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Research report

Glutamate receptor-induced toxicity in neostriatal cells

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Abstract

Infrared differential interference contrast (IR DIC) videomicroscopy was used to measure and characterize cell swelling induced by activation of glutamate receptors (GluR) in a neostriatal brain slice preparation. This swelling is, in many cases, a prelude to necrotic cell death. Activation of *N*-methyl-D-aspartate (NMDA) and non-NMDA ionotropic GluRs caused cell swelling. The concentration-response relationships and the time courses of the onset of agonist-induced swelling were very similar for NMDA and kainate (KA). However, cells were able to recover from KA but not NMDA-induced swelling. Results from ion substitution experiments suggest that sodium, chloride and to a lesser extent calcium ions play critical roles in this swelling. Heterogeneity in the response to NMDA occurred within cells of the neostriatum. Approximately 15% of the cells did not swell when exposed to NMDA. The magnitude of the NMDA-induced swelling also varied depending on the region of the nervous system. Swelling was greater in the neostriatum and neocortex than in the hippocampus and it did not occur in the suprachiasmatic nucleus. In conclusion, IR DIC videomicroscopy can be used to follow quantitatively the dynamics of GluR-evoked responses in single cells and should be instrumental in determining the factors capable of modifying excitotoxicity.

Keywords: Excitotoxicity; Hippocampus; Infrared videomicroscopy; Kainate; *N*-Methyl-D-aspartate; Neostriatum; Suprachiasmatic nucleus

1. Introduction

It is well-established that neostriatal cells are innervated by a major glutamate-containing projection from the neocortex [20,39,41,54]. Both NMDA and non-NMDA subtypes of GluRs have been localized to the neostriatum [1,43,62] and both subtypes of ionotropic GluRs contribute to excitatory post-synaptic potentials recorded in neostriatal neurons [7,9,25,29]. Dysfunctions in this excitatory cortical input have been suggested to play a role in the pathology of diseases affecting this region of the brain [2,17,58]. Thus, there is considerable interest in understanding GluR-induced toxicity in the neostriatum.

Previous work, primarily on cultured cells, has shown that an initial consequence of the application of GluR agonists is cell swelling and that this swelling is an early step in a cascade of events which can eventually lead to cell death [12,50]. Since it is possible to visualize unstained cells in brain slice preparations using infrared (IR) illumination, differential interference contrast (DIC) optics, and videomicroscopy [18,19,36], these techniques can be used to examine the dynamic physical changes as a rapid measure of toxicity in the brain slice preparation. At least two previous studies have used this technique in this manner [15,18].

In order to further understand the phenomenon of cell swelling in a brain slice, the present study was designed to: (1) characterize GluR-induced swelling of cells in the neostriatal brain slice; (2) examine the ionic dependence of NMDA-induced swelling; (3) explore the possibility of regional variation in the magnitude of this swelling.

2. Materials and methods

2.1. Preparation of neostriatal slices

Male Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN) 13–15 days old were used. After animals were killed by decapitation, brains were dissected and placed in cold oxygenated artificial cerebral spinal fluid (ACSF) containing (in mM) NaCl 130, $NaHCO_3$ 26, KCl 3, $MgCl_2$ 2, NaH_2PO_4 1.25, $CaCl_2$ 1.0, glucose 10 (pH 7.2–7.4). After cutting, transverse sections (350 μm) were placed in ACSF at 25–27°C for at least 1 h (in this

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solution $CaCl_2$ was increased to 2 mM, and 4 mM lactate was added). Individual tissue sections were then transferred to the perfusion chamber in which the slice was held down with thin nylon threads. The slice was submerged in continuously flowing oxygenated ACSF (25°C) at a rate of 2 ml/min.

2.2. IR DIC videomicroscopy

The brain slice was viewed with an upright compound microscope (Zeiss Axioskop) using a 40 × water immersion lens (Zeiss, achroplan, numerical aperture 0.75) and DIC optics. Slices were illuminated with near infrared light by placing an infrared bandpass filter (790 nm, Ealing Optics, Hollston, MA) in the light path. This filter allowed passage of light between 750–1050 nm and thus cut off much of the longer wavelength infrared radiation which would heat the tissue. The image was detected with an infrared-sensitive CCD camera (Hamamatsu C2400, Tokyo, Japan) and displayed on a video monitor. Analog contrast enhancement and gain control were provided by the camera controller. Digital images were stored on a computer/optical disk for subsequent analysis and additional digital contrast adjustment when necessary. Cells could be visualized to a depth of about 100 μm below the surface of the slice. In order to quantify changes in response to activation of GluRs, image analysis software (Optimas, BioScan, Edmonds, WA) was used to measure cross-sectional somatic area (the perimeter, maximal length and width were also measured in some cells) prior to, during and after experimental treatments. Each measurement was made twice and the average value recorded. Measurements were only taken from cells which exhibited clear borders, concave shapes, and phase brightness (Fig. 1). The identity of cells from the suprachiasmatic nucleus (SCN) was confirmed by histological analysis (Nissl stain) after data had been collected.

2.3. Solutions and drugs

The composition of solutions in ionic substitution experiments is shown in Table 1. Osmotic pressure was measured and when necessary was maintained by the addition of sucrose (osmolarity 290–300 mOsm). Only the low chloride solution required such adjustments. Pharmacological agents (unless otherwise noted were purchased from Research Biochemicals, Natick, MA) included amino-3-hydroxy-5-methyl-4-isoazole (AMPA),

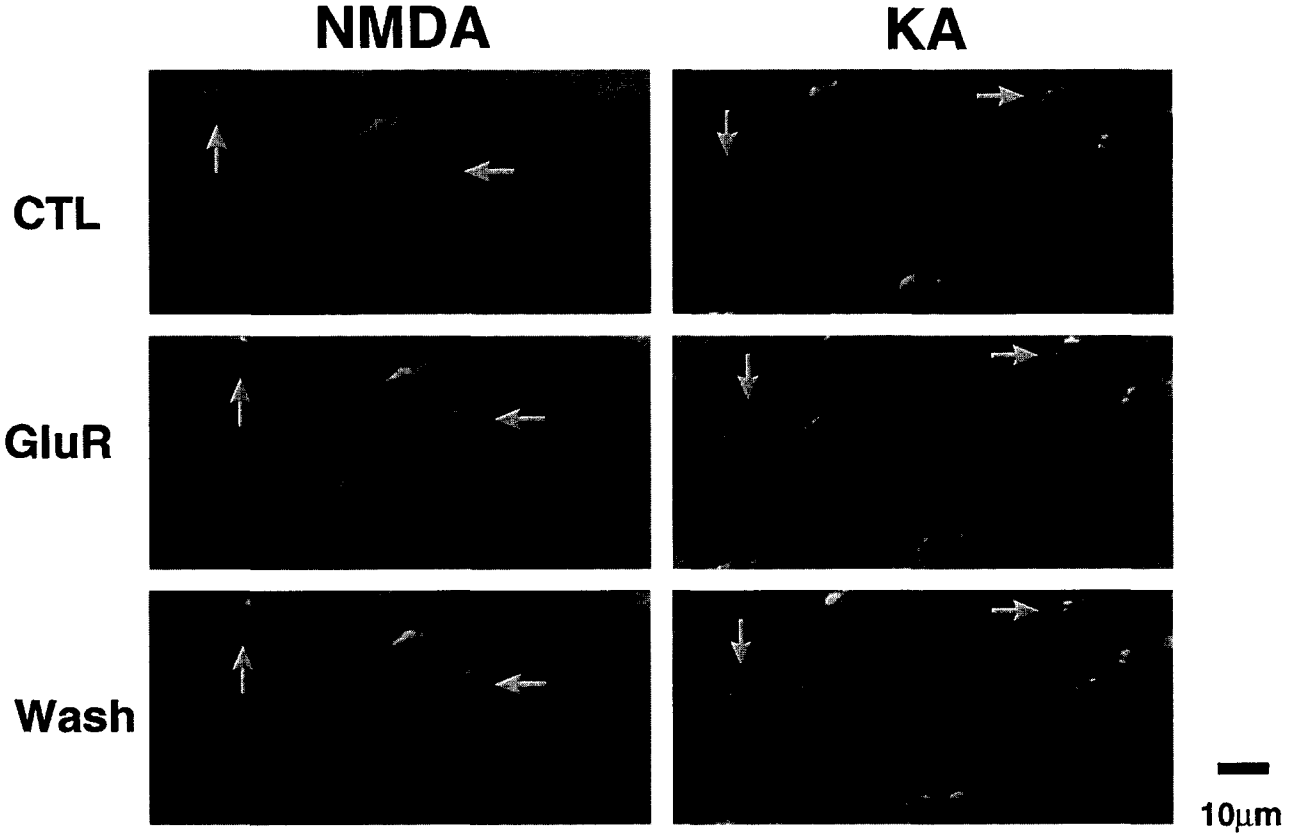

Fig. 1. Neurons in a neostriatal brain slice from a 14-day-old rat visualized by IR DIC videomicroscopy. Top panels: images of cells under control conditions. Middle panels: same cells after 10 min exposure to 100 μM NMDA (left) or 100 μM KA (right). Both GluR agonists cause cells to swell. Bottom panels: cells after 10 min wash in normal ACSF. In general, cells 'recovered' from KA − but not NMDA − induced swelling. For reference, arrows were placed next to two cells in each panel.

DL-2-amino-5-phosphonopentanoic acid (AP5), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), glutamic acid (glutamate, Sigma), KA (Sigma), 2,3 pyridinedicarboxylic acid (quinolinic acid, Sigma) and NMDA (Sigma). The AMPA/KA GluR antagonist CNQX was dissolved in dimethyl sulfoxide (10 mM stock solution) prior to final dilution. Concentrations were chosen based on our experience with electrophysiological studies in neostriatal slices.

2.4. Statistical analyses

Differences between average values for experimental and control groups were evaluated using *t*-tests or Mann–Whitney rank sum tests when appropriate. Values were considered significantly different if $P < 0.05$. All tests were performed using Sigmastat (Jandel, San Rafael, CA). In the text, values are shown as mean ± S.E.M.

3. Results

A total of 1834 neostriatal cells were visualized using DIC IR videomicroscopy. An example of a field of neostriatal cells is shown in Fig. 1 (top panels). The effects of experimental and control manipulations were determined by measuring cell area before and after treatments (results are presented in the text and figures as percent change in area). Measurements of cell area did not change under control conditions in which the cells were continuously perfused with ACSF (after 30 min, 1 ± 1% swelling, $n = 44$).

3.1. GluR-mediated cell swelling

Bath application of NMDA, AMPA, KA and glutamate caused swelling of neostriatal cells (Figs. 1 and 2). The concentration–response relationships were similar for the GluR agonists with the exception of glutamate which produced significantly ($P < 0.05$) less swelling than the other agonists at concentrations greater that 100 μM. This swelling was limited to ionotropic glutamate receptor agonists as the metabotropic glutamate receptor agonist 1-amino-cyclopentane-1,3-dicarboxylic acid (tACPD) did not cause swelling when applied alone, and limited NMDA-induced swelling [15].

NMDA-induced swelling (100 μM, 10 min) was blocked by treatment with the competitive antagonist AP5 but not by the AMPA/KA GluR antagonist CNQX (NMDA: 27 ± 2% swelling, $n = 94$; 50 μM AP5 + NMDA: 0 ± 3% swelling, $n = 30$; $P < 0.001$; 5 μM CNQX + NMDA: 27 ± 4% swelling, $n = 21$). Similarly, the NMDA GluR agonist quinolinic acid (QA) [55] caused AP5-sensitive swelling (QA 10 μM, 10 min: 32 ± 8% swelling, $n = 18$ vs. 50 μM AP5 + QA: 3 ± 4% swelling, $n = 18$; $P < 0.001$). In contrast, KA- (100 μM, 10 min) and AMPA- (100 μM, 10 min) induced swelling was inhibited by CNQX (5 μM CNQX + KA: 1 ± 1% swelling, $n = 33$ vs. KA: 32 ± 3% swelling, $n = 87$; $P < 0.001$; 5 μM CNQX + AMPA: 14 ± 3% swelling, $n = 9$ vs. AMPA: 38 ± 4% swelling, $n = 44$; $P < 0.001$). This KA-induced swelling was not blocked by treatment with AP5 (AP5 + KA: 41 ± 7% swelling, $n = 17$). By themselves, AP5 and CNQX did not alter cell area (AP5 50 μM, 10 min: −1 ± 2% swelling, $n = 33$; CNQX 5 μM, 10 min: 3 ± 1% swelling, $n = 21$).

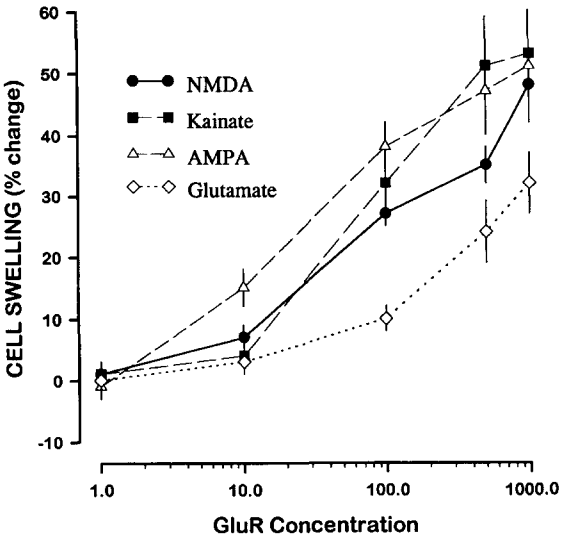

Fig. 2. GluR agonists cause concentration-dependent swelling of neostriatal neurons. Cross-sectional areas of cells were determined before and after 10-min treatments and are expressed as % change. The magnitudes of the responses evoked by the GluR agonists were similar with the exception of glutamate which produced significantly ($P < 0.05$) less swelling than the other agonists at concentrations greater than 100 μM. Concentrations of 100 μM or greater of each agonist produced swelling which was significantly different from untreated controls. Error bars indicate standard error in this and subsequent figures.

Table 1
Composition of solutions

	Solution (mM)					
Compounds	ACSF	Lo Na^+	Lo Cl^-	Lo Ca^{2+}	Lo K^+	Hi K^+
NaCl	130			130	130	33
KCl	3	3		3		100
NaH_2PO_4	1.25		1.25	1.25	1.25	1.25
$NaHCO_3$	26		26	26	26	26
$MgCl_2$	2	2		2	2	2
$CaCl_2$	2	2		1	2	2
glucose	10	10	10	10	10	10
CholineCl		130			3	
Na_2SO_4			65			
K_2SO_4			1.5			
$MgSO_4$			2			
CaGluconate		4				
EGTA						10

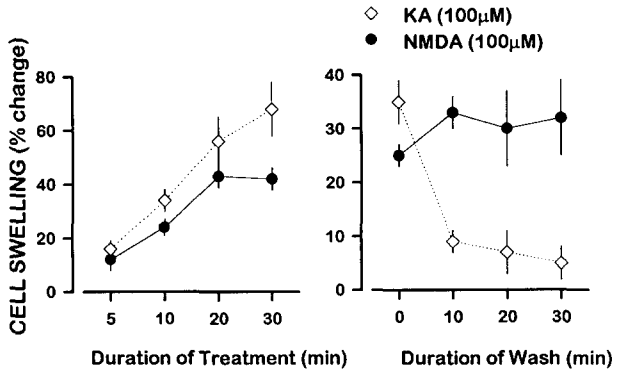

Fig. 3. Time course of NMDA- and KA-induced swelling and recovery. Left panel: cells were exposed to 30 min treatments of NMDA (100 μM) or KA (100 μM). The magnitude of cell swelling varied as a function of the duration of exposure to the agonist. Right panel: cells were exposed to 10-min treatments of NMDA or KA and then were allowed to recover for 30 min under control conditions. Cells 'recovered' from KA- but not NMDA-induced swelling.

The time course of NMDA's actions was investigated by exposing slices to NMDA for up to 30-min duration (Fig. 3, left panel). The swelling began within 5 min and generally continued to increase up to the end of the 30-min treatment. Some cells swelled to such an extent that their borders became indistinct and could no longer be visualized. More cells 'disappeared' in this manner when treated with higher concentrations or longer durations of NMDA. For example, proportionately more cells were lost (could no longer be visualized) after a 10-min treatment with 500 μM NMDA (17%, 8/47 cells) than a 10-min treatment with 50 μM NMDA (9%, 12/138 cells). Similarly, with 30-min treatments, more cells disappeared when exposed to 500 μM NMDA (78%, 36/46 cells) than when exposed to 50 μM (49%, 20/41 cells). Data from cells which disappeared were not used in the analysis.

Approximately 15% of the cells did not swell when exposed to either the low (50 μM, 10 min: 16/138 cells $< 10\%$ swelling) or high (500 μM, 10 min: 7/47 cells $< 10\%$ swelling) concentrations of NMDA. Of the cells that did swell in response to NMDA, very few showed evidence of recovery (Fig. 3, right panel). After a 10-min or 30-min wash, the overall magnitude of NMDA-induced swelling was not significantly altered (NMDA 100 μM, 10 min: $25 \pm 2\%$ swelling, $n = 88$ vs. $33 \pm 3\%$ swelling after 10-min wash and $32 \pm 7\%$ swelling after 30-min wash). After the 10-min wash, only 8 out of 88 cells returned to within 10% of their original area. In contrast, KA-induced swelling was readily reversible within 10 min of return to normal saline (KA 100 μM, 10 min: $35 \pm 4\%$ swelling, $n = 46$ vs. $9 \pm 2\%$ swelling after 10-min wash and $5 \pm 3\%$ swelling after 30-min wash). After a 10-min wash, 30 out of 49 cells returned to within 10% of their original area. Thus, there were marked differences in the ability of neostriatal cells to recover from NMDA- and KA-induced swelling.

3.2. Ionic dependence of NMDA-induced cell swelling

Since neurons appear to be particularly sensitive to toxic effects of NMDA receptor activation, ion substitution experiments were performed to better understand the mechanisms underlying NMDA-induced cell swelling (Fig. 4). When NMDA (50, 100, 500 μM, 10 min) was applied in low Na^+ or Cl^-, swelling was greatly reduced or eliminated ($P < 0.001$). These low Na^+ and low Cl^- solutions were also effective in preventing swelling when the duration of exposure to NMDA (500 μM) was increased from 10 to 30 min ($P < 0.01$). Since NMDA GluRs are permeable to Ca^{2+}, the effect of a low Ca^{2+} solution was also evaluated. Low Ca^{2+} solution blocked swelling induced by 50 μM NMDA ($P < 0.001$) and attenuated, but did not prevent, swelling induced by 100 μM NMDA ($P < 0.01$). This protective effect was completely overcome when NMDA concentration was increased to 500 μM. Finally, a low K^+ solution did not prevent NMDA-induced swelling nor did a high K^+ (100

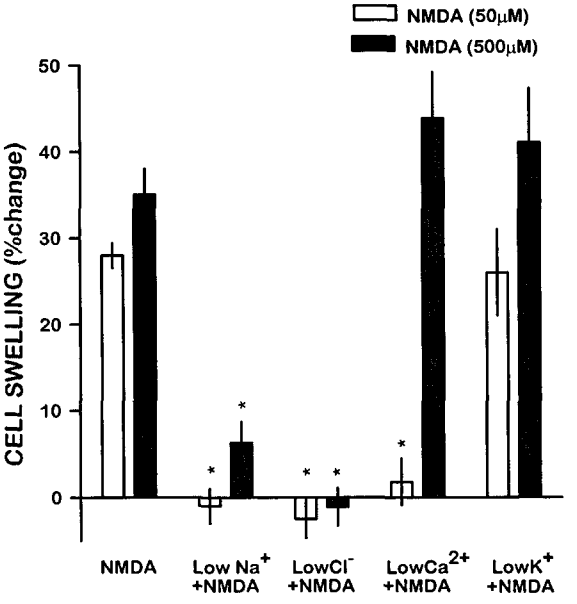

Fig. 4. Ionic dependence of NMDA-induced swelling. NMDA-induced swelling (50 μM, 10 min: open bars; 500 μM, 10 min: filled bars) was prevented by pretreatment with low Na^+ or Cl^-. Low Ca^{2+} prevented swelling induced by 50 μM NMDA but was ineffective when the NMDA concentration was raised to 500 μM. Low K^+ did not prevent swelling at either concentration of NMDA. In all cases, the test solutions were applied for 10min prior to the application of NMDA. Alone, the solutions did not alter cell area. The ionic composition of solutions is shown in Table 1. Stars indicate groups significantly different ($P < 0.001$) from NMDA alone.

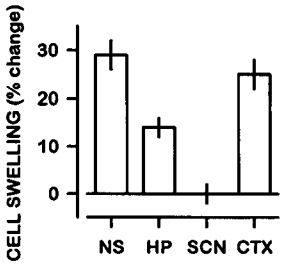

Fig. 5. Regional variation in magnitude of NMDA-induced swelling. Cells from the neostriatum (NS), hippocampus (HP), suprachiasmatic nucleus (SCN), and cortex (CTX) were exposed to NMDA (100 μM, 10 min). The HP cells were pyramidal cells of the CA1 region while the CTX cells were a mix of both non-pyramidal and pyramidal cells of the cortical region dorsal to the NS. The NMDA treatment produced significant swelling in all regions but the SCN.

mM) treatment cause swelling ($3 \pm 3\%$ swelling, $n = 30$). By themselves, low Na^+, Cl^-, K^+, or Ca^{2+} did not change cell area (low Na^+: $1 \pm 2\%$ swelling, $n = 38$; low Cl^-: $3 \pm 2\%$ swelling, $n = 74$; low K^+: $4 \pm 2\%$ swelling, $n = 38$; low Ca^{2+}: $2 \pm 2\%$ swelling, $n = 48$). These ion substitution experiments suggest central roles for Na^+ and Cl^- in mediating the NMDA-induced cell swelling, a more peripheral role for Ca^{2+}, and little role for K^+.

3.3. NMDA-induced swelling in cells from other brain regions

In order to explore potential heterogeneity in NMDA's actions, the magnitude of NMDA-induced swelling was examined in several regions of the brain (Fig. 5). Table 2 shows the average size of the cells in each region as determined by videomicroscopy. NMDA caused robust swelling in the neostriatum and cortex. In the hippocampus, NMDA (100 μM, 10 min) caused a small but significant increase in pyramidal cell area ($14 \pm 3\%$ swelling, $n = 75$, $P < 0.001$). In cells of the SCN of the basal hypothalamus, 10-min treatments with NMDA did not cause significant swelling even at the highest concentrations examined (NMDA 1000 μM, 10 min: $9 \pm 2\%$ swelling, $n = 24$). In order to produce a significant response, SCN cells had to be exposed to 1000 μM NMDA for 30 min ($20 \pm 5\%$ swelling, $n = 13$; $P < 0.01$). These hypothalamic cells were also relatively resistant to KA-induced swelling (KA 1000 μM, 10 min: $27 \pm 3\%$ swelling, $n = 21$; $P < 0.001$). Thus, there is clear evidence for regional variation in the magnitude of the GluR-induced cell swelling.

Table 2
Morphological parameters

Measurements	NS	HP	SCN	CTX
Sample size	1834	103	113	104
Area	70 ± 0.7	136 ± 5	73 ± 3	94 ± 4
Perimeter	35 ± 0.3	48 ± 1	34 ± 0.8	39 ± 1
Length	14 ± 0.1	19 ± 0.5	13 ± 0.4	14 ± 0.4
Width	8 ± 0.1	10 ± 0.2	8 ± 0.2	11 ± 0.3

4. Discussion

The present findings clearly demonstrate that IR DIC videomicroscopy can be used to measure and characterize GluR-induced cell swelling in a neostriatal brain slice preparation. Activation of NMDA and non-NMDA ionotropic GluRs caused swelling, a response that is often a prelude to necrotic cell death. The kinetics of the onset of swelling were similar for NMDA and KA but recovery occurred after exposure to KA but not NMDA. The results of the ion substitution experiments suggest that multiple ions including Na^+, Cl^-, and to a lesser extent Ca^{2+} play critical roles in the NMDA-induced swelling. Finally, the magnitude of swelling is cell-type specific as NMDA-induced swelling was robust in the neostriatum and cortex, weaker in pyramidal cells of the hippocampus, and was not observed in cells of the SCN.

Previous work, primarily in cultured neurons, has established that cell swelling is an initial step in a cascade of events which can lead to the death of a cell [12,50]. In agreement, we have recently shown that in the neostriatal brain slice preparation, exposure to NMDA causes cell swelling and increases the number of dead cells as measured by the trypan blue exclusion method [15,47]. In the present study, we have shown that IR DIC videomicroscopy can be used to follow the dynamics of GluR-mediated cell swelling and that these effects on the somatic cross-sectional area of neostriatal cells were reproducible and quantifiable. Most of the cells studied were probably medium spiny projection neurons which make up as much as 95% of the neuronal population in the neostriatum [22]. Large interneurons could easily be identified in our slice preparation and were not included in the data set. We have also filled cells in this brain slice preparation with biocytin or Lucifer yellow ([8], unpublished data). All cells of similar size to those measured in the present experiments exhibited the morphology of medium-sized neurons. However, at the ages examined in this study, it is difficult to clearly distinguish between medium-sized projection neurons and interneurons on the basis of morphology alone [30].

The data presented in this paper were collected from animals of approximately two weeks of age. This age was chosen to optimize visualization of the neurons in the brain slice and to some extent this is a potential limitation of the technique. It is harder to clearly define the borders of cells in older tissue. However, GluR agonists induced significant swelling in tissue of animals ranging from 7 to 28 days old (unpublished data). Furthermore, previous work on the development of neostriatal cells indicate that much of the extrinsic and local connectivity and electrophysiological properties of these cells mature in the first two

weeks after birth in the rat [57,59]. Thus, we feel that the findings of this study are likely to be applicable to older tissue.

A variety of GluR agonists were capable of inducing swelling in neostriatal cells. NMDA- and QA-induced swelling were blocked by the NMDA receptor agonist AP5 while KA- and AMPA-induced swelling were blocked by CNQX. This suggests that either NMDA or non-NMDA ionotropic GluR activation is sufficient to cause swelling. The concentration–response relationships and the time courses of the onsets of agonist-induced swelling were very similar for both NMDA and KA. However, there were clear differences in the recovery from swelling. Most cells treated with KA recovered within 10 min of wash. This recovery could be quite dramatic and some cells which had swollen to such an extent as to become phase invisible were able to recover to close to their original sizes. This result suggests that cell swelling is not always associated with immediate, necrotic cell death. Of course, if these cells were followed for a longer period, it is quite possible that they would eventually die through an apoptotic mechanism. In contrast, cells did not recover from NMDA-induced swelling at least during a 30-min wash. This finding is perhaps consistent with the tight association between NMDA receptor activation and cell death described previously [13,40,51]. This tight association led to our further study of NMDA-induced cell swelling.

The ionic mechanisms which underlie GluR agonist-induced toxicity have been previously studied. The initial response to GluR agonists is thought to involve Na^+ influx and membrane depolarization which in turn leads to an influx of Cl^- and eventual osmotic lysis of neurons [10,11,48,49]. The present results are consistent with these findings in cultured cells in demonstrating that the elimination of either Na^+ or Cl^- is sufficient to prevent NMDA-induced swelling. This blockade appears absolute as swelling was prevented even when the NMDA concentration was increased to 500 μM and duration of treatment lengthened to 30 min. More surprising was the finding that low Ca^{2+} also attenuated swelling produced by lower concentrations of NMDA. While removal of extracellular Ca^{2+} has been widely shown to prevent NMDA-induced toxicity [11,21,51], it is generally not thought to be involved in the acute GluR-induced cell swelling but rather to play a critical role in a temporally delayed cellular degeneration [12]. Ca^{2+} entry may only be a small part of the total ion flux carried by NMDA-gated ion channels (7–20%) [26,52], raising the possibility that Ca^{2+} influx acts as a trigger for some other as yet unknown process. Another somewhat surprising finding is that high K^+ did not alter neostriatal cell area. In cultured cortical and hippocampal cells, high K^+ mimics GluR-induced swelling [11,49]. One explanation may lie in the differences between the cell-type studied or, perhaps more interestingly, differences between slices of tissue and cells in culture. It certainly seems possible that the presence of glia in the brain slice preparation may buffer against volume changes caused by a high extracellular K^+ concentration [31,56,60]. Overall, the present data suggest that Na^+ and Cl^- play critical roles while Ca^{2+} is more peripherally involved in NMDA-induced cell swelling in the neostriatal slice.

In order to examine regional variation in the NMDA-induced cell swelling, NMDA was applied to cells visualized from several brain regions including the neocortex, hippocampus, and hypothalamus. As had been previously reported [18,49], NMDA caused swelling of hippocampal cells although the effect was proportionately smaller than that observed in neostriatal and cortical cells. Given the well-known sensitivity of hippocampal cells to excitotoxicity damage, we predicted a larger response. One possible explanation is that pyramidal cells are tightly packed in cell layers which may physically constrain their ability to swell. Even more resistant to NMDA-induced swelling were the SCN cells of the hypothalamus. These cells did not appear to be physically constrained and this finding is consistent with earlier studies which reported that cells in the SCN region are resistant to excitotoxic damage [24,46]. Despite this resistance, NMDA receptors are present in the SCN [23,28,42] and SCN neurons show electrophysiological responses to NMDA [5,27,37]. These results suggest that the electrophysiological and excitotoxic effects of NMDA receptor activation can be disassociated. Furthermore, as previously reported [3,45,53], the degree of NMDA-induced excitotoxicity appears to vary between different populations of neurons.

One of the advantages of the use of videomicroscopy and the cell swelling assay described in this paper is the ability to measure responses of single cells. Using this assay, we found evidence for heterogeneity in the cellular response to NMDA activation. In the neostriatum, approximately 15% of the cells did not swell in response to application of NMDA. This may be due to random variation or perhaps the non-swelling cells represent a resistant subpopulation of neostriatal neurons. Interestingly, a previous study reported that 17% of cultured neostriatal neurons did not show detectable responses to NMDA as measured electrophysiologically [35]. The finding that subpopulations of neurons within a particular brain region are selectively resistant to (and conversely vulnerable to) excitotoxic damage is well established in the neostriatum [3,4,16,32,38] as well as in other brain regions [6,14,33,34,44,61]. In general, the intrastriatal and regional heterogeneity in the magnitude of NMDA-evoked responses could simply be the reflection of differences in the magnitude of NMDA-induced ion flux. Differences which may simply be the result of variation in the number or subunit composition of the NMDA receptor itself. Alternatively, the variation may lie in cellular responses to the ionic influx. We hope to distinguish between these possibilities in future studies.

Acknowledgements

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