Affymetrix Gene Arrays, we found that genes known to be involved in neuronal plasticity were differentially expressed 24 h after a brief activation of NMDA receptors. The upregulation of these genes was accompanied by a sustained induction of CREB phosphorylation, and an increase in synaptophysin immunoreactivity. We conclude that NMDA receptor activation elicits expression of genes whose downstream products are involved in the regulation of early phases of the process leading to synaptogenesis and its consolidation, at least in part through sustained CREB phosphorylation.

Special issue dedicated to Dr. Anthony Campagnoni.

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## Abbreviations

|        |  |
|--------|--|
| NMDA   | N-methyl-D-aspartate   |
| AMPA   | $\alpha$ -Amino-3-hydroxy-5-methylisoxazole-4-propionic acid |
| BDNF   | Brain derived neurotrophic factor                            |
| CREB   | cAMP responsive element binding                              |
| CBP    | CREB binding protein   |
| IEGs   | Immediate early genes  |
| LTP    | Long term potentiation                                       |
| NARP   | Neuronal activity-regulated pentraxin                        |
| NF-L   | Light molecular-weight neurofilament                         |
| NGFI-B | NGF-induced factor B   |
| NOR    | Neuron-derived orphan receptor                               |
| pCREB  | Phosphorylated CREB  |

## Introduction

Mammalian brain development is modulated by the orchestrated interaction of cell intrinsic and extrinsic

factors that, in turn, can be influenced by environmental stimuli. In utero exposure to harmful substances connected with maternal behaviour and/or exposure (e.g. consumption of neurotoxic substances) may induce untimely interactions between the cell intrinsic and extrinsic signalling factors, interfering with proper brain development and increasing the risk of neurodevelopmental diseases [1–4].

Among the extrinsic factors, neurotransmitters and their receptors play key roles in central nervous system (CNS) development [5]. Glutamate is the major excitatory neurotransmitter in the brain. Its receptors are expressed early in development by neural progenitors and by selected early neuronal populations. This suggests that they might play a role in directing the development of later populations, and in the proper wiring of the developing CNS [5–7]. Glutamatergic actions are mediated through 3 types of ionotropic (ligand-gated ion channels) receptors (iGluR: N-methyl-D-Aspartate (NMDA),  $\alpha$ -Amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and Kainate) and one group of G-protein coupled metabotropic receptors (mGluR) of which several functional subunits have been identified [8, 9]. The expression of these receptor subtypes and their subunits appears to be developmentally regulated, as some of them are only expressed during embryonic or early postnatal life. In order to properly influence developmental events, each of these subtypes and some of their subunits need to be expressed at the right time. Changes in subunit composition drive their effects and are essential for proper brain development [10].

Among the iGluR, NMDA receptor activation regulates developmental programs during embryonic and perinatal periods. These receptors directly or indirectly influence cell proliferation, migration, cytoskeleton-related protein synthesis, and the formation and establishment of synapses [5–7, 10–12]. Most importantly, it was reported that blockage of NMDA receptors during brain development triggers neuronal cell death [13], further supporting the notion that in addition to support neuronal development and the development of neuronal circuits, they are important for neuronal survival. NMDA receptor activity is highly enhanced during perinatal life to promote activity-dependent synaptic connections. Immature NMDA receptor-gated channels open easily resulting in more calcium influx than in the adult brain [4]. Therefore, any event or stimulus that interferes with this “normal” level of synaptic activity will disturb brain development and may lead to life-long disabilities. For example, abnormal stimulation of glutamatergic receptors or excessive levels of glutamate can

lead to excitotoxicity and subsequent perinatal brain injury [4]. Thus, it is extremely important to understand which target genes are involved in NMDA actions as well as which factors may modulate NMDA-mediated “good” or “bad” effects [14]. In addition, knowledge on NMDA-induced gene regulation could help developing strategies for alleviating neurodevelopmental deficiencies.

In the adult brain [9, 15] NMDA receptors have been implicated in activation of signalling pathways leading to synaptic plasticity, process outgrowth and strengthening of pre-existing connections. Their activation regulates early and late phases of synaptic formation and consolidation. These cellular programs involve activation of transcription factors and immediate early genes (IEGs), up-regulation of gene expression and de novo protein synthesis [16–21]. The role of NMDA receptors on gene expression mediated by induction of IEGs and transcription factors has been extensively studied in the hippocampus [22–25], and after injury or stress [26–28] both in vivo and in vitro. However, a detailed analysis of the effects of NMDA receptor activation on regulation of gene expression in embryonic cortical neurons in the complete absence of glial cells is still lacking. Furthermore, little is known about the effects that a massive NMDA receptor activation can have on young neurons [29].

We have previously shown that massive activation of NMDA receptors in primary neuronal cultures from rat embryonic cerebral cortex elicited changes in the expression of IEGs and transcription factors such as: *cfos*, *NGFI-B*, *Krox-24*. These effects were accompanied by changes in *cfos* protein levels and sustained increase in intracellular calcium. NMDA even at the very high dose of 1 mM did not trigger apoptosis, which appeared to be mediated through AMPA receptors [30].

In the present report by using the same culture system [30] in which neuronal responses can be measured in the absence of glial cells, a cell composition resembling an immature brain, we show evidence for a link between NMDA receptor activation and gene regulation mediated, at least in part, through sustained induction of cAMP responsive element-binding (CREB) phosphorylation. Strong evidence of the involvement of the transcription factor CREB as mediator/effector of NMDA receptor action on gene expression are still lacking. Data presented in the literature remain controversial (for review: Platenik et al. 2000, [31]). We addressed the role of NMDA receptors in the program of cortical neuronal maturation, using our serum-free neuronal specific culture medium and gene array analysis. Our

work shows that the use of the combination DNA microarray-Real-time RT-PCR is a reliable and quantitative tool to assess gene regulation in cortical neurons in vitro.

## Experimental procedure

### Materials

NMDA, creatine, poly-D-lysine were purchased from Sigma, Saint Louis, MO, USA. Human recombinant bFGF, B27 supplement and DMEM-F12 were from Invitrogen Life Technologies, Carlsbad, CA, USA. The antibody directed against phosphorylated Ser133 CREB (pCREB; rabbit polyclonal) was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Anti-MAP2 (mouse monoclonal) and anti-synaptophysin (mouse monoclonal) were from Sigma (Saint Louis, MO, USA). Anti-GFAP (mouse monoclonal and rabbit polyclonal) was from Neomarkers (Fremont, CA, USA). All secondary Affinipure fluorochrome-conjugated antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA).

### Primary rat neuronal cortical culture

Cultures were prepared as previously described [30, 32] with minor modifications. E16 Sprague-Dawley rats (Charles-River, Wilmington, MA, USA) were killed following the National Institutes of Health and UCLA animal welfare guidelines. Cerebral cortices were removed, combined in DMEM-F12 containing 10% pure fetal calf serum (Atlanta Biologicals, Norcross, GA, USA) and mechanically dissociated for 2 min using a Stomacher 80 (Seward, London, UK). The dissociated cells were filtered in turn through 140  $\mu\text{m}$  and 230  $\mu\text{m}$  sieves (Collector, E-C Apparatus Corp., Holbrook, NY, USA) to remove large clusters. Sieves were washed sequentially with DMEM-F12 containing 10% pure fetal calf serum. Cells were collected by centrifugation, and cell pellet was resuspended in a neuronal specific medium [32], TII, supplemented with bFGF (10 ng ml<sup>-1</sup>), B27 (1:50), and creatine (2 mg ml<sup>-1</sup>). The cells were plated onto freshly poly-D-lysine coated dishes and cultured for 10–11 days unless differently stated. Purity of the cultures was assessed by double immunostaining with neuronal, astrocytic, oligodendrocyte and microglial markers. The cultures were 98% immunopositive for MAP2 and the remaining immunostained for astrocytic or oligodendrocyte markers. No microglial cells were detected.

### DNA array analysis

Total RNA was extracted from primary neuronal cortical cultures grown on 100 mm dishes ( $1.5 \times 10^6$  cells ml<sup>-1</sup>) for 11 days by using TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA), cleaned with RNeasy (Qiagen, Valencia, CA, USA), and its quality was monitored by micro-capillary electrophoresis (Bioanalyzer 2100, Agilent Technologies). Hybridisation and scanning of the Genechip Rat Neurobiology U34 oligonucleotide arrays (Affymetrix Inc., Santa Clara, CA, USA) was performed at the UCLA Microarray Core Facility following protocols recommended by the manufacturer. Expression values, representative of the amount of transcripts in solution, were calculated using Robust Microarray Analysis (RMA) [33]. Hybridisation experiments were repeated either 3 or 5 times using independent RNA samples. Data represent the average of all independent hybridisations in each experimental condition. Before further analysis, we selected the genes whose expression was more than 2-fold or less than 0.5-fold when compared with the control. After this selection, a *t* statistic was used to determine which genes were differentially expressed. Differences in expression were considered significant when  $p < 0.05$  for Student's *t* test. Final list of interesting genes was obtained by filtering all genes that were significantly, differentially expressed in at least one condition. The software GENECLUSTER was used to perform the mathematical calculations and to obtain convenient data visualisation [34].

### Real-Time RT-PCR

Total RNA (600 ng  $\mu\text{l}^{-1}$ ) isolated as described above was reverse transcribed using the RETROscript kit from Ambion (Austin, TX, USA), and then analysed for various transcript expressions (see Table 1) by Real-time RT-PCR on an iCycler iQ Detection System (BioRad, Hercules, CA, USA). To design Real-time RT-PCR-grade primers, cDNA encoding was first analysed for secondary structures using M-fold software [35, 36]. Portions of sequence lacking secondary structure were imported into Oligo6 software (Molecular Biology Insights) to design highly stringent primer sets (Listed in Table 1). PCR amplification resulted in the generation of single bands of sizes between 80 and 100 bp (see Table 1). To standardise the experiments we designed, using the same approach, a primer set for the rat  $\beta_2$ -microglobulin gene. Amplified bands for all the genes were cloned into PCR II using the TOPO cloning kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) and sequenced to confirm identity. Real-time RT-PCR

**Table 1** Oligonucleotide primers used for Real-time PCR

| Gene (accession #)                  | 5' primer              | 3' primer                 | Amplicon    |           | Annealing temperature (°C) |
|-------------------------------------|------------------------|---------------------------|-------------|-----------|----------------------------|
|                                     |                        |                           | Length (bp) | Position  |                            |
| NR2A (NM_012573)                    | ATACGGCAGAAGTCCACAC    | CAGGCATCACACTTGAAAAG      | 99          | 3707–3805 | 62                         |
| NR2B (NM_012574)                    | GCGCTACTTCAGGGACAAG    | AAG TCC ACG TGC TCC CAG T | 93          | 3710–3802 | 62                         |
| BDNF (NM_012513)                    | TAAAAGACTGCAGTGGACA    | CATGGATTACACTTGGTCT       | 112         | 711–822   | 60                         |
| c-fos (X06769)                      | GAGTGGTGAAGACCAATGTCA  | TCCTCTCAGGAGATAGCTGC      | 72          | 463–544   | 65                         |
| NARP (S82649)                       | TAAAGTCTGTGAGCCTCTCC   | CACACGAGACACTAAGGA        | 89          | 2660–1748 | 60                         |
| NOR-1 (NM_031628)                   | AGCCTTTTGGAGCTGTTTCTG  | CTGAAGTCGGTGCAGGACAAG     | 100         | 2121–2220 | 65                         |
| NGFI-B (U17254)                     | GCCACCTCCAACTTCTTCTC   | CTGGGAAACAATTCAGGGAAC     | 91          | 659–741   | 65                         |
| VGF (M60525)                        | CCCCATAAACACATCTTGGTG  | GGAAACCGCCAGGAATGAGCC     | 113         | 1004–1116 | 65                         |
| CBP/p300 (NM_053698)                | GCCCAATGTCATAGACATGATT | CCTTGATCGGTCCTCAAC        | 103         | 878–960   | 60                         |
| Amia-3 (XM_31707)                   | ACCAGCAAGCATGACGTTACT  | ATTTAATATTGCCCTTGGAGCC    | 96          | 373–468   | 60                         |
| NF-L (NM_031783)                    | CAGCTGGAGAACCTCGATCT   | AAGCGATCGTTGAGGTCCTGC     | 104         | 269–373   | 65                         |
| $\beta$ 2-microglobulin (NM_012512) | CAACTGCTACGTGCTCAG     | TTTGGTATCTCTTTTCCAAT      | 75          | 136–210   | 62                         |

was set up using the iQ SYBRGreen supermix (Bio-Rad, Hercules, CA, USA), for 60 cycles of a three-step procedure including a 30 s denaturation at 95°C, a 30 s annealing at temperatures between 60° and 65°C (see Table 1), followed by a 30 s extension at 72°C. Amplification specificity was assessed by melting curve analysis. Quantification utilised standard curves made from serial dilutions of control RNA sample. Differences between samples were calculated as percentage of control for the specific ratio (*gene*/ $\beta$ 2-microglobulin) calculated for each individual sample. PCR quantification was performed in triplicate. Statistical analysis was performed using GraphPad Prism 4.01 (GraphPad Software, Inc., San Diego, CA, USA).

### Immunocytochemistry

Neuronal cultures were plated onto glass coverslips (125000 cells ml<sup>-1</sup>) coated with Poly-D-Lysine and cultured for 11 days. The cells were fixed in 4% paraformaldehyde in PBS for 15 min; permeabilised for 10 min in 0.2% Triton X-100 and blocked in 10% normal goat serum for 15 min; all these steps were performed at room temperature. Primary antibodies were diluted in PBS + 1.5% goat serum and incubated overnight at 4°C. The coverslips were washed thoroughly in PBS and incubated for 30 min at room temperature with the secondary antibodies. After extensive washes, the coverslips were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA). Pictures were taken using an Olympus fluorescent microscope with a Zeiss digital camera or a Zeiss confocal microscope (LSM 510 Meta systems).

### Western blotting analysis

Following NMDA (1 mM)-treatment (see Figure legends for details), 11-day-old primary neuronal cortical cultures were washed and harvested in PBS. Western Blots were performed as previously reported [37, 38]. Equal protein loading was verified by Ponceau S solution reversible staining of the blots. Relative intensities of the protein bands were quantified by scanning densitometry using the public domain NIH Image program (NIH image, ImageJ, <http://rsb.info.nih.gov/ij/>). Statistical analysis was performed using GraphPad Prism 4.01 (GraphPad Software, Inc., San Diego, CA, USA).

### Calcium imaging

Fluorescence imaging was performed using a custom-built video-rate confocal microscope, as previously

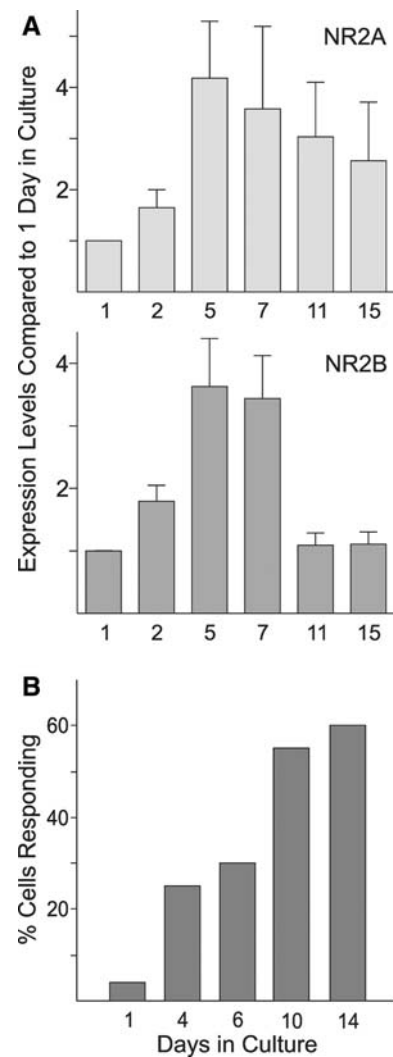
described [30]. In brief, cells on coverslips were loaded with the  $\text{Ca}^{2+}$  indicator fluo-4 by incubation in 3  $\mu\text{M}$  fluo-4 AM for 20–30 min. Cells were excited with a 475-nm diode laser and imaged with 20 $\times$  or 40 $\times$  water immersion objectives. The resulting fluorescence was bandpass filtered at 510 nm and detected by a photomultiplier tube (Hamamatsu). Excitation and emission were scanned with two oscillating mirrors (GSI Lumonics). Images were captured with a Raven board (Bit Flow, Inc.) using Video Savant software. Maximum image resolution was 980 $\times$ 730 at 15 frames per second. Analysis of imaging data was performed using ImageJ software. Cells were considered to have a response to NMDA if they showed either a >20% increase in fluorescence over background, or at least a 50% increase in the frequency of spontaneous  $\text{Ca}^{2+}$  transients in response to bath application of NMDA.

## Results

### Primary cortical neurons express NMDA receptor subunits

In the present work, we used primary cortical neuronal cultures from 16-day rat embryos. RNA extracted from primary cortical neurons at different days in culture was analysed by Real-time RT-PCR for the expression of subunits for NMDA receptors. We focus our attention on receptor subunits known to be highly represented in the cerebral cortex, and to be present since early development [8, 10].

Primary neuronal cortical cultures at 11 days in culture expressed the NMDA receptor subunit NR1 required for the formation of functional NMDA receptors (data not shown). Furthermore, since NR2A and NR2B subunit expressions are developmentally regulated and a switch in the levels of these subunits is accompanied by changes in the properties of the cationic channel coupled to NMDA receptors, we analysed the expression of these subunits by Real-time RT-PCR. Primary neuronal cultures were shown to express transcripts for both NR2A and NR2B subunits (Fig. 1A). Their levels were detectable at 24 h after plating and both subunits showed a peak at 5 days in culture. Then the expression levels of NR2A slowly decreased (Fig. 1A, upper panel), returning to levels similar to 1 day in vitro after more than 30 days in vitro (data not shown). On the other hand, NR2B expression was high between 5 and 7 days in vitro, dropping by 11 days to its level at 1 day (Fig. 1A, lower panel). Although we found some quantitative differences



**Fig. 1** Panel A: Cortical neurons in cultures express NMDA receptor subunits. Cortical neurons from E16 embryos were harvested in Trizol after different days in culture. The total RNA extracted was used as template for the RT-Reaction. The relative levels of expression of the genes of interest were normalised versus  $\beta_2$ -microglobulin and then compared to the levels at 1 day in culture. The plots are the average  $\pm$  SEM of 3–5 independent experiments performed in triplicate. Panel B:  $\text{Ca}^{2+}$  response to NMDA at different days after culture. Graph shows the percentage of cells that responded to NMDA with an increase in  $[\text{Ca}^{2+}]_i$  ( $n = 150$  total cells on 3 different coverslips for each time point). Relatively few neurons showed an increase in  $[\text{Ca}^{2+}]_i$  in response to NMDA at 1 day in culture, but this number increased progressively until day 10

between the levels of expression of NR2A and NR2B, they were both expressed at all the time points.

Consistent with the receptor subunit expression, only a small percentage of cells responded to NMDA (10  $\mu\text{M}$ ) with an increase in intracellular  $\text{Ca}^{2+}$  at day 1. The  $\text{Ca}^{2+}$  response to NMDA increased with culture age up to 10 days, at which time it remained relatively

constant through 14 days in culture (Fig. 1B). As previously reported [30], lower concentrations of NMDA (1 or 5  $\mu$ M) resulted in an increase in the frequency of spontaneous  $\text{Ca}^{2+}$  transients, whereas higher concentrations of NMDA (>10  $\mu$ M) evoked a sustained increase in  $[\text{Ca}^{2+}]_i$  in all responding cells.

#### NMDA induces time-dependent changes in gene expression in cortical neurons

Our neuronal culture system resembles the immature brain before glial development occurs. This model has allowed us to investigate glutamatergic and nicotinic actions on primary neurons, without inducing a response in other types of cells, such as oligodendrocytes and astrocytes that are known to express functional neurotransmitter receptors. Using this model, we previously reported [30] that treatment of cortical neurons with 1 mM NMDA for increasing periods of time (10–360 min) triggered changes in IEGs or transcription factors involved in the control of gene expression without inducing cell death, which appeared to be selectively elicited by activation of the glutamatergic receptor subtype AMPA.

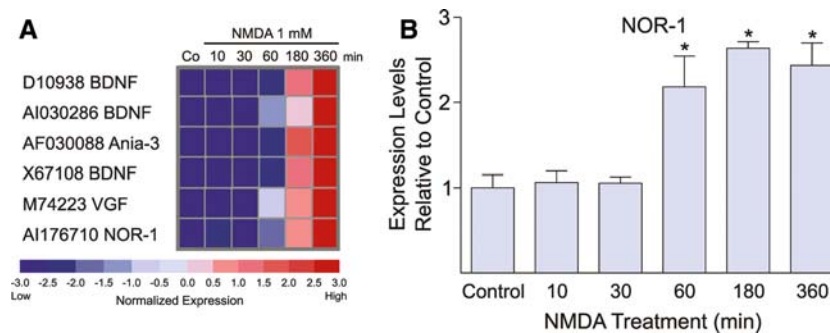
As shown in Fig. 2A, the longest treatment (360 min), in addition to the synaptic activity-regulated IEG *Ania-3* (4.5-fold over control-untreated cortical neurons), and the immediate early or stress response gene *NOR-1* (3.0-fold) triggered an increase in expression of genes known to have a role in synaptic modification, such as *BDNF* (6.4, 4.6 and 4.0-fold for three different probe sets) and *VGF* (3.0-fold). The

changes in gene expression for *NOR-1* were then examined and confirmed by Real-time RT-PCR (Fig. 2B). Changes in its expression resembled the pattern obtained by microarray analysis. However, the magnitude of the changes and the greater sensitivity of Real-time RT-PCR enabled us to detect small changes that had not been observed by microarrays (e.g. increase at 60 min).

#### Brief activation of NMDA receptors induces delayed changes in gene expression

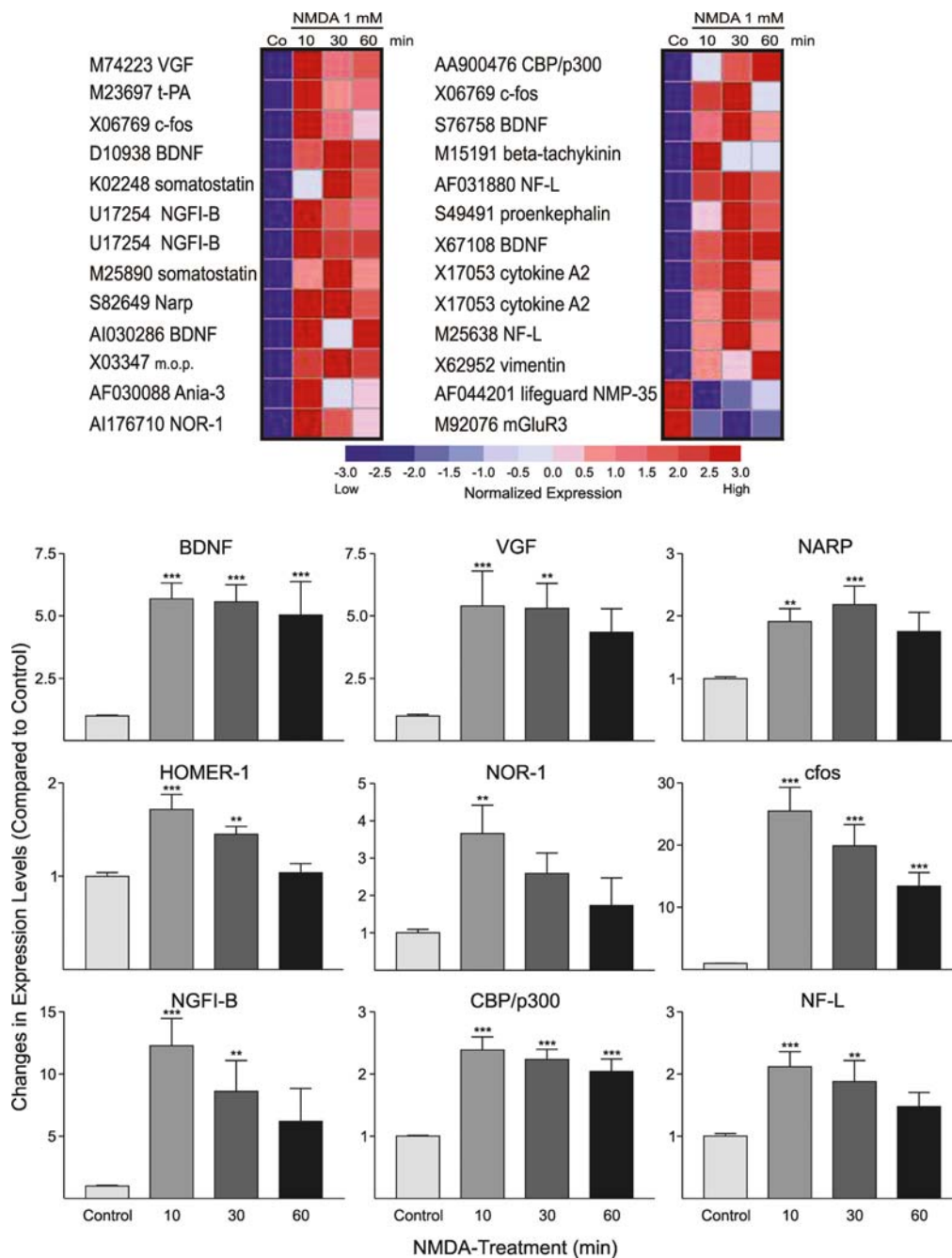
Since short treatments with NMDA (1 mM) triggered activation of IEGs and transcription factors [30], it was important to investigate any possible downstream effect that activation of these IEGs and transcription factors could have on other genes.

Hence, gene expression was analysed by DNA microarrays in neurons treated with NMDA (1 mM) for 10, 30 and 60 min, and harvested 24 h after NMDA-treatment. As shown in Fig. 3, upper panels, all the treatments had a great effect on gene expression in primary neuronal cultures. In particular, a 10 min-treatment with NMDA that did not have immediate effects on gene expression [30], did affect several genes 24 h later (Fig. 3, upper panels). Most of the genes affected by a 10 min treatment with NMDA, were also significantly (>2-fold over control-untreated cortical neurons;  $p < 0.05$ ) induced in the cultures treated for either 30 or 60 min (Fig. 3, upper panels). CREB binding protein (CBP)/p300 expression was influenced by all the treatments (2.7, 3.5 and 4.1-fold,



**Fig. 2** Time Course of the effects of NMDA (1 mM) on gene expression. Cortical neurons from E16 embryos were cultured for 10 days. NMDA at 1 mM final concentration was added to the cultures for 10, 30, 60, 180 and 360 min. The cells were harvested in Trizol after each treatment. Control cultures received an equal amount of vehicle (Water). Rat U34 Neurobiology Microarrays from Affymetrix were used to analyze differential gene expression. Panel A: List of the genes differentially expressed. The relative colour scheme shown represents how expression values move apart from the average value in terms of standard deviations relative to the mean. Blue

tones represent lower expression values and red tones represent the higher values. Student's *t* Test  $p < 0.05$ . Panel B: Real-time RT-PCR validates the changes in gene expression seen by microarrays. The total RNA extracted was used as template for the RT-Reaction. The relative levels of expression of the genes of interest were normalised versus  $\beta_2$ -microglobulin and then compared to expression levels in control. Plot represents the average  $\pm$  SEM of three independent experiments run in triplicate. Student's *t* test  $*p < 0.05$ . NOTE: For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article



**Fig. 3** A brief treatment with NMDA 1 mM triggers delayed changes in genes activated by synaptic activity and in transcription factors. Upper panels: List of the genes differentially expressed. Cortical neurons from E16 embryos were cultured for 10 days. NMDA 1 mM was added to the cultures for 10, 30 or 60 min; the drug was washed out by replacing the medium in all the plates. The control cultures received an equal amount of vehicle (Water). The cells were harvested in Trizol 24 h after fresh medium was added to the cultures. The Rat U34 Neurobiology Microarrays from Affymetrix were used to investigate gene expression. The relative colour scheme shown represents how expression values move apart from the average value in terms of standard deviations relative to the mean. Blue

tones represent lower expression values and red tones represent the higher values. Student's *t* test  $p < 0.05$ . Lower panel: Real-time RT-PCR validates the changes in gene expression seen by microarrays. The total RNA extracted was used as template for the RT-Reaction. The relative levels of expression of the genes of interest were normalised versus  $\beta_2$ -microglobulin and then compared to expression levels in control. All the transcripts were upregulated as expected from the microarray results. Plots represent the average  $\pm$  SEM of three independent experiments run in triplicate. Student's *t* test  $***p < 0.001$  and  $**p < 0.05$ . NOTE: For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article

respectively). The neuropeptide somatostatin and neuropeptide precursor proenkephalin showed the maximum increase (4.8 and 2.6-fold, respectively), using a 30 min NMDA receptor activation, while  $\beta$ -tachykinin (2.1-fold) was significantly upregulated by 10-min treatment. In addition, we found changes in genes, such as NARP (3.0-fold) that may play a role in synaptic remodelling and formation of new synapses, the light molecular-weight neurofilament (NF-L) (2.2-fold) and vimentin (2.0-fold) (Fig. 3, upper panels). Moreover, both 10 and 30 min treatments elicited a long-lasting increase in the expression of BDNF, ranging between 2.1 and 3.9-fold (Fig. 3, upper panels). VGF, whose product is thought to play a role in hippocampal plasticity and its regulation has recently been linked to BDNF [39], was the gene most upregulated by 10 min treatments (4.4-fold; 3.7 and 3.9-fold after 30 and 60 min treatment, respectively).

We found upregulation in the expression of IEGs and transcription factors. The IEG t-PA, reported to be induced during LTP, seizures and kindling [23] was increased 4.0-fold by a 10 min NMDA-treatment. Likewise, NGFI-B and c-fos (3.2 and 3.9-fold, respectively) were significantly upregulated in the samples that had been treated for 10 min with NMDA together with NOR-1 and ania-3 (both 2.7-fold over control-untreated cortical neurons, Fig. 3, upper panels). Ania-3 expression was not significantly affected by NMDA activation for 30 and 60 min (1.8 and 1.9-fold over control), whereas upregulation of other IEGs/transcription factors was sustained (c-fos: 3.3 and 2.9-fold, respectively for the other two treatments; NGFI-B: 2.8-fold at both times; and NOR-1: 2.4 and 2.1-fold). The murine osteosarcoma provirus (m.o.p.) expression (3.0-fold) was higher in the samples treated for longer time (30 min) together with another probe set for c-fos (2.9-fold).

Only two genes were significantly downregulated (<0.5-fold; Fig. 3, upper left panel) neural membrane protein 35 (NMP35) or Lifeguard and the metabotropic glutamate receptor 3 (mGluR3). The different probes for the same gene in the microarrays showed similar pattern (Fig. 3, upper panels).

Nine genes were then, selected for verification by Real-time RT-PCR (Fig. 3, bottom panel). We found a complete agreement between the changes in gene expression found with DNA arrays and Real-time RT-PCR quantification.

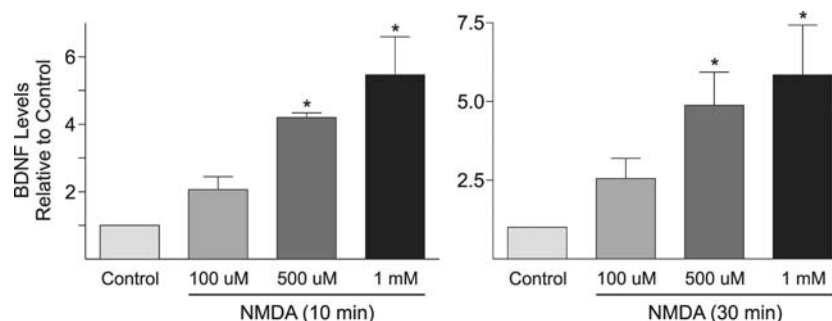
#### NMDA-induced changes in gene expression are concentration-dependent

To exclude that the changes in gene expression could be due to activation of other glutamate receptor subtypes by a very high concentration of NMDA and to demonstrate that NMDA receptors were active also at lower concentrations and there was no need to overcome a blockage of these receptors due to the magnesium present in the medium, we measured the effect of increasing concentrations of NMDA on BDNF gene expression by Real-time RT-PCR, 24 h after treatment.

As shown in Fig. 4, all the NMDA concentrations (100  $\mu$ M, 500  $\mu$ M and 1 mM) tested increased BDNF levels compared to untreated cortical neurons (control), and the increase was concentration-dependent. In addition, the increase in BDNF expression induced by the increasing concentration of NMDA was not influenced by the length of the treatment (10 or 30 min).

#### NMDA-induced CREB phosphorylation is long-lasting

#### NMDA receptor stimulation activates pathways leading to phosphorylation and activation of the transcription



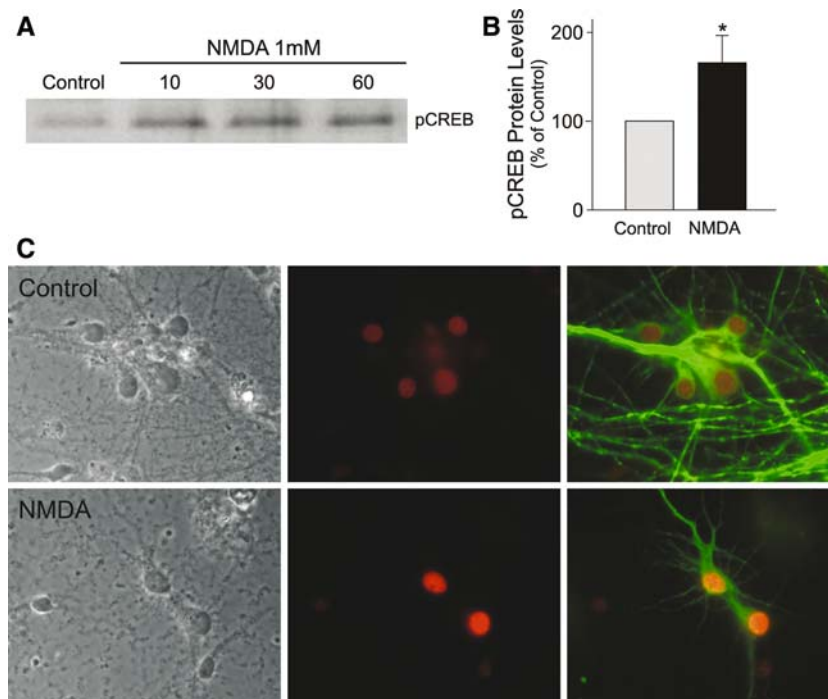
**Fig. 4** NMDA-induced changes in gene expression are concentration-dependent. Cortical neurons were treated with increasing concentration of NMDA for 10, 30 and 60 min, the medium was then changed to remove the agonist and the cells were harvested in Trizol 24 h later. The Total RNA extracted was used as template

for the RT-Reaction. The relative levels of expression of BDNF were normalised versus  $\beta_2$ -microglobulin and then compared to expression levels in control. Plots represent the average  $\pm$  SEM of three independent experiments run in triplicate. ANOVA followed by Dunnett's Multiple Comparison Test \* $p < 0.05$

factor CREB and consequently triggers changes in the expression of genes involved in neuronal growth and plasticity (for example, BDNF, c-fos etc.) [40, 41]. Therefore, we analysed the levels of pCREB 24 h after a brief treatment with NMDA (1 mM). As shown in Fig. 5A, treatment with NMDA (1 mM) for 10, 30 and 60 min elicited an increase in CREB phosphorylation that was still detectable 24 h later. The NMDA-induced increase in pCREB was about 66% over control 24 h after a 30 min NMDA-treatment (Fig. 5B). This effect was further analysed by immunocytochemistry, and in agreement with the western blot results, we found that pCREB immunoreactivity was increased in the nuclei of cortical neurons 24 h after 30 min treatment with NMDA (1 mM) (Fig. 5C). Hence, the same short NMDA-treatment that induced long lasting changes in gene expressions (Fig. 3), also triggered a sustained increase in CREB phosphorylation required for NMDA receptor activation to elicit changes in gene expression [31].

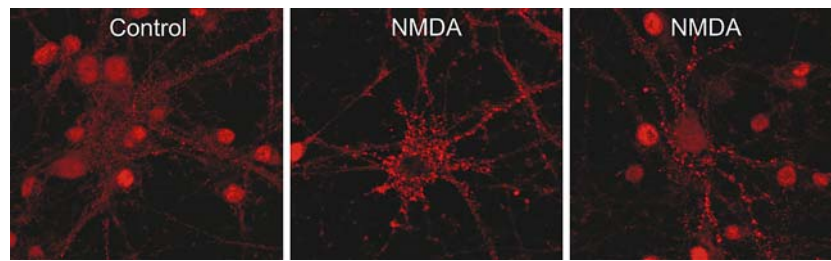
Change in gene expression is accompanied by an increase in synaptophysin immunoreactivity

To further investigate whether the alterations seen in gene expression were accompanied by plastic changes in vitro, as previously reported in vivo, we analysed the expression of markers indicative of modifications at the synapse following development and maturation. For instance, synaptophysin expression has been reported to increase in parallel with synapse formation [42, 43]. As expected, enhanced synaptophysin immunoreactivity was seen along cell processes in 10-day-old primary neurons 24 h after treatment with 1 mM NMDA for 30 min (Fig. 6). Increased expression was more evident where the processes were making contacts (Fig. 6). We speculate that NMDA receptor activation triggers the formation of new connections and changes in synaptic strength in young primary cortical neurons in vitro.



**Fig. 5** Changes in gene expression are accompanied by sustained phosphorylation of CREB. Cortical neurons from E16 embryos were cultured for 10 days on either glass coverslips or 60 mm dishes. Panel A: pCREB immunoblotting. The cortical neurons were treated with 1 mM NMDA for 10, 30 and 60 min, the medium was then changed to remove the agonist and the cells were harvested in PBS 24 h later. Control cultures received an equal amount of vehicle (Water). Changes in CREB phosphorylation were analysed with anti-pCREB antibody that specifically recognises the phosphorylated form of CREB. Forty micrograms of total proteins were loaded on the gel for each

sample. Panel B: Quantification of the effect of 30 min NMDA treatment on CREB phosphorylation. Plot represents the average  $\pm$  SEM of eight independent experiments. Paired Student's *t* test  $*p < 0.05$ . Panel C: pCREB immunostaining. NMDA (1 mM) was added to the cultures for 30 min, the medium was then changed to remove the agonist and the cells were fixed 24 h later in 4% paraformaldehyde. Fixed cells were stained for pCREB (in red) and MAP2 (in green). NOTE: For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article



**Fig. 6** Activation of NMDA receptors increases synaptophysin immunoreactivity in primary neuronal culture. Cortical neurons from E16 embryos grown for 10 days on glass coverslips were treated with 1 mM NMDA for 30 min; the medium was then changed to remove the agonist. Twenty-four hours later the cells were fixed in 4% paraformaldehyde and stained with an

antibody against synaptophysin. Several confocal images of different fields from three independent experiments were taken with a 100× objective on a Zeiss 510 Meta confocal microscope. NOTE: For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article

## Discussion

The question of how neural progenitors and immature neurons respond to the excess of glutamate released after an insult remains unresolved. In the present study, we focused on the role of NMDA receptors in the program of cortical development, using our serum-free neuronal specific culture medium and gene array analysis. An advantage of our culture system was to be able to study the effect of NMDA receptor activation in the absence of other neuromodulators released from surrounding cells, principally microglia and astrocytes, which can contribute to the excitotoxicity of glutamate and NMDA.

In an attempt to study the response(s) of young and still developing neurons to a major insult, we used a high concentration of NMDA (1 mM). Interestingly, this treatment did not result in massive cell damage followed by apoptosis [30]. Hence, we excluded a role for NMDA receptor activation in cell death and predicted that a considerable activation of NMDA glutamatergic receptors could be, on the other hand, beneficial for the developing neurons. Our contention is supported by previous studies in which blockage of NMDA receptors during development triggered increased neuronal cell death [13].

Cortical neural cells responded to NMDA receptor activation with an increase in mRNA for genes (BDNF, VGF, NARP, and IEGs such as Ania-3) known to be involved in synaptogenesis. We observed that the majority of these genes were upregulated 24 h after a brief (10 min) activation of NMDA receptors, indicating that a short event was enough to activate intracellular signal transduction pathways leading to gene transcription. Some of the IEGs and transcription factors (c-fos, NGFI-B) that we had previously reported as being significantly changed soon after 30–60 min treatment with NMDA [30], were still upregulated 24 h

after a 10 min-treatment with NMDA. In addition to NGFI-B, NMDA treatment altered the expression of another member of the nuclear orphan receptor family, NOR-1, known to be activated by stress or neurotransmitters and to play a role in CNS function and perhaps in development [44–47]. NOR-1 null mice display developmental deficiency due to abnormal axonal guidance and impaired hippocampal development [48]. NMDA treatment also increased the transcripts of the short inducible Homer family member, Ania-3, an IEG rapidly induced in response to different stimuli such as long-term potentiation, synaptic activity [49–52], traumatic brain injury [53] or drug of abuse (morphine, cocaine) [54–57]. Ania-3 expression is developmentally regulated by natural synaptic activity, and it plays a role in synaptogenesis [50, 51]. This IEG is involved in targeting of mGluRs to the plasma membrane, and binds to group 1 mGluRs [58]. Homer proteins have been implicated in the pathology of neuropsychiatric disorders, such as epilepsy, schizophrenia, depression and addiction [59].

Collectively, our results show that treatment of young neuronal cultures with a high concentration of NMDA for short periods leads to plastic changes in neurons as a consequence of upregulation of IEGs and transcription factors. They will, in turn, induce changes in downstream genes known to participate in synapse organisation and formation. It is important to consider that since IEGs are still upregulated 24 h after NMDA-treatment, they might be activated in successive waves to trigger activation of “late-response” genes. Furthermore, since (i) IEGs are the most expressed genes during brain development [17, 60]; (ii) drugs of addictions, such as morphine, not only induce activation of transcription factors and IEGs, but also alter neurotransmitter receptor expression [54, 56, 61]; and (iii) all these changes could be long-lasting, then, they will certainly affect negatively a developing

cerebral cortex. Hence, improper activation of IEGs or transcription factors may contribute to neurodevelopmental disorders following maternal exposure to neurotoxic drugs.

Activation of glutamatergic synapses *in vivo* induces rapid changes in gene expression subsequent to the activation of signaling cascades, which rapidly couples the signal of NMDA receptor activation at the synapse to the nucleus. Synaptic activity regulates gene expression through different pathways, and regulation of several genes is dependent on neuronal activity. Most of the activity-regulated genes encode for neurotrophic factors or transcription factors, which control expression of “late-response” genes [17, 21]. For example, BDNF mRNA is highly regulated by synaptic activity and the levels of neurotrophins are extremely important for cell survival during development [62].

Neurotrophin signalling is important during development for the formation and maturation of functional synapses. BDNF is rapidly upregulated by neuronal activity, and its levels are high during synaptic remodelling. Its involvement in LTP and in mediating synaptic strength and plasticity is well documented. Knockout mice for BDNF or its receptors TrkB/TrkC show reduced expression of synaptophysin, a protein associated with synaptic vesicles, reduced synaptic density, impaired LTP and deficits in presynaptic functions. These defects can be rescued by addition of exogenous BDNF. Conditional mutation in TrkB receptors have resulted in memory deficits [62–64].

Recently, VGF gene expression regulation has been linked to BDNF [39]. Alder et al. [39] showed an upregulation of both VGF proteins and gene expression in hippocampal cultures treated with BDNF for 3 h. VGF is a polypeptide secreted during neuronal differentiation in cultures [65, 66], whose expression is regulated by neuronal activity, seizures and lesions [67]. It was also reported that its mRNA colocalises with mRNA for TrkB receptors. Consequently, VGF may regulate release of other peptides [68] and may contribute to synaptic function by regulating release of BDNF and of other neuromodulators. Therefore, it is instructive to highlight the succession of the events observed in our study: activation of NMDA receptors for 180 min significantly upregulated BDNF (3.9-fold, Fig. 2A), this effect is followed by an increase in VGF expression (Fig. 2A). Both genes were still greatly expressed 24 h after a brief (10 min) activation of NMDA receptors (Fig. 3). Hence, we speculate that in cerebral cortex, BDNF may trigger changes in VGF gene expression, which in turn elicits synaptogenesis and synaptic function or simply potentiate BDNF effects. In line with this hypothesis, other genes that

may play a role in process formation and remodeling, such as NF-L and vimentin (Fig. 3), were also upregulated. Furthermore, we found upregulation of NARP (Fig. 3), an IEG rapidly induced by synaptic activity that promotes neurite outgrowth and plays a role in excitatory synaptogenesis [69]. Immunohistochemical studies have shown that NARP is expressed in hippocampus, cerebral cortex and habenula [70], and is selectively targeted to excitatory synapses, in tight association with the GluR1 subunit. Recently, it has been suggested that NARP participates in AMPA receptors clustering [71].

We also showed that NMDA receptor activation elicited a sustained increase in pCREB levels, suggesting a role for this transcription factor in mediating NMDA effects. The role of CREB in NMDA signalling is still controversial because of conflicting results reported by different groups [31]. Several lines of evidence indicate that activation of NMDA receptors leads to CREB phosphorylation and activates CREB-dependent transcription. This idea is further supported by the fact that many of the genes activated following NMDA receptor stimulation contain a CREB binding site in their promoter region [40, 72]. Since some of the genes differentially expressed in our study (for example, *c-fos*, somatostatin, BDNF, Figs. 2, 3) are known to have a CREB response element, and we detected sustained levels of pCREB (Fig. 5), necessary to elicit activity-dependent transcription, long after the NMDA receptor activation had ceased, we propose that CREB is one of the central mediator of NMDA receptor effects in primary neuronal cultures. In addition, the high levels of pCREB could in part explain the lack of NMDA-induced apoptosis. CREB and related members have been implicated in control of neuronal adaptive responses, in regulation of BDNF transcription [41] and most importantly, in preventing neuronal death and in promoting survival in both developing and adult brain [73, 74].

Since, the synaptic vesicle protein synaptophysin is considered a marker of “synaptic density” and, since during brain development, its expression parallels the formation of synapses [42, 43], the increase in synaptophysin immunoreactivity displayed by neuronal cultures after treatment with NMDA further support our conclusions. Interestingly, we did not find a significant change in synaptophysin gene expression in our studies using the gene array Rat U34 neurobiology (data not shown). This result is in agreement with a previous report [75] showing that increases in the levels of synaptophysin are regulated at the translational level.

In our model, direct activation by NMDA of the neuronal program of maturation/development occurred

at both transcriptional and post-transcriptional levels in the complete absence of astrocytes and oligodendrocytes, and therefore in the absence of glial-neuron interactions. However, by calcium imaging, we found that between 50% and 80% of the cells responded to NMDA with an increase in intracellular calcium (present paper and [30]). Thus, we could argue that the effects seen on gene expression, pCREB, cfos, and synaptophysin could be mediated by immediate and delayed responses of different types of progenitors and neuronal lineage cells present in the cultures to either glutamate or other neuromodulators (neuropeptides) released in the culture medium by the cells activated by NMDA.

In conclusion, we propose that (i) a massive activation of NMDA receptors due to high concentrations of NMDA may mimic the effects induced in vivo by repeated glutamate release following stimulation waves; (ii) like in the hippocampus, IEGs and transcription factors are key players in initiating cerebral cortical synaptic plasticity in response to NMDA; and (iii) our findings imply a key role for CREB in NMDA receptor actions, and they further support the contention for a connection between NMDA receptor, pCREB and plastic changes leading to proper synapses formation.

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## References

- Hagberg H, Mallard C (2000) Antenatal brain injury: aetiology and possibilities of prevention. *Semin Neonatol* 5(1):41–51
- Rees S, Inder T (2005) Fetal and neonatal origins of altered brain development. *Early Human Dev* 81(9):753–761
- Johnston MV (2004) Clinical disorders of brain plasticity. *Brain Dev* 26(2):73–80
- Johnston MV (2005) Excitotoxicity in perinatal brain injury. *Brain Pathol* 15(3):234–240
- Nguyen L, Rigo JM, Rocher V et al (2001) Neurotransmitters as early signals for central nervous system development. *Cell Tissue Res* 305(2):187–202
- Cameron HA, Hazel TG, McKay RD (1998) Regulation of neurogenesis by growth factors and neurotransmitters. *J Neurobiol* 36(2):287–306
- Herlenius E, Lagercrantz H (2004) Development of neurotransmitter systems during critical periods. *Exp Neurol* 190(Suppl 1):S8–S21
- Kew JN, Kemp JA (2005) Ionotropic and metabotropic glutamate receptor structure and pharmacology. *Psychopharmacology (Berl)*. 179(1):4–29
- Dingledine R, Borges K, Bowie D et al (1999) The glutamate receptor ion channels. *Pharmacol Rev* 51(1):7–61
- Lujan R, Shigemoto R, Lopez-Bendito G (2005) Glutamate and GABA receptor signalling in the developing brain. *Neuroscience* 130(3):567–580
- Komuro H, Rakic P (1998) Orchestration of neuronal migration by activity of ion channels, neurotransmitter receptors, and intracellular Ca<sup>2+</sup> fluctuations. *J Neurobiol* 37(1):110–130
- Gould E, Cameron HA (1997) Early NMDA receptor blockade impairs defensive behavior and increases cell proliferation in the dentate gyrus of developing rats. *Behav Neurosci*. 111(1):49–56
- Ikonomidou C, Bosch F, Miksa M et al (1999) Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. *Science* 283(5398):70–74
- Lipton SA, Nakanishi N (1999) Shakespeare in love—with NMDA receptors? *Nat Med* 5(3):270–271
- Sheng M, Kim MJ (2002) Postsynaptic signaling and plasticity mechanisms. *Science*. 298(5594):776–780
- Frey U, Morris RG (1997) Synaptic tagging and long-term potentiation. *Nature* 385(6616):533–536
- Kaufmann WE, Worley PF (1999) Neural activity and immediate early gene expression in the cerebral cortex. *Mental Retardation Dev Disabilities Res Rev* 5(1):41–50
- Nguyen PV, Kandel ER (1996) A macromolecular synthesis-dependent late phase of long-term potentiation requiring cAMP in the medial perforant pathway of rat hippocampal slices. *J Neurosci* 16(10):3189–3198
- Otani S, Abraham WC (1989) Inhibition of protein synthesis in the dentate gyrus, but not the entorhinal cortex, blocks maintenance of long-term potentiation in rats. *Neurosci Lett* 106(1–2):175–180
- Steward O, Schuman EM (2001) Protein synthesis at synaptic sites on dendrites. *Annu Rev Neurosci* 24:299–325
- West AE, Griffith EC, Greenberg ME (2002) Regulation of transcription factors by neuronal activity. *Nat Rev Neurosci* 3(12):921–931
- Nedivi E, Hevroni D, Naot D et al (1993) Numerous candidate plasticity-related genes revealed by differential cDNA cloning. *Nature* 363(6431):718–722
- Qian Z, Gilbert ME, Colicos MA et al (1993) Tissue-plasminogen activator is induced as an immediate-early gene during seizure, kindling and long-term potentiation. *Nature* 361(6411):453–457
- French PJ, O'Connor V, Voss K et al (2001) Seizure-induced gene expression in area CA1 of the mouse hippocampus. *Eur J Neurosci* 14(12):2037–2041
- Sala C, Rudolph-Correia S, Sheng M (2000) Developmentally regulated NMDA receptor-dependent dephosphorylation of cAMP response element-binding protein (CREB) in hippocampal neurons. *J Neurosci* 20(10):3529–3536
- Lanahan A, Worley P (1998) Immediate-early genes and synaptic function. *Neurobiol Learn Mem* 70(1–2):37–43
- Nesic O, Svrakic NM, Xu GY et al (2002) DNA microarray analysis of the contused spinal cord: effect of NMDA receptor inhibition. *J Neurosci Res* 68(4):406–423
- Ying G, Huang C, Jing N et al (2001) Identification of differentially expressed genes in the denervated rat hippocampus by cDNA arrays. *Neurosci Lett* 306(1–2):121–125

29. Sugiura N, Patel RG, Corriveau RA (2001) N-methyl-D-aspartate receptors regulate a group of transiently expressed genes in the developing brain. *J Biol Chem* 276(17):14257–14263
30. Ghiani CA, Lelievre V, Beltran-Parrazal L et al (2006) Gene expression is differentially regulated by neurotransmitters in embryonic neuronal cortical culture. *J Neurochem* 97(Suppl 1):35–43
31. Platenik J, Kuramoto N, Yoneda Y (2000) Molecular mechanisms associated with long-term consolidation of the NMDA signals. *Life Sci* 67(4):335–364
32. Espinosa-Jeffrey A, Becker-Catania SG, Zhao PM et al (2002) Selective specification of CNS stem cells into oligodendroglial or neuronal cell lineage: cell culture and transplant studies. *J Neurosci Res* 69(6):810–825
33. Irizarry RA, Hobbs B, Collin F et al (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* 4(2):249–264
34. Tamayo P, Slonim D, Mesirov J et al (1999) Interpreting patterns of gene expression with self-organizing maps: methods and application to hematopoietic differentiation. *Proc Natl Acad Sci USA* 96(6):2907–2912
35. Mathews DH, Sabina J, Zuker M et al (1999) Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J Mol Biol* 288(5):911–940
36. Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 31(13):3406–3415
37. Ghiani CA, Eisen AM, Yuan X et al (1999) Neurotransmitter receptor activation triggers p27(Kip1) and p21(CIP1) accumulation and G1 cell cycle arrest in oligodendrocyte progenitors. *Development* 126(5):1077–1090
38. Ghiani CA, Gallo V (2001) Inhibition of cyclin E-cyclin-dependent kinase 2 complex formation and activity is associated with cell cycle arrest and withdrawal in oligodendrocyte progenitor cells. *J Neurosci* 21(4):1274–1282
39. Alder J, Thakker-Varia S, Bangasser DA et al (2003) Brain-derived neurotrophic factor-induced gene expression reveals novel actions of VGF in hippocampal synaptic plasticity. *J Neurosci* 23(34):10800–10808
40. Ahn S, Riccio A, Ginty DD (2000) Spatial considerations for stimulus-dependent transcription in neurons. *Annu Rev Physiol* 62:803–823
41. Shaywitz AJ, Greenberg ME (1999) CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu Rev Biochem* 68:821–861
42. Biranowska J, Dziewiatkowski J, Ludkiewicz B et al (2002) Developmental changes of synaptic proteins expression within the hippocampal formation of the rat. *Anat Embryol (Berl)* 206(1–2):85–96
43. Knaus P, Betz H, Rehm H (1986) Expression of synaptophysin during postnatal development of the mouse brain. *J Neurochem* 47(4):1302–1304
44. Castro DS, Hermanson E, Joseph B et al (2001) Induction of cell cycle arrest and morphological differentiation by Nurr1 and retinoids in dopamine MN9D cells. *J Biol Chem* 276(46):43277–43284
45. Maxwell MA, Muscat GE (2006) The NR4A subgroup: immediate early response genes with pleiotropic physiological roles. *Nucl Recept Signal* 4:e002
46. Benoit G, Malewicz M, Perlmann T (2004) Digging deep into the pockets of orphan nuclear receptors: insights from structural studies. *Trends Cell Biol* 14(7):369–376
47. Zetterstrom RH, Solomin L, Mitsiadis T et al (1996) Retinoid X receptor heterodimerization and developmental expression distinguish the orphan nuclear receptors NGFI-B, Nurr1, and Nor1. *Mol Endocrinol* 10(12):1656–1666
48. Ponnio T, Conneely OM (2004) nor-1 regulates hippocampal axon guidance, pyramidal cell survival, and seizure susceptibility. *Mol Cell Biol* 24(20):9070–9078
49. Bottai D, Guzowski JF, Schwarz MK et al (2002) Synaptic activity-induced conversion of intronic to exonic sequence in Homer 1 immediate early gene expression. *J Neurosci* 22(1):167–175
50. Brakeman PR, Lanahan AA, O'Brien R et al (1997) Homer: a protein that selectively binds metabotropic glutamate receptors. *Nature* 386(6622):284–288
51. Kato A, Ozawa F, Saitoh Y et al (1997) vesl, a gene encoding VASP/Ena family related protein, is upregulated during seizure, long-term potentiation and synaptogenesis. *FEBS Lett* 412(1):183–189
52. Sato M, Suzuki K, Nakanishi S (2001) NMDA receptor stimulation and brain-derived neurotrophic factor upregulate homer 1a mRNA via the mitogen-activated protein kinase cascade in cultured cerebellar granule cells. *J Neurosci* 21(11):3797–3805
53. Raghavendra Rao VL, Dhodda VK, Song G et al (2003) Traumatic brain injury-induced acute gene expression changes in rat cerebral cortex identified by GeneChip analysis. *J Neurosci Res* 71(2):208–219
54. Ammon S, Mayer P, Riechert U et al (2003) Microarray analysis of genes expressed in the frontal cortex of rats chronically treated with morphine and after naloxone precipitated withdrawal. *Brain Res Mol Brain Res* 112(1–2):113–125
55. Koya E, Spijker S, Voorn P et al (2006) Enhanced cortical and accumbal molecular reactivity associated with conditioned heroin, but not sucrose-seeking behaviour. *J Neurochem* 98(3):905–915
56. de Bartolomeis A, Iasevoli F (2003) The Homer family and the signal transduction system at glutamatergic postsynaptic density: potential role in behavior and pharmacotherapy. *Psychopharmacol Bull* 37(3):51–83
57. Berke JD, Paletzki RF, Aronson GJ et al (1998) A complex program of striatal gene expression induced by dopaminergic stimulation. *J Neurosci* 18(14):5301–5310
58. Xiao B, Tu JC, Worley PF (2000) Homer: a link between neural activity and glutamate receptor function. *Curr Opin Neurobiol* 10(3):370–374
59. Szumlinski KK, Kalivas PW, Worley PF (2006) Homer proteins: implications for neuropsychiatric disorders. *Curr Opin Neurobiol* 16(3):251–257
60. Herdegen T, Leah JD (1998) Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos, Krox, and CREB/ATF proteins. *Brain Res Brain Res Rev* 28(3):370–490
61. Ammon-Treiber S, Holtt V (2005) Morphine-induced changes of gene expression in the brain. *Addict Biol* 10(1):81–89
62. Chao MV (2003) Neurotrophins and their receptors: a convergence point for many signalling pathways. *Nat Rev Neurosci* 4(4):299–309
63. Lee SH, Sheng M (2000) Development of neuron–neuron synapses. *Curr Opin Neurobiol* 10(1):125–131
64. Pozzo-Miller LD, Gottschalk W, Zhang L et al (1999) Impairments in high-frequency transmission, synaptic vesicle docking, and synaptic protein distribution in the hippocampus of BDNF knockout mice. *J Neurosci* 19(12):4972–4983

65. Benson DL, Salton SR (1996) Expression and polarization of VGF in developing hippocampal neurons. *Brain Res Dev Brain Res* 96(1–2):219–228
66. Trani E, Ciotti T, Rinaldi AM et al (1995) Tissue-specific processing of the neuroendocrine protein VGF. *J Neurochem* 65(6):2441–2449
67. Snyder SE, Cheng HW, Murray KD et al (1998) The messenger RNA encoding VGF, a neuronal peptide precursor, is rapidly regulated in the rat central nervous system by neuronal activity, seizure and lesion. *Neuroscience* 82(1):7–19
68. Salton SR, Ferri GL, Hahm S et al (2000) VGF: a novel role for this neuronal and neuroendocrine polypeptide in the regulation of energy balance. *Front Neuroendocrinol* 21(3):199–219
69. Tsui CC, Copeland NG, Gilbert DJ et al (1996) NARP, a novel member of the pentraxin family, promotes neurite outgrowth and is dynamically regulated by neuronal activity. *J Neurosci* 16(8):2463–2478
70. Reti IM, Reddy R, Worley PF et al (2002) Prominent NARP expression in projection pathways and terminal fields. *J Neurochem* 82(4):935–944
71. O'Brien RJ, Xu D, Petralia RS et al (1999) Synaptic clustering of AMPA receptors by the extracellular immediate-early gene product NARP. *Neuron* 23(2):309–323
72. Lonze BE, Ginty DD (2002) Function and regulation of CREB family transcription factors in the nervous system. *Neuron* 35(4):605–623
73. Dawson TM, Ginty DD (2002) CREB family transcription factors inhibit neuronal suicide. *Nat Med* 8(5):450–451
74. Mantamadiotis T, Lemberger T, Bleckmann SC et al (2002) Disruption of CREB function in brain leads to neurodegeneration. *Nat Genet* 31(1):47–54
75. Daly C, Ziff EB (1997) Post-transcriptional regulation of synaptic vesicle protein expression and the developmental control of synaptic vesicle formation. *J Neurosci* 17(7):2365–2375