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Timing, Short-term Plasticity, and Metaplasticity of STP

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Timing, Short-term Plasticity,

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A dissertation submitted in partial satisfaction

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by

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ABSTRACT OF THE DISSERTATION

Timing, Short-term Plasticity, and Metaplasticity of STP

by

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Doctor of Philosophy in Neurobiology

University of California, Los Angeles, 2013

Professor Dean Buonomano, Chair

Brain function depends on the communication between a vast number of neurons connected mostly via chemical synapses. The strength of these synapses changes over both short- and long-term time scales as a result of activity. Long-term changes in synaptic strength that last minutes, hours or more, including long-term potentiation (LTP) and long-term depression (LTD), have been carefully studied and are considered to be one of the neuronal bases of learning and memory. Synaptic strength also changes rapidly on the time scale of tens or hundreds of milliseconds in a use-dependent manner. This form of plasticity is termed short-term synaptic plasticity (STP). In contrast to LTP there has been significantly less work on STP.

Short-term plasticity is generally considered as a presynaptic phenomenon although its mechanism has not been fully understood. STP has generally been considered to be governed in large part by baseline synaptic strength, therefore limiting its potential functional significance. It

has been proposed, however, that STP can be "learned", specifically, that STP can be regulated by a novel learning rule in parallel to the associative learning rules governing baseline synaptic strength. Simulations in artificial neural networks have shown that their computational power is enhanced by STP plasticity. One goal of my work is to test this hypothesis using chronic stimulations in organotypic slices. I examined the induction of LTP in organotypic slices using optical pairing protocols, and tested the induction of metaplasticity of STP. Additionally, the development of STP in organotypic slices was also examined.

Another part of the dissertation focused on the function of STP in temporal processing, especially order selectivity of sensory events. Here experimental evidence of *in vitro* order-selective neurons is provided. This finding supports a potential role for STP in the formation of temporally-selective responses.

Overall my results add to the current understanding of the development, function, and regulation of short-term synaptic plasticity.

The dissertation of Weixiang Chen is approved.

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LIST OF ABBREVIATIONS

ACSF	artificial cerebrospinal fluid
AMPA	alpha-amino-3-hydroxy-5-methyl-4- isoxazole-propionic acid
ANOVA	analysis of variance
APV	(2R)-amino-5-phosphonovaleric acid
ChR2	Channelrhodopsin-2
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
EPSP	excitatory postsynaptic potential
GABA	γ-aminobutyric acid
IPSP	inhibitory postsynaptic potential
LTD	long-term depression
LTP	long-term potentiation
NMDA	N-methyl-D-aspartate
PPD	paired-pulse depression
PPF	paired-pulse facilitation
PPR	paired-pulse ratio
SEM	standard error of the mean
STDP	spike-timing dependent plasticity
STP	short-term synaptic plasticity

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Chapter 1 Introduction

Brain function depends on the communication between a vast number of neurons which are primarily connected via chemical synapses. The strength of these synapses is not constant, but rather the result of a history of activity over short- and long-term time scales (Bliss and Lomo, 1973; Markram and Tsodyks, 1996; Reyes and Sakmann, 1999; Froemke and Dan, 2002; Zucker and Regehr, 2002; Abbott and Regehr, 2004). Long lasting changes in synaptic strength have been carefully studied, and include long-term potentiation (LTP) and long-term depression (LTD). These forms of plasticity are considered to be one of the neuronal bases of learning and memory (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999; Martin et al., 2000; Lynch, 2004; Whitlock et al., 2006). In addition to these long-term forms of plasticity that last tens of minutes or longer, synaptic strength also changes rapidly on the time scale of tens or hundreds of milliseconds in a use-dependent manner. This form of plasticity is termed short-term synaptic plasticity (STP) (Gingrich and Byrne, 1985; Zucker, 1989; von Gersdorff and Borst, 2002; Zucker and Regehr, 2002; Abbott and Regehr, 2004). While the mechanisms and functional significance of long-term plasticity have been intensely studied over the last few decades, there has been significantly less work on STP. Addressing the computational role and regulation of STP is one of the main focuses of my thesis.

Long-Term Plasticity

In 1949, Donald Hebb made a famous postulate, that later became known as Hebb's rule:

"When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased."

Decades after Hebb's proposal long-term forms of plasticity that lasts hours or even days were demonstrated. This long-term plasticity was first observed extracellularly in the hippocampus using high frequency tetanic stimulation (Lomo, 1971; Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973). This form of plasticity, LTP, was later shown to obey Hebb's rule when it was demonstrated that it is associative, that is, it requires that both pre- and postsynaptic activity occur in close temporal proximity (Gustafsson and Wigstrom, 1986; Kelso and Brown, 1986; Magee and Johnston, 1997; Buonomano, 1999; Nishiyama et al., 2000; Bi and Poo, 2001; Whitlock et al., 2006). Hundreds of subsequent studies have established that long-term plasticity, including LTP and long-term depression (LTD), is one of the neural bases of learning and memory (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999; Martin et al., 2000; Lynch, 2004; Whitlock et al., 2006).

Hebb made a prediction about what conditions lead to increases in synaptic strength, he did not, however, postulate what led to decreases in synaptic strength. But experimental studies in a number of brain areas including hippocampus and cortex revealed that the relative timing between presynaptic and postsynaptic could lead to either potentiation or depression (Markram et al., 1997; Bi and Poo, 2001; Froemke and Dan, 2002; Bender et al., 2006; Froemke et al., 2006; Meliza and Dan, 2006; Caporale and Dan, 2008), which was later called spike timing-dependent plasticity (STDP). According to the STDP rule, presynaptic input which precedes postsynaptic spiking by tens of milliseconds results in long-term potentiation (LTP), while postsynaptic activity before presynaptic activity leads to long-term depression (LTD) (Froemke and Dan, 2002; Froemke et al., 2006; Caporale and Dan, 2008) (**Figure 1.1**).

In long-term plasticity studies, synaptic strength is often measured postsynaptically in response to a single presynaptic action potential every 10 seconds or longer (Bliss and

Gardner-Medwin, 1973; Bliss and Collingridge, 1993; Huang et al., 1994; Markram and Tsodyks, 1996; Froemke and Dan, 2002; Froemke et al., 2006). However, when a train of action potentials arrive at the presynaptic terminal tens or hundreds of milliseconds apart or less, the excitatory postsynaptic potentials (EPSPs) are not uniform throughout the train. Instead, synaptic strength can change rapidly in the form of either short-term depression or facilitation (Markram and Tsodyks, 1996; Reyes et al., 1998; Reyes and Sakmann, 1999; Oswald and Reyes, 2008). Because this form of plasticity occurs on the timescale of milliseconds, it is termed the short-term synaptic plasticity, or STP.

A long-standing issue relates to how LTP and STP interact. There is currently abundant evidence that depending on the cell types involved, LTP can leave STP relatively unchanged or change it dramatically (Huang et al., 1994; Markram and Tsodyks, 1996; Buonomano, 1999; Bender et al., 2006).

Short-term Plasticity

Eccles and colleagues first described STP at the neuromuscular junction over 70 years ago (Eccles JC, 1941), since then hundreds of studies have revealed that STP is a form of synaptic plasticity observed in most synapses (Zucker, 1989; Zucker and Regehr, 2002; Abbott and Regehr, 2004). Neocortical synapses exhibit robust STP in the form of short-term depression or facilitation (Markram et al., 1998; Reyes et al., 1998; Reyes and Sakmann, 1999; Rozov et al., 2001; Oswald and Reyes, 2008). Although historically not as much attention has been given to short-term synaptic plasticity as to long-term plasticity, more and more studies in recent years have been focusing on the mechanism and function of STP.

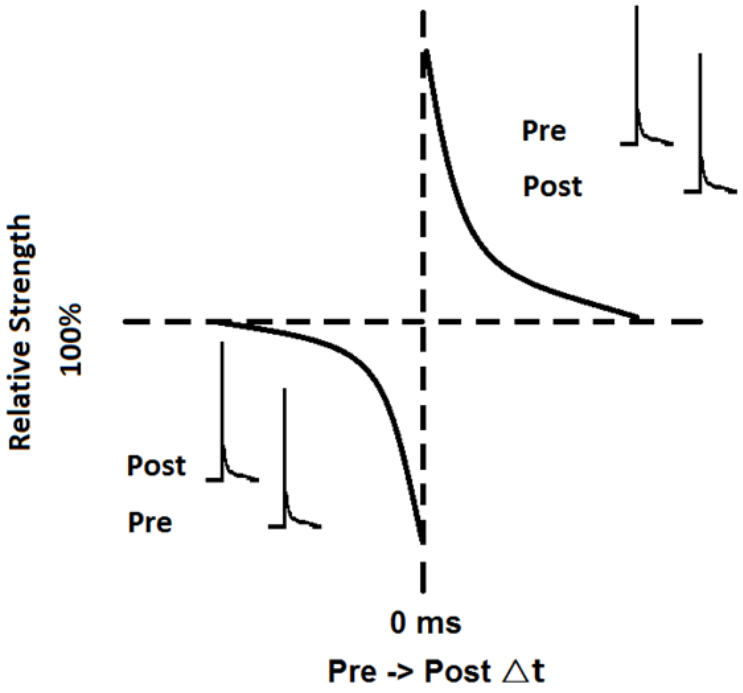

Figure 1.1. Spike timing-dependent (STDP) rule. Synaptic strength change when the presynaptic and postsynaptic action potentials fire within a few tens of milliseconds. The percentage change in the postsynaptic EPSP strength after the coincidence firing is shown as relative to the time difference between the two action potentials. When the presynaptic neuron fires before the postsynaptic neuron (upper right quadrant), the synapse is potentiated. When the postsynaptic neuron fires before the presynaptic neuron (lower left quadrant), the synapse undergoes depression. There is a short window within which the STDP rule is effective, as longer inter-spike intervals result in little or no change in synaptic strength.

Mechanisms of STP

Investigators have found that STP is a synapse-specific phenomenon that depends on the cell types of both presynaptic and postsynaptic neurons (Markram et al., 1998; Reyes et al., 1998; Rozov et al., 2001; von Gersdorff and Borst, 2002; Zucker and Regehr, 2002). The mechanisms underlying STP have not been fully unraveled, but it is generally agreed that STP is primarily a presynaptic mechanism that depends on two opposing factors: depression and facilitation (Gingrich and Byrne, 1985; Zucker, 1989; Varela et al., 1997; Markram et al., 1998; von Gersdorff and Borst, 2002; Zucker and Regehr, 2002; Abbott and Regehr, 2004).

Short-term depression is generally viewed as rising from the depletion of the readily releasable pool of synaptic vesicles (Rosenmund and Stevens, 1996; Schneggenburger et al., 2002; Zucker and Regehr, 2002). But, it is important to point out that other mechanisms can contribute to short-term depression as well (Sullivan, 2007), such as the decrease in presynaptic I_{ca} caused by the inactivation of the Ca channels (Forsythe et al., 1998; von Gersdorff and Borst, 2002; Xu and Wu, 2005; Xu et al., 2007).

Short-term facilitation is generally believed to be associated with the accumulation of residual calcium in the presynaptic terminal caused by trains of action potentials, which leads to the enhancement of subsequent transmitter release (Katz and Miledi, 1968; Zucker and Regehr, 2002; Burnashev and Rozov, 2005). In addition to residual Ca^{2+}, other mechanisms involving Ca^{2+}-dependent regulation of Ca^{2+} sensor proteins also contribute to short-term facilitation, such as the facilitation of I_{ca}, or saturation of Ca^{2+} buffers (Inchauspe et al., 2004; Ishikawa et al., 2005; Xu and Wu, 2005; Mochida et al., 2008).

Despite clear evidence of presynaptic mechanisms underlying STP, some studies also suggest that postsynaptic factors, such as the desensitization of AMPA receptors can contribute to STP as well (Rozov and Burnashev, 1999; Xu et al., 2007; von Engelhardt et al., 2010).

Although the mechanisms underlying STP are not entirely understood, it is clear that STP is observed at most synapses and likely encompasses multiple different phenomenon. At the mechanistic level STP seems to involve a complex balance and regulation of vesicle depletion, turnover, release, and mobilization.

Functions of STP

Even though STP has been widely observed at almost all neocortical synapses, its functional role in cortical computations remains unknown. On theoretical grounds it has been proposed that STP contributes to gain control (Chance et al., 1998; Galarreta and Hestrin, 1998; Abbott and Regehr, 2004; Rothman et al., 2009), working memory (Maass and Markram, 2002; Mongillo et al., 2008; Barak et al., 2010; Deco et al., 2010; Deng and Klyachko, 2011), and network stability (Galarreta and Hestrin, 1998; Sussillo et al., 2007). Importantly, STP is also hypothesized to contribute to temporal processing (Buonomano and Merzenich, 1995; Buonomano, 2000; Fortune and Rose, 2001). For example, theoretical results using artificial neural networks have demonstrated that STP improves the ability of neurons to discriminate spoken digits. Specifically, when STP was present in the network, neurons were more selective to whether digits were presented in the forward or backward direction, a cardinal feature of vocalization-sensitive neurons *in vivo* (Lee and Buonomano, 2012). It is noteworthy to point out that so far the proposed functional roles of STP rely mostly on theoretical hypothesis.

It is reasonable to postulate that the functional role of STP is likely to be in part determined by whether or not STP is itself plastic. Historically STP has often been viewed as solely an epiphenomenon of baseline synaptic strength. For example, strong synapses are often reported to be more likely to have paired-pulse depression (PPD), while weak synapses are more likely to display paired-pulse facilitation (PPF) (Katz and Miledi, 1968; Thomson et al., 1993; Debanne et al., 1996; Dobrunz and Stevens, 1997; Atzori et al., 2001; Zucker and Regehr, 2002; Boudkkazi et al., 2007). Additionally, it is often the case that the induction of LTP increases short-term depression, while induction of LTD favors short-term facilitation (Markram and Tsodyks, 1996; Buonomano, 1999; Bender et al., 2006). If STP is solely determined by baseline synaptic strength, then it may only have limited computational functions. If, however, STP can be independently regulated then STP may take a more active role in performing computations.

Long-term and Developmental Plasticity of Short-term synaptic plasticity

Despite the traditional view that short-term plasticity is determined by baseline synaptic strength, there is evidence suggesting a decoupling between unitary EPSP strength and STP. For example, investigators have failed to find a correlation between EPSP strength and paired-pulse ratio in different preparations (Thomson and Bannister, 1999; Brody and Yue, 2000; Hanse and Gustafsson, 2001; Chen et al., 2004; Fuhrmann et al., 2004; Oswald and Reyes, 2008; Boudkkazi et al., 2011; Chen and Buonomano, 2012). Additionally, after LTP induction in the mossy fibers, it has been found that early-phase LTP leads to a decrease in the paired-pulse ratio, but during late-phase LTP the paired-pulse ratio returns to baseline (Huang et al., 1994). Furthermore, in hippocampal cell cultures, it is found that increasing the calcium binding

protein neuronal calcium sensor-1 (NCS-1) can switch paired-pulse depression to facilitation without changing basal synaptic transmission (Sippy et al., 2003). Lastly, in gold fish brainstem M-axons it has been shown that each of the two EPSPs of a paired-pulse pattern can be independently regulated without affecting the other (Waldeck et al., 2000), providing a strong evidence for the plasticity of short-term synaptic plasticity itself.

STP is also plastic in the sense that it undergoes developmental changes. For example, observations in acute slices from somatosensory, auditory, and prefrontal cortex reveal a progressive increase in paired-pulse ratio (PPR) over development, generally from strong paired-pulse depression (PPD) to little PPD or mild paired-pulse facilitation (PPF) (Reyes and Sakmann, 1999; Kumar and Huguenard, 2001; Zhang, 2004; Frick et al., 2007; Oswald and Reyes, 2008; Cheetham and Fox, 2010; Takesian et al., 2010). It is believed that this developmental switch from paired-pulse depression to facilitation is to enhance the spread of excitation between pyramidal neurons (Reyes and Sakmann, 1999). In Chapter (2) I further confirmed that short-term plasticity itself undergoes developmental changes.

Although the mechanisms regulating the profile of STP are not fully understood, it is now recognized that STP is itself plastic. That is, as mentioned above STP is altered by the induction of LTP as well as through development (Song et al., 2000; Froemke et al., 2006; Caporale and Dan, 2008). But what has not been carefully examined is whether STP is plastic in the sense that there are specific learning rules in place to change it independently of baseline synaptic strength. In other words, it has not been addressed whether STP is "learned": is STP regulated by specific learning rules to optimize the computations performed at synapses?

Metaplasticity of STP: Is STP Learned?

One hypothesis that I focus on in my thesis is the novel question of the plasticity of short-term synaptic plasticity, that is, whether synapses learn to adopt different forms of STP. Specifically, can a synapse learn when it should be weakest or strongest during a train of pulses.

Hebb answered the question of what determines "which" synapses should be strong and which should be weak. Metaplasticity of STP asks the question of "when" (during a train of presynaptic spikes) a synapse should be strong (**Figure 1.2**). Specifically, it is proposed that if postsynaptic action potential is paired with the first of a train of presynaptic action potentials short-term depression should be favored. In contrast if a postsynaptic action potential is paired with the last spike of a presynaptic train, short-term facilitation should be favored. This notion extends Hebbian plasticity, because it proposes that a synapse should be stronger at the time it participates in the firing of a postsynaptic neuron.

Our lab has previously hypothesized that the plasticity of short-term synaptic plasticity, or the metaplasticity of STP, can be "learned" -- that is, STP can be regulated by learning rules that operate in parallel of the associative learning rules governing baseline synaptic strength so that the computations performed at synapses is optimized (Carvalho and Buonomano, 2011). For example, simulations in neural network demonstrate that the discrimination of complex spatiotemporal stimuli is enhanced if the parameters controlling STP can be adjusted during training according to a learning rule that governs STP (“temporal synaptic plasticity” learning rules). That is when STP undergoes plasticity the neural network is best at discriminating complex temporal stimuli, especially between the forward versus backward spatiotemporal patterns (**Figure 1.3**) (Carvalho and Buonomano, 2011).

One of the main goals of my thesis will be to test the hypothesis that short-term plasticity is governed by specific learning rules, as well as examine the developmental changes in STP. Toward these goals I have also examined for the first time the induction of LTP in cortical organotypic slices and the existence of order selective neurons in the cortical network.

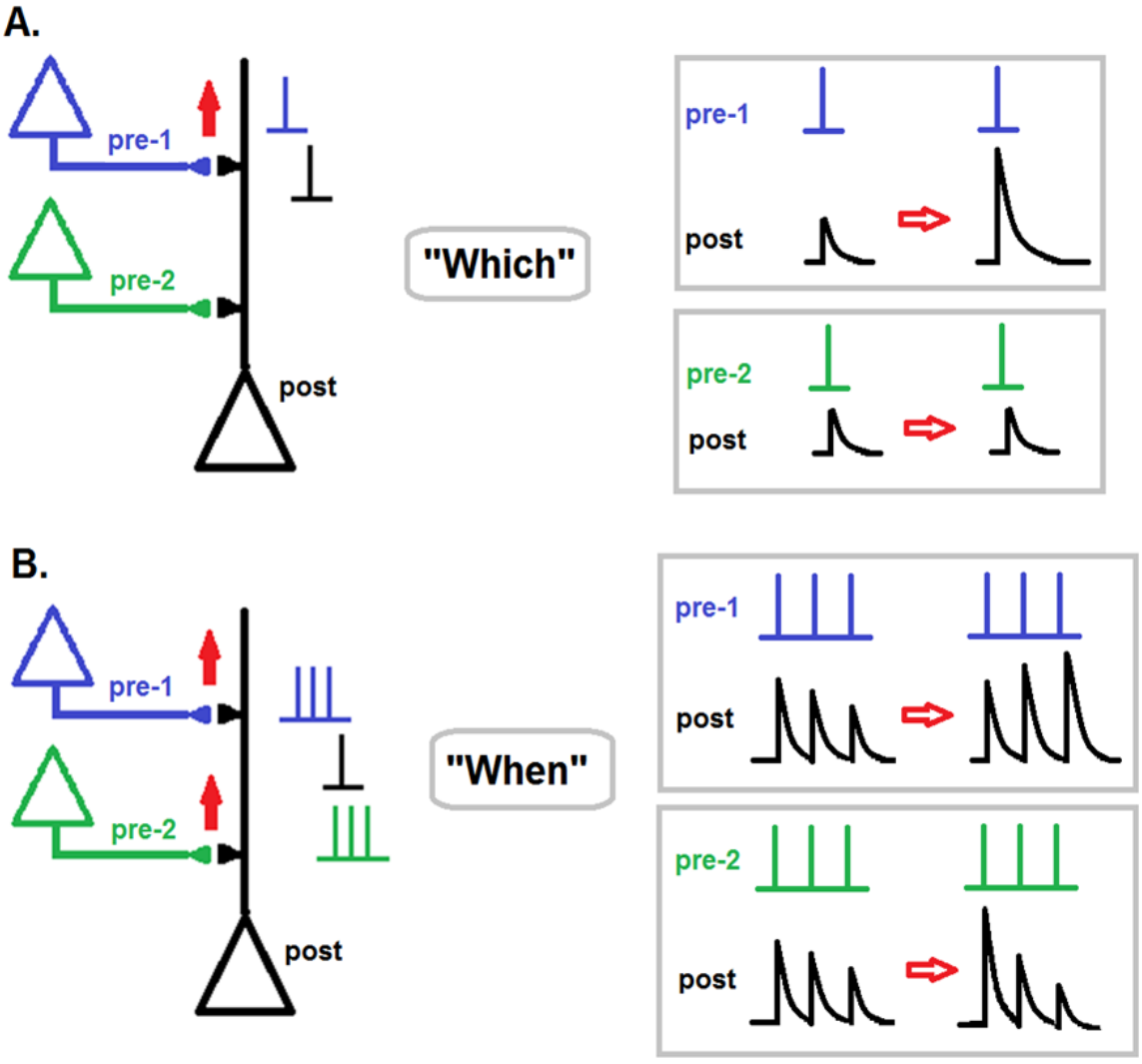

Figure 1.2. Hebb's rule and the Metaplasticity of Short-term synaptic plasticity. **(A).** Hebb's rule of "fire together, wire together" answers the question of "which" synapse should be stronger. **(B).** Metaplasticity of STP answers the question of "when" during a train of action potentials should the synapse be at its strongest point.

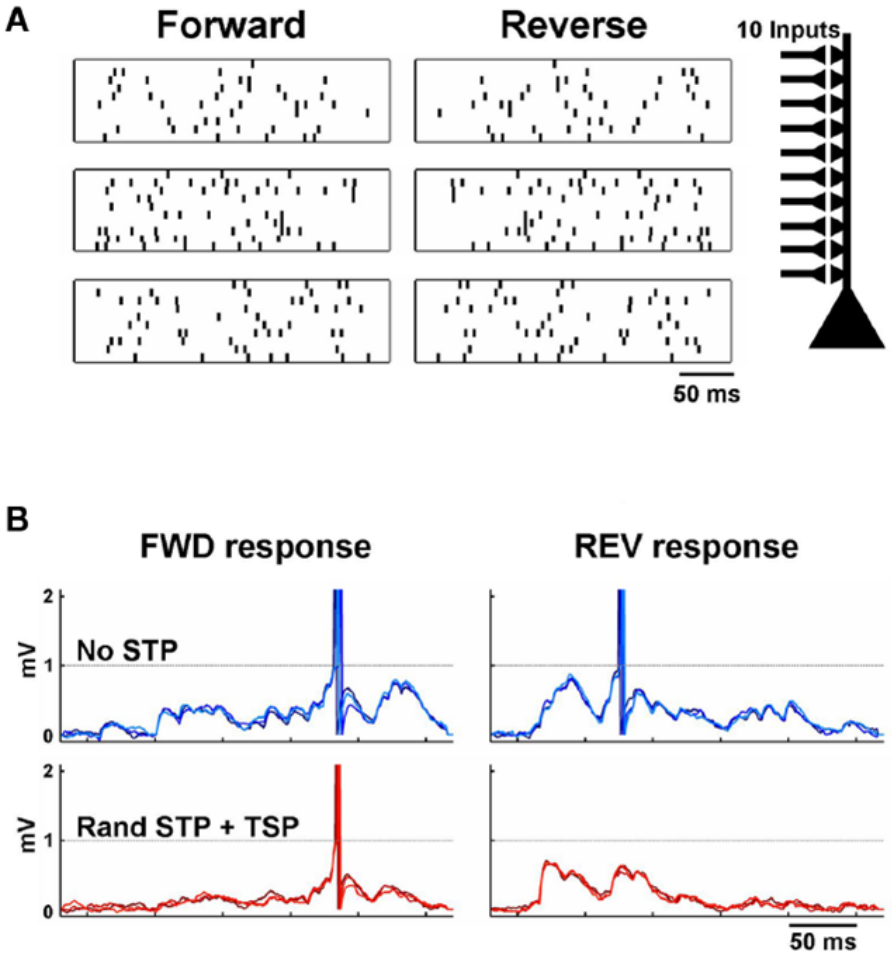

Figure 1.3. Metaplasticity of Short-term synaptic plasticity enhances the discrimination of complex spatiotemporal patterns. (A). Training the postsynaptic neuron to discriminate between inputs from 10 presynaptic neurons, both in the forward and reserved order. **(B).** Upper panel: without metaplasticity of STP (or temporal synaptic plasticity, TSP) the postsynaptic neuron cannot discriminate between forward and reversed stimuli. Lower panel: with STP independently regulated, the postsynaptic neuron learn to respond only to the forward stimulus pattern but not the reversed. (Carvalho and Buonomano 2011)

Outline of Dissertation

The studies performed here are aimed at examining a number of questions revolving around STP, metaplasticity of STP, and it's role in temporal and order selectivity. Towards this end I have used organotypic cultures.

The idea of metaplasticity of STP has previously been tested in acute slices but failed (Buonomano et al., 1997). We hypothesized that metaplasticity of STP may be protein-synthesis dependent, therefore would require longer training. The most suitable preparation to deliver chronic patterned stimulation is the cortical organotypic slices, which largely preserved the structure and properties of *in vivo* setup (for reviews, see Bolz, 1994; Gahwiler et al., 1997). But the short-term synaptic plasticity has never been studied or mapped out in the cortical organotypic slices.

In **Chapter 2**, I investigated the development of short-term plasticity in organotypic slices prepared from rat auditory cortex. I found an age-dependent decrease in short-term depression ratio (Chen and Buonomano, 2012), which largely reflects what has been observed in the *in vivo* development (Reyes and Sakmann, 1999; Zhang, 2004; Cheetham and Fox, 2010; Takesian et al., 2010). This work lays out the foundation of the experiments in the following chapters.

In **Chapter 3**, I provide an experimental demonstration of the function of short-term synaptic plasticity. Using acute cortical slices, I was able to show that there are neurons exhibiting order selectivity as a result of STP without any form of learning. This is also a collaborative work to provide experimental support for a computational simulation project.

To test the metaplasticity hypothesis I wanted to develop an associative LTP induction protocol that could be chronically applied over the course of hours. Towards this end, in

Chapter 4, I established that channelrhodopsin-2 (ChR2) can be used as an effective stimulation tool to induce LTP in organotypic slices. This study was the first to demonstrate associative LTP in cortical organotypic slices.

In **Chapter 5**, I provide experimental evidence of the induction of metaplasticity of STP using a combination of electrical stimulation and channelrhodopsin-2 stimulation. Additionally, I explored the basic mechanism of this novel form of experience-dependent plasticity.

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Chapter 2 Developmental Shift of Short-term Synaptic Plasticity in Cortical Organotypic Slices

DEVELOPMENTAL SHIFT OF SHORT-TERM SYNAPTIC PLASTICITY IN CORTICAL ORGANOTYPIC SLICES

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Abstract—Although short-term synaptic plasticity (STP) is ubiquitous in neocortical synapses its functional role in neural computations is not well understood. Critical to elucidating the function of STP will be to understand how STP itself changes with development and experience. Previous studies have reported developmental changes in STP using acute slices. It is not clear, however, to what extent the changes in STP are a function of local ontogenetic programs or the result of the many different sensory and experience-dependent changes that accompany development *in vivo*. To address this question we examined the *in vitro* development of STP in organotypic slices cultured for up to 4 weeks. Paired recordings were performed in L5 pyramidal neurons at different stages of *in vitro* development. We observed a shift in STP in the form of a decrease in the paired-pulse ratio (PPR) (less depression) from the second to fourth week *in vitro*. This shift in STP was not accompanied by a change in initial excitatory postsynaptic potential (EPSP) amplitude. Fitting STP to a quantitative model indicated that the developmental shift is consistent with presynaptic changes. Importantly, despite the change in the PPR we did not observe changes in the time constant governing STP. Since these experiments were conducted *in vitro* our results indicate that the shift in STP does not depend on *in vivo* sensory experience. Although sensory experience may shape STP, we suggest that developmental shifts in STP are at least in part ontogenetically determined.

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Key words: short-term synaptic plasticity, organotypic, development, pyramidal neurons

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Abbreviations: ACSF, artificial cerebrospinal fluid; EMEM, Eagle's minimal essential medium; EPSP, excitatory postsynaptic potential; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LTD, long-term depression; LTP, long-term potentiation; PPD, paired-pulse depression; PPF, paired-pulse facilitation; PPR, paired-pulse ratio; STP, short-term synaptic plasticity.

INTRODUCTION

Short-term synaptic plasticity (STP) is a virtually universal form of use-dependent synaptic plasticity (Zucker and Regehr, 2002; Abbott and Regehr, 2004). Since Eccles and colleagues first described STP at the neuromuscular junction over 70 years ago (Eccles et al., 1941), hundreds of studies have revealed that the strength of a synapse can change dramatically over the course of hundreds of milliseconds as a result of recent activity (Zucker, 1989; Zucker and Regehr, 2002; Abbott and Regehr, 2004). Neocortical synapses exhibit robust STP in the form of short-term depression or facilitation (Markram et al., 1998a; Reyes and Sakmann, 1999; Rozov et al., 2001). Despite the fact that STP is observed at essentially all neocortical synapses the contribution of short-term plasticity to cortical computations remains unknown. On theoretical grounds it has been suggested that short-term plasticity plays a role in gain control (Abbott et al., 1997; Chance et al., 1998; Galarreta and Hestrin, 1998; Rothman et al., 2009), working memory (Maass and Markram, 2002; Mongillo et al., 2008), and temporal processing (Buonomano and Merzenich, 1995; Buonomano, 2000; Fortune and Rose, 2001).

STP is primarily a presynaptic phenomenon that relies on the balance of two opposing factors: depression and facilitation (Gingrich and Byrne, 1985; Varela et al., 1997; Markram et al., 1998a; Zucker and Regehr, 2002; Abbott and Regehr, 2004). Depression is viewed as rising from the depletion of the readily releasable pool of synaptic vesicles (Schneggenburger et al., 2002), while facilitation is associated with the accumulation of residual calcium in the presynaptic terminal, which can enhance subsequent transmitter release (Katz and Miledi, 1968; Burnashev and Rozov, 2005). It is also known, however, that postsynaptic factors, such as desensitization of AMPA receptors, can also contribute to STP (Rozov and Burnashev, 1999; von Engelhardt et al., 2010).

The functional role of STP is likely to be in part determined by whether or not STP is itself plastic. Specifically, is STP carefully regulated by development and experience, or, as is often implicitly assumed, is the flavor of STP (e.g., depression versus facilitation) essentially an epiphenomenon of baseline synaptic strength. One indication that STP is not simply an epiphenomenon of initial synaptic strength is that there are differential interactions between STP and long-term potentiation (LTP) at different synapses. For example, while LTP and long-term depression (LTD) produce dramatic changes in STP in neocortical synapses, these forms of long-term plasticity produce

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little if any alterations in STP of CA1 synapses (Markram and Tsodyks, 1996; Buonomano, 1999; Selig et al., 1999; Bender et al., 2006; Cheetham et al., 2007; Hardingham et al., 2007). STP also varies significantly between different cortical areas (Atzori et al., 2001; Cheetham and Fox, 2010).

A number of studies have demonstrated that STP undergoes developmental changes (Reyes and Sakmann, 1999; Zhang, 2004; Cheetham and Fox, 2010; Takesian et al., 2010). Some studies have examined the role of sensory experience in the developmental changes of STP (Finnerty et al., 1999; Finnerty and Connors, 2000; Cheetham and Fox, 2011). For example, Cheetham and Fox (2011) reported that sensory deprivation in the visual cortex did not affect the developmental profile of STP. In contrast, they reported that whisker deprivation in the barrel cortex significantly affects STP, however a different study in the barrel cortex did not observe significant effects of sensory deprivation (Finnerty et al., 1999; Finnerty and Connors, 2000). A challenge inherent to examining the effects of sensory deprivation on the development of STP is that even with sensory deprivation there are numerous cross-modal developmental changes that can be altering activity in the deprived area. Indeed it is well established that cross-modal reorganization can take place in response to sensory deprivation in both juvenile and adult animals (Rauschecker, 1995; Sadato et al., 1996; Buonomano and Merzenich, 1998; Kujala et al., 2000; Bavelier and Neville, 2002; Feldman and Brecht, 2005). For example, visual deprivation induces homeostatic plasticity in both the visual and somatosensory cortex (Goel et al., 2006). To avoid potential confounding effects of cross-modal developmental changes in other brain areas, here we examine the developmental changes in STP in organotypic cultures; thus ensuring that potential changes in STP should be independent of sensory, motor, or behavioral experience.

Organotypic slices have proven to be a valuable preparation to study neuronal and synaptic function because the laminar, neuronal, and synaptic properties are relatively well conserved (for reviews see, Bolz, 1994; Gähwiler et al., 1997). Furthermore developmental changes *in vitro* seem to recapitulate aspects of *in vivo* development (Annis et al., 1993; Dantzker and Callaway, 1998; De Simoni et al., 2003; Uesaka et al., 2005; Johnson and Buonomano, 2007). However, to date, organotypic slices have not been used to study the development of STP. Towards this goal, we used rat organotypic slices from primary auditory cortex to study the ontogenetic development of STP *in vitro* in the absence of any form of sensory experience. Paired whole-cell recordings were performed in connected Layer 5 pyramidal neurons in slices that were cultured for 2–4 weeks. We found that the connection ratio and mean amplitude of unitary EPSPs are within the range previously reported in acute slices. We observed a decrease in short-term depression ratio from week 2 to week 4 *in vitro*, in the absence of a significant change in initial EPSP amplitude. There was a parallel developmental decrease in intrinsic excitability and resting membrane potential. Fitting the short-term plasticity data to a quantitative model suggests a decrease in the probability of release but no changes in the time constant of recovery from depression. We show that the developmental switch in STP profile in cortical synapses happens in organotypic slices. These results provide strong evidence that developmental shifts in STP are part of a local ontogenetic program—which does not imply that sensory experience does not influence development of STP.

EXPERIMENTAL PROCEDURES

Organotypic slice preparation

Organotypic slices were prepared using the interface method as previously described, and in accordance with the animal care and use guidelines of the UCLA Animal Research Committee (Stoppini et al., 1991; Johnson and Buonomano, 2007). Briefly, 7-day old Sprague–Dawley rats were anesthetized with isoflurane and decapitated. The brain was removed and placed in chilled cutting media. Coronal slices (400 μm thick) containing primary auditory cortex were cut using a vibratome and transferred onto cell culture inserts (Millipore, 0.4 μm pore size) with 1 ml of culture media. Culture media was changed 1 and 24 h after cutting and every 2–3 days thereafter. Cutting media was composed of EMEM (MediaTech cat. #15-010) plus 3 mM $MgCl_2$, 10 mM glucose, 25 mM Hepes, and 10 mM Trisbase. Culture media consisted of EMEM plus 1 mM glutamine, 2.6 mM $CaCl_2$, 1.85 mM $MgSO_4$, 30 mM glucose, 30 mM Hepes, 0.5 mM ascorbic acid, 20% horse serum, 10 units/L penicillin, and 10 μg/L streptomycin. Slices were incubated in 5% CO_2 and 95% O_2 at 35 °C for 8–28 days before recording.

Electrophysiology

Paired recordings were made from regular-spiking, infragranular pyramidal neurons (average depth 730 μm) using IR-DIC visualization. Experiments were performed at 30 °C in external solution composed of: 125 mM NaCl, 5.1 mM KCl, 2.6 mM $MgSO_4$, 26.1 mM $NaHCO_3$, 1 mM NaH_2PO_4, 25 mM glucose, and 2.6 mM $CaCl_2$. Note that in accordance with most organotypic slice experiments the external Ca^{2+} concentration is higher than the typical ACSF of acute slices (Stoppini et al., 1991; Musleh et al., 1997)—this is standard and is in part necessary to match divalent cation concentrations between the culture media and the external recording solution (Debanne et al., 1996; Hayashi et al., 2000; Johnson and Buonomano, 2007; Tominaga-Yoshino et al., 2008). The internal solution for whole-cell recordings contained 100 mM K-gluconate, 20 mM KCl, 4 mM ATP-Mg, 10 mM phospho-creatine, 0.3 mM GTP-Na, 10 mM Hepes, and was adjusted to pH 7.3 and 300 mOsm. EPSPs were elicited by triggering spikes using current injection: trains of five pulses at 50-, 100-, or 200-ms intervals. All cell pairs were bidirectionally tested for connections. All analyses were performed using software custom written in MATLAB. The average distance between connected neurons was 19.1 μm (range: 8.1–32.8 μm), estimated using the built-in micrometer of the MP-285 micromanipulators (Sutter).

Intrinsic excitability

To measure intrinsic excitability 250-ms current steps (0.05, 0.1, 0.15, 0.2, and 0.3 nA) were applied, and the number of spikes elicited were counted. The input–output (current–spike number) curve of each cell was fit to a sigmoid function (Marder and Buonomano, 2004). Excitability was defined as the E50 (the intensity eliciting half of the maximal number of spikes), and compared between age groups using a *t*-test.

Fitting data to model of STP

In order to quantitatively characterize STP we fit the short-term plasticity data to a model of STP (Tsodyks and Markram, 1997; Markram et al., 1998a; Maass and Markram, 2002). In the absence of short-term facilitation this model is characterized by two parameters: U, which defines the fraction of available transmitter that is released at each synaptic event; and τ_{rec}, the time constant that governs the recovery of synaptic efficacy from depression. This time constant is meant to capture the dynamics of the processes responsible for reversing the synaptic depression—τ_{rec} is often interpreted as relating to the replenishment of the readily releasable pool of vesicles. High values of U, which is bounded between 0 and 1, favor depression and low values facilitation. Although U is a simplified representation of the probability of release, if one were to assume the presence of many synaptic boutons U would be related to the probability of vesicle release (Markram et al., 1998b). Synaptic efficacy in response to successive action potentials is controlled by the variable R:

$$EPSP_n = A \times R_n \times U \quad (1)$$

$$R_{n+1} = 1 + (R_n - R_n \times U - 1) \times e^{\frac{-\Delta t}{\tau_{rec}}} \quad (2)$$

where n is the number of the current synaptic event, $n + 1$ is the next event, and Δt is the interval between nth and the $n + 1$th spike. The variable $EPSP_n$ represents the strength of the nth EPSP. The variable A is essentially a scaling factor directly dependent on the amplitude of the first EPSP. However, since the precise value of A does not alter in any way the STP estimates we normalized the value of $EPSP_1$ to 1 by imposing $A = 1/U$ (note that the baseline EPSP data are provided in Fig. 1). R_n represents the fraction of synaptic efficacy available for the nth spike. R's initial value is 1, it decreases with each EPSP, and recovers with the time constant τ_{rec}. The Tsodyks–Markram model of STP also incorporates a facilitation term (τ_{fac}) that accounts for short-term facilitation, however, since we did not observe facilitation at the synapses analyzed here this term was not included.

RESULTS

Paired recordings were performed in Layer 5 pyramidal neurons in organotypic slices of rat primary auditory cortex as previously described. To determine if a pair of neurons was connected, a train of five spikes (10 Hz) were elicited in one neuron and the averaged voltage traces of the other neuron was examined. Then the pair was tested in the opposite direction (Fig. 1A and B). Out of 161 pairs of recorded neurons 35 were connected (connection probability of 21.7%), and out of these 35 connected pairs 4 were reciprocally connected. While the connection probability is higher than some previous reports it is within previously observed ranges, and consistent with the data that the organotypic slices have higher connection probabilities than acute slices (see Discussion; De Simoni et al., 2003). The mean amplitude of unitary EPSPs was 1.01 ± 0.12 mV, ranging from 0.16 to 3.96 mV, also comparable to acute slices (Markram et al., 1997; Debanne et al., 2008).

Developmental changes in short-term synaptic plasticity

To determine if STP in organotypic slices also undergoes developmental change *in vitro*, we analyzed STP during the second week (8–15 days) and fourth week (22–27 days) *in vitro*. STP was characterized by examining the change in EPSP amplitude in response to five presynaptic action potentials at 5, 10 and 20 Hz. Trains were presented every 5 s in alternation. Synapses exhibited varying degrees of paired pulse depression, yet, from the second week to the fourth week there was a significant increase in the paired-pulse ratio (PPR = $EPSP_2$/$EPSP_1$) from 44 ± 6% to 67 ± 6% (10-Hz data). The mean amplitude of unitary EPSPs remained unchanged (1.07 ± 0.24 mV compared to 0.91 ± 0.12 mV, at 2 and 4 weeks, respectively). A two-way analysis of variance of age versus pulse number (repeated measures factor) revealed a significant interaction between age × pulse number ($F_{4,80} = 3.9$, $p = 0.006$), while the main effect of age was not significant. When EPSP amplitudes were normalized to EPSP1 there was a significant main effect of age ($F_{1,20} = 12$, $p = 0.002$) (Fig. 1C and D). Quantification of the 5- and 20-Hz data produced similar results (data not shown). Thus, while there was no significant change in mean initial EPSP amplitude, there was a significant increase in the PPR (less paired-pulse depression).

In order to examine the relationship between initial EPSP amplitude and STP we analyzed the correlation between EPSP amplitude and PPR. For week 2 synapses there was a significant correlation between EPSP amplitude and PPR ($p = 0.039$), however this correlation was not observed in week 4 ($p = 0.319$) (Fig. 1E). These results suggest that over the course of maturation there may be progressively more factors involved in the regulation of STP.

Developmental changes in cellular properties

Since previous studies have also reported developmental changes in the intrinsic properties of pyramidal neurons in acute and organotypic slices (Zhang, 2004; Johnson and Buonomano, 2007) we also analyzed changes in excitability and membrane properties between week 2 and 4 slices. To measure intrinsic excitability, 250-ms duration current steps were injected into individual cells every 10 s (with amplitudes of 0.05, 0.1, 0.15, 0.2, 0.3 nA) and the number of spikes elicited were counted (Fig. 2A). There was a significant decrease in intrinsic excitability, as measured by the shift in the input–output curve (see methods, $t_{143} = 3.57$, $p = 0.0005$, $n = 61$, 84 respectively) (Fig. 2B). In addition, there was a significant decrease in the resting membrane potential during the same development period (−58.4 ± 0.7 mV and −61.3 ± 0.5 mV, week 2 and 4 respectively; $t_{143} = 3.3$, $p = 0.001$) (Fig. 2C). The input resistance was not significantly different (202.8 ± 6.5 MΩ, 189.3 ± 6.5 MΩ) (Fig. 2D).

Quantitative modeling analysis of STP

While a number of studies have reported developmental changes in STP, what has not yet been examined is whether these changes are mostly consistent with developmental alterations related to probability of release and/or changes in the temporal profile of STP. Quantitative models often characterize STP with a parameter U that captures initial release probability and a time constant that

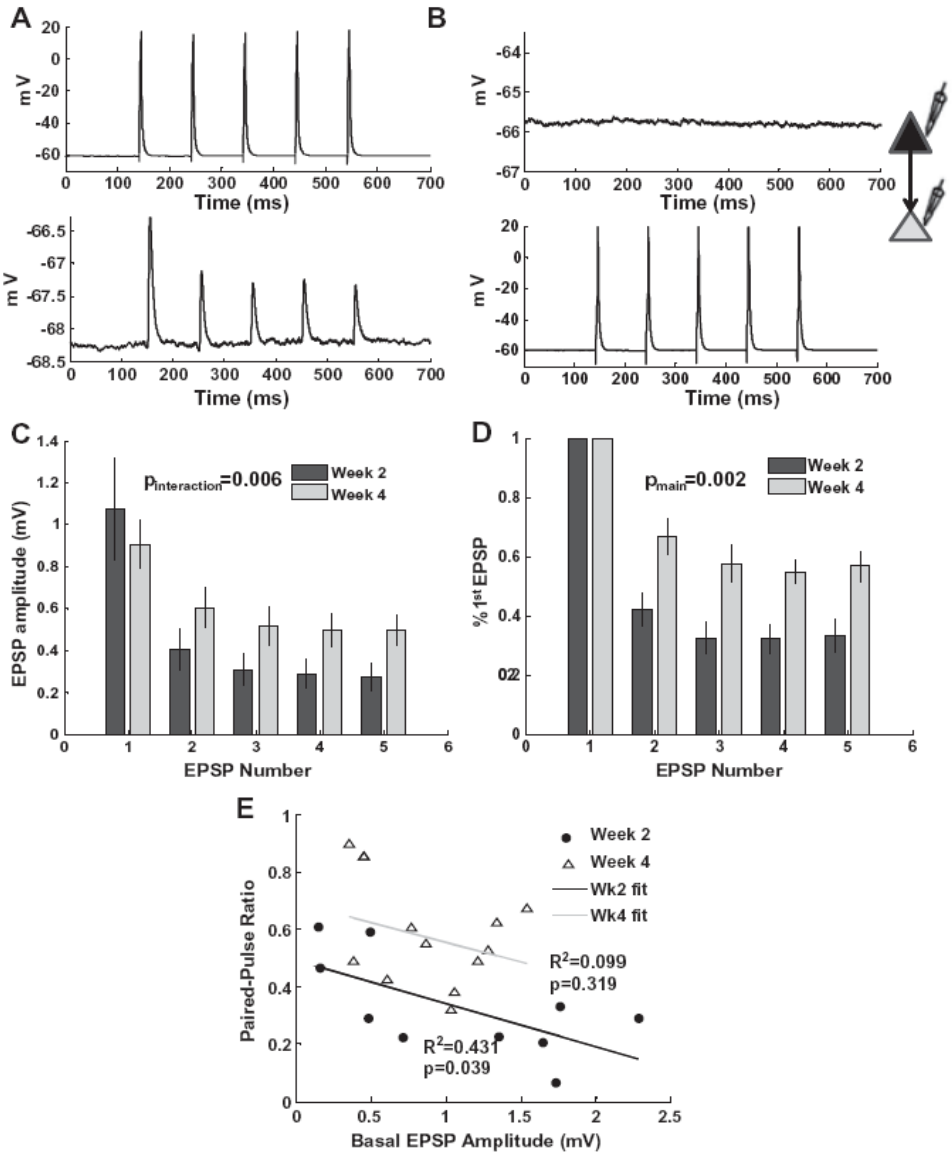

Fig. 1. Paired recordings in L5 pyramidal neurons reveals a developmental increase in paired-pulse ratio from week 2 to week 4 *in vitro*. (A) Example of a synaptically-connected pair of neurons. Spikes in the upper neuron elicit EPSPs in the lower neuron. Traces correspond to an average of 30 sweeps. (B) Testing of the other direction of the same pair. Spikes in the lower neuron do not elicit EPSPs in the upper neuron. (C) Absolute EPSP amplitude at week 2 and week 4 *in vitro* (n = 10, 12). $F_{4,80}$ = 3.9, p = 0.006 (interaction). (D) Normalized EPSP amplitude. $F_{1,20}$ = 12, p = 0.002 (main effect of week). (E) Correlation between PPR and EPSP amplitude is age dependent. There is a significant correlation at week 2 (p = 0.039) but not at week 4 (p = 0.319).

reflects the rate of recovery from depression (Tsodyks and Markram, 1997; Varela et al., 1997; Markram et al., 1998a). Specifically, we wanted to determine if the time constants of STP also underwent developmental changes. EPSP amplitudes in response to 5-, 10- and 20-Hz stimulation were fitted to the Tsodyks–Markram STP model (see Experimental procedures). This model captured the STP data at all intervals tested (mean R^2 = 0.88, range: 0.56–0.99) (Fig. 3A). The fits revealed a significant decrease in U from the second to fourth week *in vitro* (0.72 ± 0.08 versus to 0.39 ± 0.05, respectively; $t_{1,17}$ = 3.75, p = 0.0016) (Fig. 3B). Interestingly, there was no change in the variable that captures the time constant of the recovery from depression (τ_{rec}; 552 ± 74 versus 559 ± 68 ms) (Fig. 3C).

DISCUSSION

The current results revealed a robust developmental increase in PPR. Since these changes were observed in

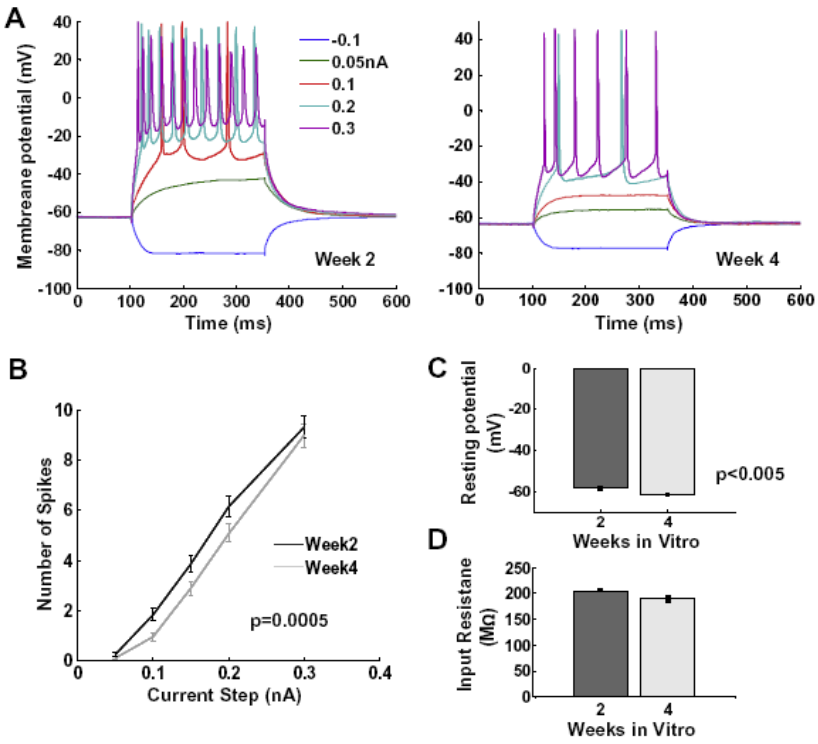

Fig. 2. Developmental change in cellular properties. (A) Examples of intrinsic excitability in week 2 (left) and week 4 (right). (B) There was a significant developmental decrease in intrinsic excitability ($p = 0.0005$). (C) There was a significant decrease in resting membrane potential ($t_{143} = 3.282$, $p = 0.001$). (D) There was no significant change in input resistance.

culture, we can conclude that they do not rely on sensory experience, and therefore are likely to be part of an ontogenetic program. Additionally, our quantitative analysis suggests that this developmental shift seems to be best accounted for by changes in release parameters, but not by changes in the time constant governing the recovery from depression. As discussed below this has implications for the computational function of STP.

Connectivity

The connection probability between local cortical neurons relates to the influence neurons have on their neighbors and the "sparcity" of local cortical circuits (Song et al., 2005). Experimental studies have reported a wide range of connection probabilities between neocortical pyramidal neurons. In acute cortical slices, the reported connection probabilities have ranged from 9% (Mason et al., 1991; Markram et al., 1997) to more than 30% (Boudkkazi et al., 2007).

The 21.7% connection probability observed here was a bit higher than most reports in acute cortical preparations, nevertheless our results are consistent with studies suggesting that organotypic cultures exhibit increased synaptic connectivity. For example, in acute hippocampal slices, the connection probability between CA3 → CA3 and CA3 → CA1 is reported to be between 1% and 5% (Miles and Wong, 1986; Sayer et al., 1990; Scharfman, 1994; Bolshakov and Siegelbaum, 1995). But in organotypic hippocampal slices connections were found in 56% of CA3 → CA3 pairs, and 76% of CA3 → CA1 pairs (Debanne et al., 1995). To the best of our knowledge however, connection probabilities have not been reported for neocortical organotypic slices. Our observations suggest that there is only a mild, if any, increase in connection probability—in contrast to the large increase reported in hippocampal connectivity. Thus, our results further suggest that the basic circuitry architecture is fairly faithfully preserved in neocortical organotypic preparations (Gähwiler et al., 1997; De Simoni et al., 2003).

Relationship between EPSP amplitude and PPR

It is often implicitly assumed that the PPR is governed mostly by initial synaptic strength, this view is supported by observations indicating that PPR is inversely correlated with the probability of release or initial EPSP amplitude in both the hippocampus (Debanne et al., 1996; Dobrunz and Stevens, 1997) and neocortex (Thomson et al., 1993; Atzori et al., 2001; Boudkkazi et al., 2007). However, it is noteworthy that numerous studies have failed to observe any clear relationship between paired-pulse plasticity and the probability of release or initial EPSP amplitude (Reyes and Sakmann, 1999; Waldeck et al., 2000; Sippy et al., 2003; Frick et al., 2007; Oswald and Reyes, 2008). Furthermore, it has been shown that

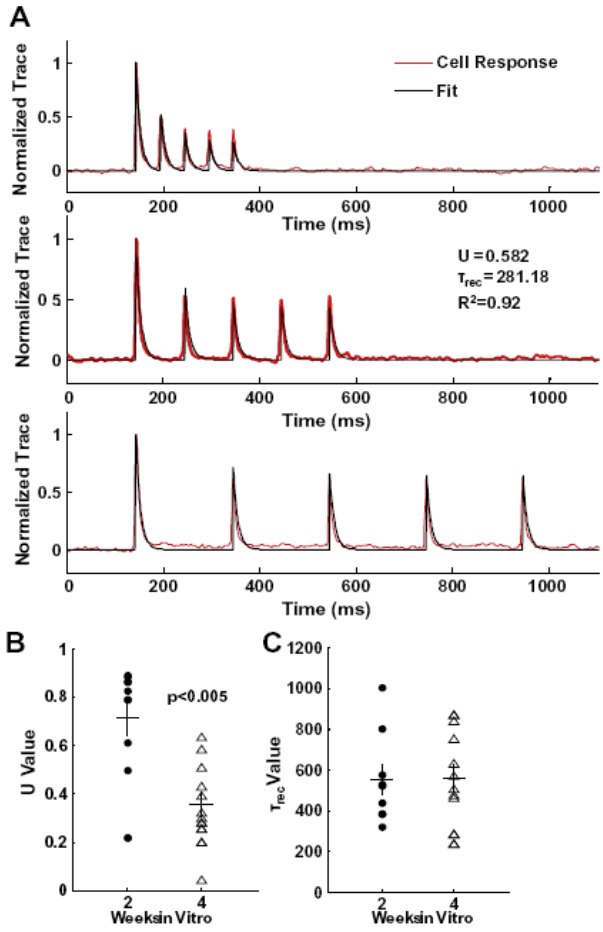

Fig. 3. Quantitative analysis of STP. (A) Example of a fit of the data to the Tsodyks–Markram model of STP (upper, middle, and lower panels correspond to 20 Hz, 10 Hz, and 5 Hz respectively). (B) There was a significant developmental decrease in the U parameter ($t_{17} = 3.75$, $p = 0.0016$). (C) There were no significant changes in the value of τ_{rec}.

increased expression of the calcium binding protein NCS-1 in hippocampal cell cultures can switch paired-pulse depression to facilitation without altering basal synaptic transmission (Sippy et al., 2003). Here we observed that the correlation between EPSP amplitude and PPR was dependent on age. For week 2 synapses, there was a significant correlation between EPSP amplitude and PPR ($p = 0.039$), but this correlation was not observed in week 4 ($p = 0.319$) (Fig. 1E). Furthermore, as shown in Fig. 1 the change in PPR from developmental weeks 2–4 was not accounted for by the initial EPSP amplitude. These observations further argue that STP is not a simple epiphenomenon of basal synaptic strength.

Developmental plasticity of STP

Numerous studies have reported an increase in PPR over the course of development. For example, observations in acute slices from somatosensory, auditory, and prefrontal cortex reveal a progressive increase in PPR, generally from strong paired-pulse depression (PPD) to little PPD or mild paired-pulse facilitation (PPF) (Reyes and Sakmann, 1999; Kumar and Huguenard, 2001; Zhang, 2004; Frick et al., 2007; Oswald and Reyes, 2008). Here we described for the first time that a similar change is observed in the *in vitro* development of cortical organotypic slices. Additionally, the developmental changes in intrinsic properties are also consistent with previous studies in acute slices (Kasper et al., 1994; Zhang, 2004; Oswald and Reyes, 2008).

As mentioned in the Introduction, previous studies have examined the role of sensory experience in the developmental shift in STP (Finnerty et al., 1999; Finnerty and Connors, 2000; Cheetham and Fox, 2011). However, there have been some conflicting results, perhaps due to the fact that even when one sensory area is deprived of its normal input, non-local or cross-modal experience-dependent changes can still influence the non-deprived cortical circuits (Rauschecker, 1995; Sadato et al., 1996; Buonomano and Merzenich, 1998; Kujala et al., 2000; Bavelier and Neville, 2002; Feldman and Brecht, 2005). For example, visual deprivation has been reported

to increase mEPSP amplitude in the visual cortex while decreasing mEPSP amplitude in the somatosensory cortex (Goel et al., 2006)—suggesting that sensory areas are not independent of each other and that normal developmental changes in non-deprived cortex could influence deprived cortical circuits. Consequently, previous developmental studies of PPR have not been able to fully dissociate whether the observed changes in short-term plasticity reflect experience-dependent plasticity or are primarily a product of an ontogenetic program (Reyes and Sakmann, 1999; Zhang, 2004; Cheetham and Fox, 2010; Takesian et al., 2010). Because developmental changes in organotypic slices take place in the absence of sensory input, our results suggest that this shift is at least partially a result of an ontogenetic program that is independent of experience. Nevertheless, it is also clear that experience can alter STP, but this experience-dependent effect may be accounted for by changes in baseline synaptic strength (Finnerty et al., 1999; Finnerty and Connors, 2000; Cheetham et al., 2007).

Regarding the mechanisms of the developmental STP shift our quantitative analysis suggests that the shift may be attributed to changes in the probability of release. A decrease in release probability together with an increase in postsynaptic responsiveness could account for the absence of a decrease in initial EPSP amplitude—which would be expected to be observed from a pure decrease in probability of release. An increase in postsynaptic responsiveness could be attributed to postsynaptic receptors or an increase in the number of synaptic contacts. Indeed, given the known developmental and activity-dependent changes in spine density it seems likely there may be an increase in the number of contacts between connected neurons (Gähwiler et al., 1997; De Simoni et al., 2003; Zuo et al., 2005; Holtmaat and Svoboda, 2009).

It should also be noted that STP can also be modulated on a time scale much faster than that observed over development as a result of network activity (Crochet et al., 2005, 2006; Reig et al., 2006). This issue is an important consideration in the current study because both organotypic and dissociated cultures exhibit developmental increases in network activity over time (Johnson and Buonomano, 2007; Sun et al., 2010). However, while spontaneous activity in organotypic slices does increase, such network effects are unlikely to influence our cross-age measurements for a number of reasons. First, even at 4 weeks Up state frequency remains less than 0.1 Hz (Johnson and Buonomano, 2007); second the mean recovery time constant of STP was well below 1 s; and third, we eliminated any traces that clearly occurred during Up states.

Computational function of STP

Although STP is observed in most types of synapses (Zucker, 1989; Zucker and Regehr, 2002) its functional role continues to be debated. Theoretical proposals regarding the function of STP include a role in gain control (Abbott et al., 1997; Chance et al., 1998; Galarreta and Hestrin, 1998; Rothman et al., 2009), working memory (Maass and Markram, 2002; Mongillo et al., 2008), and temporal processing (Buonomano and Merzenich, 1995; Buonomano, 2000; Fortune and Rose, 2001). For example, it has been postulated that STP may play an important role in temporal processing in the range of tens to hundreds of milliseconds as it provides a short-term memory of recent activity (Buonomano and Merzenich, 1995; Buonomano, 2000). Specifically, STP changes the internal state of networks of neurons in a time-dependent manner, thus allowing networks to discriminate the temporal features of sensory stimuli (Buonomano and Maass, 2009).

The issue of how STP changes with development and with synaptic plasticity is critical to understanding the computational function of STP. If it has an explicit computational role it should be possible to observe instances in which it is altered by experience. Indeed, it has been suggested that STP may undergo metaplasticity—that is, there may be specific mechanisms in place to control STP as a result of experience in a manner independent of initial synaptic strength (Waldeck et al., 2000; Sippy et al., 2003; Carvalho and Buonomano, 2011).

In addition to the developmental decrease in PPR our results demonstrated for the first time that this change in STP does not seem to be accompanied by changes in the temporal profile of STP (that is, the time constant of recovery was unaltered). This is a particularly important point in the context of the role of STP in temporal processing. Using computational models it has been recently shown that the plasticity of short-term plasticity enables circuits to solve computational problems that would be otherwise unsolvable (Carvalho and Buonomano, 2011). Specifically, by allowing synapses to "learn" to exhibit PPF or PPD simple circuits can discriminate specific spatiotemporal patterns. In this model the shifts were implemented as changes in the presynaptic release (the *U* parameter in the Markram–Tsodyks model). But the issue of whether the temporal profile of STP could also be regulated is also raised. This would take place by directed changes in the time constants governing recovery from depression and facilitation. The absence of a development change in recovery from depression observed here, however, would argue against this form of plasticity.

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Chapter 3. Order Selectivity *in vitro*

Generated by

Short-term Synaptic Plasticity

Introduction

Discriminating the order in which different sensory events are presented is of fundamental importance to many sensory computations, including speech discrimination in humans, song discrimination in birds, echolocation in bats, and direction selectivity in the visual system (Hirsh, 1959; Barlow and Levick, 1965; Doupe and Kuhl, 1999; Mossbridge et al., 2006; Simmons, 2012). For example, the meaning in language is often determined by the order of consecutive phonemes, as in “de-lay” versus “la-dy” or “mi-st” versus “mi-tts”. More generally, in the auditory domain mammals can easily learn to discriminate whether a high-frequency tone precedes a low frequency tone, or vice-versa. Indeed, there is evidence suggesting that deficits in this type of order-discrimination task contribute to certain types of language-based learning disabilities (Tallal and Piercy, 1973; Merzenich et al., 1996; Tallal, 2004).

Consistent with the clear importance of temporal order in general sensory processing and particularly in auditory processing, a large number of studies have reported order-selective and sequence sensitive neurons in rodents (Kilgard and Merzenich, 2002; Zhou et al., 2010), cats (Brosch and Schreiner, 2000), bats (Suga et al., 1978; Suga et al., 1983; Razak and Fuzessery, 2009), songbirds (Margoliash and Fortune, 1992; Lewicki and Arthur, 1996; Doupe, 1997), and monkeys (Brosch et al., 1999; Bartlett and Wang, 2005; Yin et al., 2008; Sadagopan and Wang, 2009). Yet, there has been relatively little emphasis on the mechanisms underlying order-selectivity.

Here we are primarily focusing on a general form of order-selectivity, namely the temporal selectivity in the auditory domain. Consider a stimulus composed of two consecutive sensory events such as a low (A) and high (B) frequency tone to create the tone pair AB. A

“strong” order-selective neuron would respond preferentially to AB, over BA, AA, or BB (or of course, A or B by themselves) (Lewicki and Konishi, 1995; Lewicki and Arthur, 1996; Brosch and Schreiner, 2000; Bartlett and Wang, 2005; Yin et al., 2008; Sadagopan and Wang, 2009). From a computational perspective the formation of an AB-selective neuron requires that information from both A and B pathways converge onto the selective neuron; additionally, in order to respond selectively to B when it is preceded by A (but not single B or BB), there must be a “memory” of A at the time of B. For example, if A and B were separated by 100 ms, there must be a “memory” of A that lasts at least 100 ms. Previous models of auditory order selectivity proposed that this "memory" of the first input is achieved by invoking specific circuit mechanisms, activating long-lasting currents or employing recurrent circuitry (Lewicki and Konishi, 1995; Drew and Abbott, 2003). For example, some previous models achieved order sensitivity by assuming the presence of delayed inputs (e.g. dendritic conduction delay) from one pathway, so that both inputs will converge onto a common unit at the same time. However, so far there is little direct experimental evidence supporting such delay mechanisms (Anderson et al., 1999; Baker and Bair, 2012).

Our lab proposed a simple general model of order-selectivity based on virtually universal principles of feedforward disynaptic circuits and synaptic properties such as short-term synaptic plasticity, or STP (**Figure 3.1**). STP is virtually a universal form of synaptic plasticity observed in most synapses and it can adopt the form of either facilitation or depression (Zucker, 1989; Zucker and Regehr, 2002; Abbott and Regehr, 2004). Previous theoretical work has proposed that STP contributes to gain control (Chance et al., 1998; Galarreta and Hestrin, 1998; Abbott and Regehr, 2004; Rothman et al., 2009), working memory (Maass and Markram, 2002; Mongillo et al., 2008; Barak et al., 2010; Deco et al., 2010; Deng and Klyachko, 2011), network stability (Galarreta and Hestrin, 1998; Sussillo et al., 2007), as well as temporal processing

(Buonomano and Merzenich, 1995; Buonomano, 2000; Fortune and Rose, 2001). Here we propose that STP embedded in the most basic neural circuits enables the emergence of order selective neurons without invoking any sorts of delay or requiring prior learning.

The model of order selectivity is composed of a simple feedforwad disynaptic circuit, in which two inputs (A and B) converge onto the same inhibitory neuron (Inh) as well as the excitatory neuron (Ex) (**Figure 3.1A**). An example of when Ex neuron displays AB selectivity (respond to AB, but not to BB, BA, AA, or single A or B) as well as the voltage traces revealed by computational simulations are shown in **Figure 3.1B,** left column. Computational simulations demonstrate the order-selectivity can emerge through the following mechanism: input from pathway A activates Ex neuron as well as the feedforward Inh neuron. Input from pathway B comes in at an interval (e.g. 100 ms) later and converges onto Ex neuron as well as the same Inh neuron. The IPSP onto Ex neuron undergoes heterosynaptic paired-pulse depression. Therefore when stimulus B arrives it is able to elicit a suprathreshold respond in the Ex neuron because the balance of excitation and inhibition has shifted towards excitation as a result of PPD of the IPSP. Note that single A or single B stimuli only elicit subthreshold responses in Ex. The Ex neuron will not respond to AA or BB as well as a result of paired-pulse depression of excitatory input. The voltage responses to all four input patterns (AB, BA, AA, BB) in Ex and Inh neurons as well as the excitatory and inhibitory conductance onto the Ex neuron is shown as colored traces below (**Figure 3.1B**, left column). Additionally, the computational simulations reveal that it is possible for the Ex neuron to switch from AB to BB selectivity by adjusting the synaptic weights and STP profile (**Figure 3.1B**, right column).

One of the critical concepts of our model of order selectivity is that IPSP onto Ex neuron undergoes heterosynaptic STP, e.g. if the activation of Inh neuron by input A is immediately

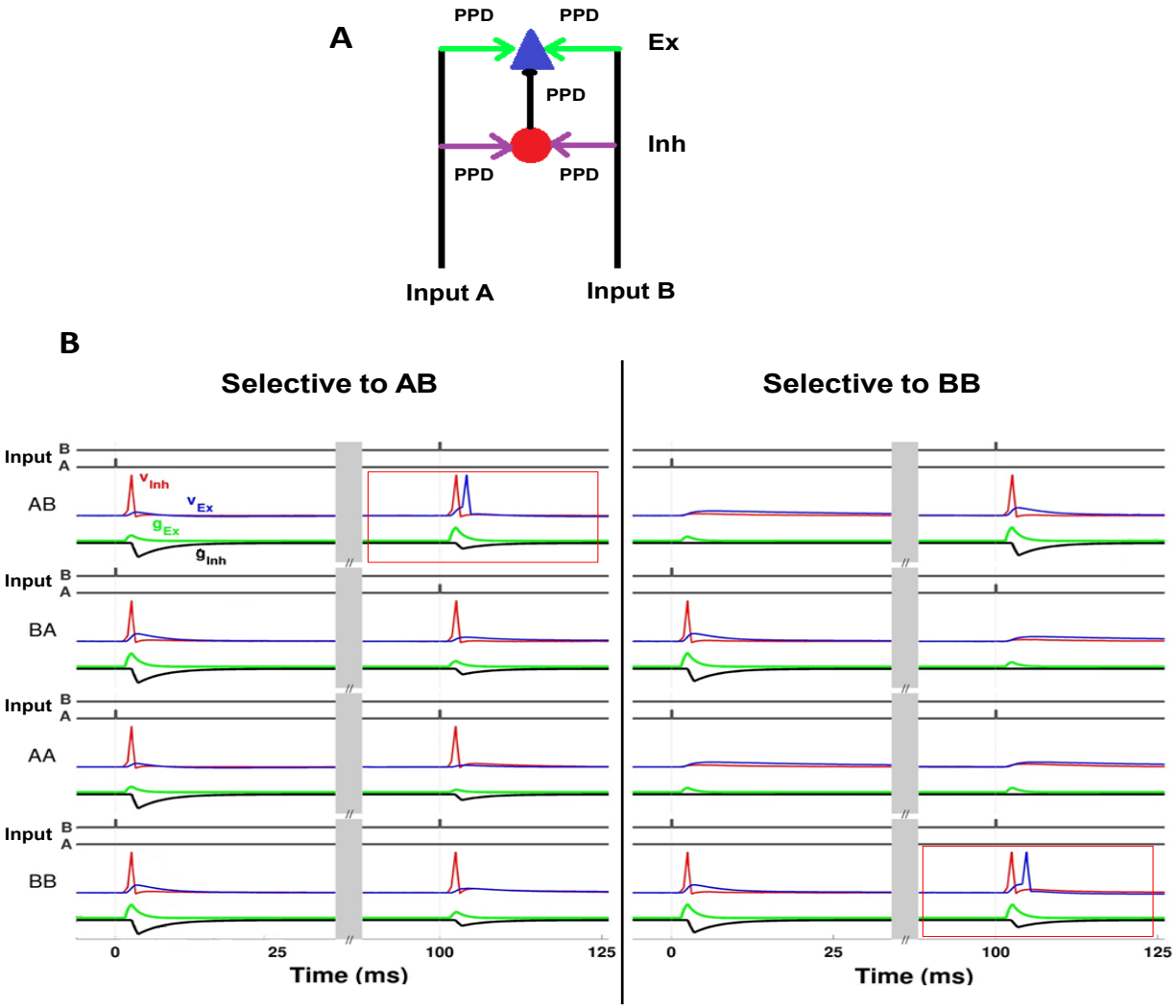


Figure 3.1. Example of AB and BB selectivity in a disynaptic circuit composed of one excitatory and one inhibitory neuron. We proposed that such a simple circuit is potentially capable of displaying order selectivity based on virtually universal principles such as STP. **(A)** Cartoon diagram of the circuit: two inputs (A and B) converge onto the same inhibitory (Inh) neuron as well as the excitatory (Ex) neuron. The heterosynaptic paired-pulse depression of the IPSPs onto Ex neuron would allow order selectivity, i.e. for AB selectivity the Ex neuron respond to input B only if it was preceded by A (but not to AA, BA, BB, or single A and B inputs). **(B)** Mechanisms of order selectivity demonstrated by computational simulations. Blue and red traces showing the voltage response of Ex and Inh neurons, respectively. Green and black traces showing the conductance of excitatory and inhibitory input onto Ex neuron, respectively. Left column: AB selectivity (red box). Right column: BB selectivity (red box). See main text for details.

followed by the activation of this Inh neuron by input B, the IPSP onto the Ex neuron will undergoes paired-pulse depression (PPD), despite of the fact that the inhibitory neuron is activated by a different input at the second time. Paired-pulse depression (PPD) of IPSPs is a conventional homosynaptic form of STP, meaning that short-term plasticity of IPSP is usually examined by delivering paired pulses from the same pathway. To the best of our knowledge, there is no experimental demonstration of heterosynaptic STP, in which stimulation of one pathway changes the temporal profile and balance of excitation/inhibition in response to a second pathway. Here I address this question experimentally to validate the assumption and test predictions of our model of order selectivity.

This project is a collaborative work composed of experimental and computational parts, and I have been responsible for the experimental component by examining heterosynaptic STP. My results revealed heterosynaptic STP and order selective neurons in acute auditory cortex slices.

Results

Heterosynaptic STP in Acute Auditory Slices

One of the concepts critical to the STP-based model of order selectivity is that changes in the balance of excitation and inhibition imposed by STP is “heterosynaptic”. That is, from the perspective of an excitatory neuron in a disynaptic circuit STP of the IPSPs can be expressed heterosynaptically as a result of the convergence of different inputs onto the same inhibitory neuron. Paired-pulse depression of IPSPs is conventionally a homosynaptic property and has been characterized by the expression of the broadening of successive EPSPs (Buonomano and Merzenich, 1998; Pouille and Scanziani, 2001; Carvalho and Buonomano, 2009). Furthermore it has been demonstrated that fast-spiking inhibitory neurons in Layers 2/3 and Layer 4 are driven by a broad range of thalamocortical and intracortical inputs (Gabernet et al., 2005; Hull et al., 2009; Oswald and Reyes, 2011), therefore validating the idea that different inputs might converge onto the same inhibitory neuron. But to the best of our knowledge it has never been experimentally demonstrated that PPD of IPSP (expressed as broadening of EPSPs) can be observed heterosynaptically: i.e, the width of an EPSP from one pathway can be modified by the preceding stimulation of another pathway.

To validate our assumption that different pathways interact on the order of tens-to-hundreds of milliseconds so that IPSPs undergo heterosynaptic STP, whole-cell recordings were performed in acute slices from rat auditory cortex. To parallel *in vivo* paired-tone experiments we recorded from Layer 2/3 pyramidal neurons while stimulating two pathways: a “vertical” pathway through an electrode placed in the underlying Layer 6/white matter, which would approximate the principal thalamocortical input to a cortical column; and a “horizontal”

pathway through electrode placed laterally in Layer 2/3, which approximates the lateral synaptic connection believed to underlie lateral suppression and enhancement (**Figure 3.2A**).

Heterosynaptic STP was examined by recording evoked EPSPs of L2/3 pyramidal neurons in response to single V or HV inputs (**Figure 3.2B,C**). The interval between H (horizontal) and V (vertical) stimulation was varied between 50 and 400 ms (H50V, H100V, H200V, H400V). Because we were interested in the effective interaction between EPSPs and IPSPs we measured changes in IPSPs indirectly through the width of the EPSPs. Specifically, the width of evoked PSPs is determined in part by the strength of the IPSPs (Buonomano and Merzenich, 1998; Pouille and Scanziani, 2001; Carvalho and Buonomano, 2009). Results revealed that the half-maximal width of EPSPs elicited by the V pathway were significantly broadened by the preceding H stimulation (**Figure 3.2D**; $F_{4,32}=15.16$, $p<10^{-6}$, n=9). This indicates that both pathways converged onto some common inhibitory neurons which underwent heterosynaptic paired-pulse depression. Posthoc tests revealed significant differences between the width of the single V EPSP and V EPSPs of H200V, H400V responses (paired t-test: unadjusted p values are p=0.1231, 0.0487, 0.0058, 0.0032 between single V and H50V, H100V, H200V, H400V respectively. See **Methods** for statistical details).

However, a potential concern relates to possible overlap between the axonal fibers activated by the two pathways. This issue was examined by normalizing EPSP slopes of V response at 50 ms interval (V of H50V and second V of V50V inputs) to the EPSP slope of single V input, at this interval paired-pulse depression or facilitation should be at maximum. At 50 ms inter-pulse interval there is also less $GABA_B$ effect activated by the first stimulus to confound the following EPSPs of V response. The "normalized" EPSP slopes of the V pathway were significantly different in H50V compared to V50V (paired t-test, $t_8=7.03$, $p<10^{-4}$) (**Supplementary Figure S3.1A**). In fact, V responses in H50V stimulation have slope values

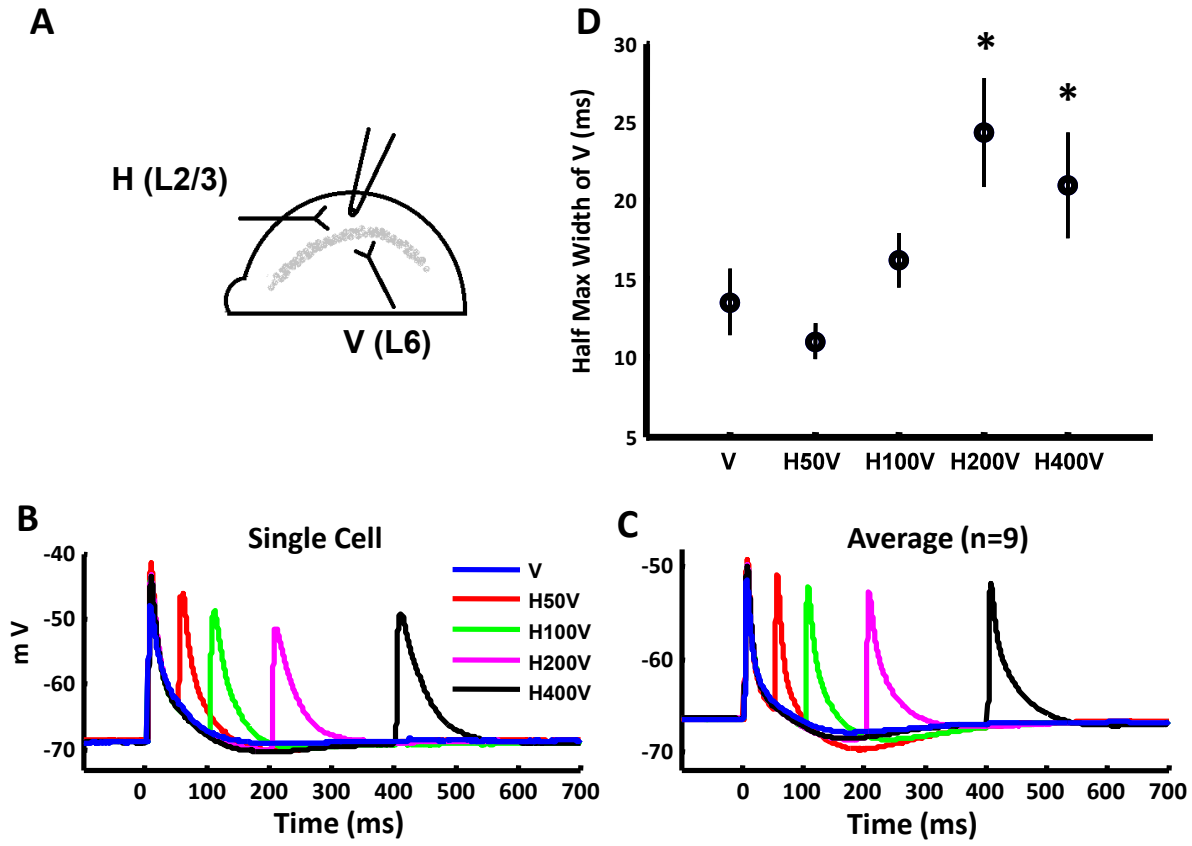


Figure 3.2. Heterosynaptic STP of IPSPs. Broadening of EPSP responses to V stimulation of HV input compared to EPSP of single V input, suggesting an interaction between the two pathways, possibly via heterosynaptic paired-pulse depression of IPSPs. H: horizontal (Layer2/3) input, V: vertical (Layer6/white matter) input. **(A)** Cartoon diagram indicating location of the two stimulus electrode as well as the recording electrode. **(B)** Sample traces of a single cell response to different inputs overlapped. V: response to a single vertical input. H50V/H100V/H200V/H400V: response to horizontal followed by vertical input, separated by 50/100/200/400 ms, respectively. **(C)** Average traces of all cells (n=9). **(D)** Half maximum width measured at the vertical responses (V) were significantly different ($F_{4,32}$=15.16, $p<10^{-6}$) (*: $p<0.0125$, paired t-test between V response of HV input and the response of single V input).

comparable to EPSP slope of a single V stimulation ("normalized" slope value: 98.07 ± 3.05%, n=9) while V50V elicited robust paired-pulse facilitation (normalized slope value 114.50 ± 4.54%, n=9). Additionally, "time to peak" value is also significantly different in the (second) V response of H50V and V50V input (paired t-test, t_8=2.46, p=0.039) (**Supplementary Figure S3.1B**). Furthermore, the use of a vertical and horizontal pathway makes it very unlikely that the electrodes are directly activating the same fibers. Indeed, out of 45 recorded neurons 6 were having antidromic responses to one of the pathways, but not for the other -- providing another strong support that overlap between pathways was minimal.

The above results showed that IPSPs in a disynaptic circuit with two input pathways undergo heterosynaptic PPD in the *in vitro* setup that we examined. This experimental result validates the critical assumption of heterosynaptic STP in our model underlying order selectivity.

Order-Selectivity *in Vitro*

Our model proposes that order-selectivity can in principle emerge from the most basic and widespread neural circuits and synaptic properties such as STP. That is, dynamic changes of the excitatory-inhibitory balance produced by STP in simple disynaptic circuits is sufficient to generate order-selective neurons—provided that the synaptic weights and STP are in an appropriate regime. Thus a prediction arising from the model is: because the mechanisms underlying order selectivity reflect general and robust properties of simple feed-forward disynaptic circuits, we may be able to observe order selectivity in *in vitro* preparations. It is important to point out that as demonstrated by computational simulations, the regime of the

circuits—meaning the dynamics of STP and the respective weights—is critical to whether order selectivity can be observed.

Based on numerous *in vitro* and *in vivo* studies it is clear that the most common "default" regime is one in which neuron respond preferentially to the first of a pair of sensory events—which makes sense particularly in early sensory areas where stimulus onset is critical to rapid behavioral responses and subsequent sensory processing. Nevertheless *in vivo* studies demonstrate that there are a significant, albeit minority of neurons that do exhibit order-selectivity (Brosch et al., 1999; Brosch and Schreiner, 2000; Kilgard and Merzenich, 2002; Yin et al., 2008; Sadagopan and Wang, 2009) -- note that here the definition of "strong" order-selectivity would be neurons that respond preferentially to AB (mostly likely to the second pulse of "B") over BA, AA, or BB (or of course, A or B by themselves). Therefore we asked if order selectivity can be observed in acute slices of rat auditory cortex.

As above we recorded from L2/3 pyramidal neurons while stimulating two pathways: a "vertical" pathway (V) and a "horizontal" pathway (H) (**Figure 3.2A**). The two pathways were stimulated in pairs: HV, VH, HH, VV, with an interval of 100 ms between them, and the four patterns of input were sequentially looped. EPSPs elicited by external orthodromic stimulation generally do not elicit spikes in pharmacologically intact acute slices, thus neurons were depolarized with a 1 s depolarizing pulse during the presentation of the stimulus pairs. The strength of the stimulation of the V and H pathways (60-800 μA) were specifically adjusted "online" to attempt to create suprathreshold responses that were in an interesting regime—that is, in which the neurons fire action potentials but not to all stimuli. Towards this end each "stimulus" consisted of an electrical shock (or a "burst" of two, which increased the number of

neurons that fired, and also increased the window of integration of the EPSPs and thus the expression of the depression of IPSPs) from one of the pathways.

Out of 17 (from of a total of 26 recorded) neurons in which stimulation was able to elicit action potentials, the majority (13/17) did not spike consistently, spiked primarily to the first pulse of the V or H stimulus (**Supplementary Figure S3.4**), or had moderate but non-significant selectivity. Four neurons (23%), however, exhibited statistically significant selective responses ($p<0.05$, χ^2-test) to only one of the four patterns (see **Methods** for statistical details). **Figure 3.3A,B** show an example of 10 successive stimulus cycles of a neuron that responded preferentially to VH. **Figure 3.3C,D** provide an example of a neuron that responded preferentially to HH. **Supplementary Figure S3.2-S3.3** provide additional examples of stimulus selective responses. In conclusion, our observation of *in vitro* order-selective neurons provide experimental support to the notion that order selectivity can emerge from general and robust properties of simple feed-forward disynaptic circuits.

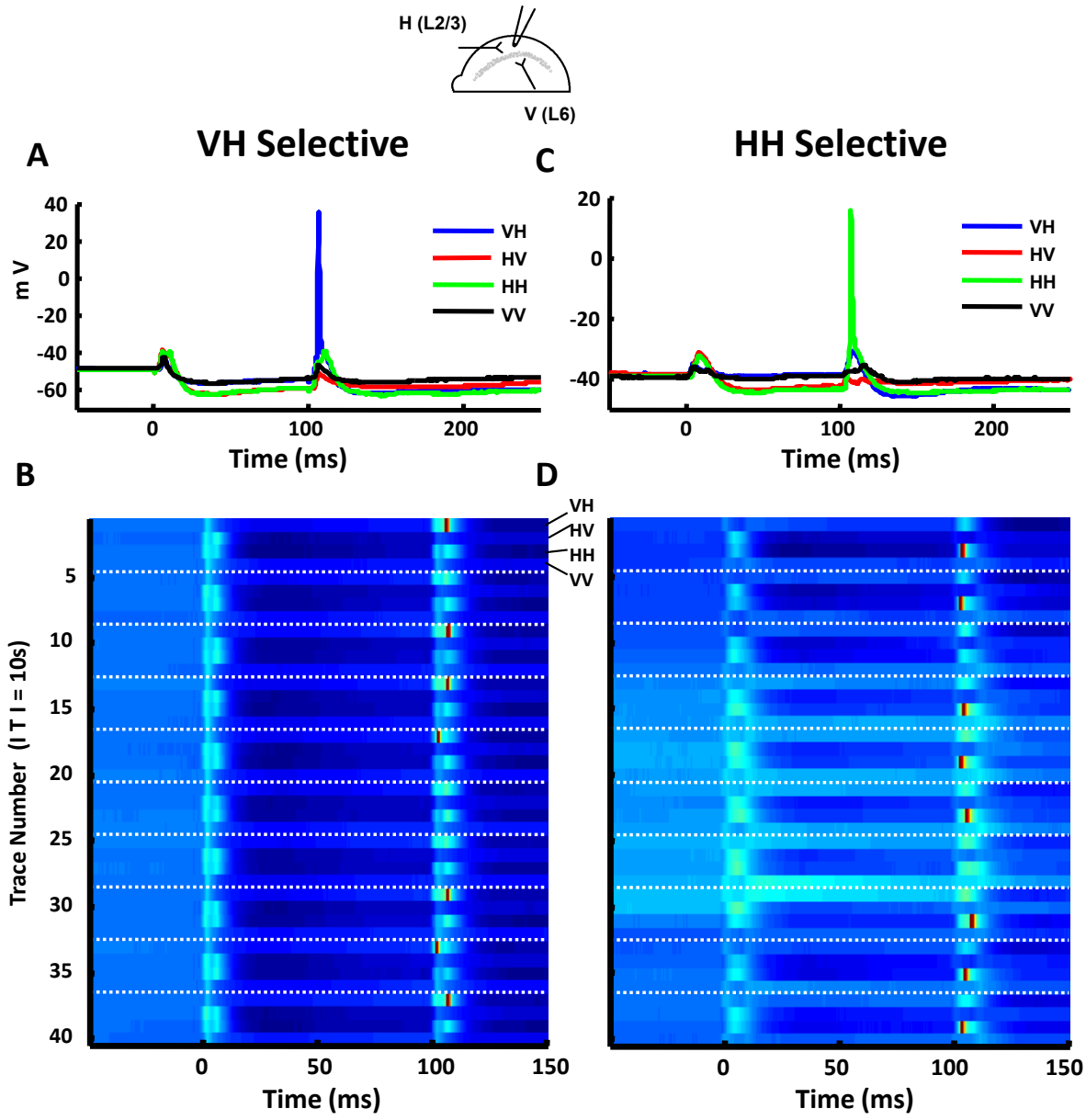


Figure 3.3. Examples of order selective cells in *in vitro* cortical network. Four stimuli patterns were given to a single cell alternatively: V (vertical, L6) – H (Horizontal, L2/3), HV, HH, VV. The cell is considered as pattern selective if it fires an action potential to the second pulse of one pattern but not to the other patterns. **(A-B)** VH selective cell. **(C-D)** HH selective cell. **(A)** and **(C)**: sample traces of response to one cycle of four stimuli pattern, with traces overlapped. **(B)** and **(D)**: heat map of the ten successive cycles. Voltage range in **(B)** is -62.7 - 0 mV, in **(D)** is -49.6 - 0 mV.

Discussion

Other critical experimental observations underlying STP-based order selectivity

One of the critical concepts in our model of order selectivity relies on the heterosynaptic STP of IPSP onto the excitatory neuron, and have been validated above. There are assumptions contributing to order-selectivity as described in the model shown in **Figure 3.1**. Two of the most important assumptions of the model are: 1) IPSPs undergo robust paired-pulse depression; and 2) despite the fact that in a disynaptic circuits, such as the thalamocortical projection, IPSPs are somewhat delayed in comparison to EPSPs because of the extra synaptic step, that they still interact with the ascending slope of the EPSPs. Both these observations have robust experimental support, as discussed briefly below.

1. The presence of STP in the excitatory and inhibitory synapses of a disynaptic circuit ensures that the balance of excitation and inhibition will change in response to consecutive stimuli. In a simple feedforward disynaptic circuit (**Figure 3.1A**) the balance of excitation and inhibition in response to consecutive inputs is shaped by at least three forms of STP that are universally observed: Ex→Ex, Ex→Inh, Inh→Ex. STP in all these synapses is variable both in terms of magnitude and time course, and sometimes even in direction (facilitation versus depression). As a general rule, Inh (fast spiking, FS)→Ex IPSPs exhibit robust depression (Gupta et al., 2000; Kapfer et al., 2007; Reyes, 2011; Ma et al., 2012). Ex→Inh synapses exhibit both depressing and facilitating synapses, however there is a sharp distinction between different types of inhibitory neurons: excitatory synapses onto FS/parvalbumin inhibitory neurons generally exhibit depression (Kapfer et al., 2007; Lu et al., 2007; Reyes, 2011; Levy and Reyes,

2012). The excitatory synapses onto pyramidal neurons exhibit a much wider range of STP ranging from robust depression to mild facilitation.

2. In a feedforward excitatory/inhibitory disynaptic circuits inhibition is rapid enough to "veto" a suprathreshold EPSP. In response to a feedforward stimulation input, excitatory neurons receive a monosynaptic EPSP and a disynaptic IPSP. Even though the IPSP is delayed because of the additional interneuron, the IPSP robustly interacts with the rising slope of the monosynaptic EPSP and can prevent a suprathreshold EPSP from producing a spike (McCormick et al., 1993; Pouille and Scanziani, 2001; Marder and Buonomano, 2004; Daw et al., 2007; Carvalho and Buonomano, 2009). Indeed, cortical circuits seems to be "designed" precisely to allow fast spiking (FS) inhibitory neurons to implement this computational feature (Mason et al., 1991; Markram et al., 1997a; Oswald and Reyes, 2008, 2011; Ma et al., 2012). Specifically, Ex→Inh(FS) and Inh(FS) →Ex synapses have shorter latency (0.6-0.9 ms) (Oswald and Reyes, 2011; Ma et al., 2012) compared to Ex→Ex synapses (1.2-1.8 ms) (Mason et al., 1991; Markram et al., 1997b; Oswald and Reyes, 2008). Secondly, fast-spiking Inh neurons generally synapse on the cell soma or proximal dendrites of excitatory pyramidal neurons, and that fast-spiking Inh neurons have low thresholds and tend to received larger thalamocortical EPSPs than excitatory neurons (Holmgren et al., 2003; Daw et al., 2007; Hull et al., 2009).

Our model of order selectivity is consistent with the above experimental observations in addition to the heterosynaptic STP of IPSP validated in **Results** part. We believe this model will add to current understanding of the biological mechanism underlying order selectivity.

Conclusion

Together the above results provide the experimental evidence for “heterosynaptic STP”: as a result of the convergence of inputs onto inhibitory neurons one pathway can broaden the evoked PSP of another pathway. Furthermore, experimental evidence of order-selective neurons *in vitro* was provided. Overall, these results support the notion that order selectivity can emerge from general and robust properties of simple feed-forward disynaptic circuits. Importantly the proposed model of order-selectivity, in contrast to previous models, relies on experimentally well established circuit and synaptic properties.

Methods

Acute slice preparation

Acute slices were prepared as previously described (Marder and Buonomano, 2003; Carvalho and Buonomano, 2009). Briefly, 22-31 day old Sprague-Dawley rats were anesthetized with isoflurane and decapitated. The brain was removed and placed in ice-cold oxygenated (95% O_2-5% CO_2) ACSF (in mM: 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 1.0 NaH_2PO_4, 26.2 $NaHCO_3$, 2.5 $CaCl_2$, and 10 dextrose) (all chemicals from Sigma). Coronal slices (300-350 μm thick) containing primary auditory cortex were cut using a vibratome and maintained in oxygenated ACSF at room temperature for 1-2 hour prior to recording.

Electrophysiology

Whole-cell recordings were made from regular-spiking, infragranular pyramidal neurons using IR-DIC visualization (average distance from the cortical surface was 260 ± 6 μM). Experiments were performed at 28-30°C in external solution composed of (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 1.0 NaH_2PO_4, 26.2 $NaHCO_3$, 2.5 $CaCl_2$, and 10 dextrose. The internal solution for whole-cell recordings contained (in mM): 130 K-gluconate, 5 KCl, 2 $MgCl_2$, 4 ATP-Mg, 10 phospho-creatine, 0.3 GTP-Na, 10 mM HEPES, and was adjusted to pH 7.3 and 300 mOsm (Oswald and Reyes, 2011). All analyses were performed using software custom written in MATLAB.

In Vitro Stimulation protocol

Two pathways were stimulated by extracellular electrodes: a “vertical” pathways through an electrode placed in the underlying L6/white matter, which would approximate the principal

thalamocortical input to a cortical column; and a “horizontal” pathway through electrode placed laterally in L2/3, which approximates the lateral synaptic connection believed to underlie paired-pulse suppression and enhancement. Recordings were done from L2/3 pyramidal neurons.

In the heterosynaptic STP experiments, evoked EPSPs in response to both single V or HV inputs were recorded. The interval between H (horizontal) and V (vertical) stimulations was varied between 50 and 400 ms (H50V, H100V, H200V, H400V). Stimulations were delivered in the sequence of V, H50V, H100V, H200V, H400V with 10 s interval in between and looped. After acquiring all the data for HV input, stimulations were switched to looped through V, V50V, V100V, V200V, V400V stimuli with 10 s interval and EPSP responses were recorded.

In the order-selectivity experiments, the stimulating pathways were activated in pairs: HH, HV, VH, VV and with an interval of 100 ms between them. The four patterns were delivered sequentially in loops with 10 s interval in between. To elicit orthodromic action potentials, neurons were depolarized with a 1 s depolarizing pulse during the presentation of the stimulus pairs. The strength of the stimulation of the V and H pathways (60-800 μA) were specifically adjusted “online” to attempt to create suprathreshold responses that were in an regime interesting to us (see above). Each “stimulus” consisted of an electrical shock (or a “burst” of two to increased the number of firing neurons, as well as the window of integration of the EPSPs and thus the expression of the depression of IPSPs).

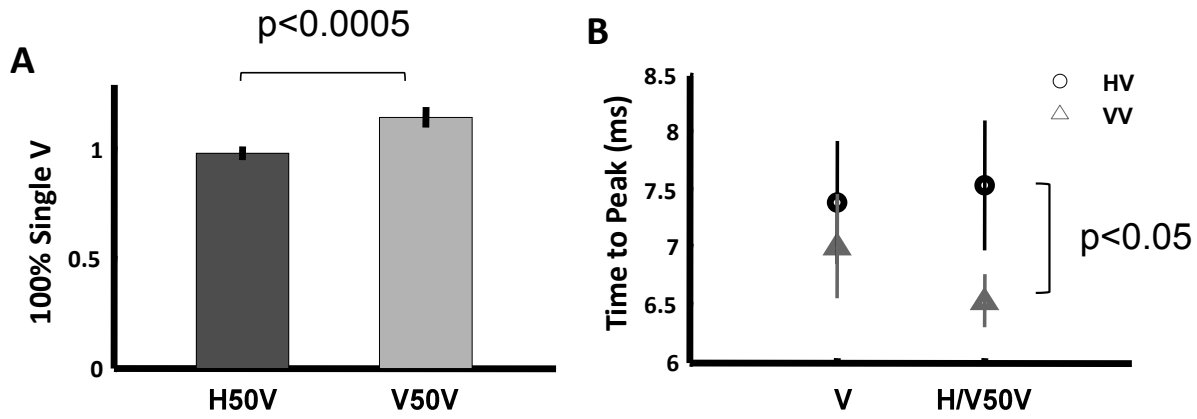
Statistics

In the heterosynaptic STP experiments, broadening of half-maximal width of EPSPs elicited by V pathway when preceded by H stimulation is quantified by two-way analysis of variance (ANOVA) with repeated measures on input patterns. Specifically, half-maximal width of

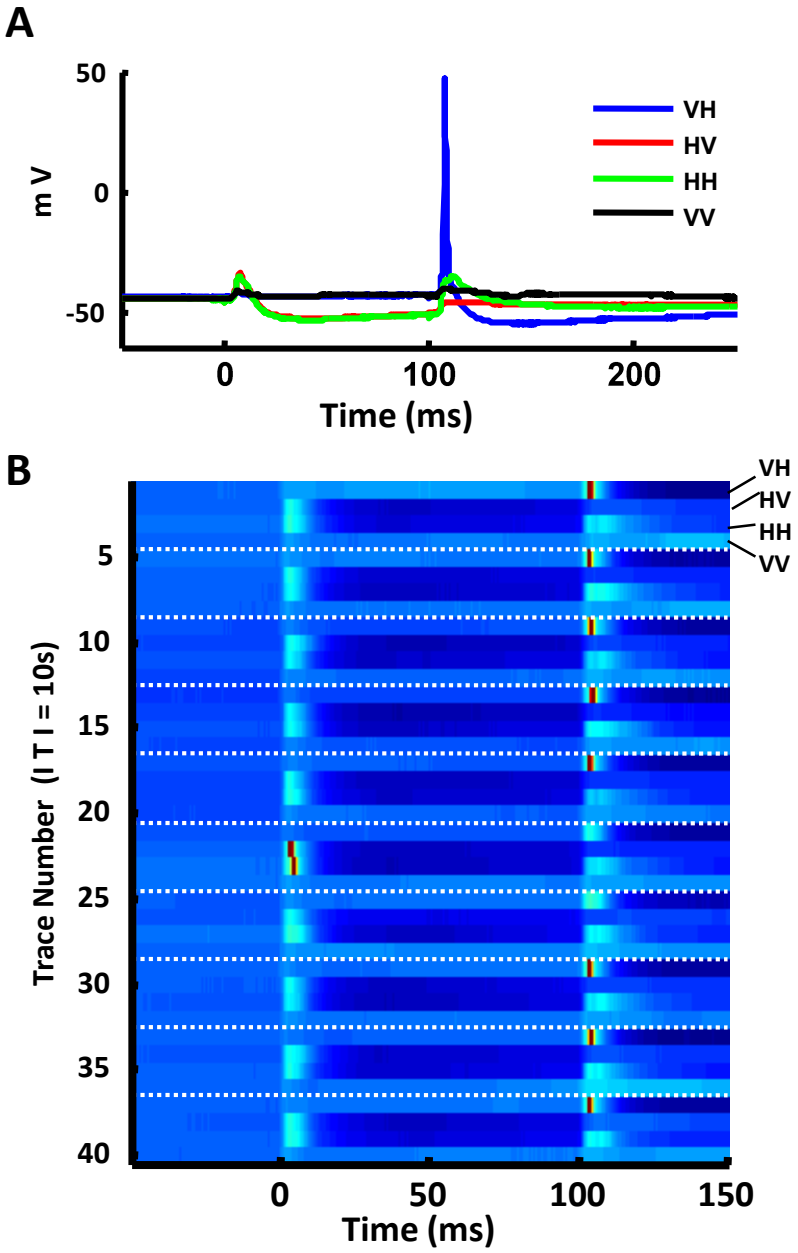
EPSP response to a single V stimuli is compared to the response to V stimuli in H50V, H100V, H200V, and H400V inputs. In posthoc tests, raw p value obtained by paired t-tests is compared to an adjusted p value of 0.05/4=0.0125 to avoid multiple comparison artifact.

In the order-selectivity experiments, definition of selectivity was based on 10 consecutive trials (with the same stimulation and interval parameters), where there was a statistically significant response to the second input of one pattern, for example responding with 7 or 8 times out of 10. Significance of the selectivity is tested with Chi-square test.

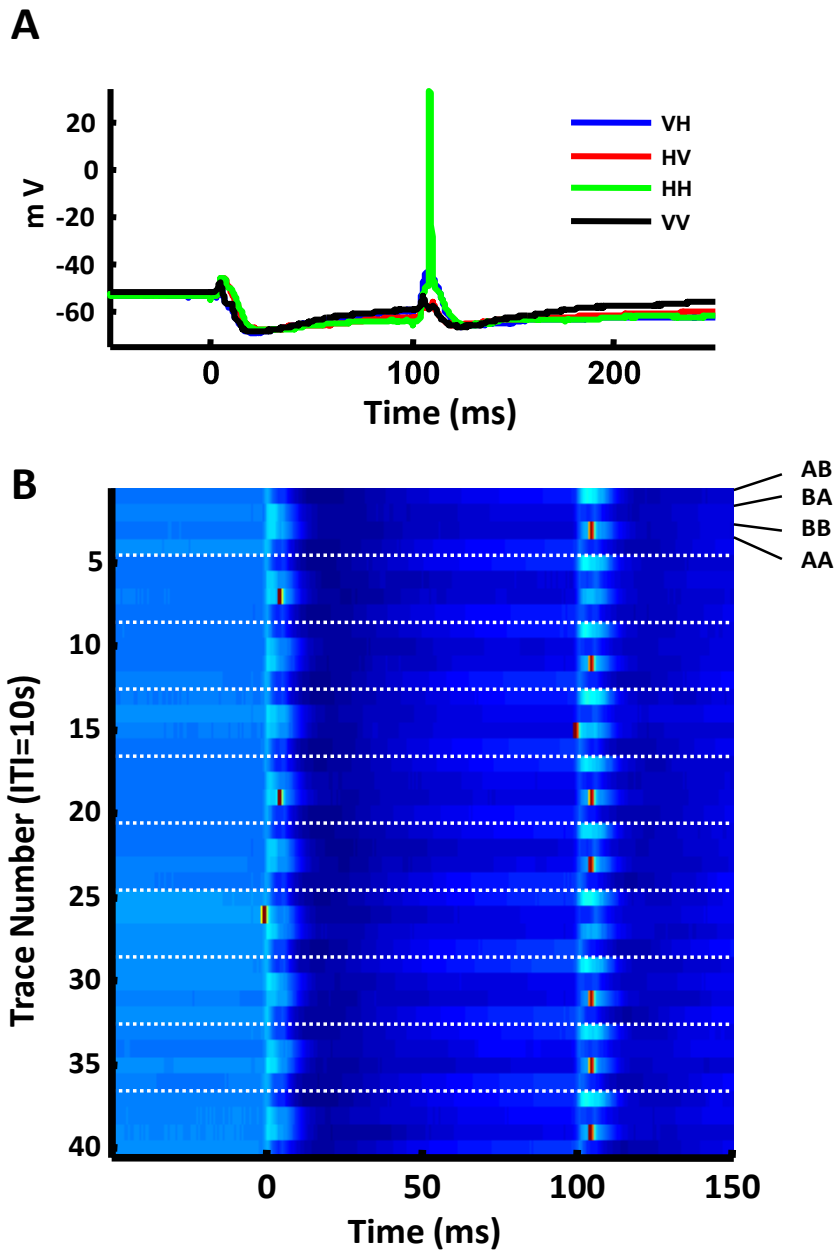
Supplementary Figures



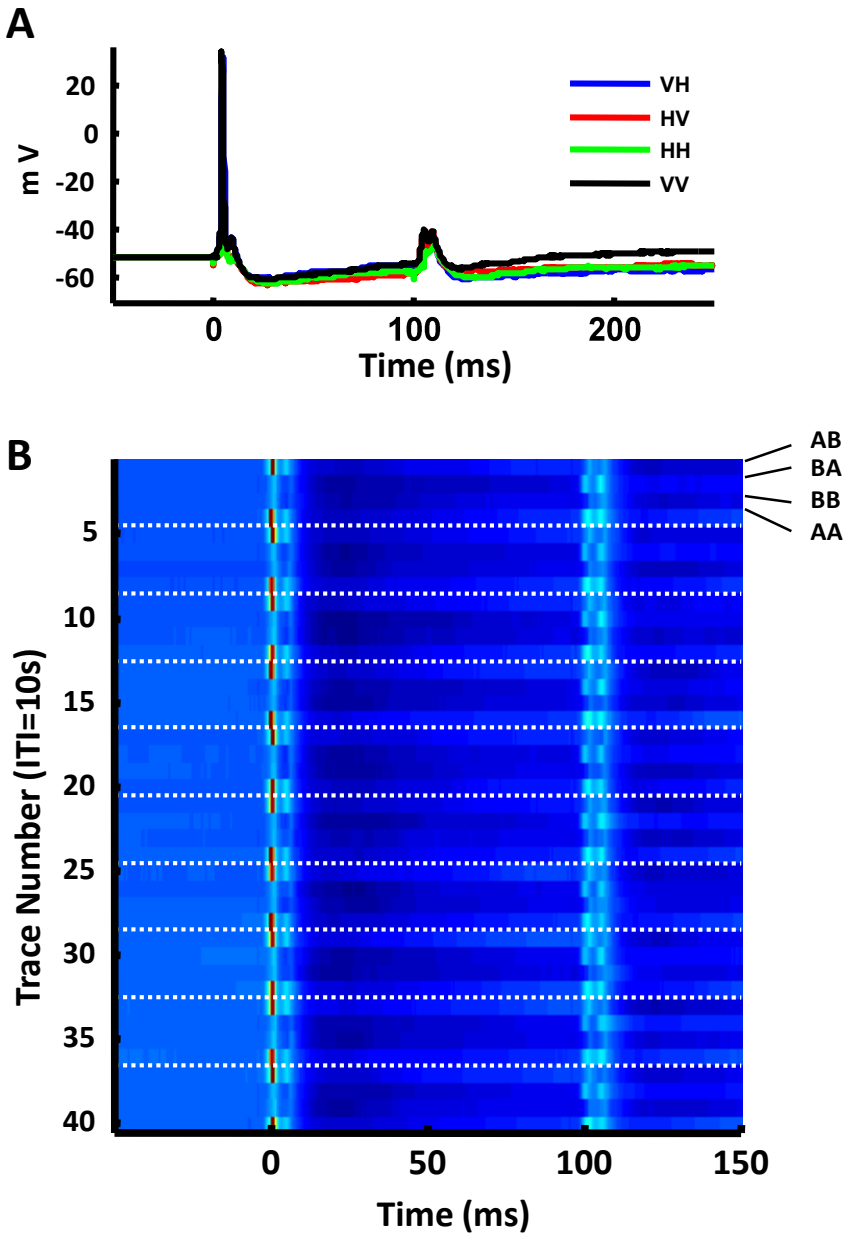
Supplementary Figure S3.1. There is little pathway overlap between H (horizontal, L2/3) and V (vertical, L6) inputs, suggested by quantifying EPSP slope and time to peak. (A) "Normalized" EPSP slope of V response is calculated by normalizing the EPSP slope of the V response from H50V or the second V response of V50V to the EPSP slope of a single V stimulation. There is a significant difference between normalized V slope from H50V and V50V (paired t-test, t_8=7.03, p=0.0001, n=9). **(B)** Time to peak for single V, V response of H50V and second V response of V50V input. The (second) V response of H50V and V50V stimuli are significantly different (t_8=2.46, p=0.039).



Supplementary Figure S3.2. **Additional example of a VH-selective neuron, responding 8 out of 10 times. (A)** Sample traces of response to one cycle of stimuli pattern. **(B)** Heat map of the ten successive cycles. Voltage range in **(B)** is -56.2 - 0 mV.



Supplementary Figure S3.3. Additional example of a HH-selective neuron, responding 7 out of 10 times. (A) Sample traces of response to 1 cycle of stimuli pattern. **(B)** Heat map of the ten successive cycles. Voltage range in (B) is -69.5 - 0 mV.



Supplementary Figure S3.4. **Example of a neuron responding to the first pulse of V pathway stimuli, responding 10 out of 10 times. (A)** Sample traces of response to one cycle of stimuli pattern. **(B)** Heat map of the ten successive cycles. Voltage range in **(B)** is -63.9 - 0 mV.

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Chapter 4. LTP in cortical organotypic slices

Introduction

Long-term potentiation, or LTP, is a form of long lasting synaptic plasticity that is considered to be one of the fundamental contributors to learning and memory (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999; Martin et al., 2000; Lynch, 2004; Whitlock et al., 2006; Ho et al., 2011). LTP was first observed in *in vivo* hippocampal experiments when Bliss and Lomo demonstrated increases in the size of the field EPSPs (and decrease in the latency of the population spike) in response to high frequency (tetanic) stimulation (Lomo, 1971; Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973; Douglas and Goddard, 1975). More than a decade later it was demonstrated that this form of LTP was associative and conformed to Hebb's postulate that paired pre- and postsynaptic activity would increase synaptic strength (Gustafsson and Wigstrom, 1986; Kelso et al., 1986; Larson and Lynch, 1986; Sastry et al., 1986).

Decades of subsequent research have demonstrated that there are a number of different forms of LTP, and that long-term synaptic plasticity is governed by the temporal relationship between pre- and postsynaptic spikes, as well as the presence of neuromodulators. Specifically, spike-timing dependent plasticity (STDP) reveals that when presynaptic input precedes postsynaptic spiking within a few tens of milliseconds, LTP is induced. In contrast, when postsynaptic spiking occurs before the presynaptic spike, long-term depression (LTD) can be generated (Debanne et al., 1994; Markram et al., 1997; Bi and Poo, 1998) (**Chapter 1, Figure 1.1**). In addition to the timing of pre- and postsynaptic spikes, it is now clear that neuromodulators also play an important role in gating the magnitude and polarity of synaptic plasticity (Auerbach and Segal, 1996; Kilgard and Merzenich, 1998; Lin et al., 2003; Froemke et al., 2007; Seol et al., 2007; Caporale and Dan, 2008; Pawlak and Kerr, 2008; Zhang et al., 2009; Buchanan et al., 2010; Pawlak et al., 2010; Gu and Yakel, 2011; Huang et al., 2012). For

example, it has been shown that β-adrenergic receptors coupled to the adenylyl cyclase signaling cascade gate the LTP side of STDP while muscarinic receptors coupled to the phospholipase C cascade were necessary for LTD (Seol et al., 2007). In these studies, pairing is not sufficient to induce LTP or LTD without neuromodulators.

The great majority of LTP studies, including all the early demonstrations of associative LTP have been carried out in acute slices — an ideal preparation to perform intra- and extracellular recordings while carefully controlling the stimulation pathways and performing pharmacological manipulations. A number of papers have also studied LTP in organotypic hippocampal slices (Stoppini et al., 1991; Collin et al., 1997; Gahwiler et al., 1997; Debanne et al., 1998; Shi et al., 1999; Pavlidis et al., 2000; Tominaga-Yoshino et al., 2008; Zhang et al., 2009). The advantage of studying LTP in organotypic slices is that long-term genetic, pharmacological, and training manipulations over the course of hours or days can be used. For example, early studies demonstrating that LTP is in part a result of the insertion of AMPA receptors were performed in hippocampal organotypic cultures because they afforded a means to transfect cells with viral vectors before performing plasticity experiments (Shi et al., 1999; Hayashi et al., 2000; Makino and Malinow, 2009). It is the ability to perform long-term experiments that drives the current research of studying plasticity in cortical organotypic slices.

Despite the advantage of using organotypic slices for long-term plasticity experiments a limitation is that it has remained challenging to use associative induction protocols that last many hours—which are likely more physiological and would provide a means to study slower forms of plasticity that rely on protein-synthesis. Specifically, it is difficult to use intracellular pairing protocols that last hours because in addition to the challenges of performing long-lasting recordings, intracellular dialysis generally prevents the induction of LTP (Pavlidis et al., 2000;

Staff and Spruston, 2003; Xu and Wu, 2005; Tanaka et al., 2008; Zhang et al., 2008). Indeed, the “wash-out” problem, is even more rapid in organotypic slices, which is why organotypic LTP studies have often used very short baselines (Hayashi et al., 2000; Pavlidis et al., 2000; Makino and Malinow, 2009).

The recent development of optogenetic tools offer a potential way to use long-term induction protocols and avoid the intracellular dialysis during induction (Zhang et al., 2008; Schoenenberger et al., 2011). Specifically, algae-derived Channelrhodopsin-2 (ChR2) which can be activated by 470 nm blue light provides a manner to depolarize neurons (Boyden et al., 2005; Zhang et al., 2006; Zhang et al., 2007; Zhang et al., 2010). When expressed in neurons, ChR2 allows precise control of neuron activity mediated by light. Therefore, it offers a unique strategy for non-invasive LTP induction: the postsynaptic depolarization required for the induction of associative LTP can now be introduced by light instead of traditional electrical stimulation. Thus the short time window for LTP induction in conventional LTP experiments before intracellular dialysis occurs can be greatly expanded if ChR2 is employed (Zhang and Oertner, 2007; Zhang et al., 2008; Kohl et al., 2011; Schoenenberger et al., 2011).

Because my ultimate goal is to use cortical organotypic slices to study the interaction between LTP and STP over the course of hours (Carvalho and Buonomano, 2011), and because LTP has never been described in cortical organotypic slices, my first objective was to demonstrate that LTP can be observed in cortical organotypic slices. My second objective was to determine that ChR2-based light-pairing induction protocols are effective, and characterize the properties of LTP using these methods.

Here I was able to induce associative LTP for the first time in cortical organotypic slices, both with conventional pairing protocols and optogenetic methods. My results suggest that the

presence of pronounced inhibition generally impairs the induction of LTP in cortical organotypic slices, but associative LTP can be induced in the presence of local Bicucculine ($GABA_A$ receptor antagonist) or small dose of CNQX (AMPA receptor antagonist) to reduce feed-forward inhibition (Doi et al., 1990; Nishigori et al., 1990; Auerbach and Segal, 1996; Fukuda et al., 1998). Additionally, I demonstrate pairing presynaptic inputs with optical stimulation of ChR2-expressing neurons over the course of minutes or hours was effective in inducing LTP and avoiding the problem of intracellular dialysis (Zhang et al., 2008; Schoenenberger et al., 2011).

Results

Reduced inhibition (Bicuculline) helps inducing LTP after short training on the rig

Associative LTP protocols typically pair presynaptic inputs with postsynaptic depolarization during intracellular whole-cell recordings. In my initial pilot experiments in L2/3 pyramidal neurons of cortical organotypic slices, the traditional pairing protocols proved unsuccessful in inducing LTP. Since it is well established that pronounced inhibition can suppress the opening of NMDA channels and the induction of LTP, it is possible that LTP was blocked by pronounced inhibition in cortical organotypic slices (Miles and Wong, 1986; Sayer et al., 1990; Scharfman, 1994; Bolshakov and Siegelbaum, 1995; Debanne et al., 1995; Chen and Buonomano, 2012). Consistent with this possibility the presence of the $GABA_A$ receptor antagonist bicuculline (500 μM) revealed a dramatic increase in EPSP amplitude and spikes, reflecting the "unmasking" of the EPSP (**Supplementary Figure S4.1**).

Based on this hypothesis, a whole-cell pairing protocol was used to induce LTP in the presence of the $GABA_A$ antagonist bicuculline. Baseline EPSPs were recorded from two different pathways with whole-cell recordings. During pairing, 500 μM of bicuculline in low-Mg ACSF (1.3 mM) was locally applied through pipette near the recording electrode to reduce inhibition without eliciting epileptic activity. Training consisted of a single pulse from one pathway paired with current injection that elicited 2-3 spikes. Pairing was delivered every 2 seconds for 2 minutes before EPSP strength was recorded from both pathways (**Figure 4.1A**). LTP was observed in the paired pathway (EPSP slope rise to 164.62 ± 30.40% of baseline at 18 minutes after pairing. Between the pathways paired t-test t_4=3.39, p=0.028), while EPSPs remain relatively unchanged in the unpaired pathway (93.02 ± 11.23% of baseline)

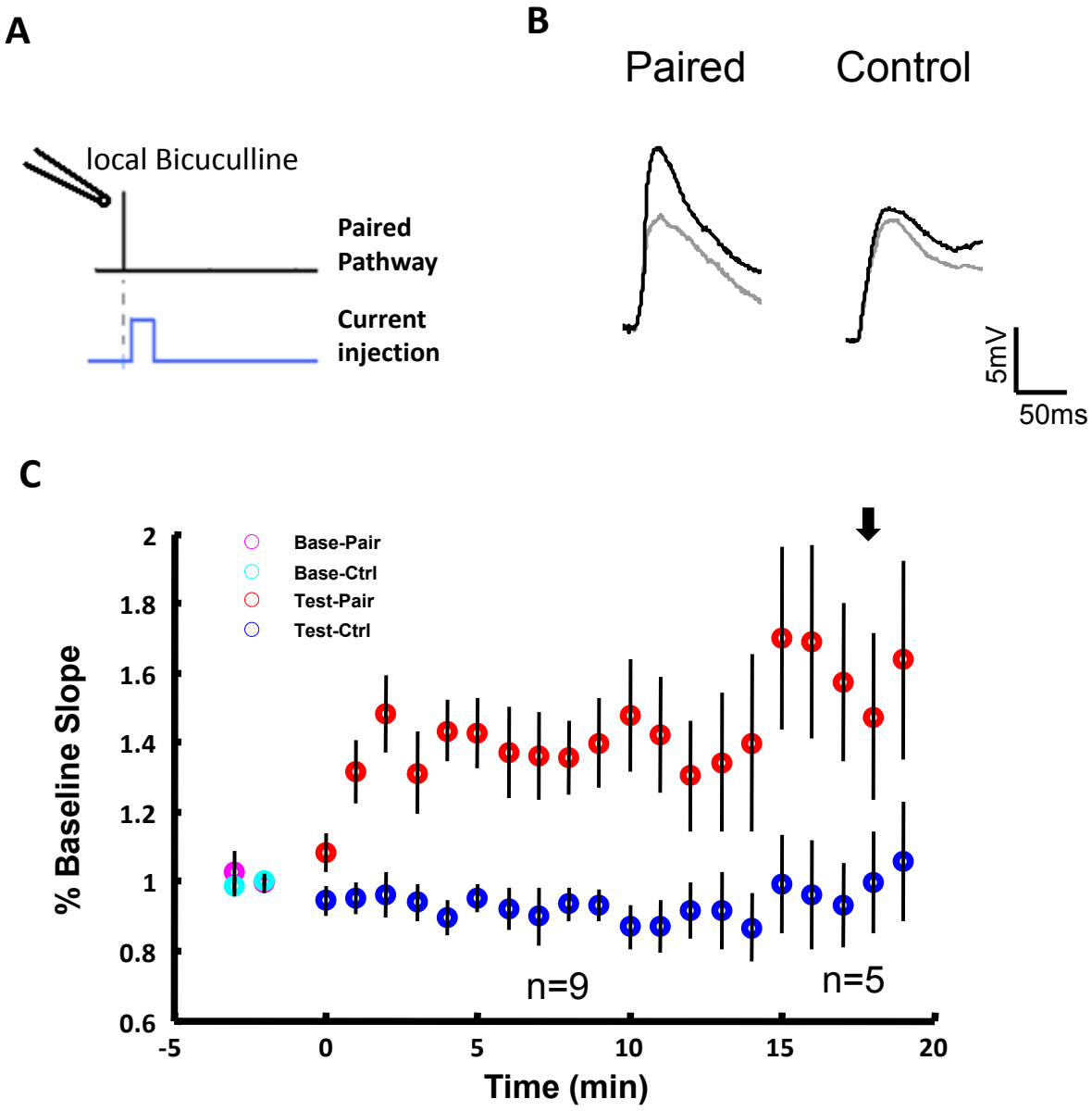


Figure 4.1. Pairing protocol under Reduced inhibition induces LTP. **(A)** Pairing protocol: pair postsynaptic depolarization with a single pulse in one pathway, every 2s for 2min on the rig. Bicuculline was locally applied near the recording electrode. **(B)** Sample traces at 18min after pairing (grey: pre-pairing; black: post pairing). **(C)** LTP induced (at 18min, t_4=3.39, p=0.028, n=5). Arrow indicated position of sample traces in **(B)**.

(**Figure 4.1B, C**). These experiments suggest that pronounced inhibition was preventing the induction of LTP in the previous experiments. This interpretation was further confirmed by experiments designed to address the potential concern that intracellular dialysis might have prevented LTP in early experiments (Pavlidis et al., 2000; Staff and Spruston, 2003; Xu and Wu, 2005; Tanaka et al., 2008; Zhang et al., 2008; Schoenenberger et al., 2011). Specifically, a pairing protocol using extracellular depolarization to avoid dialysis also failed to induce LTP (**Supplementary Figure S4.2**).

To the best of our knowledge, this is the first demonstration of associative LTP in cortical organotypic slice. However, the use of intracellular depolarization and local bicuculline is not an ideal protocol for long-term training and testing over hours because it requires long-lasting whole-cell recordings and is subject to intracellular dialysis. To overcome these problems I developed a protocol using Channelrhodopsin-2 (ChR2) to depolarize individual postsynaptic cells in a non-invasive fashion (Zhang et al., 2008; Schoenenberger et al., 2011).

Expression of ChR2 in cortical organotypic slices

Channelrhodopsin-2 (ChR2) and the fluorescent protein EYFP were expressed in cortical organotypic slices using an AAV5 viral vector (See Methods) (Zhang et al., 2006; Arenkiel et al., 2007; Zhang et al., 2007; Zhang et al., 2010). Two to three weeks after transfection neurons expressing EYFP could be visualized and patched (**Figure 4.2A**). When activated by 470 nm blue light, light-induced action potentials could be recorded both intracellularly or extracellularly (**Figure 4.2B**).

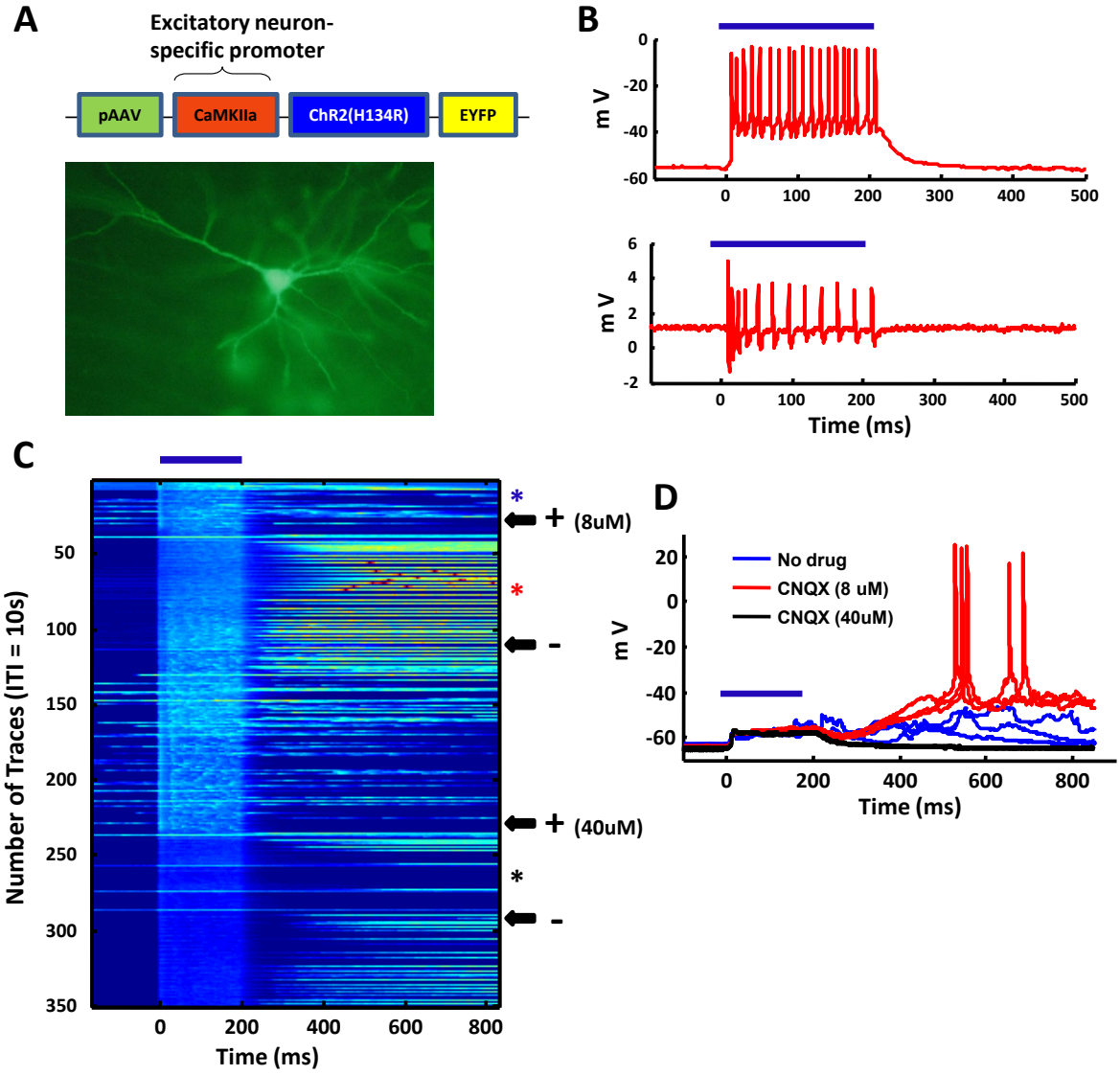


Figure 4.2. ChR2 expression in the cortical organotypic slices induces light-activated neural activity. **(A)** Construct of the virus and visualization of ChR2 expression with EYFP. **(B)** Whole-cell recording (upper) and lose-cell attach recording (lower) confirmed robust light-activated action potentials. **(C,D)** Light-evoked polysynaptic network activity can be block by high dose (40 μM) of CNQX, but low dose CNQX (8 μM) leads to more network activity by reducing feed-forward inhibition. Blue bar indicates light, asterisk indicates location of each of the sample traces in **(D)**.

Because cortical circuitry is recurrent, light activation of ChR2 in a subset of cells is expected to induce indirect activity in neurons expressing little or no ChR2. **Figure 4.2C** provides an example of a neuron in which light elicited indirect polysynaptic activity. To demonstrate that this activity is polysynaptic in nature, light evoked responses were also examined in the presence of CNQX. Interestingly, a low-dose of CNQX (8 μM) actually increased the amount of light-evoked network activity, presumably by decreasing the excitatory drive onto inhibitory neurons therefore shifting the excitation/inhibition balance in the slice (Doi et al., 1990; Fukuda et al., 1998; Menuz et al., 2007) (see below, **Figure 4.2C, D**). As expected a high-dose of CNQX (40 μM) abolished all polysynaptic activity, leaving a small direct light-activated depolarizing current. Network activity recovered after washing off CNQX. The effectiveness of optical stimulation was also verified by demonstrating that chronic stimulation (2 days) of the ChR2-expressing slices with blue light induced homeostatic plasticity, quantified by measuring either the total time of spontaneous UP states or the area under evoked responses (**Supplementary Figure S4.3**).

Optical-pairing protocol with intact inhibition does not induce LTP

Having established the effectiveness of ChR2 expression and light induced depolarization I next examined whether optically evoked depolarization could be used to induce LTP with a long-lasting (1.5 hr) pairing protocol. In these experiments organotypic slices were "implanted" with two stimulating electrodes (Johnson and Buonomano, 2009; Johnson et al., 2010) (**Figure 4.3A**). These experiments were performed under intact pharmacology, and to avoid wash-out these experiments were performed with a between-cell baseline: "baseline" EPSPs were first recorded from control cells by randomly patching neurons in the slice.

Subsequently, a single pulse was delivered through one of the electrodes to provide presynaptic activity, while 50 ms of blue light was applied with a 15 ms delay to provide postsynaptic depolarization in the ChR2-positive cells. The other implanted electrode was activated with a single pulse 2 or 5 seconds after the paired pathway and served as the unpaired control (**Figure 4.3B**). The pairing was repeated every 10 s for 1.5 hours on the electrophysiology rig. After pairing ChR2 positive (ChR+) and negative (ChR-) cells were patched and EPSP responses to the two pathways were recorded.

Pairing electrical stimulation with light did not induce robust LTP in ChR+ (nor ChR-) neurons (**Figure 4.3C**). Despite a trend, there was no significant difference in EPSP slopes between two pathways in the ChR2+ cells (2.63 ± 0.57 $mV \cdot ms^{-1}$ and 1.84 ± 0.30 $mV \cdot ms^{-1}$ for the paired and unpaired pathway, respectively. t_{11}=2.01, p=0.070, n=12). There was also no difference between the pathways in the baseline or ChR- groups (Baseline group: 2.31 ± 0.20 $mV \cdot ms^{-1}$ and 2.11 ± 0.29 $mV \cdot ms^{-1}$ for the paired and unpaired pathway, t_{11}=1.24, p=0.241, n=12. ChR- group: 2.40 ± 0.27 $mV \cdot ms^{-1}$ and 1.97 ± 0.23 $mV \cdot ms^{-1}$ for the paired and unpaired pathway, t_{13}=1.51, p=0.156, n=14). Additionally, pairing in the presence of the adrenergic agonist isoproterenol or the cholinergic agonist carbachol did not result in the induction of LTP either (data not shown). Therefore, these data suggest that even with a 1.5 hr pairing protocol inhibition may be largely preventing the induction of LTP with optical-pairing protocol.

Induction of LTP with optical-pairing protocol and reduced inhibition

We next examined the ability of the optical-pairing protocol to induce LTP in the presence of reduced inhibition. However, blocking $GABA_A$ transmission in these long-lasting

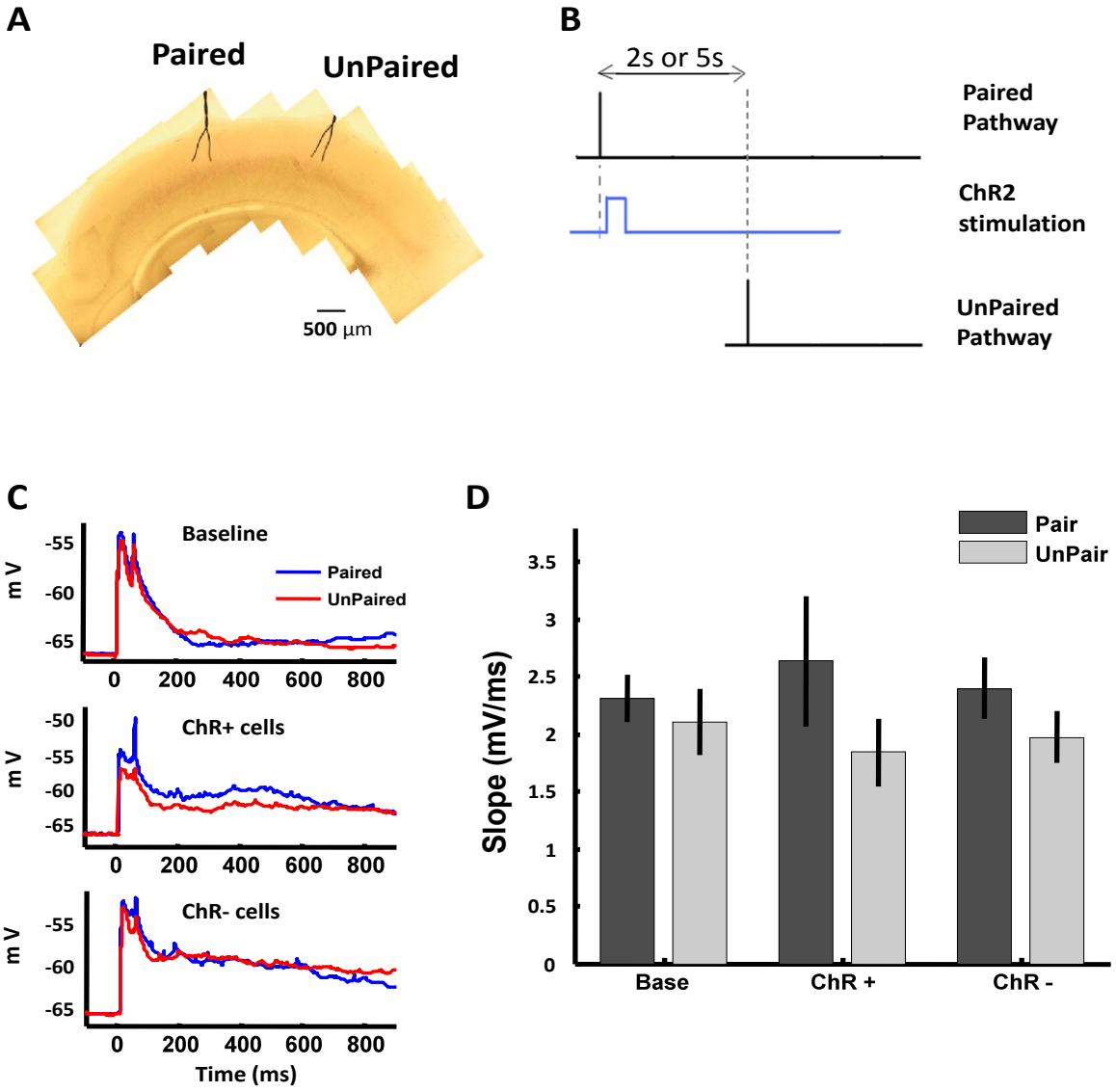


Figure 4.3. Optical-Pairing Protocol does not induce LTP in cortical organotypic slices. **(A)** Montage of an organotypic slice with the implanted electrodes, indicating paired and unpaired pathways. (Johnson and Buonomano, 2009) **(B)** Pairing protocol. **(C)** Averaged traces for baseline (upper), ChR+ cells (middle) and ChR- cells (lower) (n=12, 12, 14 respectively). **(D)** Pairing itself does not induce LTP in the ChR+ group (paired t-test, t_{11}=2.01, p=0.070).

experiments is challenging: the application of local bicuculline is difficult to control—as chronically applied bicuculline easily induces epileptic activity in the cortical network. In contrast, low-dose of CNQX (3 μM) which is a AMPA/kainate receptor antagonist, can reduce feed-forward inhibition without causing epileptic activity, specifically at low doses the decrease in AMPAergic drive to inhibitory neurons shift the excitatory/inhibitory balance towards excitation (**Figure 4.2C,D**) (Doi et al., 1990; Nishigori et al., 1990; Auerbach and Segal, 1996; Fukuda et al., 1998; van Drongelen et al., 2005; Maclean and Bowie, 2011). Therefore, I determined if the optical-pairing protocol induced LTP in the presence of low doses of CNQX.

These experiments were first performed using a short-induction protocol during whole-cell recording. In these within-cell CNQX-LTP experiments, baseline EPSPs were first recorded. Pairing was then delivered by a single pulse from one of the implanted electrodes coupled with 50 ms pulse of blue light (**Figure 4.4A**). Pairing was delivered every 3-5 s for 3-6 min (**Figure 4.4B**). A low-dose of CNQX (3 μM) was bath applied throughout the experiment. After pairing, there was a gradual increase in EPSP slope in response to the paired pathway (EPSP slope rise to 248.08 ± 60.70% of baseline at 25 minutes after pairing, between the pathways paired t-test t_8=2.62, p=0.031, n=9) while the unpaired pathway remained relatively unchanged (104.48 ± 17.45% of baseline) (**Figure 4.4C, D, E**).

These results establish that LTP can be induced using an optical pairing protocol. But because of the fast washout effect in organotypic slices, LTP must be induced within a short time window (typically 5-10 min) after breaking into the neuron (Hayashi et al., 2000; Pavlidis et al., 2000; Staff and Spruston, 2003; Xu and Wu, 2005; Makino and Malinow, 2009). To study the interaction between LTP and STP over longer timescales, I next examined an optical-pairing protocol that could be applied for hours in the incubator.

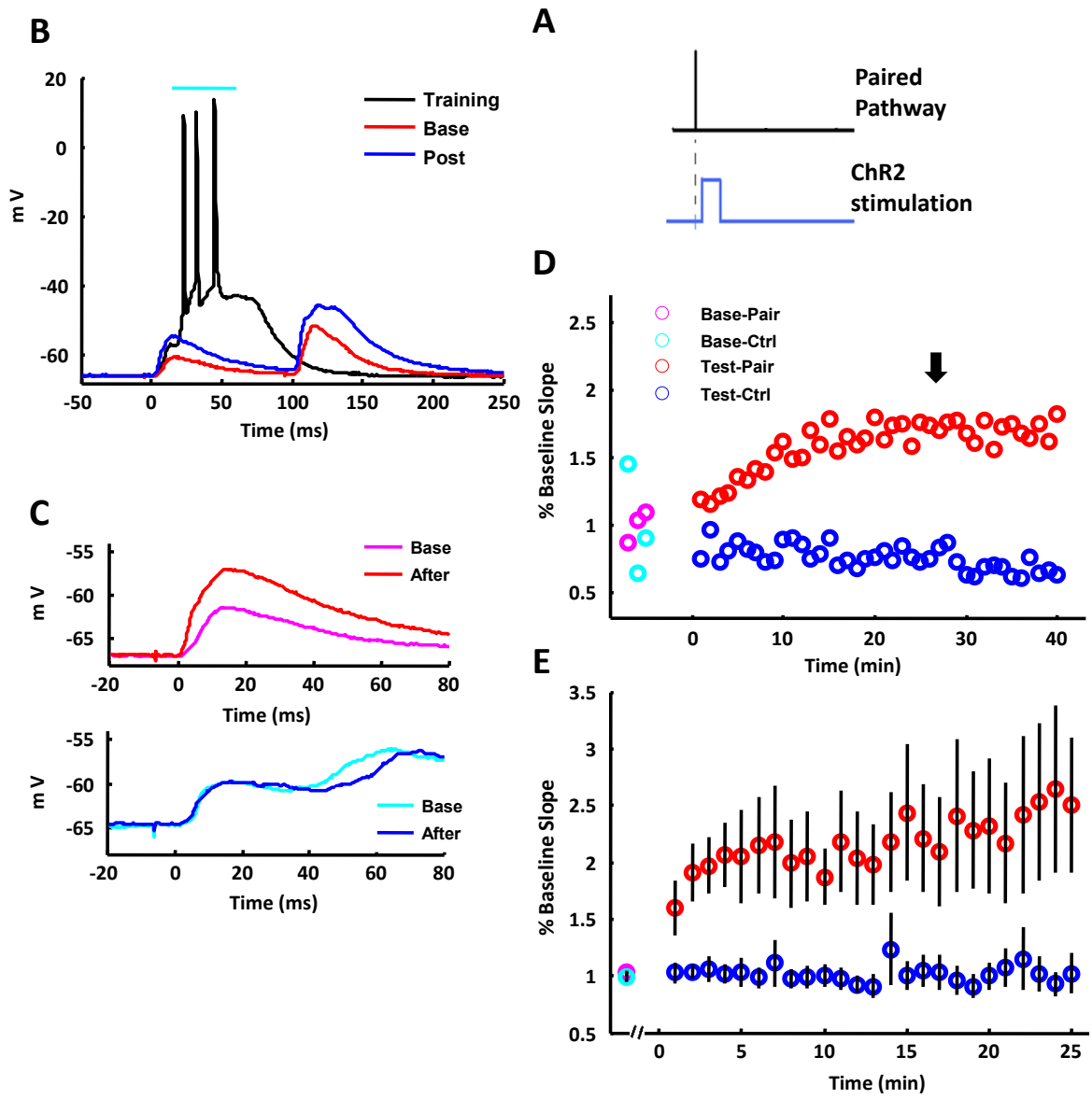


Figure 4.4. Reduced inhibition (low-dose CNQX) plus optogenetics helps induce LTP on the rig. **(A)** Pairing protocol: pair 50 ms blue light with a single pulse in one pathway, every 3-5 s for 3-6 min on the rig. 3 µM CNQX was bath applied throughout the experiment. **(B)** Sample traces of training (black), overlapped on baseline (red) and post-training (blue) of the paired pathway. Cyan bar indicates light. **(C)** Sample traces for the paired pathway (upper) and control pathway (lower) before and after LTP induction. **(D)** % Baseline slope plot to time (min). **(E)** Averaged data for all cells (between pathways, paired t-test t_8=2.62, p=0.031, n=9 at 25 min after pairing).

Induction of LTP with a long-lasting optical-pairing protocol

Our hypothesis that metaplasticity of STP relies on long-term training for hours requires that the training sessions be performed in the incubator. Thus I next used optical-pairing protocol with low-dose CNQX to induce LTP in the incubator. Pairing consisted of 40 ms pulse of light presented 20 ms after electrical stimulation of the paired pathway (every 10 seconds), while the unpaired pathway was activated 5 seconds later. A "spaced" protocol was used: a single training session lasted for 30 minutes, followed by 30 minutes of rest, for a total of 3 training sessions lasting 2.5 hours. After training ended, the slice was moved to the recording rig and both ChR2 positive (ChR+) and negative (ChR-) cells were patched (**Figure 4.5B**). Both training and testing were done in the presence of 4 μM CNQX. To analyze the difference in EPSP amplitude between the paired and unpaired pathways, and between ChR+ and ChR- neurons two-way ANOVAs were performed (with repeated measures across pathways). There was a significant difference between paired and unpaired pathways ($F_{1,20}$=11.46, p=0.003), and no significant interaction between Cells (ChR+, ChR-)×Pathway (paired, unpaired) ($F_{1,20}$=0.01, p=0.914). These results suggest a significant increase in the EPSP slope in the paired pathway compared to unpaired pathway for both ChR+ and ChR- cells. This observation was confirmed with the t-tests for both ChR+ and ChR- cells (ChR+: 0.99 ± 0.19 $mV \cdot ms^{-1}$ and 0.41 ± 0.05 $mV \cdot ms^{-1}$ in the paired and unpaired pathway, respectively; Paired t-test: t_8=2.69, p=0.028, n=9. ChR-: 1.14 ± 0.21 $mV \cdot ms^{-1}$ and 0.60 ± 0.10 $mV \cdot ms^{-1}$ in the paired and unpaired pathway, respectively; t_{12}=2.35, p=0.037, n=13 cells) (**Figure 4.5C**). To ensure any potential differences in EPSP amplitudes were not a function of the distance to the stimulating and control electrodes, I verified that there was no significant difference between the two distances (unpaired t-test, ChR+: t_{16}=0.89, p=0.389. ChR-: t_{24}=1.62, p=0.119).

Since these were the first experiment to demonstrate LTP in the incubator using an optical-pairing protocol and they generated an unexpected result of LTP in the ChR- neurons as well as in the ChR+ neurons, I replicated this study in an independent set of experiments under "blind" conditions. The same concentration of CNQX (4 μM) was bath applied during both training and testing. Again, two-way ANOVA showed a significant effect of pairing ($F_{1,22}$=22.86, $p=8.97\times10^{-5}$), along with no significant interaction between Cells (ChR+, ChR-) × Pathway (paired, unpaired) ($F_{1,22}$=0.27, p=0.607) — meaning again that the paired pathway in the ChR+ neurons exhibited LTP in addition to the ChR- neurons. Paired t-tests revealed a significant increase in EPSP slope of the paired pathway in both ChR+ and ChR- group (ChR+: 1.87 ± 0.33 $mV\cdot ms^{-1}$ and 1.15 ± 0.22 $mV\cdot ms^{-1}$ in the paired and unpaired pathway, respectively; paired t-test, t_6=2.72, p=0.035, n=7. ChR-: 1.16 ± 0.15 $mV\cdot ms^{-1}$ and 0.59 ± 0.07 $mV\cdot ms^{-1}$ in the paired and unpaired pathway, respectively; t_{16}=4.22, p=0.001, n=17) (**Figure 4.5D**). There was no significant differences in the cells' distances to the two electrodes in either groups (unpaired t-test, ChR+: t_{12}=0.98, p=0.347. ChR-: t_{32}=0.56, p=0.577).

Together these two experiments reveals robust LTP as demonstrated by the difference in EPSP slope between the paired and unpaired pathways. Surprisingly, however, both sets of experiments revealed that LTP was induced in ChR- cells in addition to the ChR+ cells.

LTP induction with short-term training protocols in the incubator

The above results raise the critical question of why we observed LTP in ChR- neurons. In the above experiments, ChR2+ cells were defined as having the light-evoked depolarization induces at least one spike. Therefore, some of the negative cells defined here respond to blue

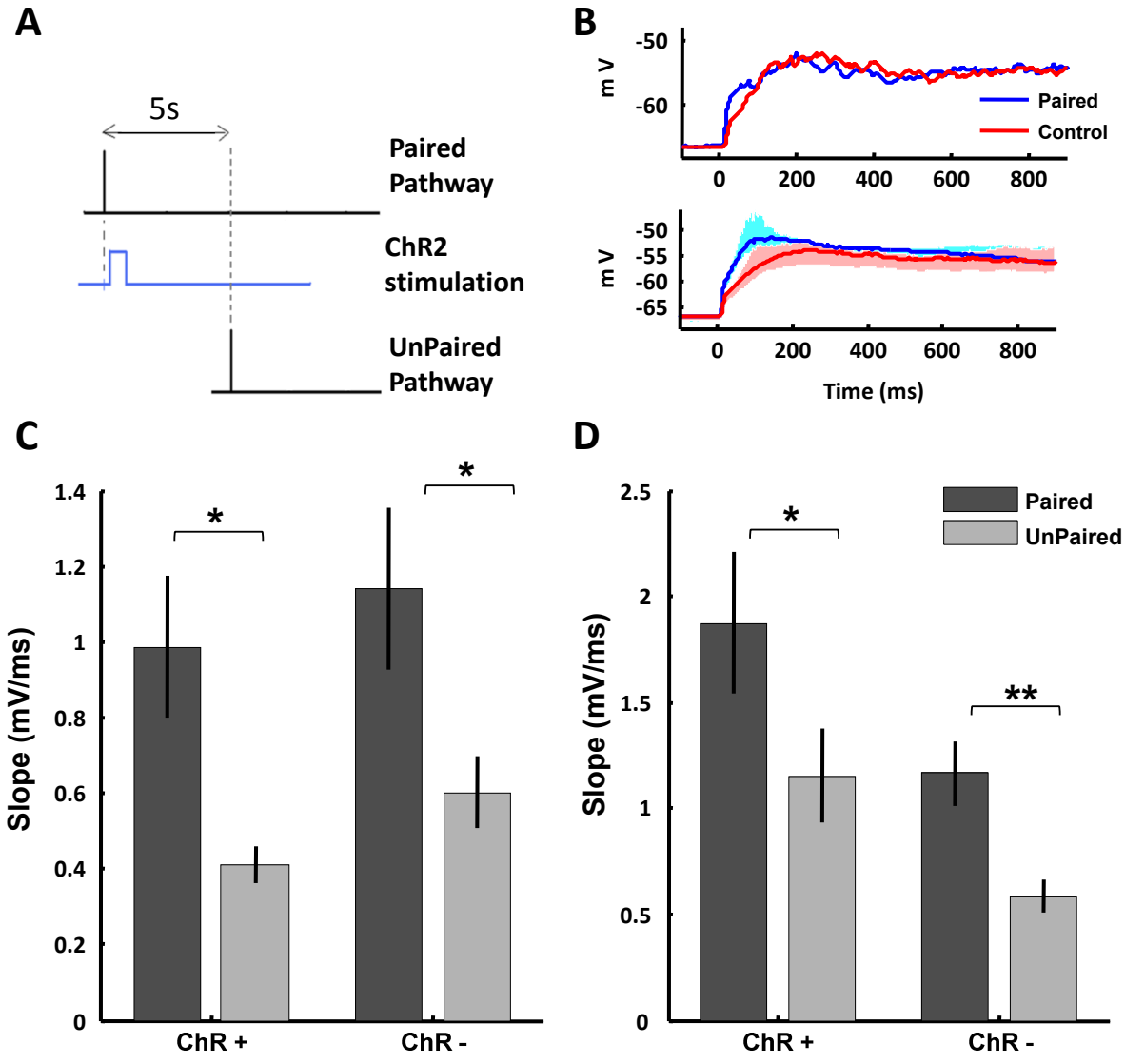


Figure 4.5. 2.5 hr optical-pairing in incubator induced LTP. (A) Pairing protocols : pair 40 ms blue light with a single pulse, with control pathway activated 5 s later, repeats every 10 s for 2.5 hr (with 30 min ON/OFF sessions) in the incubator. 4 µM CNQX was bath applied during both training and testing. **(B)** Sample traces after training, for a single cell (upper) and average for all cells (lower). SEM shown in shaded area. **(C)** After 2.5 hr of spaced training, LTP was induced in both ChR+ and ChR- cells (n=9,13 respectively). **(D)** Similar experiments as in **(C)**, with paired/unpaired pathways blinded (n=7,17). (*: $p<0.05$. **: $p<0.005$)

light as well but to a smaller degree, as a result of the high background expression of ChR2 in our slices. Because the training occurred in the incubator and the testing on the rig, we expect that there may be some differences in the light-evoked responses in two locations as a result of differences in media, temperature, and light sources. Thus it is possible that some of the ChR- cells underwent more depolarization in response to light during training in the incubator (See **Discussion** and **Supplementary Figure S4.4A, B**). Nevertheless it might still be expected that the degree of LTP in the ChR- cells would be correlated with the light-induced depolarization. However this was not the case as the correlation were not significant (**Supplementary Figure S4.5**). On the other hand it is possible our results indicate that late-phase LTP does not exhibit "presynaptic pathway specificity" in cortical slices—that is from the perspective of the presynaptic neuron, any presynaptic mechanisms contributing to LTP may not be synapse specific (Bonhoeffer et al., 1989; Schuman and Madison, 1994; Engert and Bonhoeffer, 1997).

If the lack of presynaptic specificity (meaning the presence of LTP in both ChR+ and ChR- neurons from the paired pathway) was protein-synthesis dependent, training at a shorter time in the incubator should reduce or eliminate the LTP effect in the ChR- cells. In contrast if LTP in the ChR- cells was a result of direct or indirect depolarization of the ChR- neurons during pairing, LTP should also be observed with a shorter protocol. We thus carried out another set of experiments in which the same pairing protocol was delivered to the slice, but only trained for 15 minutes in the incubator (**Figure 4.6A**). Similarly, 4 μM of CNQX was bath applied during both training and testing. Pairing still revealed a significant increase in the EPSP slope of the paired pathway (two-way ANOVA, $F_{1,26}$=12.89, p=0.001), however, the interaction between Cells (ChR+, ChR-) x Pathway (paired, unpaired) was also significant ($F_{1,26}$=10.44, p=0.003). Paired t-tests revealed that LTP was only induced in the ChR+ group (1.25 ± 0.28 $mV \cdot ms^{-1}$ and 0.67 ± 0.16 $mV \cdot ms^{-1}$ in the paired and unpaired pathway, respectively; t_9=3.39, p=0.008) but not the

ChR- group (0.79 ± 0.09 $mV \cdot ms^{-1}$ and 0.76 ± 0.11 $mV \cdot ms^{-1}$ in the paired and unpaired pathway, respectively; t_{17}=0.36, p=0.722) (**Figure 4.6B, C, D**). Distance to the two electrodes are not significantly different in either group (unpaired t-test, ChR+: t_{18}=0.21, p=0.840. ChR-: t_{34}=0.33, p=0.744). Moreover, this LTP was confirmed to be NMDA receptor dependent, as training in low-dose CNQX plus bath applied 100 μM APV abolished LTP induction in ChR+ neurons (n=16) (**Supplementary Figure S4.6**). These results support the hypothesis that the pairing-induced LTP in the ChR2 negative cells after longer training sessions may be a result of potential protein-synthesis mechanisms.

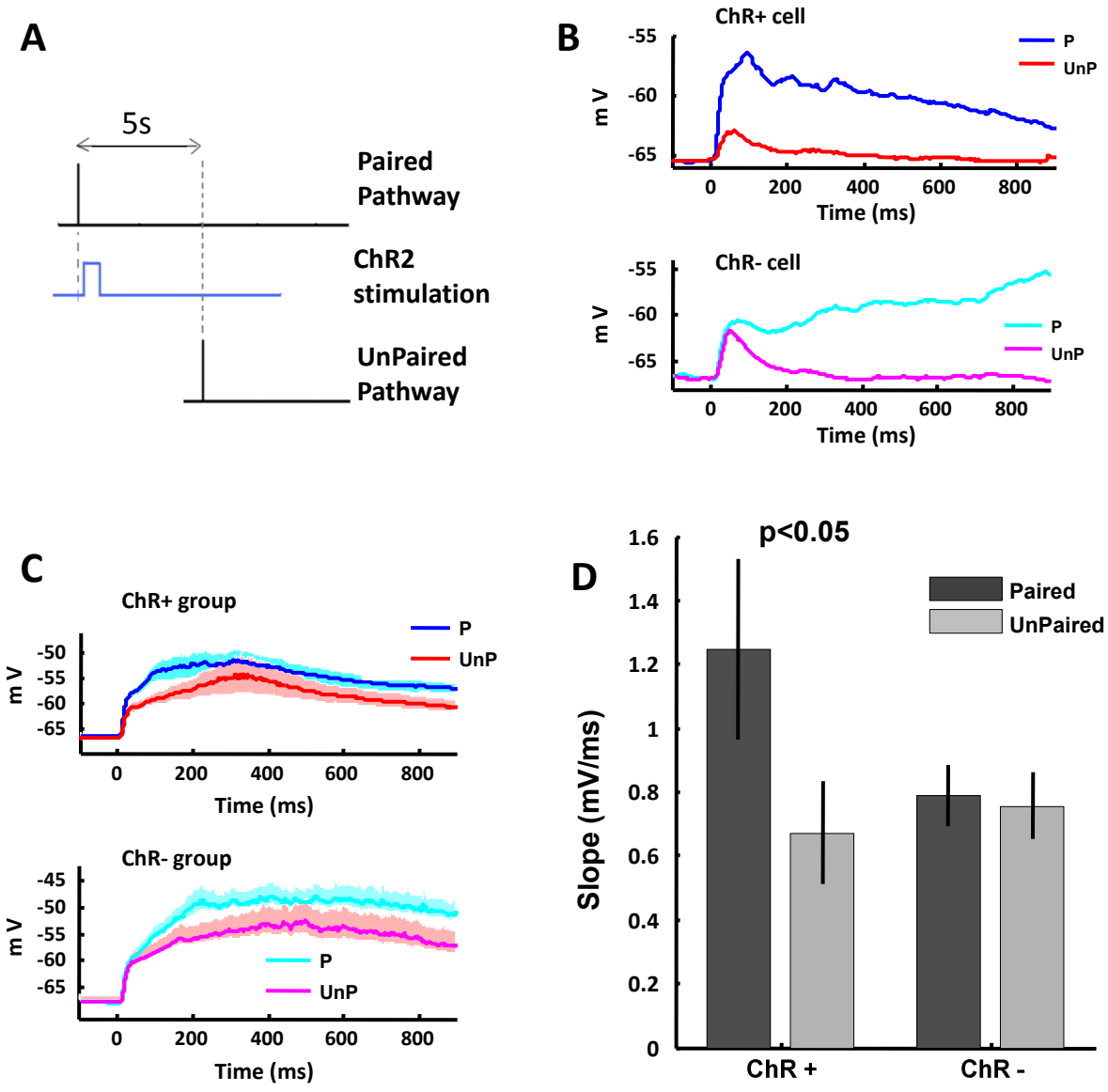


Figure 4.6. 15min optical-pairing in incubator induced LTP. (A) Pairing protocols, same as in **Figure 4.5A** but only trained for 15 min. **(B)** Sample traces after training in a ChR+ cell (upper) and a neighboring ChR- cell (lower). **(C)** Average traces for all ChR+ cells (upper) and ChR- cells (lower). SEM shown in shaded area. **(D)** 15 min training only induced LTP in the ChR+ group but not the ChR- group (n=10, 18 respectively).

Discussion

Together the above results establish that LTP can be successfully induced in cortical organotypic slices after reducing inhibition level in the network with either bicuculline or low dose of CNQX. To the best of our knowledge, this is the first time that associative LTP has been reported in cortical organotypic slices. Surprisingly, I observed a potential lack of presynaptic specificity, in which LTP as measured by the increase in synaptic strength in the paired pathway in relation to the unpaired pathways was present in both ChR+ and ChR- cells when training lasted for hours. Most importantly, however, I was able to demonstrate the effectiveness of an optical-pairing protocol necessary to test my metaplasticity of STP experiments described in Chapter 5.

Reducing inhibition is critical for LTP induction

Experiments using depolarization induced by extracellular or optical depolarization (**Figure 4.3 and Supplementary Figure S4.2**) revealed that no robust LTP was induced under intact inhibition. In contrast, LTP was induced in the presence of local bicuculline or low-doses of bath applied CNQX (**Figure 4.1 and 4.4**). These results suggest that pronounced inhibition in cortical cultures suppresses the induction of LTP. This is consistent with a large body of data from hippocampal and cortical acute slices demonstrating that decreasing inhibition enable or enhances the induction of LTP by augmenting the postsynaptic depolarization and Ca^{2+} entry through voltage-gated NMDA channels.

LTP induction in ChR-negative cells

One of the surprising results was that longer training sessions (2.5 h) resulted in the induction of LTP in both ChR2+ cells and ChR2- cells. There are a number of possible explanations for this finding. First, as reported some ChR- cells also exhibit subthreshold levels of direct light-induced depolarization or, of course, received indirect inputs from neighbouring ChR+ neurons (**Figure 4.2C, D**). However the fact that the 15 min pairing protocol did not result in LTP in the ChR- cells suggest this is not the explanation—nevertheless the ChR- could received weaker depolarization thus requiring longer induction protocols. Second, the LTP in the ChR- cells could reflect presynaptic pathway nonspecific LTP. Note that when considering the issue of pathway and synapse specificity a distinction should be made between postsynaptic and presynaptic specificity. A large number of both early and late-LTP studies have established that LTP is generally postsynaptic specific. That is, different synapses on the same postsynaptic neuron are independently modulated—although this might break down at short distances (Frey and Morris, 1997, 1998b; Govindarajan et al., 2011). Very few studies have addressed the issue of presynaptic specificity, and many of those have revealed that the non-paired presynaptic terminals of potentiated synapses may also be potentiated (Bonhoeffer et al., 1989; Schuman and Madison, 1994; Engert and Bonhoeffer, 1997; Volgushev et al., 2000). This issue has not been carefully examined in neocortical synapses, and it is possible that experiments over the course of hours which presumably allow for protein synthesis-dependent expression mechanisms do not exhibit presynaptic pathway specificity. This effect could be the result of the transport or diffusion of synaptic tags for LTP induction (Schuman and Madison, 1994; Frey and Morris, 1998a; Martin and Kosik, 2002; Redondo and Morris, 2011). In support of our hypothesis, short-term training (15min) under the same condition revealed a significant difference between ChR+ and ChR- groups, so that LTP was induced only in the ChR2 positive cells.

Conclusion

In conclusion, the results provided in **Chapter 4** demonstrate one of the first example of associative LTP in cortical organotypic slices. These results support the notion that cortical organotypic slices largely preserved the circuitry and physiological properties of acute slices. Equally importantly, these results establish a protocol in which it was possible to induce and test LTP hours after the beginning of pairing. This provides a potentially valuable preparation to study late-phase LTP using associative protocols, which has rarely been done, as well as establish the foundation for my metaplasticity of STP experiments in **Chapter 5**.

Methods

Organotypic slice preparation with implanted stimulating electrodes

Cortical organotypic slices were prepared using the interface method as previously described (Stoppini et al., 1991; Johnson and Buonomano, 2007; Chen and Buonomano, 2012) and maintained on culture inserts with implanted microelectrodes (Johnson and Buonomano, 2009; Johnson et al., 2010). Briefly, 7 day old Sprague-Dawley rats were anesthetized with isoflurane and decapitated. The brain was removed and placed in chilled cutting media. Coronal slices (400 μm thick) containing primary auditory cortex were cut using a vibratome and transferred onto cell culture inserts (Millipore, 0.4 μm pore size) with 1 ml of culture media. Culture media was changed 1 and 24 hrs after cutting and every 2-3 days thereafter. Cutting media was composed of EMEM (MediaTech cat. #15-010) plus 3 mM $MgCl_2$, 10 mM glucose, 25 mM Hepes, and 10 mM Trisbase. Culture media consisted of EMEM plus 1 mM glutamine, 2.6 mM $CaCl_2$, 1.85 mM $MgSO_4$, 30 mM glucose, 30 mM Hepes, 0.5 mM ascorbic acid, 20% horse serum, 10 units/L penicillin, and 10 μg/L streptomycin. Slices were incubated in 5% CO_2 and 95% O_2 at 35°C for 8-28 days before recording.

Electrophysiology

Whole-cell recordings were made from regular-spiking, infragranular pyramidal neurons using DIC visualization (in the 2.5 hr experiments the average distance from the cortical surface was 730 ± 21 μM and 616 ± 18 μM). Experiments were performed at 30°C in external solution composed of: 125 mM NaCl, 5.1 mM KCl, 2.6 mM $MgSO_4$, 26.1 mM $NaHCO_3$, 1 mM NaH_2PO_4, 25 mM glucose, and 2.6 mM $CaCl_2$. The internal solution for whole-cell recordings contained 100 mM K-gluconate, 20 mM KCl, 4 mM ATP-Mg, 10 mM phospho-creatine, 0.3 mM GTP-Na,

10 mM HEPES, and was adjusted to pH 7.3 and 300 mOsm. For experiments with Channelrhodopsin-2, a fluorescent dye is included in the whole-cell solution inside the recoding electrode (Alexa Fluor 488 carboxylic acid, Invitrogen cat. #A10438, at $1.1x10^{-5}$ μM) to help visualize the tip of the electrode during patching. All analyses were performed using software custom written in MATLAB.

Drug application

1(S),9(R)-(−)-Bicuculline methiodide (Sigma, cat. #14343) was first dissolved in ACSF to 2.5 mM as stock solution. To use, the stock solution was diluted with ACSF to a final concentration of 500 μM. The stock solution was made fresh daily.

CNQX disodium salt hydrate (Sigma, cat. #C239) was first dissolved into Dimethyl sulfoxide (DMSO, Sigma cat. #D2650) to make a stock solution at 2 mM. To use, the stock solution was diluted with ACSF to a final concentration as indicated in text then bath applied to the organotypic slices. The stock solution was made daily to ensure best quality of the drug.

DL-2-Amino-5-phosphonopentanoic acid (APV) (Sigma, cat.#A5282) was first dissolved with dH_2O to 10 mM as stock solution. To use, the stock solution was diluted in the culture media to a final concentration of 100 μM.

Viral transfection and ChR2 expression

rAAV5/CamKIIa-hChR2(H134R)-eYFP constructs were kindly provided by Dr. Karl Deisseroth from Stanford University and amplified and assembled by University of North Carolina vector core, with a titer of $4x10^{12}$. Each organotypic slice is transfected with about 0.5 μl of virus solution after 3 days of *in vitro* culturing. Expression can be seen as early as 10 days

after transfection, but peaks after 15 days. Experiments are typically carried out between 15-21 days after transfection.

Before transfection, each slice was separated into a single 6-well culture plate (Costar, cat. #3516). 1 µl virus solution was transferred into a glass patch pipette using a MicroFil needle (World's Precision Instruments, cat. #MF28G67-5) and split between 2 slices. Using a stereoscope the pipette is then lowered to near the slice with a manual micromanipulator, until the pipette tip is gently touching the surface of the slice. We sometimes break the tip of the glass pipette by gently touching the culture membrane near the slice before transfection, so that virus solution is easier to be pushed out. Then a small amount of positive pressure is applied to the pipette, so that virus solution is injected into the slice. The spreading of the virus solution on slice surface can be visually detected under the stereoscopic microscope (Nikon, SMZ-2B). We usually inject virus in 1-2 adjacent spots near the center between the two implanted electrodes. The slice is then put back into incubator and changed media every 2-3 days as usual.

Expression of Channelrhodopsin-2 in individual cells is visualized by imaging to eYFP under yellow light. During either training or testing, ChR2 channels are activated with 470 nm of blue light.

LTP induction protocols

For the within cell on-rig bicuculline experiments, baseline EPSPs from two pathways were first recorded in whole-cell mode for 1-3 min. This is immediately followed by pairing, during which a single pulse from the paired pathway is paired with 50 ms of current injection into the cell to elicit 2-3 spikes. Pairing was repeated every 2 s for 2 min, with locally applied Bicuculline (500 µM) in low-Mg ACSF (1.3 mM) through a second glass pipette near the

recording electrode. After pairing, the Bicuculline electrode was removed and EPSP response to the pathways were recorded in the same cell for 12-30 minutes. EPSP slope to the two pathways were quantified.

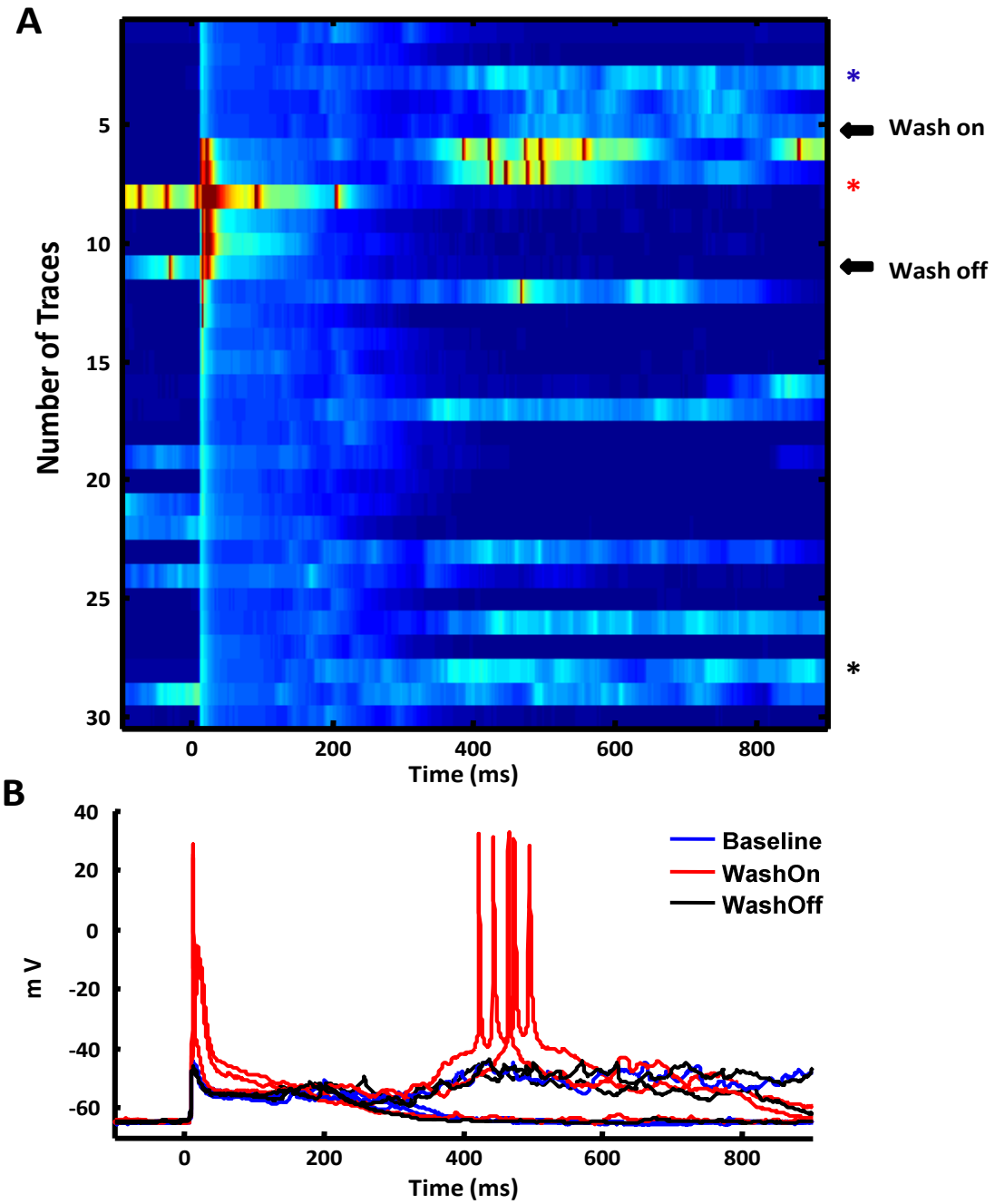
For the within cell on-rig CNQX experiments, baseline EPSPs to two pathways were first recorded for 1-3 min. Immediately afterwards, 1 pulse from the paired pathways was paired with 50 ms blue light and the pattern was repeated every 3-5 s for 3-6 min. Post-pairing EPSPs to the two pathways in the same cell were recorded for 25-60 minutes. 3 μM of CNQX was bath applied throughout the experiment.

For the cell-attached LTP experiments, 4 pulses at 50 Hz were delivered through the paired pathway, followed by 100 ms current injection through loose cell attached electrode to elicit 5-12 extracellular spikes (delay =10 ms). Unpaired pathway was activated 1 s later. Pairing was repeated every 2 s for 2 min. After 15 min of waiting, the cell was broken into and EPSPs were record in whole-cell mode for 15 min. Analysis showed that in the last 5 min of recording EPSPs were not significantly different compared to the first 5 min after break-in. Therefore EPSP slope and amplitudes were averaged within 15 minutes.

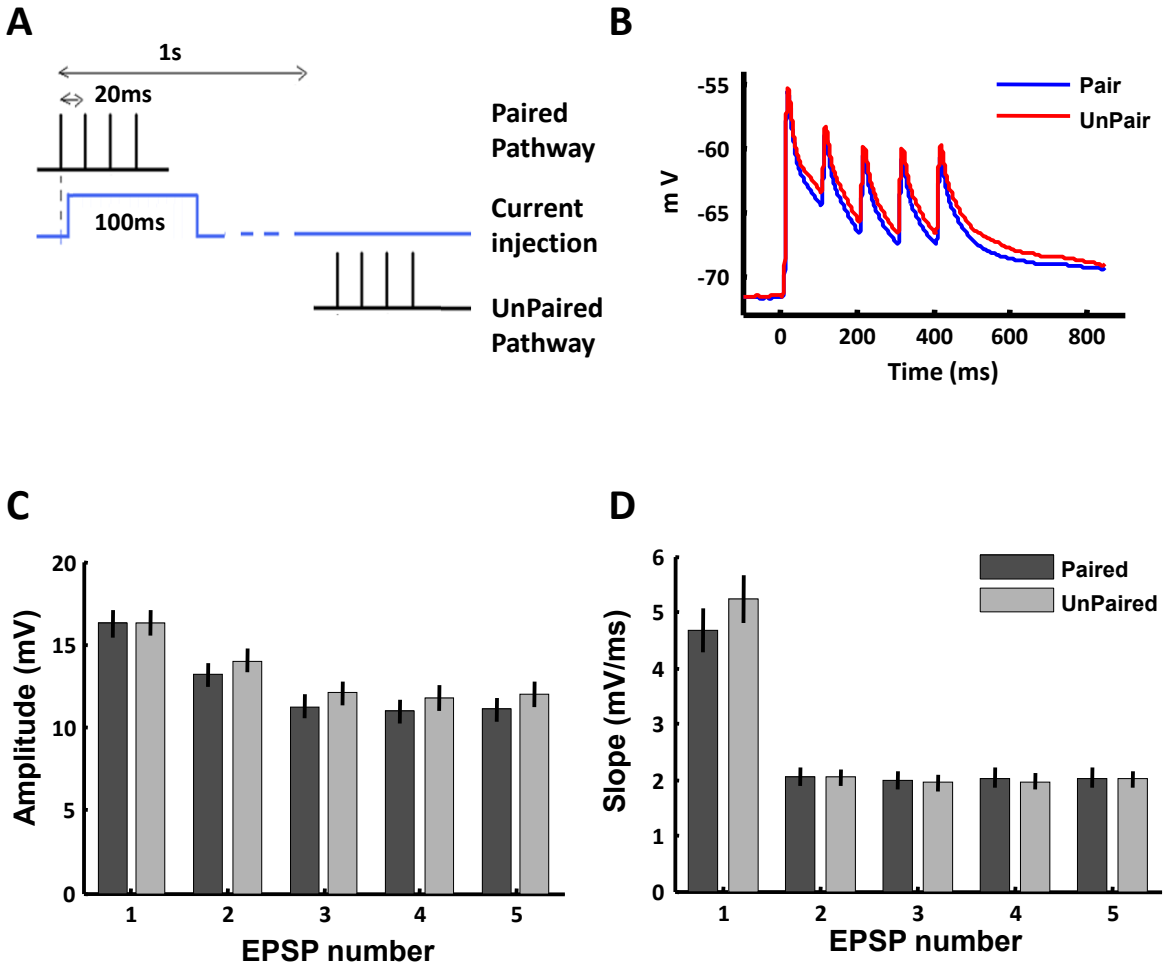
For the incubator-trained CNQX optical pairing experiments, pairing was composed of a single pulse delivered through one of the implanted electrodes to provide presynaptic activity. 40 or 50 ms of blue light was shined at the whole slice at a delay of 15 or 20 ms as stated in the **Results** part, to provide postsynaptic pairing. A second electrode was either activated or not during pairing to serve as the unpaired pathway. Stimulation intensity was at 80 μA for all three incubator CNQX-LTP experiments induced with optical pairing (2.5 hr unblind, 2.5 hr blind, 15 min unblind). The entire pairing protocol was repeated every 10 s for long (1.5, 2.5 hr) or short (15 min) period of time. For the 2.5 hours in-incubator training experiments, a spaced training

protocol was used, so that each pairing session lasted for 30 min followed by a 30 min rest period. After training, slices were moved to the recording rig. Recordings happened 5-10 min after the last training session ended. After pairing, EPSPs in response to the two pathways were recorded from both ChR2 positive (ChR+) and negative (ChR-) cells. In all experiments, ChR2+ cells were defined as having the light-evoked depolarization induces at least one spike, while the ChR- cells with light response smaller than 20mV. Low-dose of CNQX (3 μM or 4 μM) was bath applied throughout the experiment in a concentration indicated in the text. In the APV-15 min LTP experiments, stimulation intensity was at 100 μA. Only ChR+ neurons were recorded in the APV experiments.

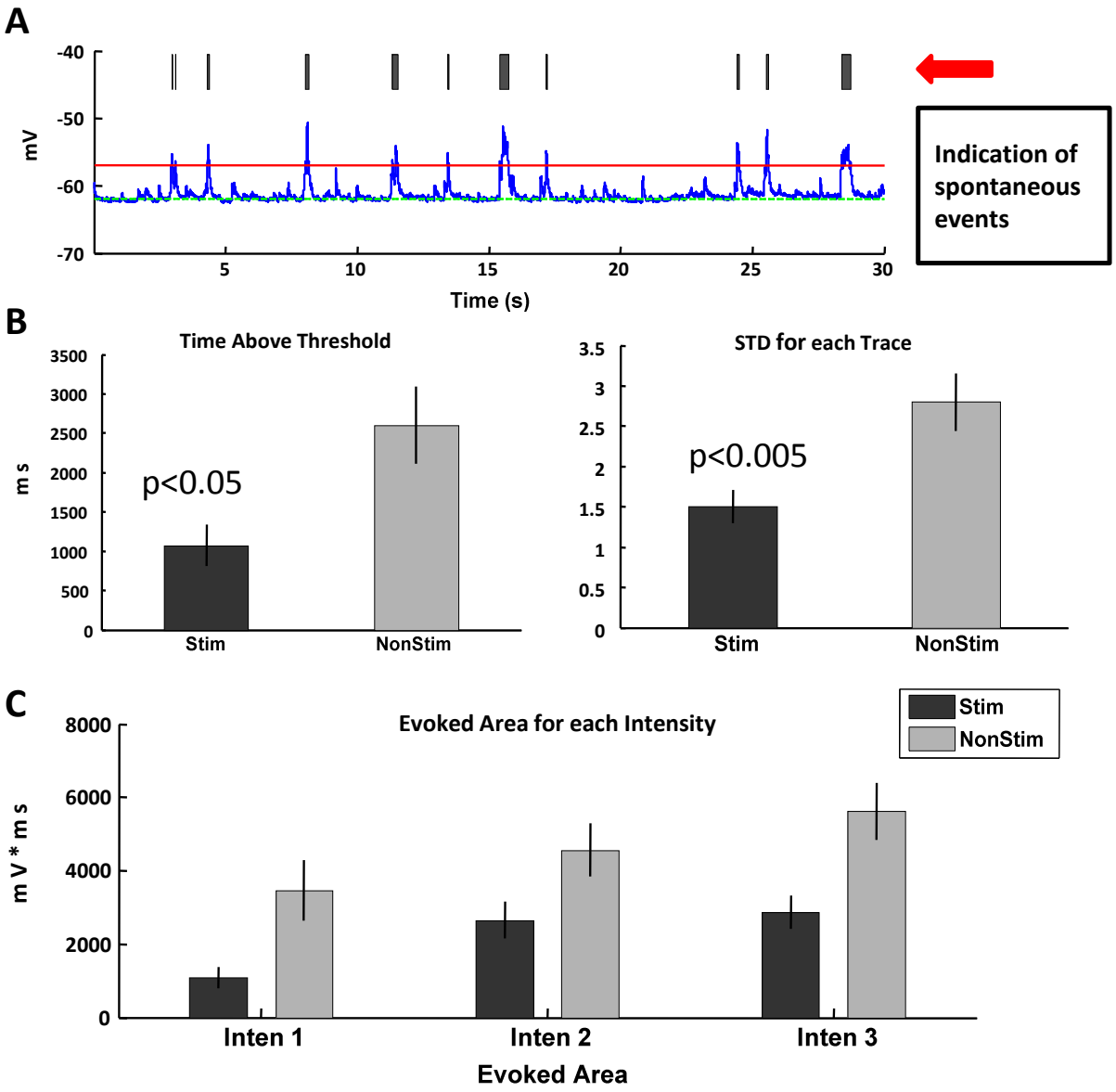
Supplementary Figures



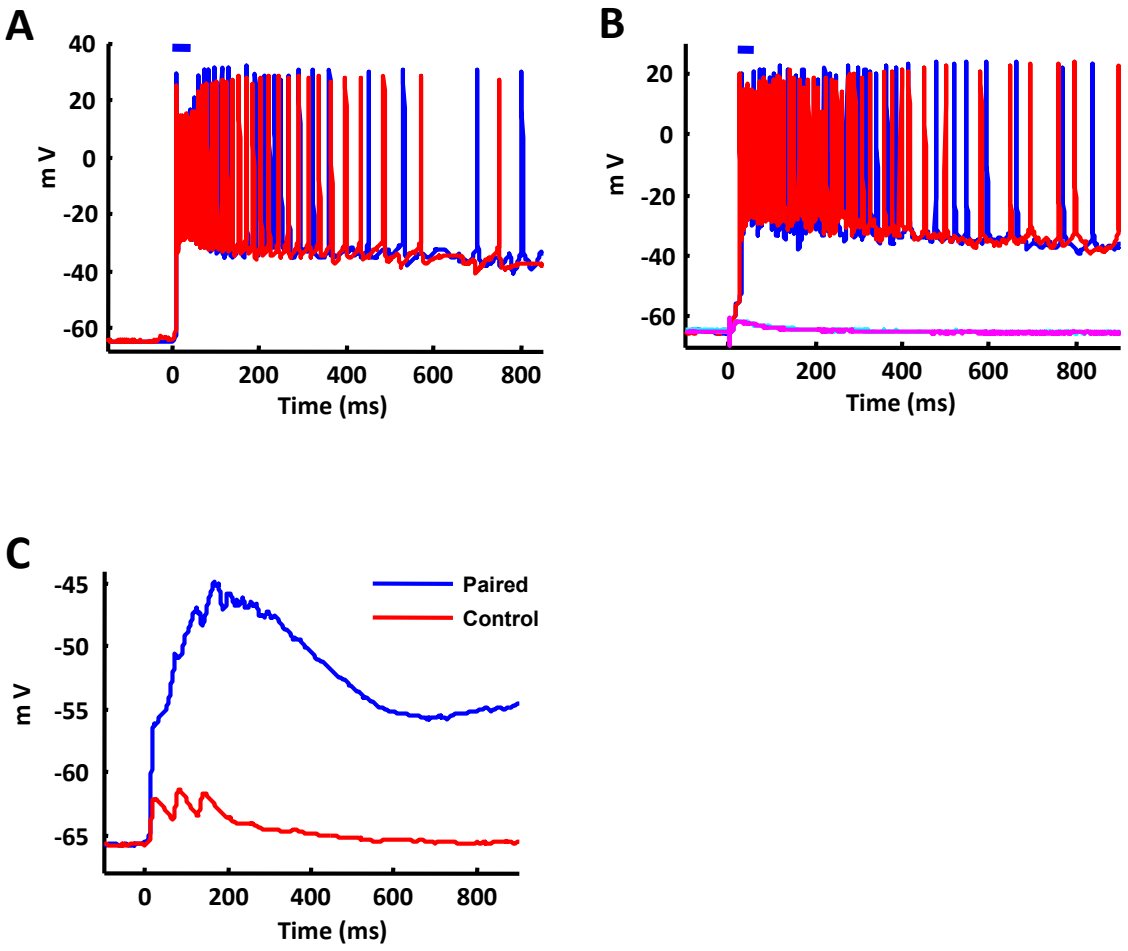
Supplementary Figure S4.1. Bicuculline induces large increase in evoked EPSP response, indicating that inhibition level is high in the slice. (A) Voltage gram of traces, with Baseline, washing on bicucullin (500 µM) and washing off conditions as indicated. Colored asterisks indicate location of sample traces. **(B)** Sample traces of each condition.



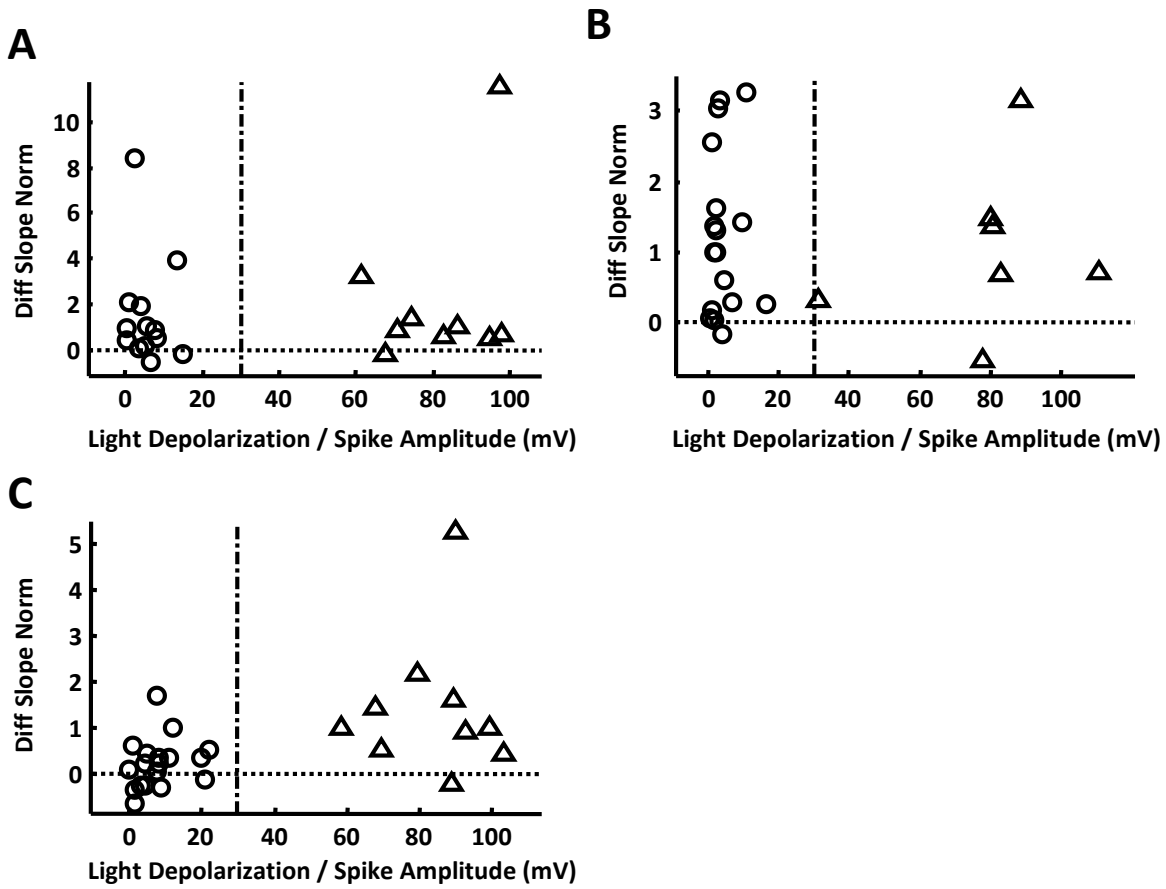
Supplementary Figure S4.2. Pairing alone is not sufficient to induce LTP. (A) Pairing protocol: 4 pulses at 50 Hz were delivered through the paired pathway, followed by 100 ms current injection through loose cell attached electrode to elicit 5-12 extracellular spikes (delay=10 ms). Unpaired pathway was activated 1 s later. Pairing was repeated every 2 s for 2 min. After 15 min waiting, cell was broken into and EPSPs were record in whole-cell mode. **(B)** Averaged traces for the paired pathway and control pathway (all cells grouped, n=34). **(C)** EPSP amplitude is not significantly different between pathways ($F_{1,66}$=0.44, p=0.510). **(D)** EPSP slope is not significantly different between pathways ($F_{1,66}$=0.08, p=0.777).



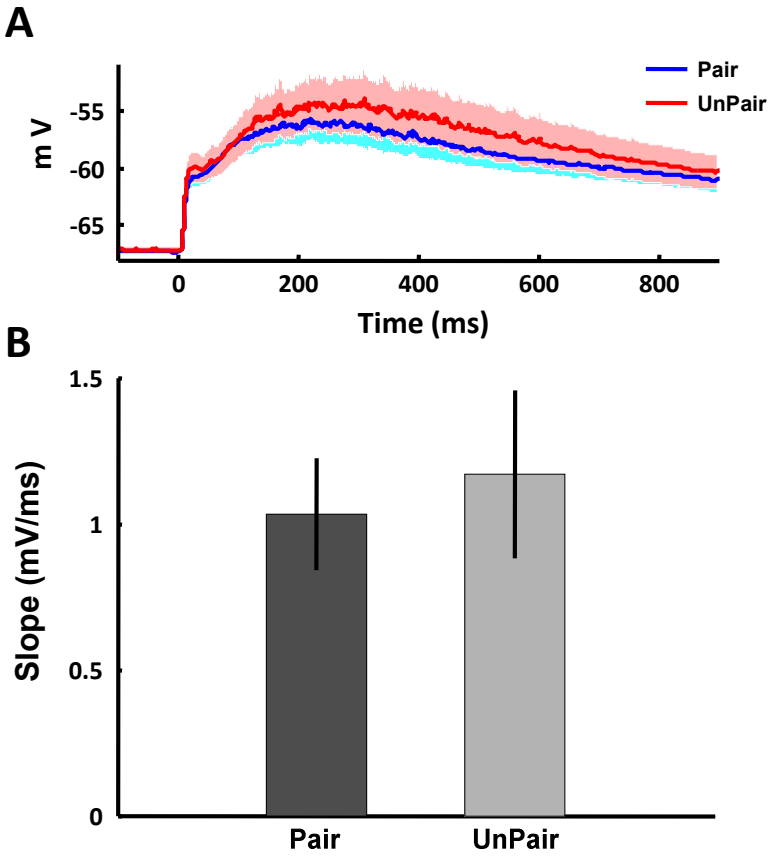
Supplementary Figure S4.3. Chronic stimulation of ChR2 in the incubator induces homeostatic plasticity in organotypic slices. Stimulation protocol: 200 ms of blue light every 1 min, for 2 days. **(A)** Sample trace of spontaneous activity, which is defined as 5 mV above baseline (black bars). **(B)** In light-stimulated group, both total time above threshold (left) and standard deviation of the trace (right) are decreased (n=24, 29). **(C)** A single pulse is delivered through implanted electrode. The areas under the evoked response to stimulation at 3 intensities (30, 40, 60 µA) were significantly lower in the stimulated group (p=0.007, ANOVA. n=24, 29). Similar results if using red light-stimulated slices as control (data not shown).



Supplementary Figure S4.4. Increased Light-Triggered Firing at Higher Temperatures. **(A)** Two sample traces of one cell's response to 40 ms blue light, recorded at elevated temperature (32℃). Light evoked sustained activity, which may reflect what happened in the incubator. **(B)** Sample traces of Training response, at elevated temperature. Two sample trials of light pairing (blue, red) elicits sustained activity while the control pathway does not (cyan, magenta). **(C)** Response to the paired pathway (blue) is significant larger than control pathway (red). Same cell as **(A,B)**.



Supplementary Figure S4.5. Relationship between LTP induction index and light-evoked depolarization. LTP index ("DiffSlopeNorm") is calculated as the difference between EPSP slopes of the two pathways normalized to the EPSP slope of the control pathway: (EPSP $slope_{paired}$ - EPSP $slope_{unpaired}$)/EPSP $slope_{unpaired}$. **(A)** Incubator 2.5 h LTP experiments. Neurons have a light-evoked depolarization larger than 30 mV are categorized as ChR+ cells. There is no significant correlation for neither ChR+ (triangle, R^2=0.102, p=0.402) nor ChR- (circle, R^2=0.015, p=0.688) cells. **(B)** Incubator 2.5 h experiments, blind. There is no significant correlation for neither ChR+ (triangle, R^2=0.081, p=0.537) nor ChR- (circle, R^2=0.0006, p=0.926) cells. **(C)** Incubator 15 min experiments. There is no significant correlation for neither ChR+ (triangle, R^2=0.0007, p=0.941) nor ChR- (circle, R^2=0.052, p=0.362) cells.



Supplementary Figure S4.6. LTP induced in ChR+ cells after 15 min training in the incubator is NMDA receptor dependent. The same LTP experiment was repeated as in Figure 4.6, while bath applied 100 µM APV during training. LTP induction was abolished in ChR+ neurons. **(A)** Averaged traces for the two pathways in all ChR+ neurons (n=16). SEM shown in shaded area. **(B)** EPSP slope is not significantly different between pathways (paired t-test, t_{15}=0.72, p=0.485).

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Chapter 5. Metaplasticity of Short-term Synaptic Plasticity

Introduction

Short-term synaptic plasticity (STP) refers to use-dependent changes in synaptic strength over the time scale of tens to hundreds of milliseconds (Gingrich and Byrne, 1985; Zucker, 1989; von Gersdorff and Borst, 2002; Zucker and Regehr, 2002; Abbott and Regehr, 2004). When a train of action potentials within this interval range arrives at the presynaptic terminal, postsynaptic EPSPs can display facilitation or depression (Markram et al., 1998; Reyes et al., 1998; Reyes and Sakmann, 1999; Rozov et al., 2001; Oswald and Reyes, 2008). Since Eccles and colleagues first described this form of plasticity at the neuromuscular junction over 70 years ago (Eccles JC, 1941), hundreds of studies have revealed that STP is a virtually universal form of synaptic plasticity. The mechanisms underlying short-term synaptic plasticity are not yet fully understood, but it is generally agreed that it involves a complex balance and regulation of the depletion, turnover, release, and mobilization of presynaptic vesicles (Gingrich and Byrne, 1985; Zucker, 1989; Varela et al., 1997; Markram et al., 1998; von Gersdorff and Borst, 2002; Zucker and Regehr, 2002; Abbott and Regehr, 2004; Xu and Wu, 2005; Xu et al., 2007).

Although STP has been widely observed at almost all neocortical synapses, its functional role in cortical computation remains unknown. On theoretical grounds it has been proposed that STP contributes to gain control (Chance et al., 1998; Galarreta and Hestrin, 1998; Abbott and Regehr, 2004; Rothman et al., 2009), working memory (Maass and Markram, 2002; Mongillo et al., 2008; Barak et al., 2010; Deco et al., 2010; Deng and Klyachko, 2011), and network stability (Galarreta and Hestrin, 1998; Sussillo et al., 2007). Importantly, STP is also hypothesized to contribute to temporal processing (Buonomano and Merzenich, 1995; Buonomano, 2000; Fortune and Rose, 2001). It is reasonable to postulate that the functional role of STP is likely to be in part determined by whether or not STP is itself plastic. If STP is a

“hardwired” property of synapses, it may only have limited computational functions. If, however, STP itself undergoes significant plasticity then it may take a more active role in performing computations.

Short-term plasticity is sometimes viewed as being determined by baseline synaptic strength. For example, strong synapses are often reported to be more likely to have paired-pulse depression (PPD), while weak synapses are more likely to display paired-pulse facilitation (PPF) (Katz and Miledi, 1968; Thomson et al., 1993; Debanne et al., 1996; Dobrunz and Stevens, 1997; Atzori et al., 2001; Zucker and Regehr, 2002; Boudkkazi et al., 2007). Additionally, it is often the case that the induction of LTP in neocortical synapses increases short-term depression, while induction of LTD favors short-term facilitation (Markram and Tsodyks, 1996; Buonomano, 1999; Bender et al., 2006). However, there is also significant evidence that the magnitude and direction of STP is not solely a function of baseline synaptic strength (Huang et al., 1994; Thomson and Bannister, 1999; Brody and Yue, 2000; Hanse and Gustafsson, 2001; Chen et al., 2004; Fuhrmann et al., 2004; Oswald and Reyes, 2008; Boudkkazi et al., 2011). For example, experimental manipulations can change paired-pulse ratio independent of baseline synaptic strength (Waldeck et al., 2000; Sippy et al., 2003), suggesting that STP may itself be plastic.

One question that has not been addressed, is whether STP can be "learned"—that is, is there specific training parameters that can "teach" a synapse to adopt different forms of STP? In other words, is STP regulated by specific learning rules to optimize the computations performed at synapses? Our lab previously proposed a form of metaplasticity of STP, termed temporal synaptic plasticity, which operates independently and in parallel of the conventional associative learning rules governing baseline synaptic strength (Carvalho and Buonomano, 2011). The

proposal is that there are mechanisms in place to keep track of the activity history of both pre- and postsynaptic side, so that STP profile can be adjusted according to the associative activity across the synaptic terminal. For example, given a train of presynaptic spikes, synapses may come to express short-term depression or facilitation depending on whether the postsynaptic cell consistently fires early or late in the train, respectively. This work further demonstrated, using computer simulations, that with such a learning rule in place the computational power of neural network was enhanced (Carvalho and Buonomano, 2011).

In order to explicitly test the metaplasticity of STP hypothesis, my goal was to induce LTP by using protocols that paired the early or late presynaptic action potentials of a train with postsynaptic depolarization. For example as schematize in **Figure 5.1**, a train of three pulses is delivered to two pathways in alternation. In the “Early-pairing Pathway” we pair the first pulse with postsynaptic depolarization, while in the “Late-pairing Pathway” the last pulse of the train of three is paired with postsynaptic depolarization. This pairing protocol should induce LTP in both pathways. The prediction is that the "Early-pairing" pathway will be more likely to display paired-pulse depression as pairing comes early in the train, while "Late-pairing" pathway will have more facilitation because pairing comes late. This prediction makes sense computationally, and it serves as an extension of Hebb’s hypothesis because it predicts that a synapse should be at its maximal strength *when* the postsynaptic neuron fires.

Previous experiments that have indirectly examined the notion of metaplasticity of STP did not observe metaplasticity of STP (Buonomano et al., 1997). Because of this, and previous experimental evidence indicating that STP can change over the course of hours after the induction of LTP (Huang et al., 1994), we hypothesized that metaplasticity of STP might be a slowly developing process that depends on protein synthesis. For this reason the current

experiments focus on long induction protocols and rely on cortical organotypic slices to induce the metaplasticity of STP.

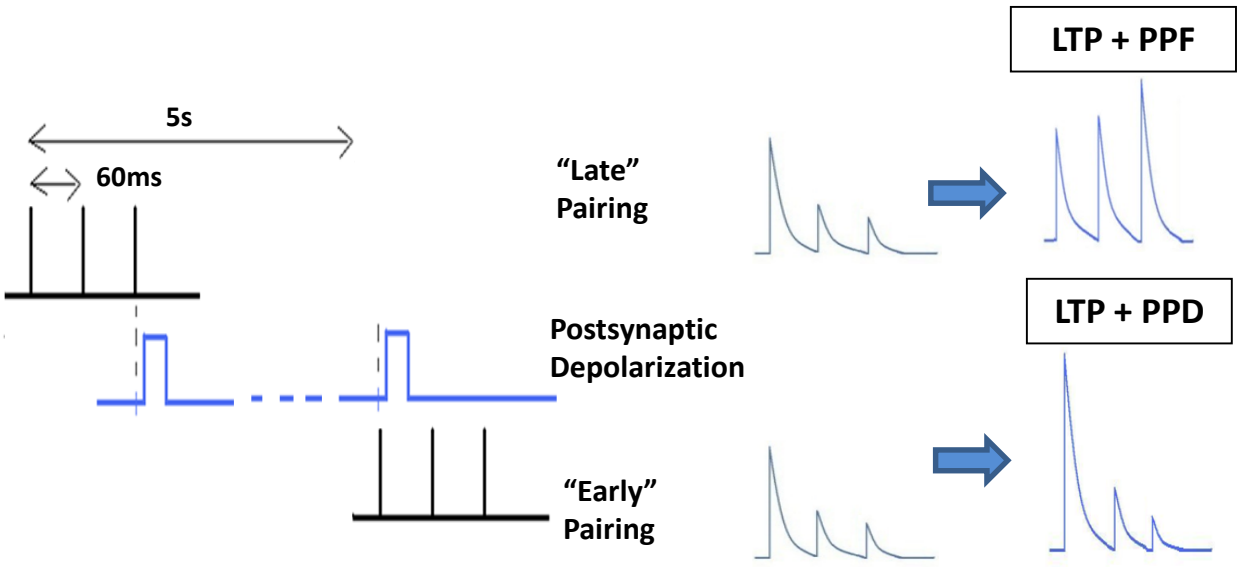


Figure 5.1. Hypothesis: Metaplasticity of STP can be induced with different associative protocols. Proposed pairing protocol: pair postsynaptic depolarization with the first pulse of "Early-pairing" pathway and the last pulse of "Late-pairing" pathway. We proposed that "Late-pairing" will result in LTP induction in addition to PPF, while "Early-pairing" will result in LTP and PPD.

Results

Induction of the Metaplasticity of STP after long-term training

Stimulation in cortical slices regularly triggers polysynaptic activity, network activity expressed as compound PSPs are even more pronounced when using paired-pulse stimulation. The presence of polysynaptic activity can impair the ability to quantify STP of the monosynaptic EPSPs. However, because we were initially interested in determining if there are any observable differences between early and late pairing protocols we performed the initial experiments under intact pharmacology. In later experiments (see below) we isolated monosynaptic EPSP by blocking most polysynaptic activity with bath applied muscimol.

Based on the LTP induction experiments in **Chapter 4**, a similar training protocol was used to induce the metaplasticity of STP: while slices were in the incubator a train of 3 pulses separated by 60 ms was delivered to either pathway (separated by 5 s). A 40 ms blue light was paired with either the first pulse of pathway one ("Early-pairing") or the last pulse of pathway two ("Late-pairing") with a delay of 20 ms (**Figure 5.2A**). The whole pattern was repeated every 10 s. Each “session” lasted 30 min, and there were a total of 3 sessions (total training time of 2.5 hr with 30 min ON/OFF spaced training sessions). After 2.5 hr of training in the incubator, the slice was moved to the electrophysiology rig (the pathways were “blinded”). Three pulses separated by 60 ms interval were delivered through either pathways, and recordings were done in ChR2 positive (ChR+) cells (**Figure 5.2B, C**) (I also recorded from ChR- neurons, see **Supplementary Figure S5.1**). Based on my previous experiments 4 μM of CNQX was bath applied during both training and testing in order to decrease inhibition and enable the induction of LTP (see **Chapter 4**). A cell was considered to be ChR2+ cells when light-evoked depolarization induced at least

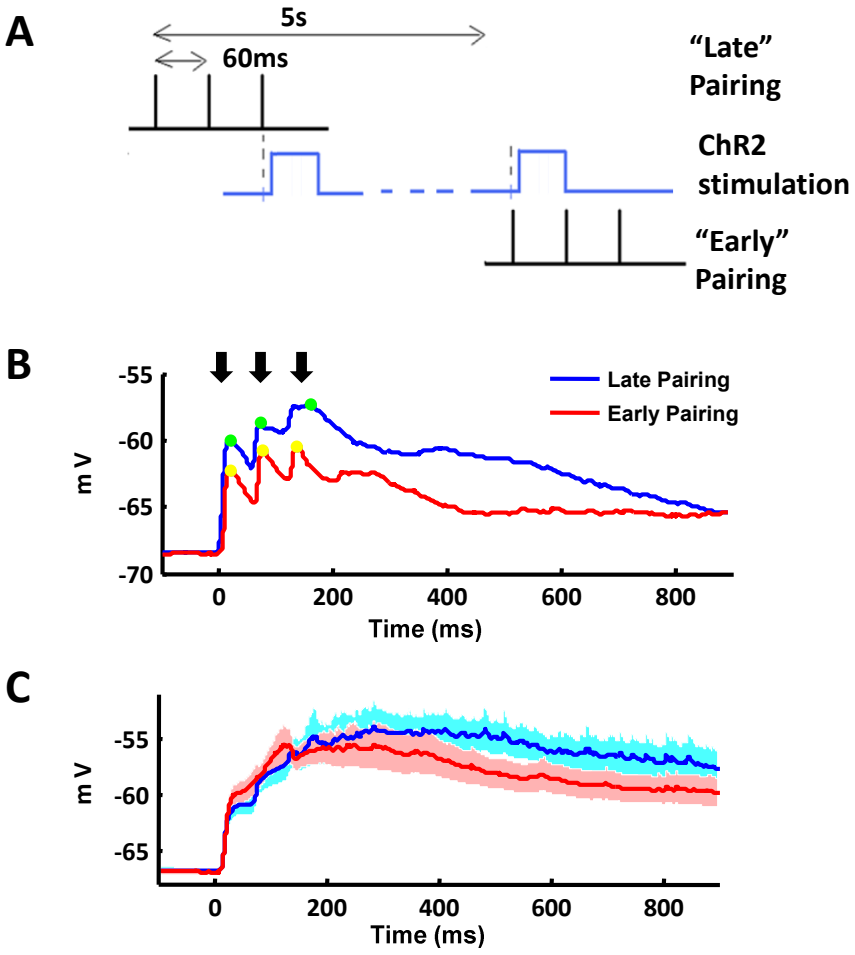


Figure 5.2. Metaplasticity of STP, blind experiment. (A) Pairing protocol: pair 40 ms blue light with the first pulse of "Early-pairing" pathway and the last pulse of "Late-pairing" pathway. The two pathways were blinded. Inter-pulse interval=60 ms, pathways separated by 5 s. The whole pattern was repeated every 10 s, lasted 2.5 hr in the incubator, with 30 min ON/OFF spaced training sessions. 4 μM CNQX was bath applied throughout experiment. **(B)** Sample traces of testing in a ChR+ cell. Green and yellow dots indicate the "peak PSP" measured for the three segments (each 60 ms in length), substituting for the real EPSP amplitudes. Arrows indicate timing of pulses. **(C)** Averaged traces for all ChR+ cells (n=15). SEM shown in shaded area.

one spike (as in **Chapter 4**). Electrical pulses elicited significant polysynaptic activity (see also **Figure 4.2C, D**), therefore it is difficult to discriminate the three EPSPs. We quantified the peak response by measuring the maximal voltage during the 3 different windows corresponding to each pulse (each 60 ms in length)—thus providing a net measure of depolarization produced by an overlap of both monosynaptic and polysynaptic network mechanisms (**Figure 5.2B, C**).

After 2.5 hours of training in the incubator, the "peak PSPs" in ChR+ neurons for the Late-pairing pathway was 5.95 ± 0.93, 9.00 ± 1.07, 10.66 ± 0.95 mV for the three segments, respectively (n=15). In the Early-pairing pathway the "peak PSPs" were 7.47 ± 0.85, 10.28 ± 0.94, 11.40 ± 1.00 mV, respectively. A two-way analysis of variance with repeated measures on both pathways (Late, Early) and pulse number (1 to 3) showed neither a significant main effect of pathway ($F_{1,14}$=1.31, p=0.272) nor a significant interaction between Pathway (Late, Early) ×Pulse number ($F_{2,28}$=0.70, p=0.507). (**Figure 5.3A**). When the PSP peaks were normalized to the first one, the normalized PSP peaks in the Late-pairing pathway were 170.40 ± 15.01%, 223.44 ± 27.25% in the 2nd and 3rd segments, respectively. Normalized PSP peaks in the Early-pairing pathways were 147.20 ± 9.07%, 166.97 ± 13.29% in the 2nd and 3rd segments, respectively. A two-way analysis of variance with repeated measures on both pathways (Late, Early) and pulse number (2^{nd} and 3^{rd}) showed a significant main effect of pathway ($F_{1,14}$=8.00, p=0.013) but not significant interaction between Pathway×Pulse number ($F_{1,14}$=3.35, p=0.088). (**Figure 5.3B**). EPSP slope measured at the first pulse was not significantly different between the two pathways for ChR+ cells (0.65 ± 0.20 $mV \cdot ms^{-1}$ and 0.74 ± 0.11 $mV \cdot ms^{-1}$ in the Late and Early pairing pathway, respectively. Paired t-test, t_{14}=0.62, p=0.547) (**Figure 5.3C**). Results for ChR- cells can be found in **Supplementary Figure S5.1**.

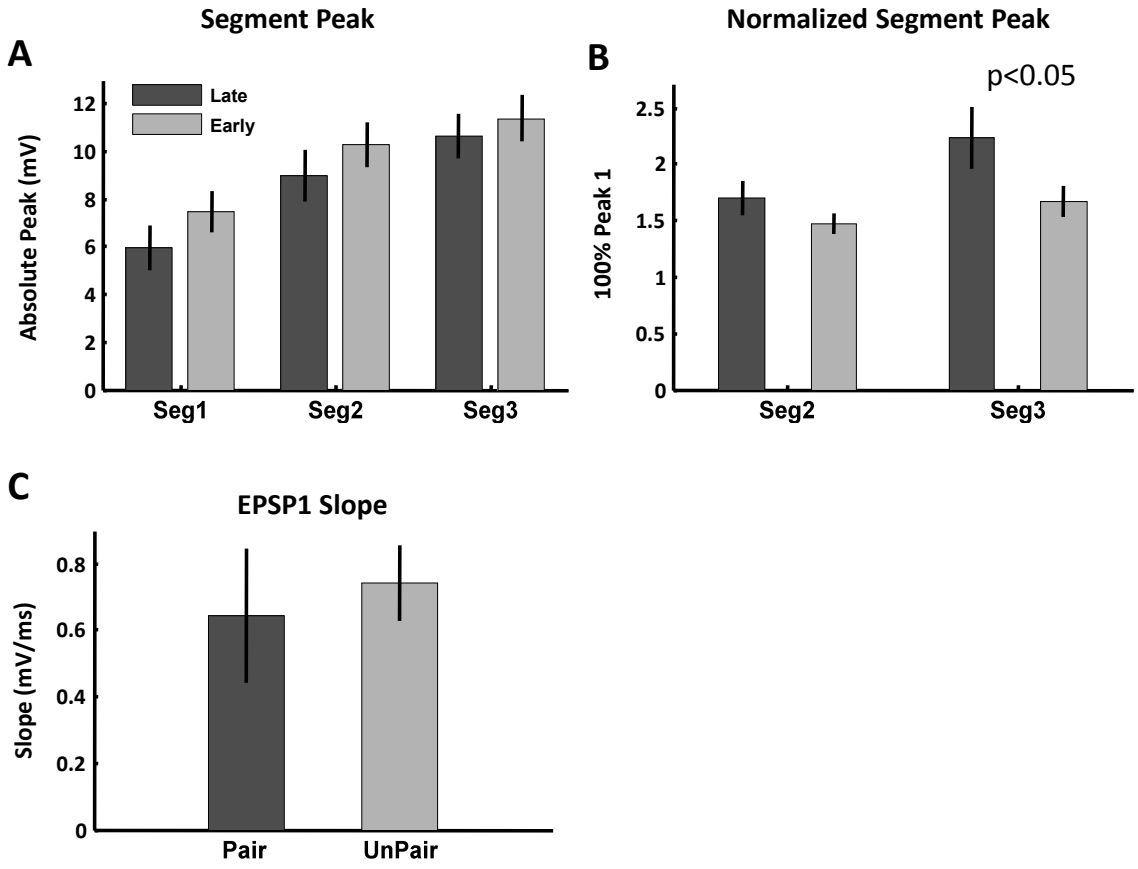


Figure 5.3. Metaplasticity of STP, blind experiments. (A) Absolute PSP peak value for each segment in ChR+ cells. There was neither a significant main effect of pathway, nor significant interaction between pathway and pulse number (n=15). **(B)** Normalized peak value for ChR+ cells. Two-way ANOVA showed a significant main effect of pathway ($F_{1,14}$=8.00, p=0.013) but not significant interaction between pathway and pulse number ($F_{1,14}$=3.35, p=0.088). **(C)** EPSP1 slope in ChR+ neurons showed no significant difference between the two pathways (paired t-test, t_{14}=0.62, p=0.547).

Therefore, the initial experiments with intact pharmacology suggested that the meta-STP protocol induced significant difference in the change of the temporal profile of the PSPs in the Late and Early pairing pathways, while the absolute peak amplitude was not significantly different in the two pathways. In other words, different LTP pairing protocols in the two pathways lead to the induction of metaplasticity of STP as we expected. However, the presence of polysynaptic activity impaired our ability to quantify STP of the monosynaptic EPSPs. Therefore, we next repeated the same experiments but tested for EPSPs in bath applied muscimol, a $GABA_A$ agonist after training in the incubator.

Directly Testing Metaplasticity of STP after Early and Late Pairing

The above initial experiments under intact pharmacology was aimed to determine if there are any observable differences between early and late pairing protocols. In order to isolate monosynaptic EPSP the same experiments were repeated but tested while blocking most polysynaptic activity with bath applied $GABA_A$ agonist muscimol (Martin and Ghez, 1999; van Duuren et al., 2007; Baptiste et al., 2010; Ludvig et al., 2011). As expected, there was a significant decrease in input resistance of individual cells (combining all neurons (ChR+ and ChR-) in the meta-STP experiments with or without muscimol, unpaired t-test $t_{134}=9.57$, $p<10^{-4}$, n=70 (no drug), 66 (muscimol) respectively) (**Supplementary Figure S5.2**), which leaded to less neuronal firing and enabled the measurement of "clean" EPSPs during testing (**Figure 5.4A, B**).

In the meta-STP muscimol experiments, the same training protocol was used to induce metaplasticity of STP as before (**Figure 5.2A**, but at higher stimulation intensity. See **Methods**).

After training 2.5 hr in the incubator, the slice was moved to the electrophysiology rig. ChR+ cells were patched, and EPSPs from both pathways were tested in the presence of 0.5-0.6 μM muscimol in addition to 4μM CNQX.

After 2.5 hours of training in the incubator, the slope in ChR+ neurons for the Late-pairing pathway were 1.34 ± 0.21, 1.11 ± 0.16, 0.93 ± 0.15 $mV \cdot ms^{-1}$ for the three EPSPs, respectively (n=25). In the Early-pairing pathway the EPSP slopes were 1.68 ± 0.20, 1.22 ± 0.17, 1.03 ± 0.16 $mV \cdot ms^{-1}$, respectively. A two-way analysis of variance with repeated measures on both pathways (Late, Early) and pulse number (1 to 3) showed neither a significant main effect of pathway ($F_{1,24}$=1.17, p=0.291) nor a significant interaction between Pathway (Late, Early) ×Pulse number ($F_{2,48}$=2.71, p=0.076). (**Figure 5.4C**). When the EPSP slopes were normalized to the first to obtain paired-pulse ratio (PPR), the normalized slopes in the Late-pairing pathway were 95.71 ± 10.49%, 84.65 ± 12.87% in the 2nd and 3rd segment, respectively. Normalized EPSP slopes in the Early-pairing pathway were 84.00 ± 9.52%, 71.64 ± 9.77% in the 2nd and 3rd segment, respectively. A two-way analysis of variance with repeated measures on both pathways (Late, Early) and pulse number (2 to 3) showed a significant main effect of pathway ($F_{1,24}$=5.81, p=0.024) but non-significant interaction between Pathway ×Pulse number ($F_{1,24}$=0.23, p=0.635). (**Figure 5.4D**). Paired t-test showed that normalized slope at both the second and third EPSPs were significantly different between the two pathways for ChR+ cells (t_{24}=2.47, p=0.021; and t_{24}=2.24, p=0.035 for the two EPSPs, respectively) (**Figure 5.4D**). These experiments were not designed to contrast effects between ChR+ and ChR- cells, however data was also collected in ChR- and is shown in **Supplementary Fig S5.3**.

The above cells were from experiments in which the EPSPs were not “contaminated” by the presence of polysynaptic activity, and thus enabled “clean” measurement of the slopes of all

three EPSPs of a train. Based on the 25 neurons (out of 29) that were included in this analysis there was not a significant difference between the absolute EPSP slopes from the two pathways using two way ANOVA analysis. There was however, a trend in the interaction between pathway$\times$Pulse number ($p=0.076$) and in the difference between the first EPSPs in the two pathways ($p=0.091$, paired t-test). This difference was significant when all 29 cells were included in the analysis (paired t-test, $t_{28}=2.42$, $p=0.022$)—note that the slope of the initial EPSP should not be "contaminated" by polysynaptic activity. We postulated that this slight difference in the 1st EPSP slope might be accountable for the difference in the STP ratio in the 2nd and 3rd EPSPs (see **Discussion** below).

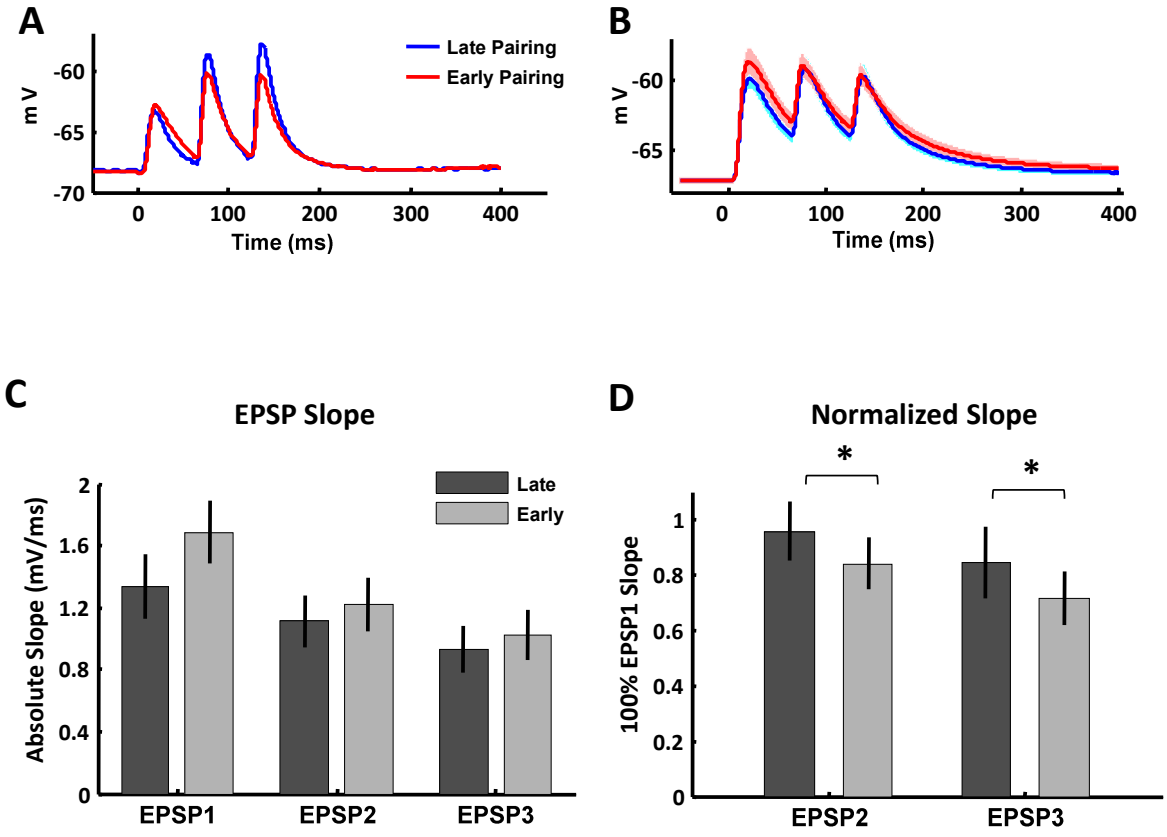


Figure 5.4. Metaplasticity of STP, testing in muscimol. Training protocol the same as Figure 5.2A, but at higher stimulation intensity. After 2.5 hr training in the incubator, slices were tested for EPSP response on the rig while bath applying 0.5-0.6 µM muscimol and 0.4 µM CNQX. **(A)** Sample testing traces in a single ChR+ cell. **(B)** Averaged traces for all ChR+ cells (n=25). SEM shown in shaded area. **(C)** Absolute slope value for three EPSPs in ChR+ cells. There was neither a significant main effect of pathway, nor significant interaction between pathway and pulse number. **(D)** Normalized slope value for ChR+ cells. Two-way ANOVA showed a significant main effect of pathway ($F_{1,24}$=5.81, p=0.024) and non-significant interaction between pathway and pulse number ($F_{1,24}$=0.23, p=0.635). Paired t-test showed significance in both EPSPs (p=0.021 and 0.035, respectively). (*: p<0.05)

Discussion

The above results suggest that when Early and Late pairing protocols are applied to two pathways onto the same neuron, moderate difference in the temporal profile of the evoked EPSPs are observed. Specifically, in ChR+ neurons, Early pairing produced relatively less depolarization later in the train, while Late pairing produced relatively more depolarization. In the isolated EPSP experiments this was reflected as increased PPD in the Early-pairing pathway, and decreased PPD in the Late-pairing pathway. While these results are consistent with the overall prediction of the meta-STP hypothesis, these results could be explained by differences in the magnitude of LTP induced with the different protocols—as discussed below.

Different potency of LTP induction in the two pathways

Here to induce the metaplasticity of STP, postsynaptic depolarization was paired at different timing with presynaptic train of action potentials (**Figure 5.1**). Note that in **Chapter 4**, I was able to show the induction of associative LTP after reducing inhibition with low dose of CNQX. In other words, here in the metaplasticity of STP experiments, a different LTP pairing protocol was delivered through either Late or Early pairing pathways. Thus one important question relates to whether one protocol is more effective than the other in inducing LTP.

There are arguments that would suggest that Early-pairing might induce more LTP than Late-pairing, as well as for the notion that Late-pairing might induced more LTP than Early-pairing. Several experimental studies have shown that synaptic plasticity may depend on properties other than the timing of the spike pairs, for example high frequency bursts of pre- and

postsynaptic spikes lead to LTP, regardless of the relative spike timing (Markram et al., 1997; Boettiger and Doupe, 2001; Sjostrom et al., 2001; Froemke and Dan, 2002; Tzounopoulos et al., 2004; Wang et al., 2005). One study specifically examined the effect of individual spikes within each burst by systematically varying the frequency and number of spikes in both the pre- and postsynaptic burst (Froemke et al., 2006). They found that pairing with the first spike in the presynaptic burst has the largest efficacy in LTP induction, compared to pairing with the successive spikes. A possible mechanism might be that the later spikes activate the postsynaptic NMDA receptors to a lesser extent as a result of presynaptic short-term depression (Froemke et al., 2006), therefore is less effective in inducing synaptic modification. According to this notion, Early-pairing might be more effective than Late-pairing in inducing LTP.

On the other hand, it is also possible that Early-pairing might induce less LTP. Studies on the effect of bursting activity in synaptic modification are often based on the simplified assumption that all pre/post spike pairs contribute equally and independently to synaptic modification, and the effect of each pair depends only on its pre-post interspike interval following the spike-timing dependent rule (STDP) (see also **Figure 1.1**) (Kempter R, 1999; Roberts, 1999; Song et al., 2000; van Rossum et al., 2000). According to this notion, in the meta-STP experiments Early-pairing could result in less or no LTP induction compared to pairing with a single presynaptic pulse, because the later two presynaptic spikes in the Early-pairing protocol would lead to LTD according to the standard STDP rule (post-pre pairing) (**Figure 5.1**).

Our observations are consistent with the notion that pairing in the Early-pairing pathway is more effective in LTP induction compared to the Late-pairing pathway. In the first set of meta STP experiment without muscimol, the 1st EPSP slope of ChR+ neurons in the Early-pairing pathway is slightly higher than the Late-pairing pathway although not significantly different

(**Figure 5.3C**). Additionally in the meta STP experiments testing in muscimol, pooled data from all ChR+ neurons showed a significantly higher 1st EPSP slope in Early-pairing pathway compared to Late-pairing pathway (p=0.022, see **Results**). In ChR+ neurons that are measurable for all three EPSP slopes (25 out of 29), paired t-test showed a trend of higher slope in the 1st EPSP of the early pathway (t_{24}=1.76, p=0.091) although two way ANOVA revealed no significant difference between the three EPSPs from two pathways (**Figure 5.4C**). In other words, our results support the notion that the first spike in presynaptic train is more effective in LTP induction (e.g. early pairing) compared to the later spike (e.g. late pairing).

The effect of metaplasticity of STP might be accountable for by a slight difference in 1st EPSP strength

Historically STP has often been viewed as an epiphenomenon of baseline synaptic strength. For example, strong synapses are often reported to be more likely to have paired-pulse depression (PPD), while weak synapses are more likely to display paired-pulse facilitation (PPF) (Katz and Miledi, 1968; Thomson et al., 1993; Debanne et al., 1996; Dobrunz and Stevens, 1997; Atzori et al., 2001; Zucker and Regehr, 2002; Boudkkazi et al., 2007). Additionally, it is often the case that the induction of LTP increases short-term depression, while induction of LTD favors short-term facilitation (Markram and Tsodyks, 1996; Buonomano, 1999; Bender et al., 2006). However, there is also evidence suggesting a decoupling between unitary EPSP strength and STP. For example, investigators have failed to find a correlation between EPSP strength and paired-pulse ratio in different preparations (Thomson and Bannister, 1999; Brody and Yue, 2000; Hanse and Gustafsson, 2001; Chen et al., 2004; Fuhrmann et al., 2004; Oswald and Reyes, 2008; Boudkkazi et al., 2011).

Because of the method we used for eliciting EPSPs (e.g. extracellular stimulation) in the meta-STP experiments, we cannot explicitly examine the issue of whether or not there is an inverse correlation between the strength of the 1st EPSP and STP. For example, in our experiments baseline EPSPs might be attributed by a small number of strong presynaptic fibers (with high probability of release), which will lead to more short-term depression; or equally the EPSPs might be the result of activating a large number of weak fibers (with low probability of release), which could lead to more facilitation. Our design of current experiment is not able to distinguish between these two possibilities. Interestingly, however, even with paired recordings between individual cells, the inverse correlation between 1st EPSP slope and paired-pulse ratio was found to be only hold true in week 2 organotypic slices but not in week 4 slices (see **Chapter 2**, **Figure 1E**), strongly suggesting that there are many interrelated factors contributing to STP.

As discussed above, it is within our expectation that 1st EPSP strength is slightly larger in the Early-pairing pathway than the Late-pairing pathway. Although when all the ChR+ and ChR- cells in both meta-STP experiments (with or without muscimol) were grouped together, there was not a significant difference in 1st EPSP slopes between the Early and Late pairing pathways (1.24 ± 0.10 $mV \cdot ms^{-1}$ and 1.39 ± 0.09 $mV \cdot ms^{-1}$ in the Late and Early pairing pathway, respectively. Paired t-test, t_{135}=1.45, p=0.150, n=136) (**Supplementary Figure S5.3B**). In the presence of muscimol there was a significant difference in the amount of LTP induced between the Early and Late pairing pathways in all ChR+ neurons grouped (paired t-test, p=0.022, n=29). Taken together, these results suggest that the difference in STP ratio from the two pathways in ChR+ neurons of the muscimol experiments (**Figure 5.4D**) might be accountable for by the small difference in 1st EPSP slope, i.e. a small number of strong presynaptic fibers with high

probability of release might be activated in the Early-pairing pathway which leads to more short-term depression that was observed.

Possible mechanisms of metaplasticity of STP

The mechanisms underlying STP have not been fully unraveled yet, but it is generally agreed that STP is primarily a presynaptic mechanism (Gingrich and Byrne, 1985; Zucker, 1989; Varela et al., 1997; Markram et al., 1998; von Gersdorff and Borst, 2002; Zucker and Regehr, 2002; Abbott and Regehr, 2004). Generally, short-term depression is viewed as rising from the depletion of the readily releasable pool of synaptic vesicles (Rosenmund and Stevens, 1996; Schneggenburger et al., 2002; Zucker and Regehr, 2002), while short-term facilitation is believed to be associated with the accumulation of residual calcium in the presynaptic terminal (Katz and Miledi, 1968; Zucker and Regehr, 2002; Burnashev and Rozov, 2005). Additionally, in some systems depression and facilitation may be caused by common mechanisms involving Ca^{2+}-dependent regulation of Ca^{2+} sensor proteins which regulate the presynaptic calcium channels responsible for triggering transmitter release (Forsythe et al., 1998; von Gersdorff and Borst, 2002; Xu and Wu, 2005; Sullivan, 2007; Xu et al., 2007; Mochida et al., 2008).

Here the idea of metaplasticity of STP is tested in cortical organotypic slices. It is proposed that the relative timing of pre- and postsynaptic spikes not only modulate long-term plasticity as predicted by associative learning rules such as STDP (Debanne et al., 1994; Markram et al., 1997; Bi and Poo, 1998), but also serve as a "teacher" signal to determine whether the synapse should undergo depression or facilitation. Therefore metaplasticity of STP would require mechanisms in place that regulate STP independently of baseline synaptic

transmission, therefore it would likely rely on regulations of the change of presynaptic Ca^{2+} while keeping track of the activity history of both pre- and postsynaptic terminals.

Since short-term plasticity is primarily governed by presynaptic mechanisms, we propose that the mechanisms underlying metaplasticity of STP would be presynaptic in nature. Specifically, a retrograde signal triggered by a postsynaptic spike could interact with presynaptic Ca^{2+} levels (determined by the number of presynaptic action potentials): low levels of Ca^{2+} at the time of the retrograde messenger would shift STP towards depression, while high levels of Ca^{2+} would favor facilitation. Possible candidate molecules responsible for this retrograde communication between pre- and postsynaptic terminals include endocannabinoids and nitric oxide (Sjostrom et al., 2003, 2004; Bender et al., 2006; Sjostrom et al., 2007). Interestingly, one study found that in the rat visual cortex, purely postsynapticly induced LTP activated the retrograde system and altered the presynaptic release indices, in a fashion depending on the initial properties of presynaptic input (Volgushev et al., 2000).

In parallel with these interactions happening at the presynaptic terminals which might be responsible for the changes in STP, one key component of the metaplasticity of STP relies on that synapses still must keep track of the order of pre- and postsynaptic spikes, as required by STDP. It has been proposed that order sensitivity of STDP might rely on coincidence detectors sitting on the postsynaptic terminals (Karmarkar and Buonomano, 2002; Shouval and Kalantzis, 2005; Bender et al., 2006). To the best of our knowledge so far there is no data supporting the interaction between the mechanisms of STDP and presynaptic probability of release. However, given the large number of presynaptic proteins involved in Ca^{2+} regulation (Zucker and Regehr, 2002; Burnashev and Rozov, 2005; Mochida et al., 2008), it seems possible that such mechanisms might be in place, so that a retrograde signals would be generated by the

postsynaptic spike, diffuse to the presynaptic terminal, and interact with the level of presynaptic Ca^{2+}. Indeed, there is evidence that presynaptic mechanisms are in place to regulate short-term plasticity independent of baseline synaptic strength. For example, it is found in hippocampal cell cultures that increasing the calcium binding protein neuronal calcium sensor-1 (NCS-1) can switch paired-pulse depression to facilitation without changing basal synaptic transmission (Sippy et al., 2003). Additionally in gold fish brainstem M-axons it has been shown that each of the two EPSPs of a paired-pulse pattern can be independently regulated without affecting the other (Waldeck et al., 2000). Furthermore, there is evidence that there may be as yet undiscovered mechanisms in place to regulate short-term facilitation, such as recently identified presynaptic NMDA receptors (Larsen et al., 2011). It will not be surprising if future investigations discover that these or other mechanisms regulating STP also interact with the mechanisms responsible for STDP.

Conclusion

Here I set out to test the hypothesis that short-term synaptic plasticity might undergo a form of metaplasticity in which the temporal relationship between presynaptic train of action potentials and postsynaptic depolarization governs magnitude of STP. The use of a novel optogenetic pairing protocol allowed the induction and testing of synaptic plasticity over the course of hours. It is found in cortical organotypic slices that after 2.5 hr of training *in vitro*, pairing postsynaptic depolarization at different timing of presynaptic spike trains induced a differentiation of short-term plasticity that was consistent with the notion of metaplasticity of STP (Carvalho and Buonomano, 2009). However, at this stage we are unable to determine whether or not the differences in STP are primarily an effect of differences in the magnitude of induced LTP or rather of some as yet undescribed synaptic learning rule.

Methods

Organotypic slice preparation (with implanted stimulating electrodes), electrophysiology recordings, and ChR2 expression methods are the same as in **Chapter 4**.

Drug application

CNQX disodium salt hydrate (Sigma, cat. #C239) was first dissolved into Dimethyl sulfoxide (DMSO, Sigma cat. #D2650) to make a stock solution at 2 mM. To use, the stock solution was diluted with ACSF to a final concentration of 4 μM then bath applied to the organotypic slices. The stock solution was made daily to ensure best quality of the drug.

Muscimol (Sigma, cat. #M1523) was first dissolved in dH20 to make a 1mM stock solution. Then it was diluted in ACSF to a final concentration of 0.5 - 0.6 μM upon use.

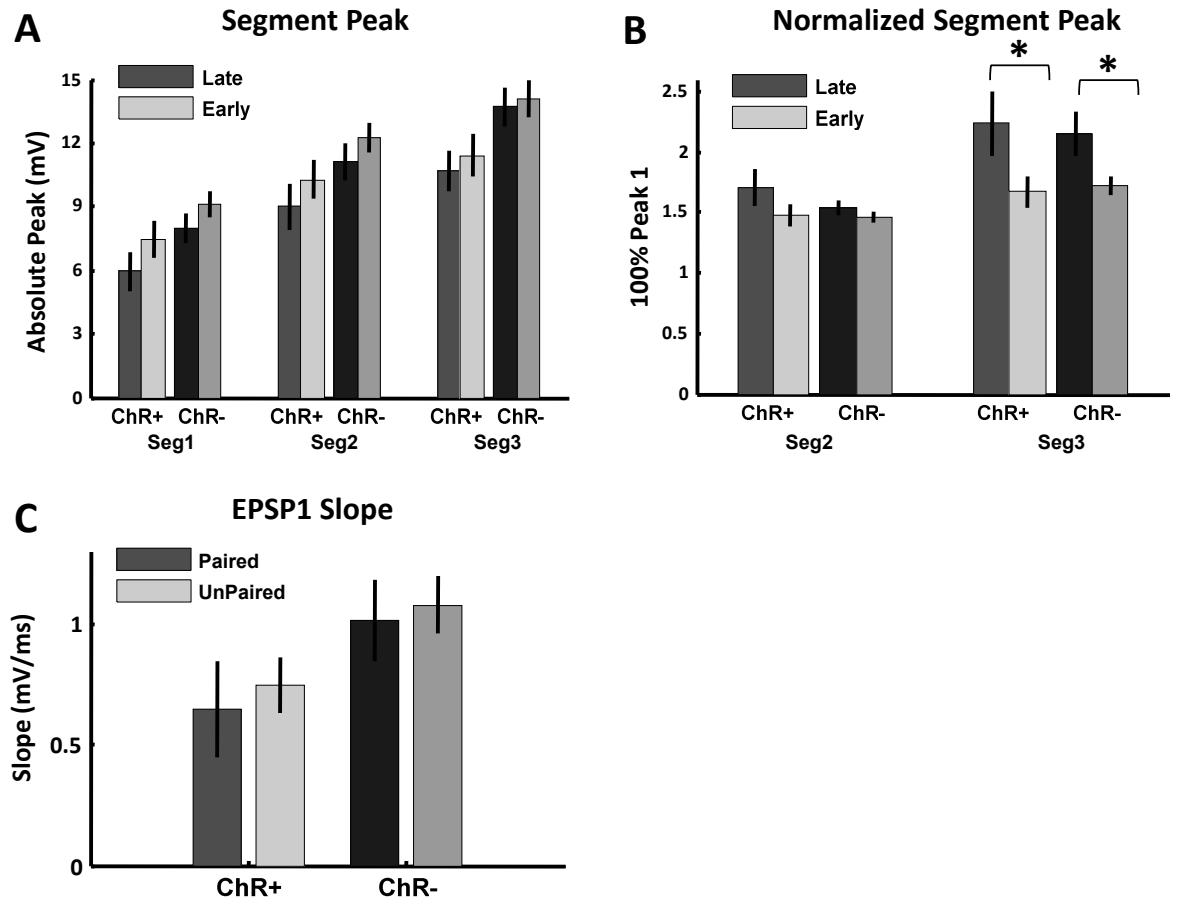
Metaplasticity of STP protocols

Metaplasticity of STP was induced with the same protocol and trained in the incubator. Briefly, a train of 3 pulses with 60 ms interval was delivered to either of the two pathways separated by 5 s alternatively, and 40 ms blue light was paired with either the first pulse of pathway one ("Early-pairing") or the last pulse of pathway two ("Late-pairing") with a delay of 20 ms. Stimulation intensity was at 80 μA for the meta-STP no drug experiments, and 120 μA for the muscimol experiments. We used lower intensity in no drug experiments to avoid excessive network activity after bath applied CNQX. The two pathways were counterbalanced (and also blinded in the meta-STP no drug experiments) -- i.e. half of the slices were trained with the Late-pairing pathway stimulated early, half of the slices with the Early-pairing pathway stimulated early. The whole pattern was repeated every 10 s. Each training “session” lasted 30 min, and

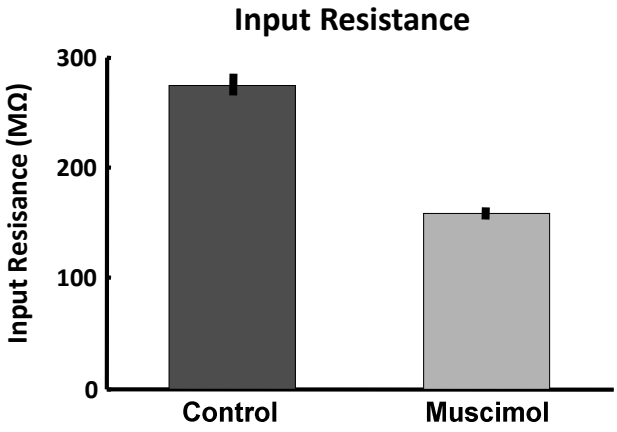
there were a total of 3 sessions (total training time of 2.5 hr with 30 min ON/OFF spaced training sessions). After 2.5 hr of training in the incubator, the slice was moved to the electrophysiology rig. Three pulses separated by 60 ms interval were delivered through both pathways (alternating and separated by 5 s), and recordings were done in both ChR2 positive (ChR+) cells and ChR2 negative (ChR-) cells. In all experiments, ChR2+ cells were defined as having the light-evoked depolarization induces at least one spike, while the ChR- cells with light response smaller than 20 mV. Low-dose of CNQX (4 μM) was bath applied throughout the experiment.

In the meta-STP muscimol experiments, muscimol (0.5 - 0.6 μM) was bath applied during testing period in addition to CNQX. Because ChR+ neurons were consistently recorded first, when ChR- neurons were recorded later the slices have had a longer perfusion time in muscimol. This difference in exposure time to muscimol might underlie the difference between the normalized EPSP slopes between ChR+ and ChR- cells in the muscimol experiments (**Supplementary Figure S5.2B**).

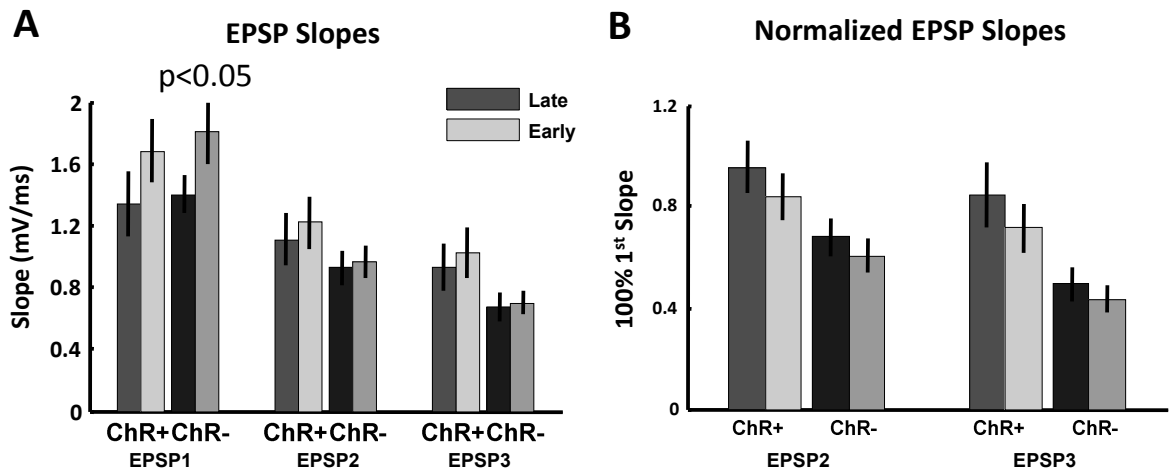
Supplementary Figures



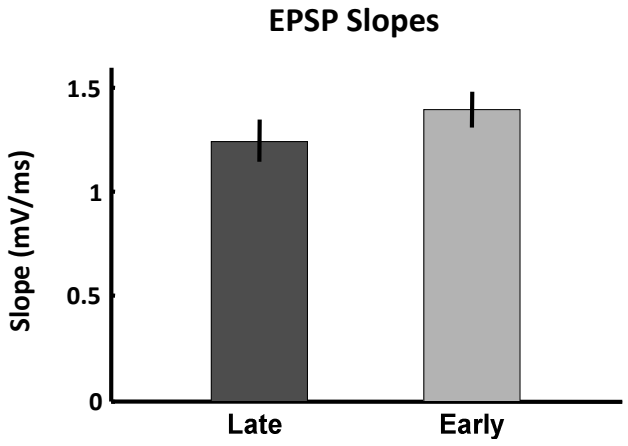
Supplementary Figure S5.1. Metaplasticity of STP blind experiments, showing both ChR+ and ChR- neurons. All statistics are from two-way ANOVA with repeated measure on pathways (Late, Early) and segments (1 to 3). **(A)** Absolute peak value for each segment. For ChR- cells (n=53), main effect of pathway $F_{1,52}$=1.96, p=0.168. Interaction Pathway × Pulse number: $F_{2,104}$=1.98, p=0.143. No significant difference between ChR+ and ChR- cells ($F_{1,66}$=2.89, p=0.094). **(B)** Normalized peak value in ChR- neurons: Main effect of pathway, $F_{1,52}$=5.95, p=0.018. Interaction, $F_{1,52}$=5.84, p=0.019. Paired t-test showed significant difference in the 3rd PSP, t_{52}=2.54, p=0.014. No significant difference between ChR+ and ChR-: $F_{1,66}$=0.10, p=0.757. **(C)** EPSP1 slope in ChR- neurons showed no significant difference between the two pathways (paired t-test, t_{52}=0.32, p=0.747). Difference between ChR+ and ChR- were not significant ($F_{1,66}$=2.69, p=0106). (* p<0.05)



Supplementary Figure S5.2. Muscimol induces a decrease in input resistance. In the meta STP experiments, bath applied muscimol leads to a significant decrease in input resistance, which helps decrease polysynaptic activity. Combining ChR+ and ChR- neurons, unpaired t-test, $t_{134}=9.57$, $p<10^{-4}$, n=70 (no drug), 66 (muscimol) respectively.



Supplementary Figure S5.3. Metaplasticity of STP testing in muscimol, showing both ChR+ and ChR- neurons. All statistics are from two-way ANOVA with repeated measure on pathways (Late, Early) and EPSPs (1 to 3). **(A)** Absolute slope value for three EPSPs in ChR- cells (n=24). Main effect of pathway $F_{1,23}$=2.16, p=0.155. Interaction Pathway × Segment: $F_{2,46}$=6.40, p=0.004. Paired t-test showed significant difference in the 1st EPSP, t_{23}=2.42, p=0.024. No significant difference between ChR+ and ChR- cells ($F_{1,47}$=0.77, p=0.386). **(B)** Normalized slope value. ChR-: Main effect of pathway, $F_{1,23}$=3.44, p=0.077. Interaction, $F_{1,23}$=0.13, p=0.718. There is a significant difference between ChR+ and ChR-: $F_{1,47}$=5.54, p=0.023, maybe as a result of different perfusion time in muscimol.



Supplementary Figure S5.4. 1st EPSP slope between Late and Early pathways, collapsing between Meta-STP non drug and muscimol experiments, combining ChR+ and ChR- neurons. There is no significant difference between pathways, paired t-test, t_{135}=1.45, p=0.150, n=136.

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Chapter 6. General Discussion

Overall the experiments described here lend further support to the notion that STP is itself plastic. First, as described in Chapter 2 STP undergoes developmental changes in vitro, suggesting that plasticity of STP is at least in part determined by an ontogenetic program. Second, as described in Chapter 5, I provided further evidence that in the cortex there is an interaction between long-term synaptic plasticity and STP. This plasticity is at least consistent with the hypothesis of metaplasticity of STP because early pairing resulted in more short-term synaptic depression than late pairing. Below I discuss in more details the implications of these findings and a number of additional questions relating to their meaning and mechanisms.

Experimental evidence supporting the metaplasticity of STP

In the **Discussion** of **Chapter 5**, I briefly discussed the possible mechanisms underlying metaplasticity of STP. We proposed that a retrograde message could be activated by postsynaptic activity, diffuse to the presynaptic terminal, and then interact with presynaptic Ca^{2+} levels to regulate release probabilities and change STP. As the presynaptic Ca^{2+} level is determined by the number of presynaptic action potentials "Early-pairing" (as described in **Chapter 5**) will result in low levels of Ca^{2+} at the time of the retrograde messenger, and shift STP towards depression; while "Late-pairing" would result in high levels of Ca^{2+} and would favor facilitation. Thus, one hypothetical model for metaplasticity of STP would be composed of three major steps: 1) generation of a retrograde messenger by postsynaptic activity; 2) interaction between retrograde messenger and presynaptic release machinery; 3) regulation of STP via probability of release--possibly without changing baseline synaptic transmission. There is potential experimental support for each of these steps from separate studies, as discussed below.

First, the notion that neural activity can generate retrograde messengers which diffuse through the synaptic cleft and influence cortical synaptic plasticity has been verified by a number of studies. Candidate signal molecules include endocannabinoids (Sjostrom et al., 2003, 2004, 2007), arachidonic acid (Williams et al., 1989), carbon monoxide (Stevens and Wang, 1993; Zhuo et al., 1993), nitric oxide (Bohme et al., 1991; O'Dell et al., 1991; Schuman and Madison, 1991; Haley et al., 1992; Schuman and Madison, 1994), and platelet-activating factor (Clark et al., 1992; Haley et al., 1992). Among these, nitric oxide has received wider attention in inducing plasticity. For example, it has been shown that associative LTP induction in CA1 neurons of hippocampus is accompanied by heterosynaptic LTP induction in neighboring neurons. This nonspecific LTP is proposed to be mediated by NO-based process as it is blocked by NO synthase inhibitor (Schuman and Madison, 1994). Additionally, studies supporting step 2) of our proposed mechanism also indicated a role of NO in mediating synaptic plasticity (see below). Therefore, based on past literature nitric oxide is a potential candidate molecule that could contribute to metaplasticity of STP.

The retrograde messenger we proposed would diffuse to the presynaptic side and interact with release probabilities in a way that is dependent on the Ca^{2+} level at the time it arrives. Although there is little direct evidence supporting this mechanism, previous studies have observed that retrograde signaling molecules can regulate presynaptic release probabilities in a manner that is related to initial STP ratio (Volgushev et al., 2000). For example, investigators found that in neocortical synapses, purely postsynaptically induced LTP was associated with alterations of release indices. Moreover, the direction and magnitude of these changes depend on the initial properties of the presynaptic input, and is at least partially depending on NO-dependent retrograde signaling systems (Volgushev et al., 2000). Although we cannot infer at

this stage if such mechanisms could contribute to metaplasticity of STP, it seems possible that similar mechanisms could be employed.

The third and last step of the metaplasticity of STP that we proposed involved regulation of STP ratio in a way that is independent on changes affecting baseline synaptic transmission. Although STP is traditionally believed to be determined by baseline synaptic strength (Katz and Miledi, 1968; Thomson et al., 1993; Debanne et al., 1996; Markram and Tsodyks, 1996; Dobrunz and Stevens, 1997; Buonomano, 1999; Atzori et al., 2001; Zucker and Regehr, 2002; Bender et al., 2006; Boudkkazi et al., 2007), a number of studies clearly support a dissociation between STP and 1st EPSP amplitude (Huang et al., 1994; Thomson and Bannister, 1999; Brody and Yue, 2000; Hanse and Gustafsson, 2001; Chen et al., 2004; Fuhrmann et al., 2004; Oswald and Reyes, 2008; Boudkkazi et al., 2011). Furthermore, a couple of studies reported that STP can be regulated independently of baseline synaptic strength. For example in cultured hippocampal neurons, overexpression of calcium binding protein neuronal calcium sensor-1 (NCS-1) switch paired-pulse depression to facilitation without altering basal synaptic strength or initial release probabilities (Sippy et al., 2003). Additionally in gold fish brainstem M-axons it has been shown that each of the two EPSPs of a paired-pulse pattern can be independently regulated without affecting the other (Waldeck et al., 2000). Based on these studies, and given the large number of presynaptic proteins involved in Ca^{2+} regulation (Zucker and Regehr, 2002; Burnashev and Rozov, 2005; Mochida et al., 2008) as well as potentially undiscovered mechanisms in place to regulate STP such as recently identified presynaptic NMDA receptors (Larsen et al., 2011), it is reasonable to postulate that at the final step of the metaplasticity of STP the retrograde messenger would interact with one of these target molecules such as NCS-1 to regulate STP independent of baseline transmission.

In summary, although there is no direct evidence supporting the mechanisms underlying metaplasticity of STP, based on previous investigations each step that we proposed seems to be biological plausible. There is a number of experiments that we can do to test for possible mechanisms underlying metaplasticity of STP, which will be discussed below.

Consistency of synaptic activation by successive stimulation

When we tested for the metaplasticity of STP, a train of three pulses is delivered through each of the pathways at 60 ms interval. One question that arises, therefore, is whether the same synapse gets activated for all three stimuli. For example, it is possible that low probability terminals are paired with depolarization in the Late-pairing and high probability terminals are paired with postsynaptic activity in the Early-pairing pathway. Our current experiment cannot rule out the possibility that different synapses might be activated by successive train of stimuli as a result of transmission failure. However, during paired-recording studies in **Chapter 2**, we found that in our cortical organotypic slices failures were generally not observed. This is possibly resulting from the high Ca^{2+} concentration in ACSF we used (2.6 mM) compared to *in vivo* (1.0 - 1.2 mM). Traditionally the Ca^{2+} concentration used in culturing organotypic slices is higher than the ACSF of acute slices (but it generally remains in balance with Mg^{2+}, Stoppini et al., 1991; Musleh et al., 1997). It is therefore important to "match" the ACSF used for organotypic electrophysiology with the ionic concentration of the culture media—otherwise the ACSF in itself induces changes in dynamics and homeostatic plasticity. For this reason the ACSF of organotypic slices tends to have higher concentrations of Ca^{2+} (and often Mg^{2+}) than acute slice ACSF (e.g., Debanne et al., 1996; Hayashi et al., 2000; Johnson and Buonomano, 2007; Tominaga-Yoshino et al., 2008).

Finally, since probability of release is often assumed to be a constant throughout a train, and to be a stochastic process, it may also be the case that such variations should be averaged out during our training protocol. But as stated above we cannot rule out the possibility a potential differential effect of different synapses being activated.

Possible future pairing protocols

One major issue that arises from the metaplasticity of STP studies in **Chapter 5** is that in the 2.5 hr muscimol experiments, we observed a difference in STP accompanied by a trend of difference in the absolute value of 1st EPSP slope (**Figure 5.4C, D**). Therefore, although the differentiation of short-term plasticity we observed was consistent with the notion of metaplasticity of STP, it was not possible to determine whether or not the differences in STP are primarily an effect of differences in the magnitude of induced LTP. In **Chapter 5** we discussed the different potency of LTP induction for the two pairing protocols of the two pathways, and our observations are consistent with the notion that pairing in the Early-pairing pathway is more effective in LTP induction compared to the Late-pairing pathway. What we observed in Early-pairing pathway (i.e. more LTP induction and more PPD) might be explainable by the common observation that stronger synapses usually display more paired-pulse depression. Similarly, Late-pairing which resulted in less LTP induction together with less PPD might be accountable by the notion that weak synapses tend to have more paired-pulse facilitation.

To address this issue, we reasoned that future experiments should be designed in a way so that a similar amount of LTP would be induced in the two pathways, then the STP ratio could be compared according to the metaplasticity of STP hypothesis. One approach would be to deliver a train of four pulses through each of the pathway, and "double" pair postsynaptic

depolarization with the last two pulses of "Late-pairing" pathway while keep the "Early-pairing" pathway unchanged (**Figure 6A**). We hypothesis that by pairing two pulses in "Late-pairing" pathway, more LTP should be induced, hopefully to a similar level as in "Early-pairing". According to metaplasticity of STP, more paired-pulse depression should be observed in "Late-pairing" compared to "Early-pairing" pathway.

Another possible pairing protocol is shown in **Figure 6B**: "Early-pairing" pathway could be compared to "Single-pairing", where a single presynaptic pulse is paired with postsynaptic depolarization. We proposed that a similar amount of LTP should be observed for both pathways. During testing phase, a train of three pulses will be delivered through each pathway and more paired-pulse depression will be expected in "Early-pairing" according to metaplasticity of STP.

Overall here I proposed two possible variations of pairing protocols that could be used to further test the metaplasticity of STP hypothesis in the future. If these two experiments do not result in the desired result of similar LTP induction in two pathways, then the protocols could be further adjusted so that the Late-pairing produces equal (or more) LTP than the Early-pairing pathway.

Possible future experiments to explore the mechanisms of metaplasticity of STP

Assuming that metaplasticity of STP does exist and that we established protocols that induce robust effects, then a number of experiments can be done to explore possible mechanisms underlying metaplasticity of STP.

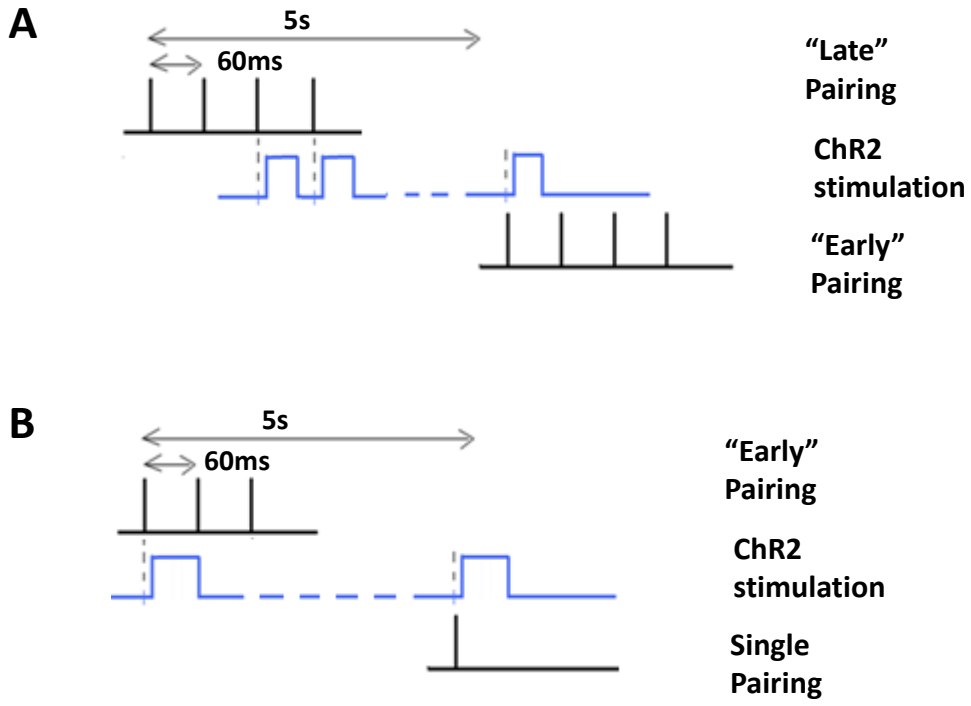

Figure 6. Possible future experiments. We proposed these pairing protocols to address the issue of different LTP induction after previous "Late" and "Early" pairing. **(A)** A train of 4 pulses will be delivered at 60 ms interval. Two postsynaptic depolarization sessions will be paired with the last two pulses of "Late-pairing" pathway while one with the first pulse of "Early-pairing" pathway. We proposed that now "Late" and "Early" pairing should result in similar degree of LTP induction in the 1st EPSP. According to the metaplasticity hypothesis, we are expecting to see more PPF in "Late-pairing" pathway and more PPD in "Early-pairing" pathway. **(B)** Alternatively, we can compare the "Early-pairing" pathway to a "Single-pairing" pathway where postsynaptic depolarization is paired with a single pulse. If the two pathways have similar LTP induction, then according to metaplasticity of STP we are expecting to see more PPD in "Early-pairing" pathway compared to "Single-pairing" pathway.

First we can test if the effect of metaplasticity of STP depends on protein synthesis as we proposed in **Chapter 5**. During training, protein synthesis inhibitors such as anisomysin (Frey et al., 1993; Huang et al., 1994; Barea-Rodriguez et al., 2000) can be bath applied to see if metaplasticity effect is abolished. Additionally we can test if metaplasticity of STP depends on retrograde signal molecules such as nitric oxide. During training, nitric oxide synthase (NOS) inhibitor such as N-methyl-L-arginine (L-Me-Arg) or NG-nitro-L-arginine methylester (L-NAME) can be bath applied to block NO-dependent retrograde signal systems (Schuman and Madison, 1994; Aonuma and Newland, 2001, 2002). But as both protein synthesis and NO system could likely to affect late-phase LTP induction, precautions should be made before they are concluded to affect metaplasticity of STP.

Conclusion

Overall the results of the experiments described in my dissertation add to the current understanding of the development, function, and regulation of short-term synaptic plasticity. I provided further evidence that STP is plastic, including both developmental plasticity as well as training-induced plasticity. Additionally, I established that order-selective neurons can be observed in acute cortical slices. This experimental evidence provides indirect support for an important computational role for STP, and is consistent with a role of STP in temporal processing. Furthermore, I experimentally tested the novel learning rule of the metaplasticity of STP and found that different pairing protocols result in changes in STP that is consistent with this hypothesis. But at this stage, it is not clear if synapses use metaplasticity for computations.

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