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Photoinactivation of Native AMPA Receptors Reveals Their Real-Time Trafficking

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Summary

AMPA receptors mediate the majority of the fast excitatory transmission in the central nervous system. Much evidence suggests that the fast trafficking of AMPA receptors into and out of the postsynaptic membrane underlies changes in synaptic strength thought to be necessary for higher cognitive functions such as learning and memory. Despite the abundance of research conducted in this area, a direct, real-time functional assay that measures the trafficking of native AMPA receptors has been lacking. Toward this aim, we use a photoreactive, irreversible antagonist of AMPA receptors, ANQX, to rapidly silence surface AMPA receptors and investigate directly the trafficking of native AMPA receptors in real time. We find that the most dynamic movement of AMPA receptors occurs by lateral movement across the surface of neurons. Fast cycling of surface AMPA receptors with receptors from internal stores does occur but exclusively at extrasynaptic somatic sites. The cycling of synaptic AMPA receptors only occurs on a much longer timescale with complete exchange requiring at least 16 hr. This cycling is not dependent on protein synthesis or action potential driven network activity. These data suggest a revised model of AMPA receptor trafficking wherein a large internal store of AMPA receptors exchanges rapidly with extrasynaptic somatic AMPA receptors, and these newly inserted AMPA receptors then travel laterally along dendrites to reside stably at synapses.

Introduction

Ionotropic neurotransmitter receptors mediate all fast chemical transmission in muscle and nerve. The rapid trafficking of these proteins to and from synapses is the subject of intense research, but remains incompletely understood. AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors, a major subtype of ionotropic glutamate receptors, mediate fast excitatory synaptic transmission in the brain. These receptors, although expressed throughout neurons, are highly clustered at the postsynaptic density ensuring rapid responses to synaptically released glutamate. Glutamatergic synapses undergo activity dependent long-lasting changes in synaptic strength, a process considered to underlie aspects of learning and memory (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999). Changes in synaptic strength are thought to involve rapid movement of AMPA receptors into and out of synapses, depending on the frequency of synaptic activity.

A number of studies have suggested that AMPA receptors, unlike NMDA, GABA, and nicotinic acetylcholine receptors, rapidly and constitutively cycle between intracellular stores and the cell surface (Bredt and Nicoll, 2003; Collingridge et al., 2004; Malinow and Malenka, 2002; Sheng and Kim, 2002; Song and Huganir, 2002). This cycling may allow for rapid, regulated changes in synaptic AMPA receptor number and thus provide a mechanism for synaptic plasticity. A variety of optical, biochemical, and electrophysiological approaches have been used to investigate AMPA receptor trafficking. For example, AMPA receptors were tracked optically by tagging them with either Green Fluorescent Protein (GFP) (Shi et al., 1999), an α-bungarotoxin binding site (Sekine-Aizawa and Huganir, 2004), or receptor-specific antibodies conjugated to fluorophores (Beattie et al., 2000; Lin et al., 2000; Passafaro et al., 2001; Lee et al., 2002). AMPA receptor trafficking has also been studied biochemically by bulk biotinylation of surface proteins and subsequent immunopurification (Ehlers, 2000; Lin et al., 2000) or by exogenous introduction of receptor subunits containing an extracellular protease cleavage site (Passafaro et al., 2001). Finally, AMPA receptors have been monitored electrophysiologically by pharmacologically blocking either exocytosis or endocytosis and recording the changes in AMPA receptor-mediated synaptic currents (Luscher et al., 1999; Luthi et al., 1999; Nishimune et al., 1998). The general conclusion from these studies is that AMPA receptors are remarkably dynamic, constitutively trafficking between intracellular stores and the cell surface at rates on the time scale of 10–20 min.

The real-time lateral diffusion of receptors has also been investigated optically by monitoring the movement of fluorescently tagged surface receptors (via receptor antibodies attached to fluorophores or quantum dots) across the membrane surface (Borgdorff and Choquet, 2002; Tardin et al., 2003). These studies suggested that, whereas extrasynaptic receptors appear to be highly mobile, synaptic receptors represent a comparatively immobile pool under basal conditions.

These approaches have provided valuable insight into the trafficking of receptors, but they have a number of limitations. Most importantly, optical and biochemical studies cannot unequivocally distinguish between synaptic and extrasynaptic receptors. Further, many of these studies relied on the use of antibodies, which are large divalent proteins that have been shown to affect the trafficking of receptors (Grunfeld, 1984; Weissman et al., 1986), or they required the use of overexpressed AMPA receptor subunits, which may perturb normal trafficking. To permit the direct, quantitative, real-time measurement of native AMPA receptor trafficking in live neurons, we have used a membrane-impermeable, photoreactive AMPA receptor antagonist to photoinactivate surface receptors (Chambers et al., 2004). The photoreactive antagonist, 6-azido-7-nitro-1,4-dihydroquinoxaline-2,3-dione (ANQX), is an aryl azide that, when irradiated with

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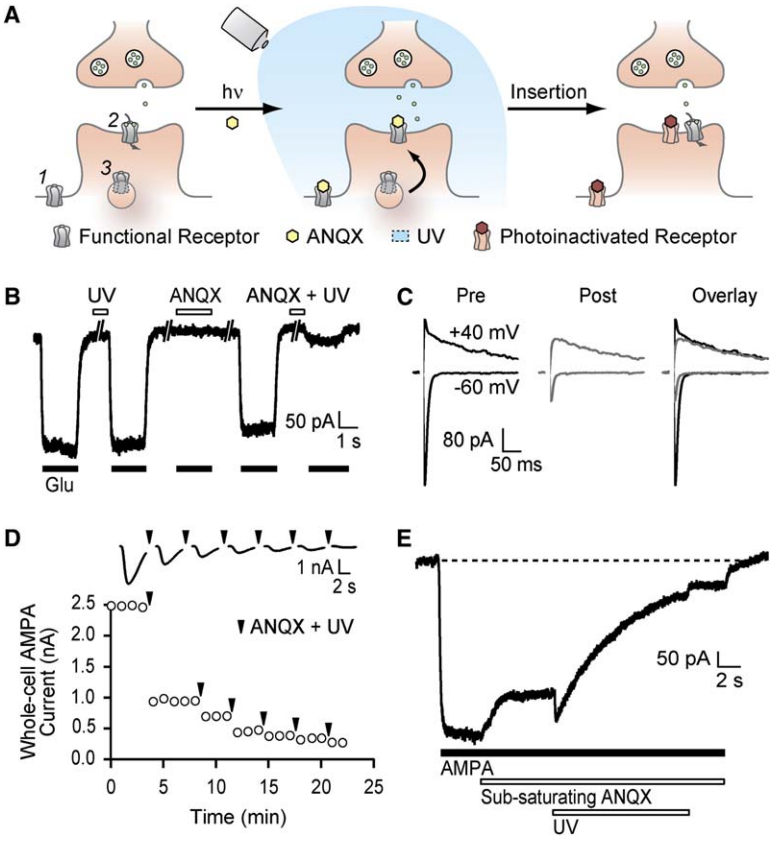

Figure 1. ANQX Is a Photoreactive, Specific, Irreversible Inhibitor of Native, Surface-Exposed AMPA Receptors

(A) ANQX rapidly binds to surface exposed extrasynaptic (1) and synaptic (2) but not intracellular (3) receptors. Global irradiation of ANQX bound receptors with UV light irreversibly antagonizes surface receptors and permits the real-time monitoring of AMPA receptor insertion from intracellular stores to the cell surface (depicted). Focal irradiation of ANQX-bound receptors permits the real-time monitoring of AMPA receptor diffusion across the cell surface.

(B) Outside-out patch recording from a cultured hippocampal neuron (10 mM glutamate, 100 μM cyclothiazide) after the indicated treatments. UV light (3 s) alone has no effect on the glutamate-evoked current. ANQX (100 μM) alone reversibly blocked the glutamate-evoked current. ANQX and UV light applied together irreversibly antagonizes AMPA receptors (n = 8).

(C) ANQX-mediated irreversible antagonism is selective for AMPA receptors. After the brief application of ANQX (100 μM) and UV light (2 s), the synaptic AMPA-mediated EPSC at −60 mV is permanently reduced, but the NMDA EPSC recorded at +40 mV is unchanged (n = 7). Stimulus artifacts have been blanked.

(D) Serial applications of ANQX (100 μM) and UV light (1 s) reduced the whole-cell AMPA (10 μM) evoked current in a stepwise fashion (n = 3).

(E) Continuous application of UV light in the presence of nonsaturating doses of ANQX (2 μM) progressively reduces the whole-cell AMPA current (n = 7).

ultraviolet light, becomes a highly reactive nitrene that can covalently cross link to and, as a result, irreversibly antagonize AMPA receptors. Thus, ANQX provides a means of rapidly silencing surface-exposed AMPA receptors, permitting the real-time monitoring of AMPA receptor insertion from intracellular stores to the cell surface as well as the lateral diffusion of receptors across the plasma membrane (see Figure 1A). Specifically, by recording the "recovery" of AMPA receptor-mediated currents electrophysiologically immediately after the global or focal photoinactivation of surface receptors, we present a direct and quantitative measurement of the exocytosis and lateral diffusion of native AMPA receptors on live neurons. In agreement with previous reports, we observed rapid trafficking of AMPA receptors from internal stores to the cell surface. Surprisingly, however, this fast exocytosis occurred only at nonsynaptic sites. Synaptic receptors did exchange with intracellular receptors, but on a much longer timescale, and this cycling did not require activity, protein synthesis, or intact microtubules. Finally, the most rapid form of AMPA receptor mobility was through the lateral movement of receptors across the neural surface.

Results

Characterization of ANQX

Previous work described the design, synthesis, and preliminary characterization of 6-azido-7-nitro-1,4-dihydroquinoxaline-2,3-dione (ANQX), a photoreactive analog of the high affinity AMPA receptor antagonist, DNQX. When ANQX is irradiated with ultraviolet light, it becomes a highly reactive nitrene that covalently cross links to and, thereby, irreversibly antagonizes AMPA receptors (Chambers et al., 2004). Here, we characterize the effect of ANQX on native neuronal synaptic and extrasynaptic AMPA receptors in cultured hippocampal neurons. First, we tested ANQX on AMPA receptor responses from outside-out patches (Figure 1B). Brief exposure (3 s) to UV light (350 nm) alone had no effect on glutamate-evoked currents, and in the absence of UV light, the antagonizing effects of ANQX (100 μM, 2 s) were rapidly reversible. Only in the presence of UV light does ANQX become an irreversible antagonist, permanently reducing the evoked current in the patch for the duration of the recording (n = 8). To examine the selectivity of ANQX and its effect on synaptic currents, we recorded EPSCs evoked extracellularly in dissociated culture and rapidly applied ANQX from a local flowpipe (Figure 1C). Two seconds of UV irradiation in the presence of ANQX (100 μM, 10 s application) irreversibly blocked AMPA currents without altering postsynaptic NMDA receptors (n = 7, AMPA block 66% ± 5%, NMDA block 0% ± 6%). The fact that ANQX did not affect NMDA currents indicates that photoinactivation with ANQX is selective for AMPA receptors and, further, that it has no presynaptic effect on transmitter release.

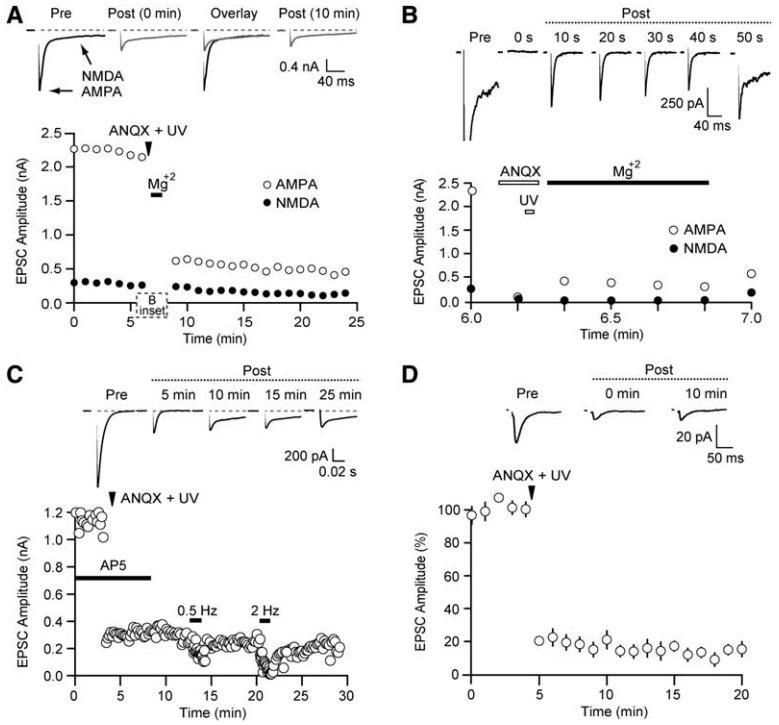

Figure 2. Synaptic AMPA Receptors Do Not Rapidly Cycle with an Intracellular Pool of Receptors on the Timescale of Minutes

(A) After 10 s application of ANQX (10 μM) and UV light (2 s) the synaptic AMPA EPSC in an autaptic cultured hippocampal neuron is permanently reduced. The NMDA EPSC measured at 100 ms after the stimulus is unchanged (n = 6). The action potentials in the traces have been blanked and responses have been averaged in 1 min bins.

(B) Expansion of minute 6–7 of the recording in (A). Mg^{+2} was rapidly applied for 40 s immediately after inactivation to isolate the AMPA response. No recovery on this timescale is apparent. Traces of each EPSC are plotted above the corresponding point in the expansion. The first trace has been truncated and the action potentials blanked.

(C) After photoinactivation of surface AMPA receptors, synaptic AMPA receptor currents do not recover despite repetitive stimulation (0.5 Hz and 2 Hz) of synaptic NMDA receptors (0 mM Mg^{+2}). APV was present until after photoinactivation to isolate the AMPA receptor current.

(D) An average of five experiments similar to in (A) (mean ± SEM) but with extracellulary evoked EPSCs in standard dissociated cultures and in gramicidin-perforated patch mode (n = 5). Stimulus artifacts in the traces have been blanked.

Next, we tested whether ANQX has an additional feature expected of a photoreactive irreversible antagonist when in great excess of its target: namely, that the amount of irreversible block depends primarily on the time of exposure to UV light. Indeed, serial applications of ANQX (100 μM, 10 s) with UV light (1 s) caused stepwise reductions in the evoked current (Figure 1D), and continuous application of subsaturating concentrations of ANQX (2 μM) in the presence of UV light progressively reduced the evoked current beyond the level of steady-state block (Figure 1E). These results also highlight the fact that although the photoinactivation of AMPA receptors is quite robust, the photocrosslinking requires repeated application of "fresh" nonphotolyzed ANQX (for a complete analysis see Supplemental Data). These findings demonstrate that ANQX can be used to rapidly, selectively, and irreversibly block surface-exposed native neuronal AMPA receptors and, thereby, provides a means of monitoring the rates of insertion or lateral diffusion of these receptors in real time.

Trafficking of AMPA Receptors from Internal Stores

To examine the rate of delivery of native AMPA receptors from internal stores to the neuronal surface, we photoinactivated surface-exposed AMPA receptors on dissociated cultured hippocampal neurons by full-field UV irradiation in the presence of ANQX and monitored the recovery of AMPA currents over time. The insertion of synaptic receptors was monitored selectively by recording evoked AMPA EPSCs in autapses (Figures 2A and 2B). The NMDA EPSCs were monitored simultaneously (in 0 mM extracellular Mg^{+2}), providing a measure of the stability of the recording. Immediately after the application of a saturating dose of ANQX (10 μM) and brief (2 s) irradiation with UV light, the synaptic AMPA EPSC (isolated by briefly washing in extracellular Mg^{+2}) was strongly and irreversibly reduced, whereas the NMDA EPSC was unchanged (after washout of Mg^{+2}). Unexpectedly, the synaptic AMPA current did not recover from photoinactivation during the recording, indicating that intracellular AMPA receptors are not trafficked to synapses on the minute timescale, even when synaptic NMDA receptors are repetitively activated (Figure 2C). Consistent with these observations, the amplitude and frequency of mEPSCs recorded in a separate set of experiments did not recover after photoinactivation (Figure S1). We noted that the application of this high dose of ANQX with UV light reversibly antagonized the synaptic NMDA EPSC, presumably because of the low affinity of quinoxalinediones for the glycine binding site on the NMDA receptor (Kleckner and Dingledine, 1989; Lester et al., 1989). We repeated this experiment on evoked EPSCs in standard dissociated culture in perforated-patch mode to rule out any consequences of whole-cell dialysis or peculiarities unique to autaptic cultures. Again, we found no rapid recovery of AMPA receptor-mediated synaptic currents after photoinactivation of surface AMPA receptors (Figure 2D). It is conceivable that the recycling of inactivated receptors back to the synapse could mask the overall level of recovery of the synaptic EPSC. However, if this were the case, we still should have seen some rapid recovery of synaptic currents before the reinsertion of inactivated receptors. But because we saw no recovery during these continuous recordings, we must rule out a process of active cycling of synaptic AMPA receptors with receptors in an internal store on this timescale.

Because our results unexpectedly indicated that synaptic AMPA receptors do not exchange with an internal pool on the minute timescale, we next investigated if

these receptors cycle on the hour timescale. To address this question, we inactivated all surface AMPA receptors on entire cover slips of neurons (100 μM ANQX, 2–3 min application, see Experimental Procedures) and returned the cultures to a 34ºC incubator for various periods of time before assaying the extent of recovery of synaptic AMPA receptor responses. To avoid the problem with signal-to-noise associated with recording mEPSCs, we evoked EPSCs by a brief application of hypertonic sucrose, a reliable method for simultaneously activating all synaptic AMPA receptors (Rosenmund and Stevens, 1996). We observed a 33% ± 5% (n = 14) recovery in sucrose-evoked EPSCs at 6 hr and 79% ± 12% (n = 12) recovery at 16 hr (Figure 3A). It should be noted that although sucrose-evoked EPSCs were evident at 6 hr, mEPSCs did not become readily apparent until 16 hr (Figures 3B and 3C), presumably because of the difficulty of resolving these miniature currents from baseline noise, unless cyclothiazide was included to facilitate the detection of these miniature events (Figure S2). Interestingly, incubating the cultures in tetrodotoxin to block action-potential-driven network function had no effect on the recovery of AMPA receptors at synapses, indicating that this cycling is independent of activity (Figure 3D).

Having established that the time course of synaptic AMPA receptor cycling is slow, we next examined the cycling of extrasynaptic receptors. Toward this end, we repeated the previous serial sampling experiments and instead examined specifically the recovery of extrasynaptic AMPA receptors by recording AMPA receptor currents from outside-out somatic patches. In striking contrast to the slow recovery observed for synaptic AMPA currents, the extrasynaptic currents exhibited fast recovery after photoinactivation, regaining 36% ± 8% (n = 25) of their initial value within 0.5 hr, and ultimately showing 79% ± 19% (n = 9) recovery in 18 hr (Figure 4A). As would be expected, if the cultures were maintained at room temperature instead of at 34ºC, the extent of recovery at 0.75 hr was moderately decreased (23% ± 3%, n = 11, instead of 44% ± 5%, n = 9, $p < 0.05$, data not shown).

To determine if the apparent recovery of synaptic and extrasynaptic AMPA receptors is due to the synthesis of new receptors or to the trafficking of a preexisting pool of receptors, we examined the effect of the protein synthesis inhibitor cycloheximide over an 18 hr period on the size of mEPSCs and glutamate-evoked currents in outside-out patches. Cycloheximide had no effect on either synaptic (Figure 4B) or extrasynaptic currents (Figure 4C), nor on the amplitude of recovery of mEPSCs recorded 18 hr after photoinactivation (Figures 4D and 4E), indicating that a pool of preexisting receptors is the primary source of AMPA receptors inserted into the plasma membrane in an 18 hr period. Additionally, we tested whether the delivery of new synaptic AMPA receptors might require intact microtubules. Incubating cultures in the microtubule polymerization inhibitor colchicine (20 μM) did not affect the recovery of sucrose-evoked synaptic currents 6 hr after photoinactivation (control = 21% ± 5%, n = 13; colchicine = 19% ± 6%, n = 11, p = 0.71), nor did it alter the size of mEPSCs over a 6 hr period (control = 14 ± 2 pA, n= 4; colchicine = 15 ± 2 pA, n = 6, p = 0.76), consistent with previous results (Bouron, 1997).

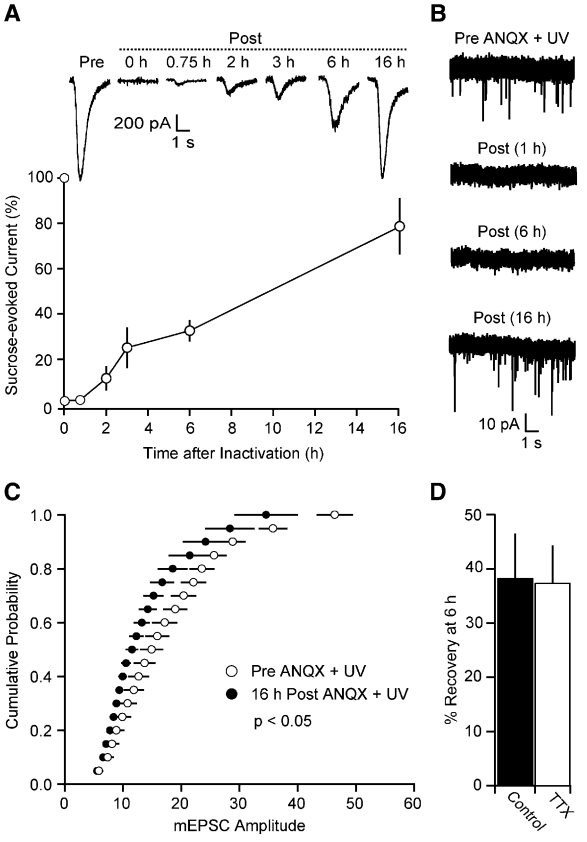

Figure 3. Synaptic AMPA Receptors Cycle with an Intracellular Pool of Receptors on the Timescale of Hours

(A) Average sucrose-evoked EPSCs (mean ± SEM) from neurons at various time points after global photoinactivation of all surface AMPA receptors. Representative traces from the indicated times are presented above the graph.

(B) Representative recordings of mEPSCs at the indicated times after photoinactivation.

(C) Cumulative distribution of mEPSC amplitudes (mean ± SEM) before (n = 6) and 16 hr after (n = 7) photoinactivation of total surface AMPA receptors.

(D) Incubation of neurons with TTX to block action potential driven activity does not affect the recovery of sucrose-evoked EPSCs (mean ± SEM) at the 6 hr time point (n = 11 in TTX, n = 9 control).

To measure the dynamics of the fast cycling of extrasynaptic receptors in real time, we uniformly inactivated all surface AMPA receptors with full-field UV irradiation (1 s, 350 nM, ANQX 50 μM) (Figure 5A, see graphic) while we simultaneously focally uncaged glutamate only at the cell body (spot diameter ~20 μm). This allowed us to monitor the recovery of somatic currents because of the cycling of surface somatic receptors with internal receptors. It should be noted that the two photolysis processes (photoinactivation with ANQX and glutamate uncaging) were separated in time by alternating the application of the two photoreactive molecules to the preparation with a local flowpipe. Consistent with the serial sampling result, we observed a rapid recovery of the uncaged glutamate response indicating that somatic receptors cycle with an internal pool of AMPA receptors on the minute timescale (Figure 5A). Notably, when this experiment was repeated with whole-cell instead of perforated-patch recording, essentially no recovery was

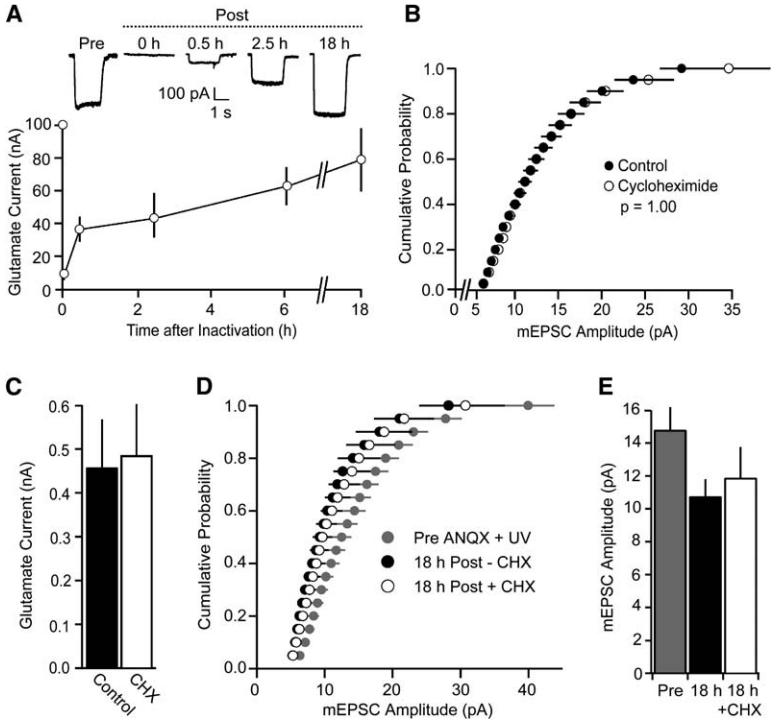

Figure 4. Extrasynaptic AMPA Receptor Currents Initially Recover More Quickly than Synaptic Currents from Photoinactivation

(A) Average glutamate-evoked (10 mM, 100 μM cyclothiazide) outside-patch currents (mean ± SEM) measured at various time points after photoinactivation of all surface AMPA receptors. Representative traces from the indicated times are presented above the graph.

(B) The amplitude of AMPA receptor mEPSCs (mean ± SEM) is not affected by incubation of neuronal cultures for 18 hr with the protein synthesis inhibitor cycloheximide (100 μM).

(C) Average response (mean ± SEM) of somatic outside-out patches to glutamate is not affected by incubation of neuronal cultures for 18 hr with cycloheximide (100 μM).

(D) Cumulative distribution of mEPSC amplitudes (mean ± SEM) before ANQX treatment (n = 7) and 18 hr later with or without incubation in cycloheximide (n = 8 each, p = 0.38, K-S test).

(E) Average mEPSC amplitudes (mean ± SEM) before ANQX treatment (n = 7) and 18 hr later with or without incubation in cycloheximide (n = 8 each, p = 0.62, Student's t test).

observed (Figure S3). This suggests that under our conditions, the typical whole-cell recording configuration disrupts somatic receptor cycling, perhaps because of rapid whole-cell dialysis of the cell body, and highlights the importance of using the noninvasive perforated-patch and serial sampling assays to investigate somatic AMPA receptor trafficking.

To test if dendritic AMPA receptors, like somatic receptors, undergo cycling on the minute timescale, we repeated the previous experiment, uniformly inactivating all surface AMPA receptors with full-field UV irradiation, but we uncaged glutamate on dendrites 75–100 μm from the soma. In this experiment, no significant recovery of the uncaged glutamate response was observed during the duration of the recording (up to 25 min), ruling out the possibility that dendritic AMPA receptors, unlike those at the cell body, are rapidly cycling with an internal pool (Figure 5B). Thus, the primary source of new functional AMPA receptors is likely to come from exocytosis at the cell body followed by lateral movement out dendrites toward synapses.

Lateral Diffusion of AMPA Receptors

Recently, optical tagging of AMPA receptors has revealed that these proteins laterally diffuse within the plane of the membrane at relatively high rates (Borgdorff and Choquet, 2002; Tardin et al., 2003). To test this idea electrophysiologically, we photoinactivated a small patch (~5 μm diameter) of AMPA receptors on the soma of neurons with focused irradiation from a UV laser in the presence of ANQX (50 μM) and then monitored the recovery of AMPA-mediated currents in the identical

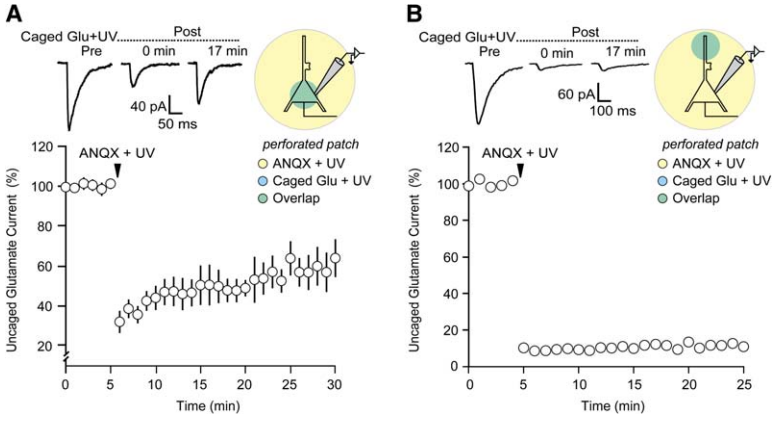

Figure 5. Somatic AMPA Receptors Rapidly Cycle with an Intracellular Pool of Receptors on the Timescale of Minutes

(A) Fast recovery of focally evoked uncaged glutamate responses (mean ± SEM) from somatic AMPA receptors is observed on the timescale of minutes after global photoinactivation of all surface AMPA receptors (n = 6). Representative traces from the indicated times are presented above the graph. Recording scheme graphic: the cell is recorded in perforated patch mode. Full-field (global) UV irradiation of the entire neuron in the presence of ANQX (yellow) inactivates surface receptors. Focal UV irradiation of the cell body in the presence of caged glutamate (blue) activates somatic AMPA receptors. Overlap of inactivated receptor region (yellow) and uncaged glutamate region (blue) is shown in green.

(B) When the uncaging spot is moved to a dendritic region 75–100 μm from the soma, no fast recovery of current (mean ± SEM) is observed (n = 6). Error bars are too small too see.

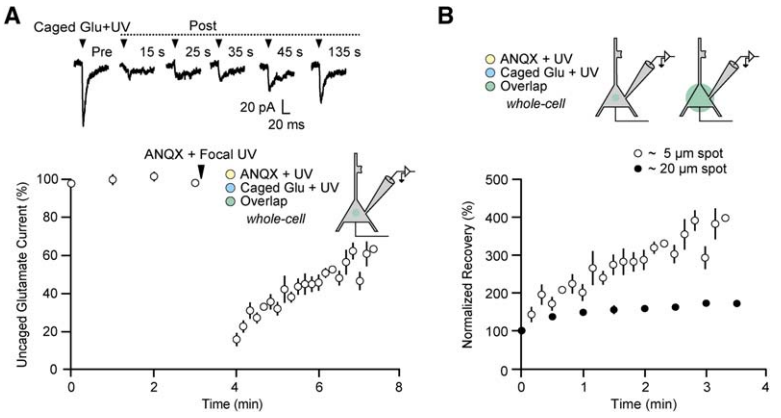

Figure 6. Somatic AMPA Receptors Laterally Diffuse on the Surface of the Neuron on the Timescale of Seconds

(A) Somatic AMPA receptor responses to focally uncaged glutamate rapidly recover after focal photoinactivation of AMPA receptors in a defined (~5 μm spot) region on the cell body. Uncaged glutamate-evoked responses (mean ± SEM) were measured in the identical region (see graphic, n = 7). Representative traces from the indicated times are presented above the graph. Recording scheme graphic: the cell is recorded in whole-cell patch mode. Focused UV irradiation of a 5 μm spot on the cell body in the presence of ANQX (yellow) inactivates a fraction of the surface receptors. Focused UV irradiation of a 5 μm spot on the cell body in the presence of caged glutamate (blue) activates somatic AMPA receptors. Overlap of inactivated receptor region (yellow) and uncaged glutamate region (blue) is shown in green. (B) Normalized recovery rate (mean ± SEM) of somatic AMPA receptor responses to focally uncaged glutamate. The rate of recovery after focal photoinactivation is reduced when the diameter of the photolysis region (spot size) is increased from 5 μm to 20 μm (n = 10). The 5 μm spot size data is replotted from (A).

region by focal uncaging of glutamate with the same UV laser spot (Figure 6, see graphic). The blockade of uncaged glutamate responses after photoinactivation confirms that both photochemical reactions occurred on the same receptors. These experiments were intentionally performed in whole-cell mode to minimize the insertion of receptors from internal stores at the cell body (see above) so that any recovery could be solely attributed to the lateral movement of surface receptors into the photoinactivated zone. In this recording scheme, the focally activated AMPA current began recovering within tens of seconds (τ = 35.4 s for an exponential fit). As expected, when the same experiment was repeated with a larger diameter spot (~20 μm) to deplete the cell-body surface of much more of its functional receptors, the recovery was slowed (τ = 82.8 s) (Figure 6B). Additionally, none of the lateral diffusion required activity because these experiments were conducted in the presence of tetrodotoxin. These results confirm the idea that extrasynaptic AMPA receptors are moving extremely rapidly across the surface of neurons.

Discussion

In the present study, we employed a photochemical approach to characterize directly the constitutive trafficking of native AMPA receptors in live neurons. We used the photoreactive AMPA receptor antagonist ANQX to specifically and irreversibly antagonize surface AMPA receptors, permitting the real-time monitoring of the insertion of AMPA receptor from intracellular stores to the cell surface as well as the lateral diffusion of receptors across the plasma membrane. Owing to the improved spatial and temporal resolution of this approach, we found that the cycling rate of surface AMPA receptors depends primarily on their location: the insertion of receptors at synaptic sites is slow, occurring on the timescale of hours, whereas the insertion of receptors to extrasynaptic somatic sites is rapid, occurring on the timescale of minutes. Lateral diffusion of receptors across the cell surface occurred on the timescale of seconds, in agreement with previous reports with antibody-tagged receptors. Importantly, neither receptor insertion nor lateral diffusion required network activity because both were unaffected by the application of tetrodotoxin.

Notably, our finding that the inhibition of protein synthesis had no effect on the stability of either synaptic or extrasynaptic AMPA currents or the recovery of mEPSCs after photoinactivation suggests that the intracellular pool of AMPA receptors is considerably larger than the total surface pool. That is, if the number of intracellular receptors were small compared to the number of surface receptors, the observed recovery after photoinactivation would have been much more incomplete because of the recycling of photoinactivated receptors. However, we observed near total recovery of surface currents (79% ± 12%) in the same time period that protein synthesis inhibition had no effect.

Our results highlight the power of using an irreversible antagonist to study receptor trafficking. Along the same lines, a number of previous studies have used irreversible antagonists of NMDA receptors (Tovar and Westbrook, 2002), nicotinic acetylcholine receptors (Akaaboune et al., 2002), and modified GABA receptors (Thomas et al., 2005) to study the dynamics of these classes of ion channels, and their results have proven invaluable to our understanding of how neurotransmitter receptors traffic in neurons. We were able to take advantage of the photoreactivity of ANQX to determine the contributions of both receptor insertion and lateral diffusion to AMPA receptor trafficking.

Although the use of a photoreactive receptor antagonist to irreversibly silence surface AMPA receptors has several advantages over previous approaches used to study AMPA receptor trafficking, a number of potential limitations should be considered. One possible problem is that irreversibly antagonized AMPA receptors could traffic differently than unblocked receptors. However, given the evidence that antagonizing AMPA receptors does not alter receptor trafficking under basal conditions or during synaptic plasticity, this concern seems unjustified (Kauer et al., 1988; Muller et al., 1988). It should be pointed out that because we only studied receptor trafficking on a comparatively short timescale (<18 hr) the homeostatic compensatory insertion of AMPA receptors observed after more prolonged activity

blockade (~48 hr) is unlikely to have influenced our results (Turrigiano et al., 1998). Indeed, under our conditions, incubation of neurons with saturating NBQX for 18 hr did not significantly enhance mEPSC amplitude (data not shown).

Another potential limitation arises if receptor cycling at synapses is both extremely rapid and involves a very small pool of intracellular receptors. If this were the case, we might underestimate the rate of receptor trafficking at synapses because of the eventual recycling of inactivated receptors. However, if this were true, we still should have observed the initial insertion of functionally intact receptors that were inside the cell at the time of photoinactivation. But because we did not see any recovery of synaptic currents immediately after photoinactivation of surface receptors, there cannot be any considerable cycling of synaptic receptors on the minute timescale. It is possible that the recycling of inactivated receptors slowed the observed recovery of synaptic and extrasynaptic currents at much longer time points. Nonetheless, the minimal recovery of synaptic currents observed in the first few minutes after photoinactivation and the significant recovery observed at 16 hr provides upper and lower limits for the rate of replacement of AMPA receptors at synapses.

Why is the rate of AMPA receptor insertion to synapses that we measure with photoinactivation so much slower than that suggested by previous studies? There are a variety of potential factors. First, it is possible that the recovery of surface AMPA receptors observed in previous studies, which could not unequivocally distinguish between trafficking of receptors to synaptic and extrasynaptic sites, was actually due to the cycling of extrasynaptic receptors. Alternatively, studies with overexpressed AMPA receptor subunits may have led to overestimates of receptor trafficking rates. Finally, the rapid changes in synaptic AMPA currents observed in studies with peptides thought to specifically disrupt endo- and exocytosis of AMPA receptors has recently been called into question by another study that demonstrated that one of the drugs, the NSF binding fragment, may also inhibit the lateral movement of receptors into synapses (Gardner et al., 2005). Thus, it is possible that the selectivity of some of these drugs for disrupting vesicular traffic has been overestimated. In fact, another study found that blockade of endosomal trafficking had no effect on basal synaptic transmission, although it did reduce the ability to induce LTP (Park et al., 2004).

Our data is best compared with the results from the study by Passafaro et al. (Passafaro et al., 2001), which used overexpressed receptors with an extracellular thrombin cleavage tag to address directly the rate of exocytosis of AMPA receptors from internal stores. This study found that the exocytosis rate depended on the AMPA receptor subunit overexpressed. Because we studied native receptors, which are thought to be heteromers of GluR1/2 and GluR2/3 subunits (Wenthold et al., 1996), our data is best compared with their results when GluR1 and GluR2 were coexpressed. Under these conditions, they observed about 25% recovery of surface fluorescence in 1 hr. We observed ~35% recovery of extrasynaptic currents in 30 min and ~25% recovery of synaptic currents in 3 hr (see Figures 3 and 4). The slightly faster recovery rates observed with overexpressed receptors by Passafaro et al. (2001) may be due to the substantial overproduction of AMPA receptor subunits.

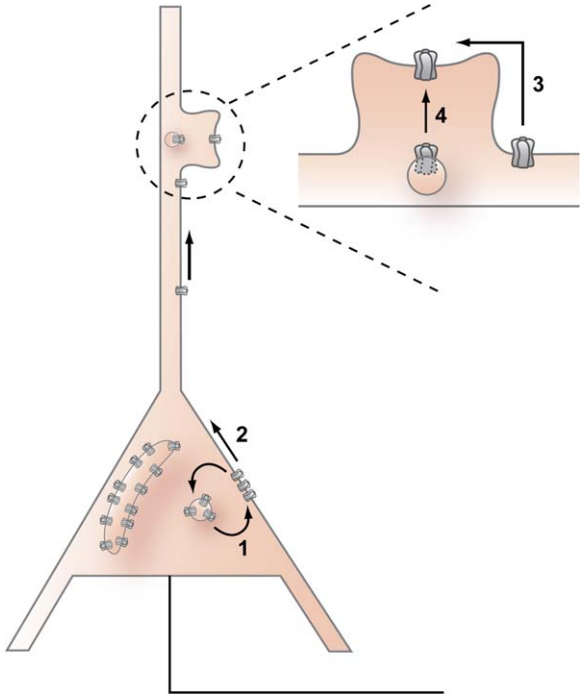

Figure 7. A Model of Basal AMPA Receptor Trafficking

A large intracellular pool of AMPA receptors exchanges rapidly (1) with extrasynaptic somatic AMPA receptors, and these newly inserted AMPA receptors then travel laterally (2) out to dendrites to reside stably at synapses. The lateral diffusion of perisynaptic receptors into the synapse may be regulated by accessory synaptic proteins (3). The exchange of intracellular receptors with synaptic receptors is slow (4).

An obvious experiment to conduct with ANQX is to irreversibly antagonize surface AMPA receptors and then induce long-term potentiation (LTP) because LTP is thought to involve the rapid insertion of AMPA receptors into synapses, possibly from an intracellular pool. Unfortunately, despite considerable effort, we were unable to obtain a reliable induction protocol for LTP in cultured neurons. Conducting such an experiment in acute hippocampal slices, in which LTP is a robust phenomenon, also was not possible because of several technical limitations of this system (for a full discussion see Supplemental Data).

A fundamental question in molecular neuroscience is how neurotransmitter receptors synthesized within the cell body are targeted to synapses. Are receptors first exocytosed at the cell body and then trafficked along the cell surface to synapses, or are they trafficked intracellularly to dendrites and then exocytosed directly at synapses? Our data support the former model (Figure 7). First, we established that there is a large intracellular pool of AMPA receptors that are mostly inserted at the cell body. Second, this pool of receptors rapidly supplies the neuronal surface with functional receptors at the cell body but not at dendrites or synapses—suggesting that the bulk of the cycling intracellular pool is restricted to the soma. Third, the lateral diffusion of receptors at the cell body is rapid, and according to previously published

reports, the lateral diffusion at dendrites is also rapid (Tardin et al., 2003). This observation supports the idea that diffusion along the surface is a primary route for targeting receptors to the synapse, but this must be confirmed with future experiments involving highly focused inactivation of AMPA receptors selectively at postsynaptic densities. Additionally, because disruption of microtubules did not affect recovery of synaptic current after photoinactivation, nor did it alter synaptic transmission over a 6 hr period, it is unlikely that transport of AMPA receptors along microtubules contributes significantly to synaptic AMPA receptor cycling.

Given that somatic exocytosis is fast and the mobility of surface receptors is high, why does it still takes hours to see appreciable recovery of synaptic currents? Based on the measured coefficient of diffusion (*D*) for extrasynaptic AMPA receptors from optical studies (Tardin et al., 2003) and a simple model of Brownian motion along a one-dimensional surface (in which $x^2 = 2Dt$ and $D = 0.45\ \mu m^2/s$), an AMPA receptor at the cell body could take up to 3 hr to travel 100 μm, the range of most proximal synapses. Even at synapses close to the cell body, the replacement of photoinactivated receptors with functional receptors from internal stores will still be limited by the relative immobility of synaptic receptors as observed in the optical study. Thus, we suggest that the rate-limiting step in the exchange of synaptic receptors could be a regulated process in which accessory synaptic proteins "free" a synaptic receptor, making the site available for a new receptor to diffuse inward and become fixed. Future studies will be required to quantitatively determine the rate at which laterally diffusing dendritic AMPA receptors supply synapses with functional receptors.

The present study highlights the power of using a photoreactive antagonist to study AMPA receptor trafficking. By irreversibly inactivating all surface AMPA receptors, we were able to track the movement of receptors from internal stores to the cell surface. By irreversibly inactivating AMPA receptors focally, we were able to monitor the rapid movement of receptors across the membrane surface. We anticipate that future work with ANQX, as well as other classes of photoreactive receptor ligands, will help answer fundamental questions in neuroscience related to receptor trafficking, synaptic plasticity, and neural circuit function.

Experimental Procedures

Cell Culture

Dissociated hippocampal cultures were prepared from P0 Sprague-Dawley rat pups. Hippocampi were dissected, and the dentate gyrus was carefully removed. The tissue was enzymatically digested with papain (Worthington) mechanically triturated and plated on poly-D-lysine-coated coverslips at a density of 100,000 cells/well. Cells were cultured in Neurobasal-A media (Gibco) supplemented with B27, FBS, and pencillin/streptomoycin for 3 to 5 days, and then half the media was replaced with serum-free media containing the mitotic inhibitor FUDR (10 μM). Cultures were used for physiology between 12–24 DIV.

Patch-Clamp Electrophysiology

Recordings were performed at room temperature with an Axopatch-1B or 1D amplifier and patch pipettes of 3–5 MΩ. Series resistances ranged between 10 and 25 MΩ. Cells were visualized by IR-DIC on an upright BX50WI or BX51WI Olympus microscope fitted with a 40× objective (LumplanFI). The external solutions contained (in mM) 140 NaCl, 2.4 KCl, 10 HEPES, 10 glucose, 2–4 $CaCl_2$, 0–4 $MgCl_2$, 0.01 glycine, and 0.1 picrotoxin (pH 7.27). TTX (500 nM) was included for analysis of mEPSCs, sucrose EPSCs, outside-out patches, and all caged glutamate experiments. The internal solution contained (in mM) 115 $CsMeSO_4$, 20 $CsCl_2$, 10 HEPES, 2.5 $MgCl_2$, 4 NaATP, 0.4 NaGTP, 10 NaCreatine, 0.6 EGTA, and 5 QX314 (pH 7.2). For recording of autaptic EPSCs, K-gluconate was substituted for $CsMeSO_4$. For perforated-patch recording, 100 μg/ml gramicidin and 10 μM Alexa-fluor 488 were included in the internal solution. mEPSCs (about 100 events per cell) were automatically detected with in-house software. Picrotoxin and TTX were from Sigma; MNI-caged glutamate, AMPA, AP-V, and QX-314 were from Tocris. ANQX was synthesized in-house according to Chambers et al. (2004). Drugs were dissolved in HEPES buffer or DMSO, and concentrated stocks were stored at −20ºC. Glutamate, AMPA, ANQX, hypertonic sucrose (1M), and MNI-glutamate were delivered by a local flow pipe (350 μm tip) connected to a manifold fed by four reservoirs (Automate Scientific). Sucrose-evoked EPSCs and glutamate-evoked currents in outside-out patches were recorded in the presence of cyclothiazide (100 μM) to prevent desensitization, making measurements more rigorously quantitative. Synaptic and whole-cell currents were recorded without cyclothiazide. Solutions were switched by alternately opening and closing valves attached to each reservoir, and solutions could be completely exchanged in 1–2 s. Student's t test was used for all statistical analysis, except for analysis of mEPSC cumulative distributions, for which the Kolmogorov-Smirnov test was used. Sucrose-evoked responses in a given experiment were normalized to an average of responses in at least eight control cells in that same experiment to account for variability of the amplitude of this response between different sets of cultures, presumably because of different survival densities of cells. Each data point presented for serially sampled experiments (Figures 3 and 4) represents the averaged response of at least eight cells or patches recorded in 15 min bins. Extracellularly evoked EPSCs were induced by placing a glass monopolar pipette within 100 μm of the recorded cell. This form of stimulation frequently yielded a measurable monosynaptic EPSC.

Photolysis of ANQX and Caged Glutamate

For continuous recordings, ANQX (10–100 μM) was locally applied for 5–10 s at a flow of 1 ml/min and activated by 0.5–3 s of continuous UV light (as indicated) from a mercury arc lamp (100 W Olympus) filtered by a 330–385 nm bandpass UV filter (Olympus). This always gave reliable irreversible block of AMPA currents and minimal reduction in NMDA currents with or without the presence of cyclothiazide or under any other condition. Cyclothiazide was only included in the photoinactivation buffer in Figure 1A but was absent in all other cases. UV exposure of these durations alone had no lasting effect on the amplitude of AMPA or NMDA currents. More prolonged inactivation with focused UV irradiation often resulted in increases in holding current, decreases in input resistance, and nonspecific reductions in NMDA currents. For inactivation of a complete coverslip of cultured neurons, ANQX (100 μM) was bath applied for 2–3 min at 5 ml/min with continuous unfocused UV irradiation (no objective). Under these conditions, we observed no detectable phototoxicity as evidenced by no decrement in the averaged response to exogenously applied glutamate when UV light was applied alone. Additionally, there was no change in the average input resistance or basic morphological features of the cells. For focal activation of ANQX and glutamate uncaging, a pulsed UV laser (UVILA, 355 nm, Rapp Optics) was used coupled to a 25 or 50 μm quartz fiber and launched into a spot illumination adaptor (Rapp Optics) housed in the epifluorescence port of the microscope. The beam was focused through a 40× objective to ~5 or ~20 μm spot measured with a red He/Ne laser also coupled to the same fiber. UV laser activation of ANQX required between 15 and 30 pulses, given at 1 Hz. Caged glutamate was locally applied from an alternate valve at a concentration of 0.2–0.5 mM at 0.033 Hz for 2–3s in the presence of TTX to reduce spontaneous activity. Cyclothiazide (100 μM) was sometimes included with MNI-glutamate to increase the amplitude of the response, but no difference in recovery rate in CTZ was observed, so the data were pooled. Although we generally did not observe any movement of the preparation during recordings, to ensure that drift

of the preparation did not lead to artifactual changes in uncaged glutamate responses, we compared photographs of each cell before and at the end of each recording. Any experiments in which drift was apparent were excluded from analysis. We ensured that UV photolysis of ANQX and MNI-glutamate occurred at the same spot for experiments in Figure 6 by, in some cases, moving the laser laterally across the cell body after photoinactivation of the receptors in the first spot. Uncaged glutamate current was always observed at the lateral site (at comparable amplitudes to the first site) indicating that photocrosslinking was limited to the region being irradiated.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/48/6/977/DC1/.

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