Lysosomal Dysfunction Produces Distinct Alterations in Synaptic α-Amino-3-Hydroxy-5-Methylisoxazolepropionic Acid and N-Methyl-D-Aspartate Receptor Currents in Hippocampus

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INTRODUCTION

The efficiency of excitatory synaptic transmission in the CNS depends heavily on uncompromised glutamate receptor-mediated communication between neurons (1–3). Regulated release of the glutamate from the presynaptic terminals and the concomitant activation of glutamate receptors in the postsynaptic sites are important for normal synaptic transmission (4–6). The glutamatergic transmission is mediated mainly via α-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors (7, 8). Functional decline of NMDA and AMPA receptors arising from altered receptor properties is thought to be responsible for memory impairment in aging and cognitive disorders including Alzheimer disease (AD) (9, 10). AMPA receptor subunits have been shown to decrease with aging and Alzheimer-type pathology (11–15), so are the binding sites for AMPA and NMDA receptors in hippocampus and other brain regions (12, 16).

In the aged mouse brain, glutamate receptor 1 (GluR1) immunoreactivity as well as AMPA receptor binding activity was found to be significantly reduced compared with young brain tissue, whereas other neurotransmitter receptors (dopamine, serotonin, and α-aminobutyric acid (GABA)) were unchanged (12). The reduction in AMPA receptors selectively occurred in the telencephalon, a large brain region responsible for many types of higher order behaviors. More pronounced decreases in protein and mRNA levels for GluR1 and GluR2/3 have been measured in the hippocampal slice model of protein accumulation (17, 18) and in AD (14, 19). Thus, reduction of glutamatergic communication is a major issue in the aged brain and in AD. It should be noted that although there is evidence for decreases in AMPA receptor subunits and their binding sites, a few reports indicate no change or increase in AMPA receptors in aging and AD (16, 20, 21).

Aging and age-related disorders such as AD result in gradual deterioration of the endosomal lysosomal system, which includes an increase in the number of secondary lysosomes and abnormalities in lysosomal chemistries (22–24). Lysosomal dysfunction has been suggested as an early manifestation of neurodegeneration (25, 26), resulting in Alzheimer-type formation of meglaneurites, neurofibrillary...
tangles, and β-amyloid-containing peptides (17, 27–30). Studies with experimentally induced lysosomal dysfunction in hippocampal cultures using the lysosomotropic agent chloroquine (CQN) have shown characteristics similar to those found in aged human brain and AD (17). CQN application has been shown to result in ultrastructural changes in the lysosomal system (31). Some of the features such as an increase in the number of secondary lysosomes, an increase in cathepsin D, amyloid precursor protein fragments, and induction of acyl hydrolases are considered primary responses to lysosomal inhibition, whereas neuronal dystrophy and loss of synaptic proteins are likely to be secondary responses. However, the overt signs of neuronal degeneration occurred only after 10 to 15 days of CQN treatment in the slice model (17). The loss of the presynaptic marker synaptophysin was remarkable and observed as early as 48 hours after CQN treatment and was followed by decreases in AMPA receptor subunits GluR1, GluR2/3, and NMDA receptor subunit NR1 48 to 96 hours later (17, 18, 31). Although these reports suggest that such changes may manifest in reduced glutamatergic synaptic transmission, most of the studies have focused mainly on biochemical and structural changes due to lysosomal inhibition. The putative alterations in glutamatergic synaptic transmission associated with lysosomal inhibition have not been investigated in detail.

In view of the above findings, we hypothesize that chemically induced lysosomal dysfunction downregulates hippocampal glutamatergic synaptic transmission, causing a time-dependent functional decline. Therefore, in the current study we investigated the effects of lysosomal dysfunction on the functional properties of hippocampal synaptic AMPA and NMDA receptors. We have used a combination of electrophysiologic analyses measuring whole-cell synaptic currents of cultured hippocampal slices and single channel currents of glutamate receptors from isolated synaptosomes to determine how changes in specific channel properties of synaptic receptors contribute to an altered amplitude and time course of synaptic currents. The results from this study suggest that both synaptic AMPA and NMDA receptor functions are progressively altered after CQN-induced lysosomal dysfunction.

**MATERIALS AND METHODS**

**Animals and Chemicals**

Sprague-Dawley rats (dams with pups) were obtained from Charles River Laboratories (Wilmington, MA) and housed with food and water provided ad libitum. All salts and reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless specified. The following drugs and chemical reagents were purchased from Invitrogen (Carlsbad, CA): horse serum, Hanks’ balanced salts, Earle’s balanced salt, minimal essential medium, penicillin/streptomycin, Fungizone, and glutamine. Culture plates, membrane inserts, microfilters and sterile pipettes were purchased from Fisher Scientific (Pittsburgh, PA).

**Antibodies**

Monoclonal antibodies against cathepsin D and NR1 were obtained from Upstate (Charlottesville, VA). Anti-GluR1 was prepared as described previously (32). The secondary antibodies were obtained from Amersham Biosciences (Piscataway, NJ).

**Immunoblotting**

Cultured hippocampal slices were collected and homogenized in 50 mM Tris, pH 7.5; 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 60 mM octyl-glucoside, and protease inhibitors. Protein content was determined using the DC Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Aliquots of each homogenate (30 µg) were diluted with equal amounts of sample buffer containing 2% sodium dodecyl sulfate, 50 mM Tris-HCl (pH 6.8), 10% 2-mercaptoethanol, 10% glycerol, and 0.1% bromphenol blue. Samples were boiled for 5 minutes and then were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 4% to 12% polyacrylamide gradient gels (Bio-Rad). Proteins were electrophoretically transferred onto nitrocellulose. After transfer, nitrocellulose membranes were blocked in 5% non-fat dry milk (NFDM) in Tris-buffered saline (TBS, pH 7.4) containing 0.1% Tween 20 for 1 hour at room temperature. Primary antibody incubations were carried out in 1% NFDM in TBS plus 0.1% Tween 20 overnight at 4°C. The antibodies used were GluR1 (1:1000), cathepsin D (1:200), and actin (1:5000). After overnight incubation, membranes were washed with 1% NFDM in TBS plus 0.1% Tween 20. Membranes were then incubated with either anti-mouse or anti-rabbit IgG (1:2000–1:10,000) for 1 hour at room temperature. After 3 washes in 1% NFDM in TBS plus 0.1% Tween 20 (10 minutes each), the blots were developed via enhanced chemiluminescence using ECL Plus (Amersham Biosciences).

**Organotypic Slice Cultures**

Organotypic hippocampal slice cultures were prepared according to the procedure of Stoppini et al with slight modifications (33). Briefly, the whole brains from 5- to 7-day postnatal Sprague-Dawley rats were isolated and placed in ice-cold Hanks’ balanced salt solution and sliced into 400-µm-thick sections using a Vibratome (Warner Instruments, Hamden, CT). The hippocampal slices were separated from the cortex and midbrain region and placed at the air-medium interface of humidified semipermeable (0.4 µm) Millicell membrane inserts (Millipore Corp., Bedford, MA) that rested on a 6-well culture plate (Falcon Multiwell; Becton Dickinson, Franklin Lakes, NJ) containing 1 mL of culture media in each well. The culture medium was composed of 25% Earle’s balanced salt, 50% minimal essential medium, and 25% heat-inactivated horse serum and was supplemented with 1 mM glutamine and 36 mM glucose. Penicillin/streptomycin and Fungizone were also added in a 1:100 ratio of the medium (1%). Before use, the culture medium was sterile filtered with 0.22 µm pore filters. Cultures were maintained in an incubator at 36°C, 100% humidity, and 5% carbon dioxide, and the medium was changed every other day. Under these conditions, nerve cells continued to proliferate, differentiate, and develop into a tissue organization that closely resembled that observed in...
situ. Treatment with CQN was initiated after the slices were allowed to recover for 10 days in culture. Following a standard protocol for the slice model of protein accumulation (17, 18), culture medium supplemented with CQN (60 μM) was added every other day. This particular dose of CQN was chosen on the basis of previous studies that assessed the compromise to lysosome-mediated protein processing by measuring the resultant protein accumulation events, in which 50 to 60 μM CQN was the optimal range to influence lysosomal processes without affecting glycosylation, tyrosine sulfation, protein synthesis, and secretion (17, 34, 35). CQN treatment continued for 3, 6, and 9 days, respectively, for each of the 3 treatment groups.

Electrophysiologic Recordings in Slices

Cultured slices were harvested for electrophysiologic recordings after 3, 6, and 9 days of CQN treatment. The hippocampal slices were isolated from the insert by cutting the membrane around the tissue. Slices were then transferred to a submerged-type recording chamber and held between 2 nylon nets. Throughout the recording the slices were perfused with oxygenated (95% O2/5% CO2) artificial cerebral spinal fluid containing 130 mM NaCl, 3 mM KCl, 2.5 mM CaCl2, 2.5 mM MgCl2, 26 mM NaHCO3, 1.25 mM KH2PO4, and 10 mM glucose (pH 7.4 and osmolarity of 310 mOsm). Cells were visualized using an Olympus BX-51WI upright microscope equipped with a video monitor. Whole-cell recordings were made from hippocampal CA1 pyramidal neurons using patch pipettes (5–10 MΩ) pulled from thick-walled borosilicate glass capillaries. The pipettes were back-filled with intracellular solution containing 122.5 mM Cs-glucocate, 10 mM HEPES, 2 mM MgCl2, 20 mM KCl, 1 mM EGTA, 2 mM Na2-ATP, 2 mM QX-314, 0.25 mM Na3-GTPx3H2O and pH 7.4. AMPA-mediated miniature excitatory postsynaptic currents (mEPSCs) were recorded in the continuous voltage-clamp mode at a holding potential of −80 mV using an Axopatch 200B amplifier (Molecular Devices, Union City, CA), in the presence of 50 μM picrotoxin (GABA receptor antagonist), 1 μM tetrodotoxin (TTX) (sodium channel blocker), and 50 μM aminophosphonovalerate (APV) (NMDA receptor antagonist) to the isolate the AMPA receptor-mediated mEPSCs responses. AMPA receptor-mediated spontaneous excitatory postsynaptic currents (sEPSCs) were recorded using the same conditions but in the absence of TTX. Access resistance and cell input resistance were monitored throughout the experiment; no series compensation was used to maximize the signal-to-noise ratio. Access resistance below 15 MΩ was accepted for analysis; otherwise the experiments were rejected. Offline analyses of mEPSCs and sEPSCs were done using the Mini Analysis program (Synaptosoft, Inc., Decatur, GA). The recordings of NMDA receptor-mediated mEPSCs and sEPSCs were performed with Mg2+-free solution containing 160 mM NaCl, 2.5 mM KCl, 0.2 mM CaCl2, 10 mM HEPES, 10 mM glucose, and 0.2 mM EDTA. The APV was replaced with 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione to block AMPA receptor currents and the voltage was clamped at −70 mV.

Preparation of Synaptosomes and Single Channel Recordings

The slices were harvested after 3, 6, and 9 days of CQN treatment. Tissues were isolated from the insert membranes by scraping with a spatula. Synaptosomal preparations from these tissues were made following previously described methods (36). Single channel recordings by tip-dip artificial lipid bilayer methods from these synaptosomes were done as described elsewhere (31). AMPA receptor currents were evoked by addition of 290 nM AMPA (Tocris Bioscience, Ellisville, MO) to the cis-side of the bilayer in the presence of 1 μM TTX, 2 μM tetraethylammonium, 50 μM APV, 1 μM (2S,4R)-4-methylglutamate (SYM 2081), and 100 μM picrotoxin to block sodium channels, potassium channels, NMDA receptors, kainate receptors and GABA and glycine receptors, respectively. At the end of each experiment, AMPA receptor-mediated currents were blocked by 10 μM concentrations of the potent AMPA receptor specific antagonist (1S)-4-(4-aminocephalyl)-1,2-dihydro-1-methyl-2-propylcarbomyl-6,7-methylenedioxyphthalazine (SYM 2206). To isolate NMDA single channel currents, recordings were performed with APV being replaced by SYM 2206 to block AMPA channel currents. At the end of the experiments, NMDA-mediated currents were blocked by the addition of APV.

The synaptosomal single channel currents clamped at various voltages were amplified (Axopatch 200B; Molecular Devices), filtered at 2 kHz, digitized at 5 kHz and recorded in videocassettes. pClamp 9 software was used for both online data acquisition and offline analysis. For single channel analysis only recordings showing consistent single channel current fluctuations were selected. Peaks in all points amplitude histograms were fitted with the bimodal Gaussian method and dwell time distributions were analyzed using time distribution histograms fitted by the Marquardt least-squares method. Single channel conductances were calculated by plotting AMPA evoked currents as a function of membrane voltage as described previously (36). During each experiment membrane capacitance and resistance were monitored continuously to ensure the formation and stability of membranes. The data from 3, 6, and 9 days of CQN treatments were compared with those of corresponding control cultures that were maintained in parallel.

RESULTS

Expression of Synaptic Proteins and Cathepsin D

To verify lysosomal dysfunction in the cultured hippocampal slices, Western immunoblot studies were performed to quantify the lysosomal enzyme cathepsin D. Our results show a progressive increase in cathepsin D in 3, 6, and 9 days of CQN treatment. Immunoblot experiments were also performed to determine the level of glutamate receptor subunits. Levels of these proteins in CQN-treated slices were compared with those in corresponding untreated control slices that were maintained in parallel. Because there were no time-dependent changes in the levels of glutamate receptor subunits in the control cultures (3, 6, and 9 days), the data
shown indicate 0 day as representative of all other controls. The results indicate a progressive decline in glutamate receptor subunits GluR1 and NR1 corresponding to the number of days of CQN treatment. The levels of actin, used as a control, did not change during the course of the experiment (Fig. 1). This slice model has been extensively characterized as exhibiting stable levels of glutamate receptor subunits and other synaptic markers once the tissue recovers from the preparation procedure over the first 6 to 8 days in culture (37, 38).

**Effect of Chloroquine on α-Amino-3-Hydroxy-5-Methylisoxazolepropionic Acid Receptor-Mediated Miniature and Spontaneous Excitatory Postsynaptic Currents**

To determine the effects of lysosomal dysfunction on the AMPA receptor-mediated mEPSCs, whole-cell electrophysiologic recordings in hippocampal slices were performed on 3-, 6-, and 9-day CQN-treated slices. Spontaneous currents in the absence of TTX were first recorded followed by the mEPSCs recordings. Representatives of gap-free recordings of membrane currents at a holding potential of −80 mV are illustrated in Figure 2A and B. CQN treatment resulted in progressive reductions in both miniature and spontaneous current frequencies in a treatment duration-dependent manner corresponding to the number of days of treatment. The mEPSCs in slices after 3 days of treatment resulted in an insignificant decrease in frequency (Table 1). The AMPA-mediated mEPSCs from 6- and 9-day slices exhibited prolonged interevent intervals compared with the control (Fig. 2D), indicating a significant reduction (p < 0.05, n = 10 for 6 days and p < 0.01, n = 10 for 9-day

**FIGURE 1.** Chloroquine (CQN) treatment increased expression of lysosomal enzyme cathepsin D (CD), indicative of protein accumulation stress, and correspondingly reduced glutamate receptor subunits. Hippocampal slice cultures were incubated with 60 μM CQN for 0 to 9 days and levels of CD, α-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA) receptor subunit glutamate receptor 1 (GluR1), NMDA receptor subunit NR1, and actin were determined across equal protein aliquots of homogenate samples by immunoblotting. (A) Representative immunoblot showing increased levels of CD and decreased levels of GluR1 and NR1. Molecular weight standards are shown in parentheses for each protein. (B) Quantitative analysis shows that strong increments of CD levels with duration of CQN exposure and decreases in glutamate receptor subunits (*p < 0.05). The data were obtained from 25 to 30 slices from each group. Data shown are representative of 4 to 5 experiments.

**FIGURE 2.** Effects of lysosomal dysfunction on α-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA)-mediated spontaneous excitatory postsynaptic currents (sEPSC) and miniature excitatory postsynaptic currents (mEPSC) on cultured hippocampal slices. Lysosomal dysfunction depresses the frequency and amplitude of mEPSCs and sEPSCs recordings in cultured hippocampal slices after 3, 6, and 9 days of chloroquine (CQN) treatment. (A) Representative traces of AMPA sEPSCs in the absence of tetrodotoxin and in the presence of NMDA blockers. (B) Representative traces of AMPA mEPSCs in controls and after 3, 6, and 9 days of CQN treatment. (C) CQN treatment on hippocampal slices resulted in a reduction in peak amplitude. Ci, Cii, Ciii, and Civi represent average traces of 50 seconds from control slices and culture slices treated with CQN for 3, 6, and 9 days, respectively. (D) Cumulative fraction of amplitude (Di) and interevent intervals (Dii) of mEPSCs. CQN treatment resulted in a shift of the curve to the right, representing a decrease in the amplitude corresponding to the number of days of CQN treatment. (C, D) Data from 10 slices were used with recordings from 3 to 4 cells in each slice.
CQN-treated slices) in frequencies (Table 1). In addition, mean mEPSC amplitude was also significantly reduced in the 6-day (p < 0.05, n = 10) and 9-day (p < 0.01, n = 10) treated culture slices (Table 1; Fig. 2C). There were no significant differences in either amplitude or frequencies among 3-, 6-, and 9-day controls (data not shown). The frequencies and amplitudes of the sEPSCs were also significantly reduced in 6-day (p < 0.05) and 9-day (p < 0.01) treated slices (Table 1).

We also investigated the consequences of CQN treatment on the decay and the rise kinetics of AMPA-mediated EPSCs. Our results indicate that the treatment groups showed an appreciable difference in decay kinetics but not in the rise time. The 2 exponential fittings of decay times ($D_T1$ and $D_T2$) indicate a significant change (p < 0.05) in $D_T2$ but not in $D_T1$ in the 6- and 9-day treated slices (Table 1). The 3-day CQN-treated slices did not show significant changes in decay times. To ensure that EPSCs recorded were AMPA receptor-mediated, the currents were blocked by SYM 2206 in all experiments (data not shown).

### Effects of Chloroquine on Single Channel Properties of Synaptosomal $\alpha$-Amino-3-Hydroxy-5-Methylisoxazolepropionic Acid Receptors

To determine whether the reduction in AMPA-mediated miniature and spontaneous currents in CQN-treated slices were due to changes in the channel properties of synaptic AMPA receptors, we investigated the single channel properties of AMPA receptors in synaptosomes. The resulting data analyzed using pClamp 9 software indicated significant reductions (p < 0.05) in the probability of channel openings (Po) for the 6- and 9-day CQN-treated slices compared with the controls (Fig. 3) without any changes in single channel conductance.

The single channel currents, recorded at 96 mV, were subjected to dwell time analysis and fitted using 1 to 2 exponentials. The results indicate that the mean open time ($T_o$) decreased significantly (Table 2). We observed shorter and longer mean open times ($T_o1$ and $T_o2$), both of which decreased significantly after 6 days of CQN treatment. However, after 9 days of CQN treatment, the longer mean...
open time ($\tau_{o2}$) was absent. The mean closed times were fitted with 2 exponential fittings ($\tau_{c1}$ and $\tau_{c2}$). The data indicate a significant increase of $\tau_{c2}$ as the CQN treatment progressed for 6 and 9 days. The mean closed times for 6 and 9 days were best fitted by 1 exponential (Table 2). Single channel currents were blocked with SYM2206 at the end of all experiments (data not shown).

### Effects of Chloroquine on N-Methyl-D-Aspartate-Mediated Miniature and Spontaneous Excitatory Postsynaptic Currents

We conducted electrophysiologic recordings in CQN-treated and control slices to determine the NMDA component of mEPSCs and sEPSCs. To isolate the NMDA-mediated mEPSC, 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione was added to the extracellular solution to block the AMPA receptor-mediated responses. The sEPSCs were measured in the absence of TTX. The representative sEPSCs and mEPSCs traces of each treatment group are shown in Figure 4A and B, respectively. Our data indicate that the NMDA currents were significantly altered in a trend corresponding to the time of duration of CQN treatment. However, in general, the differences in AMPA receptor-mediated current amplitudes and frequencies between treatment groups were larger when compared with the corresponding differences in the NMDA receptor components. In addition, there was no significant difference ($p > 0.05$, n = 10) between the average amplitudes of the control and those of 3-day CQN-treated slices (Table 3). The average traces of the amplitudes are shown in Figure 4C, and their cumulative amplitudes and interevents intervals are shown in Figure 4Di and 4Dii, respectively. A summary of the NMDA mEPSCs and sEPSCs data is shown in Table 3.

The rise and decay kinetics of the NMDA component of mEPSCs showed significant changes ($p < 0.05$, n = 10) in decay times but not in rise time. These changes correlated with the days of CQN treatment. The decay time was fitted by 2 exponentials. $\tau_{d2}$ but not $\tau_{d1}$ showed a significant decrease for the 6- and 9-day CQN-treated slices. Similarly, the $\tau_{d2}$ of sEPSC was significantly less in 6- and 9-day CQN-treated slices (Table 3). At the end of all experiments, EPSCs were blocked by the NMDA channel blocker APV (data not shown).

### Effects of Chloroquine on Single Channel Properties of Synaptosomal N-Methyl-D-Aspartate Receptors

Single channel recordings of isolated synaptosomes indicate that NMDA receptor currents were remarkably altered in CQN-treated samples. The Po for the 3-, 6-, and 9-day treated slices showed significant decreases from the control level (Fig. 5). Investigation of the mean open and closed time kinetics indicates that the mean open time decreased with days of CQN treatment, whereas the mean closed time increased. The data fitted with 2 exponential fittings ($\tau_{o1}$ and $\tau_{o2}$) for the mean open time indicate

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**TABLE 2.** Effects of CQN on Single Channel Properties of AMPA Receptors

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>3 days of CQN</th>
<th>6 days of CQN</th>
<th>9 days of CQN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conductance (pS)*</td>
<td>28 ± 2</td>
<td>27 ± 4</td>
<td>27 ± 3</td>
<td>29 ± 2</td>
</tr>
<tr>
<td>Mean open time in ms ($\tau_{o1}$)†</td>
<td>2.1 ± 0.9</td>
<td>1.9 ± 0.7</td>
<td>0.4 ± 0.2†‡</td>
<td>0.3 ± 0.2*‡</td>
</tr>
<tr>
<td>Mean open time in ms ($\tau_{o2}$)†</td>
<td>5.9 ± 1.1</td>
<td>5.4 ± 1.2</td>
<td>3.3 ± 0.6†‡</td>
<td>—</td>
</tr>
<tr>
<td>Mean closed time in ms ($\tau_{c1}$)</td>
<td>27 ± 15</td>
<td>21 ± 17</td>
<td>49 ± 16†‡</td>
<td>78 ± 26†‡</td>
</tr>
<tr>
<td>Mean closed time in ms ($\tau_{c2}$)</td>
<td>4.1 ± 1.4</td>
<td>3.2 ± 1.1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Open probability</td>
<td>0.39 ± 0.06</td>
<td>0.41 ± 0.04</td>
<td>0.10 ± 0.04†‡</td>
<td>0.03 ± 0.01†‡</td>
</tr>
</tbody>
</table>

* Holding potential was 96 mV. All values are expressed as means ± SD (n = 6).
†, Determined from current-voltage graph.
‡, Values determined from 1 to 2 exponential fittings.
* Significant level of p < 0.05; Student t-test. Each group comprised synaptosomes prepared from 20 to 30 slices, and 9 channel recordings were performed on synaptosomes of each group.

**TABLE 3.** Effects of Lysosomal Dysfunction on NMDA Receptor-Mediated mEPSCs and sEPSCs

<table>
<thead>
<tr>
<th></th>
<th>mEPSC</th>
<th>sEPSC</th>
<th>mEPSC</th>
<th>sEPSC</th>
<th>mEPSC</th>
<th>sEPSC</th>
<th>mEPSC</th>
<th>sEPSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency (/s)</td>
<td>4.4 ± 0.7</td>
<td>3.2 ± 0.6</td>
<td>4.3 ± 0.7</td>
<td>3.1 ± 0.6</td>
<td>4.4 ± 0.7</td>
<td>3.2 ± 0.6</td>
<td>4.3 ± 0.7</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td>Amplitude (pA)</td>
<td>14.9 ± 2.6</td>
<td>30.3 ± 5.2</td>
<td>14.9 ± 2.6</td>
<td>30.3 ± 5.2</td>
<td>14.9 ± 2.6</td>
<td>30.3 ± 5.2</td>
<td>14.9 ± 2.6</td>
<td>30.3 ± 5.2</td>
</tr>
<tr>
<td>Decay $\tau_1$(ms)</td>
<td>28.4 ± 3.8</td>
<td>31.3 ± 2.1</td>
<td>28.4 ± 3.8</td>
<td>31.3 ± 2.1</td>
<td>28.4 ± 3.8</td>
<td>31.3 ± 2.1</td>
<td>28.4 ± 3.8</td>
<td>31.3 ± 2.1</td>
</tr>
<tr>
<td>Decay $\tau_2$(ms)</td>
<td>142.2 ± 8.3</td>
<td>140.1 ± 15.3</td>
<td>142.2 ± 8.3</td>
<td>140.1 ± 15.3</td>
<td>142.2 ± 8.3</td>
<td>140.1 ± 15.3</td>
<td>142.2 ± 8.3</td>
<td>140.1 ± 15.3</td>
</tr>
<tr>
<td>Rise time (ms)</td>
<td>4.2 ± 0.4</td>
<td>3.9 ± 0.5</td>
<td>4.2 ± 0.4</td>
<td>3.9 ± 0.5</td>
<td>4.2 ± 0.4</td>
<td>3.9 ± 0.5</td>
<td>4.2 ± 0.4</td>
<td>3.9 ± 0.5</td>
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</tbody>
</table>

* Significant level of p < 0.05.
†, Significant level of p < 0.01.

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FIGURE 4. Effects of lysosomal dysfunction on N-methyl-D-aspartate (NMDA) receptor-mediated currents. Chloroquine (CQN) treatment on hippocampal slices results in a decrease in the NMDA component of sEPSCs and mEPSCs. Representative sEPSC (A) and mEPSC (B) traces exhibiting reductions in both frequency and amplitude. (C) Superimposed average traces from 1-minute recordings of sEPSCs and mEPSCs in control (i) and 3-day (ii), 6-day (iii), and 9-day (iv) CQN-treated slices. All recordings were performed at a membrane potential of −70 mV. (D) Cumulative fraction of amplitudes (i) and of interevent intervals (ii) A shift to the left in (Di) represents a decrease in the NMDA mEPSC amplitude corresponding to the number of days of CQN treatment. Likewise a shift to the right in the interevents interval graph (Dii) depicts a decrease in the frequency of NMDA-mediated mEPSC with the increase in the number of days of CQN treatment. (C, D) Results consist of data from 10 slices with recordings from 3 to 4 cells in each slice.

FIGURE 5. Lysosomal dysfunction results in a decrease in open probabilities (Po) of N-methyl-D-aspartate (NMDA) single channels in isolated synaptosomes. The decrease in Po correlates with the time period of chloroquine (CQN) treatment. Representative amplitude histograms (A–D) show the Po of NMDA currents in control and 3-, 6-, and 9-day CQN-treated samples, respectively. Representative traces recorded at a holding potential of +54 mV are shown on right of their corresponding histograms.
TABLE 4. Effects of CQN on Single Channel Properties of NMDA Receptors

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
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</thead>
<tbody>
<tr>
<td>Conductance (pS)*</td>
<td>37 ± 6</td>
<td>35 ± 7</td>
<td>38 ± 4</td>
<td>36 ± 2</td>
</tr>
<tr>
<td>Mean open time in ms (τo1)*</td>
<td>1.6 ± 0.5</td>
<td>0.9 ± 0.4</td>
<td>0.3 ± 0.1‡</td>
<td>0.3 ± 0.2‡</td>
</tr>
<tr>
<td>Mean open time in ms (τo2)*</td>
<td>6.1 ± 0.3</td>
<td>5.8 ± 1.1</td>
<td>2.7 ± 0.4‡</td>
<td>1.9 ± 0.7‡</td>
</tr>
<tr>
<td>Closed time in (ms)†</td>
<td>35 ± 7</td>
<td>38 ± 9</td>
<td>57 ± 18‡</td>
<td>85 ± 35‡</td>
</tr>
<tr>
<td>Open probability*</td>
<td>0.25 ± 0.04</td>
<td>0.21 ± 0.05</td>
<td>0.06 ± 0.02‡</td>
<td>0.02 ± 0.02‡</td>
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</table>

Holding potential was 54 mV. All values are expressed as means ± SD (n = 10).
*, Determined from current voltage graph.
†, Values determined from 1 to 2 exponential fittings.
‡, Significant differences with the corresponding values in control (p < 0.05; Student t-test). Each group comprised synaptosomes prepared from 20 to 30 slices and 10 channel recordings were performed on synaptosomes of each group.
CQN, chloroquine; NMDA, N-methyl-D-aspartate.

Our results indicate strong modulation of both synaptic AMPA and NMDA receptors resulting from lysosomal dysfunction induced by CQN. This modulation is temporally progressive and alters single receptor channel properties and thereby modifies synaptic transmission. In addition, we found that levels of glutamate receptor subunits continued to decrease after exposure to CQN. Remarkably, declines in AMPA and NMDA receptor subunits were paralleled by an increase in the proteolytic lysosomal enzyme cathepsin D. Increases in members of the cathepsin family of hydrolases are indications of protein accumulation stress that can lead to synaptic compromise (31, 32).

Our whole-cell current experiments showed declines in the amplitude and frequency of AMPA and NMDA receptor-mediated mEPSCs and sEPSCs in 6- and 9-day CQN-treated tissues. The reduction in amplitude of miniature and spontaneous currents can result from a reduced number of receptors on the postsynaptic membrane (4). Accordingly, this finding is supported by our biochemical studies showing reduced AMPA and NMDA receptor levels. Although there were reductions in glutamate receptor subunits in 3-day CQN-treated samples, no changes in whole-cell currents were noted. It is possible that this moderate reduction may not represent substantial changes in synaptic receptors and therefore did not produce any significant changes in synaptic currents. In addition, this observation supports the previous report showing that CQN has no acute effects on synaptic responses (17). In general, our observations with EPSCs are supported by previous findings that showed a decline in field potentials in hippocampal slices after CQN treatment (17).

The mean current amplitude of single channel currents is a product of single channel conductance (g) and probability of openings (Po). Therefore, a reduction in conductance or open probability will reduce the mean current amplitude of the single synaptic receptors. If this is the case, the amplitude of the miniature and spontaneous currents will be reduced if any one of these parameters decreases for single synaptic receptors. Our single channel recordings of isolated synaptosomes from CQN-treated slices showed a progressive decline in open channel probabilities of AMPA and NMDA receptors without any significant changes in the conductance. Therefore, the reduction in single channel open probabilities and expression of synaptic AMPA and NMDA receptors could have contributed to the reduction in amplitude of the synaptic currents observed in slice experiments.

Interestingly, other toxic species generated during lysosomal dysfunction can also contribute to the reductions in glutamatergic synaptic currents. The accumulation of amyloid β has been shown to result about 48 hours after lysosomal dysfunction (17). The reduction in AMPA and NMDA receptor currents could have also resulted from amyloid β interaction with these receptors. Recent studies demonstrate modulation of synaptic glutamatergic neurotransmission by amyloid β peptides (34). Therefore, reduced AMPA and NMDA receptor-mediated currents in lysosomal dysfunction are apparently governed by more than one mechanism.

Data from our single channel experiments provide evidence that both synaptic AMPA and NMDA receptors exhibit functional downregulation as lysosomal dysfunction progressed. In particular, single channel probabilities and mean channel open and closed times of both receptors show a close relationship with decay time of EPSCs. Reductions in open times and concurrent increases in closed times probably contributed to the reduced decay times of EPSCs. As per our results, changes in single channel properties could be one of the initial and primary factors responsible for reduced AMPA and NMDA receptor-mediated glutamatergic synaptic transmission during lysosomal inhibition. Later, as the lysosomal dysfunction continues unabated, reduced levels of receptors could further exacerbate the weakening of glutamatergic synaptic transmission.

The synaptic AMPA and NMDA receptor levels and their altered functional properties could also have resulted from defective interaction with regulatory proteins. One
such mechanism could be reduced phosphorylation of these receptors. The single channel open probability of AMPA receptors is known to be positively modulated by phosphorylation (39). Interestingly, our results showed a reduced single channel open probability that may have resulted from reduced phosphorylation. Other possibilities include deregulatory processes affecting scaffold proteins associated to glutamate receptor subunits. For example synapse-associated protein 97 has been shown to increase the number of postsynaptic AMPA receptors as well as frequency of mEPSCs (40). Investigations aiming to resolve changes, if any, in scaffold proteins would be an attractive direction for future research and might provide valuable information on the underlying mechanisms responsible for reduced receptor functionality. The major emphasis of this report, however, is that lysosomal dysfunction targets glutamatergic synaptic transmission in hippocampus and specific changes in receptor functionality.

The reduction in the frequency of mEPSCs and sEPSCs may have resulted from reduced transmitter release, the size of the quantal content, or reduced CA3–CA1 connectivity (41). Although in this study we did not examine the release probability, a reduction in synaptophysin by lysosomal disruption indicates a possible reduction in synaptic terminals, which probably contributed to the decrease in transmitter release and thus reduced current frequencies. Additional support for this notion comes from a study indicating a significant reduction in synaptic terminals of hippocampal slices exhibiting lysosomal dysfunction (17, 42). Interestingly, elevated levels of cathepsin D also could have indirect effects on glutamatergic presynaptic function in the CA1 region. This hypothesis is supported by a recent finding that reduced levels of cathepsin D could account for the dysfunction of GABAergic interneurons in the CA3 region due to lysosomal degradation of glutamate decarboxylase (43). Therefore, elevated levels of cathepsin D may strengthen the inhibitory currents in CA3 region and thereby reduce presynaptic excitatory function in the CA1 region, which in turn reduces EPSC frequencies.

In summary, our results demonstrate that lysosomal dysfunction leads to a progressive decline in glutamatergic synaptic transmission in the hippocampus. In addition to reduced subunit levels and altered enzyme (cathepsin D) levels, the reduction in mEPSC and sEPSC amplitudes and the decay times are paralleled by the reduction in single channel open probability and mean open times for both AMPA and NMDA receptors. These findings indicate that changes in specific single channel properties could lead to modification of amplitude and time course of synaptic currents. Therefore, results of our study strongly suggest that lysosomal dysfunction, which is believed to underlie the early neurodegenerative processes in diseases such as AD, potently inhibits the AMPA and NMDA receptor-mediated neurotransmission in the hippocampal CA1 region. Thus, lysosomal modulation in cultured hippocampal tissues is a novel approach for acute evaluation of functional modifications in glutamatergic synaptic transmission and to study the effects of therapeutic compounds in age-related neuropathogenesis.

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