MECHANISMS OF ACTIVITY-DEPENDENT PLASTICITY INVESTIGATED AT INDIVIDUAL SYNAPTIC CONTACTS

by

David William Nauen

B.A., Harvard University, 1994

Submitted to the Graduate Faculty of the School of Medicine in partial fulfillment of the requirements for the degree of Doctor of Philosophy University of Pittsburgh 2008
This dissertation was presented

by

David William Nauen

It was defended on December 3, 2007

and approved by

Committee Chair: Stephen D. Meriney, Ph.D.
Elias Aizenman, Ph.D.
Karl Kandler, Ph.D.
Robert E. Kass, Ph.D.
Nathaniel N. Urban, Ph.D.
Venkatesh N. Murthy, Ph.D.
Doctoral Advisor: Guo-Qiang Bi, Ph.D.
MECHANISMS OF ACTIVITY-DEPENDENT PLASTICITY INVESTIGATED AT INDIVIDUAL SYNAPTIC CONTACTS

David W. Nauen, Ph.D.

University of Pittsburgh, 2008

ABSTRACT

Understanding the mechanisms of synaptic transmission and plasticity is among the primary challenges facing neuroscience, but the small size of mammalian central synapses and the large number contributing to most recordings present obstacles to observation of the operation of individual sites. These considerations are compounded by probabilistic behavior, making it difficult or impossible to infer individual synapse function from measurements of the ensemble. This thesis presents an experimental method for, and results from, studies of short- and long-term plasticity at very few synapses.

Our investigation of synaptic transmission in cultured hippocampal neurons reveals the independent function of neighboring synapses along the axon, but no evidence for the contribution of neurotransmitter depletion to short-term plasticity and no evidence for encoding of the recent history of activity in the responses of few synaptic sites.

Our studies of the mechanisms of spike-timing-dependent long-term potentiation (STDP) demonstrate that synaptic activity during the induction protocol is required for potentiation. The state of the synapse at baseline influences the manner in which the synapse potentiates, and release probability and the size of non-failure responses can change differentially after induction. Changes in short-term plasticity occur at times corresponding to the change in size of non-failure responses. Asynchronous release events
can be unchanged for extended periods, but when they do change, this also occurs near the
time of the increase in size of successful evoked responses. Taken together these results
indicate that following the induction protocol, STDP usually unfolds through a sequence of
steps that begins with persistent change in presynaptic function and is followed by the
addition of new synapses.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS .............................................................................................................. xi

1.0 INTRODUCTION: SHORT-TERM PLASTICITY AND SPIKE-TIMING-DEPENDENT LONG-TERM PLASTICITY ............................................................................................................. 1

1.1 FUNCTION AND SHORT-TERM PLASTICITY OF INDIVIDUAL SYNAPSES ................................................. 2

1.1.1 Depletion as a cause of short-term depression ................................................................. 2

1.1.2 History dependence ........................................................................................................ 4

1.1.3 Functional independence of neighboring synapses .......................................................... 4

1.1.4 Role for dopamine in rapid synaptic modification ............................................................ 5

1.2 MECHANISMS OF SPIKE-TIMING-DEPENDENT LONG-TERM POTENTIATION ....................................................................................................................... 6

1.2.1 Relation to initial release probability ............................................................................. 6

1.2.2 Potency and reliability ................................................................................................... 7

1.2.3 Time of potentiation ...................................................................................................... 8

1.2.4 Change in short-term dynamics .................................................................................... 10

1.3 SUMMARY .................................................................................................................................... 11

2.0 MICROPERFUSION METHOD FOR MEASURING ACTIVITY AND PLASTICITY AT SMALL NUMBERS OF SYNAPSES .................................................................................. 13

2.1 OVERVIEW ...................................................................................................................................... 13

2.2 INTRODUCTION .................................................................................................................................. 13

2.3 MICROPERFUSION METHOD ........................................................................................................ 15

2.3.1 Modifications of the culture technique ........................................................................... 15

2.3.2 Modifications of the patch technique .............................................................................. 15

2.3.3 Microscope ....................................................................................................................... 16

2.3.4 Single-cell labeling and imaging ..................................................................................... 16

2.3.5 Micropertusion .................................................................................................................. 18

2.3.5.1 Positive pressure ........................................................................................................ 20

2.3.5.2 Negative pressure ..................................................................................................... 21

2.3.6 Measuring flow ................................................................................................................ 22

2.4 RESULTS ........................................................................................................................................ 24

2.5 DISCUSSION .................................................................................................................................... 40

2.5.1 The approach ................................................................................................................... 40

2.5.2 Depletion as cause of short-term depression ................................................................. 42

2.5.3 History dependence ........................................................................................................ 43
4.2.3.1 Dependence of STDP on activity at synapses during induction ................................................................. 90
4.2.3.2 Effect of initial conditions ................................................................. 91
  4.2.3.2.1 Relation of change in release probability to initial release probability ................................................. 91
  4.2.3.2.2 State-dependence ................................................................. 93
4.2.3.3 Time of change .................................................................... 93
4.2.3.4 Locus ......................................................................................... 95
  4.2.3.4.1 Differential change in potency and reliability — pre- and postsynaptic contributions to STDP .......... 95
  4.2.3.4.2 Combined pre- and postsynaptic mechanisms ........ 96
  4.2.3.4.3 Silent synapses ................................................................. 98
  4.2.3.4.4 Trans-synaptic signaling .................................................. 99
4.2.3.5 Multiple time scales ................................................................. 100
  4.2.3.5.1 Change in short-term plasticity following long-term plasticity .............................................................. 100
  4.2.3.5.2 LTP as stabilizing the changes created transiently with facilitation ..................................................... 101
  4.2.3.5.3 Effect of induction on AP propagation ................. 101
  4.2.3.5.4 The influence of the inter-trial interval on synaptic dynamics ............................................................... 102
4.2.3.6 Change in asynchronous release following induction ....... 102
4.2.3.7 Accelerated synaptic maturation as the mechanism of potentiation ......................................................... 103
4.3 CODA ......................................................................................... 109
5.0 EPILOGUE .................................................................................... 111
APPENDICES ..................................................................................... 119
  APPENDIX A. MICROPERFUSION PROTOCOL AND TECHNICAL NOTES ..... 121
  APPENDIX B. METHODS OF MEASURING ACTIVITY AT FEW SYNAPSES .... 123
  APPENDIX C. CHANGES IN SHORT-TERM SYNAPTIC DYNAMICS FOLLOWING INDUCTION OF LONG-TERM PLASTICITY ................................................. 145
  APPENDIX D. STIMULATION AND ACQUISITION USING MATLAB ........ 149
  APPENDIX E. LIST OF CUSTOM SOFTWARE ROUTINES USED ............ 153
  APPENDIX F. STRUCTURE OF DATABASE USED .................................. 157
BIBLIOGRAPHY .................................................................................. 171
LIST OF FIGURES

Figure 2.1: The microperfusion system ................................................................. 19
Figure 2.2: Pipette mounting configuration for the microperfusion system .......... 20
Figure 2.3: Use of the acoustic signal to monitor microperfusion flux .................. 23
Figure 2.4: Reduction of the number of active synapses with microperfusion .......... 24
Figure 2.5: Local application of normocalcemic solution is necessary for evoked synaptic transmission ......................................................................................... 25
Figure 2.6: Functional stability of the microperfusion system ................................ 27
Figure 2.7: Comparison of PSC waveforms .......................................................... 29
Figure 2.8: Method for measuring latency ............................................................. 30
Figure 2.9: Latency measurements at individual synapses ................................... 32
Figure 2.10: Depletion does not influence short-term plasticity at hippocampal synapses in culture ................................................................................................. 34
Figure 2.11: The inter-pulse interval (IPI) before the most recent IPI does not affect the synaptic response ............................................................................................... 36
Figure 2.12: Adjacent synapses on an axon function independently .................... 38
Figure 2.13: Dopamine exposure increases synaptic strength in part by increasing release probability ............................................................................................ 39

Figure 3.1: Induction of STDP in the focal perfusion configuration ...................... 57
Figure 3.2: Summary of focal perfusion STDP results ........................................ 58
Figure 3.3: A threshold level of activity at the synapse during induction is needed for potentiation ........................................................................................................ 58
Figure 3.4: Absence of potentiation during induction ............................................ 59
Figure 3.5: Potentiation involves increases in both potency and reliability .......... 60
Figure 3.6: Distributions of identified success and failures .................................. 62
Figure 3.7: Example of detected change points for potency and reliability .......... 63
Figure 3.8: Controls for change point analysis ...................................................... 65
Figure 3.9: Reliability can change prior to or without change in potency .......... 66
Figure 3.10: Short-term dynamics are frequently altered by STDP ..................... 68
Figure 3.11: Preinduction facilitation predicts postinduction facilitation .............. 70
Figure 3.12: Time of change in potency predicts time in synaptic dynamics .......... 71
Figure 3.13: Asynchronous release event sizes can remain unchanged for extended periods following successful induction ............................................................ 73
Figure 3.14: Asynchronous release changes after a delay at some synapses .......... 74
Figure 3.15: Cumulative histograms of latency before (blue) and after induction of STDP .... 75
Figure A.1: Siphon-suction junction used in the microperfusion system ................................ 127

Supplementary Movies:
http://www.cnbc.cmu.edu/~david_nauen/microperfusion_movies_dwn.htm
ACKNOWLEDGEMENTS

Thanks to my advisor, Guo-Qiang Bi, for encouraging an atmosphere of thorough enquiry, clear thinking, and good humor. Thanks to all members of the Bi lab, especially Pakming Lau, Huaixing Wang, Jichuan Zhang, Rick Gerkin, Xianyi Tang and Chad Noble-Tabiolo for creating such a collaborative and exciting research environment. Nyob zo! Thanks to the Department of Neurobiology and the Center for Neuroscience at the University of Pittsburgh, and to the University of Pittsburgh/Carnegie Mellon University Center for the Neural Basis of Cognition and Medical Scientist Training Program. Thanks to the members of my Thesis Committee: Steve Meriney, who served as chair; Elias Aizenman; Karl Kandler; Robert E. Kass, who was also my IGERT project advisor; Nathan Urban; and Venkatesh N. Murthy, who served as outside examiner. Thanks to everyone else who trained me in formal or informal capacities or provided information, knowledge or wisdom during my time in graduate school, including Patti Argenzio, Jim Buhrman, Jason Castro, Barb Dorney, Bard Ermentrout, Jay Jacobs, Jon Johnson, Abigail Kalmbach, Gunsoo Kim, Hanmi Lee, Jay McClelland, Jon Rubin, Hunter Simpson, Manjit Singh, Peter Strick, Floh Thiels, Dave Touretzky, Frank Valentech, and Clayton Wiley.

Thanks to the Department of Education, the MSTP, the CNUP, the CNBC and the NSF for support.

Thanks to my friends, my brothers, my sister, my parents, the Rapaka family, and Rekha, who was with me.
1.0 INTRODUCTION:
SHORT-TERM PLASTICITY AND SPIKE-TIMING-DEPENDENT
LONG-TERM PLASTICITY

Synapses were visualized in the latter part of the 19th century by anatomists including Ramon y Cajal, who postulated their role in directional transfer of information on structural grounds (Ramon y Cajal 1911). In the 20th century researchers including Lucas and Adrian characterized the action potential (AP; see Bradley and Tansey 1996) while others determined that despite the importance of this electrical activity, the ultimate bearer of information from one neuron to the next is often chemical (Dale 1934). Experiments at the neuromuscular junction revealed that these chemical neurotransmitters are released in quantal packets (Fatt and Katz 1952, Del Castillo and Katz 1954). After release, neurotransmitters bind to proteins on the surface of neurons, increasing their permeability to certain ions (Neher and Sakmann 1976). For contemporary neuroscience, this outline continues to serve as the framework for studies of the mechanisms both of synaptic transmission and of the synaptic basis of learning, assumed to be reflected in the phenomenon of long-term potentiation (Bliss and Lomo 1973). The investigations presented in this thesis address synaptic plasticity by considering two distinct but related topics: short-term plasticity, correlated function, and rapid activation of individual synapses; and mechanisms of spike-timing-dependent long-term potentiation. To provide context for the reports of these investigations presented in later chapters, this chapter serves as an overview of the relevant scientific literature, focusing on those studies most germane to the approach and findings of the present thesis.
1.1 FUNCTION AND SHORT-TERM PLASTICITY OF INDIVIDUAL SYNAPSES

The need for calcium in transmitter release (Katz and Miledi 1965) suggests that repeated action potentials could result in its accumulation in presynaptic terminals, leading to increased probability of release during trains of action potentials (Feng 1941). The extrusion of a chemical transmitter from the presynaptic terminal implies the possibility of reduced synaptic strength over time. These two classes of mechanisms, cumulative addition and cumulative subtraction, are hypothesized to underlie two classes of effects at the synapse, short-term facilitation and depression. Over time the balance of the two and their respective opposing processes, calcium sequestration and vesicle replenishment, gives rise to the short-term dynamics of the synapse.

1.1.1 Depletion as a cause of short-term depression

Short-term depression of evoked synaptic current is usually viewed as determined by the release of transmitter and the resultant depletion of the available pool of vesicles. This assumption is the basis for measurement of the paired-pulse ratio as an indicator of release probability. Depletion clearly contributes to the depression seen at some stimulation rates at some synapses (Goutman and Glowatzki 2007, Foster and Regehr 2004), but its role at other synapses is less clear (Kraushaar and Jonas 2000). Apart from depletion, other mechanisms of decreased release probability due to activity have been reported (Burrone and Lagnado 2000). For example, presynaptic calcium influx acts on synaptotagmin I to permit rapid evoked
release (Nishiki and Augustine 2004), but the same protein can also have an inhibitory influence on vesicle release (Tamura et al. 2007).

If depletion causes depression, in depressing conditions the response to the second of a pair of stimuli should be smaller following a successful release than following a failure. In some studies, however, this not seen (Cabezas and Buno 2006). Further calling into question the importance of depletion for short-term depression, in cultured hippocampal neurons the paired-pulse ratio is the same before and after a massive exocytosis provoked with hyperosmotic solution (Sullivan 2007). Additional evidence against a major impact of vesicle depletion on depression is the finding that vesicles are reused more rapidly than had been assumed (Pyle et al. 2000), and the discovery that some vesicles appear to be released twice before adjacent vesicles are released once (Rizzoli and Betz 2004). Given the conflict between these recent results and long-held views, further tests on the role of depletion in short-term depression are needed.

An important consideration in studies of short-term depression is the inter-pulse interval (IPI) used in stimulation. The effect of depletion will ultimately depend on the rate of replenishment, so the IPIs chosen for an experiment are critical for determining its contribution. Refilling of the ‘immediately releasable site’ likely requires approximately 5 ms (Hanse and Gustafsson 2001) to 20 ms (Stevens and Wang 1995). At synapses between cultured hippocampal neurons, postsynaptic receptor desensitization influences responses within 20 ms of a previous response (Forti et al. 1997). Endocytosis is considerably slower and has been shown at some synapses to operate on time constants on the order of 1-10 s (Neves et al. 2001). The ratio of the responses to a pair of evoked APs is a function of the time courses of these and other contributing processes and the IPIs used. Variable IPIs permit sampling from a larger portion of this parameter space. This allows the cumulative effect on synaptic strength of the different processes to be assessed at multiple time
points, providing a more comprehensive view than would be possible with a single inter-pulse interval.

1.1.2 History dependence

A number of theoretical studies have reported that short-term fluctuations of synaptic strength may have high information content (Maass and Markram 2002). In this view the processes set in motion when the action potential invades the terminal, leading to facilitation or depression of the subsequent response, would accumulate across multiple responses, potentially providing information about the number and timing of recent APs. Theoretical work suggests that such fluctuations could contain information about the durations of as many as 8 preceding inter-pulse intervals (Fuhrmann et al. 2002). However, given the stochastic nature of synaptic function, the extent to which information about history can be extracted from short-term dynamics at synapses consisting of few sites is unclear. Essentially the question is whether the function of synapses is sufficiently deterministic for such information to be reliably transmitted. The larger the number of synaptic contacts formed by the presynaptic neuron onto the postsynaptic, the more deterministic their collective behavior. In many brain regions, however, the synapse consists of few contacts (Deuchars and Thomson 1996) or even a single one (Gulyas et al. 1993). The stochastic function of these synapses would reduce the fidelity with which information on recent activity history could be transmitted, but the extent of this reduction can only be determined by further experiments.

1.1.3 Functional independence of neighboring synapses
When an AP invades the axon, nearby synaptic sites having low probability might be expected to function with some degree of correlation to one another. The metabolic state of nearby regions is likely to be similar, and autocrine and paracrine influences are likely to overlap. Moreover, the activity at one synaptic terminal on the axon influences subsequent activity at nearby terminals through overspill of neurotransmitter (Vogt and Nicoll 1999). However, it has been argued that the information storage and transmission capacities of the brain are maximized when synapses release or fail to release independently (Barbour 2001), and modeling has supported the idea that nearby synapses do function independently (Franks et al. 2002). At inhibitory synapses, independence is suggested by studies showing that blockade of gamma-aminobutyric acid (GABA) transport only prolongs inhibitory postsynaptic currents (PSCs) when the number of stimuli is large or the anatomical spacing of sites is dense (Overstreet and Westbrook 2003). To our knowledge independence has not been tested directly at excitatory synapses.

1.1.4 Role for dopamine in rapid synaptic modification

The monoamine neuromodulator dopamine affects many aspects of synaptic function (Schultz 2007, Tecuapetla et al. 2007). Given the evidence indicating that dopaminergic reward circuitry plays a role in addiction (Schroeder et al. 2008) and increasing indications that the process of addiction shares mechanisms with learning and memory (Redish 2004), further evaluation of the influence of dopamine on short- and long-term synaptic plasticity could reveal cellular processes common to both.

---

1 Under experimental conditions, imperfect space clamp could also make nearby regions function more similarly than distant regions. Branch failure (Debanne et al. 1996) could be another source of correlation of local activity.
1.2 MECHANISMS OF SPIKE-TIMING-DEPENDENT LONG-TERM POTENTIATION

If LTP fascinates neuroscientists, how much more so STDP, in which synapses become stronger not through chemical treatment or high-frequency shocks, but simply by their neurons firing action potentials at lifelike rates for a short period (Bi and Poo 1998)? The apparent simplicity of STDP has attracted interest from modelers and theoreticians seeking rules for the network mechanisms of various forms of learning. Mindful of the diverse patterns of action potentials fired by neurons in the brain, experimenters have probed the properties of integration of more complex stimuli than the single pre- and postsynaptic spike originally identified (Sjostrom et al. 2001, Wang et al. 2005, Froemke et al. 2006). Some of the component signaling cascades involved in STDP have been identified (Wang et al. 2005, Sjostrom et al. 2003), but many questions on the relative contributions of the presynapse and the time course of synaptic changes following induction have been difficult to resolve with conventional experimental techniques.

1.2.1 Relation to initial release probability

In seeking the factors that shape a given synapse’s responses to LTP induction, the role of preinduction release probability has been addressed by a number of studies. An inverse relationship between initial release probability \( (p_r) \) and change in \( p_r \) was observed in studies of long-term potentiation that measured \( p_r \) by estimating quantal content (Larkman et al. 1992, Liao et al. 1992, O’Connor et al. 2007). To the extent that paired-pulse facilitation reflects release probability, an inverse
relationship between initial release probability and potentiation was also found by Volianskis and Jensen (Volianskis and Jensen 2003). In cultured hippocampal neurons, following tetanic stimulation only low probability synapses exhibit change in probability (Ninan et al. 2006), and both facilitation and increase in external calcium increase release probability disproportionately at low $p_r$ synapse (Murthy et al. 1997). On the other hand, some studies have found a selective potentiation of high probability synapses (Rosenmund et al. 1993)\(^1\).

The influence of $p_r$ on induction indicates that the baseline state of the synapse shapes its response. This is also suggested by studies of serial inductions in which the presynaptic release probability changes only after a second potentiation (Ward et al. 2006). The optical methods used in that study to estimate $p_r$ on the basis of the calcium transient in the bouton are more equivocal than direct measurement, because calcium influx may not guarantee transmitter release and because the linkage between calcium influx and transmitter release is likely to be affected by the potentiation protocol. Nonetheless it is clear that in the organotypic preparation presynaptic calcium influx increases more due to a second induction than to a first. In the case of STDP, the influence of initial release probability on induction is not known.

1.2.2 Potency and reliability

Separation of synaptic current into ‘reliability’ and ‘potency’ components has been performed to assess the mechanisms of LTP (Stevens and Wang 1995, Bolshakov and

\(^1\) These apparently contradictory findings could be reconciled by consideration of ‘low $p_r$’ and ‘high $p_r$’ as relative to the preparation and experimental conditions. If some activity at the synapse during induction is required for change, but synapses with already-high $p_r$ are unable to further increase, the result would be an ‘inverse u’ function, where synapses in the middle of the overall range would be best suited to undergo increases in $p_r$. Whether synapses in this overall middle fall on the high end or low end of the distribution seen in a given experiment would depend on the preparation and conditions.
Siegelbaum 1995). Reliability refers to the average release probability over a train of stimuli, while potency indicates the average size of non-failure responses. In the slice preparation, minimal stimulation of axon bundles is generally used for this type of study to evoke release at a small number of sites, preferably one. Previous studies have reached conflicting results with regard to changes in reliability and potency following potentiation. LTP using minimal stimulation in some cases is associated with an increase in potency with no change in release probability (Isaac et al. 1996), while in other experiments change in release probability (closely related to reliability) but not potency is reported (Stevens and Wang 1995). Other groups have reported that change in potency and change in reliability both occur, and at nearly the same time (O'Connor et al. 2007), though this study considered only 5-10 minutes after induction. With the minimal stimulation used in these experiments, the potency result must be considered in light of the fact that the excitability of the fiber bundle could change (Collingridge 1994), although when potency does not change, change in axon excitability is not a concern. Experiments with both the pre- and postsynaptic neurons patched, where sodium currents can be identified to confirm AP initiation, could reduce concerns about changes in the excitability of the axon during long-term potentiation, suggesting revisitation under such conditions of the question of change in potency and reliability as mechanisms of LTP.

1.2.3 Time of potentiation

The onset time of changes in synaptic function following LTP induction has been studied as an indication of the mechanisms of plasticity. An early investigation found that sensitivity to externally applied glutamate receptor ligand increased beginning roughly 15 minutes after induction by tetanic stimulation and continued to increase for approximately 2 hours (Davies et al. 1989). In hippocampal slice a
delay of up to 15 minutes before locally-induced LTP was maximal was seen in some cases (Engert and Bonhoeffer 1997). Candidate mechanisms of change in synaptic strength such as receptor phosphorylation are unlikely to require such a long interval.

A result of the focus on time course of synaptic change following potentiation has been the division of early and late LTP (Frey et al. 2001). Early LTP is considered protein synthesis independent, while late LTP is blocked by anisomycin (Straube et al. 2003). Synaptic growth during the late phase may lead some synapses to develop new release sites (Bolshakov et al. 1997).

In studies using minimal stimulation, more rapid change has been observed (O’Connor et al. 2005, Petersen et al. 1998). The fast, quasi-binary change observed in these studies suggests a process such as simultaneous insertion of many postsynaptic receptors or ‘activation’ of a presynaptic release site. The latency for this change varied across synapses but averaged about 50 seconds in both studies, a value similar to that found in a postsynaptic-only study using glutamate uncaging (Bagal et al. 2005).

Rapid presynaptic change is seen in other forms of potentiation. A brain-derived neurotrophic factor (BDNF)-mediated change in release probability ‘awakens’ synapses following a train of theta-burst stimulation in culture (Yang et al. 2003). LTP due to application of BDNF causes change in the frequency of spontaneous events almost immediately after application (Alder et al. 2005). In that study synaptic charge changed in biphasic fashion following application of BDNF, with a drop at around 10 minutes followed by an increase. Interestingly, similar nonmonotonic change was also reported by other investigators. Zakharenko et al. report drop-rise kinetics of potentiation in the presence of amino-pentavalerate (APV), suggesting that different pathways, possibly N-methyl-D-aspartate (NMDA)-type glutamate receptor-dependent LTP and calcium channel-dependent LTP, correspond to the different temporal components of LTP, expressed initially through
change in the presynapse and then through change in the postsynapse (Zakharenko et al. 2001). To date, however, little work has directly examined the time course of potentiation due to the spike-timing protocol.

1.2.4 Change in short-term dynamics

Changes in synaptic strength due to activity occurring on the timescale of tens of milliseconds, hypothesized to contribute to neural information processing (Destexhe and Marder 2004), is altered at many synapses by LTP (Appendix C). Changes in this short-term plasticity (also called synaptic dynamics, SD) that follow the induction of long-term potentiation may represent alterations in pathways common to both forms of plasticity and provide a means for identification of cellular processes involved. Intersection of the pathways could occur at essentially any stage in the process of transmission. SD is heavily influenced by presynaptic calcium (Dittman and Regehr 1998), though there is evidence that LTP causes change downstream of calcium influx (Schiess et al. 2006). Facilitation persists after calcium becomes undetectable (Atluri and Regehr 1996), suggesting that calcium modifies some component of the release machinery in a manner that persists after calcium has been sequestered. Similar, but longer-lasting, modification of release apparatus could be a mechanism of long-term potentiation. To date, the effect on SD of STDP has not been measured.

1.3 SUMMARY

Many questions in synaptic transmission and plasticity have resisted resolution
because the stochastic nature of the processes involved and the presence of multiple synapses obscure activity at any one site. The dominant causes of short-term plasticity, the influence of previous activity at a synapse on ongoing activity, and the independence of nearby boutons on the one hand and the mechanisms of spike-timing-dependent long-term potentiation on the other both bear further investigation with methods that reduce the number of synapses contributing to measured responses.
2.0 MICROPERFUSION METHOD FOR MEASURING ACTIVITY AND PLASTICITY AT SMALL NUMBERS OF SYNAPSES

2.1 OVERVIEW

The probabilistic character of synaptic function makes it difficult or impossible to determine the mechanisms involved in synaptic transmission when the number of synapses contributing to measured activity is too large. To limit activity to few synapses a method is presented that builds on the class of technique pioneered by Katz and Miledi, localized perfusion. The method is spatially precise and temporally stable, as demonstrated by several control experiments. The method is used to address mechanisms of short-term plasticity, to investigate the independence of nearby synapses on the same axon, and, in a preliminary experiment, to measure the rapid effect of neuromodulatory input on synaptic function.

2.2 INTRODUCTION

Cell culture is an important research tool in neurobiology because the culture approach is well suited to imaging (Ehlers et al. 2007), electrophysiology (Lau and Bi 2005) or both (Murphy et al. 1995). While the overall number of synapses per neuron formed in culture is generally less than in the brain (Schikorski and Stevens 1997),
neurons in culture are constrained in a roughly two-dimensional environment, resulting in the formation of increased numbers of synapses between pairs of neighboring cells. As a result electrophysiological studies of a unitary synaptic connection in culture can involve hundreds of synaptic contacts from the soma to the distal dendrites of the postsynaptic neuron. The postsynaptic current generated when the presynaptic cell spikes is thus a sum of the activity at all of these sites. The averaging of responses across sites can distort interpretations of synaptic function: the greater spatial density of sites near the postsynaptic soma would bias measured responses toward a ‘proximal’ phenotype (Froemke et al. 2005), and differences in function and plasticity at individual contacts, likely to be large (Rosenmund et al. 1993), would not be resolvable in somatic recordings.

Restricting activity to small numbers of synaptic sites can minimize the distortions present when average population activity is recorded. This class of method was used at the neuromuscular junction to determine definitively that calcium was not necessary for the propagation of the nerve impulse but was required for neurotransmitter release (Katz and Miledi 1965). More recently it has been used to measure the saturation of glutamate receptors (McAllister and Stevens 2000) and to determine the spatial specificity of dendritic protein synthesis (Sutton et al. 2006). Reasoning that focal enabling of synapses could provide improved mechanistic resolution for the study of short- and long-term action potential-dependent synaptic plasticity, we adapted the method to our system as presented in this chapter. Improvements to the method are also described that result in a constant laminar flow stream and unintrusive, continual measurement of flux through the microperfusion system. After presenting the method, this chapter also reports the results of experiments exploiting the acuity of the method. These experiments demonstrate that depletion is not a primary determinant of short-term plasticity at these synapses. They show that the responses of individual synapses contain little record of recent activity. They reveal that synapses on the same axon
function independently, as theory had predicted (Franks et al. 2002). They also show that neuromodulatory input with dopamine can lead to rapid and persistent activation of synapses.

2.3 MICROPERFUSION METHOD

2.3.1 Modifications of the culture technique

The culture protocol was slightly modified from that generally used (Wilcox et al. 1994; http://bilab.neurobio.pitt.edu/dwn/protocols_dwn/). The changes were designed to achieve optimal neural density for microperfusion experiments and dendritic ‘splay’ sufficient for finding isolated axonal-dendritic connections on healthy glial beds. Poly-l-lysine (1 mg/ml in H2O, Peninsula Labs) was stamped using custom stamps (Allegheny Marking) or sprayed using an Airbrush (Aztec) onto 12 mm #0 cover slips (Karl Hecht). Cover slips were dried for 24 hours, soaked for 24 hours in culture water, and dried again before pre-incubation with culture medium for 12 hours prior to cell plating. Cells were plated at 15-25,000/ml. Given the size of the polylsine islands this density results in 5-15 cells/island, which in turn results in a distance of 5-10 μm along a given dendrite between boutons formed by the same presynaptic neuron at the age at which cells were used for experiments, 11-17 DIV. Mitotic inhibitors such as cytosine arabosinoside were not used.

All experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

2.3.2 Modifications of the patch technique
Perforated patch-clamp (Rae et al. 1991) is well suited to long-duration plasticity experiments, and is also ideal for holding patches in the low (0.2 mM) calcium external solution used in microperfusion experiments to prevent evoked release. 3 μl of amphotericin B (Sigma, 40 μg/μl in DMSO) was mixed with 600 μl internal solution and dissolved thoroughly, then back filled into pipettes (Kimax, pulled to 2.0-2.2 MΩ) after tip-fill with amphotericin-free internal solution. Internal solution contained (in mM, Fisher Scientific) K-gluconate 136.5, KCl 17.5, NaCl 9, MgCl₂·6H₂O 1, HEPES 10, EGTA 0.2 at pH 7.4 and 326 mOsm. External bath solution contained NaCl 150, KCl 3, CaCl₂·2H₂O 3, MgCl₂·6H₂O 2, HEPES 10, and Glucose 5 at pH 7.4 and 328 mOsm.

Patches were obtained in normocalcemic solution on cells with processes oriented roughly normal to the direction of perfusion flow. For microperfusion, low calcium external bath solution, as above but with 4.8 mM Mg²⁺ and 0.2 mM Ca²⁺, was used.

2.3.3 Microscope

Experiments were done on a Leica inverted scope (DMIRB) with a moveable support base and a freestanding stage to permit independent movement of the scope and the sample. The stage holding the perfusion chamber was rotated for orienting neuritic processes normal to the microperfusion stream prior to recording. A 20x DIC objective was used for patching and a 40x non-DIC objective was used for imaging labeled boutons.

2.3.4 Single-cell labeling and imaging
Experiments could be done blindly or with labeling. For labeling, SynaptoGreen (Biotium) was perfused at 12.5 μM in normocalcemic solution supplemented with 10 μM CNQX (Sigma) to prevent synaptic current through AMPAR. Because the dye adhered to the microperfusion pipettes they were held above the solution during labeling. The presynaptic cell was stimulated to fire Poisson-distributed trains of APs at a mean rate of 5 Hz. 7 trains of 25 pulses were evoked with one minute between trains and following the final train. Because only one cell was stimulated only boutons from that cell were labeled, simplifying the search for synaptic contacts between the patched cells. After the staining protocol, dye was cleared from the perfusion chamber with a fast 30 second rinse with bath solution. Following this a second rinse with 200 μM dye-scavenging cyclodextran Advasep7 (Cydex; Kay et al. 1999) was carried out in a reduced calcium (1.5 μM Ca²⁺, 3.5 μM Mg²⁺) bath solution to minimize loss of signal from boutons due to spontaneous release. Advasep was essential for reducing background sufficiently to allow visualization of single boutons. Because FM fluoresces when bound to Advasep, an additional fast rinse with normal solution to clear away residual Advasep was used. During rinses the green filter and the 40x lens were moved into place and the camera light path was opened.

A green bandpass filter (Chroma) was used to visualize fluorescently-labeled boutons. The fluorescent light source (Ludl) was gated with a shutter (Uniblitz) under control of the CCD camera driver software. Labeling was checked by eye then captured using the CCD (PCO). 800-1000 ms exposure was used for capturing fluorescence without causing detectable phototoxicity. Fluorescent images were subjected to an intensity threshold using Matlab (Mathworks), then rendered partially transparent (Glass2k, Chime) and overlaid on the ongoing visible light
image to guide placement of microperfusion pipettes. For automated serial imaging, custom software was used to trigger the shutter and the exposure.

When performed, single-neuron labeling (Murthy et al. 1997) simplified the task of finding isolated boutons (Liu and Tsien 1995). Moderate intensity boutons were selected (Chen et al. 2004) as these were considered likely to contain only a small number of release sites. The drawback to single-neuron labeling was that it required holding the cells during fairly intense stimulation in the presence of dye, which could compromise patch quality slightly, and it delayed the start of recording. As an alternative, when a single, properly oriented dendrite was visible, it was straightforward to identify release sites in low Ca$^{2+}$ bulk perfusion solution without labeling by running the positive perfusion tip along the length of the dendrite while stimulating the presynaptic neuron. When a PSC was seen the negative tip was used to isolate responses. In this way visual information about the bouton, such as size, was unavailable, but this was offset by the better neuron health, higher patch quality and longer recording durations.

2.3.5 Microperfusion

Focal perfusion was established with flow from a positive pressure pipette containing normocalcemic solution across the tissue into a negative pressure pipette capturing the normocalcemic solution and removing it from the bath (Figure 2.1). The two pipettes were placed such that flow passed over only one visible neurite, a dendrite accompanied by a presumptive axon invisible at this magnification.
**Figure 2.1**: The microperfusion system. A process of the presynaptic neuron (top) meets a process of the postsynaptic neuron (bottom) in the middle of the frame. The positive pressure microperfusion tip (right) is apposed to the neurites. It delivers normocalcemic solution to the cells. The solution is then removed by the negative pressure tip (left). Scale bar 25 μm.

Headstages were on manipulators in the upper left/lower right corners of the stage, while the microperfusion tips were placed lower left and upper right (Figure 2.2). With right-to-left bulk perfusion, the right-hand microperfusion tip was set for positive pressure and the direction of the microperfusion flow was made nearly parallel to that of bulk flow by the orientation of the tips to minimize turbulence and mixing. The microperfusion pipettes were mounted to move vertically during replacement so as not to interfere with patch pipettes, which swung horizontally; this permitted changing patch electrodes without moving the microperfusion pipettes. The stability of the microperfusion electrodes was crucial to ensure constant microperfusion of the same site over the course of the experiment (Supplementary Movies 1 and 2\(^1\)). Bulk perfusion during the experiment was from a constant-flow syringe at 1-1.5 ml/min. The bath liquid height could influence the

---

\(^1\) Supplementary movies are at http://www.cnbc.cmu.edu/~david_nauen/microperfusion_movies_dwn.htm
siphon pressure and thus was kept constant throughout the experiment. Solution height in the perfusion chamber was kept low, and bulk solution removal was done with a standard height-based vacuum system (Warner).

**Figure 2.2:** Pipette mounting configuration for the microperfusion system. A) Top view. Back right mount holds the positive microperfusion tip, which is connected to source of pressurized air (not shown). Front left mount holds the negative microperfusion tip, connected to the siphon (not shown). B) These two mounts swing vertically (arrows). Front right and back left mounts hold headstages for patch electrodes. These two mounts swing horizontally, toward the experimenter. Any tip can thus be removed or changed without interfering with others. (Mechanical drawing by Jay Jacobs.)

2.3.5.1 Positive pressure: Positive-pressure solution was filtered (0.2 um) and spun (12,000 RPM, 3 minutes; this did not alter osmolarity) before use to prevent occlusion by precipitate. The air pressure to the positive tip was taken from the building compressed air supply, filtered through 5.0 and 0.1 um filters and a low-precision protective regulator, then passed through a high-precision regulator (filters and positive pressure regulators, Wilkerson) that permitted fine control of the pressure on the solution and high stability. Before reaching the perfusion liquid the
pressurized air was also passed through an electronically controlled valve (Picospritzer) that permitted software controlled opening and closing.

During set-up for each experiment, confirmation of positive pressure was obtained visually by placing the tip near the edge of a glial island and manually triggering the pressure to cause movement of cellular debris. The positive pressure was then set to on for the duration of the experiment using a software-controlled digital high TTL to the Picospritzer. Constant pressure of either 2 or 3 psi was used for focal delivery of solution.

2.3.5.2 Negative pressure: Collection and removal of normocalcemic solution was performed by a siphon of bath solution flowing from the perfusion well to a collection point beside the stage and a suction line removing solution at the collection point. The negative pressure tip was a pipette pulled to low resistance (~200 kΩ) and fire-polished to smoothness, held in a microinjection-style holder (Warner). The siphon line (Tygon 3606, 1/32” I.D.) attached to the holder was sealed at all junctions against pressure loss. The shortest feasible tube was used to reduce the siphon priming time and to eliminate the possibility of a turn in the tube affecting suction pressure.

Negative pressure was regulated at the junction of the siphon outflow with a vacuum line (Appendix A). Observation of dye streams on glial beds and time-lapse movies of flow (Supplementary Movies 1 and 2) indicated that negative pressure was effectively constant over at least 80 minutes, while much lower and more finely regulable negative pressures were obtained than would be possible with suction alone. The presence of a stable laminar flow ensured a constant microenvironment for the microperfused bouton. The constancy of the stream was a precondition to investigations of synaptic plasticity. Positive flux was ~0.05 μl/min and negative flux was ~0.75 μl/min. Negative flux was thus ~15 times positive flux, which was
sufficient to ensure total capture but gentle enough for long-lasting seals and healthy cells.

2.3.6 Measuring flow

The siphon flow was only visible on phase contrast optics when the global bath solution was water and the microperfusate was extracellular solution or vice-versa, so optical measurements could not be made during experiments without adding dye. To confirm that siphon outflow, and hence negative microperfusion pressure, was constant during each experiment, the sound created as perfusate was removed from the siphon outflow by the vacuum line was recorded each sweep (Figure 2.3) with a precision microphone (Piezotronic) placed near the aperture in the housing for the siphon-suction junction.
Figure 2.3: Use of the acoustic signal to monitor microperfusion flux. A microphone placed at the aperture of the siphon-suction junction housing records liquid removal. All traces represent 10 s of recording. A) Recordings at 15 minute intervals show stability over time. B) The negative pressure of the siphon is regulated by varying the height of the s-s junction. Recordings at three negative pressures confirm that the audio signal reflects the pressure presented to the cell bed by the negative pressure tip. C) Audio traces recorded at 30 s intervals. An induced block occurred between the 30 second and 60 second sweeps.

A protocol for microperfusion experiments is provided in Appendix A.
2.4 RESULTS

Postsynaptic currents (PSCs) recorded in the focal perfusion configuration were consistent with transmitter release from at most few boutons and with inactivity of the majority of synaptic sites involved in responses under conventional conditions (Figure 2.4). The reduction of peak PSC relative to standard perfusion was generally from 10-50 fold and the possibility of polysynaptic activity was eliminated.

![Image](image.png)

**Figure 2.4**: Reduction of the number of active synapses with microperfusion. Left: Overlay of two trials in the same experiment, one under standard perfusion and one under microperfusion. Right: Close-up of the microperfusion trial showing the area overlaid with the gray box on the left. Stimulus artifact has been blanked for clarity.

To confirm that microperfusion of normocalcemic solution was necessary for release of transmitter and thus that release originated from within the

---

1 Note: The studies reported in this thesis investigated glutamatergic synaptic transmission, which is depolarizing. By convention this current is often plotted such that greater values are lower. Hence the ‘rising phase’ of a PSC waveform, for example, refers to that portion at the beginning of the curve getting lower over time and ‘peaking’ at the trough.
microperfusion stream we removed the positive pressure to the microperfusion system for individual trials (Figure 2.5). Without local perfusion no responses were seen, and when local perfusion was resumed, responses returned as soon as could be assessed. At this synapse, as in most synapses studied, short-term synaptic facilitation was greater after brief inter-pulse intervals, indicating that had positive pressure not been stopped the second trial (Figure 2.5) would have been the most likely to cause release. Turning off microperfusion reliably silenced release at all synapses tested.

**Figure 2.5:** Local application of normocalcemic solution is necessary for evoked synaptic transmission. Three consecutive trials are shown. Positive pressure to the microperfusion tip was stopped between the first and the second trial, then resumed following the second trial.

To assess the physical stability of the system we performed time-lapse imaging of the focal perfusion stream. Using water as the bulk perfusion solution, the extracellular bath solution used in the microperfusion stream becomes visible on phase-contrast optics. Pipette tip positions and stream characteristics were stable for at least 80 minutes (Supplementary Movies 1 and 2). During time-lapse imaging of patch-clamp experiments a small amount of thermal drift of the patch pipettes on
the cell bodies is generally observed. We compared the stability of the patch pipettes, which were sufficient for recordings of 2 hours or more, to that of the microperfusion pipettes and found the latter appeared equally or more stable over time (data not shown). The acoustic signal generated by perfusate removal was measured (Methods) for monitoring the stability of the microperfusion flux.

To confirm the functional stability of the configuration we recorded postsynaptic responses to brief trains of 3-4 presynaptic action potentials at inter-trial intervals of 40-50 s for periods of 25-55 minutes. As expected due to the stochastic quality of the function of few synapses, PSC responses varied from trial to trial (Figure 2.6), but no trend was observed. To quantify the stability we fit the values of the first response in each train with a line. When responses are fit linearly the resulting slopes range from -0.0868 to 0.0867, with a mean of 0.0015. When change point analysis (Chapter 3) is conducted on these PSC values the range is from 17.3 to 80.6% probability of there being a change point, with the mean 51.1%, all consistent with no change. *t*-test comparison of the first half versus the second half of each recording yielded no significant difference, with the range of p-values from 0.46 to 0.83 with a mean of 0.62. To test the contribution of the negative pressure component of the microperfusion system to the observed fluctuations in responses, that component was not used in some experiments (Figure 2.6E). Responses to presynaptic APs at this synapse showed similar fluctuation to others, indicating that the fluctuations are not due to variations in negative pressure but rather to variability at the synapse itself. Because the negative pressure component of the microperfusion system was necessary to get responses consistent with single or few synaptic contacts in most experiments it was in place during all experiments reported in this document apart from this test.
Figure 2.6: Functional stability of the microperfusion system tested at five synapses. Responses are to the first presynaptic AP in each train unless those responses were essentially all synaptic failures, in which case the second response in each train is shown. Dotted line shows linear fit. The data in panel E were recorded with the positive, but not the negative, component of the microperfusion system in place.
We then sought to test for any distortion of synaptic responses due to the microperfusion configuration. Glutamate receptor antagonists have been used in some localized activation studies to block synaptic currents at non-enabled sites, but if mixing occurs, this could alter the waveforms recorded from the active site. Conversely, reduction of calcium for disabling synaptic release, as done here, would not be expected to affect waveforms. To test for distortion in our system we compared responses from 4 classes of experiments: evoked APs with localized perfusion, evoked APs with standard perfusion at weak synaptic connections, evoked synaptic release with local application and removal of hyperosmotic solution using the microperfusion system, and AP-independent events recorded during bath application of the sodium channel antagonist tetrodotoxin (Figure 2.7). The action potential-evoked events recorded under small synapse standard perfusion and microperfusion were qualitatively similar, suggesting that microperfusion does not distort waveforms. Synaptic events recorded following sucrose application were also similar, as were those recorded in TTX. The distribution of event sizes recorded under conditions of AP block, taken in some studies as an indicator of the quantal size, is comparable to that of the microperfusion PSCs in the experiments shown here (Figure 2.7D, cf. Figure 2.7B and sizes shown in Figure 2.6).

---

1 Because of the small contribution of Ca\(^{2+}\) flux to PSCs at the hyperpolarized postsynaptic holding potential used here (Skeberdis et al. 2006), lower [Ca\(^{2+}\)] due to potential mixture of global perfusate into the microperfusion stream (Johnson and Ascher 1987) is likely to have little measurable effect on PSC.
Figure 2.7: Comparison of PSC waveforms. In each case 20-30 typical waveforms are shown with the distribution of peak amplitudes and a single representative trace. A) Evoked events from a weak connection recorded in normocalcemic solution. B) Evoked events recorded in microperfusion configuration. C) Events due to application of hyperosmotic solution in microperfusion configuration (single 10 sec application). D) Spontaneous events recorded in 1.5 μM TTX, 10 μM BMI (540 sec recording time).
To determine the latency of locally perfused synapses the interval between the presynaptic AP and the onset of the PSC was measured. AP onset was taken to be the first down-going sample in the $I_{\text{Na}}$ notch and PSC onset (Figure 2.8) was taken as the first sample to cross threshold for template-fit (Clements and Bekkers 1997). Apart from occasional biphasic cases (discussed below), values for latency were narrowly distributed (Figure 2.9, bar graphs), though they showed long tails (Figure 2.9, cumulative histograms), as expected considering the possibility of asynchronous release. There was no clear trend for latencies to become longer or shorter over the successive responses to each train (Figure 2.9).

![Figure 2.8: Method for measuring latency. A) Detection of onset of presynaptic sodium current (green circle), taken as time zero. B) The artifact in the postsynaptic membrane current recording due to the end of the presynaptic stimulation (cyan) is removed to eliminate a potential source of error. C) Membrane current (black) and detection criterion (red) with threshold (blue). Crossing of threshold is used to indicate successful release, and the time of threshold crossing is taken as the event onset time.](image)

In infrequent cases (12 of 199 pairs of neurons) postsynaptic currents had two components separated by large and consistent differences in latency (Figure 2.9). These biphasic responses may reflect the presence of two separate axonal branches of the presynaptic neuron within the focal perfusion stream. The width of latency
distributions were comparable around each peak to the monophasic distributions observed at other synapses.
Figure 2.9: Latency measurements at individual synapses. A) Cumulative histograms of latencies following the first (red), second (green), third (blue) and if applicable fourth (black) pulses in a train, with mean values in bar graphs. Error bars show SEM. B) Double response seen in one case (A, first column, third down), leading to bimodal appearance of cumulative histograms.
We next performed experiments in the system to determine the importance of neurotransmitter depletion in short-term depression. Both facilitating and depressing processes (Zucker and Regehr 2002) are likely to occur at central synapses. Known mechanisms of short-term facilitation, primarily calcium accumulation in the presynaptic terminal, are release-independent. On the other hand the most commonly considered cause for depression is depletion, which requires release. With few release sites, if depletion affects transmission, the depletion due to a successful release would render a second release less likely to succeed. The presence and extent of facilitation, which does not depend on release, would have the same effect on the state of the synapse at the time of second response regardless of the success or failure of the first response.

To evaluate whether depletion of vesicles causes short-term depression at excitatory hippocampal neurons we considered the success rate for the second response based on the success or failure of the first response with a fixed IPI of 50 ms (Figure 2.10A). In no synapse studied was there a significant difference. As the failure rate of the first pulse was generally higher, we then considered this rate based on the success or failure of the second pulse as a test for a fluctuating synaptic state that could affect the release dependence result (Figure 2.10A). The absence of such a relationship is consistent with fluctuations in synaptic state having no effect on responses given the 50 ms IPI used here.

---

1 Fluctuation in release probability (Volynski et al. 2006) is unlikely to contribute across the range of IPIs used here, mostly exerting measurable effects on the time scale of 20 ms or less (Dobrunz et al. 1997).
Figure 2.10: Depletion does not influence short-term plasticity at hippocampal synapses in culture. A) Green bars show mean and error (Agresti-Coull binomial CI) proportion of PSC2 successes depending on whether PSC1 succeeded (left) or failed (right) at 5 synapses. Purple shows the success proportion for PSC1, depending on whether PSC2 that trial succeeded (left) or failed (right). The success of either PSC does not predict that of the other. 50 ms IPI. B) Cumulative histogram of the size (pA) of PSC2 depending on the success (black) or failure (red) of PSC1 at 5 synapses. 50 ms IPI. C) Size of PSC1 versus size of PSC2 by IPI at 7-10 synapses. PSC1 values are normalized to <PSC1>, PSC2 to <PSC2>. Each color represents a synapse, each dot represents a pair of values, and each line represents a linear fit to the value pairs for that synapse.

Despite our efforts to limit release to at most a few sites, it was still possible that several synaptic contacts were release-capable, each having a low $p_r$. In this limiting case even if depletion affected individual sites, the large number of sites could mask
the effect; success could frequently be followed by success due to release from one of the other sites. However in this case if depletion influences responses the second response should still tend to be smaller when the first response succeeded. Hence we examined the size distribution of second responses based on whether the first response succeeded or failed (Figure 2.10 B). Kolmogorov-Smirnov (K-S) tests of the distributions showed no evidence at 5 synapses tested that depletion played any role in determining the size of the subsequent responses. The probability of the values of the two distributions, PSC₂ when PSC₁ succeeded and PSC₂ when PSC₁ failed, actually being drawn from the same underlying distribution, as calculated by the K-S statistic, was 0.60, 0.25, 0.94, 0.95, and 0.51 at the five synapses studied.

To consider the effect of depletion across a range of intervals, we varied the IPI to be either 25, 50, 100, 200 or 400 ms and compared first and second PSC values (Figure 2.10C). Responses were normalized to the mean for that response at each synapse. In the scatter plots, separated by interval, each individual pair of responses is represented by a dot, with color representing each synapse. The responses from each synapse were fit linearly. If depletion influences synaptic responses, with size terms taken as relative to the average for that response, large PSC₁ would tend to be followed by small PSC₂, and small PSC₁ by large PSC₂. In our experiments no clear trend was observed.

We next sought to determine the extent to which postsynaptic currents contain a record of the short-term activity at the synapse. For this purpose we used stimulation trains containing a variable inter-pulse interval (IPI) followed by a fixed IPI. We compared synaptic responses after the last IPI (50 ms) based on the duration of the penultimate, variable IPI (50 or 400 ms, Figure 2.11). Some asynchronous release is observed because these are responses to the fourth pulse in the train. No visually detectable difference between the waveforms of the two groups is apparent. Similarly, Kolmogorov-Smirnov comparisons of the cumulative distributions of
charge at each synapse showed no difference: The K-S test p-value for the response to the fourth AP separated by the second IPI at the synapse in Figure 2.11A was 0.19 and the synapse in Figure 2.11B it was 0.93. Across the remaining synapses tested values were 0.60, 0.52, and 0.14. This suggests that when few synapses are present there is no clear mechanism by which the postsynaptic cell could "read out" the recent history of activity in the presynaptic neuron from the PSC.

Figure 2.11: The inter-pulse interval (IPI) before the most recent IPI does not affect the synaptic response. A) Left, Waveforms elicited by the fourth pulse in a train of four when the IPIs were 50, 50, 50 ms. Because this is the final pulse in the train asynchronous release is evident. Middle: Waveforms elicited when the IPIs were 50, 400, 50 ms. Right, Cumulative histogram of the charge values for the two stimulus trains. Responses to the 50, 400, 50 trains are shown with red line. B) As A, for different synapse.

In rare cases synapses formed by the presynaptic cell onto both the postsynaptic
and the presynaptic (autaptic, Van der Loos and Glaser 1972) neuron were present within the microperfusion stream (3 of >225 synaptic sites studied, 1 case sufficient duration for analysis). This presented an opportunity to gauge the activity of the two sites simultaneously. When the presynaptic cell was stimulated in brief trains, successes were observed in the autaptic and synaptic currents, indicating release from neighboring focally perfused sites on the axon of the presynaptic neuron (Figure 2.12). Both currents showed failures and successes but interestingly we observed that these did not tend to co-occur. To quantify this we calculated the co-occurrence relative to that expected by chance given the success rates of each synapse. For the first pulse in the train 0.72 simultaneous successes were expected and 1 event was observed. For the last pulse in the train, 7.56 simultaneous successes were expected due to chance, 7 were observed. The co-occurrence values indicate that the nearby sites function independently. The greater latency of autaptic responses, Figure 2.12B, likely reflects the onset masking effect of the nonsynaptic current following the evoked AP in the presynaptic neuron.

The presence of the simultaneous synapse and autapse within the stream also provides information on whether failure of AP propagation at branch points contributes to the responses seen here. AP initiation was confirmed by observation of presynaptic sodium current for all events. For the last pulse in the train, the failure of both synapse and autapse occurs on only 44% percent of trials, considerably lower than the 81% for the synapse alone. This is consistent with the view that the bulk of failures seen at individual synapses in this preparation are failures of release rather than propagation.
Finally, as a test of the microperfusion system’s ability to monitor changes in synaptic strength, we exposed neurons in microperfusion to dopamine. After characterization of baseline synaptic responses for 30 minutes (4 AP trains, IPIs of 50 or 400 ms, 45 s ITI), dopamine (20 μM) was added to the extracellular bath solution for 15 minutes as stimulation of the presynaptic neuron to fire APs continued at the same rate. Dopamine was then washed out and the synapse was re-characterized for 30 minutes. Then the dopamine treatment was repeated and characterization continued (Figure 2.13). The postsynaptic neuron was held in voltage clamp and did not spike at any point. The first post-treatment epoch showed LTP, with mean

Figure 2.12: Adjacent synapses on an axon function independently. A) Top, Autaptic (red) and synaptic (blue) current both arise within the focally perfused region, indicating proximity on the axon. Middle, Success of synapse in isolation. Bottom, Success of autapse in isolation. B) Histogram of latencies of the two classes of events.
(standard deviation) of the first epoch 74.2845 fC (79.5588) and of the second characterization epoch 213.7779 fC (130.6649), *t*-test p-value of 4.3558e-006. 30 minutes later, DA was washed in again, resulting in further potentiation with values for the third characterization epoch of 387.2779 fC (144.2476), *t*-test relative to baseline p= 6.5109e-017, relative to second epoch p=5.5151e-008. The reason for the drop in charge during the second dopamine treatment is not known.

![Figure 2.13: Dopamine exposure increases synaptic strength in part by increasing release probability. A) Charge of the first response each train over time. B) The mean waveform (first response in train) from each epoch, epochs colored as in A.](image)

In this recording the focal region of high calcium included a second synapse active at long latency (Figure 2.13). Initially this long-latency site was essentially silent in the absence of facilitation. During the first DA treatment the short-latency response changed little but the long-latency response required less facilitation, becoming active after the first AP in the train on some trials. After the second dopamine treatment both sites increased in size and the failure rate of the long-latency component decreased. If only the first response is considered by measuring peak PSC within the window ending 14 ms after the AP, the mean (standard
deviation) values each characterization epoch are 65.6 pA (63.0), 147.1 pA (77.3), 217.0 pA (64.5). If only the second response is considered by measuring from 14 to 31 ms after the AP, the values are 7.4 pA (31.9), 49.4 pA (56.0), 137.7 pA (95.9). While it is important to avoid speculation on the basis of this single case, this evidence is consistent with the interpretation that exposure to dopamine can cause potentiation at least in part by increasing release probability.

2.5 DISCUSSION

2.5.1 The method

With 5-15 neurons per glial island present in our culture conditions, neurons receive less dense projections from each individual presynaptic cell than expected in conditions with only 2 neurons per island (Bekkers and Stevens 1995), making it easier to limit activity to few presynaptic boutons. Given the spacing between synapses along axons of cultured hippocampal neurons (Schikorski and Stevens 1997, Liu and Tsien 1995), the microperfusion stream used in these studies likely permits at most 2-3 boutons from the presynaptic neuron to be active (Figure 2.4). Dye labeling permits identification of individual boutons of a single neuron in the experiments where it is used. The siphon technique permits milder and more finely controllable suction than would be possible with vacuum suction, with no damage to neurons or glia. With double filtering of the pressurized air into the positive tip blocks are not observed. With fire-polishing of the negative pressure pipette, blocks are rare; the high flow rate relative to the surrounding medium means that any debris is quickly pulled fully into the pipette. Long baselines establish that drift is
minimal and the system is stable (Figure 2.6). The rapid appearance of synaptic events when calcium microperfusion is turned on and rapid disappearance when it is turned off (Figure 2.5) indicate that the puffed calcium acts directly at the synapse and is necessary for transmitter release. The use of low calcium rather than glutamate receptor antagonists to block activity at non-perfused regions avoids distortion of waveforms (Figure 2.7). The acoustic signature method (Figure 2.3) permits measurement of stream flux without the use of dye, which has the potential for neuroactivity (Augustine and Levitan 1980) or toxicity; measuring the acoustic signal does not affect the system. Perforated patch-clamp permits stable recording for several hours from synaptically connected pairs of neurons. The elimination of polysynaptic activity with focal perfusion removes a major potential confound to experiments in dissociated primary neural culture. These elements together make it possible to record action potential-evoked release and short- and long-term plasticity at individual synaptic contacts.

The accessible geometry of the culture configuration promotes rapid diffusion, and it is likely that studies requiring application and removal of local calcium on finer time scales would also be possible with this system. The system could be used to measure the latency from somatic $I_{Na}$ to the onset of synaptic current from any site along the axon (Figure 2.9), thus permitting a direct measure of the speed of AP propagation. In cases where two release sites, potentially on different axonal branches, lie within the microperfusion stream (Figure 2.9B), the covariance of the latencies could be used to estimate the variability of axonal propagation velocity. The laminar stream microperfusion configuration and acoustic method for flux measurement described are straightforward and can be adapted for any cell

---

1 Katz and Miledi used focal perfusion to discover the requirement for calcium in transmitter release and also to determine the rate of AP propagation (Katz and Miledi 1965). Due to the invisibility of the axon, propagation could not be measured in our preparation, but with vital dye labeling of the axon that would be possible. This would permit testing of the effect of STDP on reliability of the propagation machinery and rate of propagation (Daoudal and Debanne 2003).
biological investigation where a restricted portion of the cell surface is to be exposed to a solution different from that applied globally.

Despite our efforts to correctly identify successful versus failed PSCs, at the range of event sizes in the present study (see Figure 3.6) it is inevitable that errors are made. Due to our interest in correctly characterizing successful release events we adopted criteria favoring specificity at the expense of sensitivity (relative to visual assessment) throughout the study. Because presynaptic APs were separated by at least 25 ms\(^1\), longer than the time required for decay of synaptic current, the response to the first pulse had no influence on the evaluation of success/failure for the second pulse, so error in success/failure classification would exert no systematic bias on the measurements across these groups.

2.5.2 Depletion as cause of short-term depression

Responses to a second pulse are not influenced by the success or failure of release following the first AP, indicating that vesicle depletion is not a major influence on short-term plasticity in our system at the IPIs used (Figure 2.10A, B). Given the restricted number of release sites available in microperfusion, depletion would manifest as a trend for large responses to be followed by small responses; this was not seen. The assumption that successful release at a site temporarily reduces the likelihood of subsequent release underlies interpretations of the paired-pulse ratio, but recent evidence has challenged this assumption (Sullivan 2007, Brody and Yue 2000). The evidence of the present study is consistent with factors other than depletion being the dominant causes of short-term depression.

However depletion could still contribute if replenishment of the readily-

---

\(^1\) In one case an interval of 12.5 ms was used 1 of every 7 trials.
releasable vesicle pool happens more rapidly than could be detected with the IPIs used in this study (Hanse and Gustafsson 2001) yet more slowly than the recovery of the neuron from refractoriness. This suggests further tests with very short IPIs, though the greater likelihood of AP initiation/propagation failure at short IPI (Chacron et al. 2007, Brody and Yue 2000) must be considered. The results presented here establish that at the 50-100 ms intervals generally used to test paired-pulse ratio to determine whether $p_r$ has increased — because decreased PPR is assumed to reflect higher $p_r$ and greater depletion — depletion plays little role.

2.5.3 History dependence

The ability of synaptic dynamics to convey information about presynaptic activity history (Maass and Markram 2002, Fuhrmann et al. 2002) at single or few sites was tested by measuring responses after a fixed interval that followed a varied interval, grouping responses according to the varied interval (Figure 2.11). While the varied interval clearly influenced responses that immediately followed it (e.g., Figure 3.10) at these synapses, one pulse later this effect was gone 1. This suggests that limited information about history is contained in the short-term dynamics of individual hippocampal synaptic contacts. In the nervous system, multiple synaptic contacts may still carry such information in their collective function. This would suggest that the larger the number of contacts comprising a synapse the more information on recent activity history potentially present in individual PSCs, which could influence the coding mechanisms available to different neural circuits.

2.5.4 Independent function of nearby synapses

1 Figure 3.10 and others also show that the ordinal value of the pulse in the train has considerable additional influence on the size of the response.
The independence of nearby synapses implies the absence of metabolic or other local coordination of their activity. It may also influence the transfer of information between neurons (Franks et al. 2002). Activity at neighboring sites could be distinguished in a microperfusion experiment due to the chance occurrence of an autaptic and a synaptic contact within the local stream, allowing testing of independence (Figure 2.12). Because of the limited extent over which AP-evoked exocytosis is possible in the microperfusion configuration, it is assumed that the two sites lie along the same axon. Biphasic responses (Figure 2.9B) that may arise from different axonal branches could underlie the responses measured here, but those were seen in only ~6% of synapses, so this is unlikely. Comparison of synaptic and autaptic responses in microperfusion indicates that nearby synapses formed by the same axon function in an uncorrelated manner following each action potential. The responses were also consistent with limited influence of AP propagation failure in this system (Debanne et al. 1997).

The synaptic independence suggested by the present data would be reduced by crosstalk among sites through overspill of transmitter (Rusakov and Kullmann 1998). The degree of crosstalk will depend on the glial ultrastructure (Piet et al. 2004) which physically constrains diffusion and also determines transmitter removal from the cleft through transporter action. Neuronal, as opposed to astrocytic, transmitter transporters could also play a role (Brasnjo and Otis 2001). The rate of activity and the proximity of release sites will further influence independence; independent function is seen at inhibitory synapses unless the number of stimuli is large or the anatomical spacing of the sites is dense (Overstreet and Westbrook 2003). While excitatory and inhibitory synapses located several μm from one another on the axon function independently at low firing rates, the extent to which release sites within a single terminal (Schikorski and Stevens 1997) release independently is not known.
2.5.5 Dopaminergic activation of synapses

Previous studies have indicated that the neuromodulator dopamine may increase synaptic strength by altering presynaptic function (Geldwert et al. 2006) and modifying short-term plasticity, also through presynaptic changes (Tecuapetla et al. 2007). The application of dopamine provided a good means of testing the ability of the microperfusion configuration to monitor presynaptic change. In our preliminary experiment both the first and second treatments with dopamine caused considerable potentiation of the synapse (Figure 2.13). By chance the microperfusion stream in this experiment contained a second synapse from the presynaptic neuron to the postsynaptic, distinguishable by its greater latency and thus presumed position along another axonal branch, at further remove from the presynaptic soma. Initially this synapse was weak, though it responded late in the train due to facilitation. Following DA treatment the synapse became considerably more likely to release. This suggests that one mechanism by which dopamine potentiates neurons may be accelerating the maturation of synapses, increasing their release probability. Such an effect is likely to contribute to the change in short-term dynamics seen with DA treatment (Tecuapetla et al. 2007).

2.6 CONCLUSION

The stochastic character of synaptic transmission makes it desirable in studying synaptic function to measure responses from as few synapses as possible. This chapter presents a method, developed on the basis of techniques put forward by a
number of other researchers, for long-term, stable recordings of action potential-evoked transmission at very few synapses. The method includes modular elements (positive focal perfusion, negative focal perfusion, acoustic detection of liquid flow) that can be combined in the manner best suited to answering the question at hand and should be straightforward to implement.

Experiments with the method presented here demonstrate that at excitatory synapses between hippocampal neurons dopaminergic input can lead to rapid, persistent activation of synaptic sites, nearby sites on an axon release or fail to release independently, depletion does not determine short-term depression, and the function of individual synapses contains little record of the recent activity history.
3.0 MECHANISMS OF SPIKE-TIMING-DEPENDENT LONG-TERM POTENTIATION INVESTIGATED THROUGH STUDY OF ACTIVITY AT INDIVIDUAL SYNAPSES

3.1 OVERVIEW

STDP is a physiologically realistic model for learning and memory, but the cellular mechanisms involved are not fully known. In most investigations of this and other forms of synaptic plasticity, responses measured are nonlinear sums of activity at many synapses, which may obscure the underlying mechanisms. Using a method of local perfusion to reduce the number of active synapses we studied spike-timing-dependent LTP at excitatory synapses between hippocampal neurons in primary dissociated culture. Our results confirm that activity during induction is needed for individual synapses to potentiate. For synapses that potentiate, the state of the synapse prior to induction predicts the manner in which the synapse changes. The reliability (number of successes in a brief train) and potency (mean size of successful responses) change differentially following induction, with reliability generally changing earlier when both values change. Short-term plasticity was also altered, and the time of this change correlated well with the time of change in potency, suggesting that a single cellular change underlies both processes. Finally asynchronous release waveforms, which can be taken to represent individual quanta, were unchanged at a number of synapses for up to 20 minutes following induction, but at synapses where asynchronous release did change, the time of change also correlated with the time of change in potency and synaptic dynamics. Taken
together these results suggest that the form of potentiation initially involves change in the function of the existing presynapse followed at a delay by the addition of new synaptic sites.

3.2 INTRODUCTION

A number of studies have focused on determining the timing rules of STDP (Froemke and Dan 2002, Sjostrom et al. 2001, Nevian and Sakmann 2004, Wittenberg and Wang 2006., Bender et al. 2006), but the underlying cellular modifications caused by induction of spike-timing-dependent potentiation are not fully known. In the case of timing-dependent long-term depression, a presynaptic expression mechanism has been identified (Sjostrom et al. 2003), and in the case of potentiation it is known that distinct signaling cascades play a role (Wang et al. 2005), but the relative contributions of pre- and postsynaptic change are unclear. It is also not know what functional and structural changes take place at the synapse when STDP is induced.

Some studies of classical LTP find largely presynaptic contributions to potentiation, at least in young animals (Lauri et al. 2006), though in other studies the fraction of potentiation driven by presynaptic change can vary considerably across experiments (Liao et al. 1992) and may depend on the preinduction release probability (Larkman et al. 1992). Other reports implicate postsynaptic change as the primary mechanism of potentiation (e.g., Manabe et al. 1992, Isaac et al. 1998). One process subserving postsynaptic change is the insertion of AMPA-type glutamate receptors into previously NMDA-only ‘silent synapses’ (Kullmann 2003, Kullmann 1994). A recent study (Ward et al. 2006) found that silent synapses are presynaptically active prior to induction, and their release probability is not affected
by a first induction of LTP, though in other studies, alteration in the function of presynaptically silent (“mute”) synapses has been found to underlie LTP (Gasparini et al. 2000). For spike-timing-dependent long-term potentiation the relative contributions of presynaptic and postsynaptic change and the degree of involvement of silent synapses are not known.

Short-term dynamics contributes to neural circuit function (Destexhe and Marder 2004), but the effects on these dynamics of long-term synaptic change is variable (Appendix C). In comparing responses to brief trains of APs before and after conventional LTP, in some cases only the first response increases, suggesting a presynaptic locus (Markram and Tsodyks 1996), while in other cases all responses to the train are scaled up, consistent with a postsynaptic locus (Pananceau et al. 1998). In addition to providing information on the mechanisms of plasticity, comparisons of short-term dynamics before and after induction can offer insight into how the synaptic change could affect circuit processing in the brain. As with the locus of plasticity, it is increasingly appreciated that different brain regions and different plasticity protocols have different effects on synaptic dynamics (Buonomano 1999). This may be due to the experience of the synapse (Ward et al. 2006) or to the precise synaptic state at the time of the induction (Ismailov et al. 2004) that could dramatically alter the response to induction through nonlinear dynamic processes.

In paired recordings, both cells can be voltage-clamped, but the synaptic currents measured from multiple synaptic contacts reflect the net variability of those contacts. To improve our resolution in studies of the mechanism of plasticity we have developed a means of recording action-potential evoked activity from a small number of synaptic boutons based on the method used by Katz and Miledi (Chapter 2). Here we report results of experiments involving few synapses to investigate the mechanisms of STDP.

---

1 Stochastic fluctuations in synaptic state as part of cellular metabolism would seem likely.
3.3 METHODS

3.3.1 Culture

Dissociated primary embryonic rat hippocampal neurons were cultured as described previously (Wilcox et al., 1994, http://bilab.neurobio.pitt.edu/dwn/protocols_dwn/). Briefly, the hippocampi of E18 embryos were removed and placed in a trypsin solution (0.25% in HBSS) at 37°C for 15 minutes. Cells were then mechanically dissociated by trituration and plated at 15-25,000 cells/ml on glass cover slips (Karl Hecht) in 35 mm Petri dishes (Falcon). Prior to plating cover slips were patterned with microislands of poly-l-lysine (Peninsula Labs) applied using custom rubber stamps (Allegheny Marking) or an airbrush (Aztec). The culture medium was DMEM with glutamine (BioWhittaker) supplemented with 10% heat-inactivated bovine serum (Hyclone), 10% Ham’s F-12 with glutamine (BioWhittaker), 50 units/ml penicillin/streptomycin (Sigma) and 2% B27 supplement (Gibco). In some cases previously cultured glial cells were preplated onto the cover slips 24 hours before neuron plating. 24 hours after culture, one third of the medium was replaced by medium supplemented with 20 mM KCl solution to stimulate activity. Cells were fed 25-50 μl of culture medium each week. Dishes were kept at 37°C in 7% CO2 incubators (NuAire). After 11-17 days in vitro neurons were used for experiments.

All experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.
3.3.2 Electrophysiology

Cover slips were placed on an inverted microscope (Leica DMIRB) and perfused continually (~1 ml/min) with solution of the following, in millimolar: 150 NaCl, 3 KCl, 3 CaCl2·2H2O, 2 MgCl2·6H2O, 10 HEPES, 5 glucose. Intracellular solution contained 136 K-gluconate, 17.5 KCl, 9 NaCl, 1 MgCl2·6H2O, 10 HEPES, 0.2 EGTA. All compounds used in these solutions were from Fisher. Internal and external solutions were titrated to 326-8 mOsm concentration at pH 7.4. All experiments were performed at room temperature.

Patch electrodes (Kimax) were pulled (Narishige) to 2.0-2.2 MΩ resistance, tip-filled with internal solution and back-filled with internal solution mixed with Amphotericin B (200 µg/ml, Sigma) for perforation. Visually identified neurons on glial microislands were patched and test pulses were delivered to determine synaptic connectivity and transmitter phenotype. Stimulation and recording was performed with an Axon 700B amplifier (Molecular Devices) controlled by custom software in Matlab (Mathworks). Pairs of glutamatergic neurons having at least one monosynaptic connection were used in experiments. Sampling was at 10 kHz and filtering was with a 4-pole Bessel at 3 kHz.

3.3.3 Labeling

In some experiments the release sites of the presynaptic neuron were labeled. Single cell labeling was performed with wash-in of Synaptogreen (12.5 µM, Biotium) while the presynaptic cell was made to fire aperiodic trains of action potentials (20 Hz mean rate, 25 pulses/train, one train/minute, for 7 minutes). After labeling, background signal was reduced by wash-in of Advasep-7 (1 mM, Biotium) for approximately 5 minutes. Cell images were captured using a CCD camera (PCO
Coolsnap, CamWare driver); bouton fluorescence was elicited using an arc lamp (Ludl), a software-controlled shutter (Uniblitz) and a fluorescence filter (Chroma). In other cases where the dendritic arborization suggested that boutons might be found by blind test placements of the microperfusion stream, labeling was not performed.

### 3.3.4 Microperfusion

Extracellular solution containing sufficiently low calcium to prevent evoked release from the 3-4 pulse trains used in this study (0.2 mM Ca$^{2+}$) was washed in globally. Locally, a laminar flow of normocalcemic solution was established using positive and negative pressure pipettes. For the positive tip, continuous pressure at either 2 or 3 psi, constant over the experiment, was delivered to a pipette pulled to a tip size of 5-7 MΩ filled with extracellular solution containing 3 mM Ca$^{2+}$. For the negative tip, a ~200 kΩ pipette, fire-polished for smoothness, was connected to a siphon column to generate negative pressure. Control experiments (Figure 2.6, and Supplementary Movies 1 and 2) established that the normal calcium microperfusion stream was constant and approximately 2-3 μm wide and that release from a bouton depended on the presence of the stream.

For experiments with labeling, the fluorescence image was superimposed on the live camera image using shareware (Glass2k) and the microperfusion system was apposed to visually identified boutons. For blind experiments the microperfusion system was applied to various locations through the dendrites of the postsynaptic neuron and test pulses were evoked in the presynaptic cell until responses consistent with single or very few boutons were obtained.

### 3.3.5 Characterization and potentiation
Trains of 3 or 4 action potentials were evoked in the presynaptic cell for characterization of synaptic dynamics. One inter-pulse interval was parametrically varied each trial. Variation was random but constrained such that no timing could repeat \( n+1 \) times until all had been presented \( n \) times. During baseline, both pre- and postsynaptic cells were held in voltage clamp at -70 mV. Inductions of STDP were performed by switching the postsynaptic cell into current clamp with injection of sufficient current to yield a postsynaptic \( V_m \) of -65 mV +/- 1 mV. APs were evoked in the presynaptic neuron then 10 ms later in the postsynaptic neuron. This pairing was repeated at 1 Hz for 60 s. After this the postsynaptic neuron was returned to voltage clamp and dynamics was re-characterized. The microperfusion configuration was used throughout the baseline, induction and postinduction periods. Potentiation was evaluated by comparing the mean charge of either the first response in the train or all responses over at least 10 minutes of consecutive recording just before induction and at least 10 minutes of consecutive recording beginning at least 15 minutes after induction using \( t \)-tests.

3.3.6 Analysis

In all experiments the presence of presynaptic sodium current was confirmed for each action potential.

Determination of the presence or absence of postsynaptic current was performed algorithmically with a template-detection method (Clements and Bekkers 1997). The criterion was set empirically for each synapse.

In figures where multiple postsynaptic currents are overlaid, the y-offset of each was subtracted. In some figures membrane current is smoothed for clarity using a 1-
ms boxcar average. Binomial confidence intervals were performed with the Agresti-Coull method (Agresti and Coull 1998) implemented in Matlab. Cumulative histograms were compared using the Kolmogorov-Smirnov test.

Peak PSC was measured by finding the lowest-amplitude sample within a window following each presynaptic AP. This value was averaged with the 0.5 ms of activity that preceded and followed it to determine the amplitude measurement. Charge was calculated by adding all samples, with no filtering apart from the Bessel filter of the amplifier, after the AP over a fixed window for each synapse.

The slope of the bouton EPSP (bEPSP) during induction was found by taking the slope of the steepest one millisecond portion of the PSP waveform each pairing. The maximal slopes measured from each of the 60 pairings of the induction protocol were averaged to yield the slope value for that synapse.

To determine whether the induction protocol resulted in a change of reliability or potency two epochs were assumed and the change point separating the epochs was identified as the minimum in a vector containing a cumulative sum of differences from the mean (Taylor 2000). The change point was not constrained and could occur at any point from the second trial of baseline to the end of the experiment. Confidence intervals for the occurrence of a change point were calculated by randomly permuting the recorded sequence of observations, identifying the change point in this new sequence as the break point between two epochs, and comparing the deviation of points in each epoch from the mean of that epoch. Total deviation from the epoch means of the unpermuted data was compared to the total deviation from the epoch means of each permutation and the proportion of permuted cases having lower error was taken as the false-positive rate for the change point. 1,000 random permutations were performed.

When all pulses in a train fail, reliability is zero, but potency has no value. The use of no value was not compatible with the change point analysis used here, but
replacing these no values with the means of the two adjacent potency values allowed change point analysis and did not affect results.

Based on the observation that following induction responses after the short interval changed at a different time than did responses following the long interval, inter-pulse intervals were separated into two groups, short (25, 50, 100 and in one case 12.5 ms) and long (200 and 400 ms). The PSC size following the variable interval each trial was averaged with the preceding value from the same group, and values were interpolated across trials where the interval was from the other group. The time of change in short-term plasticity was identified as the onset of the 5 minute window during the postinduction period where the difference between the groups was greatest.

To determine the probability of a divergence of the magnitude observed being due to chance, the sequence of postinduction trials was permuted and the magnitude of the maximal divergence was re-measured. The number of iterations needed to observe a single instance of a magnitude greater than or equal to that in the recorded data (mean over 10 repetitions of up to 10,000 iterations each) was used a confidence interval for the likelihood of observing such change due to chance.

Asynchronous release events were detected in the 20-220 ms following the last pulse in each train using the template-matching method and the standard template used for determining success/failure but with a higher threshold, 4, to avoid false positives. Background events were measured over 1.4 s prior to the first stimulus in the train, and cases where this rate was high were not further analyzed.

All stimulation, recording, analysis and modeling software is available as Matlab m-files from http://bilab.neurobio.pitt.edu/dwn/matlab_m_files_dwn/
3.4 RESULTS

In order to confirm that the spike-timing-dependent protocol could result in potentiation in the microperfusion configuration we identified pairs of glutamatergic neurons, established focal perfusion, and characterized baseline responses, then applied a pre-post spike-timing protocol with a 10 ms interval (Figure 3.1). After induction the postsynaptic neuron was returned to voltage clamp and characterization of synaptic function was resumed. In 10 out of 15 experiments in this configuration potentiation was induced (Figure 3.2). In 8 of the 10 experiments potentiation occurred when the first pulse was compared, whereas 8 showed potentiation when all pulses were compared; the statistical noninductions for Q1 showed significant change in Qall at p=0.0375 and p=3.102exp-7 and the nond inductions for Qall showed significant change for Q1 at p=0.0412 and p=0.0170 (values by t-test). For the failed induction experiments there was no significant potentiation or depression when either the first (p-values: 0.6424, 0.4233, 0.1685, 0.1445, 0.0678) or all pulses (p-values: 0.9809, 0.5508, 0.1743, 0.1511, 0.1403) were considered (values by t-test).
Figure 3.1: Induction of STDP in the focal perfusion configuration. A) Diary plot of responses over time. STDP was induced at time 0. B) Five consecutive responses before (left) and after induction, recorded during the periods marked with colored bars in A. Scale bars: 10 ms, 20 pA.

Figure 3.2: Summary of focal perfusion STDP results. Mean charge transferred by the first pulse each train before v. after induction for all induction experiments, mean and SEM. Dotted line shows equality. Significant potentiation cases are assigned a color while failures are shown in black.

In examining differences between those synapses where potentiation was effective and those where it was not we hypothesized that activity at the synapse
during induction could contribute to this outcome. We compared the mean greatest slope of each natural EPSP prior to the postsynaptic current injection during the induction with the amount of potentiation ultimately observed (Figure 3.3). Activity during induction was low in all the failed induction cases (mean 0.11 mV/ms, max 0.14 mV/ms) while in the potentiation cases it was at least 0.19 mV/ms (mean 0.45 mV/ms), confirming that activity at the synapse is needed during induction for change in synaptic strength. Above this apparent threshold, though, there was no clear relationship between voltage and potentiation.

![Figure 3.3](image)

Figure 3.3: A threshold level of activity at the synapse during induction is needed for potentiation. A) The average of the peak slope of each EPSP during induction plotted versus the ratio of synaptic charge (first response in train) during the postinduction period to charge at baseline. Dotted lines separate successful from failed inductions. B) EPSP traces (left) and mean of all PSC waveforms before and after induction (right).

We sought to evaluate the possibility that the greater activity during induction at these synapses was due either to facilitating dynamics at 1 Hz or a positive feedback “real-time” induction (Figure 3.4). However the mean peak slope of the natural
PSPs showed no increasing trend over the course of the 60 pre-post pairings, consistent with the view that neither process contributes.

**Figure 3.4:** Absence of potentiation during induction. Panels show the maximum natural postsynaptic EPSP slope for each of the 60 pre-post pairings of the STDP induction protocol at each of the 10 synapses that ultimately showed potentiation. Values are divided by the mean for that synapse (horizontal line). "Real-time" potentiation would appear as an increase in the values over time.

Because few synapses were active in the localized perfusion protocol the number of failures was high, permitting division of the response into the probability of success of the first response \((p_{r1})\) and the size of successful first responses (potency\(_1\)) (Stevens and Wang 1995). The change in synaptic strength (ratio of mean \(Q_{1post}\) to mean \(Q_{1pre}\)) did not correlate with the baseline strength (Figure 3.5). The baseline potency\(_1\) was also not predictive of change in potency\(_1\). However the change in \(p_{r1}\) appeared to relate to initial \(p_{r1}\). Because of the variability inherent in the function of individual synapses the error in measurements was large when only first responses were considered. We therefore examined synaptic strength, potency and release probability as average values for all pulses in the 3- or 4-pulse train. Changes in synaptic strength and potency were again unrelated to baseline values, but change in reliability (# successful transmissions/# APs) was inversely correlated with initial
reliability and could be approximated by an exponentially decaying relation, ratio=$2.2^*(e^{(-\text{baseline}/1.2)})$ ($R=0.86$, $p<0.002$) (Figure 4.6C).

**Figure 3.5:** Potentiation involves increases in both potency and reliability. A) First pulse in train: mean, potency, and proportion releases successful before and after induction. B) As A, but considering all pulses in the 3- or 4-pulse trains. C) Ratios of postinduction to preinduction all-pulse value versus preinduction all-pulse value, fitted with exponentially decaying dependence.

Evaluation of reliability and potency assumes accurate identification of successes and failures. At these event sizes perfect detection is not currently possible, so the criterion for each synapse was chosen so as to favor specificity over sensitivity.
Figure 3.6: Distributions of identified success and failures. Each row contains data from one synapse. A) Values of successes (green) and failures (blue) over the entire experiment. B) Values of failures before (cyan) and after (magenta) induction of STDP.
Because the peak PSC measurement was based on the event peak, values even for failure were often slightly positive (Figure 3.6A). The distributions of charge values for failures were centered at zero. The size distributions for failures were consistent before and after induction at all synapses tested (Figure 3.6B).

The predictive value of reliability and potency suggested that the two measures of synaptic function might change differently following the STDP pairing protocol, so we examined the two over time (Figure 3.7). We observed that there was indeed a difference in the time of change.

![Figure 3.7](image)

**Figure 3.7:** Example of detected change points for potency and reliability. Reliability, the number of successes per train (black dots), and potency (blue line), the average size of successful responses, change at different points following induction. The detected change point for reliability is marked with a black arrow. The detected change point for potency is marked with a blue arrow. When all pulses in a train fail to elicit a postsynaptic response the potency has no value; here for visual clarity it is represented by zero.

To quantify the time of change we applied change point analysis to the reliability and potency over time (Methods). Confidence intervals on the occurrence of the change were determined with a permutation test (Methods). The change point was not constrained and could occur at any point in the experiment after the first preinduction trial. In 7 out of 10 experiments a change in reliability occurred with >
90% confidence, and all of these changes occurred after induction (Figure 3.8A). For failed induction experiments, used as a control, the values of confidence for a change in reliability were varied, as were the occurrence times (Figure 3.8A). Similar results were found for potency, with 8 out of 10 potentiation experiments showing a change, and all of these being classed as occurring either within one trial of induction, in one case, or after induction, for the remaining cases (Figure 3.8A). Several lines of evidence reinforce the change point findings (Figure 3.8): confident change points occurred in all induction cases, but non-potentiated synapses, used as controls, never showed confident change points (Figure 3.8A); change points could occur at any time, yet when they occurred with confidence it was always at or after the induction protocol (Figure 3.8B); both potency and reliability were always larger after their respective change points (Figure 3.8C and D), and in many cases showed no increase after induction prior to the change.

**Figure 3.8** (Next page): Controls for change point analysis. A) Confidence levels (mean and SEM, with values from individual synapses as dots) for the occurrence of a change in reliability and for the occurrence of a change in potency. Left bars show values for three classes of synapse (left to right): LTP and at least 90% confidence of change in reliability, LTP and < 90% confidence of a change in reliability, and failed induction. Right bars show values for the occurrence of a change in potency for the same three classes with respect to potency. B) The time of the change point for the same 3 classes. Induction is time zero. The analysis searched for change at any time after the first baseline trial. C) Potency values (mean and SEM) and cumulative histograms for baseline, postinduction but before the potency change point, and after the potency change point (periods 1, 2 and 3 respectively). Cumulative histograms show these epochs as red, green and blue respectively. D) Reliability values (successes/pulses each trial, mean and SEM) before induction, after induction but before the change in reliability, and after the change in reliability (periods 1, 2 and 3 respectively). E) Potency before the change in reliability (left bar) and after the change in reliability but before the change in potency (right bar). F) Reliability before the change in potency and after the change in potency but before the change in reliability.
The fact that reliability often changed soon after induction meant few trials for the middle category, resulting in large variability.

In 5 cases changes in both reliability and potency occurred, with change in reliability preceding change in potency 4 of the 5 synapses (Figure 3.9). Change in the reliability of synaptic release without change in the size of successful responses would be inconsistent with synaptic potentiation due to increased postsynaptic conductance so we further checked this result by measuring potency before and after the change point in reliability (Figure 3.8E). Potency was unchanged before the change in reliability versus after it, confirming the independence of these two measures and verifying that in several synapses studied change in reliability could occur up to tens of minutes before change in potency. At 2 synapses a change point for reliability but not for potency was seen.

**Figure 3.9:** Reliability can change prior to or without change in potency. Time of change for potency (upper rows) and reliability (lower rows). In 5 cases, confident change points were identified for both parameters (middle rows). Of these, change in reliability preceded change in potency in 4 (solid lines).
In 3 cases potency changed without change in reliability. Two of these synapses were at the upper end of the reliability distribution initially and thus unlikely to undergo increase in reliability (Figure 3.5C) or to exhibit a change point for reliability.

The use of stimulation trains with variable inter-pulse intervals permitted us to examine the short-term plasticity of responses at few synapses before and after induction of STDP. PSC waveforms showed facilitation and depression at short and long intervals (Figure 3.10). In most cases the PSC following the short IPI was larger on average than that following the long IPI; the third case in Figure 3.10A was the only exception. In 7 of the 10 synapses potentiated by STDP, short-term dynamics appeared altered (Figure 3.10B), as for the second and third cases shown in Figure 3.10A, in which the proportional size of responses was changed by induction. At 3 of 10 synapses, however, the proportional size of responses was roughly conserved following induction (Figure 3.10B), as shown in the first case in Figure 3.10A. The three synapses at which short-term dynamics appeared unchanged exhibited either no change or very delayed change in reliability (Figure 3.9).
Figure 3.10: Short-term dynamics are frequently altered by STDP. A) Mean waveforms from preinduction (black) and postinduction periods for first pulse in train, pulse immediately after the variable IPI when that IPI was short, and pulse immediately after the variable IPI when that IPI was long. (Scale bars 10 ms, 10 pA.) B) Rung plot of the average charge for the three responses shown in (A) before and after induction for all induction cases, normalized to the first response in the train.

Nonproportional change ("redistribution") suggests increased postsynaptic conductance at existing synapses alone is unlikely to underlie the observed potentiation.

To examine more closely the changes in short-term dynamics following induction of STDP we considered the 3 induction cases where a fixed 50 ms inter-pulse interval was used between the first two pulses, yielding a large number of responses separated by the same interval (Figure 3.11). For the synapse shown in Figure 3.11A, facilitation was present at baseline, with the distribution of second responses larger than that of first responses (p=3.97e-004, K-S test). The first response was changed by induction (p= 0.018, K-S test) whereas the second response was not (p=0.18, K-S test). The increase of the first response reduced but did not
eliminate the difference in the distributions as the as the first and second responses were still significantly different after induction (p=0.023, K-S test). For the synapse shown in Figure 3.11B, neither the first response (p=0.89, K-S test) nor the second response (p=0.68, K-S test) was altered by induction; this synapse also exhibited no change point for reliability. For the case shown in Figure 3.11C, the responses were not different at baseline (p=0.95, K-S test), indicating that there was no facilitation. After induction the first and second response distributions were different (p=2.83e-004, K-S test). This difference came about because the second response increased after induction (p=0.001), while the first did not (0.95, K-S test).

Thus for the two of these synapses (Figure 3.11A and C) that exhibited a reliability change point (Figure 3.9), the change in reliability had contrasting effects on facilitation: in the case that facilitated at baseline, first responses grew following induction, so facilitation decreased. In the case with no facilitation at baseline, only the second pulse facilitated postinduction. In the first of these cases, a site available at baseline only with facilitation appeared to become available without facilitation following induction. In the second case, when no facilitation occurred at baseline, suggesting no nearby low-p_r site, induction of STDP is likely to have made a release site available, but only when facilitation was present.
Figure 3.11: Preinduction facilitation predicts postinduction facilitation. Cumulative histograms for the three induction experiments where a fixed IPI of 50 ms between the first two pulses was used. First response values in black, second response values in color. Left column shows preinduction values, right postinduction.

To determine when after induction the changes in short-term dynamics observed at potentiated synapses arose, we plotted the responses following the variable inter-pulse interval, considering separately the responses after short and long intervals by taking a 2-point moving average of each (Figure 3.12, Methods). The short-term dynamics of the two groups was similar after induction then diverged, with the facilitating intervals resulting in larger responses. The divergence was quantified by finding the onset of the window where the net difference between the responses following short intervals and those following long intervals was greatest (Methods). Considering the indications that STDP involved making new sites available to facilitation (Figure 3.11), and because the change in potency (Figure 3.9) could involve the activation of new sites, we compared the time of greatest divergence in the short-term dynamics with the time of the potency change point, finding good agreement between the two (Figure 3.12).
Figure 3.12: Time of change in potency predicts time of change in synaptic dynamics. A) Identification of time of change of SD after induction. Responses after short intervals (black line) and after long intervals (gray line), both relative to first response, are plotted over time using interpolation (Methods). Responses were often similar after induction then diverging at delay. The onset time of the 5-minute window of maximal difference between the two types of response (colored vertical bar) is taken as time of change of SD. B) Time of change in potency versus time of change in SD for those synapses showing a significant change in potency. In one case, marked by *, the first change in potency was at induction so the change point considering only postinduction was found.

Confidence intervals for the assessment of the time of divergence of SD were obtained by permuting the postinduction SD values randomly and counting the number of permutations needed to observe a jump in the difference of the SD of equal or greater magnitude. Over 10 iterations the mean number of permutations (maximum 10,000) required for all synapses having a confident change point for potency was 1.7825e+003, 5.5104e+003, 5.7500e+001, 1.0000e+004, 3.5100e+001,
3.0884e+003, 4.8220e+002, and 7.1646e+003, providing confidence that this sustained jump was unlikely (p < .05 in all cases) to occur by chance.

In Figure 3.12 the divergence in SD is generally shortly after the time of change in potency. The method for identifying the time of change in SD finds the onset of the window of largest difference, so in some cases it may lag behind the true onset of the divergence as suggested visually (Figure 3.12A, right). Moreover, the SD values are calculated from the penultimate response in each train whereas all responses are included in the analysis of potency; the response to the last pulse generally exhibits the most facilitation, so an increase in the degree of facilitation of this pulse could be detected by the potency metric prior to the divergence detected with the SD change metric. Because the last response in a train does not show differential SD (Chapter 2), the SD analysis cannot be applied to that response.

Many synapses showed some degree of asynchronous release during activity trains. Because this form of transmission is not directly controlled by action potentials, and it is rare, few sites are likely to be active, and in the microperfusion preparation many of the events are likely to reflect the postsynaptic response to individual quanta of neurotransmitter. For 6 of the focal perfusion STDP experiments, the background activity was sufficiently low to permit examination of asynchronous release after the last pulse in the stimulation train. In several cases there was essentially no change in the size of asynchronously released events, consistent with no change in postsynaptic responses following induction (Figure 3.13).
Figure 3.13: Asynchronous release event sizes can remain unchanged for extended periods following successful induction. Each panel shows all asynchronous events recorded at a synapse. Preinduction events are shown in black and postinduction events in color. The numerical value above each panel gives the time, in minutes since induction, of the recorded postinduction events. A) Events were unchanged after induction. B) Asynchronous events occurred during the decay phase of the previous synaptic current, and their size increased shortly after induction.

For the three cases shown in Figure 3.13A there was no increase in asynchronous event size for as much as 10-20 minutes postinduction. This suggests that the number or conductance of postsynaptic receptors had not been altered. In the synapse shown in Figure 3.13B asynchronous events occurred during the decay phase of the previous synaptic current. Here the size of asynchronous events appeared to increase immediately following induction by ~20-25%. This synapse had high reliability at baseline (Figure 3.5C) and had no change point for reliability (Figure 3.9). Its short-term dynamics changed proportionally following induction (Figure 3.10A, top).

In two cases asynchronous release was similarly unchanged from baseline for several minutes following induction but then increased (Figure 3.14; the first of these synapses showed a smaller asynchronous event size immediately after induction that then reappeared approximately 15 minutes later). For the first case, events became somewhat more frequent after induction, and their peak size
increased at approximately 10 minutes. For the second synapse, at approximately 10 minutes postinduction asynchronous events could newly be detected.

**Figure 3.14:** Asynchronous release changes after a delay at some synapses. A) Left: Waveforms of asynchronous release events after the last pulse in the train following induction (earlier events in front and in blue). Inset shows the occurrence times of these events over 200 ms (width) following each train for the 30+ minutes (depth) postinduction. Right: Diary plot of the time and size of asynchronous events. Insets show all preinduction and postinduction waveforms. B) As in A. There were no asynchronous events prior to induction for this case.

The time of this change correlated well with the time of change in potency (at 9 minutes postinduction in both cases; Figure 3.9), suggesting the same process underlies both changes. For the synapse shown in 3.14B, at 20 minutes postinduction the events’ maximum size increased. The large size of these late
events may suggest a marked increase in postsynaptic conductance or multivesicular release.

To consider additional mechanisms of potentiation we measured latency (Figure 2.8) and compared values before and after induction (Figure 3.15). There was no clear trend for latency to be altered following induction.

**Figure 3.15:** Cumulative histograms of latency before (blue) and after induction of STDP. Bar graphs, mean values, error bars, SEM.
3.5 DISCUSSION

We have investigated the mechanisms of spike-timing-dependent long-term potentiation at small numbers of synaptic contacts between hippocampal neurons. In our experiments STDP depended on the amount of activity at the synapse during induction and this form of potentiation could involve increases in both the number of successful responses in a train and the size of successful responses. The size of successful responses (potency) before induction had no systematic relation with the size of successful responses after induction, but the proportion of successful responses (reliability) was inversely related to the change in this proportion. Examination of the time course of potentiation following induction showed that potency and reliability could change at different times following induction. Change in reliability could precede change in potency by more than 15 minutes, and in some cases where a change point for reliability occurred, a change point in potency did not. This suggests that persistent change in the presynapse, when such change has not already been saturated, contributes to this form of potentiation. In examining short-term plasticity we found that in most cases it was altered by induction; changes in short-term facilitation suggested that sites weakly present at baseline became more strongly present following induction, and an increase in the difference between facilitating and non-facilitating responses was found to occur at nearly the same time after induction as the change in potency, further suggesting that a new synaptic site became available at this time. Finally, in cases where asynchronous release at the microperfused synapse changed, it did so in a way that was consistent with involvement of a new synapse at nearly the same time as suggested by change point analysis and by analysis of short-term dynamics. In other cases, no change in asynchronous release was detected, and quantal events were remarkably similar before and after induction, indicating that at the sites where these events occurred,
no change in postsynaptic properties occurred for as long as 20 minutes following induction.

3.5.1 The experimental system

Although the focal perfusion method has several advantages for investigations of synaptic function, there are several potential limitations to consider. Magnesium from the bulk perfusion solution may diffuse into the local perfusion stream and increase the block of NMDA receptors within the active region. STDP is still seen, indicating that to the extent $I_{\text{NMDAR}}$ is necessary for potentiation it is still present. However the success rate for induction in microperfusion may be lower than for standard experiments, and higher local Mg$^{2+}$ could be one reason. Alternatively if cooperativity between nearby synapses (Harvey and Svoboda 2007) plays a role in this form of LTP, the absence of such cooperativity would make potentiation more unlikely. If release of calcium from the endoplasmic reticulum requires a threshold level of extracellular calcium influx (Emptage et al. 1999), this may be less likely when nearby synaptic contacts are outside the microperfusion stream. However, there is increasing evidence that plasticity is synapse specific (Steward et al. 1998, Sajikumar et al. 2007), suggesting that the local machinery that modifies synaptic strength can function in isolation. Another consideration with focal-perfusion STDP is the possibility of different calcium handling in the postsynaptic neuron due to lower external calcium (Camodeca et al. 1998). The lower $[\text{Ca}^{2+}]_{\text{ext}}$ would further reduce the low calcium influx through the NMDAR at room temperature (Chung and Kuyucak 1995) as was used for these experiments. There is also the possibility that a retrograde messenger such as endocannabinoid (Di Marzo et al. 1994) could be washed away, pushing the synapse toward one particular type of potentiation,
though such a consideration seems unlikely to bias the synapse toward the presynaptic change observed at a number of synapses in the present investigation.

3.5.2 Dependence on slope of bEPSP during induction

Spike-timing-dependent potentiation depends on the slope of the postsynaptic potential during the induction protocol (Figure 3.3), confirming that the signaling cascades involved in this form of potentiation require a certain amount of current (and presumably calcium) influx for initiation (Bienenstock et al. 1982). Once a threshold level of ionic influx is reached, the amount of change at the postsynaptic neuron is not directly predicted from the current influx. This unpredictability suggests a critical process and may point to intra-synaptic cooperativity in this form of potentiation.

The dependence of potentiation on activity during induction suggested that change might occur during induction itself. However we found no evidence for such real-time change (Figure 3.4). Other studies, in contrast, find that potentiation can occur within 30-40s of induction, with induction itself lasting less than 1 s (Petersen et al. 1998, Bagal et al. 2005). One key difference between STDP and the pairing protocol used in these studies is the long depolarization of the postsynaptic neuron in the pairing protocol. This could differentially influence activity at the postsynapse and alter the release of retrograde signals (Magby et al. 2006).

An additional factor that could influence responses to the spike-timing protocol is the location of the active synaptic site relative to the postsynaptic soma (Sjostrom and Hausser 2006, Froemke et al. 2005). In our system synapses at considerable distance potentiated, and some synapses that failed to potentiate were closer to the postsynaptic soma than some synapses that succeeded (not shown). In our
preparation distance from the postsynaptic soma does not dictate success or failure of STDP.

The observation that that activity during induction determines the ability of the synapse to potentiate but not the amount of potentiation suggests that a threshold of activity is needed but other factors determine the amount of synaptic change once the threshold is met. The initial state of the synapse (Montgomery and Madison 2002, Ismailov et al. 2004) could be one such factor.

3.5.3 Relation to initial release probability

Reliability at baseline was inversely related to the change in reliability caused by induction (Figure 3.5). Conversely, neither efficacy nor potency of preinduction responses had a relationship with change in these values. This suggests that one of the primary mechanisms for change available at these synapses may be increase in release probability or reliability, and that that mechanism once used is no longer available. To the extent that increases in the size of the postsynapse subserve change (Ostroff et al. 2002) or new space is needed for potentiation (Lisman and Raghavachari 2006), the spatial constraints on activity in the present study could have restricted potentiation at some synapses.

The inverse relation of initial value and change ratio would be trivially due to a ceiling effect if the final reliability values were 1, but most synapses studied did not approach perfect reliability after induction, suggesting that the ceiling on reliability is biologically determined (Murthy et al. 1997).

3.5.4 Differential change in potency and reliability
In those cases where reliability changed long before or without change in potency (Figure 3.9) it was argued that this reflects change in the existing presynapse, but it is not impossible that the change in reliability without change in potency detected in the experiments reported here could still reflect addition of a new synapse (Malinow and Mainen 1996). However, as discussed by Bolshakov and Siegelbaum in their response to the previously cited communication, this would require a number of assumptions (Siegelbaum and Bolshakov 1996) that seem unlikely to all be true. Specifically, it is unlikely that a site newly participating after induction could be small enough not to change potency, but large enough to be detected and thus change reliability.

Change in glial (Genoud et al. 2006) or neuronal (Brasnjo and Otis 2001) glutamate transporter function could also be involved in the observed potentiation. However such mechanisms are unlikely to play a major role in the changes seen here as they are unlikely to affect reliability, short-term dynamics, or asynchronous release.

Even with the presynaptic neuron patched, when AP initiation can always be verified, a concern with analyses of reliability is whether all failures are due to release failure, or action potential propagation failure (“branch failure”) could also contribute. Other investigators found evidence for branch failure in cultured hippocampal neurons (Brody and Yue 2000), but they used longer stimulation trains at the higher frequency of 50 Hz, which could bias toward failure. Comparison of autaptic and synaptic responses in microperfusion suggests that branch failure is uncommon (Chapter 2), but without direct measurement of propagation it cannot be excluded.

3.5.5 Time of change
The change in reliability prior to change in potency seen in a number of cases is inconsistent with several candidate mechanisms of potentiation — increase in the number (Luscher et al. 1999) or conductance (Benke et al. 1998) of AMPA-type glutamate receptors at the synapse would increase potency, as would activation of a formerly silent synapse, regardless of whether the synapse had been ‘mute’ or ‘deaf’ (Voronin and Cherubini 2004). Change in reliability alone is most parsimoniously explained as altered function at the presynapse. In most cases where both parameters change, the change in reliability is followed by change in potency. This delay suggests a multistage process of synaptic plasticity with change at the existing presynaptic terminal, when not saturated, completed first, and processes underlying the increase in potency requiring ~5-15 minutes for completion. The increase in potency could reflect an ability of STDP to accelerate the developmental time course (Friedman et al. 2000). In this scenario synapses in various stages of maturity lie at baseline within the focal perfusion stream, initially releasing only when facilitated (Figure 3.11) or not at all.

3.5.6 Change in short-term dynamics

Short-term plasticity changes at most synapses following induction. This indicates some process apart from an increase in the number or conductance of postsynaptic glutamate receptors at the existing synapse, though at a subset of synapses proportional potentiation of all responses in the train is observed (Figure 3.10). The amount of facilitation at baseline relates to the amount of change following induction in a manner suggesting that the response to the induction protocol is determined by the availability of a release site to be facilitated (Figure 3.11). If the amount of facilitation at these synapses reflects the extent to which new sites are
available to be potentiated (Gardner-Medwin 1989, Jensen et al. 2007), following induction their availability earlier within the stimulus train would tend to reduce the amount of short-term facilitation, as observed. In order to track the possible emergence of new sites the time of maximal difference between facilitation and non-facilitating responses was identified (Figure 3.12). This time was close to that of the change point in potency (Figures 3.8 and 3.9) identified using different data and a different analytical method, suggesting that a single process alters both parameters.

3.5.7 Change in asynchronous release

While the number of asynchronous events in the microperfusion configuration is low, the likelihood that they originate from the same release site and reflect current flow through the same postsynaptic receptor cluster increases their informativeness. Following potentiation, asynchronous release waveforms remained virtually unchanged from their preinduction shapes at some synapses for as long as 20 minutes (Figure 3.13). This argues that the postsynaptic receptors impacted by the asynchronously released transmitter are not altered in number or conductance (Hayashi et al. 2000, Luthi et al. 2004) by the potentiation protocol. At a synapse with high baseline reliability, asynchronous release events increased in size soon after induction. At other synapses, change in asynchronous release did occur, but only after a delay comparable to that observed for change in potency and change in short-term dynamics (Figure 3.14). The similarity of these time courses suggests that the same underlying cellular change underlies all three processes.

For spike-timing-dependent long-term potentiation, where the relative contributions of the pre- and postsynapse to long-term change were unknown, our results suggest that at hippocampal synapses having low release probability (Figure 3.5) new synaptic sites become available after existing sites are modified (Figure 3.9).
The initial modifications are often presynaptic in nature, with increases in $p_r$ and reliability prior to change in potency. Additionally there are indications (Figure 3.11) that the “added” sites can be weakly present prior to induction and become more likely to release transmitter due to induction.

3.5.8 Latency before and after induction of STDP

We identified no clear trend for change in latency following induction of spike-timing-dependent LTP (Figure 3.15), a finding in contrast to recent studies that have reported change in latency after potentiation (Boudkkazi et al. 2007). However, our results indicate that STDP involves activation of new synaptic contact sites (Figure 3.14). Thus, in addition to changes in the excitability of the presynaptic neuron that follow potentiation (Aizenman and Linden 2000, Armano et al. 2000, Ganguly et al. 2000), the recently reported of changes in latency, and the accompanying reported changes in $p_r$, could be due to the activation of synaptic contacts close to the postsynaptic soma.

3.6 CONCLUSION

This chapter reports several findings on the mechanism of spike-timing-dependent long-term potentiation. Activity during induction of STDP is required for potentiation at the level of individual synapses but does not predict the magnitude of potentiation. As long as a threshold level of activity is present during induction, low initial reliability predicts a large change in reliability. Reliability and potency change in many experiments, but often at different times, with reliability changing
well before change in potency in a number of cases. Short-term dynamics is changed in a manner suggesting low probability sites become more likely to release after induction. Change in short-term dynamics happens at nearly the same time as the measured change in potency, though there is no a priori reason the difference between facilitating and depressing responses should become greater at this time. Asynchronous release is unchanged as far as can be measured at several synapses, suggesting the effect of a quantum of transmitter is not altered by potentiation. At other synapses in the study the rate or size of asynchronous release changes following induction, and these changes occur with delays similar to those found by the other metrics used here, indicating that following induction sequential changes at the synapse — change in the function of the existing presynapse followed by addition of new sites — can be observed as they occur.
4.0 SUMMARY AND CONCLUSIONS

4.1 SUMMARY

This thesis has presented a new experimental method and findings on synaptic function and short-term plasticity made possible by that method with results of investigations using the method to determine the cellular changes effected by induction of spike-timing-dependent long-term plasticity. These results are briefly summarized as follows:

4.1.1 Measuring synaptic transmission at individual synaptic contact sites

A method for recording from very few synaptic contact sites formed between neurons in culture was described. The function of the system was characterized and modular extensions including labeling the boutons of a single neuron and the use of acoustic methods for measuring focal perfusion flow were presented. The system was used to study several aspects of single synapse function including the role of depletion in short-term depression and the influence of previous inter-pulse intervals on synaptic responses. Independent behavior of neighboring release sites on an axon was tested and confirmed and the role of dopaminergic input in rapid synaptic activation was observed in experiments made possible with the method.

4.1.2 Mechanisms of spike timing dependent potentiation
Using the microperfusion method to measure activity at individual synapses before, during and after spike-timing-dependent long-term potentiation, it was discovered that the response to the induction protocol depends on activity during induction. Responses could be separated into potency and reliability components, and among successful inductions, initial values for reliability predicted change in reliability, which was not the case for potency. The occurrence of change in potency or reliability varied, as did their respective times, but in a number of cases a change in reliability preceded a change in potency by more than ten minutes, consistent with change at the existing presynapse alone. Changes in short-term plasticity suggested presynaptic change as well. When analyzed over time, short-term dynamics following induction showed a change near the time when change in potency was found to occur. Finally, at a number of synapses, asynchronous release was unchanged for as much as 20 minutes or more following induction, consistent with no change in postsynaptic properties, while at those synapses where change was seen, it occurred at nearly the same time as the changes in potency and synaptic dynamics. Taken together these results indicate consistent change in the existing presynapse followed at delay by the addition of new synapses.

4.2 CONCLUSIONS AND IMPLICATIONS

4.2.1 The localized perfusion method

The method developed and used in this study has several advantages over conventional techniques. Because all active synapses are formed by the same
presynaptic neuron, which is patched, it is possible to be certain that the action potential was successfully evoked. Many of the processes underlying short-term dynamics are likely to depend on the steps between action potential firing and exocytosis, and stimulation by AP invasion of the bouton initiates these processes in a realistic fashion. Study of spike-timing-dependent plasticity requires both pre- and postsynaptic action potentials which are ensured in the dual-patch configuration. Some of the boutons visualized and selected for study when all neurons are labeled and field stimulation is used may be inhibitory, likely influencing the properties under study (Gitler et al. 2004), but with single-cell labeling and stimulation there is certainty as to the phenotype and cell of origin for each bouton. With focal perfusion single bouton synaptic current appears or disappears essentially instantly when the puffer is turned on or off, confirming a high degree of control over the synapse’s milieu. The consistent microenvironment at the bouton makes it possible to study short- and long-term plasticity, and the siphon-generated negative pressure is low and causes no detectable damage to neurites.

These conditions permit recordings from very few synapses in intact neurons without drugs or toxins in stable environments for several hours. The paucity of participating synapses and the equal effect of variables such as dendritic filtering (Smith et al. 2003) and distance from the soma (Froemke et al. 2005) on all responses in an experiment make for highly informative PSC recordings.

4.2.2 Findings and implications — Short-term plasticity and dopaminergic input

4.2.2.1 Little role for depletion in short-term depression: Depletion of neurotransmitter vesicles is generally assumed to be a principal cause of short-term depression, but recent experiments have called this into question (Sullivan 2007).
The focal perfusion method was used to test the importance of depletion by comparing responses at few synaptic sites to pairs of APs separated by variable intervals. If depletion were a primary determinant of responses, successful transmission events would be disproportionately likely to be followed by failures, and after larger-than-average responses, smaller-than-average responses would be expected. However, when many focal perfusion experiments were considered, no clear trend was observed at any of a number of intervals, suggesting that on the time scale of 25-400 ms depletion has little influence on short-term plasticity at these synapses.

4.2.2.2 Synapse function is too stochastic to encode recent activity history: When the synapse between two neurons consists of many contacts, the relatively deterministic collective behavior of these sites could permit encoding of the recent activity history of the neuron in the size of the PSC (Maass and Markram 2002, Fuhrmann et al. 2002). For those synapses made up of few contacts, it is clear that the interval immediately preceding an AP influences the size of the resulting PSC, but the extent to which prior IPIs could be encoded in the synaptic responses was not known. Using brief trains of 4 APs having intervals either of 50, 50, 50 ms or 50, 400, 50 ms, PSC waveforms after the final AP were separated by interval used. At all synapses studied, no significant difference between the groups existed. Encoding of recent activity in PSC size is unlikely to be feasible at synapses having few sites.

4.2.2.3 Neighboring synapses on the same axon function independently: Neighboring release sites on an axon are likely subject to similar metabolic and modulatory inputs both from within and from outside the neuron, implying that their activity could covary. Such covariation, however, would affect the transmission of information by the neuron. Using microperfusion of sites that
released transmitter onto both pre- and postsynaptic neurons the correlation of adjacent sites could be measured directly. The degree of similarity in the function of the sites was low, indicating that each is little influenced by activity at the other.

One interpretation of this independence is that synapse function is not significantly affected by possible regional variables within the axon such as metabolic state or local fluctuation in $V_m$. A further interpretation is that the independence of operation indicates that a single axon transfers information to postsynaptic targets individually, in such a way as to be maximally informative (Markram et al. 1998, Franks et al. 2002).

4.2.2.4 Dopaminergic input can lead to rapid, persistent activation of synapses: To examine the consequences of dopaminergic input for hippocampal synaptic function we measured its effects on transmission in the microperfusion preparation, where the size and success rate of synaptic events could be separately determined. Exposure to 20 μM DA for 15 minutes caused long-term potentiation of the synapse, and further potentiation was seen with a second exposure. Though DA increases AMPAR expression (Gao et al. 2006), at least part of the effect we observed was presynaptic (Tecuapetla et al. 2007), as a long-latency release site, seen initially only with the facilitation brought about by multiple APs, appeared earlier in the train and showed an increasing success rate over time.

In the striatum dopamine regulates long-term synaptic plasticity (Calabresi et al. 2000, Kerr and Wickens 2001), and exposure to dopamine accelerates some aspects of synaptic development (Porter et al. 1999). Dopaminergic input alters short-term dynamics at excitatory synapses in cortex (Gonzalez-Burgos et al. 2005) through activity-dependent mechanisms. The experiment presented here shows that at hippocampal synapses, DA rapidly activates new synaptic contacts and increases their release probability. This synaptic change may be one mechanism by which DA
4.2.3 Findings and implications – Spike-timing-dependent potentiation

4.2.3.1 Dependence of STDP on activity at synapses during induction: Results presented here demonstrate that activity at a synapse during induction, long thought to be critical to conventional LTP, is also necessary for STDP. This confirms that at individual synapses current flow during the induction protocol is needed to set in motion the cellular changes that underlie potentiation. The success of STDP with normocalcemic bulk perfusion and in vivo may therefore depend on the extent to which each individual synaptic contact is active during induction, which would in turn depend primarily on the ability of the contact to release transmitter consistently at the stimulation frequency used.

In the BCM model of synaptic plasticity (Bienenstock et al. 1982), moderate postsynaptic calcium influx results in depression and larger calcium influx results in potentiation. Though several synapses which failed to potentiate showed smaller responses on average following induction, none were statistically depressed from baseline, suggesting either that the amount of calcium influx was too small for depression or that the potentiating bias of positive timing STDP (Wang et al. 2005) negated the depressing processes initiated by low calcium influx, resulting in insignificant net change. The reason for the lower activity of these synapses during the induction protocol is unclear, but short-term dynamics unable to support repeated release at the 1 Hz rate of induction is a likely factor.

Synapses that tend to fail will transmit no charge during the induction protocol (Yang et al. 2003) and will consequently be unavailable to be undergo plasticity. It is a strength of the intermittent STDP protocols (e.g., O’Connor et al. 2005) that the participation of a given synapse during induction can be directly inferred from its
activity at baseline because the stimulation frequencies are the same. In such an experimental design short-term dynamics cannot influence presynaptic properties differently during induction than was the case at baseline. With the use of current-clamp throughout the experiment, moreover, there is no concern that bias in the measurement could distort responses recorded during induction.

In the protocols used in the present study, where characterization is performed in voltage-clamp and induction in current-clamp, it is important to consider the possibility that the apparent differences in activity are actually due to differences in electrotonic distance of the activated site from the postsynaptic soma. Due to differences in space clamp, activity at sites electrotonically distant from the soma could appear smaller in current clamp than in voltage clamp, so sites appearing inactive during the induction protocol might simply be farther from the postsynaptic soma. Previous studies of STDP showed that the result of a potentiation protocol depends on the dendritic location of the synapse (Sjostrom and Hausser 2006, Letzkus et al. 2006). The smaller ratio of PSP to PSC at the nonpotentiated synapses in the present study could reflect the poorer space clamp and greater signal attenuation in current clamp of electrotonically distant inputs, or alternatively short-term dynamics that led to low release probability during induction. However, synaptic sites at considerable distance from the postsynaptic soma were capable of potentiation (not shown), so distance from the postsynaptic soma is not the only factor determining response to induction.

4.2.3.2 Effect of initial conditions

4.2.3.2.1 Relation of change in release probability to initial release probability: The present study has demonstrated that in STDP, initial $p_r$ is inversely related to change
in $p_r$. In other studies of LTP, baseline release probability predicts the change in release probability or the extent of potentiation. Greater change at low $p_r$ synapses is often seen (e.g., Malgaroli et al. 1995) while increased potentiation at high $p_r$ synapses has also been observed (Rosenmund et al. 1993). This apparent discrepancy is likely explained by considering the distribution of $p_r$ present in each experiment; see 1.2.1.

The initial release probability has a twofold influence on potentiability: by influencing the extent of the site's activity during induction and thus its availability for potentiation$^2$, and by reflecting the state of the synapse and how labile the synapse may be. These two influences are opposed inasmuch as low $p_r$ suggests lability (Figure 3.5) but also indicates low likelihood of firing during induction. As a result, long-term increase in probability due to STDP may be available only when the release probability is in an intermediate range. Nascent synapses would be too weak to be consistently active during the STDP pairing, while highly mature synapses would be unable to further increase $p_r$. The limit of $p_r$ is likely biologically imposed (Murthy et al. 1997) rather than an artifactual ceiling effect due to saturation of the measurement, because no perfectly reliable synapses were observed.

Beyond the threshold of activity needed for potentiation, the level of activity during induction does not predict the magnitude of potentiation (Figure 3.3). This unpredictability may reflect the variability of time of activation of new synaptic contacts following induction (Figures 3.9 and 3.14). If postinduction synaptic strength is measured prior to the presumptive addition of the new site, and thus reflects only the change in function at the existing site(s), the correlation would be expected to be closer. If the addition of new sites occurs before or during the

---

$^1$ In Chapter 3 reliability, the mean success rate, was the primary metric of presynaptic function. Reliability is shaped by $p_r$, and discussion of $p_r$ permits ready comparison with the many studies of long-term plasticity that examined this parameter.

$^2$ Low $p_r$ synapses would, on average, be active fewer times during induction. Second-order effects such as facilitation would in principle influence the result of STDP, but in practice facilitation is not seen at 1 Hz in the synapses studied here.
measurement window, on the other hand, the change would be larger than predicted. The experimental configuration may play a role; if the microperfusion stream happens to include an immature synapse that can become activated, or if it is wide enough to permit a new release site to enter the normocalcemic area (Supplementary Movie 3), this could influence the delay.

4.2.3.2.2 State-dependence: The influence of the initial reliability on the potentiation outcome in the experiments reported here reflects a form of state dependence. The influence of the synapse’s initial state on its response to plasticity is further suggested by the relation between short-term facilitation before induction and its change after induction (Figure 3.11). State dependence was found in other studies where single synapse measurements were made (Montgomery and Madison 2002, Ward et al. 2006). The silent synapses reported in the latter study also displayed short-term facilitation, just as the short-term plasticity data shown here are consistent with synapses inactive for the first pulse in the train being made active by the facilitation of the second pulse (Figure 3.11). Other studies have found that the ability of a synapse to undergo long-term change depends on its ability to undergo short-term modulation (Perea and Araque 2007), that is, its state. Indeed, it would be surprising if the result of a potentiation protocol were not influenced by the state of the synapse prior to the protocol, but in most studies the state of individual synapses at the time of potentiation is not known. Previous authors have highlighted the fact that baseline synaptic strength is a poor indicator of the state of the synapse (Montgomery and Madison 2004), in part because it reflects events long prior to the experiment that may not markedly impact synaptic change. Our data suggest that initial pr or reliability is a more informative measure of synaptic state than mean response size, the general indicator of synaptic condition before baseline (Figure 3.6). Nonetheless the unpredictability of the magnitude of overall potentiation, once threshold has been exceeded, suggests that additional parameters
of the initial state in the present experiments may remain unidentified; see Figure 3.3.

4.2.3.3 Time of change: In the experiments of the present study, following the STDP protocol synaptic function generally changed at delay. Similar delayed change was recently reported in another single-synapse potentiation study, where waveforms did not change for at least 10 minutes following induction (Perea and Araque 2007). In contrast, other forms of plasticity such as high-frequency stimulation have their effects at the synapse within a few seconds (Gustafsson and Wigstrom 1990) and calcium elevation in the postsynaptic cell from tetanus must occur within two seconds for the tetanus to have an effect (Malenka et al. 1992). The STDP protocol appears to be slower to initiate the signaling cascades that will ultimately lead to potentiation. The nonlinearity of the contribution of each pre-post pairing (Tsodyks 2002) is a factor in STDP; pairings with small numbers of pulses have no detectable effect on the synapse (Wittenberg and Wang 2006), whereas increasing the number of pairings above ~80 yields no additional potentiation (GQ Bi, personal communication). These nonlinearities may affect the time course of the process in that ‘strong’ protocols can lead to ‘rapid’ potentiation. This hypothesis is consistent with results from reports that LTP could be evoked with a single pairing of glutamate uncaging and postsynaptic depolarization (Bagal et al. 2005), where potentiation occurred in less than 45 s. In contrast, our studies of spike timing dependent plasticity showed no evidence during any case of systematic change in strength during the 60 seconds of induction (Figure 3.4), and change generally occurred at considerable delay.

The insertion of postsynaptic receptors has been found to require SNARE-

---

1 It is possible that change is occurring during induction but is not detected because of the reduced signal-to-noise ratio in I_{clamp}. For the single case in our study where synaptic strength is seen to be changed immediately after the pairing, the change may occur during the ~30-45 second period following induction when the postsynaptic neuron is returned to voltage clamp.
mediated exocytosis (Lu et al. 2001), and the lengthy depolarization of the postsynaptic neuron used in the “pairing protocol” could permit sufficient calcium influx (when coupled with glutamate receptor activation) to yield exocytotic receptor insertion, which would have the binary character reported in some studies (Petersen et al. 1998, O’Connor et al. 2005). The brief single BPAP in the spike timing protocol employed here might be insufficient to activate this mechanism. The pattern of presynaptic activity during induction can determine the necessity of postsynaptic depolarization (Urban and Barrionuevo 1996) or spiking (Linden 1999) for potentiation, and it may be that the pattern of activity at the postsynaptic neuron can determine the number of presynaptic impulses needed for potentiation and the delay that will precede it.

Overall, the STDP protocol appears to be relatively weak, in that a large number of pairings are needed to effect synaptic change, and STDP tends to occur at delay. The weakness of the STDP protocol is also likely to be exhibited by synaptic potentiation processes at work in the brain.

4.2.3.4 Locus

4.2.3.4.1 Differential change in potency and reliability — pre- and postsynaptic contributions to STDP: An advantage of the focal perfusion method is that the restricted number of synapses makes it highly likely that failures will occur. This permits consideration of the proportion of responses that succeed (reliability) as distinct from the mean size of success responses (potency). In analyzing these two parameters using a bootstrap-based method to detect the presence and the time of change points, it was found that when both change, reliability generally changes prior to potency. Change in reliability with no change in potency suggests presynaptic-only change, so the time course indicates that change at the existing presynapse is the first effect of potentiation.
In other studies of LTP demonstrations of presynaptic change consistent with greater release probability and reliability are numerous. In cultured neurons LTP induced by glutamate application causes an increase in the frequency of miniature synaptic events with no change in their amplitude (Malgaroli and Tsien 1992). Using quantal analysis other groups have shown no change in quantal size, with an increase in release probability and number of sites (Sokolov et al. 1998). Change in presynaptic function suggesting increased release probability was also seen following a chemical LTP protocol with tetraethyl ammonium, and using high-frequency tetanus (Zakharenko et al. 2001). Conversely, in LTD, reduction of release probability has been shown to play a role (Stevens and Wang 1994), and in spike-timing-dependent LTD, presynaptic mechanisms have been identified (Sjoström et al. 2003).

4.2.3.4.2 Combined pre- and postsynaptic mechanisms: While the studies described above indicated presynaptic components of LTP, many others highlighted the involvement of postsynaptic processes such as AMPAR insertion in potentiation. In considering the possible cellular changes underlying LTP it is important to bear in mind that many different permutations of the values of various pre-and postsynaptic parameters could in principle result in similar consequences for synaptic strength. Holmes and Grover investigated the interdependent ranges of these parameters using simulations (Holmes and Grover 2006). Interestingly, these authors found that neither activation of silent synapses nor addition of AMPA receptors to existing synapses would alone be sufficient to generate LTP at magnitudes reported in many studies, whereas increasing vesicle release probability could, in isolation, be sufficient to generate the magnitudes of long-term potentiation.

---

1 The increase in reliability due to induction may initially be due to elevated calcium, but other mechanisms must ultimately intervene, as the elevation in calcium is transient (Wu and Saggau 1994).
reported in the literature\(^1\).

A number of methods have been employed to disambiguate pre- and postsynaptic contributions to synaptic change, among them application of exogenous glutamate to the postsynaptic dendrites to test for changes in sensitivity. One study using this method reported that LTP was not accompanied by a change in dendritic response to glutamate (Eder et al. 2002), implicating presynaptic change though another study, using laser uncaging of glutamate coupled with postsynaptic depolarization, found that sensitivity to glutamate changed sharply following pairing and remained elevated (Bagal et al. 2005). However, the protocols differed: the former study used high-intensity electrical stimulation of presynaptic axons at 5 Hz and the latter used glutamate uncaging coupled with postsynaptic depolarization.

In fact it is likely that divergent findings in the literature on the relative contributions of pre- and postsynaptic change to LTP are in many cases due to differences in protocol. Many studies depolarize the postsynaptic neuron while the presynaptic cell is stimulated, but, interestingly, potentiation can also result from depolarization of the postsynaptic neuron alone (Sjostrom et al. 2004)\(^2\). The mechanism of change in synaptic response elicited by depolarization of the postsynaptic neuron alone may involve increase in the release probability at previously silent (mute) boutons (Voronin et al. 2004). Neither presynaptic nor postsynaptic spiking in isolation generally cause long-term potentiation (but see Volgushev et al. 1994), so the mechanisms of synaptic change due to prolonged postsynaptic depolarization appear to be different than those available during STDP, when APs and BPAPs, rather than protracted depolarization, are the stimulus. The different stimuli may set in motion different mechanisms that result in increased

\(^1\) Increasing AMPAR single-channel conductance was also sufficient in the simulations to cause LTP (Derkach et al. 1999, Poncer et al. 2002).

\(^2\) This effect is mediated by L-type calcium channels, not by NMDA receptors, and is dependent on PI3 kinase activity (Baxter and Wyllie 2006).
synaptic strength, in which case the relative contributions of the pre- and postsynaptic components are likely to differ as well. When the difference in protocols explains the difference in results, one implication is that multiple paths leading to the ‘potentiated state’ exist for the synapse. The path taken by synapses in the nervous system would depend on the activity history prior to and during potentiation.

4.2.3.4.3 Silent synapses\(^1\): One mechanism for the involvement of new synapses would be the activation of almost-mature synapses, most notably by insertion of AMPA receptors into previously AMPA-free postsynaptic receptor clusters (Kullmann 1994, Isaac et al. 1995). This phenomenon is referred to as the activation of silent synapses, though at baseline such synapses may have a low but non-zero success rate (Kullmann 2003). Thus the silent synapses reported by other groups may involve initially low-p\(_r\) synapses, which are suggested in the present study. Apart from proper stimulation frequency a number of pathways for rapid awakening of mute synapses have been identified, including activation of nicotinic receptors (Maggi et al. 2003) and BDNF stimulation (Du and Poo 2004)\(^2\). BDNF stimulation works through a presynaptic pathway to increase release probability (Schinder et al. 2000), and knockout of the trkB receptor reduces the number of morphological synapses (Luikart et al. 2005). Interestingly, the release of BDNF has been shown to occur with single-cell depolarization of hippocampal neurons (Magby et al. 2006).

---

\(^1\)Though they are widely believed to contribute to LTP, measurement of silent synapses is contentious. Some groups argue that the postsynaptic depolarization itself is responsible for the change in release probability, in other words, that measurement of silent synapses that causes them to appear (Voronin and Cherubini 2004) The frequency of presynaptic stimulation will affect the success rate of presynaptic release, with too-rapid stimulation (Saviane et al. 2002) leading to short-term depression of release probability that could mimic the appearance of a silent synapse.

\(^2\) Conversion of mute synapses to loquacity or deaf synapses to hearing could explain the binary plasticity seen in other studies (O’Connor et al. 2007, Petersen et al. 1998).
STDP requires concerted spiking of both neurons (Bi and Poo 1998) and synaptic transmission (Figure 3.3), so it is not possible to study this form of plasticity at completely silent synapses. Also, the experimental configuration employed in the present study requires identification of electrophysiologically active synapses at the beginning of the experiment in order to record the baseline, meaning that the primary synaptic conductance must be mediated by receptors active at the holding potential, i.e., AMPAR. Even with such selection of active synapses, it is still possible and in some experiments may in fact be likely that the microperfusion stream encompasses, at baseline, both active and silent synapses. The silent synapses could be either mute or deaf. If previously non-transmitting synapses within the stream are activated by the spike-timing-dependent protocol (see above), it is ultimately necessary to determine whether they are activated presynaptically (Krueger et al. 2003), postsynaptically (Kullmann 1994), or both. (The dynamics of these newly functional synapses may be different from that of the original synapse, Figure 3.10, but this does not distinguish presynaptic from postsynaptic mechanisms1.)

The jump in facilitation following induction at delay in the present study is most simply explained as the involvement of new synapses. The fact that this change is in turn simultaneous with the change in potency and, in those cases where it changes, with change in asynchronous release, suggests that this is the time when the silent synapses become active.

4.2.3.4.4 Trans-synaptic signaling: Changes in presynaptic function requiring postsynaptic activity suggest the involvement of a retrograde messenger. In an imaging study of conventional LTP that reported change in both release probability

---

1 Pseudo-silent synapses are also a possibility; even when no synaptic current is measured, release of a small amount of glutamate may have occurred; given the higher affinity of NMDA receptors for glutamate this may have been enough to open these receptors and cause calcium influx (Renger et al. 2001), setting in motion synaptic change.
and in the effect of release, it was found that the postsynaptic change was blocked by
tetanus toxin, but the presynaptic change was not (Ward et al. 2006), indicating that
the retrograde signal leading to increase in the presynaptic calcium transient is
generated independently of SNARE-based exocytosis.

The notion that long-term change at the synapse involves processes that span the
synaptic left has long had advocates (Colley and Routtenberg 1993) and detractors
(Collingridge et al. 1989.) Recent studies support the existence of trans-synaptic
signals (Reid et al. 2004, Contractor et al. 2002)\(^1\).

4.2.3.5 Multiple time scales

4.2.3.5.1 Change in short-term plasticity following long-term plasticity:
Considerable diversity of SD across synapses was observed in the present study.
Similar heterogeneity of short-term plasticity is found in a number of systems
(Saviane and Silver 2006, Dobrunz and Stevens 1997). This heterogeneity correlates
with the heterogeneous release probabilities of individual boutons (Trommershauser
et al. 2003), likely reflecting their different maturational state. Comparison of short-
term dynamics before and after induction has been used to indicate the mechanisms
of long-term potentiation in a number of studies, with widely varying results
(Appendix C). Probably the most influential of these studies considered responses
to brief periodic trains before and after LTP and found an increase in the first
response with little change in later responses, a phenomenon the authors called
"redistribution" as it was taken to reflect an alteration in the transfer function
between neurons (Markram and Tsodyks 1996)\(^2\). Most synapses in our study did

\(^1\) To the extent both sides of the synapse are involved in long-term change, it may be more probable that
both potentiating and depressing changes can co-occur (Tzounopoulos et al. 2007) in the different
compartments. Thus what is measured experimentally is the dominant, rather than the only, change.
\(^2\) As in all studies using paired-pulse ratio to assess vesicle depletion, the study by Markram and Tsodyks
assumed that the increase of first responses relative to subsequent responses after potentiation reflected
increased depletion. In our system the success or failure of release following an AP has little effect on the
show a change in short-term dynamics following potentiation (Chapter 3), which is generally taken to signify change in presynaptic properties. However, multiple lines of evidence point to involvement of new synapses at some delay STDP (Figures 3.8 and 3.14), though the change in short-term plasticity coinciding with the activation of these sites does not distinguish between awakening of ‘presynaptically silent’ or ‘postsynaptically silent’ synapses.

4.2.3.5.2 LTP as stabilizing the changes created transiently with facilitation: At many synapses, the second response in a train is more consistently successful than is the first response (Stevens and Wang 1995) due to short-term facilitation of $p_r$. The increase in reliability following induction thus serves a similar role to facilitation, and in this sense potentiation is a longer-lasting facilitation. The similarity in the effects on the synapse of the two processes, short-term facilitation and long-term potentiation, is highlighted by comparison of the distributions of preinduction PSC$_2$ to postinduction PSC$_1$ (Figure 3.11). It may be that the temporary elevation of release probability by facilitation effectively foreshadows the structural changes at the site initiated by LTP (Gardner-Medwin 1989).

4.2.3.5.3 Effect of induction on AP propagation: Successful initiation was verified for all APs recorded during the present study. However, even with initiation at the soma, it is still possible that an axonal branch between the soma and the terminal fails to propagate the impulse. This seems unlikely because the inter-pulse intervals used in the study were never shorter 12 ms and in the majority of experiments were never shorter than 25 ms; these intervals are longer than those typically expected to trap sodium channels in inactivated states to the degree that would be necessary to measurably increase the probability of axonal branch conduction failure (Chacron et al. 2000).

---

response to a subsequent AP (Chapter 2). This absence of release- or history-dependence indicates that the increased PSC early/PSC late seen in redistribution could not be explained in hippocampal neurons as an increase in release probability.
The trains used in the present experiments contained only 3 or 4 APs, also unlikely to fatigue the AP propagation apparatus. Even if branch failures did occur, the only influence on the results would be if the rate of failure changed following induction. Change of the branch failure rate following induction (Debanne et al. 1997) as a mechanism of increased reliability seems unlikely given that the cellular signal for such change would have to travel considerable distance from the active axon terminal along the axon in the short amount of time needed for reliability changes to become visible.

4.2.3.5.4 The influence of the inter-trial interval on synaptic dynamics: Short-term synaptic plasticity is generally thought of in terms of the interval between pulses within a trial, but it is important to recognize that the inter-trial interval will also influence the dynamics, and the interaction of the two intervals will have an additional effect. “Low-frequency mute” synapses, that are inactive when the stimulation rate is too low (Hanse and Gustafsson 2001) as well as synapses that become silent when the stimulation rate is too high (Saviane et al. 2002) have both been identified. Change of the sensitivity of the synapse to the speed of stimulation following induction cannot be excluded as an explanation for the increased reliability observed in the present study. In fact, such an increase in the robustness of the response to stimulation could be a component of the increased reliability; this would have a dramatic effect on neuronal communication in vivo.

4.2.3.6 Change in asynchronous release following induction: A number of groups have performed quantal analysis before and after long-term potentiation. Some studies found no difference in quantal size (Voronin et al. 1992), while others reported increases (Liao et al. 1992). Quantal analysis is analogous in some respects to the analysis in the present study of asynchronous release events before and after induction at a single site, as these events likely represent true quantal activity.
Changes in the rate of asynchronous release following induction indicate that new synaptic contacts have become active, presynaptic calcium handling at the existing terminals has changed, or both. In the present study asynchronous event size was not changed for tens of minutes at several synapses. Increase in the conductance or number of postsynaptic receptors would have been likely to change the size of asynchronous responses, so the absence of change in size (Figure 3.13) is important as an indication that the postsynapse was not altered on the time frame in question. At other synapses studied, the rate of asynchronous release increased following induction, but not immediately (Figure 3.14). Because reliability at these synapses changed shortly after induction, the change in asynchronous release is less likely to reflect change at existing presynapses, and more likely to signify the involvement of new sites.

Change in the size of asynchronous events was observed after conventional LTP (Oliet et al. 1996). The larger asynchronous events seen at delay following induction at some synapses in the present investigation could reflect late changes in AMPAR number or conductance, or increased coordination of presynaptic release. Rates of spontaneous and evoked release at a site are correlated (Prange and Murphy 1999, though the vesicles contributing to each may be drawn from different pools, Sara et al. 2005), but it is unknown if a similar correlation exists for asynchronous and phasic release, and if so, how the relation is affected by STDP. What the asynchronous data of the present study demonstrate is that the postsynapse is unchanged for at least 10 minutes after induction, and possibly indefinitely.

4.2.3.7 Accelerated synaptic maturation as the mechanism of potentiation: Taken jointly, the data presented in Chapter 3 suggest that spike-timing-dependent potentiation involves two processes: an increase in release probability at existing synapses, and, later, a change in potency that likely reflects the addition of a new
synapse\textsuperscript{1}. Developmental studies have indicated that as synapses develop in culture, anatomical formation can precede full functionality (Grantyn \textit{et al.} 1995), suggesting that a portion of synapses would be nearly mature, but still functionally silent. Our results that synapses with low release probability became more likely to release following induction and that potentiation appears to involve new synapses suggested similarities between the processes of potentiation and maturation (Bolshakov and Siegelbaum 1995).

A number of studies have reported rapid change in the status of release sites following activity. In some systems modification can occur on the time scale of short-term plasticity (Humeau \textit{et al.} 2007). Evidence for rapid maturation of synaptic release sites in hippocampal neurons has also been reported (Maggi \textit{et al.} 2003), though the effect may be short-lived. The participation of an individual release site in a given event, which varies stochastically (Quastel 1997) might be made more likely by a maturational process initiated by the pairing of pre and postsynaptic activity.

A role for activity in synaptic assembly during development has been postulated (Friedman \textit{et al.} 2000, Zhai \textit{et al.} 2001). Other studies suggest that dramatic increases in release competence are effected by a single-step modification of synapses (Mozhayeva \textit{et al.} 2004). Developmentally, the presence of constitutively active CaMKII, a protein shown to be necessary in its wild-type form for STDP (Wang \textit{et al.} 2005) increases the number of synaptic contacts in culture (Pratt \textit{et al.} 2003). The activation of release sites in or near originally-active boutons may underlie the observation of more ‘extra large’ synapses after perturbations that increase synaptic strength (Thiagarajan \textit{et al.} 2005). Actin disruption affects immature synapses more than mature ones (Zhang and Benson 2001), indicating that skeletal rearrangements

\textsuperscript{1} Whether the newly active synapse is added by unsilencing (Kullmann 2003) or by activation of a previously inactive release site (Voronin and Cherubini 2004) is difficult to determine from the present results. The changes in short-term dynamics and asynchronous release observed would be explained equally well by activity of previously ‘deaf’ or ‘mute’ synapses.
may be involved in the final maturational steps.

The connection between potentiation and synaptic growth was highlighted in studies of other forms of LTP (Antonova et al. 2001, Geinisman et al. 2001, Geinisman 1993, Toni et al. 1999). A recent study indicates that presynaptic release sites can both become release-capable and increase in release probability during potentiation (Ninan et al. 2006). Interestingly, in this study the formation of new synapses as judged by GluR1 GFP intensity seemed to require about 10 minutes following induction. Potentiation as the recruitment of additional release sites is also suggested by single-synapse studies (O’Connor et al. 2005).

If synapses must be active during induction to be potentiated (Figure 3.3), the question arises as to how low probability synaptic contacts would be eligible. In the present study, because of the small area of the microperfusion stream such synapses would have lain in close proximity to the higher probability synapses that were presumably active throughout the induction protocol. This spatial proximity may have permitted local cytoplasmic spread (Harvey and Svoboda 2007) of the induction cascade from the higher probability synapse to the lower probability synapse. Vesicles and other components of the presynaptic cytoplasm can be shared by nearby release sites (Darcy et al. 2006), and the spread of ‘activated’ (e.g., phosphorylated) proteins or other compounds could hasten the maturation of nearby synapses. An earlier study using local perfusion found that potentiation spreads over an area of approximately 70 μm (Engert and Bonhoeffer 1997). At a neuromuscular junction, transmitter release probability was highly variable at adjacent boutons, and the presence of release-related proteins was also variable (Knight et al. 2005), suggesting that the maturational state of en passant boutons along the same neuron vary in maturity. In our preparation a juvenile bouton may exist side-by-side with the mature one identified at the onset of the experiment and

---

1 Dopamine’s activation of new release sites (Chapter 2) may share similarities with its ability to accelerate developmental processes (Porter et al. 1999).

2 For example, cyclic AMP alone is capable of inducing functional boutons (Ma et al. 1999).
chosen for study. This second site could then undergo change. Selection of the microperfusion site during set-up would tend to pick boutons with low-release-probability neighboring boutons or none at all.

Alternatively, the change in reliability observed shortly after induction at most synapses may be driven by an increase in presynaptic calcium conductance. Calcium ions admitted at one active zone could spread to influence active zones in physical proximity\(^1\). The elevation of presynaptic calcium that follows a bout of activity (Habets and Borst 2006) may not persist during long-term potentiation (Wu and Saggau 1994), but changes in the release apparatus may persist after calcium levels drop. A third possibility is that short-term facilitation at the 1 Hz frequency of the induction protocol is sufficient to activate these low- \(p_r\) sites during induction, and the occurrence of the simultaneous BPAP leads to release of a retrograde signal that modifies them, making them active at longer ITI following induction. This seems unlikely though because almost all synapses showed short-term depression at intervals greater than 200 ms (Figure 3.10).

An alternative to the activation of new sites would be splitting of existing sites following induction (Edwards 1995). This mechanism would presumably halve the current receptor content, decreasing potency, while potentially allowing for an increase in reliability by generating two release sites. However, if both release sites had access to the receptor cluster present initially, this mechanism would be compatible with an increase in reliability with no change in potency. (It should be noted that activation of a nearby site releasing transmitter onto the same receptors, without splitting, could also have the same effect.) The splitting could involve both presynaptic and postsynaptic components: in keeping with the predictions of the model, LTP causes short-lived increase in the proportion of postsynaptic densities that are perforated and an increase in the proportion of boutons contacting two or

\(^1\) Such spread could be direct or involve the initiation of a calcium-induced calcium release wave (Ashby et al. 2002).
more spines (Toni et al. 1999). A drop in potency after a delay could be seen when continued structural change at the synapse restricts access to some of the receptors for each release site (Edwards 1995, Lisman and Raghavachari 2006). Some cases reported here do show a drop in potency following induction, but there are indications in a number of experiments that the synapse involved in a change in potency following induction is actually present before induction but is too low-probability to release without short-term facilitation (Figure 3.11), arguing against the splitting hypothesis.

For the first pulse in a train, low probability sites would appear to be essentially silent (“mute”), becoming active later, and the facilitation often observed (Figures 3.10 and 3.11) could reflect recruitment of such a site. By increasing the $p_r$ of such sites, STDP could enable them to respond to the first AP in a train. Alternatively, the activation of postsynaptically silent (“deaf”) receptor clusters presents a third possible mechanism for the addition of new synapses reported here. Indeed, there may be some correlation between the two types of synaptic silence: other researchers have found that when measured at positive holding potential, using $I_{\text{NMDAR}}$ to detect presynaptic release, AMPAR-containing synapses tend to have a higher release probability than synapses lacking AMPAR (Cabezas and Buno 2006). The probability of presynaptic calcium influx is also lower at presynaptic terminals apposed to AMPA-deficient synapses (Ward et al. 2006), and mute synapses are less sensitive than functional synapses to drugs affecting release of calcium from intracellular stores (Cabezas and Buno 2006), further suggesting reduced maturity. Thus postsynaptic AMPA receptor insertion may be accompanied by increased presynaptic reliability during LTP.

Early after induction of STDP, when potency has not changed, the mechanism of LTP is most likely increased reliability at the synapse active at baseline, and potency change initially seems to involve increased $p_r$ at formerly low-$p_r$ sites, because these sites tend to become available to facilitation. However, later changes in potency may
also involve postsynaptic insertion of AMPA receptors: during development, AMPAR clustering requires glutamate input (Rao et al. 2000), so new presynaptic release of glutamate might promote AMPAR insertion.

Still other mechanisms may contribute to LTP. Because boutons in these neurons can be mobile (Supplementary Movie 3), and inappropriate apposition of pre- and postsynaptic densities is observed in hippocampal culture (Rao et al. 2000, Krueger et al. 2003), re-alignment of pre- and postsynaptic structures could also be a mechanism of potentiation. It is interesting to note that in the cortex, even in adults, presynaptic boutons can be highly labile (Stettler et al. 2006). Migration of mitochondria (Supplementary Movie 4) into the stream is also a possibility (Li et al. 2004).

As a further consideration, the spike timing protocol may be potentiating release sites at the edge of the microperfusion stream, where calcium may be somewhat lower than in the bulk of the stream. Prior to induction the calcium admitted during the action potential may be insufficient to evoke exocytosis from such sites, but following induction a maturation process could lead to slow change in formerly ‘mute’ sites, making them release capable.

4.2.4 Temperature

Physical systems, including the synapse, operate differently at different ambient temperature. Differences in the thermal energy available to the system between experiments conducted at room temperature and those done at physiological temperature have dramatic influences on the kinetics of synaptic enzymes and synaptic transmission. Higher temperature results in minis of larger amplitude and faster kinetics (Postlethwaite et al. 2007). Short-term facilitation is increased at elevated temperatures (Klyachko and Stevens 2006), likely because both vesicle recruitment (Kushmerick et al. 2006) and exocytosis (Nouvian 2007, Micheva and
Smith 2005) are accelerated at higher temperatures.

In the present thesis, room temperature was used for all experiments. Elevated temperatures are likely to increase the metabolism of the neurons under study, possibly leading to greater metabolic stress, which would shorten the time frame for experiments. The stress on the cells would likely further increase given the low calcium concentration of the extracellular solution (0.2 mM) used for focal perfusion experiments. Moreover, most physiological studies of cultured neurons, and essentially all studies of activity-dependent long-term potentiation at synapses between cultured neurons, have been performed at room temperature, so comparison with other studies is facilitated by maintaining this condition.

4.3 CODA

Ultimately knowledge of the molecular biology and biochemistry of synaptic transmission and plasticity will require integration with knowledge obtained through imaging and electrophysiological studies. This process will be facilitated by methods that bridge the gaps between approaches, allowing physiological processes to be observed on scales increasingly closer to those of the molecules in question. At the same time, these reductionist approaches will be strengthened by experimental and analytical techniques increasingly able to account for simultaneous change in the many unobserved interacting components of the system.
Cellular neurophysiology as currently practiced frequently involves a good deal of engineering in order to develop the apparatus or method for addressing the question at hand. This can highlight the differences between engineering, where it can be difficult to make something work, but once it works the task is complete, and science, where the mechanism must ultimately be understood. In this sense engineering is like medicine, where there may be little time or need to figure out exactly why a given treatment works for a given patient after others have failed, as long as one that works is identified.

In studying the mechanisms of synaptic function it may be helpful to bear in mind that the nervous system and all of its components are products of evolution. While good enough for survival, and probably better than most other possibilities available at each stage of development and change, they are unlikely to be optimal in any strict sense (Linden 2007). Thus reasoning about biological data by considering why a given mechanism exists is not in general likely to be a productive strategy. It is probably more useful to ask what and how and where and when.

Hundreds of person-years and tens or hundreds of millions of dollars have been spent in pursuit of understanding of the mechanisms of long-term potentiation and depression. This effort has revealed many facets of the processes, but there remain numerous fundamental disagreements in the field.

As pointed out by Edwards (Edwards 1995), small differences in experimental variables generally not considered significant and in some cases not even reported
could be sufficient to bias the processes of synaptic change set in place by an induction protocol toward one among multiple possible mechanisms, ultimately resulting in the disparate outcomes that build disagreement among groups. Slight differences in temperature, osmolarity, pH, ion concentrations, oxygenation, and other variables are almost certain to affect the signaling cascades that bring about synaptic change. For example, in studies of STDP, an important technical detail that was widely overlooked was the use of cesium versus potassium as the internal cation during patch-clamp recordings, which was shown to dramatically influence the ability of somatic APs to back-propagate (Wittenberg and Wang 2006). Another difference likely to affect mechanistic conclusions obtained by studies of long-term synaptic plasticity is the induction protocol used: LTP due to 200 Hz tetanus can involve both pre and postsynaptic neurons, while in the same preparation LTP due to 50 or 100 Hz tetanus involves only postsynaptic change (Zakharenko et al. 2001, further discussed in Lisman and Raghavachari 2006). Postsynaptic depolarization, frequently a component of induction protocols, can itself alter presynaptic release probability (Voronin et al. 2004), possibly through a process analogous to the depolarizing suppression of inhibition seen at GABAergic synapses (Wilson and Nicoll 2001). Developmental factors must also be considered; for example, in younger animals LTP in hippocampal CA1 is mediated by increases in potency and release probability, whereas in older animals the change is accounted for by a change in potency alone (Palmer et al. 2004).

Perhaps the many seemingly conflicting results in synaptic plasticity research will ultimately be traced to as-yet unappreciated variables, but the process will be slower than necessary due to other obstacles within the field. One such impediment is imprecise terminology (Lazebnik 2004): the term ‘LTP’ has been useful among neuroscientists as a shorthand referring to a set of experimental phenomena, but it may be now that what one researcher means when using the term is not the same as
what another means\(^1\). The more detailed scientists’ understanding of the mechanisms of ‘LTP’ becomes, the more important it is that the same underlying phenomenon is referred to when the term is used. If multiple phenomena are referred to by the same term, its use could cause confusion and even spurious disagreement. Only from the remove of a future time when synaptic plasticity is better understood will it be possible to determine if and when LTP ceased to be a productive concept around which to frame experiments.

In the present, though, the biggest obstacle facing the field of synaptic plasticity is more sociological than terminological. Is LTP presynaptic or postsynaptic? One group shows definitively it must be presynaptic. Shortly thereafter, another group shows that it has to be postsynaptic. How long would such confusions persist if, rather than battling in the pages of journals and at conferences, the researchers involved were compelled to do publication-quality experiments together? Those who finance the research (i.e., the public) should expect that efficient methods of resolving controversies take precedence over personal animosities. In most cases the natural process of debate within the scientific community is effective at resolving such disagreements, but in extreme cases it may necessary for funding agencies to step in and accelerate the process\(^2\).

The situation is reminiscent of that discussed by Richard Feynman in “Cargo Cult Science,” in that many investigators are aware of other findings which call into question assumptions on which their experiments are premised but which they

---

\(^1\)Some investigators use the term LTP to refer to a process lasting 5-7 minutes (O'Connor et al. 2007), while others use it for change present hours after an induction protocol. Other examples are numerous. As pointed out by Burgoyne and Barclay, for some investigators ‘kiss-and-run’ refers to a process occurring on the timescale of milliseconds, whereas for others it refers to a process 10- or 100-fold slower (Burgoyne and Barclay 2002). As evidenced by the present thesis, the term ‘synapse’ is used to refer to both individual synaptic contacts and the hundreds of contacts that can make up the connection between two neurons.

\(^2\)However, such a top-down approach is unlikely to work; for the Manhattan Project, to give an example of a large, top-down research project, there were infinite wrong ways to solve the problem of building a bomb but also infinite right ways, increasing the odds for a well-managed research team to arrive at one successful method, while for science there are infinite wrong ways, but only one right answer to each question, and even the best management might not pick the right directions in the search for that solution. With independent teams, the probability that at least one will take the right tack is probably greater.
ignore out of the sense that this is the only way to obtain experimental results in a reasonable amount of time, publish, and renew funding (Feynman 2007). Adding new pebbles to an already crowded beach — rather than seeking to integrate existing findings, reconcile apparent contradictions, and synthesize a larger understanding — is the path to scientific stability encouraged by the existing structure.

At the same time, truly contradictory findings may be not overlooked but rather nonexistent. A report of X phenomenon at synapse Y is not contradicted by a finding of not X when the synapse in question is not Y; in few cases are all particulars of an experiment ever reproduced in another experiment outside the original lab. When conditions are different, contradiction cannot occur—reproducibility, which in theory serves as the final check on all scientific discoveries, is difficult or impossible to assess. The system of disproving accepted hypotheses through failure to reproduce findings works imperfectly because reproducibility is seldom considered a realistic criterion (Pike 1933, Giles 2006). Experimental methods are complex and take years to learn, further reducing the likelihood of true tests of replicability. Besides, replication is neither intellectually engaging nor prestigious; even if funding were available, who would want to do it? Additionally, once a finding is accepted, it is generally far easier to withhold data that contradict the finding than to mount a full assault against the established dogma. Such withholding need not be malicious, or even conscious, as evidence contravening dogma can almost always be explained by a known or presumed change in experimental conditions. Far from a Popperian world where a new theory is subject to multiple attempts at disproof, failing which it is accepted (Popper 1951), if an interpretation of a biological phenomenon provides a satisfactory account of most known data it is accepted, and may gradually become dogma, assumed by those not intimately familiar with the field to be unquestionably true.

At times though it seems that no dogma is sufficiently stable to serve as a basis
for a conceptual framework. Quantal analysis (Butler 2007), depletion as the source of short-term depression (Bellingham and Walmsley 1999, Sullivan 2007), the readily releasable pool of vesicles as being those vesicles anatomically closest to the synaptic cleft (Rizzoli and Betz 2004), the stable docking of vesicles prior to release (Degtyar et al. 2007)... each of these long-accepted tenets has recently been called into question, in turn calling into question discoveries which assumed the truth of the ‘lower-level’ dogma.

Neuroscience may function not by clear rejection of theories but rather by gradual moving away from the framework in which the theory was put forward. As the framework becomes passé (as did such concepts as engrams, operant-only mental function, or complete encoding of the plan for neural development in the genetic material), the catchwords of that time are left behind, quietly taking their implicit framework with them. New experimental results attract attention to a new set of questions and the field moves on, the previous theories not disproved so much as widely held to be implausible, or wrongheaded in approach1.

If dogmas are not disproved and are known only to initiates to be false, or incomplete, other researchers, who rely on these truths in interpreting their own studies, may be reasoning from false premises. Perhaps the ideal stance for a scientist is to recognize that assumptions on which current arguments and current conclusions are drawn may later be found to be false, so it is best to a) be mindful of assumptions, and question them, b) stay aware of new findings that could challenge or invalidate the assumptions, and when this occurs, react accordingly, rather than ignoring the problem until reviewers demand it be addressed, and c) base findings on multiple lines of evidence and reasoning. It requires judgment to decide when a given assumption is no longer tenable, but as long as a good-faith effort is made that should be sufficient, as long as one does not say, as the researchers criticized in

---

1 Disproof, and direct testing, of theories may play a relatively small part in neuroscience due to a paucity of theory, in the sense of a unifying explanation for a wide range of observed phenomena.
“Cargo Cult Science” said, “I can still publish this way, so I will.” What a disappointing application of the decades of education and training invested in each scientist that attitude represents!

For individuals, academic and intellectual attainment is closely linked to mastery of fundamentals and unaddressed weaknesses hinder progress. The same is probably true for scientific disciplines. As science gradually enlarges humanity’s understanding, the greatest obstacle to advancement is not the absence of knowledge, but the presence of inaccurate knowledge.

Sensory systems physiologists increasingly appreciate that the choice of stimulus set and presentation structure can influence experimental results designed to determine neural representations of the outside world (Brincat and Connor 2006). Similarly it is likely that synaptic physiologists will develop greater recognition of the importance of the interaction of experimental variables such as inter-pulse and inter-trial interval. In the present study a simplistic attempt at improved mapping of the parameter space was made with the use of variable stimulus trains presented randomly but uniformly. This permitted a wider search for the effects of a given IPI on the synaptic response that follows it along with testing of the degree to which the effect can propagate in time. In a perfectly deterministic system, this propagation could be infinite, and it would necessarily be detectable as long as measurement resolution were also infinite. With an imperfectly deterministic process such as synaptic transmission, the large parameter space implies that mapping of the parameters will only be feasible with well-designed closed-loop control. Ultimately it is desirable to develop stimuli that probe interactions of parameters in the process of characterizing neural function with enough analytical power to productively guide experiments in real time.

The eventual success of biology in the effort to understand living organisms
mechanistically, to know for example what all molecules (ions, water, proteins, lipids, RNA) in the presynapse and postsynapse do, how they interact and regulate one another during development, plasticity, and aging, in health and disease, is not guaranteed. Its achievement would require continual effort on a massive scale to develop new experimental methods, more powerful experimental software, improved semantic databases, and other necessary tools. However it may be that in the end humanity does not want this knowledge enough to bear the costs of obtaining it. If it is known what goes wrong with a synapse in disease and how to fix it, or how to let nature fix it, for example with stem cells, that may be good enough. At some point the detailed mechanisms of synaptic biology could become something of a curiosity, with reduced practical importance, when satisfactory repairs or workarounds for dysfunction exist. Alternatively, it is possible that only through decades or centuries of continued pursuit of knowledge of synaptic mechanisms will understanding be sufficient to cure the diseases that neurology and psychiatry seek to treat. Until the outcome is clear scientists will keep seeking to understand the function of the biological machinery, in the hope not only for knowledge for its own sake but also that the findings will soon be of greater utility to those who ultimately support them.
APPENDICES
MICROPERFUSION PROTOCOL AND TECHNICAL NOTES

Protocol

The sequence of the experiment is as follows: Begin bulk perfusion, place the microperfusion tips and patch pipettes and prime the siphon. Place the cover slip and identify candidate pairs of neurons — healthy-looking cells with no neurons between them are most promising. Get the cells in the camera view, place the positive pressure tip and confirm it is unobstructed, start positive pressure1, and place the negative pressure tip to confirm access to the intended site. Place the patch pipettes near the neurons intended for patching, if indicated rotate the stage to get the predicted dendrites of interest in the right orientation for local perfusion (microperfusion across a dendrite being much more focal than puffing the length of a dendrite), and lift the perfusion pipettes from the solution to avoid dye. Switch the light path to the camera, use the camera and the oscilloscope to patch, check connectivity and cell types, and if cells are synaptically connected and both are glutamatergic, proceed.

If single-neuron labeling will be performed, add normocalcemic solution with FM 1-43 and CNQX, evoke 7 trains of 25 APs at roughly 5 Hz in the presynaptic cell, with one minute between trains, leave dye flowing for ~1 min following this

---

1 Notes: The pressure regulators standard with the Picospritzer device suffered from low precision and low stability. The positive pressure tip should be as small as practicable; at or above 5.5 MΩ is generally sufficient for obtaining focal recordings. Given the small tip diameter, impurities in either the solution or the pressurized air could cause blocks in the positive pressure pipette; before filling, the positive pressure pipette should be tapped with tip up to remove any debris. For tip geometry, a long taper may reduce the spread of microperfusion solution leaving the pipette.
stimulation to ensure dye is present during remaining exocytosis. During staining switch to the 40x objective and the correct filter cube for the dye. Wash quickly with normal solution to remove free-floating dye (note that seal quality often drops during rinses), wash with Advasep 7 for ~5 min using fast perfusion, wash briefly again with normal solution to remove Advasep, darken the room, focus using visible light, and capture then export the visible light image for records. Focus up ~10° for fluorescence, turn off the incandescent light, open the shutter, capture the fluorescence image, close the shutter, and export the file and threshold it to highlight boutons. Make this figure window transparent, resume visible light imaging, lay the transparent fluorescence image over the ongoing visible light movie and align them manually. Return the perfusion pipettes to the bath, re-prime the siphon line briefly with the tip far from cells to confirm air entered, place the perfusion pipettes near the desired bouton\(^1\) using the camera image and the labeled overlay, switch bath solution to low Ca\(^{2+}\), and evoke APs in the presynaptic neuron.

If boutons were not labeled, move the positive tip along the postsynaptic dendrite to find responses then place the negative tip to sharpen responses until event sizes and failure rates, and the placement of microperfusion tips, are consistent with enabling of few or single sites. When searching for active boutons it is better to use several stimulation pulses at brief IPI due to the low release probability. Ongoing camera input can be used while manipulating the tips for simultaneous visualization of the tip positions, the bouton position, and the recorded bouton EPSCs. To confirm that microperfusion is working once a candidate bouton is identified, turn puffing off for a trial or move the microperfusion stream away from the bouton.

For the experiment, place the tips, especially the positive tip, as close as possible to the dendrite of interest. Place the negative tip as close to the glial surface as possible.

---

\(^1\) Recordings should be made from boutons of moderate size and brightness to reduce the likelihood of recording from a contact with multiple active zones.
possible without touching and the positive tip almost as close. Judging positions in the z-axis is best done using DIC or other depth-providing optics. Once an active synapse is identified, begin recording.

**Configuring the local perfusion system**

During modification of the rig to include microperfusion, preliminary experiments with inks (Trypan Blue) and dyes (FM, which fluoresces only if it contacts a lipid membrane) can be used to fine-tune angles, check perfusion of the glial bed, and confirm that the negative tip is trapping all puffed solution. If inert ink or food coloring is included in the local puffer solution for assessment of the extent of spread (Bekkers and Stevens 1995; Engert and Bonhoeffer 1997) particles must be very small and not viscous to avoid blockage of the tip. Some colorants may affect neural responses (Augustine and Levitan 1980). If the bath liquid height is correct, the local perfusion stream can be visualized on phase contrast optics when water is used for bulk perfusion, which is useful for calibrating the system prior to experiments.

In theory the negative tip could be omitted if the positive pressure tip is applied to neurites far ‘downstream’ of the two patched cells, because in this configuration it is likely that the normocalcemic solution will only be available to small numbers of release sites. However, this approach could be problematic for studies of activity-dependent plasticity, presumably because of the possibility of several release sites present in the spray area. The normocalcemic solution could be diluted by being spatially spread, resulting in each site having a little activity during induction, likely making potentiation impossible (Figure 4.3). Thus while the primary function of the negative tip is to collect normocalcemic solution, ensuring it is not available beyond a very small region, restricting the normocalcemic spray domain is likely to also locally increase \([\text{Ca}^{2+}]\).
The positioning of and pressure to the tips requires optimization of several possibly competing variables. For example, a more vertical positive pressure tip would help ensure that the synapse, lying on the glial bed, is well perfused by the local flow, but this could also lead to greater local spread due to turbulence. Relevant variables include positive pressure tip size, positive pressure, height of positive pressure tip above the glial bed, and angle of positive pressure tip relative to the glial bed; size and height of negative pressure tip, angle, and amount of negative pressure; and angles of tips relative to each other and to overall flow, inter-tip distance and global perfusion flow rate and bath height. The results reported in this document were obtained with somewhat vertical positive pressure tip (≈60°) for good perfusion and a more shallow angle for the negative pressure tip (≈30°) for good collection. Practice is recommended for gauging best tip orientation and positioning.

Mechanical stability of the patch electrodes was particularly important in low calcium solution owing to the reduced membrane tolerance for stress. Stability of the microperfusion pipettes for constant local activation over the experiment is indispensable.

**Labeling**

Phototoxicity and photobleaching depend on the combination of [dye], [Advasep], load time, rinse time, exposure time, aperture settings and signal intensity; the optimal parameters for single-cell imaging will need fine-tuning for each preparation. An alternate method of labeling is to stain all neurons’ boutons using high potassium and dye loading prior to patching. This is simpler but can make it harder to find boutons formed by the patched presynaptic cell. Use of transfected constructs such as synaptophysin-GFP (electroporation or lipofection) may also be suitable for single-bouton experiments. Labeling of freshly dissociated neurons by electroporation of genetic material for fluorescently-labeled protein is
followed by ~10 days of recovery, so cell health should not be affected, but efficiency is low. Compounded by the low probability of finding two morphologically promising neurons in correct orientation to one another, this renders candidate pairs scarce.

**Noise**

Noise reduction is also critical. The optical and microperfusion systems can both increase recorded noise if precautions are not taken. The setup described here uses an enclosed Faraday cage with the front face being a bolt of conducting cloth pulled across the opening during experiments. The arc lamp power supply is passed through a 1:1 isolating transformer (Triad) to separate this from the remainder of the rig, with a styrofoam sheet under the transformer to dampen its mechanical vibrations. The shutter is grounded and the inductive kick created by its mechanical vibrations. An opto-isolater was added to the shutter-camera card connection to prevent noise from the camera card from entering the rig. In keeping with general noise-reduction strategies the setup uses trees off power strips ordered from most to least sensitive, avoiding daisy chains, and makes use of an aluminum-block ground mecca. The air pressure controller that gates the positive pressure can also contribute significant noise, ameliorated by grounding. The fan of the CCD introduces a small amount of high-frequency noise that is difficult to eliminate; water cooling might be preferable. (This fan could also introduce mechanical instability, but because the stage is separated from the scope, which holds the camera, this is minimized.) The positive and negative pressure tips bring noise into the perfusion well, so their holders are grounded. Dimming flat screen monitors below a certain level can create noise, so colored cellophane is used on the monitor to reduce brightness.

**Siphon control**
The siphon tube abuts the suction tube inside a larger tube (a modified Warner Instruments pipette holder, ends sealed with pipette holder gaskets; Figure A.1) open to the air by a hole approximately 8 mm in diameter. The negative pressure can be finely adjusted by altering the height of the siphon outflow using the screwplate holding the siphon-suction junction\(^1\). To prevent the small change in siphon pressure that would occur due to falling of drops at the siphon outflow (effectively changing siphon height), the vacuum pressure is apposed to the siphon outflow such that drops were removed as they form. The outflow end of the siphon tubing is joined to a beveled cylinder (partly blunted 18-gauge hypodermic); this is apposed to a non-beveled tube (sawed-off 18-gauge hypodermic) connected to the suction system. Removal of liquid from the siphon outflow occurred nearly continuously.

While the siphon outflow height is a primary determinant of negative pressure, the contact between the suction tube and the siphon efflux may increase the siphon negative pressure through the cohesion of the liquid, so suction regulation (Fairchild) should be considered. An alternative to apposing the end of the siphon tube to the suction tube is the use of a single inner tube with holes drilled around the perimeter. The size and spacing of these holes will determine the influence of the suction on the siphon.

If precise control of suction is not available, the distance between the suction tip and the siphon outflow should be increased sufficiently to allow drops (appx. 5 \(\mu l\)) to form and fall naturally, eliminating the influence of the suction on the negative pressure. The negative pressure created by the siphon at the cell bed should be moderate, as too strong could slowly damage neurites, while too low could permit dispersal of normocalcemic solution outside the intended laminar flow area. If negative pressure is too low this could also permit an oscillatory suction pressure

---

\(^1\) There was very little hysteresis of the siphon; when the outflow end was moved above its equilibrium point flow reversed essentially instantaneously and it reverted instantly when outflow was moved below this point.
dynamic with fluctuations in microperfusion confinement (Supplementary Movie 5). This problem is readily detected by the experimenter as event sizes fluctuate periodically and dramatically.

**Figure A.1:** Siphon-suction junction used in the microperfusion system. The siphon outflow tubing (a) is connected to a pipe (1/16" inner diameter) that enters the housing (b) from above, with the apparatus vertical during experiments. As solution exits the siphon it is removed by the vacuum tubing, also connected to a pipe (c). This removal minimizes variation in the siphon outflow point. The gap between the siphon and the suction is exposed to the air through the aperture (d). To prime the siphon d is closed, which converts the siphon to an extension of the suction line because the housing is airtight at either end.

Prior to each experiment the siphon is primed by placing the negative pressure tip in the perfusion well before cells are introduced and closing the hole on the siphon-suction junction housing (Figure A.1), functionally connecting the vacuum suction line to the siphon. Filling requires ~20 seconds, and after the line is filled the hole is reopened. Reopening is done gently to prevent air from being pulled into the siphon from the back end during the drop in negative pressure that effectively acts
momentarily as positive pressure. Filling can also be done from the back using a screw tip connected to a liquid-filled syringe. Occasionally the siphon becomes blocked by a large piece of debris; these can generally be removed by surface tension through lifting the negative pressure tip out of the solution. (The function of this siphon deviates from that of an ideal siphon in a serendipitous way: if the siphon tip is briefly pulled above the liquid line then returned, no air bubble will be introduced and the siphon effect is not interrupted. This is presumably due to capillary action at the tip.) In cases of recalcitrant block, bulk perfusion and vacuum suction are briefly stopped and an air-filled 10 cc syringe connected to a threaded tip is screwed in to the aperture of the siphon-suction housing. A small amount of air injected into the line cleared the siphon, which is then re-primed. In practice, such blocks occur during set-up, when tips are being introduced into the bath, as large debris was not cellular but low density material that floated. Between experiments the negative pressure tip is cleaned with xylenes or replaced whenever debris is visible.

As judged from imaging of the focal perfusion stream, siphon pressure does not fully stabilize until several minutes after negative tip filling and placement, so experiments should not begin until after this period.
APPENDIX B

METHODS OF MEASURING ACTIVITY AT FEW SYNAPSES

B.1 INTRODUCTION

The probabilistic character of synaptic activity makes it desirable to increase the power of physiological experiments by observing the function of the smallest possible number of synapses. The relevance of this undertaking to improved knowledge of neural communication is highlighted by the fact that single synaptic contact sites often serve to link neurons in the brain (Gulyas et al. 1993, Henze et al. 2002). Many approaches have been taken toward the goal of measuring activity at few synapses. Consideration of these methods permits a review of the contribution of each to the present state of understanding of synaptic function.

B.2 REVIEW OF METHODS

B.2.1 Patching boutons
Likely the most direct method of studying activity at single boutons is to label and patch the bouton itself. To this end Forti et al. visualized synapses formed by hippocampal neurons in culture with the lipophilic dye FM 1-43 then patched them in ‘loose patch’ configuration (Forti et al. 1997). The group was able to record AMPAR-mediated current due to exocytosis and receptor activation, apparently by measuring from the ‘outside’ as ions flowed into the postsynaptic neuron. Quantal amplitude varied from PSC to PSC, a fact the authors cited as evidence for the non-saturation of glutamate receptors. They found that although the single-site events were variable in size, their time course was consistent, suggesting the mechanism of release varies little. Forti et al. put an upper bound of 20 ms on the time for recovery of glutamate receptors from desensitization by considering the correlation of event size and inter-event interval. They also performed simultaneous somatic clamp and found that the distortion of events reaching the soma is nearly linear. The coefficient of variation for events recorded at the single site was smaller than that for all somatic events, pointing to inter-site variation (Frerking and Wilson 1996) and differential dendritic filtering (Smith et al. 2003) as important contributors to quantal variation. The recordings of Forti et al. show inhomogeneity (“burstiness”) in event occurrence times (cf. Meiri and Rahamimoff 1978) and a Gaussian distribution of response sizes, which may reflect single vesicle variability or variable coordination of multivesicular release. Such coordination could be strengthened, for example, due to the high \([\text{Ca}^{2+}]_{\text{pipette}}\) employed (Llano et al. 2000).

Related methods involve patching the large mossy fiber boutons (Alle and Geiger 2006) or the ‘blebs’ formed by the cut axon in slice (Shu et al. 2006). Both approaches found that somatic voltage changes, including fluctuations due to

---

1 Despite their correction of current measurements for loss through the loose patch seal, the increase in noise due to the increased current across the loose seal would seem likely to have reduced the signal-to-noise ratio of their recordings. At the same time this may have had the benefit of rendering other events along the same dendrite undetectable.
activity in the circuit, propagate down the axon much further than had been assumed. Interestingly, the membrane voltage of the mossy fiber bouton varies considerably from trial to trial due to population activity in dentate gyrus, indicating an influence on synaptic transmission. With current techniques it is not possible to measure the effects of presynaptic somatic $V_m$ on local $V_m$ at synaptic contacts in smaller hippocampal synapses, but it seems likely that the propagation observed by these investigators would occur at these sites as well.

B.2.2 Restricting perfusion to selectively enable release ("microperfusion")

In the classic study of Katz and Miledi (Katz and Miledi 1965) local perfusion with calcium-containing solution in a global bath of calcium-free medium was used to establish the necessity of local calcium for release of transmitter at the neuromuscular junction. Calcium was present in the recording pipette placed on a visually identified synaptic contact and held within the pipette or allowed to diffuse by changing current. When calcium was withheld, no end plate potential was observed following stimulation of the nerve; when calcium was released the EPP appeared.

This method has since been adapted by a number of investigators. In order to address quantal size and the number of receptors at a postsynaptic density Kraszewski and Grantyn (Kraszewski and Grantyn 1992) used a spatially restricted perfusion method to record synaptic currents from collicular neurons grown in culture, with low calcium perfused globally. They measured an effective quantal size of 5-10 pA and counted the number of channels opened by each single synapse event, concluding that a single quantum opened 5-15 GABA receptors.

Veselovsky et al. (Veselovsky et al. 1996) reported a technique using two local perfusion pipettes, source and sink. The source tip had two internal channels, one
with positive and one with negative pressure. Using the negative pressure within the source tip, the flow from the source to the bath could be spatially constrained and the solution could be changed quickly, with speed presumably limited only by the acceptable negative pressure at the external mouth of the source tip. Similar methods were developed by other groups (Maconochie and Knight 1989, Ogata and Tatebayashi 1991). Modifying the technique of Veselovsky et al. by removal of the second pipette, Engert and Bonhoeffer (Engert and Bonhoeffer 1999) tested the spatial specificity of LTP in dendrites. With positive and negative pressure inside the source tip they limited their local perfusion stream width to ~30 μm and had the advantage of only having to manipulate a single perfusion tip. Working with slice cultures, they used a low calcium bath containing cadmium to block exocytosis at non-puffed sites (though Cd²⁺ may be toxic with extended application, Biagioli et al. 2007), and elevated calcium in the local stream. Using the solution exchange capacity of their system along with the NMDAR open-channel antagonist MK-801 they established the spatial specificity of the configuration. With this technique Engert and Bonhoeffer concluded that LTP spreads about 70 μm from the synapse of induction, though without knowing the cell of origin of presynaptic terminals they could not distinguish spread within the cytoplasm from spread by extracellular diffusion.

In order to study local protein synthesis, the Shuman group used microperfusion to apply the NMDA receptor antagonist APV to a small region of a neuron’s dendrites (Sutton et al. 2006). With local perfusion their finding that blockade of

---

1 Changes in the liquid junction potential were used to measure the speed of solution exchange; both onset and offset of fluid flow were rapid, requiring only several milliseconds.

2 Solution exchange was slow; synaptic potentials could only be observed ~30 seconds after moving the superfusion tip and only vanished ~20 seconds after the tip was removed. This sluggishness is presumably due to the dense packing of neuropil in slice culture; for Katz and Miledi, after calcium was re-applied to the NMJ, release resumed rapidly.

3 This finding has recently been challenged, however, with experiments using more spatially precise optical techniques that indicate that LTP is synapse specific, but does reduce the threshold for LTP at synapses within a ~10 μm radius (Harvey and Svoboda 2007).
NMDAR leads to rapid membrane insertion of the GluR1 subunit of the AMPA receptor was confirmed to be due to local signaling.

B.2.3 Local stimulation of release with high potassium or sucrose

Local stimulation of release from individual boutons permits highly focal activation and avoids the possibility of failures of AP initiation or propagation, though the release process may be altered to some extent. A method for local activation of labeled boutons was developed by Liu and Tsien (Liu and Tsien 1995a, and companion article Liu and Tsien 1995b). In this set of studies the authors used local application of high K+/Ca2+ solution with low calcium global perfusion to activate visualized synapses in cultured hippocampal neurons1. Interestingly, PSCs were not observed for several hundred milliseconds after the onset of solution application, suggesting different mechanisms from those involved in AP-initiated depolarization and release. Notably, even for this spatially restricted release no clear quantization was visible in the waveforms, a frequent finding in single-bouton studies (Forti et al. 1997, Hanse and Gustafsson 2001). By counting events released during each ‘puff’ of high potassium over multiple trials the authors estimated that ~90 events could be generated without vesicle recycling. The large size of the recorded events (>80 pA in some instances) suggests that local depolarization may permit synchronous release of multiple vesicles, though the authors found that the amplitude distributions of evoked and spontaneous events onto a postsynaptic cell were quite similar, indicating either coordinated spontaneous release (Liley 1957) or large effective size of some single vesicles. The single-bouton studies of Liu and Tsien also revealed that the amplitudes of events at different sites in the dendrites of the same

---

1 The authors indicate that the combination of two necessary factors being only locally available likely accentuated the spatial narrowness of their stream, though it is difficult to be sure how spatially restricted the flow was without a collection tip.
postsynaptic cell are quite similar, suggesting the existence of a normalization process. A related finding was that as the number of synapses onto a given postsynaptic cell increases, the average strength of each individual synapse decreases and the average response to iontophoresed glutamate also drops, indicating combined pre- and postsynaptic adaptations to new synapse formation.

An additional use of techniques based on synaptic activation through local perfusion has been to address the question of receptor saturation. Staining the release sites of hippocampal neurons in culture with FM dye, McAllister and Stevens (McAllister and Stevens 2000) used local application of a high-osmolarity solution in conjunction with local collection to cause large amounts of exocytosis at individual synaptic contacts. (Given the 500 ms inter-event interval used to isolate individual events, overlapping events were likely rare, but events were still fairly large, on the scale of 50 pA, suggesting either large univesicular release, or, again, some form of AP-independent coordination.) The authors argued that because responses to synaptic transmission were highly variable, whereas responses to local iontophoretic glutamate application were highly constant, synapses are not saturated by the release of transmitter by a presynaptic neuron.

The effect of short-term activity on quantal size has also been studied using local bouton activation (Chen et al. 2004). The authors used local electrical stimulation of labeled boutons to evoke release, in the presence of TTX to prevent APs. They found that paired-pulse facilitation, as represented by the second pulse’s lower likelihood of failure, did not change quantal size. From this they inferred that the events they recorded were, if not due to fusion of a single vesicle, at least as monoquantal as are spontaneous events. The fact that EPSC sizes were not different in 1 mM and 3 mM external calcium, even though p_ increased considerably in the latter condition, further indicated that these were true monoquantal events. The study found that in

---

1 AMPAR-antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) was used globally to block other synapses, and zero magnesium was used locally to increase the NMDAR current. Additionally, the width of the microperfusion stream was roughly 2 μm, indicating that a fairly high negative pressure was used.
[Ca\textsuperscript{2+}]_{\text{external}} generating short-term depression, successful second responses tended to be smaller than successful first responses, regardless of the success or failure of the first stimulus, suggesting an activity-dependent, release-independent reduction in quantal size or release mode.

Microperfusion with both AP- and hyperosmotically-induced exocytosis was used to study synaptic properties by Bekkers and Stevens (Bekkers and Stevens 1995). Their positive pressure pipette was double-barreled, with one barrel containing high calcium extracellular solution and the other at the same ionic composition, but much higher osmolarity\textsuperscript{1}. The authors found a large variability in quantal sizes and a low release probability at individual release sites. Comparing electrophysiological responses to histological counts of boutons they observed that responses at most synapses were not well fit by binomial models taking the number of release sites as N (cf. Korn et al. 1982)\textsuperscript{2} though the uniquantal hypothesis would have predicted a good fit (see also Ninio 2007).

B.2.4 Activation of synapses with a toxin

\textsuperscript{1} Of all studies discussed in this review of single-synapse methods, this is the closest in methodology to that employed in experiments for the present thesis, because the authors used action potentials to evoke many of the release events recorded, and because both source and sink local perfusion tips were used. Nonetheless, the events recorded by Bekkers and Stevens were considerably larger than those reported here. This is likely due to two technical limitations that would have reduced spatial specificity of the earlier study: the absence of a means of visually identifying boutons, and the use of neurons grown in isolated microislands of just two cells, in which the number of synaptic contacts on a swath of postsynaptic membrane formed by the patched presynaptic neuron (and thus within the microperfusion stream) would have been high. With a stream width of 30 μm, they estimated four to 14 synapses within the stream using immunohistochemical methods: response amplitudes, apart from one outlier experiment, ranged from ~50 to several hundred pA.

\textsuperscript{2} In terms of methodology, Bekkers and Stevens use charge rather than amplitude as a measure of synaptic size in some cases, and discuss the merits of charge versus peak as measures of small events: charge may be less distorted by passive cable filtering in the dendrites, but slight inaccuracies in the determination of the baseline can have much larger effects on the measurement of the charge than would be seen with measurement of peak PSC. Indeed, to minimize cable filtering, the authors often puffed at sites near or on the postsynaptic soma, permitting more faithful measurement of events at the expense of inclusion of many sites.
α-latrotoxin, derived from black widow spider venom, causes bursts of exocytosis when applied to neurons. Auger and Marty (Auger and Marty 1997) used these long bursts of events to study receptor saturation and concluded that that receptors were not saturated by a single event. The rapid rate of α-latrotoxin-evoked events allows testing of the influence of the previous event on the current event, an approach pioneered by Tang et al. (Tang et al. 1994) for study of receptor desensitization. When the burst frequency was high, Auger and Marty observed receptor desensitization. Using both latrotoxin and high-frequency trains of APs, desensitization and fast recovery were also identified by Crowley et al. (Crowley et al. 2007), who argued similarly that events evoked by α-latrotoxin originated at a single synaptic site.

B.2.5 Activation of a single synaptic contact on mechanically dissociated neurons

Using an acutely dissociated preparation with presynaptic terminals still attached to the patched cell, Akaike et al. stimulated individual boutons with brief applications of current, finding that bouton stimulation is all-or-none, is blocked by TTX, and depends on the external calcium concentration (Akaike et al. 2002). Interestingly, in

---

1 Events within latrotoxin bursts have similar amplitudes whereas between bursts the amplitudes are different, and on this basis it is argued that the bursts occur at a single release site. EPSCs obtained with this method are highly consistent, suggesting low within-site variance (Frerking and Wilson 1996), but it seems difficult to be sure that the toxin is not altering the mechanism of release — the toxin is believed to form a cation-selective pore in the presynaptic terminal — such that response sizes are more consistent than they would be otherwise. Additionally the large number of synaptic events recorded during each burst raises the issue of whether the pool of vesicles would be sufficiently large, or refill sufficiently quickly, to enable the recorded activity, if it truly is from a single site. With a total of 100 vesicles in the readily releasable pool (Schikorski and Stevens 1997), at a release rate of 20 Hz the pool would be exhausted in 5 seconds, while the bursts recorded last much longer, though rapid endocytosis and refilling of the type observed at cerebellar synapses (Saviane and Silver 2006, Crowley et al. 2007) could make this possible.

2 Presumably because of the high input resistance of the dissociated cell and the low pipette capacitance possible with this preparation, current noise is enviably low. For example, see Jin and Andersen, SFN Abstracts 2005.
this very reduced preparation, amplitude histograms of IPSCs did not show discrete peaks.

B.2.6 Minimal stimulation of axon bundles to evoke release from a single site in slice

Since its development as a tool for determining the number of quanta underlying release at a single synapse (Raastad et al. 1992), the method of minimal stimulation has been used by a number of investigators to test the properties of release at a single site (for example, Dobrunz and Stevens 1997, Isaac et al. 1998). A series of studies using minimal stimulation revealed that the pool of immediately available vesicles at individual terminals is small and begins to refill almost immediately on stimulation (Hanse and Gustafsson 2001a, Hanse and Gustafsson 2001b). Change of responses following long-term potentiation has also been addressed using minimal stimulation: at the Schaeffer collateral synapse Peterson et al. (Petersen et al. 1998) identified step-like transitions in synaptic strength following induction at single synaptic sites, suggesting that potentiation can be mediated by binary changes in synaptic strength (O’Connor et al. 2005, O’Connor et al. 2007).

The high failure rate in minimal stimulation allows separation of the probability of success from the size of successful responses (potency), which offers the possibility of increased insight into the mechanisms of synaptic function. However, as with many studies of the mechanisms of LTP, different groups obtained conflicting results: one study found a change in reliability (proportion of stimuli that successfully result in current) with no change in potency (Stevens and Wang 1995), while another found that change in potency, assumed to reflect postsynaptic change, always occurred while change in failure rate occurred only in some experiments (Isaac et al. 1998).
B.2.7 Paired recordings from neurons connected by a single contact

More precise control of presynaptic action potential firing than is available with the minimal stimulation approach can be had from paired recordings. Generally these are difficult to perform because the sparse connectivity of randomly chosen pairs of neurons in acute slice or in vivo makes it difficult to patch connected pairs, but otherwise unavailable results on cortical anatomy and connectivity are obtained when brute force searches for connected pairs are conducted (Song et al. 2005, Mercer et al. 2006). In organotypic slice culture, the rate of connectivity increases sharply, to the point where it becomes feasible to use the preparation to study synaptic plasticity (Poncer and Miles 1995, Pavlidis and Madison 1999). By monitoring the responses of a single synaptic contact over sequential potentiation, depotentiation and depression protocols, Montgomery and Madison characterized a number of phenomenological rules regarding state transitions of synaptic strength (Montgomery and Madison 2004).

B.2.8 Co-existence of autapses and synapses in paired recordings from cultured cells

In cultured retinal amacrine neurons, synapses form in which pre- and postsynaptic receptor clusters appear to be activated by transmitter released from a single presynaptic site (Frerking et al. 1995). The amplitudes of simultaneous spontaneous events measured in the two neurons are strongly correlated, consistent with a presynaptic locus for most of the variation in quantal size, and possibly, depending on the ultrastructure of this synapse, indicating that the location of vesicle release within the active zone is not a major determinant of quantal variability (cf. Raghavachari and Lisman 2004, Franks et al. 2003).
B.2.9 Microchamber for exposure of different cellular regions to different environments

A powerful technique permitting separate measurement of the effects of activity at distal dendrites and that at sites near the soma was used by Sherff and Carew to study integration of inputs across synapses (Sherff and Carew 1999). Using Aplysia neurons grown in culture, serotonin presented to distal synapses or to the soma separately did not affect synaptic strength. When the compound was presented to both soma and synapses simultaneously, however, the synapse underwent long-term facilitation.

B.2.10 Optical activation of synapses

Optical stimulation methods have been used for local synaptic activation before and after induction of LTP to isolate the postsynaptic component of synaptic strength. One such study (Eder et al. 2002) concluded that induction does not alter postsynaptic properties. Optical uncaging of glutamate has also been used as a component of the induction protocol itself, either for LTD (Kandler et al. 1998) or LTP (Harvey and Svoboda 2007, Bagal et al. 2005). Apart from the surprising finding of postsynaptically-determined short-term dynamics, the latter study indicated that rapid, seemingly binary changes in synaptic strength occur after pairing of glutamate uncaging with postsynaptic depolarization when the depolarization is prolonged. However, despite the extraordinary spatial resolution of this experimental approach, the waveforms of synaptic currents due to evoked uncaging showed slower rise times than did those due to evoked exocytosis, suggesting that
some functions of synaptic glutamate release (e.g., Renger et al. 2001) may not be duplicated by the uncaging. Additionally, the depolarization used was considerably larger than any that could likely due to postsynaptic spiking activity; see Chapter 4, Summary and conclusions.

B.2.11 Imaging calcium transients

Imaging of calcium in individual boutons has been a powerful technique for measuring calcium dynamics during exocytosis (Neher 1994) and short-term plasticity (Atluri and Regehr 1996). With simultaneous calcium imaging and patch clamp recording in zero magnesium conditions, Murphy et al. (Murphy et al. 1995) compared the calcium transient in the dendrites due to AP-independent spontaneous transmission with the current recorded at the soma. The time of calcium signal crossing a threshold was used as a trigger for identifying the corresponding synaptic current. The amplitudes of the two signals were correlated, as expected, and synaptic currents associated with calcium transients in spines tended to be smaller than those associated with calcium transients in the dendritic shaft, indicating that the resistance of the spine neck may serve to reduce the influence of activity within the spine on the soma.

Single-synapse calcium imaging has also been used in studies of long-term plasticity. At imaged boutons in slice culture, the first induction of LTP did not change the probability of presynaptic calcium influx, while further potentiation led to an increase in peak [Ca^{2+}]_{presyn} (Ward et al. 2006).

B.2.12 Imaging vesicle activity
Other single-synapse imaging studies have used dyes or genetic constructs to visualize vesicle cycling (Ryan et al. 1997). A strength of these approaches is the ability to monitor many individual sites at once. Imaging of dye-loaded vesicles within boutons was used to determine that synaptic vesicle endocytosis does not involve the endosome (Murthy and Stevens 1998) and that docking at the active zone is reversible (Murthy and Stevens 1999). Using the rate of FM 1-43 release to indicate the rate of vesicle release events, Prange and Murphy (Prange and Murphy 1999) found evidence for release of multiple quanta from single boutons. The release of more than one quantum per action potential is seen at larger synapses, as would be expected given the presence of multiple ultrastructural active zones at some sites (Schikorski and Stevens 1997).

In studies of LTP, increases in vesicle cycling in cultured neurons were observed following potentiation, with the greatest change at synapses with low initial turnover (Malgaroli et al. 1995). Similarly in acute slice, using high frequency trains or TEA to induce potentiation, alterations in presynaptic activity were seen following LTP (Zakharenko et al. 2001): chemical LTP and potentiation with high-frequency trains caused dye (loaded into vesicles) to be released from boutons more rapidly when a second stimulus is applied, but lower intensity stimuli sufficient to cause electrophysiological LTP did not cause changes in presynaptic release.

B.2.13 Electrophysiology at the Calyx of Held

The unique morphology of this large synapse makes it well suited to studies of single-synapse function. Patching directly onto the extracellular face of the

---

1 Additionally Murthy and Stevens found that a given terminal tended to release the same amount of dye every time it was stimulated. This may have been simply due to the limits of resolution of the optical method but on the other hand it may indicate that in those terminals where multivesicular release occurs it is consistent, and thus potentially coordinated.
presynaptic terminal, He et al. (He et al. 2006) measured capacitance changes due to single-vesicle exocytosis and endocytosis events. Some capacitance jumps were followed by rapid drops while others were slower to decay, in some cases dramatically so, reflecting multiple modes of endocytosis. Another group, patching directly onto the back of the Calyx terminal, dialyzed glutamate into the presynaptic cytoplasm, increasing the vesicular glutamate concentration. The observed increases in spontaneous event size proved that receptors are not saturated at baseline by spontaneous events (Ishikawa et al. 2002). This technique also revealed that synaptic events rise more quickly with higher [glu]_{cleft}.

B.2.14 Amperometric measurements of single vesicle release

Amperometry offers a high temporal and spatial resolution method for monitoring exocytosis of oxidizable compounds. This method revealed that small dopamine-containing vesicles release approximately 3000 molecules over 200 μs, and that both glia-derived neurotrophic factor and the dopamine precursor levodopa increase the measured quantal size (Pothos et al. 1998), suggesting that vesicle loading or fusion is altered by the signaling cascades initiated by these stimuli.

B.3 SUMMARY

Many methods have been developed for studying synaptic transmission at individual synapses, and each has contributed to understanding of synaptic function.

---

1 As exogenous neurotransmitters can be loaded into synaptic vesicles during stimulation for later AP-evoked release (Bekkers 2005), it is interesting to consider a future method involving a pharmacologically inert, hydrophilic, oxidizable synthetic species. Loading of such a compound and measurement of its release with amperometry would permit high resolution electrophysiological measurement of exocytosis at glutamatergic and GABAergic central synapses.
The flat geometry of the dissociated culture preparation is well suited to methods based on spatial isolation of synaptic transmission and for simultaneous imaging and patch recording, or alternatively for methods involving imaging of many synapses simultaneously. Specialized slice preparations such as those including the Calyx of Held or mossy fiber boutons offer structural advantages. For generating activity, evoking action potentials in the presynaptic neuron offers the greatest confidence that vesicles contributing to recorded responses originate from the cell in question, but local stimulation at the terminal eliminates the potential confound of initiation or branch failure. As those investigating synaptic function seek more comprehensive knowledge of the synapse’s enzymatic machinery, it is clear that new methods, both variations on the classes discussed here and entirely new approaches, will continue to play a critical part.
# APPENDIX C

## CHANGES IN SHORT-TERM SYNAPTIC DYNAMICS FOLLOWING INDUCTION OF LONG-TERM PLASTICITY

<table>
<thead>
<tr>
<th>Reference</th>
<th>Preparation</th>
<th>Protocol</th>
<th>Change in SD</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lei and McBain, J. Neuroscience 24(9): 2112-2121 (2004)</td>
<td>Hipp. Granule cell to CA3 interneurons</td>
<td>HFS of mossy fibers (100 Hz, 1 sec, repeat 3 times at 10 second intervals)</td>
<td>LTD presyn if AMPARs were Ca-permeable (reduced pr), postsyn if AMPARs were Ca-impermeable</td>
<td></td>
</tr>
<tr>
<td>Markram and Tsodyks, Nature 382(6594): 807-810</td>
<td>Layer 5 pyramidal neurons</td>
<td>Injection of current into both cells for 200 ms, postsyn injection delayed 1-5 ms, repeat 30 times every 20 s</td>
<td>The classic “redistribution.” The first response in a train grew, later ones grew less or none</td>
<td></td>
</tr>
<tr>
<td>Reid et al., J. Neuroscience 24(14): 3618-26</td>
<td>Mossy fiber to CA3, transverse slice, 8 do Wistar male, cultured 12-28 days</td>
<td>3 1 s trains, 20 s apart (minimal stimulation of axon bundle)</td>
<td>Increased pr, recruitment of new sites</td>
<td>“Optical quantal analysis”</td>
</tr>
<tr>
<td>Yasui et al., J. Physiology 566(Pt 1): 143-60 (2005)</td>
<td>CA1 transverse slices, 17-26 day old Wistar rats</td>
<td>Redistribution is presynaptic, involves changes in I(h)</td>
<td>Reversal protocols (e.g. LTD after LTP) also change SD</td>
<td></td>
</tr>
<tr>
<td>Ryan et al. Neuron 17: 125-134 (1996)</td>
<td>Culture from CA3-CA1 (3-5 day old rats),</td>
<td>900 APs at 10 Hz (extracellular)</td>
<td>“Potentiation of evoked vesicle turnover”</td>
<td>Extracell stim, room temperature,</td>
</tr>
<tr>
<td>Study</td>
<td>Condition Description</td>
<td>Stimulus Details</td>
<td>Measurement</td>
<td></td>
</tr>
<tr>
<td>-----------------------------------------</td>
<td>---------------------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------</td>
<td>----------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Zakharenko et al. Nature Neuroscience 4(7): 711-717 (2001)</td>
<td>Acute slice, looked at Schaeffer collateral synapses</td>
<td>TEA or 50 Hz (1 s, ½ maximal intensity) 100 Hz (4 trains, 1 s, 20 s apart, ½ intensity) 200 Hz (10 trains, 200 ms, every 5 s, 75% intensity)</td>
<td>TEA leads to faster unload of FM 50 and 100 Hz, NC in unload 200 Hz, also faster unload</td>
<td>APV and Nitrendipine block TEA effect</td>
</tr>
<tr>
<td>Malgaroli et al. Science 268(5217) 1624-1628 (1995)</td>
<td>CA3-CA1 hippocampal culture from 3-5 do rats, maintained 10-20 days</td>
<td>Glutamate is applied</td>
<td>Faster uptake of antibodies (vesicles stained with antibodies to luminal domain)</td>
<td>Nature 357 134-139 same prep and protocol, get more frequent minis, amplitude little changed</td>
</tr>
<tr>
<td>Kullmann and Nicoll Nature 357: 240-244 (1992)</td>
<td>Hipp acute slice</td>
<td>Presynaptic firing, postsynaptic depolarization</td>
<td>Increase in quantal content, quantal amplitude, or both</td>
<td></td>
</tr>
<tr>
<td>Anwyl and Rowan Neuroscience 100(2) 213-220 (2000)</td>
<td>Urethane-anesthetized and freely moving rats</td>
<td>10 trains of 20 pulses at 200 Hz</td>
<td>Reduction in paired pulse facilitation (40 ms IPI), lasts for 2 hours</td>
<td>Field recordings Reduction correlated with magnitude of LTP (Also with initial PPF)</td>
</tr>
<tr>
<td>Stricker et al. J Physiology 490 (Pt 2) 443-54 (1996)</td>
<td>Hipp slice?</td>
<td>Extracellular stim and postsynaptic depolarization</td>
<td>Increased quantal content, increased “quantal current,” increased number of sites</td>
<td>“Quantal analysis” of 11 EPSCs?</td>
</tr>
<tr>
<td>Finnerty et al.</td>
<td>Barrel slice</td>
<td>Sensory (D=deprived,</td>
<td>They</td>
<td></td>
</tr>
<tr>
<td>Nature 400:367-371 (1999)</td>
<td>deprivation S=spared) S to D more depression S to S intermed. D to D less depr.</td>
<td>downplayed this somewhat in later papers.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>---------------------------------------------------------------------------------</td>
<td>-----------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selig et al., J. Neuroscience 19(4): 1236-1246</td>
<td>Hippocampal slice, 13-18 do Sprague-Dawley</td>
<td>“Uniform potentiation at both Schaffer-CA1 and CA3-CA3 synapses” but use of first and seventh responses (rather than first and second, e.g.) obscures differences</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>120 stim, 1 Hz, postsyn cell clamped at +10mV</td>
<td>Used paired recordings for CA3-CA3 synapses Nicoll and Malenka are also authors</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sokolov et al, Brain Research 957 61-75 (2002)</td>
<td>Transverse hippoc slice from 5-7 week old male Wistar rats</td>
<td>Increase in quantal size and quantal content</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Three trains, 1s, 100 Hz, 20 s between trains, 20 mV dep of postsyn cell</td>
<td>Quantal analysis by noise deconvolution</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poncer and Malinow, Nature Neuroscience 4 (10) 989-996</td>
<td>Hippocampal slice from 10-15 do rats, 29-31 °C</td>
<td>Argument: AMPA PPR and NMDA PPR are different at baseline, but become more alike following LTP b/c AMPA responses change; presyn modifications would change both equally QED silent synapses underlie LTP and have diff dynamics</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>200-300 stimuli at 2 Hz with postsyn cell depolarized to 0 mV</td>
<td>Perforated patch</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Pananceau *et al.*  
| J Physiology  
| 508.2 503-514 (1998) | Field recording guinea pig CA1 slice | Five trains of 20 APs at 200 Hz at “twice test strength” — first 13 (of 44) expts had APV in the bath at 20 μM — They say this made no difference | On average, NC response to 5 pulse train (20 or 50 Hz) is as facilitating before and after. Indiv experiments changes in facil were seen, but these were unrelated to baseline p, or to LTP magnitude | They also saw changes in facilitation at control inputs not subjected to LTP |
| Buonomano J Neuroscience  
| 19(16):6478-6754 (1999) | L II/III auditory cortex and CA1 pyramidal cells, 18-30 do Sprague-Dawley | Postsyn depolarization, 100-150 ms, applied together with 40 Hz train, at end of train (ctx) or at either beginning (early pairing) or end (late pairing) of train (hippocampus) | Hippocampus, no redistribution, cortex redistributed; if IPI of pair of APs is < 100 ms, first pulse is preferentially enhanced |
STIMULATION AND ACQUISITION USING MATLAB

All stimulus generation, output, and recording, as well as all analysis of electrophysiological responses in the experiments reported here, was done in Matlab (Mathworks). The process is summarized as follows:

Acquisition of baseline/postinduction sweeps is run by an m-file (Matlab program) which calls the parameters file in which values including the stimulus class, pulse duration, pulse intensity, and the channel to treat as presynaptic have been set by the user. Here the user also specifies the category of experiment being performed. The main m-file calls the appropriate program to generate stimuli. In some cases stimuli are generated randomly each trial with constraints based on what stimuli were presented previously. In these cases the stimulus-generating program will receive a record of the previous stimuli used from the main program, update the record when the stimulus is generated, and pass the stimulus and the record back to the main program. To output stimuli, an “Analog Output Object” is invoked in the main program. Each trial this object is loaded with the stimulus and then triggered to output it.

The flow of output stimulation is

Matlab → Matlab DAQ Toolbox → NI-DAQ Driver → DAQ Board → Connector Block → Amplifier → Headstages → Neurons

For input an analogous “Analog Input Object” is used and data flows the stimulation path in reverse. Once the waveforms reach the DAQ Toolbox’s analog input object, the recordings are stored as .daq files, with each sweep as a separate file.
The name of the .daq file and additional information about the file (sweep number, stimulation voltage and duration for each channel, number of the channel used as presynaptic, etc.) are sent from the main routine running the experiment to a subroutine charged with updating the patch file. This m-file opens the .daq file, which contains membrane current data only, opens the .hdf file, and stores the membrane trace and the additional information into the .hdf file as a new pair of columns\(^1\). Each trial becomes a ‘dataset’ within the .hdf file.

A separate m-file is used to coordinate basic analysis. This program opens the .hdf file, opens each dataset within the file and sends it to the inner routine that handles all basic processing. This inner m-file reads the metadata appended to the beginning of each trace and stores the relevant settings for use in processing then strips the metadata. It then scales the trace based on the feedback resistor and gain used during recording and calls subprograms to find stimulus times, access and input resistances and several synaptic parameters, depending on flags set in the front end analysis file. The analysis always includes detection of the peak PSC, charge, latency, rise time and decay time following each event as well as asynchronous release following the last event. These six values can be measured across any of the four possible synapses in a paired-patch recording (A to B, B to A, A to A, and B to B). On the last trial, the measured values are stored in text files named as the original .hdf file with the parameter in question appended to the end of the file name. Control data are saved to a file named by the .hdf file name with ‘controls’ appended. A continuous strip of the raw recording combining the PSCs of each sweep is saved as tab-delineated ascii text with the name suffix ‘raw’.

The text files are used for analysis by a routine designed for comparing pre- and post-induction data — this program calls subroutines for sorting raw traces

---

\(^1\) NSF developed the hierarchal data format for compact storage of large data sets. Apart from compactness, an advantage of .hdf is the file integrity conferred by the requirement for specialized commands to manipulate the files. The present study was done using .hdf 4. For more information, see [http://www.hdfgroup.org/](http://www.hdfgroup.org/).
according to interval and sequence, for checking the stability of the input and access resistances, and for generating diary, overlay, and other plots. Figures output from these m-files make up the summary files for each case which are stored as graphical files.

The ascii text files created by the basic analyses form what amounts to a database. For comparison of results from multiple experiments an Access database (Appendix E), which is in effect an index to the true database of text files, is queried from within Matlab and the names of experiments matching the query are returned. The names are appended to the parameter to be analyzed, the text files created by the initial analysis are opened, and data are extracted and processed.
APPENDIX E

LIST OF CUSTOM SOFTWARE ROUTINES USED

Running experiments:
dwn_oscope.m
run_expt_dwn.m
  run_expt_dwn_parametersfile.m
generate_log_filename.m
generate_poisson_trains.m
generate_train.m
timesincemidnightinms.m
update_patchlog.m
update_patchlog_threechannel.m
stdp3.m
random_train0.m
random_train1.m
poisson_train_a.mat
onedim_randomizer.m
irregularly_spaced_pairs_dwn.m
make_ramp_stimulus.m
create_patchlog_name.m
date_time_stamp.m
b16_reader.m
triggerspritzer.m, stopspritzer.m

Initial analysis:
  single epoch
create_storage_matrices.m
  patchfile_analysis.m
    fitting_to_fast_and_slow_templates.m
    template_based_mini_detector.m
    mini_waveform_extractor.m
    find_access_and_input_resistances_from_test_pulse.m
    find_average_around_minimum_of_events.m
find_peaks_within_threshold_crossing_regions.m
patchfile_analysis_specialforplateaus.m
audio_signal_analysis.m
cosine_template_generator.m
create_basic_text_file.m
lsqfit_raccess_decay_curve_generator.m
lsqfit_raccess_decay_finder.m
presyn_I_Na_checker.m
bump_counter.m

multi-epoch
convert_stored_matrix.m
    analyze_stored_controls.m
    find_time_windows.m
stsd_grid_plotter.m (> 20 other methods of sd analysis)
template_loop_diver.m
gaussianwindowsmooth.m, movingwindowsmooth.m
historical_histogram.m
leafmapper.m, pathfinder.m, matchmaker.m

miscellaneous
anothermoviemaker.m
image_registration_dwn.m

Analysis from database:
manual
find_repetitions_in_a_cell_array_input.m
fix_flagged_file_facts_from_access.m
simple_database_querier.m

automated
analyze_across_sd_stdp_expts.m
    query_generator.m
    agresti_coull_binomial_confidence_interval.m
    analyze_and_plot_mean_responses.m
    analyze_autocorrelation.m
    analyze_correlation_between_successive_pscs.m
    analyze_diary.m
    analyze_history_dependence.m
    analyze_realtime.m
    analyze_release_dependence.m
analyze_sd_over_time.m
   replace_zeros.m
   class_membership_assigner.m
   interpolate_with_smoothing.m
   mean_with_preference_for_precedents.m
autocorrelation_dwn.m
rectangles_with_error_bars.m
psp_slope_intensity_measurement.m
poor_mans_waterfall.m
pr_fluctuation_rd_hd.m
list_all_nonredundant_permutations_upto_lengthn.m
longest_run_variable_thres.m
iterative_curve_fit_singledecay.m
exp_decay.m
monoexpreisdecay_template_generator.m
change_point_analysis_deviance_method.m
change_point_analysis_formal_method.m
change_point_analysis_trend_method_with_bootstrap.m
change_point_data_plotter.m
recursive_search_for_balanced_bootstrap.m
residual_bootstrap_dwn.m
balanced_bootstrap_array_maker.m
balanced_bootstrap_dwn.m
basic_bootstrap_dwn.m
find_change_point_using_sum_of_squares.m
find_change_point_using_trend_method.m

careful_asynch_measurer.m
careful_charge_calculator.m
careful_charge_calculator_core.m

cumulative_histogram.m
find_detection_crits_and_latencies_generalizedform.m
find_flagged_filenames_from_access.m
find_greatest_n_consecutive_vals.m
generalized_rung_plotter.m
standard_err.m
simple_induction_plotter.m
simple_stdp_slope_calculator.m

ccluster_asynch_based_on_phasic.m
ccompare_asynch_waveform_clusters_to_phasic_waveform.m
cvm_curve_fitter.m
cwaveform_segmental_variance.m
cfind_all_n_possible_contiguous_groupings.m
cfind_riseplateau.m
csegment_seeker.m
cshuffle_randomization.m
cmontecarlo_jittered_events.m
cmontecarlo_noisy_events.m
cnoise_autocorrelation_structure.m
cnoise_recombinase.m
clsqfit_vm_parabola_generator.m
cdouble_errorbar_plotter_dwn.m
cdual_errorbar_plot.m
cfit_comparison_lsq.m

Models:
color_is_time.m
cfacilitation_depression_model.m
csynaptic_dynamics_model_optimizer.m
cs_s_stsd.m
csingle_synapse_stsd_solve.m
csingle_syn_coupled_equations.m
csingle_syn_stsd.m
csingle_syn_stsd_for_montecarlo.m
clability_exposures_stdp_model.m

Note that many routines are called in multiple contexts. All software is available for download at http://bilab.neurobio.pitt.edu/dwn/matlab_m_files_dwn/
APPENDIX F

STRUCTURE OF DATABASE USED

The Access database contains filenames and other file data such as type of experiment, sequence, relation to other files, culture info and image/movie/audio/summary files available. File names and file information, but not patch or image data, are stored in the database. When a query is sent to the database it returns the names of the appropriate text files. Matlab then loads these files, containing data previously extracted from the .hdf files, and processes the data as instructed. A record of the query sent, the file names returned, and the time and date of the query is also automatically generated; this can be stored, off the printable area, of any figure made with the data from the filenames returned by the query as the accurate record of data included in a figure each time a figure is modified.

All experiments from Jan 1, 2005 through the end of the PhD are included in the database except for the experiments for the NR2 project and the experiments on okadaic acid’s effects on release for the vesicle dynamics study. Any experiment deleted or not saved because seals were of very poor quality is also not represented in the database.

The database contains two tables, one for experiments and one for epochs. Experiments are defined as all recordings from a given neuron or pair of neurons, that is, as the files listed between slashes in the lab notebook. The primary key for patchlog_expts_table is first_patchlogfile_this_expt. The patchlog_epochs_table uses first_patchlogfile_this_expt (the foreign key), epoch_class_and_sequence and suffix as primary keys. Because some epochs include multiple patchlogfiles, the file names
in patchlogfiles_this_epoch are enclosed within brackets in epochs to allow them to be separated and loaded within Matlab. Trials_to_ignore and suffix are enclosed within brackets as well for the same reason. In some cases, for example when the positive pressure is increased during a recording to a new set point, the same log file belongs to multiple epochs with one epoch corresponding to each setting; the trials_to_ignore entry will allow the trials from a patchlogfile belonging to each epoch to be loaded. A few microperfusion recordings include two separate release sites reliably distinguishable by latency; these were analyzed and stored separately using a suffix such as ‘_2to10ms’ and this is reflected in the table. The relationship of the expts table to the epochs table is one-to-many, but there are some expts that have only one matching epoch entry, and, when it is unlikely that a given experiment will ever be analyzed, sometimes the information is entered only into the expts table, not into the epochs table; these expts table entries thus have no matching epochs table entry. Epochs table entries must have a corresponding entry in the experiments table.

Each experiment in the experiments table is classed according to main and secondary stimulus class and main and secondary configuration.

**Allowed stimulus classes are:**

stdp (used as expt_main_stimulus_class when the induction is a useful part of the expt; hence roughly synonymous with expt_free_of_significant_technical_problems and induction_can_be_measured both being 1 — if stdp was attempted but these conditions are not met, stdp should be expt_secondary_stimulus_class)

sd (used as expt_main_stimulus_class when synaptic dynamics is the main focus of the expt; when stdp is the main stim class, the presence of useful sd information is implied. Any period of recording suitable for sd analysis is also suitable for release dependence and history dependence analysis)

rrp (readily releasable pool size experiments)

reverb (experiments on reverberatory networks)
total_pool_size (local puffing with high K or sucrose)
brief_tetanus_var_recovery
test (during building the rig and working out staining, noise, camera, movie, etc.)
network_ID
minis_in_TTX
truncated (when not even any SD is available)
gaba_sd
dopamine

As stated, whenever stdp is attempted, stdp is listed as the main stimulus class. In such experiments, when good trials exist but there are too few of them to measure stdp, induction_can_be_measured is 0. The sd from such experiments may still be valuable; when this is the case, expt_secondary_stimulus_class will be sd. When technical problems are present throughout enough of the experiment to make evaluation impossible, expt_free_of_severe_technical_problems is 0; this means the experiment yields no useful information. If expt_main_stimulus_class is stdp and induction_can_be_measured and expt_free_of_severe_technical_problems are both 1, then expt_shows_induction can be determined; if it is 1, then the experiment is a successful induction, and if it is zero, the synapse failed to potentiate; either of these are suitable for charge transfer and related analyses. Later, expt_shows_LTP can be quantified more precisely using t-tests. Within a potentially useful experiment, a technically bad epoch can be marked by setting epoch_free_of_severe_technical_problems to 0.

Secondary stimulus class becomes important in situations where for example a brief train variable recovery (secondary class) experiment is done both before and after stdp (primary class). Because the vast majority of stdp experiments consist of the sequence sd-induction-sd, sd is not listed as a secondary stimulus class for these
experiments. Secondary experiment class is used in situations such as RRP characterization followed by sd characterization at the same bouton.

**Configurations possible are:**

macroperfusion  Note: If microperfusion is the expt_main_configuration_class and macroperfusion is listed as expt_secondary_configuration_class, that means there is something more than M1 available from the macroperfusion recording period(s).

microperfusion

microperfusion_PPO (positive pressure only)

natural_oligobouton — these are essentially very small synapses under macroperfusion, so the two categories may be merged in the future as the cutoff is nebulous. These expts are useful for comparison to microperfusion expts for testing the effect of microperfusion on PSC waveforms.

dendritic_patch

**Coded notes in the comment fields** (Procedural notes and potential confounds in italics):

*CannotBeUsed* –

*CBU* – can be used (generally as part of a check statement).

*CHECK* –

ChgAsynch – suspected change in asynchronous release following induction

ChgAudio – suspected change in the audio signal over time

ChgAutapse – suspected change in autaptic responses

ChgExcit – induction seems to require less current to make the postsyn cell spike than was needed during a previous induction

ChgJitter – suspected

ChgPotency – suspected

ChgPr – suspected change in successful transmission rate
**ChgProtocol** – the experimental protocol was changed beginning with (or around the time of) this experiment

**ChgRacc** –

**ChgSD** – suspected change in short-term synaptic dynamics (with or without LTP)

**ClumpedEvents** – (generally during RRP-type expts) successes in response to trains of APs tend to occur in a temporally nonrandom fashion

**Clusterable** – waveforms suggest suitability for clustering

**CompareMacroMicro** – waveforms in global and microperfusion Ca bear comparison

**CtrlNoPosPressure** – positive microperfusion pressure was off for one or more trials to show that without it, release cannot occur

**DiffPuffLocations** – over the course of the expt or epoch

**DiffReleaseSitesCoOccur** – more than one waveform seen with consistent latency.

Latency should be included as a parameter when clustering. (The first dopamine experiment is an example.) This differs from InterestingAsynch, MultiPeaks in that here the two latencies are very consistent.

**DiffReleaseSitesOverTime** – for example, the first 10 sweeps show PSC with latency of ~5 ms, then suddenly a latency of ~10 ms is seen for ~10 sweeps — the simplest explanation is pipette drift, but more interesting possibilities exist, especially if a given site (i.e. latency) disappears then reappears, as is often the case. Latency should be included as a parameter when clustering in these cases.

**DiffSDAutSyn** – suspected difference in the short-term dynamics at the autapse and the synapse

**DoublePostsynSpike** – during induction, the postsyn cell spikes twice

**ElevBgrd** – suspected elevation of background following induction

**EstMPT** – minutes per trial obtained from expt start/stop time and trial count
EventSizeShapeDendriticLocation – if the distance from the postsyn soma is known because image of micropuff location is available, questions of the relation event size and rise time to dendritic distance can be addressed

FNP – fluctuating negative pressure is thought to have been a problem during the experiment

Frenzy – for lack of better term, one or both of the cells goes into a form of ‘frenzy’ of tremendous activity. Usually seen only with low patch quality.

Garbage – No informative traces.

GlobalNormCaInduc – 3 mM Ca\textsuperscript{2+} present globally during the induction

HD – history dependence bears closer analysis

HighBgrd – high activity not related to stimulation or induction, a potential confound

HighFailureRate – low pr synapse

HighKUsed – generally for staining

ImgUseful – potentially useful image(s) or movie(s) associated with this file

InterestingAsynch – MultiPeaks is one type, waveforms frequently show two or more peaks with variable latencies, suggesting multiple vesicles being released from one or more release sites

InterestingSD – Ufunction is one possible type, Monotonic [with IPI] is another

ITI\_TS – inter-trial interval likely too short

LargeEventsAndFailures – At the same micropuff site, failures and large events alternate

LargeLateLTP – suspected

LargeMicroperfArea – many sites could be exposed

LowCaLongLatency –

LTD – suspected

LTDBecomesLTP – suspected

LTP – suspected LTP
Mixing – useful for technical evaluation of mixing in microperfusion between zero and 0.2 mM Ca, or between with/without CNQX

MultiBumpbEPSP –

MultipleValsAPsPerTrial – more than one value of APs per trial exists in the epoch; should only have one such expt

MVAM – suspected multi-vesicle asynch a/o mini release, for questions of AP-independent coordination of release

NeedCrit (or Ctrl, Pscclass, Raw, or INa) – need template match or other information

NoHistoryDep – responses to third or fourth pulse show no clear correlation with earlier responses in the sweep (this finding is strongest if there is dependence from o to a, and if b or c show no trend over the expt)

OverSpill – waveform suggests transmitter overspill and extrasynaptic activation

PostIndStun – suspected ‘stunning’ (~ diminished responses for several minutes) after induction

PrCorrelatesWithinTrials – suspected

ProbsINa – uncertainty re evoking the presynaptic AP; cannot be included in any group analyses

PSCaHypervariable – the second response in the train tends to have a huge variability, suggesting that after the first response the state of the synapse is poised at a criticality.

PSCb400 – shows large, consistent difference in PSCb50 and PSCb400, providing info on Facilitation/Depression

PTP – suspected post-induction potentiation

PuffingHighCa – microperfused [Ca^{2+}] higher than the usual 3 mM

RD – release dependence at this synapse merits investigation

Redistribution –

SD – short-term synaptic dynamics

SDOfAsynch –
SDOfClusters – a given release site may be preferentially activated by a given stimulation history

SDOfJitter –

SDOfLatency –

SDOfPr – Facil is one type

SettingsChgDuringEpoch – the epoch is anisotropic in terms of pos or neg pressure, tip positions, etc.; analysis must be limited to constant portion

ShortIPIDestabilizes – after a (very) short IPI, the responses to later APs are multi peaked and ‘choppy’

StableBaseline – example of long, stable baseline

StrgWav – strange waveforms (Shoulder is one type, may be special case of DiffReleaseSitesCoOccur; VerySlow is another type, may be due to elevated R_acc, DecayBend is another type)

TargSpec – contains information for SD of E to I connections

TestCoop – multiple induction attempts at the same site differing in the number of boutons in the stream due to changes in pos pressure, as a means of testing cooperativity of stdp

TransposedCells – postsyn cell becomes presyn, presyn cell becomes postsyn

TrueSingleVes – a (putative) true single vesicle event

UnblockableCurrent –

Unquantized – data in the experiment are inconsistent with quantized responses

UnstableBaseline –

VariableLatency –

Each epoch in the epochs table is assigned a class, given by a letter:

M – macrosynaptic, single pulse tests of each cell to establish transmitter phenotype, generally before low calcium wash-in

L – loading of FM dye
C – characterization (reserved for stdp or sd expts, in either micro or macroperfusion) Note: if stimulus_class is sd or stdp and config class is microperfusion, C1 is the first characterization epoch in microperfusion.

I – induction (in either micro or macroperfusion)

T – test

N – network

R – reverb

P – pool

S – search

F – facilitation and depression

X – minis in ttx

WC, WI – characterization or induction with the cells sWitched (presyn cell used as postsyn...)

Epochs are also assigned a number. Numbering is as follows: M1 is the “macroperfusion” recording just prior to C1, which in turn is the characterization just prior to I1, the first induction. Immediately following I1 is C2. Earlier characterization epochs, with different microperfusion placements for example, are of the form Cneg1, Cneg2... with neg1 being the epoch just prior to the real first characterization epoch, going backward in time from that point.

In epoch entries, information relevant to inductions is also storable, as a subset of epochs are inductions. pairing_special_circumstance is used when the formerly postsyn cell is used as presyn, for example. For stdp expts, info is returned from epochs C1 and C2 sharing the same first patchlogfile this expt, after checking that C1 and C2 both exist for that experiment. When trials_to_ignore or suffix are crucial in specifying epochs, these must be referenced in the query as well. To ensure filenames are consistently returned in the same order each time a query is sent,
queries specify the ORDER BY portion of the return. For an example query, see below.

For an epoch to be marked free of significant technical problems, a) R_acc must be stable and moderate, b) no runup or rundown can be present, c) seal quality must be high, d) presyn sodium current must be present all pulses, all trials (for meaningful analysis of success rate, release dependence, etc.), e) the pscclass, crit, ctrl, raw and I_Na records for the patchlogfile(s) in that expt must have the same number of columns and be in register, and f) the epoch must have been mechanically stable and free of known or suspected problems.

Note on columns with numeric-type data: Null, rather than 0, should generally be the default value, as this permits distinction between ‘no value entered’ and a value of zero. Recall that Matlab views Null as NaN.

Related note, on yes/no situations: as structured query language does not distinguish between ‘No’ and ‘Don’t Know’, yes/no situations should generally be represented with Numeric data, where 1 is yes, 0 is no, and Null is Don’t Know (the default).

Other considerations with regard to the database: give variables the same name in the database and in analysis files as much as possible; skipping a bad-seal sweep during initial analysis in Matlab creates trouble later because the skip is handled differently by some saves than others (PSC, q etc. and controls save a zero column for the skipped sweep, while raw and criterion do not).

An example of the query used to retrieve the preinduction baseline from STDP experiments (with curly brace replaced in this document by exclamation point for compatibility with the reference manager):

```matlab
function selection_clauses=query_generator
database_query_pure_form=0
```
if database_query_pure_form
    desired_characterization_epoch_info=SELECT
        patchlogfiles_this_epoch, trials_to_ignore, has_baseline_and_postinduction.suffix, event_detection_threshold, minutes_per_trial, APs_per_trial, color_for_plotting FROM (';
    desired_induction_epoch_info=SELECT
        patchlogfiles_this_epoch, conversion_to_mV_or_pA_postsyn_channel, induction_interval_prepost_insamples, color_for_plotting, has_baseline_and_postinduction.suffix FROM (';
    first_epochal_check=SELECT first_patchlogfile_this_expt, suffix FROM
        patchlog_epochs_table WHERE epoch_class_and_sequence = "C1" AND
        next_epoch_class_and_sequence = "I1";
    second_epochal_check=SELECT first_patchlogfile_this_expt, suffix FROM
        patchlog_epochs_table WHERE epoch_class_and_sequence = "I1" AND
        induction_stimulus = "RO" AND next_epoch_class_and_sequence = "C2";
    third_epochal_check=SELECT first_patchlogfile_this_expt, suffix FROM
        patchlog_epochs_table WHERE epoch_class_and_sequence = "C2";
    charac_epochs_check=strcat('SELECT
        first_epochal_check.first_patchlogfile_this_expt, first_epochal_check.suffix FROM
        (', first_epochal_check, ') AS first_epochal_check INNER JOIN (',
        third_epochal_check,) AS third_epochal_check ON
        first_epochal_check.first_patchlogfile_this_expt =
        third_epochal_check.first_patchlogfile_this_expt AND
        first_epochal_check.suffix = third_epochal_check.suffix'));
    charac_and_induc_epochs_check=strcat('SELECT
        charac_epochs_check.first_patchlogfile_this_expt, charac_epochs_check.suffix FROM
        (', charac_epochs_check, ') AS charac_epochs_check INNER JOIN (',
        second_epochal_check,) AS second_epochal_check ON
        charac_epochs_check.first_patchlogfile_this_expt =
        second_epochal_check.first_patchlogfile_this_expt AND
        charac_epochs_check.suffix = second_epochal_check.suffix'));
    joint_exptal_epochal_check=strcat('SELECT
        charac_and_induc_epochs_check.first_patchlogfile_this_expt, charac_and_induc_epochs_check.suffix, patchlog_expts_table.color_for_plotting FROM
        (', charac_and_induc_epochs_check, ') AS charac_and_induc_epochs_check INNER JOIN
        patchlog_expts_table ON patchlog_expts_table.first_patchlogfile_this_expt =
        charac_and_induc_epochs_check.first_patchlogfile_this_expt WHERE
        patchlog_expts_table.expt_main_stimulus_class = "stdp" and
        patchlog_expts_table.expt_main_configuration_class = "microperfusion"');
    baseline_select_clause=') AS has_baseline_and_postinduction INNER JOIN
    patchlog_epochs_table ON
has_baseline_and_postinduction.first_patchlogfile_this_expt =
patchlog_epochs_table.first_patchlogfile_this_expt AND
has_baseline_and_postinduction.suffix = patchlog_epochs_table.suffix WHERE
patchlog_epochs_table.epoch_class_and_sequence = "C1" ORDER BY
patchlogfiles_this_epoch ASC;
    induction_select_clause=') AS has_baseline_and_postinduction INNER JOIN
patchlog_epochs_table ON
has_baseline_and_postinduction.first_patchlogfile_this_expt =
patchlog_epochs_table.first_patchlogfile_this_expt AND
has_baseline_and_postinduction.suffix = patchlog_epochs_table.suffix WHERE
patchlog_epochs_table.epoch_class_and_sequence = "I1" ORDER BY
patchlogfiles_this_epoch ASC;
    postinduction_select_clause=') AS has_baseline_and_postinduction INNER JOIN
patchlog_epochs_table ON
has_baseline_and_postinduction.first_patchlogfile_this_expt =
patchlog_epochs_table.first_patchlogfile_this_expt AND
has_baseline_and_postinduction.suffix = patchlog_epochs_table.suffix WHERE
patchlog_epochs_table.epoch_class_and_sequence = "C2" ORDER BY
patchlogfiles_this_epoch ASC;

selection_clauses!1!=!%selecting epoch C1, 'characterization1', aka baseline
strcat(desired_characterization_epoch_info,joint_exptal_epochal_check,baseline_select_clause)
;

selection_clauses!2!=!%selecting epoch I1, 'induction1', aka first induction
strcat(desired_induction_epoch_info,joint_exptal_epochal_check,induction_select_clause)
;

selection_clauses!3!=!%selecting epoch C2, 'characterization2', aka the first postinduction period
strcat(desired_characterization_epoch_info,joint_exptal_epochal_check,postinduction_select_clause)
;
elseif ~database_query_pure_form
    from_expts_table='SELECT first_patchlogfile_this_expt, color_for_plotting FROM
patchlog_expts_table WHERE expt_main_stimulus_class ="stdp" AND
expt_main_configuration_class = "microperfusion" AND
expt_free_of_significant_technical_problems = 1 AND induction_can_be_measured = 1'

    from_epochs_table="SELECT first_patchlogfile_this_expt,patchlogfiles_this_epoch,
epoch_class_and_sequence,trials_to_ignore,suffix,next_epoch_class_and_sequence,
next_C_epoch_if_C_epoch_and_epochs_can_be_compared,
event_detection_threshold,minutes_per_trial,APs_per_trial,conversion_to_mV_or_p
A_postsyn_channel,induction_interval_prepost_insamples FROM
patchlog_epochs_table WHERE epoch_free_of_significant_technical_problems =1
AND next_C_epoch_if_C_epoch_and_epochs_can_be_compared IS NOT NULL'

lotsa_info_table=strcat('SELECT
from_epochs_table.first_patchlogfile_this_expt,color_for_plotting,
patchlogfiles_this_epoch,epoch_class_and_sequence,trials_to_ignore,suffix,
next_epoch_class_and_sequence,
next_C_epoch_if_C_epoch_and_epochs_can_be_compared,
event_detection_threshold,minutes_per_trial,APs_per_trial,conversion_to_mV_or_p
A_postsyn_channel,induction_interval_prepost_insamples FROM
(from_epochs_table) AS from_epochs_table INNER JOIN (from_expts_table) AS
from_expts_table ON from_epochs_table.first_patchlogfile_this_expt =
from_expts_table.first_patchlogfile_this_expt'

selected_preinduction_table=strcat('SELECT
patchlogfiles_this_epoch,trials_to_ignore,suffix,event_detection_threshold,minutes_
per_trial,APs_per_trial,color_for_plotting FROM (lotsa_info_table) ORDER BY
patchlogfiles_this_epoch'

selected_induction_table=strcat('SELECT
patchlog_epochs_table.patchlogfiles_this_epoch,patchlog_epochs_table.conversion_to_mV_or_p
A_postsyn_channel,patchlog_epochs_table.induction_interval_prepost_insamples,lotsa_info_table.color_for_plotting,patchlog_epochs_table.suffix FROM
patchlog_epochs_table INNER JOIN (lotsa_info_table) AS lotsa_info_table ON
patchlog_epochs_table.first_patchlogfile_this_expt = lotsa_info_table.first_patchlogfile_this_expt AND
patchlog_epochs_table.suffix = lotsa_info_table.suffix AND
patchlog_epochs_table.epoch_class_and_sequence = lotsa_info_table.next_epoch_class_and_sequence ORDER BY
patchlog_epochs_table.patchlogfiles_this_epoch'

selected_postinduction_table=strcat('SELECT
patchlog_epochs_table.patchlogfiles_this_epoch,patchlog_epochs_table.trials_to_ignore,
patchlog_epochs_table.suffix,patchlog_epochs_table.event_detection_threshold,
patchlog_epochs_table.minutes_per_trial,patchlog_epochs_table.APs_per_trial,lotsa
_info_table.color_for_plotting FROM patchlog_epochs_table INNER JOIN
(lotsa_info_table) AS lotsa_info_table ON
patchlog_epochs_table.first_patchlogfile_this_expt
= lotsa_info_table.first_patchlogfile_this_expt AND patchlog_epochs_table.suffix
= lotsa_info_table.suffix AND patchlog_epochs_table.epoch_class_and_sequence
= lotsa_info_table.next_C_epoch_if_C_epoch_and_epochs_can_be_compared ORDER
BY patchlog_epochs_table.patchlogfiles_this_epoch)

    selection_clauses!1!=!%selecting epoch C1, 'characterization1', aka baseline
    selected_preinduction_table
    !;

    selection_clauses!2!=!%selecting epoch I1, 'induction1', aka first induction
    selected_induction_table
    !;

    selection_clauses!3!=!%selecting epoch C2, 'characterization2', aka the first
    postinduction period
    selected_postinduction_table
    !;
end
BIBLIOGRAPHY


Isaac, J. T., A. Luthi, et al. (1998). "An investigation of the expression mechanism of LTP of AMPA receptor-mediated synaptic transmission at hippocampal CA1


