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The Role of Intracellular Sodium in the Regulation of NMDA-Receptor-Mediated Channel Activity and Toxicity

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Abstract

Sodium (Na^+) is the major cation in extracellular space and, with its entry into cells, may act as a critical intracellular second messenger that regulates many cellular functions. Through our investigations of mechanisms underlying the activity-dependent regulation of *N*-methyl-D-aspartate (NMDA) receptors, we recently characterized intracellular Na^+ as a possible signaling factor common to processes underlying the upregulation of NMDA receptors by non-NMDA glutamate channels, voltage-gated Na^+ channels, and remote NMDA receptors. Furthermore, although Ca^{2+} influx during the activation of NMDA receptors acts as a negative feedback mechanism that downregulates NMDA receptor activity, Na^+ influx provides an essential positive feedback mechanism to overcome Ca^{2+}-induced inhibition, thereby potentiating both NMDA receptor activity and inward Ca^{2+} flow. NMDA receptors may be recruited to cause excitoxicity through a Na^+-dependent mechanism. Therefore, the further characterization of mechanisms underlying the regulation of NMDA receptors by intracellular Na^+ is essential to understanding activity-dependent neuroplasticity in the nervous system.

Index Entries: NMDA channel gating; sodium and calcium; single-channel activity; excitability; toxicity.

Introduction

Sodium (Na^+) is the major cation in extracellular space. Through activated ligand- (e.g., glutamate) and voltage-gated cation channels, membrane exchangers, and/or gradient-driven cotransport, Na^+ can enter cells and, therefore, alter neuronal functions *(1,2)*. Voltage-gated Na^+ channels, which initiate fast-action potentials and shape subthreshold electrical properties *(2–6)*, are essential for neuronal excitability. The expression of voltage-gated Na^+ channels is developmentally and dynamically regulated,

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exhibiting extensive plasticity at both the transcriptional and posttranscriptional levels *(3–6).* Na^+ entry may regulate the functions of both pre- and postsynaptic machinery in the central nervous system (CNS; refs. *7–11*). For example, an increase in intracellular Na^+ concentration ($[Na^+]_i$) may speed up the turnover of vesicular pools in presynaptic terminals, thus affecting neurotransmitter release *(10).*

Stuart and Sakmann *(9)* demonstrated that local Na^+ influx produces an amplification of excitatory postsynaptic potentials in neocortical pyramidal neurons. Linden and colleagues *(7,8,11)* showed that inhibition of Na^+ influx into postsynaptic neurons blocks the induction of long-term depression of synaptic responses in cultured cerebellar neurons. The postsynaptic spike bursts, and long-term potentiation induced by tetanus stimulation at the θ-frequency (5 Hz × 150 pulses) in hippocampal neurons can be blocked by application of voltage-gated Na^+ channel blockers such as QX-314 or tetrodotoxin (TTX) *(12).* Researchers have consistently demonstrated that electrical activity—particularly bursting activity—dramatically increases $[Na^+]_i$ in neurons *(13–15).* Specifically, $[Na^+]$ may be increased up to 40 m*M* in spines and their adjacent dendrites by short bursts of synaptic stimulation and by up to 100 m*M* during tetanic stimulation that induces synaptic long-term potentiation *(15,16).*

Interestingly, Na^+ influx and efflux may be differentially regulated by dopaminergic D1 and D2 receptors *(17),* and the $[Na^+]$ increases in spines and dendrites induced by synaptic stimulation, which are concurrent to synaptic potentials and back-propagating action potentials, are mainly mediated by Na^+ entry through *N*-methyl-D-aspartate (NMDA) type glutamate channels *(15,16).*

This article focuses on essential findings that are relevant to the actions of intracelluar Na^+ in the regulation of NMDA receptor functions. To provide some background knowledge, the actions of Na^+ as an important second messenger and its involvement in the pathological process of tissue injury are briefly reviewed.

Na^+ Acts as an Important Second Messenger

A growing number of studies have shown that intracelluar Na^+ may act as a second messenger to regulate many cellular functions. For example, intracelluar Na^+ may downregulate the activity of amiloride-sensitive epithelial Na^+ channels, forming a negative feedback control mechanism of these channels *(18–21).* Further mechanistic studies have demonstrated that Na^+ activates the ubiquitin–protein ligase Nedd4, which binds to the C-termini of the β- and γ-subunits of the channels through its WW domains, and consequently inhibits channel activity *(19–21).*

Intracellular Na^+ may also directly activate ion channels that are permeable to other ions, such as potassium (K^+) *(22–31).* An increase in $[Na^+]_i$ by 10 to 30 m*M* may activate *Slo* gene-encoded K^+ channels *(26,27,29,30,32),* which are inhibited by increases of intracellular K^+ *(30,32).* D226N, a single-point mutation in the Na^+-activated K^+ channel, abolishes the Na^+-dependent activation of both homo- and heteromeric K^+ channels *(33).* Phosphatidylinositol bisphosphate (PIP_2) dramatically increases the open probability of the K^+ channels in the absence of Na^+ but does not preclude further activation by Na^+, suggesting that Na^+ might simply act to promote PIP_2 binding to the channels *(33).* Dascal and colleagues showed that increases in intracellular Na^+ may promote dissociation of α- and βγ-subunits of G proteins and that via this mechanism, an increase in intracellular Na^+ either may upregulate G protein-gated K^+ channels *(28,31)* or downregulate N-type voltage-gated Ca^{2+} channels *(34).* Data also exist to suggest that Na^+ and K^+ may compete for a superficial site on permeation pathway of K^+ channels *(30,32).* Na^+-mediated K^+ channels are widely distributed throughout the nervous system and are involved in both the regulation of the after potential following action potentials *(30,35)* and the protection of neurons from hypoxic stimulation *(27,29,30).*

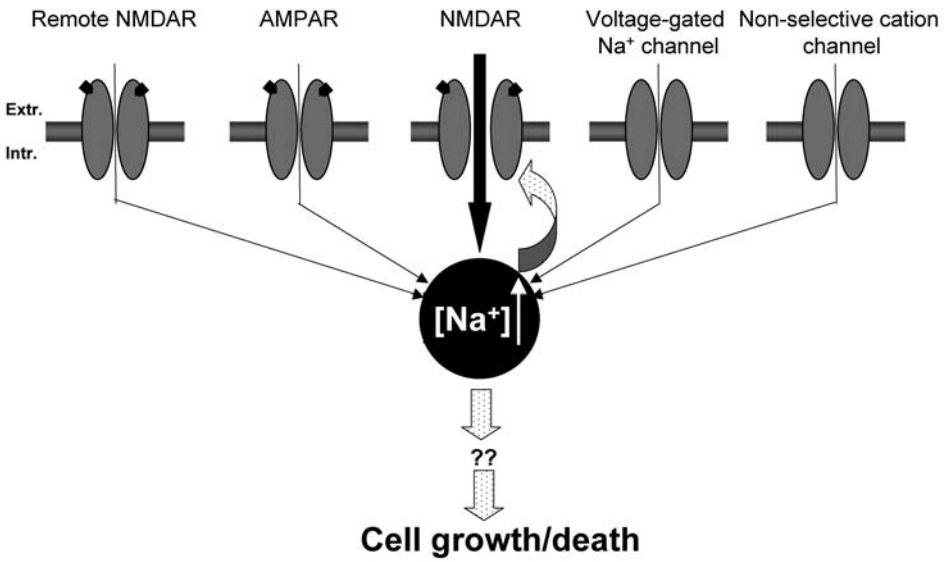

Fig. 1. Intracellular Na^+ may act as common signaling factor upregulating NMDA receptors by other receptor/channels such as AMPA receptors, voltage-gated Na^+ channels, nonselective cation channels, and remote NMDA receptors. NMDAR, NMDA receptor; AMPAR, AMPA receptor; Extr., extracellular; Intra., intracellular.

Some have found that intracellular Na^+ plays an important role in the regulation of receptor–ligand and ligand–transporter interactions *(36–40).* Cox and colleagues *(39)* reported that intracellular Na^+ at 10 to 30 m*M* may significantly reduce binding of agonists to opioid receptors on guinea pig cortical neuron membranes. Maximal inhibition of μ-, δ-, and κ-opioid receptor binding by Na^+ is approx 60, 70, and 20%, respectively. They further demonstrated that intracellular Na^+ does not reduce the density of μ-opioid receptor binding but, rather, reduces the density of δ-opioid receptor binding with no change in agonist affinity. Additionally, the κ-receptors appear to be less sensitive to Na^+ than the μ- and δ-opioid receptors *(39).*

Intracellular Na^+ and extracellular Na^+ can have opposing effects on ligand–transporter interactions. For example, intracellular Na^+ inhibits, whereas extracellular Na^+ facilitates, the binding of dopamine to dopamine transporters *(41–44).* These may associate with the opposite effects of intracellular Na^+ and extracellular Na^+ on the formation of the inward-facing state of dopamine transporters *(45).*

Na^+ is also implicated in processes of cell proliferation. Glutamate receptor activation inhibits mitogenic-factor-induced proliferation of cortical oligodendrocyte progenitor cells through an increase in intracellular Na^+ *(25).* Agents that increase intracellular Na^+ may also inhibit mitogenic-factor-induced proliferation *(25).* The mechanisms underlying these effects of intracellular Na^+ have yet to be elucidated. One possibility may be that Na^+ entry may cause an increase in cytosolic Ca^{2+} through either Na^+–Ca^{2+} exchanger or activation of voltage-gated Ca^{2+} channels *(46,47),* thereby activating Ca^{2+}-dependent mechanisms. Another suggestion is that Na^+ entry, via Na^+–H^+ exchange, may cause changes in intracellular pH *(48–51),* thereby regulating many cellular functions, including enzyme activity, neuronal growth, and death (Fig. 1).

Na^+ in the Process of Tissue Injury

A significant increase in $[Na^+]_i$ has been found to be a characteristic event associated with tissue injury, as found in cardiac cells after burn injury

(52) and in central neurons following hypoxic and/or traumatic insults *(53–57).* Banasiak and colleagues *(57)* showed that hypoxia induces increases of intracellular Na^+ and neuronal apoptosis in cultures of rat neocortical neurons, which can be detected by electron microscopy, annexin V staining, and terminal UDP nick-end labeling staining. Application of voltage-gated Na^+ channel blocker reduces both Na^+ entry and apoptotic neuronal death *(57).* Conversely, increasing Na^+ entry using the voltage-gated Na^+ channel activator veratridine induces neuronal apoptosis and caspase-3 activation *(57).* Sheldon et al. *(58)* showed that Na^+ entry during anoxia apparently occurs through Gd^{3+}-sensitive pathways and $Na^+/K^+/2Cl^-$ cotransport in cultured hippocampal neurons. Together, these findings imply that multiple Na^+ entry pathways may be activated during tissue injury.

It is well-known that an accompanying influx of Cl^- and water during large amounts of Na^+ influx may lead to acute neuronal swelling and damage *(59,60).* Interestingly, a recent study demonstrated that cell shrinkage (or the loss of cell volume), which is a ubiquitous characteristic of programmed cell death, can be reversed in Jurkat cells by a blockade of Na^+ influx and that the initial Na^+ influx may play an important role in the onset of anti-Fas-induced apoptosis *(61).* Although mechanisms underlying ischemia-induced injury remain elusive, evidence exists to show that the inhibition of Na^+–H^+ exchange may attenuate ischemia-induced cell death *(62, 63)* and that the blockade of voltage-gated Na^+ channels protects neurons from traumatic injury to both the spinal cord *(53,64–68)* and peripheral nerves *(67,69).* Application of voltage-gated Na^+ channel blockers can reduce the loss of white matter resulting from injury *(65,67),* minimize the sensitization associated with chronic pain *(70),* and prevent seizures during kindling development *(71).* Therefore, finding effective therapeutic approaches targeting voltage-gated Na^+ channels has been one of the major focuses in pharmaceutical research *(56).*

Na^+ in the Regulation of NMDA Receptors

The NMDA receptor is a subtype of a receptor activated by glutamate, and is highly permeable to both mono- and divalent cations such as Na^+ and Ca^{2+} *(72–74)* and critically involved in the regulation of many physiological and pathophysiological processes, such as synaptic plasticity *(75–79),* neuronal survival, and death *(80–86).* NMDA receptor proteins are found to be associated with a large number of other proteins including neurotransmitter receptors, enzymes, cytoskeletal and adaptor protein *(87–93).*

Based on previous findings that: (a) NMDA receptor activation stimulates protein tyrosine phosphorylation *(94),* (b) protein tyrosine phosphorylation may be enhanced by cell depolarization *(95),* and (c) NMDA receptor activity may be regulated by receptor-associated protein tyrosine kinases *(96,97),* we initially hypothesized that NMDA receptor-associated protein tyrosine kinases might be activated by cell depolarization and/or by the activation of NMDA receptors and, therefore, upregulate NMDA receptor activity. To examine this hypothesis, we recorded NMDA single-channel activity evoked by including 10 μ*M* of NMDA in a standard extracellular solution that filled recording electrodes in neurons bathed with the extracellular solution containing: 100 m*M* of Na_2SO_4, 10 m*M* of Cs_2SO_4, 25 m*M* of HEPES, 1.3 m*M* of $CaCl_2$, 33 m*M* of glucose, 0.001 m*M* of TTX, 0.003 m*M* of glycine, pH 7.35 and osmolarity 310 to 320 m*M* in cell-attached patches (Fig. 2A) before and during bath application of the same extracellular solution, with the exception of including 30 m*M* of KCl or the NMDA receptor agonist L-aspartate (500 μ*M*) or NMDA (300 μ*M*) *(98).* Interestingly, we found that although both the 30 m*M* of KCl and NMDA receptor agonist applications produced similar neuronal depolarization (approx 20 mV), the application of NMDA receptor agonists—but not KCl—induced a significant change in NMDA single-channel activity *(98,99).* Therefore, we deduced that the alteration of NMDA channel gating during application of NMDA receptor agonist is not

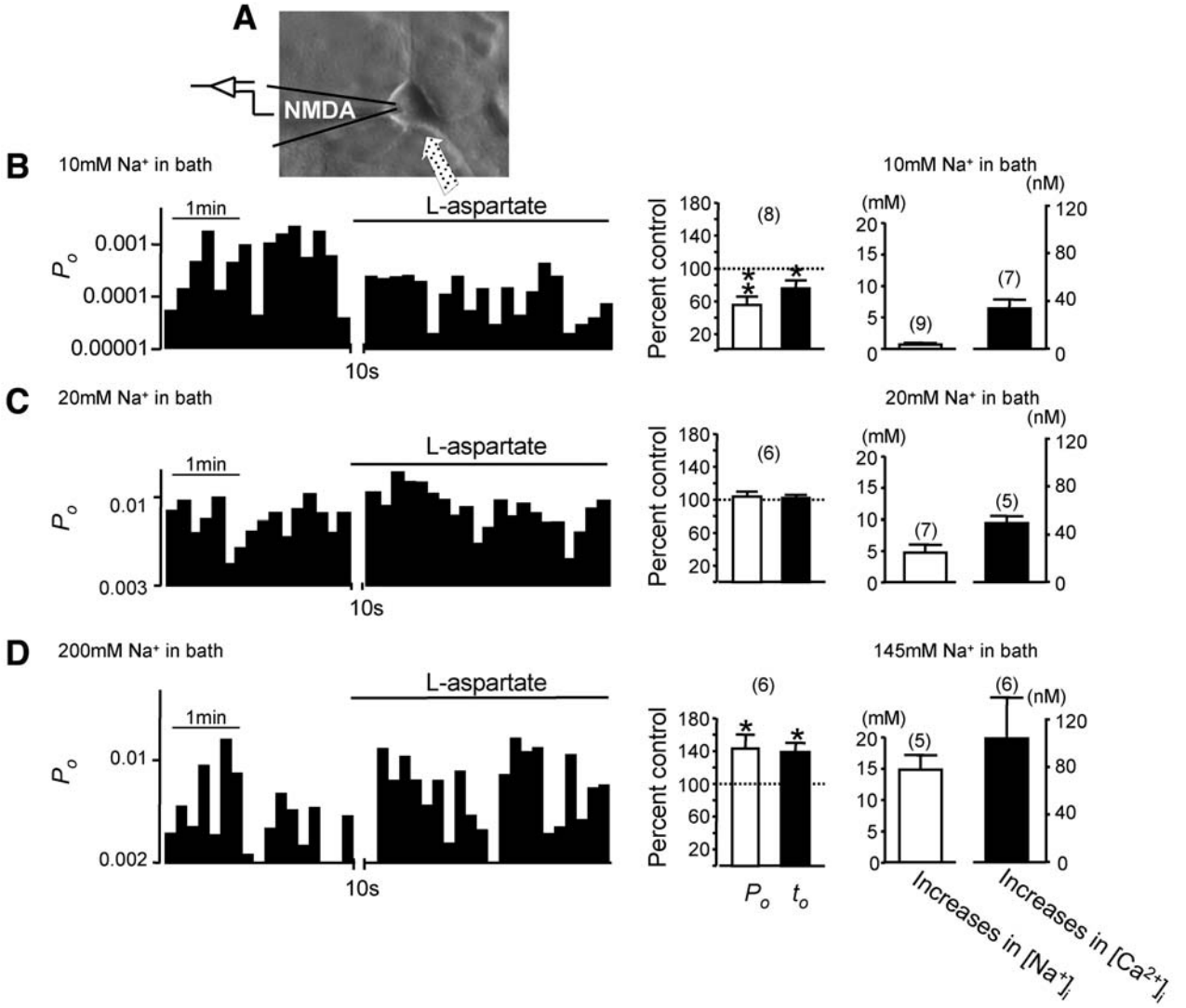

Fig. 2. Functional relationship of Na^+ and Ca^{2+} influx in the regulation of NMDA receptor gating by remote NMDA receptors. **(A)** The cell-attached recording configuration. **(B–D)** Effects of bath application of the NMDA receptor agonist L-aspartate (500 μ*M*) on NMDA single-channel activity and intracellular Na^+ and Ca^{2+} recorded from neurons bathed with the extracellular solution containing 10, 20, 200, or 145 m*M* of Na^+, respectively. The left panels in B, C, and D show examples of NMDA-channel open probability (P_o, bin: 10 sec) before (control) and during the activation of remote NMDA receptors. The break during the recording was required for re-adjusting the patch potential to 70 mV from the NMDA channel reversal potential after L-aspartate was applied to neurons. The middle panels show summarized data (mean ± SEM) outlining overall P_o and mean open time (t_o) during remote NMDA receptor activation. The right panels show summarized data (mean ± SEM) that indicate increases in $[Na^+]_i$ and $[Ca^{2+}]_i$ induced by bath application of 500 μ*M* of L-aspartate. Values in brackets indicate the number of patches/neurons tested. *, **: $p < 0.05$, 0.01 (Wilcoxon test) in comparison of P_o or t_o before (=100%, dashed line) and during the activation of remote NMDA receptors.

induced by cell depolarization. The observation that bath application of NMDA receptor antagonist DL-2-amino-5-phosphonopentanoic acid (APV) alone did not significantly affect NMDA channel activity recorded in cell-attached patches but, rather, prevented the effects of NMDA receptor agonists co-applied indicated that the upregulation of NMDA single-channel activity is induced by activation of remote NMDA channels *(98,100).*

Further experiments have shown that the upregulatory effects induced by the activation of remote NMDA channels were not altered by the removal of extracellular Ca^{2+} but by blockade of Na^+ influx. These data implicate that Na^+ may act as a signaling factor to upregulate NMDA channel activity. This suggestion was subsequently confirmed by experiments examining the effects of neuronal application of Na^+ on NMDA-receptor-mediated single-channel currents, whole-cell currents, and synaptic responses *(98,99).* In particular, application of Na^+ ionophore monensin to neurons bathed with an extracellular solution containing various [Na^+] (from 0 to 50 m*M*) showed that an increase in $[Na^+]_i$ of more than 10 m*M* produced detectable increases in NMDA single-channel activity *(98).*

Na^+ can enter neurons through multiple pathways, including voltage-gated Na^+ channels and non-NMDA glutamate channels. Therefore, via a Na^+-dependent mechanism, the activation of these pathways may directly regulate NMDA receptors. To verify this possibility, we examined the effects of Na^+ influx through activated non-NMDA glutamate channels as well as voltage-gated Na^+ channels *(98).* We found that activating non-NMDA receptors by bath application of kainate (1 m*M*) increased NMDA single-channel activity recorded in cell-attached patches in neurons bathed in an extracellular solution containing 200 m*M* of Na^+, but it inhibited the channel activity if Na^+ was removed from the extracellular solution *(98,99).* To determine whether influx of Na^+ through voltage-gated Na^+ channels may affect NMDA channel function, we applied veratridine (10 μ*M*), which prevents the inactivation of these channels *(98,99).* After the cell-attached configuration was established in neurons, the bath solution was replaced with standard extracellular solution that was modified to block both the main ionotropic neurotransmitter receptors and the release of glutamate and other transmitters evoked by depolarizing presynaptic terminals with veratridine *(98,99).* We found that bath application of veratridine significantly increased NMDA single-channel gating, whereas no such effect was found in presence of TTX *(98).* Therefore, this is the first demonstration that multiple types of receptor/channels, such as α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, voltage-gated Na^+ channels, and nonselective cation channels, may regulate NMDA receptor activity by increasing $[Na^+]_i$ (Fig 1; refs. *98, 100,* and *101*).

It is well-known that activated NMDA receptors are highly permeable to K^+, Cs^+ Na^+, and Ca^{2+} *(72–74)* and that the activation of NMDA receptors produces prolonged increases of both intracellular Ca^{2+} and Na^+ in neurons *(13–15, 53,72–74,100).* Previous studies focusing on how Ca^{2+} affects NMDA receptor activity have convincingly demonstrated that increases in intracellular Ca^{2+} may downregulate NMDA channels, thereby acting as a negative feedback mechanism to control NMDA receptor activity *(72–74, 102–110).* Therefore, both intracellular Ca^{2+} and intracellular Na^+ may regulate the activity of NMDA receptors, but they may do this in an opposing manner. To clarify how NMDA channels may be regulated when both ionic species flow into neurons during the same time through activated NMDA receptors, we investigated the functional relationship of Na^+ and Ca^{2+} influxes in the regulation of NMDA channel gating by the activation of remote NMDA receptors *(100).*

Individual NMDA Receptors Can Be Up- and Downregulated by the Activation of Remote NMDA Receptors

We found that bath application of the NMDA receptor agonist L-aspartate may change NMDA

channel activity recorded in cell-attached patches in a concentration-dependent manner. A significant increase in NMDA channel gating occurred when 100 μ*M* of L-aspartate was applied by bath *(100).* Our initial study *(98)* showed that the activation of remote NMDA receptors may inhibit NMDA channel activity when Na^+ in the extracellular solution is replaced with the membrane-impermeant cation *N*-methyl-D-glucamine. However, the mechanism underlying this effect remained unclear.

By recording the ratio of 346 vs 380 nm of fluorescence in the Na^+-sensitive dye (sodium-binding benzofuran isophthalate) and the Ca^{2+}-sensitive dye (Fura-2) in the soma region of neurons, we found that the basal $[Ca^{2+}]_i$ and $[Na^+]_i$ of neurons were approx 84 n*M* and 16 m*M*, respectively, when the Na^+ gradient across the cell membrane was decreased by reducing extracellular Na^+ concentration ($[Na^+]_e$) to 20 m*M* and including the Na^+ ionophore monensin (10 μ*M*) in the extracellular solution. Under this condition $[Ca^{2+}]_i$ increased by 66 n*M*, whereas $[Na^+]_i$ decreased by 5.8 m*M*, and NMDA single-channel gating was significantly reduced during bath application of L-aspartate *(100).*

The average overall channel open probability and mean open time were reduced to 64 and 77% of controls, respectively. The burst and cluster lengths were also significantly reduced. These inhibitory effects produced by the bath application of L-aspartate could be effectively prevented by application of APV, indicating that the activation of remote NMDA receptors may also downregulate NMDA channel activity. Interestingly, we found that blocking Ca^{2+} influx by removal of Ca^{2+} from the extracellular bath solution may also prevent the inhibition of NMDA channel gating induced by the activation of remote NMDA receptors *(100),* suggesting that Ca^{2+} influx may play an important role in the downregulation of NMDA channel gating induced by the activation of remote NMDA receptors when Na^+-influx is reduced. Bregestovski and colleagues *(109)* found that NMDA single-channel activity recorded in cell-attached patches could be downregulated after wash-out of NMDA, which was bath-applied to neurons. Together, these findings demonstrate that the activity of individual NMDA channels may be regulated by their neighboring NMDA receptors (Fig. 1; ref. *100*).

NMDA receptors on the cell surface are located on both the synaptic and extrasynaptic regions of central neurons. Because there is a lack of direct evidence showing the effects of the activation of remote NMDA receptors on the activity of synaptic NMDA receptors, simulation experiments were performed to estimate the potential effects of the activation of remote NMDA receptors on synaptic responses mediated by NMDA receptors. Supercluster current traces recorded before and during the activation of remote NMDA receptors were aligned at the start of the first openings and were subsequently summed to produce an ensemble current *(100).* We found that depending on the amount of Na^+ and Ca^{2+} influx through activated remote NMDA receptors, the decay of ensemble currents could be increased or decreased *(100).* Therefore, we believe that the crosstalk between NMDA receptors, which is mediated by a functional Na^+–Ca^{2+} interaction, may be an important mechanism regulating synaptic responses.

Na^+ Influx Enhances Ca^{2+} Influx and Masks the Ca^{2+}-Dependent Inhibition of NMDA Channels

We then measured the $[Na^+]_i$ and $[Ca^{2+}]_i$ of neurons bathed with the standard extracellular solution containing a $[Na^+]$ of 10, 20, and 145 m*M* before and during the activation of NMDA receptors induced by bath application of L-aspartate. We found that with an increase in $[Na^+]_e$, the activation that NMDA receptors produced increased $[Na^+]_i$ as expected but also unexpectedly increased $[Ca^{2+}]_i$ (Fig. 2). Excluding the effect of Ca^{2+}-influx-induced Ca^{2+} release from intracellular stores, the increase in $[Ca^{2+}]_i$ of neurons bathed with extracellular solution containing 145 m*M* of Na^+ was significantly higher than that of neurons bathed with

extracellular solution containing 10 m*M* of Na^+ *(100).* This strongly suggests that Na^+ influx may enhance Ca^{2+} influx during NMDA receptor activation; consequently, further investigations are required to clarify the functional relationship between Ca^{2+} and Na^+ influx in the regulation of NMDA channel activity by remote NMDA receptors.

When extracellular Na^+ was reduced to 10 m*M,* we found that the activation of NMDA receptors produced increases in $[Na^+]_i$ and $[Ca^{2+}]_i$ by around 0.8 m*M* and 35 n*M,* respectively, and that the activation of remote NMDA receptors produced significant inhibition of NMDA channel gating (ref. *100;* Fig. 2). When $[Na^+]$ of the extracellular bath solution was increased to 20 m*M,* NMDA receptor activation produced an increase of 5 m*M* in $[Na^+]_i$ and 50 n*M* in $[Ca^{2+}]_i$, but there was no significant change in NMDA channel gating (ref. *100;* Fig. 2). We also found that increasing extracellular K^+ concentration by 30 m*M* in an extracellular solution containing 170 m*M* of Na^+ and 1 μ*M* of TTX produced increases in $[Na^+]_i$ and $[Ca^{2+}]_i$ by around 7 m*M* and 48 n*M,* respectively, but there were no significant changes in NMDA single-channel gating *(98,100).* Therefore, an approx 5-m*M* increase in $[Na^+]_i$ appears to be a critical concentration for masking the inhibitory effects induced by Ca^{2+} influx on NMDA channels during the activation of remote NMDA receptors in cultured hippocampal neurons *(100).*

In our previous electrophysiological experiments, we found that removal of extracellular Ca^{2+} did not produce any effect on the upregulation of NMDA channels by remote NMDA receptors in neurons bathed with the standard extracellular solution containing 200 m*M* of Na^+ *(98).* Our current study demonstrated that a modest increase of $[Ca^{2+}]_i$ by approx 35 n*M* could inhibit NMDA channel activity when $[Na^+]_e$ was reduced to 10 m*M* (Fig. 2; ref. *100*). These findings suggest that on one hand, Na^+ influx may enhance Ca^{2+} influx and, on the other hand, may also mask the inhibitory effects of Ca^{2+}. To confirm this finding, we recorded NMDA single-channel activity before and during the activation of remote NMDA receptors in cell-attached patches with (a) pipets filled with a Ca^{2+}-free extracellular solution containing 200 m*M* of Na^+ from neurons that had been pretreated with BAPTA-AM (10 μ*M* for 4 h) and bathed with the same Ca^{2+}-free extracellular solution, and (b) pipets filled with extracellular solution containing 0.3 or 1.2 m*M* of Ca^{2+} (which were determined by a Ca^{2+}-selective electrode, Thermo Electron Corporation, Beverly, MA) from neurons bathed with the extracellular solution containing the same amount of Ca^{2+}, respectively. Our observation that the activation of remote NMDA receptors produced a similar upregulation of NMDA channel activity when local and bath $[Ca^{2+}]$ was set at 0, 0.3, and 1.2 m*M* implies that the effects of Ca^{2+} influx in the regulation of NMDA receptors by remote NMDA receptors are overcome by Na^+ under normal conditions *(100).*

An ongoing Ca^{2+} inhibition of NMDA receptors accompanied by the activation of NMDA channels may serve as a negative feedback mechanism to control NMDA channels *(72–74, 108).* The effect of Na^+, which overcomes Ca^{2+}-induced inhibition, provides an essential positive feedback mechanism to enhance NMDA receptor activity and the inward flow of Ca^{2+} to regulate neuronal functions.

Na^+ Influx: A Novel Mechanism Underlying the Recruitment of NMDA Receptors in Neurotoxicity

Glutamate is a principle excitatory neurotransmitter in the CNS. Based on findings that glutamate concentration increases occur in both humans *(111,112)* and animals following nervous system injury *(113)* and that application of NMDA receptor antagonists may protect neurons in animal stroke/CNS trauma models *(59,114),* it has been proposed that NMDA-receptor-mediated excitoxicity may underlie neuronal death associated with stroke/traumatic CNS injury. However, this theory has been challenged by negative findings in clinical trials testing NMDA receptor

antagonists *(115–118),* and until recently, it remained unclear how and when NMDA receptors can be recruited to cause neuronal injury.

We examined the effects of extracellular Ca^{2+} depletion and reperfusion (which may occur in patients with stroke) on cultured hippocampal neurons bathed initially with an extracellular solution containing: 140 m*M* of NaCl, 5 m*M* of CsCl, 1.8 m*M* of $CaCl_2$, 33 m*M* of glucose, 25 m*M* of HEPES; pH 7.35; osmolarity: 310 to 320 mOsm. We found that neuronal injury produced characteristic morphological changes (such as swelling, beading, or process disintegration; Fig. 3), a reduction of 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide, and an increase in caspase-3 activity when extracellular Ca^{2+} was reduced from 1.8 to 0.5 or 0 m*M.* Interestingly, application of NMDA receptor antagonists APV (100 μ*M*) and MK801 (2 μ*M*) were neuroprotective only when applied concomitantly to the reduction of $[Ca^{2+}]_e$ from 1.8 to 0 m*M.* No protective effects of the drugs could be found when applied during Ca^{2+} reperfusion or when $[Ca^{2+}]_e$ was reduced from 1.8 to 0.5 m*M (101).*

These data provide the first piece of experimental evidence indicating that the depletion of extracellular Ca^{2+} may evoke NMDA-receptor-mediated neurotoxicity and that NMDA receptor antagonists can be protective only when applied to neurons within a critical time-period (Fig. 3; ref. *101*). These findings also raise an important issue regarding how and when NMDA receptor activity is recruited to cause neuronal injury by the removal of extracellular Ca^{2+}.

To address this question, we recorded NMDA single-channel activity before and during a depletion of extracellular Ca^{2+} from 1.8 to 1.3, 0.5, or 0 m*M* in cell-attached patches from cultured hippocampal neurons bathed with the standard extracellular solution, in which Cl^- was replaced by SO_4^{2-} to prevent cell damage during the reduction of extracellular Ca^{2+} *(59,98,100,101).* We found that bath application of a low-$[Ca^{2+}]$ solution to neurons caused a parallel shift of the current–voltage relationship in NMDA single-channels, indicating that there is a cell depolarization but no change in single-channel conductance *(100,101).* Therefore, the holding potential was re-adjusted to maintain a 70-mV patch-potential from the reversal potential of recorded channels. No significant change in channel gating was observed unless $[Ca^{2+}]_e$ was reduced from 1.8 to 0 m*M.* Application of the NMDA receptor antagonist MK801 abolished channel activity and confirmed that a $[Ca^{2+}]_e$ reduction from 1.8 to 0 m*M* produced an increase in single-channel currents mediated by NMDA receptors *(98,100,101).*

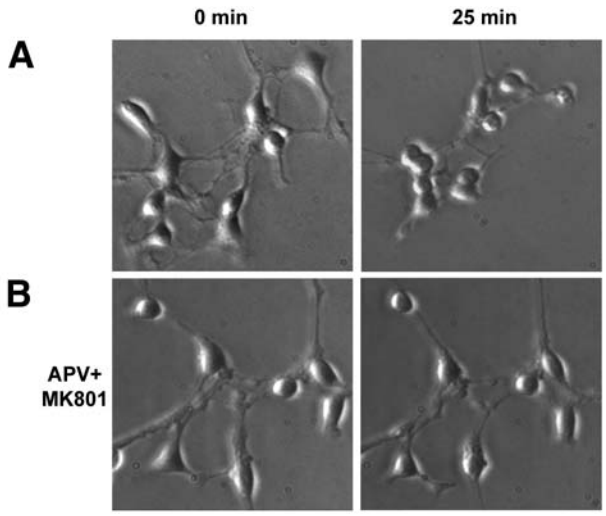

Fig. 3. NMDA receptor antagonists reduced neuronal injury induced by reduction of $[Ca^{2+}]_e$ to 0 m*M.* Images of same neurons were recorded immediately (0 min) and at 25 mins after the culture medium was changed to the extracellular solution containing 0 Ca^{2+}. **(A)** Neurons were bathed in a Ca^{2+}-free extracellular solution. **(B)** Neurons were bathed in the Ca^{2+}-free extracellular solution containing MK801 (2 μ*M*) and APV (100 μ*M*). Compared to the morphology of neurons bathed with the Ca^{2+}-free extracellular solution, including NMDA receptor antagonists in the extracellular solution significantly reduced the number of neurons showing swelling, beading, and/or process disintegration.

Because NMDA single-channel activity was enhanced only when $[Ca^{2+}]_e$ was reduced from 1.8 to 0 m*M* and blocking NMDA receptors

concomitantly to the reduction of $[Ca^{2+}]_e$ from 1.8 to 0.5 m*M* may actually increase the number of injured neurons *(101),* there are indications that the upregulation of NMDA receptors is essential to trigger toxicity mediated by NMDA receptors and that application of NMDA receptor antagonists in the Ca^{2+} reperfusion model may be protective only when NMDA receptors are recruited.

Several recent studies have demonstrated that removal of extracellular Ca^{2+} may induce inward currents, which are mainly carried by Na^+ *(119,120),* through nonselective cation channels *(119)* such as TRPM7 channels *(120).* To identify the mechanisms that may underlie the upregulation of NMDA receptors by the removal of extracellular Ca^{2+}, we measured $[Ca^{2+}]_i$ and $[Na^+]_i$ in cultured hippocampal neurons before and during reductions of $[Ca^{2+}]_e$. We found that reductions of $[Ca^{2+}]_e$ from 1.8 to 1.3, 0.5, 0.1, and 0 m*M* induced a $[Ca^{2+}]_e$-reduction-dependent increase in $[Na^+]_i$ and a decrease in $[Ca^{2+}]_i$ *(101).* These studies not only provided another piece of quantitative evidence confirming that an increase in $[Na^+]_i$ of less than 10 m*M* may not upregulate NMDA receptor activity *(98)* but also showed that a reduction of extracellular Ca^{2+} from 1.8 to 0 m*M* might produce increases in $[Na^+]_i$, which sufficiently enhances NMDA channel gating *(98,100,101).*

It is known that the removal of extracellular Ca^{2+} to 0 m*M* may increase NMDA single-channel conductance *(73,74,121)* and that reducing intracellular Ca^{2+} may reduce the Ca^{2+}-dependent inhibition of NMDA receptors, thereby enhancing NMDA channel activity *(73,74,107,122–127).* Therefore, it is possible that NMDA receptor gating may be enhanced by the removal of extracellular Ca^{2+} through Na^+- and/or Ca^{2+}-dependent mechanisms. We examined the effects of blocking Na^+ influx by bathing neurons in extracellular solution containing 20 m*M* of Na^+ and monensin. We found that blocking Na^+ influx prevented the upregulation of NMDA channel gating induced by extracellular Ca^{2+} reduction *(101).* By comparing the ensemble currents produced by the summation of consecutive superclusters recorded before (1.8 m*M*) and after reducing $[Ca^{2+}]_e$ to 0 m*M,* we found that the removal of extracellular Ca^{2+} can significantly increase the decay time of ensemble currents and that this effect can be abolished by blocking Na^+ influx, which suggests that the removal of extracellular Ca^{2+} may affect NMDA-receptor-mediated whole-cell responses via the action of Na^+.

Because large reductions in $[Ca^{2+}]_e$ may occur during instances of high neuronal activity *(128,129),* the development of seizures *(130),* hypoglycemic coma *(131),* and periods of hypoxia and ischemia *(132,133),* the Na^+-dependent enhancement of NMDA channel activity induced by lowering $[Ca^{2+}]_e$ may be an important mechanism underlying the increase in neuronal excitability and neurotoxicity in the CNS.

Src Family Kinases Act as Switches That Control the Effects of Intracellular Na^+

Interestingly, we noted that an increase in $[Na^+]_i$ larger than 10 m*M* can upregulate NMDA single-channel activity recorded in cell-attached patches *(98,100,134),* whereas larger increases in $[Na^+]$ (>50 m*M*) on the cytoplasmic site of inside-out patches are required for such an effect *(98,134).* Furthermore, we found that application of the broad-spectrum protein kinase inhibitor staurosporine (1 μ*M*) prevented the increase in channel activity induced by monensin applied to neurons bathed with an extracellular solution containing 50 m*M* of Na^+. Therefore, we hypothesized that protein kinases might be critically involved in the regulation of NMDA receptors.

Because protein tyrosine kinase Src was known to both associate with and upregulate NMDA channels *(97),* we examined the effects of activating Src family kinases (SFKs)with the phosphopeptide EPQ(pY)EEIPIA, which binds to the Src SH2 domain to enhance Src activity *(135,136).* After the patches were treated with EPQ(pY)EEIPIA for 5 to 8 min and the channel activity reached a stable level, Na^+ was applied to the cytoplasmic face of the membrane. We

found that NMDA channel activity recorded from such patches can be upregulated by a significantly smaller increase in Na^+ concentration than in patches without the peptide treatment or those treated with the dephosphorylated peptide EPQYEEIPIA, which has been shown not to activate SFKs *(98,134,135).* Therefore, we have concluded that Na^+-dependent regulation of NMDA receptors is controlled by protein tyrosine kinases such that enhancing or reducing the activity of protein tyrosine kinases can potentiate or depress the regulation of NMDA channel gating by intracellular Na^+ *(98,99,134).*

Questions and Future Studies

Because we found that the effects of Na^+ applied to cytoplasmic face of the membrane on NMDA channel gating may be significantly enhanced by treatment with Src kinase activator peptide, we examined whether Na^+ can enhance Src kinase activity. We performed in vitro kinase assays and found that an increase in [Na^+] from 0 to 140 m*M* produced no change in Src enzyme activity (Zhao and Yu, unpublished data, 2003). Therefore, it is unlikely that Na^+ upregulates NMDA channel activity through the enhancement of Src enzyme activity. However, it remains unknown how protein tyrosine kinases regulate the actions of Na^+ on NMDA channels.

There is an abundance of data demonstrating that the regulation of NMDA receptors by SFKs plays very important roles in many physiological and pathophysiological processes *(93,136–146).* SFKs are critically involved in signal transduction mediated by growth factors, G protein-coupled receptors, and/or ligand-gated ion channels in almost all cell types, including neurons and cancer cells *(147–157).*

Several tyrosine residues in NR2A and NR2B subunits are involved in the regulation of the phosphorylation and channel activity of NMDA receptors by SFKs *(93,136,158–161).* Therefore, another possibility resulting from the regulation of Na^+ actions by SFKs may be that the regulation of NMDA receptors by Na^+ depends on phosphorylation status of the receptors. Because Na^+ influx is closely regulated by neuronal discharge activity *(1,2)* and SFKs are important signaling factors regulating many functions from neuronal development to synaptic plasticity *(93,136–146),* the elucidation of mechanisms underlying the regulation of the actions of Na^+ by SFKs will be necessary for understanding the regulation of neural excitability and toxicity.

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References

1. Nicholls D. and Attwell D. (1990) The release and uptake of excitatory amino acids. *Trends Pharmacol. Sci.* **11,** 462–468.
2. Hille B. (1992) In: *Ionic Channels of Excitable Membranes,* Sinauer, Sunderland.
3. Waxman S. G., Dib-Hajj S., Cummins T. R., and Black J. A. (2000) Sodium channels and their genes: dynamic expression in the normal nervous system, dysregulation in disease states(1). *Brain Res.* **886,** 5–14.
4. Cantrell A. R. and Catterall W. A. (2001) Neuromodulation of Na+ channels: an unexpected form of cellular plasticity. *Nat. Rev. Neurosci.* **2,** 397–407.
5. Goldin A. L. (2001) Resurgence of sodium channel research. *Annu. Rev. Physiol.* **63,** 871–894.
6. Catterall W. A., Goldin A. L., and Waxman S. G. (2003) International Union of Pharmacology. XXXIX. Compendium of voltage-gated ion channels: sodium channels. *Pharmacol. Rev.* **55,** 575–578.
7. Linden D. J., Smeyne M., and Connor J. A. (1993) Induction of cerebellar long-term depression in

culture requires postsynaptic action of sodium ions. *Neuron* **11,** 1093–1100.
8. Linden D. J. (1994) Long-term synaptic depression in the mammalian brain. *Neuron* **12,** 457–472.
9. Stuart G. and Sakmann B. (1995) Amplification of EPSPs by axosomatic sodium channels in neocortical pyramidal neurons. *Neuron* **15,** 1065–1076.
10. Bouron A. and Reuter H. (1996) A role of intracellular Na+ in the regulation of synaptic transmission and turnover of the vesicular pool in cultured hippocampal cells. *Neuron* **17,** 969–978.
11. Linden D. J. (1999) The return of the spike: postsynaptic action potentials and the induction of LTP and LTD. *Neuron* **22,** 661–666.
12. Thomas M. J., Watabe A. M., Moody T. D., Makhinson M., and O'Dell T. J. (1998) Postsynaptic complex spike bursting enables the induction of LTP by theta frequency synaptic stimulation. *J. Neurosci.* **18,** 7118–7126.
13. Callaway J. C. and Ross W. N. (1997) Spatial distribution of synaptically activated sodium concentration changes in cerebellar Purkinje neurons. *J. Neurophysiol.* **77,** 145–152.
14. Rose C. R. and Ransom B. R. (1997) Regulation of intracellular sodium in cultured rat hippocampal neurones. *J. Physiol. (Lond.)* **499,** 573–587.
15. Rose C. R. and Konnerth A. (2001) NMDA receptor-mediated Na+ signals in spines and dendrites. *J. Neurosci.* **21,** 4207–4214.
16. Rose C. R. 2002. Na+ signals at central synapses. *Neuroscientist* **8,** 532–539.
17. Aizman O., Brismar H., Uhlen P., et al. (2000) Anatomical and physiological evidence for D1 and D2 dopamine receptor colocalization in neostriatal neurons. *Nat. Neurosci.* **3,** 226–230.
18. Komwatana P., Dinudom A., Young J. A., and Cook D. I. (1996) Cytosolic Na+ controls and epithelial Na+ channel via the Go guanine nucleotide-binding regulatory protein. *Proc. Natl. Acad. Sci. USA* **93,** 8107–8111.
19. Dinudom A., Harvey K. F., Komwatana P., et al. (2001) Roles of the C termini of alpha -, beta -, and gamma -subunits of epithelial Na+ channels (ENaC) in regulating ENaC and mediating its inhibition by cytosolic Na+. *J. Biol. Chem.* **276,** 13,744–13,749.
20. Cook D. I., Dinudom A., Komwatana P., Kumar S., and Young J. A. (2002) Patch-clamp studies on epithelial sodium channels in salivary duct cells. *Cell Biochem. Biophys.* **36,** 105–113.
21. Fotia A. B., Dinudom A. , Shearwin K. E., et al. (2003) The role of individual Nedd4-2 (KIAA0439) WW domains in binding and regulating epithelial sodium channels. *FASEB J.* **17,** 70–72.
22. Bader C. R., Bernheim L., and Bertrand D. (1985) Sodium-activated potassium current in cultured avian neurones. *Nature* **317,** 540–542.
23. Dryer S. E. (1994) Na^+-activated K^+ channels: A new family of large-conductance ion channels. *Trends Neurosci.* **17,** 155–160.
24. Zhainazarov A. B. and Ache B. W. (1997) Gating and conduction properties of a sodium-activated cation channel from lobster olfactory receptor neurons. *J. Membr. Biol.* **156,** 173–190.
25. Knutson P., Ghiani C. A., Zhou J. M., Gallo V., and McBain C. J. (1997) K+ channel expression and cell proliferation are regulated by intracellular sodium and membrane depolarization in oligodendrocyte progenitor cells. *J. Neurosci.* **17,** 2669–2682.
26. Bhattacharjee A., Joiner W. J., Wu M., Yang Y., Sigworth F. J., and Kaczmarek L. K. (2003) Slick (Slo2.1), a rapidly-gating sodium-activated potassium channel inhibited by ATP. *J. Neurosci.* **23,** 11,681–11,691.
27. Dryer S. E. (2003) Molecular identification of the Na+-activated K+ channel. *Neuron* **37,** 727–8.
28. Rishal I., Keren-Raifman T., Yakubovich D., et al. (2003) Na+ promotes the dissociation between Galpha GDP and Gbeta gamma, activating G protein-gated K+ channels. *J. Biol. Chem.* **278,** 3840–3845.
29. Yuan A., Santi C. M., Wei A., et al. (2003) The sodium-activated potassium channel is encoded by a member of the Slo gene family. *Neuron* **37,** 765–773.
30. Bhattacharjee A. and Kaczmarek L. K. (2005) For K(+) channels, Na(+) is the new Ca(2+). *Trends Neurosci.* **28,** 422–428.
31. Yakubovich D., Rishal I., and Dascal N. (2005) Kinetic modeling of Na(+)-induced, Gbetagamma-dependent activation of G protein-gated K(+) channels. *J. Mol. Neurosci.* **25,** 7–19.
32. Niu X. W. and Meech R. W. (2000) Potassium inhibition of sodium-activated potassium (K(Na)) channels in guinea-pig ventricular myocytes. *J. Physiol.* **526(pt 1),** 81–90.
33. Ho I. H. and Murrell-Lagnado R. D. (1999) Molecular determinants for sodium-dependent activation of G protein-gated K+ channels. *J. Biol. Chem.* **274,** 8639–8648.

34. Blumenstein Y., Maximyuk O. P., Lozovaya N., et al. (2004) Intracellular Na+ inhibits voltage-dependent N-type Ca2+ channels by a G protein betagamma subunit-dependent mechanism. *J. Physiol.* **556,** 121–134.
35. Liu X. and Stan L. L. (2004) Sodium-activated potassium conductance participates in the depolarizing afterpotential following a single action potential in rat hippocampal CA1 pyramidal cells. *Brain Res.* **1023,** 185–192.
36. Pert C. B., Snowman A. M., and Snyder S. H. (1974) Localization of opiate receptor binding in synaptic membranes of rat brain. *Brain Res.* **70,** 184–8.
37. Pert C. B., Aposhian D., and Snyder S. H. (1974) Phylogenetic distribution of opiate receptor binding. *Brain Res.* **75,** 356–361.
38. Bloch R. J. (1986) Loss of acetylcholine receptor clusters induced by treatment of cultured rat myotubes with carbachol. *J. Neurosci.* **6,** 691–700.
39. Werling L. L., Brown S. R., Puttfarcken P., and Cox B. M. (1986) Sodium regulation of agonist binding at opioid receptors. II.Effects of sodium replacement on opioid binding in guinea pig cortical membranes. *Mol. Pharmacol.* **30,** 90–95.
40. Puttfarcken P., Werling L. L., Brown S. R., Cote T. E., and Cox B. M. (1986) Sodium regulation of agonist binding at opioid receptors. I.Effects of sodium replacement on binding at mu- and delta-type receptors in 7315c and NG108-15 cells and cell membranes. *Mol. Pharmacol.* **30,** 81–89.
41. Pifl C., Agneter E., Drobny H., Reither H., and Singer E. A. (1997) Induction by low Na+ or Cl- of cocaine sensitive carrier-mediated efflux of amines from cells transfected with the cloned human catecholamine transporters. *Br. J. Pharmacol.* **121,** 205–212.
42. Chen N., Trowbridge C. G., and Justice J. B. Jr. (1998) Voltammetric studies on mechanisms of dopamine efflux in the presence of substrates and cocaine from cells expressing human norepinephrine transporter. *J. Neurochem.* **71,** 653–665.
43. Pifl C. and Singer E. A. (1999) Ion dependence of carrier-mediated release in dopamine or norepinephrine transporter-transfected cells questions the hypothesis of facilitated exchange diffusion. *Mol. Pharmacol.* **56,** 1047–1054.
44. Khoshbouei H., Wang H., Lechleiter J. D., Javitch J. A., and Galli A. (2003) Amphetamine-induced dopamine efflux. A voltage-sensitive and intracellular Na+-dependent mechanism. *J. Biol. Chem.* **278,** 12,070–12,077.
45. Chen N. and Reith M. E. (2003) Na+ and the substrate permeation pathway in dopamine transporters. *Eur. J. Pharmacol.* **479,** 213–221.
46. Koch R. A. and Barish M. E. (1994) Perturbation of intracellular calcium and hydrogen ion regulation in cultured mouse hippocampal neurons by reduction of the sodium ion concentration gradient. *J. Neurosci.* **14,** 2585–2593.
47. Blaustein M. P., Fontana G., and Rogowski R. S. (1996) The Na(+)-Ca2+ exchanger in rat brain synaptosomes. Kinetics and regulation. *Ann. NY Acad. Sci.* **779,** 300–317.
48. Boonstra J., Moolenaar W. H., Harrison P. H., Moed P., van der Saag P. T., and de Laat S. W. (1983) Ionic responses and growth stimulation induced by nerve growth factor and epidermal growth factor in rat pheochromocytoma (PC12) cells. *J. Cell Biol.* **97,** 92–98.
49. Moolenaar W. H., Tsien R. Y., van der Saag P. T., and de Laat S. W. (1983) Na+/H+ exchange and cytoplasmic pH in the action of growth factors in human fibroblasts. *Nature* **304,** 645–648.
50. Moolenaar W. H., Defize L. H., and de Laat S. W. (1986) Ionic signalling by growth factor receptors. *J. Exp. Biol.* **124,** 359–373.
51. Baxter K. A. and Church J. (1996) Characterization of acid extrusion mechanisms in cultured fetal rat hippocampal neurones. *J. Physiol. (Lond.)* **493,** 457–470.
52. Ballard-Croft C., Carlson D., Maass D. L., and Horton J. W. (2004) Burn trauma alters calcium transporter protein expression in the heart. *J. Appl. Physiol.* **97,** 1470–1476.
53. Strichartz G., Rando T., and Wang G. K. (1987) An integrated view of the molecular toxinology of sodium channel gating in excitable cells. *Annu. Rev. Neurosci.* **10,** 237–267.
54. Friedman J. E. and Haddad G. G. (1994) Anoxia induces an increase in intracellular sodium in rat central neurons in vitro. *Brain Res.* **663,** 329–334.
55. Bauer R., Walter B., Fritz H., and Zwiener U. (1999) Ontogenetic aspects of traumatic brain edema—facts and suggestions. *Exp. Toxicol. Pathol.* **51,** 143–150.
56. Schwartz G. and Fehlings M. G. (2002) Secondary injury mechanisms of spinal cord trauma: a novel therapeutic approach for the management of secondary pathophysiology with the sodium channel blocker riluzole. *Prog. Brain Res.* **137,** 177–190.
57. Banasiak K. J., Burenkova O., and Haddad G. G. (2004) Activation of voltage-sensitive sodium channels during oxygen deprivation

leads to apoptotic neuronal death. *Neuroscience* **126,** 31–44.
58. Sheldon C., Diarra A., Cheng Y. M., and Church J. (2004) Sodium influx pathways during and after anoxia in rat hippocampal neurons. *J. Neurosci.* **24,** 11,057–11,069.
59. Choi D. W. (1993) NMDA receptors and AMPA/kainate receptors mediate parallel injury in cerebral cortical cultures subjected to oxygen-glucose deprivation. *Prog. Brain Res.* **96,** 137–143.
60. Choi D. W. (1995) Calcium: still center-stage in hypoxic-ischemic neuronal death. *Trends Neurosci.* **18,** 58–60.
61. Bortner C. D. and Cidlowski J. A. (2003) Uncoupling cell shrinkage from apoptosis reveals that Na+ influx is required for volume loss during programmed cell death. *J. Biol. Chem.* **278,** 39,176–39,184.
62. Vornov J. J., Thomas A. G., and Jo D. (1996) Protective effects of extracellular acidosis and blockade of sodium/hydrogen ion exchange during recovery from metabolic inhibition in neuronal tissue culture. *J. Neurochem.* **67,** 2379–2789.
63. Mentzer R. M. Jr., Lasley R. D., Jessel A., and Karmazyn M. (2003) Intracellular sodium hydrogen exchange inhibition and clinical myocardial protection. *Ann. Thorac. Surg.* **75,** S700–S708.
64. Fehlings M. G. and Agrawal S. (1995) Role of sodium in the pathophysiology of secondary spinal cord injury. *Spine* **20,** 2187–2191.
65. Agrawal S. K. and Fehlings M. G. (1996) Mechanisms of secondary injury to spinal cord axons in vitro: role of Na+, Na(+)-K(+)-ATPase, the Na(+)-H+ exchanger, and the Na(+)-Ca2+ exchanger. *J. Neurosci.* **16,** 545–552.
66. Agrawal S. K. and Fehlings M. G. (1997) The effect of the sodium channel blocker QX-314 on recovery after acute spinal cord injury. *J. Neurotrauma* **14,** 81–88.
67. Teng Y. D. and Wrathall J. R. (1997) Local blockade of sodium channels by tetrodotoxin ameliorates tissue loss and long-term functional deficits resulting from experimental spinal cord injury. *J. Neurosci.* **17,** 4359–4366.
68. Hains B. C., Saab C. Y., Lo A. C., and Waxman S. G. (2004) Sodium channel blockade with phenytoin protects spinal cord axons, enhances axonal conduction, and improves functional motor recovery after contusion SCI.E*xp. Neurol.* **188,** 365–377.
69. Cummins T. R. and Waxman S. G. (1997) Downregulation of tetrodotoxin-resistant sodium currents and upregulation of a rapidly repriming tetrodotoxin-sensitive sodium current in small spinal sensory neurons after nerve injury. *J. Neurosci.* **17,** 3503–3514.
70. Appelgren L., Janson M., Nitescu P., and Curelaru I. (1996) Continuous intracisternal and high cervical intrathecal bupivacaine analgesia in refractory head and neck pain [see comments]. *Anesthesiology* **84,** 256–272.
71. Loscher W. and Ebert U. (1996) The role of the piriform cortex in kindling. *Prog. Neurobiol.* **50,** 427–481.
72. Mayer M. L. and Westbrook G. L. (1987) The physiology of excitatory amino acids in the vertebrate central nervous system. *Prog. Neurobiol.* **28,** 197–276.
73. McBain C. J. and Mayer M. L. (1994) N-methyl-D-aspartic acid receptor structure and function. *Physiol. Rev.* **74,** 723–760.
74. Dingledine R., Borges K., Bowie D., and Traynelis S. F. (1999) The glutamate receptor ion channels. *Pharmacol. Rev.* **51,** 7–61.
75. Bredt D. S. and Nicoll R. A. (2003) AMPA receptor trafficking at excitatory synapses. *Neuron* **40,** 361–379.
76. Blitz D. M., Foster K. A., and Regehr W. G. (2004) Short-term synaptic plasticity: a comparison of two synapses. *Nat. Rev. Neurosci.* **5,** 630–640.
77. Cull-Candy S. G. and Leszkiewicz D. N. (2004) Role of distinct NMDA receptor subtypes at central synapses. *Sci. STKE.* 2004:re16.
78. Carlisle H. J. and Kennedy M. B. (2005) Spine architecture and synaptic plasticity. *Trends Neurosci.* **28,** 182–187.
79. Xia Z. and Storm D. R. (2005) The role of calmodulin as a signal integrator for synaptic plasticity. *Nat. Rev. Neurosci.* **6,** 267–276.
80. Choi D. W. (1988) Calcium-mediated neurotoxicity: Relationship to specific channel types and role in ischemic damage. *Trends Neurosci.* **11,** 465–469.
81. Lipton S. A. and Rosenberg P. A. (1994) Excitatory amino acids as a final common pathway for neurologic disorders [see comments]. *N. Engl. J. Med.* **330,** 613–622.
82. Rothman S. M. and Olney J. W. (1995) Excitotoxicity and the NMDA receptor—still lethal after eight years. *Trends Neurosci.* **18,** 57,58.
83. Arundine M. and Tymianski M. (2003) Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. *Cell Calcium* **34,** 325–337.
84. Vanhoutte P. and Bading H. (2003) Opposing roles of synaptic and extrasynaptic NMDA

receptors in neuronal calcium signalling and BDNF gene regulation. *Curr. Opin. Neurobiol.* **13,** 366–371.
85. Hardingham G. E. and Bading H. (2003) The Yin and Yang of NMDA receptor signalling. *Trends Neurosci.* **26,** 81–89.
86. Waxman E. A. and Lynch D. R. (2005) N-methyl-D-aspartate receptor subtypes: multiple roles in excitotoxicity and neurological disease. *Neuroscientist* **11,** 37–49.
87. Westphal R. S., Tavalin S. J., Lin J. W., et al. (1999) Regulation of NMDA receptors by an associated phosphatase-kinase signaling complex. *Science* **285,** 93–96.
88. Husi H., Ward M. A., Choudhary J. S., Blackstock W. P., and Grant S. G. (2000) Proteomic analysis of NMDA receptor-adhesion protein signaling complexes. *Nat. Neurosci.* **3,** 661–669.
89. Sheng M. and Pak D. T. (2000) Ligand-gated ion channel interactions with cytoskeletal and signaling proteins. *Annu. Rev. Physiol.* **62,** 755–778.
90. Lee F. J. S, Xue S., Pei L., et al. (2002) Dual regulation of NMDA receptor functions by direct protein-protein interactions with the dopamine D1 receptor. *Cell* **111,** 219–230.
91. Lei G., Xue S., Chery N., et al. (2002) Gain control of N-methyl-D-aspartate receptor activity by receptor-like protein tyrosine phopshatase alpha. *EMBO J.* **21,** 2977–2989.
92. Ehlers M. D. (2003) Activity level controls postsynaptic composition and signaling via the ubiquitin-proteasome system. *Nat. Neurosci.* **6,** 231–242.
93. Salter M. W. and Kalia L. V. (2004) Src kinases: a hub for NMDA receptor regulation. *Nat. Rev. Neurosci.* **5,** 317–328.
94. Bading H. and Greenberg M. E. (1991) Stimulation of protein tyrosine phosphorylation by NMDA receptor activation. *Science* **253,** 912–914.
95. Woodrow S., Bissoon N., and Gurd J. W. (1992) Depolarization-dependent tyrosine phosphorylation in rat brain synaptosomes. *J. Neurochem.* **59,** 857–862.
96. Wang Y. T. and Salter M. W. (1994) Regulation of NMDA receptors by tyrosine kinases and phosphatases. *Nature* **369,** 233–235.
97. Yu X.- M., Askalan R., Keil G. J. I., and Salter M. W. (1997) NMDA channel regulation by channel-associated protein tyrosine kinase Src. *Science* **275,** 674–678.
98. Yu X.- M. and Salter M. W. (1998) Gain control of NMDA-receptor currents by intracellular sodium. *Nature* **396,** 469–474.
99. Salter M. W. (1999) A pinch of salt for NMDA receptors. *Mol. Psychiatry* **4,** 209–211.
100. Xin W.- K., Kwan C. L., Zhao X.- H., et al. (2005) A functional interaction of sodium and calcium in the regulation of NMDA receptor activity by remote NMDA receptors. *J. Neurosci.* **25,** 139–148.
101. Xin W.- K., Zhao X.- H., Xu J., et al. (2005) The removal of extracellular calcium: a novel mechanism underlying the recruitment of N-methyl-D-aspartate (NMDA) receptors in neurotoxicity. *Eur. J. Neurosci.* **21,** 622–636.
102. Legendre P., Rosenmund C., and Westbrook G. L. (1993) Inactivation of NMDA channels in cultured hippocampal neurons by intracellular calcium. *J. Neurosci.* **13,** 674–684.
103. Rosenmund C. and Westbrook G. L. (1993) Calcium-induced actin depolymerization reduces NMDA channel activity. *Neuron* **10,** 805–814.
104. Hollmann M. and Heinemann S. (1994) Cloned glutamate receptors. *Annu. Rev. Neurosci.* **17,** 31–108.
105. Rosenmund C., Feltz A., and Westbrook G. L. (1995) Calcium-dependent inactivation of synaptic NMDA receptors in hippocampal neurons. *J. Neurophysiol.* **73,** 427–430.
106. Rosenmund C., Feltz A., and Westbrook G. L. (1995) Synaptic NMDA receptor channels have a low open probability. *J. Neurosci.* **15,** 2788–2795.
107. Ehlers M. D., Zhang S., Bernhardt J. P., and Huganir R. L. (1996) Inactivation of NMDA Receptors by direct interaction of calmodulin with the NR1 subunit. *Cell* **84,** 745–755.
108. Kyrozis A., Albuquerque C., Gu J., and MacDermott A. B. (1996) Ca(2+)-dependent inactivation of NMDA receptors: fast kinetics and high Ca2+ sensitivity in rat dorsal horn neurons. *J. Physiol. (Lond.)* **495,** 449–463.
109. Medina I., Filippova N., Bakhramov A., and Bregestovski P. (1996) Calcium-induced inactivation of NMDA receptor-channels evolves independently of run-down in cultured rat brain neurones. *J. Physiol. (Lond.)* **495,** 411–427.
110. Sucher N. J., Awobuluyi M., Choi Y. B., and Lipton S. A. (1996) NMDA receptors: from genes to channels. *Trends Pharmacol. Sci.* **17,** 348–355.
111. Baker A. J., Moulton R. J., MacMillan V. H., and Shedden P. M. (1993) Excitatory amino acids in cerebrospinal fluid following traumatic brain injury in humans. *J. Neurosurg.* **79,** 369–372.
112. Persson L. and Hillered L. (1992) Chemical monitoring of neurosurgical intensive care patients using intracerebral microdialysis. *J. Neurosurg.* **76,** 72–80.

113. Benveniste H., Drejer J., Schousboe A., and Diemer N. H. (1984) Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *J. Neurochem.* **43,** 1369–1374.
114. Lipton P. (1999) Ischemic cell death in brain neurons. *Physiol. Rev.* **79,** 1431–1568.
115. Morris G. F., Bullock R., Marshall S. B., Marmarou A., Maas A., and Marshall L. F. (1999) Failure of the competitive N-methyl-D-aspartate antagonist Selfotel (CGS 19755) in the treatment of severe head injury: results of two phase III clinical trials. The Selfotel Investigators. *J. Neurosurg.* **91,** 737–743.
116. Albers G. W., Clark W. M., Atkinson R. P., Madden K., Data J. L., and Whitehouse M. J. (1999) Dose escalation study of the NMDA glycine-site antagonist licostinel in acute ischemic stroke. *Stroke* **30,** 508–513.
117. Davis S. M., Lees K. R., Albers G. W., et al. (2000) Selfotel in acute ischemic stroke : possible neurotoxic effects of an NMDA antagonist. *Stroke* **31,** 347–354.
118. Ikonomidou C. and Turski L. (2002) Why did NMDA receptor antagonists fail clinical trials for stroke and traumatic brain injury? *Lancet Neurol.* **1,** 383–386.
119. Xiong Z., Lu W. and MacDonald J. F. (1997) Extracellular calcium sensed by a novel cation channel in hippocampal neurons. *Proc. Natl. Acad. Sci. USA* **94,** 7012–7017.
120. Aarts M., Lihara K., Wei W.- L., et al. (2003) A key role for TRPM7 channels in anoxic neuronal death. *Cell* **115,** 863–877.
121. Westbrook G. L., Krupp J. J., and Vissel B. (1997) Cytoskeletal interactions with glutamate receptors at central synapses. *Soc. Gen. Physiol. Ser.* **52,** 163–175.
122. Lieberman D. N. and Mody I. (1994) Regulation of NMDA channel function by endogenous Ca^{2+}- dependent phosphatase. *Nature* **369,** 235–239.
123. Mulkey R. M., Endo S., Shenolikar S., and Malenka R. C. (1994) Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. *Nature* **369,** 486–488.
124. Tong G., Shepherd D., and Jahr C. E. (1995) Synaptic desensitization of NMDA receptors by calcineurin. *Science* **267,** 1510–1512.
125. Zhang S., Ehlers M. D., Bernhardt J. P., Su C. T., and Huganir R. L. (1998) Calmodulin mediates calcium-dependent inactivation of N-methyl-D-aspartate receptors. *Neuron* **21,** 443–453.
126. Wechsler A. and Teichberg V. I. (1998) Brain spectrin binding to the NMDA receptor is regulated by phosphorylation, calcium and calmodulin. *EMBO J.* **17,** 3931–3939.
127. Krupp J. J., Vissel B., Thomas C. G., Heinemann S. F., and Westbrook G. L. (1999) Interactions of calmodulin and alpha-actinin with the NR1 subunit modulate Ca2+-dependent inactivation of NMDA receptors. *J. Neurosci.* **19,** 1165–1178.
128. Vassilev P. M., Mitchel J., Vassilev M., Kanazirska M., and Brown E. M. (1997) Assessment of frequency-dependent alterations in the level of extracellular Ca2+ in the synaptic cleft. *Biophys. J.* **72,** 2103–2116.
129. Rusakov D. A. and Fine A. 2003. Extracellular Ca2+ depletion contributes to fast activity-dependent modulation of synaptic transmission in the brain. *Neuron* **37,** 287–297.
130. Heinemann U. and Hamon B. 1986. Calcium and epileptogenesis. *Exp. Brain Res.* **65,** 1–10.
131. Ekholm A., Kristian T., and Siesjo B. K. 1995. Influence of hyperglycemia and of hypercapnia on cellular calcium transients during reversible brain ischemia. *Exp. Brain Res.* **104,** 462–466.
132. Nicholson C., Bruggencate G. T., Steinberg R., and Stockle H. 1977. Calcium modulation in brain extracellular microenvironment demonstrated with ion-selective micropipette. *Proc. Natl. Acad. Sci. USA* **74,** 1287–1290.
133. Harris R. J., Symon L., Branston N. M., and Bayhan M. 1981. Changes in extracellular calcium activity in cerebral ischaemia. *J. Cereb. Blood Flow Metab.* **1,** 203–209.
134. Yu X.- M. and Salter M. W. 1999. Src, a molecular switch governing gain control of synaptic transmission mediated by N-methyl-D-aspartate receptors. *Proc. Natl. Acad. Sci. USA* **96,** 7697–7704.
135. Liu X., Brodeur S. R., Gish G., et al. 1993. Regulation of c-Src tyrosine kinase activity by the Src SH2 domain. *Oncogene* **8,** 1119–1126.
136. Ali D. W. and Salter M. W. 2001. NMDA receptor regulation by Src kinase signalling in excitatory synaptic transmission and plasticity. *Curr. Opin. Neurobiol.* **11,** 336–342.
137. Grant S. G., O'Dell T. J., Karl K. A., Stein P. L., Soriano P., and Kandel E. R. 1992. Impaired long-term potentiation, spatial learning, and hippocampal development in fyn mutant mice. *Science* **258,** 1903–1910.

138. Miyakawa T., Yagi T., and Kitazawa H., et al. 1997. Fyn-kinase as a determinant of ethanol sensitivity: relation to NMDA-receptor function. *Science* **278,** 698–701.
139. Kojima N., Ishibashi H., Obata K., and Kandel E. R. 1998. Higher seizure susceptibility and enhanced tyrosine phosphorylation of N-methyl-D-aspartate receptor subunit 2B in fyn transgenic mice. *Learn. Mem.* **5,** 429–445.
140. Woolf C. J., and Salter M. W. 2000. Neuronal plasticity: increasing the gain in pain. *Science* **288,** 1765–1769.
141. Zhao W., Cavallaro S., Gusev P., and Alkon D. L. 2000. Nonreceptor tyrosine protein kinase pp60c-src in spatial learning: synapse-specific changes in its gene expression, tyrosine phosphorylation, and protein-protein interactions. *Pro. Natl. Acad. Sci. USA* **97,** 8098–8103.
142. Guo W., Zou S., Guan Y., et al. 2002. Tyrosine phosphorylation of the NR2B subunit of the NMDA receptor in the spinal cord during the development and maintenance of inflammatory hyperalgesia. *J. Neurosci.* **22,** 6208–6217.
143. Sato E., Takano Y., Kuno Y., Takano M., and Sato I. 2003. Involvement of spinal tyrosine kinase in inflammatory and N-methyl-D-aspartate-induced hyperalgesia in rats. *Eur. J. Pharmacol.* **468,** 191–198.
144. Guo W., Wei F., Zou S., et al. 2004. Group I metabotropic glutamate receptor NMDA receptor coupling and signaling cascade mediate spinal dorsal horn NMDA receptor 2B tyrosine phosphorylation associated with inflammatory hyperalgesia. *J. Neurosci.* **24,** 9161–9173.
145. Kawasaki Y., Kohno T., Zhuang Z. Y., et al. 2004. Ionotropic and metabotropic receptors, protein kinase A, protein kinase C, and Src contribute to C-fiber-induced ERK activation and cAMP response element-binding protein phosphorylation in dorsal horn neurons, leading to central sensitization. *J. Neurosci.* **24,** 8310–8321.
146. Khan A. M., Cheung H. H., Gillard E. R., et al. 2004. Lateral Hypothalamic Signaling Mechanisms Underlying Feeding Stimulation: Differential Contributions of Src Family Tyrosine Kinases to Feeding Triggered Either by NMDA Injection or by Food Deprivation. *J. Neurosci.* **24,** 10,603–10,615.
147. Bromann P. A., Korkaya H., and Courtneidge S. A. 2004. The interplay between Src family kinases and receptor tyrosine kinases. *Oncogene* **23,** 7957–7968.
148. Gauld S. B. and Cambier J. C. 2004. Src-family kinases in B-cell development and signaling. *Oncogene* **23,** 8001–8006.
149. Geahlen R. L., Handley M. D., and Harrison M. L. 2004. Molecular interdiction of Src-family kinase signaling in hematopoietic cells. *Oncogene* **23,** 8024–8032.
150. Kalia L. V., Gingrich J. R., and Salter M. W. 2004. Src in synaptic transmission and plasticity. *Oncogene* **23,** 8007–8016.
151. Luttrell D. K. and Luttrell L. M. 2004. Not so strange bedfellows: G-protein-coupled receptors and Src family kinases. *Oncogene* **23,** 7969–7978.
152. Palacios E. H. and Weiss A. 2004. Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. *Oncogene* **23,** 7990–8000.
153. Parsons S. J. and Parsons J. T. 2004. Src family kinases, key regulators of signal transduction. *Oncogene* **23,** 7906–7909.
154. Playford M. P. and Schaller M. D. 2004. The interplay between Src and integrins in normal and tumor biology. *Oncogene* **23,** 7928–7946.
155. Reynolds A. B. and Roczniak-Ferguson A. 2004. Emerging roles for p120-catenin in cell adhesion and cancer. *Oncogene* **23,** 7947–7956.
156. Shupnik M. A. 2004. Crosstalk between steroid receptors and the c-Src-receptor tyrosine kinase pathways: implications for cell proliferation. *Oncogene* **23,** 7979–7989.
157. Silva C. M. 2004. Role of STATs as downstream signal transducers in Src family kinase-mediated tumorigenesis. *Oncogene* **23,** 8017–8023
158. Zheng F., Gingrich M. B., Traynelis S. F., and Conn P. J. 1998. Tyrosine kinase potentiates NMDA receptor currents by reducing tonic zinc inhibition. *Nat. Neurosci.* **1,** 185–191.
159. Cheung H. H. and Gurd J. W. 2001. Tyrosine phosphorylation of the N-methyl-D-aspartate receptor by exogenous and postsynaptic density-associated Src-family kinases. *J. Neurochem.* **78,** 524–534.
160. Grosshans D. R. and Browning M. D. 2001. Protein kinase C activation induces tyrosine phosphorylation of the NR2A and NR2B subunits of the NMDA receptor. *J. Neurochem.* **76,** 737–744.
161. Yang M. and Leonard J. P. 2001. Identification of mouse NMDA receptor subunit NR2A C-terminal tyrosine sites phosphorylated by coexpression with v-Src. *J. Neurochem.* **77,** 580–588.