AWARD NUMBER: W81XWH-12-1-0320

TITLE: Cellular Basis for Learning Impairment in Fragile X Syndrome

PRINCIPAL INVESTIGATOR: John R. Larson

CONTRACTING ORGANIZATION: University of Illinois at Chicago
Chicago, IL 60612-7205

REPORT DATE: August 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2015	Annual	1 Aug. 2014 – 31 Jul. 2015

4. TITLE AND SUBTITLE
Cellular Basis for Learning Impairment in Fragile X Syndrome

5a. CONTRACT NUMBER
W81XWH-12-1-0320

5b. GRANT NUMBER
PR110362; IIRA

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
John Larson

E-Mail:jrlarson@uic.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES)
University of Illinois at Chicago
Chicago, IL 60612-4305

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
This research combines behavioral, electrophysiological, and molecular approaches to elucidate the cellular basis for learning impairment in Fragile X Syndrome (FXS), using olfactory learning in Fmr1-KO mice as a model system. We hypothesize that FMRP, the protein missing in FXS, participates in two aspects of circuit function that are critical to learning: synaptic plasticity and the generation and survival of new neurons in the adult brain. Efforts in the third year of support were directed toward establishing a method to specifically upregulate neurogenesis in adult fragile X mice, test fragile X mice for learning deficits in hippocampal-independent tasks, and determine how synaptic plasticity is disrupted in fragile X model mice. Significant advances have been made in the establishment of behavioral paradigms to test both hippocampal -dependent and -independent forms of olfactory learning. Experimental paradigms have also been refined for the regulation of neurogenesis in the hippocampus and olfactory bulb as well as to determine the effect of FMRP deficiency on inhibitory synaptic transmission in the olfactory cortex. These studies are likely to advance our understanding of intellectual disability and autism in addition to the specific condition of fragile X syndrome. This knowledge will be necessary for the development of rational strategies for prevention and treatment of cognitive impairments from a variety of causes.

15. SUBJECT TERMS
Fragile X Syndrome, synaptic plasticity, long-term potentiation, glutamate receptors, neurogenesis, olfactory learning

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	53	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

1. **Introduction……...4**
2. **Keywords……....5**
3. **Accomplishments……..…….6**
4. **Impact……...……10**
5. **Changes/Problems...…..……11**
6. **Products…….……..…..…….12**
7. **Participants & Other Collaborating Organizations……...13**
8. **Special Reporting Requirements……15**
9. **Appendices……17**

1. INTRODUCTION

Fragile X syndrome is the most common inherited form of intellectual disability. The disorder is caused by mutation in the *FMR1* gene that transcriptionally silences the gene and results in lack of production of the encoded protein, FMRP. A mouse model of the human syndrome (the *Fmr1*-KO mouse) reproduces the protein (FMRP) deficiency and exhibits abnormalities of synaptic structure and function as well as learning impairments. This research combines behavioral, electrophysiological, and molecular approaches to elucidate the cellular basis for learning impairment in *Fmr1*-KO mice using olfactory learning as a model system. We hypothesize that FMRP, the protein missing in FXS, participates in two aspects of circuit function that are critical to learning: synaptic plasticity and the generation and survival of new neurons in the adult brain (neurogenesis). First, we use molecular tools to study the cellular basis for neurogenesis deficits in adult *Fmr1*-KO mice and the signaling pathways by which FMRP regulates neurogenesis in both the olfactory bulb and the hippocampus of the adult brain. We test the hypothesis that selective down-regulation of the pro-apoptotic gene, *Bax*, in neurogenic niches will reverse neurogenic deficits in *Fmr1*-KO mice. Second, electrophysiological, biochemical, and pharmacological methods are used to characterize synaptic dysfunction in the olfactory-hippocampal circuit in *Fmr1*-KO mice. We hypothesize that absence of FMRP disrupts trafficking of NMDA receptors to synapses, resulting in impairments in NMDA-dependent synaptic plasticity mechanisms such as long-term potentiation (LTP). Third, using behavioral analyses, we attempt to determine the contributions of neurogenic and synaptic dysfunction to learning impairments in *Fmr1*-KO mice. Finally, we test the hypothesis that impaired neurogenesis underlies the learning impairment in *Fmr1*-KO mice by experimentally stimulating neurogenesis in the mice. In summary, this project will exploit the advantages of the olfactory system to study the cellular basis for learning impairment in a mouse model for fragile X syndrome. This is likely to also have impact on our understanding of developmental intellectual disability syndromes in general as well as learning impairments in other autism spectrum disorders. This knowledge will be necessary for the development of rational strategies for prevention and treatment of cognitive impairments from a variety of causes.

2. KEYWORDS

Fragile X Syndrome
Autism
Learning
Memory
Synaptic Plasticity
Neurogenesis
Synaptic Excitation
Synaptic Inhibition
Long-Term Potentiation
Glutamate Receptors
GABA Receptors

3. ACCOMPLISHMENTS

A) Major goals of the project.

1) Use molecular tools to study the cellular basis for neurogenesis deficits in adult *Fmr1*-KO mice and the signaling pathways by which FMRP regulates neurogenesis in both the olfactory bulb and the hippocampus of the adult brain.
2) Use electrophysiological, biochemical, and pharmacological methods to characterize synaptic dysfunction in the olfactory-hippocampal circuit in *Fmr1*-KO mice.
3) Use behavioral analyses to determine the contributions of neurogenic and synaptic dysfunction to learning impairments in *Fmr1*-KO mice.

B) Progress toward accomplishment of major goals.

1) Is olfactory bulb neurogenesis impaired in *Fmr1*-KO mice? (SOW task 2a)

Adult neurogenesis occurs in two areas of the mammalian brain: (i) the subgranular layer (SGL) of the dentate gyrus of the hippocampal formation produces excitatory, glutamatergic granule neurons incorporated into the dentate gyrus and (ii) the subventricular zone (SVZ) produces inhibitory, GABAergic granule and periglomerular cells incorporated into the olfactory bulb. Both of these areas have prominent roles in olfactory learning. We already showed, using molecular markers for dividing cells as well as immature and mature neurons, that proliferation and survival of adult-generated neurons are suppressed in the Fmr1-KO mouse brain, resulting in fewer mature granule cells in the dentate gyrus of one year-old mice (Lazarov et al., 2011). In the past year, we have begun to examine these processes in the SVZ and olfactory bulb. To examine the proliferation history and cell fate of neural progenitor cells (NPCs) in the SVZ of *Fmr1-KO* and *WT* mice, mice were injected with the thymidine analog, bromodeoxyuridine (BrdU), one month before sacrifice. Brain sections were processed for immunohistochemistry to identify proliferating cells (labeled with BrdU), immature neurons, mature neurons, and astrocytes. Proliferating cells in an early stage of neuronal differentiation were identified in the subventricular zone (SVZ) and olfactory bulb (OB) using antibodies raised against the early neuronal marker, doublecortin (DCX). For the identification of glia we used antibodies raised against glial fibrillary acidic protein (GFAP). Our preliminary data shows that the number of proliferating cells (BrdU+) is significantly reduced in the SVZ of Fmr1-KO mice, compared to WT mice (please see **APPENDIX Figure 1** and **APPENDIX 4**). The number of neuroblasts (BrdU+, DCX+) was also reduced, although we do not have adequate samples as yet for statistical significance. Ongoing studies are aimed at confirming these preliminary results and determining if any of these processes are differentially affected by age in *Fmr1*-KO mice. In addition, ongoing experiments are aimed at determining the rate of survival of new neurons, their migratory pathway, and functional integration in the olfactory bulb of these mice.

2) Restoration of neurogenesis in *Fmr1*-KO mice by selective down-regulation of Bax in neurogenic niches (SOW task 2c).

It is not clear whether stimulation of neurogenesis is sufficient for the amelioration of cognitive deficits in Fragile X syndrome, and whether enhanced neurogenesis has therapeutic value for these cognitive deficits. In our experimental plan we proposed to use lentiviral vectors to knockdown Bax and enhance neurogenesis. We decided to take a much more advanced and reliable genetic approach (Sahay et al., 2011) by using NestinCreER$^{T2/+}$/Bax$^{f/+}$ mice (obtained from Dr. Rene Hen, Columbia University). To achieve gain of neurogenic function in *Fmr1-KO* mice, *Fmr1*-KO mice are bred with Bax$^{lox/lox}$ followed by breeding of Bax$^{lox/+}$/*Fmr1-KO* mice and

NestinCreER$^{T2/+}$/Bax$^{lox/lox}$ to achieve *Fmr1-KO*/nestinCreER$^{T2/+}$/ Bax$^{lox/lox}$ mice. We have now obtained some of the required mice and are breeding them to obtain populations sufficient for behavioral and electrophysiological testing.

3) Comparison of synaptic plasticity (LTP) in different components of the olfactory-hippocampal circuit in *Fmr1*-KO and WT mice (re: SOW task 3b).

Since there is increasing evidence that deficits in LTP may only appear in fragile X mice when stimulation conditions do not produce a saturation of synaptic potentiation (Lauterborn et al., 2007; Seese et al., 2014), we have been exploring optimal conditions to use in the different components of the olfactory-hippocampal circuit (piriform cortex, entorhinal cortex, dentate gyrus, CA3, and CA1). A robust deficit in hippocampal LTP in *Fmr1*-KO mice will also be necessary to test for rescue of LTP after stimulation of neurogenesis in *Fmr1*-KO mice (SOW task 3c). Several experiments has been attempted. In the first, we compared a stimulation pattern that produces maximal LTP – theta-burst stimulation (10 high frequency bursts of 4 pulse each at 100 Hz, repeated at 5 Hz) with a submaximal pattern (10 bursts repeated at 1 Hz) on LTP in the final stage of the circuit, field CA1. In WT slices, 5 Hz bursts produced a stable LTP effect of about 30-35%. Similar results were obtained in slices from *Fmr1*-KO mice. We also tested 1 Hz bursts in the same slices and found that submaximal LTP was induced in WT slices (10-15%). Somewhat larger effects were induced in slices from *Fmr1*-KO mice, but the difference between WT and KO mice was not statistically significant (please see **APPENDIX Figure 2**).

In the second experiment, we tested for LTP in the medial (non-olfactory) and lateral (olfactory) components of the performant path input to the dentate gyrus. To our surprise, larger LTP was seen in the dentate synapses of the fragile X mice at both of these pathways (**APPENDIX Figure 3**), although with the small samples we have so far, the enhancement was only statistically significant for the lateral (olfactory pathway).

In a third experiment, we tested for effects of two episodes of TBS spaced one hour apart on LTP in field CA1 of WT and *Fmr1*-KO mice. Prior studies have suggested that spaced TBS may allow elevation of maximal LTP (saturation), possibly *via* a protein synthesis-dependent mechanism (Kramar et al., 2012). In our experiments (**APPENDIX Figure 4**), a first episode of TBS induced comparable LTP in both WT and *Fmr1*-KO mice. A second TBS one hour later induced only short-term potentiation, without an additional long-term component. No differences between WT and *Fmr1*-KO mice were seen.

4) Comparison of inhibitory synaptic transmission in anterior piriform cortex of *Fmr1*-KO and WT mice (re: SOW task 3b).

The experiments of Specific Aim 2 (SOW task 3a and 3b) are designed to uncover the synaptic basis for deficits in long-term potentiation in the anterior piriform cortex of *Fmr1*-KO mice. Since synaptic inhibition antagonizes LTP induction, we are measuring the efficacy of GABAergic inhibitory synaptic transmission in this system in WT and *Fmr1*-KO mice. Andrew Widmer (doctoral student) is using whole-cell patch-clamp recordings to analyze the frequency and amplitude characteristics of spontaneous inhibitory postsynaptic currents (sIPSCs) and mIPSCs in these mice. The studies are in progress; some example data are presented in **APPENDIX Figure 5**. Slices prepared as in our previous publications (Gocel and Larson, 2012, 2013) are patched with electrodes containing 120 mM CsCl and voltage-clamped at -70 mV in the presence of CNQX (AMPA-type glutamate receptor antagonist, 25 μM) to block excitatory synaptic transmission. Under these conditions, sIPSCs are inward currents that occur frequently (~100/min) and average about 30-70 pA in amplitude. Moderate hyperpolarization (to -90mV) increases sIPSC amplitude without altering frequency. TTX (1 μM) only slightly reduces the frequency of the larger events, indicating that most of the sIPSCs are mIPSCs. Addition of

bicuculline ($GABA_A$ receptor antagonist, 25 mM) abolishes all synaptic activity in the presence of CNQX. A comparison of these IPSCs in *Fmr1*-KO and WT mice at different ages is in progress.

5) Comparison of olfactory learning in *Fmr1*-KO and WT mice in a successive-cue olfactory discrimination task (re: SOW task 4b).
Experiment 4b calls for the testing of *Fmr1*-KO and WT control mice in a non-hippocampal dependent olfactory discrimination task. We ran several pilot experiments during the first year of this project to optimize a successive-cue discrimination paradigm to compare learning rates in mutant and control mice. The best paradigm (tested with C57Bl/6J control mice) used a series of eight separate odor discrimination problems as follows: Mice (3 months old) were trained on each discrimination problem in 100-trial sessions in which each trial involved presentation of one of the discriminative odors to both sniff ports in our standard discrimination chamber. Each trial was signaled by the extinguishing of a lamp and the presentation of the odor for 5 sec. A nose poke in either port within the 5 sec presentation period constituted a "GO" response; no response in the same period constituted a "NO-GO" response. GO responses to the positive cue (S+) or NO-GO responses to the negative cue (S-) are scored as correct; GO responses to S- or NO-GO responses to S+ are scored as errors. Only Go responses to S+ are rewarded with a drop (.01 ml) of water. The inter-trial interval (ITI) after correct responses was 10 sec and the ITI after errors was 30 sec. In the second year of this award, we tested a cohort of *Fmr1*-KO and WT littermates at 6-12 months of age on this task. Each mouse was trained to criterion performance of eight consecutive correct responses within a single 100-trial training session. Note that, since no more than three S+ or S- trials can occur consecutively, this criterion requires the mouse to respond correctly to at least two S+ trials and at least two S- trials within the sequence of eight consecutive correct responses. The probability of 8 consecutive correct responses by chance is 1/256 or ~0.4%.) Sessions were repeated on subsequent days if criterion performance was not met.

The main finding was that *Fmr1*-KO mice made significantly more errors while learning the first two-odor discrimination problem. Since other evidence indicates that this successive-cue olfactory discrimination task does not require participation of the hippocampus, the new results suggest that the learning deficit may be due to impaired synaptic plasticity in the olfactory cortex or defective neurogenesis in the olfactory bulb (SOW task 4c).

These results were presented at the 2014 Annual Meeting of the Society for Neuroscience (please see **APPENDIX 3**) and a manuscript is in preparation for publication (please see **APPENDIX 2**).

6) Comparison of learning rates at different ages in *Fmr1*-KO and WT mice (SOW task 4a). The experiment described immediately above will be repeated in mice ranging in age from 3 to 24 months. Preliminary findings related to this aim were obtained from the animals already tested at 5-12 months of age (please see *Figure 6* of **APPENDIX 2**). In the data collected so far, there is a trend of poorer performance in *Fmr1*-KO mice as they get older; no such trend was evident in the WT mice. If this trend is confirmed, it would strongly suggest that the learning impairment is due to LTP impairment in piriform cortex, since the LTP impairment also does not appear until six months of age (Larson et al., 2005). A new cohort of WT (n=5) and *Fmr1*-KO (n=8) is currently being tested on this task at 15 months of age, with another cohort to be tested immediately thereafter, at the same age.

C) Opportunities for training and professional development provided by the project.

1) Andrew Widmer, B.A., UIC Graduate (MD/PhD) student is conducting his PhD dissertation research by analyzing synaptic function in piriform cortex of *Fmr1*-KO mice for this project (2013-2015).
2) Patricia Dykas, B.S., research assistant, is learning new experimental methods, analyzing LTP in the olfactory-hippocampal circuit of *Fmr1*-KO mice (2014-2015).
3) Samantha Keil, B.S. was accepted into the UIC Graduate Program in Neuroscience, in part because of research experience obtained working on this project (2013-2014).
4) Tiffany Cheng, UIC undergraduate student, obtained laboratory research experience in this project by assisting with the genotyping of mice in the fragile X colony (2014-2015).
5) Maribell Heredia, UIC undergraduate student, is obtaining laboratory experience by assisting with the behavioral studies of *Fmr1*-KO mice (2015).
6) Camelia Saha, UIC undergraduate student, is gaining laboratory experience by assisting with the behavioral studies of *Fmr1*-KO mice (2015).
7) David Nai, UIC medical student, gained laboratory experience in summer of 2015, helping to analyze inhibitory synaptic function in *Fmr1*-KO mice.
8) Caryn Davis, B.S., UIC Graduate (PhD) student is analyzing adult neurogenesis in hippocampal dentate gyrus and olfactory bulb as part of her PhD dissertation research.
9) Carolyn Hollands, Ph.D., UIC postdoctoral scholar, is developing genetic model mice in which neurogenesis can be altered in the context of brain disorders, including fragile X syndrome.

D) Dissemination of results to communities of interest.

The results obtained so far have been presented in posters at the Annual Meetings of the Society for Neuroscience and other local and National meetings. As the project concludes, we plan to publish the findings in scientific journals for the benefit of the scientific community.

E) Plans for next reporting period.

Our goals for the next year are to (1) verify the genetic strategy for altering adult neurogenesis in fragile X knockout mice, (2) test for behavioral effects of altered neurogenesis in these mice, (3) complete the study of the age-dependent cognitive deficits in fragile X mice, and (4) to conduct an analysis of inhibitory synaptic transmission in piriform cortex of these mice. These studies will allow us to establish a cellular basis for certain aspects of the cognitive disability that characterizes the human fragile X syndrome.

4. IMPACT

A) Impact on development of principal disciplines of the project.
The existence of age-dependent changes in cognition and synaptic plasticity in fragile X syndrome and autism more generally is beginning to receive attention in the neuroscientific community. The contribution of adult neurogenesis to learning and other cognitive functions is also receiving increasing attention.

B) Impact on other disciplines.
Nothing to report.

C) Impact on technology transfer.
Nothing to report.

D) Impact on society beyond science and technology.
Nothing to report.

5. CHANGES/PROBLEMS

A) Changes in approach.

We had originally planned to use surgical introduction of lentiviral vectors to enhance adult neurogenesis in fragile X mice. With the development of more powerful, specific, and efficient genetic strategies to do this in other disease models, we decided to adapt the genetic approach to our fragile X syndrome model mice. With the departure of postdoctoral researcher, Giovanni Lugli PhD, we suspended biochemical studies of glutamate receptor trafficking. When MD/PhD student Andrew Widmer joined the lab, we began studies of inhibitory synaptic transmission in piriform cortex of fragile X mice. These studies are important for the overall success of the project, since both excitatory (glutamate) and inhibitory synapses contribute to the circuitry that regulates synaptic plasticity (LTP).

B) Problems and delays and plans for resolution.

We encountered delays in the breeding of mice for genetic enhancement of adult neurogenesis in fragile X model mice. This breeding is in progress but insufficient mice are as yet available for behavioral and electrophysiological testing. Therefore we requested and were granted a no-cost extension of the award for an additional one-year period (see below).

C) Changes with impact on expenditures.

We requested and were granted a one year no-cost extension to complete the experiments described in the Statement of Work. We plan to use the remaining funds in the extension period to maintain the mouse colony and pay scientific staff (Patricia Dykas) to complete the experiments. During the no-cost period, the PI will supervise all activity and prepare manuscripts for publication.

D) Changes in use and/or care of vertebrate animals.

With the change in approach to regulation of neurogenesis, different strains of mice are used. The necessary modifications to our animal use protocol were submitted and approved by the UIC Animal Care Committee (IACUC) and the ACURO.

6. PRODUCTS

Publications

1. Larson, J. Munkácsy, E. Theta-Burst LTP. *Brain Research*, 1621 (2015) 38-50. (**APPENDIX 1).**

Manuscripts

1. Larson, J., Keil, S.A., Heredia, M., & Saha, C. Impaired olfactory discrimination learning in fragile X knockout mice. Manuscript in preparation (**APPENDIX 2**)

Abstracts

1. Keil, S.A. & Larson, J. Impaired olfactory discrimination learning in fragile X knockout mice. *Abstracts, Society for Neuroscience* (2014) Program no. 699.10. (**APPENDIX 3**)
2. Davis, C., Demars, M., Larson, J., & Lazarov, O. Deficits in adult neurogenesis in fragile X syndrome. *Abstracts, Society for Neuroscience* (2015) Program No. 491.06. (**APPENDIX 4**)

7. PARTICIPANTS

Name:	**John Larson, PhD**
Project Role:	**Principal Investigator**
UIC ID:	**674804260**
Person-Months:	**4**
Contribution:	**The PI is supervising all research in the project**
Funding Support:	**UIC Department of Psychiatry**

Name:	**Orly Lazarov, PhD**
Project Role:	**Co-Investigator**
UIC ID:	**675343841**
Person-Months:	**1**
Contribution:	**Dr. Lazarov is supervising the neurogenesis experiments.**
Funding Support:	**UIC Department of Anatomy & Cell Biology; National Institute on Aging**

Name:	**Andrew Widmer, BA**
Project Role:	**Graduate Student (MD/PhD)**
UIC ID:	**674125340**
Person-Months:	**12**
Contribution:	**Mr. Widmer is analyzing synaptic receptor function in Fmr1-KO mice.**
Funding Support:	**UIC Center for Clinical and Translational Science (Aug., 2015-Present)**

Name:	**Neil Smalheiser, MD,PhD**
Project Role:	**Consultant**
UIC ID:	**674669197**
Person-Months:	**1**
Contribution:	**Dr. Smalheiser provides consulting advice on biochemical analysis of synaptic proteins.**
Funding Support:	**UIC Department of Psychiatry; National Library of Medicine**

Name:	**Patricia Dykas, BS**
Project Role:	**Research Assistant**
UIC ID:	**663171617**
Person-Months:	**12**
Contribution:	**Ms. Dykas is testing for LTP in the olfactory-hippocampal circuit in Fmr1-KO mice.**
Funding Support:	

Name:	**Carolyn Hollands, PhD**
Project Role:	**Postdoctoral Researcher**

UIC ID:	**677858472**
Person-Months:	**12**
Contribution:	**Dr. Hollands is manipulating neurogenesis in Fragile X mice.**
Funding Support:	**National Institute on Aging**

Name:	**Caryn Davis**
Project Role:	**Graduate Student (PhD)**
UIC ID:	
Person-Months:	**3**
Contribution:	**Ms. Davis is analyzing neurogenesis in Fmr1-KO mice.**
Funding Support:	**UIC Department of Anatomy & Cell Biology**

Name:	**Maribell Heredia**
Project Role:	**Undergraduate Student**
UIC ID:	**652274879**
Person-Months:	**2**
Contribution:	**Ms. Heredia is assisting with behavioral training experiments.**
Funding Support:	

Name:	**Camelia Saha**
Project Role:	**Undergraduate Student**
UIC ID:	**672194445**
Person-Months:	**2**
Contribution:	**Ms. Heredia is assisting with behavioral training experiments.**
Funding Support:	

Name:	**Amy Saulnier**
Project Role:	**Undergraduate Student**
UIC ID:	**652251471**
Person-Months:	**2**
Contribution:	**Ms. Saulnier is assisting with genotypic of the fragile X mouse colony.**
Funding Support:	

8.SPECIAL REPORTING REQUIREMENTS

None.

REFERENCES

Gocel, J., and Larson, J. (2012). Synaptic NMDA receptor-mediated currents in anterior piriform cortex are reduced in the adult fragile X mouse. Neuroscience *221*, 170-181.

Gocel, J., and Larson, J. (2013). Evidence for loss of synaptic AMPA receptors in anterior piriform cortex of aged mice. Frontiers in aging neuroscience *5*, 39.

Kramar, E.A., Babayan, A.H., Gavin, C.F., Cox, C.D., Jafari, M., Gall, C.M., Rumbaugh, G., and Lynch, G. (2012). Synaptic evidence for the efficacy of spaced learning. Proc Natl Acad Sci U S A *109*, 5121-5126.

Larson, J., Jessen, R.E., Kim, D., Fine, A.K., and du Hoffmann, J. (2005). Age-dependent and selective impairment of long-term potentiation in the anterior piriform cortex of mice lacking the fragile X mental retardation protein. JNeurosci *25*, 9460-9469.

Lauterborn, J.C., Rex, C.S., Kramar, E., Chen, L.Y., Pandyarajan, V., Lynch, G., and Gall, C.M. (2007). Brain-derived neurotrophic factor rescues synaptic plasticity in a mouse model of fragile X syndrome. J Neurosci *27*, 10685-10694.

Lazarov, O., Demars, M.P., Da Tommy Zhao, K., Ali, H.M., Grauzas, V., Kney, A., and Larson, J. (2011). Impaired survival of neural progenitor cells in dentate gyrus of adult mice lacking FMRP. Hippocampus.

Sahay, A., Scobie, K.N., Hill, A.S., O'Carroll, C.M., Kheirbek, M.A., Burghardt, N.S., Fenton, A.A., Dranovsky, A., and Hen, R. (2011). Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. Nature *472*, 466-470.

Seese, R.R., Wang, K., Yao, Y.Q., Lynch, G., and Gall, C.M. (2014). Spaced training rescues memory and ERK1/2 signaling in fragile X syndrome model mice. Proc Natl Acad Sci U S A *111*, 16907-16912.

APPENDIX 1

BRAIN RESEARCH 1621 (2015) 38–50

Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/brainres

Brain Research

Review

Theta-burst LTP

John Larson[a,*], Erin Munkácsy[b]

[a]Psychiatric Institute, Department of Psychiatry, University of Illinois College of Medicine, Chicago, IL 60612, United States

[b]Barshop Institute for Longevity and Aging Studies, Department of Cell and Structural Biology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78245, United States

ARTICLE INFO

Article history:
Accepted 18 October 2014
Available online 27 October 2014

Keywords:
Long-term potentiation
LTP
Theta burst stimulation
NMDA
AMPA
GABA
Hippocampus
CA1

ABSTRACT

This review covers the spatial and temporal rules governing induction of hippocampal long-term potentiation (LTP) by theta-burst stimulation. Induction of LTP in field CA1 by high frequency stimulation bursts that resemble the burst discharges (complex-spikes) of hippocampal pyramidal neurons involves a multiple-step mechanism. A single burst is insufficient for LTP induction because it evokes both excitatory and inhibitory currents that partially cancel and limit postsynaptic depolarization. Bursts repeated at the frequency (~5 Hz) of the endogenous theta rhythm induce maximal LTP, primarily because this frequency disables feed-forward inhibition and allows sufficient postsynaptic depolarization to activate voltage-sensitive NMDA receptors. The disinhibitory process, referred to as "priming", involves presynaptic GABA autoreceptors that inhibit GABA release. Activation of NMDA receptors allows a calcium flux into dendritic spines that serves as the proximal trigger for LTP. We include new data showing that theta-burst stimulation is more efficient than other forms of stimulation for LTP induction. In addition, we demonstrate that associative interactions between synapses activated during theta-bursts are limited to major dendritic domains since such interactions occur within apical or basal dendritic trees but not between them. We review evidence that recordings of electrophysiological responses during theta burst stimulation can help to determine if experimental manipulations that affect LTP do so by affecting events antecedent to the induction process, such as NMDA receptor activation, or downstream signaling cascades that result from postsynaptic calcium fluxes. Finally, we argue that theta-burst LTP represents a minimal model for stable, non-decremental LTP that is more sensitive to a variety of experimental manipulations than is LTP induced by other stimulation paradigms.

This article is part of a Special Issue entitled SI: Brain and Memory.

*Corresponding author.
E-mail address: jrlarson@uic.edu (J. Larson).

http://dx.doi.org/10.1016/j.brainres.2014.10.034
0006-8993/

Contents

1. Introduction . . . 39
2. Multiple-step induction mechanism and peculiarity of the theta frequency . . . 40
3. "Economy" of theta-burst stimulation . . . 42
4. Spatial limits of associative theta-burst LTP . . . 43
5. A within-burst timing effect . . . 44
6. Theta-burst responses as diagnostics . . . 45
7. Sensitivity of theta-burst LTP to various experimental manipulations . . . 46
8. Conclusions . . . 47
Acknowledgments . . . 47
References . . . 48

1. Introduction

During the first decade after the discovery of long-term potentiation (LTP) was announced (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973), there was some skepticism that it could serve as a memory mechanism, given the conditions that seemed necessary to induce the effect. At the same time, it seemed too many that memory could only be studied neurobiologically in simple nervous systems in which stimulus and response could be traced directly. In this time period (the first decade after 1973), there were 49 papers published on LTP; in the same interval, Eric Kandel's lab alone published 77 papers on mechanisms of learning in *Aplysia*. Of course, there were many factors that contributed to the lag in appreciation of LTP (and the decline in popularity of the invertebrate model system approach), but an important one was the question of whether or not LTP could be produced by physiological processes during learning.

The discovery that "theta-burst stimulation" (TBS) was an effective stimulus for LTP (Larson et al., 1986) was significant for three reasons: *first*, it showed that patterns of neuronal firing (complex-spikes) that occurred spontaneously during behavior could, if appropriately timed, induce LTP; *second*, the optimal repetition rate corresponded to the frequency of the hippocampal theta rhythm, an EEG pattern previously related indirectly to memory storage processes; and *third*, patterned stimulation paradigms allowed the uncovering of multiple events that contribute to LTP induction.

We will begin with a historical review, then present some original data pertaining to a few unresolved issues, and conclude with a discussion of some theoretical and practical implications. LTP can be divided into two sets of processes which we will refer to as *induction* and *expression*. The induction events are transient and very brief (<1 s), involve both presynaptic and postsynaptic responses to unusual patterns of activation, and are modulated by local circuit mechanisms. Expression refers to the synapse-specific and enduring (>1 hour) enhancement of synaptic transmission. In addition, persistence of expression requires mechanisms for stabilization or continued maintenance. This review will focus on the mechanisms by which theta burst stimulation induces LTP and will not say much about the cellular events downstream from the induction events. The post-induction events are most likely common to LTP induced by TBS and other stimulation paradigms; i.e., they are not particular to theta-burst LTP.

The theta-burst paradigm mimics two characteristics of hippocampal physiology: complex-spike discharges of the pyramidal neurons (Ranck Jr., 1973) and the rhythmic modulation of excitability of those cells during theta rhythm (Rudell et al., 1980). Hippocampal theta was originally described as the hippocampal "arousal rhythm" (Green and Arduini, 1954) since it was correlated with the neocortical desynchronization characteristic of awake, attentive states. Follow-up studies attempted to relate theta to specific psychological concepts such as attention, learning, memory encoding, etc.; however, Vanderwolf (1969), in a very influential paper, argued effectively that theta should be considered as a hippocampal correlate of voluntary movement. This conclusion invites the question: why is the hippocampus concerned with movement? One explanation is that movement in space necessitates updating cognitive maps of the environment and the spatial activity of hippocampal place cells (O'Keefe and Nadel, 1978). A second explanation is that much of voluntary movement is exploratory in nature, involving the gathering of both spatial and non-spatial information relevant to goal-directed behaviors. Perhaps one of the most impressive examples of the coupling of behavior to neural activity in mammals occurs during exploratory sniffing in rodents: rhythmic respiration is coupled to theta rhythm throughout the "olfactory–hippocampal circuit" (olfactory bulb, piriform cortex, entorhinal cortex, dentate gyrus, and hippocampus) both at the gross behavioral level and with synchronization of the behavioral rhythm with the EEG pattern on a cycle-by-cycle basis (Macrides, 1975; Macrides et al., 1982; Rojas-Líbano et al., 2014; Tsanov et al., 2014). This suggests that a stimulus sampling pattern organizes neuronal activity throughout a brain circuit that processes the information obtained by the sampling behavior. The synchronization of olfactory sampling with hippocampal theta may be only the most direct and striking example of this: sniffing in rodents is also synchronized with active tactile sensation using the mystacial vibrissae (Deschenes et al., 2012; Welker, 1964), although the pathways for somatosensory information to reach the hippocampus are far less direct than those for olfaction. It is also intriguing that visual saccades in humans and non-human primates occur at frequencies in the theta range and may be coupled to hippocampal theta (Hoffman et al., 2013).

The efficacy of stimulation patterns mimicking complex-spike discharges was first tested in the *in vivo* dentate gyrus (Douglas and Goddard, 1975; Douglas, 1977) and eight-pulse,

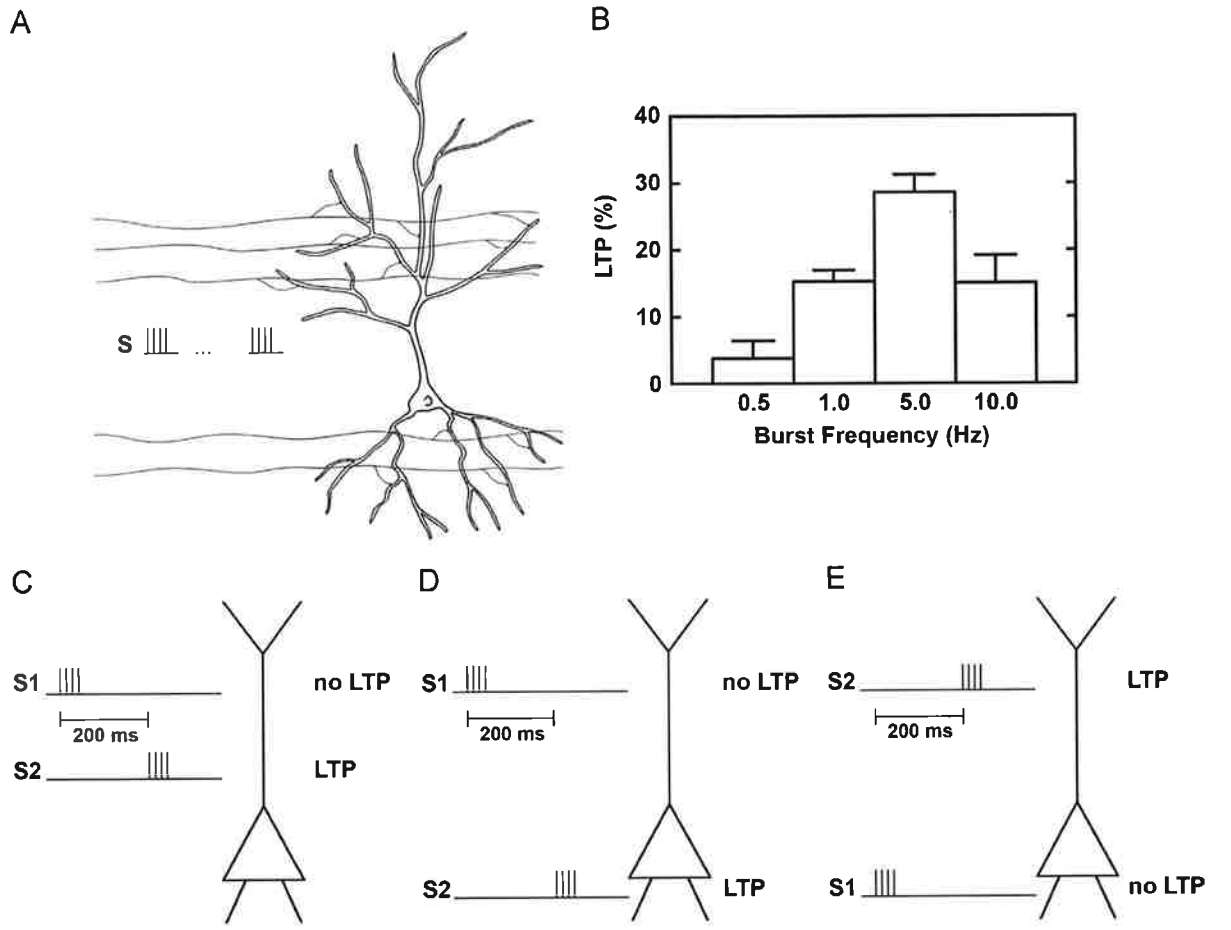


Fig. 1 – Timing rules for theta-burst LTP. (A) CA1 pyramidal neuron with apical and basal dendritic trees that receive excitatory synaptic connections from CA3 via Schaffer collaterals and commissural inputs to both apical and basal dendritic trees. Bursts of high frequency stimulation (S) are used to simulate complex-spike discharges in the afferent neurons. Timing rules are established by varying inter-burst frequency and spatial location of the activated synapses. (B) At apical dendritic synapses, bursts repeated at 5 Hz produce optimal LTP. Histogram plots the magnitude of LTP induced by 5–20 bursts repeated at the frequencies indicated on the abscissa. Figure modified from Larson et al. (1986). A similar frequency dependence is seen at basal dendritic synapses (Capocchi et al., 1992). (C) A burst to one apical dendritic input (S1) does not itself produce LTP at those synapses, but primes the cell so that a second burst to a separate input (S2) 200 ms later triggers LTP at only the synapses activated by the second burst. (D) A burst to an apical dendritic input (S1) can prime LTP induction at a basal dendritic input (S2). (E) A burst to a basal dendritic input (S1) can prime LTP induction at an apical dendritic input (S2). Results depicted in panels C–E are from Larson and Lynch (1986).

400 Hz bursts for LTP induction became common in studies of this structure. In this system, each burst was thought to induce an increment of LTP; repetition served only to summate these increments and little regard was placed on the repetition rate (Douglas, 1977). However, due to limited effectiveness, this pattern never caught on for studies of CA1 in hippocampal slices, where the most common induction pattern was a 1–2 s-long 100 Hz train (tetanus). Combining the complex-spike pattern with a theta frequency repetition rate produced a robust, reliable, and stable LTP in the CA1 field of hippocampal slices (Larson et al., 1986). These experiments used a four-pulse burst at 100 Hz to mimic the complex-spike burst and repeated it ten times at five bursts per second to approximate the theta rhythm frequency. The significance of LTP induced by this pattern was not immediately apparent until it was found that burst repetition frequencies lower or higher that 5 Hz were significantly less effective for LTP induction. The frequency-dependence of burst-induced LTP is reproduced in Fig. 1A, B.

2. Multiple-step induction mechanism and peculiarity of the theta frequency

What accounts for this frequency-dependence? Many mechanisms contribute. The first clue was that either a single burst or multiple bursts repeated at long intervals (≥ 2 s) were almost always ineffective (Larson and Lynch, 1986; Rose and Dunwiddie, 1986). However, a burst to one input could "prime" the circuit so that a second burst to a separate input 200 ms

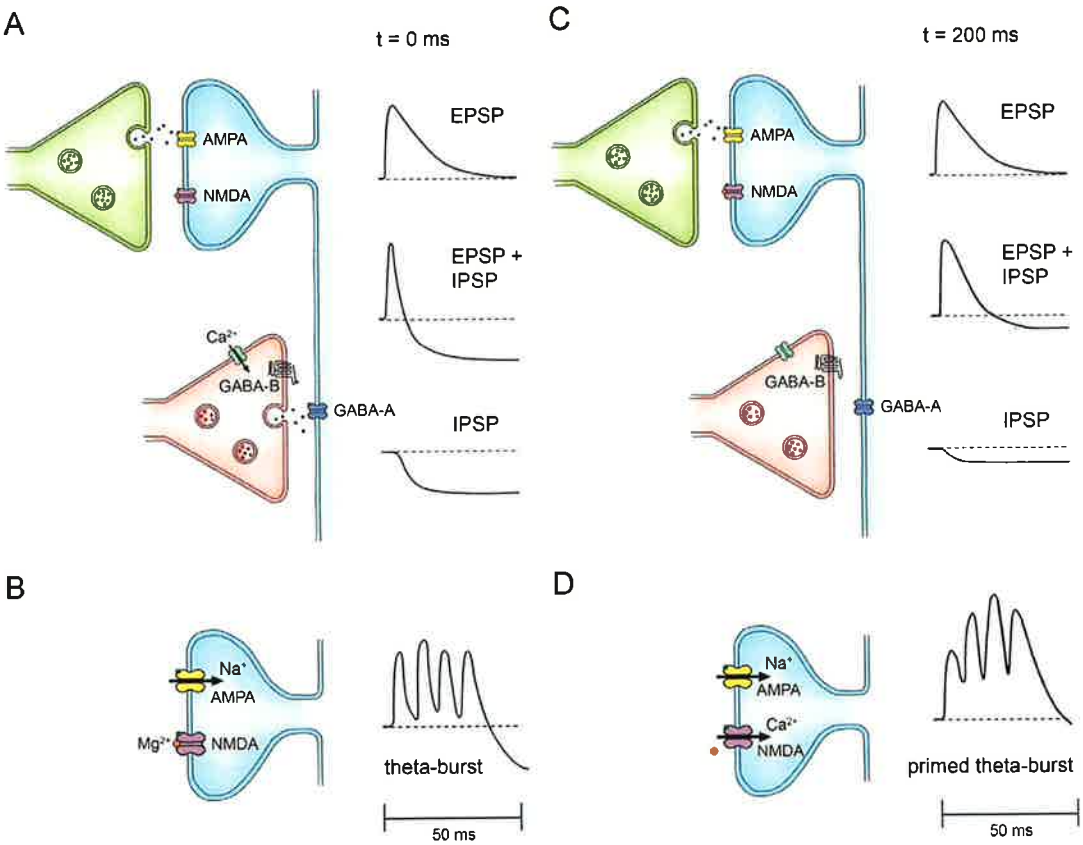


Fig. 2 – Multiple-step induction mechanism for theta-burst LTP in field CA1 of hippocampus. (A) In the "unprimed" state (t=0 ms), afferent stimulation activates a monosynaptic glutamatergic (excitatory) axo-spinous synapse and a disynaptic GABAergic (inhibitory) axo-dendritic synapse on the same pyramidal neuron. Glutamate activates postsynaptic AMPA receptors to depolarize the pyramidal neuron (EPSP) while co-expressed NMDA receptors are blocked by Mg^{2+}. GABA activates postsynaptic $GABA_A$ receptors that hyperpolarize the neuron (IPSP). The IPSP curtails the depolarization evoked by the EPSP (EPSP+IPSP). The GABA released at the inhibitory synapse also activates a slow, metabotropic $GABA_B$ receptor on the presynaptic inhibitory terminal. (B) A high frequency burst (theta-burst) under these conditions results in poor temporal summation of the depolarization due to the feed-forward inhibition. (C) In the "primed" state (t=200 ms), there is little change in the synaptic action at the excitatory synapse, but the action of the presynaptic $GABA_B$ receptor produces a use-dependent suppression of GABA release. The diminished IPSP causes the EPSP to have a prolonged depolarizing effect (EPSP+IPSP). (D) A high frequency burst in the primed state (primed theta-burst) shows enhanced temporal summation of excitation; the enhanced depolarization allows relief of the Mg^{2+} channel block and a calcium influx through the NMDA receptor.

later would reliably induce LTP, but only on the synapses activated by the second burst (Fig. 1C). A single burst activates a feed-forward inhibitory postsynaptic potential (IPSP) that truncates the excitatory postsynaptic potentials (EPSPs) evoked by that burst or a subsequent burst within 100–150 ms. The key to the theta frequency optimum is that the feed-forward inhibition becomes suppressed after its own activation and does not recover for about a second (Davies et al., 1990; McCarren and Alger, 1985). Because of this disinhibition, a second burst at 200 ms after the first burst evokes maximal postsynaptic depolarization in the pyramidal neuron. Bursts that occur too early are shunted by the already-present IPSP; bursts that are delayed too long encounter a recovering IPSP. We initially referred to the IPSP suppression as "priming" the circuit and showed that it could be effectuated by a single pulse (no burst required) and that it was spatially widespread since priming of apical synapses could be induced by basal dendritic stimulation and vice-versa (Fig. 1D, E) (Larson and Lynch, 1986). The IPSP suppression was verified (Pacelli et al., 1989, 1991) and shown to be mediated by metabotropic $GABA_B$ autoreceptors that result in diminished GABA release (Davies et al., 1991; Mott and Lewis, 1991, 1992). The enhanced burst-evoked depolarization of "primed" bursts allows measurable NMDA receptor-dependent currents to be activated (Larson and Lynch, 1988). There is evidence that the IPSP suppression is restricted to the feed-forward inhibitory circuit and absent in the feedback (recurrent) circuit, but this needs to be more firmly established (Arai et al., 1995). Interestingly, this may explain how theta rhythm is sustained if the GABAergic inhibitory interneurons ("theta" cells) are mainly the feedback rather than feed-forward interneurons. The main contributors to LTP induction by TBS are diagramed in Fig. 2.

Other factors that contribute to the pattern of dendritic depolarization and synaptic NMDA receptor activation are agents that enhance AMPA receptor activity by enhancing glutamate release or by allosteric modulation of AMPA receptors (ampakines): the latter drugs magnify the depolarization without changing the temporal pattern of burst response enhancement. Consequently, ampakines magnify the LTP induced by suboptimal burst repetitions (i.e., after 5 bursts) but do not alter the maximal LTP expressed (Arai and Lynch, 1992). In contrast, an agent such as forskolin that antagonizes the calcium-dependent AHP between theta bursts elevates the LTP "ceiling" without changing the fractional increment produced by suboptimal bursts (Arai and Lynch, 1992), possibly by extending the duration of NMDA receptor currents.

A four-pulse burst was used in these experiments because it is within the common range of hippocampal complex-spikes and because three-pulse bursts are rarely sufficient – see also Arai et al. (2004) and Diamond et al. (1988). We have used 100 Hz as the intra-burst frequency because it allows resolution of responses to the individual pulses. Other variations on pulse number and intra-burst frequency have not been studied systematically. It is likely that intra-burst frequency effects such as presynaptic facilitation and depletion (Pan and Zucker, 2009) interact with pulse number to determine the synaptic cleft glutamate concentration available to interact with postsynaptic NMDA receptors.

The theta frequency optimum has been questioned by Grover and colleagues who tested the efficacy of four-pulse bursts repeated 20 times at frequencies from 0.05 to 10.0 Hz, with maximal LTP induced at 2–3 Hz (Grover et al., 2009), making the interesting point that the lower frequency maximum may correspond more closely to the delta waves that are present in the hippocampal EEG during slow-wave sleep rather than the theta waves of behavioral arousal and REM sleep. It is an intriguing possibility to consider that the frequency–plasticity relationship may be altered by changes in "circuit kinetics", perhaps dictated by brainstem modulatory systems, associated with different behavioral states. On the other hand, the frequency–plasticity relationship may also be distorted during regular repetition of bursts to a single input. A pair of appropriately timed bursts is much more likely to occur naturally during behavior than a series of 10–20 bursts repeated at regular intervals. Studies using the primed-burst paradigm (homosynaptic priming) or the two-input paradigm showed significant LTP maxima at 140 ms (~7 Hz) (Diamond et al., 1988) and 100–200 ms (5–10 Hz), respectively (Larson, 1987). Since a single (primed) burst is sufficient to induce a small increment of stable LTP that is additive (Larson and Lynch, 1986), this frequency-dependence seems more likely to reflect the conditions *in vivo* than those that obtain when many bursts are repeated in a long series. In chronic single-unit recording studies, Otto and Eichenbaum observed that complex-spike burst discharges of CA1 pyramidal cells were often preceded by spike or burst activity at theta periodicity during odor discrimination or spatial learning (Otto et al., 1991), validating the occurrence of theta-burst activity in freely-behaving animals.

Theta-burst LTP exhibits three other canonical features of LTP: cooperativity (associativity), saturation, and stability. The requirement for adequate postsynaptic depolarization to activate voltage-sensitive NMDA receptors was discussed above; normally, this is provided by simultaneous stimulation of many afferents (cooperativity). How realistic is this? It is comforting perhaps that detectable and stable LTP is induced by a single appropriately-primed four-pulse burst (Larson and Lynch, 1986; Rose and Dunwiddie, 1986). In situ, some of the necessary depolarization may be provided by the theta rhythm itself: experiments show that single bursts *in vivo* can be effective when timed to occur at the peak depolarization of endogenous theta waves and are ineffective when evoked at the trough (Holscher et al., 1997; Hyman et al., 2003; McCartney et al., 2004; Orr et al., 2001; Pavlides et al., 1988). Thus, LTP induction may not require unrealistically massive synchronization of many bursting inputs to a cell. We will return to the spatial limits of within-burst spatial summation in a later section. With regard to saturation, LTP resulting from a single episode of TBS seems to reach a maximum after about 10 bursts (Larson et al., 1986); however, this limit may be exceeded with widely-spaced episodes (Kramar et al., 2012), suggesting that synapses may differ in LTP threshold, with later episodes recruiting less susceptible synapses (Lynch et al., 2013). Finally, theta-burst LTP in slice preparations typically is non-decremental after the first 15 min. This also appears to be true *in vivo*, where the potentiation is maintained at a stable level for weeks (Staubli and Lynch, 1987).

3. "Economy" of theta-burst stimulation

It seems evident from the frequency-dependence of patterned burst-induced LTP that long continuous trains (tetanic stimulation) would be less efficient than TBS in the sense that each stimulus pulse would contribute less to the LTP effect. However,

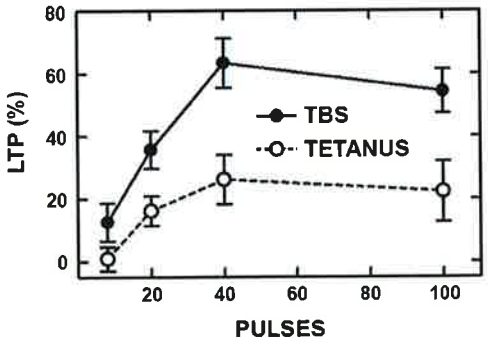


Fig. 3 – Theta-burst stimulation is more efficient than continuous high frequency stimulation for LTP induction in hippocampal field CA1. LTP at apical dendritic synapses was measured as the percent increase in field EPSP slope 60 min after LTP induction by stimulation of Schaffer-commissural fibers with either a continuous, 100 Hz train consisting of 8–100 pulses (TETANUS) or the same total number of pulses given as bursts of four repeated at 200 ms intervals (TBS). Significantly greater LTP was evoked by TBS with 20 pulses (5 bursts, $p<0.05$), 40 pulses (10 bursts, $p<0.01$), and 100 pulses (25 bursts, $p<0.01$) than tetani consisting of the corresponding number of pulses. Results are from unpublished data by J. Larson and J. Crouch.

this has received little systematic study. One study of field CA1 in rat hippocampal slices (Hernandez et al., 2005) reported no statistically-significant differences between the amounts of LTP induced by tetani and theta-bursts containing the same total stimulation pulses. The minimal TBS stimulus consisted of ten theta bursts in that study, so the submaximal stimulation range was not investigated. Furthermore, the pulse number-LTP magnitude curves for both TBS and tetanic stimulation were fairly flat, suggesting that a ceiling effect may have obtained with the minimal conditions used.

We re-examined this issue using a two-input paradigm in field CA1 of male adult (2–3 months) mouse (CD-1) hippocampal slices. Slices were prepared as described (Larson et al., 1999) and maintained at the interface between perfused (1 mL/min) aCSF (in mM: NaCl 124, KCl 3, KH_2PO_4 1.2, $NaHCO_3$ 26, $MgSO_4$ 2.5, $CaCl_2$ 3.4, D-glucose 10, Na-L-ascorbate 2) and an atmosphere of 95%O_2/5% CO_2, at 34±1 °C. Stimulation electrodes (bipolar twisted nichrome wires) were placed in *stratum radiatum* of CA1a and CA1c and field potentials recorded via a glass microelectrode (2 M NaCl) in *s. radiatum* of CA1b. Test pulse intensity was set to evoke a 2.0 mV field EPSP using a stimulus pulse duration of 0.1 ms. Each slice tested was given a theta-burst stimulus on one pathway and a tetanus (continuous 100 Hz train) consisting of the same total number of stimulus pulses on the other pathway (in random order and with position counter-balanced) with at least 30 min delay between LTP induction episodes. Pulse duration was increased to 0.2 ms during the induction stimuli. The degree of LTP induced was quantified at 60 min post-stimulation for each pathway. The total number of induction pulses used in different slices was 8 (2 theta-bursts or an 80 ms long tetanus), 20 (5 theta-bursts or a 200 ms tetanus), 40 (10 theta-bursts or a 400 ms tetanus), or 100 (25 theta-bursts or a 1 s tetanus). As shown in Fig. 3, no significant LTP was induced by the shortest tetanus, although significant LTP was induced by the same number of pulses (8) given as two theta-bursts (however, due to large variances, the direct comparison of TBS to tetanus was not significant). For greater numbers of pulses, theta-bursts yielded more LTP than the same number of pulses given as a single tetanus. A two-way ANOVA on the percent change persisting at 60 min post LTP stimulation showed significant main effects of stimulus pattern ($F_{1,124}=25.65$, $p<0.0001$) and pulse number ($F_{3,124}=11.39$, $p<0.0001$) but no significant interaction between the two independent variables ($F_{3,124}=1.37$, $p>0.25$). These results confirm that TBS is indeed more economical for LTP induction than un-patterned stimulation. In fact, the 100-pulse tetanus yielded somewhat less LTP than a theta-burst pattern of only 20 pulses. More is not necessarily better! A possible explanation for the weaker effect of tetanic stimulation is that depletion of glutamate available for release occurs during continuous trains, thus reducing NMDA receptor activation.

4. Spatial limits of associative theta-burst LTP

Classic "associative LTP" experiments in the CA1 *s. radiatum* (apical dendritic layer) showed that a single stimulus train of intensity or duration too weak to induce LTP on its own can do so if simultaneously paired with a stronger train to a separate input (Barrionuevo and Brown, 1983; Gustafsson and Wigstrom, 1986; Kelso and Brown, 1986; Kelso et al., 1986). Further studies provided evidence that these associative interactions are most effective when the two inputs terminate in spatially-overlapping regions of the dendritic tree (Hardie and Spruston, 2009; White et al., 1988, 1990) or individual branches (Govindarajan et al., 2011), although there is one report that strong stimulation of a basal dendritic (*s. oriens*) input can facilitate LTP in a simultaneously-stimulated apical dendritic input (Gustafsson and Wigstrom, 1986).

We investigated associative interactions between basal and apical dendritic synapses using TBS in field CA1 of hippocampal slices from adult male CD-1 mice, prepared and maintained as described above. Two stimulating electrodes were used: one was designated as the test electrode (S1; weak) and positioned either in *s. radiatum* (apical dendrite) or *stratum oriens* (basal dendrite) of CA1c; the other electrode was designated the conditioning electrode (S2; strong) and was positioned either in *s. radiatum* or *s. oriens* of CA1a. The recording electrode was positioned in CA1b in the same lamina as the test electrode. Stimulus intensity for the S1 (weak) electrode was always set to evoke a fEPSP about 1 mV in amplitude (typically about 20–25% of the maximal population spike-free fEPSP) while the S2 (strong) intensity was always set to evoke a population spike. In the first set of experiments (see Fig. 4A–D), S1 was positioned in *s. radiatum*. After establishing a baseline, two episodes of five theta-bursts (4 pulses at 100 Hz with 200 ms between the bursts) separated by 5 s were given to the test electrode (S1) alone. Recording was continued for 30 min to monitor the LTP induced. The conditioning (S2) stimulation electrode was then placed in *s. oriens* and S1 responses monitored for a 10 min baseline before both test (S1) and conditioning (S2) electrodes were given the same theta-bursts simultaneously. After an additional 30 min of recording, the conditioning (S2) electrode was moved to *s. radiatum*, the combined theta-burst stimulation (S1+S2) repeated, and LTP monitored on S1 for an additional 30 min. Stimulus pulse duration was *never* increased during TBS. In the second set of experiments (see Fig. 4E–H), S1 was located in *s. oriens* and S2 was first in *s. radiatum* and subsequently in *s. oriens*.

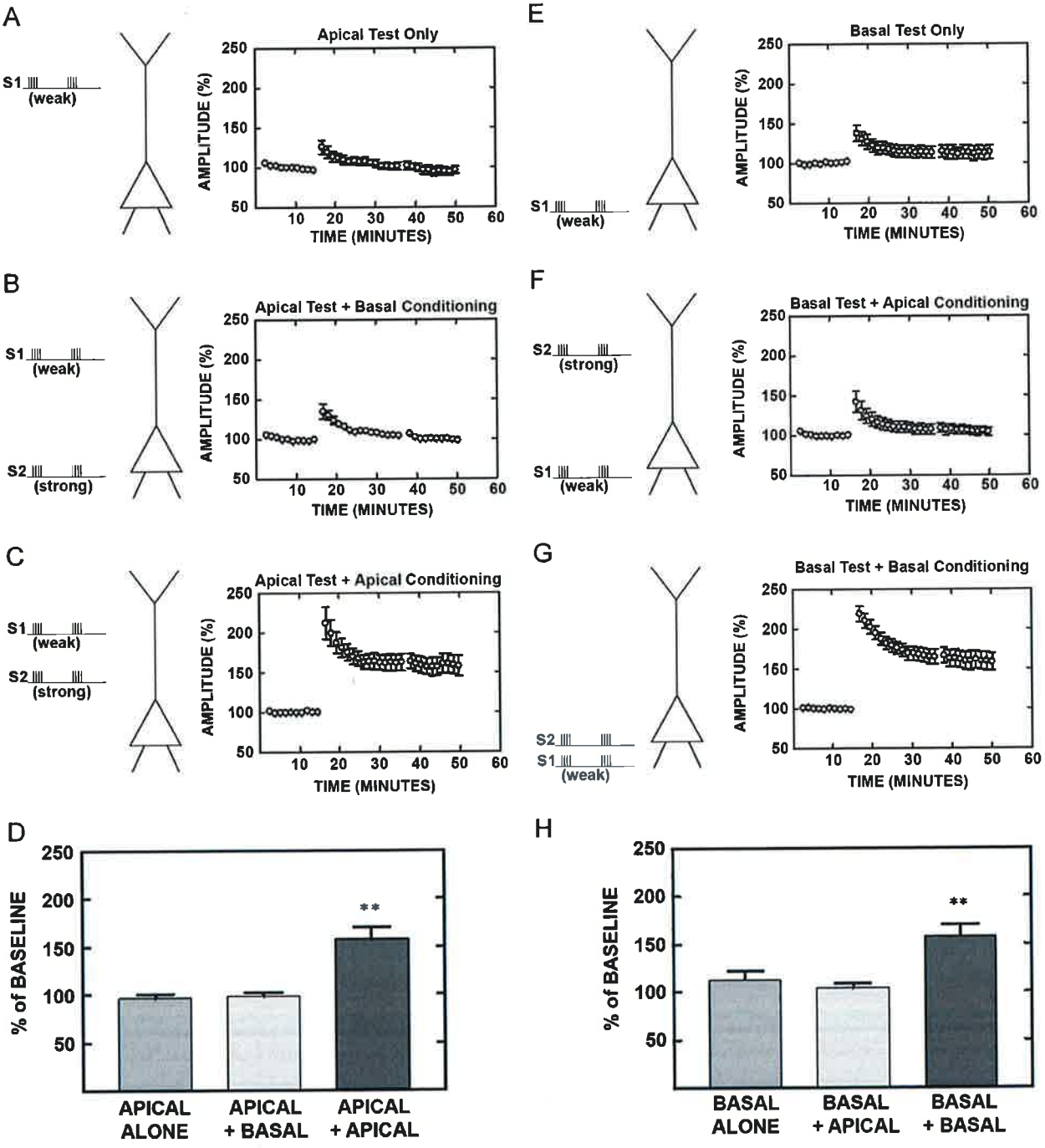
Conditioning stimulation of an apical dendritic input effectively facilitated LTP in the apical dendritic test input. In contrast, no associative LTP was induced in the apical test input when the conditioning stimulation was a basal dendritic input. Likewise, the basal dendritic test input was facilitated by a basal conditioning stimulus, but not by an apical input.

These results suggest that associative interactions during LTP induction by theta-bursts are limited to dendritic compartments. Weak and strong synaptic inputs can cooperate for LTP induction within the same dendritic tree but not between dendrites separated by the soma. The conventional interpretation of such interactions is that they require spatial summation of depolarization to allow activation of synaptic NMDA receptors. This suggests that the spatial summation can occur within apical or basal dendritic trees but not between them. One possibility is that shunting inhibition at the soma limits the spread of depolarization from apical to basal dendrites and vice-versa. While feed-forward IPSPs are suppressed during theta-bursts, these may be preferentially

located in the dendrites (Alger and Nicoll, 1982); somatic feedback IPSPs, $GABA_B$-dependent postsynaptic IPSPs, and calcium-activated after-hyperpolarizations appear to be intact during theta-bursts. The only experiment in which associative interactions between apical and basal dendritic synapses were found was conducted with $GABA_A$ receptors blocked pharmacologically (Gustafsson and Wigstrom, 1986). Alternatively, if associative LTP depends on diffusion and summation of chemical signals, this process also appears to be compartment specific (Govindarajan et al., 2011). In any case, it appears that the conditions of our experiment allow effective summation of synaptic signals within branches of the apical or basal dendritic trees, but not between the apical and basal compartments.

5. A within-burst timing effect

The possible role of asynchrony within theta-bursts was studied using a three-input paradigm in field CA1 of hippocampal slices (Larson and Lynch, 1989). The paradigm and results are summarized in Fig. 5. Three separate apical



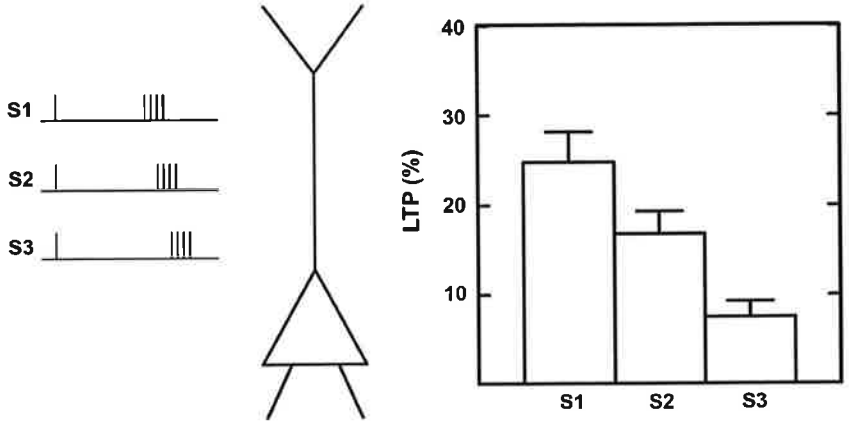


Fig. 5 – Within-burst timing and LTP. Hippocampal slices were prepared with a recoding electrode and three stimulating electrodes in s. *radiatum* of CA1. After a baseline period of recording responses to all three inputs, patterned stimulation was applied as depicted at left. All three inputs received a priming pulse followed by asynchronous bursts. The first pulse in the burst (4 pulses, 100 Hz) to S1 occurred 180 ms after the priming pulse, the first pulse to S2 coincided with the third pulse in the S1 burst, and the first pulse in the S3 burst coincided with the third pulse in the S2 burst. The pattern was repeated ten times at 5 s. intervals. The LTP induced at all three sets of synapses were statistically different from each other ($p<0.05$). Modified from Larson and Lynch (1989).

dendritic inputs were stimulated using stimulus intensity too low for any one input to be potentiated by TBS independently. For LTP induction, each input was stimulated with a simultaneous "priming" pulse followed by a 4-pulse burst to each input in a staggered but partially overlapping sequence. The spatial position of the inputs was counter-balanced *vis a vis* the timing pattern in different slices. The pulse-burst pattern was repeated ten times at five-second intervals. The degree of LTP induced was significantly related to the position of the input in the temporal sequence, with the synapses stimulated first in the sequence showing the most potentiation and the last-stimulated input being potentiated the least. One explanation for this timing effect is that the later arriving bursts can maintain depolarization longer at the synapses activated first and sustain NMDA channel activity at those synapses (Larson and Lynch, 1989). There is some similarity between this within-burst rule and the rules that govern spike timing-dependent plasticity (Feldman, 2012). The functional significance of the within-burst timing rule was investigated in network models; this work indicated that it contributes to encoding and recall of cues consisting of temporal sequences (Granger et al., 1994).

6. Theta-burst responses as diagnostics

Measuring postsynaptic responses to theta-bursts can be used to determine if experimental manipulations such as drugs or mutations affect LTP by interfering with the electrophysiological responses of the circuit to TBS or, alternatively,

Fig. 4 – Associative LTP induced by theta-burst stimulation is restricted to major dendritic domains. (A) A test electrode (S1) placed in the s. *radiatum* of field CA1 was used to study the spatial rules governing associative LTP induced by theta-burst stimulation. Stimulus intensity was set to evoke a field EPSP too weak (~1 mV in amplitude) to induce LTP when stimulated with TBS alone (see text for details). Graph shows field EPSP amplitude measured at 20 s intervals for 15 min before and 35 min. after TBS (each point is the mean ± S.E.M. of 4 consecutive trials) in seven different slices, each from a different mouse. (B) The same test input (S1) was then given TBS simultaneous with TBS to a strong input (S2) to the basal dendrites. The graph shows the S1 response amplitude (as in A) before and after TBS to S1 and S2 ($n=7$). (C) The test input was then given TBS simultaneous with stimulation of a strong input to the apical dendritic field (S2). This resulted in robust LTP to the S1 input ($n=6$, one slice rejected from analysis due to instability). (D) Histograms show field EPSP amplitude measured 30 min after TBS, relative to the pre-TBS baseline, in the three stimulation conditions. LTP was significantly greater after the apical-apical pairing than in the other two conditions ($p<0.01$). (E) as in (A), except that the test input (S1) was to the basal dendrites ($n=6$ experiments in slices from different mice). Weak stimulation to the basal dendritic input did not induce LTP when stimulated alone (E) or in combination with an apical dendritic input (F), but did show robust LTP after combined stimulation of a strong (S2) basal dendritic input (G). Histograms (H) show that significantly greater LTP was induced after combined basal-basal stimulation than in the other two conditions (**$p<0.01$). One slice in (E) was rejected from analysis due to instability. In both (C) and (G), the physical location of weak (S1) and strong (S2) electrodes were in subfields CA1c and CA1a, respectively (recording electrode in CA1b), and paired-pulse tests confirmed that the activated fibers were independent. In all other cases, the stimulation electrodes were in CA1c (i.e., the subfield closest to CA3). Results are from unpublished experiments by E. Munkácsy, N. Bartolotti, and J. Larson.**

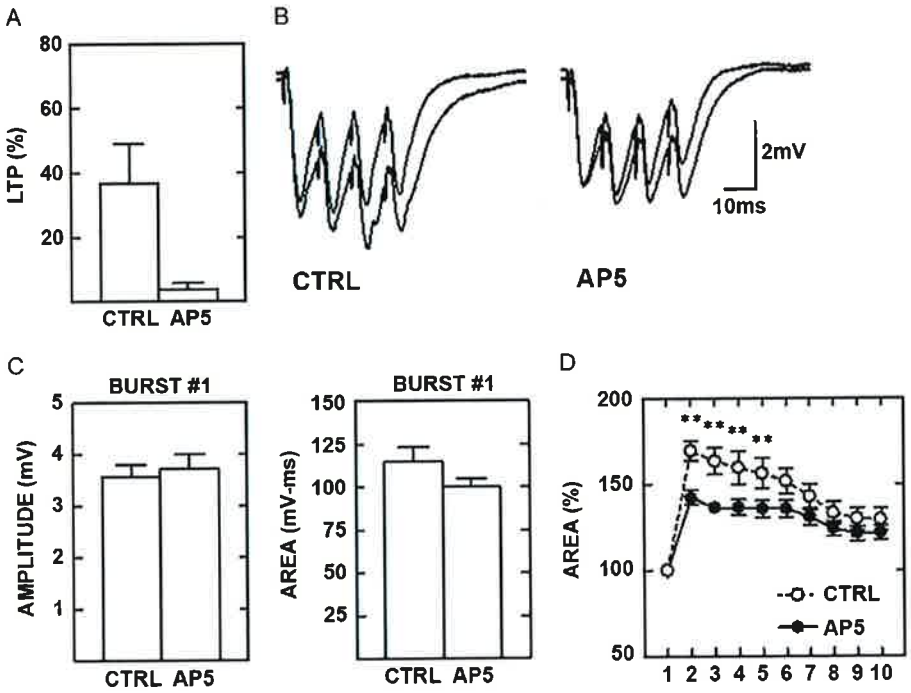


Fig. 6 – Activation of NMDA receptors during theta-burst stimulation. (A) The NMDA receptor antagonist, D-AP5 (50 μM) blocks LTP induction by TBS consisting of ten bursts (4 pulses, 100 Hz) repeated at 5 Hz. LTP was measured as the percent increase in field EPSP slope 60 min after TBS in the absence of D-AP5 (CTRL) or 60 min after TBS given in the presence of the drug (AP5). Data are means+S.E.M. ($n=7$). (B) Field EPSP waveforms evoked by the first two bursts in the TBS under control conditions (CTRL) and in the presence of the antagonist (AP5). The second burst response is larger than the first in both conditions. (C) Measurements of the amplitude of the first response within the first burst (left panel) and the total area under the response to the first burst (right panel) were not significantly affected by AP5. (D) The areas of bursts 2–10 were calculated and expressed as a percentage of the area of the first burst in both the AP5 condition and the control condition. The NMDA receptor antagonist significantly reduced the area of responses to second through fifth bursts of the TBS ($^{**}p<0.01$). Unpublished data from J. Larson and J. Crouch.

affect downstream signaling cascades. The activation of NMDA receptors by theta patterned stimulation was first demonstrated using a two-input paradigm (Larson and Lynch, 1988), but is also evident in the standard TBS paradigm, as shown in Fig. 6. Mouse hippocampal slices were prepared as described above with two stimulation electrodes and an extracellular recording electrode in *s. radiatum* of field CA1. Slices were first stimulated with 10 theta-bursts on one pathway and synaptic transmission was monitored for 60 min. post-TBS. Slices were then perfused with the competitive NMDA receptor antagonist, D-AP5 (50 μM), and stimulated with an identical TBS on the second pathway. Synaptic transmission was again monitored for one hour post-TBS. Field potentials evoked by the theta bursts were recorded and measured (see Fig. 6 legend for details). The NMDA antagonist, while blocking LTP, had very little effect on the response to the first ("unprimed") burst, but reduced the envelope of depolarization evoked by several of the succeeding bursts. With repeated theta-bursts, synaptic facilitation and depletion probably contribute to the inverted U shape of the burst response enhancement curves.

Other conditions in which alterations of LTP are associated with altered burst responses include suppressed LTP in transgenic mouse models for Alzheimer's disease (Larson et al., 1999), suppressed LTP by diazepam (del Cerro et al., 1992), reduced LTP after neonatal lesions of contralateral hippocampus (van Praag et al., 1998), reduced LTP after inhibition of matrix metalloproteinases (Meighan et al., 2007), enhanced LTP in mice overexpressing brain protease nexin-1 (Luthi et al., 1997), facilitated LTP by an M1 muscarinic antagonist (Boddeke et al., 1992), and enhanced LTP after environmental enrichment (Malik and Chattarji, 2012), among others. Recording and analyzing the burst response pattern can help to provide a first-pass evaluation of whether an experimental manipulation affects events before or after the NMDA receptor-mediated influx of calcium that serves as the essential trigger for LTP induction.

7. Sensitivity of theta-burst LTP to various experimental manipulations

There is considerable overlap between the downstream molecular events triggered by TBS and other high-frequency tetanic stimulation methods; however, there are many conditions in which theta-burst LTP is more sensitive to experimental manipulations than is the LTP induced by long trains of 100 Hz stimulation. For example, theta-burst LTP is more

vulnerable to aging (Moore et al., 1993; Rex et al., 2005) and experimentally-induced stress (Diamond et al., 1989, 1992, 1994) than is tetanus-induced LTP and appears to be more sensitive to manipulations that affect BDNF (Chen et al., 1999, 2010; Kang et al., 1997; Pang et al., 2004; Patterson et al., 2001; Skucas et al., 2011), serotonin (Corradetti et al., 1992), endocannabinoid (Pan et al., 2011), or adenosine receptor-dependent (Costenla et al., 1999) signaling mechanisms. Experiments on a number of mutant mouse models show selective or larger disruptions of LTP induced by theta-bursts than of LTP induced by tetanic stimulation (Evers et al., 2002; Gong et al., 2009; Jedlicka et al., 2009; Luthi et al., 1997; Shalin et al., 2006). Many of these experimental conditions also affect learning and/or memory processes, suggesting that theta-burst LTP may be a better model for the memory effects than tetanus-induced LTP.

Few of the manipulations described above have been resolved into effects on events antecedent or consequent to the LTP induction triggering event. The preponderance of evidence suggests that theta-burst LTP represents a minimalist model for stable (non-decremental) LTP. If so, then we might expect that theta-burst LTP would be more sensitive to perturbations of events antecedent to LTP induction. Theta-burst stimulation exploits the endogenous circuit properties to maximize NMDA receptor activation with the least amount of afferent stimulation. Under some circumstances, long trains may simply be less efficient for triggering LTP (Fig. 3). However, this inefficiency may be overcome by high intensity trains or multiple repetitions at short (<1 min) intervals. Some paradigms may even engage mechanisms in addition to those minimally essential for stable LTP. For example, trains of 200 Hz stimulation are reported to activate voltage-dependent calcium channels as well as synaptic NMDA receptor-mediated calcium fluxes (Grover and Teyler, 1990). Clearly, manipulations that alter the circuit dynamics exploited by theta-bursts, such as blockade of inhibition, have a major effect on the sensitivity of theta-burst LTP as compared to tetanus-induced LTP (Pacelli et al., 1989).

If we consider the events consequent to the minimal trigger for LTP – NMDA receptor-mediated calcium flux into the dendritic spine – the situation is more complex. The biochemical mechanisms for insertion of AMPA receptors (LTP expression) and cytoskeletal reorganization essential for LTP stabilization must be common to all stimulation paradigms that produce a stable effect. Under the mild induction conditions of TBS, these mechanisms may be more sensitive to disruption by experimental manipulations. On the other hand, more intense or protracted stimulation patterns may engage additional pathways that would be sensitive to disruptions that have little effect on theta-burst LTP (Smith et al., 2009).

8. Conclusions

The forty-odd years of LTP research have been filled with debates, even arguments, about its cellular mechanism and significance for learning and memory. We have reviewed here one aspect of this history: the relationship between naturally-occurring cell activity patterns and LTP induction. The traditions that have influenced attitudes about synaptic plasticity have been argued elsewhere (Larson et al., 1991) and will not be elaborated here except to say that one approach considers all manifestations of plasticity to be potential participants in brain substrates of experience, or at least worthy of study in their own right, whereas a second approach emphasizes the plastic changes that are triggered by neuronal activity patterns that actually occur during learning events. Theta-burst LTP emerged from the latter tradition. The former tradition has its own merits; indeed, the discovery of LTP arose from an exploration of the frequency-dependence of synaptic transmission (Andersen, 1991).

Most of the work reviewed here has used the field CA1 of hippocampus as a model system. However, theta-bursts induce LTP in other cortical networks, including the dentate gyrus (Greenstein et al., 1988; Yeckel and Berger, 1998), subiculum (Commins et al., 1999), perirhinal cortex (Cousens and Otto, 1998), piriform cortex (Jung et al., 1990; Kanter and Haberly, 1990), entorhinal cortex (de Curtis and Llinas, 1993; Yun et al., 2002), as well as visual (Heynen and Bear, 2001; Kirkwood et al., 1995), somatosensory (Castro-Alamancos and Connors, 1996; Hardingham et al., 2003), auditory (Hogsden et al., 2011), anterior cingulate (Liu and Zhuo, 2014), prefrontal (Maroun and Richter-Levin, 2003; Vickery et al., 1997), and insular (Liu et al., 2013) neocortices. Whether or not theta-burst LTP in these areas reflects resonance with local circuit operation and the same cellular mechanisms as in CA1 is not clear. Neocortical neurons show intrinsic rhythmogenesis (Silva et al., 1991) and circuit resonance (Castro-Alamancos, 2013) in the theta frequency range. Furthermore, there is now a large literature of studies using transcranial magnetic stimulation protocols based on theta burst stimulation for therapeutic purposes in neurological disease in which long-term enhancement of cortical function is indicated (Huang et al., 2005; Nettekoven et al., 2014).

We have presented a model for the means by which theta burst stimulation leads to NMDA receptor activation for LTP induction, tested the efficiency of theta-burst LTP, described the limitations of spatial summation of synaptic activity during theta bursts, and discussed the special sensitivity of theta-burst LTP to a variety of experimental manipulations. We argued that theta-burst LTP represents a minimal model for the LTP process. The discussion has emphasized cellular mechanisms and has omitted detailed consideration of the role of theta and bursting in learning and memory. A few studies have reported parallel effects of experimental manipulations on spontaneous theta rhythm *in vivo* and theta-burst LTP (Maren et al., 1994; Staubli and Xu, 1995). An alternative approach, showing that theta-burst stimulation *in vivo* produces synaptic potentiation as its behavioral significance is learned (Roman et al., 1987) was instrumental in the development of the theta-burst LTP paradigm.

Acknowledgments

We thank Nancy Long Bartolotti and Joel Crouch for assistance with some of the experiments reported here. JL was supported by grants from the NIH (DC005793) and Department of Defense (USAMRMC 10917352). EM was supported by the UIC Honors College, UIC College of Liberal Arts and

Sciences, the Glenn Foundation for Medical Research, and NIH (T32 AG021890).

REFERENCES

Alger, B.E., Nicoll, R.A., 1982. Feed-forward dendritic inhibition in rat hippocampal pyramidal cells studied in vitro. J. Physiol. 328, 105–123.

Andersen, P., 1991. Foreword: LTP—an exciting and continuing saga. In: Baudry, M., Davis, J.L. (Eds.), Long-term Potentiation: A Debate of Current Issues. MIT Press, Cambridge, MA, pp. xiii–xvii.

Arai, A., Lynch, G., 1992. Factors regulating the magnitude of long-term potentiation induced by theta pattern stimulation. Brain Res. 598, 173–184.

Arai, A., Silberg, J., Lynch, G., 1995. Differences in the refractory properties of two distinct inhibitory circuitries in field CA1 of the hippocampus. Brain Res. 704, 298–306.

Arai, A.C., Xia, Y.F., Suzuki, E., 2004. Modulation of AMPA receptor kinetics differentially influences synaptic plasticity in the hippocampus. Neuroscience 123, 1011–1024.

Barrionuevo, G., Brown, T.H., 1983. Associative long-term potentiation in hippocampal slices. Proc. Natl. Acad. Sci. USA 80, 7347–7351.

Bliss, T.V., Gardner-Medwin, A.R., 1973. Long-lasting potentiation of synaptic transmission in the dentate area of the unanaestetized rabbit following stimulation of the perforant path. J. Physiol. 232, 357–374.

Bliss, T.V., Lomo, T., 1973. Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. J. Physiol. 232, 331–356.

Boddeke, E.W.G.M., Enz, A., Shapiro, G., 1992. SDZ ENS 163, a selective muscarinic M1 receptor agonist, facilitates the induction of long-term potentiation in rat hippocampal slices. Eur. J. Pharmacol. 222, 21–25.

Capocchi, G., Zampolini, M., Larson, J., 1992. Theta burst stimulation is optimal for induction of LTP at both apical and basal dendritic synapses on hippocampal CA1 neurons. Brain Res. 591, 332–336.

Castro-Alamancos, M.A., Connors, B.W., 1996. Short-term synaptic enhancement and long-term potentiation in neocortex. Proc. Natl. Acad. Sci. USA 93, 1335–1339.

Castro-Alamancos, M.A., 2013. The motor cortex: a network tuned to 7–14 Hz. Front. Neural Circuits 7, 21.

Chen, G., et al., 1999. Relative contribution of endogenous neurotrophins in hippocampal long-term potentiation. J. Neurosci. 19, 7983–7990.

Chen, L.Y., et al., 2010. Learning induces neurotrophin signaling at hippocampal synapses. Proc. Natl. Acad. Sci. USA 107, 7030–7035.

Commins, S., et al., 1999. The effects of single and multiple episodes of theta patterned or high frequency stimulation on synaptic transmission from hippocampal area CA1 to the subiculum in rats. Neurosci. Lett. 270, 99–102.

Corradetti, R., et al., 1992. Serotonin blocks the long-term potentiation induced by primed burst stimulation in the CA1 region of rat hippocampal slices. Neuroscience 46, 511–518.

Costenla, A.R., de Mendonca, A., Ribeiro, J.A., 1999. Adenosine modulates synaptic plasticity in hippocampal slices from aged rats. Brain Res. 851, 228–234.

Cousens, G., Otto, T.A., 1998. Induction and transient suppression of long-term potentiation in the peri- and postrhinal cortices following theta-related stimulation of hippocampal field CA1. Brain Res. 780, 95–101.

Davies, C.H., Davies, S.N., Collingridge, G.L., 1990. Paired-pulse depression of monosynaptic GABA-mediated inhibitory postsynaptic responses in rat hippocampus. J. Physiol. 424, 513–531.

Davies, C.H., et al., 1991. GABA autoreceptors regulate the induction of LTP. Nature 349, 609–611.

de Curtis, M., Llinas, R.R., 1993. Entorhinal cortex long-term potentiation evoked by theta-patterned stimulation of associative fibers in the isolated in vitro guinea pig brain. Brain Res. 600, 327–330.

del Cerro, S., Jung, M., Lynch, G., 1992. Benzodiazepines block long-term potentiation in slices of hippocampus and piriform cortex. Neuroscience 49, 1–6.

Deschenes, M., Moore, J., Kleinfeld, D., 2012. Sniffing and whisking in rodents. Curr. Opin. Neurobiol. 22, 243–250.

Diamond, D.M., Dunwiddie, T.V., Rose, G.M., 1988. Characteristics of hippocampal primed burst potentiation in vitro and in the awake rat. J. Neurosci. 8, 4079–4088.

Diamond, D.M., et al., 1989. Adrenalectomy reduces the threshold for hippocampal primed burst potentiation in the anesthetized rat. Brain Res. 492, 356–360.

Diamond, D.M., et al., 1992. Inverted-U relationship between the level of peripheral corticosterone and the magnitude of hippocampal primed burst potentiation. Hippocampus 2, 421–430.

Diamond, D.M., Fleshner, M., Rose, G.M., 1994. Psychological stress repeatedly blocks hippocampal primed burst potentiation in behaving rats. Behav. Brain Res. 62, 1–9.

Douglas, R.M., Goddard, G.V., 1975. Long-term potentiation of the perforant path-granule cell synapse in the rat hippocampus. Brain Res. 86, 205–215.

Douglas, R.M., 1977. Long lasting synaptic potentiation in the rat dentate gyrus following brief high frequency stimulation. Brain Res. 126, 361–365.

Evers, M.R., et al., 2002. Impairment of L-type Ca^{2+} channel-dependent forms of hippocampal synaptic plasticity in mice deficient in the extracellular matrix glycoprotein tenascin-C. J. Neurosci. 22, 7177–7194.

Feldman, Daniel E., 2012. The spike-timing dependence of plasticity. Neuron 75, 556–571.

Gong, N., et al., 2009. GABA transporter-1 activity modulates hippocampal theta oscillation and theta burst stimulation-induced long-term potentiation. J. Neurosci. 29, 15836–15845.

Govindarajan, A., et al., 2011. The dendritic branch is the preferred integrative unit for protein synthesis-dependent LTP. Neuron 69, 132–146.

Granger, R., et al., 1994. Non-Hebbian properties of long-term potentiation enable high-capacity encoding of temporal sequences. Proc. Natl. Acad. Sci. USA 91, 10104–10108.

Green, J.D., Arduini, A., 1954. Hippocampal electrical activity in arousal. J. Neurophysiol. 17, 533–557.

Greenstein, Y.J., Pavlides, C., Winson, J., 1988. Long-term potentiation in the dentate gyrus is preferentially induced at theta rhythm periodicity. Brain Res. 438, 331–334.

Grover, L.M., Teyler, T.J., 1990. Two components of long-term potentiation induced by different patterns of afferent activation. Nature 347, 477–479.

Grover, L.M., et al., 2009. LTP in hippocampal area CA1 is induced by burst stimulation over a broad frequency range centered around delta. Learn. Mem. 16, 69–81.

Gustafsson, B., Wigstrom, H., 1986. Hippocampal long-lasting potentiation produced by pairing single volleys and brief conditioning tetani evoked in separate afferents. J. Neurosci. 6, 1575–1582.

Hardie, J., Spruston, N., 2009. Synaptic depolarization is more effective than back-propagating action potentials during induction of associative long-term potentiation in hippocampal pyramidal neurons. J. Neurosci. 29, 3233–3241.

Hardingham, N., et al., 2003. Neocortical long-term potentiation and experience-dependent synaptic plasticity require alpha-

calcium/calmodulin-dependent protein kinase II autophosphorylation. J. Neurosci. 23, 4428–4436.
Hernandez, R.V., et al., 2005. Differences in the magnitude of long-term potentiation produced by theta burst and high frequency stimulation protocols matched in stimulus number. Brain Res. Brain Res. Protoc. 15, 6–13.
Heynen, A.J., Bear, M.F., 2001. Long-term potentiation of thalamocortical transmission in the adult visual cortex in vivo. J. Neurosci. 21, 9801–9813.
Hoffman, K.L., et al., 2013. Saccades during visual exploration align hippocampal 3–8 Hz rhythms in human and non-human primates. Front. Syst. Neurosci. 7, 1–10 (Article 43).
Hogsden, J.L., Rosen, L.G., Dringenberg, H.C., 2011. Pharmacological and deprivation-induced reinstatement of juvenile-like long-term potentiation in the primary auditory cortex of adult rats. Neuroscience 186, 208–219.
Holscher, C., Anwyl, R., Rowan, M.J., 1997. Stimulation on the positive phase of hippocampal theta rhythm induces long-term potentiation that can be depotentiated by stimulation on the negative phase in area CA1 in vivo. J. Neurosci. 17, 6470–6477.
Huang, Y.Z., et al., 2005. Theta burst stimulation of the human motor cortex. Neuron 45, 201–206.
Hyman, J.M., et al., 2003. Stimulation in hippocampal region CA1 in behaving rats yields long-term potentiation when delivered to the peak of theta and long-term depression when delivered to the trough. J. Neurosci. 23, 11725–11731.
Jedlicka, P., et al., 2009. Impairment of in vivo theta-burst long-term potentiation and network excitability in the dentate gyrus of synaptopodin-deficient mice lacking the spine apparatus and the cisternal organelle. Hippocampus 19, 130–140.
Jung, M.W., Larson, J., Lynch, G., 1990. Long-term potentiation of monosynaptic EPSPs in rat piriform cortex in vitro. Synapse 6, 279–283.
Kang, H., et al., 1997. Neurotrophins and time: different roles for TrkB signaling in hippocampal long-term potentiation. Neuron 19, 653–664.
Kanter, E.D., Haberly, L.B., 1990. NMDA-dependent induction of long-term potentiation in afferent and association fiber systems of piriform cortex in vitro. Brain Res. 525, 175–179.
Kelso, S.R., Brown, T.H., 1986. Differential conditioning of associative synaptic enhancement in hippocampal brain slices. Science 232, 85–87.
Kelso, S.R., Ganong, A.H., Brown, T.H., 1986. Hebbian synapses in hippocampus. Proc. Natl. Acad. Sci. USA 83, 5326–5330.
Kirkwood, A., Lee, H.K., Bear, M.F., 1995. Co-regulation of long-term potentiation and experience-dependent synaptic plasticity in visual cortex by age and experience. Nature 375, 328–331.
Kramar, E.A., et al., 2012. Synaptic evidence for the efficacy of spaced learning. Proc. Natl. Acad. Sci. USA 109, 5121–5126.
Larson, J., Lynch, G., 1986. Induction of synaptic potentiation in hippocampus by patterned stimulation involves two events. Science 232, 985–988.
Larson, J., Wong, D., Lynch, G., 1986. Patterned stimulation at the theta frequency is optimal for the induction of hippocampal long-term potentiation. Brain Res. 368, 347–350.
Larson, J., Lynch, G., 1988. Role of N-methyl-D-aspartate receptors in the induction of synaptic potentiation by burst stimulation patterned after the hippocampal theta-rhythm. Brain Res. 441, 111–118.
Larson, J., Lynch, G., 1989. Theta pattern stimulation and the induction of LTP: the sequence in which synapses are stimulated determines the degree to which they potentiate. Brain Res. 489, 49–58.
Larson, J., Ambros-Ingerson, J., Lynch, G., 1991. Sites and mechanisms for expression of long-term potentiation. In: Baudry, M., Davis, J.L. (Eds.), Long-Term Potentiation: A Debate of Current Issues. MIT Press, Cambridge, MA, pp. 121–139.
Larson, J., et al., 1999. Alterations in synaptic transmission and long-term potentiation in hippocampal slices from young and aged PDAPP mice. Brain Res. 840, 23–35.
Larson, J.R., 1987. Activity Patterns, Postsynaptic Events, and the Induction of Synaptic Plasticity in the Hippocampus. University of California, Irvine, Irvine, CA152.
Liu, M.G., et al., 2013. Long-term potentiation of synaptic transmission in the adult mouse insular cortex: multielectrode array recordings. J. Neurophysiol. 110, 505–521.
Liu, M.G., Zhuo, M., 2014. No requirement of TRPV1 in long-term potentiation or long-term depression in the anterior cingulate cortex. Mol. Brain 7, 27.
Luthi, A., et al., 1997. Endogenous serine protease inhibitor modulates epileptic activity and hippocampal long-term potentiation. J. Neurosci. 17, 4688–4699.
Lynch, G., et al., 2013. Differences between synaptic plasticity thresholds result in new timing rules for maximizing long-term potentiation. Neuropharmacology 64, 27–36.
Macrides, F., 1975. Temporal relations between hippocampal slow waves and exploratory sniffing in hamsters. Behav. Biol. 14, 295–308.
Macrides, F., Eichenbaum, H.B., Forbes, W.B., 1982. Temporal relationship between sniffing and the limbic theta rhythm during odor discrimination reversal learning. J. Neurosci. 2, 1705–1717.
Malik, R., Chattarji, S., 2012. Enhanced intrinsic excitability and EPSP-spike coupling accompany enriched environment-induced facilitation of LTP in hippocampal CA1 pyramidal neurons. J. Neurophysiol. 107, 1366–1378.
Maren, S., et al., 1994. Parallel augmentation of hippocampal long-term potentiation, theta rhythm, and contextual fear conditioning in water-deprived rats. Behav. Neurosci. 108, 44–56.
Maroun, M., Richter-Levin, G., 2003. Exposure to acute stress blocks the induction of long-term potentiation of the amygdala-prefrontal cortex pathway in vivo. J. Neurosci. 23, 4406–4409.
McCarren, M., Alger, B.E., 1985. Use-dependent depression of IPSPs in rat hippocampal pyramidal cells in vitro. J. Neurophysiol. 53, 557–571.
McCartney, H., et al., 2004. Theta reset produces optimal conditions for long-term potentiation. Hippocampus 14, 684–687.
Meighan, P.C., et al., 2007. Effects of matrix metalloproteinase inhibition on short- and long-term plasticity of schaffer collateral/CA1 synapses. J. Neurochem. 102, 2085–2096.
Moore, C.I., Browning, M.D., Rose, G.M., 1993. Hippocampal plasticity induced by primed burst, but not long-term potentiation, stimulation is impaired in area CA1 of aged Fischer 344 rats. Hippocampus 3, 57–66.
Mott, D.D., Lewis, D.V., 1991. Facilitation of the induction of long-term potentiation by GABAB receptors. Science 252, 1718–1720.
Mott, D.D., Lewis, D.V., 1992. GABAB receptors mediate disinhibition and facilitate long-term potentiation in the dentate gyrus. Epilepsy Res. Suppl. 7, 119–134.
Nettekoven, C., et al., 2014. Dose-dependent effects of theta burst rTMS on cortical excitability and resting-state connectivity of the human motor system. J. Neurosci. 34, 6849–6859.
O'Keefe, J., Nadel, L., 1978. In: The Hippocampus as a Cognitive Map. Oxford University Press, New York.
Orr, G., et al., 2001. Hippocampal synaptic plasticity is modulated by theta rhythm in the fascia dentata of adult and aged freely behaving rats. Hippocampus 11, 647–654.
Otto, T., et al., 1991. Learning-related patterns of CA1 spike trains parallel stimulation parameters optimal for inducing hippocampal long-term potentiation. Hippocampus 1, 181–192.

Pacelli, G.J., Su, W., Kelso, S.R., 1989. Activity-induced depression of synaptic inhibition during LTP-inducing patterned stimulation. Brain Res. 486, 26–32.
Pacelli, G.J., Su, W., Kelso, S.R., 1991. Activity-induced decrease in early and late inhibitory synaptic conductances in hippocampus. Synapse 7, 1–13.
Pan, B., Zucker, R.S., 2009. A general model of synaptic transmission and short-term plasticity. Neuron 62, 539–554.
Pan, B., et al., 2011. Alterations of endocannabinoid signaling, synaptic plasticity, learning, and memory in monoacylglycerol lipase knock-out mice. J. Neurosci. 31, 13420–13430.
Pang, P.T., et al., 2004. Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. Science 306, 487–491.
Patterson, S.L., et al., 2001. Some forms of cAMP-mediated long-lasting potentiation are associated with release of BDNF and nuclear translocation of phospho-MAP kinase. Neuron 32, 123–140.
Pavlides, C., et al., 1988. Long-term potentiation in the dentate gyrus is induced preferentially on the positive phase of theta-rhythm. Brain Res. 439, 383–387.
Ranck Jr., J.B., 1973. Studies on single neurons in dorsal hippocampal formation and septum in unrestrained rats. I. Behavioral correlates and firing repertoires. Exp. Neurol. 41, 461–531.
Rex, C.S., et al., 2005. Long-term potentiation is impaired in middle-aged rats: regional specificity and reversal by adenosine receptor antagonists. J. Neurosci. 25, 5956–5966.
Rojas-Líbano, D., et al., 2014. The olfactory bulb theta rhythm follows all frequencies of diaphragmatic respiration in the freely behaving rat. Front. Behav. Neurosci. 8, 1–12 (Article 214).
Roman, F., Staubli, U., Lynch, G., 1987. Evidence for synaptic potentiation in a cortical network during learning. Brain Res. 418, 221–226.
Rose, G.M., Dunwiddie, T.V., 1986. Induction of hippocampal long-term potentiation using physiologically patterned stimulation. Neurosci. Lett. 69, 244–248.
Rudell, A.P., Fox, S.E., Ranck Jr., J.B., 1980. Hippocampal excitability phase-locked to the theta rhythm in walking rats. Exp. Neurol. 68, 87–96.
Shalin, S.C., et al., 2006. Kinase suppressor of Ras1 compartmentalizes hippocampal signal transduction and subserves synaptic plasticity and memory formation. Neuron 50, 765–779.
Silva, L.R., Amitai, Y., Connors, B.W., 1991. Intrinsic oscillations of neocortex generated by layer 5 pyramidal neurons. Science 251, 432–435.
Skucas, V.A., et al., 2011. Impairment of select forms of spatial memory and neurotrophin-dependent synaptic plasticity by deletion of glial aquaporin-4. J. Neurosci. 31, 6392–6397.
Smith, J.P., et al., 2009. Stimulus pattern dependence of the Alzheimer's disease amyloid-beta 42 peptide's inhibition of long term potentiation in mouse hippocampal slices. Brain Res. 1269, 176–184.
Staubli, U., Lynch, G., 1987. Stable hippocampal long-term potentiation elicited by 'theta' pattern stimulation. Brain Res. 435, 227–234.
Staubli, U., Xu, F.B., 1995. Effects of 5-HT3 receptor antagonism on hippocampal theta rhythm, memory, and LTP induction in the freely moving rat. J. Neurosci. 15, 2445–2452.
Tsanov, M., et al., 2014. Respiratory cycle entrainment of septal neurons mediates the fast coupling of sniffing rate and hippocampal theta rhythm. Eur. J. Neurosci. 39, 957–974.
van Praag, H., et al., 1998. Unilateral hippocampal ablation at birth causes a reduction in contralateral LTP. Brain Res. 795, 170–178.
Vanderwolf, C.H., 1969. Hippocampal electrical activity and voluntary movement in the rat. Electroencephalogr. Clin. Neurophysiol. 26, 407–418.
Vickery, R.M., Morris, S.H., Bindman, L.J., 1997. Metabotropic glutamate receptors are involved in long-term potentiation in isolated slices of rat medial frontal cortex. J. Neurophysiol. 78, 3039–3046.
Welker, W.I., 1964. Analysis of sniffing of the albino rat. Behaviour 22, 223–244.
White, G., Levy, W.B., Steward, O., 1988. Evidence that associative interactions between synapses during the induction of long-term potentiation occur within local dendritic domains. Proc. Natl. Acad. Sci. USA 85, 2368–2372.
White, G., Levy, W.B., Steward, O., 1990. Spatial overlap between populations of synapses determines the extent of their associative interaction during the induction of long-term potentiation and depression. J. Neurophysiol. 64, 1186–1198.
Yeckel, M.F., Berger, T.W., 1998. Spatial distribution of potentiated synapses in hippocampus: dependence on cellular mechanisms and network properties. J. Neurosci. 18, 438–450.
Yun, S.H., Mook-Jung, I., Jung, M.W., 2002. Variation in effective stimulus patterns for induction of long-term potentiation across different layers of rat entorhinal cortex. J. Neurosci. 22, RC214.

APPENDIX 2

Impaired olfactory discrimination learning in Fragile X knockout mice

(DRAFT: manuscript in preparation)

John Larson, Samantha Keil, Maribell Heredia, and Camelia Saha

Psychiatric Institute
Department of Psychiatry
College of Medicine
Graduate Program in Neuroscience
University of Illinois at Chicago
Chicago, Illinois

This work was supported by the Office of the Assistant Secretary of Defense for Health Affairs, through the Peer Reviewed Medical Research Program under Award No. W81XWH-12-1-0320. Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the Department of Defense.

INTRODUCTION

Fragile X Syndrome (FXS), due to mutation of the *FMR1* gene, is the leading cause of inherited intellectual disability and results in cognitive impairment, hyperactivity, attention deficits, seizure disorders, and autistic features (Bhakar et al., 2012). Neurobiological studies using mouse models of FXS (*Fmr1*-KO mice) have focused on synaptic function, development, and plasticity, since synaptic communication is critical for the cognitive functions affected in the human disorder. Many studies have now identified synaptic plasticity deficits involving both long-term potentiation (LTP) (Larson et al., 2005; Lauterborn et al., 2007; Li et al., 2002; Wilson and Cox, 2007; Zhao et al., 2005) and long-term depression (LTD) (Hou et al., 2006; Huber et al., 2002) in hippocampus and other cortical regions in *Fmr1*-KO mice. However, the molecular mechanisms responsible for these plasticity deficits in mice lacking FMRP are not well understood.

Directly relating cellular dysfunction to cognitive impairments is challenging in mouse models. Olfactory learning has a number of advantages for this type of work. First, the earliest neural processing stages of the olfactory system occur in forebrain networks that are directly connected to systems critical for human cognition, including the hippocampus, amygdala, and prefrontal cortex. Second, the first two stages of the olfactory system, the olfactory bulb and olfactory cortex are telencephalic structures with stratified cell layers and laminated input systems that greatly facilitate electrophysiological studies of synaptic function and plasticity. Third, sophisticated behavioral paradigms (Slotnick, 2001) have exploited the natural tendency of mice to attend to odor cues and demonstrated features of olfactory learning that resemble the human declarative memory system.

Our first behavioral studies used a simultaneous-cue, two-odor, olfactory discrimination task that depends on the integrity of both piriform cortex and hippocampus for optimal learning. In two independent experiments, *Fmr1*-KO mice showed significant impairment of learning rate compared to WT littermate controls (Larson et al., 2008). The deficit was specific for *acquisition* of odor-reward associations since long-term memory for acquired odor-reward associations was unaffected. Tests of olfactory sensory thresholds did not distinguish *Fmr1*-KO mice from WT controls, suggesting that the learning deficit was not simply a failure of odor detection.

Comparisons of LTP induced by theta burst stimulation (TBS), a paradigm that mimics cellular activity in the olfactory-hippocampal circuit during odor sampling behavior (Larson and Munkacsy, in press) were made in anterior piriform cortex (APC) slices prepared from WT and *Fmr1*-KO mice at ages ranging from three to eighteen months (Larson et al., 2005). Input-output curves for ASSN synapses did not reveal any differences in AMPA receptor-mediated basal excitatory synaptic transmission between *Fmr1*-KO and WT mice. In slices from mice 6-18 months old, TBS-induced LTP decayed more rapidly in the *Fmr1*-KO mice than in their age-matched WT controls. The impairment of APC LTP in *Fmr1*-KO mice was age-dependent and progressive: potentiation was normal in mice aged 3-6 months, failed to stabilize in mice aged 6-12 months, and was reduced at all post-TBS time points in 12-18 month-old mice. Given the age-dependence of the LTP deficit in APC, we examined LTP in hippocampal field CA1 from *Fmr1*-KO and littermate WT mice at ages ranging from three to twelve months. LTP was unaffected in *Fmr1*-KO mice at all ages (Larson et al., 2005).

In order to determine if the olfactory learning deficits in *Fmr1*-KO mice are due to impaired LTP in olfactory (piriform) cortex or to disturbance in hippocampal function, the present experiments were designed to assess learning in an olfactory-guided task that depends on

piriform cortex function but does not depend on hippocampal function. A successive-cue, two-odor discrimination task, in which only one discriminative cue is present on each trial and the mouse is required to respond (GO) when the 'positive' cue is present, but not respond (NO GO) when the negative cue is present, is such a task (Eichenbaum et al., 1988). Therefore, we assessed acquisition of a series of two-odor discriminations in *Fmr1*-KO and WT littermates in the successive-cue task.

METHODS

Subjects

Ten male *Fmr1*-KO and ten WT littermate mice (C57BI/6J background; 5-12 months old) were tested. Mice were bred on-site from stock obtained from Jackson Labs (Bar Harbor, ME) and housed in a climate controlled animal colony with a normal 14:10 light-dark cycle. They were maintained on a water deprivation schedule with access to 0.5-2.0mL of water once per day for a week prior to and throughout training. This schedule reduced and sustained the subjects' body weight at 80% of the ad libitum levels. Behavioral experimentation was performed during the light stage.

Apparatus

Four behavior testing chambers were made from black acrylic as described previously (Larson and Sieprawska, 2002). Chambers were constructed with 60 X 10 X 30 cm panels; side panels were set 15º off vertical along the length and vertically at each end. A single small cup in the floor on both the "East" and "West" ends functioned for a water delivery system. Similarly, two cylindrical "sniff ports" at both the East and West end enabled recording of nose poke responses. The sniff ports on the West end were connected to individual air-dilution olfactometers for odor stimulus delivery (Patel and Larson, 2009). Custom software activated and controlled odor and water delivery as well as recording infrared photo beam breaks.

Odor Pairs

Eight odor pairs were utilized throughout experimentation. Odorant was dissolved in a solvent of ddH_2O, propylene glycol or mineral oil. One odor from each pair was randomly chosen as the positive stimulus.

Discrimination	Odor Pair
1	16+ (Banana) / 14- (Strawberry)
2	101+ (Hexyl octanoate) / 102- (Propionic acid)
3	23+ (Root Beer) / 22- (Almond)
4	98+ (Methyl salicylate) / 97- (Ethyl lactate)
5	18+ (Cherry) / 30- (Lemon)
6	107+ (Terpinyl acetate)/ 106- (Anisole)
7	20+ (Vanilla) / 25- (Butter)
8	29+ (Maple) / 17- (Coconut)

Procedure

Shaping

The procedure consisted of daily 40-trial sessions, with 10µL water reinforcement for a nose poke to the sniff port on either end of the chamber. A lamp at each end of the alley was lit

during the inter-trial interval (ITI) and was extinguished over the ports at which the reinforcement was available during the trials. Each trial was a maximum of 120 seconds long, with a 10sec ITI. Mice were required to traverse the chamber repeatedly, as sequential reinforcement was received for a nose poke at the end of the alley opposite the last correct response. Mice were trained to criterion of three sessions in which 90% of the last 20 trials were correct before beginning odor discrimination.

Odor Discrimination

The mice were presented with eight distinct two-odor discrimination problems in 100-trial sessions. Each trial involved presentation of one discriminative odor. Trials were signaled by extinguishing of a lamp and the presentation of the odor for 5 seconds. A nose poke to either port within the presentation period constituted a "GO" response; no response in the same period constituted a "NO-GO" response. GO responses to the positive cue (S+) or NO-GO responses to the negative cue (S-) were scored as correct. Similarly, GO responses to S- or NO-GO responses to S+ were scored as errors. Only GO responses to S+ were rewarded water. The mice were trained to criterion performance of eight consecutive correct responses within a single 100-trial training session, for each of the eight discrimination problems. The eight discrimination problems were presented to half of the mice in each group (randomly chosen) in one order, and in reverse order for the other half.

Memory Retention

The mice were tested on memory retention of their fifth odor discrimination problem. Memory retention was tested after all mice had completed each of the eight odor discriminations and at least one week after finishing this particular odor pair.

Partial Reinforcement

The last odor pair of the original eight discriminations was used. Each mouse was "retrained" to criterion for these odors with a 1:1 reward to response ratio. Once meeting criterion, reward response was changed to 1:2, and 1:4 respectively.

Binary Odor Mixture Discrimination

Two pure chemical odorants, eucalyptol (odor A) and heptanol (odor B), were chosen for mixture discrimination based on previously tested glomerular activation patterns (Johnson and Leon, 2007). Mice were first trained to discriminate the pure odors, i.e., 100% odor A vs. 100% odor B. Whether odor A or B was the rewarded cue (S+) was counter-balanced across mice within each genotype. After reaching learning criterion, mice were trained to discriminate a mixture of 80% A and 20% B from a mixture of 20% A and 80% B. After learning the 80/20 mixture discrimination mice were trained to discriminate mixtures in which there was substantially more overlap between the discriminanda, i.e., 60% A and 40% B vs. 40% A and 60% B (60/40 mixture discrimination).

RESULTS AND DISCUSSION

Odor Discrimination Learning

All mice were trained to a strict behavioral criterion (eight consecutive correct trials) on each of eight two-odor discrimination problems. Fragile X KO mice learned the first discrimination problem more slowly than did WT mice. *Fmr1*-KO mice (n=10) required more trials to reach criterion in the first discrimination than did wild type (n=10) littermates (Figure 1A); however this effect only approached statistical significance (t_{18} = 1.95, p = .07). Total errors for the first discrimination problem (Fig. 1B) were significantly different between WT and KO mice (t_{18} = 2.13, p<.05). Errors were classified as misses or false alarms. The number of misses (Fig. 1C) was significantly higher in *Fmr1*-KO than in WT mice (t_{18} = 2.38, p<.05) but the number of false alarms (Fig. 1D) did not differ (t_{18} = 1.13, p>.25). The average number of errors made during acquisition of all eight discrimination problems in WT and KO mice are shown in Fig. 2. Two-way, repeated measures analysis of variance showed a significant main effect of genotype ($\mathbf{F}_{1,18}$ = 5.94, p<.05) as well as a significant main effect of problem number ($\mathbf{F}_{7,126}$ = 27.57, p<.0001), but no significant interaction between genotype and problem number ($\mathbf{F}_{7,126}$ = 2.04, p>.05). Multiple comparisons tests using the Newman-Keuls method showed that KO mice made significantly more errors (p<.01) before reaching learning criterion only on the first discrimination problem. Both WT and *Fmr1*-KO mice achieved criterion performance on the remaining discrimination problems in fewer trials and with fewer errors, and did not differ in this regard.

These results indicate that *Fmr1*-KO mice are impaired in acquisition of a novel odor discrimination task that does not require normal hippocampal function. The improvement in odor discrimination learning rate across multiple discrimination problems, known as learning set acquisition (Larson and Sieprawska, 2002; Slotnick and Katz, 1974), appeared to be unaffected in *Fmr1*-KO mice. This was also the pattern of results seen in *Fmr1*-KO mice trained using a hippocampal-dependent simultaneous-cue discrimination task (Larson et al., 2008). Together, these results suggest that the learning impairment in *Fmr1*-KO mice may be due to dysfunction in piriform cortex, perhaps because of an LTP impairment (Larson et al., 2005) and/or deficiency of NMDA receptors (Gocel and Larson, 2012).

Memory Retention

The mice were tested on memory retention of the fifth odor discrimination problem, after having learned three additional problems, and at least one week after the last training session for discrimination five. The retention test consisted of a training session with the same odors as in discrimination five, but with the significance (valences) of the odors reversed. If the mice remember the initial significance of the cues, reversal learning should require more trials than the initial learning. As shown in Figure 3, both WT and KO mice required many more trials to meet learning criterion (8 consecutive correct trials) during cue reversal than during initial learning. A difference score was computed for each mouse by subtracting the trials required for initial learning from the trials required for reversal learning, to provide an estimate of the strength of memory for the initial cue valences. WT and KO mice did not differ in memory strength (t_{18} = 1.40, p>.15).

Together with those of the previous study (Larson et al., 2008), these results indicate that *Fmr1*-KO show normal long-term memory for the significance of olfactory cues, provided that they are trained long enough to establish high rates of discriminative responding during training.

Partial Reinforcement

In an effort to test whether or not *Fmr1*-KO mice may be more or less prone to extinction relative to WT mice, both groups were trained using partial reinforcement schedules. After the memory retention test, all mice were retrained to criterion on discrimination eight. As in all previous training, water reinforcement was given after every correct trial (i.e., a fixed ratio 1 reinforcement schedule). After reaching criterion at FR1, the mice were retrained to criterion with a FR2 reinforcement schedule in effect. After criterion was made, mice were trained again with a FR4 (i.e., reinforcement after every fourth correct trial) schedule. The results are shown in Figure 4. Retraining at FR1 and FR2 required about the same number of trials; however, switching to FR4 resulted in increased errors and an increase in trials needed to reach criterion performance. Nevertheless, there were no significant differences in the response of WT and KO mice to partial reinforcement. Two-way, repeated measures ANOVA yielded no significant genotype ($\mathbf{F}_{1,18} = 0.92$, $p>.30$) or reinforcement ratio ($\mathbf{F}_{2,36} = 1.03$, $p>.35$) main effect nor an interaction ($\mathbf{F}_{2,36} = 0.60$, $p>.55$). Since there was a large variance in the data for FR4, due to a few extreme values, a non-parametric test was conducted on these data alone; however, this test was insignificant as well (Mann-Whitney U-test, $p>.05$).

These results suggest that *Fmr1*-KO mice do not differ from WT mice in reponse to changes in cue-reward contingencies.

Binary Odor Mixture Discrimination

Discriminating mixtures of odorants containing overlapping common elements can be a particularly challenging task for the olfactory system (Abraham et al., 2004). In animals that have already been extensively trained using odor cues, discrimination of binary mixtures represents a sensitive test of sensory ability rather than a test of learning *per se*. The same *Fmr1*-KO and WT mice were tested for discrimination of two odors that each contain the same mono-molecular odors in different proportions. Mice were first trained to discriminate the pure odors from each other, that is, to discriminate eucalyptol from heptanol. The mice were then trained to discriminate an 80%/20% mixture (eucalyptol/heptanol) from a 20%/80% mixture of the same two monomolecular odors. Mice were then trained to discriminate 60%/40% mixtures of the two odors. The results are displayed in Figure 5. There was no significant effect of genotype across modes ($\mathbf{F}_{1,18} = 0.56$, $p>.45$) or interaction between genotype and mode ($\mathbf{F}_{2,36} = 0.00$, $p>.95$). There was a highly significant main effect of mode ($\mathbf{F}_{2,36} = 13.58$, $p<.0001$), reflecting the fact that the 60/40 mixture was learned faster than either the pure two-odor discrimination or the 80/20 mixture discrimination.

These results do not support a sensory deficit interpretation of the impairment in odor discrimination learning, at least for well-trained mice. Both *Fmr1*-KO mice seemed to treat the 80/20 mixture as a novel discrimination, requiring about as many trials to reach criterion performance on the 80/20 mixture as for the pure odors. The 60/40 discrimination required many fewer trails to reach criterion performance, as if the significance of the discriminative odors had already been learned.

Effects of Age on Learning of Discrimination Problem One

The mice tested in these experiments were between 5.5 and 11.5 months old at the outset of training. Since the only significant learning impairment in *Fmr1*-KO mice was on the first discrimination problem, we examined the number of errors on this problem as a function of age

(Fig. 6). WT mice at different ages showed no obvious trends in error scores across ages. However, the KO mice showed increases in error scores as age increased. Although the sample sizes are too small for these trends to be statistically significant, if confirmed they would suggest that the age-dependent LTP impairment in these mice may play an important role in the initial learning deficit.

CONCLUSIONS

Combined with previous work, the present study indicates that *Fmr1*-KO mice show similar impairments in learning olfactory discriminations, regardless of whether the discriminative cues are present together (simultaneous-cue task) or separately (successive-cue task) on individual trials. Both paradigms depend on the integrity of the piriform cortex but are differentially affected by hippocampal damage. This suggests that the impaired learning in *Fmr1*-KO mice may be a result of LTP impairments in the primary olfactory (piriform) cortex. Learning set formation (Larson and Sieprawska, 2002; Slotnick, 2001) or "rule learning" (Quinlan et al., 2004) appears to be unaffected in *Fmr1*-KO mice trained in either paradigm. Long-term memory for the significance of odors also appears to be normal in *Fmr1*-KO mice. Finally, we obtained no evidence that the ability to detect odors (Larson et al., 2008) or to process odor mixtures is impaired in the *Fmr1*-KO mice, suggesting that the learning deficit is not sensory in nature. An extensive study of the effect of age on olfactory learning will be necessary to determine if this learning impairment is due to a deficiency of NMDA receptors in piriform cortex or to possibly related disturbances in synaptic plasticity such as LTP at synapses in this structure.

Figure 1

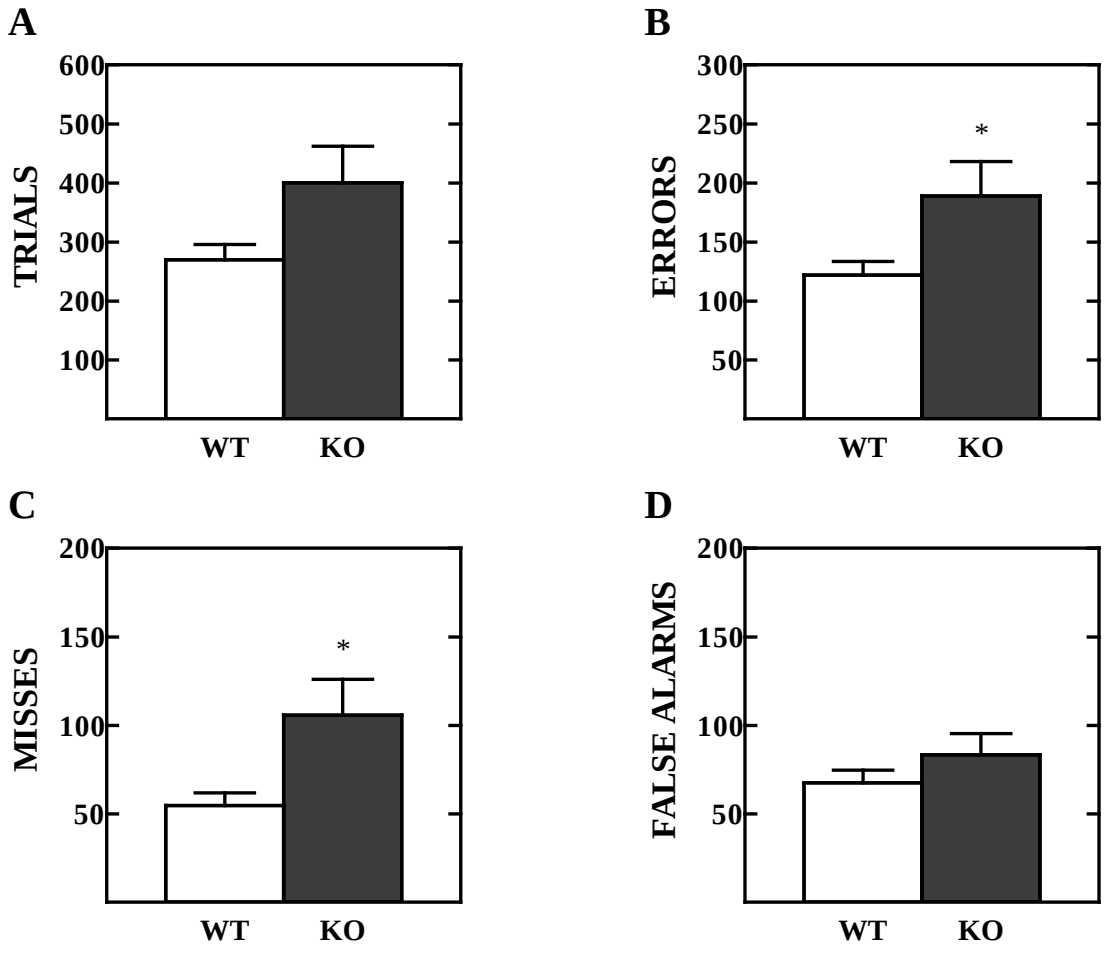


Figure 1. *Fmr1*-KO mice show impaired learning of an appetitively-motivated, successive-cue, two-odor discrimination task. A) The total trials (mean + s.e.m.) required to achieve criterion performance on discrimination 1 were higher in *Fmr1*-KO (n = 10) than in WT (N = 10) mice, although the difference only approached statistical significance. B) The number of errors made during acquisition of the first discrimination problem were significantly higher in KO than in WT mice (*: $p<.05$). C) Misses (failure to respond to presentation of the positive cue, S+) were significantly higher in KO than in WT mice (*: $p<.05$). D) False alarms (Responding to the negative, unreinforced S- cue) were not different between KO and WT mice.

Figure 2

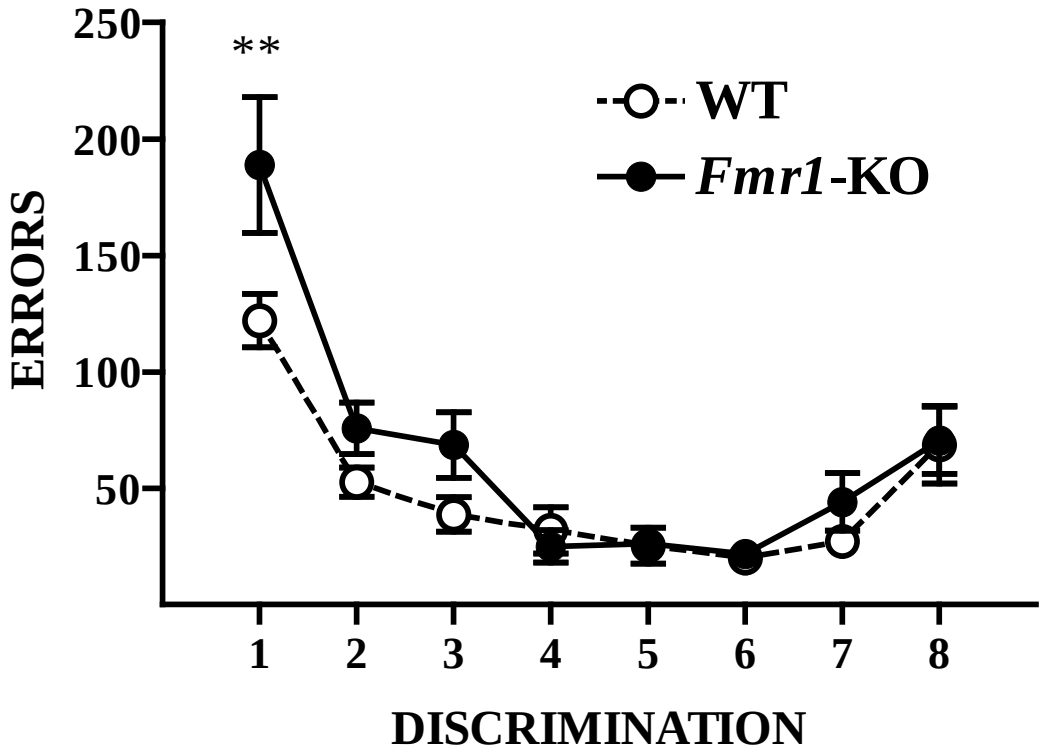


Figure 2. Learning curve for successive-cue olfactory discrimination acquisition in WT (C57Bl/6, n = 10) and *Fmr1*-KO (n = 10) mice. Mice were trained on eight distinct two-odor discrimination problems. The graph plots the average number of errors made before reaching the learning criterion for each problem. **: $p<.01$

Figure 3

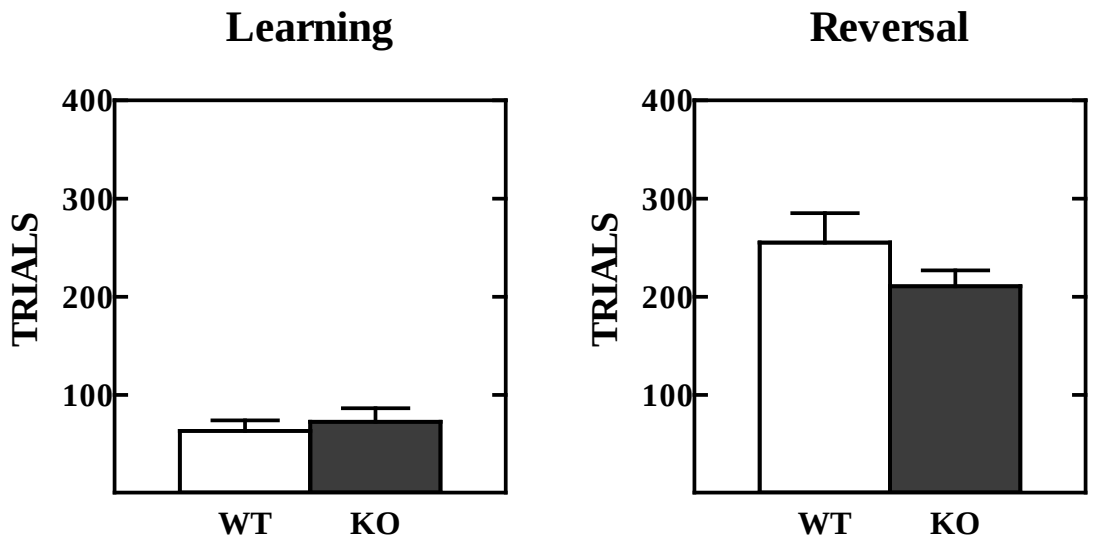


Figure 3. Mice were tested for long-term memory by retraining on one of the odor discrimination problems and comparing the trials needed to acquire the reversal (right) with the trials needed for initial learning (left). WT (n = 10) mice showed a significant increase in trials needed for reversal, relative to initial learning (paired $t_9 = 5.78$, p<.001), as did *Fmr1*-KO (n = 10) mice (paired $t_9 = 7.18$, $p<.0001$). The relative increase in trials necessary to learn the reversal compared to initial learning did not differ between *Fmr1*-KO and WT mice ($t_{18} = 1.40$, $p>.15$).

Figure 4

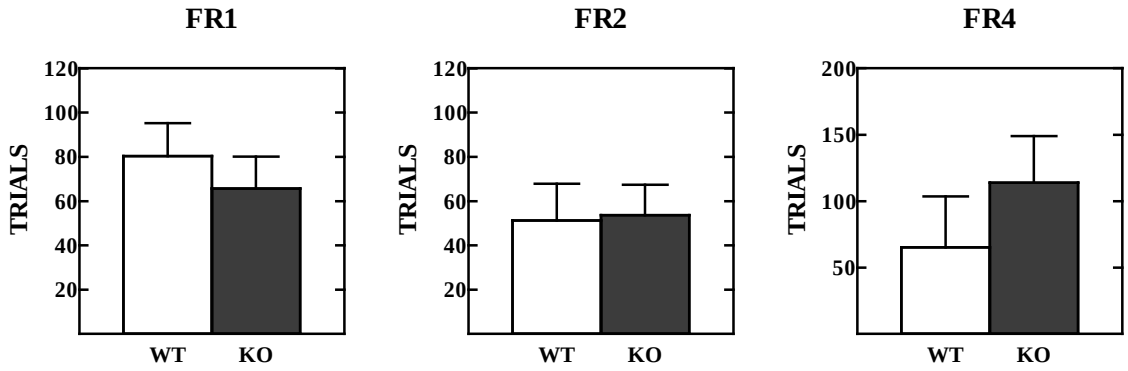


Figure 4. Response to partial reinforcement in WT (n = 10) and *Fmr1*-KO (n = 10) mice. Mice were first tested for performance of a familiar discrimination problem (#8) on a FR1 reinforcement schedule; after performing at criterion (8 consecutive correct trials), the next session was conducted with a FR2 reinforcement session(s) until criterion was again reached, followed by sessions at FR4. Shown are the means (+s.e.m.) trials to criterion for each reinforcement contingency. Although, the *Fmr1*-KO mice tended to require more trials at FR4, this effect was not statistically significant.

Figure 5

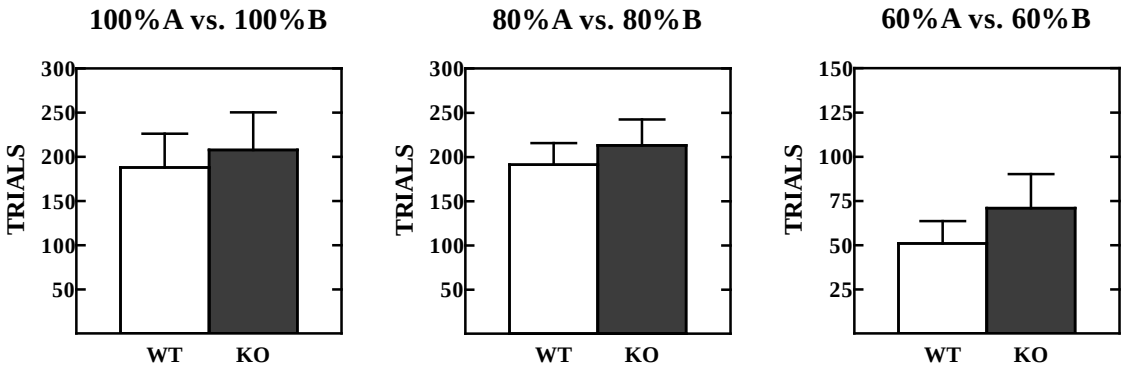


Figure 5. Odor mixture discrimination in *Fmr1*-KO (n = 10) and WT (n = 10) mice. Histograms display mean (+ s.e.m.) trials required to reach criterion on discriminations involving pure eucalyptol and heptanol (100% A vs. 100% B; left panel), the two odors in a mixture of either 80% eucalyptol and 20% heptanol versus 20% eucalyptol and 80% heptanol (middle panel) or the two odors in 60%/40% mixtures (right panel). There were no statistically significant differences between KO and WT mice on any of these discriminations.

Figure 6

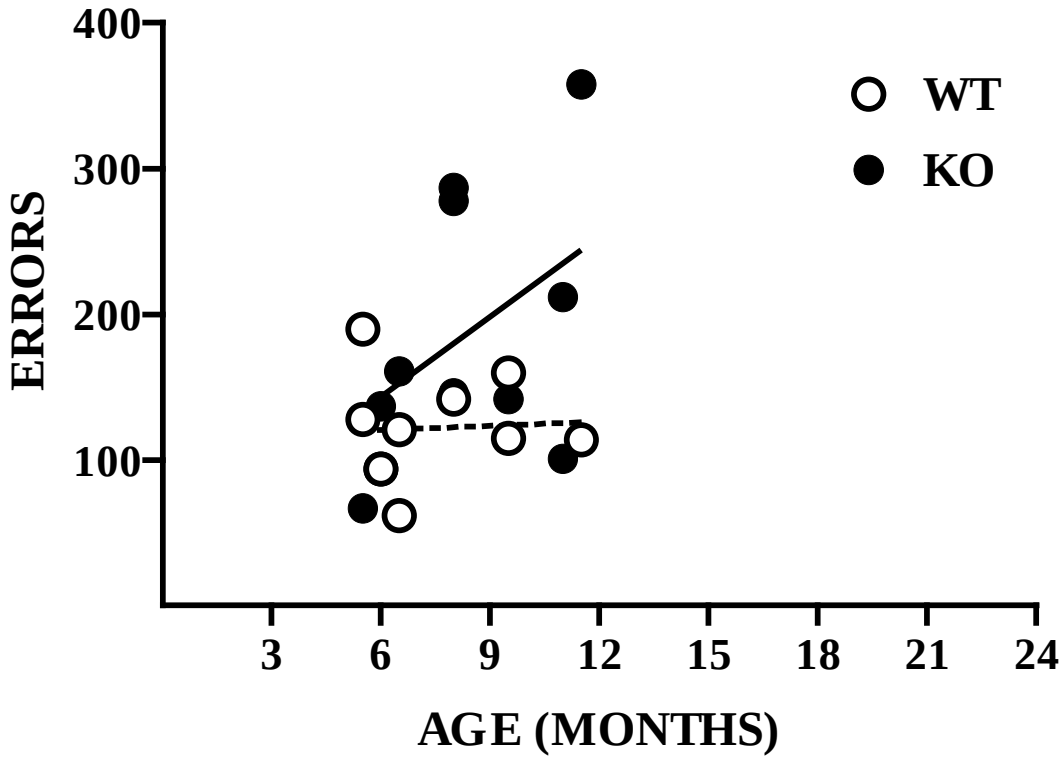


Figure 6. Effect of age on discrimination learning in WT (n = 10) and *Fmr1*-KO (n = 10) mice. Scatter plot shows the number of errors committed before acquiring criterion performance on discrimination problem #1 for individual mice. The dotted line shows the best-fitting regression line for WT subjects whereas the solid line shows the best-fitting regression line for KO subjects.

REFERENCES

Abraham, N.M., Spors, H., Carleton, A., Margrie, T.W., Kuner, T., and Schaefer, A.T. (2004). Maintaining accuracy at the expense of speed: stimulus similarity defines odor discrimination time in mice. Neuron *44*, 865-876.

Bhakar, A.L., Dolen, G., and Bear, M.F. (2012). The pathophysiology of fragile X (and what it teaches us about synapses). Annual review of neuroscience *35*, 417-443.

Eichenbaum, H., Fagan, A., Mathews, P., and Cohen, N.J. (1988). Hippocampal system dysfunction and odor discrimination learning in rats: impairment or facilitation depending on representational demands. BehavNeurosci *102*, 331-339.

Gocel, J., and Larson, J. (2012). Synaptic NMDA receptor-mediated currents in anterior piriform cortex are reduced in the adult fragile X mouse. Neuroscience *221*, 170-181.

Hou, L., Antion, M.D., Hu, D., Spencer, C.M., Paylor, R., and Klann, E. (2006). Dynamic translational and proteasomal regulation of fragile X mental retardation protein controls mGluR-dependent long-term depression. Neuron *51*, 441-454.

Huber, K.M., Gallagher, S.M., Warren, S.T., and Bear, M.F. (2002). Altered synaptic plasticity in a mouse model of fragile X mental retardation. ProcNatlAcadSciUSA *99*, 7746-7750.

Johnson, B.A., and Leon, M. (2007). Chemotopic odorant coding in a mammalian olfactory system. J Comp Neurol *503*, 1-34.

Larson, J., Jessen, R.E., Kim, D., Fine, A.K., and du Hoffmann, J. (2005). Age-dependent and selective impairment of long-term potentiation in the anterior piriform cortex of mice lacking the fragile X mental retardation protein. JNeurosci *25*, 9460-9469.

Larson, J., Kim, D., Patel, R.C., and Floreani, C. (2008). Olfactory discrimination learning in mice lacking the fragile X mental retardation protein. NeurobiolLearnMem *90*, 90-102.

Larson, J., and Munkacsy, E. (in press). Theta-Burst LTP. Brain research.

Larson, J., and Sieprawska, D. (2002). Automated study of simultaneous-cue olfactory discrimination learning in adult mice. BehavNeurosci *116*, 588-599.

Lauterborn, J.C., Rex, C.S., Kramar, E., Chen, L.Y., Pandyarajan, V., Lynch, G., and Gall, C.M. (2007). Brain-derived neurotrophic factor rescues synaptic plasticity in a mouse model of fragile X syndrome. J Neurosci *27*, 10685-10694.

Li, J., Pelletier, M.R., Perez Velazquez, J.L., and Carlen, P.L. (2002). Reduced cortical synaptic plasticity and GluR1 expression associated with fragile X mental retardation protein deficiency. MolCell Neurosci *19*, 138-151.

Patel, R.C., and Larson, J. (2009). Impaired olfactory discrimination learning and decreased olfactory sensitivity in aged C57Bl/6 mice. Neurobiology of Aging *30*, 829-837.

Quinlan, E.M., Lebel, D., Brosh, I., and Barkai, E. (2004). A molecular mechanism for stabilization of learning-induced synaptic modifications. Neuron *41*, 185-192.

Slotnick, B. (2001). Animal cognition and the rat olfactory system. Trends Cogn Sci *5*, 216-222.

Slotnick, B.M., and Katz, H.M. (1974). Olfactory learning-set formation in rats. Science *185*, 796-798.

Wilson, B.M., and Cox, C.L. (2007). Absence of metabotropic glutamate receptor-mediated plasticity in the neocortex of fragile X mice. ProcNatlAcadSciUSA *104*, 2454-2459.

Zhao, M.G., Toyoda, H., Ko, S.W., Ding, H.K., Wu, L.J., and Zhuo, M. (2005). Deficits in trace fear memory and long-term potentiation in a mouse model for fragile X syndrome. JNeurosci *25*, 7385-7392.

APPENDIX 3

Impaired Olfactory Discrimination Learning in Fragile X Knockout Mice

Samantha A. Keil and John R. Larson

Graduate Program in Neuroscience and Department of Psychiatry, University of Illinois at Chicago

Abstracts, Society for Neuroscience Annual Meeting (2014), Program No. 699.10.

Fragile X Syndrome (FXS), caused by mutation of the FMR1 gene, is the leading cause of inherited intellectual disability and is known to cause cognitive impairment, hyperactivity, attention deficits and seizure disorders. Previous studies using a mouse model for FXS (Fmr1-KO) have described impairments in acquisition of simultaneous-cue olfactory discriminations and a specific deficit in LTP in primary olfactory cortex. The present study tested for impaired olfactory discrimination learning in fragile X mice, using a hippocampus-independent successive-cue training procedure.

Male Fmr1-KO and WT littermate mice (C57Bl/6J background; 5-12 months old) were trained to criterion (8 consecutive correct responses) in a fully-automated, successive-cue, go/no-go, two-odor discrimination task. Each mouse was trained in 100-trial sessions on eight separate discrimination problems. Each trial consisted of the presentation of one of two discriminative odors. The eight discrimination problems were presented to half of the mice in each group (randomly chosen) in one order, and in reverse order for the other half. Mice were run blind with respect to genotype.

Fmr1-KO mice made significantly more errors than WT littermates before acquiring the first discrimination problem. Both WT and Fmr1-KO mice made fewer errors on the remaining discrimination problems and did not differ in this regard.

Combined with previous work, the present study indicates that Fmr1-KO mice show similar impairments in learning olfactory discriminations, regardless of whether the discriminative cues are present together (simultaneous-cue task) or separately (successive-cue task) on individual trials. Both paradigms depend on the integrity of piriform cortex but are differentially affected by hippocampal damage. This suggests that the impaired learning in Fmr1-KO mice may be a result of LTP impairments in the primary olfactory (piriform) cortex.

APPENDIX 4

Deficits in Adult Neurogenesis in Fragile X Syndrome

Caryn Davis, Michael Demars, John Larson, and Orly Lazarov

Departments of Anatomy & Cell Biology and Psychiatry, University of Illinois at Chicago

Abstracts, Society for Neuroscience Annual Meeting (2015), Program No. 491.06.

Fragile X Syndrome (FXS) is the most prevalent inheritable form of mental retardation in humans and is the most common known genetic cause of autism. FXS is an X-chromosome-linked disorder characterized by hypermethylation of a CGG repeat expansion in the promoter region of the Fmr1 gene causing gene silencing and subsequently a deficiency of the fragile X mental retardation protein (FMRP). Studies have demonstrated impaired synaptic function and plasticity in the brain in the absence of FMRP. However, the mechanism underlying mental retardation is not fully elucidated. Adult neurogenesis is implicated in learning and memory and in brain plasticity. Neurogenesis takes place in the subgranular layer (SGL) of the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ). Here, we examined whether deletion of fmr-1 affects neurogenesis in adult Fmr1 knockout (Fmr1-KO) mice. We show that the number of fast-proliferating cells is dramatically reduced in the SVZ and the SGL in Fmr1-KO mice when compared to wild type littermates. Additionally, the rate of survival of neuroblasts in the SGL was dramatically compromised. Finally, we show that these impairments result in significantly fewer mature neurons in the DG of the Fmr1-KO mice. Taken together, these results suggest that compromised neurogenesis may cause reduced plasticity and may underlie learning deficits in FXS.

APPENDIX Figure 1

Reduced adult neurogenesis in the olfactory system of *Fmr1*-KO mice.
(C. Davis, M. Demars, J. Larson, & O. Lazarov, unpublished data).
To determine the effect of fast proliferating cells in the subventricular zone (SVZ), the source for adult-born granule cells of the olfactory bulb, 5-month-old littermate *Fmr1*-KO and WT mice were injected once with bromodeoxyuridine (BrdU) one month prior to sacrifice. The number of proliferating cells was significantly greater in WT than *Fmr1*-KO mice (Figure 1). There was also a trend toward reduced numbers of neuroblasts labeled with doublecortin (DCX) in the *Fmr1*-KO mice.

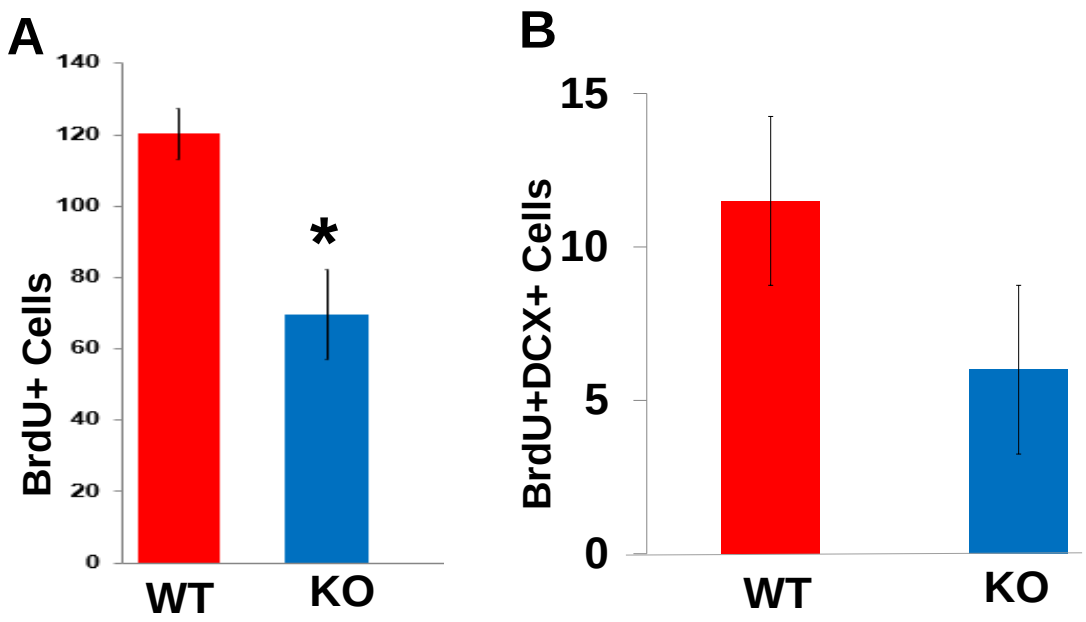


Figure 1. Neurogenesis in the olfactory system of WT and Fragile X knockout mice. **A)** Number of BrdU-labeled cells in SVZ one month after injections (WT: n=4; *Fmr1*-KO, n=7). *: p<.05 (*t*-test). **B)** Number of cells co-labeled with BrdU and DCX in WT (n=4) and *Fmr1*-KO (n=7). The trend for reduced neuroblast numbers in the *Fmr1*-KO mice was not statistically significant.

APPENDIX Figure 2

Comparison of LTP induced by high frequency bursts repeated at theta frequency (5 Hz) and a non-theta frequency (1 Hz) in hippocampal field CA1 of WT and *Fmr1*-KO mice
(P. Dykas, S. Corwin, & J. Larson, unpublished data).

Hippocampal slices were prepared from WT (n = 11) and *Fmr1*-KO (n = 7) mice and a recording electrode placed in stratum radiatum of Field CA1b to monitor field excitatory postsynaptic potentials (fEPSPs). Stimulation electrodes were placed in stratum radiatum of field CA1a and CA1c to activate separate sets of afferent synapses. The two synaptic pathways were stimulated alternately at 10 sec intervals for 13 minutes before and 70 minutes after episodes of LTP-inducing stimulation. One of the two pathways in each slice was randomly selected to receive high frequency bursts at 5 Hz (theta-burst stimulation); the other was stimulated with the same number of bursts at 1 Hz. At least 30 min separated the 5 Hz and 1 Hz stimulation episodes. LTP was quantified as the degree of potentiation of fEPSP slope 60 minutes after burst stimulation. The average results from all slices are plotted in Figure 2.

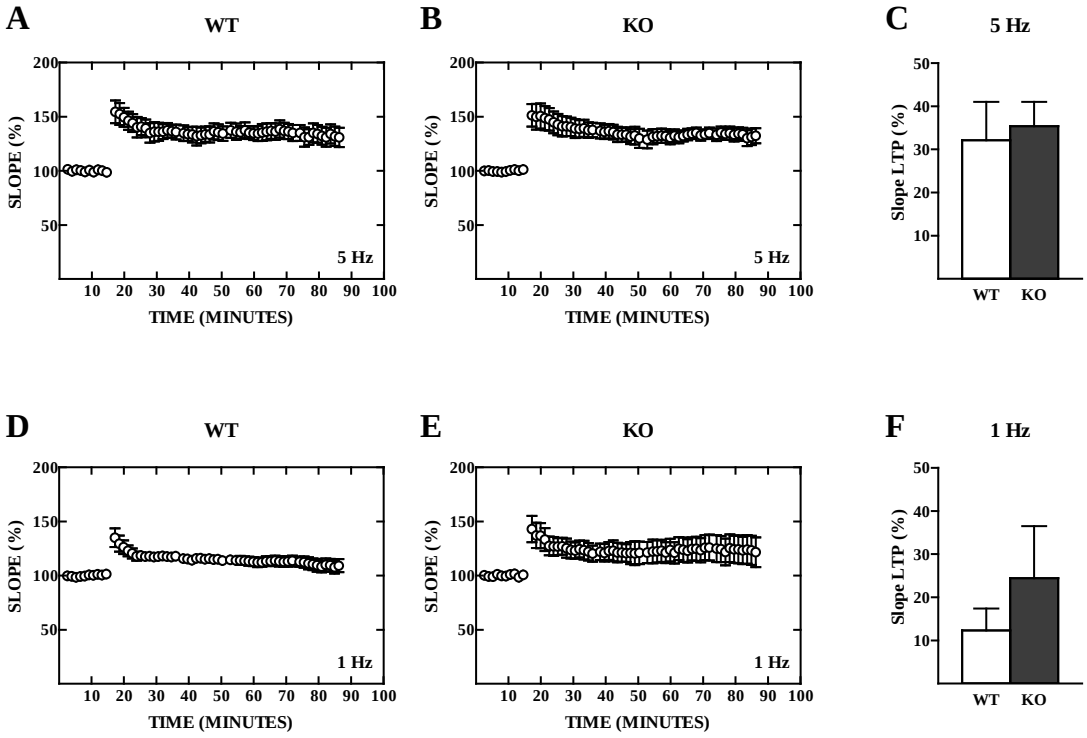


Figure 2. LTP after ten high frequency bursts repeated at 5 Hz (standard theta-burst stimulation) or 1 Hz in WT and *Fmr1*-KO mice. **A)** fEPSP slope recorded before and after 5 Hz bursts in slices from WT mice (n = 11). The graph shows mean (± s.e.m.) for 11 experiments, each for a different mouse. **B)** As in (A), for 7 experiments, each from a different *Fmr1*-KO mouse. **C)** Histograms show mean (+ s.e.m.) potentiation measured 60 minutes after burst stimulation at 5 Hz. There was no significant difference in LTP in WT and KO mice (t_{16} = 0.35, p>.70). **D)** As in (A), but for bursts repeated at 1 Hz in the same WT slices shown in (A). **E)** As in (D), but for 1 Hz bursts in the same *Fmr1*-KO slices shown in (B). **F)** Histograms show mean (+ s.e.m.) potentiation measured 60 minutes after burst stimulation. The difference between WT and KO was not statistically significant (t_{16} = 1.06, p>.30).

APPENDIX Figure 3

Comparison of LTP induced by TBS in medial (MPP) and lateral (LPP) performant path synapses in dentate gyrus of WT and *Fmr1*-KO mice

(P. Dykas & J. Larson, unpublished data).

Hippocampal slices were prepared from WT (n = 7) and *Fmr1*-KO (n = 8) mice. Stimulation electrodes were placed either in the outer molecular layer (LPP) or middle molecular layer (MPP) and recording electrodes in the corresponding layers to record evoked field excitatory postsynaptic potentials (fEPSPs). Slices were perfused with 10 μM picrotoxin to block $GABA_A$ receptor-mediated synaptic inhibition. Paired-pulse stimulation (50-800 msec inter-pulse intervals, IPIs) was used to verify the stimulation sites as lateral (facilitation) or medial (depression) performant paths. After a baseline period of more than 10 min., the slice was given TBS and responses recorded for one hour thereafter. LTP was quantified as the degree of potentiation of fEPSP slope 60 minutes after burst stimulation. The average results from all slices are plotted in Figure 3.

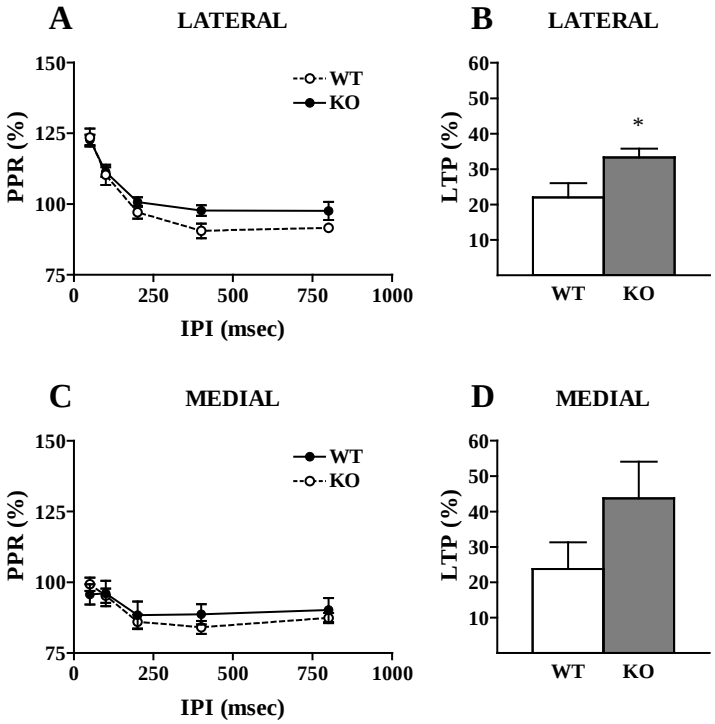


Figure 3. LTP in lateral and medial performant path synapses in hippocampal slices from WT and *Fmr1*-KO mice. **A)** Paired-pulse stimulation produces synaptic facilitation at lateral perforant path synapses. Graphs show responses to the second stimulus of each pair at indicated inter-pulse intervals (IPIs). **B)** Magnitude of LTP evoked by TBS of lateral path synapses. Bars show mean (+s.e.m.) percent increase measured 60 min. after TBS in WT (n=5) and *Fmr1*-KO (n=8) slices. Significantly greater LTP was seen in *Fmr1*-KO mice (t_{11}=2.48, p<.05). **C)** Paired-pulse stimulation produces synaptic depression at medial perforant path synapses. Graphs show responses to the second stimulus of each pair, expressed as a percentage of the first response, at indicated IPIs. **D)** Magnitude of LTP evoked by TBS of medial path synapses. Bars show mean (+s.e.m.) percent increase measured 60 min. after TBS in WT (n=8) and *Fmr1*-KO (n=7) slices. The enhanced LTP in *Fmr1*-KO mice was not statistically significant (t_{13}=1.61, p>.1).

APPENDIX Figure 4

Comparison of LTP induced by two episodes of TBS spaced one hour apart in field CA1 of WT and *Fmr1*-KO mice

(P. Dykas & J. Larson, unpublished data).

Hippocampal slices were prepared from WT (n = 5) and *Fmr1*-KO (n = 4) mice and a recording electrode placed in stratum radiatum of Field CA1b to monitor field excitatory postsynaptic potentials (fEPSPs). Stimulation electrodes were placed in stratum radiatum of field CA1c to activate Schaffer-commissural synapses. After a baseline period of more than 10 min., the slice was given TBS and responses recorded for one hour. Then, a second TBS was given and responses again monitored for one hour afterward. LTP was quantified as the degree of potentiation of fEPSP slope 60 minutes after each episode of burst stimulation. The average results from all slices are plotted in Figure 4.

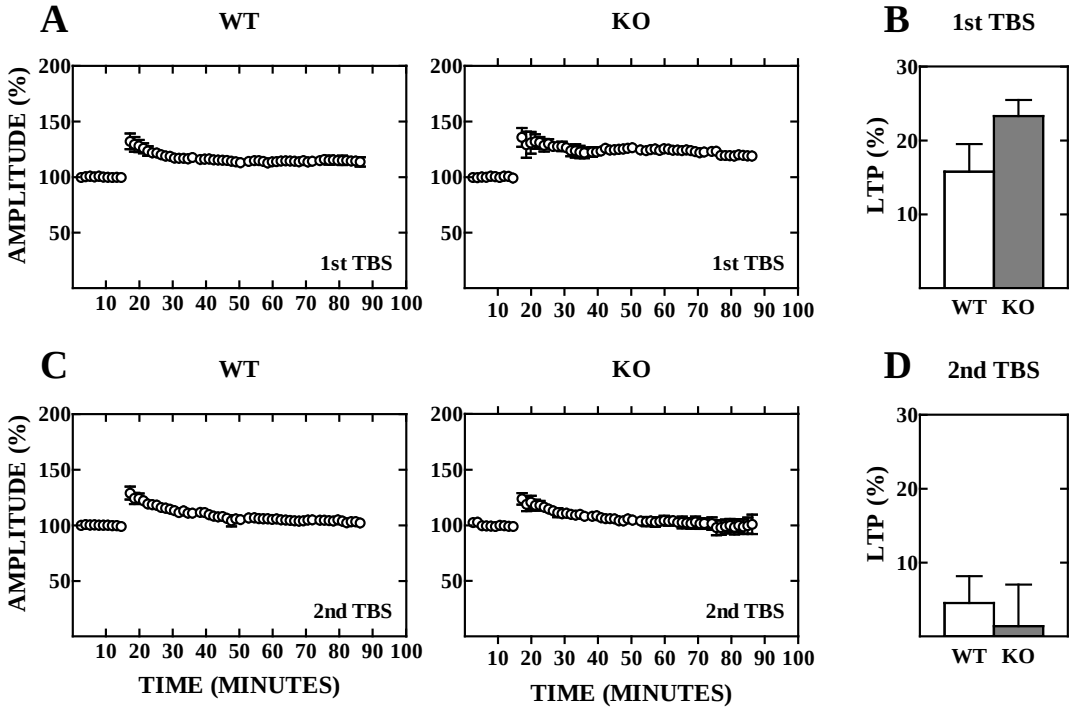


Figure 4. LTP after two episodes of TBS spaced one hour apart in WT and *Fmr1*-KO mice. **A)** fEPSP amplitude recorded before and after the first TBS in slices from WT (n=4) or Fmr1-KO (n=4) mice. The graph shows mean (± s.e.m.) for 4 experiments, each from a different mouse. **B)** Bar graph shows mean (+s.e.m.) potentiation one hour after TBS in each group (*t*-test, $p>.05$). **C)** As in (A), for the second episode of TBS in the same slices. **D)** Histograms show mean (+ s.e.m.) potentiation measured 60 minutes after the second TBS. (*t*-test, $p>.05$).

APPENDIX Figure 5

Spontaneous inhibitory post-synaptic currents (sIPSCs) in layer II pyramidal neurons of anterior piriform cortex.

(A. Widmer & J. Larson, unpublished data).

Slices of anterior piriform cortex are prepared, incubated, and maintained as in our previous publications; layer II pyramidal neurons are identified visually and patched with pipettes containing 120 mM CsCl. In artificial cerebrospinal fluid (aCSF) containing CNQX to block excitatory synaptic transmission, GABA-mediate chloride currents in cells patched with CsCl pipettes are inward at -70 mV holding potential. Recordings of spontaneous inward currents (sIPSCs) are made for 5 min in each of several conditions: control (Control: -70mV, CNQX), in a mildly hyperpolarized state (Hyperpolarized: -90mV, CNQX), after addition of 1 μM TTX (TTX: -70 mV, CNQX, TTX), and after addition of bicuculline (10 μM) to block $GABA_A$ receptors (TTX+BIC). Recordings under these conditions from one cell are shown in Figure 5.

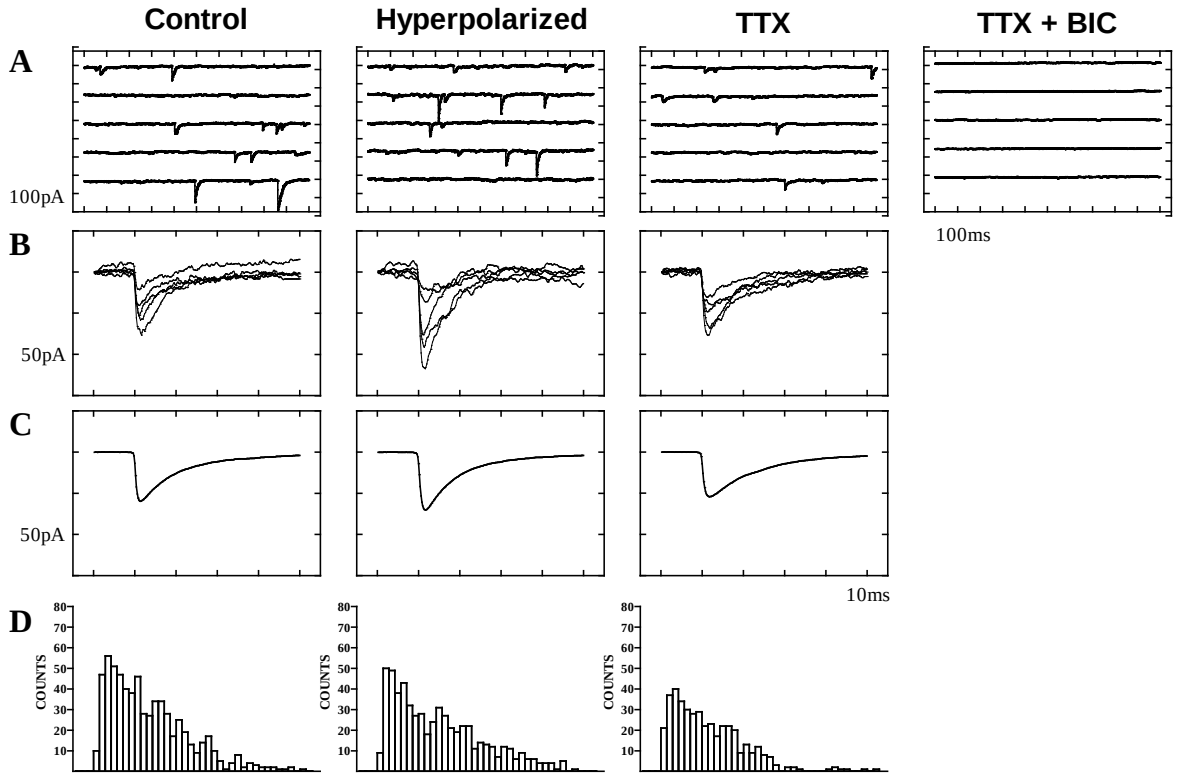


Figure 5. IPSCs in pyramidal neurons of anterior piriform cortex. **A)** Spontaneous inhibitory currents recorded under indicated conditions. Each panel shows five consecutive 1 sec-long sweep. Note that bicuculline abolishes all activity. Tic marks: 100 pA and 100 msec. **B)** sIPSCs identified using a threshold detection method and aligned in time. Each panel shows five events captured during the recording epochs shown in (**A**). Tic marks: 50 pA and 10 msec. **C)** Averaged waveforms of all events detected during a five-min. recording period under each condition. Tic maks: 50 pA and 10 msec. **D)** Amplitude histograms show the size distributions of all IPSCs recorded under each condition. Bin size is 5 pA and the abscissa extends from 0 to 200 pA.