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NMDA receptor-dependent long-term potentiation of slow synaptic inhibition in hippocampus

Author
Huang, Shen Cindy

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NMDA receptor-dependent long-term potentiation of slow synaptic inhibition in hippocampus

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Shen Cindy Huang

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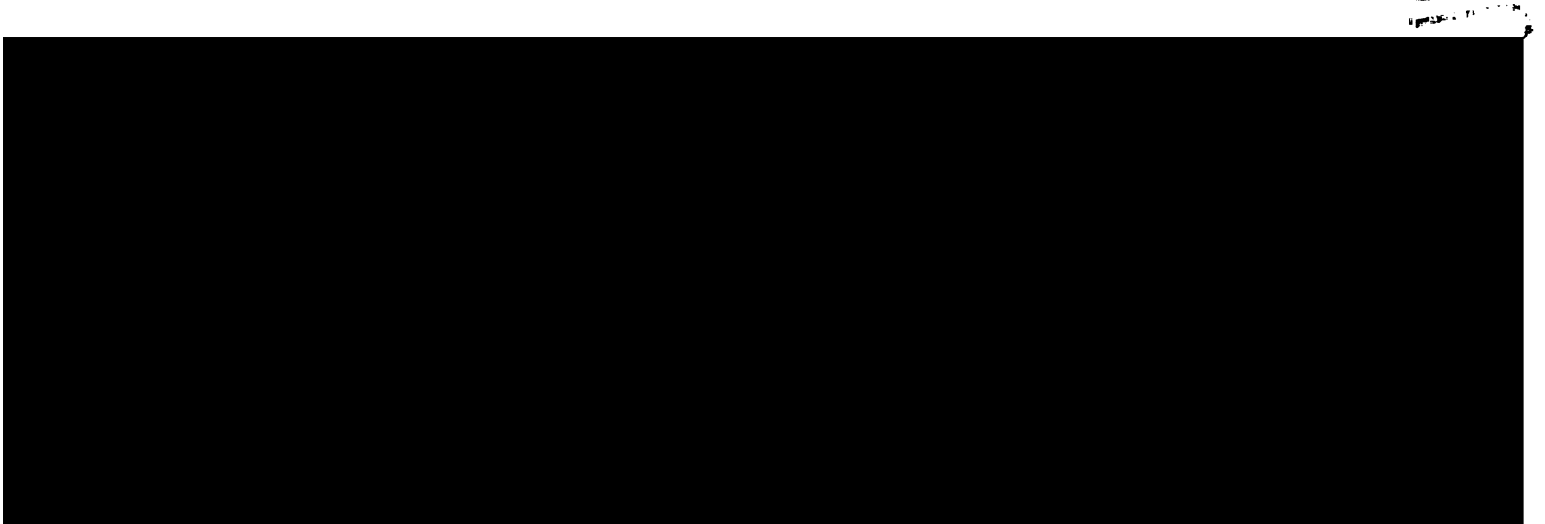

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Abstracts

Synaptic plasticity, the cellular correlate for learning and memory, involves a number of molecules that reside in the dendritic spine. For example, long-term potentiation (LTP) of the excitatory postsynaptic current (EPSC) is induced by postsynaptic NMDA receptor and Ca^{2+}/calmodulin-dependent kinase II (CaMKII) activity. Intrigued by recent findings that dendritic spines also harbor the metabotropic $GABA_B$ receptor for the major inhibitory transmitter GABA, as well as its downstream effector the G protein-activated inwardly rectifying K^+ (GIRK) channel, we wondered whether pairing glutamate release with postsynaptic depolarization would affect the slow inhibitory postsynaptic current (sIPSC) mediated by $GABA_B$ receptors and GIRK channels. Remarkably, we found that the same signaling pathway for inducing LTP of the EPSC also potentiated the sIPSC. Moreover, in mice lacking Nova-2, which is targeted in paraneoplastic opsoclonus myoclonus ataxia (POMA) patients and binds RNAs for synaptic proteins, the hippocampal CA1 neurons exhibited no LTP of slow synaptic inhibition.

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Introduction

Long-term potentiation of excitatory synapses at CA1 hippocampal neurons

Much attention over the past decades has focused on the plasticity of excitatory synaptic transmission (Lisman et al., 2002 ; Malenka et al., 1989; Malenka et al., 1988; Malinow and Malenka, 2002; Malinow et al., 1989; Nicoll, 2003), as a cellular correlate of learning and memory (Bliss and Collingridge, 1993). In most cases, LTP requires the activation of *N*-methy-*D*-Aspartate (NMDA) receptors, however, several mechanisms have been proposed for synaptic potentiation at different types of synapses, such as the involvement of presynaptic I_h channels and PKA in the expression of mossy fiber LTP (Mellor et al., 2002; Malenka and Bear, 2004). The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors open upon binding to glutamate and conduct monovalent cations like Na^+ and K^+, which mediate the majority of the fast excitatory postsynaptic current (EPSC). Optimal NMDA receptor activation during the induction of LTP requires glutamate release from the presynaptic nerve terminals concurrent with depolarization of the postsynaptic neuron to relieve block of the channel pore by external Mg^{2+} ions. Depolarization of postsynaptic cells is accomplished experimentally by high frequency stimulation for field recording or by direct depolarization of the postsynaptic cell under whole cell patch clamp paired with presynaptic low frequency stimulation. In addition, LTP can also be induced by appropriately pairing weak synaptic inputs with action potentials in the postsynaptic cell which backpropagated into the dendrites in a manner that is regulated by K^+ channel on the dendrites (Bi and Poo, 2001; Bi and Wang, 2002; Hoffman et al., 1997; Johnston et al., 2000; Paulsen and Sejnowski, 2000).

Dissociation of Mg^{2+} from its binding site within NMDA receptors/channels allows Ca^{2+} to enter the dendritic spines. A rise in Ca^{2+} within dendritic spines for a very short time period (a few seconds) is the critical trigger for LTP (Lynch et al., 1983; Malenka et al., 1988, 1992). The structure of dendritic spines provides compartmentalization of the rise of intracellular Ca^{2+} hence the input specificity of LTP.

A variety of molecules can be activated by Ca^{2+} that result in an increase in synaptic strength. There has been much debate whether the increase in synaptic strength during LTP is due to postsynaptic modification in AMPA receptor function or altered probability of presynaptic transmitter release or both. Strong evidence has been obtained supporting the notion that the mechanisms for the expression of LTP involve an increase in the number of AMPA receptors in the plasma membrane at synapses by activity-dependent trafficking (Malenka and Nicoll, 1999; Song and Huganir, 2002). Calcium/calmodulin-dependent protein kinase II (CaMKII) is one of the most important factors to modulate AMPA receptors for inducing LTP (Daw et al., 1993; Malenka et al., 1989; Malenka et al., 1988; Malinow et al., 1989; McBain and Mayer, 1994); LTP induction can be blocked by pharmacologically inhibiting CaMKII or genetically removing it. CaMKII exerts at least two effects on AMPA receptor activity (Fink and Meyer, 2002). Increase of phosphorylation on Ser831 by CaMKII enhances transmission by increasing single channel conductance in both native hippocampal cells and expression systems (Derkach et al., 1999; Barria et al., 1997; Mammen et al., 1997). Additionally, the catalytic domain of CaMKII has been shown to increase AMPA synaptic transmission in experiments using eletrophysiologically tagged AMPA receptors. Moreover, CaMKII- and tetanic

stimulation-induced synaptic delivery of the AMPA receptor subunit GluR1 is abolished by elimination of the PDZ consensus sequence at the GluR1 COOH-terminus, but not Ser831Ala mutation (Hayashi, et al., 2000). CaMKII also binds to the NR2B subunit of NMDA receptors and a number of postsynaptic density (PSD) proteins, such as PSD-95 and SAP97. Interaction between CaMKII and NMDA receptors increases binding affinity between calmodulin (CaM) and CaMKII and maintains the kinase in an active configuration (Bayer et al., 2001). On the other hand, CaMKII interaction with NMDA receptors and PSD proteins may increase anchoring sites for AMPA receptors at the synapses, thereby enhancing synaptic transmission. Not only is CaMKII activation required for LTP induction, CaMKII activation is also sufficient for LTP. Overexpression of the constitutively active form of CaMKII enhances synaptic transmission by inducing delivery of AMPA receptors into synapses and also occludes further LTP induction (Hayashi, et al., 2000).

The Ras-MAPK has emerged as a major postsynaptic signaling mechanism in the synaptic plasticity of EPSC (Sheng and Kim, 2002). NMDA receptor activation stimulates Ras activity, possibly by activating Ras-guanine-nucleotide releasing factor (Ras-GRF) and calcium- and diacylglycerol-related guanine nucleotide exchange factor (CalDAG-GEF) family members, and by recruiting the Grb2-SOS RasGEF complex via the calcium- and PKC-activated tyrosine kinase PYK2(CAKβ). Activation of CaMKII, downstream of NMDA receptor activation, is also important to Ras activity (Zhu et al., 2002). Moreover, the dominant-negative form of Ras blocks the potentiating effect of CaMKII as well as LTP, and active forms of Ras mimic and occlude LTP. Therefore, Ras

likely acts downstream of CaMKII, and Ras activity is necessary and sufficient to relay the CaMKII signaling that produces synaptic potentiation (Zhu et al., 2002). Moreover, NMDA receptor-containing complexes harbor CaMKIIβ subunit, Ras, Raf, MEK, and ERK : the MAP kinase cascade that is activated by Ras (Husi et al., 2000). It has become clear that ERK activation can be stimulated by Ras and some other kinases and is involved in both early- and late-phase hippocampal LTP (English et al., 1997; Impey et al., 1998; McGahon et al., 1999).

Other kinases have also been implicated in playing a role in LTP induction (Lynch, 2004). PKA, cAMP-dependent protein kinase, is required for LTP in neonatal rats that are younger than P9 (Yasuda et al., 2003) and older animals (Esteban et al., 2003). Activity-dependent phosphorylation of GluR1 and GluR4 by PKA leads to the incorporation of the receptors into synapses. PKC is important for LTP as well, because PKC inhibitors block LTP (Hu et al., 1987; Malinow et al., 1989). Phosphatidylinositol 3-kinase (PI3 kinase) and tyrosine kinase Src also play role in LTP induction by modulating AMPA and NMDA receptors, respectively (Malenka and Bear, 2004; Man et al., 2003; Salter and Kalia, 2004)

Activity-dependent AMPA receptor trafficking is a prominent mechanism of LTP, involving kinases, but also a number of scaffold proteins, as shown in both organotypic hippocampus slices and dissociated hippocampal neurons (Contractor and Heinemann, 2002; Lüscher, et al., 1999; Shi et al., 2001). Endogenous AMPA receptors are heter-oligomeric complexes composed of GluR1/GluR2 and GluR2/GluR3 (Wenthold et al.,

1996). GluR1 and 4 have long cytoplasmic carboxyl termini, GluR2 and 3 have short cytoplasmic carboxyl termini (Köhler et al., 1994). AMPA receptors containing long carboxyl termini are delivered to synapses by activity-dependent mechanisms (Shi et al., 1999; Hayashi et al., 2000). The activity-dependent process driving GluR1 into synapses requires the interaction between GluR1 carboxyl termini and PDZ domain proteins. GluR1 subunits with mutations at their PDZ interaction site or truncated GluR1 without its carboxyl terminus are not delivered to synapses. In contrast to the necessity of synaptic activity or CaMKII activity to the surface delivery of GluR1, GluR2, with a short carboxyl terminus, is constitutively inserted into synapses (Shi et al., 2001). This insertion relies on the interaction between GluR2 carboxyl termini and N-ethylmaleimide-sensitive factor (NSF) and group II PDZ domain proteins (Shi et al., 2002; Lüscher et al., 1999). Strikingly, two mechanisms for the synaptic delivery of AMPA receptors contribute to important aspects of synaptic function. GluR2/GluR3 receptors constitutively recycle in the synapse, whereas GluR1/GluR2 receptors undergo regulated delivery to modify synaptic strength. Those scaffold proteins that interact with GluR1 and GluR2 may serve as "slots" to maintain or alter the number of receptors at the synapses (Shi et al., 2001).

Late phase LTP involves mechanisms responsible for the increase of synaptic strength one hour after induction or even days later. It is believed that late phase LTP requires new protein synthesis and gene transcription, in contrast to the early, short-term, phase of LTP (Kelleher et al., 2004; Pittenger and Kandel, 2003; Tang and Schuman, 2002). Interestingly, protein synthesis is needed at the time of induction, although the protein

synthesis-dependent component of LTP appears 1-2 hours later (Otani et al., 1989). In addition, dendrites severed from their cell bodies display only short term potentiaion, suggesting that mRNA or proteins transported from the soma are needed to sustain LTP (Frey et al., 1989). The mechanisms of late phase LTP will be discussed in detail below.

Long-term plasticity at GABAergic synapses

Because most CNS regions contain both principal neurons that release glutamate and interneurons that release GABA (Markram et al., 2004; McBain et al., 1999), the complex circuitry involving synapses between these glutamatergic and GABAergic neurons presents a considerable challenge in discerning the precise sites of synaptic plasticity induced by nerve stimulation (McBain et al., 1999; Perez et al., 1999). To overcome this problem, researchers have devised ways specifically to activate the NMDA receptors of a single CA1 neuron under whole-cell patch-clamp, by pairing stimulation of its presynaptic glutamatergic nerve fibers with depolarization of the CA1 neuron (Hayashi et al., 2000; Kauer et al., 1988; Nicoll et al., 2003). The stimulation electrode placed in the dendritic field of CA1 neurons, however, will activate not only glutamatergic nerve fibers but also inhibitory interneurons so as to induce inhibitory synaptic potentials in the CA1 neuron, either di-synaptically due to innervation of these interneurons by the stimulated glutamatergic nerve fibers or monosynaptically due to placement of the stimulation electrode near the soma or processes of these inhibitory neurons. Indeed, stimulation paradigms that induce LTP of the EPSC may cause long-term depression (LTD) of fast inhibitory postsynaptic current (fIPSC) in the CA1 neuron (Caillard et al., 1999, 2000; Chevaleyre and Castillo, 2003; Gaiarsa et al., 2002; Lu et al.,

2000) as well as LTD of the EPSC in the GABAergic interneurons (Laezza et al., 1999; McMahon and Kauer, 1997). Not surprisingly, several different mechanisms have been reported to contribute to the induction and maintenance of inhibitory synapses or excitatory synapses on interneurons. For instance, the high frequency stimulation protocol which is commonly used to induce LTP in excitatory synapses in the CA1 area, also triggers a group I mGluR-dependent heterosynaptic LTD at inhibitory synapses, mediated by retrograde endocannabinoid signaling, but independent of postysynaptic NMDA receptor activation (Chevaleyre and Castillo, 2003). The tetanic field stimulation protocol could also induce LTD of the fIPSP in CA1 pyramidal neurons a process that requires NMDA receptor activation and calcineurin activity (Lu et al., 2000). These different outcomes in synaptic plasticity could be attributed to different conditioning protocols or the use of AMPA receptor antagonist or even different age of animals used in those studies. No synaptic specificity seems to be a common characteristic in most studies of plasticity at both GABAergic synpases onto pyramidal neurons and excitatory synapses onto interneurons (Chevaleyre and Castillo, 2003; McMahon and Kauer, 1997). It is possible that the special morphological feature of the axons of hippocampal interneurons makes it difficult to conduct two independent stimulation pathways to examine synaptic specificity. These and other studies (Perez et al., 1999) reinforce the notion that the synaptic plasticity monitored in a given neuron could arise from altered synaptic efficacy of the excitatory and/or inhibitory synaptic potentials in glutamatergic neurons as well as GABAergic interneurons in the central neuronal circuitry.

The only study that examines synaptic plasticity of slow inhibition was done by Lacaille and colleagues (Perez et al., 1999). Slight potentiation of both fast inhibitory response mediated by $GABA_A$ receptors and slow inhibition response mediated by $GABA_B$ receptors and G protein-activated inwardly rectifying K^+ channels (GIRK) was observed at CA1 pyramidal neurons by theta-burst stimulation protocol at Shaffer collaterals. The plasticity requires NMDAR activation.

Two types of transmitter receptors mediate synaptic transmission

Neurotransmitters mediate signaling between neurons by activating two different types of receptors: ionotropic transmitter receptors, ligand-gated ion channels that mediate fast synaptic potentials, and metabotropic transmitter receptors, G protein-coupled receptors that generate slow synaptic potentials (Hille, 1992). Localization of these two receptors is quite different too. Ionotropic transmitter receptors usually localize right opposite to the presynaptic boutons in the postsynaptic density (PSD) in the case of excitatory synapses, however, metabotropic transmitter receptors are relativly far from neurotransmitter release sites, outside of synaptic clefts (Huang, 1998; Isaacson, 2000). It is believed that diffusion of neurotransmitters is required for activating postsynaptic metabotropic transmitter receptors (Scanziani, 2000). Both types of receptors for glutamate, the major excitatory transmitter in the mammalian central nervous system (CNS), are present in the spines, small protrusions from dendrites that harbor the great majority of the excitatory synapses (Harris et al., 1999; Sheng and Kim, 2002). In contrast, dendritic shafts provide the setting for inhibitory synapses involving GABA, the major inhibitory transmitter in the mammalian brain, with the notable exception of a few examples of inhibitory

synapses on the spines (Freund and Buzsaki, 1996; Somogyi et al., 1998; Tamas et al., 2003).

GIRK channels

Inward rectifier potassium (Kir) channels constitute a large family of proteins possessing essential functions in the brain and other tissues. Within this family, G protein-activated inward rectifier potassium (GIRK) channels (also known as Kir3 channels) mediate the effects of some neurotransmitter effect in the central nervous system (CNS) and heart, controlling heart rate, neuronal signaling and membrane excitability (Dascal, 1997; Stanfield et al., 2002; Yamada et al., 1998). Furthermore, GIRK channels are involved in opioid– and ethanol– induced analgesia, which is absent in GIRK2 null and *weaver* mice (Ikeda et al., 2000, 2002, 2003; Kobayashi et al., 1999; Mitrovic et al., 2003). Activation of GIRK channels via G protein-coupled receptor (GPCR) stimulation is mediated by heterotrimeric G proteins ($G_{i/o}$) that are sensitive to pertussis toxin (PTX). Inhibitory transmitters (such as GABA, dopamine) activate the receptors, resulting in the dissociation of the G protein into the α- (Gα) and the βγ- (βγ) subunits; direct binding of Gβγ and GIRK channels, in turn, increases activity of GIRK channels. In this way, these channels produce a hyperpolarization in response to transmitters. In cardiac myocytes, activation of the heart muscarinic m2 receptor slows the heart rate by activating GIRK1/4 hetero-tetrameric channels. In the brain, a variety of receptors, such as A_1 adenosine, D2 dopamine, μ-, δ-, κ-opioid, 5HT 1A serotonin, somatostatin, m-GluR, $GABA_B$, substance P and neurotensin receptors have been shown to activate GIRK channels (Dascal 1997; Lüscher et al., 1997; Yamada et al., 1998).

Four members of GIRK subfamily, GIRK1-4, have been cloned in mammals, including several splice variants (Inanobe et al., 1999; Wickman et al., 2002; Wei et al., 1998; Yamada et al., 1998); these protein can form homotetramers or heterotetramers in vivo, due to their diverse trafficking patterns and different distribution in vivo (Ma, 2002). GIRK1 alone does not produce functional channels when expressed in most cell lines tested, except for *Xenopus* oocytes. Hedin et al. presented conclusive evidence that functional GIRK channels in *Xenopus* oocytes injected with GIRK1 RNA are formed by the combination of the expressed GIRK1 and an endogenous subunit, GIRK5 (Hedin et al., 1996). GIRK2-4 are necessary to coassemble with GIRK1 to produce functional channels with nearly identical single-channel properties in the heterologous system (Jelacic et al., 1999). However, GIRK2 and GIRK4 alone are directed to the cell surface efficiently, due to the presence of forward trafficking motifs – ER export motif and Golgi surface-promoting motif (Ma et al., 2002). Significantly, when GIRK1 is coexpressed with GIRK2 or GIRK 4, the trafficking of GIRK1/GIRK2 or GIRK1/GIRK4 heterotetramers is dictated by the forward trafficking motifs of GIRK2 or GIKR4. Four alternative splicing forms of GIRK2 that have different C termini (GIRK2C and GIRK2B compared to GIRK2A), or different N termini (GIRK2D vs. GIRK2A) show different trafficking patterns corresponding to their sequences. For instance, GIRK2C has extra 11 amino acids containing a PDZ domain binding motif at the extreme C terminus compared to GIRK2A, and hence exhibits similar trafficking behavior though more surface channels were formed by GIRK2C probably due to the presence of a PDZ domain

binding motif (Ma et al., 2002). However, interaction between GIRK2C and PSD95 is still controversial (Inanobe et al., 1999; Nehring et al., 2000; Ma et al., 2002).

Localization of GIRK isoforms has been extensively studied (DePaoli et al., 1994; Karschin et al., 1996; Kobayashi et al., 1995; Lesage et al., 1994; Liao et al., 1996; Morishige et al., 1996; Murer et al., 1997; Ponce et al., 1996; Spauschus et al., 1996). In general, GIRK1-3 mRNA are abundantly expressed throughout the brain, although GIRK4 mRNA is expressed in the brain to a much lesser extent than other GIRK transcripts. In the rat hippocampus, all GIRK mRNAs and proteins are strongly expressed by dentate gyrus granule cells and CA1-CA3 pyramidal neurons. (Karschin et al., 1996; Liao et al., 1996). GIRK2A and GIKR2C but not GIRK2B or GIRK2D are expressed in hippocampus assayed by single cell RT-PCR (Leaney, 2003). In electron microscopic analysis, stratum lacunosum-moleculare has the most intense labeling, indicating the highest GIRK1 expression, followed by stratum radiatum and stratum pyramidale in CA1 region of the hippocampus (Drake et al., 1997). GIRK1 is present exclusively in neurons and predominantly located in spiny dendrites of pyramidal neurons. Surprisingly, GIRK1 labeled spines are observed much more frequently than GRIK1 labeled shafts in both stratum lacunosum-moleculare and stratum radiatum. In dendritic spines and thin shafts, GIRK1 labeling is present with striking frequency near the PSD of asymmetric type of synapses usually associated with excitatory neurotransmission. These results demonstrate the localization of GIRK1 mainly on the spine, around the PSD of excitatory synapses in stratum lacunosum-molecurlare. Functional demonstration of the postsynaptic localization of GIRK has been shown by Nicoll and colleagues in their study of the

GIRK2 null mice (Lüscher et al., 1997). Upon presynaptic stimulation, the slow inhibition (sIPSC) following the $GABA_A$ mediated response (fIPSC) is absent in mutant mice in the presence of AMPA and NMDA receptor antagonists. In contrast, presynaptic inhibition of excitatory and inhibitory transmitter release by a number of presynaptic G protein coupled receptors is unaltered in these mutant mice.

A number of signaling molecules modulate GIRK function, including cytoplasmic ATP, Mg^{2+}, Na^{+}, and unsaturated fatty acid (Dascal 1997; Sui, et al., 1996; Petite-Jacques et al., 1999; Kim et al., 2000), as well as phosphotidylinositol 4,5-biphosphate (PIP2) (Kim and Bang, 1999; Han et al., 2003; Huang et al., 1998). PIP2 direct binds to GIRK channels, and the interaction between GIRK and PIP2 is stabilized by the βγ subunits of the G protein (Gβγ) (Huang et al., 1998). In addition, several kinases are important to GIRK channel function, such as PKA, PKC and protein phosphatase 2 (Median et al., 2000; Müllner et al., 2000, 2003; Sharon et al., 1997). The atrial GIRK channels (GIRK1/GIRK4) are assembled in a signaling complex with Gβγ, G protein-coupled receptor kinase (GRK), PKA, two protein phosphatase, PP1 and PP2A, receptor for activated C kinase 1 (RACK1) and actin (Nikolov and Ivanova-Nikolova, 2004). This complex can even further recruit PKC to the channel following α-adrenergic receptor stimulation. Thus, this complex would enable the GIRK channels to rapidly integrate β-adrenergic and m2 acetycholine receptor signaling in the membrane. The formation of a stable macromolecular complex maybe a common feature for G protein-mediated signal transduction (Lavine et al., 2002).

$GABA_B$ receptor

GABA, the predominant inhibitory neurotransmitter in the mammalian nervous system, signals through ionotropic $GABA_A$ and metatropic $GABA_B$ receptors. Binding of GABA to $GABA_B$ receptors results in GDP/GTP exchange in the associated G-protein and dissociation of Gα and Gβγ, which affect to a wide variety of intracellular targets, including adenylyl cyclase, GIRK channels and voltage-gated Ca^{2+} channels (Billinton et al., 2001; Mott and Lewis, 1994). In the hippocampus, eletrophyiological and pharmacological studies have provided characterization of the presynaptic and postsynaptic inhibitory actions of $GABA_B$ receptors. Presynaptic $GABA_B$ receptors decrease excitatory and inhibitory neurotransmitter release by depressing Ca^{2+} influx through voltage dependent Ca^{2+} channels, via PTX-insensitve G proteins (Dutar and Nicoll, 1988; Harrison, 1990; Wu and Saggau, 1995). The presynaptic $GABA_B$ receptors have been shown to regulate the induction of LTP in the hippocampus (Davies et al., 1991). Activation of postsynaptic $GABA_B$ receptors elicits the slow synaptic inhibition mediated by GIRK channels, through PTX-senstive G proteins (Lüscher et al., 1997; Kaupmann et al., 1998b).

Similar to metabotropic glutamate receptors (mGluR), $GABA_B$ receptors are dimmers formed by $GABA_{B1}$ and $GABA_{B2}$ (Jones et al., 1998; Kaupmann et al., 1998a; White et al., 1998; Kuner et al., 1999). In the $GABA_B$ heterodimers, the $GABA_{B1}$ subunit binds GABA and all competitive $GABA_B$ ligands, whereas the $GABA_{B2}$ is important for surface trafficking and G protein coupling (Calver et al., 2001; Margeta-Mitrovic et al., 2000; Pagano et al., 2001; Robins et al., 2001). Mice lacking $GABA_{B1}$ and $GABA_{B2}$

exhibit similar phenotypes, such as spontaneous seizures, hyperalgesia, hyperlocomotor activity, and memory impairment (Gassmann et al., 2004; Schuler et al., 2001). The pre- and postsynaptic $GABA_B$ responses are absent in $GABA_{B1}$ null mice, as expected; however, $GABA_{B2}$ null mice showed atypical eletrophysiological $GABA_B$ responses and relocation of $GABA_{B1}$ proteins to soma and proximal dendrites from distal neuronal sites. Both $GABA_{B1}$ and $GABA_{B2}$ exist in different splice forms (Billinton et al., 2001). Furthermore, mRNA localization studies suggest that different splicing forms ($GABA_{B1a}$ and $GABA_{B1b}$) of $GABA_{B1}$ might involve different composition of pre- and postsynaptic $GABA_B$ receptors (Billinton et al., 1999), although both of them could couple to K^+ or Ca^{2+} channels in transfected cells (Fillippov et al., 2000; Schwarz et al., 2000).

$GABA_{B1}$ exhibits a broader cellular expression pattern than $GABA_{B2}$ (Clark et al., 2000; Kim et al., 2003; Margeta-mitrovic et al., 1999). Both subunits are highly expressed in stratum lacunosum-moleculare, and weakest in stratum radiatum in hippocampal CA1 region (Kulik et al., 2003). Subcellular localization shown by electron microscopy demonstrates that both subunits are more abundant on postsynaptic elements, in the dendritic layer of the hippocampus (Kulik et al., 2003). Presynaptically, they are mainly detected in the extrasynaptic membrane and occasionally over the membrane specializations of putative glutamatergic and, to a lesser extent, GABAergic axon terminals. Postsynaptically, $GABA_{B1}$ and $GABA_{B2}$ are most abundant at the edge of asymmetrical synapses of pyramidal cell spines. $GABA_B$ receptors form a gradient with the strongest expression at the glutamatergic synapses, though not at PSD. Around

inhibitory synaspes, however, $GABA_B$ receptors are evenly distributed on dendritic shafts (Kulik et al., 2003).

Synaptic plasticity of slow inhibition

Whereas the ionotropic $GABA_A$ receptor resides primarily on the soma, dendritic shafts and axon initial segments (Farrant and Nusser, 2005; Klausberger et al., 2002; Macdonald and Olsen, 1994; Nyiri et al., 2001), recent studies have revealed that, remarkably, the metabotropic $GABA_B$ receptors and GIRK channels are located not only on dendritic shafts but also on the spines (Drake et al., 1997; Kulik et al., 2003). These findings raise the intriguing question of whether the slow synaptic inhibition mediated by $GABA_B$ receptors and GIRK channels (Luscher et al., 1997; Marshall et al., 1999) is affected by the machinery in the spine for inducing synaptic plasticity of the EPSC mediated by ionotropic glutamate receptors (Harris, 1999; Malinow, 2003; Nicoll, 2003; Sheng and Kim, 2002).

To address the open question whether postsynaptic NMDA receptor activation causes synaptic plasticity of the sIPSC, we have used the pairing protocol to activate NMDA receptors in the CA1 neuron under whole-cell patch-clamp recording. Remarkably, we found that coincidence detection of synaptic release of glutamate and postsynaptic depolarization of the CA1 neuron caused LTP of the sIPSC. Not only is this synaptic plasticity of slow inhibition dependent on NMDA receptor activation, it also requires postsynaptic Ca^{2+} increase and CaMKII activity. Indeed, expression of the constitutively

active form of CaMKII potentiated the sIPSC and occluded further potentiation of the sIPSC via the pairing protocol. It thus appears that the same signaling pathway mobilized by NMDA receptor activation for eliciting LTP of the EPSC also causes LTP of the sIPSC. To explore the functional requirement for this novel form of synaptic plasticity further, we examined the role of the RNA-binding protein Nova-2, an autoimmune target likely to be important for cognitive functions (Albert and Darnell, 2004; Yang et al., 1998), which exhibits intriguing interactions and regulation of molecules crucial for synaptic transmission (Ule et al., 2003; Ule et al., 2005).

RNA binding protein Nova-2

Paraneoplastic opsoclonus myoclonus ataxia (POMA) patients, often with latent breast cancer, fallopian cancer or small cell lung cancer, display not only ataxic movements but also cognitive loss, probably due to the autoimmune responses they developed against neuronal specific antigens expressed by their cancer cells (Buckanovich et al., 1993; Hormigo et al., 1994; Luque et al., 1991; Pranzatelli, 1992). The proteins recognized by their autoimmune antibodies, Nova-1 and Nova-2, are RNA-binding proteins that control alternative splicing of a number of gene products specifically expressed in the CNS and the first mammalian tissue-specific splicing factor identified (Jensen et al., 2000; Ule et al., 2005). Alternative splicing has been demonstrated to determine the subcellular location, molecular interactions, or function of proteins from single RNA precursors to regulate neuronal activity in hippocampus and sex determination in Drosophila (Tacke and Manley, 1999; Ehlers et al., 1995, 1998; Ferns et al., 1992). Interestingly, many of the targets of Nova-1 and Nova-2 contribute to inhibitory synaptic transmission and/or

synaptic plasticity. For instance, Nova binds an intronic UCAU-rich GlyRα2 pre-mRNA upstream of the mutually exclusive exons 3A and 3B, leading to preferential utilization of exon 3A, although the physiological significance of this alternative splicing of glycine receptors remains to be established. Whereas Nova-1 is mainly restricted to the diencephalon, brainstem and motor neurons of the ventral spinal cord, Nova-2 is expressed mostly in the cerebral cortex, hippocampus and dorsal spinal cord (Jensen et al., 2000; Ule et al., 2003; Yang et al., 1998). Nova-1 null mice exhibit apoptotic death of motor neurons in brainstem and spinal cord, indicating that Nova-1 is required for neuronal survival (Jensen et al., 2000; Dredge et al., 2001). This expression pattern of the Nova-2 protein is likely to underlie the development of cognitive deficits in some POMA patients. UV cross-linking and immunoprecipitation (CLIP) was developed and used by Darnell and colleagues to identify transcript targets of Nova-2 (Ule et al., 2003). It is observed that most of CLIP tags are located in the first three introns of target RNA and some are located within 3' UTR. The interaction between Nova-2 and target RNA is sequence-specific and of high affinity. One-third of Nova-2 target RNAs are involved in inhibitory synaptic transmission including GIRK2 and $GABA_BR2$, which both have some alternative splicing forms. Therefore, it would be interesting to know if Nova-2 null animals have different splicing forms of GIRK2 and $GABA_BR2$ compared to wild type animals in the hippocampus, and how this altered expression pattern might further affect synaptic transmission or plasticity of sIPSC. On the other hand, fractionation of mouse brain into nuclear and cytoplasmic fractions revealed that two-thirds of total Nova protein is present outside of the nucleus, although when normalized to protein mass, the highest concentration of Nova protein is in the nucleus (unpublished data, Darnell). Furthermore,

both immunofluorescence and electron microscopy demonstrate that Nova protein is present at inhibitory synapses, vicinity of gephyrin labeling region in spinal cord neurons. The distribution of endogenous Nova-2 is further confirmed by immunoflurorescence staining, which suggest Nova-2 not only localizes in the soma, but also within dentrites in dissociated mature hippocampal neurons (unpublished data, Darnell). The cytoplasmic location of Nova protein suggests that it may also regulate mRNA within the dendrite, either by affecting their localization, translation, or half-life. Most likely, the interaction between mature RNA and Nova-2 protein within dendrites is through low-affinity binding site in the exons of mRNA derived from precursors containg high affinity binding sites in the intron (personal communication, Darnell). These findings may reflect analogus phenomena to those reported for localized mRNA (*oskar*) in *Drosophila*, where splicing appears to be a pre-requisite for mRNA localization (Hachet and Ephrussi, 2004).

The discovery of synapse-associated polyribosome complexes (SPRCs), clusters of polyribosomes and associated membranous cisterns selectively located beneath postsynaptic sites on the dendrites of CNS, has raised the possibility of protein synthesis outside of the neuronal soma (Kiebler and DesGroseillers, 2000; Kloc et al., 2002; Martin et al., 2000; Steward and Levy, 1982; Steward and Schuman, 2001; Steward and Schuman, 2003; Tang and Schuman, 2000). This idea is appealing because it provides the neuron with a means of rapidly changing protein composition in a spatially restricted manner. *In situ* hybridization methods indicate that several mRNAs, including αCaMKII, MAP2, glutamate receptors, Fragile X mental retardation protein, and Arc, can be localized within dendrites (Sheetz et al., 2000; Steward and Schuman, 2003; Weiler et al.,

1997; Zalfa et al., 2003). Experiments using imaging studies and synaptosome preparations provide evidence that localized mRNA could be locally translated into active proteins, thus changing the composition of synaptic components in response to specific physiological conditions such as alteration in synaptic activity due to the action of neurotrophic factor brain-derived neurotrophic factor (BDNF), mGluR, or depolarization (Aakalu et al., 2001; Weiler et al., 1997; Greenough et al., 2001). Taking advantage of new methodology to discriminate preexisting protein and newly synthesis protein, Malenka and colleagues have demonstrated that AMPA receptors are locally synthesized in dendrites and delivered to synapses, and this process can be regulated by activity, thereby contributing to activity-dependent changes in synaptic strength (Ju et al., 2004). In support of these findings, there is redistribution of polyribosome to dendritic spines and large synapses after LTP induction by tetanic stimulation, as shown by serial electron microscopy (Ostroff et al., 2002). Certain neuronal mRNAs contain dendritic targeting signals in their 3'-UTR (Kislauskis et al., 1994; Mayford et al., 1996; Blichenberg et al., 1999; Miller et al., 2002). Disruption of 3'-UTR of αCaMKII, which contains two cytoplasmic polyadenylation elements, largely reduces the protein level in dendritic layers of the hippocampus, especially in the PSD fraction (Miller, et al., 2002; Wells, et al., 2001). mRNA level is also significantly reduced in CA1 stratum radiatum compared to CA1 stratum pyramidale and whole hippocampus. Subtle but significant deficits in late-phase LTP and memory in the mutant animals have been observed by physiological and behavioral studies, suggest that local synthesis of CaMKII protein is important for these processes.

Dependence of sIPSC LTP on Nova-2 function

By comparing the sIPSC before and after the pairing protocol in Nova-2 null mice and their heterozygous siblings, we found that, while the Nova-2 null mice exhibited basal sIPSC comparable to that in control mice, they failed to show LTP of the sIPSC, indicating that one of the physiological functions of Nova-2 in the mammalian brain is to enable activity-dependent modulation of the strength of slow synaptic inhibition. Because the Nova-2 null mice still exhibited LTP of the EPSC, Nova-2 appears to be specifically involved in the NMDA receptor-mediated synaptic plasticity of slow inhibition. Given that $GABA_{B1}$mRNA is abundant in synaptosomes, comparable with MAP2, and to a lesser extent, $GABA_{B2}$, GIKR1 and GIRK2 as well, it would be very interesting to determine whether the loss of synaptic plasticity of slow inhibition in Nova-2 null mice is due to altered alternative splicing of GIRK or $GABA_BR$ or any other molecules in the signal transduction pathway, or altered distribution of mRNA of GIRK or $GABA_BR$ at the synapses.

Materials and Methods

Hippocampal cultures and immunocytochemistry

Dissociated hippocampal cultures from 18-day-old embryonic rats were prepared as described (Brewer et al., 1993). For endogenous GIRK2 immunostaining, neuronal cultures were transfected with EGFP constructs by Sinbis virus at DIV 3-4 weeks and fixed with 4% paraformaldehyde and 4% sucrose in phosphate buffered saline (PBS) at 4°C for 20 minutes, permeabilized with 1% Triton in PBS and exposed to GIRK2 antibodies (Alamone, Jerusalem) for immunofluorescence. For surface GIRK1 staining, neuronal cultures were cotransfected GIRK1-ha-EGFP (HA epitope-YPYDVPDYA, was fused to the extracellular loop of GIRK1, between amino acid 115 and 116) and GIRK2 constructs by gene gun (BioRad) at DIV3-4 weeks. After 48 hours, cells were fixed with 4% paraformaldehyde and 4% sucrose. After wash, cells were incubated with anti-HA antibody (HA.11, Babco) for 1 hour, and then biotin-conjugated secondary antibody, followed by cy5- conjugated strepavidin. Repeated wash with PBS was necessary between incubations. The visual field was blindly moved to a random site on a coverslip, the first transfected pyramidal neuron was sampled by a digital camera and analyzed with Image J. Five areas of similar size from 5 dendrites of the same neuron were randomly picked to calculated the amount of total protein (EGFP, green) and surface protein (Cy5, red) by Image J. For all experiments, control and treated converslips were obtained from the same culture preparation and immunocytochemistry was processed in parallel. The ratio of surface protein over total protein was determined after subtracting background fluorescent intensity (Carroll et al, 1999).

Slice culture and eletrophysiology

Rat hippocampal organotypic slice cultures were prepared according to published protocols (Hayashi et al., 2000). Briefly, transverse hippocampal slices (400μm-thick) were obtained from postnatal 6- to 8- day-old Sprague-Dawley rats (Charles River) with tissue copper and explanted onto a membrane (Millicell-CM, 0.4μm pore size) placed in 0.75ml of culture medium (MEM) containing 3 mM glutamine, 30 mM Hepes, 5 mM $NaHCO_3$, 30 mM D-glucose, 0.5 mM L-ascorbate, 2 mM $CaCl_2$, 2.5 mM $MgSO_4$, 1 μg insulin, and 20 % horse serum (Musleh et al., 1997). The slices were cultured for 2-3 weeks at 35°C. In a recording chamber, superfused with a solution containing 119 mM NaCl, 2.5 mM KCl, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 26.2 mM $NaHCO_3$, 1 mM NaH_2PO_4, 11 mM glucose and equilibrated with 5% CO_2 and 95% O_2, CA1 pyramidal neurons were analyzed via whole-cell recordings at room temperature with patch electrodes (3-5 MΩ) filled with pipette solution containing 140 mM K-Gluconate, 5 mM HEPES, 2 mM $MgCl_2$, 1.1 mM EGTA, 2 mM MgATP and 3 mM Na_3GTP. Recordings were amplified with Axonpatch 1D, filtered at 1 kHz and sampled using programs written in Igor Pro (Wavematrics, OR). To evoke synaptic responses, a cluster electrode (FHC, ME) was placed ~300-500 μm from the layer of pyramidal neurons in the CA1 region and stimuli of ~0.1 msec duration were delivered. Data were analyzed using macros written in Igor Proand Microsoft Excel. For recordings from cell pairs, two cells with cell bodies within ~20 μm were selected, one showing EGFP fluorescence and the other one not fluorescent. Drugs used were NBQX (Tocris), picrotoxin (Tocris), SCH23390 (Tocris), SCH50911

(Tocris), AM251 (Tocris), tertiapin (Alamone), APV (Tocris), KN-93 (Calbiochem), DRB (Calbiochem), Chelerythrine (Tocris) and adenosine 3', 5'-cyclic Monophosphorothioate (Sigma). Data are expressed as means ± SEM and statistical differences of the means were determined using student's t–test.

Sinbis virus

Sinbis viruses were constructed for infection of neurons as reported previously (Hayashi et al., 2000), and recordings were made 24 to 36 hours after infection. The CaMKII(1-290)-EGFP construct was made by inserting the CaMKII(1-290) fragment amplified via polymerase chain reaction into the pEGFP-N1 vector (Clontech) as described (Hayashi et al., 2000). CaMKII (1-290)-EGFP fragment was cut from pEGFP-N1 constructs and cloned into pSinReb5 vector. After sequencing for verification, linearlized clones were transcribed into RNA with in vitro SP6 transcription kit (Ambion). Target RNA and virus structural RNA were cotransfected into BHK cells by eletroporation. After transfection, cells and medium were collected 48-72 hours later. High titer virus was obtained by high speed centrifuge (20,000 rpm) for 2 hours. Virus was injected into S. Pyramidale in CA1 region by picospritzer.

Acute slices

Brains from 25-30 days old Sprague-Dawley rats (Charles River, CA) were removed and immersed into cold dissection buffer containing 87 mM NaCl, 25 mM $NaHCO_3$, 75 mM Sucrose, 10 mM Glucose, 2.5 mM KCl, 1 mM NaH_2PO_4, 0.5 mM $CaCl_2$, 7 mM $MgCl_2$ and equilibrated with 95% O_2 and 5% CO_2. Hippocampal slices of ~400 μm thickness

were prepared with vibratome (Leica), transferred to a holding chamber with the ACSF solution containing 119 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, 26.2 mM $NaHCO_3$, 1 mM NaH_2PO_4, 11 mM glucose and bubbled with 95%O_2/5% CO_2 at 37°C for ~1 hour and then incubated at room temperature for at least 30 minutes before recording. Stimulation electrode was placed at stratum lacunosum-moleculare during recording.

Nova-2 mice

Nova-2 null mice were generated in the CD-1 stain (Ule et al., 2003). Two pairs of primers used for genotyping are designed to target the wildtype locus and mutant locus. The primers targeted to the wildtype locus are N2GT-F1 and N2GT-R2, and the sequences are: GGATCCTCTAGAGTCACACC and GGGTGACATGGAAGAAAGGG respectively. A band size of 450 bp is expected. The primers targeted to the mutant locus are Cre-4 and S6-b, and the sequences are: TTTCCGTCTCTGGTGTAGC and GTGCACACACACATGTCC respectively. A band size of 550 bp is expected. PCR protocol is 94°C for 2'30", 45 cycles of 94°C for 30", 60°C for 45", and 72°C for 45". The final extension is 72°C for 7' followed with 4°C.

Hippocampal organotypic cultured slices were prepared from heterozygous and homozygous sibling mice with the same procedure as for rat slice culture. For recording miniature fIPSC and miniature EPSC, the patch pipette was filled with an internal solution containing 115 mM cesium methanesulfonate, 20 mM CsCl, 10 mM HEPES, 2.5

mM $MgCl_2$, 4 mM Na_2ATP, 0.4 mM Na_3ATP, 10 mM sodium phosphocreatine, 0.6 mM EGTA, pH 7.25. The neuron was held at –70 mV for recording miniature fIPSC and at –60 mV for recording miniature EPSC. The external solution containing blockers for glutamate receptors (for recording mIPSC) or $GABA_A$ blocker (for recording mEPSC) and sodium channels: NBQX (5 μM), APV (100 μM), PTX (100 μM) and TTX (2 μM). All recordings were at room temperature. The peak amplitudes of AMPA-R EPSC and NMDA-R EPSC were measured at 10-15 msec (at –60 mV) and 200 msec (at +40 mV), respectively, from the onset of the synaptic currents.

Results

GIRK2 also resides in the dendritic spines of hippocampal neurons

Given that $GABA_B$ receptors and GIRK1 are located at spines, which house 90% of excitatory synapses, the distribution of GIRK2 was first characterized. GIRK2 antibody was applied to mature rat dissociated hippocampal culture transfected with the green fluorescent protein EGFP. The dendritic spines that were predominantly marked by the EGFP also exhibited strong GIRK2 immnunofluorescence (Fig 1). This result provided further evidence that components for slow inhibition reside in the spines. The proximity of slow inhibition and excitatory synapses (probably on the same spines) raises the possibility that the same machinery - NMDA activation and downstream CaMKII activity, which induce excitatory postsynaptic current (EPSC) LTP - may affect slow inhibition mediated by $GABA_B$ receptors and GIRK channels (Fig 2).

The sIPSC mediated by $GABA_B$ receptors and GIRK channels in CA1 neurons

To study sIPSC, whole cell patch clamp recording was carried out in CA1 neurons in cultured rat hippcampal slices. The stimulation electrode was placed 300-500 μm away from stratum pyramidale – likely within stratum lacunosum-moleculare, which harbor that interneurons innervated dendritic shafts as well as spines of CA1 pyramidal neurons (Vida et al., 1998). To isolate the sIPSC component, which is much smaller and slower than fast synaptic responses, I recorded sIPSC at –60 mV in the presence of NBQX and picrotoxin to block AMPA and $GABA_A$ currents. A biphasic current was obtained with presynaptic stimulation. The later outward current with a peak latency of 320 ± 81 msec

was blocked by GIRK channel blocker SCH23390 (10 μM, n=6; Fig 3, blue) (Kuzhikandathil and Oxford, 2002). To elicit a detectable sIPSC, I used a much stronger stimulation strength than what is usually used in the study of fast synaptic responses, to activate more presynaptic glutamatergic and GABAergic nerve fibers. Therefore, a quite large inward NMDA-R EPSC with a peak latency of 31 ± 9 msec, was detected even with the membrane potential held at –60 mV and with 4 mM extracellular Mg^{2+}. As expected, it could be blocked by APV, an NMDA receptor antagonist (100 μM, Fig 3, red). The sIPSC was further verified by its sensitivity to a structurally unrelated GIRK channel blocker, tertiapin (100nM, n=4, Fig 4) (Bichet et al., 2004), and the $GABA_B$ receptor antagonist SCH50911 (25μM, n=7, Fig 5) (Bolser et al., 1995). There was little correlation between the size of the NMDA-R EPSC and the amplitude of the sIPSC (linear correlation coefficient R^2=0.5, n= 13, p>0.1, Fig 6). The large amplitude of NMDAR-mediated currents (Fig 3) suggested that interneurons might fire in response to the strong stimulation paradigm we employed. Stimulation would thus result in both a monosynaptic sIPSC, triggered by direct stimulation of interneurons, and a disynaptic sIPSC, triggered by the synaptic stimulation of interneurons. Evidence for such disynaptic sIPSCs came from the demonstration that sIPSCs were partially blocked by adding the NMDA antagonists APV and CPP (Fig 7).

The pairing protocol causes potentiation of the sIPSC

The amplitude of sIPSC and NMDA-R EPSC remained relatively stable throughout the duration of the experiment (~ 30 minutes, Fig 8), when sampled at 0.33 Hz with the CA1

neuron held at –60 mV. To induce plasticity of sIPSC, I used the pairing protocol, which has been extensively used for inducing NMDA receptor-dependent LTP in whole cell configuration in many studies to activate postsynaptic NMDA receptors as the coincidence detector (Malenka and Bear, 2004). To avoid washout of synaptic plasticity, a short period of baseline (~ 3 mins) was obtained by holding CA1 neuron at –60 mV and delivering stimuli at 0.33 Hz stimulation, followed by 3 Hz stimulation paired with postsynaptic depolarization for 2 minutes. After the pairing protocol, 0.33 Hz stimulation was resumed to monitor the synaptic current at –60 mV. Strikingly, sIPSC was gradually increased 5-10 minutes after pairing. This potentiation was robust and long lasting; the sIPSC amplitude increased by ~4 fold (415 ± 40% at 20-25 min after pairing, n=12, $p<0.001$), and the potentiation persisted for ~20 min throughout the duration of experiment (Fig 9). This was accompanied by stable input resistance and series resistance, both monitored through the experiments (Fig 9). Change of NMDA-R EPSC amplitude or time course by pairing protocol could affect the sIPSC peak amplitude measured from the biphasic response. However, no significant change of amplitude (136 ± 50% at 20-25 mins after pairing, n= 12, p= 0.2) or decay time (measured in the presence of SCH50911, NBQX and picrotoxin to isolate NMDA-R EPSC, 97 ± 17 msec before pairing, n=7; 90 ± 10 msec after pairing, n=10, p=0.7) of NMDA-R EPSC was found (Fig 10). By measuring the area of sIPSC response instead of the sIPSC peak amplitude, I still observed a persistent and robust pairing-induced potentiaion (normalized area: 2.9 ± 0.5 at 20-25 mins after pairing, n=12, $p< 0.05$, Fig 11). There was no correlation between the extent of the sIPSC potentiation and the baseline amplitude of the sIPSC (linear

correlation index R^2=0.04, n=19, p>0.5, Fig 12) or the NMDA-R EPSC (linear correlation index R^2=0.1, n=19, p>0.5, Fig 13).

To characterize this novel form of potentiation of sIPSC, it is important to ascertain whether this plasticity can be observed in hippocampal acute slices. Interneurons in the distal apical dendritic field of CA1 neurons have a high target preference for not only dendritic shafts but also spines (Vida et al., 1998), and the spines in stratum lacunosum-moleculare are particularly rich in the immunoreactivity of GIRK1 and both subunits of $GABA_B$ receptors (Drake et al., 1997; Kulik et al., 2003). Therefore we delivered nerve stimuli to the perforant path at stratum lacunosum-moleculare. I started the acute slices experiments with 15-20 day-old rats whose developmental age is similar to those for the cultured slices (7 days old animals plus in vitro culture 2-3 weeks) (De Simoni et al., 2003). However, basal sIPSC showed gradual depression from CA1 pyramidal neurons in acute hippocampal slices (25.1 ± 9.3% at ~20 mins, n=10, p<0.01) (Fig 14). No sIPSC potentiation was observed (80.2 ± 11.2% at 20-25 mins after pairing, n=19, p=0.08) (Fig 15). Because the absence of sIPSC potentiation was probably due to serious rundown of baseline after 20 mins, older animals (p25-30 days old) were tested to see whether they gave rise to more stable baseline. The baseline sIPSC sampled with 0.33 Hz stimulation exhibited slight rundown; the sIPSC amplitude 20-25 min after initiation of whole-cell patch-clamp recording was 80 ± 27% of the starting value (n=13, p=0.02) (Figure 16). In spite of this slightly reduction of the basal sIPSC with time, the pairing protocol induced potentiation of the sIPSC (142 ± 13% at 20-25 min after pairing, n=14, p<0.01) (Figure 17).

Whereas the pairing protocol induced LTP of the sIPSC in acute hippocampal slices as well as cultured slices, both the sIPSC amplitude and the extent of its potentiation were much greater in cultured slices, probably because the more exuberant innervation established by glutamatergic nerve fibers that remain in the slice culture made it more likely for the stimulation electrode to be situated near the interneurons and/or their presynaptic glutamatergic nerve fibers. Perhaps because of the relatively large stimulation strength necessary for a single stimulus to elicit an sIPSC in the acute slice, the pairing protocol caused some potentiation of the NMDA-R EPSC, similar to what has been observed in previous studies (Aniksztejn and Ben-Ari, 1995; Bashir et al., 1991; Watt et al., 2004). The long-lasting potentiation of the NMDA-R EPSC underscores that pairing postsynaptic depolarization with perforant path stimulation is effective in inducing synaptic plasticity in the acute slice. An increase in the NMDA-R EPSC after pairing, as well as the gradual rundown of the sIPSC, likely would cause under-estimation of the extent of sIPSC potentiation in the acute slice. For these reasons, we have concentrated our efforts on characterizing the sIPSC potentiation in the slice culture for the rest of this study. The slice culture also made it possible to study the Nova-2 null mutant mice with early lethality (see below).

Potentiation of the sIPSC requires NMDA receptor activation in the postsynaptic CA1 neuron, but exhibits no pathway specificity

To further establish that the pairing protocol caused NMDA receptor activation leading to long-term potentiation of the sIPSC, we first showed that the potentiated sIPSC after the

pairing protocol was sensitive to the $GABA_B$ receptor antagonist SCH50911 (Figure 18) as well as the GIRK channel blocker SCH23390 (Figure 19).

Pathway specificity is one of the important properties of EPSC LTP. Excitatory LTP is only elicited at the synapses stimulated by afferent activity but not at adjacent synapses on the same postsynaptic cell. To test whether sIPSC LTP has the same property, I placed 2 stimulation electrodes at two separated locations on the slice, one without stimulation during the pairing protocol for the control pathway, the other one with 3Hz stimulation during the pairing protocol, for the pairing pathway. No pathway specific of sIPSC potentitaion was observed (pairing pathway: 320 ± 50% 20-25 mins after pairing, n=8; control pathway: 290 ± 100% 20-25 mins after pairing, n=8, p=0.87) (Fig 20). Similar result has been observed for the interneuron plasticity (McMahon et al., 1997). The difference characteristic between EPSC LTP and sIPSC LTP could be due to different distribution of axons; whereas the Schaffer collaterals are highly orientated, the axons of interneurons are dispersed over the dendirtic field of CA1 neurons.

Next, we tested the dependence of the sIPSC potentiation on pairing-induced NMDA receptor activation. When we applied the NMDA receptor antagonist APV (100 μM) as well as 5 μM NBQX to block AMPA receptors and 100 μM picrotoxin to block $GABA_A$ receptors, we found no pairing-induced LTP of the sIPSC (102 ± 20% at 20-25 min after pairing, n=7, p=0.1) (Figure 21). In the presence of these glutamate receptor antagonists, a very small residual inward synaptic current remained probably due to the very strong stimulation strength. Moreover, the sIPSC amplitude was greatly reduced as well, likely

due to inefficient synaptic excitation of the inhibitory interneurons for the generation of the di-synaptic sIPSC. To get around this problem, we performed two additional control experiments.

First, we stimulated the presynaptic nerve fibers at 3 Hz without depolarizing the postsynaptic CA1 neuron, and found there was no persistent potentiation of the sIPSC (123 ± 26% at 20-25 min after pairing, n=6, p=0.1) (Figure 22). Second, we treated the cultured slices only with antagonists of the $GABA_A$ receptors and NMDA receptors. Under this condition, there was no pairing-induced potentiation of the biphasic response composed of AMPA receptor-mediated EPSC (AMPA-R EPSC) and the sIPSC (90 ± 12%, n=6, p=0.3) (Figure 23). Thus, notwithstanding the difficulty to assess precisely the extent of dendritic depolarization while the CA1 neuron was clamped to between –5 to 0 mV in K^+-based internal solution, there must have been sufficient dendritic depolarization during the pairing protocol to induce NMDA receptor-dependent synaptic plasticity of the sIPSC. These experiments confirm that NMDA receptor activation via the pairing protocol induced LTP of the sIPSC. However, no correlation of between NMDA-R EPSC and fold of sIPSC potentiation (Fig 13) indicates that there are more than one group of synapses recruited to obtain NMDA-R EPSC and sIPSC. Only NMDA mediated EPSC or sIPSC was obtained from some of the connections, since only excitatory or inhibitory presynaptic was stimulated. No correlation of NMDA mediated EPSC and folds of potentiation of sIPSC was expected within this type of synapses. However, in the synapse that receives both excitatory and inhibitory neurotransmitter activation, correlation between NMDA mediated EPSC and fold of potentiation of sIPSC

would be expected, because NMDA receptor activation was required for inducing sIPSC potentiation (Fig 21). The results may imply the majority of synapses recruited was the former type of synapses.

Induction of the sIPSC LTP is dependent on postsynaptic Ca^{2+} increase

Ca^{2+} entry through NMDA receptors is essential for inducing LTP of the EPSC (Malenka et al., 1988). To determine whether a rise in postsynaptic Ca^{2+} is also required for the sIPSC potentiation, we introduced Ca^{2+} chelators to the patch pipette solution to buffer the intracellular Ca^{2+} concentration. Whereas the pairing protocol still caused potentiation of the sIPSC when the intracellular Ca^{2+} level was moderately buffered by 1.1 mM EGTA including in normal internal solution, chelating Ca^{2+} within the CA1 neuron with 10 mM BAPTA eliminated pairing-induced LTP of the sIPSC (87 ± 19% at 20-25 min after pairing, n=6, p=0.6) (Figure 24). Thus, an increase in postsynaptic Ca^{2+} is essential for the induction of LTP of the sIPSC.

Even though pairing-induced potentiation of the sIPSC requires NMDA receptor activation and Ca^{2+} rise in the postsynaptic CA1 neuron, *a priori*, it remains possible that this potentiation could result from increased GABA release. For example, certain forms of synaptic plasticity involve retrograde signaling via endocannabinoids that are released from the postsynaptic neuron to activate presynaptic cannabinoid receptors (Carlson et al., 2002;Chevaleyre and Castillo, 2003; Piomelli, 2003; Wilson and Nicoll, 2001). Cannabinoid receptor-1 (CB1) is mainly expressed at the axon termininal of GABA-

mediated inhibitory interneurons in the hippocampus (Wilson and Nicoll, 2001). Release of endogenous cannabinoids from pyramidal neurons by depolarization or activation of mGluR activates presynaptic CB1 receptors, which further suppress GABA-mediated transmission in a Ca^{2+}-dependent manner. However, we found that with a selective antagonist for the CB1 cannabinoid receptor, AM251, potentiation of sIPSC can still be induced by pairing protocol (370 ± 33% at 20-25 min after pairing, n=5, p<0.001) (Figure 25). Moreover, the pairing protocol did not alter GABA release to an extent that affected the paired-pulse ratio of the fIPSC (0.74 ± 0.04 before pairing versus 0.74 ± 0.05 at 10 min after pairing, p=0.99; and 0.7 ± 0.04 at 20 min after pairing, p=0.5; n=5) (Figure 26). These experiments show that the pairing-induced LTP of the sIPSC could not be attributed to endocannabinoid retrograde signaling or increased GABA release. As a control, paired-pulse ratio of fIPSC was altered by changing from 0.5 mM [Ca^{2+}] to 4 mM (0.5 mM Ca^{2+}: 1.3 ± 0.2, 4 mM Ca^{2+}: 0.95 ± 0.1, n= 6, p<0.01). Consistent with previous studies, slight depression of fIPSC was also observed after pairing (68.5 ± 20.2, n=5, p=0.15) (Fig 27).

PKC is required for sIPSC potentiation maintenance

PKC is important for the induction LTP of EPSC (Malenka and Nicoll, 1999; Malenka and Bear, 2004). However, an atypical protein kinase C isozyme, protein kinase M zeta (ζ), is necessary and sufficient for LTP maintenance but not LTP induction (Ling et al., 2002; Serrano et al., 2005). The PKC inhibitor chelerythrin (20μM), which blocks conventional and novel PKCs at this concentration, was applied to see whether sIPSC LTP can still be elicited. No persistent potentiation was found with PKC inhibitor (106 ±

24% at 20-25 min after pairing, n=9, p=0.5) (Fig 28). It would be interesting to test whether PKMζ has a similar role in sIPSC potentiation by selectively blocking it with very low concentration of chelerythrin. The acidic cluster of GIRK2 contains a threonine residue that can be potentially phosphorylated by casein kinase II (Ma et al., 2002). Mutation of this site alters the surface expression level of homotermeric GIRK2. It is possible that an increase of casein kinase II activity would potentiate slow inhibition by increasing GIRK channel surface expression. However, treatment of slices with 20 μm DRB, a selective inhibitor of casein kinase II, potentiation of sIPSC can still be induced (373 ± 24% at 20-25 mins after pairing, n=9, p<0.005) (Fig 29).

Activation of CaMKII is both necessary and sufficient for inducing LTP of the sIPSC

Ca^{2+} influx through the NMDA receptor is known to activate CaMKII, leading to potentiation of the EPSC (Lisman et al., 2002; Malenka et al., 1989; Malinow et al., 1989). To test whether CaMKII activation is also necessary for inducing LTP of the sIPSC, we treated the slice with 10 μM KN-93, a selective inhibitor of CaMKII. We found that this CaMKII inhibitor eliminated pairing-induced LTP of the sIPSC (90 ± 15% at 20-25 min after pairing, n=11, p=0.6) (Figure 30). Thus, like LTP of the EPSC, potentiation of the sIPSC requires CaMKII activation.

To ask whether postsynaptic CaMKII activity is sufficient to potentiate the sIPSC, we utilized the Sindbis virus to introduce into CA1 neurons a constitutively active form of this enzyme fused with EGFP, CaMKII(1-290)-EGFP, which is effective in causing

potentiation of the EPSC (Hayashi et al., 2000). We note that the great majority of the infected neurons are glutamatergic pyramidal neurons. For an internal control, we simultaneously recorded from an uninfected neuron and a nearby neuron expressing CaMKII(1-290)-EGFP (Figure 31). By comparing two adjacent neurons subjected to the same condition for synaptic stimulation, we found that the constitutively active CaMKII potentiated the sIPSC (6.1 ± 0.5 pA for neurons expressing CaMKII(1-290)-EGFP versus 3.0 ± 1 pA for neighboring control neurons, n=16, $p<0.0005$) (Figure 32) but not the NMDA-R EPSC (46.0 ± 10.7 pA for neurons expressing CaMKII(1-290)-EGFP versus 47.5 ± 12.5 pA for neighboring control neurons, n=17, p=0.4) (Figure 32). In control experiments, neurons expressing EGFP and their neighboring uninfected neurons yielded similar sIPSC (2.9 ± 0.7 pA for neurons expressing EGFP versus 3.1 ± 0.9 pA for neighboring control neurons, n=18, p=0.8) and NMDA-R EPSC (38.8 ± 5.1 pA for neurons expressing EGFP versus 40.9 ± 6.0 pA for neighboring control neurons, n=24, p=0.4) (Figure 33). Therefore, the CaMKII activity was sufficient for the potentiation of the sIPSC.

If NMDA receptor activation via the pairing protocol causes potentiation of the sIPSC by activating CaMKII in the postsynaptic CA1 neuron, expression of constitutively active CaMKII in the CA1 neuron not only should potentiate the sIPSC—as we have observed, it ought to occlude further potentiation of the sIPSC via the pairing protocol. Indeed, neurons expressing the constitutively active CaMKII showed no further potentiation of the sIPSC after the pairing protocol (113 ± 54%, n=7, p=0.1), while control neurons expressing EGFP still exhibited pairing-induced LTP of the sIPSC (287 ± 43%, n=4,

$p<0.01$) (Figure 34). These experiments demonstrate that the sIPSC was potentiated by the activation of CaMKII in the postsynaptic CA1 neurons.

The function of the RNA-binding protein Nova-2 is important for the potentiation of the sIPSC

Having established that the sIPSC mediated by $GABA_B$ receptors and GIRK channels can be potentiated by activation of NMDA receptors and CaMKII in the CA1 neurons, we wondered whether this synaptic plasticity of slow inhibition might be subjected to concerted modulation of relevant synaptic proteins. One candidate for coordinating such modulation is Nova-2, because it regulates a network of synaptic proteins and one third of its targets are molecules involved in inhibitory synaptic transmission, including $GABA_B$ receptors and GIRK channels (Ule et al., 2003; Ule et al., 2005).

To characterize synaptic transmission and plasticity in the Nova-2 null mice (M. Ruggiu and R. B. Darnell, unpublished data), which lived for 2-3 weeks after birth, we first measured the resting potential of CA1 neurons in cultured hippocampal slices, and found no difference between Nova-2 null mice (-60.6 ± 3.5 mV, n=8) and their heterozygous siblings (–59.4 ± 3.1 mV, n=5, p=0.5). In these experiments, PCR with specific primers determined the genotype of the litters (Fig 35). To test whether Nova-2 function is important for inhibitory synaptic transmission, we compared the null mutants with their heterozygous siblings and found no difference in the miniature fIPSC amplitude (5.3 ± 0.4 pA for Nova-$2^{-/-}$ versus 5.2 ± 0.7 pA for Nova-$2^{+/-}$, n=5, p=0.8) or frequency (1.4 ± 0.3 Hz for Nova-$2^{-/-}$ versus 1.6 ± 0.4 Hz for Nova-$2^{+/-}$, n=5, p=0.4) (Figure 36). Moreover,

by measuring the amplitudes of the sIPSC and fIPSC generated by nerve stimulation with increasing strengths, we found the null mutants and their heterozygous siblings exhibited a similar ratio of the slow and fast IPSC amplitudes (0.07 ± 0.02 for Nova-$2^{-/-}$ versus 0.08 ± 0.02 for Nova-$2^{+/-}$, n=8, p=0.2) (Figure 37). Finally, in cultured slices treated with NBQX and picrotoxin to block AMPA receptors and $GABA_A$ receptors, the ratio of the sIPSC and NMDA-R EPSC amplitudes was also comparable between Nova-2 null mice and controls (0.4 ± 0.1 for Nova-$2^{-/-}$ versus 0.5 ± 0.1 for Nova-$2^{+/-}$, n=15, p=0.4) (Figure 38). Normal mIPSC, basal transmission of fIPSC and sIPSC implied the normal morphology of inhibitory synapses in Nova-2 null animals. Interestingly, whereas their heterozygous siblings yielded normal potentiation of the sIPSC (338 ± 39% at 20-25 min after pairing, n=6, p<0.01), the Nova-2 null mutants exhibited no pairing-induced LTP of the sIPSC (130 ± 38% at 20-25 min after pairing, n=8, p=0.5, Figure 39).

Given that the same machinery for inducing LTP of the EPSC also is required for the generation of LTP of the sIPSC, we further tested whether the Nova-2 function is necessary for pairing induced LTP of the EPSC. We examined the miniature excitatory postsynaptic currents and found the amplitude and frequency of these unitary responses to be normal in the null mutants (amplitude: 3.5 ± 0.3 pA for Nova-$2^{-/-}$ versus 3.9 ± 0.6 pA for Nova-$2^{+/-}$, p=0.5; frequency: 0.5 ± 0.2 Hz for Nova-$2^{-/-}$ versus 0.4 ± 0.2 Hz for Nova-$2^{+/-}$, p=0.7, n=7) (Figure 40). We then measured the AMPA-R EPSC at –60 mV and NMDA-R EPSC at +40 mV and found similar ratios of these glutamate receptor-mediated synaptic currents in Nova-2 null mice and control mice (2.3 ± 0.9 for Nova-$2^{-/-}$, n=8, versus 2.5 ± 0.8 for Nova-$2^{+/-}$, n=7; p=0.91) (Figure 41). Next, we recorded the

NMDA receptor-mediated EPSC at different membrane potentials in the presence of the AMPA receptor antagonist NBQX, and found comparable voltage-dependence of the NMDA-R EPSC in Nova-2 null mutants and their sib controls (Figure 42). Finally, the pairing protocol elicited LTP of the EPSC in both heterozygous control mice (171 ± 14% for the pairing pathway, n=10, $p<0.01$; 99 ± 8% for the control pathway, n=5, p=0.8) and Nova-2 null mice (177 ± 14% for the pairing pathway, n=9, $p<0.01$; 99 ± 13% for the control pathway, n=4, p=0.93) (Figure 43). These experiments thus revealed no significant differences between Nova-2 null mice and control mice in excitatory synaptic transmission. Since the basal synaptic transmission appeared normal for excitation (Figure 40 and 41) and inhibition (Figure 36 and 37), and the capacity of inducing LTP of the EPSC remained in the Nova-2 null mutant (Figure 43), it appears that the machinery essential for potentiating the slow inhibition mediated by the $GABA_B$ receptors and GIRK channels is specifically impaired in Nova-2 null mice.

Increase of GIRK channel surface expression by chemically induced LTP in hippocampal dissociated cultures

To determine the expression level of receptor/channel on the membrane, immunostaining dissociated cultures without permeabilization is a more direct way compared to electrophysiology. However, there are no antibodies against the extracellular part of GIRK channel, making it difficult to tract endogenous protein. Coexpression of GIRK2A and GIRK1, with an HA epitope insertion into extracellular loop (Ma et al., 2002) and green fluoresence protein fusion at the carboxyl terminus in mature hippocamal cultures (DIV 3-4 weeks) by gene gun showed very little surface staining of GIRK1, which was

quantified and normalized with total GIRK1 protein level (Fig 44 and 47). To detect activity-dependent surface trafficking of GIRK channel, neurons were bathed in APV-containing culture medium for weeks, then changed to NBQX-containing culture medium for 2 hours, which increases insertion of AMPA receptors to induce LTP (Liao et al., 1999). However, it might be needed to further verify in our culture condition. This chemical LTP protocol significantly increased surface expression of GIRK channels (165 ±10% normalized to control, n=7, $p<0.05$) (Fig 45 and 47). However, similar effect was not observed in depolarized neurons with 25 mM KCl for 30 minutes (158 ± 17%, normalized to control, n=6, p=0.09) (Fig 46). Strikingly, punctate and spine located GIRK surface staining was also induced in an activity–dependent manner (Fig 45, arrow). These results indicate GIRK channel surface insertion is activity-dependent, a possible mechanism underlying the sIPSC potentiation deserved in hippocampal slices.

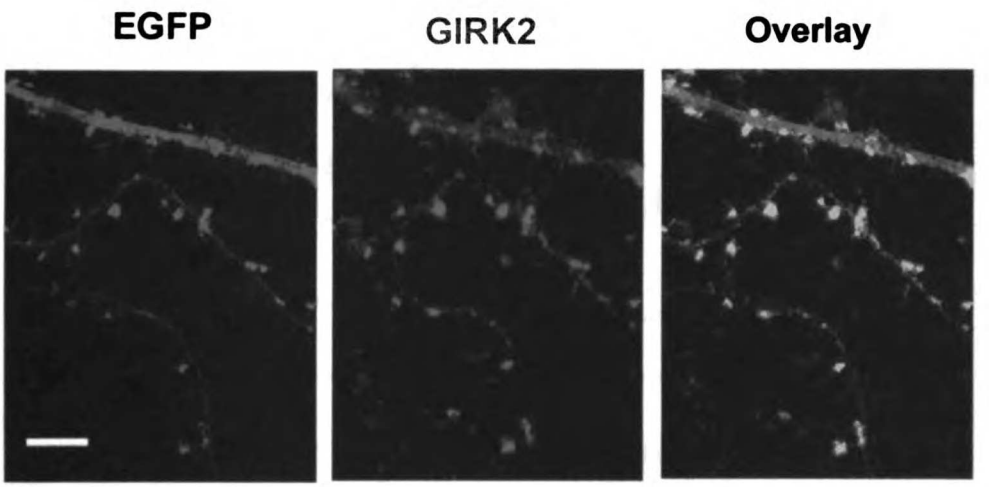

Fig 1.

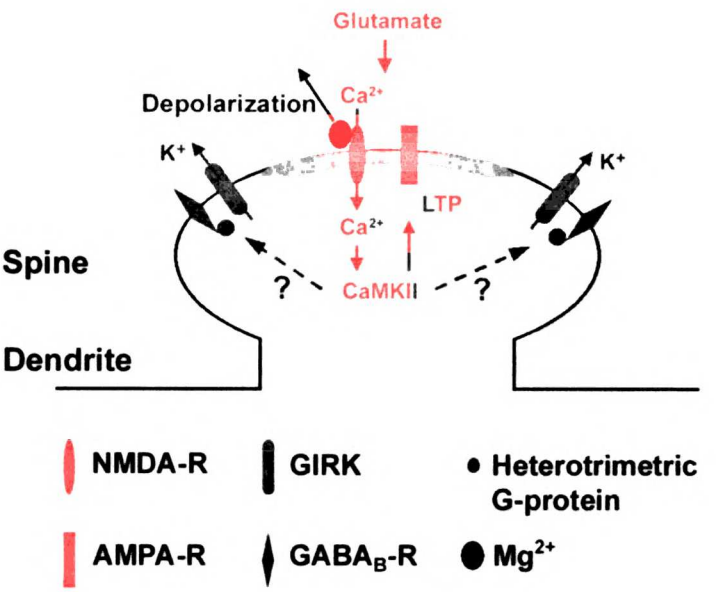
Glutamate
Depolarization
Ca2+
K+
K+
LTP
Ca2+
Spine
?
CaMKII
?
Dendrite
NMDA-R
GIRK
Heterotrimetric G-protein
AMPA-R
GABAB-R
Mg2+

Fig 2.

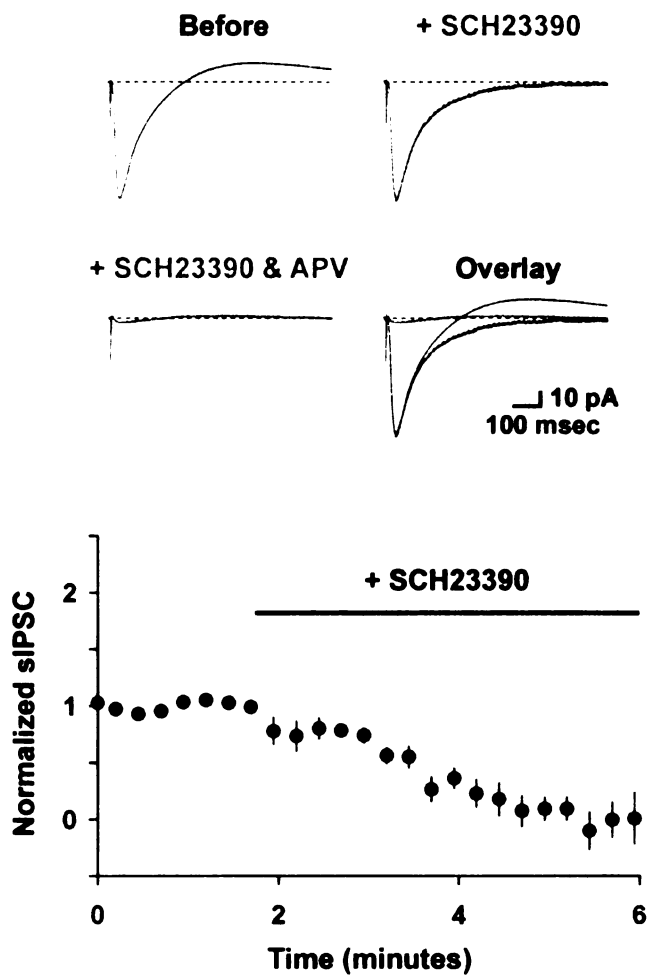
Before
+ SCH23390
+ SCH23390 & APV
Overlay
10 pA
100 msec
+ SCH23390
Normalized sIPSC
2
1
0
0
2
4
6
Time (minutes)

Fig 3.

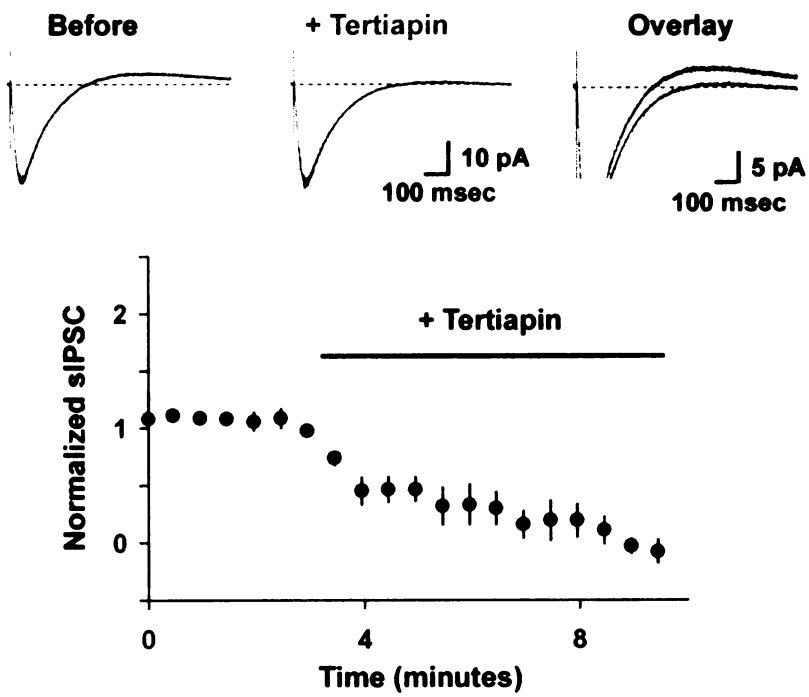
Before
+ Tertiapin
Overlay
10 pA
100 msec
5 pA
100 msec
+ Tertiapin
Normalized sIPSC
2
1
0
0
4
8
Time (minutes)

Fig 4.

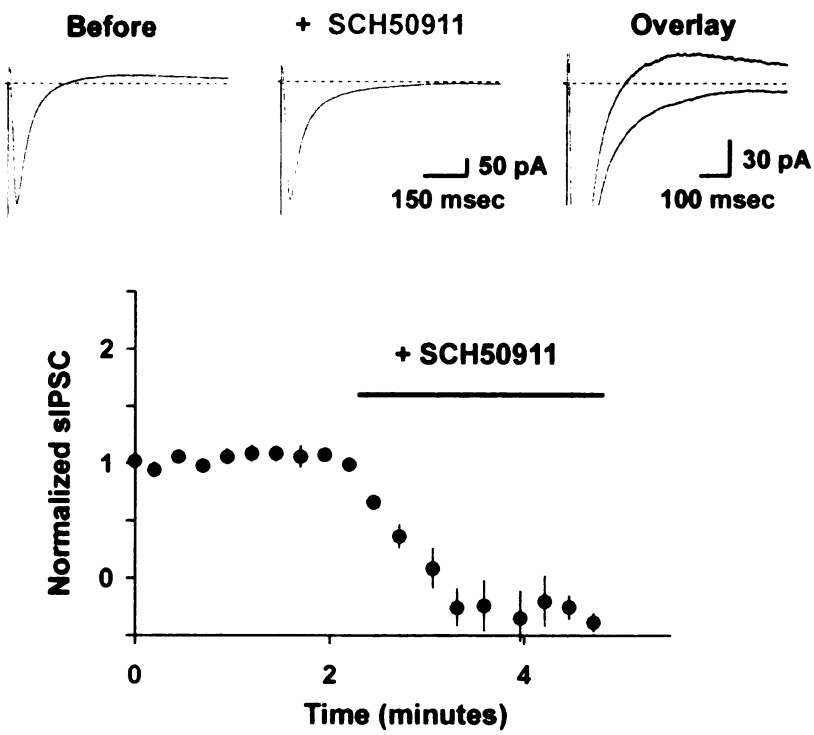
Before
+ SCH50911
Overlay
50 pA
150 msec
30 pA
100 msec
+ SCH50911
Normalized sIPSC
2
1
0
0
2
4
Time (minutes)

Fig 5.

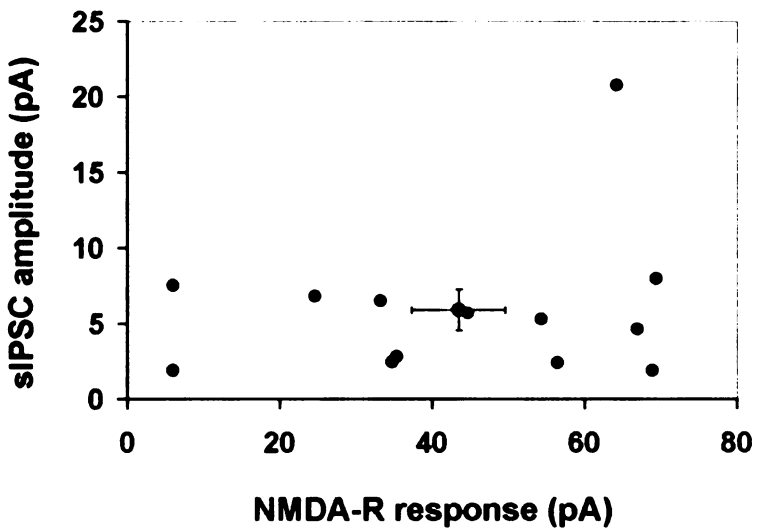
25
20
15
10
5
0
sIPSC amplitude (pA)
0
20
40
60
80
NMDA-R response (pA)

Fig 6.

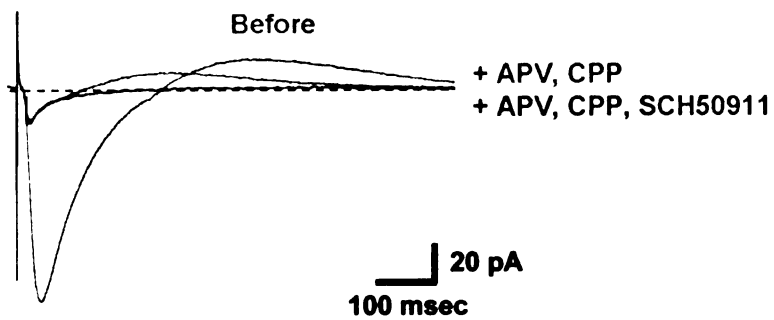

Fig 7.

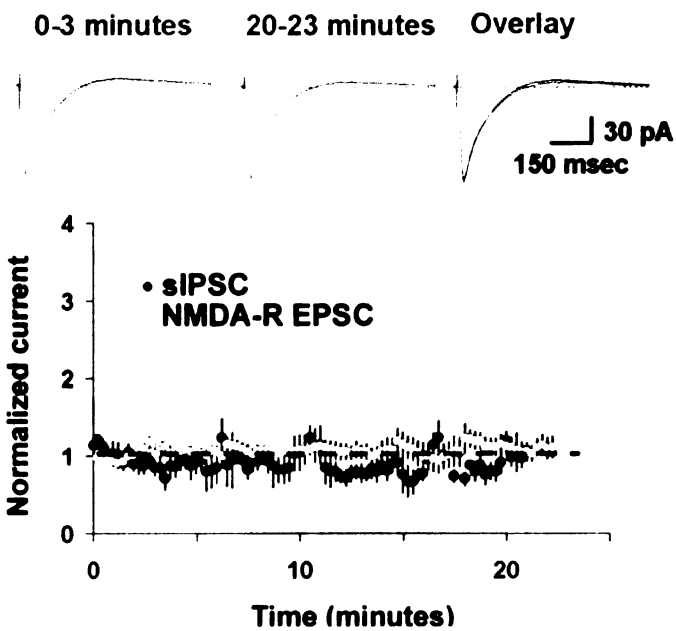
0-3 minutes
20-23 minutes
Overlay
30 pA
150 msec
• sIPSC
NMDA-R EPSC
Normalized current
4
3
2
1
0
0
10
20
Time (minutes)

Fig 8.

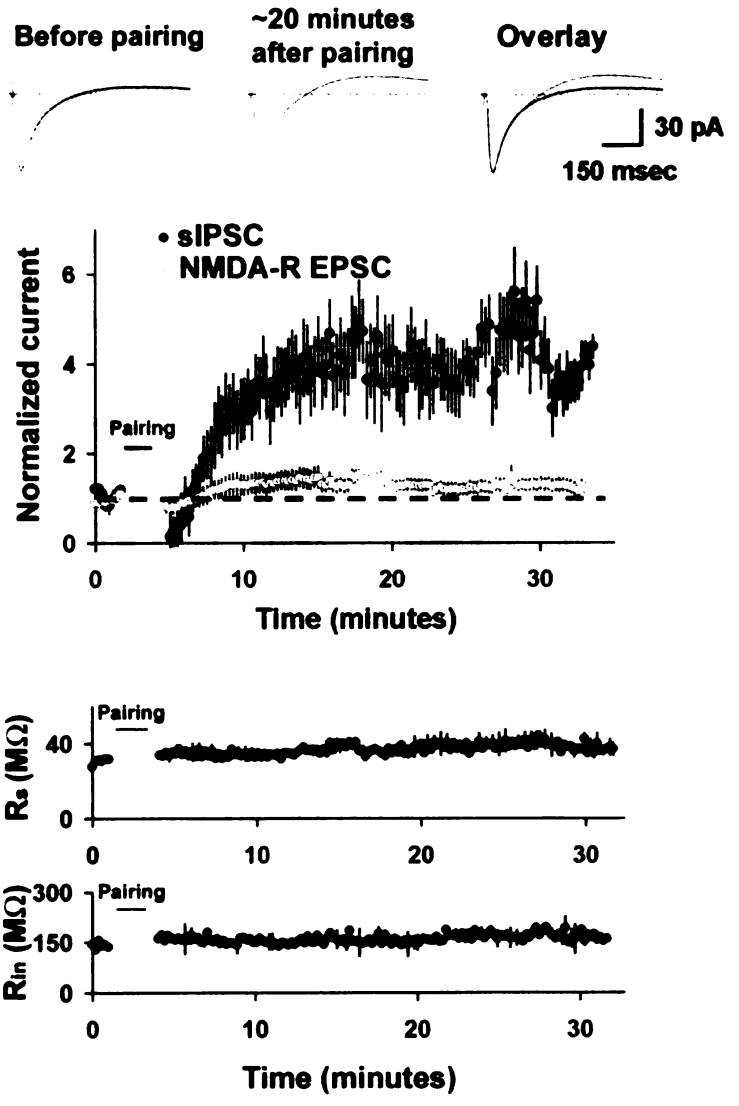
Before pairing
~20 minutes after pairing
Overlay
30 pA
150 msec
• sIPSC
NMDA-R EPSC
Normalized current
Pairing
Time (minutes)
Rs (MΩ)
Rin (MΩ)

Fig 9.

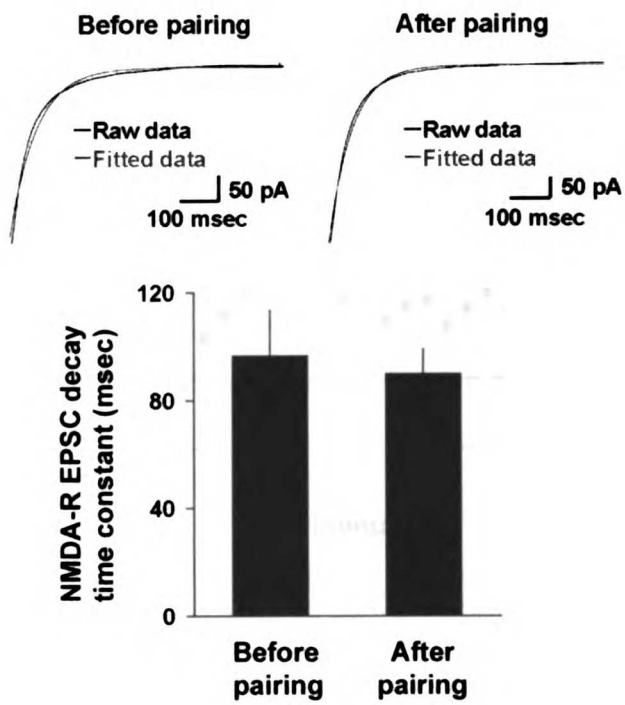
Before pairing
After pairing
Raw data
Fitted data
50 pA
100 msec
Raw data
Fitted data
50 pA
100 msec
NMDA-R EPSC decay time constant (msec)
120
80
40
0
Before pairing
After pairing

Fig 10

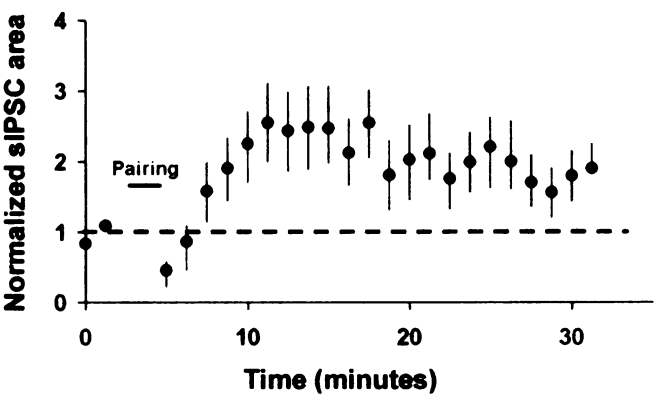

Fig 11.

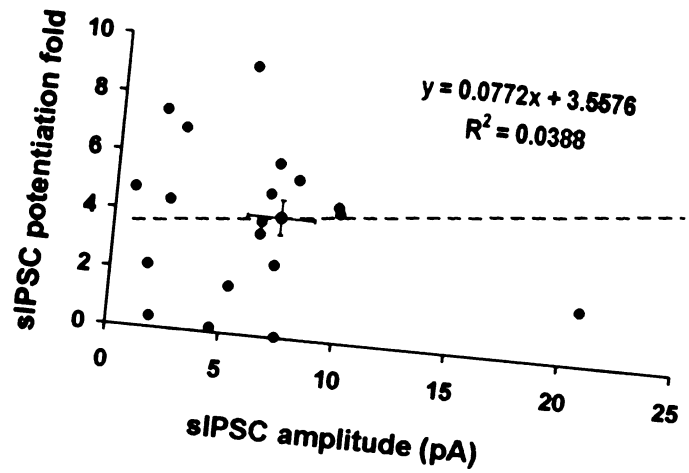
sIPSC potentiation fold
10
8
6
4
2
0
0
5
10
15
20
25
sIPSC amplitude (pA)
y = 0.0772x + 3.5576
R2 = 0.0388

Fig 12.

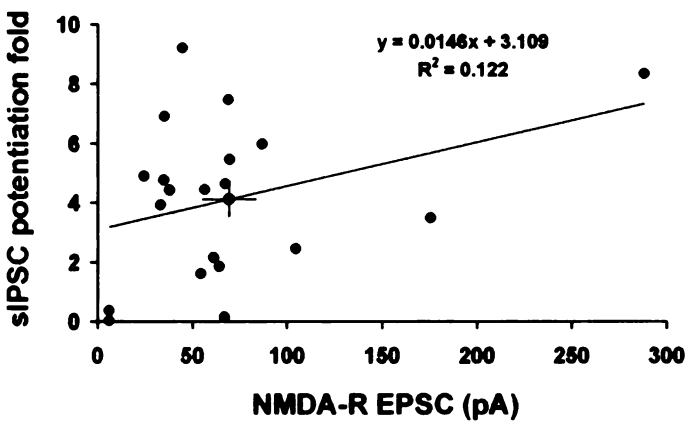
sIPSC potentiation fold
10
8
6
4
2
0
y = 0.0146x + 3.109
R² = 0.122
0
50
100
150
200
250
300
NMDA-R EPSC (pA)

Fig 13.

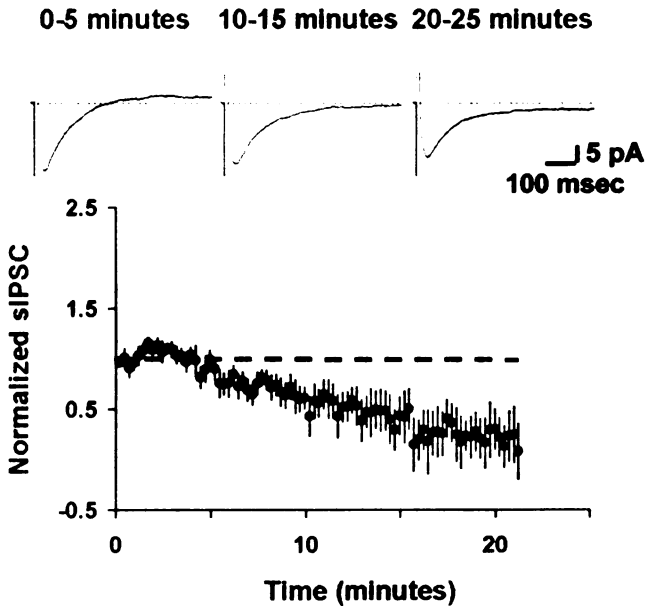
0-5 minutes
10-15 minutes
20-25 minutes
5 pA
100 msec
Normalized sIPSC
2.5
1.5
0.5
-0.5
0
10
20
Time (minutes)

Fig 14

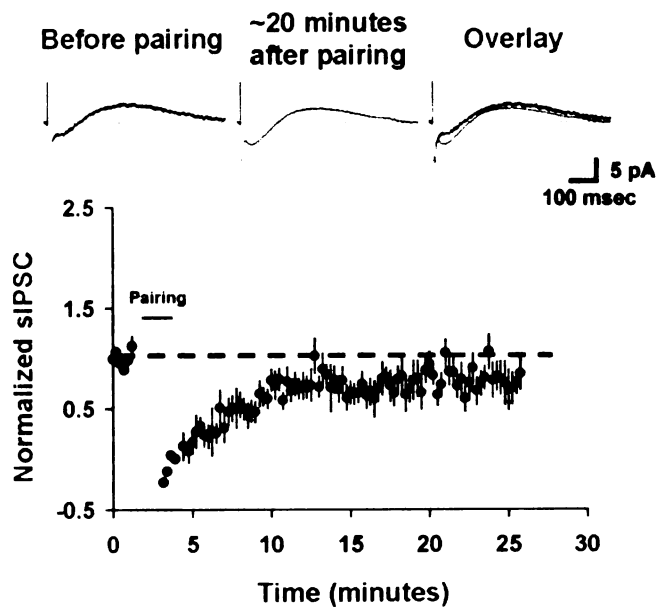
Before pairing
~20 minutes after pairing
Overlay
5 pA
100 msec
Normalized sIPSC
2.5
1.5
0.5
-0.5
Pairing
0
5
10
15
20
25
30
Time (minutes)

Fig 15

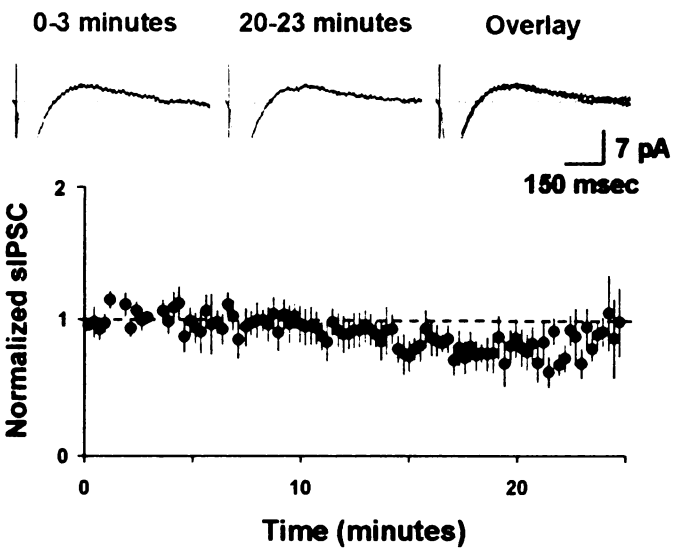
0-3 minutes
20-23 minutes
Overlay
7 pA
150 msec
Normalized sIPSC
2
1
0
0
10
20
Time (minutes)

Fig 16.

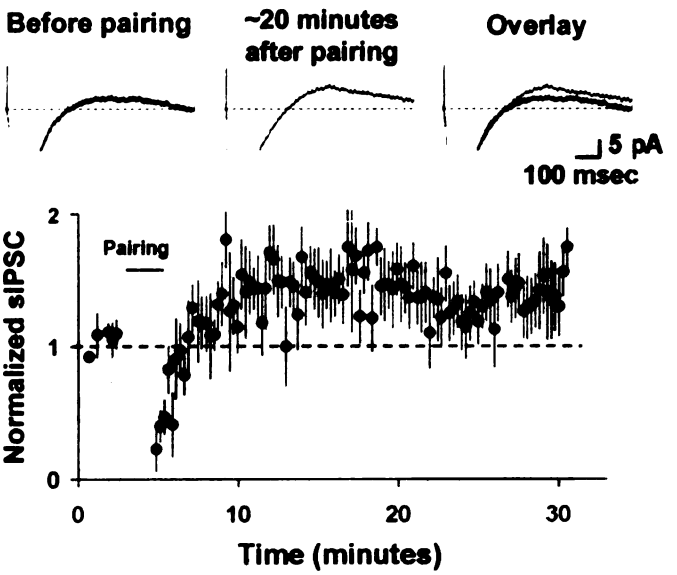
Before pairing
~20 minutes after pairing
Overlay
5 pA
100 msec
Pairing
Normalized sIPSC
2
1
0
0
10
20
30
Time (minutes)

Fig 17.

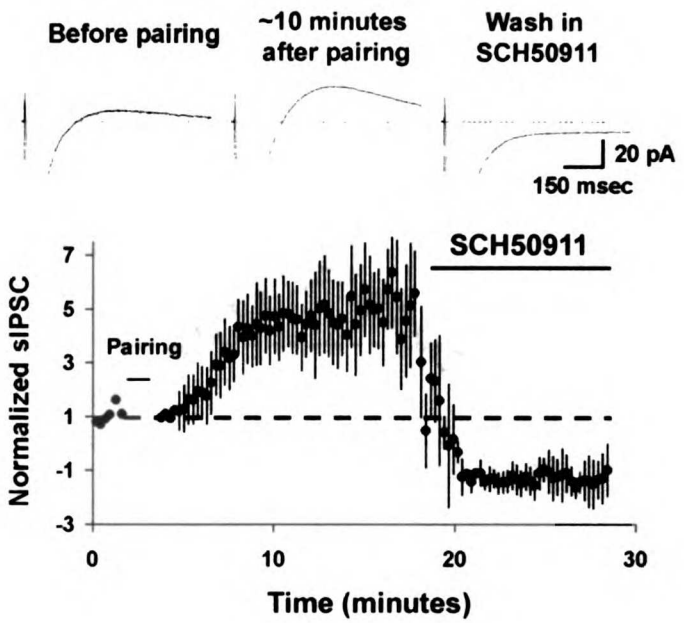
Before pairing
~10 minutes after pairing
Wash in SCH50911
20 pA
150 msec
SCH50911
Pairing
Normalized sIPSC
7
5
3
1
-1
-3
0
10
20
30
Time (minutes)

Fig 18.

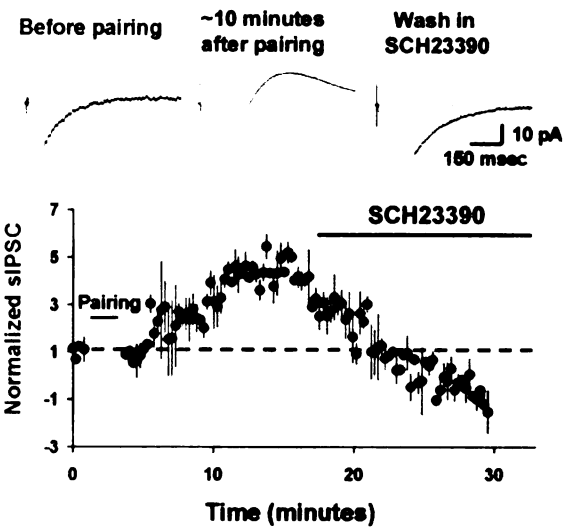
Before pairing
~10 minutes after pairing
Wash in SCH23390
10 pA
160 msec
SCH23390
Pairing
Normalized sIPSC
7
5
3
1
-1
-3
0
10
20
30
Time (minutes)

Fig 19.

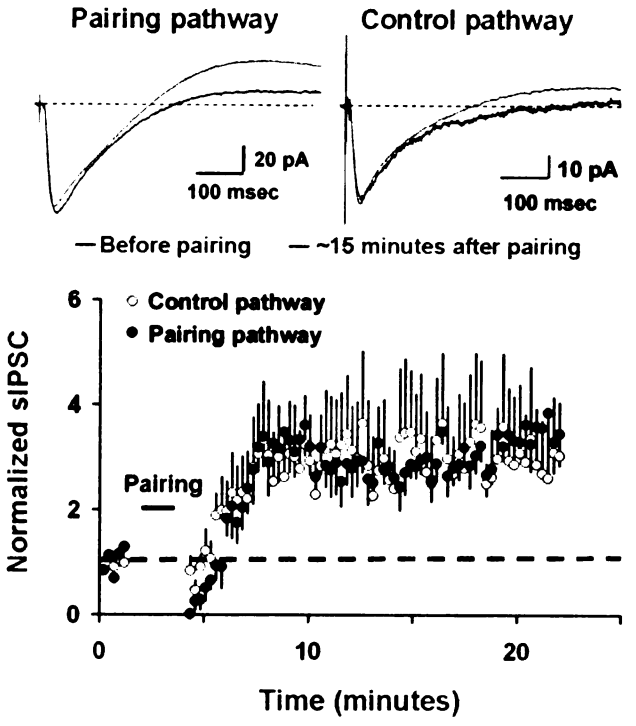
Pairing pathway
Control pathway
20 pA
100 msec
10 pA
100 msec
—Before pairing
— ~15 minutes after pairing
Control pathway
Pairing pathway
Pairing
Normalized sIPSC
0
2
4
6
0
10
20
Time (minutes)

Fig 20.

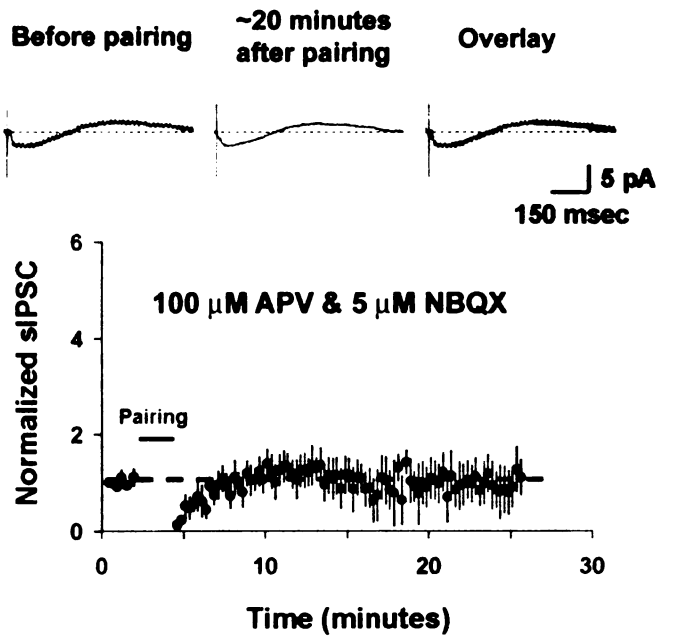
Before pairing
~20 minutes after pairing
Overlay
5 pA
150 msec
100 μM APV & 5 μM NBQX
Pairing
Normalized sIPSC
0
2
4
6
0
10
20
30
Time (minutes)

Fig 21.

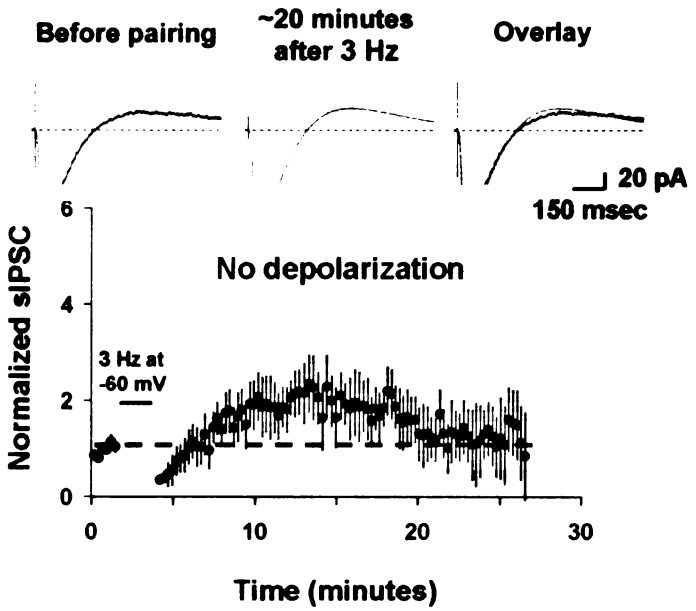
Before pairing
~20 minutes after 3 Hz
Overlay
20 pA
150 msec
No depolarization
3 Hz at -60 mV
Normalized sIPSC
6
4
2
0
0
10
20
30
Time (minutes)

Fig 22.

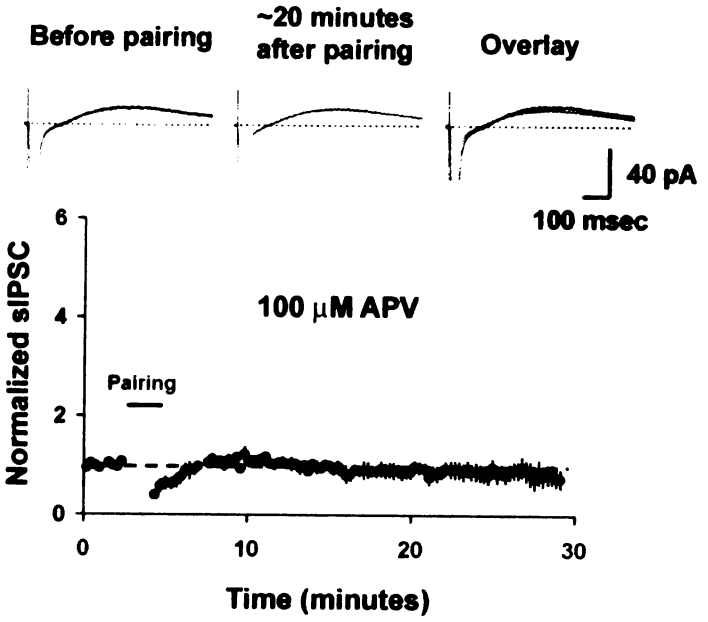
Before pairing
~20 minutes after pairing
Overlay
40 pA
100 msec
100 μM APV
Pairing
Normalized sIPSC
6
4
2
0
0
10
20
30
Time (minutes)

Fig 23.

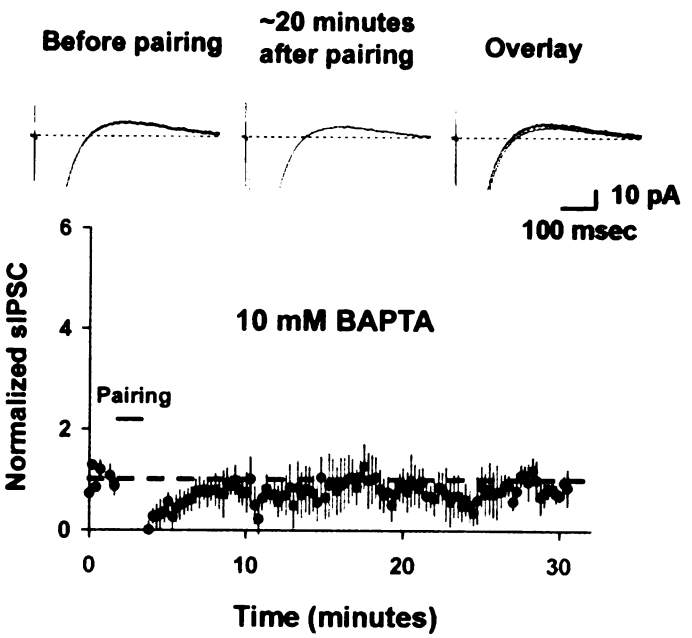
Before pairing
~20 minutes after pairing
Overlay
10 pA
100 msec
10 mM BAPTA
Pairing
Normalized sIPSC
6
4
2
0
0
10
20
30
Time (minutes)

Fig 24.

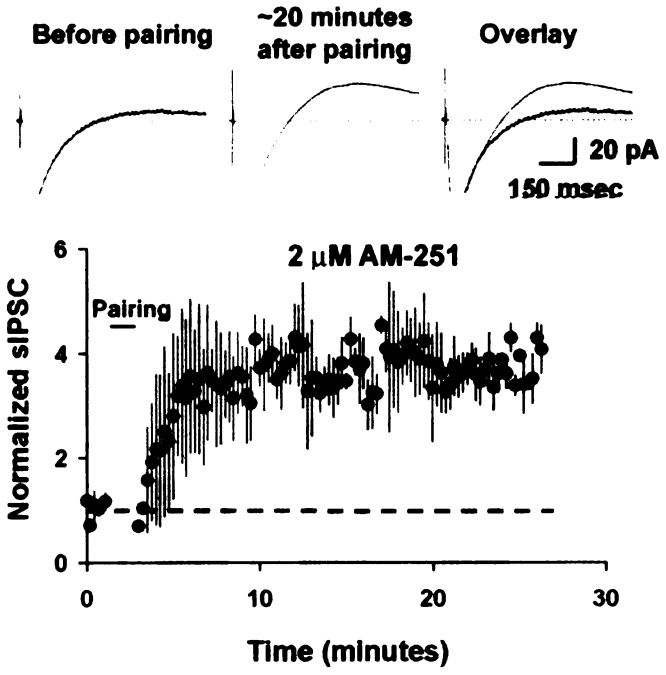
Before pairing
~20 minutes after pairing
Overlay
20 pA
150 msec
2 μM AM-251
Pairing
Normalized sIPSC
6
4
2
0
0
10
20
30
Time (minutes)

Fig 25.

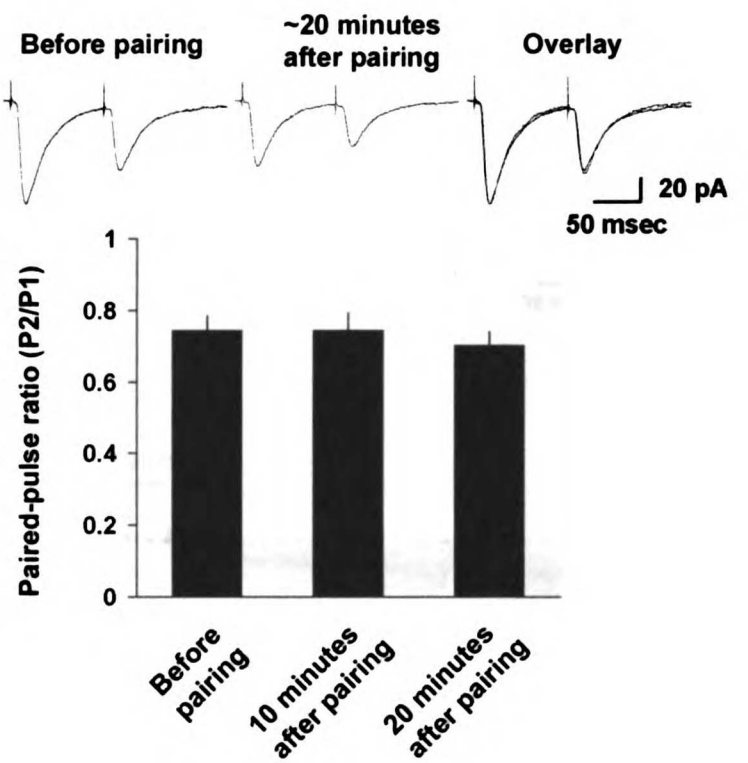
Before pairing
~20 minutes after pairing
Overlay
20 pA
50 msec
Paired-pulse ratio (P2/P1)
1
0.8
0.6
0.4
0.2
0
Before pairing
10 minutes after pairing
20 minutes after pairing

Fig 26.

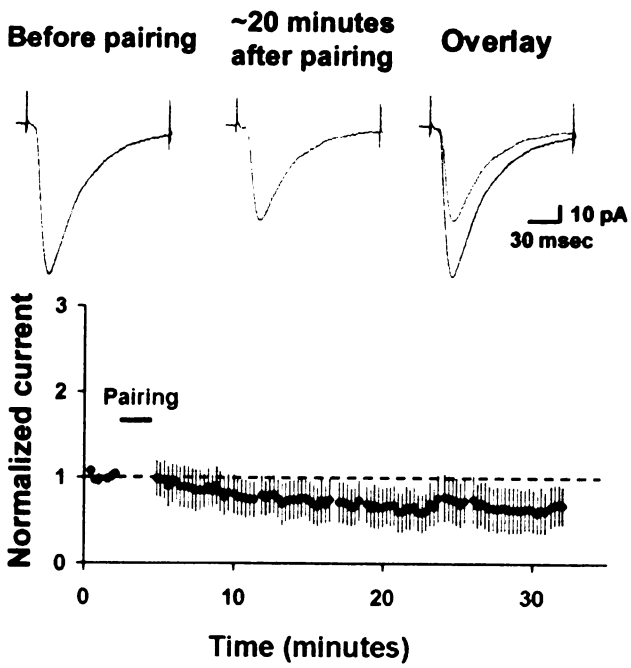
Before pairing
~20 minutes after pairing
Overlay
10 pA
30 msec
Normalized current
3
2
1
0
Pairing
0
10
20
30
Time (minutes)

Fig 27.

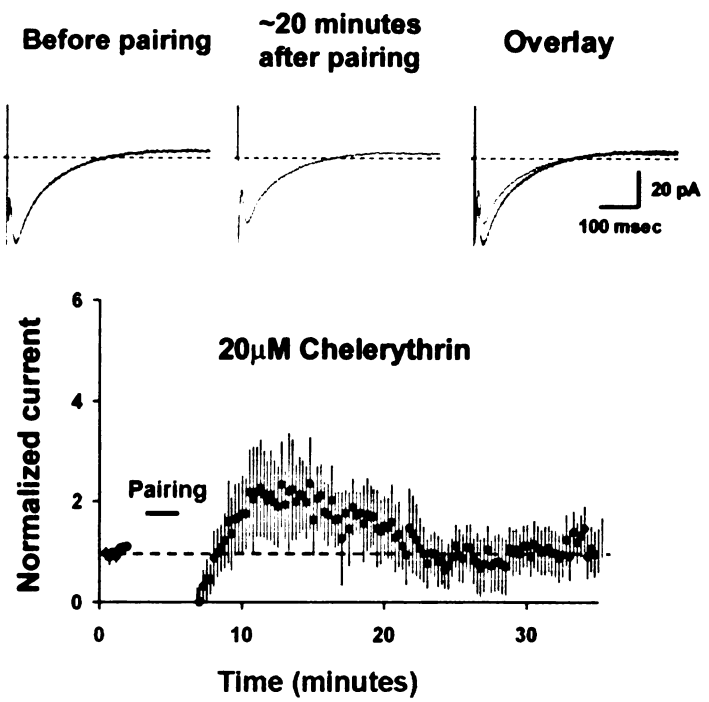
Before pairing
~20 minutes after pairing
Overlay
20 pA
100 msec
20μM Chelerythrin
Normalized current
Pairing
0
2
4
6
0
10
20
30
Time (minutes)

Fig 28.

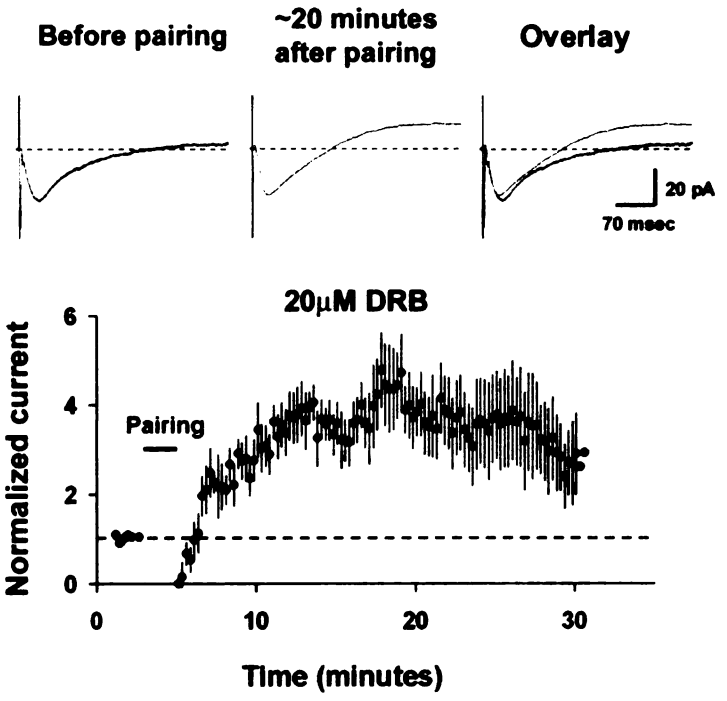
Before pairing
~20 minutes after pairing
Overlay
20 pA
70 msec
20μM DRB
Normalized current
Pairing
0
2
4
6
0
10
20
30
Time (minutes)

Fig 29.

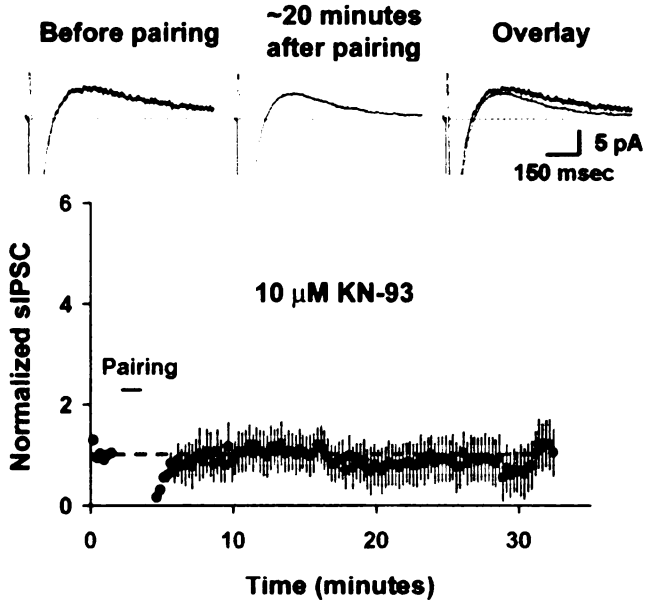
Before pairing
~20 minutes after pairing
Overlay
5 pA
150 msec
Normalized sIPSC
6
4
2
0
10 μM KN-93
Pairing
0
10
20
30
Time (minutes)

Fig 30.

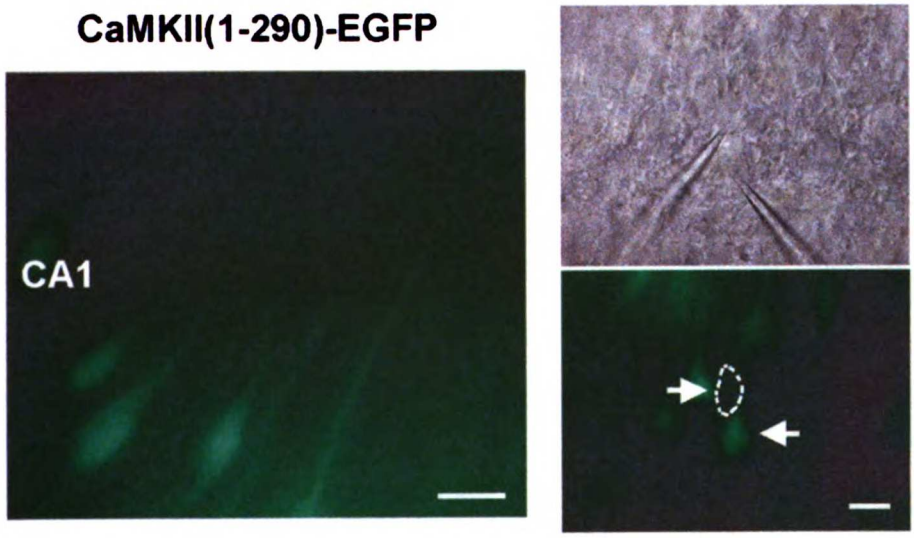

Fig 31.

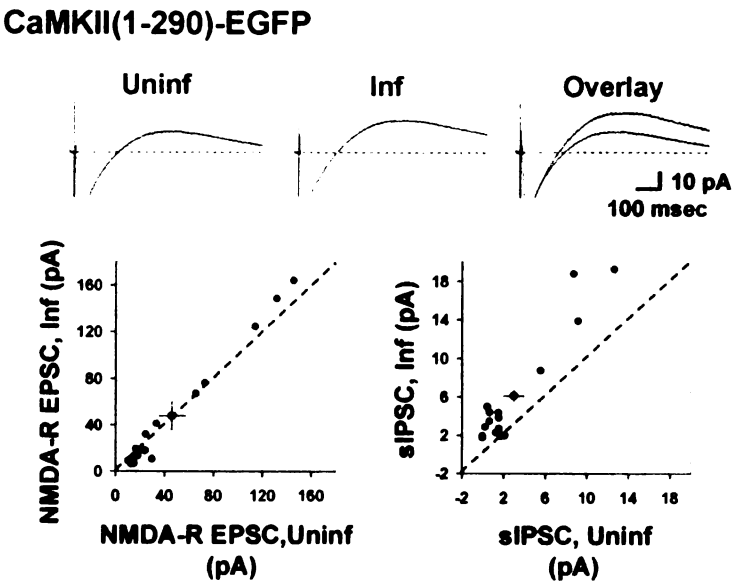
CaMKII(1-290)-EGFP
Uninf
Inf
Overlay
10 pA
100 msec
NMDA-R EPSC, Inf (pA)
NMDA-R EPSC,Uninf (pA)
sIPSC, Inf (pA)
sIPSC, Uninf (pA)

Fig 32.

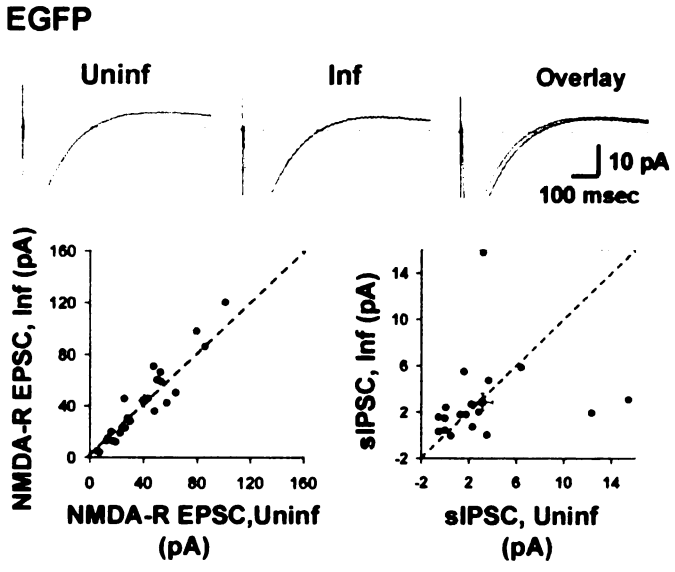
EGFP
Uninf
Inf
Overlay
10 pA
100 msec
NMDA-R EPSC, Inf (pA)
NMDA-R EPSC,Uninf (pA)
sIPSC, Inf (pA)
sIPSC, Uninf (pA)

Fig 33.

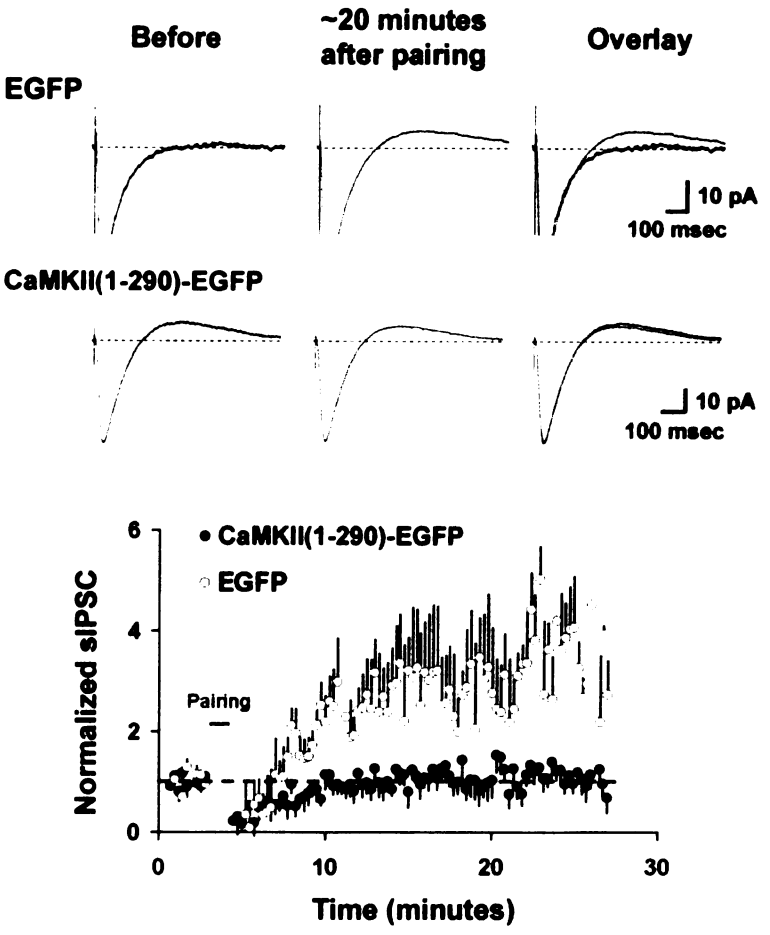
Before
~20 minutes after pairing
Overlay
EGFP
10 pA
100 msec
CaMKII(1-290)-EGFP
10 pA
100 msec
• CaMKII(1-290)-EGFP
○ EGFP
Pairing
Normalized sIPSC
0
2
4
6
0
10
20
30
Time (minutes)

Fig 34

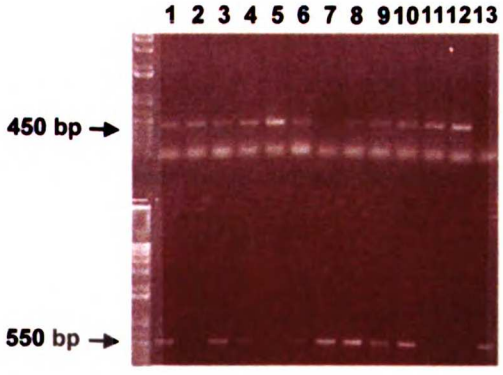

Fig 35.

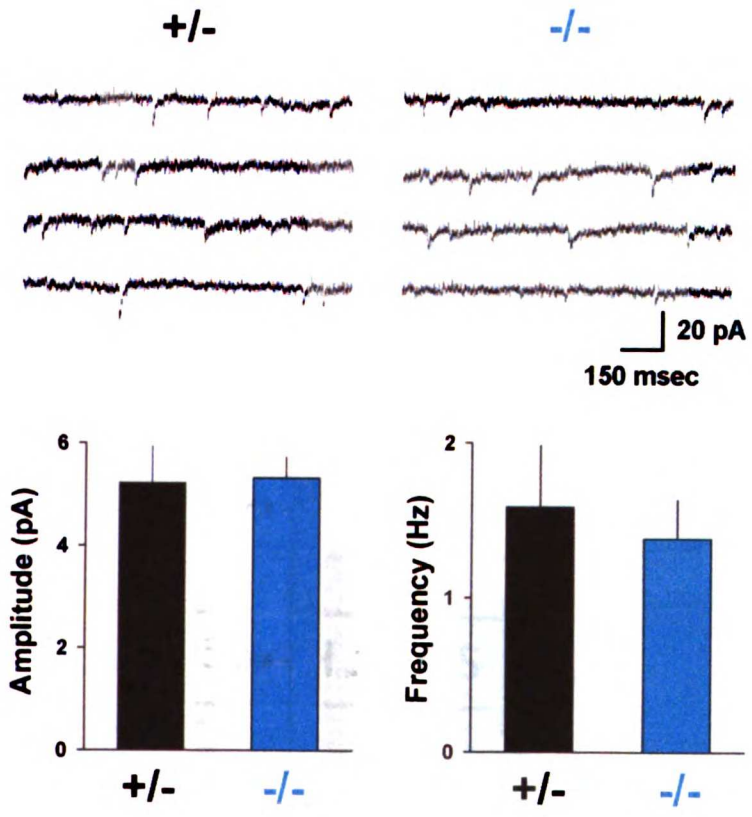

Fig 36.

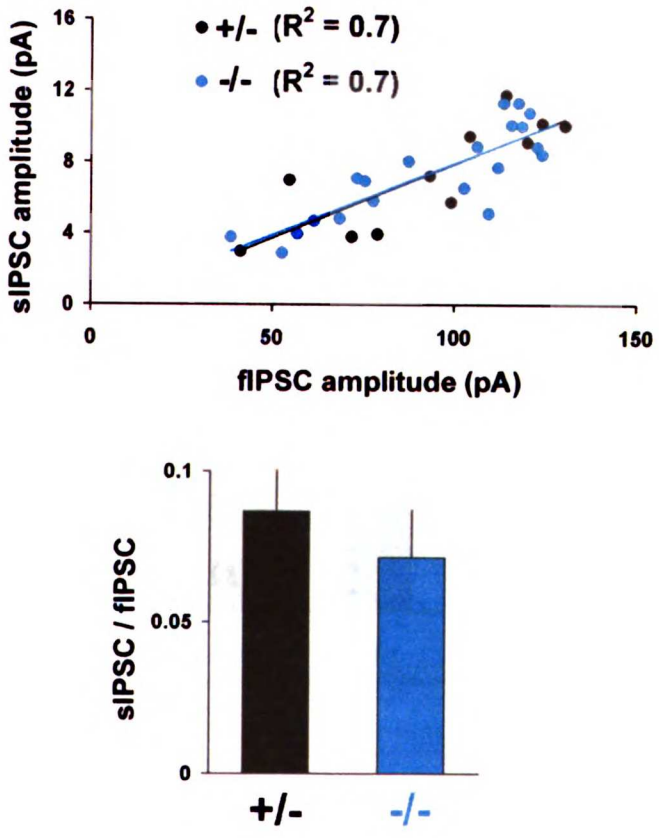
+/- (R^2 = 0.7)
-/- (R^2 = 0.7)
sIPSC amplitude (pA)
16
12
8
4
0
0
50
100
150
fIPSC amplitude (pA)
sIPSC / fIPSC
0.1
0.05
0
+/-
-/-

Fig 37.

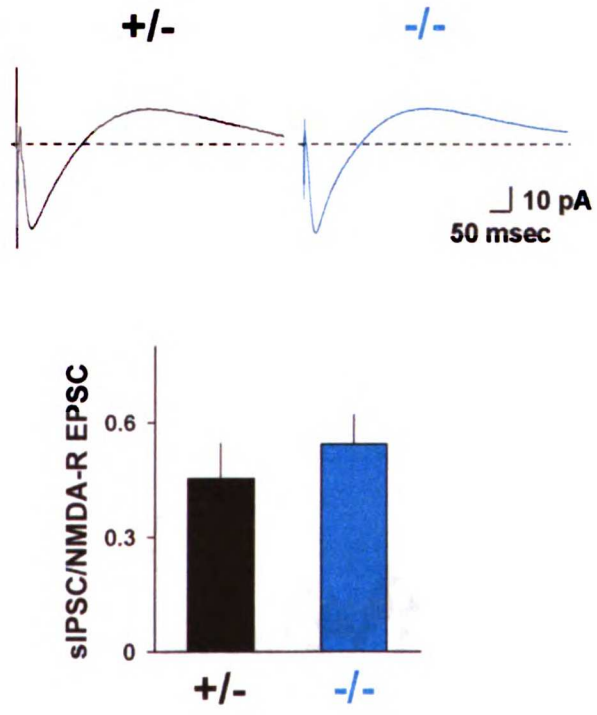

Fig 38.

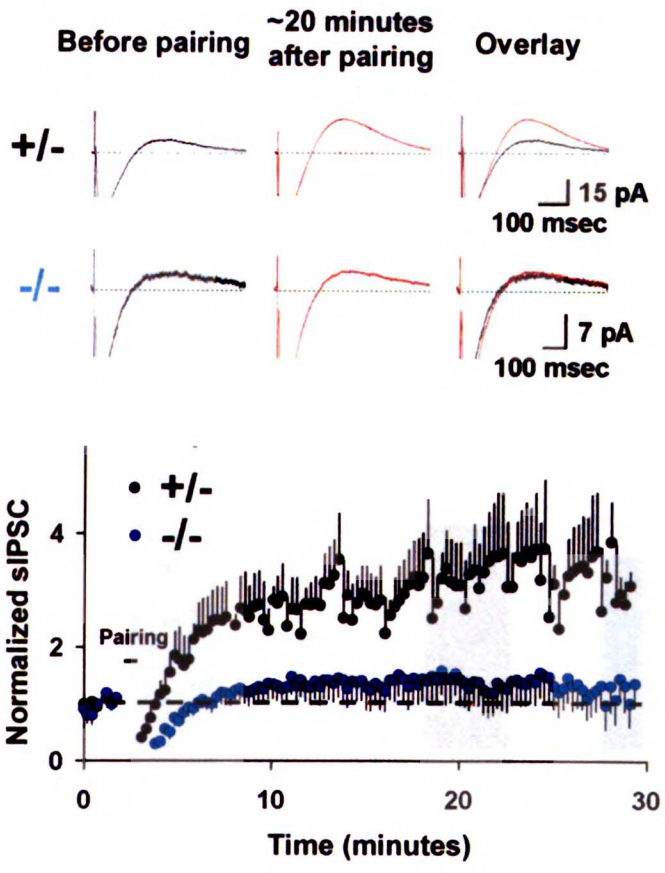
Before pairing
~20 minutes after pairing
Overlay
+/-
15 pA
100 msec
-/-
7 pA
100 msec
+/-
-/-
Pairing
Normalized sIPSC
0
2
4
0
10
20
30
Time (minutes)

Fig 39.

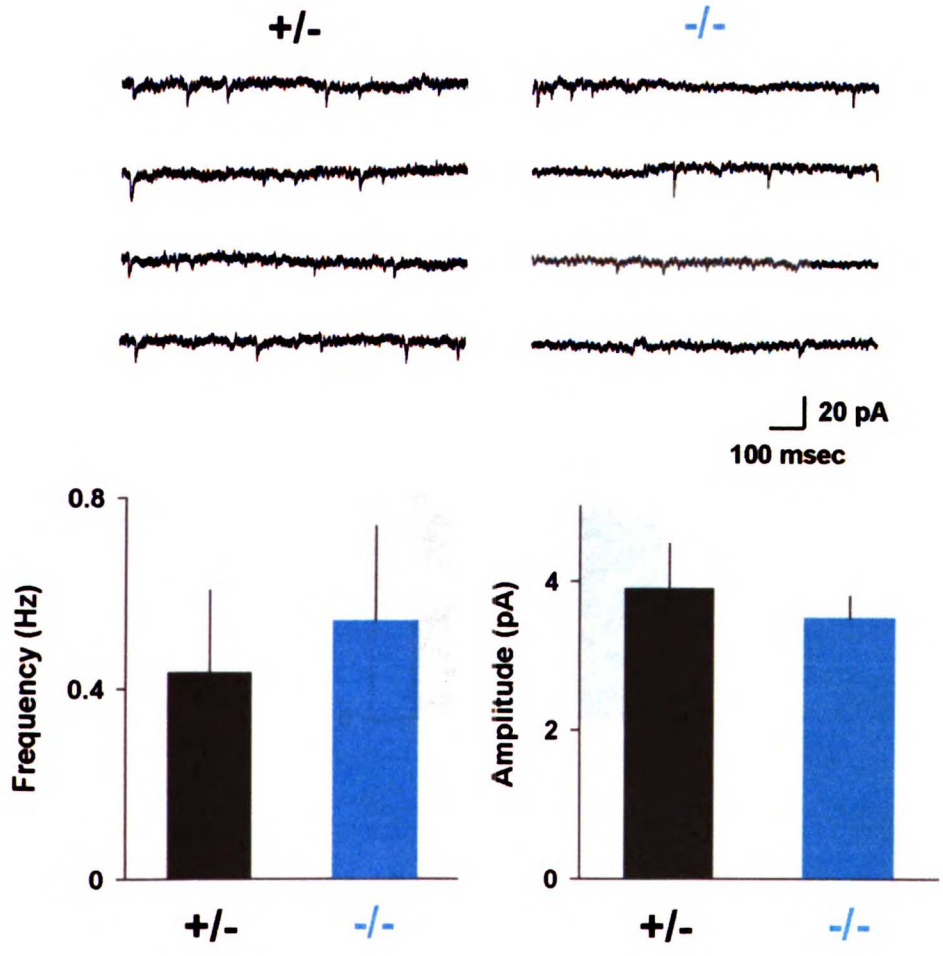

Fig 40.

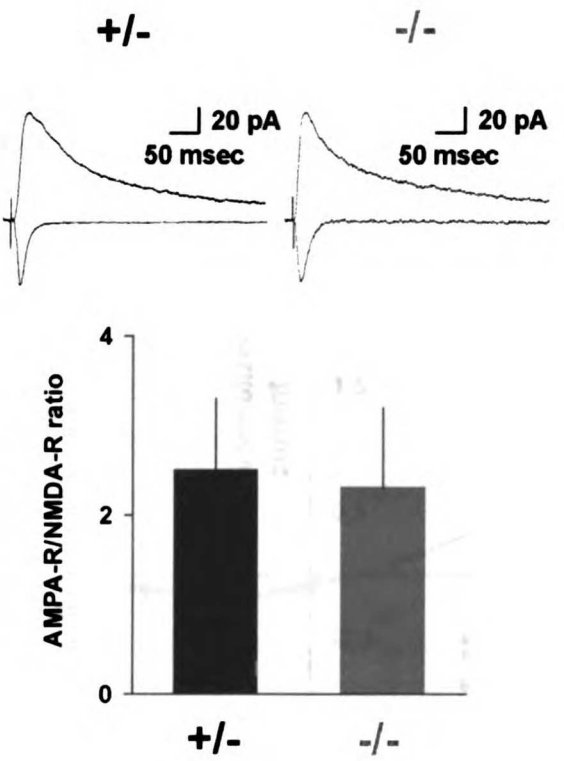

Fig 41.

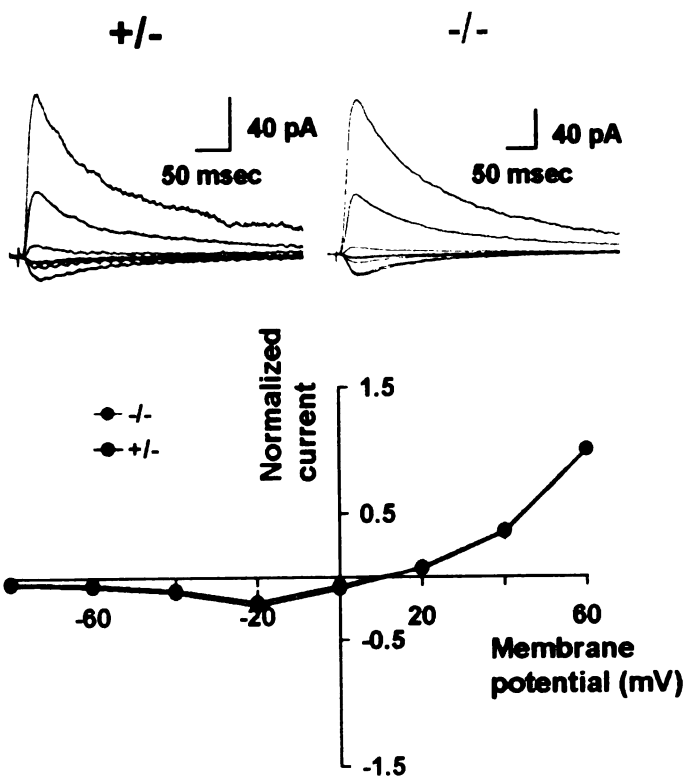
+/-
-/-
40 pA
50 msec
40 pA
50 msec
-/-
+/-
Normalized current
1.5
0.5
-0.5
-1.5
-60
-20
20
60
Membrane potential (mV)

Fig 42.

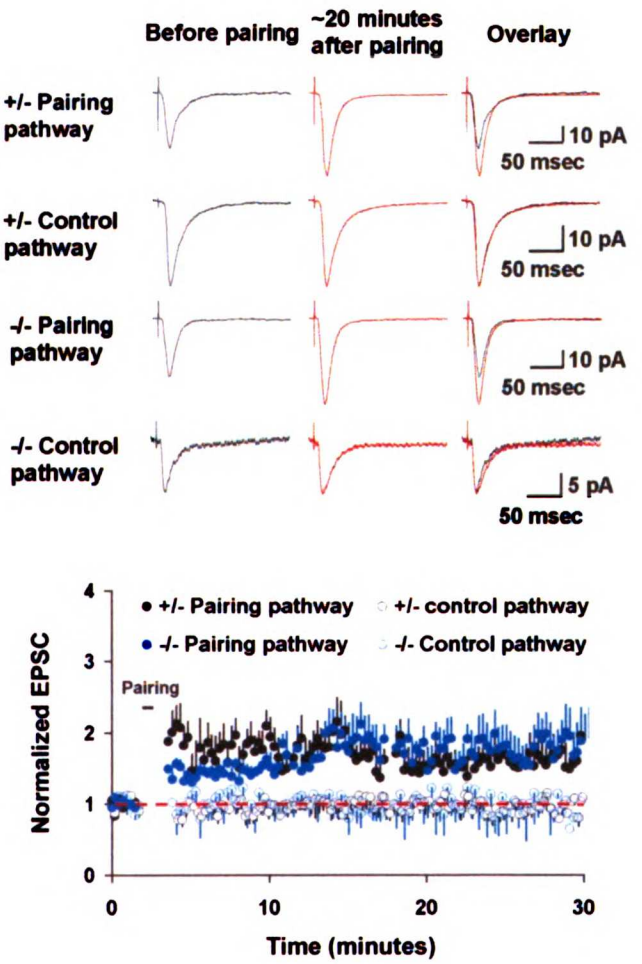
Before pairing
~20 minutes after pairing
Overlay
+/- Pairing pathway
10 pA
50 msec
+/- Control pathway
10 pA
50 msec
-/- Pairing pathway
10 pA
50 msec
-/- Control pathway
5 pA
50 msec
+/- Pairing pathway
+/- control pathway
-/- Pairing pathway
-/- Control pathway
Pairing
Normalized EPSC
0
1
2
3
4
0
10
20
30
Time (minutes)

Fig 43.

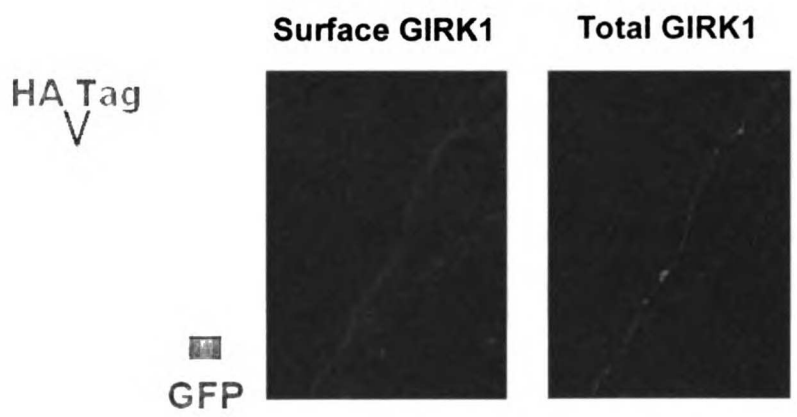

Fig 44

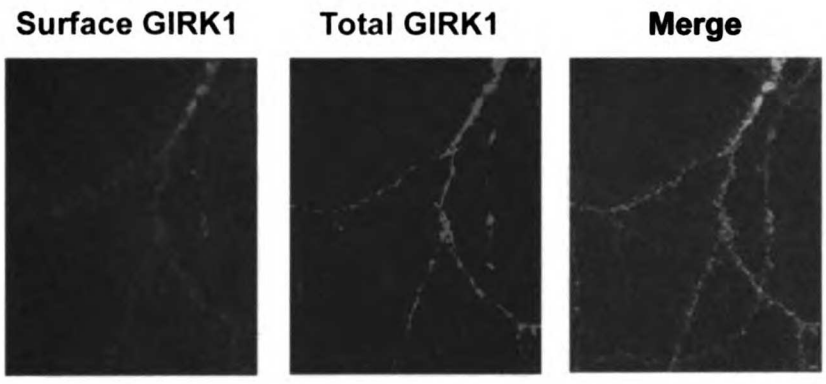

Fig 45.

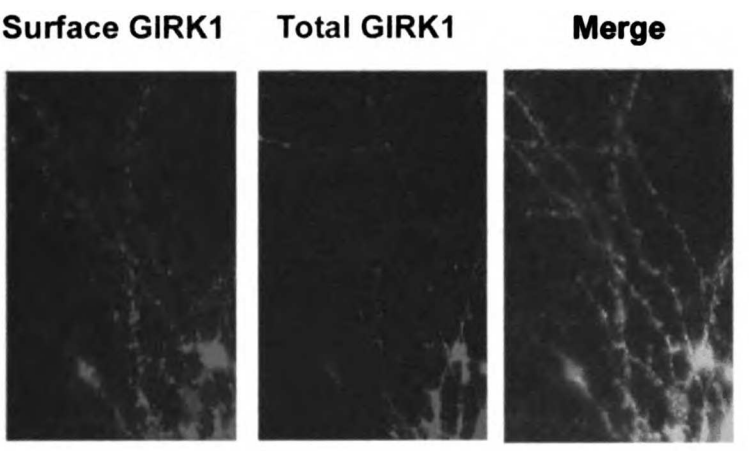

Fig 46.

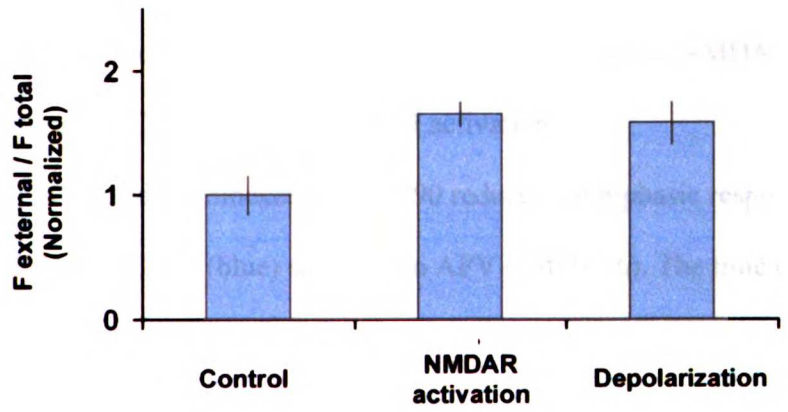
F external / F total
(Normalized)
2
1
0
Control
NMDAR
activation
Depolarization

Fig 47.

Figure Legends

Figure 1. Prominent GIRK2 immunofluorescence (red) in spines of EGFP-expressing neurons (green). Scale bar: 10 μm.

Figure 2. Pathway in the spine for inducing LTP of the EPSC: Glutamate concurrent with depolarization relieving the Mg^{2+} block causes optimal NMDA receptor activation, Ca^{2+} entry and CaMKII activation.

Figure 3. GIRK channel blocker SCH23390 reduces the biphasic response (black) to the NMDA-R EPSC (blue) sensitive to APV (red) (n=6). The time course of sIPSC reduction is also shown.

Figure 4. Another GIRK channel blocker, Tertiapin, also eliminates the sIPSC (n=4).

Figure 5. The sIPSC is sensitive to the $GABA_B$ receptor antagonist SCH50911 (n=7). The overlay of the last two panels are shown at a different scale to highlight the sIPSC.

Figure 6. There was relatively little correlation between the size of the NMDA-R EPSC and the amplitude of the sIPSC (linear correlation coefficient R^2= 0.5)

Figure 7. The amplitude of the sIPSC was reduced to 25.7 ± 4.3% ($p<0.01$) by blocking NMDA receptors mediating EPSC.

Figure 8. Hippocampal slice culture with fairly stable baseline of sIPSC (filled circles) and NMDAR-EPSC (open circles) sampled at 0.33 Hz. Example traces (average of ~40-60 episodes) shown above plots of peak amplitudes (mean ± standard error) in this and subsequent figures.

Figure 9. Pairing 3 Hz stimulation with depolarization (-5 to 0 mV) for ~2.5 min potentiated the sIPSC (filled circle, 415 ± 40%, n=12, $p<0.001$) but not NMDA-R EPSC (open circle, 136 ± 50%, n=12, p=0.2) in slice culture. The series resistance R_s (tope) and input resistance R_{in} (bottom) remained constant after pairing.

Figure 10. The NMDA-R EPSC decay time constant (97 ± 17 msec, n=7), measured in the presence of SCH50911, NBQX and picrotoxin, was not altered after pairing (90 ± 10 msec, n=10, p=0.7) (bottom). Sample traces (black) are compared with idealized traces generated with the average decay time constant (red) (top).

Figure 11. Pairing induced LTP of the sIPSC as assessed by integrating the sIPSC amplitude over time (normalized area: 2.9 ± 0.5, n=12, $p<0.05$).

Figure 12. There is no correlation between the amplitude and the extent of potentiation of the sIPSC (linear correlation index $R^2=0.4$)

Figure 13. There is also no correlation between sIPSC potentiation and the NMDA-R EPSC amplitude ($R^2=0.1$).

Figure 14. Acute hippocampal slice from p15-20 animals exhibited gradual depression of sIPSC (25.1 ± 9.3% at ~20 mins, n=10, $p<0.01$).

Figure 15. No sIPSC potentiation was observed in acute slices from p15-20 animals (80.2 ± 11.2% at 20-25 mins after pairing, n=19, p=0.08).

Figure 16. Acute hippocampal slice from p25-30 animals exhibited slight rundown of the sIPSC (80 ± 27%, n=13, p=0.02).

Figure 17. Pairing-induced potentiation of the sIPSC (142 ± 13% at 20-25 min after pairing, n = 14, $p<0.01$).

Figure 18. The potentiated sIPSC is sensitive to the $GABA_B$ receptor antagonist SCH50911.

Figure 19. The potentiated sIPSC is also sensitive to the GIRK channel blocker SCH23390.

Figure 20. No pathway specific of sIPSC potentiation was observed (pairing pathway: 320 ± 50%, 20-25 mins after pairing, n=7; control pathway: 290 ± 100% 20-25 mins after pairing, n=8, p=0.87).

Figure 21. Treatment of slice culture with APV as well as NBQX reduced both EPSC and sIPSC amplitudes and eliminated LTP of the sIPSC (102 ± 20%, n=7, p=0.1).

Figure 22. 3 Hz stimulation without concurrent postsynaptic depolarization failed to induce sustained potentiation of the sIPSC (123 ± 26%, n=6, p=0.1).

Figure 23. Treatment with the NMDA receptor antagonist APV but not the AMPA receptor antagonist NBQX eliminated LTP of the sIPSC (90 ± 12%, n=6, p=0.3) while preserving its size.

Figure 24. Chelating postsynaptic Ca^{2+} with 10 mM BAPTA in the pipette solution abolished LTP of the sIPSC (87 ± 19%, n=6, p=0.6).

Figure 25. Pairing-induced sIPSC potentiation persisted in the presence of the CB1 receptor antagonist AM-251 (370 ± 33%, n=5, p<0.001).

Figure 26. Pairing did not alter the paired pulse ratio of the fIPSC (0.74 ± 0.04 before pairing versus 0.74 ± 0.05 at 10 min after pairing, p=0.99; and 0.7 ± 0.04 at 20 min after pairing, p=0.5, n=5) (bottom). Because the pairing protocol caused depression of the fIPSC as previously reported, the overlay compares the time course of traces before pairing with normalized traces after pairing (top).

Figure 27. Slightly depression of fIPSC was observed after pairing (68.5 ± 20.2, n=5, p=0.15)

Figure 28. No persistent potentiation was found with PKC inhibitor (106 ± 24% at 20-25 mins after pairing, n=9, p=0.5).

Figure 29. sIPSC potentiation was still observed with casein kinase II inhibitor (373 ± 24% at 20-25 mins after pairing, n=9, p<0.005)

Figure 30. The CaMKII inhibitor KN-93 prevented pairing-induced sIPSC potentiation (90 ± 15%, n=11, p=0.6).

Figure 31. Images of CA1 neurons expressing CaMKII(1-290)-EGFP (left) and pair-wise recording from one infected neuron and a nearby uninfected neuron (dotted outline) seen with transmitted light (upper right) and fluorescence microscopy (lower right). Scale bars: 25 μm.

Figure 32. Expression of constitutively active CaMKII(1-290)-EGFP potentiated sIPSC (right, 6.1 ± 0.5 pA for inf, 3.0 ± 1 pA for uninf; n=16, p<0.0005) but not NMDA-R EPSC (left, 46.0 ± 10.7 pA for inf, 47.5 ± 12.5 pA for uninf; n=17, p=0.4).

Figure 33. Pair-wise comparison between control infected (inf) neurons expressing EGFP and nearby uninfected (uninf) neurons revealed no difference in sIPSC (right, 2.9 ± 0.7 pA for inf, 3.1 ± 0.9 pA for uninf; n=18, p-0.8) or NMDAR-EPSC (left, 40.9 ± 6.0 pA for inf, 38.8 ± 5.1 pA for uninf; n=24, p=0.4).

Figure 34. No further potentiation of the sIPSC in neurons expressing constitutively active CaMKII(1-290)-EGFP (113 ± 54%, n=7, p=0.1), in contrast to control neurons expressing EGFP (287 ± 43%, n=4, p<0.01).

Figure 35. PCR with 2 pairs of specific primers determined the genotype of litters, wild type locus gave the band around 450bp (upper), targeted locus gave the band around 550 bp (lower). Therefore, No. 2, 4, 11 and 12 were wildtype, No. 7 and 13 were knockout, the rest were heterozygous.

Figure 36. The miniature fIPSC of Nova-2 null mice has normal amplitude (5.3 ± 0.4 pA in mutant mice versus 5.2 ± 0.7 in control, p=0.8) and frequency (1.4 ± 0.3 Hz in mutant mice versus 1.6 ± 0.4 Hz in control, p=0.4).

Figure 37. The ratio of sIPSC and fIPSC in Nova-2 null mice (0.07 ± 0.02, n=8) is similar to that in control mice (0.09 ± 0.02, n=8, p=0.2).

Figure 38. The ratio of sIPSC and NMDA-R EPSC in Nova-2 null mutant (0.5 ± 0.1n=15) is comparable to that in control mice (0.4 ± 0.1, n=15, p=0.4).

Figure 39. Nova-2 null mice exhibited no pairing-induced sIPSC potentiation (130 ± 38%, n=8, p=0.5), unlike their heterozygous siblings (338 ± 39%, n=6, p<0.01).

Figure 40. The miniature EPSC of Nova-2 null mice has normal amplitude (3.5 ± 0.3 pA in mutant mice versus 3.9 ± 0.6 in control, n=7, p=0.5) and frequency (0.5 ± 0.2 Hz in mutant mice versus 0.4 ± 0.2 Hz in control, n=7, p=0.7)

Figure 41. The ratio of AMPA-R EPSC and NMDA-R EPSC appears normal in Nova-2 null mice (2.3 ± 0.9 for Nova-2 $^{-/-}$ versus 2.5 ± 0.8 for Nova-2 $^{+/-}$ n=7; n=8, p=0.9).

Figure 42. The voltage-dependence of the NMDA-R EPSC is comparable in Nova-2 null mutants and controls.

Figure 43. Similar pairing-induced LTP of the EPSC in Nova-2 null mice (177 ± 14%, n=9, p<0.01 for the pairing pathway; 99±13%, n=4, p=0.9 for the control

pathway) and their heterozygous siblings (171 ± 14%, n=10, $p<0.01$ for the pairing pathway; 99 ± 8%, n=5, p=0.8 for the control pathway).

Figure 44. Cultured hippocamal neuron (DIV3-4 weeks) transfected with GIRK1-ha-EGPF and GIRK2 by gene gun. Without any treatment, very few of GIRK protein was on the cell surface (red) relative to total protein (green).

Figure 45. With same transfection as figure 44, same cultured neurons were treated with NBQX for 3-4 weeks than APV for 2 hours. This protocol significantly increased surface expression of GIRK1 (red) relative to total protein (green).

Figure 46. With same transfection as figure 44, same culture neurons were treated with 25mM of KCl for 30 minutes which also increased surface GIRK expression (red) relative to total protein (green).

Figure 47. Quantitation analysis showed increase of surface GIRK over total protein in NMDA activation and KCl treatment (control: 100 ± 15.7%, n=5; NMDA activation: 165 ±10%, n= 7, $p<0.05$; KCl depolarization: 158 ± 17%, n=6, p=0.09).

Discussion and Future directions

For neurons to communicate with one another, ionotropic transmitter receptors generate fast synaptic responses whereas metabotropic transmitter receptors generate slow synaptic potentials that are long lasting partly due to the mobilization of G proteins and downstream second messengers and effectors (Hille, 2001). Moreover, the ability of metabotropic receptors to respond to transmitter released at a distance not only further prolongs the slow synaptic potentials, it also provides one venue for neurons to integrate and process synaptic inputs. In this study, we have explored the physiological implication of the unexpected placement of both $GABA_B$ receptors and GIRK channels in the dendritic spines (Drake et al., 1997; Kulik et al., 2003).

Our study has uncovered a new form of synaptic plasticity, LTP of the sIPSC mediated by $GABA_B$ receptors and GIRK channels (Figure 2), due to NMDA receptor activation via concurrent glutamate release and depolarization of the postsynaptic CA1 neuron (Figure 21-23). Like LTP of the EPSC (Malenka et al., 1989; Malenka et al., 1988; Malinow et al., 1989), LTP of the sIPSC requires elevation of postsynaptic Ca^{2+} level (Figure 24) and activation of postsynaptic CaMKII (Figure 30). Moreover, expression of constitutively active CaMKII in CA1 neurons was sufficient to potentiate the sIPSC (Figure 33), and to occlude the pairing-induced LTP of the sIPSC (Figure 34). This novel form of synaptic plasticity of the slow synaptic inhibition is specifically affected in the Nova-2 null mice (Figure 36 - 43), raising the intriguing possibility that one of the

physiological functions of the Nova-2 RNA-binding protein is to enable central neurons to adjust their slow synaptic inhibition based on neuronal activity.

What might be the physiological significance of potentiating slow synaptic inhibition via the pairing protocol for coincidence detection?

Potentiation of the sIPSC could narrow the time window for the coincidence detection of excitatory synaptic inputs; late arriving excitatory inputs would be at a disadvantage imposed by both hyperpolarization and increased membrane conductance due to the potentiated slow inhibition. Importantly, in small structures like the dendritic spines, slow synaptic inhibition mediated by K^+ channel activation via $GABA_B$ receptors is likely to be more effective in dampening the excitatory inputs—in principle—than the fast synaptic inhibition due to Cl^- current through $GABA_A$ receptors, because the Cl^- concentration rise in small structures is predicted to significantly decrease the driving force for Cl^- ions (Qian and Sejnowski, 1990). Thus, the localization of both $GABA_B$ receptors and GIRK channels in dendritic spines (Figure 1) (Drake et al., 1997; Kulik et al., 2003) not only enables the same signaling pathway for synaptic plasticity of excitatory synaptic potentials to induce long-lasting changes of slow synaptic inhibition, potentiation of slow inhibitory synaptic potentials due to GIRK channel activity also represents one highly effective way of harnessing excitatory synaptic inputs.

In addition to reducing excitation at dendritic spines, slow synaptic inhibition may also induce failures of action potential propagation along axons, particularly at axonal branch points (Debanne, 2004; Debanne et al., 1997; Kopysova and Debanne, 1998). Thus,

potentiation of the sIPSC could sharpen the coincidence detection of synchronous excitatory synaptic inputs, a hallmark for learning and memory, in a variety of ways—by reducing the impact of late arriving excitatory inputs within the spines that receive the synaptic excitation, and by decreasing the likelihood that excitation by late arriving synaptic inputs will be productive in causing transmitter release from the nerve terminals.

Long-term potentiation of slow synaptic inhibition may also modulate rhythmic activities such as the theta oscillation (4-7 Hz) important for learning and memory (Hyman et al., 2003; O'Keefe, 1993; Sederberg et al., 2003; Seidenbecher et al., 2003). Interneurons are remarkably effective in synchronizing this oscillatory firing pattern of hippocampal pyramidal neurons (Cobb et al., 1995). It is of interest to note that blockade of $GABA_B$ receptors increases the frequency of oscillation, which can be reversed by the GABA uptake blocker (Scanziani, 2000). If theta modulation indeed depends on postsynaptic $GABA_B$ receptors, LTP of the sIPSC is likely to slow theta oscillation hence shift its phase, a crucial temporal parameter in filtering out specific synaptic activities for potentiation or depression (Hyman et al., 2003).

How might Nova-2 function to enable synaptic plasticity of slow inhibition?

Nova proteins bind with high affinity to known sequence motifs in the RNA to regulate alternative splicing (Dredge and Darnell, 2003; Jensen et al., 2000; Musunuru and Darnell, 2001; Ule et al., 2003; Ule et al., 2005). As paraneoplastic neurologic disease antigens, these RNA-binding proteins are probably responsible for the POMA patients' reduced inhibitory control of movements and dementia (Albert and Darnell, 2004;

Buckanovich et al., 1993; Hormigo et al., 1994; Pranzatelli, 1992; Yang et al., 1998). Interestingly, a significant fraction of the RNAs that bind Nova-2 code for proteins that mediate synaptic inhibition, including $GABA_B$ receptors and GIRK channels (Ule et al., 2003). The ability of hippocampal neurons from Nova-2 null mice to exhibit LTP of the EPSC (Figure 43) but not the sIPSC (Figure 39) strongly suggests that Nova-2 exerts its functions specifically on the machinery for slow synaptic inhibition and endows it with the capacity to respond to NMDA receptor activation with long lasting changes.

How might Nova-2 contribute to synaptic plasticity of slow inhibition? Multiple variants for both $GABA_B$ receptors and GIRK channels exist due to alternative splicing (Isomoto et al., 1998; Martin et al., 2001; Pfaff et al., 1999; Wei et al., 1998) or alternative promoter usage (Steiger et al., 2004), to diversify their function and their traffic pattern (Charles et al., 2001; Ma et al., 2002; Martin et al., 2004). As an RNA-binding protein known to regulate splicing of molecules important for synaptic transmission and plasticity (Ule et al., 2005), Nova-2 may function in CA1 neurons to dictate appropriate representations of the splice variants of $GABA_B$ receptors, GIRK channels or other associated molecules (Couve et al., 2001; Couve et al., 2004; Nehring et al., 2000; Vernon et al., 2001; White et al., 2000), perhaps to ensure that these molecules in dendritic spines and other synaptic sites are suitably equipped to respond to signaling downstream of the coincidence detector, the NMDA receptor. Especially, RNA of numbers of important molecules which are required for potentiation of slow synaptic inhibition are also targets of Nova-2 protein, such as NMDA receptors and αCaMKII (Ule et al., 2005). While altered splicing patterns are evident from analyses of different

CNS regions of Nova-1 and Nova-2 null mice (Dredge and Darnell, 2003; Jensen et al., 2000; Ule et al., 2005), further studies will be necessary to uncover cell type-specific functions of Nova proteins. In this regard, it is worth noting that regulation of splicing of various K^+ channels and Ca^{2+} channels in individual neurons, or specific subsets of neurons, plays physiologically important roles in fine tuning excitability (Baranauskas et al., 2003; Bell et al., 2004; Fettiplace and Fuchs, 1999).

Whereas Nova proteins are known to regulate alternative splicing, given the intriguing coupling between RNA splicing and cytoplasmic RNA targeting and regulation (Gu et al., 2002; Hachet and Ephrussi, 2001; Hachet and Ephrussi, 2004; Kataoka et al., 2000; Le Hir et al., 2000; Palacios, 2002), a physiological role of Nova-2 outside the nucleus—in neuronal soma or processes—remains an interesting possibility for future studies. Unpublished data from Darnell's group demonstrated by immnocytochemistry EM the cytoplasmic localization of Nova-2, close to inhibitory synapses. Additionally, $GABA_{B1}$ mRNA is enriched in hippocampus synpatosome preparations compared to whole hippocampus and so as $GABA_{B2}$ and GIRK2 mRNAs (Personal communication, Raab-Graham). Recent studies have shown that synaptic activity can trigger the transport of new mRNA transcripts to synaptic sites and modulate the translation of mRNA already in place (Ju et al., 2004; Ouyang et al., 1997; Smith et al., 2005). Therefore, it is conceivable that Nova-2 protein affects the plasticity of slow synaptic inhibition by interacting with target mature RNAs (such as GIRK, $GABA_B$ receptors or other molecules) outside of nucleus as well, and this RNA-protein interaction could be regulated by neuronal activity. To test whether the local protein synthesis is required for

potentiation of slow inhibition, protein translation inhibitors such as cycloheximide, anisomycin, or rapamycin, could be applied during slice recording first. Other tests may involve overexpression of GIRK or $GABA_B$ receptors fused with tetracysteine motif (EAAAREACCRECCARA), and application of FlAsH-EDT_2 and ReAsH-EDT_2 before and after inducing synaptic activity in dissociated neuronal cultures (Ju et al., 2004). These two fluorescent dyes, when applying sequentially, would help to determine preexisting and newly synthesis protein (Gaietta et al., 2002; Zhang et al., 2002). To test for the involvement of such activity-induced local protein synthesis, similar experiments could also be carried out using Nova-2 null.

What is the mechanism underlying slow synaptic inhibition downstream of CaMKII activation?

To determine the expression level of receptor/channel on the membrane, immunostaining without permeabilization or biochemistry by directing labeling surface protein in dissociated neurons is more straightforward compare to electrophysiology. However, destruction of neuronal networks in dissociated neuronal cultures makes it difficult to induce plasticity by activating a specific group of synapses. Different protocols of chemical induced LTP were developed by many labs to change neuronal activity in entire networks for a timescale of days or even weeks (Liao et al., 1999; Lu et al., 2001). The mechanisms underlying this homeostatic plasticity may not be exactly the same as Hebbian modification, which only happens in selective pre- and postsynaptic sites with no overall modification in total synaptic weight over a timescale of hours (Burrone et al., 2002; Royer and Pare, 2003). Therefore, it needs to be scrutinized by additional

experiments to further test whether increase of GIRK trafficking to the membrane is the mechanism underlying plasticity of slow inhibition, whereas activity depedent GIRK trafficking was observed in dissociated cultured system. Eletrophysiological tags with different electrophysiological property (such as rectification) have been used in some studies to discriminate these tagged proteins from endogenous protein (Shi et al., 2001). Therefore, an eletrophysiological tag altering rectification, but without changing any other channel properties, might be a plausible way to pursue this question. Mutation of the negative charged E224 and E299 in Kir2.1 reduces inward rectification due to loss of docking sites for cations (Stanfield et al., 2002; Taglilatela et al., 1995; Yang et al., 1995). However, mutation of both or either one of two sites on GIRK1 and/or GIRK2, which are equivalent to E224 and E299 on Kir2.1, had no effect on GIRK channel rectification (Data not shown). Overexpression of both GIRK1 and GIRK2 or only GIRK2 in GIRK2 null background will allow further test whether GIRK channel trafficking is the mechanism of plasticity of slow synaptic inhibition. Moreover, expression of truncated or other mutation forms of the channel would be useful to identify the motif important to this activity dependent trafficking. However, alteration of $GABA_B$ receptor function or trafficking cannot be excluded. Similar methods could be applied to determine the role of $GABA_B$ receptors in the plasticity of slow inhibition.

Why would the pontentiation of sIPSC only happen in st. lacunosum-moleculare?

Due to the high expression of GIRK1 and $GABA_B$ receptors on the dendritic spines in st. lacunosum-moleculare (Kulik et al., 2003; Drake et al., 1997), stimulation electrodes were placed on this layer in both slice culture and acute slice experiments. Interneurons

in the hippocampus are very diverse and almost impossible to classify them into functional or anatomical sub-populations (Parra et al., 1998; Jonas et al., 2004; McBain and Fisahn, 2001). However, perforant pathway-associated interneurons, which stratify in st. lacunosum-moleculare synapse onto the dendritic spines and shafts of CA1 neurons(Vida et al., 1998). Therefore, it is likely that potentiation of sIPSC can only be triggered with specific groups of interneurons which make synaptic contact onto the spines where GIRK1 and $GABA_B$ receptors and glutamatergic synapses colocolize.

In addition, different subtypes of NMDA receptors may be activated in the schaffer collateral and perforant pathway inputs (Arrigoni and Greene, 2004). A greater NR2B subunit contributes the NMDA component of the schaffer collateral EPSC, compared to the NMDA component of the perporant pathway EPSC. Interestingly, the NR2B subunit appears to be important to the induction of LTD but not LTP, whereas NR2A subunit is critical for the induction of LTP but not LTD (Liu et al., 2004). The different distribution of NMDA subunits in these two pathways could further affect the induction of sIPSC potentiation. This hypothesis can be tested by inducing plasticity of sIPSC with different NMDAR subunit specific antagonists (Liu et al., 2004).

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