RECIPROCAL INTERACTIONS BETWEEN SYNAPTIC AMPA RECEPTORS AND
ACTION POTENTIAL FIRING IN CEREBELLAR STELLATE CELLS

A Dissertation in
Biology
by
Iaroslav Savtchouk

Submitted in Partial Fulfillment
of the Requirements
for the Degree of
Doctor of Philosophy

August 2011
The dissertation of Iaroslav Savtchouk was reviewed and approved* by the following:

James H. Marden  
Professor of Biology  
Chair of Committee  
Interim Assistant Department Head for the Department of Biology

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

Timothy J. Jegla  
Assistant Professor of Biology

Dezhe Jin  
Associate Professor of Physics

Matthew Whim  
Associate Professor of Cell Biology and Anatomy, LSU HSC  
Special Member

S. June Liu  
Associate Professor of Cell Biology and Anatomy, LSU HSC  
Special Member

*Signatures are on file in the Graduate School
ABSTRACT

The cerebellum controls motor learning and is also implicated in higher cognitive functions including fear conditioning and emotional response. Inhibitory interneurons such as stellate cells are emerging as central players in cerebellar learning and function. These neurons receive their excitatory input through mostly GluR2-lacking, calcium permeable AMPA receptors. However, GluR2-lacking receptors can be replaced with GluR2-containing, calcium impermeable receptors by synaptic activity. GluR2 insertion at the Ca-permeable synapse typically increases the decay time of a synaptic current while reducing the amplitude. An increase in GluR2-lacking receptors occurs during both learning and pathological experience, in neurons that normally express GluR2-containing receptors. Despite the fact that this GluR2 subtype switch was observed in many brain regions, the functional implications of such change are unknown. Here I investigated how GluR2 incorporation affected the ability of synaptic input to evoke an action potential (AP) in the stellate cells. Firstly, we find that fear exposure increases GluR2 gene transcription and synaptic GluR2 incorporation in stellate cells. Secondly, I show that a fear-induced increase in GluR2-containing receptors results in a significant enhancement of evoked AP probability, and a change in the firing pattern of the interneurons due to a change in post-synaptic facilitation.

Changes in pre-synaptic activity are known to modify the composition of AMPA receptors. Here, we tested whether changes in post-synaptic APs alone can alter the synaptic AMPAR phenotype. First, we show that increasing the AP duration can facilitate the incorporation of synaptic GluR2-containing receptors in a transcription-dependent manner. Second, I observe that dendritic synapses are mostly GluR2-lacking close to the soma, but mainly GluR2-containing at the distal dendrites, which requires protein synthesis. Broadening the action potentials results in a decrease in GluR2-containing receptors along the dendrite. Therefore the APs can have antagonistic effects on synaptic GluR2 levels.

In conclusion, I show in stellate cells, that the switch in AMPAR phenotype can change the probability of APs, and conversely, that changes in AP duration can alter the AMPAR phenotype. This reciprocal interaction between AMPAR remodeling and AP generation may result in a stable dendritic GluR2 gradient that may be altered by emotional experience.
# TABLE OF CONTENTS

List of Figures ........................................................................................................................................ viii
Acknowledgements .................................................................................................................................... x

## Chapter 1 Introduction ................................................................................................................................. 1

- General overview ........................................................................................................................................ 2
- Cerebellar structure and function .................................................................................................................. 2
  - Anatomy of the cerebellum .......................................................................................................................... 2
  - Circuitry of the cerebellar cortex ................................................................................................................ 3
  - Evidence of cerebellar involvement in emotional stress, anxiety, spatial learning, and fear conditioning .................................................................................................................. 3
  - PF-PC LTD as a model of cerebellar learning ............................................................................................. 5
  - Incompleteness of the Albus-Marr (PF-PC LTD) model of cerebellar learning..................................... 6
- The role of interneuron network in the cerebellar learning ............................................................................. 7
  - Evolutionary expansion of cerebellar cortex recruits interneurons ............................................................ 7
  - Cerebellar interneurons shape the sensory fields of Purkinje cells ............................................................. 7
- The role of synaptic plasticity at inhibitory terminals ....................................................................................... 8
- Mechanism of Stellate-to-Stellate cell iLTP in the cerebellum ...................................................................... 9
- AMPA receptors and their role in excitatory transmission .......................................................................... 10
- Role of Ca-Permeable (GluR2-lacking) receptors in synaptic plasticity ..................................................... 10
  - Ca permeability ....................................................................................................................................... 11
  - AMPAR polyamine block ............................................................................................................................. 11
  - Conductance and kinetics ............................................................................................................................. 12
  - Effect of GluR2 on the functional output of neurons .................................................................................. 12
  - Regulation of subunit trafficking .................................................................................................................. 13
- Changes in action potential (AP) firing due to changes in kinetics and amplitude ...................................... 14
- Synopsis of our investigation ......................................................................................................................... 15
Chapter 2  A single fear-inducing stimulus causes transcription-dependent increase in GluR2-containing AMPA receptors ................................................................. 16

Abstract ................................................................................................................................. 17
Introduction .......................................................................................................................... 17
Materials and Methods: ....................................................................................................... 18
   Behavioral experiments: ................................................................................................. 18
   Behavioral video recording and analysis ........................................................................ 19
   Single cell real time RT-PCR: ....................................................................................... 20
   In situ hybridization ........................................................................................................ 20
   Slice preparation and electrophysiology: ....................................................................... 20
   Statistics: ......................................................................................................................... 21
Results .................................................................................................................................. 22
   Exposure to the smell of a predator increases freezing response in mice ............... 22
   Emotional stress alters synaptic AMPAR subtype by increasing GluR2-containing receptors ........................................................ 22
   Stress-induced decrease in sEPSC rectification supports the observed GluR2 increase .................................................................................................................. 22
   Beta-adrenergic receptors are needed for stress-mediated GluR2 increase..... 23
   Emotional stress causes an increase in GluR2 mRNA in stellate cells ................. 23
   Norepinephrine increases the level of GluR2 mRNA expression ......................... 24
   Action potential duration may control transcription of mGluR2 ......................... 24
Discussion .............................................................................................................................. 25

Chapter 3 Remodeling of Synaptic AMPA Receptor Subtype Alters the Probability and Pattern of Action Potential Firing ........................................................................ 33

Introduction .......................................................................................................................... 34
Materials and Methods: ....................................................................................................... 35
Results: .................................................................................................................................. 35
   A stress-induced increase in EPSC duration enhances EPSP–AP coupling:..... 35
   Transcription-independent switch in AMPAR subtype reduces EPSP–AP coupling .......................................................... 36
   Replacing GluR2-lacking with GluR2-containing receptors abolishes postsynaptic paired-pulse facilitation: ................................................. 38
Paired-pulse facilitation enhances the probability that stellate cells will fire AP doublets in response to two consecutive stimuli .......................... 39

Discussion.................................................................................................................. 44

References.................................................................................................................. 43

Chapter 4 Inhibition of Ca\(^{2+}\)-activated large-conductance K\(^{+}\) channel activity alters synaptic AMPA receptor phenotype in mouse cerebellar stellate cells ....................... 46

Introduction ............................................................................................................... 47

Methods: .................................................................................................................. 48

Results: .................................................................................................................... 50

Stellate cells express a large amount of large-conductance Ca\(^{2+}\) activated potassium (BK) channels: ................................................................. 50

Prolonged BK block increases synaptic GluR2-AMPARs in stellate cells ........ 51

Action potential (AP)-induced Ca\(^{2+}\) influx determines GluR2-AMPAR phenotype: ................................................................................................................. 51

Intracellular Ca\(^{2+}\) stores and Ca\(^{2+}\)-induced Ca\(^{2+}\) release may influence synaptic GluR2 content ........................................................................................................ 52

Discussion.................................................................................................................. 53

Chapter 5 Dendritic synapses show distance-dependent variation in the inclusion of GluR2-containing AMPA receptors in cerebellar stellate cells ......................................................... 59

Abstract: .................................................................................................................... 60

Introduction: ............................................................................................................. 61

Materials and Methods: .......................................................................................... 62

Results ....................................................................................................................... 64

Synaptic GluR2 increases at distal dendrites of stellate cells ............................ 64

Action potential back-propagation sets up a master gradient for determining receptor composition ................................................................................. 65

Ongoing protein synthesis but not transcription is necessary for maintaining the distance-dependent synaptic GluR2 gradient ......................... 67

Discussion: ............................................................................................................... 69

Distant-dependent differences in AMPAR plasticity at the proximal and distal synapses .............................................................................................................. 69

The origin of synaptic GluR2 gradient within dendrites .................................... 70

The role of Ca\(^{2+}\) in determining AMPA receptor composition .................... 70
The role of TARP and other auxillary proteins in receptor distribution .......... 71
The role of distal receptors in the lateral inhibition in the cerebellum .......... 71

Chapter 6 General discussion ........................................................................... 78
Emotional stress causes increase in GluR2 transcription and
synaptic insertion in stellate cells ................................................................. 79
The significance of increased current decay time after GluR2-insertion .......... 80
Demonstration of functional role of the post-synaptic facilitation
at stellate cells ............................................................................................... 80
The role of stellate cell sensitivity in the cerebellar processing ................. 81
The role of Action Potential duration in determining
GluR2 APMAR phenotype ........................................................................... 81
Conclusion .................................................................................................... 83

References .................................................................................................... 84
LIST OF FIGURES

Figure 1-1. The diagram of major neuronal cell types in the cerebellar cortex. .......................... 4

Figure 2.1. Fox urine increases freezing response in juvenile mice. .......................... 27
Figure 2.2. Fear exposure resulted in an increase in synaptic GluR2-containing AMPARs in stellate cells ................................................................. 28
Figure 2.3. Beta-adrenergic receptors are required for fear-induced GluR2 increase .......... 29
Figure 2.4. Fear exposure causes an long-term increase in GluR2 mRNA. .................... 30
Figure 2.5. Noradrenaline and TEA treatment increase the level of GluR2, but not GluR1 mRNA expression in situ ................................................................. 32

Figure 3.1. Emotional stress prolonged EPSC decay time without changing the current amplitude in stellate cells ................................................................. 36
Figure 3.2. Increase in EPSC decay time enhanced action potential probability in stellate cells ................................................................................................. 37
Figure 3.3. Theta-burst stimulation of parallel fibers induced a switch in AMPAR phenotype. ... 38
Figure 3.4. The theta-burst stimulation-induced change in synaptic conductance reduced the efficacy of EPSP–AP coupling ................................................................. 39
Figure 3.5. Presynaptic stimulation-induced incorporation of GluR2 receptors abolished spermine-dependent postsynaptic facilitation ................................................................. 40
Figure 3.6. Changes in postsynaptic facilitation altered the pattern of AP firing evoked by paired inputs ................................................................................................. 41
Figure 3.7. The stress-induced incorporation of GluR2 receptors did not alter the ability of the paired conductance waveform to evoke an action potential doublet .... 42
Figure 3.8. Supplementary Figure 1 (examples of trace selection criteria) .................. 45

Figure 4.1. BK channels mediate a large portion of voltage-gated K currents in stellate cells.... 55
Figure 4.2. Block of BK channels induces an increase in synaptic GluR2 in stellate cells ...... 56
Figure 4.3. Block of L-type Ca\textsuperscript{2+} channels can prevent IBTX-induced increase in synaptic GluR2 ................................................................................................. 57
Figure 4.4. Ryanodine induces an increase in synaptic GluR2-containing AMPA receptors. .... 58
Figure 5.1. Outward currents increase at distal synapses .........................................................73
Figure 5.2. Distant synapses exhibit properties consistent with GluR2 increase .......................74
Figure 5.3. Rectification differences disappear in the absence of spermine .............................75
Figure 5.4. Action potential back-propagation determines synaptic AMPAR phenotype .........76
Figure 5.5. Ongoing protein synthesis is required for synaptic GluR2 maintenance at distal synapses ..............................................................................................................................................77
ACKNOWLEDGEMENTS


These chapters were re-written to focus on this author’s contribution, but in the interest of maintaining a logical reasoning flow, I have also included some data collected by the collaborators. Some figures contain electrophysiological data from Dr. Yu Liu as indicated in the legend. The in situ hybridization experiment was performed by Drs. Luigi Formisano Yukihiro Takayasu in R. Suzanne Zukin’s lab at Albert Einstein College of Medicine. Real time PCR reactions were performed by Dr. D. Grove, Penn State RNA facility. Dr. Gábor Szabó has generated the transgenic GAD-GFP mice.

I would like to express my gratitude to Dr. S. June Liu, my research advisor. Thank you for all your guidance and patience. Thank you for your personal involvement and dedication. Thank you for teaching me to be a scientist!

I would like to thank Dr. Matthew Whim for continued scientific and personal help.

I would also like to thank the many scientific collaborators over my years at Penn State and LSU-HSC.

I am grateful to all the folks over at Penn State Biology and LSU-HSC Anatomy for making my graduate experience unforgettable. I thank Dr. Chuck Fisher, and Ms. Kathryn McClintock for helping me make up my mind; I never looked back!

I thank my teachers who guided me over the years. I also thank Ronald and Lisa Dusablon for their friendship.

Finally, I would like to acknowledge the US taxpayers/NSF/NIH for providing the financial support for this work.
I am grateful to my parents for their sacrifice that allowed me to accomplish this work.
Chapter 1. Introduction
General overview

The cerebellum is a part of the brain responsible for simple motor learning and Pavlovian conditioning, but is also implicated in higher cognitive functions and memory formation. Cerebellar cortex is an extremely attractive preparation because of an amazingly simple (for any brain region) structure and well-defined circuitry. It is a good model for electrophysiologists and cognitive researchers alike due to the fact that it has only a few neuronal cell types that are morphologically distinct and whose connections are well described (Albus, 1971; Ito, 2000). A number of models describing cerebellar function have been constructed and are currently undergoing experimental evaluation. The role of inhibitory interneurons in both cerebellum and the entire brain is now beginning to be recognized as of central importance in information processing by the brain. In this work, we try to improve our understanding of how the changes at excitatory afferents onto the inhibitory interneurons can shape the functioning of the feed-forward inhibitory loop, and of the entire cortical circuit. In particular, the changes in the subunit composition of AMPA receptors appear to be of a deep functional significance in shaping the output of the interneurons, and thus serve as a focus of our work.

Cerebellar structure and function

Anatomy of the cerebellum

The cerebellum develops from the telencephalon at the point of the cranial flexure of the pons. Visually, the cerebellum appears as an egg-shaped corrugated object located at the back of the brain, adjacent to the pons. Structurally, the cerebellum can be separated into deep cerebellar nuclei and the cortex. The amount of cortex increases greatly from the lower to the higher vertebrates, resulting in a comparatively much greater amount of folding in the mammals (Herrick, 1891). Rostro-caudally, such cortical folding results in an emergence of distinct lobes. Laterally, there is a greater expansion of the hemispheres as compared to the vermal (middle) part, with the ultimate case being that of a human cerebellum, where there is an absolute preeminence of the hemispheres. Despite apparently large differences in the final appearance, there is a great similarity in the development of the cerebellum in mammals (Stroud, 1895). Early subdivisions of the cerebellum by the primary, secondary, and postnodular fissures are identical in all mammals (Smith, 1903), but later differences in development make the number of folds somewhat variable. Nevertheless, the direct matching of the cerebellar folds can be made at least in the vermis (Larsell, 1953).

The cortex is composed of the four distinct layers: the granule cell layer, the Purkinje cell layer, the molecular layer containing the stellate and basket cells, and the external granular cell layer where new granule cells are created and thereafter migrate into the internal granule cell layer.
Circuitry of the cerebellar cortex

The cerebellum receives a number of inputs via cerebellar peduncles composed of cephalic, ventral/pontine, and caudal tracts (Stroud, 1895). The cerebellar cortex receives its excitatory somato-sensory input from the mossy fibers synapsing onto granule cells. The granule cells send their axon into the molecular layer where they provide the excitatory drive onto the Purkinje cell dendrites as well as the Golgi cells and the interneurons (stellate and basket cells) (Figure 1.1). Golgi cells form a feed-back inhibitory loop onto the granule cells, while the molecular layer interneurons provide the feed-forward inhibition onto the PCs. Purkinje cells in turn provide inhibition of the cerebellar nuclei neurons.

Deep cerebellar nuclei cells provide the main output of the cerebellum to the premotor neurons and the brainstem nuclei, with their output shaped by the excitatory pontine projections, the inhibitory Purkinje cells of the cerebellar cortex, and the excitatory inferior olive collaterals that also innervate Purkinje cells as climbing fibers (Albus, 1971; Ito, 2000; Zhang et al., 2004). The coincident activation of the parallel fibers and the climbing fibers is generally believed to be the underlying mechanism of the cerebellar learning (Ito, 2000). Studies of Lurcher mutant with degenerating Purkinje cells indicate that deep cerebellar nuclei alone appear to be sufficient for basic motor learning, but more demanding tasks require cerebellar cortex input (Caston et al., 1995).

Evidence of cerebellar involvement in emotional stress, anxiety, spatial learning, and fear conditioning

Motor involvement of the cerebellum is well established and is a central theme of most models of the cerebellar learning (Albus, 1989; Dean et al., 2010; Ito, 2000). However, the researchers begin to appreciate the importance of cerebellum in other forms of learning and behavior. Early indications of the cerebellar involvement in emotional response and other “higher” cognitive functions came from a study of mutants with localized cerebellar pathologies (Lalonde and Strazielle, 2007). In a Lurcher mutant, a degeneration of Purkinje and granule cells leads to a decrease in motor performance and visual coordination, but actually increases exploratory activity and time spent exploring open arms of the elevated plus maze, suggesting a perturbation in emotional response. When the Lurcher mice were subjected to stressful test in the plus maze, their corticosterone levels increased, indicating an appropriate response of HPA axis, yet their behavior showed much less anxiety (Hilber et al., 2004), suggesting that cerebellum plays a vital role in transforming stressful stimuli into behavioral changes.

Morris water maze tests performed on cerebellectomized wild-type and lurcher mice indicated that spatial information can be learned in the absence of the cerebellum (Hilber et al., 1998). However, if the learning is performed with a cerebellum intact, a consequent cerebellectomy abolishes the stored memory of the learned escape task in the mouse (Hilber et al., 1998), suggesting that under normal conditions the cerebellum is involved in either acquisition, processing, long-term storage, or retrieval of the information. When neuronal
Figure 1-1. The diagram of major neuronal cell types in the cerebellar cortex

Parallel Fibers (PF) are the axons of the granule cells that are located in the granule cell layer. These form synapses on both stellate cells (SC) and Purkinje cells (PC).

A parallel fiber to stellate sell excitatory synapse carries sensory information to the stellate cell. We study the effects of the changes from GluR2-lacking to GluR2-containing AMPA receptors reported at this location.

Stellate cell to Purkinje cell inhibitory synapse provides feed-forward inhibition and has a powerful influence on PC excitability.

(Golgi cells, Basket cells, Stellate-to-stellate cell inhibitory synapses, and certain other cells are omitted for clarity)
communication in cerebellar vermis or DCN was temporarily blocked with localized TTX injections at different time-points post acquisition, it became apparent that there is a critical time window of up to one week when the cerebellum is required for both contextual and acoustic fear memory consolidation (Sacchetti et al., 2002). This group also reported post-synaptic potentiation at the (excitatory) PF-to-PC synapse following fear conditioning (Sacchetti et al., 2004). The potentiation is present in the lobules V and VI but not IX and X (Sacchetti et al., 2004), consistent with the nature of the inputs to these regions. A follow-up study by the same group has shown that the pre-synaptic release probability at the inhibitory synapses onto PCs is also enhanced by fear conditioning (Scelfo et al., 2008). In the most recent study, this group has shown that fear conditioning and the Morris water maze learning required an increase in the number of excitatory connections onto the interneurons in the cerebellum and the hippocampus (Ruediger et al., 2011).

**PF-PC LTD as a model of cerebellar learning**

Climbing fibers provides error input or indication of an unexpected stimulus (Bloedel and Bracha, 1998), which, when coactivated together with sensory inputs from the parallel fiber leads to long-term depression at the parallel fiber-Purkinje cell synapse (Ekerot and Kano, 1985; Ito and Kano, 1982). This heterosynaptic plasticity of the PF-PC synapse causes Purkinje cells to become less excitable by the sensory input (Ito and Kano, 1982), which causes disinhibition on the deep cerebellar nuclear cells presumably enabling the motor response (Ito, 2000). This experimentally observed phenomenon served as a mechanistic implementation of the Marr-Albus hypothesis proposed decades before, wherein climbing fiber error signals reduce the strength of PF-PC synapses (Albus, 1971; Marr, 1969). The contextual learning interpretation of the Marr-Albus model now relied heavily on the idea that inferior olive’s climbing fibers carried error input that actually triggered the learning (Bloedel and Bracha, 1998). This hypothesis received more support after Gellman et al. (1985) experiments in behaving cats that were trained to perform repetitive walking task. Passive and (unexpected) touch to the hide of the cat caused excitation in IO cells but touch stimulation caused by active walking did not, which suggested IO cells respond mostly to unexpected behavior (Gellman et al., 1985). Moreover, in a different experiment with ladder climbing cats, complex spikes (css, triggered by the climbing fiber discharges) were observed in response to random touch of the body, and spontaneously occurred randomly during walk at a frequency of about 1 to 2 css per step (Andersson and Armstrong, 1987). When the ladder step was made to give way unexpectedly and without warning, a time-locked css occurred on average 2 out of 5 steps, during the initial phase of the step suggesting the climbing fiber was relaying an error indication, whenever there was a difference between an expected and actual movement (Andersson and Armstrong, 1987).

How exactly could the IO cells know what is an expected or an unexpected movement (i.e. what is an error)? In vivo recordings from mice suggest that there are distinct subpopulations of IO cells whose firing frequency and oscillation frequency are remarkably constant (Khosrovani et al., 2007). Such cells are capable of being electronically coupled by gap junctions, with the junctions controlled by a GABA input, possibly from cerebellum (Khosrovani
et al., 2007), so that these cells could be coupled into units with distinct I/O relationship based
on the ‘authorized’ action from the cerebellum, forming distinct units cued into the “expected”
sensory input, and discharging once it is not received with the right sequence and timing.
Notably, differential IO activity is observable on a macroscopic scale with fMRI and event-driven
recordings, such as performed by Xu et al. (2006) who noticed that the inferior olive/climbing
fiber is more active in response to unexpected movements and less active in response to the
expected ones.

Incompleteness of the Albus-Marr (PF-PC LTD) model of cerebellar learning

Despite the fact that the CF-triggered long term depression of PF-PC synapses appears
to be a plausible model of cerebellar learning, and that it elegantly incorporates the strong
evidence for inferior olive error detection functionality, there have been some difficulties
obtaining the direct experimental in vivo confirmation for Marr-Albus (Ito, 2000). For one, a
parallel fiber to Purkinje cell LTD was heavily studied in cultured preparations (Chung et al.,
2003) or in ex vivo brain slices (Linden and Connor, 1995); whole-animal studies have been
limited to either anesthetized (Ito and Kano, 1982) or un-anesthetized (Ekerot and Kano, 1985)
de-cerebrate animals. As such, the Parallel fiber-Purkinje cell LTD was never observed in
behaving animals (Bloedel and Bracha, 1998; Ito, 2000) with the exception of one early
experiment by Gilbert and Thach (Gilbert and Thach, 1977). Additionally, newer studies have
indicated that unitary parallel fiber input may already be too weak to evoke Purkinje cell
response, so further reducing the strength of the sub-critical synapse would not be expected to
have any functional effect. In one experiment, dynamic current clamp was used to deliver
modeled excitatory and inhibitory conductances to the Purkinje cell which showed that
excitatory conductances have only a minimal effect on the Purkinje cell action potential timing,
while the inhibitory input onto the cell was much more influential in determining the output
(Jaeger and Bower, 1999). Recordings from Purkinje cell soma from slices at physiological
temperatures could not detect individual excitatory events from the parallel fibers, instead
producing a steady tonic-like level of excitation (Carter and Regehr, 2002). Indeed, it is
estimated that up to 85% of PF-PC synapses do not evoke any detectable voltage response
(Isope and Barbour, 2002). Moreover, a detailed compartmental model of Purkinje cell has
demonstrated that individual EPSPs have synaptic conductances in ranges that are particularly
susceptible to dendritic filtering, and even though an EPSC from a single fiber can have a large
local effect in part of the dendrite, this effect will not propagate to the soma other than a small
part of the tonic excitatory input (Santamaria et al., 2002). Direct experimental evidence
undermining the importance of PF-PC LTD as a learning mechanism comes from the
preliminary reports in the conditional knockout mice lacking Purkinje cell LTD. In these animals
there appear to be no sort-term memory effects and normal motor performance and long-term
learning (Schonewille et al., 2011). On the other hand, experimenters have found that inhibitory
transmission onto Purkinje cells may have a large role in shaping the cell output, with individual
events being both detectable at soma and powerful enough to affect the spike timing (Carter
and Regehr, 2002; Jaeger and Bower, 1999; Santamaria et al., 2002). Perhaps one of the major
The drawbacks of the current implementations of Marr-Albus model is that they almost entirely disregard the inhibitory interneurons (Bower, 2010; Dean and Porrill, 2008). Interneurons house approximately 40% of all inhibitory synapses in the molecular layer (Briatore et al., 2010), which is consistent with the understanding that interneuron synapses are important (albeit so far neglected) players in the cerebellar plasticity (Bower, 2010; Briatore et al., 2010; Dean et al., 2010).

The role of interneuron network in the cerebellar learning

**Evolutionary expansion of cerebellar cortex recruits interneurons**

The evolutionary expansion of cerebellar cortex in “higher” vertebrates was recognized early on (Herrick, 1891). Such expansion is evident both in the total area and in the number of synaptic inputs reaching individual cells (Wilcox et al., 2009). While the matter is still under active debate, it has been suggested that such expansion in birds and mammals resulted in a much greater need for cerebellar interneurons in order to maintain a reasonable excitation/inhibition balance (Bower, 2010). Interestingly, while many fish, amphibians and reptiles lack basket cells and only possess stellate cell connections (Bower, 2010; Waks and Westerman, 1970), the elephant fish (that live in cloudy water and extensively use electro-sensory navigation) appear to have basket-like connections similar to that of the more “advanced” species. An evolutionary expansion in cortical inhibition suggests that interneurons are an essential part of the cerebellar circuit.

**Cerebellar interneurons shape the sensory fields of Purkinje cells**

Cerebellar interneurons have long been an elusive target for the electro-physiologists due to their small size (Jorntell et al., 2010) and fragility. Initial evidence for importance of interneurons in cortical processing comes via exploration of the so called “beam hypothesis”. In the cerebellar cortex, the ascending granule cell axon will bifurcate into two parallel fibers which proceed to form many synapses across hundreds of Purkinje cells along the folium. Therefore, the activation of a granule cell would be expected to activate a strip of cells lying along the same PF – the “beam hypothesis” (Bower, 2010; Braintenberg and Atwood, 1958). This idea has been verified in vitro using focal stimulation of parallel fibers (Gao et al., 2006), but was not observable in vivo (Cohen and Yarom, 1998). Instead, experimenters have noted a much more limited “patchy” activation of PCs immediately above the stimulated granule cells (Rokni et al., 2007). It has been proposed that this could be due to the fact that the ascending granule cell axon synapses are more powerful than the parallel fiber synapses (Santamaria and Bower, 2005) or because the APs de-synchronise as they propagate over long distances, thus losing the ability to activate distal cells (Bower, 2010). Recent experimental evidence obtained via photochemical and electric stimulation of granule cells suggests there is in fact no difference between the ascending and parallel fiber inputs (Walter et al., 2009). However, the dendrites of
the molecular layer inhibitory interneurons (stellate and basket cells) are located within the same sagittal plane as the PC dendrites and thus receive same PF input as the neighboring PCs. Correspondingly, the presence of feed-forward inhibition modulated by the interneurons can explain the severely limited longitudinal spread of PC activation; conversely, the application of GABAergic antagonists restores the full-beam PC excitation (Bower, 2010; Santamaria et al., 2007) expected of the pure GC-PC circuit.

Feed-forward inhibition can serve several roles in a circuit, such as sharpening the population response by restricting the activation spread, providing gain control to maintain the response of PC in the linear range to a widest-possible range of inputs (activation of 1 to 150,000 synapses: 5 orders of magnitude variability in input), and varying the integration response window of the PC. Additionally, the presence of interneurons activated within the same “beam” is predicted to form a neighboring lateral zone of inhibition where a PC receives only a feed-forward interneuron drive but not the PF drive, essentially inverting the response to the input. The asymmetrical, eccentric nature of the dendritic and axonic fields of the interneurons (Sultan and Bower, 1998) makes the existence of lateral inhibition fields an expected feature of the cortical circuitry. The ability of the same group of inputs to cause excitation and inhibition in two adjacent populations of PCs would helpful in motor control which involves co-activation of the paired flexor-extensor muscle groups (Garwicz, 2000). A direct demonstration of the importance of the feed-forward synaptic inhibition on the cerebellar learning was provided by a transgenic animal without functioning GABA-A synapses onto the PCs (Wulff et al., 2009). The mice had no large baseline motor deficiencies but exhibited a severe lack of gain and phase adaptation in the vestibular ocular reflex test (VOR) (Wulff et al., 2009). In general, the ability of the interneurons to shape the sensory fields, shape the response, and even reverse the polarity of the response of the PCs highlights the central role of interneurons in cortical computation.

The role of synaptic plasticity at inhibitory terminals

The long and short-term changes in the glutamate release probability at excitatory synapses are extensively studied and described (Nicoll and Malenka, 1995). Such changes were believed to be the main foundation of learning and memory. Even after the discovery of inhibitory neurotransmission, the latter was mostly relegated only the routine, maintenance roles such as controlling excitability of glutamatergic networks (Abbott and Nelson, 2000). With the more recent discovery of multiple forms of plasticity present at the inhibitory synapses (Chevaleyre and Castillo, 2003; Kano et al., 1992; Stelzer et al., 1994), there came an emerging appreciation for the role the inhibitory interneurons could play in cognitive processes, including some forms of behavior learning (Scelfo et al., 2008).

Inhibitory innervation has been shown to have a powerful effect of the firing pattern of stellate and Purkinje cells (Hausser and Clark, 1997). Other researchers have shown that synaptic inhibition has a large effect on action potential timing, and has the power to synchronize action potential firing across entire subpopulations of neurons both in vivo and in vivo.
silico under specific conditions (Matsuyama, 1976; Prescott and De Koninck, 2003; Tuckwell, 1986; Vida et al., 2006). The changes in inhibition of PCs was observed during eyeblink conditioning and extinction (Jirenhed et al., 2007). When the inhibitory synapses onto PCs were selectively and acutely potentiated using a combination of transgenic/pharmacological manipulation, the mice suffered a large deterioration in the rotarod and walking performance (Wulff et al., 2007), which highlights the role of inhibitory interneurons in the cerebellar motor function. Very fast oscillations (200 Hz) of Purkinje cells due to innervation by PC axon collaterals have been proposed to be an intrinsic characteristic of the cerebellar information processing system (de Solages et al., 2008), and arguably not requiring any input from stellate cell interneurons. However, the synchronicity of stellate cell firing is likely to lead to a timed increase in concurrent inhibition on Purkinje cells resulting either in improved Purkinje cell synchronicity, a possible effect on stimulus-induced spike-timing dependent processing outcome, or even changes in the carrier frequency of the VFOs. An increased synchronicity of interneurons has been shown to result in a large increase of inhibition of pyramidal cells in hippocampus, including the appearance of giant IPSCs (Yang and Michelson, 2001).

Mechanism of Stellate-to-Stellate cell iLTP in the cerebellum

In our lab, Lachamp and Liu have identified of a novel type of stellate-to-stellate cell plasticity, where the activation of NMDA receptors by either repetitive PF stimulation or agonist application can cause a long-term increase of GABA release probability, resulting in long-term potentiation of inhibition (iLTP) (Liu and Lachamp, 2006). Detailed investigation of the mechanism has since been undertaken, and of a particular interest is the evidence implicating RIM1alpha protein in the induction of iLTP (Lachamp et al., 2009). RIM1alpha is an active zone protein that interacts with a number of other proteins to form a scaffold that is needed for normal neurotransmitter release (Schoch et al., 2002) and has been shown to be required for long-term potentiation at some excitatory synapses (Castillo et al., 2002). Liu and Lachamp show that a knockout of RIM1alpha lacks the ability to induce this type of iLTP, which provides a useful tool to dissect the cause and effect changes taking place at the synapse. In future, this knockout could be used to study the effects of SC-to-SC plasticity on Purkinje cell firing, and for monitoring concurrent mitochondrial changes.

The existence of inhibitory-to-inhibitory synapses in the cerebellum provides for a rather unique situation wherein a response to spontaneous EPSCs can be greater than to the “real” (evoked) EPSCs due to the changes in the membrane decay constant caused by coincident feed-forward IPSCs. The existence of plasticity at the SC-to-SC synapse would therefore allow for an additional layer of control wherein the SC network itself can be tuned to recognize certain patterns of input (e.g. shunt away the periodic, repetitive activation, but pick out the unmatched outliers in the excitatory inputs).

Overall, it appears that the SC-to-SC iLTP phenomenon has physiological relevance since it can be induced not only by repetitive PF stimulation and chemical induction, but also by moderate burst stimulation of bundles of parallel fibers under physiological conditions (Lachamp
et al., 2009) suggesting another mechanism for cerebellar memory and reinforcing the functional importance of interneuron coordination. Reports of a similar type of potentiation at the stellate cell to Purkinje cell synapses suggests the iLTP phenomenon in general might have profound behavioral implications, with inhibitory synapse “memory” being important in some forms of associative learning (Scelfo et al., 2008). In summary, the existence of a large number of interneurons in the molecular layer, a number of “memory” mechanisms observed at the inhibitory synapses, and the recent reports of stellate cell involvement in fear learning all suggest that these feed-forward and feedback networks may play a role in cerebellar signal processing.

**AMPA receptors and their role in excitatory transmission**

Both Purkinje cells and stellate cell interneurons receive sensory input through the parallel fiber excitatory synapses. A bulk of fast excitatory transmission in the central nervous system is mediated by α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPARs), which are tetrameric ion channels composed of four possible kinds of subunits numbering GluR1 to GluR4 (Burnashev, 1993; Dingledine et al., 1999). Changes in AMPAR activity and trafficking underlie short and long-term synaptic plasticity, which is thought to be important in learning and memory (Song and Huganir, 2002). A novel type of synaptic plasticity that involves changes in the receptor subunit composition, rather than in receptor number or conductance, has recently been reported (Liu and Cull-Candy, 2000). This subunit remodeling is accomplished by activity-dependent incorporation of GluR2 subunits into synaptic AMPARs (Gardner et al., 2005; Liu and Cull-Candy, 2000). Stellate cells of the cerebellum normally express Ca\(^{2+}\) permeable, GluR2 lacking AMPA receptors, that may be switched to Ca\(^{2+}\) impermeable, GluR2 containing receptors, exhibiting prolonged kinetics and reduced amplitude (Liu and Cull-Candy, 2002). Activation of extra-synaptic receptors can also induce a similar change in subunit composition (Sun and Liu, 2007). Finally, this type of plasticity may also be triggered by mGluR activation (Kelly et al., 2009). Because the cerebellar interneurons are electrically compact (Carter and Regehr, 2002), it is proposed that GluR2 incorporation may have a drastic effect of the input-output processing of small cerebellar interneurons.

**Role of Ca-Permeable (GluR2-lacking) receptors in synaptic plasticity**

The GluR2 incorporation in AMPA receptors has two distinct but profound effects on synaptic plasticity, namely changes in current conductance and in calcium entry. Firstly, GluR2 incorporation significantly decreases the amplitude and increases gating kinetics of the AMPA receptors. Secondly, GluR2-lacking receptors can mediate long-term changes at the synapse. Synaptic activation of such receptors has been shown to induce LTD (Kelly et al., 2009; Liu and Cull-Candy, 2000) or LTP (Lamsa et al., 2007; Oren et al., 2009).
**Ca²⁺ permeability**

By default, an AMPA receptor formed by any of the GluR1, R3, or R4 subunits is highly Ca²⁺ permeable, but incorporation of even a single GluR2 subunit into the tetramer renders the entire receptor Ca-impermeable (Isaac et al., 2007; Washburn et al., 1997). Studies of recombinant receptors in *Xenopus* oocytes have shown for example, that homomeric GluR1, GluR3, or heteromeric GluR1/GluR3 receptors are highly permeable to Calcium, but GluR1/GluR2 or GluR3/GluR2 receptors were essentially impermeable to Ca²⁺, indicating that GluR2 incorporation attenuates intrinsic calcium permeability of the receptors (Hollmann et al., 1991). Single-cell mRNA studies in brain slices have revealed that cells with Ca²⁺ impermeable AMPA receptors had high level of GluR2 mRNA, indicative of the subunit’s importance (Jonas et al., 1994). Comparing cDNA sequences of GluR1-4 subunits have shown that the GluR2 subunit contains a positively charged arginine in place of a neutral glutamine residue in all the other subunits in a portion of the pore region named Q/R site (Jonas and Burnashev, 1995). Remarkably, it was later found that this residue substitution is not present in genomic DNA but happens post-transcriptionally by editing GluR2 mRNA. (Jonas and Burnashev, 1995). Controlling GluR2 incorporation into the receptor could allow cells to control calcium entry and the resulting activation of signaling cascades.

**AMPA polyamine block**

One remarkable property of a AMPARs is that GluR1, 3 or 4-containing receptors show a complex double-rectifying steady-state current-voltage relationship indicating reduced currents at certain positive and negative potentials, while GluR2-containing receptors show simple outward rectification (Verdoorn et al., 1991). Such behavior is explained by the fact that GluR2 lacking receptors are selectively blocked by the naturally-occurring intracellular polyamines both at positive and, to a lesser degree, at negative potentials, imparting a previously puzzling double-rectification behavior initially assumed to be an intrinsic property of the proteins (Koh et al., 1995). This phenomenon of a polyamine-mediated voltage-dependent block is also observed in an inwardly-rectifying potassium channels (Ficker et al., 1994) and even neuronal nicotinic acetylcholine receptors (nAChR) (Haghighi and Cooper, 1998). Interestingly, at negative (resting) potentials, the polyamine block can be partially relived by a fast repetitive receptor activation, providing for a post-synaptic event facilitation that is frequency dependent (Burnashev, 2005; Rozov and Burnashev, 1999). Such property allows GluR2-lacking synapses to “remember” previously encountered transmission, and to vary current strength based on prior synaptic history. Among other things, such actions could compensate for the overall signal reduction during high-frequency events caused by the receptor desensitization or pre-synaptic depression (Burnashev, 2005), thereby improving reliability and signal fidelity of synaptic transmission.
Conductance and kinetics

Another consequence of GluR2 incorporation is the changes in current amplitude and kinetics. In general, a time-course of AMPAR-mediated synaptic transmission is determined by both the receptor de-activation properties and the length of glutamate presence (and receptor desensitization) at the synapse. Studies of recombinant receptors have also shown relatively fast deactivation or desensitization kinetics of AMPA receptors (Mosbacher et al., 1994). Early recombinant AMPAR experiments that employed transfection of human embryonic kidney cells indicated that ionic conductance and kinetic qualities are dependent on the subunit composition (Burnashev, 1993; Lomeli et al., 1994; Swanson et al., 1997). These recombinant studies further indicated that GluR2-containing, Ca$^{2+}$ impermeable receptors exhibit both a lower single-channel conductance as well as prolonged kinetics (Swanson et al., 1997). Interestingly, GluR2 incorporation can reduce the single channel conductance of AMPARs by as much as two-thirds without however increasing the probability of channel opening (Swanson et al., 1997). However, the GluR2-containing channels would no longer be subject to the resting polyamine block (at negative potentials) which can range anywhere from about 40 to 70% at resting potentials (as calculated at -70 to -50 mV for a GluR1 homotetrameric channel) (Bowie and Mayer, 1995). Overall, in the presence of intracellular polyamines we expect GluR2 insertion to result in a modest reduction of amplitude at resting potentials and an increase in the deactivation time of the channel.

When the agonist is present at the synapse for a longer time, the receptor closing is controlled by its desensitization properties. Under prolonged application of glutamate, all AMPA receptors desensitize relatively quickly with a time constant of 1 ms and up to 10 ms, but the exact parameters are determined by the subunit composition and the splice isoforms such as “flip” (slower desensitization) and “flop” (fast desensitization) that change during development (Mosbacher et al., 1994). Flip/Flop isoform duality allows further fine-tuning of receptor kinetics and may play a similar role as GluR2 presence under sustained stimulation or glutamate spillover conditions.

Effect of GluR2 on the functional output of neurons

Given that GluR2 presence can change individual synaptic currents, could massive GluR2 incorporation change the response of interneurons? Over-expressing GluR2 in mutant mice under a GAD67 promoter resulted in slower EPSCs observed in stratum pyramidale fast-spiking interneurons (normally low in GluR2), which lead to a decrease in synchronicity of gamma oscillations of the interneuron network (Fuchs et al., 2001). In response to a single pulse on a stimulating electrode, the authors observed that 6 out of 6 mutant (GluR2 expressing) interneurons fired a doublet action potential, while 0 out of 5 wild-type neurons fired a doublet (Fuchs et al., 2001). While the data is not actually shown and further investigation would be prudent, one could argue that this observation does suggest that the prolonged kinetics of EPSCs mediated by GluR2 receptors have an increased ability to evoke action potentials in the interneurons (Fuchs et al., 2001)
Experiments with a brief application of glutamate to the outside-out membrane patches of various cells in the mouse cochlear nucleus have shown that GluR2-lacking AMPA receptors along the auditory nerve synapses show significantly faster deactivation kinetics (decay tau=0.4-0.5 ms) compared to those excised from around the parallel fiber synapses (decay tau=1.2-2.0 ms, all at room temperature) which is thought to have an important role in audio signal processing and stereo-positioning of the sound (Gardner et al., 2001). In hippocampal interneurons, two distinct inputs onto the same cell exhibited different levels of GluR2 incorporation as evidenced by the rectification index, and the differential sensitivity to philanthotoxin that selectively blocks Ca\(^{2+}\) permeable receptors (Tóth and McBain, 1998). The mossy fiber input was shown to contain GluR2-lacking synapses while the CA3 pathway had GluR2-containing, philanthotoxin insensitive receptors (Tóth and McBain, 1998). Interestingly, while the authors did not perform the comparison of the kinetics and amplitude of the two inputs, the examples shown in figure suggest a markedly faster kinetics and larger amplitude for the GluR2-lacking pathway (Fig. 1 in (Tóth and McBain, 1998)). Although (to the best of my knowledge) a systematic study of GluR2 effects on interneuronal activity and response had not been undertaken prior to our investigation, it appeared likely that GluR2 incorporation would have a large functional significance.

**Regulation of subunit trafficking**

Phosphorylation of glutamate receptors is a common mechanism for controlling their function (Raymond et al., 1993). In particular, AMPA receptor subunits have multiple known phosphorylation sites located on the intracellular C terminal (Song and Huganir, 2002). This process can affect both the conductance properties and the membrane trafficking, including the insertion or endocytosis due to the dynamic interaction with such proteins as GRIP (interacting with GluR2, 3, 4 subunits) and NSF (interacting with GluR2) (Song and Huganir, 2002).

A PDZ-domain containing GRIP (glutamate receptor interacting protein) interacts with a C terminal of GluR subunits and may contribute to AMPA receptor clustering at the synapse (Dong et al., 1997). PICK1 (protein interacting with C kinase) was similarly shown to interact with some GluR2 subunits (Xu et al., 2006). In particular, PICK1 facilitated GluR2 recruitment to the synapse (Xu et al., 2006). The interactions of AMPAR subunits with GRIP and PICK were shown to control the level of GluR2 incorporation to a cerebellar parallel fiber- stellate cell synapses where the repeated stimulation lead to an internalization of the GluR2-lacking receptors by disrupting their interactions with GRIP while recruiting GluR2-containing receptors due to action of PICK (Gardner et al., 2005; Liu and Cull-Candy, 2005). To sum up, there is a large number of subunit-interacting proteins that can differentially bind to different subunits (and their isoforms). Therefore, all of this biochemical machinery may potentially allow the neurons to directly control receptor properties and implement independent adjustment for different input pathways.
Cerebellar stellate cells could be particularly sensitive to the effects of GluR2 plasticity. As opposed to the larger primary neurons that require multiple coincident inputs to reach threshold, cerebellar stellate cells are electrically compact and are potentially capable of firing action potentials in response to several or even a single quantum of neurotransmitter with very precise millisecond timing (Carter and Regehr, 2002). However, this observation was questioned by other experimental reports that the actual link between the incident excitatory input and stellate cell firing is quite weak (Mann-Metzer and Yarom, 2002). A possible explanation is provided by Suter and Jaeger who suggest that only rapid changes in excitatory/inhibitory balance input are capable of evoking stellate cell spikes with a millisecond-level precision, while the prolonged, tonic excitatory input has no effect (Suter and Jaeger, 2004). The task of responding to individual events is further complicated by the fact that a single stellate cell receives inputs from approximately 2000 parallel fibers, each of which can give rise to spontaneous EPSCs which would essentially constitute input signal noise. One might postulate the existence of some mechanism to distinguish between single, quantum-sized spontaneous events, and the real sensory input that comes as a short train of 2-4 EPSCs (Chadderton et al., 2004). Post-synaptic facilitation due to temporary polyamine un-block would be one possible mechanism.
Synopsis of our investigation

In chapter 2 of this manuscript, we begin by examining the effects of acute emotional stress on the AMPAR subunit composition of the cerebellar stellate cells. After the animals are exposed to a strong fearful stimulus, there is an increase in transcription of GluR2 mRNA, and a sustained increase in the synaptic GluR2 content. This has the effect of prolonging the decay time of the synaptic currents.

In chapter 3, we proceed to examine these functional effects of the AMPAR-GluR2 subunit switch on the cerebellar circuit. While the GluR2 switch was commonly observed, the functional consequences were not known. We report that the probability of evoking an action potential in the stellate cells increases with a post-stress GluR2-containing AMPAR current waveform. The firing pattern of the stellate cell is likely to change due to the changes in post-synaptic facilitation.

In chapter 4, we take a closer look at the reasons behind an increase in GluR2 transcription and insertion at the synapse. Stellate cells express high levels of large conductance Ca\(^{2+}\)-activated potassium channels (BK), fire short-duration spontaneous APs, and have mostly GluR2-lacking synapses. We find that a partial block of BK (among other things) can lead to an AP broadening, which in turn causes an increase in Ca\(^{2+}\) influx, leading to an increase in GluR2 transcription.

In chapter 5, we ask if the proximal and the distal dendrites of the stellate cells exhibit different synaptic properties. We find that in addition to a boost in the number of receptors at the distal dendrites, their actual subunit composition switches from GluR2-lacking to GluR2-containing. This has the potential to increase the chance of an AP through the distal inputs. We report that the dendritic gradient may be shifted by varying the duration of the back-propagating somatic AP in the stellate cells, and that protein synthesis plays a role in maintaining synaptic GluR2 gradient. The difference in subunit composition opens up intriguing possibilities for divergence in plasticity, strength, and spatio-temporal integration properties between the proximal and the distal synapses.
Chapter 2. A single fear-inducing stimulus causes transcription-dependent increase in GluR2-containing AMPA receptors
Abstract

Emotional experience can modify neuronal activity and affect learning and memory formation. Long-term persistence of such plasticity is believed to involve changes in gene transcription. In this work we report that an acute stressful experience, exposing mice to the odor of a predator, can increase GluR2 mRNA and the amount of synaptic GluR2-containing AMPA receptors the cerebellar stellate cells. This increase requires the activation of beta-adrenergic receptors. An increase in the action potential duration is sufficient to induce this transcription-dependent GluR2 synaptic change.

Introduction

The regulation of gene transcription is necessary for the brain to transform a transient stimulus into a persistent synaptic modification (West et al., 2002). The best studied postsynaptic modification involves changes in the number and phosphorylation state of AMPA receptors (Barry and Ziff, 2002; Malinow and Malenka, 2002; Song and Huganir, 2002). An additional way to modify synaptic AMPAR properties such as conductance, kinetics, and Ca^{2+} permeability is by changing the AMPAR subunit composition (Cull-Candy et al., 2006; Isaac et al., 2007; Liu and Zukin, 2007). These changes may occur via both controlled receptor trafficking, and the local dendritic protein synthesis (Bredt and Nicoll, 2003; Ju et al., 2004; Mameli et al., 2007; Sutton et al., 2006; Traynelis et al., 2010). However, it is not so far unclear whether experience can alter AMPAR subtype by differentially regulating the transcription of AMPAR subunit genes.

The stress hormone norepinephrine mediates memory consolidation by emotion (Cahill et al., 1994). Norepinephrine plays a central role in motor learning and fear-related memories, which affect synaptic transmission in the cerebellum (Cartford et al., 2004; Sacchetti et al., 2002; Sacchetti et al., 2004; Scelfo et al., 2008). During emotional arousal, norepinephrine can be released in the cerebellum from the projections of locus coeruleus (Bickford-Wimer et al., 1991; Siggins et al., 1971). These fibers terminate in the cerebellar cortex where norepinephrine activates beta-adrenergic receptors to increase the action potential (AP) firing rate of inhibitory stellate cells (Kondo and Marty, 1998; Saitow et al., 2000) and alter the spontaneous firing of Purkinje cells (Bickford-Wimer et al., 1991; Siggins et al., 1971). Additionally, norepinephrine can modulate synaptic plasticity which is widely believed to the molecular foundation of learning and memory. In the hippocampus and visual cortex, norepinephrine lowers the threshold for induction of long-term potentiation by facilitating phosphorylation and synaptic delivery of GluR1-containing AMPARs (Hu et al., 2007; Seol et al., 2007). However, the ability of norepinephrine to alter synaptic AMPAR composition is uncertain.

Cerebellar stellate cells express GluR2-lacking, Ca-permeable AMPARs, which are often observed in inhibitory interneurons (Isaac et al., 2007; Jonas et al., 2004). Without the edited
GluR2 subunit, synaptic AMPARs typically show high Ca\(^{2+}\) permeability, fast decay kinetics, and are blocked by intracellular polyamines (Geiger et al., 1995). The polyamine sensitivity is particular allows us to directly monitor the incorporation of GluR2-containing receptors at the GluR2-lacking synapses of the cerebellar stellate cells. High Ca\(^{2+}\) permeability of the GluR2-lacking receptors may be of great physiological and pathological significance to neurons (Liu and Zukin, 2007). Faster kinetics and other properties of GluR2-lacking receptors may also be functionally important, since an over-expression of GluR2 in inhibitory interneurons was shown to disrupt long-range synchrony of gamma oscillations in the hippocampus (Fuchs et al., 2001). Therefore, a change in GluR2 gene (GRIA2) expression could have a large impact on neuronal function and information processing in neuronal networks. In the current study we studied the effects of acute emotional stress on subunit composition of AMPA receptors. We show that a single fear-inducing stimulus increases GluR2 mRNA and alters synaptic AMPAR phenotype in cerebellar stellate cells. This transcription-dependent form of synaptic plasticity may be important for neuronal processing of fear-inducing stimuli.

Materials and Methods:

**Behavioral experiments:**

The fox-urine treatment was performed as previously described (Kopec et al., 2007). About 4 hours after the onset of the dark cycle, the C57BL/6J mice between the ages of P18-24 were placed in a transparent plastic container covered with glass. The container was elevated and had small regularly spaced openings drilled in the floor. The entire apparatus was contained within a larger covered cardboard box with a pre-positioned digital camera and a light source consisting of a dim red-colored LED array; the video of the entire experiment was stored for offline analysis. The movement of each mouse was then calculated as the amount of motion that occurs between two successive frames, using a custom written program (see below).

After a control period of 3 minutes, a filter paper disk soaked with fox urine (about 5 ml) was inserted below the perforated floor to let the odor permeate the chamber. After an additional 5 minute interval, the animal was removed from the chamber and kept in the cage overnight (~15 hours) for electrophysiological recording.

In some experiments mice were injected with propranolol (20 mg/kg) or saline (IP) about 30 min before entering the chamber. Care was taken to minimize the handling stress of the mice, but young animals (P18) typically showed excessive immobility following the injection and were therefore excluded from the analysis.

It should be emphasized that the mice were extremely sensitive to the latent smell of fox urine. We therefore used disposable covers (aluminum foil and Saran plastic wrap, topped by paper) for any of the exposed surfaces that could come in contact with fox urine. After each...
experiment, the plastic container, the cover glass, and the tray were also washed with detergent (Labtone), and the room was well ventilated.

Behavioral video recording and analysis

The activity of mice was monitored with an infrared CCD camera (LCL-902K, Black & White 1/2", Watec Corp. camera attached to the vari-focal-IR lens TG3Z2910AFCS-IR, CBC Corp.) for the entire 8 min period. The video stream was digitized using consumer-grade Composite/USB converter (Kworld DVD maker USB2.0) and stored on the hard disk using Windows Media Encoder software with 320x240 resolution at 30 FPS using the Windows Media Video 9 codec on the 100/100 “high quality” VBR setting. This produced video files of ~ 40 megabytes/minute; while smaller sizes would be achievable with lower quality settings, the high quality setting was selected to avoid codec compression artifacts.

The video analysis was performed with a custom program [written in C++ using Visual Studio 2005 PE, Windows SDK v6.1 and wmvcore (Windows Media) libraries]. The software was designed to implement an analysis algorithm similar to the previously described one (Kopec et al., 2007). To that end, the successive frame images were extracted at 5 Hz frame rate and subtracted. The resultant image was subdivided into the 80x60 pixel tiles and the mean and standard deviation calculated for each tile; the tile with the lowest mean (i.e. with the least difference) was assigned as the background. The difference image was then processed with a Gaussian digital filter (sigma=0.5 pixels, 3x3 pixel window). The number of pixels outside the 10 standard deviations of the background was counted and represented the significant motion pixels (SMPs). When the number of SMPs remained below a pre-determined threshold for at least 1 second, such time was counted as freezing. To determine the SMP threshold, the freezing responses were scored by a lab member blind to the experimental conditions. The % freezing was calculated as the total freezing time divided by the length of the recording for either the control or the stress periods.

Single cell real time RT-PCR:

The horizontal cerebellar slices were cut (350 um) from P18- 23 GAD-65 GFP mice (Fiszman et al., 2005). This mouse expressed GFP in the basket and stellate, but not Purkinje cells of the cerebellum. The cerebellar slices were digested in the extracellular solution (in mM: 135 NaCl, 3 KCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 HEPES, 11 Glucose; pH adjusted to 7.3 with 1M NaOH) containing 32 units/ml papain at 37°C for 40 min, washed and then triturated through a glass pipette. Individual GFP positive cells with a diameter of 6–10 µm were picked up using a fire-polished glass electrode (that was back-filled with DEPC treated water containing 0.5 u RNAase inhibitor) under visual guidance. We were able to attain the finely tuned changes in the suction pressure by gently hand-warming or cooling the closed-off suction syringe.
The pipette containing the fluorescent cell was then emptied (and the tip crashed) into
the RT solution (10 µl) prepared for reverse transcription (high capacity cDNA RT kit, Applied
Biosystems). The probes for GluR1, 2 and 3 (TagMan gene expression assays) were obtained
from Applied Biosystem. Two house-keeping protein genes, GAPDH and cyclophilin, were used
as the “loading” controls because they showed no detectable change following fox urine
exposure. Total cerebellar RNA was used as positive control. Negative control samples included
the bath solution without cells and the reaction solution omitting the reverse transcriptase, and
both showed no detectable signals. Real time PCR was performed using the ABI 7300
Sequence Detection System (Applied Biosystems) at Penn State Nucleic acid facility. All of the
cells were selected within 1 hour of dissociation.

In situ hybridization

The in situ hybridization experiments were performed in R. Suzanne Zukin’s lab at Albert
Einstein College of Medicine. The transverse cerebellar slices (400 µm) from P18–21 Sprague
Dawley rats were incubated with noradrenaline or TEA and inhibitors in ACSF for 3 hours. After
incubation, slices were chilled at 4˚C, embedded, and 20 µm slices were cut by cryostat
(Shandon) and fixed with paraformaldehyde (4% for 30 min). A digoxigenin (DIG)-labeled RNA
probe was prepared by in vitro transcription of GluR1 and GluR2 cDNAs with T3 and T7 RNA
polymerases in the presence of dig-labeled UTP (Roche Molecular Biochemicals).

In situ hybridization was performed as previously described (Calderone et al., 2003). Signals were
detected by immunocytochemistry with an alkaline phosphatase-conjugated anti-DIG antibody,
according to the DIG RNA Detection kit (Roche Molecular Biochemicals). For each treatment
condition, several areas (5-10) of the molecular layer (100 µm X 160 µm area) of the vermis
(lobules IV to VIII) from multiple slides were sampled.

The analysis of the images was performed by the author in S. June Liu’s lab at Penn
State University, blind to the experimental conditions. The labeling that had the intensity higher
than background within an outline of the soma of typical stellate cells (a circle of 10 µm
diameter) was selected using ImageJ (version 1.38x, NIH). The background was measured by
selecting 14–32 (average, 24 ± 4) cell-free areas from each image. Since GluR1 mRNA level
did not change in cerebellar stellate cells as shown by our single cell RT-PCR experiment,
GluR1 was used as a control. Both the labeling and subsequent quantification was done in a
blind and unbiased manner.

Slice preparation and electrophysiology:

Please refer to chapter 3 for the electrophysiological and slice procedure details.
Statistics

All values are expressed as mean ± SEM. Statistical significance was assessed by a two-tailed Student’s t-test. Kolmogorov-Smirnov test was used for in situ image data analysis where indicated.

In addition to the statistical analysis performed in the manuscript (Liu et al. 2010), I have now re-analyzed the multiple groups data using ANOVA with the following results:

For mRNA distribution data in Figure 2.4 C, a one-way ANOVA shows significant changes in GluR2 mRNA (GRIA2 gene transcript) between the control, 15 hour, and 3 hour groups due to fox urine exposure ($F_{(2,47)} = 3.669$, $P = 0.033$). A post-hoc (Tukey) test indicates that the "15 hours" group shows a statistically significant increase compared to control, at 0.05 cutoff.

Analysis of the freezing data in figure 2.3 using a two-way ANOVA shows a significant difference in freezing behavior between the uninjected, saline, and propranolol injected groups ($F_{(2,40)}=3.647$, $P=0.035$), and between the pre-exposure and post-urine exposure periods ($F_{(1,40)}=5.564$, $P=0.023$). Post-hoc (Tukey) suggests that the propranolol and saline-injected animal responses are statistically different (at 0.05 level).

Analysis of the cell counts from the in situ experiments shows that the detectable cell number is significantly increased (Figure 2.5 I, ANOVA: $F_{(2,12)}=29.353$, $P=0.0002$) in the noradrenaline-treated group compared to both the control and the noradrenaline + Acinomycin D groups (both significant at 0.001 using Tukey posthoc). Similarly, the analysis in of data presented in Figure 2.5 J indicated a significant difference between the control, TEA, TEA + ActD, and TEA + U0126 groups (ANOVA: $F_{(3,29)}= 10.084$, $P= 0.0001$). TEA-treated group shows statistically significant increase in counts compared to the control and TEA+ActD groups (at 0.001 level using Tukey post hoc) and to TEA + U0126 (at 0.05 level, Tukey post hoc).
Results

Exposure of mice to fox urine, an olfactory sign of a natural predator, causes unconditional fear and promotes norepinephrine release in the brain (Hayley et al., 2001; Hu et al., 2007). To examine whether emotional stress can induce changes in synaptic AMPAR properties, we exposed mice to fox urine for 5 min and monitored their locomotor activity. Following the exposure, AMPAR phenotype was accessed by analysing spontaneous EPSCs recorded from the stellate cells in the cerebellum.

Exposure to the smell of a predator increases freezing response in mice

About 4 hours after an onset of the dark cycle, the juvenile mice (P18-24) were placed in a transparent container. Animal movement was captured with a night vision camera for offline analysis. Mice are spontaneously active during the dark phase as evidenced by their exploratory behaviour under the control conditions (Figure 1A, B). After three minutes in the enclosure, a filter paper disk soaked with ~5 ml of fox urine was placed under the perforated container floor, and the mice were exposed to the predator smell for 5 minutes. Upon introduction of fox urine under the cage, the animals exhibited a detectable freezing response, indicative of fear (Figure 1C, D). On average, after the introduction of fox urine, the freezing response of mice doubled from 14.6 ±3.2% to 34.4 ±6.3% (n=5, p<0.01).

Emotional stress alters synaptic AMPAR subtype by increasing GluR2-containing receptors

To assess the AMPAR properties, we prepared acute cerebellar slices from the stressed and control mice, and examined the excitatory postsynaptic currents (EPSCs) at parallel fibre to stellate cell synapses. We first used IEM-1460, a subunit-selective blocker of Ca²⁺-permeable AMPARs, to determine the subunit composition of the receptors. In control cells at physiological temperature (36 °C), the application of IEM-1460 (100 µM) reduced the EPSC amplitude at –60 mV by ~60% (n=3 from 3 mice, Figure 2A), suggesting the AMPARs were predominantly GluR2-lacking. In slices prepared 15 hours after fox urine exposure, the current inhibition by IEM was significantly reduced (36 ± 9%; n=3 from 3 exposed mice, p<0.05, Figure 2 A and B ), consistent with a stress-induced increase in GluR2-containing AMPARs at the synapse.

Stress-induced decrease in sEPSC rectification supports the observed GluR2 increase

We proceeded to use a different technique to verify our observation of a stress-induced increase in GluR2-containing AMPA receptors. To do this, we measured spontaneous EPSCs (sEPSCs) at different membrane holding potentials in the presence of the polyamine spermine.
Inclusion of spermine in the patch pipette solution produces a voltage-dependent block of GluR2-lacking AMPARs, and thus shows a characteristic inwardly rectifying I-V relationship (Cull-Candy et al., 2006; Isaac et al., 2007; Liu and Zukin, 2007). Synaptic incorporation of GluR2-containing AMPARs is detected as decreased rectification (i.e. larger outward currents at positive potentials) of AMPAR-mediated EPSCs. Because sEPSCs originate from all the active synapses onto a stellate cell, we used the average of the synaptic currents to assess the global effects of fear-inducing stimulus on receptor composition.

In stellate cells from control mice, the average sEPSC amplitude was attenuated at positive membrane potentials. This inwardly rectifying I-V relation confirms previous observations that the stellate cell sEPSCs are mediated mainly by GluR2-lacking, Ca\(^{2+}\)-permeable AMPARs (Figure 2C1). In contrast, in stellate cells from mice that were exposed to fox urine, synaptic currents recorded at 3 hours after exposure showed a near linear I-V relation (Figure 2C2), indicating that they are mediated mainly by GluR2-containing AMPARs. Whereas fox urine increased the sEPSC amplitude from 10.7 ± 0.9 pA (control) to 18.2 ± 0.8 pA at +40 mV (n = 5, P < 0.001), there was no significant change in the sEPSC amplitude at −60 mV. Fox urine increased the rectification index from predominantly inwardly rectifying (0.34 ± 0.03; n = 5) to nearly linear (0.82 ± 0.11; n = 5; P < 0.01, Figure 2D). The switch in synaptic AMPAR phenotype was long lasting since even after 15 hours post fear exposure, the synaptic currents still exhibited a near linear I-V relationship (0.78 ± 0.08; n = 4; P < 0.01 vs. Control, Figure 2C3,D) and the elevated amplitude at +40 mV (16.6 ±1.7 pA; P < 0.01).

**Beta-adrenergic receptors are needed for stress-mediated GluR2 increase**

We reasoned that during emotional arousal norepinephrine released onto cerebellar stellate cells might activate β-adrenergic receptors, which are expressed by stellate cells and mediate fear-induced freezing behavior (Hu et al., 2007; Saitow and Konishi, 2000). The β-adrenergic receptor blocker propranolol injected 30 min prior to fox urine exposure prevented a typical freezing response to the stressful predator odor (Figure 3A). Correspondingly, propranolol also largely abolished stress-induced increase in GluR2-containing AMPARs at stellate cell synapses (stress+propranolol, 0.37 ± 0.03; n = 6, P < 0.01 vs. stress alone; stress+saline, 0.80 ± 0.07, n = 6; P < 0.0005 vs. stress+propranolol, Figure 3B and C). Thus, norepinephrine and β-adrenergic receptors likely mediate the stress-induced change in synaptic AMPAR phenotype.

**Emotional stress causes an increase in GluR2 mRNA in stellate cells**

The long delay between the fear-inducing stimulus and the increase in synaptic GluR2 is consistent with a need for transcription-dependent modifications. To examine whether fox urine exposure alters GluR2 mRNA expression, we measured GluR2 mRNA levels in individual GFP-positive stellate cells from glutamic acid decarboxylase (GAD)-65-GFP knock-in mice using quantitative single cell RT-PCR (Figure 4A). Cerebellar tissue from control and stressed mice
was dissociated, and single fluorescent cells were picked under visual guidance (Figure 4B). Fox urine exposure increased GluR2 mRNA in stellate cells by 63 ± 19% ($P < 0.05$) at 3 hours and 103 ± 36% ($P < 0.05$) at 15 hours (Figure 4C). The stress-induced increase in GluR2 mRNA was subunit-specific since GluR1 and GluR3 mRNA levels were unaltered (Figure 4C). Therefore, insertion of GluR2-containing AMPARs at synapses is associated with an increase in GluR2 mRNA levels in individual stellate cells. These results do not, however, distinguish between the transcription of new mRNA and the enhanced stability of the existing GluR2 mRNA.

*Norepinephrine increases the level of GluR2 mRNA expression.*

We next examined whether norepinephrine increases the expression of GluR2 mRNA in stellate cells. Toward this end, we incubated acute rat cerebellar slices in norepinephrine (3 h, with kynurenic acid and picrotoxin) and then processed cerebellar sections for in situ hybridization. Norepinephrine markedly increased the number of cells expressing GluR2 mRNA and intensity of GluR2 mRNA expression in individual cells within the molecular layer (Figure 5A,B,I,L). Actinomycin D prevented the norepinephrine-induced increase in GluR2 mRNA (Figure 5 C,I,L). At this age (P18-21), the vast majority of neurons in the molecular layer of the cerebellar cortex are inhibitory basket/stellate cells. These data indicate that norepinephrine acts via transcription of new GluR2 mRNA to increase the number of GluR2-containing AMPARs at parallel fiber-stellate cell synapses.

*Action potential duration may control transcription of mGluR2*

We have shown that the duration of action potentials can control Ca$^{2+}$ influx and change synaptic AMPAR phenotype (Liu et al., 2011). Here we investigated whether lengthening the duration of postsynaptic action potential is sufficient to selectively enhance the expression of GluR2 mRNA. The slices were incubated in 1 mM TEA, a broad spectrum K channel blocker, that increases AP duration in stellate cells at this concentration. A three hour incubation in TEA has induced a marked increase in GluR2 mRNA expression in individual stellate cells and in the number of cells expressing GluR2 mRNA (Figure 5 E,F,J,M). The change in GluR2 expression was subunit-specific since GluR1 mRNA expression was unaltered (Figure 5 E,F,K,N). As predicted actinomycin D abolished the TEA-induced increase in GluR2 mRNA expression (Figure 5 G,J,M). Moreover, the ERK/MAPK inhibitor U0126 also blocked the TEA-elicited increase in GluR2 mRNA expression in stellate cells (Figure 5 H,J,M). These findings indicate that lengthening the action potential duration, which enhances Ca$^{2+}$ entry, stimulates the Ca$^{2+}$-sensitive ERK/MAPK pathway, which in turn elevates GluR2 mRNA and promotes a switch in AMPAR phenotype at stellate cell synapses.
Persistent storage of synaptic modifications generally requires alterations of gene transcription (Bailey et al., 1996; Kandel, 2001). Long-term changes in glutamatergic synaptic transmission require activation of transcription factors such as CREB (Barco et al., 2002; West et al., 2002). However, it is unclear whether learning and experience can directly regulate glutamate receptor gene expression. Here we report that norepinephrine released during emotional stress can cause a selective increase in $GRIA2$ transcription and the corresponding increase in synaptic GluR2-containing AMPA receptors, leading to a switch in AMPAR phenotype at cerebellar stellate cell synapses. This provides direct evidence for an activity-dependent, subunit-specific regulation of in $GRIA2$ gene transcription and synaptic AMPAR composition. The net result is a lasting increase in GluR2-containing receptors and a reduction in $\text{Ca}^{2+}$ entry through synaptic AMPARs in stellate cells.

Our results demonstrate the involvement of ERK/MAPK in norepinephrine or TEA-induced GluR2 increase. This is consistent with the well established observations that $\text{Ca}^{2+}$ entry through L-type $\text{Ca}^{2+}$ channels can activate ERK/MAPK signaling and promote phosphorylation and activation of the transcription factor CREB (Deisseroth et al., 1998; Dolmetsch et al., 2001). Both CREB and RE1-element silencing transcription factor (REST) are implicated in regulation of GluR2 expression in response to neuronal activity and neuronal insults (Calderone et al., 2003; Liu et al., 2004; Myers et al., 1998). It is therefore possible that either one or both of these transcription factors are responsible for up-regulation of $GRIA2$ mRNA transcription in response to norepinephrine. However, our findings do not rule out the possibility that norepinephrine can also enhance the stability of the $GRIA2$ transcript, which further increase the detected level of mRNA.

An important mechanism responsible for changes in synaptic strength involves regulation of AMPAR trafficking (Barry and Ziff, 2002; Bredt and Nicoll, 2003; Cull-Candy et al., 2006; Isaac et al., 2007; Liu and Zukin, 2007; Malinow and Malenka, 2002; Song and Huganir, 2002). Arc mRNA, which is induced by neuronal activity and targeted to stimulated synaptic area, plays a critical role in AMPAR trafficking (Chowdhury et al., 2006; Rial Verde et al., 2006). Repetitive activation of synaptic glutamate receptors can also regulate local dendritic protein synthesis of AMPARs in a synapse specific manner (Ju et al., 2004; Mameli et al., 2007; Sutton et al., 2006). In contrast to these localized trafficking and translation changes that affect the strength of individual synapses, a modification in AMPAR gene transcription should produce a long term change in synaptic AMPARs throughout an entire neuron. Regulation of AMPAR gene transcription affects the properties of the global synaptic cohort of the neuron, and therefore represents a powerful means to modulate the activity of neuronal cells and circuits.

Neuronal activity can regulate the synaptic incorporation of GluR2-containing receptors by facilitating their targeting to the parallel fiber-stellate synapse without an alteration in GluR2 gene transcription. This mechanism requires activation of synaptic glutamate receptors and
involves interactions with PICK1, NSF and GRIP (Gardner et al., 2005; Liu and Cull-Candy, 2000; Liu and Cull-Candy, 2005; Sun and Liu, 2007). Synaptic activity also selectively suppresses the synthesis of GluR1 (but not GluR2) subunits in hippocampal neurons (Sutton et al., 2006) and enhances the synthesis of GluR2 in the dopamine neurons in the ventral tegmental area (Mameli et al., 2007). Our results demonstrate that norepinephrine increases GRIA2 gene transcription. In cerebellar stellate cells, elevated Ca\(^{2+}\) influx through L-type channels during a postsynaptic action potential elevates GRIA2 mRNA and thereby increases the number of synaptic GluR2-containing receptors (Liu et al., 2010). Thus, newly synthesized AMPARs can ultimately be incorporated via receptor trafficking at stellate cell synapses, a mechanism may involve interactions with PICK1 and GRIP (Gardner et al., 2005; Liu and Cull-Candy, 2005). In addition to changing synaptic current decay kinetics, this modification would be expected to reduce the Ca\(^{2+}\) permeability of synaptic AMPARs and may alter post-synaptic short-term plasticity (Geiger et al., 1995; Rozov and Burnashev, 1999), producing a qualitative change in synaptic transmission. Dynamic regulation of AMPAR transcription may provide a mechanism for homeostatic control of synaptic transmission (Ibata et al., 2008). Overall, the observation that norepinephrine can control AMPAR subunit expression via a transcription-dependent mechanism reveals a significant role for neuromodulators and postsynaptic action potentials properties in determining synaptic AMPAR phenotype.
Figure 2.1. Fox urine increases freezing response in juvenile mice.

A high sensitivity “surveillance” camera was used to record the movement of mice under dim red light conditions.

A. An example single video frame showing a mouse in motion under the control conditions. An algorithm analyzes the sequential frames and outputs a difference image with significant motion pixels (SMPs) highlighted in color (red: pixels changed from bright to dark, green: pixels changed from dark to bright). A composite overlay shows the direction of locomotion (forward).

B. A single example of a plot of mouse movement (significant motion pixels) vs time. Blue lines indicate the detected “freezing” episodes where a mouse remained motionless for at least 1 second (5 consecutive frames at 5 Hz).

C. Fox urine caused, a natural olfactory stimulus, caused fear evidenced as a freezing response. A single frame grab shows a representative period of low mobility (note that some motion is still detected in this frame).

D. A plot of movement vs time shows an increase in stationary periods interspersed with running bouts.

E. Group data shows an increase in a freezing response. The (% freezing) was calculated during the 3 min control and 5 min fox urine exposure period (n = 5; *, P < 0.01). Error bars: SEM
Figure 2.2. Fear exposure resulted in an increase in synaptic GluR2-containing AMPARs in stellate cells [Panels A and B were previously published in (Savtchouk and Liu, 2011). The data in panels C and D for this figure were collected by Dr. Liu Yu]

A. Inhibition of sEPSCs at 60 mV by IEM-1460 (100 µM), a selective Ca2+-permeable AMPAR blocker, is reduced 15 hours after fox urine exposure

B. A decrease in IEM-1460 block of synaptic currents indicates a long-term increase in synaptic GluR2-containing receptors (n = 3 in each group, *: p<0.05). This experiment was performed at physiological temperature.

C. Synaptic currents (at +40 and -60 mV, left) and an I-V relationship of sEPSCs recorded from stellate cells when spermine was included in the patch pipette. The control cells (C1) displayed an inwardly rectifying I-V relationship with positive currents showing pronounced attenuation, suggesting the presence of GluR2 lacking receptors. Mice were exposed to fox urine, a fear-inducing olfactory stimulus, for 5 min. (C2). Three hours following fox urine exposure the synaptic current in stellate cells showed a near linear I-V relationship (n = 5, P < 0.01), indicating that it was mediated mainly by GluR2-containing AMPARs. (C3). When slices were prepared 15 hours after fox urine exposure the synaptic current still showed a near linear I-V relationship (n = 4).

D. Group summary of the rectification index data (*, P < 0.01; **, P < 0.005). Error bars: SEM
Figure 2.3. Beta-adrenergic receptors are required for fear-induced GluR2 increase
[experimental data in panels B and C was collected by Dr. Yu Liu]

A. Mice were injected with either propranolol (a beta-adrenergic receptor blocker) or a vehicle (saline) about 30 minutes before exposure to the smell of fox urine. Introduction of fox urine has resulted in about a two-fold increase in the freezing time for the control (x2.4 increase, n=5, p<0.05) and saline-injected (x1.9 increase, n=9, p<0.05), but not propranolol-injected animals (apparent decrease, n=8, p=0.15). Propranolol significantly reduced fear-induced freezing in mice compared to the control (p<0.005) and saline-injected (p<0.01) groups, yet did not affect the baseline freezing before the smell exposure (p=0.55 vs control and p=0.93 vs saline).

B. Example sEPSC and I-V relations obtained from the saline pre-injected (left) and propranolol pre-injected mice (right) about 15 hours after injection and fox urine exposure. Current rectification is decrease in saline-injected but not propranolol-injected animals.

C. Group data shows that propranolol, but not saline, preserves inward rectification and therefore prevents an increase in GluR2-containing synaptic receptors (saline vs propranolol, n=6 and 6, ***, P < 0.0005). Error bars: SEM
Figure 2.4. Fear exposure causes an long-term increase in GluR2 mRNA.
We examine the changes in mRNA level in individual cerebellar molecular layer interneurons after fox urine exposure.

A. A sagittal slice from a transgenic mouse expressing GFP under GAD65 promoter shows that the green fluorescence is specific to stellate and basket cells (but not Purkinje cells) in the molecular layer. The cerebellar tissue from the naïve and stressed mice was acutely dissociated and individual cells examined under the microscope. (scale bar: 200 µm)

B. We used a patch pipette with a large opening to pick up the individual GFP positive neurons (~8 µm in diameter). Representative example shows 4 interneurons inside the pipette before being transferred to their separate reaction tubes. (scale bar: 20 µm)

C. The GluR1, 2 and 3 mRNA level in individual stellate cells was determined using real time single cell RT-PCR (control, 23 cells from 4 animals; 3 hr following fox urine exposure, 18 cells; 15 hr following fox urine exposure, 15 cells; *, P < 0.05). Error bars show SEM.
Figure 2.5. Noradrenaline and TEA treatment increase the level of GluR2, but not GluR1 mRNA expression in situ (n=5).

A-H. DIG-labeled RNA antisense probes for GluR1 and GluR2 mRNA (A-C; E-H) or the sense probes (D) were used.

A and E. Control.

B and C. Norepinephrine treatment increased the level of GluR2 mRNA expression and actinomycin D (Act D) blocked the NA-induced increase in GluR2 expression.

F. TEA treatment increased the level of GluR2, but not GluR1 mRNA expression.

G and H. Actinomycin D (Act D) and U0126 prevented the TEA-induced increase in GluR2 mRNA expression in stellate cells. ML, molecular layer; PL, Purkinje cell layer; GL, granule cell layer; KYNA, kynurenic acid; picrotoxin, picrotoxin. The labeling that has intensity higher than background within an outline of typical stellate cells (~8 µm) was selected as regions of interest. Images are typical of n = 5 in each group.

I, G and K. The number of labeled stellate cells that express high level of GluR2 mRNA under each condition relative to control. We used the mean of background intensity plus two standard deviations as threshold for positive labeling. (*, P < 0.05, **, P<0.001, by unpaired Student's t-test).

L, M, N. Cumulative distribution of the labeling intensity of selected regions of interest after background subtraction illustrated the changes in staining intensities of positive stained cells under each treatment condition (the number of ROIs ranged from 104 to 365 under each condition; noradrenaline vs. control or noradrenaline + Act D, TEA vs. control, or TEA + Act D, or TEA + U0126, Kolmogorov-Smirnov test, P < 0.0001). Scale bars, 200 µM (left panels), and 50 µM (right panels).
Chapter 3. Remodeling of Synaptic AMPA Receptor Subtype Alters the Probability and Pattern of Action Potential Firing
Remodeling of Synaptic AMPA Receptor Subtype Alters the Probability and Pattern of Action Potential Firing

Iaroslav Savtchouk and Siqiong June Liu
Department of Cell Biology and Anatomy, Louisiana State University Health Sciences Center, New Orleans, Louisiana 70112, and Department of Biology, Pennsylvania State University, University Park, Pennsylvania 16802

Changes in the subunit composition of postsynaptic AMPA-type glutamate receptors can be induced at CNS synapses by neural activity and under certain pathological conditions. Fear-induced incorporation of GluR2-containing receptors at cerebellar synapses selectively prolongs the decay time of synaptic currents, whereas a switch from GluR2-lacking to GluR2-containing receptors induced by parallel fiber stimulation reduces the amplitude in addition to lengthening the duration of EPSCs. Although it is often assumed that these two forms of synaptic plasticity will alter action potential (AP) firing in the postsynaptic neuron, this has not been directly tested. Using a dynamic current-clamp approach, we now show that the fear-induced increase in EPSC duration increases the size of EPSPs and thereby markedy enhances the AP firing probability. In contrast, the parallel fiber stimulation-triggered switch in GluR2 expression reduces the EPSP–AP coupling because of the decrease in the synaptic current amplitude. The switch also abolished the paired-pulse facilitation that arose from an activity and spermine-dependent unblock of GluR2-lacking receptors and hence reduced the ability of paired stimuli to evoke two consecutive APs. Therefore, fear-induced incorporation of GluR2 receptors enhances the EPSP–AP coupling, but the parallel fiber stimulation-triggered switch reduces both the EPSP–AP coupling and evoked AP doublets. In contrast to long-term potentiation and depression, which modify the amplitude of synaptic currents, this activity-induced change in AMPA receptor phenotype alters synaptic conductance waveform and postsynaptic short-term plasticity. These changes modulate both the probability and pattern of evoked AP firing via a fundamentally different mechanism from long-term potentiation and long-term depression.

Introduction

Experience-induced modifications of synaptic transmission can alter information processing within a neuronal circuit. The best understood postsynaptic modification involves a change in the number or phosphorylation state of AMPA receptors (AMPARs), so altering the amplitude of the synaptic current and giving rise to long-term potentiation and depression (Barry and Ziff, 2002; Malinow and Malenka, 2002; Song and Huganir, 2002). However, neuronal activity can also alter the subunit composition of synaptic AMPARs and thereby change the kinetics of the synaptic current (Geiger et al., 1995). The most commonly observed AMPAR subtype remodeling involves changes in the abundance of GluR2 subunits. Such changes can occur after repetitive synaptic stimulation, sensory deprivation, emotional stress, drug addiction, and under certain pathological conditions, such as epilepsy (Fitzgerald et al., 1996; Grooms et al., 2000; Liu and Cull-Candy, 2000; Opitz et al., 2000; Bellone and Lüscher, 2006; Clem and Barth, 2006; Goel et al., 2006). Therefore, it is critical to determine the functional consequences of the AMPAR phenotype change on signal transmission in a neuronal network.

The waveform of the synaptic current is a key element that shapes the EPSP (Trussell, 1998). Inhibitory interneurons often express Ca2+-permeable GluR2-lacking AMPARs that exhibit several unusual features (Cull-Candy et al., 2006; Isaac et al., 2007; Liu and Zukin, 2007). First, the synaptic current of these neurons displays particularly fast kinetics. We have recently found that stress can enhance GluR2 gene expression in cerebellar stellate cells and selectively prolongs the decay time of synaptic currents (Liu et al., 2010). The second characteristic of GluR2-lacking AMPARs is the paired-pulse facilitation that arises from an intracellular polyamine blockade and that can be released in a use-dependent manner (Rozov and Burnashev, 1999). Given these characteristics, we would expect that an activity-dependent switch in AMPAR subtype would abolish postsynaptic short-term plasticity. Cerebellar stellate cells are electrically compact and the activation of single parallel fiber (PF) inputs can strongly influence stellate cell firing (Carter and Regehr, 2002). The activity-induced change in current amplitude, kinetics, and facilitation that results from the switch in AMPAR subtype would thus be predicted to alter the efficacy of EPSP–action potential (AP) coupling in stellate cells and to influence the timing and firing frequency of Purkinje cells, which are the only cortical output.

The present study was undertaken to determine whether synaptic AMPAR subtype remodeling by two physiologically relevant stimuli (emotional stress and repetitive parallel fiber
stimulation) actually does lead to an alteration in the AP firing of stellate cells. We found that the AMPAR subtype remodeling alters both the probability and the pattern of evoked APs in stellate cells. Unexpectedly, these two stimuli had different effects on AP firing. The emotional arousal-induced incorporation of GluR2 receptors potentiated AP firing, whereas the parallel fiber stimulation-induced switch in AMPAR phenotype suppressed AP firing and reduced the ability of paired stimuli to evoke two consecutive APs. Thus, a bidirectional alteration in the AP probability and pattern that results from AMPAR subtype remodeling strongly depends on the nature of the stimulus.

Materials and Methods

Slice preparation and recordings. Horizontal and sagittal slices (250 μm) were cut from cerebella of C57BL/6J mice at postnatal day 18 (P18) to P22 on a Leica VT1000S vibratome microlicer in ice-cold slicing solution (in mM: 125 NaCl, 2.5 KCl, 1 CaCl₂, 7 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and 25 glucose, pH 7.4, with a 5% CO₂/95% O₂ mix). Stellate cells located in the outer two-thirds of the molecular layer were visually identified using differential interference contrast with a 60× water-immersion objective. Stellate cells were identified by their spontaneous action potential firing (in cell-attached mode) and by the presence of spontaneous synaptic currents (in whole-cell mode). All recordings were performed at near physiological temperature (34–37°C) unless otherwise indicated.

Electrophysiology. Voltage and dynamic current-clamp recordings were made using an Axopatch 700A or a 200B amplifier (Molecular Devices). Recordings were low-pass filtered at 10 kHz and digitized at 20 kHz. Whole-cell recordings were performed using 5–7 MΩ borosilicate glass pipettes.

Voltage-clamp recordings. All voltage-clamp experiments were performed using a cesium-based internal solution [in mM: 135 CsCl, 10 EGTA-Cs, 10 HEPES, 4 ATP-Na, 4 MgCl₂, 5 TEA, 1 lidocaine N-ethyl bromide (QX-314), and 0.1 spermine; pH adjusted to 7.3 with 1 M CsOH]. Spermine (0.1 mM) was included in the patch electrode to block GluR2-lacking AMPA receptors at depolarized potentials and was used to determine the subunit composition of synaptic AMPA receptors. The series resistance was monitored throughout the experiment. The collected data were not used if the value changed by >20%. To characterize the properties of unitary synaptic currents mediated by GluR2-lacking and GluR2-containing receptors, spontaneous EPSCs (sEPSCs) were determined at various holding potentials before, and after, parallel fiber stimulation, which was delivered via a bipolar electrode placed across the molecular layer. The stimulation protocol consisted of 10 repetitions of four pulses (12 V, 200 μs) at 50 Hz, every 2 s. The average EPSC waveform and I–V relationship were constructed as previously described (Liu and Cull-Candy, 2000). The cells were voltage-clamped at −70 mV (n = 3) or at −30 mV (n = 2) during the burst stimulation. The decay time constants of spontaneous EPSCs were obtained by fitting the decay phase of individual EPSC events with single- and double-exponential decay functions. The weighted decay time constants were similar to those obtained with a single-exponential fit.

To determine whether the switch in AMPAR subtype is associated with a change in paired-pulse facilitation, EPSCs were evoked by a focal threshold stimulation of parallel fibers using a 100–200 μs voltage pulse (5–15 V) delivered via a monopolar electrode (5–7 MΩ) positioned −100 μm from the cell. Paired pulses were delivered at a 10 ms interval. The evoked EPSCs were measured at −60 and +40 mV before and 15 min after high-frequency parallel fiber stimulation (three trains of 100 pulses at 50 Hz each, every 20 s) performed at −60 mV and at room temperature. Short-term postsynaptic facilitation was characterized by the postsynaptic paired-pulse ratio (PPR). To remove any presynaptic contribution, each current trace was reviewed to remove failures and multiple peak events, and each EPSC was manually aligned based on the current rise time using N, version 4.0 (written by Stephen Traynelis, Emory University, Atlanta, GA). Postsynaptic paired-pulse ratio was calculated by dividing the amplitude of the average current evoked by pulse 2 by the average current evoked by pulse 1.

Dynamic current-clamp experiments. Action potentials were recorded in current-clamp mode while injecting small negative currents of up to −30 pA to keep the membrane potential at −60 to −70 mV. Action potentials were recorded using a potassium-based internal solution containing the following (in mM): 115 KMeSO₄, 10 HEFES, 0.5 K-EGTA, 0.16 CaCl₂, 2 MgCl₂, 10 NaCl, 4 ATP- Na, 14 Tris-creatine phosphate, 0.4 GTP- Na. Series resistance was periodically monitored, and the data were discarded if this changed by >40%. All recordings were performed in the presence of 200 μM picotoxin and either 2–5 μM kynurenic acid or 10 μM NBQX [6-nitro-7-sulfamoylbenzo(f)quinazoline-2,3-dione] and 10 μM 4-(3-phosphonopropyl)pyrazine-2-carboxylic acid [(R)-CPP] to block inhibitory and glutamergic synaptic transmission. Cells were not used if the amplitude of action potentials was <40 mV. The geometric mean of the capacitance of stellate cells was 5.96 pF (n = 321; geometric SD, 1.47) when fitted with a log normal distribution. Therefore, cells with a capacitance between 2.74 and 12.9 pF (mean ± SD) were included in our analysis.

For artificial synaptic event delivery, an SM-1 analog computer was used in combination with an Axopatch 700A or 200B amplifier and a Digidata 1320 digitizer. Conductance templates were constructed by dividing the recorded current traces by the membrane potential under voltage-clamp recording. The SM-1 used the membrane voltage provided by the amplifier and the conductance template from the Digidata 1320 to modify the command current. To minimize the effect of systematic changes, conductance templates were applied at 1 Hz and different conductance template waveforms were alternated throughout the recording. The cell response was determined by counting the number of action potentials evoked by the template within a 20 ms window starting at the time of injection or within a 10 and 60 ms window for paired conductance waveforms. In some cells, 0.3 μM TTX was added to record the passive EPSP response to injected conductances.

Fox urine treatment. Fox urine treatment was performed as previously described (Kopeck et al., 2007). The mouse was placed inside a plastic cage (16 × 9 × 6 inch) with an elevated floor containing regularly spaced small openings. After 3 min, a paper towel soaked in 5 ml of fox urine was placed under the floor for 5 min (Liu et al., 2010).

Chemical reagents. Fox urine was obtained from Green Sense Organic Gardening, (R)-CPP from Tocris Bioscience, Tris-creatine phosphate and sodium ATP from Sigma-Aldrich, potassium methylsulfonate from Alfa Aesar, sodium GTP from Calbiochem, spermine from MP Biomedicals, picrotoxin from Indofine, and kynurenic acid and QX-314 from Ascent. A two-tailed Student’s paired t test, ANOVA, and Kolmogorov–Smirnov test were used as indicated. All data are presented as mean ± SEM.

Results

A stress-induced increase in EPSC duration enhances EPSP–AP coupling

AMPArs lacking GluR2 subunits are present at the parallel fiber to stellate cell synapse (Liu and Cull-Candy, 2000). In a recent study, we have found that a fear-inducing stressor enhances GluR2 gene transcription and promotes the expression of synaptic GluR2-containing AMPARs in stellate cells (Liu et al., 2010). Here, we investigated the functional consequences of the fear-induced change in AMPAR subtype. We exposed mice to fox urine for 5 min, a paradigm that induces a fear response, and then monitored spontaneous EPSCs at the stellate cell synapse in cerebellar slices taken from mice 15 h later. N,N,N',N'-Trimethyl-5-[(tricyclo[3.3.1.3.7]dec-1-yl)methylamino]-1-pentanaminiumbromide hydrobromide (IEM-1460), a Ca²⁺-permeable AMPAR blocker, was used to determine the subunit composition of synaptic AMPARs. In control cells, application of IEM-1460 (100 μM) blocked the EPSC amplitude at −60 mV by −60% (n = 3 cells from 3 control mice) (Fig. 1A). At 15 h, after fox urine exposure, the degree of inhibition by IEM-1460 was significantly reduced (36 ± 9%; n = 3 cells from 3 fox urine-treated mice; p < 0.05) (Fig. 1A). These data suggest that a single exposure to a fear-inducing stimulus alters the AMPAR subtype from GluR2-lacking to GluR2-containing at the parallel fiber-to-stellate cell synapse.
Emotional stress prolonged EPSC decay time without changing the current amplitude in stellate cells. A, Mice were exposed to fox urine for 5 min to elicit a fear response. EPSCs were measured at 36°C 15 h after a mouse was exposed to fox urine. Inhibition of EPSCs at −60 mV by IEM-1460 (100 μM), a selective Ca^{2+}-permeable AMPAR blocker, was reduced after fox urine exposure (n = 3 mice from 3 different mice in each group; p < 0.05). B, Example showing the increase in decay time of average sEPSCs after fox urine exposure. C, Median decay time constant of individual sEPSCs increases from 0.57 to 0.71 ms after fox urine exposure (control, 562 synaptic events, n = 7 cells from 7 mice; fox urine treated, 558 events, n = 6 cells from 3 mice; p < 0.00001, Kolmogorov–Smirnov test). D, Average sEPSC amplitude did not change after fox urine exposure (control, n = 7; fox urine treated, n = 6; p > 0.05, unpaired t test). Error bars indicate SEM. *p < 0.05.

To examine the impact of the change in synaptic AMPAR phenotype on the kinetics of EPSCs, we measured the decay time constant of spontaneous EPSCs at −60 mV at 36°C in cerebellar stellate cells (Fig. 1B). This allowed us to determine the properties of the quantal synaptic currents, and to sample synaptic currents from a large number of synapses. Fox urine exposure significantly prolonged the decay time of EPSCs (control, 562 individual EPSCs from 7 cells; fox urine, 558 events from 6 cells; p < 0.00001, by Kolmogorov–Smirnov test) (Fig. 1C). However the amplitude of synaptic currents at −60 mV in stellate cells from mice that were briefly exposed to fox urine was indistinguishable from control (control: −62.7 ± 2.8 pA, n = 7; 15 h after fear-inducing stimulus: −65.0 ± 8.4 pA, n = 6) (Fig. 1D). The average conductance of somatic AMPAR channels was 30% smaller in stellate cells expressing predominantly Ca^{2+}-impermeable AMPARs than in those having a mixed population of GluR2-containing and -lacking receptors (Li and Cull-Candy, 2005). Thus, the total number of synaptic receptors is estimated to increase by 36% after fear stimulus. A mixture of receptors is predicted to give rise to a synaptic current that contains both fast and slow components mediated by GluR2-lacking and -containing receptors, respectively. Indeed, when fitted with a two-exponential function, the fast component comprised ~60% control EPSCs, but only ~30% of the total synaptic current after fear-inducing stimulus (p = 0.13). These results suggest that fear stimulus markedly promotes the synaptic incorporation of additional GluR2-containing receptors and moderately reduces GluR2-lacking receptors, giving rise to a prolonged EPSC decay time and unaltered EPSC amplitude.

Using the dynamic current-clamp technique, we next examined the effects of the prolonged EPSC decay time on the waveform of EPSPs in stellate cells. We constructed conductance templates to mimic the synaptic current in control and after emotional stress. These templates had the same rise time as the control quantal events, but with a single-exponential decay phase with time constants of 0.5 and 0.7 ms (which corresponds to the median decay time constants of EPSCs in control and after emotional stress, respectively) (Fig. 1C) and an amplitude of double the unitary conductance. We reasoned that the prolonged decay kinetics would allow a greater number of ions to enter the cell and would evoke a larger EPSP. To test this possibility, we injected these conductance templates into stellate cells and measured membrane potentials in the presence of TTX (to block Na channels). Indeed prolongation of the EPSC decay kinetics from 0.5 to 0.7 ms gave rise to an increase in the EPSP amplitude (9.6 ± 0.7 vs 11.1 ± 0.8 mV; n = 4; p < 0.0005) (Fig. 2A). To determine the effect of the prolonged EPSC decay time on the ability to evoke action potentials, we injected the conductance templates into stellate cells and monitored the membrane potential in the absence of TTX. Increasing the EPSC decay kinetics from 0.5 to 0.7 ms resulted in a marked increase in the evoked AP probability from 0.42 ± 0.08 to 0.62 ± 0.08 (n = 7; p < 0.005) (Fig. 2A–C). Note that a small increase in decay time is sufficient to alter EPSP amplitude and leads to enhanced AP firing in stellate cells. Thus, the change in decay time associated with the insertion of GluR2 receptors markedly promotes EPSP–AP coupling in stellate cells.

To determine the dynamic range over which EPSC decay time alters AP firing probability, we tested a series of templates that covered the distribution of decay time constants of naturally occurring synaptic events that were recorded under physiological conditions. These ranged from 0.3 to 1.1 ms in steps of 0.2 ms (Fig. 1C). As shown in Figure 2, B and C, prolonging the EPSC decay time markedly increased the probability of evoking an AP in stellate cells. The raster plot illustrates that the templates with longer decay time are more successful at evoking action potentials with virtually no failures at the longest duration (1.1 ms). We then examined the effects of background spontaneous EPSPs on AP firing, using templates with unitary conductance (control: 1.12 nS, equivalent to −67 pA). In contrast to using the double conductance, the AP firing probability was low and gradually increased as the decay time became longer (Fig. 2C,D). Thus, prolonging the decay time of synaptic currents enhances AP firing probability at double unitary conductance but has little effect as background spontaneous unitary events.

Transcription-independent switch in AMPAR subtype reduces EPSP–AP coupling

Whereas emotional stress promotes GluR2 gene transcription in stellate cells on the order of hours, repetitive parallel fiber stimulation (300–400 stimuli) induces a rapid change in synaptic AMPAR subtype from GluR2-lacking to GluR2-containing re-
AMPA receptors that lack GluR2 subunits (Fig. 3A). After PF stimulation, the $I-V$ relationship changed from being inwardly rectifying to nearly linear, and the rectification index (RI) (defined as $-1.5 * I_{\text{raw}} / I_{\text{sh}}$) increased from 0.38 ± 0.04 (control; $n = 5$) to 0.74 ± 0.09 (30 min after PF stimulation; $n = 5$; $p < 0.05$ vs control) (Fig. 3B). The amplitude of synaptic currents at +40 mV increased from 15.4 ± 0.5 to 20.3 ± 1.9 pA ($p < 0.05$; $n = 5$), indicative of an increase in GluR2-containing receptors. The EPSC amplitude at −60 mV decreased from 15.7 ± 0.7 pA ($p < 0.05$; $n = 5$) to 12.2 ± 0.04 pA ($p < 0.05$, control; $n = 5$) (Fig. 3C). These results suggest that GluR2-lacking synaptic AMPARs had been replaced by GluR2-containing receptors. The decay time constant of EPSCs at −60 mV was prolonged from 0.68 ± 0.04 to 0.83 ± 0.05 ms ($p < 0.05$; $n = 5$) (Fig. 3D). When fitted with a two-exponential function, the fast component (mediated by GluR2-lacking receptors) comprised ~60% of control EPSCs but contributed little to currents after PF stimulation ($n = 5$; $p < 0.05$). Thus, a physiologically relevant stimulation paradigm can induce a switch in AMPAR subtype from GluR2-lacking to GluR2-containing receptors. In contrast to the stress-induced incorporation of GluR2 receptors that prolonged EPSC decay time, the synaptic activity-induced switch is associated with a reduced synaptic conductance as well as a prolonged current decay time.

Whereas lengthening EPSC duration increased action potential firing probability (Fig. 2), a decrease in synaptic current amplitude is predicted to reduce its ability to evoke an AP. We constructed conductance templates from an average synaptic AMPA receptors evoked a burst of action potentials at an interburst interval of 2 ms at 36°C. 

We stimulated parallel fibers with a theta-burst-like train of four depolarizing pulses at 50 Hz, which was repeated 10 times with an equal number of GluR2-containing receptors, the current amplitude is reduced and the duration of EPSCs is prolonged (Liu and Cull-Candy, 2000).

In vivo recordings of cerebellar granule cells show that sensory stimulation evokes a burst of action potentials at ~80 Hz (Chaderton et al., 2004). We determined whether a physiologically relevant stimulus can alter the synaptic AMPAR phenotype. We stimulated parallel fibers with a theta-burst-like train of four depolarizing pulses at 50 Hz, which was repeated 10 times with an interburst interval of 2 s at 36°C.

To monitor changes in the subunit composition of synaptic AMPA receptors, we included spermine in the patch electrode. Spermine blocks currents via GluR2-lacking receptors at positive membrane potentials, giving rise to an inwardly rectifying current–voltage relationship, whereas synaptic currents via GluR2-containing receptors display a linear relationship. We measured spontaneous EPSCs at different potentials before and after PF stimulation. Control synaptic currents displayed an inwardly rectifying $I-V$ relationship, indicating the presence of synaptic receptors in a transcription-independent manner (Liu and Cull-Candy, 2000; Liu et al., 2010). This switch is triggered by the repetitive activation of synaptic glutamate receptors and requires interaction between PICK (protein interacting with C kinase) and GluR2 subunits (Liu and Cull-Candy, 2000, 2005; Gardner et al., 2005; Sun and Liu, 2007). Incorporation of GluR2 subunits into an AMPAR is known to reduce the channel conductance (Swanson et al., 1997). When GluR2-lacking receptors are replaced with an equal number of GluR2-containing receptors, the current amplitude is reduced and the duration of EPSCs is prolonged (Liu and Cull-Candy, 2000).

Figure 2. Increase in EPSC decay time enhanced action potential probability in stellate cells. A, EPSP waveforms and action potential distributions evoked by two artificial conductance templates approximating the control and poststress EPSC decay time constant (control, 0.5 ms; poststress, 0.7 ms). The AP probability plot represents instantaneous AP probability within 0.5 ms bins. B, Increasing EPSC decay time constant (from 0.3 to 1.1 ms) increased the likelihood of evoking an action potential at the doubled conductance amplitude (equivalent to coactivation of two inputs). The raster plot shows the action potentials recorded during 60 repetitions of each conductance template (arrows). The bar graph on the right, AP probability measured in 10 ms bins. The middle of the gray bar corresponds to the time of template application. C, Summary of AP probability evoked by synaptic templates with different decay times ($n = 7$; two-way ANOVA: $p < 0.0001$ between unitary and double conductance; $p < 0.0001$ between decay times). D, The effect of increasing the decay time on the probability of evoking an action potential with the unitary conductance. Raster plot shows 60 repeats of each stimulus waveform. Error bars indicate SEM.
current before PF stimulation and reduced its amplitude to the conductance of poststimulation EPSCs without altering the decay time. As expected, we found that the AP firing probability decreased from $0.63 \pm 0.14$ to $0.38 \pm 0.16$ ($n=4; p<0.05$) (Fig. 4A) as the conductance was reduced from the prestimulation to the poststimulation amplitude ($2 \times$ unitary conductance). The unitary conductance of GluR2-lacking receptors (prestimulation, 1.12 nS) was then scaled by multiplying it by a factor of 0.5–5 to cover the wide range of EPSC amplitudes that are likely to be encountered by stellate cells. Increasing the amplitude over this range consistently enhanced the ability of synaptic events to evoke APs in stellate cells (Fig. 4A). Thus, the changes in EPSC amplitude and duration that are associated with an AMPAR subtype switch produce two opposing effects on AP firing in stellate cells.

We next determined the overall effect of a switch in AMPAR subtype that is characterized by a prolonged EPSC decay time and a reduction in amplitude, on its ability to evoke AP firing in stellate cell. We used the average of EPSCs recorded before and after PF stimulation to construct conductance templates. The prestimulation (GluR2-lacking) template (2× unitary conductance) elicited a larger EPSP than the poststimulation (GluR2-containing) templates (prestimulation, 11.5 ± 0.8 mV, vs poststimulation, 10.2 ± 0.7 mV; n = 4; p < 0.0005). The poststimulation template evoked APs at a rate that was lower than that seen with the prestimulation templates (poststimulation, 0.46 ± 0.10, vs prestimulation, 0.54 ± 0.10; n = 9; p < 0.05) (Fig. 4B). This was primarily attributable to the reduced EPSP amplitude as the AP probability increased with the increasing size of EPSPs ($r = 0.87$) (Fig. 4C). Since a prolonged EPSC decay time promotes AP firing (Fig. 2), the decrease in EPSP–AP coupling associated with an AMPAR switch arises as a consequence of the reduced current amplitude. To test the effects of background spontaneous EPSPs on AP firing, an equal number (300) of natively occurring EPSC quantal events from several cells were recorded before and after PF stimulation and were converted to a conductance waveform. The prestimulation quantal templates evoked ~20% more APs than the poststimulation templates ($p < 0.01; n = 6$) (Fig. 4D). Thus, GluR2-containing quantal events evoked fewer action potentials than GluR2-lacking events.

Replacing GluR2-lacking with GluR2-containing receptors abolishes postsynaptic paired-pulse facilitation

One of the possible consequences of GluR2 incorporation stems from the observation that GluR2-lacking receptors are partially blocked by intracellular polyamines at resting membrane potentials (Bowie and Mayer, 1995). This blockade can be relieved for short periods after receptor opening, giving rise to a postsynaptic paired-pulse facilitation that is absent at synapses expressing GluR2-containing receptors (Rozov et al., 1998; Rozov and Burnashev, 1999; Bagal et al., 2005). This action underlies the facilitation of the synaptic current through GluR2-lacking (but not through GluR2-containing) receptors during high-frequency synaptic stimulation. Given that the majority of parallel fiber-to-stellate cell synapses have GluR2-lacking receptors, it is possible that polyamine-dependent postsynaptic facilitation could also be present at these synapses. We therefore determined whether incorporation of GluR2 attenuates postsynaptic paired-pulse facilitation and alters short-term plasticity at the parallel fiber-to-stellate cell synapse.

The extent of postsynaptic paired-pulse facilitation was assessed by delivering paired stimuli with a 10 ms interval to the parallel fibers and recording the evoked synaptic currents in stellate cells. The PPRs of evoked paired EPSCs were determined at the same synapse before and after repetitive PF stimulation (Fig. 5A). It is known that the paired-pulse ratio is also sensitive to changes in presynaptic release (Zucker and Regehr, 2002). Thus, to focus on the postsynaptic component, EPSCs were evoked by threshold stimulation of presynaptic parallel fiber stimulus to minimize the potential activation of multiple inputs. The failure rate for the first of the paired pulses was 47 ± 6%, and the average EPSC amplitude was $-61.1 \pm 3.4$ pA, which is within the range of the unitary EPSC amplitudes previously reported for stellate cells (Liu and Cull-Candy, 2000, 2005; Sun and Liu, 2007; Liu et al., 2010). We then also removed sweeps containing stimulation failures or multiple release events (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). If this selection method does remove the presynaptic contribution to PPR, we predicted that in the absence of intracellular spermine postsynaptic paired-pulse facilitation would be absent at the parallel fiber-to-stellate cell synapse. As expected, PPR without spermine in the patch electrode was 0.99 ± 0.04 ($n = 5$) (Fig. 5B).

When we included spermine in the patch electrode, naive synapses had a paired-pulse ratio of 1.15 ± 0.05 ($n = 6$), characteristic of a GluR2-lacking synapse (Fig. 5A,C). This value is in agreement with that reported previously (Rozov and Burnashev, 1999; Burnashev, 2005; Soto et al., 2007). After high-frequency parallel fiber stimulation, the PPR decreased to 0.96 ± 0.07 ($p < 0.05$ vs before PF stimulation; $n = 6$) (Fig. 5A,C), consistent with the absence of polyamine block of GluR2-containing receptors. This reduction in PPR after the high-frequency parallel fiber
stimulation supports the idea that a switch from GluR2-lacking to GluR2-containing receptors abolished postsynaptic paired-pulse facilitation.

The synaptic event selection method we used would not eliminate the potential presynaptic contribution because of multiple release sites. To rule out the possibility that some change unrelated to use-dependent polyamine unblock may have contributed to the attenuation of the paired-pulse ratio, we performed a control experiment in the absence of intracellular spermine. As expected, before stimulation, the recorded currents showed neither inward rectification nor paired-pulse facilitation (Fig. 5B, D). After the high-frequency parallel fiber stimulation, the rectification index and paired-pulse ratio were unchanged (before stimulation: RI, 1.06 ± 0.16; PPR, 0.99 ± 0.04; after stimulation: RI, 1.04 ± 0.11; PPR, 1.06 ± 0.05; n = 5) (Fig. 5D). This suggests that the decrease in PPR in the presence of intracellular spermine is most likely of a postsynaptic polyamine-sensitive origin, although we cannot completely rule out an additional presynaptic contribution. Thus, the activity-induced switch to GluR2-containing synapses abolishes postsynaptic facilitation at the parallel fiber-to-stellate cell synapse. This indicates that the AMPAR switch is an unusual form of plasticity because in addition to evoking a long-term change in the kinetics and amplitude of synaptic currents it also affects short-term synaptic plasticity.

Paired-pulse facilitation enhances the probability that stellate cells will fire AP doublets in response to two consecutive stimuli

An in vivo study showed that sensory stimulation evokes a burst of action potentials in cerebellar granule cells that innervate stellate cells (Chadderton et al., 2004). Such burst presynaptic activity would facilitate the postsynaptic response at GluR2-lacking receptor synapses, but not at GluR2-containing synapses. We therefore examined whether these two different postsynaptic responses affected their ability to evoke AP.

Paired conductance waveforms were constructed by spacing two prestimulation GluR2-lacking or post-PF stimulation GluR2-containing average waveforms obtained from sEPSC recordings at a 10 ms interval. The second pulse of the GluR2-lacking receptor template was scaled 1.15 times to account for the paired-pulse facilitation. The paired GluR2-lacking template of unitary conductance evoked a greater number of APs than the paired GluR2-containing template (Fig. 6A). When the first pulse evoked an AP, the second pulse nearly always failed to evoke an action potential.

We then tested whether multiple action potentials can be evoked by paired conductance waveforms that mimic coactivation of a number of inputs, using two times the unitary conductance (Fig. 6B). An action potential doublet is defined as having
Savinckouk and Liu • Synaptic AMPA Receptor Subtype Controls Action Potential Firing

**Figure 5.** Presynaptic stimulation-induced incorporation of GluR2 receptors abolished spermine-dependent postsynaptic facilitation. **A,** When spermine was included in the pipette solution, the amplitude of the synaptic current at +40 mV was attenuated, indicating the presence of GluR2-lacking AMPA receptors at the parallel fiber-to-stellate cell synapse. High-frequency presynaptic stimulation (HFS) induced an increase in the EPSC amplitude at +40 mV and a decrease at −60 mV. HFS also abolished postsynaptic paired-pulse facilitation that was observed at the naive synapse (A2/A1). **B,** In the absence of spermine, the synaptic current exhibited a linear I–V relationship even before stimulation. HFS did not alter the paired-pulse ratio. **C,** Summary of HFS-induced changes in rectification index (n = 6; *p < 0.01) and paired-pulse ratio (n = 6; *p < 0.05) in the presence of intracellular spermine. **D,** Summary of rectification index and paired-pulse ratio changes in the absence of intracellular spermine (n = 5). Error bars indicate SEM.

Figure 5. Presynaptic stimulation-induced incorporation of GluR2 receptors abolished spermine-dependent postsynaptic facilitation. A, When spermine was included in the pipette solution, the amplitude of the synaptic current at +40 mV was attenuated, indicating the presence of GluR2-lacking AMPA receptors at the parallel fiber-to-stellate cell synapse. High-frequency presynaptic stimulation (HFS) induced an increase in the EPSC amplitude at +40 mV and a decrease at −60 mV. HFS also abolished postsynaptic paired-pulse facilitation that was observed at the naive synapse (A2/A1). B, In the absence of spermine, the synaptic current exhibited a linear I–V relationship even before stimulation. HFS did not alter the paired-pulse ratio. C, Summary of HFS-induced changes in rectification index (n = 6; *p < 0.01) and paired-pulse ratio (n = 6; *p < 0.05) in the presence of intracellular spermine. D, Summary of rectification index and paired-pulse ratio changes in the absence of intracellular spermine (n = 5). Error bars indicate SEM.

an action potential in a 10 ms window after the first pulse, and another AP within a 60 ms window after the second pulse. These criteria were chosen from inspection of the peristimulus time histogram (Fig. 6C). We observed that the probability that a GluR2-lacking paired conductance waveform evoked an action potential doublet was greater than the GluR2-containing waveform (28.5 ± 6.4 vs 10.3 ± 3.2%; p < 0.001; n = 7) (Fig. 6B, D). This is primarily attributable to a greater amplitude of the EPSP elicited by the GluR2-lacking paired conductance waveform, relative to GluR2-containing waveform (second EPSP: GluR2-lacking, 15.3 ± 0.9 mV, vs GluR2-containing, 12.6 ± 0.8 mV; n = 4; p < 0.001) (Fig. 6B). Thus a PF stimulation-induced switch in AMPA receptor phenotype can reduce the frequency and alter the firing pattern of evoked action potentials in stellate cells.

The AMPAR switch not only abolishes postsynaptic facilitation but also reduces the current amplitude and prolongs the decay time. To test the importance of postsynaptic facilitation in evoking double action potentials, we systematically scaled up the second pulse of the GluR2-lacking templates from 0 to 20%. As shown in Figure 6D, the presence of postsynaptic facilitation significantly enhanced the probability of evoking a doublet. Overall, EPSP amplitude of the second pulse appears to determine the probability of evoking a doublet action potential (linear fit to several tested conditions, r = 0.997) (Fig. 7C).

Stress-induced incorporation of GluR2 receptors prolongs EPSC decay time but does not alter current amplitude. To examine the effect on the action potential firing pattern, we constructed a poststress paired conductance template from an average EPSC (rather than a template with a decay time that corresponds to the median decay time constant after fear stimulus). This template had a prolonged decay time (0.75 ms; control, 0.65 ms) but the same conductance amplitude as the control without facilitation (Fig. 7). We reasoned that the probability of evoking a doublet was likely to be reduced because of the lack of facilitation, but enhanced by the prolonged decay time, and that these two opposing effects may cancel each other out. Indeed, the probability for the paired poststress conductance waveform to evoke an action potential doublet was not significantly different from the control GluR2-lacking waveform (poststress, 31.6 ± 7.7%; control, 33.0 ± 6.8%; p = 0.3, paired t test; n = 7) (Fig. 7B). This was primarily attributable to the amplitude of the membrane depolarization being similar under the two tested conditions (second EPSP, poststress: 16.9 ± 1.7 mV; control GluR2-lacking waveform with 15% facilitation: 17.1 ± 1.7; n = 4) (Fig. 7C). However, the doublet action potentials evoked by the poststress paired conductance displayed a slightly longer delay of the second AP relative to control (GluR2-lacking and 15% facilitation: 40.3 ± 4.0 ms; poststress, 42.6 ± 4.2 ms; n = 7; p < 0.05).

Therefore, these two physiological stimulus-induced changes in EPSC waveforms produce distinct alterations in action potential firing. The stress-induced incorporation of GluR2 receptors markedly enhances the action potential firing probability but...
does not alter the firing pattern in response to two consecutive synaptic inputs. In contrast, the PF stimulation-induced switch in AMPAR phenotype reduces the action potential firing probability and abolishes the ability of the paired conductance waveform to evoke an action potential doublet.

Discussion

Experience can alter synaptic AMPAR subtype in several brain regions (Liu and Cull-Candy, 2000; Bellone and Lüscher, 2006; Clem and Barth, 2006; Goel et al., 2006; Plant et al., 2006). In cerebellar stellate cells, two different physiological stimuli, a fear-inducing stimulus and PF activity that mimic sensory stimulation, both increase synaptic GluR2-containing receptors but produce distinct changes in EPSC waveforms. Fear-inducing stimulus increases GluR2 gene transcription and promotes synaptic GluR2-containing receptor expression on the order of hours (Liu et al., 2010). This prolongs the decay time of the EPSCs without changing the current amplitude at −60 mV. Here, we show that the prolonged decay time produced a marked increase in AP firing probability. In contrast, a theta-burst-like PF stimulation induced a rapid change from GluR2-lacking to GluR2-containing AMPARs at the PF-to-stellate cell synapse. The switch in AMPAR phenotype reduced the amplitude and lengthened the decay kinetic of EPSCs (Liu and Cull-Candy, 2000), and led to a reduction in the EPSP–AP coupling frequency, which was primarily attributable to the decrease in EPSC amplitude. Furthermore, postsynaptic paired-pulse facilitation, a form of short-term plasticity, at cerebellar synapses enabled stellate cells to fire doublet APs. The switch in AMPAR subtype abolished postsynaptic paired-pulse facilitation, and thus two consecutive EPSCs evoked only single APs. Therefore, two physiologically relevant stimuli alter synaptic AMPAR phenotype via different mechanisms and produce distinct functional consequences; a stress-induced incorporation of GluR2 receptors enhances the EPSP–AP coupling, whereas a PF stimulation-triggered switch in AMPAR phenotype reduces EPSP–AP coupling and alters the pattern of APs in response to a burst of synaptic activity.

PF stimulation reduced EPSC amplitude at −60 mV by 32%. The similarity between this and a 30% reduction in the average single-channel conductance of Ca²⁺-impermeable AMPARs, relative to that of a mixture of both types of receptors (Liu and Cull-Candy, 2005), suggests that the total number of synaptic receptors is not greatly altered (“switch”). In keeping with this idea, control EPSCs mediated by a mixture of receptors contain both fast (−60%) and slow components, but only the slow component contributed to currents after PF stimulation. In contrast, fear stimulus did not alter EPSC amplitude at −60 mV, which suggests an increase in the total number of synaptic receptors by ~36% (“incorporation”). This is probably attributable to the insertion of newly synthesized GluR2 receptors since the current amplitude at +40 mV increased by 70% (Liu et al., 2010). The fast component still comprised ~30% of the total synaptic current. These results indicate that fear stimulus markedly promotes the insertion of GluR2 receptors and moderately reduces Ca²⁺-permeable receptors, therefore preserving the EPSC amplitude at −60 mV.

The spontaneous synaptic conductances that were recorded in physiologically relevant conditions displayed a wide range of amplitudes, all of which were found to be within the linear range of the input–output curve for stellate cells. The majority of single spontaneous events fail to evoke an action potential. However, simultaneous activation of multiple inputs (two or more coincident inputs) can more reliably trigger AP firing, consistent with previous reports (Carter and Regehr, 2002). The coincident re-

![Figure 6](image-url)
lease of two quanta increased the likelihood of AP firing from 20 to 60%, and four synchronous quantal events further enhanced the efficacy to >90%. Thus, the stellate cell input–output curve is tuned to a range that allows them to mostly “ignore” spontaneous release noise (small sEPSCs) yet fire reliably in response to as few as two coincident inputs.

We find that incorporation of GluR2-containing receptors at a synapse that normally expresses GluR2-lacking receptors prolongs the EPSC decay time constant. This is consistent with earlier observations that GluR2-containing receptors have slower kinetics (Burnashev et al., 1992; Mosbacher et al., 1994; Geiger et al., 1995; Gardner et al., 1999). This change in kinetics contrasts with long-term potentiation and depression that is associated with alterations in the amplitude but not the decay time of synaptic currents. The small increase in decay time (from 0.5 to 0.7 ms) that is associated with the insertion of GluR2 produced a marked increase in the probability of evoking an AP (by ~50%). Thus, stellate cells are tuned to the physiologically relevant range of EPSC kinetics. They are particularly sensitive to changes in decay time that are mediated by the insertion of GluR2. We have recently found that a fear-inducing stimulus, which causes the release of noradrenaline, activates β-adrenergic receptors and drives GluR2 gene expression and synaptic incorporation of GluR2-containing AMPARs (Liu et al., 2010). This gives rise to a prolonged decay time of EPSCs and thereby enhances AP firing in stellate cells.

Whereas fear-inducing stimulus selectively lengthens EPSCs, PF stimulation-induced switch in AMPAR subtype not only prolongs EPSC decay time but also reduces the current amplitude at −60 mV. Indeed, poststimulation synaptic current via GluR2-containing receptors became less effective at triggering an AP and consequently an increased number of coincident quantal events (from 2 to 2.5 quanta) would be required to trigger stellate cell firing ~60% of the time. However, no difference was observed in response to more than four synchronous quanta as the probability of evoking AP reached a plateau (data not shown). Thus, a switch in AMPAR phenotype is expected to alter the synaptic computation in response to a few synaptic inputs.

Granule cells fire a burst of APs in response to sensory stimulation (Chaderton et al., 2004). Although short-term presynaptic facilitation is known to enhance synaptic transmission in stellate cells (Chen and Regge, 1999), our results indicate that paired presynaptic stimuli can also produce different postsynaptic responses in stellate cells depending on the subunit composition of AMPARs (although the relative contribution from presynaptic and postsynaptic loci is unclear). Postsynaptic facilitation was observed at cerebellar synapses containing GluR2-lacking receptors but was abolished after PF stimulation when the synaptic receptors changed to GluR2-containing receptors. This is consistent with the reports that postsynaptic paired-pulse facilitation only occurs at synapses expressing GluR2-lacking receptors (Rozov et al., 1998; Rozov and Burnashev, 1999; Bagal et al., 2005). The postsynaptic facilitation not only enhances the AP probability but also enables the postsynaptic stellate cells to fire two APs in response to paired stimuli. In contrast, paired EPSCs that mimic the GluR2-containing receptor current after the PF stimulation evoked fewer APs and failed to evoke doublets. The generation of two APs by a pair of stimuli would therefore enhance GABA release from stellate cells and produce more powerful inhibitory effects on postsynaptic Purkinje cells and other synaptically connected stellate cells (Häusser and Clark, 1997; Lachamp et al., 2009).

Postsynaptic facilitation at Ca²⁺-permeable synapses occurs only during repetitive activation of the same synapse that triggers the use-dependent polyamine unblock. This contrasts with the purely coincident inputs coming from different synapses that give rise to two consecutive, but independent synaptic events (e.g., GluR2-lacking paired conductance waveform without facilitation). Thus, the large effect of postsynaptic facilitation on the firing pattern may allow stellate cells to distinguish between two randomly coincident EPSCs and a burst input originating from the same synaptic input.

Inhibitory transmission in the cerebellum is thought to be critically involved in cerebellar learning and memory (Sclaf et al., 2008; Wulff et al., 2009). The firing pattern of stellate/basket cells controls the timing and firing frequency of Purkinje cells (Midtgaard, 1992; Häusser and Clark, 1997; Jaeger and Bower, 1999). Our results indicate that an activity-dependent remodeling of AMPAR subtype can alter the stellate cell AP firing probability and pattern in response to a few synaptic inputs. Emotional stress-induced incorporation of GluR2 receptors increases the AP firing probability without compromising its ability to evoke AP doublets. This is expected to enhance the feedforward inhibition of Purkinje cell activity. In contrast, PF stimulation induced
a switch in AMPAR phenotype that reduced the AP firing probability and the ability of paired stimuli to evoke two consecutive APs. These changes would reduce inhibitory transmission onto Purkinje cells in response to the burst activity of granule cells that occurs during sensory stimulation (Chaderton et al., 2004). Thus, alterations in synaptic AMPAR phenotype in stellate cells are likely to influence the AP firing of Purkinje cells and would be expected to have a profound effect on cerebellar network activity.

AMPAR subtype remodeling has been widely observed in several brain regions under physiological and pathological conditions (Liu and Zukin, 2007). Recent studies show that the association of AMPARs with transmembrane AMPA receptor regulatory protein (TARP) and CKAMP44 also prolongs the decay kinetics and alters postsynaptic paired-pulse facilitation of AMPA currents (Tomita et al., 2005; Cho et al., 2007; Milstein et al., 2007; Morimoto-Tomita et al., 2009; von Engelhardt et al., 2010). Thus, our results suggest that enhanced AMPAR–TARP interactions could also strengthen EPSP–AP coupling. Our finding that both the waveform and postsynaptic paired-pulse facilitation of synaptic conductance are critical in controlling AP firing highlights the functional importance of activity-dependent changes in AMPAR phenotype and of AMPAR regulatory proteins.

References


used to avoid activating multiple synapses. Through a narrow opening electrode (5-7 MΩm pipette) was threshold stimulation. Multiples were also discarded. In addition, multiple peaks indicative of multisynaptic EPSC, the entire sweep was discarded. Therefore, whenever either of the pulses failed to evoke an

Suppl Fig 1. Post-synaptic facilitation may only be observed when the same synapse is activated by both pulses.

Acceptable

Multiple Release

Release Failures

Unacceptable traces:

Satchour and Liu Suppl. Fig 1
Chapter 4. Inhibition of Ca$^{2+}$-activated large-conductance K+ channel activity alters synaptic AMPA receptor phenotype in mouse cerebellar stellate cells
Abstract

Many fast-spiking inhibitory interneurons, including cerebellar stellate cells, fire brief action potentials (APs) and express GluR2-lacking, Ca\(^{2+}\)-permeable AMPA receptors. Recently we have shown that norepinephrine can increase AP duration and thereby promote GRIA2 transcription and synaptic GluR2 insertion in stellate cells. In the current project we tested whether the activity of potassium channels that control AP duration can suppress the expression of GluR2-containing AMPARs at stellate cell synapses. We report that large-conductance Ca\(^{2+}\)-activated potassium channels (BK) mediate a large portion of depolarization-evoked non-inactivating potassium current in stellate cells. Pharmacological block of BK channels has resulted in a synaptic AMPAR phenotype switch from GluR2-lacking, to GluR2-containing, Ca\(^{2+}\)-impermeable receptors. Nifedipine, an L-type Ca\(^{2+}\) channel blocker, has prevented the BK blocker-induced switch in AMPAR phenotype. Therefore, the availability of BK channels may suppress the expression of GluR2-containing synaptic AMPARs, and conversely a block of BK may lead to a phenotype switch from the GluR2-lacking to GluR2-containing receptors.

Introduction

Memories associated with learning and experience are stored as persistent modifications of both the neuronal synapses and genes (Kandel, 2001). At the synaptic level, much of the research effort has been dedicated to the changes involving AMPA receptors (Malinow and Malenka, 2002; Song and Huganir, 2002; Traynelis et al., 2010). One of the most profound changes to AMPAR properties is accomplished by re-assembling the receptor with or without inclusion of the GluR2 subunit. The presence of GluR2 makes the receptor impermeable to Ca\(^{2+}\), insensitive to polyamines, and generally reduces peak conductance while prolonging conducting time (Cull-Candy et al., 2006; Hansen et al., 2007; Isaac et al., 2007; Liu and Zukin, 2007; Traynelis et al., 2010).

The ratio of GluR2 to other AMPAR subunits varies between cell types in the brain. In general, inhibitory interneurons have lower levels of GluR2 mRNA compared to glutamatergic excitatory cells (Geiger et al., 1995; Sugino et al., 2006). Since GluR2-lacking receptors are Ca\(^{2+}\) permeable and have faster decay kinetics, this difference in GluR2 transcription could be partially due to the differences in the Ca\(^{2+}\) buffering properties of the interneurons or in their ability to respond to the faster currents through the GluR2-lacking receptors. For instance, a substitution of GluR2-containing for GluR2-lacking receptors in interneurons has been shown to alter long range synchrony (Fuchs et al., 2001). However, it is not well understood why the inhibitory interneurons have less GluR2.

Many inhibitory interneurons also express greater levels of high-threshold non-inactivating K channels such as Kv3 or large-conductance Ca\(^{2+}\)-activated K channels (BK) (Perney et al., 1992; Rudy and McBain, 2001; Sugino et al., 2006). The properties of these channels allow the neurons to discharge with high frequency, short duration action potentials
(Rudy and McBain, 2001; Salkoff et al., 2006). The effect of K channels on reducing action potential (AP) duration may be critical for limiting Ca\(^{2+}\) influx through the voltage-gated Ca\(^{2+}\) channels (Bailey et al., 1996; Womack et al., 2009). Since many of the GABA interneurons have high levels of high-threshold non-inactivating K channels and also have lower levels of GluR2, one can surmise that brief AP duration down-regulates GluR2.

Norepinephrine is known to prolong the AP duration in stellate cells by enhancing I\(_{\text{h}}\) current (Liu et al., 2010; Saitow and Konishi, 2000). We have previously shown that in stellate cells, norepinephrine can increase GRIA2 (GluR2 gene) transcription and GluR2 synaptic incorporation (Liu et al., 2010). Since GRIA2 transcription can be modified by changing AP duration, it is possible that K-channel expression/activity of the cell (which also changes AP duration) can by itself determine GluR2 phenotype. In this work, we tested whether high BK channel activity is responsible for low level of GluR2 expression in the cerebellar stellate cells.

Using pharmacological tools, we demonstrate that in stellate cells, a large portion of non-inactivating voltage-gated K currents is mediated by the BK channels. A prolonged (3 h) block of these BK channels causes a switch in synaptic AMPAR phenotype from predominantly Ca\(^{2+}\)-permeable, GluR2-lacking, to Ca\(^{2+}\)-impermeable, GluR2-containing receptors. Most likely this is because of the resulting AP broadening and an increase in Ca\(^{2+}\) influx through the L-type Ca\(^{2+}\) channels (Liu et al., 2011). Finally, we demonstrate that the switch can be prevented by co-incubation of the slice in nifedipine, an L-type Ca\(^{2+}\) channel blocker. Altogether, these results suggest a novel mechanism of synaptic plasticity, whereby a pattern of voltage-gated channel activity may directly influence synaptic AMPA receptor subunit composition.

**Methods**

**Slice preparation and recordings.** Slice preparation and recordings were performed as previously described (see Chapter 3). Briefly, the horizontal and sagittal slices (250-300 µm) were cut from cerebella of C57BL/6J mice at P18-22 on a Leica VT1000S vibrating microslicer in ice cold slicing solution (in mM: 125 NaCl, 2.5 KCl, 1 CaCl\(_2\), 7 MgCl\(_2\), 1.25 NaH\(_2\)PO\(_4\), 26 NaHCO\(_3\), and 25 glucose, pH 7.4 with a 5% CO\(_2\)/95%O\(_2\) mix). Stellate cells were located in the outer two thirds of the molecular layer as visualized using DIC with a x60 water immersion objective. Stellate cells were identified by their spontaneous action potential firing (in cell-attached mode) and by the presence of spontaneous synaptic currents (in whole-cell mode). All recordings were performed at room temperature.

**Electrophysiology.** Voltage and dynamic current clamp recordings were made using an Axopatch 700A, 700B, or a 200B amplifier (Molecular Devices, Foster City, CA). Recordings were low-pass filtered at 2-6 kHz and digitized at 20 kHz. Whole-cell recordings were performed using 5-7 M\(\Omega\) borosilicate glass pipettes.

**Voltage-clamp recordings.** All voltage clamp recordings measuring K currents were performed using a pipette solution contained (in mM) 135 KCl, 4.6 MgCl\(_2\), 0.1 CaCl\(_2\), 10 HEPES, 1 EGTA, 4 ATP-Na, 0.4 GTP-Na, pH 7.3 (75 - 95% composition; series resistance =20.3 ± 1.5
The bath solution contained 300 nM tetrodotoxin (TTX), 20 µM ZD7288, 1 mM kynurenic acid (KYNA) and 100 µM picrotoxin (PTX) to block Na\(^+\) channels, h currents, ionotropic glutamate receptors and inhibitory transmission, respectively. Amphotericin B (∼0.6 mg/ml) was added to the potassium-based pipette solution for perforated patch recordings (series resistance = 25 ± 3 MΩ, n = 4).

Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded from stellate cells using a cesium-based internal solution (in mM: 135 CsCl, 10 EGTA-Cs, 10 HEPES, 4 ATP-Na, 4 MgCl\(_2\), 5 TEA, 1 QX314 and 0.1 spermine; pH adjusted to 7.3 with 1M CsOH). Spermine (0.1 mM) was included in the patch electrode to block GluR2-lacking AMPA receptors at depolarized potentials and was used to determine the subunit composition of synaptic AMPA receptors. We calculated the rectification index (RI) as a measure of the GluR2 content at the synapse. RI was defined as the actual current recorded at +40 mV vs the one predicted based on the linear fit to the currents recorded at the negative holding potentials. Since the GluR2-lacking but not containing AMPARs are blocked by spermine at +40 mV, a synapse with greater ratio of GluR2 containing receptors will have a larger RI as compared to the synapse with mostly GluR2-lacking receptors. The series resistance was monitored throughout the experiment. The collected data was not used if the value changed by more than 20%.

Cerebellar slices were incubated for 3 hours in the presence of 1 mM KYNA and 100 µM PTX in aCSF (same as slicing solution except for 2 mM CaCl\(_2\) and 1 mM MgCl\(_2\)) at room temperature. Depending upon the experimental condition, the incubation solution also contained 100 nM Iberiotoxin (3 hours), 20 µM nifedipine, or 100 µM ryanodine as indicated. sEPSCs were recorded in aCSF in presence of 100 µM PTX. KYNA was washed out for at least 15 minutes before recording.

Synaptic events that did not have a smooth rise and decay phase were rejected. Average sEPSCs at each holding potential (typically average of 50-100 events over 10-15 min) were measured using N version 4.0 (written by Steve Trayer, Emory University) as described previously (Liu and Cull-Candy, 2000). The rectification index of sEPSC I-V relationship was defined as the ratio of the measured current amplitude at +40 mV to the predicted linear value at +40 mV (extrapolated from linear fitting of the currents at negative potentials). The decay time constant of individual synaptic events were determined by fitting the decay phase of an EPSC with a single exponential function, since the weighted decay time constants calculated from a double exponential decay fit are similar to those obtained with a single-exponential fit (Savtchouk and Liu, 2011).

Statistics. All values are expressed as mean ± SEM. Statistical significance was assessed by a two-tailed Student’s t-test. TTX, iberiotoxin and ZD7288 were obtained from Tocris Bioscience. Water soluble amphotericin B was obtained from Sigma.
Results

**Stellate cells express a large amount of large-conductance Ca\(^{2+}\)-activated potassium (BK) channels**

Potassium currents were measured in stellate cells using the perforated patch technique under voltage clamp in the presence of synaptic channel blockers picrotoxin and kynurenic acid, voltage-gated Na channel blocker TTX, and \(I_h\) blocker ZD7288. The cells were clamped at the resting potential of -100 mV to reduce K channel inactivation, and the currents were triggered by increasing the command voltage in 10 mV increments, up to +60 mV. Starting at about -30 mV, we observed prominent outward currents comprised of a larger transient peak followed by a smaller steady-state current (Figure 1 A). The largest steady-state conductance on the order of 10s of nS was reached at around -10 mV (Figure 1 B). The currents were very similar when recorded under the perforated patch and whole cell conditions, suggesting minimal impact of intracellular dialysis on the recorded currents (Figure 1 A). We therefore used the simpler whole cell method for further measurements.

Cerebellar stellate cells fire brief action potentials suggesting the presence of BK or Kv3 channels. We therefore tested for the presence of these channels by applying 1 mM triethylammonium chloride (TEA) solution, a compound that selectively blocks BK and Kv3 channels at this concentration (Rudy and McBain, 2001; Wang et al., 1998). Indeed, the application of TEA inhibited a large portion of voltage sensitive K current (Figure 1 A). At 0 mV, the transient peak current was reduced by 53.2 ± 1.8% and the steady-state non-inactivating current decreased by 42.8 ± 6.2% (n = 4; \(P < 0.05\), Figure 1 B, E). Hence a large portion of outward K current is mediated by either BK or Kv3 channels.

To further narrow down the K channel subtype, we used a scorpion toxin iberiotoxin (IBTX) that selectively blocks BK channels with high affinity (Salkoff et al., 2006). Accordingly, the application of 100 nM IBTX inhibited a large portion of the steady-state currents (70.4±4.8%; n = 4; \(P < 0.005\), Figure 1 C, E). This indicated that BK channels mediate a large portion of K currents in stellate cells. Further application of TEA on top of IBTX resulted in some minor additional block (Figure 1 C, E), consistent with the idea that much of the TEA sensitive currents are in fact conducted through the BK channels, but also suggesting a small contribution from Kv3 channels.

Since Kv3 channels are selectively blocked by the toxin 4-aminopyrididine (4-AP), we used this compound to examine the contribution of Kv3 channels to the currents in stellate cells (Coetzee et al., 1999). The application of 4-AP resulted in only a 13.3 ± 3.6% block of the voltage-gated K current (n = 5, \(P < 0.05\), Figure 1 D, E), suggesting that Kv3 channels do not make a large impact on non-inactivating K currents in stellate cells. This finding is similar to the previous report that 4-AP does not inhibit K current in basket cells, the cerebellar molecular layer interneurons (Southan and Robertson, 1998).
Stellate cells typically express GluR2-lacking Ca\(^{2+}\)-permeable synaptic AMPA receptors that can be switched to GluR2-containing receptors by synaptic activity (Liu and Cull-Candy, 2000), but also by postsynaptic AP broadening (Liu et al., 2010). Since BK channels are activated by the APs and can thus alter the AP duration in stellate cells (Liu et al., 2011), we asked whether the availability of BK channels is important for maintaining the GluR2-lacking phenotype. To answer that, we incubated the cerebellar slices with a BK blocker IBTX and examined the effects of BK blockade on GluR2-AMPAR inclusion.

GluR2-lacking and GluR2-containing receptors can be distinguished by their sensitivity to intracellular polyamines, with GluR2-lacking receptors showing a characteristic current reduction at positive potentials, and therefore an inwardly-rectifying current-voltage (IV) relationship. As previously reported, the stellate cells exhibited inwardly rectifying sEPSCs under the control (PTX+KYNA) conditions (rectification index: 0.29 ± 0.02 (n = 8), Figure 2 A). However, after three hour incubation with the addition of a BK blocker IBTX, there was a relative increase in the amplitude of sEPSCs at the positive potentials consistent with a greater incorporation of GluR2-containing receptors at the synapse (Figure 2 B). Accordingly, the rectification index of the sEPSCs significantly increased following the IBTX incubation from 0.29 ± 0.02 to 0.75 ± 0.07 (n = 3; P < 0.05 vs control, Figure 2 C). Likewise, the fitted decay time of the sEPSCs increased (Kolmogorov-Smirnov test, P < 0.0001, Figure 2 D) consisted with an increase in synaptic GluR2.

We have confirmed the switch of the GluR2 AMPAR subtype using the application of 1-naphthyl acetyl spermine (NASPM), a selective Ca\(^{2+}\)-permeable AMPAR blocker. This toxin reduces the currents through the GluR2-lacking but not GluR2-containing receptors. Under the control conditions, 500 nM NASPM application reduced the sEPSC amplitude by approximately 80% (n=6, Figure 2 E). However, when the slices were incubated in IBTX, the NASPM block was reduced to 47 ± 3% (n = 4; P < 0.0002, Figure 2 E, F), indicating an increase in the relative abundance of GluR2-containing synaptic AMPA receptors.

**Action potential (AP)-induced Ca\(^{2+}\) influx determines GluR2-AMPAR phenotype**

Cerebellar stellate cells are spontaneously active even in the absence of synaptic input. Such ongoing activity depolarizes a cell multiple times per second, and may result in a significant influx of Ca\(^{2+}\) through voltage-gated channels. Our group has shown that block of BK channels prolongs the duration of the action potentials and can thereby increase the influx of Ca\(^{2+}\) through the L-type voltage gated Ca\(^{2+}\) channels (Liu et al., 2011). We therefore tested whether AP-triggered Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels is important for determining GluR2-AMPAR phenotype.

We used 20 µM nifedipine, an L-type voltage-gated Ca\(^{2+}\) channel blocker to test whether these channels are involved in IBTX-induced GluR2 increase. The incubation of the slice with
BK blocker IBTX switches normally inwardly rectifying synapses to linear (Figure 2). However, co-incubation in the presence of nifedipine partially prevented this increase in the GluR2-containing receptors, with EPSCs remaining more inwardly rectifying compared to IBTX-treatment group (RI = 0.52 ± 0.02, n = 4; P < 0.05, IBTX + nifedipine vs IBTX alone, Figure 3). A 3 hour incubation in nifedipine alone actually slightly increased the GluR2 content of synapses compared to control (RI = 0.42 ± 0.02, n = 4, P<0.005, Figure 3C), but the overall currents were still inwardly rectifying. From this we conclude that the activation of L-type voltage-gated Ca\(^{2+}\) channels is necessary for IBTX-induced increase in synaptic GluR2 receptors.

**Intracellular Ca\(^{2+}\) stores and Ca\(^{2+}\)-induced Ca\(^{2+}\) release may influence synaptic GluR2 content**

We have previously shown that Ca\(^{2+}\) influx can change GRIA2 transcription in stellate cells via activation of Erk/MAPK pathway (Liu et al., 2010). However, the source of Ca\(^{2+}\) needed for this signaling is not known. In addition to the direct influx of extracellular Ca\(^{2+}\) through the L-type voltage gated channels, such influx can also trigger a Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from the intracellular stores by activating IP3 and ryanodine receptors (Berridge, 1998; Rose and Konnerth, 2001). Since CICR can potentially cause a substantial amplification in Ca\(^{2+}\) signaling, we decided to investigate whether the release from intracellular stores is required for increasing synaptic AMPAR GluR2 inclusion after BK block. To this end, we incubated the slices in 100 µM ryanodine, an alkaloid that blocks ryanodine receptors at this concentration (Verkhratsky and Shmigol, 1996).

After the slices were incubated in ryanodine for 3 hours to block Ca\(^{2+}\)-induced Ca\(^{2+}\) release, the rectification index of sEPSCs increased from 0.29 ± 0.04 (control, n = 8) to 0.72 ± 0.07 (n = 4; P < 0.005, Figure 4). This indicates that ryanodine caused the increase in GluR2-containing receptors at the synapse. However, it is not clear whether this is due to the block of CICR, since lower concentration of ryanodine (~10 µM), such as would occur at the beginning of the incubation) can also lock the ryanodine receptors at the half-open state (Verkhratsky and Shmigol, 1996). This would actually cause a transient release of Ca\(^{2+}\) from the intracellular stores (Llano et al., 2000), until the ryanodine concentration elevated to the level sufficient for the receptor block. Since the available CICR inhibitor already changes the GluR2-AMPAR phenotype, we are unable to rule out the involvement of CICR in IBTX-induced GluR2 increase.
**Discussion**

It is well established that synaptic activity is able to alter the prevalence of GluR2-containing AMPA receptors at the synapse (Gardner et al., 2005; Kelly et al., 2009; Liu and Cull-Candy, 2000). It is less clear whether the action potential characteristics alone can also affect the AMPAR phenotype. Here we demonstrate that the ongoing activation of BK channels limits Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels, which prevents GluR2-AMPAR incorporation at the stellate cell synapse. A three hour pharmacological block of these BK channels is sufficient to cause an increase in synaptic GluR2, probably by increasing GRIA2 transcription and the GluR2 synthesis and insertion at the synapse (Liu et al., 2010).

Ca\(^{2+}\) is an important signaling molecule able to change gene transcription in neurons (Berridge, 1998; Bito et al., 1997; Sheng and Greenberg, 1990). The duration of APs can control Ca\(^{2+}\) influx through the voltage-gated Ca\(^{2+}\) channels, which in turn may affect gene expression. In *Aplysia*, a reduction in the outward K current has been shown to broaden the AP, resulting in an increased Ca\(^{2+}\) influx and altered short-term neurotransmitter release probability (Bailey et al., 1996). In vertebrates, studies using *in vitro* neuronal culture preparation suggested that a reduction of Ca\(^{2+}\) influx through somatic channels may result in increased gene transcription and cause synaptic scaling (Ibata et al., 2008). Similarly, homeostatic plasticity mechanisms are known to affect synaptic strength of excitatory synapses in response to decrease in postsynaptic firing or a block of synaptic Ca\(^{2+}\) influx (Ibata et al., 2008; Sutton et al., 2006). In this work, we demonstrate that the change in the waveform of postsynaptic APs by itself is sufficient to cause a change in the AMPA receptor composition.

Ca\(^{2+}\) influx through ligand- or voltage-gated channels can trigger further Ca\(^{2+}\) release from intracellular stores (Berridge, 1998; Rose and Konnerth, 2001; Verkhratsky and Shmigol, 1996). Conversely, intracellular store release can control external Ca\(^{2+}\) influx by both modulating L-type Ca\(^{2+}\) channels (Adachi-Akahane, 2004) and hyperpolarizing the membrane potential via the activation of the Ca\(^{2+}\)-sensitive potassium channels (Berridge, 1998). Our ryanodine incubation results show that interfering with intracellular Ca\(^{2+}\) release can by itself increase the level of GluR2 at the stellate cell synapses. Since ryanodine can both transiently increase and suppress Ca\(^{2+}\) release depending on the temporal concentration profile, we are not able to perform an in-depth examination of the role that CICR may play in GluR2 increase caused by AP broadening.

Previous attempts to measure BK in molecular layer interneurons, basket cells, were not successful in observing significant BK currents, based on the absence of charybdotoxin sensitivity (Southan and Robertson, 1998). This is in contrast to our observation that a large portion of steady-state K currents are mediated by BK channels in molecular layer interneurons stellate cells (current study). One possible explanation for the discrepancy is due to the fact that the previous observations were performed using high 10 uM EGTA intracellular solution which would buffer the cytoplasmic Ca\(^{2+}\) concentration and therefore BK activation. The current study’s use of a lower EGTA concentration appears more physiological because the perforated patch recordings and the whole-cell current recordings produced indistinguishable results. An
alternative explanation could be the presence of beta-4 or possibly beta-1 BK subunits in basket but not stellate cells, which could decrease the sensitivity of the BK channels to the block by charybdotoxin or iberiotoxin (Lippiat et al., 2003; Meera et al., 2000).

Stellate cells are paradoxical because they are electrically compact, yet receive a large number of coincident inputs. We and others have previously shown that stellate cells readily fire APs in response to as few as 2 co-incident inputs (Carter and Regehr, 2002; Savtchouk and Liu, 2011). However, the stellate cells have approximately 2000 PF synapses, a large portion of which could be activated in a behaving animal. The presence of a large amount of BK conductance in stellate cells may allow them to dynamically adjust the synaptic response curve. Under low BK activation, these cells can react to 2-3 inputs, yet still remain linearly responsive to hundreds of inputs after larger BK recruitment. Recent in vivo experiments reported sustained elevation of $\text{Ca}^{2+}$ signal in cerebellar molecular layer interneurons during locomotion (Ozden et al., 2010), which is consistent with the idea of BK activation dynamically shaping the integrating properties of the cerebellar stellate cells.

We have previously reported that increasing AP duration via modulation of h-currents by norepinephrine can elevate synaptic GluR2 in stellate cells (Liu et al., 2010). In our current work we demonstrated that the expression of BK channels limits the duration of APs and restricts the expression of synaptic GluR2-containing AMPARs in the cerebellar stellate cells. Conversely, a block of BK channels can increase GluR2-AMPAR via an AP broadening that leads to an increase in $\text{Ca}^{2+}$ influx through L-type $\text{Ca}^{2+}$ channels. Therefore, the findings that K channel phenotype and/or AP duration can directly control AMPAR subunit gene expression may represent a novel type of synaptic plasticity. Such mechanisms may have important functional implications in light of the fact that BK channel activity itself can be readily modulated by multiple second messengers, hormones, and metabolic indicators (Hou et al., 2009).
Figure 4.1. BK channels mediate a large portion of voltage-gated K currents in stellate cells.

A. K+ current traces recorded from a stellate cell using perforated and whole cell patch clamp. Stellate cells were voltage-clamped at -100 mV and stepped from -90 mV to +60 mV, in 10 mV increments. Current-voltage relationship of the peak and non-inactivating currents (perforated patch, n = 4; whole cell patch, n = 10).

B and C. Depolarization from -100 mV to 0 mV elicited an outward current. TEA (1 mM, n = 4) and iberiotoxin (100 nM, n = 4) inhibited a large portion of the potassium current. Non-inactivating conductance-voltage relation shows that TEA and iberiotoxin (IBTX) sensitive current (= I_{control} - I_{TEA} or I_{IBTX}) are activated at -30 and -20 mV, respectively.

D. 4-AP (1 mM) moderately reduced potassium currents.

E. Summary of K+ current inhibition at 0 mV by TEA, iberiotoxin and 4-AP. (*, P < 0.05; current amplitude, control vs inhibitor; #, P < 0.05, IBTX vs IBTX+TEA).
**Figure 4.2. Block of BK channels induces an increase in synaptic GluR2 in stellate cells.**

Cerebellar slices were incubated with KYNA and PTX for 3 hours with or without a BK blocker IBTX. sEPSCs were recorded in presence of intracellular spermine.

[A. Without BK blocker IBTX (control), the average sEPSCs were attenuated at positive holding potential and displayed an inwardly rectifying IV relationship indicating the prevalence of Ca2+-permeable, GluR2-lacking AMPARs (n = 8).](#)

[B. When IBTX was included in the incubation solution, the synaptic current in stellate cells showed a near linear I-V relationship, indicating an increase in GluR2-containing AMPARs.](#)

[C. Summary data showing RI under control and IBTX treatment conditions.](#)

[D. Cumulative distribution of fitted decay time constants of individual EPSCs at -60 mV (Kolmogorov-Smirnov test, P < 0.0001).](#)

[E. Application of GluR2-lacking AMPAR blocker Naspm (500 nM) resulted in a greater inhibition of sEPSCs in control (n = 4) compared to IBTX-incubated cells (n = 4).](#)

[F. Summary data for Naspm block (***, P < 0.0005).](#)
Figure 4.3. Block of L-type Ca\(^{2+}\) channels can prevent IBTX-induced increase in synaptic GluR2.

A. sEPSCs recorded from control cells show inward rectification indicative of predominantly GluR2-lacking synaptic receptors.

B. Co-application of Nifedipine (NF, 20 µM) can largely prevent IBTX-induced increase in GluR2, preserving an inward IV relationship (n = 4).

C. Summary of rectification index of EPSCs under control and IBTX-treated conditions, with or without NF (*, P < 0.05; **, P < 0.005; ***, P < 0.0005).
Figure 4.4. Ryanodine induces an increase in synaptic GluR2-containing AMPA receptors.

A. Average sEPSCs recorded from control cells show inward rectification.

B. After a 3 hour incubation in ryanodine, the currents became linear (IV relationship, n=4) indicating a relative increase in the GluR2-containing AMPA receptors.

C. Group data showing an increase in RI of sEPSCs after 3 h incubation in 100 µM ryanodine (***: P<0.005)
Chapter 5. Dendritic synapses show distance-dependent variation in the inclusion of GluR2-containing AMPA receptors in cerebellar stellate cells
Abstract:

As the currents travel from the distal dendrites towards the soma, they may suffer a reduction in amplitude and an increase in kinetics, compared to the currents from the nearby synapses. Cells typically counteract this problem (dendritic filtering) by boosting the number of receptors at distal sites.

Here we report that the cerebellar stellate cells present a novel type of solution for maintaining “dendritic democracy.” In addition to an increase in the number of AMPA receptors located at the distal synapses, but also a change in the receptor subunit composition. Synapses located at proximal dendrites contain predominantly GluR2-lacking, Ca\(^{2+}\) permeable AMPA receptors. However, the rectification index of EPSCs evoked at the distal sites (>60 um) shows a significant increase in the presence of polyamines, consistent with a larger number of GluR2-containing receptors. The synaptic gradient requires an on-going protein synthesis and can be abolished by translation blocker cycloheximide, but does not rely on short-term transcription of DNA. This gradient can be pharmacologically shifted towards the distal sites by enhancing action potential duration (and back-propagation). Functionally, the synapses at distal dendrites may produce larger somatic response. In addition, the differences in receptor subtype between the proximal and the distal sites could allow for differential allocation of synaptic plasticity mechanisms within the interneurons of the cerebellar cortex.
Introduction:

Proper brain functionality requires faithful transmission of information across synapses. However, individual neurons may face severe topological constrains that force synapses to be located at different distances along the dendrite. As a consequence, synaptic currents through distal synapses can suffer from reduced amplitude and prolonged rise and decay kinetics compared to proximal synapses due to dendritic filtering (Hausser, 2001). This presents a problem for information processing. Experiments in pyramidal cells have revealed one possible compensatory mechanism which consists of a large increase in the number of AMPA receptors at distal dendrites (Andrasfalvy et al., 2003; Ito and Schuman, 2009).

Another way to modulate synaptic weight could be through changes in the receptor subunit composition (Cull-Candy et al., 2006). An AMPA receptor is composed of four different subunits (GluR1-4) (Dingledine et al., 1999). In particular, GluR2 subunit incorporation into the AMPA receptor is known to have a drastic effect on channel properties, including alterations in amplitude, an increase in decay time, decrease in desensitization, a reduction in Ca\(^{2+}\) permeability and polyamine sensitivity (Jonas and Burnashev, 1995; Swanson et al., 1997; Verdoorn et al., 1991). We have previously shown that GluR2 incorporation causes an increase in the current decay time and a decrease in post-synaptic facilitation, which enhances the action potential probability and alters the firing pattern in stellate cells (Savtchouk and Liu, 2011). Thus an increase in GluR2 at a distal synapse might actually increase the chances of evoking an action potential. Conversely, the more rapid kinetics of GluR2-lacking synapses might be useful for counteracting the effects of synaptic filtering. While it is not known whether there is a difference in the subunit composition between the proximal and the distal synapses of stellate cells, such difference would have a large impact on synaptic transmission.

Unlike glutamatergic neurons in the CNS, many GABAergic interneurons have Ca\(^{2+}\)-permeable AMPA receptors at the post-synaptic sites (Isaac et al., 2007). Afferent-specific targeting of different AMPAR subtypes has been previously reported in inhibitory interneurons (Tóth and McBain, 1998). Several types of plasticity involving a subunit switch from GluR2-lacking to GluR2-containing receptors have also been identified in cerebellar stellate cells in response to activation of Ca-permeable AMPA, beta-adrenergic, and mGlu receptors (Kelly et al., 2009; Liu and Cull-Candy, 2000; Liu et al., 2010). If there is a subunit difference between the proximal and distal synapses, this would imply that some of these forms of plasticity would become restricted to specific regions of the dendrite.

In this project we report our observation of the difference in GluR2 expression between the proximal and distal synapses on stellate cells. We examined the differences in synaptic properties that result from this distance-dependent distribution gradient. Finally, we examined the possible mechanism for establishing and maintaining the difference between the synapses. Our observations put into question whether “dendritic democracy” is enforced in the cerebellar stellate cells.
Materials and Methods:

Slice preparation and electrophysiology:

Horizontal and sagittal slices (300 µm) were cut from cerebella of C57BL/6J mice at P18-22 on a Leica VT1200 vibrating microslicer in ice cold slicing solution (in mM: 125 NaCl, 2.5 KCl, 1 CaCl2, 7 MgCl2, 1.25 NaH2PO4, 26 NaHCO3, and 25 glucose, pH 7.4 with a 5:95 % CO2/O2 mix). All recordings were performed at room temperature. Stellate cells located in the outer 2/3 of the molecular layer were visually identified using DIC with a x60 upright water immersion objective.

Voltage and current clamp recordings were made using Axopatch 700B amplifier. The recordings were low-pass filtered at 6 kHz and digitized at 20 kHz using Digidata 1320. Whole-cell recordings were performed using 5-7 MΩ borosilicate glass pipettes, and the cell series resistance was monitored throughout the experiment. The collected data was not used if the values changed by over 20%. Voltage clamp experiments were performed using a cesium-based internal solution (in mM: 135 CsCl, 10 EGTA-Cs, 10 HEPES, 4 ATP-Na, 4 MgCl2, 5 TEA, and 1 QX314 pH adjusted to 7.3). To measure GluR2 content of the synapse, the spermine was included in the internal solution at 100 uM unless otherwise indicated.

Stellate cells send out their dendrites radially within a sagittal plane of the cerebellar folium. Parallel fibers extend orthogonally to the stellate cell plane. Therefore, there is expected to be a reasonable correlation between the dendritic distance and stimulation distance within a sagittal plane, with stimulation sites more distant from the stellate cell body corresponding to the more distal synapses.

Therefore, synaptic AMPAR currents were evoked by placing a monopolar glass stimulating electrode filled with aCSF (5-7 MOhm) at various distances from the patched cell body and applying brief (~400 us) voltage pulses (5-25 V) in presence of 200 uM PTX and 10 uM R-CPP at 0.3 Hz.

IV relationship was recorded by pseudo-randomly clamping a cell at -60 to +40 mV potentials while evoking the synaptic current. Between 70 and 200 sweeps were collected at each potential at each site. The current amplitude at each potential was measured using several methods: 1) peak of the average of all sweeps at each potential, 2) average of the current peaks (within a 2-3 ms window) of all sweeps at that potential, 3) same as 2, but with failure removal. All three methods produced very similar results, but we ultimately used method 2 for calculating the RI because this minimized the error caused by the synaptic jitter, and produced the best linear fit at negative potentials. Method number 3 was not used for RI because of possibility of a bias toward the results due to the removal of sub-failure threshold events. We used the failure removal results from the method 3, however, to measure the relative failure rates at the positive and negative potentials; this was not found to vary between the proximal and the distal synapses (p=0.5), suggesting no distance-dependent pre-synaptic mechanism differences under our recording conditions. In addition to RI, we also calculated Rectification Ratio (I @
+40 / I @ -40 mV), and voltage-normalized Rectification Ratio (I @ +40 / I @ -60 mV x 1.5); in most cases all three produced very similar results; when a proper fit to the negative potential data could not be obtained, the data was not used and the synapse discarded from our analysis. Only the spontaneously firing stellate cells in the outer half of the molecular layer, with at least 2 measured stimulation sites were included in our analysis.

Images of the recording (patch) and stimulation electrode locations were captured at different focal planes and stored for off-line analysis. Since the parallel fibers run orthogonally to the dendritic plane of the stellate cell, we calculated the synaptic distance as a Cartesian distance between the z-projections of the patch electrode and each stimulation electrode onto the same plane. In a few pilot experiments we included Alexa or Rhodamine-based intracellular fluorescent dyes to visualize the dendrites and more accurately measure the synaptic distance; however, due to a large increase in synapse linearity with intracellular dyes, the RI could not be accurately measured and the data was not included in our analysis.

In order to obtain the accurate amplitude and decay measurements of synaptic currents, we manually selected individual events exhibiting a smooth rise and decay phases to avoid including multiple release cases (holding: -60 mV). Additionally, our use of threshold stimulation helped further minimize the chance of recruiting multiple synapses. The manually selected EPSCs were aligned and the resulting average waveform was fitted with a single component exponential decay function. multiple exponential fit generally produced similar results as previously reported (Savtchouk and Liu, 2011).
Results

Synaptic GluR2 increases at distal dendrites of stellate cells

To measure the amount of GluR2 incorporation into the receptors at the synapse, we took advantage of the fact that the intracellular polyamines selectively reduce the currents through GluR2-lacking (but not GluR2-containing) AMPA receptors at positive membrane potentials (Kamboj et al., 1995; Koh et al., 1995). We therefore included spermine in our patch pipette and used a focal stimulating electrode to activate the parallel fibers and measure the polyamine block at several synaptic locations along the dendrite (Figure 5.1A). Under control conditions at the proximal dendrites (closest to the soma), we observed a strong reduction in the evoked synaptic currents at positive membrane potentials (i.e. an inward rectification of currents), consistent with the presence of GluR2-lacking receptors (Figure 5.1B). However, at the distal sites the inward rectification decreased, consistent with an increase in GluR2. The full current-voltage (I-V) relationship (Figure 5.1C) shows an overall reduction in polyamine-dependent block at the distal sites suggesting an increase in GluR2 incorporation.

To systematically compare the amount of GluR2 between individual synapses in multiple cells, we calculated the rectification index value (RI) for each location. RI is defined as the actual current measured at +40 mV divided by the predicted value based on the linear fit to the current amplitudes measured at negative holding potentials. RI value of 1 corresponds to a purely GluR2-containing, non-rectifying synapse. A value of below 1 would indicate some inward rectification caused by the presence of GluR2-lacking receptors.

As shown in Figure 5.2, the observed RI values ranged from 0.31 to 0.94 (n=52, 16 cells). In other words, the receptors ranged from mainly GluR2-lacking (0.31) to almost purely GluR2-containing (0.94). When we plotted average RI value vs the stimulation distance from the cell body, it became apparent that the more distal synapses exhibit an increase in linearity (Figure 5.2A). The rectification index for proximal (<40 um) synapses was 0.47 ±0.02 (n=29), which increased to 0.64 ±0.06 (n=11, p=0.0005) for the intermediate (40-60 um) synapses, and stayed at 0.66 ±0.04 (n=12, p=0.0005) for the most distal synapses (>60 um, average 88 ± 7 um). The distance dependent increase in rectification index in the presence of spermine is consistent with an increase in GluR2 inclusion in AMPARs at distal synapses.

GluR2-lacking and GluR2-containing receptors can have different deactivation rates, affecting the decay kinetics of the synaptic currents through these receptors (Fuchs et al., 2001; Lomeli et al., 1994). We therefore examined whether the eEPSC decay time also increased at the distal dendrites. In order to obtain an accurate decay time, we used threshold stimulation to minimize the probability of activating multiple synapses and triggering multiple release. In addition, we only selected eEPSCs exhibiting a smooth rise and decay phases that indicated a unitary release. The selected events were aligned and the resulting average waveform was fitted with a single component exponential decay function since multiple exponential fit generally produced similar results (Savtchouk and Liu, 2011). The decay time of the average eEPSC
increased from 0.92±0.05 (n=27) ms for the proximal synapses to 1.38±0.14 ms (n=12) for the distal synapses (p<0.01, Figure 5.2D). This increase in decay time is consistent with an observed increase in GluR2 at the distal synapses; however, we cannot rule out the effects of dendritic filtering on modifying the waveform.

GluR2-containing receptors typically show reduced current amplitude. Unexpectedly, we have observed no significant difference between the proximal (-68.7 ± 3.6 pA, n=29) and distal synapses (-72.9 ±7.5 pA, n= 12, P=0.61, Figure 5.2C). Because the GluR2-containing receptors have a ~30% smaller single channel conductance compared to the GluR2-containing receptors in stellate cells (Liu and Cull-Candy, 2005), we can propose an increase in the total number of receptors at the distal sites. Other explanations for no decrease in the distal amplitude, such as semi-active propagation, are less likely due to the absence of voltage-gates Na currents in cerebellar stellate cell dendrites (Myoga et al., 2009).

Does the RI increase actually reflect the increase in GluR2 or is this perhaps due to an unrelated phenomenon such as a poor space clamp or a difference in dendritic filtering/attenuation at different potentials? To address this issue, we reasoned that if the rectification is due to differences in GluR2, then in the absence of intracellular polyamines we should not be able to observe the changes in rectification index. Figure 5.3A shows a representative experiment conducted without spermine in the patch pipette. The currents recorded under these conditions were not attenuated at positive potentials and were actually outwardly rectifying at the proximal dendrites (Figure 5.3B). As expected of the group data, we observed no significant differences in rectification between the proximal and the distal synapses (RI proximal vs distal: 1.19 ± 0.09 vs 1.14 ± 0.06, n=8 and 7, P=0.66, Figure 5.3C). However, we saw a slight increase in the amplitude at the proximal dendrites consistent with the removal of resting polyamine block at negative potentials (Rozov and Burnashev, 1999). It is less clear why there is an apparent decrease in the amplitude at the distal sites under spermine-free conditions; it is possible that this is due to the small sample size and may not be representative. There could also be distance-dependent differences in the distribution of transmembrane AMAR regulatory proteins (TARPs) that we did not address (see Discussion). Overall, the distance-dependent changes in rectification are in fact caused by intracellular polyamines, which strongly suggests the differences in GluR2 incorporation between the proximal and the distal synapses.

In summary, the changes in the polyamine-dependent rectification with synaptic distance indicate an increase in the GluR2 content at the distal synapses. The changes in decay time are consistent with the observed increase in GluR2. The absence of change in amplitude, however, suggests an additional distance-dependent mechanism regulating the number of receptors.

Action potential back-propagation sets up a master gradient for determining receptor composition

Stellate cell dendrites can extend over 100 um from the cell body. How can the cellular components accurately determine the dendritic distance in order to change the receptor
composition? A master gradient established by a different mechanism is typically necessary for setting up protein distribution over large-scale distances. One such mechanism could be a chemical diffusion gradient of a compound. Another mechanism could involve a voltage gradient which has recently been shown to affect the distribution of AMPA receptors in pyramidal cells (Ito and Schuman, 2009). Localized differences in the voltage-gated channel distribution could determine the properties of the nearby synapses (Andrasfalvy et al., 2008; Johnston et al., 1996; Magee and Johnston, 1997).

Cerebellar stellate cells are spontaneously active (i.e. fire action potentials) even in the absence of synaptic input. This spontaneous firing results in a periodic Ca\(^{2+}\) influx through the voltage-gated Ca\(^{2+}\) channels (Liu et al 2011 in press), which in turn may determine the AMPAR phenotype. The APs are generated close to the soma and propagate passively throughout the dendrites without the observable voltage-gates Na channel facilitation (Myoga et al., 2009). Therefore, as the dendritic distance increases, we speculated the amplitude of the propagating depolarization pulse (and Ca\(^{2+}\) influx) should be reduced (Figure 5.4A). Such a reduction could cause a spatial switch in the AMPA receptor subtype GluR2-lacking (close to soma) to GluR2-containing (far from soma).

We proceeded to test whether AP backpropagation acts as a master gradient for setting up the synaptic receptor subtype. AP back-propagation was pharmacologically enhanced by incubating the slice in 1 mM TEA (tetraethylamine chloride salt) for 3 hours (Figure 5.4B). We have previously shown that at this low concentration TEA is selective for BK/Kv3 channels and increases the duration of action potential in stellate cells (Liu et al., 2011). We therefore predicted that TEA incubation will enhance the spike back-propagation towards the distal dendrite by counteracting the dendritic attenuation of depolarization. Additionally, TEA has secondary effect of enhancing GluR2 transcription during long-term incubation (Liu et al., 2010). Therefore, actinomycin D (in DMSO) was co-applied together with TEA to counteract transcriptional changes. The propagation of APs was thus selectively enhanced for 3 hours by incubating the slice in TEA with actinomycin D. As a control, we kept the slice in actinomycin D alone without K-channel blockade.

Incubation in actinomycin D alone does not alter the dendritic GluR2 gradient. After a 3 hour transcription block, the distal sites still showed a greater rectification index (0.69 ± 0.07, n=7) compared to the proximal sites (0.54 ± 0.03, n=7, P=0.064, Figure 5.4C). A slight increase in the RI at the proximal sites, while not statistically significant compared to the control values for the same distance (P=0.11) may be due to the effect of DMSO.

After the TEA was used with actinomycin D to enhance AP back-propagation, the difference between the proximal and the intermediate synapses disappeared (p=0.52). Accordingly, the proximal sites under TEA incubation remained inwardly rectifying compared to the actinomycin D control (TEA+AcD vs AcD RI: 0.58±0.06 vs 0.54±0.03, n=13 and 7, p=0.65, Figure 5.4C). However, we have observed a significant decrease in the rectification index at the synapses located between 40 and 60 microns from the soma (TEA+AcD vs AcD: 0.52±0.05 vs 0.69±0.07, n=7 and 7, p=0.026, Figure 5.4B and C). This is consistent with the idea that
broadened AP would travel further within the dendrite and may result in an increase Ca\(^{2+}\) influx, and thereby reduce GluR2 at the intermediate synapses. No comparison between the distant (>60 um) regions was made because of the low n values obtained so far (preliminary data suggests an RI increase even in after TEA treatment).

We next examined how AP broadening affected the fitted decay time constant of unitary eEPSCs. We did not detect any differences in decay time at the proximal (<40 um) synapses (K-S test p=0.61, n=362 unitary eEPSCs after TEA+AcD vs n=265 for AcD alone). However, there was a large decrease in the decay time at the intermediate (40-60 um) synapses (K-S test p=0.0006, n=203 TEA+AcD eEPSCs vs n=248 AcD eEPSCs, Figure 5.4C). This decrease in the decay time is consistent with a decrease in GluR2. Therefore, the decay constant data supports the idea that enhancing AP back-propagation turns the intermediate region from the distal-like to a more proximal-like AMPAR phenotype. Moreover, a significant decrease in the decay time after TEA incubation suggests that the subunit composition, and not dendritic filtering, is the primary cause of longer decay time at the distal synapses. This suggests that AP back-propagation does in fact determine the switchover point for the GluR2 incorporation at AMPARs. We therefore proceeded to determine the mechanism that translates localized Ca\(^{2+}\) influx into changes in AMPAR phenotype.

**Ongoing protein synthesis but not transcription is necessary for maintaining the distance-dependent synaptic GluR2 gradient**

The examination of Ca\(^{2+}\) signal timecourse suggests a very limited diffusion of Ca\(^{2+}\) from the within a dendrite, possibly due to the presence parvalbumin and the overall diffusion properties in the stellate cells (Soler-Llavina and Sabatini, 2006). Since the Ca\(^{2+}\) influx is unlikely to travel far within a dendrite, localized machinery is needed to sense this influx and implement the GluR2-AMPAR switch-over. A number of mechanisms may regulate GluR2 ratio at the excitatory synapses of the stellate cells. AMPAR receptor composition may be altered within 10s of minutes via receptor trafficking mechanisms (Gardner et al., 2005; Liu and Cull-Candy, 2000). Additionally, AMPAR subunit gene transcription and translation is tightly controlled (Dingledine et al., 1999; Traynelis et al., 2010). The synaptic levels of GluR2 readily respond to changes in transcription (Liu et al. 2010) and localized translation of AMPAR subunits (Mameli et al., 2007; Sutton et al., 2006). Do the differences in the GluR2 distribution arise due to differential trafficking of subunits, or due to the differences in the local protein synthesis?

To test the importance of DNA transcription, we examined the results of actinomycin D incubation control from our TEA experiment. Since the dendritic gradient was preserved, we concluded that the transcription of new mRNA is not needed to maintain high GluR2 at distal synapses, at least during the span of several hours.

We proceeded to examine whether protein synthesis is needed for maintaining the differences in the GluR2 between the proximal and the distal synapses. We have measured the rectification ratio in proximal and distal synapses of the cerebellar stellate cells after incubating
the slice in the presence of protein synthesis blocker cycloheximide (100 uM) for 3 hrs (Figure 5.5). After the protein synthesis was blocked for at least 3 hours, the typical GluR2 gradient was no longer observable. Rectification index differences between the proximal and the distal synapses have disappeared (RI 0.45±0.07 for proximal vs RI 0.53±0.04 for distal synapses, p=0.43, Figure 5.5C). Compared to the control slices, the RI did not change at the proximal (RI 0.45±0.07 vs 0.47±0.02 control, p=0.79) or the intermediate synapses (RI 0.56±0.04 vs 0.64±0.06, p=0.34), but was significantly decreased at the distal sites (RI 0.53±0.04 vs 0.66±0.04, p=0.03).

It therefore appears that the trafficking mechanisms alone are not sufficient for maintaining the synaptic GluR2 gradient, and require an ongoing synthesis of new protein.
Discussion:

In this work we report an increase in the GluR2 at the distal synapses of the cerebellar stellate cells. The distal AMPARs show a decrease in polyamine sensitivity and an increase in the current decay time, but without any decrease in the current amplitude expected of the GluR2-containing receptors. Increasing the duration of spontaneous APs shifts the GluR2 gradient towards the distal dendrites, suggesting a possible role of voltage-gated Ca\(^{2+}\) influx. The establishment and maintenance of the GluR2 gradient requires protein synthesis. A block of protein synthesis abolishes the gradient while a block of transcription has no impact.

Distant-dependent differences in AMPAR plasticity at the proximal and distal synapses

Stellate cell receptors may be switched from GluR2-lacking to GluR2-containing, by repetitive PF stimulation, extrasynaptic NMDAR, or mGluR activation (Liu and Cull-Candy 2000, Sun and Liu 2007, Kelly et al. 2009). Since the GluR2-lacking receptors are localized predominantly at the proximal synapses, these types of plasticity are expected to be restricted to the dendritic regions closest to the stellate cell soma. We have previously shown that a substitution of GluR2-containing for GluR2-lacking receptors results in a 20% decrease in the chance of evoking an action potential (Savtchouk and Liu, 2011), which probably results from an LTD at the proximal but not distal dendrites. It remains to be seen, however, if the reverse of this process (substitution of GluR2-lacking instead of GluR2-containing receptors) might happen at the distal sites, and whether that would result in changes to the receptive field of the stellate cell (i.e. the area of inputs that reliably trigger stellate cell APs). Jorntell and Ekerot reported a large expansion of sensory fields in stellate cells following repetitive co-activation of the parallel fiber and the climbing fiber inputs (Jorntell and Ekerot, 2003). While only a small number of sensory inputs triggered large responses in stellate cells before the stimulation, the population was expanded to include many more synapses afterwards. This would be consistent with a distance-dependent plasticity idea, e.g. an insertion of additional (perhaps GluR2-lacking) receptors at distal dendrites.

The presence of GluR2 lacking receptors at only the proximal dendrite regions may also impart distance-dependent variation in pre-synaptic mechanisms (e.g. retrograde cannabinoid signaling). Activation of Ca-permeable AMPARs coupled with metabotropic receptors can lead to long-term depression involving cannabinoid (Soler-Llavina and Sabatini, 2006). This LTD would presumably not occur at the GluR2-containing, distal synapses.

On a separate note, GluR2 incorporation may be a preferable way for the cell to increase synaptic weight at remote, low-volume processes, because it prevents the large Ca\(^{2+}\) influx commonly observed during the opening of the GluR2-lacking AMPARs.
The origin of synaptic GluR2 gradient within dendrites.

Ca$^{2+}$ and Na$^+$ imaging experiments have shown that stellate cells do not have active propagation of action potentials in the dendrites, yet the depolarization (and Ca$^{2+}$ influx) may spread efficiently to the range of up to 60 um (Myoga et al 2009). Interestingly, 60 microns is the Cartesian range at which we observe a complete switch-over of the AMPAR subtypes. The direct comparison of these measurements is difficult however, since the dendrites do always not spread out in a straight line, and therefore our slice distance is expected to under-estimate the actual dendritic distance of the synapse. Nevertheless, the remarkable similarity between our observed switchover distance and the distance of the previously reported spike-induced Ca$^{2+}$ influx supports the role of somatic APs in setting up the synaptic GluR2 gradient.

What limits the influx of Ca$^{2+}$ at distal dendrites? One possibility is the passive dendritic filtering that reduces the spread of AP depolarization. In addition, a differential distribution of potassium channels has been shown to regulate voltage spread in other cell types (Ito and Schuman, 2009). Additionally, a change in the distribution of low threshold Ca$^{2+}$ channels may affect localized response to the propagating AP. Low threshold Ca$^{2+}$ channels (L, N type) were shown to be concentrated close to the soma while other channels (R, T type) located at the distal dendrites (Johnston et al., 1996). Our observation that TEA-induced AP broadening produces a shift in GluR2 lends support to the passive AP attenuation model, but the role of K, VG-Ca, or other channels or proteins cannot be ruled out.

The role of Ca$^{2+}$ in determining AMPA receptor composition

Our observations regarding the role of AP duration suggest that Ca$^{2+}$ influx may determine the amount of GluR2 at the synapse. The role of Ca$^{2+}$ influx with regards to GluR2 is convoluted, possibly leading to an increase or a decrease depending on the circumstances. Ca$^{2+}$ influx through synaptic GluR2-lacking AMPARs or extrasynaptic NMDARs may cause changes in insertion of the GluR2-containing AMPARs at the stellate cell synapse (Liu and Cull-Candy, 2005; Sun and Liu, 2007). Conversely, Ca$^{2+}$ influx through NMDARs may lead to a pullout of GluR2 containing receptors (Hanley and Henley, 2005). The levels and timecourse of Ca$^{2+}$ influx (large but transient vs moderate and prolonged) has been shown to play a role in glutamate receptor plasticity (Cummings et al., 1996; Yang et al., 1999), and thus may affect the exact outcome with regards to GluR2 incorporation. In addition, GluR2 degradation is sensitive and can be upregulated by Ca$^{2+}$ influx (Li et al., 2003). Finally, the involvement of intracellular stores may affect AMPAR phenotype (Rose and Konnerth, 2001). We have previously reported that the incubation of a slice in ryanodine (a toxin affecting Ca$^{2+}$ release from the intracellular stores) may increase synaptic GluR2 balance in stellate cells (Liu et al., 2011).

In addition to changing receptor trafficking, Ca$^{2+}$ influx has been shown to modulate GluR2 transcription and translation in stellate cells. Changes in AP-triggered Ca$^{2+}$ influx can lead to an increase in GluR2 transcription which also results in more synaptic GluR2 (Liu et al., 2010; Liu et al., 2011). However, a decrease in Ca$^{2+}$ influx, such as caused by blocking APs
with TTX, can also paradoxically lead to an increase in synaptic GluR2 (Gainey et al., 2009), (Sun and Liu, unpublished observations). In our model (see Figure 5.4A), localized Ca\(^{2+}\) influx due to the back-propagating AP affects localized GluR2 translation at the proximal, but not distal synapses. Ca\(^{2+}\) influx/AP block by e.g. TTX incubation in effect turns all the synapses into proximal and increases GluR2 synthesis.

The role of TARP and other auxiliary proteins in receptor distribution

AMPAR can interact with transmembrane AMAR regulatory proteins (TARPs) that can affect channel properties, as well as AMPAR expression and trafficking (Traynelis et al., 2010). TARPs have been shown to affect localization of the AMPARs (Kessels et al., 2009; Tomita et al., 2005). This suggests a possible contribution of TARP to the GluR2 gradient we observed in the dendrites. Perhaps more importantly, TARPS can affect polyamine sensitivity or decay kinetics of AMPARs (Cho et al., 2007; Soto et al., 2007). If one of the TARP isoforms is more tightly associated to the AMPA receptors at distal synapses, this could provide an alternative explanation of our observed changes in rectification and decay time of the currents at distal synapses.

The role of distal receptors in the lateral inhibition in the cerebellum

Early examinations of molecular layer interneurons have divided these cells into populations with either a short or a long axon (Fox et al., 1967; Palay and Chan-Palay, 1974). The interneurons with a shorter axon would be co-activated by the same beam of parallel fibers as the PCs they inhibit, and would thus provide a feed-forward “on-beam” inhibition onto these PCs. The longer axon interneurons would synapse onto distal PCs that would not receive the corresponding excitation from the PFs, thereby providing lateral inhibition (“off-beam”). Imaging and electrophysiological studies have verified the presence of lateral inhibition in reduced cerebellar preparation, with lateral inhibition beams exhibiting a very low threshold of activation and saturation expected of the electrically compact interneurons (Cohen and Yarom, 2000). While the morphological reconstructions of molecular layer interneurons do not support clear division based on the axonal size, it is nonetheless expected that about 25% of PF inputs will evoke only inhibitory currents in the PCs (Sultan and Bower, 1998). Based on the reported asymmetry and overlaps of the dendritic and axonal fields of interneurons (Sultan and Bower, 1998), it appears that the lateral inhibition-driving inputs may come exclusively from the distal AMPAR synapses, while the on-beam inhibition is driven by both the proximal and the distal inputs. The fact that distal synapses service a separate set of target PCs may explain why it may be advantageous for stellate cells to use a distinct population of receptors at distal locations.

If the lateral inhibition is preferentially triggered via the distal interneuron synapses, then the dendritic GluR2 gradient may explain the differences in the temporal summation response between the on-beam and the off-beam inhibition. The lateral inhibition is recruited at a lower
threshold but exhibits a large paired pulse suppression (Cohen and Yarom, 2000). This observation is consistent with our previous reports that GluR2-containing synapses may increase the chance of evoking a single AP by ~50%, but reduce the probability of a doublet by 300% due to the changes in decay time and post-synaptic facilitation by polyamines (Savtchouk and Liu, 2011).
Figure 5.1. Outward currents increase at distal synapses

A. Synaptic EPSCs were evoked by stimulating parallel fibers with a monopolar electrode. The example figure shows a patched stellate cell (open white circle, right electrode) along with several stimulation locations (colored circles). The tips of stimulating electrode were z-projected to the patch focal plane for easier visualization.

B. An example of the average current traces recorded at the stimulation sites as labeled in part A (currents normalized to the amplitude at -60 mV). At the distal sites there is a relative increase in the outward currents recorded at +40 mV.

C. The normalized I-V relationship for EPSCs evoked at each of the labeled sites. The red line is fitted to the currents at negative potentials (-60 to 0 mV) (error bars: S.E.M.)
Figure 5.2. Distant synapses exhibit properties consistent with GluR2 increase

A. The rectification index (RI) increases at distal synapses. Open circles represent the individual measurements (52 locations from n=16 stellate cells) and the line shows the segmented average (error bars: SEM). RI is defined as actual current measured at +40 mV over the current extrapolated from a negative potential fit (I@+40 mV/ I predicted).

B. The rectification index at the intermediate and distal synapses is significantly larger than RI at the proximal synapses (unpaired t-test, ***: p<0.001, error bars here and in other panels: SEM)

C. The unitary current amplitude does not change significantly with stimulation distance (unpaired t-test: proximal vs intermediate, p=0.26, proximate vs distal, p=0.61).

D. The synaptic EPSC decay constant (tau) increases at distal synapses consistent with the presence of more GluR2-containing receptors (unpaired t-test, **: p<0.01). The distribution of decay constants for individual fits to unitary eEPSCs shows a significant increase in decay at the intermediate and distal synapses (non-parametric Kolmogorov-Smirnov test, p<0.0001 between proximal (n=732 events, K-S statistic= 0.224) vs intermediate (n=300, K-S S=0.333), proximal vs distal (n=381, K-S S=0.197), and intermediate vs distal).
Figure 5.3. Rectification differences disappear in the absence of spermine

A. We evoked EPSCs in a stellate cell patched with a spermine-free pipette solution. The images of the stimulation pipette tips (openings marked with colored circles) were z-projected onto the focal plane of the stellate cell/patch electrode (open circle).

B. Example average current traces and the full I-V plots for each of the stimulation sites in panel A. Without spermine, the currents are no longer attenuated at the positive potentials at any of the sites.

C. The rectification index of eEPSCs evoked under spermine-free conditions is significantly greater than the control recordings (spermine-free proximal: 8 sites from 5 cells, distal: 7 sites from 4 cells, unpaired t-test vs spermine-containing recordings; **: p<0.001; ***: p<0.00005) but is not different between the proximal and the distal sites.

D. The amplitude of spermine-free eEPSCs is smaller at the distal sites (*; p<0.05).
Figure 5.4. Action potential back-propagation determines synaptic AMPAR phenotype

A. A working model of how action potential propagation could determine AMPAR phenotype. Action potential should be reduced in amplitude (“filtered”) as it propagates passively within the dendrites. As a result, distal regions may experience reduced Ca\(^{2+}\) influx resulting in greater GluR2 production. Increasing AP duration should increase back-propagation, and thereby shift the dendritic GluR2 gradient towards more distal regions.

B. A slice was incubated in K-channel blocker (TEA) in presence of actinomycin D for 3 hours. An example of a recording showing a decrease in outward current (at +40 mV) at the intermediate stimulation distance.

C. The rectification index of intermediate synapses is significantly reduced following the incubation with TEA (TEA+AcD, n=7 from 7 cells, AcD alone, n=6 from 5 cells, p< 0.05). Actinomycin D alone does not abolish the gradient (proximal vs distal, p=0.064, in progress).

D. The decay times for individual eEPSCs are significantly reduced at the intermediate dendrites consistent with a decrease in synaptic GluR2.
Figure 5.5. Ongoing protein synthesis is required for synaptic GluR2 maintenance at distal synapses
A. Synaptic currents were evoked in a slice incubated in the presence of protein synthesis blocker cycloheximide (3 hours).
B. An increase in the rectification index is abolished at the distal synapses.
Chapter 6. Discussion
A large portion of neuronal communication occurs via release of glutamate onto fast excitatory AMPA receptors. Learning and memory formation at the level of a single synapse could occur by changing the pre-synaptic neurotransmitter release probability or varying the total number and conductance of the post-synaptic receptors. An additional way to change the properties of the synapse is by varying the composition of AMPA receptors. Of the many subunits, GluR2 has the most profound influence on AMPAR properties (Isaac et al., 2007). GluR2 incorporation reduces Ca\(^{2+}\) permeability and current amplitude, prolongs the decay time, and decreases polyamine sensitivity (Traynelis et al., 2010). Changes in GluR2 content can be triggered by normal learning and experience (associative learning, sensory deprivation, emotional experience) as well as by many pathologic conditions such as ischemic assault and drug addiction (Bellone and Luscher, 2006; Clem and Barth, 2006; Goel et al., 2006; Kauer and Malenka, 2007; Opitz et al., 2000). Two issues remained unaddressed. First, despite the profound impact of GluR2 incorporation on the AMPAR properties, relatively little was known about how the change in AMPAR phenotype would affect the probability of evoking an action potential in the post-synaptic neuron. Secondly, while the synaptic activity is known to alter GluR2 content, it was not clear whether post-synaptic action potential activity can change the synaptic GluR2 expression. In this work we address these issues by examining the functional consequences of GluR2 increase via physiologically-relevant induction paradigms (chapters 2 and 3). We also aim to understand how the action potential duration and back-propagation affects the change from the synaptic GluR2-lacking to GluR2-containing AMPARs in stellate cells (chapters 4 and 5).

*Emotional stress causes increase in GluR2 transcription and synaptic insertion in stellate cells.*

*In vitro* experiments have shown that repetitive PF stimulation can induce an increase in GluR2-containing receptors by activating GluR2-lacking AMPARs, NMDARs, and metabotropic glutamate receptors (Kelly et al., 2009; Liu and Cull-Candy, 2000; Sun and Liu, 2007). A decrease in synaptic GluR2-containing receptors was associated with sensory stimulation in barrel cortex (Clem and Barth, 2006), or cocaine addiction in accumbens (Conrad et al., 2008). We now report the opposite effect in the cerebellar stellate cells, where an increase in synaptic GluR2-containing AMPARs is induced by a brief stressful stimulus (Liu et al., 2010; Savtchouk and Liu, 2011). We observed that norepinephrine acts via beta-adrenergic receptors to enhance the h-currents leading to a broadened action potential and an increase in Ca\(^{2+}\) influx, which causes an increase in the GluR2 transcription (chapter 2) (Liu et al., 2010). In addition, spontaneous action potential broadening alone (e.g. by blocking the BK channels with IBTX or TEA) is sufficient to cause an increase in synaptic GluR2 (chapter 4). These results suggest a direct link between the emotional experience of the animal and the synaptic AMPA receptor phenotype change across multiple synapses. Moreover, the GluR2 increase after stress exposure is predicted to be functionally different from the GluR2 substitution triggered by the repetitive PF activation (chapter 3).
The significance of increased current decay time after GluR2-insertion

The time-course of synaptic currents can determine how the postsynaptic cell responds to pre-synaptic input. The well-studied LTP/LTD processes entail changes in the current amplitude (Abbott and Nelson, 2000; Barry and Ziff, 2002). In contrast, AMPAR phenotype switch from the GluR2-lacking to GluR2-containing receptors can also increases the decay time-course of the currents. We observed that the acute fear exposure to the smell of a predator caused a long-lasting increase in the decay time of sEPSCs, but no change in the current amplitude. This is caused mainly by the insertion of the additional GluR2-containing receptors at the synapse. On the contrary, repetitive PF activation produced a reduction in the amplitude of EPSCs together with an increase in the decay time – a phenomenon best explained by a substitution of GluR2-lacking with the GluR2-containing receptors. These two different induction paradigms produced the current waveforms that had very different ability to evoke an action potential in a stellate cell. Stress-induced waveform had a 50% greater chance of evoking an AP compared to the control cell, while a repetitive activation waveform resulted in a 20% decrease in AP probability. These results suggest that following the acute stress exposure, there should be a large overall increase in the amount of feed-forward and lateral inhibition onto the Purkinje cells by the interneurons. Similar increase in inhibition of Purkinje cells was already reported in fear-conditioned animals (Scelfo et al., 2008). Since an increase in inhibition would result in the delay of PC spiking and the dis-inhibition of the deep cerebellar nuclei, we speculate that after the acute stress, the threshold for action, movement, or even cognitive processing could be lowered. In contrast, after the repetitive activation of the PF-SC synapses (such as a repetitive sensory stimulation without any emotional context), the stellate cells will fire fewer evoked APs in response to the stimulus, thus leading to a predicted decrease of PC inhibition, and the opposite action/movement effects.

Demonstration of functional role of the post-synaptic facilitation at stellate cells

A unique feature of the GluR2-lacking receptors is the availability of the postsynaptic facilitation via temporary relieve of intracellular polyamine block (Koh et al., 1995; Rozov and Burnashev, 1999). Paired pulse facilitation (PPF) or depression (PPD) are widely accepted pre-synaptic phenomena whose strength differs between brain regions and even individual synaptic pairs. These pre-synaptic effects strongly interfere with measuring post-synaptic facilitation (PSF) predicted to occur at the GluR2-lacking synapses. In the past, the post-synaptic facilitation at the GluR2-lacking synapses could only be demonstrated in vitro using fast glutamate application or ex vivo using photo-uncaging or complex paired neuronal recordings (Bagal et al., 2005; Rozov and Burnashev, 1999; Rozov et al., 1998). For the first time (chapter 3), we apply a new method to 1) observe the presence of post-synaptic facilitation at the stellate cell synapse, 2) demonstrate that the GluR2 insertion causes a decrease in the paired pulse ratio at the same synapse, and 3) show that this decrease in the facilitation causes an alteration in the evoked action potential pattern, a reduction in doublet probability.
In the present work we reported that the postsynaptic facilitation is observed at the control (GluR2-lacking) synapses of the stellate cells, and can be reduced following the substitution of the GluR2-containing receptors at the synapse. While the overall effect is relatively small (~20% facilitation), it may be particularly relevant for the PF-SC synapse because in vivo sensory input travels down the PFs as a short high-frequency burst (Chadderton et al., 2004) that is ideally suited for triggering the post-synaptic facilitation. By comparing the effect of facilitation of the paired pulse inputs, we observed a significant increase in the likelihood of evoking a doublet action potential in the stellate cell in the presence of facilitation. We conjecture that post-synaptic facilitation may allow the cells to distinguish between the sensory input burst, and the two randomly coincident “noise” inputs coming from different fibers (without facilitation). In addition, GluR2 “switch” would abolish the facilitation and severely decrease the resulting feed-forward inhibition onto the Purkinje cell.

The role of stellate cell sensitivity in the cerebellar processing

Stellate cells are distinct in that they are electrically compact and can be responsive to even individual (quantal) synaptic events (Carter and Regehr, 2002). We report that as few as two to three coincident inputs are sufficient to reliably activate a stellate cell, yet a single sEPSC will generally not evoke an action potential (Savtchouk and Liu, 2011). This is in contrast with a Purkinje cell that will require co-activation of hundreds of PFs to trigger an action potential (Santamaria and Bower, 2005). Given that the interneurons have a powerful inhibitory drive onto the PCs (Hausser and Clark, 1997; Jaeger and Bower, 1999) and are in turn very sensitive to the excitatory input, it stands to reason that sensory information arrives at the PCs primarily through the interneurons (SC-to-PC) and not through the granule cell/PF-to-PC pathway. What would then be the function of the PF-to-PC synapse? Perhaps this could act as a counter-balance to inhibition (Bower and Woolston, 1983; Jaeger and Bower, 1999), a kind of background subtraction, a “volume control” used to filter out the situations where the large numbers of PFs are activated by irrelevant input. Additionally, the inhibitory cell response could become saturated during large PF activation, while the PF-PC connection may remain in the linear range. The PF-to-PC synapse may provide the information about how many PFs are active at any given time, while the individual fiber/input processing and learning happens at the more powerful PF-SC and SC-PC synapses.

The role of Action Potential duration in determining GluR2 APMAR phenotype

Our group and others have previously reported that Ca\(^{2+}\) influx through the GluR2-lacking AMPA receptors or NMDA receptors can increase the level of synaptic GluR2-containing AMPARs in cerebellar stellate cells (Liu and Cull-Candy, 2000; Sun and Liu, 2007). Conversely, blocking the synaptic activity can increase the expression of GluR2-lacking AMPARs in response to the loss of synaptic Ca\(^{2+}\) influx in other brain regions (Sutton et al., 2006; Thiagarajan et al., 2005). Blocking post-synaptic action potentials also results in a homeostatic upregulation of synaptic AMPARs (Ibata et al., 2008). Therefore it has been shown that synaptic
activity can change receptor composition, and postsynaptic excitability can change the receptor number. However, it is not known whether postsynaptic APs alone can affect AMPAR phenotype.

Here we report that prolonged post-synaptic action potentials can induce substitution of GluR2-containing AMPARs at the GluR2-lacking synapses, even in the absence of any synaptic input. After the AP duration was increased by fear exposure or BK channel block, the synaptic GluR2 level went up in a transcription-dependent manner (chapters 2 and 4). However, the same increase in AP duration also caused a decrease in GluR2 at the proximal dendritic synapses, in a translation-dependent fashion (chapter 5). The combination of these processes is predicted to result in a transcription-dependent shift of the translation-derived GluR2 dendritic gradient. Unlike the previously described GluR2 changes in response to a repetitive synaptic activation (Liu and Cull-Candy, 2002) or neuron activity (Gainey et al., 2009; Thiagarajan et al., 2005), the AP-dependent dendritic gradient is neither restricted to the individual synapses, nor uniformly imposed at all synapses.

It is interesting that the amount of Ca$^{2+}$ influx during an individual AP is more important than the changes in the action potential firing rate, since the increase in frequency alone is not sufficient to cause the switch, but an increase in duration is (Liu et al., 2010; Liu et al., 2011). Similarly, the switch may be prevented by reducing the extracellular Ca$^{2+}$ during AP broadening protocol (Yu Liu, unpublished observations). We can speculate that the intracellular Ca$^{2+}$ buffers might be cleared rapidly in between the individual action potentials (Soler-Llavina and Sabatini, 2006), so the only way to overcome the threshold is either by increasing the instantaneous influx (changing duration of depolarization) or perhaps by applying a high frequency train of APs. In addition, it remains to be seen if the greater chance of synaptically evoked AP would also affect GluR2 gradient.

How can the predicted phenomenon of emotionally-shifted dendritic GluR2 gradient be incorporated in our understanding of plasticity mechanisms? Neurons maintain the synaptic strength by increasing the number of receptors at distant synapses – a function described as “dendritic democracy” (Andrasfalvy and Magee, 2001; Andrasfalvy et al., 2008; Hausser, 2001). Conversely, “homeostatic plasticity” exerts a global shift in the strength of all the synapses in response to changes in cell activity (Turrigiano et al., 1994). Our observed GluR2 gradient is in fact a hybrid phenomenon involving distance-dependent homeostatic “democracy”, which might also be shifted by the experience (stress). Since the described mechanisms work at the level of synaptic cohorts rather than individual inputs (e.g. not Hebbian) and provides some form of distance-dependent strength adjustment, yet may also be altered by learning (e.g. not purely homeostatic), perhaps a fitting term for this type of plasticity would be “heterostatic democracy”.
Conclusion

In summary, the cerebellum is an evolutionarily conserved structure responsible for motor learning and coordination, but also involved in higher cognitive responses. Despite the amazing simplicity of the cerebellar circuit, we still do not possess a satisfactory model for cerebellar learning. The currently accepted model that focuses on the LTD at the PF-PC synapse is not borne out by the experimental evidence. Recently, more attention has been drawn towards the role of inhibitory interneurons in cerebellar learning. Our research examines the effects of the changes in the excitatory synaptic drive onto these molecular layer interneurons – the stellate cells. We observed that a change in GluR2 content of AMPA receptors can be controlled not only by synaptic activation, but also by an acute emotional experience, as well as homeostatic/synaptic scaling processes. Calcium influx during an action potential can both set up a gradient of synaptic GluR2, and cause an overall composition change in the global synaptic cohort. In this work we report that post-synaptic changes in synaptic GluR2 have a profound effect on probability of evoking an action potential in the stellate cell. Our results may be helpful in understanding the role of GluR2 in the development of learning, addiction, and disease. The results have an important implication on understanding the functioning of the cerebellar network.
References


Andrasfalvy, B.K., and Magee, J.C. (2001). Distance-dependent increase in AMPA receptor number in the dendrites of adult hippocampal CA1 pyramidal neurons. Journal of Neuroscience 21, 9151-9159.


Herrick, C.L. (1891). Contributions to the comparative morphology of the central nervous systems. Journal of Comparative Neurology 1, 5-37.


Iaroslav Savtchouk

EDUCATION:
The Pennsylvania State University: PhD in Biology (Neuroscience); 08.2011
Advisor: Dr. SQ June Liu
University Park, PA 16802 USA
Franklin and Marshall College: B.A. in Biology, Minor: Chemistry; 05.2004
Lancaster, PA 17604 USA

PUBLICATIONS:

RESEARCH AND LABORATORY EXPERIENCE:
• Research assistant to Dr. SQ June Liu, LSU Health Sciences Center; 2009-2011
• Research assistant to Dr. SQ June Liu, The Penn State University; 2005-2009
• Analytical Chemist in QC lab, Pfizer Inc. (via Kelly Scientific); 2004-2005

TEACHING:
The Penn State University, State College, PA:
• Teaching assistant – Lab instructor in “Molecular and Cell Biology” Fall 2005
• Teaching assistant – Lab assistant in “Techniques in Neuroscience” Spring 2006
• Teaching assistant – “Neurobiology” Fall 2008

AWARDS:
• Braddock Award, The Penn State University 2005, 2008
• J. Ben and Helen D. Hill Award, The Penn State University 2008
• Global Scholar, Marshall Scholar, Franklin and Marshall College 2000-2004

CONFERENCE PRESENTATIONS:
• 2010 Society for Neuroscience Annual Meeting (San Diego, CA)
  Savtchouk, IA, Liu, SQ Distance-dependent changes in expression of synaptic GluR2 containing AMPA receptors in cerebellar stellate cells
• 2009 Society for Neuroscience Annual Meeting (Chicago, IL)
  Savtchouk, IA, Liu, SQ Changes in AMPA receptor phenotype alter the probability and pattern of action potential firing in cerebellar stellate cells
  Yu, L, Savtchouk, IA, Liu, SQ Emotional stress induces GluR2 gene transcription and a switch in synaptic AMPA receptor phenotype
• 2008 Society for Neuroscience Annual Meeting (Washington, DC)
  Savtchouk, I, Liu, J Activity-dependent switch in AMPA receptor phenotype alters the excitability of cerebellar stellate cells

LANGUAGES: English, Russia, Norwegian

PERMANENT MAILING ADDRESS: Kouzbasskoi divizii str.; h. 50, ap. 229; Pskov, 180024 Russia
Alternative name spelling: Yaroslav Savchuk — direct transliteration from Cyrillic (Ярослав Савчук Алексеевич)