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Expression and regulation of NMDA receptor subunit R1 and neuronal nitric oxide synthase in cortical neuronal cultures: Correlation with cytochrome oxidase

CHENYANG ZHANG and MARGARET WONG-RILEY*

Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, WI 53226, USA

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Summary

Our previous studies showed a differential distribution of the glutamatergic terminals in cytochrome oxidase-rich and -poor regions of the visual cortex. The NMDA type of glutamate receptors have been proposed to be involved in the activation of nitric oxide synthase to produce nitric oxide, the neurotransmitter. In the present study, we hypothesized that the expressions of glutamate receptor, NMDA receptors (NMDAR1) and neuronal nitric oxide synthase (nNOS) were colocalized and were also correlated with that of cytochrome oxidase (CO) in a subset of neurons. We used primary cultures of postnatal rat visual cortical neurons as a model system, so that we could examine both the somatic and dendritic expressions of these neurochemicals in individual neurons. We found a difference in the sequence of developmental expressions of NMDAR1, nNOS, CO, and Na^+/K^+ ATPase. Triple labeling showed that all nNOS-positive neurons were immunoreactive for NMDAR1, and a subpopulation of them had high CO activity. The expression of NMDAR1 was positively correlated with CO activity. This is consistent with our previous finding that CO activity is strongly governed by excitatory glutamatergic synapses. After 40 hours of depolarizing potassium chloride treatment, CO activity was increased, and NMDAR1and nNOS levels were up-regulated in parallel. One week of tetrodotoxin significantly decreased the expression of NMDAR1, nNOS, and CO activity. Our results demonstrate that NMDA receptors and nNOS do co-exist in a subset of neurons that have high CO activity and their expressions are under the control of neuronal activity.

Introduction

The NMDA type of glutamate receptors belongs to a family of voltage-dependent ion channels. In response to glutamate, the postsynaptic membrane is depolarized, the magnesium block is removed, and calcium is allowed to enter the cell through NMDA receptor channels. According to the hypothesis of Garthwaite (1991), this influx of calcium is needed for the activation of neuronal nitric oxide synthase (nNOS) and the production of nitric oxide (NO); nitric oxide can then function as a signal molecule through the cGMP pathway (Garthwaite, 1991; Dawson & Dawson, 1996). Therefore, the nNOS cascade may be functionally linked to an energy-dependent process. The postsynaptic membrane depolarized by glutamate needs to be actively repolarized to the resting state by Na^+/K^+ ATPase (Sokoloff, 1974; Beal *et al.*, 1993). This pumping activity requires energy supplied by oxidative metabolism through the respiratory chain, of which cytochrome oxidase (CO) is the terminal enzyme (Erecinska & Silver, 1989).

The activation of NMDA receptors has been linked to many aspects of brain development (Constantine-Paton *et al.*, 1990; Goodman & Shatz, 1993; Bear, 1996). Nitric oxide has been suggested to play important roles in neuronal differentiation and maturation (Kalb & Agostini, 1993; Bredt & Snyder, 1994; Yan *et al.*, 1994). Additionally, neuronal activity can influence the survival, differentiation, and synapse formation of neurons during certain stages of their development (Harris, 1981; Gallo *et al.*, 1987; Lo & Poo, 1991; Fields & Nelson, 1992). However, whether nNOS and NMDA receptors were co-localized in neurons during development was unknown. Whether increased neuronal activity would

* To whom correspondence should be addressed.

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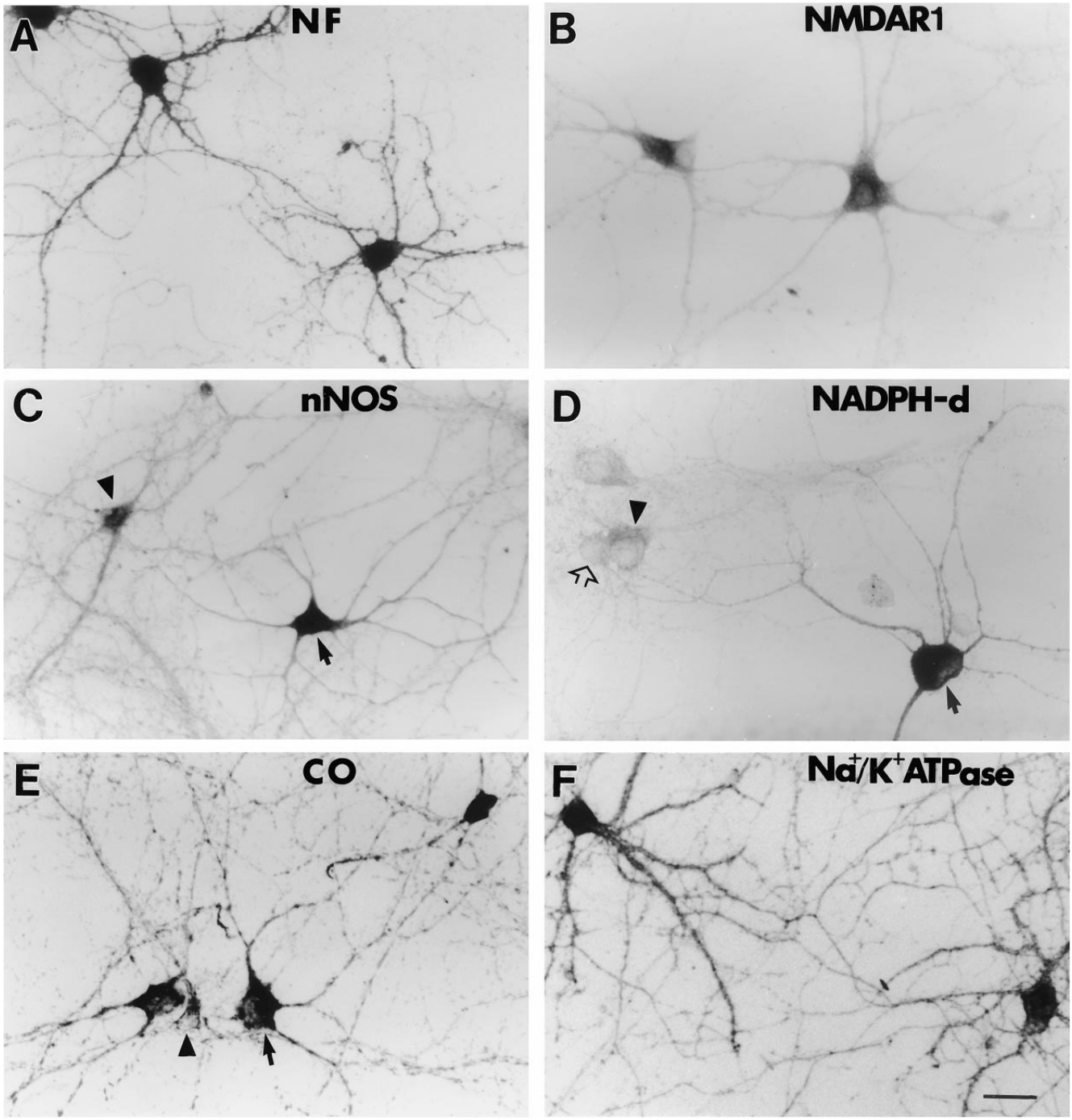


Fig. 1. Examples of neurons at 14 DIV reacted for neurofilament 200 (NF, A), NMDAR1 (B), nNOS (C), NADPH-diaphorase (NADPH-d, D), cytochrome oxidase (CO, E) and Na^+/K^+ ATPase (F). Note that a very darkly NADPH-d reactive neuron (solid arrow, D) coexists with a moderately reactive one (arrowhead, D) and a non-reactive neuron (open arrow, D) in the same field. Examples of neurons darkly labeled for nNOS (arrow, C) or CO (arrow, E) co-existing with moderately labeled ones (arrowheads in C and E) are also shown. Scale bar = 50 m.

affect the levels of nNOS and NMDA receptor proteins in neurons was also not well understood.

Neurons in culture can provide a better resolution at the single cell level for studying the expression and regulation of neurochemicals in different neuronal compartments, i.e., cell bodies and dendrites. It also allows us to study the effect of increasing or decreasing neuronal activity on individual neurons and their processes. In cultures, we can easily control neuronal activity by manipulating the contents of the medium.

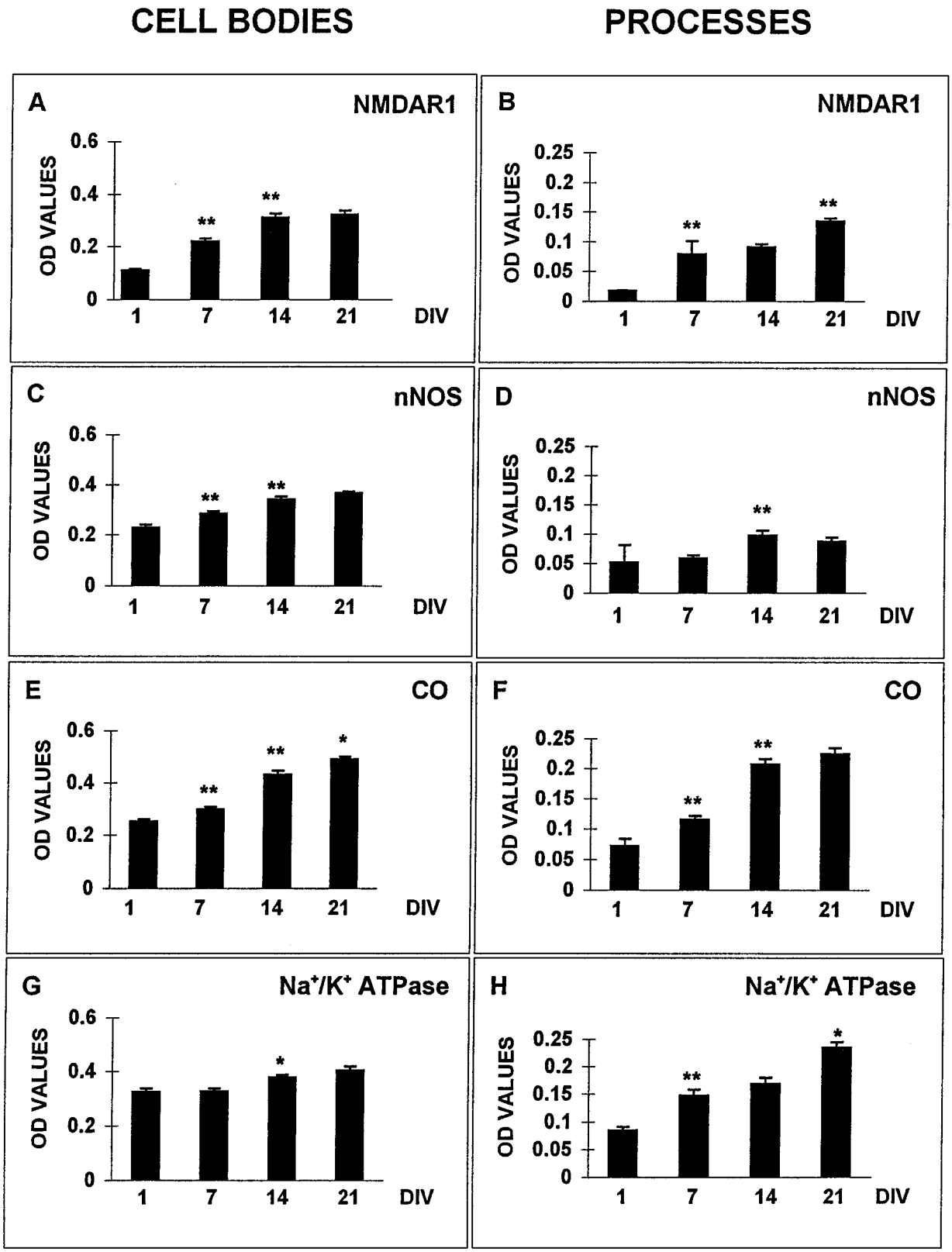
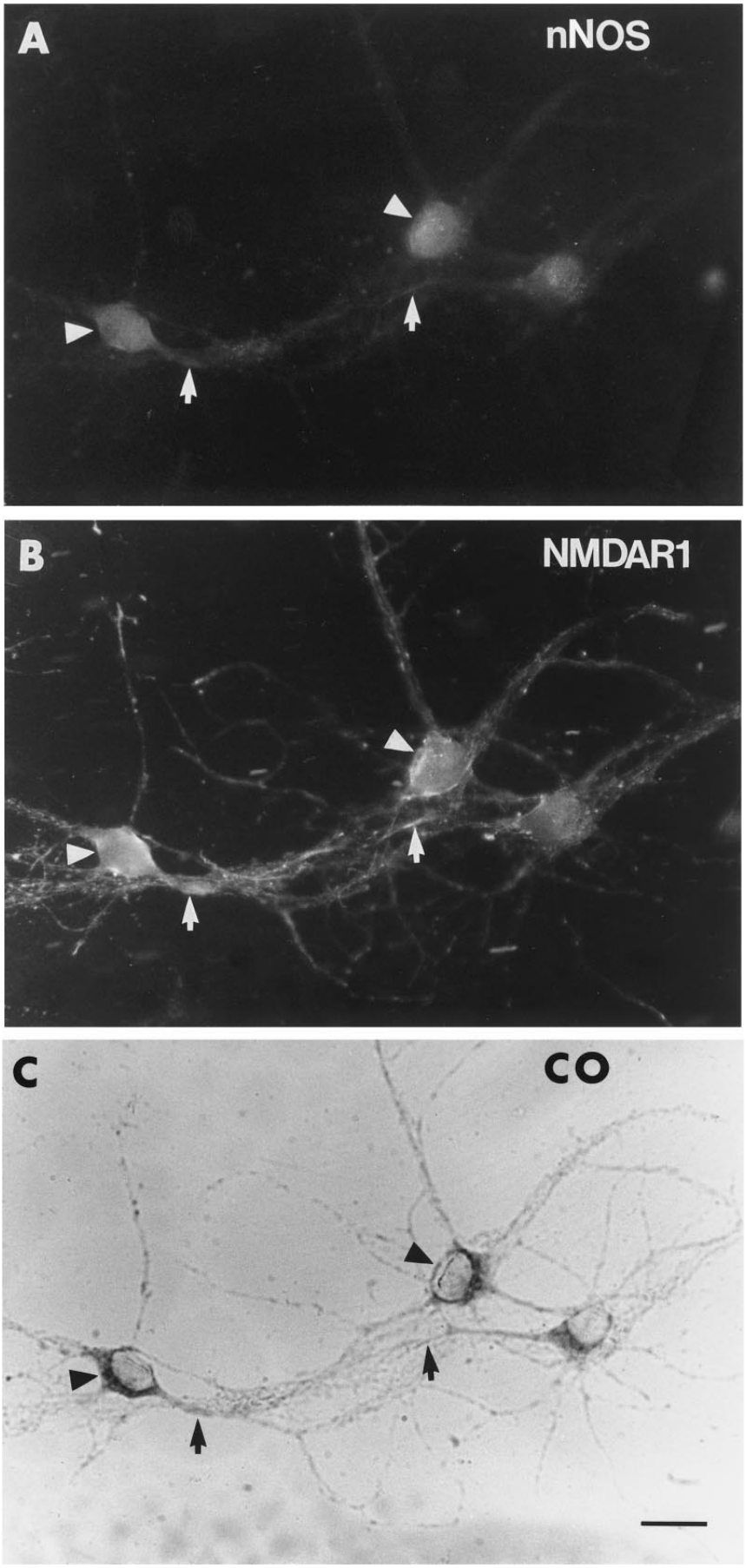


Fig. 2. Quantitative optical densitometric analyses of changes in the staining intensity of various neurochemicals within neuronal cell bodies and processes from 1 to 21 DIV. Note that the level of NMDAR1 increases significantly at 7 and 14 DIV within cell bodies and at 7 and 21 DIV in neurites (**, $P < 0.001$). There is a significant increase at 7 and 14 DIV within cell bodies and at 14 DIV in neurites for nNOS (**, $P < 0.001$). The CO activity is significantly higher at 7 and 14 DIV in cell bodies and neurites (**, $P < 0.001$). At 14 DIV, the level of Na^+/K^+ ATPase is increased significantly within cell bodies (*, $P < 0.05$). OD values: optical densitometric values.


A
nNOS
B
NMDAR1
C
CO

We were able to maintain the survival of neurons that originated from the visual cortex of postnatal rats for up to four weeks.

The energy demand of a neuron is largely dependent on the type of synapse, i.e., excitatory or inhibitory, that it receives. The level of CO activity is found to be higher in neurons that receive predominantly glutamatergic synapses on their cell bodies than those that receive exclusively GABAergic synapses (Nie & Wong-Riley, 1996). Glutamate is the major excitatory neurotransmitter in our present culture system (Ramakers *et al.*, 1993). Whether NMDA receptors are distinctly expressed in these neurons is not known. If the NMDA receptor-nNOS pathway is involved in neuronal development, and if there is a positive correlation between frequent membrane depolarization and NMDA receptor activation in a subset of postsynaptic neurons, then it is reasonable to expect a heightened energy requirement in those neurons. Therefore, we tested the following hypotheses: (1) the developmental expressions of nNOS and NMDA subunit R1 (NMDAR1, the key subunit for the structural and functional NMDA receptor (Moriyoshi *et al.*, 1991)) and metabolic activity can be correlated in a subset of neurons; and (2) neuronal activity would play an important role in regulating the expression of NMDAR1 and nNOS at the protein level. Both CO activity and Na^+/K^+ ATPase expression were used as markers for neuronal metabolic activity, because CO is involved in energy generation, while Na^+/K^+ ATPase is a major consumer of energy in neurons (Beal *et al.*, 1993).

Methods

PRIMARY NEURONAL CULTURE FROM POSTNATAL RAT VISUAL CORTEX AND TREATMENTS

To prepare primary neuronal cultures, glial cells from postnatal 2-day-old rat cortex were cultured in 60 mm dishes as described by Goslin & Banker (1991). After about two weeks, when this glial feeder layer was subconfluent, visual cortex from postnatal 5–6 day-old rat pups was dissected as described by Baughman *et al.* (1991). Briefly, pups were anesthetized with carbon dioxide and decapitated. Their brains were removed from the skull, followed by the removal of the meninges and choroid plexus. The primary visual cortex, about 2×3 mm in size, was removed from the dorsal surface of the occipital cortex. Neurons were dissociated by 15 minutes of incubation in 1.6% trypsin at 37°C, triturated and plated at a density of $1–3 \times 10^5$ cells per coverslip coated with poly-L-lysine. Three coverslips were then co-cultured with the subconfluent glial cells in a 60 mm culture dish, and maintained in minimal essential medium (MEM) containing N_2 supplements (Bottenstein, 1985). Cytosine arabinoside (Ara-C) was added one day after the plating of neurons to inhibit the replication of non-neuronal cells. Neuronal cultures were maintained by replacing one-third of the medium once a week.

Cultures of different ages (1, 7, 14, and 21 days *in vitro*, DIV) were reacted in parallel for different markers at the same time. Sister cultures at each age group or experimental group were processed for CO histochemistry and immunocytochemistry for nNOS, NMDAR1 and Na^+/K^+ ATPase. For each marker, cultures were incubated in identical solutions for the same amount of time at the same temperature. Three independent experiments were performed using exactly the same procedures.

In experimental groups, we compared changes in nNOS, NMDAR1, CO, and Na^+/K^+ ATPase in 10 day-old cultures under different effective treatments, which increased or decreased neuronal activity. Cultures were treated with 20 mM KCl in the last 40 hours before they were harvested. The TTX treatment was given when cultures were 4 days old at a concentration of 0.2 M. This was refreshed every 3 days and the cells were harvested when they were 10 days old. Shorter times of exposure to 0.2 M TTX did not have any effect, and higher concentrations of TTX rapidly killed the cells.

CO HISTOCHEMISTRY

After fixation in 4% paraformaldehyde for 1 hour on ice, cultures were incubated in phosphate-buffered saline (PBS), pH 7.2, with 0.05% (weight/volume) diaminobenzidine (DAB, Sigma) and 0.03% (weight/volume) cytochrome C (Sigma) for 2 hours at 37°C (Wong-Riley, 1979). This reaction time was chosen because the enzymatic reaction had reached its plateau and could reproducibly reflect a differential staining intensity among neurons in the series of cultures at different ages.

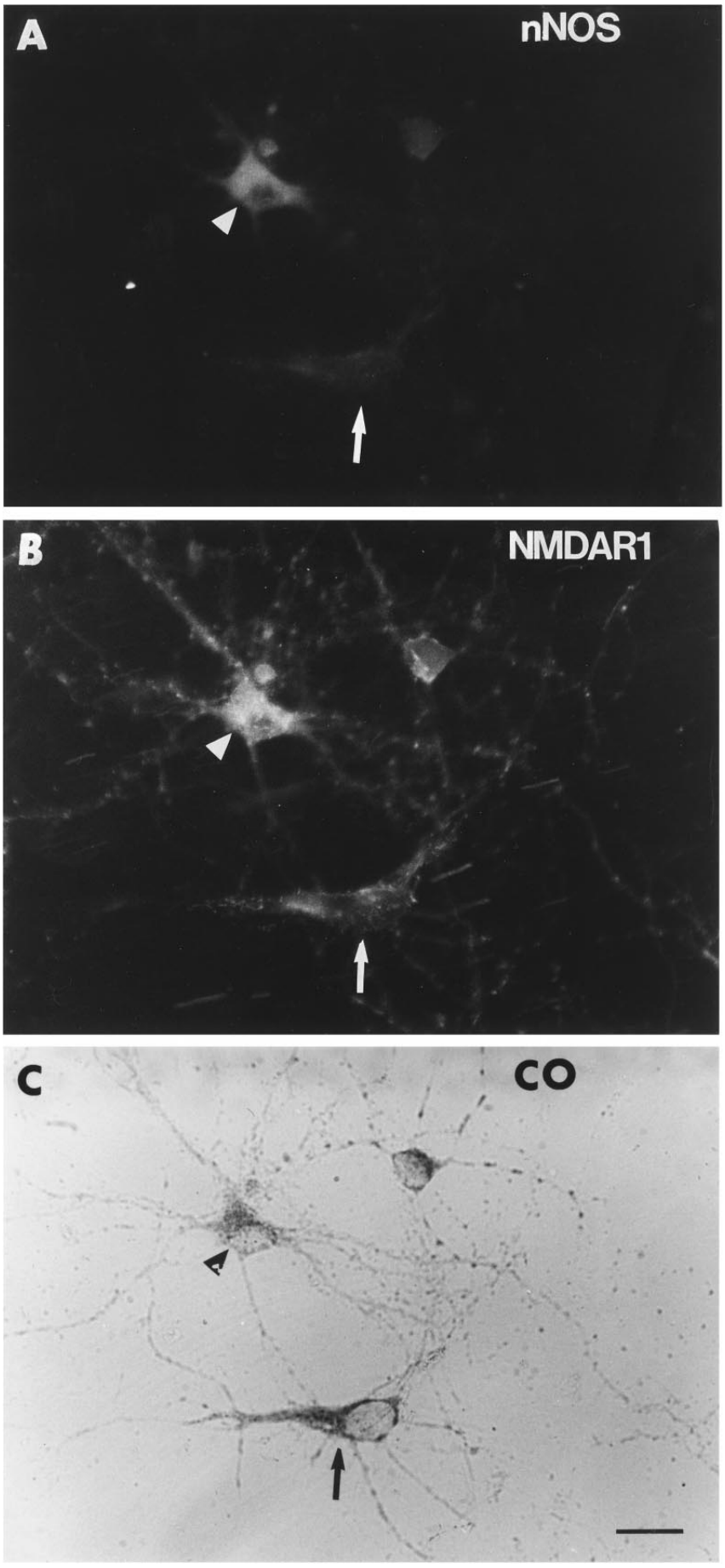
NADPH-DIAPHORASE HISTOCHEMISTRY

Cultures at 7 and 14 DIV were fixed with 4% paraformaldehyde for 10 minutes on ice, and were incubated in PBS, pH 7.2, containing 0.3% Triton X-100, 0.1 mg/ml nitroblue tetrazolium and 1.0 mg/ml -NADPH at 37°C for 90 minutes.

IMMUNOCYTOCHEMISTRY

Antibodies: (1) Anti-Na^+/K^+ ATPase (Affinity BioReagents, Golden, CO): Monoclonal antibodies against 3 subunit of canine heart Na^+/K^+ ATPase were used at 1:3000 dilution. (2) Anti-nNOS (Transduction Lab, Lexington, KY): Polyclonal antibodies generated against a 22.3 kDa protein fragment corresponding to amino acids 1095–1289 of human nNOS, were used at 1:800 dilution. (3) Anti-NMDAR1 (Chemicon, Temecula, CA): Polyclonal antibodies against a 30-mer synthetic peptide corresponding to the C-terminus of rat NMDA receptor subunit R1, which can recognize the major splice variants NR1-1a, NR1-1b, NR1-2a, and NR1-2b in the rat brain, were used at 1:1000

Fig. 3. Examples of triple-labeled neurons at 14 DIV. Dark field micrographs (A, B) show neurons that were doubly immunofluorescent-labeled for nNOS (A) and for NMDAR1 (B) in both cell bodies (arrowheads) and processes (arrows). These neurons also had high CO activity shown by bright field micrograph (C). Scale bar = 60 m.


A
nNOS
B
NMDAR1
C
CO

dilution. (4) Polyclonal antibodies against neurofilament 200 (Sigma, St. Louis, MO) were used at 1 : 400 dilution. Neurons were immersion fixed with 4% paraformaldehyde, blocked with 5% normal goat serum, and incubated with primary antibodies overnight at 4°C followed by 4 hour incubation with goat anti-rabbit or goat anti-mouse IgG conjugated to HRP at 1 : 100 at room temperature (RT). Reaction products were visualized by DAB as the chromagen.

TRIPLE LABELING WITH CO HISTOCHEMISTRY AND INDIRECT DUAL IMMUNOFLUORESCENCE

Neurons from 14-day-old cultures were fixed and reacted as described before (Liu & Wong-Riley, 1990) with modifications. Briefly, after CO histochemistry, they were blocked in PBS containing 0.1% bovine serum albumin (BSA) for 1 hour at RT, and immuno-stained overnight at 4°C with monoclonal antibodies against NMDAR1 (1 : 200, PharMingin, San Diego, CA) and polyclonal antibodies against nNOS (1 : 200, Transduction Lab, Lexington, KY). Following rinses in PBS-BSA, the cultures were incubated with secondary antibodies (Chemicon, Temecula, CA) designed for dual labeling: fluorescein-conjugated goat anti-mouse IgG at 1 : 10 for NMDAR1 antibodies and rhodamine-conjugated goat anti-rabbit IgG at 1 : 40 for nNOS antibodies for 1 hour in the dark at RT. Differential labeling was observed with a fluorescence microscope using specific excitation/barrier filters for fluorescein and rhodamine.

QUANTITATIVE OPTICAL DENSITOMETRIC ANALYSES

Optical densitometric measurements of reaction product were made by means of a computer-assisted image processor with a Zeiss photometer attached to a Zeiss compound microscope as described previously (Liu & Wong-Riley, 1990). Cultures reacted for CO histochemistry or nNOS, NMDAR1 and Na^+/K^+ ATPase immunocytochemistry were analyzed. Optical densities reflected the intensities of reaction product. The slides were numbered so that the age and treatment parameters were unknown to the observer. All lighting conditions, magnifications, and reference point (collagen substratum) were kept constant. An effective measuring spot of 2 m^2 was used at a magnification of 40×. The spot was centered on the cytoplasm of individual somata or single neurites. The background was a blank region on the coverslip and was set to zero. Each value given by the computer represented an average of 15 automatic readings. Readings were taken from 100 neurons in consecutive fields of view in two non-overlapping bands that spanned the width of the coverslip. The mean value ± SEM for each marker at each time point represented the readings from either cell bodies or neurites of three independent experiments. In the experiments where KCl or TTX was added, readings were taken from five separate experiments for paired control and KCl- or TTX-treated cultures. Data were analyzed using Microsoft® Excel and statistical analysis was done using two-tailed Student's *t*-test, which compared two consecutive age groups or control and experimental groups for each marker. A P value of 0.05 or less was considered significant.

WESTERN BLOT ANALYSIS

Cultures for this purpose were plated at a higher density (8×10^5). Cell lysates from equal numbers of control and treated cultures were solubilized in the same volume of extraction buffer (10 mM Tris-HCl buffer, pH 7.4, containing 2 mM EDTA, 1 mM -mercaptoethanol, 1 mM PMSF, and 10 g/ml leupeptin) and the same amount of lysates were subjected to 7.5% SDS-PAGE. Proteins were transferred to nitrocellulose membrane, and incubated overnight using the above-described nNOS and NMDAR1 antibodies at 1 : 1000. Monoclonal antibodies against neurofilament 68 (NF 68, Sigma. This antibody reveals a 68 kD band that is easily distinguishable from the 155 kD band for nNOS and the 120 kD band for NMDAR1) were used at 1 : 2000 as a control for equal sample loading in each lane. Subsequently, membranes were incubated for 3–4 hours with the secondary antibodies (1 : 2000) at room temperature and the labeled bands were visualized on a film with enhanced chemiluminescence (Amersham). For quantification, the films were scanned into the computer and the density of each band was measured by means of an AlphaImager (Alpha Innotech Corp., San Leandro, CA) following manufacturer's instructions.

Results

Neurons dissociated from postnatal rat visual cortex were attached to poly-L-lysine-coated coverslips. Within the first day of culture, freshly seeded cells began to form extensions from rounded cell bodies. The size of cell bodies visibly increased during the first two weeks in culture. Processes lengthened with time and formed multiple contacts among neurons. The outgrowths of neurites transformed cells to bi- and multipolar neurons that resembled the developing neurons *in vivo*, and led to a well-developed neuronal network after the first week. Cultures maintained in this way not only allowed neurons to survive up to four weeks, but also separated neurons from the glial feeder layer. By using polyclonal antibodies against neurofilament 200 (Sigma), a neuron-specific marker, we found that more than 95% of cells grown on the coverslips were neurons at all periods examined (Fig. 1A). After 3 weeks *in vitro*, the cultures started to show signs of degeneration, such as retraction of neurites and abundance of fine cell debris. Therefore, our experiments were performed on neurons only during the first 3 weeks in culture. We compared the developmental expressions of

Fig. 4. Examples of neurons after triple labeling. Dark field micrographs (A, B) show that a neuron (arrowheads) was doubly immunofluorescent-labeled for nNOS (A) and NMDAR1 (B), and moderately reactive for CO histochemistry (C). A neuron (arrows in A–C) that was immunofluorescent-labeled for NMDAR1 (B) was not doubly labeled for nNOS (A), but was darkly reactive for CO (C). Scale bar = 60 m.

NMDAR1 and nNOS immunocytochemically in sister cultures. Four age groups (1, 7, 14, 21 DIV) were reacted simultaneously, allowing us to examine developmental expressions and quantitatively compare optical densitometric values of reaction product in cell bodies and neurites. There was no positive correlation between the optical densitometric values and cell sizes at any given age, in that small cells can be more immunoreactive than larger cells. We then analyzed the co-localization of NMDAR1, nNOS and CO activity by triple labeling. Finally, we showed the effects of KCl or TTX on the protein levels of NMDAR1 and nNOS by Western blots.

DEVELOPMENTAL EXPRESSION OF NMDAR1 AND nNOS IMMUNOREACTIVITY

All neurons examined between 1 and 21 DIV were immunoreacted for NMDAR1, but their intensities increased with time *in vitro*. There was a two-fold increase in the staining intensity of neuronal cell bodies and almost a four-fold increase in the processes between 1 and 7 DIV, both of which were significant ($P < 0.001$). At 14 DIV (Fig. 1B), staining of both cell bodies and processes continued to increase and was significantly more intense in neuronal cell bodies ($P < 0.001$, Fig. 2A). While the level of somatic NMDAR1 plateaued at 14 DIV, that of neuritic NMDAR1 increased further by 21 DIV (Fig. 2B). This implies that proteins made in neuronal cell bodies were partially transported to the processes.

The levels of nNOS immunoreactivity increased progressively from 1 to 21 DIV, and by 7 DIV, approximately 50% of neurons were immunoreactive for nNOS. The increase was not only in the number of immunoreactive cells, but also in the level within each reactive cell. By 14 DIV, 60–70% of neurons was nNOS-positive; however, only 1% of these was darkly immunoreactive (Fig. 1C). These increases were significant in the cell bodies at 7 and 14 DIV ($P < 0.001$, Fig. 2C). Within the processes, nNOS level increased significantly at 14 DIV and remained high at 21 DIV ($P < 0.001$, Fig. 2D).

Another marker for NO-producing neurons is NADPH-diaphorase (Dawson *et al.*, 1991; Hope *et al.*, 1991). At 7 DIV, several very darkly NADPH-diaphorase-labeled cells that were similar to those darkly NOS-reactive neurons were seen in each coverslip, and the majority of neurons did not react for NADPH-diaphorase. The absolute numbers of reactive neurons, especially the moderately reactive ones, increased dramatically from 7 to 14 DIV. At 14 DIV, about 60–70% of neurons was reactive for NADPH-diaphorase, and only about 1% of these was darkly reactive, all of which had long reactive processes (Fig. 1D).

DEVELOPMENTAL CHANGES IN CO ACTIVITY AND Na^+/K^+ ATPase IMMUNOREACTIVITY

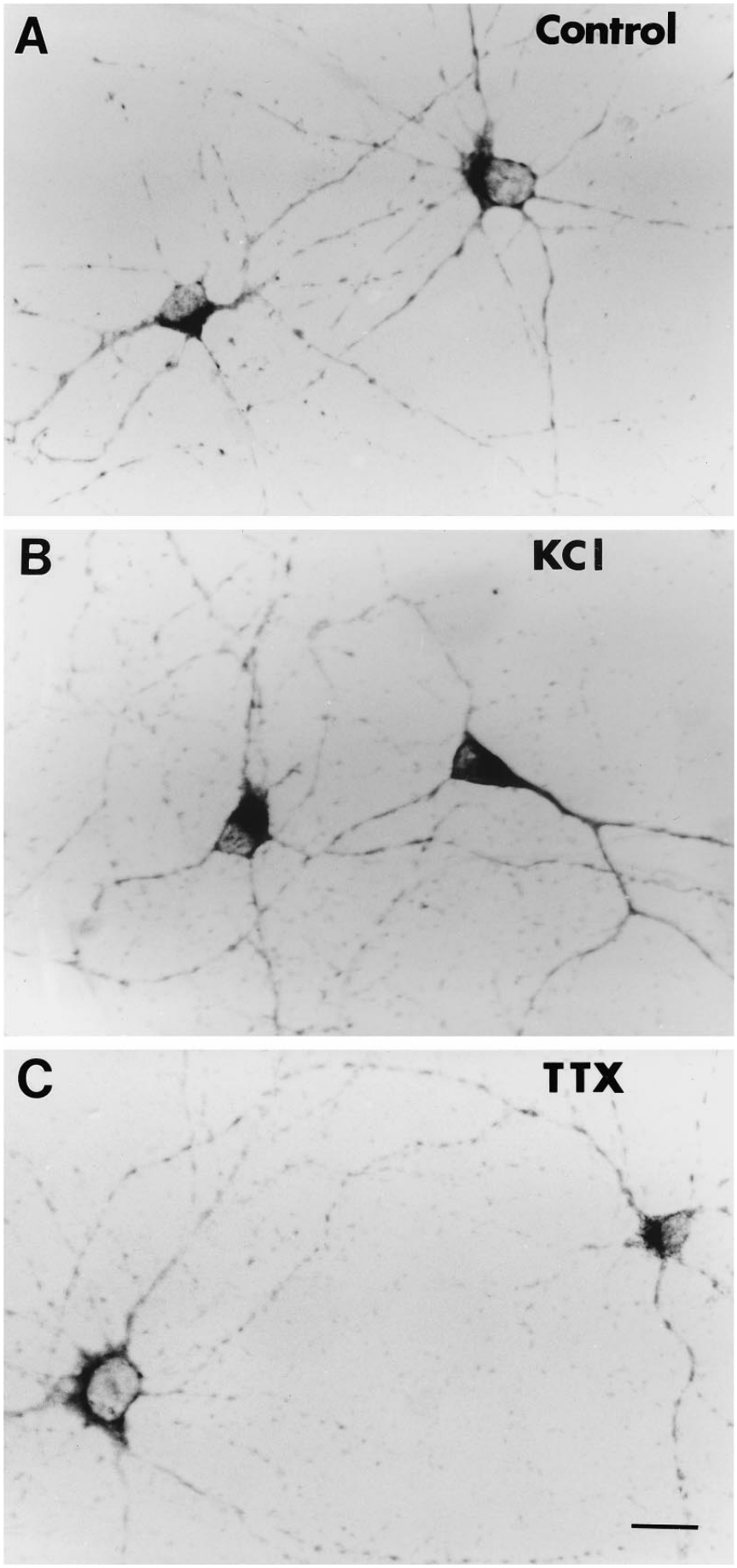
From the first day in culture and at all the time points examined, all of the neurons had a detectable level of reaction product for CO in their cell bodies and processes (Fig. 1E). The intensity among neurons was not homogeneous, and could be described as dark, moderate and light according to their optical densitometric values, which ranged anywhere from 0.1 to 0.6 by our measurements. At 14 DIV, $28 \pm 5\%$ of neurons had high CO activity, $14 \pm 3\%$ of neurons were lightly reactive, while the rest were moderately reactive. The mean intensities at later ages were significantly higher than those at younger ages ($P < 0.001$, Fig. 2E). The level of CO activity in processes increased significantly at 7 DIV ($P < 0.001$) and continued to rise with time in culture (Fig. 2F).

The immunoreactivity of the energy-consuming enzyme, Na^+/K^+ ATPase, was evident in both cell bodies and processes (Fig. 1F). The level was clearly detectable and relatively high in cell bodies from the first day in culture and it increased significantly at 14 DIV ($P < 0.05$, Fig. 2G). In the neuronal processes, the intensity was relatively low at 1 DIV, but became significantly higher between 7 and 21 DIV (Fig. 2H).

CORRELATION OF THE IMMUNOREACTIVITY OF NMDAR1 AND nNOS WITH CO ACTIVITY

To assess the relative level of metabolic activity in individual NMDAR1- and nNOS-immunoreactive neurons, a triple labeling approach combining CO histochemistry and indirect dual immunofluorescence was used in neurons at 14 DIV. The intensity of reaction product for CO histochemical reaction in NMDAR1 and/or nNOS-immunoreactive neurons was divided into dark, moderate, and light categories. Fluorescent signals were very bright, moderately bright, or negative in neurons within the same field of view. All nNOS-immunoreactive neurons were reactive for NMDAR1- and $33 \pm 4\%$ of them had high CO activity in both neuronal cell bodies and processes (Fig. 3). Other nNOS- and NMDAR1-immunolabeled neurons had moderate levels of CO activity (Fig. 4, arrowheads). The NMDAR1-immunolabeled neurons were not always double-labeled for nNOS but their intensities were highly correlated with CO activity in both neuronal cell bodies and processes (Figs. 3 and 4). However,

Fig. 5. Changes in CO activity after KCl or TTX treatments. (A) CO histochemical staining in a control (no treatment) culture. (B) CO histochemical staining in a KCl-treated culture. Note the increase in staining intensity within both cell bodies and processes in KCl-treated cultures in comparison to control cultures. (C) CO histochemical staining in a TTX-treated culture. Note the reduction in staining intensity as compared to the control. Scale bar = 60 m.


A
Control
B
KCl
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nNOS-nonreactive neurons could still have high CO activity (Fig. 4, arrows). Neurons that had high CO activity were all NMDAR1-immunoreactive (Figs. 3 and 4).

EFFECT OF CHANGING NEURONAL ACTIVITY ON NMDAR1, nNOS, CO AND Na^+/K^+ ATPase

To determine if nNOS and NMDAR1 immunoreactivity was regulated by neuronal activity, we treated cultures either with 20 mM KCl, which depolarized neuronal membrane, or with 0.2 M TTX, which blocked voltage-dependent sodium channels. The number of neurons in treated cultures was the same as that of control cultures, and the viability did not significantly decrease during these treatments (not shown). To circumvent the fact that the fluorescent signals from the triple labeling did not permit precise quantitative comparison between the control and treated cultures, we used single peroxidase-based immunocytochemistry as well as Western blots to compare the effects of these treatments.

Studies using cell recordings have demonstrated changes in the spiking activity with KCl and TTX treatments (Balázs *et al.*, 1992; Ramakers *et al.*, 1993). Under KCl-treated conditions, CO activity was significantly increased (Fig. 5B). At the same time, the level of Na^+/K^+ ATPase immunoreactivity was also increased in neuronal cell bodies (Fig. 6B). These changes in neuronal cell bodies were confirmed quantitatively by optical densitometry (Fig. 8A and B). The increase indicated that neurons treated with KCl required more ion pumping activity, and thus more ATP. In Western blots, the protein levels of NMDAR1 (Fig. 7A, lane 2) and nNOS were up-regulated (Fig. 7B, lane 2) as compared to untreated control cultures (Fig. 7A, lane 1; Fig. 7B, lane 1). KCl treatment did not alter the proportion of nNOS-positive neurons in culture. On the other hand, in TTX-treated cultures, decreases in CO activity (Fig. 5C) and Na^+/K^+ ATPase immunoreactivity (Fig. 6C) were found, and the expressions of NMDAR1 (Fig. 7A, lane 3) and nNOS proteins (Fig. 7B, lane 3) were clearly down-regulated in Western blots. As a control, the level of neurofilament 68 was not significantly altered with these treatments (Fig. 7C). These results were quantified and confirmed by the normalization of the density of each band for NMDAR1 (Fig. 8C) and nNOS (Fig. 8D) to that of NF 68. Similar changes were found in immunocytochemical staining for nNOS and NMDAR1 (not shown).

Discussion

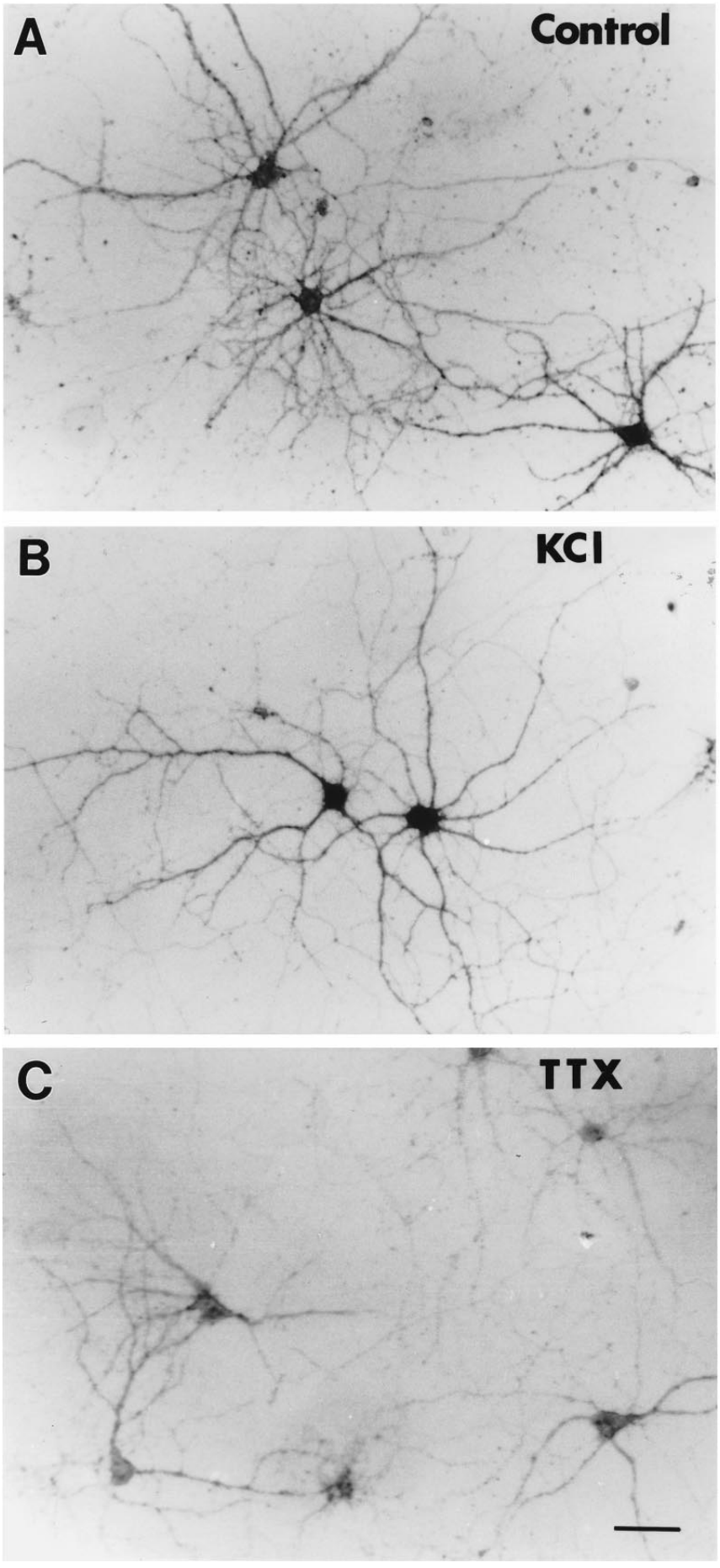
Our results indicate that neurons in culture continue to express increasing amount of NMDA receptors and nNOS proteins during the dynamic growth/regrowth of processes and the formation of new interactions. The positive correlation between the expression of NMDAR1 and neuronal activity indicates that glutamatergic input may be one of the driving forces in heightening the energy demand of neurons during development. The expression of NMDAR1 and nNOS proteins is also regulated by neuronal activity.

THE EXPRESSION OF NMDAR1, nNOS, AND CO ACTIVITY

We demonstrated the anatomical co-localization of nNOS and NMDAR1 in both cell bodies and neurites of the same visual cortical neurons in culture. This is consistent with Garthwaite's hypothesis (Garthwaite, 1991) that nNOS is linked to NMDA receptor-activation and NO formation. The developmental increase in the expression of NMDAR1 and nNOS paralleled the increase in CO activity. Moreover, those neurons that are immunoreactive for both NMDAR1 and nNOS and have high CO activity probably have a heightened energy requirement during development. The rationale for this positive correlation is based on the depolarizing requirement for the activation of NMDA receptors, which are postsynaptic to glutamatergic inputs. ATP is needed for repolarization subsequent to depolarization, and this contributes to a major energy consuming function of neurons (Sokoloff, 1974; Beal *et al.*, 1993). Previously, we found that in the adult rat central nervous system, certain tonically active nNOS-positive neurons were also immunoreactive for NMDAR1 and had high levels of CO activity (Zhang & Wong-Riley, 1996a). In the visual cortex of adult monkeys, metabolically active regions with high CO activity also had an enrichment of glutamate-related neurochemicals, such as NMDA receptors and nNOS (Wong-Riley *et al.*, 1998). A recent report (Aoki *et al.*, 1997) also demonstrated that nNOS and NMDA receptors could coexist within dendrites and at sites receiving excitatory inputs in the adult rat cerebral cortex. The present study further demonstrates that this correlation exists in developing neurons. Therefore, nNOS could be activated, presumably through the activation of NMDA receptor, and function in neuronal development.

However, not all nNOS-positive neurons have high CO activity, and neurons with high CO activity are not necessary nNOS-immunoreactive. Neuronal NOS-immunoreactive neurons would show low levels of CO activity if they received strong inhibitory input or they might not have been tonically active. In addition, calcium entry through NMDA receptors and/or voltage-sensitive calcium channels can also serve as

Fig. 6. Changes in the level of Na^+/K^+ ATPase immunoreactivity after KCl or TTX treatments. (A) Na^+/K^+ ATPase immunoreactivity in a control (no treatment) culture. (B) Na^+/K^+ ATPase immunoreactivity in a KCl-treated culture. Note the increased labeling in both cell bodies and processes. (C) Na^+/K^+ ATPase immunoreactivity is clearly reduced in a TTX-treated culture. Scale bar = 100 m.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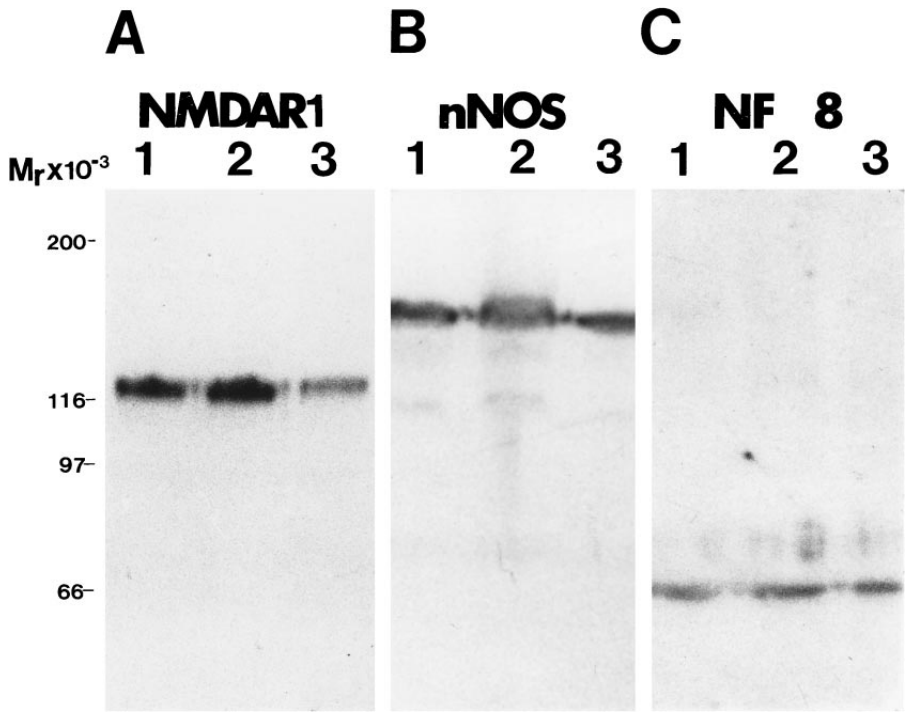

Fig. 7. Western blot analyses of control (Lanes 1 in A to C), KCl- (Lanes 2 in A to C), and TTX-treated cultures (Lanes 3 in A to C). These blots were probed with antibodies against NMDAR1 (A), nNOS (B) and NF 68 (C), respectively. Equal amounts of lysates were loaded in each lane. Note the wider and denser bands in lanes 2 and the narrower and lighter bands in lanes 3 in A and B as compared to controls (lanes 1), indicating that the protein levels of NMDAR1 and nNOS were increased in KCl-treated but decreased in TTX-treated cultures. No significant alterations were found for NF 68. Molecular weight markers are shown on the left. These experiments were replicated five times.

a trigger in other second messenger signal cascades instead of activating nNOS, and result in influencing neuronal survival and neurite outgrowth (Ghosh & Greenberg, 1995).

The activation of NMDA receptors has been reported to be involved in various aspects of neuronal development (Mattson, 1988; Constantine-Paton *et al.*, 1990; Goodman & Shatz, 1993; Bear, 1996). The proportion of neurons in cerebellar granule cell cultures that grow neurites was found to be regulated by the activation of NMDA receptors, the spontaneous NMDA current, and the release of glutamate (Pearce & Dutton, 1981; Pearce *et al.*, 1987; Kilic *et al.*, 1991). Alterations in proteins involved in neurite extension have been seen in cortical neurons in response to excitatory amino acids (Bigot *et al.*, 1991). These findings suggest that neuronal development is likely to be stimulated by depolarization and/or the activation of NMDA receptors. Our results are consistent with this notion. Given the important roles that NMDA receptors can play in developing neurons, we speculate that the activation of NMDA receptors in these postsynaptic neurons may be one of the key factors that drive the high-energy demand, as demonstrated by their level of CO activity.

CO ACTIVITY AND ENERGY METABOLISM

The level of energy metabolism in a neuron is closely coupled to its functional activity (Wong-Riley, 1989). Previous studies have shown that active ion transport, synaptic activity and/or the rate of spontaneous discharges are the main factors that consume energy during neuronal development. On the other hand, certain cellular functions, such as protein synthesis, are not the major users of energy (Woodward *et al.*, 1969; Lowry, 1975). Cytochrome oxidase activity shown histochemically is a useful marker for the level of energy requirement in neurons. In the present study, there is a significant increase in the mean level of CO activity in neurons at 7 and 14 DIV, corresponding in time to the formation of morphologically mature synapses (Huettner & Baughman, 1988; Ichikawa *et al.*, 1993) and physiologically recordable synaptic currents in cultured visual cortical neurons (Huettner & Baughman, 1988). We also examined the expression of CO subunit mRNAs, and found a consistent increase with time in culture (Zhang & Wong-Riley, 1997). However, neurons do show varying levels of CO activity, which may indicate their differential energy requirements in

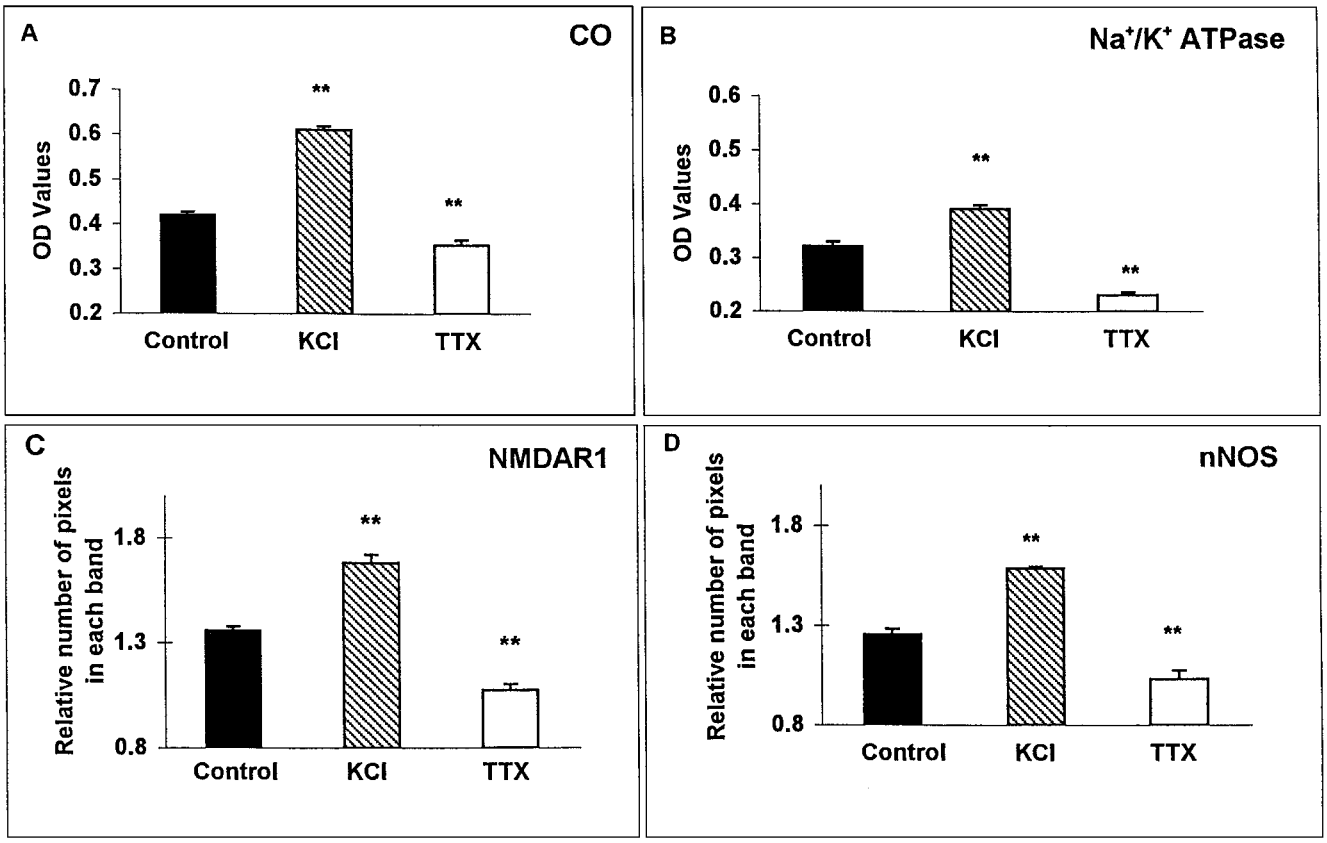

Fig. 8. (A and B) Quantitative optical densitometric analysis indicates a significant (**, $P < 0.001$) increase in KCl-treated cultures and a decrease in TTX-treated cultures for both CO (A) and Na^+/K^+ ATPase (B). (C and D) Quantitative analysis of the density of each band in Fig. 7 detected from the original films exposed to immunoblots. The density of each band for NMDAR1 and nNOS was normalized to that of NF 68. There is an increase in KCl-treated and a decrease in TTX-treated cultures for NMDAR1 (C) and nNOS (D) as compared to untreated controls.

their course of development. Is it possible that the increase in CO activity in cultured neurons is a response to an intrinsic signal independent of the functional requirement of neuronal activity? We found that KCl-induced depolarization could readily increase the level of CO activity, whereas blocking the voltage-gated sodium channels with TTX could decrease CO activity, indicating that the level of CO activity in these neurons is regulated primarily by their neuronal activity. Therefore, the increase in CO activity with time in culture suggests that there is an increased energy demand, presumably an increased need for ATP generation to fuel ion pumps, such as Na^+/K^+ ATPase. The tight regulation of Na^+/K^+ ATPase immunoreactivity in response to changing neuronal activity by KCl or TTX also represents a close relationship between neuronal activity and active ion transport.

REGULATION OF NMDAR1 AND nNOS BY NEURONAL ACTIVITY

In the present study, we found that chronic depolarization by high concentrations of potassium chloride or chronic blockage of impulse activity by TTX can modulate the levels of nNOS and NMDAR1 protein expression. Neural activity is required for pattern formation and synaptic plasticity in the neocortex (Katz & Shatz, 1996). Spontaneously occurring bioelectric activity has also been shown to be important in morphological and functional development of the central nervous system (Stryker & Harris, 1986; Fields & Nelson, 1992). The molecular mechanism of the effects of changing neural activity on neuronal development is not clear. Some studies have suggested that a number of substances, such as arachidonic acid, calcium, and nitric oxide may be involved in this process (Gallo *et al.*, 1987; Fields & Nelson, 1992). In our study, neuronal membrane depolarization induced by excess extracellular K^+ resulted in an increased immunoreactivity to NMDAR1. This finding suggests that the regulation of NMDA receptors can happen at its protein level, and an increase in the number of NMDA receptors is a way that neurons adapt to their increased depolarizing activity. On the other hand, the properties of NMDA receptor channels can also be modified at the transcriptional level and involves subunits such as NR2A in addition to NMDAR1

(Bessho *et al.*, 1994; Tascedda *et al.*, 1996). Increasing neuronal activity also resulted in an up-regulation in the protein level of nNOS, the enzyme downstream of NMDA receptor activation. This effect is important because it indicates that an external stimulation may be converted to an adjustment in the formation of a neurotransmitter, NO. The up-regulation of nNOS could be limited because excess production of NO would mediate glutamate toxicity (Dawson & Dawson, 1996).

We also found that TTX treatment dramatically decreased the protein levels of NMDAR1 and nNOS. TTX treatment has been shown to significantly retard synapse formation and the maturation of synaptic junctions in neuronal cultures (Van Huizen *et al.*, 1985). Neuronal activity was suppressed under such a condition. The exact role that glutamate-NMDA receptor-nNOS-NO pathway plays in synaptogenesis and neuronal maturation remains to be determined. However, their regulation by neuronal activity suggests that they may influence activity-dependent synaptic plasticity and neuronal survival during development. Neurons normally exhibit varying metabolic demands, but the regulations of their neurochemicals, like that of CO, are exquisitely controlled by neuronal activity. The mechanism(s) controlling the activity-dependent regulation of neurochemicals and metabolic capacity are not well understood. However, our culture system is useful in pursuing the study of these mechanisms.

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