LONG-TERM SYNAPTIC PLASTICITY IN MOUSE CEREBELLAR STELLATE CELLS

A Dissertation in Biology by Lu Sun

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The dissertation of Lu Sun was reviewed and approved* by the following:

Si-Qiong Liu  
Assistant Professor of Biology  
Dissertation Adviser  
Chair of Committee

Bernhard Luscher  
Professor of Biology, Biochemistry & Molecular Biology, and Psychiatry

Gong Chen  
Associate Professor of Biology

Matthew Whim  
Assistant Professor of Biology

Steven Schiff  
Professor of Neurosurgery, Engineering Science & Mechanics, Physics

Douglas Cavener  
Professor of Biology  
Head of Department of Biology

*Signatures are on file in the Graduate School.
Abstract

The cerebellum is a brain structure essential for motor control and coordination, as well as motor learning and memory. The highly organized anatomy of the cerebellum makes it a good model for the study of network function. As the only output of the cerebellar cortex, Purkinje cells are considered as the cellular basis for certain types of motor learning. Purkinje cells receive excitatory synaptic inputs from parallel fibers and climbing fibers, and inhibitory inputs from GABAergic interneurons located at the molecular layer of the cerebellum. Since the activity of Purkinje cells is largely regulated by the synaptic integration, knowledge about cerebellar granule cells and interneurons is necessary for the understanding of the mechanism of motor learning and memory.

Interneurons including stellate cells and basket cells obtain afferent excitatory inputs from parallel fibers and project inhibitory inputs onto Purkinje cells, and thus form a feed-forward inhibition network. The inhibition from the interneurons counteracts the excitatory effects from parallel fibers and prevents the Purkinje cells from being over excited. However, the synaptic plasticity of the interneurons remains elusive. Using stellate cell as a model, we investigated the function of glutamate receptors in the synaptic plasticity in interneurons and the consequent impact on the pattern of GABA release from interneuron axonal terminals, which directly determines the inhibition of Purkinje cells. We observed that the activation of extrasynaptic NMDA receptors could induce a new form of synaptic plasticity at the parallel fiber-to-stellate cell synapse, including a subtype switch of AMPA receptors from naturally GluR2-lacking (Ca^{2+}-
permeable) to GluR2-containing (Ca\(^{2+}\)-impermeable). This plasticity is probably postsynaptically induced and requires protein kinase C (PKC) and the activity of protein interacting with PRKCA 1 (PICK1). In addition, previous studies showed that the activation of NMDA receptors directly triggered a long-lasting potentiation of GABA release at axonal terminals. Our work about the characterization of NMDA receptors in stellate cells suggested the possible expression of NR2B and NR2D subunits. However, blockade of single subtype of NMDA receptors did not affect the basal level of GABA release.

Changes in synaptic transmission would alter the excitability of a cell and therefore affect the action potential firing pattern. We explored if action potential firing would in return regulate the synaptic efficacy. We found that blockade of spontaneous action potentials (sAPs) in stellate cells induced an increased expression of GluR2-containing AMPA receptors at the parallel fiber-to-stellate cell synapse. This effect might be transcription-independent, but requires intact protein synthesis machinery. Moreover, inhibition of calmodulin mimicked the effect of sAP blockade, indicating the sAP blockade-induced GluR2 expression may be mediated by a reduced calmodulin activity.

Our study revealed mechanisms underlying long-term plasticity of AMPAR subtype at the parallel fiber-to-stellate cell synapse, and the potential functional significance. Our findings would gain the insight into cerebellar interneuron functions and their contribution to motor learning and memory.
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Chapter 1

Introduction

The cerebellum is an essential brain structure involved in motor acquisition and refinement. Coordination of movement, especially at high speed, is impossible without intact cerebellar functions. The subtle control of movement should inevitably require learning and memory process. Cerebellar cortex is considered to have an intrinsic motor learning capability. Purkinje cells are the sole output of cerebellar cortex and exert an inhibitory action on the target cell in deep cerebellar nuclei (DCN) (Ito et al., 1964). Purkinje cells are a convergence point of two excitatory inputs: parallel fibers from cerebellar granule cells and climbing fibers from inferior olive (Eccles et al., 1966d, b). It is suggested that decreased inhibitory output from Purkinje cells to DCN neurons might underlie the cellular mechanisms of motor learning (Raymond and Lisberger, 2000).

Paired stimulation of climbing fibers and parallel fibers at a low frequency can induce a long-lasting suppression of synaptic strength at the parallel fiber-Purkinje cells (PF-PC) synapses, which is called cerebellar long-term depression (cerebellar LTD) (Ito et al., 1982; Wang and Linden, 2000). Cerebellar LTD would attenuate the excitatory input from parallel fibers to Purkinje cells and thus reduce Purkinje cell excitability. Therefore cerebellar LTD may cause the disinhibition of DCN neurons from Purkinje cells, and be involved in the mechanism of motor learning.
On the other hand, Purkinje cells receive inhibitory synaptic inputs from interneurons in cerebellar molecular layer, including stellate cells and basket cells (Szentagothai, 1965; Eccles et al., 1966c). Enhanced GABA release from the interneurons would also decrease the action potential firing probability of Purkinje cells (Hausser and Clark, 1997), and weaken the inhibition to DCN neurons. Therefore study of the GABA release pattern from interneurons in molecular layer would help understand the mechanism of motor learning.

Stellate cells are a type of GABAergic interneurons in the molecular layer that is of our interest. They receive excitatory inputs from parallel fibers. Parallel fibers are distributed in the molecular layer in parallel with the Purkinje cell layer, and form glutamatergic synapses on both interneurons and Purkinje cells. They are the axonal terminals rising from granule cells in granule cell layer. Granule cells are a group of small numerous cells located in the granule cell layer. They receive excitatory afferent inputs from mossy fibers that originate from precerebellar nuclei, and inhibitory inputs from GABAergic Golgi cells in granule cell layer (Eccles et al., 1966a). Stellate cells receive inhibitory presynaptic inputs from other adjacent interneurons (e.g. stellate cell-to-stellate cell synapses) in molecular layer. Their axons form inhibitory synapses onto Purkinje cells.

The firing pattern of action potentials in stellate cells directly determines GABA release onto Purkinje cells. The pattern of action potential firing is largely dependent on the integration of synaptic transmission. Excitatory and inhibitory synaptic transmission onto stellate cells is mediated by parallel fiber-to-stellate cell (PF-SC) synapses and
stellate/basket cell-to-stellate cell synapses respectively. Most fast excitatory synaptic transmission at PF-SC synapses is mediated by $\alpha$-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate type glutamate receptors (AMPA receptors or AMPARs). AMPA receptors are composed of four subunits (GluR1-GluR4). Among them GluR2 subunit bear unique properties due to the RNA editing from Glutamine to Arginine at the Q/R site (Mishina et al., 1991; Burnashev et al., 1992; Burnashev et al., 1996). Incorporation of GluR2 subunit abolishes the permeability to calcium of AMPA receptors (Burnashev et al., 1992; Jonas and Burnashev, 1995). GluR2-containing receptors display a smaller single channel conductance and no sensitivity to polyamine compared to GluR2-lacking receptors (Koh et al., 1995; Swanson et al., 1997). As a result, the subtype of AMPA receptors (GluR2-lacking or containing) at least partially determines the strength of excitatory synaptic transmission.

In hippocampus, GABAergic interneurons were reported to predominantly express GluR2-lacking AMPARs at the excitatory synapses (Geiger et al., 1995). Similarly, cerebellar stellate cells were also shown to naturally express GluR2-lacking receptors at the PF-SC synapses (Liu and Cull-Candy, 2000; Gardner et al., 2005). These receptors have been proved to be composed of GluR3 and GluR4 subunits (Keinanen et al., 1990; Sato et al., 1993; Petralia et al., 1997; Gardner et al., 2005). Although a recently study in our lab suggested the presence of GluR1 (Liu et al., submitted). In 2000 Liu and Cull-Candy reported a novel form of synaptic plasticity at PF-SC synapses: high-frequency stimulation of the parallel fibers induced the AMPAR subtype switch from GluR2-lacking to GluR2-containing (Liu and Cull-Candy, 2000). $Ca^{2+}$ entry through GluR2-lacking receptors was sufficient to induce this plasticity. Subsequent study demonstrated
that the association of protein interacting with PKC (PICK) and GluR2-containing receptors is required for the delivery of GluR2-containing receptors into synapses; and the dissociation of glutamate receptor interacting protein (GRIP) and GluR2-containing receptors is necessary for the removal of GluR2-lacking receptors from the synapses (Liu and Cull-Candy, 2005, Gardner et al., 2005). However, the molecule(s) that link Ca^{2+} entry and PICK-driven GluR2-containing receptor trafficking are not well known.

The high frequency stimulation of parallel fibers that induced the AMPAR subtype switch can cause glutamate spillover. The acute elevation of glutamate concentration saturates glutamate transporters and increases ambient glutamate concentration. In this case, the extrasynaptic glutamate receptors would be activated. N-methyl-D-aspartic acid (NMDA) receptors are located outside of PF-SC synapses, and can be activated during the high-frequency stimulation of the parallel fibers (Carter and Regehr, 2000; Clark and Cull-Candy, 2002). We hypothesized that the activation of NMDA receptors would also regulate the subtype of AMPARs at the synapse.

Our hypothesis was based on two facts: first, NMDA receptors have high binding affinity to glutamate that allows the activation of NMDARs during the glutamate spillover; second, NMDA receptors have high permeability to Ca^{2+}, and the robust Ca^{2+} entry seems to be an initiating factor for the induction of AMPAR subtype switch. NMDA receptors are composed of NR1 subunit and at least one type of NR2 subunit. The subtype of NR2 subunit (NR2A-2D) determines the functional properties of NMDA receptors in terms of the glutamate binding affinity, the sensitivity to Mg^{2+}, the
activation/deactivation kinetics, and pharmacology (Cull-Candy et al., 2001). The types of NR2 subunits expressed in stellate cells were not yet clearly profiled. Previous immunohistochemical and in situ hybridization studies gave rise to different results (Akazawa et al., 1994; Thompson et al., 2000; Duguid and Smart, 2004). Nonetheless, all of them suggested the expression of NR2D subunit in stellate cells.

Compared to other NR2 subunits, recombinant NR1/NR2D receptors showed a distinct low sensitivity to Mg\(^{2+}\) blockade (Momiyama et al., 1996). Macroscopic currents recorded from these receptors also displayed a slow deactivation time constant of several seconds (Vicini et al., 1998; Cull-Candy et al., 2001). Consistently, NMDAR-mediated currents in stellate cell can be activated at hyperpolarized potentials in the presence of 1mM Mg\(^{2+}\), and showed a similar kinetics (Sun and Liu, 2007). Since the electrophysiology data are in support of the previous study mentioned above, NR2D-containing receptors are very likely to exist in cerebellar stellate cells. We further testified the presence of NR2D subunit with the application of PPDA, a NR2D-preferred inhibitor (Hrabetova et al., 2000; Feng et al., 2004).

Cerebellar stellate cells naturally express GluR2-lacking receptors at their PF-SC synapses. We are curious about the mechanisms underlying this expression pattern. A recent study suggested that blockade of action potential firing would up-regulate \(\beta_3\) integrins which preferably stabilize GluR2-containing receptors at the synapses (Cingolani et al., 2008). This study supplemented the mechanisms of the induction of homeostatic plasticity. Moreover, it implied that action potential firing might regulate the
subunit composition of synaptic AMPA receptors. Cerebellar stellate cells fire action potentials spontaneously even in the absence of synaptic transmission (Hausser and Clark, 1997). We investigated if the spontaneous action potentials (sAPs) have a regulatory role of synaptic AMPAR subtype. Blockade of sAPs for three hours selectively increased the expression of GluR2-containing receptors, but had little effect on GluR2-lacking receptors. This implied that action potential firing might be involved in the maintenance of high levels GluR2-lacking receptors and low levels of GluR2-containing receptors. Previous studies showed that blockade of action potential firing elevated excitatory synaptic transmission by increasing the number of AMPA receptors, namely homeostatic plasticity. The subunit composition of AMPARs was not changed during this plasticity (Turrigiano et al., 1998; Wierenga et al., 2005; Ibata et al., 2008). Our observation is different from the features of homeostatic plasticity, suggesting a distinct signaling pathway and functional significance. The following study indicated the involvement of calmodulin and the requirement of protein synthesis.
Chapter 2

Materials and Methods

2.1 General materials and methods

2.1.1 Slice preparations

C57/BL6 mice (postnatal day 18–23) were used for these experiments. All experimental procedures were in accordance with the animal welfare guidelines of Penn State University. Following decapitation, cerebellar slices (250 µm) were obtained with a Leica VT1000S vibrating microslicer. Dissection and slicing of the cerebellum were performed in an ice-cold slicing solution (mM: 125 NaCl, 2.5 KCl, 0.5 CaCl₂, 7 MgCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, and 25 glucose, saturated with 95% O₂-5% CO₂, pH 7.4). Cerebellar slices were maintained in external artificial cerebrospinal fluid (ACSF, identical to the slicing solution except that 0.5 mM CaCl₂ and 7 mM MgCl₂ were replaced with 2 mM CaCl₂ and 1 mM MgCl₂) at room temperature for 30–60 min before recording.

2.1.2 Pipette solution

Potassium channels can be activated at depolarized potentials and give rise to large currents (in the order of nano-amp), which makes it impossible to measure the synaptic currents (in the order of pico-amp) of our interest. AMPARs are permeable to cesium that blocks potassium channels. Therefore Cs⁺ is an ideal replacement of intracellular K⁺ for the examination of synaptic AMPARs. Synaptic currents mediated by AMPARs consist of Cs⁺ ion flow. The Cs⁺ based pipette solution at pH 7.3 used in the experiments
contained (mM) 140 CsCl, 2 NaCl, 10 CsHEPES, 10 CsEGTA (0.5CsEGTA was used in some experiments in Chapter 3), 4 Mg-ATP, 5 1 N-(2, 6-
dimethylphenylcarbamoylmethyl) triethyl ammonium bromide (QX314), 5
tetraethylammonium (TEA), and 0.1 spermine. Spermine was excluded from the pipette solution for the experiments described in Chapter 4 Functional NR2B and NR2C/2D-containing receptors at extrasynaptic sites in mouse cerebellar stellate cells.

2.1.3 Electrophysiology

Cerebellar slices in the recording chamber was continuously superfused by a gravity fed system with external ACSF containing 0.1mM picrotoxin to block inhibitory transmission. Whole cell patch clamp recordings were obtained using an Axoclamp 700A amplifier (Axon Instruments). Recordings were made from neurons located in the outer two-thirds of the molecular layer in cerebellar slices. Stellate cells were identified by the presence of action potentials in the cell attached configuration and spontaneous synaptic currents in the whole cell configuration. Recordings began 10–15 min after obtaining the whole cell configuration to allow the perfusion of pipette solution into the cell. Series resistance was monitored every 5 min throughout the experiment. The recording was terminated if the value of series resistance changed by more than 30%.

2.1.4 Current-voltage relationship (I-V relationship)

In experiments described in Chapters 3 and 5, spontaneous excitatory postsynaptic currents (sEPSCs) were recorded at various holding potentials (from –60 mV to +60 mV). sEPSCs were filtered at 2 kHz and digitized at 20 kHz. The average current trace at each
holding potential (typically 20–40 sEPSCs) was constructed by aligning each event on its point of fastest rise using N version 4.0 (written by Dr. Steve Traynelis, Emory University). Events that did not have smooth rise and decay phases were rejected. The current amplitude was plotted at each potential producing an I–V relationship. The mean sEPSC amplitudes at negative potentials were fitted by linear regression. The rectification index (RI) of the I–V relationship was defined as the ratio of the current amplitude at +40 mV to the predicted linear value at +40 mV (extrapolated from linear fitting of the currents at negative potentials). The ratio of current amplitude at +40mV to that at -60mV was also used as an indicator of AMPAR subunit composition.

All values are expressed as mean ± S.E.M. Statistical significance was assessed using a two-tailed Student's t test. chelerythrine, GYKI 52466, ifenprodil, NBQX, NMDA, pep2-AVKI, pep2-SVKE, picrotoxin, PPDA and R-CPP (4-(3-phosphonopropyl) piperazine-2-carboxylic acid) were obtained from Tocris. Glycine and strychnine were purchased from Sigma. PKCl 19-36 was obtained from EMD Biosciences. (-)indolactam-V and (+)indolactam-V were from Alexis Biochemicals. Tetrodotoxin (TTX) was purchased from Tocris or Ascent.

2.2 Specific methods for Chapter 3

2.2.1 Stimulation of parallel fibers

The parallel fibers in horizontal or coronal slices were stimulated by a train of depolarizations to cause ‘spillover’ of glutamate, which can activate non-synaptic NMDARs. A parallel bipolar electrode (150 µm spacing) was placed across the molecular
layer about 200 µm from the recording electrode to stimulate PF inputs. The stimulus intensity was above threshold and ranged from 10 to 50 V with the stimulus duration of 100–200 µs. The stimulation protocol contained 100 trains of four depolarizations at 50 Hz, with a 2 s interval between two trains and 20 s between every 10 trains for the reload of glutamate into presynaptic terminals (Ottersen et al., 1990; Li et al., 1995). PFs were stimulated in an external solution that contained 50 µM glycine, 1 µM strychnine, and 50 µM GYKI 52466. Physiological-like stimulation experiments were performed at 32°C (further temperature elevation led to stability problems in these long-term recordings). During PF stimulation, the cell was voltage clamped at –70 mV and depolarized to 0 mV for 1 ms immediately after each burst stimulation. The brief depolarization was designed to mimic an action potential occurring in the postsynaptic cell that had been evoked by the presynaptic stimulation, and to remove the Mg\(^{2+}\) block of NMDA receptors. A few experiments were conducted in a Mg\(^{2+}\)-free external solution at room temperature, in which the postsynaptic cell was voltage clamped at –60 mV during PF stimulation (five depolarizations at 50 Hz) without a postsynaptic depolarization. In the experiment shown in Fig 3.1, an extracellular glass stimulating electrode filled with ACSF was placed about 100 µm from the recording electrode in the molecular layer. The stimulation intensity was near threshold. A pipette solution containing 10 mM EGTA was used for all PF stimulation experiments.

2.2.2 Application of NMDA and glycine

To briefly activate NMDARs, 20 µM NMDA plus 50 µM glycine was applied by a double-barreled gravity-fed perfusion system positioned just above the slice for 3 min
while the stellate cell was voltage clamped at −30 mV. Mg$^{2+}$-free ACSF was used in some of these experiments to maximize the effects. We tested two pipette solutions (containing 0.5 or 10 mM EGTA) and observed no difference in the amplitude of sEPSCs and in the NMDA/glycine-induced change in the rectification of sEPSCs. Thus the pipette solution that contained 0.5 mM EGTA was used for the chemical induction experiments (unless otherwise stated). The experiments testing PKC inhibitors and agonists were performed in an Mg$^{2+}$-free external solution.

2.3 Specific methods for Chapter 4

2.3.1 Evoked NMDAR-mediated currents

NMDAR-mediated currents were recorded at room temperature. 100 µM picrotoxin and 5 µM NBQX were added into external ACSF to inhibit GABA$_A$R and AMPAR-mediated currents. The membrane potential was voltage clamped at +40mV to remove Mg$^{2+}$ blockade of the NMDA receptors during the recording of NMDAR-mediated currents. A glass pipette with a diameter of ~10 µm was filled with ACSF and used for the stimulation of parallel fibers. This pipette was placed ~100 µm away from the recorded cell. In one train, a burst of 4 stimuli at 100Hz was delivered to the parallel fibers to evoke an NMDAR-mediated current at +40mV in the postsynaptic cell. There was a 30-second interval between two stimuli. To avoid the possible depletion of glutamate at the presynaptic terminals caused by the repetitive stimulation, we gave a 2 minute break after every 5 stimuli when the postsynaptic cell was voltage clamped at the resting potential (~60mV).

2.3.2 Application of subunit-selective/preferred antagonist
After the NMDAR-mediated currents was stably evoked (current amplitude and decay time were similar during at least 15 consecutive stimuli), a subunit-selective/preferred antagonist (CPP, ifenprodil, or PPDA) was added in the external ACSF. The antagonist was washed into the recording chamber 3 minutes before, and during, the recording of the evoked currents. The current amplitude would gradually decrease due to the blockade of NMDARs by the antagonist. After the amplitude decreased to a stable level, the antagonist was washed out with regular external ACSF. The washout process typically lasted for 3 to 5 minutes during which the stimulation was paused. After the washout of the antagonist, the parallel fibers were stimulated again to evoke NMDAR-mediated currents in the postsynaptic cell.

2.3.3 Measurements of the frequency of miniature IPSCs (mIPSCs)

In 15 minutes after the break-in, the cell was voltage clamped at -60mV, and the mIPSCs were recorded in the presence of 0.3µM TTX and 1µM strychnine contained in the external ACSF. The average mIPSC frequency was analyzed with Clampfit software every 5 minutes. Once the frequency was stable for consecutive 15 minutes, a subunit-selective/preferred antagonist (CPP, ifenprodil, or PPDA) would be washed in for 10 to 15 minutes to block certain subtypes of NMDARs. Then the antagonist would be washed out to remove the blockade of NMDARs. Recordings of mIPSCs were continuous before, during and after the application of the antagonist.

2.3.4 NMDAR-mediated currents in cerebellar granule cells at the mossy fiber-granule cell synapse
Parasagittal cerebellar slices (250µm) were obtained from C57/BL6 mice at postnatal 6 or 7 days. Whole cell configuration was obtained from granule cells located at the granule cell layer. A glass pipette containing external ACSF was placed in the white matter to stimulate the mossy fibers. Mossy fiber-evoked EPSCs were examined in an Mg\(^{2+}\)-free ACSF with 100 µM picrotoxin and 5 µM NBQX to block GABA\(\Lambda\) receptors and AMPA receptors respectively. Glycine (10 µM) was added to the external ACSF to facilitate NMDAR activation. Strychnine (1µM) was applied to block glycinergic receptors. During the recording, the postsynaptic cell was voltage clamped at -60mV. The mossy fibers were stimulated at 0.1Hz to evoke a synaptic current mediated by NMDA receptors, with a 5 minute interval between every 10 stimuli. The stimulation intensity was adjusted at just suprathreshold. The procedure of PPDA application was the same as described above (See Application of subunit-selective/preferred antagonist in 2.3.2). Average current amplitudes and charge transfer were estimated based on typically 30 to 40 current traces before, during or after PPDA application.

2.4 Specific methods for Chapter 5

2.4.1 Slice incubation

In control experiments, cerebellar slices were incubated for at least 3 hours with external ACSF that contained 1mM kynurenic acid and 0.1mM picrotoxin to block excitatory and inhibitory synaptic transmission respectively. TTX (0.5µM) was included in this incubation solution to block action potential firing. In some experiments, \(\omega\)-conotoxin GVIA (0.5µM), W7 (50µM), cycloheximide (100µM) or anisomycin (40µM) was added
either alone or with TTX in the incubation solution to inhibit different molecular pathways.
Chapter 3

Activation of extrasynaptic NMDA receptors induces a PKC-dependent switch in AMPA receptor subtypes in mouse cerebellar stellate cells

Introduction

Activity-dependent long-term plasticity of synaptic transmission has been widely studied and considered as a cellular mechanism underlying learning and memory. A number of molecular mechanisms that induce the plasticity have been recognized. A well studied mechanism in various brain areas is that the activation of postsynaptic NMDA receptors (NMDARs) allows calcium entry and triggers homosynaptic changes in AMPA receptors. The changes include the number of AMPA receptors (AMPARs) and their phosphorylation state (Nicoll and Malenka, 1999; Nicoll, 2003). It is less known about the physiological roles of NMDA receptors located outside of synaptic sites. Those receptors can be activated by bath application of NMDAR agonists, or by a “spillover” of glutamate from presynaptic terminals during high-frequency or high-intensity stimulation.

Cerebellar stellate cells predominantly express GluR2-lacking AMPARs at the PF-SC synapse (Liu and Cull-Candy, 2000). However, a high-frequency stimulation of parallel fibers (PFS) can induce a delivery of GluR2-containing receptors and a loss of GluR2-lacking receptors at this synapse (Liu and Cull-Candy, 2000). This switch of AMPAR subtypes results in a reduction in the Ca$^{2+}$ permeability of the synaptic AMPAR (Geiger
et al., 1995), as well as a decreased single channel conductance (Swanson et al., 1997). Later on this phenomenon was also discovered in other brain areas, in some cases along with other types of synaptic plasticity (Liu and Zukin, 2007). The activation of Ca\(^{2+}\)-permeable AMPARs by a high-frequency stimulation of the parallel fibers is sufficient to induce this type of plasticity in cerebellar interneuron. However, the repetitive stimulation should cause glutamate spillover and subsequently activation the extrasynaptic NMDARs in stellate cells. Thus we investigated the impact of the activation of extrasynaptic NMDARs on the regulation of synaptic AMPAR subtypes.

Previous studies demonstrated that the activity-dependent insertion of GluR2-containing receptors requires a GluR2-PICK1 protein interaction while the loss of GluR2-lacking receptors involves a disruption of GluR2-lacking receptor-GRIP complex (Gardner et al., 2005; Liu and Cull-Candy, 2005). These changes were initiated by an elevated calcium influx. However, the molecular mediator that links the increased calcium entry through glutamate receptors and the changes in the relationship between AMPARs and their interacting proteins was not clear. One candidate for the molecular mediator is protein kinase C (PKC) of which activity is enhanced by an increased intracellular calcium level. PKC has been shown essentially involved in the trafficking of AMPARs during long-term depression (LTD) in hippocampal and Purkinje neurons, and long-term potentiation (LTP) in dorsal horn neurons (Li et al., 1999; Daw et al., 2000; Xia et al., 2000; Kim et al., 2001; Leitges et al., 2004). Moreover, there is evidence that the activation of PKC facilitates the insertion of GluR2-PICK1 complexes into spines of hippocampal neurons (Perez et al., 2001). PKC phosphorylation of GluR2 also disrupts the GluR2-GRIP interaction and alters the AMPAR expression at synapses (Matsuda et al., 1999; Chung et
al., 2000; Chung et al., 2003). Therefore, we tested the role of PKC during the alteration of AMPAR composition.

In this set of experiments, we showed that the activation of extrasynaptic NMDARs can induce a long-lasting increase in GluR2-containing receptors and a loss of GluR2-lacking receptors at PF-SC synapses. This process requires an elevated intracellular calcium level, and the activation of a PKC-dependent pathway is also required. PICK1 drives the delivery of GluR2-containing receptors into synapses in this NMDAR-dependent synaptic plasticity.

**Results**

In order to determine the subtype of synaptic AMPA receptors, we included 0.1 mM spermine in the pipette solution. Polyamine is known to block GluR2-lacking receptors at depolarized potentials (Bowie and Mayer, 1995; Koh et al., 1995). Therefore, in the presence of intracellular spermine, sEPSCs at +40mV are solely mediated by GluR2-containing receptors. At a synapse where GluR2-lacking receptors are dominant, currents at depolarized potentials should be largely inhibited by intracellular spermine. This would lead to an inwardly-rectifying current-voltage relationship (I-V relationship, Fig 3.1A). In contrast, when GluR2-containing receptors are dominant, the EPSCs should not be affected by spermine and thus give rise to a linear I-V relationship. Therefore we can estimate the subunit composition of synaptic AMPARs according to the rectification of the I-V relationship.
Fig 3.1: Characterization of glutamate receptor-mediated synaptic and extrasynaptic currents in stellate cells.

(A). Spontaneous EPSCs in stellate cells exhibit a reduced amplitude at +40 mV and inwardly rectifying I-V relationship when spermine was included in the pipette solution. (B) Synaptic currents recorded in the absence of spermine have a linear I-V relationship. (C)-(F). GYKI52466 reversibly inhibited the AMPAR-mediated synaptic current, but not the extrasynaptic NMDAR current evoked by a burst of high-frequency stimulation of parallel fibers.
using near-threshold stimulation intensity. The pipette solution did not contain spermine. (C). EPSCs at -60 and +40 mV evoked by stimulation of parallel fibres with a single depolarization (left) and with a train of 5 depolarizations at 50 Hz (right). (D). The effect of GYKI52466 (50 µM) on AMPAR-mediated synaptic currents evoked by PF stimulation (5 depolarizations at 50 Hz) in the presence of 20 µM CPP. Current traces at -70 mV. (E). The extrasynaptic NMDAR-mediated current at +40 mV evoked by PF stimulation (5 depolarizations at 50 Hz) in the presence of 10 µM NBQX. 50 µM GYKI52466 and 20 µM CPP were bath applied. (F) Group data of sEPSC amplitude (normalized to the amplitude prior to the application of GYKI52466) vs time (n = 4). These recordings were made in the regular ACSF that contained 1 mM Mg$^{2+}$.

To investigate the effects of NMDA receptors on the synaptic AMPAR subtype, we first tested if NMDARs can be activated under physiological-like conditions. NMDARs in stellate cells are located at extrasynaptic sites (Carter and Regehr, 2000; Clark and Cull-Candy, 2002), and cannot be activated by a single stimulus of the parallel fibers (Fig 3.1C left). However, in vivo recordings of cerebellar granule cells showed that a sensory stimulation could evoke a burst of action potentials at ~80Hz (Chadderton et al., 2004). And our result suggested that the stimulation of parallel fibers at 50Hz was sufficient to evoke a slow current at depolarized potential. The current was sensitive to CPP, an NMDAR inhibitor (Fig 3.1C, right). The evidence indicated that NMDARs expressed in cerebellar stellate cells can be activated by physiologically relevant stimulation.

Glutamate spillover activates both NMDARs and AMPARs. To isolate the effects of the activation of NMDARs on the induction of AMPAR subtype switch, we applied GYKI 52466 during the high frequency stimulation to parallel fibers. GYKI 52466 is a selective inhibitor of non-NMDA glutamate receptors. Our data confirmed that it highly effectively antagonizes AMPA receptors (Fig 3.1D), and has little effect on NMDAR-mediated
currents (Fig 3.1E). The effects of GYKI 52466 can be washed out in 30 minutes (Fig 3.1F).

Fig 3.2: PF stimulation at 32°C induced a change in AMPAR subtypes in ACSF that contained 1 mM Mg. Spermine (100 µM) was included in the pipette solution.

(A) Schematic of the experimental procedure. (B) a: PF stimulation followed by a brief depolarization to 0 mV evoked an NMDAR current in ACSF that contained GYKI52466 (upper trace). b: this stimulation protocol did not activate the current in the presence of CPP (lower trace). Stimulation intensity was above threshold. (C-E) PF stimulation without CPP. (C) Average sEPSC current traces recorded at +40 mV and -60 mV before and 30 minutes after PF stimulation. (D) Group data of sEPSC amplitude (n = 4). (E) Ratio of sEPSC amplitude at +40 vs -60 mV of individual cells (open circles). Average values are shown in filled circles. (F)-(H) Parallel fibers were stimulated in the presence of 20 µM CPP to block NMDARs. (F) sEPSC current traces. (G) Average sEPSC amplitude (n = 4). (H) Ratio of sEPSC amplitude.
amplitude at +40 vs -60 mV of individual cells (open circles) and average values (filled circles). (* p < 0.05 paired t-test)

As illustrated in Fig 3.2A, spontaneous EPSCs (sEPSCs) were recorded at different potentials prior to the stimulation of parallel fibers to generate a control I-V relationship. Then GYKI was washed into the recording chamber 3 minutes before and during the stimulation to prevent the activation of AMPARs. After the stimulation, the inhibition of AMPARs by GYKI was removed with ACSF for 30 minutes, followed by the recordings of sEPSCs at different potentials to convey a post-stimulation I-V relationship.

3.2.1 Presynaptic stimulation activates extrasynaptic NMDARs and triggers a switch in AMPAR subtype at the synapse

We stimulated the PFs with a depolarizing train followed by a brief depolarization of the postsynaptic cell from -70mV to 0mV. The brief depolarization was designed to mimic the action potential occurring in the postsynaptic cell that had been evoked by the presynaptic stimulation. This stimulating protocol evoked an inward current that can be inhibited by CPP (Fig 3.2B), suggesting that this protocol could at least partially remove the Mg$^{2+}$ blockade of NMDA receptors. Therefore this stimulating protocol was applied to activate NMDARs in ACSF containing Mg$^{2+}$, to test whether it would induce a switch in synaptic AMPAR subtypes under physiological-like conditions (at 32°C).

Prior to the PF stimulation, sEPSC amplitude at +40mV was small in the presence of 0.1mM spermine (Fig 3.2C and D). Consequently, the ratio of the amplitude at +40mV to that at -60mV was low (Fig 3.2E). This ratio was used to estimate the subunit composition of AMPARs. After the PF stimulation, the amplitude of sEPSC at -60mV
was decreased, while the amplitude at +40mV was significantly increased (Fig 3.2C and D, n = 4, p < 0.05). As a result, the ratio increased from 0.18±0.03 to 0.30±0.05 (Fig 3.2E, p < 0.02). Since in the presence of intracellular spermine, currents at a depolarized potential are solely mediated by GluR2-containing receptors, the increase in the amplitude at +40mV indicated that the number of GluR2-containing receptors increased. The decrease in current amplitude at -60mV (via both GluR2-containing and lacking receptors) indicated a reduction in the number of GluR2-lacking receptors.

We next determined if this switch in AMPAR subtypes was induced by the activation of NMDARs. PFs were stimulated as described above except for the addition of CPP during the stimulation. In the presence of CPP the NMDAR-mediated currents were effectively inhibited (Fig 3.2Bb), under this condition sEPSC amplitudes at both potentials remained the same as prior to the stimulation (Fig 3.2F and G, n = 4, p > 0.05). As a result, the ratio of sEPSC amplitude at +40mV versus -60mV was not changed (Fig 3.2H). To verify a successful stimulation of PFs, at the end of the recording of each cell CPP was washed out and PFs were stimulated briefly again at the same position. The cell would be discarded if the stimulation failed to evoke an NMDAR-mediated current. These data indicated that the switch in synaptic AMPAR subtypes was due to the activation of extrasynaptic NMDA receptors.
Fig 3.3: Activation of non-synaptic NMDARs by PF stimulation induced a change in the subunit composition of synaptic AMPARs.
(A) NMDAR currents evoked by PF stimulation (5 depolarizations at 50 Hz) in the Mg$^{2+}$-free ACSF at room temperature. Stimulation intensity was above threshold. GYKI52466 (50 µM) was present during the PF stimulation. (B) Average sEPSC current traces recorded at +40 mV and -60 mV before and 30 minutes after PF stimulation. (C) Group data of sEPSC amplitude (n = 5). (D) Ratio of sEPSC amplitude at +40 vs -60 mV of individual cells (open circles). Average values are shown in filled circles. (E)-(H) Parallel fibers were stimulated in the presence of 20 µM CPP to block NMDARs in the Mg$^{2+}$-free external solution. (E) CPP blocked NMDAR currents evoked by PF stimulation. (F) sEPSC current traces. (G) Average current amplitude (n = 4). (H) Ratio of sEPSC amplitude at +40 vs -60 mV of individual cells (open circles) and average values (filled circles). (* p < 0.05; *** p < 0.0005).

The activation of extrasynaptic NMDARs can be enhanced by reducing the external Mg$^{2+}$ concentration. Thus an increase in NMDAR activation would be expected to produce a larger change in AMPAR subtypes. Indeed, compared to the experiment conducted at 32°C in the presence of 1mM Mg$^{2+}$, PF stimulation in an Mg$^{2+}$-free solution at room temperature evoked a larger NMDAR-mediated current (Fig 3.3A), as well as a more pronounced increase in the ratio of sEPSC amplitude (from 0.18 ± 0.02 to 0.38±0.03, Fig 3.3B, C and D, n = 5). Again the addition of CPP during the stimulation abolished the increase in current amplitude at +40mV, and the decrease at -60mV, as well as the increase in the ratio of sEPSC amplitude (Fig 3.3F, G and H). These results support the idea that the repetitive activation of NMDARs caused by glutamate spillover during a train of presynaptic activity altered the subunit composition of synaptic AMPARs in cerebellar stellate cells.
Figure 3.4: BAPTA blocked the NMDA-induced change in AMPAR subtypes.

A brief application of NMDA induced a lasting change in synaptic AMPAR subtypes (A–D). This effect was blocked when 10 mM BAPTA was included in the patch electrode (E–G). Experiments were performed in a Mg$^{2+}$-free
(A–G) or regular external (B and C) solution that contained 1 mM Mg$^{2+}$ (50 µM GYKI was present during NMDA application). A, average current traces at +40 mV and −60 mV prior to and 30 min following the application of 20 µM NMDA and 50 µM glycine. B, I–V relationship of sEPSCs prior to and following NMDA application. C left, rectification index determined from the I–V relationship of individual cells. The average rectification index (*) increased. Right, ratio of sEPSC amplitude at +40 mV versus −60 mV from individual cells. Average values increased (•, *P < 0.05). D, sEPSC amplitude was normalized to that prior to NMDA application at each potential. E–G, sEPSCs were recorded using a pipette solution that contained 10 mM BAPTA. E, BAPTA blocked the NMDA-induced change in sEPSC amplitude. F, NMDA application failed to induce a change in the I–V relationship of sEPSCs (n = 5). G, rectification index and ratio of sEPSC amplitude at +40 mV versus −60 mV remained unaltered following NMDA application. H, average sEPSC amplitude increased at +40 mV and declined at −60 mV (n = 5) using the control pipette solution; the current amplitude did not change when using a BAPTA-containing pipette solution.

3.2.2 Activation of NMDA receptors induces a lasting change in AMPAR subtypes

To determine whether the activation of NMDA receptors is sufficient to induce the change in AMPAR subtypes, NMDARs were directly activated by exogenous agonists, NMDA and glycine. Following a control recording of sEPSCs at different potentials, 30 µM NMDA and 50 µM glycine were applied for 3 minutes in an Mg$^{2+}$-free ACSF, while the postsynaptic cell was voltage clamped at -30 mV. Under this condition, the sEPSC amplitude increased at +40 mV and decreased at -60 mV (Fig 3.4A and H, n = 5, p < 0.05) following the application of NMDA and glycine. The I-V relationship of the synaptic currents changed from inwardly rectifying to nearly linear after the application of NMDA (Fig 3.4B), as shown by the increase of rectification index (Fig 3.4C left, n = 5, p < 0.02). Consistently, the ratio of current amplitude at +40 mV versus -60 mV significantly increased (Fig 3.4C right, p < 0.02). The simultaneous increase in the sEPSC amplitude at +40 mV and decrease at -60 mV are consistent with the idea that the
number of GluR2-containing receptors increased while there was a loss of GluR2-lacking receptors after the application of NMDA.

The changes of current amplitude were observed ~30 minutes after the application of NMDA, and lasted for at least another 30 minutes (Fig 3.4D). Without the activation of NMDA receptors, the current amplitudes at both potentials remained constant for at least 1 hour (data not shown). Therefore the potentiation of current amplitude at +40mV and the suppression at -60mV were triggered by the activation of NMDARs.

The activation of NMDARs may allow increased Ca\(^{2+}\) influx into stellate cells. We tested whether the elevated intracellular Ca\(^{2+}\) level was required to induce the change of AMPAR subtype. When 10mM BAPTA was included into the pipette solution to buffer the Ca\(^{2+}\) rise in the postsynaptic cell, there was no change in the current amplitude at either +40mV or -60mV (Fig 3.4E and H). The I-V relationship of synaptic currents and the ratio also remained unaltered (Fig 3.4F and G). This result indicated that the change in AMPAR subtypes was triggered by the Ca\(^{2+}\) influx through NMDA receptors.

3.2.3 Inhibition of PKC blocks the activity-dependent switch in AMPAR subtypes

We investigated whether the PKC pathway was required in the induction of changes in AMPAR subtypes. First we tested if PKC was involved in the constitutive recycling of synaptic AMPARs. When 10\(\mu\)M chelerythrine, a PKC inhibitor, was added into the pipette solution, the current amplitudes at +40mV and -60mV were not changed (data not shown). A structurally different inhibitor, PKCI 19-36 did not alter the current amplitude either.
Figure 3.5: Intracellular application of PKCI 19-36 and chelerythrine blocked the NMDA-induced switch in AMPAR subtypes.

A–F, PKCI 19-36 (20 µM) was included in the pipette solution. A, examples of average sEPSC traces at +40 mV and −60 mV. B, group data of sEPSC amplitude (n = 5). C, sEPSC amplitude normalized to that prior to NMDA application at each potential versus time. D, I–V relationship of synaptic currents. E, rectification index of individual
cells and the average value (•). F, ratio of sEPSC amplitude at +40 mV versus −60 mV of individual cells and average values (•). G–L, chelerythrine (10 µM) was added to the pipette solution. G, average sEPSCs at +40 mV and −60 mV before and after the application of NMDA. H, group data of sEPSC amplitude at these potentials (n = 5). I, sEPSC amplitude normalized to that prior to NMDA application at each potentials versus time. J, I–V relationship of sEPSCs prior to and following the application of NMDA. K, rectification index of individual cells and average value (•). L, ratio of sEPSC amplitude at +40 mV versus −60 mV of individual cells and average values (•).

We next tested whether the activity-dependent change in AMPAR subtype requires the activation of PKC. As shown in Fig 3.5A-C, the application of NMDA failed to induce a change in current amplitude at either +40mV (pre 11.00 ± 0.67pA vs. post 12.33 ± 0.91pA, p > 0.05) or -60mV (pre -52.55 ± 5.19pA vs. post -51.62 ± 2.75pA, p > 0.05) when PKCI 19-36 was present in the pipette solution. Fig 3.5D-F showed that PKCI 19-36 abolished the change in the I-V relationship (RI: pre 0.30 ± 0.03 vs. post 0.30 ± 0.02, p > 0.05). This suggested that the activation of PKC may be required for the insertion of GluR2-containing receptors and the loss of GluR2-lacking receptors at the synapse. To ensure that the effect of PKCI 19-36 was due to the inhibition of PKC, we also tested the effect of another PKC inhibitor, chelerythrine. 10µM chelerythrine included in the pipette solution also eliminated the changes in current amplitudes at both +40mV (pre 9.62 ± 0.40pA vs. post 10.18 ± 0.37pA, p > 0.05) and -60mV (pre -41.76 ± 0.84pA vs. post -41.96 ± 2.36pA, p > 0.05) following the application of NMDA (Fig 3.5G-I, n = 5). This consequently did not change the I-V relationship (Fig 3.5J-L, RI: pre 0.32 ± 0.03 vs. post 0.31 ± 0.04, p > 0.05). Therefore, these results strongly suggested that the activation of PKC is required for the NMDA-induced increase in GluR2-containing receptors and the loss of GluR2-lacking receptors at the synapse.
Figure 3.6: Activation of PKC by (−)indolactam-V or OAG changed the subunit composition of the AMPARs at the synapse.

A, I–V relationship of sEPSCs when (−)indolactam-V (300 µM) was included in the pipette solution (n = 7). B, (+)indolactam-V was used as a negative control (n = 7). C, inclusion of OAG in the patch electrode gave rise to a linear I–V relationship of sEPSCs (n = 5). D, rectification index of synaptic currents increased in the presence of (−)indolactam-V and OAG (DMSO control, n = 4; *P < 0.05).

3.2.4 Activation of PKC increases synaptic GluR2-containing AMPARs

Is activation of PKC sufficient to trigger the change in AMPAR subtypes at the synapse? To address this question, we included 300µM (-) indolactam-V, a PKC activator, into the pipette solution and recorded the I-V relationship of spontaneous EPSCs mediated by
synaptic AMPARs. sEPSCs at different potentials were recorded ~15 minutes after the formation of whole cell configuration.

In the presence of (-)indolactam-V, the I-V relationship of synaptic currents appeared to be nearly linear with a rectification index of 0.64±0.13 (Fig 3.6A, n = 7). In a control experiment, the inactive form of (-)indolactam-V, (+)indolactam-V, was used in the pipette solution. Under that condition, the I-V relationship remained strongly inwardly rectifying. And the rectification index (Fig 3.6B, 0.27±0.01, n = 7) was significantly lower than that of (-)indolactam-V (p < 0.05). A similar rectification index was found in the presence of DMSO (0.27±0.04, n = 4, data not shown). This suggested that the activation of PKC would induce a switch in AMPAR subtypes.

We next compared current amplitudes for (-)indolactam-V and its inactive analogue. The amplitude of synaptic currents at +40mV changed from 9.6±0.6 pA in (+)indolactam-V control to 12.2±0.8 pA in the presence of (-)indolactam-V (n = 7, p < 0.02). This 27% increase in the EPSC amplitude is comparable to the increase at +40mV that followed PF stimulation and NMDA application (Figs 3.2-3.4). The potentiation of synaptic currents at +40mV was consistent with the idea that the activation of PKC increases the expression of GluR2-containing receptors at the synapse. However, unlike the change following PF stimulation or NMDA application, the current amplitude at -60mV was not reduced in the presence of (-)indolactam-V. If the number of GluR2-lacking receptors remained constant at the synapse, one would expect to see an enhancement of EPSC amplitude at -60mV due to the increase of GluR2-containing receptors. Since the activation of PKC by (-)indolactam-V increased the current amplitude at +40mV by
~3pA, the current amplitude at -60mV should increase by ~4.5pA. There was indeed an obvious increase in current amplitude at -60mV (from -54.4±3.3pA to -60.3±2.5pA), which may account for the elevated level of GluR2-containing receptors at the synapse. These data implied that the activation of PKC may enhance the expression of GluR2-containing receptors, but have little effect on GluR2-lacking receptors at the synapse.

To further support the effects of the activation of PKC, we infused another PKC activator, 20μM OAG, into the pipette solution. OAG also increased the current amplitude at +40mV (n = 5, p < 0.005), but did not change the amplitude at -60mV (p = 0.33), compared to the DMSO control. The I-V relationship of the synaptic currents was linear (Fig 3.6C and D) with a rectification index of 0.89±0.02. Therefore it is very likely that the activation of PKC is both necessary and sufficient to cause an increase in the number of GluR2-containing receptors at the synapse. However, PKC activation is necessary but not sufficient for the removal of GluR2-lacking receptors from the synapse. This indicated that the removal of GluR2-lacking receptors may also require other molecular mediators.
Figure 3.7: pep2-AVKI blocked the NMDAR-induced increase in sEPSC amplitude at +40 mV.
3.2.5 PICK is involved in the NMDA-induced increase in GluR2-containing receptors

PICK is known to be associated with GluR2 subunit, and once activated by PKC it facilitates the delivery of GluR2 into synapse (Perez et al., 2001). Previous studies showed that the disruption between PICK and GluR2/3 blocked the activity-dependent increase in GluR2-containing receptors at the synapse (Liu and Cull-Candy, 2005). We investigated whether the PICK-GluR2 interaction is required for the NMDA-induced delivery of GluR2-containing receptors.

We utilized pep2-AVKI, a peptide inhibitor that selectively disrupts the interaction between PICK and GluR2/3 subunit (Osten et al., 2000). Inclusion of pep2-AVKI has been shown not to alter the basal synaptic transmission at PF-SC synapses (Liu and Cull-Candy, 2005). We included this peptide into the pipette solution and recorded sEPSCs prior to and following NMDA application, as described before. As shown in Fig 7A and C, the activation of NMDARs no longer induced any change in current amplitude at +40mV. Since currents at +40mV are solely mediated by GluR2-containing receptors, it suggested that the interaction between PICK and GluR2 is necessary for the NMDA-
induced delivery of GluR2-containing receptors. In the presence of pep2-AVKI, current amplitude at -60mV was significantly decreased following the NMDA application. This is consistent with the previous report (Liu and Cull-Candy, 2005), and indicated that PICK might not be involved in activity-dependent removal of GluR2-lacking receptors. To further confirm that the inhibitory effect of pep2-AVKI was due to its selective binding to PICK, we repeated the experiment with an inactive control peptide, pep2-SVKE that does not bind to PICK. In the presence of pep2-SVKE, we still observe a significant increase in the current amplitude at +40mV (Fig 3.7D and F, n = 6, p < 0.05), as well as a decrease at -60mV (Fig 3.7E, p < 0.05). Thus disrupting PICK-GluR2 interaction by pep2-AVKI prevented the NMDA-induced increase in the expression of GluR2-containing receptors.

Discussion

Our results suggested that the physiological-like stimulation of parallel fibers can activate the extrasynaptic NMDARs of cerebellar stellate cells. The activation of NMDARs induced a long-lasting switch in AMPAR subunit composition at PF-SC synapses. This switch involved an increase in GluR2-containing receptors and a decrease in GluR2-lacking receptors at the synapse. An elevated intracellular Ca^{2+} concentration is required in the induction of this plasticity. The activity of PKC is necessary and sufficient for the PICK-dependent recruiting of GluR2-containing receptors into the synapse; whereas PKC is necessary but not sufficient for the removal of GluR2-lacking receptors. These data indicated that the activity of extrasynaptic NMDARs can control the dynamic movement of AMPARs at the synapse via certain intracellular signaling pathways.
Activation of extrasynaptic NMDARs or postsynaptic Ca\(^{2+}\)-permeable AMPARs can induce similar changes in postsynaptic AMPAR subtypes (Liu and Cull-Candy, 2000). They indeed share similarities in the induction mechanism. First, high-frequency stimulation of the parallel fibers can induce the switch in AMPAR subtype in both experiments. Single stimulation does not activate extrasynaptic NMDARs in cerebellar stellate cells (Clark and Cull-Candy, 2002). Although postsynaptic AMPARs can be activated spontaneously, test stimulation did not induce change in subunit composition (Liu and Cull-Candy, 2000). Stimulation at 50 or 100Hz is sufficient to induce the plasticity in AMPAR subunit composition. Second, the activation of extrasynaptic NMDARs results in a 36% increase in the sEPSC amplitude at +40 mV and a 20% decrease at −60 mV. This leads to a two-fold increase in the rectification index of sEPSCs. Previous studies reported that the average conductance of Ca\(^{2+}\)-impermeable receptors (~5.5 pS) is about 23% lower than that of AMPARs that display partial inward rectification in stellate cells (~7.2 pS) (Liu and Cull-Candy, 2005). The reduction in channel conductance is likely to account for the change in sEPSC amplitude associated with the NMDAR-induced switch in AMPAR subtypes. Thus like the activation of postsynaptic Ca\(^{2+}\)-permeable AMPARs, the activation of NMDARs probably causes the replacement of some of the GluR2-lacking receptors that left the synapse with GluR2-containing receptors. Third, intracellular perfusion of BAPTA abolished the phenomenon triggered by the activation of NMDARs. Therefore, Ca\(^{2+}\) entry is necessary for both NMDAR-induced and postsynaptic AMPAR-induced switch in AMPAR subunit
composition. This also suggested that activation of NMDARs produced a change in AMPAR subtype via a postsynaptic mechanism.

NMDAR-dependent switch in AMPAR subtype also displays distinct differences compared to the plasticity induced by the activation of postsynaptic AMPARs. First, high-frequency stimulation of parallel fibers is required for the activation of NMDARs. However, cells that displayed a higher rate of spontaneous synaptic AMPAR activity already give linear I–V plots (Liu and Cull-Candy, 2002). Therefore extrasynaptic NMDARs may serve as a ‘sensor’ that detects the enhancement of presynaptic activity and regulate the postsynaptic plasticity once activated. Second, repetitive activation of synaptic AMPARs induces a change in AMPAR subtypes at the same synapse, and therefore is a form of homosynaptic plasticity. Glutamate spillover or bath application of NMDA activates NMDARs located outside of the synapse, thus the change they induce may not necessarily be input specific. This raises the possibility that the Ca\(^{2+}\) entry via extrasynaptic NMDARs could potentially alter the AMPAR subtypes not only at the nearest synapse but also synapses that are distant away. Third, activation of postsynaptic AMPARs triggers the change in I–V relationships within 15 to 30 minutes. The NMDAR-dependent change, however, displayed a comparable but slightly delayed onset (~30 minutes). We speculated that Ca\(^{2+}\) entry through synaptic AMPARs might cause an acute boost of local Ca\(^{2+}\) concentration at the same synapse. This local Ca\(^{2+}\) increase may rapidly alter the AMPAR subtype homo-synaptically. In contrast, the activation of extrasynaptic NMDARs allows the Ca\(^{2+}\) influx outside of synapses. It may require more time for the Ca\(^{2+}\) to diffuse into neighboring synapses to trigger the change in synaptic AMPAR subtypes.
PKC/PICK is a much studied signaling pathway in the mechanism of long-term plasticity. It is shown to be essential in bidirectional NMDAR-dependent hippocampal plasticity (Terashima et al., 2008). PKC activity is increased during the induction of LTP and is required for the maintenance of LTP (Reymann et al., 1988a; Reymann et al., 1988b; Colley et al., 1990). PKC is also involved in the induction of hippocampal LTD (Wang et al., 1998), and its activity is decreased in the process of LTD (Ramakers et al., 2000; Thiels et al., 2000). PKC/PICK activity is critically involved in the induction of cerebellar LTD in Purkinje cells (Xia et al., 2000; Chung et al., 2003; Leitges et al., 2004; Steinberg et al., 2006). Our study identified the role of PKC in the regulation of the subunit composition of synaptic AMPARs in cerebellar GABAergic interneurons. Consistent with previous study (Gardner et al., 2005; Liu and Cull-Candy, 2005), our data suggested that PKC could be activated by Ca$^{2+}$ entry through NMDARs, and the activated PKC triggered PICK-driven delivery of GluR2-containing receptors into synapses. However, this model contrasts with work in hippocampal and Purkinje cells. It was suggested that PKC and PICK are required for the loss of GluR2-containing receptors during LTD. One explanation of data from Purkinje cells was that PICK1 binds AMPA receptors in the internal pool, preventing them from compensatory AMPA receptor insertion (Daw et al., 2000; Xia et al., 2000; Kim et al., 2001; Leitges et al., 2004).

In the process of AMPAR subtype switch, the removal of GluR2-lacking receptors requires the disruption of the AMPAR–GRIP interaction (Liu & Cull-Candy, 2005). We
found that inhibition of PKC blocked the NMDA-induced loss of GluR2-lacking receptors. This result suggested that PKC might be required in the dissociation of AMPAR and GRIP. GRIP is known to bind to the C-terminus of GluR2/3/4c subunits (Dong et al., 1997; Srivastava et al., 1998; Dong et al., 1999). Stellate cells appear to express all three subunits (Keinanen et al., 1990; Sato et al., 1993; Petralia et al., 1997; Gardner et al., 2005), and hence GluR2-lacking receptors are likely to contain GluR3/4 subunits. PKC can phosphorylate GluR2 at Ser 880, and disrupt the GRIP–GluR2 interaction (Matsuda et al., 1999; Chung et al., 2000). PKC might also phosphorylate GluR3 at the analogous residual, Ser 885 (Chung et al., 2000; Xia et al., 2000; McDonald et al., 2001; Perez et al., 2001); thereby PKC phosphorylation might dissociate GluR3 and GRIP via a similar mechanism. However the PKC activators, (−)indolactam-V and OAG, did not reduce the current amplitude at −60 mV, indicating that the activity-dependent loss of GluR2-lacking receptors may require both PKC activation and other signaling molecules. Clathrin adaptor protein AP2 is involved in the internalization of AMPARs specifically induced by NMDAR activation (Lee et al., 2002). A recent study indicated that clathrin-dependent endocytosis mediate the internalization of GluR2-lacking receptors in cerebellar stellate cells (Gardner et al., 2005). Therefore we speculate that the loss of GluR2-lacking receptors in our experiments requires the clathrin-dependent internalization triggered by NMDAR activation. Dissociation of GRIP/GluR3 complex by PKC activity might prime for the clathrin-dependent internalization of GluR2-lacking receptors.
Changes in AMPA receptor subunit composition occur during other types of synaptic plasticity. In hippocampus, a brief glutamate release triggered LTP at single spine along with increased proportion of GluR2-containing receptors (Bagal et al., 2005). However, another research in hippocampus indicated a rapid and transient incorporation of GluR2-lacking receptors during LTP at Schaffer collateral synapses onto CA1 pyramidal neurons (Plant et al., 2006). Studies in retina ganglion cells showed a subunit change from predominantly GluR2-containing to GluR2-lacking that is caused by synaptic silence (Xia et al., 2007). Subtype switch of synaptic AMPA receptors is also involved in the induction of homeostatic plasticity in cultured hippocampal neurons (Thiagarajan et al., 2005), and in the development of the mossy fiber-pyramidal neuron synapse (Ho et al., 2007). Therefore, dynamic regulation of AMPAR subunit composition may be a general mechanism underlying synaptic plasticity.

The subtype switch of AMPARs in our work was induced by the activation of NMDAR, due to the glutamate spillover. The glutamate spillover was evoked by repetitive high frequency stimulation (50Hz) of the presynaptic parallel fibers. Since cerebellar granule cells can fire action potentials at ~80Hz (Chadderton et al., 2004), a glutamate spillover is very likely to occur in response to physiological sensory stimulation, thus induce the subtype switch in synaptic AMPAR of stellate cells by activation extrasynaptic NMDARs. Glutamate concentration can be controlled by glutamate uptake into neurons and glia by excitatory amino acid transporters (Fellin et al., 2004; Huang and Bergles, 2004). Therefore glutamate spillover can also be caused by a reduction of glutamate transporter activity. In support of our observations, decreased glutamate transporter
activity was reported to induce long-term change in synaptic AMPARs by activating extrasynaptic NMDARs. For example, pharmacological blockade of glutamate uptake can activate extrasynaptic NMDARs in cortical neurons, thereby leading to the induction of LTD (Massey et al., 2004). Conversely reducing glutamate transporter activity by lowering the temperature allows the glutamate that is released from the stimulated synapses to activate NMDARs and induces LTP at un-stimulated synapses in the amygdale (Tsvetkov et al., 2004).

NMDARs are present at many excitatory synapses as well as in extrasynaptic regions (Tovar and Westbrook, 2002; Scimemi et al., 2004). In some cells they are localized exclusively at extrasynaptic sites (Chen and Diamond, 2002; Clark and Cull-Candy, 2002). The proposed functions of extrasynaptic NMDARs involve cooperative interactions between neighboring hippocampal synapses (Arnth-Jensen et al., 2002), the regulation of cAMP response element-binding protein (CREB) activity which is coupled to cell death pathways (Hardingham et al., 2002) and the regulation of neuronal excitability via the modulation of Kv2.1 channels (Mulholland et al., 2008). Recent studies demonstrated the regulatory effects of NMDARs in GABA release from cerebellar interneuron terminals (Duguid and Smart, 2004; Fiszman et al., 2005; Liu and Lachamp, 2006; Lachamp et al., 2009). Our results revealed another role for extrasynaptic NMDARs in the induction of a long-term change in subunit composition of synaptic AMPARs. Therefore, we are interested in the features of the extrasynaptic NMDARs in cerebellar stellate cells, including the subunit composition, pharmacology,
distribution, etc. The next chapter will explain our preliminary work in the investigation of NMDAR characteristics.
Chapter 4

Functional NR2B and NR2C/2D-containing receptors at extrasynaptic sites in mouse cerebellar stellate cells

Introduction

N-methyl-D-aspartic acid receptors (NMDA receptors) are a major type of ionotropic glutamate receptors in the central nervous system (CNS) that are involved in various forms of synaptic plasticity (Nakanishi, 1992; Collingridge and Bliss, 1995). Conventional NMDA receptors are composed of obligatory NR1 subunits that bind to glycine and of NR2A to NR2D subunits that bind to glutamate (Monyer et al., 1992; Furukawa et al., 2005). NR3 subunits are also expressed in the developing mammalian CNS (Ciabarra et al., 1995; Sucher et al., 1995; Nishi et al., 2001; Chatterton et al., 2002). The type of NR2 subunit in an NMDA receptor not only determines the distinct properties of the receptor, including the binding affinity to glutamate, sensitivity to Mg$^{2+}$, current kinetics, single channel conductance and pharmacological traits; but also regulates the localization of the receptors and their function in synaptic plasticity (Cull-Candy et al., 2001).

Previous studies in the hippocampus suggested that in adult neurons NR2A is restricted to the synapses whereas NR2B was selectively located at extrasynaptic sites (Stocca and Vicini, 1998; Tovar and Westbrook, 1999; Fellin et al., 2004; Scimemi et al., 2004).
However, the extrasynaptic localization of NR2A and synaptic presence of NR2B were also reported and corrected the notion about the subunit-dependent distribution of NMDARs (Li et al., 1998; Mohrmann et al., 2002; Thomas et al., 2006). Although the segregation of NR2A and NR2B to synaptic and extrasynaptic sites is not absolute in hippocampus, the spatial distribution of different subunits was well identified in some cell types in cerebellum. In cerebellar Golgi cells, NR2B is expressed at both synaptic and extrasynaptic sites, whereas NR2D is restricted to the extrasynaptic membrane (Misra et al., 2000; Brickley et al., 2003). In granule cells, NR2C is exclusively expressed in synaptic receptors (Rumbaugh and Vicini, 1999; Cathala et al., 2000). NMDA receptors are also widely expressed presynaptically and regulate synaptic transmissions (Corlew et al., 2008).

Activation of NMDA receptors crucially regulates synaptic function and underlies the mechanisms of learning and memory. The functional roles of NMDA receptors are associated with their subunit composition. For example, the postnatal appearance of NR2A accelerates the deactivation time course of synaptic NMDA receptors (Flint et al., 1997; Barth and Malenka, 2001). Incorporation of NR2C into the synapse of cerebellar granule cells reduces the sensitivity of Mg$^{2+}$ and the single channel conductance of NMDARs (Cathala et al., 2000). Due to the differences in properties such as current decay time and single channel conductance, NMDARs containing distinct NR2 subunits may carry different amount of Ca$^{2+}$ per unit of currents that is a key to the induction of long-term plasticity. Therefore, the subunit composition of NMDARs may determine the direction of synaptic plasticity. Consistent with this idea, studies in hippocampus and
visual cortex provided evidence implying that a high NR2A/NR2B ratio favors the activity-dependent induction of LTD, and a low NR2A/NR2B ratio lower the threshold for the induction of LTP (Liu et al., 2004; Massey et al., 2004; Barria and Malinow, 2005; Berberich et al., 2005).

In cerebellar stellate cells, NMDA receptors can be effectively activated by glutamate spillover caused by the high-intensity stimulation or the inhibition of glutamate transporters (Carter and Regehr, 2000; Clark and Cull-Candy, 2002; Sun and June Liu, 2007), but not by a single quantum of glutamate release or the stimulation of a small number of parallel fibers. In addition, patch clamp recordings from interneuron presynaptic terminals indicated the existence of NMDA-activated single channels (Fiszman et al., 2005). These data suggested that NMDA receptors are localized at extrasynaptic sites and perhaps presynaptic sites as well. The functional roles of NMDARs in cerebellar stellate cells have also been revealed recently. As described in the last chapter, the activation of extrasynaptic NMDA receptors induces a long-lasting change of synaptic AMPAR subunit composition through a PKC-PICK dependent pathway (Sun and Liu, 2007). The activation of NMDARs, most likely at the presynaptic sites, induces long-lasting potentiation of GABA release that requires PKA/RIM1α signaling pathway (Liu and Lachamp, 2006; Lachamp et al., 2009). This presynaptically expressed enhancement in GABA release may directly modulate the inhibitory effects on the activity of Purkinje cells (Glitsch and Marty, 1999; Duguid and Smart, 2004; Huang and Bordey, 2004).
Given that the functions of NMDARs are determined by the subunit composition, knowledge of the subunit composition of NMDARs in cerebellar interneuron should be important to the better understanding of how NMDARs regulate the SC-PC synaptic transmission. However, the subtype of NMDARs in cerebellar stellate cells remains to be elucidated. With the in situ hybridization method, Akazawa et al. reported that there was only NR2D mRNA found in molecular layer (Akazawa et al., 1994). In contrast, an immunostaining study in 2000 suggested the existence of NR2A, NR2C/2D receptors in stellate/basket cells (Thompson et al., 2000). Duguid and Smart reported that all four types of NR2 subunits were expressed in cerebellar stellate cells (Duguid and Smart, 2004). And a recent study showed NR3B immunoreactivity in stellate/basket cells (Wee et al., 2008).

Our study utilized electrophysiology recording combined with pharmacological tools to investigate the subunit composition of extrasynaptic NMDA receptors in cerebellar stellate cells. CPP at low concentration (0.2µM), 3µM ifenprodil and 0.1µM PPDA were applied to detect NR2A/2B, NR2B and NR2C/2D respectively. By measuring the reduction of the total evoked currents in response to the subunit selective/preferred antagonists, we found that both NR2B and NR2C/2D receptors may be present at somatodendritic sites of stellate cells, with the possibility of NR1/2B/2D triheteromeric assembly. However it was not clear whether NR2A-containing receptors are present at the somatodendritic sites. On the other hand, application of PPDA at low concentration did not produce any change in the miniature IPSC frequency, indicating that NR2C/2D-containing receptors alone could not constitutively regulate GABA release.
Results

4.2.1 PPDA shows stronger inhibitory effects on NR2C/2D subunits than on NR2A/2B subunits.

Recent studies showed that 1-(phenanthren-2yl-carbonyl) piperazine-2, 3-dicarboxylic acid (PPDA) was a NR2C/2D preferred NMDA receptor antagonist (Feng et al., 2004; Lozovaya et al., 2004), and it was used to investigate the activity of NR2D subunit during the NMDAR-mediated LTP in granule cells in dentate gyrus (Harney et al., 2008). Based on the Ki values of PPDA for inhibiting the responses from recombinant NMDA receptors (Table 4.1A), we estimated the percentage inhibition of NMDAR responses by 0.1 µM PPDA with the Michaelis-Menten equation (Table 4.1B). Our calculation showed that PPDA, at the concentration of 0.1 µM, inhibits ~50% of the responses mediated by NR2C or NR2D subunits, but only blocks 15.4% and 24.4% of responses from NR2A and NR2B respectively. Since the blockade of NR2C/2D seems only 2~3 fold higher than NR2A/2B, we further tested the effectiveness of PPDA on native NR2A/2B receptors. Pharmacological study and single channel recordings showed that in mouse cerebellar granule cells, NMDARs at the mossy fiber-granule cell synapse solely contain NR2A and NR2B subunits at postnatal 6 or 7 days (Cathala et al., 2000). We examined the effects of PPDA on the NMDAR-mediated synaptic currents in animals at this age. As shown in Fig 4.1 (n = 4), there was a small but insignificant decrease in current amplitude (10.2 ± 6.4%) and charge transfer (20.2 ± 9.9%) during the perfusion of 0.1µM PPDA. The extent of blockade is similar to the estimated percentage inhibition of NR2A and NR2B by 0.1 µM PPDA (Table 4.1B). The percentage blockade of the charge transfer in our
experiment is lower than the estimated value about NR2B, and higher than that about NR2A, probably reflecting a replacement of NR2B with NR2A during the development of the synapses (Cathala et al., 2000).

<table>
<thead>
<tr>
<th>A</th>
<th>$Ki$ (µM)</th>
<th>$NR1a/NR2A$</th>
<th>$NR1a/NR2B$</th>
<th>$NR1a/NR2C$</th>
<th>$NR1a/NR2D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R)-CPP</td>
<td>0.041 ± 0.003</td>
<td>0.27 ± 0.02</td>
<td>0.63 ± 0.05</td>
<td>1.99 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>PPDA</td>
<td>0.55 ± 0.15</td>
<td>0.31 ± 0.02</td>
<td>0.096 ± 0.006</td>
<td>0.125 ± 0.035</td>
<td></td>
</tr>
</tbody>
</table>

(Feng et al., British Journal of Pharmacology, 2004)

<table>
<thead>
<tr>
<th>B</th>
<th>% Inhibition</th>
<th>$NR1a/NR2A$</th>
<th>$NR1a/NR2B$</th>
<th>$NR1a/NR2C$</th>
<th>$NR1a/NR2D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2µM CPP</td>
<td>83.0%</td>
<td>42.6%</td>
<td>24.1%</td>
<td>9.1%</td>
<td></td>
</tr>
<tr>
<td>0.1µM PPDA</td>
<td>15.4%</td>
<td>24.4%</td>
<td>51.0%</td>
<td>44.4%</td>
<td></td>
</tr>
<tr>
<td>0.2µM PPDA</td>
<td>26.7%</td>
<td>39.2%</td>
<td>67.6%</td>
<td>61.5%</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1: Low concentrations of CPP and PPDA preferentially inhibit NR2A/2B and NR2C/2D receptors, respectively.

(A) Published Ki values for inhibiting the responses of recombinant NMDA receptors expressed in xenopus oocytes (Feng et al., 2004). (B) The percentage inhibition of NMDAR responses by low concentration of CPP and PPDA was determined by the Michaelis-Menten equation ($V/V_{max} = [S]/(K+[S])$), based on data shown in (A).
Fig 4.1: PPDA (0.1 µM) did not affect synaptic currents via NMDARs that are known to contain NR2A/2B subunits. (A) Evoked EPSCs were recorded at -60 mV in an Mg$^{2+}$-free ACSF that contained NBQX, picrotoxin and glycine, and thus were mediated by NMDARs. (B) The application of 0.1 µM PPDA caused a small but insignificant reduction in the current amplitude (10.2 ± 6.4%) and the area (20.2 ± 9.9%). Data were normalized to the values before PPDA application.

4.2.2 NR2B and NR2D subunits may be expressed outside of the parallel fiber-to-stellate cell synapse
Cerebellar stellate cells express NMDA receptors at extrasynaptic sites (Carter and Regehr, 2000; Clark and Cull-Candy, 2002, Sun and Liu, 2007). We delivered a burst of 4 stimuli at 100Hz to the parallel fibers to generate glutamate spillover. This protocol evoked a slow current that was completely blocked by 10µM CPP in the postsynaptic cell when it was voltage clamped at +40mV. NMDAR-mediated currents from individual cells varied from 45.8pA to 712.3pA with an average of 286.1 ± 59.5pA (n =12), most values between 100 and 300pA (n = 7). The decay time of NMDAR-mediated currents was measured as a ratio of the current charge transfer over the amplitude. The average value of the decay time is 333.2 ± 96.2ms (n = 12). The current amplitude and decay time in our experiments are consistent with the previous report by Carter and Regehr (Carter and Regehr, 2000).

To further identify the subtypes of NMDARs, we applied chemicals that preferentially antagonize certain NR2 subunits. As shown in Table 4.1, CPP at low concentration (eg. 0.2µM) inhibits most currents through NR1/2A receptors (83%) and 42.6% of currents through NR1/2B receptors, but has limited effects on NR2C or 2D-containing receptors. CPP (0.2 µM) application reduced the current amplitudes and charge transfer by 18.5 ± 7.1% and 18.0 ± 3.9% respectively (Fig 4.2 Aa and D, p < 0.05, n = 3). On one hand, this decrease might be due to the blockade of NR2A. Given the percentage inhibition of CPP on NR2A (Table 4.1), the number of NR2A-containing receptors might be ~20% of the total receptors activated. Considering that NR1a/NR2A receptors display high single channel conductance (Cull-Candy et al., 2001), the portion of NR2A could be even lower. On the other hand, this 18% reduction may result from the inhibition of NR2B receptors.
In this case, one can estimate that the number of NR2B receptors possibly take up ~45% of the total receptors.

To investigate these possibilities, we tested the effects of ifenprodil (3 µM), a highly selective inhibitor to NR1/2B receptors (Kew et al., 1996; Allgaier et al., 1999). Interestingly, we observed a 43.5 ± 4.1% decrease in NMDAR-mediated current amplitude (Fig 4.2 Ba and D, p < 0.001, n = 5) and 39.8 ± 6.8% decrease in current area (p < 0.01, n = 5) when 3 µM ifenprodil was applied. The data indicated that NR2B-containing receptors composed at least 40% out of the total NMDA receptors. This is consistent with the second interpretation described above (that the 18% decrease was caused by the blockade of NR2B by 0.2 µM CPP). And it indirectly suggested that functional NR2A subunit is perhaps either not present in stellate cells or expressed at a very low level.

To test the existence of NR2C/2D receptors, we applied PPDA at the concentration of 0.1 µM. Amplitudes of NMDAR-mediated currents were reduced by 35% when 0.1 µM PPDA was applied. Comparably, there was a 36% decrease in current area in the presence of PPDA (Fig 4.2 Ca and D). According to previous research, 0.1 µM PPDA inhibits ~ 24.4% of currents from NR1/NR2B receptors (Table 4.1). Given the portion of NR2B receptors estimated above (>= 40%), we deduced that around 1/3 of the PPDA inhibition came from the blockade of NR2B receptors. Assuming that 2/3 of the inhibition was purely due to the blockade of NR2C or NR2D-containing receptors, one can estimate that the NR2C/2D receptors compose around 50% of overall NMDARs. A
simple explanation is that the entire NMDAR population activated by glutamate spillover consists of only NR1/NR2B receptors and NR1/NR2C or 2D receptors. However, this explanation is very unlikely given the possible existence of triheteromeric receptors.

Evidence from the single channel recording and from pharmacology study suggested that triheteromeric receptors containing two types of NR2 subunits are expressed in different brain regions (Pina-Crespo and Gibb, 2002; Brickley et al., 2003; Brothwell et al., 2008; Chamberlain et al., 2008). To test the possibility of triheteromeric receptors in stellate cells, the decay time constant of NMDAR EPSCs was measured before and after the addition of ifenprodil or PPDA. We assumed that NR2B and NR2D subunits dominate the NMDAR population at the extrasynaptic sites. If the ifenprodil-sensitive component was contributed by NR2B-containing diheteromeric receptors, the rate of decay should be slowed in ifenprodil (Brothwell et al., 2008). Similarly, the rate of decay should accelerate in the presence of PPDA if the PPDA-sensitive component was dominated by NR2D-containing diheteromeric receptors. Figure 4.2 (Bb and Cb) showed that there was no significant difference in decay time constant of NMDAR currents between in control conditions and in the presence of 3µM ifenprodil or 0.1µM PPDA. Consistently, the application of 0.2µM CPP did not change the decay time constant of the macroscopic NMDAR-mediated currents (Fig 4.2 Ab). However, there was an increase in the decay time constant after the wash-out of CPP of which the reason is unknown. Nevertheless, the data suggested that the ifenprodil-sensitive NMDAR currents or the PPDA-sensitive NMDAR currents are mediated by a similar NMDA receptor population to the total NMDAR currents. Therefore it is likely that NR2B and NR2D subunits form
triheteromeric receptors outside of PF-SC synapses in stellate cells.
Fig 4.2: CPP, ifenprodil, and PPDA partly reduced NMDAR-mediated currents in stellate cells (Aa, Ba, Ca) NMDAR-mediated currents were recorded before (black), during (red) and after (green) the application of three inhibitors. (Ab, Bb, Cb) In order to compare kinetics of currents evoked with or without inhibitors, amplitudes of sample current traces were normalized (upper panel). The decay time of currents were plotted over time (lower panel, Ab: n = 3, Bb: n = 5, Cb: n = 4). (D) Summary of the reduction in current amplitudes and area caused by individual inhibitors. Control amplitudes and area were normalized to 1.

4.2.3 Blockade of NR2C/2D-containing receptors did not affect the basal GABA release from cerebellar stellate cells

Bursts of PF activity could induce long-lasting potentiation in GABA release (I-LTP) in cerebellar stellate cells. The induction of I-LTP requires the activation of NMDARs by the glutamate spillover, as well as the downstream signals including cAMP, PKA and RIM1α (Liu and Lachamp, 2006; Lachamp et al., 2009). To further understand the essential role of NMDARs in I-LTP, it is important to identify the specific NR2 subunit(s) that regulates the GABA release.

Preliminary studies by Dr. Lachamp suggested that the NMDAR-dependent I-LTP was abolished in the presence of 0.1µM PPDA (data not shown). Therefore, the activation of NR2C/2D-containing receptor might be required for the induction of activity-dependent I-LTP. However, it is unknown whether NR2C/2D-containing receptors have constitutive effects on GABA release. Here we investigated whether the basal frequency of GABA release is regulated by NR2C/2D-containing receptors. We compared the frequency of mIPSCs prior to and during the application of 0.1µM PPDA. As shown in Figure 4.3 (n = 3), the application of PPDA did not alter the mIPSC frequency compared to without PPDA (before the PPDA application or after the washout of PPDA). Therefore it is likely
that the inhibition of NR2C/2D receptors does not affect the basal pattern of GABA release. A direct explanation is that NR2C/2D receptors are not involved in the regulation of GABA release from stellate cells. However, 0.1µM PPDA only block ~50% of NR2C/2D receptors (Table 4.1). It is not known whether complete inhibition of NR2C/2D receptors would have effects on GABA release. Another possibility is that the basal GABA release is controlled by NR2B receptors and NR2C/2D receptors together. Therefore blockade of NR2C/2D alone is not sufficient to suppress GABA release. A third explanation is that the application of PPDA had little effects on NR2C/2D receptor because they were not activated in a resting condition by the ambient glutamate.

![Figure 4.3](image)

Fig 4.3: Blockade of NR2C or NR2D alone does not affect basal pattern of GABA release. Plot of average mIPSC frequency vs. time in response to 0.1µM PPDA (p > 0.05, n = 3).

**Discussion**

Cerebellar stellate cells express NMDA receptors at extrasynaptic sites on the dendrites (Clark and Cull-Candy, 2002). As functional roles of cerebellar NMDARs have been gradually revealed, their subunit composition is not thoroughly understood. Our
electrophysiology data suggested that NMDARs at somatodendritic compartment might contain NR2B and NR2C/2D subunits. Although NR2C/2D receptors may be involved in the NMDAR-mediated I-LTP (data not shown), it is likely that they do not regulate basal GABA release from stellate cell presynaptic terminals.

Our conclusion about the NMDAR subunit composition in stellate cells was drawn from the present data. The data showed that the macroscopic NMDAR-mediated currents can be inhibited by ifenprodil and PPDA that are selective / preferred to NR2B and NR2C/2D receptors respectively. This idea was also supported by some indirect evidence from previous studies. For instance, the average decay time of the current evoked at +40mV (333ms) in our study is close to the decay time for the recombinant NR1-1a/2B receptors that is 300ms (Vicini et al., 1998). This implicated the existence of NR2B receptors outside of PF-SC synapses. On the other hand, we previously reported that NMDAR-mediated currents in cerebellar stellate cells can be evoked in the presence of 1mM Mg\textsuperscript{2+} at the hyperpolarized potential (Sun and Liu, 2007). The low Mg\textsuperscript{2+} sensitivity implied the expression of NR2C/2D subunits in cerebellar stellate cells (Momiyama et al., 1996).

However, due to the limitation of the pharmacological tools, there are a few questions not addressed in the current research. First, it is not verified whether functional NR2A subunits exist at the extrasynaptic sites. Analysis of the current data suggested that NR2A receptors possibly contribute very little to the overall NMDAR-mediated response (See Results 4.2.2). In subsequent study, the existence of NR2A-containing receptors should be further tested with the Zn\textsuperscript{2+} chelator, TPEN \((N,N',N'',N'''\text{-tetakis [2-pyridylmethyl]})\)
ethylenediamine), which enhances the NMDAR-mediated response by removing the tonic inhibition of NR2A caused by the low concentration of Zn\(^{2+}\) in the external solution (Paoletti et al., 1997). Second, PPDA was utilized in our experiment to identify NR2C/2D-containing receptors. A defect of this method is that NR2C and NR2D subunits display indistinguishable sensitivity to PPDA (Table 4.1). Although in cerebellum NR2C subunit is expressed at high levels only in granule cells (Cull-Candy et al., 2001), a few contrary studies reported the appearance of NR2C subunit in molecular layer or even in the presynaptic varicosity of stellate cells (Thompson et al., 2000; Duguid and Smart, 2004). Therefore, in the future study we could detect whether NR2C is expressed in stellate cells by examining the effect of 0.1\(\mu M\) PPDA in NR2D knockout animals.

Our current data suggested that NR2C or 2D subunit, together with NR2B, might form triheteromeric receptors extrasynaptically. The similar pattern of NR2D subunit distribution has been identified in cerebellar Golgi cells, where both NR1/2D diheteromeric receptors and NR1/2B/2D triheteromeric receptors are present out of synapses (Misra et al., 2000; Brickley et al., 2003). The extrasynaptic co-existence of NR2B and NR2D was also found in other brain regions such as hippocampus, substantia gelatinosa and substantia nigra pars compacta (SNC) (Momiyama, 2000; Lozovaya et al., 2004; Jones and Gibb, 2005). While there is no evidence about NR1/NR2D diheteromeric receptors at any central synapse (Cull-Candy, et al., 2001), a recent report showed the presence of synaptic NR1/2B/2D triheteromeric receptors in developing SNC dopaminergic neurons (Brothwell et al., 2008). Extrasynaptic NR2D-containing receptors
may also be recruited into synapses during the LTP of NMDAR-mediated synaptic transmission (Harney et al., 2008). Therefore, the distinct membrane localization of NR2D-containing receptors might be dynamically regulated during development and neuronal plasticity.

The stimulation of PFs in our experiment activates the somatodendritic NMDARs in stellate cells. However, the axonal NMDARs may not be effectively activated as well due to the effects of glutamate transporters (Huang and Bordey, 2004). Therefore, it is difficult to identify the subunit composition of axonal NMDARs with subunit selective/preferred antagonists. Axonal NMDARs have been attributed to be responsible for the induction of long-term potentiation of GABA release (I-LTP) in cerebellar interneurons (Petralia et al., 1994; Glitsch and Marty, 1999; Duguid and Smart, 2004; Huang and Bordey, 2004). This idea was supported by the detection of NMDARs located on axons. For example, first, the NMDAR immunoreactivity was shown in the molecular layer of cerebellar tissue, implying the dendritic and axonal expression in stellate/basket cells (Petralia et al., 1994). Second, in cultured stellate cells, the immunolabeling for the NMDAR subunits showed that NR1 and all four NR2 subunits are present on axon varicosities (Duguid and Smart, 2004). Third, NMDA elicited single channel currents in patches excised from axonal varicosities of cultured cerebellar interneurons (Fiszman et al., 2005). However, recently Christie and Jahr (2008) demonstrated that NMDARs are exclusively expressed at somatodendritic sites in cerebellar stellate cells. And the activation of somatodendritic NMDARs would result in the passive propagation of dendritic depolarization onto axons, which activates the axonal voltage-sensitive calcium
channels and allows the Ca\(^{2+}\) entry near the GABAergic synaptic terminals (Christie and Jahr, 2008). Thereby this finding might provide an alternative explanation on mechanisms of NMDAR-dependent I-LTP in cerebellar interneurons.

Despite the uncertainty about the membrane localization of NMDARs that are essential in the induction of I-LTP, our preliminary research suggested that the induction of I-LTP probably depends on specific subunit(s) of NMDARs. It showed that the stimulation of PFs failed to induce I-LTP in the presence of 0.1µM PPDA, but 3µM ifenprodil did not significantly affect the induction of I-LTP (data not shown, contributed by Dr. Philippe Lachamp). This indicated that NR2C or 2D, instead of NR2B-containing receptors might be required for the induction of I-LTP. However, due to the indistinguishable inhibitory effect of PPDA on NR2C and NR2D, it is difficult to identify which specific subunit is involved. To address this question, we will examine whether I-LTP can be induced in NR2D knockout animals in future studies.
Chapter 5

Action potentials regulate AMPA receptor phenotype via a calmodulin and protein synthesis-dependent pathway

Introduction

$\alpha$-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate type glutamate receptors (AMPA receptors) mediate the majority of fast excitatory synaptic transmission in the central nervous system (CNS). AMPA receptors in different neuronal circuits, as well as in different cell types display different functional properties exemplified by the single channel conductance, the permeability to $\text{Ca}^{2+}$ and the sensitivity to polyamine (Burnashev et al., 1992; Jonas et al., 1994; Raman et al., 1994; Geiger et al., 1995). The differences mainly result from the subunit composition of AMPA receptors. In the cerebellar cortex, Purkinje cells express GluR2-containing ($\text{Ca}^{2+}$-impermeable) AMPA receptors, whereas GABAergic interneurons in the molecular layer predominantly express GluR2-lacking ($\text{Ca}^{2+}$-permeable) receptors at the excitatory synapses. Similarly, GABAergic interneurons possess high level of $\text{Ca}^{2+}$-permeable receptors compared to the neighboring principal neurons, in many other brain regions such as hippocampus, visual cortex, striatum, and basolateral amygdala (Geiger et al., 1995; Racca et al., 1996; He et al., 1998; He et al., 1999; He et al., 2001; Chan et al., 2003). Compared to the principal neurons that express high-level GluR2-containing receptors, AMPA receptors in GABAergic interneurons mediate particularly fast EPSCs. This allows GABAergic
interneurons to play distinct functional roles such as the long-range synchronization of gamma oscillations in hippocampus (Fuchs et al., 2001).

Little is known about the mechanisms that determine the low level expression of GluR2 subunit in GABAergic interneurons. Work from our lab demonstrated an increased expression of GluR2 mRNA in response to the broadened action potential waveform in cerebellar GABAergic interneuron (Liu et al., submitted). This finding implied a regulatory effect of a neuron’s spiking pattern on its AMPAR subtype. In support of this idea, a recent research suggested that the deprivation of action potential up-regulates GluR2-containing AMPARs through an elevated expression of β3 integrins (Cingolani et al., 2008). Some previous studies also showed that following the blockade of action potentials and/or AMPAR activity, there was an increase in the proportion of GluR2-lacking receptors in hippocampal neurons (Ju et al., 2004; Thiagarajan et al., 2005; Sutton et al., 2006). Hence, the blockade of action potential firing possibly alters the subunit composition of AMPA receptors.

Changes in the AMPAR subunit composition mentioned above occurs during the induction of homeostatic plasticity, the best studied form of plasticity caused by the altered action potential firing. In the process of homeostatic plasticity, the neurons detect changes in their action potential firing and consequently up-or down-regulate the strength of all the synapses to stabilize the firing rate (Turrigiano et al., 1998; Turrigiano and Nelson, 2004; Wierenga et al., 2005). A number of Ca^{2+}-dependent signaling pathways have been proposed to underlie the synaptic scaling (Turrigiano, 2008). For example, a
recent study indicated that the blockade of postsynaptic firing for 4 hours triggers synaptic up-scaling that is mediated by the suppression of somatic \( \text{Ca}^{2+} \) influx, a reduction in CamKIV activity, and the activation of a transcriptional pathway (Ibata et al., 2008). It would be interesting to know whether these mechanisms also regulate the subtype of synaptic AMPA receptors.

To further understand the role of action potential firing in the regulation of AMPAR subunit composition in GABAergic interneurons, we incubated the acute cerebellar slices with TTX, kynurenic acid and picrotoxin. Following 3 hour blockade of spontaneous action potentials (sAPs) and both excitatory and inhibitory synaptic transmission, we found that the expression of GluR2-containing receptors was enhanced at PF-stellate cell synapses, while the level of GluR2-lacking receptors was unchanged. We also found that the increase in GluR2 expression may require a reduction of \( \text{Ca}^{2+} \) influx during the suppression of sAPs, the involvement of calmodulin signaling, as well as intact protein synthesis machinery. However, interruption of the transcription pathway did not significantly affect the phenomenon caused by the TTX application. Therefore, our results indicated that sAPs firing might be an important factor regulating the subunit composition of synaptic AMPARs in GABAergic interneurons.

**Results**

5.2.1 Blockade of spontaneous excitatory and inhibitory synaptic transmission did not change AMPAR subunit composition at the parallel fiber-to-stellate cell synapse
Stellate cells in the cerebellar molecular layer predominantly express AMPA-type glutamate receptors that lack GluR2 subunit (Liu and Cull-Candy, 2000). These GluR2-lacking receptors are permeable to Ca\(^{2+}\), blocked by intracellular polyamine at depolarized potentials (Mishina et al., 1991; Bowie and Mayer, 1995; Geiger et al., 1995; Kamboj et al., 1995), and therefore display an inwardly rectifying I-V relationship in the presence of spermine (Fig 5.1 A and B, n = 3, RI = 0.23 ± 0.05). To investigate the regulatory effects of spontaneous action potentials (sAPs) on AMPAR subtype at the synapse, we need to block the postsynaptic sAPs in cerebellar stellate cells with TTX. However, the bath application of TTX would also inhibit the AP firing in presynaptic cells. This would lead to a reduced presynaptic neurotransmitter release and a consequent suppression of synaptic transmission in postsynaptic stellate cells. The synaptic activity itself has impacts on the subtype of AMPARs at the PF-stellate cell synapse (Liu and Cull-Candy, 2002). Thus to detect the effects of postsynaptic sAPs on AMPAR subtype, we first inhibited both excitatory and inhibitory synaptic transmission in stellate cells with kynurenic acid and picrotoxin as control. Three hour incubation of cerebellar slices with 1mM kynurenic acid (KA) and 0.1mM picrotoxin (PTX) did not change the current amplitude, kinetics and the rectification index of the I-V relationship of sEPSCs mediated by synaptic AMPA receptors (Fig 5.1 C and D, n = 8, RI = 0.32 ± 0.04, p > 0.05). Therefore KA and PTX were included in the 3 hour incubation in the rest of experiments, and data from KA and PTX incubation was mentioned as control.
Fig 5.1: Cerebellar stellate cells predominantly express GluR2-lacking AMPARs at parallel fiber-stellate cell synapses (A and B) Spontaneous EPSCs in stellate cells showed an inwardly rectifying I-V relationship, indicating the presence of GluR2-lacking receptors at the synapse. (C and D) Cerebellar slices were incubated with 1mM kynurenic acid (KA) and 100µM picrotoxin (PTX) for 3 hours prior to the recording. The I-V relationship remained inwardly rectifying, indicating this treatment did not change the synaptic AMPAR phenotype.

5.2.2 Blockade of spontaneous action potential firing enhances the expression of Ca\(^{2+}\)-impermeable AMPARs at the parallel fiber-to-stellate cell synapse

Acute cerebellar slices were incubated with 0.5µM TTX, 1mM KA, and 0.1mM PTX to block the firing of spontaneous action potentials. After 3 hour incubation with TTX, as shown in Fig 5.2, current amplitude at +40mV was significantly increased from 8.25 ± 0.38pA to 14.40 ± 0.86pA (Fig 5.2 B, n = 10, p < 0.00005). In the presence of
intracellular spermine, currents at +40mV are solely mediated by GluR2-containing receptors, thereby the increased current amplitude suggested an enhanced expression of GluR2-containing receptors. As a result, the I-V relationship of AMPAR-mediated currents was converted from inwardly rectifying towards linear (Fig 5.2 Ab). The rectification index (RI) increased from 0.32 ± 0.04 at control level to 0.70 ± 0.06 (Fig 5.2 C, p < 0.001). Consistent with RI, ratio of the amplitude at +40mV to that at -60mV increased from 0.21 ± 0.02 to 0.35 ± 0.03 (p < 0.001). Blockade of action potential, however, did not significantly alter the amplitude at -60mV (TTX: -43.33 ± 3.33pA vs. control: -40.84 ± 2.22pA, p > 0.5). Incorporation of GluR2 subunit into the AMPARs has been shown to prolong the decay time constant of EPSCs (Liu and Cull-Candy, 2000; 2002). Consistently, as shown in Figure 5.2D TTX treatment caused a significant increase in the decay time constant of AMPAR-mediated currents recorded at -60mV (254 events from control, 450 events from TTX treatment, Kolmogorov-Smirnov test, p < 0.002). These results suggested that blockade of spontaneous action potential firing up-regulates the expression of Ca\(^{2+}\)-impermeable receptors, but does not change the expression of Ca\(^{2+}\)-permeable receptors at the parallel fiber-to-stellate cell synapse.
Fig 5.2: Suppression of postsynaptic action potentials alters synaptic AMPAR phenotype in cerebellar stellate cells.

Cerebellar slices were incubated with 0.5µM TTX in the presence of 1mM kynurenic acid and 100µM picrotoxin for 3 hours. Spontaneous EPSCs were then measured at various holding potentials. (A) Upper panel: average EPSCs at +40 and -60mV. Lower panel: I-V relationship of EPSCs became nearly linear following TTX treatment (right) compared to control (left), indicating an increase in synaptic AMPA receptors that contained the GluR2 subunit. (B) Average EPSC amplitudes of control and TTX-treated cells. (C) Summary of rectification index. (D) Comparison of kinetics of currents between control (solid) and TTX treatment (dot) (Kolmogorov-Smirnov test, p < 0.002).
5.2.3 Spontaneous action potentials regulate the AMPAR subtype through a calmodulin-dependent pathway

We further explored the molecular mechanism underlying the TTX-induced enhancement in the expression of Ca\(^{2+}\)-impermeable AMPAR subtype. A direct effect of the suppression of sAPs is that voltage-gated calcium channels would not be activated normally to allow Ca\(^{2+}\) influx and the increase in intracellular calcium concentration.

According to Dr. Yu Liu’s experimental data, 3 hour incubation with 0.5\(\mu\)M \(\omega\)-conotoxin GVIA, an N-type calcium channel blocker, caused the I-V relationship of AMPAR-mediated currents to change from inwardly rectifying towards linear compared to the control. The rectification index (RI) is 0.53 ± 0.13 (Fig 5.3A, n = 4, p < 0.05), and the ratio is 0.35 ± 0.06 (n = 4, p < 0.005). This suggested that blockade of spontaneous action potential firing might regulate AMPAR subtypes by reducing Ca\(^{2+}\) entry through voltage-gated calcium channels.

Calmodulin is a ubiquitously expressed calcium-binding protein which regulates a number of cellular functions. Therefore, it is likely to be a target modulated by calcium level and regulates the AMPAR subtype. In experiments conducted by Dr. Yu Liu, cerebellar slices were incubated with 50\(\mu\)M W7, a calmodulin antagonist, for 3 hours. It was found that inhibition of calmodulin activity caused an increase in current amplitudes at +40mV that is similar to TTX treatment (W7: 14.26 ± 2.76pA vs. TTX: 14.40 ± 0.86pA). There was no difference in current amplitude at -60mV between W7 and TTX treatment (W7: -42.36 ± 7.44pA vs. TTX: -43.33 ± 3.33pA). Also, the RI of I-V relationship for W7 treatment is 0.56 ± 0.06 (n = 5), and the ratio is 0.34 ± 0.02. Neither
of them is significantly different from that produced by TTX treatment (Fig 5.3B). The slightly lower RI of EPSCs following W7 treatment than that from TTX treatment might be caused by a reversal potential closer to 0mV. More interestingly, co-incubation with TTX and W7 did not produce further changes in the I-V relationship or the ratio (Fig 5.3B). Thus, we deduced that blockade of action potential firing might enhance the expression of Ca\(^{2+}\)-impermeable AMPARs by suppressing the calmodulin activity.

Fig 5.3: Suppression of Ca\(^{2+}\)/calmodulin activity increased the rectification index of I-V relationship.

Cerebellar slices were incubated with 500nM \(\omega\)-CTX GVIA to inhibit N-type Ca\(^{2+}\) channels (A) for 3 hours. Following these treatments the I-V relationship of spontaneous EPSCs became more linear than the control, indicating an increase in the expression of GluR2-containing receptors. (B) Cerebellar slices were incubated with 50\(\mu\)M W7, a calmodulin inhibitor, or with both W7 and TTX, for 3 hours. Following W-7 treatment the I-V relationship of sEPSCs became
more linear compared with control. Inclusion of W7 during TTX incubation did not cause any further change in the rectification of sEPSCs.

5.2.4 Protein synthesis is required for the action potential-dependent regulation of the AMPAR subtype

The prolonged blockade of action potentials and miniature synaptic transmission has been shown to enhance dendritic protein synthesis (Sutton et al., 2004; Sutton et al., 2006). Considering that the enhanced expression of Ca\(^{2+}\)-impermeable receptors was induced by a similar treatment (blockade of action potential and synaptic transmission), we studied whether protein synthesis plays a role in the action potential-dependent regulation of AMPAR subtypes. Two types of protein synthesis inhibitors were separately co-applied with TTX.
Fig 5.4: The TTX-induced change in synaptic AMPA receptor phenotype requires protein synthesis. Cerebellar slices were incubated with protein synthesis inhibitors (100µM cycloheximide (CHX) or 40µM anisomycin (ASM)) with or without TTX for 3 hours prior to EPSC recordings. (A) Following the treatment with cycloheximide and TTX, EPSCs showed an inwardly rectifying I-V relationship. (B) Anisomycin also prevented the TTX-induced change in
We first tested the effect of Cycloheximide (CHX), a eukaryotic protein synthesis inhibitor that blocks tRNA translocation to and from ribosomes. The treatment with TTX and CHX together did not change the current amplitude at -60mV (Fig 5.4 A, -48.59 ± 2.49pA, n = 4, p > 0.05). However, the average amplitude at +40mV is 11.88 ± 0.31pA (Fig 5.4 A, n = 4, p < 0.005), which is significantly lower than that after TTX treatment. As a result, the I-V relationship of CHX and TTX treatment displayed an inwardly rectifying pattern with a RI of 0.40 ± 0.05 (Fig 5.4A and C, n = 4), and a ratio of 0.25 ± 0.01. This RI is different from TTX treatment (p < 0.02), but similar to the control. To verify the role of protein synthesis in the TTX-induced phenomenon, we used a second protein synthesis inhibitor, anisomycin (ASM) that prevents peptide bond formation. Co-incubation with TTX and ASM resulted in a similar inwardly rectifying I-V relationship (Fig 5.4 B and C). Thus, it is likely that inhibition of protein synthesis at least partly abolishes the increase in Ca\textsuperscript{2+}-impermeable receptors induced by action potential blockade. To be cautious about possible side effects from these two protein synthesis inhibitors, cerebellar slices were then incubated with CHX or ASM alone. The treatment with protein synthesis inhibitors alone did not alter the rectifying pattern of I-V relationship compared to the control (Fig 5.4 A and B, n = 4, p > 0.05). Therefore, the reduced protein synthesis activity by itself may not alter the native AMPAR subtypes at the parallel fiber-to-stellate cell synapse. Instead, a simple explanation could be that the protein synthesis activity is augmented by the blockade of action potentials, and thus the enhances expression of Ca\textsuperscript{2+}-impermeable AMPARs.
Discussion

Activity blockade could increase the intrinsic excitability of GABAergic interneurons via a BDNF-dependent pathway (Desai et al., 1999). In the current study, we demonstrated a new type of synaptic plasticity induced by the blockade of spontaneous action potentials. Mouse cerebellar stellate cells naturally express mainly Ca\(^{2+}\)-permeable AMPARs at PF-stellate cell synapses. Three hour treatment with TTX enhanced the expression of Ca\(^{2+}\)-impermeable receptors at the PF-stellate cell synapses. This process could be mimicked by the blockade of calmodulin activity. It is likely that calmodulin is a downstream signal of the action potential firing. Interestingly, protein synthesis machinery is required for the increase in Ca\(^{2+}\)-impermeable receptors that is triggered by the blockade of sAPs. Our study revealed that a blockade of spontaneous action potentials in cerebellar GABAergic interneurons triggered the switch of AMPAR subtype from Ca\(^{2+}\)-permeable to Ca\(^{2+}\)-impermeable. This suggested that spontaneous action potential firing may be involved in the maintenance of a high level expression of Ca\(^{2+}\)-permeable AMPARs.

Action potential firing is essential for many aspects of neuronal development and physiology. In the mammalian neocortex, formation of horizontal axon branches is associated with an increased frequency of spontaneous action potential (sAP) firing, and a blockade of sAPs by TTX significantly suppressed axonal branching (Uesaka et al., 2005). Moreover, during the process of development, a blockade of spontaneous action potential firing also increased the number of functional excitatory synapses, indicated by enhanced expression of presynaptic markers, GluR1 subunit, as well as associated increase in mEPSC frequency and amplitude (Lauri et al., 2003). sAPs also play a role in
the regulation of mature neuronal connectivity. A study with hippocampal cell culture showed that 48hrs blockade of neuronal activity with TTX promotes silent synapse formation by surface delivering NR2B-containing NMDARs in mature neurons. And these newly formed silent synapses were rapidly converted into functional synapses after the release of TTX inhibition (Nakayama et al., 2005).

sAP firing is also important for maintaining a balance between excitatory and inhibitory synaptic transmission, as well as the normal intrinsic neuronal excitability. Inhibition of neuronal activity seems to down-regulate inhibitory synaptic transmission (Swanwick et al., 2005); while studies about homeostatic plasticity demonstrated that deprivation of neuronal action potentials up-regulates the excitatory synaptic transmission (Turrigiano et al., 1998) due to the recruitment of AMPARs into synapses (Turrigiano and Nelson, 2004). There is a proportional increase in different AMPAR subtypes in spinal and neocortical neurons (O'Brien et al., 1998; Wierenga et al., 2005), and a selective elevation of GluR2-lacking receptors in hippocampal neurons (Thiagarajan et al., 2005; Sutton et al., 2006) in homeostasis. In contrast, our study suggested that the deprivation of action potential specifically increases the proportion of GluR2 subunit without changing the amplitude of EPSCs mediated by synaptic AMPARs in cerebellar interneurons. Thus the synaptic plasticity induced in our experiments probably represents a distinct mechanism.

Our study about the mechanisms underlying this synaptic plasticity revealed that the increase in Ca$^{2+}$–impermeable receptors requires the reduced Ca$^{2+}$ influx through the voltage-sensitive calcium channels. And the blockade of the activity of calmodulin, a
Ca\textsuperscript{2+}-dependent sensor, mimicked the effects triggered by action potential blockade. This implied a possible signaling pathway triggered by the TTX treatment: the blockade of spontaneous action potentials reduces Ca\textsuperscript{2+} influx and hence suppresses calmodulin, and the reduced calmodulin activity causes the elevated expression of GluR2-containing receptors. Our data also suggested that protein synthesis is involved at a certain step in this process. It is, however, not yet clear what molecular mediator(s) link the depressed calmodulin activity and the protein synthesis (perhaps of GluR2). Calmodulin binds to a variety of protein kinases including Ca\textsuperscript{2+}/Calmodulin-dependent kinase III (CamKIII). CamKIII is expressed in cerebellum (Nairn et al., 1985) and is reported to be highly concentrated at the dendritic PSD (Asaki et al., 2003). Activated CamKIII phosphorylates eukaryotic elongation factor 2 (eEF2) and renders it inactive, thus inhibits protein synthesis at the elongation stage (Nairn and Palfrey, 1987; Ryazanov and Davydova, 1989; Iizuka et al., 2007). Therefore, these facts together with our own data may imply the mechanism of the TTX-induced increase in synaptic GluR2-containing receptors. That is, calmodulin activity is suppressed by a reduction in calcium influx during sAP blockade, rendering CamKIII less active; and the decrease in CamKIII activity releases the inhibition of eEF2, allowing augmented protein synthesis (perhaps of GluR2). One defect of this scenario is that so far it is unknown whether functional CamKIII is expressed in cerebellar interneurons. Another unclear point is that whether the intact translational machinery is required for the synthesis of GluR2 subunit itself, or for other proteins that facilitate GluR2 trafficking. In addition it is unknown why enhanced protein synthesis selectively increases GluR2-containing but not GluR2-lacking receptors. While these questions need to be addressed in the future study, there are alternative explanations
about the signaling pathways. For instance, first it is possible that the protein synthesis is independent of calmodulin activity. Second, NMDA receptors might also participate in this TTX-induced increase in Ca\(^{2+}\)-impermeable receptors. In our experiment the incubation with kynurenic acid inhibits both AMPARs and NMDARs. Recent studies showed that the blockade of miniature NMDAR-mediated currents accelerates synaptic scaling up with an alteration in AMPAR subunit proportion, by affecting dendritic protein synthesis (Sutton et al., 2006).

The increase in GluR2-containing receptors induced by the blockade of sAPs did not change the amplitude of sEPSCs at -60mV. However, it caused a slower decay time constant of AMPAR-mediated EPSCs than the control. According to a study in our lab, when current amplitudes remain the same, the prolonged EPSCs significantly enhance the probability of action potential firing in cerebellar stellate cells (Savtchouk and Liu, submitted). Therefore, a predicted functional outcome of the TTX-induced synaptic plasticity is to increase the action potential firing and then probably enhance the GABA release onto Purkinje cells. Since TTX was bath applied in our experiment, it should block the neuronal activity of cerebellar granule cells as well. Assuming this blockade might cause the up-scaling of mossy fiber-granule cell synaptic transmission, one can expect a potentiated firing rate in granule cells and the enhanced glutamate release from PFs onto Purkinje cells. In this case, the increased GABA release from stellate cells might be indispensable to compensate the elevated excitatory inputs onto Purkinje cells, stabilizing the output of Purkinje cells. Therefore this AMAPR subtype switch induced
by the blockade of spontaneous action potentials may have a physiological role on the stability of the entire network in cerebellar cortex.

Overall, this study showed that a conventional homeostatic plasticity inducing protocol can trigger another type of synaptic plasticity in cerebellar GABAergic interneurons-- a switch in AMAPR subtype at parallel fiber-to-stellate cell synapses. This type of synaptic plasticity might participate in the maintenance of a stable output of cerebellar cortex.
Chapter 6
Discussion

The cerebellum is a brain region that is critically involved in the motor and emotional learning and memory (Marr, 1969; Maxwell, 1976; Sacchetti et al., 2002; Ito, 2006). As the only output of cerebellar cortex, the firing rate and pattern of Purkinje cells is believed to underlie the mechanisms of the cerebellar learning and memory. Therefore, the synaptic plasticity of Purkinje cells, especially that of parallel fiber-Purkinje cell synapses, has been extensively studied. A well studied example is that the acquisition of associative eyelid conditioning is attributed to a sustained reduction in Purkinje cell action potential firing that is caused by the attenuated synaptic transmission between parallel fiber and Purkinje cells, namely cerebellar LTD (Ito et al., 1982; Xia et al., 2000; Hansel et al., 2001). On the other hand, the inhibitory synaptic transmission between GABAergic interneurons (stellate and basket cells) and Purkinje cells (e.g. SC-PC synapse) prevents the latter from over excited, controls the synaptic integration of Purkinje cells, and enables the precise time of firing in Purkinje cells (Midtgaard, 1992; Hausser and Clark, 1997; Jaeger and Bower, 1999; Mittmann et al., 2005). The synaptic transmission of SC-PC synapses can be potentiated by the repetitive stimulation of climbing fiber through a postsynaptic \( \text{Ca}^{2+} \)-dependent signaling pathway (Kano et al., 1992; Hashimoto et al., 1996; Kano et al., 1996), or by the stimulation of the parallel fibers that triggers a sustained enhancement in GABA release from stellate cells (Liu and Lachamp, 2006; Lachamp et al., 2009). The inhibitory LTP expressed at both pre- and
postsynaptic sites suppresses the spike output of Purkinje cells, and therefore contributes to the associative cerebellar learning.

However, the synaptic plasticity of GABAergic interneuron itself is less studied. To obtain an insight into the role of inhibitory transmission in the cerebellar circuit, we investigated the long-term synaptic plasticity, particularly at the parallel fiber-stellate cell synapse (PF-SC synapse) in cerebellar GABAergic interneurons. The fast excitatory synaptic transmission at PF-SC synapses is mediated by GluR2-lacking AMPA receptors that are permeable to Ca$^{2+}$ (Liu and Cull-Candy, 2000). High-frequency stimulation of the presynaptic parallel fibers decreases GluR2-lacking receptors while increases GluR2-containing receptors at this synapse. This process is mediated by Ca$^{2+}$ influx through GluR2-lacking AMPARs themselves (Liu and Cull-Candy, 2000, 2005; Gardner et al., 2005). The alteration in the level of GluR2 subunit of synaptic AMPA receptors produces remarkable changes in synaptic transmission and neuronal circuits (Liu and Zukin, 2007). For instance, overexpression of GluR2 subunit in hippocampal GABAergic interneurons disrupts gamma oscillation synchronization (Fuchs et al., 2001). In GluR2-deficient mutant mice, the superficial dorsal horn neurons displayed an enhanced LTP induction and reduction in LTD induction (Jia et al., 1996; Youn et al., 2008). Therefore the AMPAR subtype switch following PF stimulation may markedly impact synaptic transmission and integration in cerebellar GABAergic interneurons. Our previous and current study further explored distinct mechanisms that regulate the subtype of AMPA receptors.
First, we explored the mechanism underlying the subtype switch of AMPARs induced by the stimulation of the parallel fibers. An in vivo study demonstrated that sensory stimulation could elicit a burst of action potentials in cerebellar granule cells (Chadderton et al., 2004). We applied a burst of stimulation to the parallel fibers to mimic this physiological relevant action potential firing. We found that this stimulation causes glutamate spillover that activates the NMDA receptors located outside of PF-SC synapses. The activation of NMDARs induced a long-lasting AMPAR subtype switch from GluR2-lacking to GluR2-containing at PF-SC synapses. The induction of the synaptic plasticity requires elevated concentration of intracellular Ca\(^{2+}\) that activates PKC. PKC activity is both necessary and sufficient for the PICK-dependent delivery of GluR2-containing receptors to the synapse. The activation of PKC alone, however, is required but not sufficient for the removal of GluR2-lacking receptors from the synapse.

Second, we found that the blockade of action potential firing in stellate cells also enhanced the expression of GluR2-containing receptors at the PF-SC synapse. This phenomenon is likely to involve the reduced Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels and a subsequent suppression of calmodulin activity. The transcriptional activity may not be required for this process (data not shown). Instead, protein synthesis (possibly of GluR2 subunit itself) seems to be crucial in the increase in GluR2-containing receptors induced by the blockade of action potential firing in stellate cells. Therefore, the spontaneous action potential firing in stellate cells might maintain the calmodulin activity that suppresses the synthesis of GluR2 subunit. This possibly contributes to the pattern of
The number of functional GluR2-containing receptors increases in both types of synaptic plasticity. However, comparison between these two types of synaptic plasticity suggested distinct induction mechanisms, as well as perhaps opposite functional significance. First, PF stimulation causes intense excitatory inputs onto stellate cells and the activation of the glutamate receptors that is required for the induction of AMPAR subtype switch. In contrast, TTX administration, plus kynurenic acid and picrotoxin, completely inhibit the action potential firing and synaptic activity in stellate cells. Second, although both processes depend on Ca\(^{2+}\)-sensitive mechanisms, increased Ca\(^{2+}\) entry through glutamate receptors is a must during PF stimulation-induced AMPAR subtype switch; whereas TTX-induced phenomenon requires a suppressed Ca\(^{2+}\) influx. Third, the PF stimulation-induced phenomenon occurs rapidly (no more than 30 min after stimulation), while the blockade of sAPs increases the expression of GluR2-containing receptors in a 3hr incubation with TTX. Although effects of shorter TTX application was not tested, some recordings (lasting for more than 1hr) of AMPAR-mediated EPSCs with TTX-containing perfusion showed no progressive change in current amplitudes (data not shown). A possible physiological relevance of this long-term TTX treatment is that AP firing pattern might be chronically altered under certain physiological and pathological conditions. Fourth, the PF stimulation triggers a PICK-driven GluR2 trafficking into the PF-SC synapse. The GluR2-containing receptors are probably delivered from an intracellular pool (Rubio and Wenthold, 1999; Lee et al., 2001; Fu et al., 2003) or via a lateral
migration from the perisynaptic sites (Gardner et al., 2005). However, in the TTX-induced process, it is likely that the increased GluR2-containing receptors are newly synthesized after the shutdown of sAPs. This might explain why TTX induces a slower increase in GluR2 slower than PF stimulation. Fifth, PF stimulation causes both an increase in GluR2-containing receptors and a decrease in GluR2-lacking receptors, and thus depressed the current amplitude at the resting potential (Liu and Cull-Candy, 2000; Sun and Liu, 2007). This should reduce the AMPAR channel conductance as well as prolong the decay times of EPSCs (Isaac et al., 2007). In contrast, TTX treatment increases GluR2 expression and prolongs the decay time of AMPAR-mediated currents, but not changes the expression of GluR2-lacking receptors. Current amplitudes of AMPAR-mediated EPSCs at -60mV remained unchanged after TTX treatment. A recent study in our lab demonstrated that prolonged EPSCs promote the EPSP-AP coupling in stellate cells, whereas the reduced channel conductance decreases the probability of the EPSP-coupled action potential firing in stellate cells. The net effect of the AMPAR subtype switch following PF stimulation is to reduce the EPSP-AP coupling and prevent the firing of AP doublets in response to two consecutive stimuli (Savtchouk and Liu, submitted). In contrast, the TTX treatment leads to a slower decay time and an unaltered amplitude in EPSCs, therefore would augment the action potential firing rate in stellate cells and perhaps potentiate GABA release.

This presumable functional outcome of increase in GluR2-containing and no change in GluR2-lacking receptors may have an impact on Purkinje cell spike timing following fear learning. That is because fear-inducing stimulus with fox urine triggers an altered
AMPAR subtype that is comparable to the phenomenon induced by TTX treatment (Liu et al., submitted), and thus may increase IPSC frequency at SC-PC synapses. Consistently, a recent study showed that there is an increase in frequency but not amplitude of IPSC onto PCs after fear learning. Within certain time window, two excitatory inputs from PFs are integrated in Purkinje cells to elicit a spike. The potentiated GABAergic transmission prevents the temporal fidelity of the time window from being degraded, and thus allows a greater probability of PC firing (Scelfo et al., 2008).

Our study in TTX-induced increase in GluR2 also suggested a possible mechanism that maintains a low GluR2 level in GABAergic interneurons: The spontaneous action potential firing allows Ca$^{2+}$ entry that activates calmodulin. Calmodulin activity might suppress the synthesis of proteins that are essential for expression of GluR2-containing receptors at the synapse. In contrast to this speculation, a recent work from our lab demonstrated that the broadened action potential firing in stellate cells enhances the transcription of GluR2, and consequently increases GluR2 expression at the synapses (Liu et al., submitted). Thus two opposite treatments (elimination and broadening of sAPs) result in similar increase in GluR2-containing receptors and both might cause increased probability of action potential firing in stellate cells. Hence, one can speculate that probably the specific waveform of sAPs in GABAergic interneurons plays an essential role in the low-level expression of GluR2. Width of action potential may directly control the amount of Ca$^{2+}$ influx through the voltage-gated Ca$^{2+}$ channels. Studies of bidirectional synaptic plasticity suggested that postsynaptic calcium concentration might be a key variable that favors the induction of either LTP or LTD, via the activation of
protein kinases or phosphatases (Castellani et al., 2005). It is possible that in cerebellar GABAergic interneurons, optimal Ca$^{2+}$ concentration is required for the maintenance of low GluR2 level; either higher or lower concentration due to the change in AP waveform (with an extreme case in our study, AP blockade) would probably enhance GluR2 expression. If this is the case, current data may indicate that higher Ca$^{2+}$ concentration activates GluR2 mRNA transcription, whereas lower Ca$^{2+}$ concentration possibly releases the inhibition of GluR2 synthesis.

We also identified the subtype of NMDA receptors in stellate cells which are involved in the induction of inhibitory LTP (I-LTP) (Duguid and Smart, 2004; Fiszmann et al., 2005; Liu and Lachamp, 2006; Lachamp et al., 2009). NMDA receptors in stellate cells can be activated by a burst stimulation of the parallel fibers. In our experiment the evoked macroscopic NMDAR-mediated currents are sensitive to ifenprodil and PPDA at a low concentration. We deduced that NMDARs at extrasynaptic sites contain NR2B and NR2D subunits, and they probably form triheteromeric receptors. NR2D subunit possesses higher binding affinity to glutamate and lower sensitivity to Mg$^{2+}$ blockade (Cull-Candy et al., 2001), therefore allows the activation by lower concentration of glutamate at more negative potentials. Our study showed that the activation of NMDARs (probably NR2D-containing receptors) induces a switch in AMPAR subtype, and hence may reduce action potential firing probability (Savtchouk and Liu, submitted). The activation of NMDARs in stellate cells by PF stimulation was also shown to induce a long-lasting potentiation in miniature IPSC frequency (Liu and Lachamp, 2006; Lachamp et al., 2009). Together these data suggested that the activation of extrasynaptic NMDARs
by PF stimulation probably has two opposite effects on stellate cell long-term plasticity: to suppress action potential-coupled GABA release and to promote the AP – independent GABA release.

Repetitive stimulation of parallel fibers causes long-term potentiation of excitatory synaptic transmission at the PF-Purkinje cell synapses (Hansel et al., 2001), as well as the I-LTP in stellate cells via the activation of NMDARs. Hence it seems that the long-term plasticity in stellate cells serves as a dynamic inhibitory signal to counterbalance the enhanced excitatory inputs onto Purkinje cells. This could be a mechanism that maintains the stability of the sole output of the cerebellar cortex. Noticeably, PF stimulation induces I-LTP at SC-PC synapses via a presynaptic mechanism (Lachamp et al., 2009); whereas the stimulation of climbing fibers causes an I-LTP expressed at the postsynaptic site (Kano et al., 1992; Hashimoto et al., 1996; Khodakhah and Armstrong, 1997). It is unknown if there is an additive effect on I-LTP during the co-activation of parallel fibers and climbing fibers.

Our research suggested that the synaptic AMPA receptors at the parallel fiber-stellate cell synapse undergo dynamic changes from Ca\(^{2+}\)-permeable to Ca\(^{2+}\)-impermeable form. This alteration could be in response to either external sensory stimulation (PF stimulation) or the suppression of intrinsic activity (spontaneous action potential). In addition, the release probability of GABA at the axonal terminals is also controlled by a non-synaptic mechanism via an NMDAR-mediated pathway. Further investigation of the regulatory role of NMDA receptors (eg. subunit-specific regulation) would be important for the
understanding of the feedforward inhibition of stellate cells. Overall, long-term synaptic plasticity may regulate the firing pattern of stellate cells, and have an impact on the network of cerebellar cortex. In the future study, the role of stellate cells in cerebellar learning and memory could be further tested by studies in the correlation between the synaptic plasticity and alteration in animal behaviors.
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Vita

Lu Sun

Education

**Ph.D. candidate in Biology** 08/2003-present
The Pennsylvania State University, University Park, PA.
**Dissertation** Long-term synaptic plasticity in mouse cerebellar stellate cells

**B.S. Biotechnology** 09/1999-06/2003
The Chukechen Honor College of Zhejiang University, Hangzhou, China.

Academic Services and Awards

Reviewer for the SDE/GWIS Fellowship 2008
J Ben and Helen D. Hill Memorial Fund Award, Penn State University 2008
Braddock Award, Penn State University 2007
J Ben and Helen D. Hill Memorial Fund Award, Penn State University 2007
J Ben and Helen D. Hill Memorial Fund Award, Penn State University 2005
Braddock Fellowship, Penn State University 2003

Publications and Abstracts


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