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Inducible expression of neuronal glutamate receptor channels in the NT2 human cell line

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ABSTRACT **Glutamate receptor (GluR) channels are responsible for a number of fundamental properties of the mammalian central nervous system, including nearly all excitatory synaptic transmission, synaptic plasticity, and excitotoxin-mediated neuronal death. Although many human and rodent neuroblast cell lines are available, none has been directly shown to express GluR channels. We report here that cells from the human teratocarcinoma line NT2 are induced by retinoic acid to express neuronal *N*-methyl-D-aspartate (NMDA) and non-NMDA GluR channels concomitant with their terminal differentiation into neuron-like cells. The molecular and physiologic characteristics of these human GluR channels are nearly identical to those in central nervous system neurons, as demonstrated by PCR and patch clamp recordings, and the cells demonstrate glutamate-induced neurotoxicity.**

Glutamate is the major excitatory neurotransmitter in the vertebrate central nervous system (CNS) (1, 2) and plays an important role in CNS plasticity and learning (3–5). Three classes of neuronal glutamate receptor (GluR) have been cloned, sequenced, and reconstituted in expression vectors (6–12). One class, metabotropic GluRs, transduces signals to the cell interior by a G-protein-coupled mechanism. The other two classes, *N*-methyl-D-aspartate (NMDA) receptors and non-NMDA, or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate, receptors, are transplasma membrane channels permeable to Na^+, K^+, and Ca^{2+}. Entry of excessive Ca^{2+} into the cell via intense activation of NMDA receptors, as occurs during brain ischemia, activates mechanisms that cause neuronal death (13–16).

Prior studies of the physiological properties of NMDA receptors, and of glutamate excitotoxicity, depended largely upon the use of primary cultures of neurons prepared from immature rodent (13–20). A cell line of human neurons suitable for pharmacological and molecular biological studies of neuronal GluR channels has not been available. Many neuronal cell lines have been screened for GluR channels, but direct electrophysiological recordings have not demonstrated NMDA receptor channels in any of these lines. We now report that functional GluR channels, of the NMDA and AMPA/kainate classes, are induced after neuronal differentiation of the clonal human teratocarcinoma line NT2.

MATERIALS AND METHODS

Cell Culture. NT2 cells (Fig. 1*A*) were grown in OptiMEM-I (GIBCO) supplemented with 5% (vol/vol) fetal bovine serum (FBS) and penicillin/streptomycin (P/S) as described (21–23). For differentiation, NT2 cells were treated with 1 μM retinoic acid (RA) twice weekly for 4 weeks. The cells were then replated in Dulbecco's modified Eagle's medium high glucose (DMEM/HG) with 10% FBS and P/S. One day later, the NT2-N cells (Fig. 1*B*) were mechanically dislodged and replated on Matrigel (Collaborative Research)-coated dishes. For the next 2–3 weeks, the NT2-N cells were maintained in DMEM/HG supplemented with 10% FBS, P/S, 1-β-D-arabinofuranosylcytosine (1 μM), fluorodeoxyuridine (10 μM), and uridine (10 μM). Thereafter, NT2-N cells were maintained in OptiMEM-I supplemented with 5% FBS and P/S (Fig. 2).

PCR and DNA Sequencing. Total RNA was isolated (24). The PCR amplification protocol was similar to that of Reddy and Pleasure (25). The PCR primers used were 5′ primer, 5′-AACCTGCAGAACCGCAAG-3′, and 3′ primer, 5′-GCTTGATGAGCAGGTCTATGC-3′. Five micrograms of total RNA was used for reverse transcription in each case. The amplification profile involved denaturation at 94°C for 1 min, primer annealing at 45°C for 1 min, and extension at 72°C for 1 min. This cycle was repeated 30 times. The amplified DNA was ethanol-precipitated, and the pellet was dried, dissolved in Tris/EDTA buffer, and electrophoresed in a 2.5% agarose gel. An oligonucleotide, 5′-CACAGTCAATGGTGACCCAG-3′, which spanned a portion of the region between the two primers in the reported sequence for the rat NMDAR1 cDNA (6), was kinase-labeled and used as a probe for Southern blotting (26). Exposure of the autoradiogram was for 30 min.

PCR products were extracted using GeneClean II (Midwest Scientific). Approximately 300 ng of each isolated band was sequenced with 3.2 pmol of the 5′ primer using a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). The cycle sequencing reactions were performed in a Perkin–Elmer 9600 thermal cycler for 25 cycles with a profile of 96°C for 15 s, 50°C for 1 s, and 60°C for 4 min. Following separation of the extension products on a Select-D G-50 column (5 Prime 3 Prime, West Chester, PA), the reaction mixtures were dried, resuspended in 4 ml of 5:1 formamide/50 mM EDTA, loaded on a 6% sequencing gel, and analyzed using an Applied Biosystems 373 fluorescent sequencer.

Excitotoxicity. To determine if NT2-N cells are susceptible to glutamate excitotoxicity, cultures were exposed to glutamate and then examined for loss of plasma membrane integrity [by assaying release of lactate dehydrogenase (LDH) (27)] and of mitochondrial function [by measuring their capacity to cleave 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a formazan product (28)].

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Abbreviations: GluR, glutamate receptor; CNS, central nervous system; NMDA, *N*-methyl-D-aspartate; RA, retinoic acid; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CSS, control salt solution; FBS, fetal bovine serum; APV, DL-2-amino-5-phosphonovaleric acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; P/S, penicillin/streptomycin; *I–V*, current–voltage.

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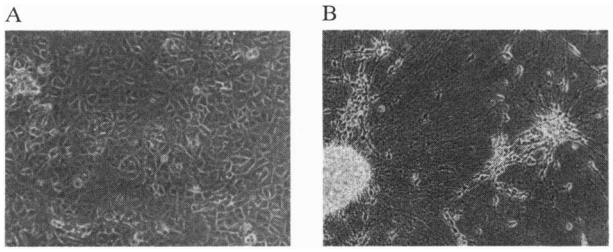

FIG. 1. Phase-contrast micrographs of undifferentiated NT2 (*A*) and differentiated NT2-N (*B*) cells. (×150.)

Cells were washed once with Dulbecco's phosphate-buffered saline (PBS; 37°C) and then exposed to the indicated medium for 15 min at 37°C. Control cells were exposed to a control salt solution (CSS) containing 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 15 mM glucose, 25 mM Hepes, and 50 μM glycine (pH 7.4); 1 mM L-glutamate was added to CSS in the presence or absence of 300 nM MK801. Following the exposure, cells were washed twice with PBS, fed with OptiMEM-I without FBS, and returned to the incubator. LDH and MTT reduction were measured 24 hr later. Previous studies indicated that the cells could survive in OptiMEM-I without FBS for longer than 48 hr without changing morphology, releasing LDH, or decreasing MTT reduction. LDH was measured in the culture medium by the colorimetric detection of pyruvate (29) using a Sigma diagnostic kit (kit 500) and the modification of Priestley *et al.* (30). MTT reduction was measured by the colorimetric detection of the formazan product of MTT cleaved in active mitochondria (Promega cell titer 96). The result was used as an estimate of the number of functioning mitochondria present in the culture dish and indirectly of the number of functionally intact cells (28).

Electrophysiology. Membrane currents were measured under voltage clamp condition in either whole-cell or outside-out configuration of the patch clamp technique (31). Recordings of single-channel currents were first filtered at 2 kHz and then digitized at 5 kHz. Rapid step changes of external solutions were achieved in <1 ms as described (32). External solutions consisted of 150 mM NaCl, 2 mM $CaCl_2$, 3 mM KCl, and 10 mM Hepes, buffered to pH 7.3. No Mg^{2+} and 10 μM glycine was present unless otherwise marked; 1 mM $MgCl_2$ and no glycine was present for recordings marked + Mg^{2+}, − Glycine. The internal pipette solution consisted of 140 mM $CsMeSO_4$, 10 mM Hepes, 5 mM bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate (sodium salt), 1 mM $MgCl_2$, and 2 mM $CaCl_2$, buffered to pH 7.3. To establish the EC_{50} for the glutamate-evoked current responses, glutamate concentrations between 0.2 and 5000 μM were used.

RESULTS

To determine if the NMDA receptor gene was expressed in NT2-N cells, we used PCR to amplify a 332-nucleotide sequence partially overlapping the putative glutamate binding domain of the rat NMDA receptor NMDAR1 (6). Fig. 3 illustrates that this amplification product was detected in 2-week-old NT2-N cells (point C on time line in Fig. 2) but not in untreated NT2 cells (point A in Fig. 2) or in NT2 cells that had been treated with RA for only 3 days (point B in Fig. 2; data not shown). The nucleotide sequence of the amplification product (Fig. 4*A*) was 90% homologous with the corresponding region of the rat NMDAR1 cDNA and encodes an amino acid sequence 97% homologous with that of the corresponding domain of the rat NMDAR1 protein (Fig. 4*B*).

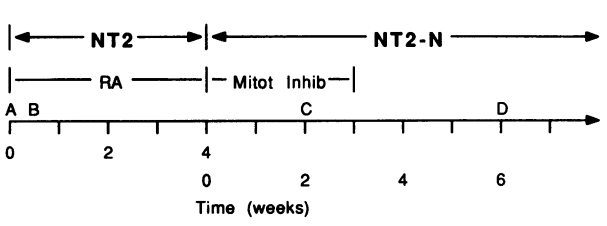

FIG. 2. The time line illustrates the method used in treating and naming the cells and the ages (points A–D) at which cells were studied. At time A, we performed PCR and cytotoxicity; at B, PCR; at C, PCR, electrophysiology, and cytotoxicity; and at D, electrophysiology and cytotoxicity. Mitot Inhib, mitotic inhibition.

Functional expression of GluR channels in NT2-N cells was examined using patch clamp techniques (31). Glutamate-evoked currents became detectable in some NT2-N cells at 2 weeks (point C, Fig. 2) and were present in all cells by 6 weeks (point D, Fig. 2). Fig. 5*A* illustrates the whole-cell currents from a 6-week-old NT2-N cell evoked with rapid step applications of glutamate (500 μM). Two kinetically distinct current components were seen. There was an initial, rapidly activating, and desensitizing current which was selectively eliminated by addition of the non-NMDA antagonist CNQX (5 μM; Fig. 5*A*). This initial current was followed by a second, slowly activating, desensitizing and deactivating current. Addition of Mg^{2+} and omission of glycine from the external perfusion solution selectively eliminated the slow current component. The latter two factors markedly attenuate current flow through NMDA channels (17–19). Fig. 5*B* shows that the slowly activating and desensitizing current is activated by the step application of the selective agonist NMDA (100 μM) and is attenuated by APV (200 μM). Further evidence that the slow current is mediated by NMDA channels is its voltage-dependent block by external Mg^{2+}. In the *I–V* relationship shown on the left of Fig. 5*C*, negligible amount of current is measured at membrane potentials held below −40 mV in the presence of 1 mM Mg^{2+}. In the absence of external Mg^{2+}, the *I–V* relationship is approximately linear. Reversal potential ranges between 10 and 15 mV under these recording conditions. On the other hand, the fast transient current remains approximately linear in the presence of external Mg^{2+} and reversal potential ranges between 0 and 5 mV. The EC_{50} for the NMDA current component with glutamate as the agonist was 3.8 μM (n = 7), which is close to that measured for the NMDA receptor in mouse hippocampal neurons (20). The EC_{50} for the peak and plateau components of the non-NMDA current were 270 and 15 μM

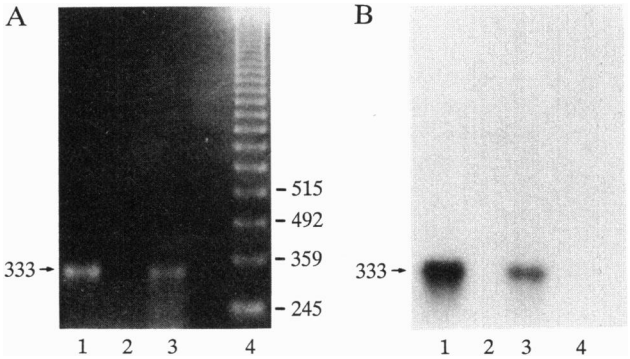

FIG. 3. PCR demonstrates NMDA receptor mRNA in NT2-N cells. (*A*) Ethidium bromide-stained agarose gel of PCR products. (*B*) Autoradiogram of a Southern blot from the same gel. Lanes 1, adult rat brain; lanes 2, NT2 cells (point A in Fig. 2*C*); lanes 3, 2-week-old NT2-N cells (point C in Fig. 2*C*); lanes 4, nucleotide sizing ladder. *A* shows that a PCR product of the same apparent size was amplified in rat brain and NT2-N cells but was not detected in NT2 cells. *B* shows that the human NT2-N cell PCR product, as well as the rat brain PCR product, hybridizes to an oligonucleotide corresponding to a sequence of the published rat NMDA receptor (6) flanked by the 5′ and 3′ primers.

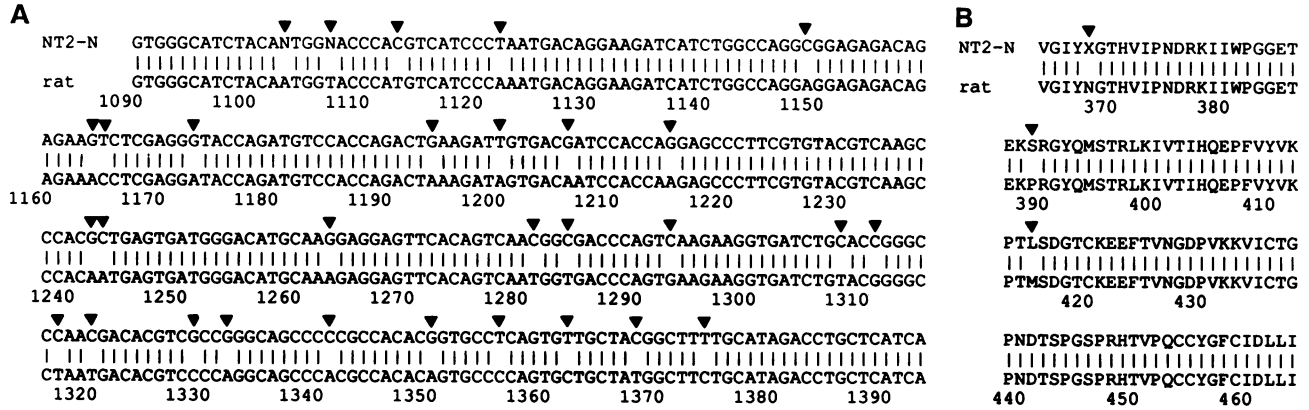

FIG. 4. The oligonucleotide (*A*) and deduced amino acid (*B*) sequences of the PCR-amplified segment of the human NT2-N and adult rat NMDA receptor channel are compared. Discrepancies between the rat and human sequences are indicated by arrowheads. Confirmation that the rat brain and human NT2-N cell PCR products were amplified from NMDA receptor mRNA was obtained by finding that the rat PCR product was identical in sequence to residues 1090 through 1393 of the rat NMDA receptor cDNA (6).

($n = 12$), respectively, results similar to those described in rat hippocampal neurons (20, 33, 34).

Identification of multiple GluR channels in NT2-N cells at the single channel level is illustrated in Fig. 6. In primary neuronal cultures, NMDA channel openings are characterized by high unitary conductance (50 pS) and slow activation, desensitization, and deactivation kinetics (35–38). Non-NMDA channel openings are characterized by a number of different but generally lower unitary conductances (32, 39, 40) that open rapidly and then desensitize rapidly. Fig. 6*A* shows NMDA and non-NMDA channels coexisting in an excised NT2-N membrane patch. After the addition of Mg^{2+} and the elimination of glycine, only the non-NMDA channels remain. A 35-pS non-NMDA channel is illustrated in Fig. 6*A*. Lower conductance non-NMDA channels are recorded in two other patches shown in Fig. 6*B*. The existence of multiple subtypes of non-NMDA channels in NT2-N cells is consistent with and supports the concept of a heterogeneous family of non-NMDA GluR channels (11, 41). Fig. 6*C* illustrates that NMDA channels in NT2-N cells have the characteristic high single channel conductance and slow gating kinetics described for CNS neurons. One subtle difference is the relatively slower activation of NT2-N channels. Fig. 6*D* illustrates rapid "flickery" block of the NT2-N NMDA channels by low concentration of Mg^{2+}. These data clearly show that the NT2-N cells express GluR channels.

To determine if NT2-N cells are susceptible to glutamate excitotoxicity, cultures were exposed to glutamate and then examined for loss of plasma membrane integrity and mitochondrial function. Exposure to 1 mM L-glutamate for 15 min (Fig. 7) in a medium containing L-glycine and devoid of Mg^{2+} reproducibly damaged NT2-N cells when they were 6 weeks old. NT2 cells and 2-week-old NT2-N cells were not injured. This time feature parallels the development of neuritic polarity (a characteristic of terminally differentiated neurons) in NT2-N cells (23). Glutamate cytotoxicity was blocked by MK801.

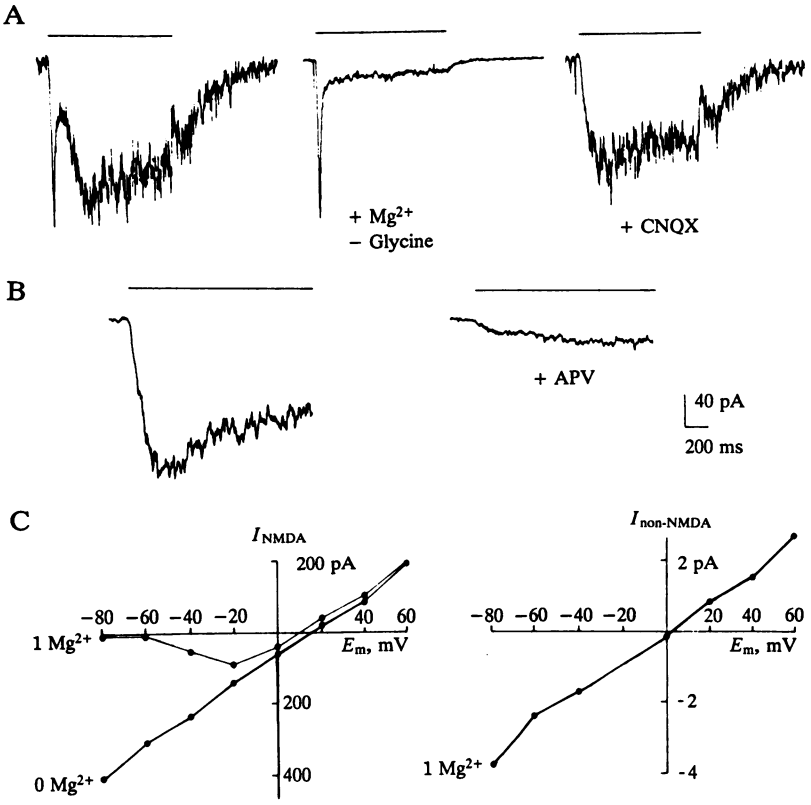

FIG. 5. Electrophysiologic and pharmacologic identification of GluR channels in NT2-N cells. (*A*) Rapid step applications of 500 μM glutamate (marked by bars) were applied to an NT2-N cell under conditions that were either permissive for current flow through NMDA and non-NMDA channels (left), selective for the non-NMDA channels (center, marked + Mg^{2+}, − Glycine), or selective for the NMDA channels [right, marked + CNQX (where CNQX indicates 6-cyano-7-nitroquinoxaline-2,3-dione)]. A fast transient current and a second slower current can be clearly distinguished by their kinetics. The fast transient current component is selectively eliminated by the non-NMDA antagonist CNQX (5 μM). The slower component is selectively eliminated by external Mg^{2+} and removal of glycine, conditions that minimize current flow through NMDA channels. (*B*) Further identification that the slow current component is mediated by the NMDA receptor is shown. Application of NMDA (100 μM) results in the selective activation of the slow current. In the presence of the selective antagonist DL-2-amino-5-phosphonovaleric acid (APV, 200 μM), this slow NMDA-activated current is attenuated. (*C*) The current–voltage (*I–V*) relationship for the NMDA-activated peak current is shown on the left with and without external Mg^{2+}. The *I–V* relationship for the fast transient glutamate-activated peak current is shown on the right. Ensemble-averaged excised patch recordings were used for the fast transient non-NMDA *I–V* relationship.

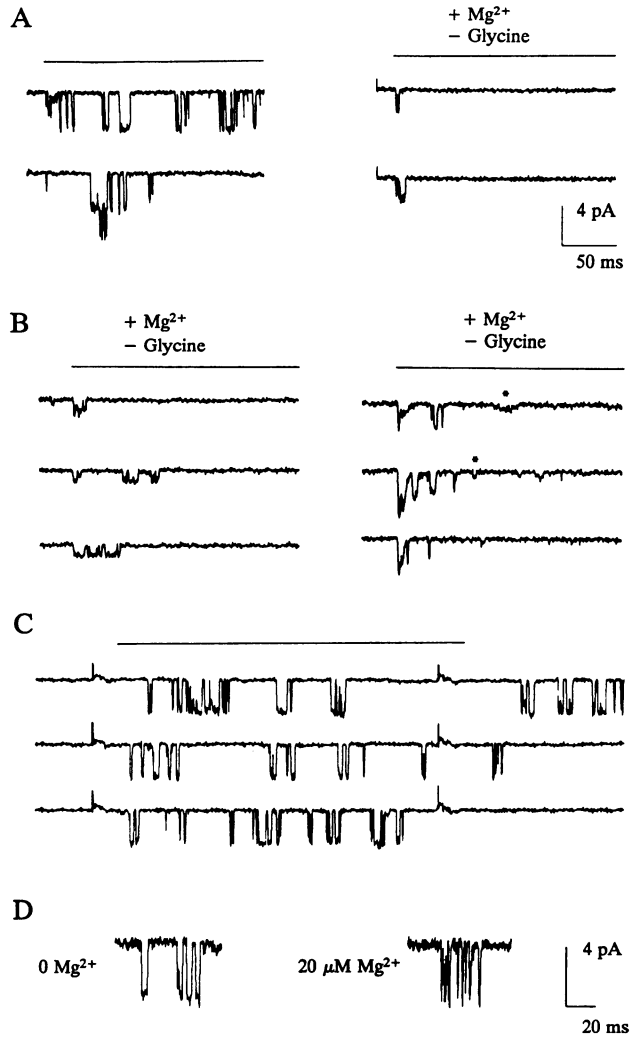

FIG. 6. Identification of NMDA and non-NMDA channels at the single channel level in response to rapid step application of glutamate (500 μM). (*A*) NMDA and non-NMDA channel openings can be seen coexisting in a single excised outside-out membrane patch. The slower and higher conductance NMDA openings are completely eliminated by addition of external Mg^{2+} (1 mM) and removal of glycine. Non-NMDA channels are characterized by lower unitary conductance, rapid initial opening, and low probability of repeated openings. (*B*) A number of different unitary conductances for the non-NMDA channels can be seen in NT2-N cells. Events marked with an asterisk (*) identify openings with conductances <10 pS. (*C*) In the absence of external Mg^{2+}, and presence of glycine (10 μM) and CNQX (5 μM), high-conductance NMDA openings can be seen. Note the slow activation and slow deactivation kinetics that are characteristic of these receptor channels. (*D*) The presence of low concentrations of Mg^{2+} induces "flicker block" of the NMDA openings. Current and time scales are the same in *A–C*. Membrane potential was held at −80 mV.

DISCUSSION

We have shown that human postmitotic NT2-N neurons derived from RA treatment of a teratocarcinoma cell line (NT2) express functional GluR channels. NT2-N cells are almost indistinguishable from primary neuronal cultures obtained from rodents (23). In earlier studies, the number of NT2-N cells after RA treatment of NT2 cells represented only a small percentage of the cells in culture, and they coexisted with a population of unidentified large flat cells and a residual number of undifferentiated stem cells (21, 22). However, we recently developed a strategy to obtain nearly pure (>95%) neuronal cultures of human neurons from RA-treated NT2 cells and have characterized these NT2-N cells extensively (23). For example, NT2-N cells are permanently postmitotic, express all well-defined CNS neuronal marker proteins, and elaborate processes that can be identified as axons or dendrites using molecular and functional criteria. NT2-N cells express α-internexin (a CNS-specific intermediate protein) but not peripherin (a peripheral nervous system-specific intermediate protein); this suggests that NT2-N cells correspond to late embryonic human CNS neurons. Since large quantities of NT2-N cells can be generated and maintained in a fully differentiated state for weeks, these cells provide a unique system whereby unlimited numbers of identical, terminally differentiated, human neuron-like cells can be used to study the regulation of GluR expression and other fundamental aspects of GluR biology.

Many other neuron-like cell lines have been screened for GluR channels. For example, glutamate cytotoxicity has been demonstrated in the neuroblastoma–embryonic retinal hybrid cell line N18-RE-105, but this is not mediated via NMDA receptor channels (42–44). The HCN-1A cell line, established from human cerebral cortical neurons, stains positively for glutamate, but GluR channels have not been reported in HCN-1A cells (45). Other neuronal cell lines, such as HT-4, demonstrate NMDA-sensitive neurotransmitter release (46), and NCB-20, a mouse neuroblastoma–Chinese hamster brain hybrid cell line, contains mRNA encoding the NMDA/phencyclidine receptor (47). However, direct electrophysio-

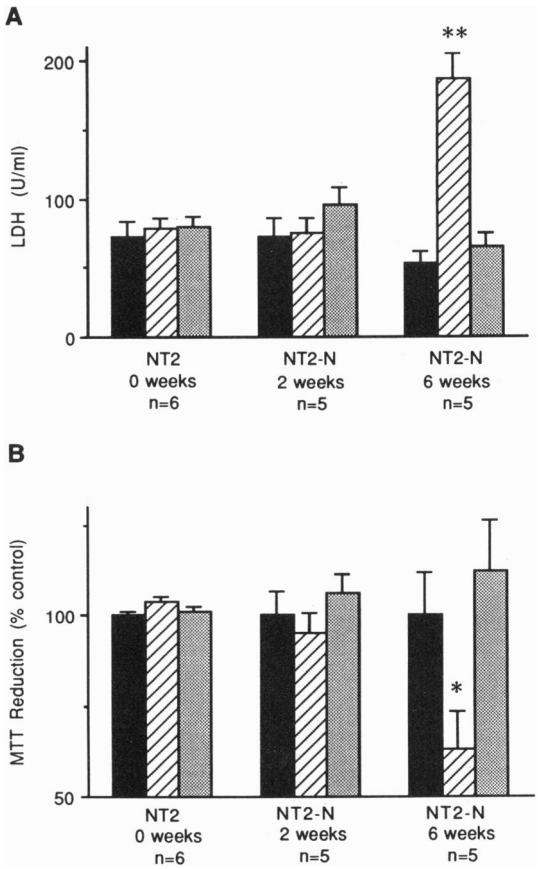

FIG. 7. Evidence of glutamate cytotoxicity in NT2-N cells. Cells were studied at three time points. NT2 cells (point A in Fig. 2) were studied prior to RA treatment. NT2-N cells were studied 2 and 6 weeks after replating on Matrigel (points C and D in Fig. 2). Values are reported as mean ± SEM. There were no differences between treatment groups with NT2 and 2-week-old NT2-N cells. In 6-week-old NT2-N cells, glutamate exposure caused increased LDH release (**, $P < 0.001$ by ANOVA) and decreased MTT reduction (*, $P < 0.05$, ANOVA). ■, Control; ▨, glutamate; ▩, glutamate plus MK801.

logical recordings, which are the signature of functional GluR channels, have not demonstrated NMDA receptor channels in any neuronal cell line except NT2-N cells.

It is puzzling that no one has directly and reproducibly demonstrated GluR channels in neuronal cell lines. The absence of cell lines stably expressing GluR channels could be explained on the basis of the results presented here. First, neuronal cells bearing GluR channels may not survive in culture, because glutamate in the culture medium may induce excitotoxic cell death. However, undifferentiated NT2 cells, which resemble neuroblasts, do not express GluR channels, and they survive and multiply in medium containing glutamate. Second, the expression of GluR channels may be linked to the commitment to terminal neuronal differentiation. Unlike NT2-N neurons, most neuronal cell lines are incapable of terminal differentiation. Although exogenously applied factors can arrest cell division and allow for the expression of more differentiated characteristics [e.g., NGF's effect on PC12 cells (48, 49)], removal of these factors usually results in reentry into the cell cycle and return to the more undifferentiated state. The ability of NT2-N cells to permanently exit the cell cycle and maintain fully differentiated neuronal characteristics after RA withdrawal could account for its unique ability to express neuronal GluR channels. Finally, the ability to express GluR channels may be specific to CNS neurons, since many neuronal cell lines, unlike the NT2-N cells, do not closely resemble postmitotic CNS neurons.

The ability to induce GluR channel expression in the NT2 human cell line is significant for several reasons. First, it provides an opportunity to study regulation of NMDA and AMPA/kainate GluR gene expression in a model system that is both clonal and neuronal. Second, because this line is amenable to molecular biological manipulations such as stable transfection and subcloning (23), it should be possible to manipulate the specific subunit composition of the various GluR channels, thus permitting correlation of molecular structure and physiologic properties. Third, because of their human origin and because prior to exposure to RA these cells are capable of rapid proliferation, the NT2 cell line will provide an unlimited supply of a homogenous population of cells for the study of GluR channels in relation to human neurological disorders.

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