August 2015

Relationship Between Learning-Related Synaptic and Intrinsic Plasticity Within Lateral Amygdala

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RELATIONSHIP BETWEEN LEARNING-RELATED SYNAPTIC AND INTRINSIC PLASTICITY WITHIN LATERAL AMYGDALA

by

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A Dissertation Submitted in

Partial Fulfillment of the

Requirements for the Degree of

Doctorate of Philosophy

in Psychology

at

The University of Wisconsin-Milwaukee

August 2015

ABSTRACT
RELATIONSHIP BETWEEN LEARNING-RELATED SYNAPTIC AND INTRINSIC PLASTICITY WITHIN LATERAL AMYGDALA

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The University of Wisconsin Milwaukee, 2015
Under the Supervision of James R. Moyer

A central question in neuroscience is to determine the mechanisms that govern formation, storage and modulation of memories. Determining these mechanisms would allow us to facilitate new memory formation as in the case of aging-related cognitive decline or weaken preexisting pathological memories such as traumatic memories and cue-induced drug craving. Pharmacological and genetic manipulation of intrinsic neuronal excitability has been demonstrated to impact the strength of memory formation, allocation of memories, and modulation of memories through retrieval and reconsolidation-dependent processes. In addition to experimental manipulations of intrinsic excitability, intrinsic plasticity, a change in neuronal intrinsic excitability, can be brought about by behavioral means such as learning. Indeed, learning-related intrinsic plasticity has been observed in many brain structures following acquisition of a variety of learning paradigms. Despite its ubiquitous nature, little is known about the functional significance of learning-induced intrinsic plasticity. Using the well-characterized lateral amygdala-dependent auditory fear conditioning as a behavioral paradigm, the current experiments investigated the time course and relationship between intrinsic and synaptic plasticity. We found that learning-

related changes in amygdala intrinsic excitability were transient and were no longer evident 10 days following fear conditioning. We also found that fear learning related synaptic plasticity was evident up to 24hr following fear conditioning but not 4 days later. Finally, we demonstrate that the intrinsic excitability changes are evident in many of the same neurons that are undergoing synaptic facilitation immediately following fear conditioning. These data demonstrate that learning related intrinsic and synaptic changes are transient and co-localized to the same neurons. These data demonstrate that memory encoding neurons are more excitable, thus more likely to capture new memories for a time after the learning event.

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LIST OF ABBREVIATIONS

AHP: afterhyperpolarization

AMPA: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA: analysis of variance

AP: Action potential

BLA: basolateral nucleus of amygdala

CR: Conditioned response

CREB: cAMP response element-binding

CS: conditioned stimulus

EC: external capsule

EPSP: excitatory postsynaptic potential

EPSC: excitatory postsynaptic potential

GluR1: glutamate receptor 1

HFS: high frequency stimulation

IL: infralimbic subdivision of medial prefrontal cortex

IR-DIC: infra-red differential interference contrast

ISI: interstimulus interval

IS: immediate shock

ITI: intertrial interval

LA: lateral amygdala

LTP: long term potentiation

MAPK: mitogen-activated protein

NMDA: N-methyl-D-aspartic acid

NMDAR: N-methyl-D-aspartic acid receptors

mPFC: medial prefrontal cortex

MTL: medial temporal lobe

MWM: Morris Water Maze

PBS: Phosphate buffered saline

PFC: prefrontal cortex

PKA: protein kinase A

PKC: protein kinase C

PL: prelimbic subdivision of mPFC

PPR: paired pulse ratio

PTSD: post-traumatic stress disorder

RMP: resting membrane potential

RN : input resistance

SK channel: small-conductance Ca2+-activated K+ channel

T-LA: thalamic-LA pathway

UR: unconditioned response

US: unconditioned stimulus

VDCC: voltage-dependent calcium cannels

VDSC: voltage-dependent sodium channels

WCR: whole-cell recording

ACKNOWLEDGEMENTS

I dedicate this dissertation to my late grandparents, Roshan Lal Sehgal and Kiran Sehgal; your unconditional love, and often unconditional pride in my every little achievement will always inspire me to be more deserving.

I am thankful to Erin Hochstetter, Alex Girgis, Joe Aitken, Tyler Bula, Natalie Fettinger, Blake Hummer, Brett Fulleylove-Krause and Kathleen Feldmann for technical assistance, this was not possible without them. Thank you to past and present graduate students in the Moyer lab, Julia Detert, Chenghui Song, Vanessa Ehlers and Andrew Nye for their help, advice and for being wonderful people. To Anjali Rameshbabu, Jim Otis, Madalyn Hafenbreidal and Swati Gupta for being friends and family. To all the esteemed residents of the 4th floor of Garland Hall, I am lucky to have worked with you.

I am thankful to my committee members, Drs. Rodney Swain and Geoffery Murphy for their valuable time and advice regarding my dissertation. A big thank you, Drs. Fred Helmstetter and Devin Mueller for advice, encouragement and criticism through the years. You have been and will always be an inspiration to me. To my parents, Sunil and Chander Prabha Sehgal for many things big and small, for letting me know that I will always have your support. Last but most importantly, I am grateful to my advisor, Dr. James R. Moyer for giving me this opportunity, for his time and patience through the years, and for his thoroughness - in science and in his mentorship.

INTRODUCTION

A fundamental question in the field of neuroscience is how memories are formed, what regulates their strength and where memories are stored. “Use it or lose it” is a popular adage often associated with use-dependent improvement in cognitive abilities. It refers to the idea that activity-dependent neural plasticity can improve performance on tasks or skills that require the relevant neural pathways. This could endow protection against aging-related cognitive decline or explain why acquisition of certain tasks is faster following learning of a very similar task.

Although the above-mentioned idea is well accepted and intuitive, little is known regarding the neural mechanisms that underlie experience-dependent facilitation of cognitive abilities. One hypothesis that explains this phenomenon is that learning can bring about a state of metaplasticity - higher order plasticity (Abraham and Bear, 1996; Abraham, 2008), where subsequent plasticity is facilitated leading to enhanced learning ability (Saar et al., 1998). Intrinsic plasticity, a change in intrinsic firing properties of a neuron, has been implicated as one such metaplasticity mechanism (Abraham and Bear, 1996; Kim and Linden, 2007; Abraham, 2008; see Sehgal et al., 2013 for a review). Specifically, it has been hypothesized that intrinsic plasticity can modulate future learning by lowering the threshold for synaptic plasticity (Sehgal et al., 2013). Indeed, various lines of research demonstrate that pharmacological and genetic manipulation of intrinsic plasticity can affect baseline synaptic transmission as well as synaptic plasticity (Sah and Bekkers, 1996; Faber et al., 2005; Zhou et al., 2009; Zaitsev and Anwyl, 2012; Sehgal et al., 2013). However, it is unknown how learning-related intrinsic plasticity interacts with synaptic plasticity

during learning. The overall aim of my dissertation is to establish the relationship between learning-related intrinsic and synaptic plasticity.

Plasticity: Forms and functions

Neural plasticity is evolutionarily conserved; even the simplest nervous systems exhibit plasticity in response to changing environmental contingencies (Milner et al., 1998). There can be many neural substrates for such plasticity e.g. changes can involve genetic or epigenetic mechanisms (Zovkic et al., 2013), plasticity of synaptic transmission (Mayford et al., 2012) as well as plasticity at non-synaptic sites throughout the neuron (Kim and Linden, 2007). Indeed, plasticity that involves a change in the number, distribution and/or properties of ion channels located outside the synapse has been extensively documented and constitutes *intrinsic plasticity* (for reviews see, Zhang and Linden, 2003; Disterhoft and Oh, 2006b; Kim and Linden, 2007; Mozzachiodi and Byrne, 2010; Sehgal et al., 2013). The term '*intrinsic*' refers to the idea that such plasticity is intrinsic to the neuron and is not a result of changes in synaptic efficacy. Importantly, such intrinsic plasticity can have dramatic consequences for information processing within a neuron (see below).

Intrinsic neuronal plasticity can be achieved in remarkably diverse ways (Marder and Goaillard, 2006). As previously mentioned, intrinsic plasticity refers to a change in the number or activation of various ion channels. Based on the location of these ion channels, intrinsic plasticity could be local (i.e., limited to a small portion of the dendrite) or global (i.e., somatic, including larger portions of proximal dendrites, thus impacting input from many synapses, Zhang and Linden, 2003). Here, we follow the course of synaptic inputs

from the dendritic spines to the axon terminal of a neuron to illustrate this idea (also see *Figure 1*).

Intrinsic plasticity (dendritic or somatic) has been linked to modulation of synaptic plasticity and *vice versa*. Modulation of dendritic intrinsic excitability can regulate the throughput of synaptic transmission in various ways (*see Figure 1*). First, it can have consequences for the dendritic integration processes that influence degradation of synaptic signals (*see Figure 1, Panel 2*; also see Spruston (2008), for an excellent review of how dendritic properties can affect synaptic integration in pyramidal neurons). For example, in pyramidal neurons (hippocampal CA1 as well as basolateral amygdala or BLA), repetitive firing activates the slow afterhyperpolarization current or sI_{AHP} (Hotson and Prince, 1980; Lancaster and Adams, 1986; Storm, 1990; Faber et al., 2001), which hyperpolarizes the somatic and proximal dendritic membrane potential (Sah and Bekkers, 1996). Activation of the sI_{AHP} reduces the amplitude and summation of EPSPs arising from stimulation of the apical dendritic tree (Sah and Bekkers, 1996; Power et al., 2011). Thus, the sI_{AHP} can act as an adjustable gain control mechanism, influencing the ability of synaptic signals from dendrites to reach the soma.

It needs to be emphasized that the above example reflects a very simplified view of dendritic information processing. Experimental and computational studies have demonstrated that dendritic information processing is highly complex (Kastellakis et al., 2015). Anatomical and biophysical plasticity can allow synaptic tagging (Govindarajan et al., 2006), synaptic clustering (Poirazi and Mel, 2001) and compartmentalized regenerative spikes (Hausser et al., 2000; Larkum et al., 2009) which are further regulated by plasticity of inhibitory signals as well as homeostatic plasticity (Turrigiano and Nelson, 2004). This

ensures that dendrites can exercise control over the synaptic information in myriad ways ultimately resulting in long term memory storage without saturating the information capacity of the neuron.

In addition to basal synaptic transmission, intrinsic excitability can alter the requirements for induction of synaptic plasticity. Inhibition of the sI_{AHP} reduces the threshold for LTP (long term potentiation) induction in CA1 neurons (Sah and Bekkers, 1996; Cohen et al., 1999). Similar effects have been observed in the amygdala as well as the medial prefrontal cortex following modulation of the sI_{AHP} (Faber et al., 2005; Power et al., 2011; Zaitsev and Anwyl, 2012). Taken together, enhanced dendritic excitability likely facilitates baseline synaptic transmission as well induction of LTP.

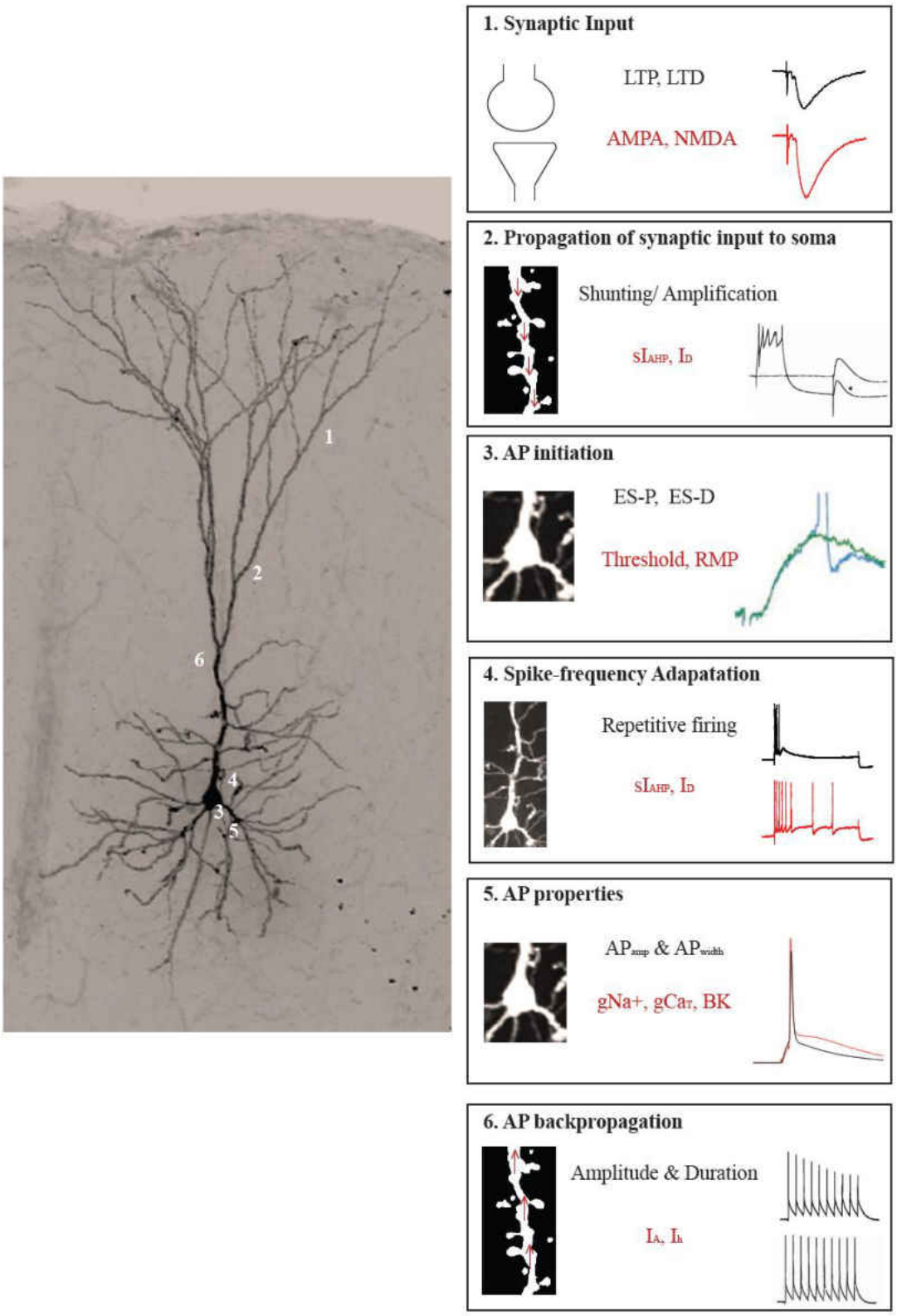
If intrinsic plasticity can alter the integration of synaptic inputs, this can in turn impact action potential (AP) generation, and thus the neuronal output. Better transmission of synaptic inputs to the soma is evident as an enhanced ability of an EPSP to generate an action potential (AP), referred to as EPSP-to-spike (ES) coupling or ES potentiation (Bliss and Lomo, 1973). As shown in *Figure 1, Panel 3,* ES coupling can undergo bidirectional plasticity following induction of long-term potentiation or depotentiation (Daoudal et al., 2002). Furthermore, environmental factors such as enrichment can also enhance ES coupling (Malik and Chattarji, 2012). Although ES plasticity can result from changes in the balance between inhibitory and excitatory synaptic drive, changes in neuronal intrinsic excitability also contribute to ES plasticity (see Daoudal and Debanne, 2003, for review). Thus, intrinsic plasticity in the form of changes in the active properties of dendrites can shape synaptic signals significantly, and thus impact ES coupling.

Once the synaptic inputs reach the soma, various intrinsic factors can contribute to AP initiation, including modulation of AP threshold (Malik and Chattarji, 2012) or local membrane potential (Rosenkranz et al., 2010). In addition to the all-or-none firing of an AP, efficient relay of neuronal information may require repetitive AP firing (*see Figure 1, Panel 4*). For example, in working memory tasks such persistent neuronal firing is critical for maintaining representations across time, and reduced excitability in the form of greater spike frequency adaptation may limit working memory performance (Durstewitz, Seamans, and Sejnowski, 2000).

Single AP characteristics also contribute to neuronal excitability (*see Figure 1, Panel 5 and Table 2*). AP amplitude and half-width are plastic intrinsic properties (Varela et al., 2012) that can influence the duration and extent of Ca^{2+} influx at the presynaptic terminal (Deng et al., 2013). In addition, when a neuron fires an AP, the AP can backpropagate into portions of the dendritic tree (*see Figure 1, Panel 6)*, which can be influenced by changes in local dendritic excitability (Frick et al., 2004). Such backpropagating APs (bAPs) are associated with Ca^{2+} influx into the dendritic compartments (Larkum et al., 1999) and are important for LTP induction (Sjostrom and Hausser, 2006). LTP induction in turn enhances local dendritic excitability through modulation of A-type K^{+} channels and results in an input-specific increase in bAP amplitude (Frick et al., 2004). Thus, APs and bAPs represent yet another example of how intrinsic neuronal excitability is closely associated with synaptic throughput and plasticity in the brain.

Figure 1. Synaptic and intrinsic properties shape neuronal information processing. Left panel depicts a medial prefrontal cortical neuron filled with biocytin during whole-cell patch clamp recording and imaged using confocal microscopy (Olympus FV1200). Numbers 1, 2, 3, 4, 5 and 6 refer to the boxes in the right panel. (1) A vast majority of neuronal input originates on the dendritic spines with smaller contributions from synapses that are made on the dendrites, soma and axons. Synaptic inputs can undergo bidirectional plasticity in the form of LTP and LTD by modulation of AMPA and NMDA receptor-mediated transmission. (2) Propagation of the synaptically generated signal (EPSP) depends upon the active and passive dendritic properties including ionic conductances that contribute to the afterhyperpolarization (AHP). (3) Once the signal reaches the soma, neuronal output is determined based on factors like AP initiation threshold, resting membrane potential, etc…, which in turn rely on ion channels within the soma. (4) Bidirectional plasticity impacting the coupling of EPSPs to spikes is referred to as ES-P and ES-D. The number of APs generated following sustained stimulation (spike frequency adaptation) can also code relevant information, and relies upon K^+ conductances, including those that underlie the AHP. (5) In addition, properties like amplitude and duration of APs can also modulate pre- and postsynaptic aspects of neuronal processing like neurotransmitter release and bAPs. (6) The magnitude and travel distance of bAPs can be influenced by I_A currents, which can ultimately modulate Ca^{2+} influx into the dendritic compartment. Abbreviations: long term potentiation, LTP; long term depression, LTD; excitatory postsynaptic potential, EPSP; afterhyperpolarization, AHP; action potential, AP; EPSP-to-Spike coupling potentiation, ES-P; EPSP-to-Spike coupling potentiation, ES-D; backpropagating APs, bAPs. Electrophysiological traces in boxes 2, 3, 5 and 6 were adapted from Sah and Bekkers (1996), Daoudal et al. (2002), Deng et al. (2013) and Tsubokawa et al. (2000) respectively, with permission. Adapted from Sehgal, Song, Ehlers & Moyer, 2013).

Figure 1


1. Synaptic Input
LTP, LTD
AMPA, NMDA
2. Propagation of synaptic input to soma
Shunting/ Amplification
sIAHP, ID
3. AP initiation
ES-P, ES-D
Threshold, RMP
4. Spike-frequency Adapatation
Repetitive firing
sIAHP, ID
5. AP properties
APamp & APwidth
gNa+, gCaT, BK
6. AP backpropagation
Amplitude & Duration
IA, Ih
1
2
6
4
3
5

Mechanisms of intrinsic plasticity

While many neuronal components are involved in intrinsic plasticity, I will largely focus on plasticity of the afterhyperpolarization (AHP) and spike frequency adaptation. The AHP is a hyperpolarizing current that follows a burst of action potentials and limits action potential firing (Hotson and Prince, 1980). Spike frequency adaptation refers to the process by which the instantaneous firing of a neuron gradually slows over time in response to sustained excitation (e.g. see Madison and Nicoll, 1984). In CA1 as well as other neurons, spike frequency adaptation is heavily influenced by the AHP (although other currents are also involved). When the AHP is small, spike frequency adaptation is also reduced, meaning that a sustained depolarization can now evoke more action potentials (Madison and Nicoll, 1984; Faber et al., 2001).

The AHP is influenced by several underlying currents mediated by Ca^{2+}-activated K^+ channels. There are several phases of the AHP, including fast, medium, and slow AHP (see Storm, 1990 for an excellent review). These are evoked as a result of action potential-elicited K^+ currents, including: 1) a voltage- and Ca^{2+}-dependent current (I_C); 2) a voltage-dependent, muscarine-sensitive current (I_M); 3) a Ca^{2+}-dependent and apamin-sensitive current (I_{AHP}); and 4) a Ca^{2+}-dependent apamin-insensitive current (sI_{AHP}) (Storm, 1989; Sah, 1996; Gasparini and DiFrancesco, 1999; Stocker et al., 1999). The fast AHP is modulated by changes in I_C; the medium AHP is modulated by changes in I_C, I_M, and the apamin-sensitive I_{AHP}; the slow AHP (sAHP) is modulated by changes in the apamin-insensitive sI_{AHP} (Storm, 1989; Sah, 1996; Gasparini and DiFrancesco, 1999; Stocker et al., 1999). Although learning-related modulation is possible for all three phases of the AHP (e.g. see Matthews et al., 2008; Santini et al., 2008; Matthews et al., 2009 for learning-

related changes in fast and medium AHP), the current discussion is limited to sAHP for two reasons: (1) learning-related changes in sAHP are more extensively described, and (2) a description of learning-related sAHP changes is adequate to support the central hypothesis of this proposal.

Intrinsic excitability: A substrate for learning

Learning involves a change in behavior in response to environmental stimuli, and depends critically on plasticity within the nervous system. Such learning-related changes include modulation of intrinsic neuronal excitability. This section summarizes such learning-related intrinsic plasticity. Finally, evidence that links aging-related deficits in learning with failure to modulate intrinsic excitability is presented.

Learning-related intrinsic plasticity. Early demonstrations of behaviorally-induced intrinsic plasticity in vertebrates came from the work of Woody *et al* in cats (Woody and Black-Cleworth, 1973). In this study, cats were classically conditioned to associate a tap to the glabella with an auditory click. Following learning, the auditory click evoked the same response as the glabella tap; i.e. a nose and an eye twitch. These behavioral changes were accompanied by a reduction in the minimum current necessary to evoke an AP (i.e., rheobase) in neurons that project to the relevant musculature (Woody and Black-Cleworth, 1973). This was closely followed by the work of Alkon and colleagues in the invertebrate *Hermissenda crassicornis* (Alkon, 1979, 1984). These organisms display positive phototaxis, but when rotation (an aversive stimulus) is paired with light the animals display reduced phototaxis. Like the work of Woody *et al.* in cats, Alkon and colleagues found that excitability of type B photoreceptor, neurons responsible for the behavior is increased following learning. In addition, they demonstrated that these

excitability changes are concomitant with decrease in outward potassium (in this case, IA and calcium-dependent K+ currents, Alkon, 1979).

Note that these early demonstrations of learning-related changes in intrinsic excitability were observed in the 1970-80s, around the same time when Bliss & Lomo described synaptic plasticity in the form of long term potentiation (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973). Since then, learning-related changes in intrinsic excitability have been demonstrated using many learning and memory paradigms and have often preceded a direct demonstration of learning-related synaptic plasticity (reviewed below). Despite this the significance of learning-related intrinsic plasticity is poorly understood, especially relative to the role of synaptic plasticity in memory formation.

The common theme that emerged from the studies of intrinsic excitability is that these intrinsic changes are evident in neurons implicated in the learning process and are often accompanied by plasticity of potassium currents. Important among these are potassium currents that contribute to postburst AHP and thus, spike frequency adaptation. Although learning-induced intrinsic plasticity has been demonstrated in vertebrate and invertebrate species (see Mozzachiodi and Byrne, 2010 for a review), for the sake of brevity, we will focus primarily on vertebrate studies.

Pavlovian conditioning paradigms. Pavlovian conditioning has proven to be a powerful model for assessing learning and memory across species (Hawkins et al., 2006; Freeman and Steinmetz, 2011; Johansen et al., 2011). In a Pavlovian conditioning paradigm, a neutral conditioned stimulus (CS) such as a tone is paired with an aversive unconditioned stimulus (US) that elicits an unconditioned response (UR, such as an air puff to the eye elicits eyeblink response). Following repeated pairings of the CS with the US

(e.g. tone with the airpuff), animals learn to associate CS with the US and the CS itself can elicit the same conditioned response (CR, e.g. eyeblink). Such associative learning has been demonstrated across species from invertebrates to humans and has become an important learning and memory model.

Based on the temporal relationship between the CS and US presentations, the Pavlovian conditioning paradigms can be classified into 2 important variants: delay and trace fear conditioning. In delay conditioning, the CS and US presentations overlap temporally; the onset of US presentation occurs during or at the CS offset. In trace conditioning, the CS offset and the US onset is separated by a small 'trace' period. Introduction of this trace period between CS and US presentations influences how quickly learning occurs (Thompson et al., 1996) and necessitates the involvement of additional brain structures for successful learning (Solomon et al., 1986; Moyer et al., 1990; Quinn et al., 2002; Kesner, 2005; Bangasser et al., 2006; Suh et al., 2011).

Early work investigating learning-related intrinsic plasticity in vertebrates exploited eyeblink conditioning. In eyeblink conditioning, a tone is repeatedly paired with, for example, an air puff to the eye that results in an eyeblink response. Following many such pairings, the tone presentation alone can elicit an eyeblink response indicating that the animal now associates the tone CS with the air puff US. In the earliest demonstration of AHP plasticity, Disterhoft and colleagues showed that following delay eyeblink conditioning, hippocampal CA1 neurons from trained rabbits have significantly smaller AHPs than neurons from naïve or psuedoconditioned animals (Disterhoft et al., 1986). This was a seminal study for two reasons. First, it demonstrated that associative learning in vertebrates is accompanied by plasticity of K^+ mediated ionic conductances. More

importantly, this was the first ever demonstration of learning-related plasticity using an *in vitro* brain slice preparation.

Since then learning-related changes in AHP plasticity have been reported extensively. For example, trace eyeblink conditioning increases intrinsic excitability of CA1 and CA3 pyramidal neurons (Moyer et al., 1996). These changes include transient reductions in AHPs as well as spike frequency adaptations that are no longer evident 14 days post-conditioning (Moyer et al., 1996). Importantly, such plasticity has been demonstrated in the presence of synaptic blockers indicating it is independent of changes in synaptic transmission and hence, reflects modulation of intrinsic ionic conductances (e.g., Coulter et al., 1989). Lastly, learning-related changes following eyeblink conditioning are not limited to the hippocampal circuit. Intrinsic excitability of Purkinje neurons within the cerebellum is also increased for up to 1 month following eyeblink conditioning (Schreurs et al., 1998). Thus, learning-related intrinsic plasticity following eyeblink conditioning is distributed within the neural circuit mediating the behavior and follows a different time course in distinct structures.

Fear conditioning is another Pavlovian conditioning paradigm that has been extensively used to study the neurobiology of emotional memories (LeDoux, 2000). In fear conditioning, a neutral CS such as a tone is paired with a noxious US such as a footshock. Fear conditioning is an extremely powerful model of emotional learning as the association is rapidly acquired and a single brief training session can be sufficient to produce a long-lasting memory. Similar to eyeblink conditioning, both delay and trace variants of fear conditioning have been employed. While both delay and trace fear conditioning require amygdala plasticity (Kwapis et al., 2011), trace fear conditioning also requires the intact

medial temporal lobe and higher cortical structures for successful learning (McEchron et al., 1998; Quinn et al., 2002; Kesner, 2005; Bangasser et al., 2006; Kholodar-Smith et al., 2008; Gilmartin and Helmstetter, 2010; Suh et al., 2011; Gilmartin et al., 2013; Kwapis et al., 2015).

Similar to trace eyeblink conditioning, trace fear conditioning also enhances intrinsic excitability of CA1 pyramidal neurons (Kaczorowski and Disterhoft, 2009; McKay et al., 2009; Song et al., 2012). These studies also demonstrate that modulation of intrinsic excitability occurs following successful acquisition of conditioned fear. For example, middle-aged mice that display low freezing in the conditioning context also display bigger AHPs (Kaczorowski and Disterhoft, 2009). In addition, even in adult rats freezing during the trace interval is correlated with the amplitude, area as well as duration of the AHP, and with spike frequency adaptation (Song et al., 2012). Such correlation is evident for trace conditioned but not pseudo-conditioned rats. These findings further demonstrate that intrinsic plasticity is specific to animals that learn and predicts the strength of acquired memories.

A potential mechanism by which conditioned associations can be disrupted is extinction training (see Quirk and Mueller, 2008 for a review). Extinction involves the repeated presentations of a CS in the absence of a US. During extinction learning, the CS loses its associative strength and the conditioned response is gradually reduced. A large body of work suggests that successful extinction of conditioned fear requires plasticity in a distributed network of brain structures including hippocampus, amygdala and parts of medial prefrontal cortex (mPFC, for example see Sierra-Mercado et al., 2011). Fewer studies have investigated extinction-related intrinsic plasticity, however a consistent theme

that emerges is that extinction reverses the conditioning-related intrinsic plasticity. For example, context fear conditioning is accompanied by an increase in CA1 neuronal excitability (Kaczorowski and Disterhoft, 2009; McKay et al., 2009). This increased excitability returns to naïve levels following behavioral extinction (McKay et al., 2009). Similarly, within the infralimbic subregion of mPFC (IL), delay fear conditioning results in reduced neuronal excitability, which is reversed to naïve levels by successful extinction (Santini et al., 2008). Trace fear conditioning also modulates mPFC intrinsic excitability – PL neurons are less excitable and IL neurons are more excitable but these changes are reversed following extinction (personal communication, Chenghui Song). As evident in previous reports, these neuronal excitability changes include plasticity of AHPs as well as neuronal firing. Taken together, these data illustrate that the strength of associative conditioning is correlated with the degree of intrinsic plasticity and behavioral extinction of these associations reverses the learning-related intrinsic plasticity.

Of course, learning-related intrinsic plasticity is not limited to associative conditioning paradigms. Extensive work by Edi Barkai's group has demonstrated intrinsic plasticity following acquisition of odor discrimination tasks in a distributed network of brain structures. Successful acquisition of these tasks requires an animal to correctly differentiate between olfactory cues in order to receive a reward. Following learning, intrinsic plasticity in the form of reductions in AHPs and spike frequency adaptation has been demonstrated in hippocampus (Zelcer et al., 2006), piriform cortex (Saar et al., 1998), and basolateral amygdala (Motanis et al., 2012). As with associative learning, intrinsic plasticity following olfactory learning is transient and predicts the behavioral performance of the animal.

To summarize, learning–related intrinsic plasticity is widespread in the vertebrate nervous system. These changes are often accompanied by plasticity of K^+ conductances. Finally, intrinsic plasticity is a strong predictor of behavioral performance making it a highly suitable target for therapeutic interventions aimed at ameliorating cognitive deficits.

Implications of intrinsic plasticity on learning

Considering that intrinsic plasticity is evolutionarily conserved (i.e. it is observed following a variety of behavioral paradigms across species), it is reasonable to assume that intrinsic plasticity plays an important role in memory formation. However, a disproportionately large body of work investigating neural correlates of learning focuses on synaptic plasticity as an underlying mechanism (Mayford et al., 2012). This is in part because of the ease with which synaptic plasticity can explain certain mnemonic functions e.g. memory and synaptic plasticity can be long-lasting and associative (Martin et al., 2000). However, it has become increasingly clear that an exclusively synaptic model for memory storage is unlikely and that intrinsic plasticity plays a critical role in memory formation (Zhang and Linden, 2003; Frick and Johnston, 2005; Kim et al., 2013). Some of the literature that implicates intrinsic plasticity as a factor regulating memory formation is reviewed below.

Aging and aberrant intrinsic excitability. Aging is associated with reduced cognitive ability. Moreover, the effect of normal aging (in the absence of disorders) varies between individuals – some elderly individuals suffer from more pronounced cognitive deficits whereas others are relatively unimpaired (Deary et al., 2009). Such heterogeneity in the normal aging process can be useful in determining factors that are responsible for

healthy aging. In addition, these mechanisms are also likely to overlap with those that predict enhanced learning in a younger population.

Normal aging leads to a decline in many brain functions, but the two brain regions consistently implicated in these functional alterations are the medial temporal lobe (MTL) and the prefrontal cortex (PFC; Burke and Barnes, 2006; also reviewed later). Hippocampal involvement in a variety of learning and memory paradigms is well documented (for a review see Squire, 2004). Although aged rodents typically perform worse than young rodents on many hippocampus-dependent tasks, aged animals can be divided into aged impaired (AI) or aged unimpaired (AU) based on their performance (Gallagher et al., 1993). Heterogeneity in acquisition of Morris water maze (MWM) in aged rodents is associated with differences in CA1 neuronal excitability. The AI rats display greater sAHP and spike frequency adaptation relative to young and AU rats, and the sAHP amplitude is correlated with behavioral performance (Tombaugh et al., 2005). Interestingly, in this study the electrophysiological recordings were performed weeks (~2-5 weeks) after MWM training suggesting that these changes were not learning-related, but rather, they reflect basal differences in CA1 excitability. It is worth noting that although the mean delay interval between behavioral testing and slice preparation varied between young (~25d), AU (~13d) and AI (~30d) rats, however, analysis using a subset of rats with comparable delay intervals also revealed similar results. Thus, it is possible that pre-learning differences in the intrinsic excitability of neurons contribute to aging-related cognitive decline and could explain the heterogeneity observed in cognitive performance during the aging process. Alternatively, it is unlikely but possible that intrinsic excitability

changes following MWM last longer than those seen following acquisition of trace eyeblink conditioning.

Normal aging also leads to pronounced deficits in acquisition of hippocampus-dependent trace eyeblink and fear conditioning tasks. These deficits are observed in aged rodents (Kishimoto et al., 2001; Knuttinen et al., 2001; Moyer and Brown, 2006), rabbits (Deyo et al., 1989; Solomon and Groccia-Ellison, 1996; Thompson et al., 1996; Moyer et al., 2000), and humans (Finkbiner and Woodruff-Pak, 1991). In addition to forming associations between the CS and the US, animals also form associations between the conditioning context and the US. Such contextual associations require hippocampal function (Kim and Fanselow, 1992) and are also impaired in aged rodents (Moyer and Brown, 2006; Kaczorowski and Disterhoft, 2009; Kaczorowski et al., 2011). Rodents, especially aging rodents, display heterogeneity in the acquisition of trace and context conditioning. Animals (young, middle-aged, or aged) that acquire context or trace conditioning have enhanced CA1 intrinsic excitability relative to age-matched control and slow learning animals (Moyer et al., 2000; Kaczorowski and Disterhoft, 2009; Song et al., 2012). These data indicate that reduced intrinsic excitability (pre- and post-learning) may be an important predictor of cognitive decline. Animals that learn well have significantly smaller AHPs than animals that learn poorly. This is seen not only in adult animals but also aged animals where the AHP is significantly larger. This bidirectional modulation of the AHP suggests that it is an important intrinsic mechanism influencing behavioral plasticity.

In addition to hippocampus, PFC function is also impaired during normal aging. The PFC is critical for working memory and executive function (Funahashi et al., 1993;

Mair et al., 1998). Impairments in acquiring working memory tasks, such as the Delayed non-matching-to-sample (DNMS) are observed across species with aging (Dunnett et al., 1988; Moss et al., 1988; Moss et al., 1997; Lyons-Warren et al., 2004). In primates, working memory tasks are dependent upon alterations in AP firing rates in dorsolateral PFC (Goldman-Rakic, 1995), and normal aging results in changes in the intrinsic properties of primate dorsolateral PFC neurons. Specifically, aging increases input resistance, decreases AP amplitude and fall time, and increases AP firing rate in layer II/III dorsolateral PFC neurons (Chang et al., 2005). Furthermore, in aged monkeys, performance on DNMS has a U-shaped quadratic relationship with the firing rate of layer II/III dorsolateral PFC neurons, where either low or very high firing rates predict poor performance (Chang et al., 2005). Interestingly, modulation of excitability of layer III dorsolateral PFC neurons by inhibiting cAMP signaling, HCN channels or KCNQ channels restores persistent firing during the delay period and leads to improved performance of DNMS task (Wang et al., 2011). These data indicate that subregion-specific alterations in intrinsic properties may underlie learning deficits, and that modulation of excitability can ameliorate aging-related cognitive decline.

PFC subregions are also critical for cognitive flexibility (Oualian and Gisquet-Verrier, 2010). One example of cognitive flexibility is behavioral extinction, which is the learned inhibition of a behavioral response as a result of a change in stimulus contingencies (for a review of extinction, see Quirk and Mueller, 2008). As previously mentioned, PFC (particularly IL) is critical for extinction of a conditioned fear response. Our laboratory has previously demonstrated that normal aging leads to impaired extinction of trace fear conditioning in both middle-aged and aged rats (Kaczorowski et al., 2012). These aging-

related extinction deficits are paralleled by a significant decrease in the intrinsic excitability of IL regular spiking neurons, and an increase in the intrinsic excitability of burst spiking neurons within the prelimbic (PL) sub-region of mPFC. Taken together, these data suggest that a redistribution of intrinsic excitability within mPFC subregions during aging may reduce IL-mediated inhibitory signaling within amygdala and thus, contribute to the observed extinction deficits. These data are clinically relevant as extinction deficits are thought to underlie a variety of disorders, including posttraumatic stress disorders (PTSD). Hence, modulation of intrinsic excitability within IL could strengthen extinction learning in aging populations and also potentially benefit patients at risk for or suffering from PTSD.

In order to develop a clinically relevant intervention that targets aging-related neuronal excitability changes, it is important to determine the ionic conductances that are altered during aging and how these relate to cognitive function. Given the wealth of data suggesting aging-related deficits in calcium regulation (for reviews see Disterhoft et al., 1996; Thibault et al., 2007; Toescu and Verkhratsky, 2007), it is noteworthy that sAHP, largely mediated by a Ca^{2+}-dependent K^+ current (Alger and Nicoll, 1980), is enhanced in aging animals (Landfield and Pitler, 1984; Moyer et al., 1992; Moyer and Disterhoft, 1994; Kumar and Foster, 2002). In aged animals, an enhanced sI_{AHP} is correlated with an enhanced sAHP and impaired learning ability (Power et al., 2002). Other K^+ channels that contribute to the AHP in hippocampal neurons include K^+ channels containing auxiliary Kvβ1.1 subunit (Giese et al., 1998) and small-conductance Ca^{2+}-activated K^+ channel type 2; SK2 and SK3 (see Stocker, 2004 for a review). Aged mice that lack Kvβ1.1 subunits have increased hippocampal neuronal excitability, exhibit a smaller AHP, lower LTP induction threshold, and improved spatial learning relative to age-matched controls

(Murphy et al., 2004). Elevated expression of SK3 channels in the hippocampus is correlated with impairment of LTP, and trace fear conditioning in aged mice (Blank et al., 2003). Finally, transgenic mice that overexpress SK2 channels also have a higher threshold for LTP induction and deficits in learning both hippocampus- and amygdala-dependent tasks (Hammond et al., 2006). Thus, aberrant intrinsic plasticity may emerge during normal aging, as a result of alterations in the numbers, distributions, or modulation of K^+ channels that underlie the AHP and impact neuronal excitability during learning.

Does intrinsic plasticity encode memory? As described above, intrinsic plasticity is strongly correlated with learning. Changes in neuronal excitability are learning-specific since they are observed in animals that learned, but not in pseudoconditioned controls or animals that failed to learn (Moyer et al., 1996; Oh et al., 2003; Song et al., 2012). Similar learning-related AHP reductions are also observed in middle-aged and aged animals (Moyer et al., 2000; Kaczorowski and Disterhoft, 2009). While these data support the hypothesis that intrinsic plasticity could underlie memory formation, a mnemonic role for intrinsic plasticity as a mechanism for maintaining a long-term memory seems unlikely for two reasons. First, these changes are transient (lasting only a few days) whereas behavioral expression of the memory can last for weeks, months, or even years. Within the hippocampus, enhanced intrinsic excitability of CA1 neurons is no longer evident 7 days following acquisition of trace eyeblink conditioning, even though behavioral expression of the memory is evident for at least 6 months (Moyer et al., 1996). It is worth mentioning that in some cases learning-related intrinsic plasticity can be persistent and hence, may very well underlie certain long-term memories (Brons and Woody, 1980; Schreurs et al., 1998; Mozzachiodi et al., 2008). However, the majority of studies that have looked at the time

course of learning-induced intrinsic plasticity have found that these changes are short-lived (Moyer et al., 1996; Saar et al., 1998; Zelcer et al., 2006; Motanis et al., 2012). Second, learning-related changes in intrinsic excitability are measured using somatic current injections. Such global intrinsic plasticity is not synapse-specific, which limits its information storage capacity. It is unlikely that such plasticity would underlie a memory trace without quickly saturating the capacity for new memory formation (Moyer et al., 1996; Zhang and Linden, 2003). Thus, although intrinsic plasticity is learning-specific, its transient and global nature suggests that it is not likely to code for the memory itself.

An alternate explanation suggests that within the hippocampus the time course of enhanced intrinsic excitability reflects a period of time when these memories are undergoing systems memory consolidation (Moyer et al., 1996). Over time, as the memory trace is transferred to higher cortical structures (Kim et al., 1995; Nadel and Moscovitch, 1997), transient intrinsic plasticity could facilitate the consolidation of memory from hippocampus to higher cortical structures, such as the prefrontal cortex (Wierzynski et al., 2009). Such a system-level consolidation process requires reactivation and replay of neuronal ensembles underlying memories (Ji and Wilson, 2007; Girardeau et al., 2009), and enhanced excitability could facilitate these processes by lowering neuronal spike firing requirements. Thus, transient enhancement of excitability may facilitate processes that allow successful memory formation without directly encoding the memory.

In order to demonstrate a clear functional role for intrinsic plasticity, it is important to determine the necessity and sufficiency of intrinsic plasticity. This is still poorly understood. It is clear that in order to advance our understanding of exactly how intrinsic plasticity contributes to cognitive functions, including learning and memory, it is important

to understand how intrinsic plasticity influences synaptic and behavioral changes. That synaptic and intrinsic plasticity also share similar signaling pathways presents unique complications to understanding how these processes interact to influence behavioral plasticity.

Interactions between synaptic and intrinsic plasticity: Chicken or the egg?

Canadian psychologist Donald Hebb postulated that changes in connection strength could be the cellular mechanism for learning and memory (Hebb, 1949). Since then, synaptic plasticity has been demonstrated *in vitro* and *in vivo* in a variety of brain regions (for review, see Lynch, 2004). Numerous experiments not only suggest that synaptic plasticity shares some of the same cellular and molecular pathways as does learning, but they also suggest that blocking synaptic plasticity impairs learning (e.g., Morris et al., 1986; Whitlock et al., 2006; Gruart and Delgado-Garcia, 2007).

Synaptic plasticity is often accompanied by intrinsic plasticity. Stimulation protocols that induce synaptic plasticity also modulate intrinsic excitability. For example, LTP induction in hippocampal CA1 neurons leads to ES potentiation (Bliss and Lomo, 1973; Daoudal et al., 2002), increases local dendritic excitability, and facilitates bAPs (Frick et al., 2004). These changes are input specific, and are NMDA receptor-dependent (Daoudal et al., 2002; Frick et al., 2004). In addition, learning can facilitate synaptic transmission as well as intrinsic excitability in hippocampus, piriform cortex, infralimbic cortex and lateral amygdala (Moyer et al., 1996; Power et al., 1997; Saar et al., 1998, 2002; Johansen et al., 2011; Sepulveda-Orengo et al., 2013). Hence, synaptic and intrinsic plasticity can be induced by the same stimuli (e.g., learning or *in vitro* stimulation). Synaptic and intrinsic plasticity are also mediated by the same intracellular signaling

pathways. For example, both types of plasticity depend on the activation of NMDARs and some intracellular cascades mediated by kinases such as PKA (protein kinase A), PKC (protein kinase C), and Ca^{2+}-Calmodulin-dependent protein kinase II or CAMKII (Daoudal and Debanne, 2003). However, it is unclear which form of plasticity comes first, whether learning-related intrinsic plasticity facilitates learning-related synaptic plasticity, or whether both are induced in parallel. This remains an open question.

Several lines of evidence suggest that intrinsic plasticity can facilitate synaptic plasticity (also discussed above). Drugs or other treatments that reduce the AHP (and thus enhance intrinsic excitability) also facilitate the induction of LTP (Cohen and Abraham, 1996; Sah and Bekkers, 1996; Cohen et al., 1999). Such facilitation is also evident in absence of any changes in baseline synaptic transmission, indicating they do not result from better propagation of synaptic signal alone. Furthermore, enhancement of synaptic plasticity can be achieved by increasing intrinsic excitability via downregulating transient A-type K^+ channels (Hoffman and Johnston, 1998; Chen et al., 2006) as well as blocking of SK2 channels with BDNF or apamin (Kramar et al., 2004). Thus, enhanced intrinsic excitability favors induction of synaptic plasticity.

Finally, recent studies suggest that intrinsic plasticity can be induced independent of synaptic plasticity, indicating that intrinsic plasticity is generated before synaptic plasticity. For example, Barkai and colleagues (Cohen-Matsliah et al., 2010) demonstrated that a high frequency synaptic stimulation (e.g., 20 stimuli at 50 Hz) although not sufficient to induce LTP at 1 hour following stimulation, was capable of reducing the post-burst AHP in CA1 pyramidal neurons 3-6 hours later. Although the study did not measure LTP induction and AHP changes at the same points, these data suggest that intrinsic plasticity,

particularly AHP changes, may be induced at lower thresholds than synaptic plasticity. Taken together, these data are consistent with the hypothesis that learning-related intrinsic plasticity precedes synaptic changes and may be instrumental in reducing the threshold for synaptic plasticity enough to allow learning to occur.

That synaptic plasticity occurs after or in the presence of intrinsic plasticity (Saar et al., 1998, 1999, 2002; Saar et al., 2012; Song et al., 2012) further supports the hypothesis that intrinsic plasticity could lead to synaptic plasticity. Recently, data from our laboratory have also demonstrated that following trace fear conditioning, learning-related intrinsic excitability (size of AHP) and magnitude of LTP induction were significantly correlated with each other in good learners (Song et al., 2012). Thus, learning-related changes in neuronal intrinsic excitability predict the strength of subsequent LTP induction. It is possible that synaptic stimulation during learning results in a change in intrinsic excitability, and the magnitude of this plasticity predicts future synaptic plasticity, however, this still remains to be proven.

Metaplasticity: Change begets change

Consistent with the above discussed role for intrinsic plasticity, learning-specific changes in intrinsic excitability can also serve a metaplasticity function. Metaplasticity refers to the higher-order plasticity that affects synaptic or intrinsic plasticity. Metaplasticity develops as a result of a series of time-dependent events. That is, an initial priming event first induces physiological or biochemical changes in neurons or synapses that can modulate plasticity induced by a subsequent event (e.g. low- or high-frequency stimulation, or learning, see Abraham and Bear, 1996). Plasticity of intrinsic excitability

is one such mechanism that could underlie metaplasticity (see Abraham, 2008 for discussion of other mechanisms implicated in metaplasticity induction).

Evidence for the role of intrinsic plasticity as a metaplasticity mechanism comes from data indicating that the duration of learning-induced enhancements of intrinsic excitability overlaps with a time period of enhanced learning (Saar et al., 1998; Zelcer et al., 2006). Using an olfactory discrimination paradigm where water-deprived rats learn to choose a particular odor for a water reward, discrimination between the first odor pair takes ~7 days whereas learning to discriminate subsequent pairs occurs more rapidly (~ 1 day). This phenomenon is called "rule learning". Following acquisition of rule learning, intrinsic excitability of piriform cortical neurons is enhanced for up to 3 days and returns to baseline levels by 5 days. Remarkably, if training is suspended after acquisition of rule learning, rats display better discrimination for 1-2 additional days, which corresponds to the aforementioned period of enhanced excitability (Saar et al., 1998). Thus, the period of enhanced excitability of piriform cortex neurons matches the period during which rats display enhanced learning abilities.

Learning-induced intrinsic plasticity within a specific structure can also facilitate the acquisition of a different learning task dependent on that structure. Olfactory learning results in transient enhancement of hippocampal intrinsic excitability. During this period of enhanced neuronal excitability, acquisition of the hippocampus-dependent Morris water maze task is facilitated (Zelcer et al., 2006). Thus, intrinsic plasticity may be a mechanism that facilitates acquisition of new learning. It should be noted, however, that learning-related enhancement of learning has not been universally observed in all reported studies. For example, simultaneous but not consecutive training on two hippocampus-dependent

tasks, trace eyeblink conditioning and MWM, facilitates acquisition of the trace eyeblink but not the water maze task (Kuo et al., 2006). These data indicate that *learning-induced facilitation of learning* may depend on additional factors, including the nature and timing of the learning paradigms. Establishing the contingencies that allow for *learning-induced facilitation of learning* should be an exciting new avenue for memory researchers.

If modulation of intrinsic excitability affects learning, might it also explain individual differences or heterogeneity in learning ability? Or is the ability to modulate intrinsic excitability an index of intelligence? On more than one occasion, smart rodents have been demonstrated to have greater learning-induced enhancement of intrinsic excitability (Cohen-Matsliah et al., 2009; Song et al., 2012). For example, rats display heterogeneity in their ability to discriminate between odors in a simple maze (Cohen-Matsliah et al., 2009). Intrinsic excitability of piriform cortex neurons in fast performers (i.e., rats that display maximum efficacy right away on exposure to the maze) is greater relative to those from control rats. In contrast, piriform cortex neurons from slow performers are less excitable than control neurons. These differences are observed early on (12 h following maze learning) and subside as the performance of slow and fast learners converge. Furthermore, these performance differences are maintained on a complex olfactory discrimination maze (Cohen-Matsliah et al., 2009). Thus, fast performers appear to modulate intrinsic excitability sooner (12h) than slow performers (3 days). Similarly, following trace fear conditioning, CA1 neurons from rats classified as good learners had higher intrinsic excitability than those from poor learners or pseudoconditioned rats (Song et al., 2012). These data indicate that individual differences in learning ability could reflect a differential capacity to modulate intrinsic neuronal excitability.

If intrinsic excitability regulates the strength of learning, then it stands to reason that interventions capable of reducing the postburst AHP or otherwise enhancing intrinsic excitability should enhance learning and *vice versa* (also see Disterhoft and Oh, 2006a). Indeed, early studies demonstrated that nimodipine, an L-type Ca^{2+} channel blocker, enhanced hippocampal intrinsic excitability by reducing the postburst AHP (Moyer et al., 1992), and facilitated the acquisition of trace eye-blink conditioning in aging rabbits (Deyo et al., 1989). More recently, administration of the SK2 channel agonist NS309 was shown to not only increase the size of the medium postburst AHP, but also impair the ability of rats to learn trace eyeblink conditioning (McKay et al., 2012), suggesting that bidirectional modulation of the AHP can enhance or impair cognitive function.

Within amygdala, β-adrenergic receptor antagonists that modulate the AHP in LA neurons (Faber and Sah, 2002) block acquisition as well as reconsolidation of fear conditioning (Debiec and Ledoux, 2004; Bush et al., 2010; Muravieva and Alberini, 2010). Modulation of CREB expression within LA enhances both intrinsic excitability (Zhou et al., 2009) and fear learning (Han et al., 2007). Furthermore, enhanced noradrenergic and cholinergic transmission decreases the sAHP, increases spike firing, and enhances mPFC-dependent learning (Mueller et al., 2008; Santini and Porter, 2010; Santini et al., 2012). Taken together, these studies support a significant role for intrinsic plasticity in modulation of cognitive function and indicate that intrinsic plasticity is an important modulator of memory acquisition and strength.

Based on the discussion so far, it is reasonable to expect that transgenic manipulations that enhance intrinsic excitability would result in facilitated learning. Paradoxically, a few studies have observed the opposite effect. For example, deletion of L-

type voltage gated Ca^{2+} channel, $Ca_{V1.3}$ impairs long term consolidation of contextual fear memories (McKinney and Murphy, 2006). Interestingly, these knockout mice have enhanced neuronal excitability but impaired long term potentiation within amygdala (McKinney et al., 2009). Furthermore, deletion of an auxiliary potassium channel subunit Kvβ2 results in smaller sAHP and higher neuronal excitability within lateral amygdala neurons (Perkowski and Murphy, 2011). However, these mice are impaired on learning amygdala-dependent associative conditioning paradigms such as context and cued fear conditioning (Perkowski and Murphy, 2011). These data would indicate that greater intrinsic excitability can also impair rather than enhance learning. Interestingly, a recent study used an inducible CREB transgenic system to demonstrate that chronic (but not acute) upregulation of CREB results in impaired classical eyeblink conditioning (Gruart et al., 2012). CREB upregulation can enhance neuronal intrinsic excitability (Benito and Barco, 2010). It is likely that for optimal learning intrinsic excitability needs to be tightly controlled; i.e. smaller discrete and transient changes in intrinsic excitability may facilitate learning while chronic upregulation of excitability may be detrimental.

Memory allocation: Engram

A fundamental question in the search for the engram is "How are neurons selected for memory formation?" For example, although about 70% of LA neurons receive sensory inputs (Romanski et al., 1993), only ~20-30% of LA neurons display learning-related synaptic plasticity that is critical for fear memory recall (Rumpel et al., 2005). How are the neurons that are incorporated into the memory trace different from the other neurons that are left out?

It is possible that as per Lashley's law of equipotentiality (Lashley, 1929), all neurons within a structure are equally capable of being incorporated into the memory trace. This supports the probabilistic view of memory formation where all (or most) neurons within a structure receive information necessary for memory encoding; however only a few end up coding for the memory. The alternate view supports the deterministic nature of the memory trace and implies that the neurons incorporated into a memory trace are predetermined – perhaps on the basis of their synaptic inputs (Squire, 1987).

In recent years, there has been considerable evidence to support the probabilistic view of memory formation (Han et al., 2007; Won and Silva, 2008; Han et al., 2009). In a notable study, a viral vector was used to inject a wildtype or dominant negative form of CREB (cyclic AMP response element-binding protein) into either transgenic CREB-deficient or wildtype mice (Han et al., 2007). These viral transfections affected ~20% of LA neurons. Neurons with higher CREB levels were more likely to be incorporated into the memory trace than the surrounding, CREB-deficient neurons. Furthermore, selective ablation of the CREB overexpressing neurons, which were preferentially incorporated into the memory trace, abolished the fear memory (Han et al., 2009). These data suggest that all LA neurons are equally likely to be incorporated into the memory trace, but certain factors e.g. CREB levels bias neurons to be preferentially recruited.

How does CREB upregulation bias memory allocation? It is well established that CREB expression can modulate neuronal excitability (Benito and Barco, 2010). Upregulation of CREB expression within a subset of LA neurons enhances their intrinsic excitability and leads to greater fear conditioning-related LTP in these neurons (Zhou et al., 2009). Thus, it is likely that enhanced excitability facilitates synaptic plasticity, which

could bias the allocation of new memories to a subset of neurons. Further support for the probabilistic nature of the memory trace and the role of intrinsic excitability in biasing this trace come from a study manipulating intrinsic excitability of hippocampal CA1 neurons during spatial exploration (Lee et al., 2012). In this study, whole-cell patch clamp recordings were obtained from CA1 neurons while the animals explored a novel environment. Hippocampal CA1 neurons represent spatial information by environment-specific spiking activity such that these place cells fire in a particular place in the animal's environment (O'Keefe and Dostrovsky, 1971). Remarkably, increasing the excitability of a neuron by injecting a small, depolarizing current injection turns a previously silent cell into a place cell. In contrast, reducing the excitability by injecting a small hyperpolarizing current changed a place cell to a silent cell (Lee et al., 2012). These data indicate that even silent CA1 neurons receive spatial information and that this information can be uncovered by enhancement of its intrinsic excitability.

Memory allocation mechanisms such as CREB or intrinsic excitability upregulation appear to be a brain-wide phenomenon. Upregulation of CREB biases spatial memories of Morris water maze to retrosplenial cortex neurons such that inactivation of these neurons impairs memory formation (Czajkowski et al., 2014). Same is true for conditioned taste aversion memory in the insular cortex and conditioned place preference memory in the lateral amygdala (Sano et al., 2014). The aforementioned studies employed CREB upregulation for biasing memory allocation. Phosphorylation of CREB is one of the earliest plasticity permissive events within a neuron; CREB phosphorylation can lead to a cascade of transcriptional and translational changes including the expression of immediate early genes. Although CREB’s central role in neuronal plasticity makes it an attractive candidate,

memory allocation may not be “CREB-centric”. Neuronal excitability can be modified in numerous ways. A recent study from Josselyn and colleagues demonstrated that bidirectional modification of excitability in a subset of neurons can enhance or impair the fear memory. Interestingly, activating these more excitable neurons using a DREADD (Designer Receptors Exclusively Activated by Designer Drugs) system was also sufficient to allow expression of the memory (Yiu et al., 2014). Together, these data demonstrate that pre-training levels of intrinsic excitability may prime neurons for successful information processing and storage.

If pre-training levels of intrinsic excitability are important, then endogenous factors that allow intrinsic plasticity would influence learning and memory. The levels of CREB, especially the active phosphorylated form of CREB, are dependent upon the previous activity of a neuron (Sheng et al., 1991). The activity-dependent modulation of CREB levels within neurons could determine the intrinsic excitability, and thus the probability with which these neurons are incorporated into the memory trace. This would mean that neurons which were previously active because they were a part of the original memory trace are more likely to be a part of a new memory trace (*see Figure 2*). Similarly, if intrinsic excitability can bias the recruitment of neurons to become part of a memory circuit, then what are the implications of learning-related intrinsic plasticity for memory allocation? For example, fear conditioning enhances intrinsic excitability within a subset of LA neurons (see preliminary data). This demonstrates that learning-related intrinsic plasticity can be restricted to a small subset of neurons within a structure. It is likely that these neurons displaying enhanced excitability have a lower threshold for synaptic plasticity and thus, are also especially likely to encode a subsequent memory trace.

Keeping in mind that learning-related intrinsic plasticity is usually transient, such learning-related bias in memory allocation should also be transient.

Unresolved questions

The data reviewed so far indicate that behaviorally, pharmacologically and genetically induced enhancement of intrinsic excitability can enhance learning and strengthen memories. *In vitro*, pharmacological enhancement of neuronal excitability can also improve basal synaptic transmission (Sah and Bekkers, 1996) and facilitate synaptic plasticity by lowering the threshold for induction of LTP (Sah and Bekkers, 1996; Faber et al., 2005; Zaitsev and Anwyl, 2012). Based on these data, we can put together a working model for the role of intrinsic excitability in memory formation (*see Figure 2*). During learning, natural variations in intrinsic excitability can bias the threshold for induction of synaptic plasticity and hence bias memory allocation to these more excitable neurons (Silva et al., 2009). Thus, more excitable neurons get allocated to the memory trace or the engram (Han et al., 2007; Won and Silva, 2008; Han et al., 2009; Silva et al., 2009). Following and/or during memory acquisition, learning-related signaling cascades (e.g. Ca^{2+} influx and CREB phosphorylation) can result in *de novo* protein synthesis and/or post translational changes that enhance intrinsic excitability by influencing the function of various ion channels in the neuronal ensembles constituting the engram (Sehgal et al., 2013). This learning-related modulation of intrinsic excitability can in turn bias or drive the allocation of a future memory into neuronal ensemble storing the original memory by lowering the threshold for induction of synaptic plasticity.

Several recent studies have demonstrated that various aspects of this model are indeed accurate. This model of memory formation also allows us to make several predictions that are henceforth untested:

a) Learning-related intrinsic plasticity is restricted to neurons that constitute the memory engram.

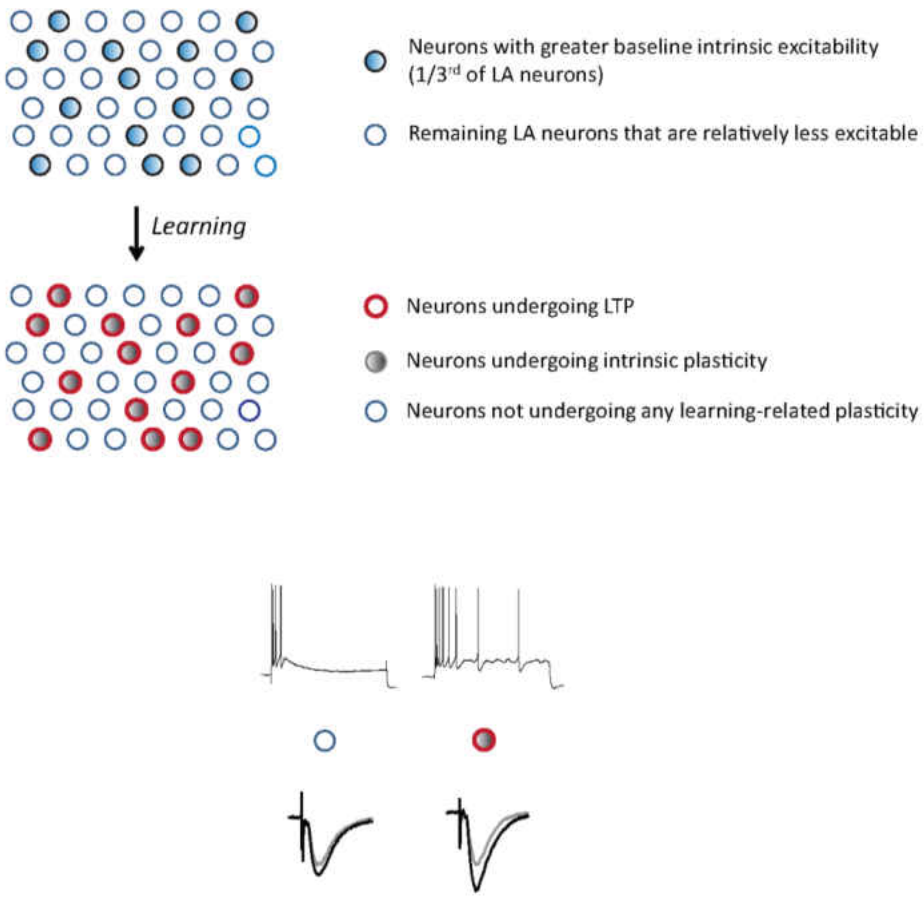
b) Learning-related intrinsic plasticity can modulate the threshold for synaptic plasticity in these neurons influencing future behavioral plasticity.

c) Learning-related enhancement of intrinsic excitability is transient and follows the same time course as the learning-related modulation of synaptic or behavioral plasticity.

The current dissertation project aims to test some of these predictions using an amygdala-dependent fear conditioning paradigm.

Figure 2. Proposed Model: Intrinsic excitability can effect memory allocation by facilitating synaptic plasticity. Naturally occurring variations in intrinsic excitability could result in lowered threshold for synaptic plasticity and bias these neurons to be preferentially recruited for memory formation. Since, learning, and intrinsic and synaptic plasticity depend upon the same signaling cascades, it is likely that learning-related synaptic and intrinsic plasticity is evident in the same neuronal population. Finally, learning-related increase in intrinsic excitability could facilitate future synaptic plasticity by reducing the threshold for LTP induction. These neurons may be further predisposed to encode future memories.

Figure 2



Facilitated LTP induction in excitable LA neurons

Fear conditioning and amygdala

As previously mentioned, in fear conditioning a neutral stimulus e.g. tone, is paired with an aversive footshock. The neural correlates of fear conditioning memories have been extensively investigated, making it an excellent model system to study factors affecting learning and memory.

LTP: a cellular correlate for learning. Bliss and Lomo first described LTP within hippocampus in 1973 (Bliss and Lomo, 1973). The LTP phenomenon immediately captured the attention of memory researchers due to the widely popular belief that learning and memory is mediated by activity-dependent changes in the strength of synaptic connections (Hebb, 1949). Additional research demonstrated that LTP is long-lasting, associative and input-specific; properties that make it an especially attractive mechanism for memory storage (Bliss and Collingridge, 1993). Since then, many of the physiological and molecular changes underlying LTP have been extensively characterized with much of this research focusing on the hippocampal trisynaptic circuit.

Despite the considerable degree of success in elucidating the molecular underpinnings of LTP (especially in the hippocampus), it took several decades before a direct proof that LTP underlies learning and memory was available. This is in part because relatively little was known about the exact synaptic connections that encode long term memory. For example, to this day, the precise role of hippocampus in long-term memory is relatively uncertain, let alone the identity of the exact synaptic connection/s that underlie these functions (Sigurdsson et al., 2007). These difficulties prompted the use of simpler models of learning in order to understand the relationship between memory and LTP. The merit of such an approach was confirmed when researchers from 2 groups simultaneously

and independently demonstrated that simple associative learning such as fear conditioning causes LTP-like changes in underlying synaptic pathways (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997). More pertinent to the current discussion, the same argument is also true regarding the role of intrinsic plasticity in learning and memory. I believe that using a simple but rather well characterized memory circuit such as the one underlying auditory fear conditioning can be instrumental in determining the precise relationship between intrinsic plasticity, memory and synaptic plasticity.

An extensive amount of research has demonstrated that the amygdala is a structure critical for fear memories (LeDoux, 2000; Johansen et al., 2011). Within the amygdala, polymodal sensory inputs first converge within the lateral nucleus (LA). The information is then processed and relayed onto various output structures including the central nucleus, resulting in different aspects of the behavioral response (LeDoux, 2000). Thus, LA acts as the gateway to the rest of the amygdala. It follows that plasticity (intrinsic or synaptic) within LA is uniquely capable of modulating behavioral responses to emotional stimuli.

As per the cellular hypothesis of fear conditioning, learning results when the synaptic strength of the pathways that relay the auditory CS information increases (Blair et al., 2001; Schafe et al., 2001; Sigurdsson et al., 2007). Prior to fear conditioning, the CS inputs are weak (akin to the weaker pathway in an LTP experiment). During conditioning, the weak CS inputs converge on the cells that also receive much stronger US inputs. As the weak CS inputs are active during the strong US-induced depolarization, the CS pathway to LA is potentiated driving the fear response. Note that this cellular hypothesis of fear conditioning is similar to an associative LTP experiment with CS and US pathways representing the weak and strong pathways respectively.

During fear conditioning, the auditory inputs reach LA from two pathways; the thalamic inputs from medial geniculate nucleus via the internal capsule (T-LA pathway) (LeDoux et al., 1984) and the inputs from auditory cortex present via the external capsule (EC-LA pathway, Romanski and LeDoux, 1993). Each of these pathways is capable of LTP using *in vitro* slice electrophysiology techniques (Chapman et al., 1990; Chapman and Bellavance, 1992; Huang and Gean, 1994) as well as following *in vivo* tetanic stimulation in anaesthetized or awake-behaving animals (Clugnet and LeDoux, 1990; Rogan and LeDoux, 1995; Yaniv et al., 2001; Doyere et al., 2003). *In vitro*, LTP has been induced by tetanic stimulation of the thalamic inputs (Huang and Gean, 1994) or the external capsule (Chapman and Bellavance, 1992). Thus, both auditory input pathways to LA can undergo synaptic potentiation.

LTP can be induced in LA neurons following a 'pairing' protocol where a weak presynaptic stimulation is paired with a strong postsynaptic depolarization (Weisskopf et al., 1999; Huang et al., 2000; Tsvetkov et al., 2004; Humeau et al., 2005). This is relevant as this pattern of stimulation is similar to the pattern of inputs during fear conditioning where a weak CS input is followed by a stronger US input onto the LA neurons. Furthermore, LTP induction using this protocol is sensitive to the same stimulus parameters as fear conditioning (Bauer et al., 2001). In addition to the contiguity, contingency of the 2 stimuli determines the strength of LTP. For example, unpaired US presentations in addition to CS-US pairings reduces the contingency while maintaining the contiguity between CS and US presentation. In this case, introduction of unpaired US presentations reduces the contingency and thus the strength of fear conditioning. Similarly, addition of unpaired postsynaptic depolarization reduces the magnitude of LTP induction (Bauer et al.,

2001). Finally, in addition to being associative, LTP in LA is input-specific and long lasting (up to 6 days, Doyere et al., 2003; Tsvetkov et al., 2004). Thus, LTP of auditory inputs to the LA neurons possesses characteristics that make it a suitable mechanism for storage of long-term memories.

Indeed, two parallel studies in 1997 demonstrated that fear conditioning leads to LTP-like potentiation of the T-LA pathway. Using an *in vivo* approach, Joseph LeDoux's group demonstrated that the auditory-evoked field responses are enhanced within LA neurons as the conditioned fear response develops (Rogan et al., 1997). Around the same time, *in vitro* studies from Patricia Shinnick-Gallagher's group demonstrated that fear conditioning enhances AMPA receptor dependent currents and reduces paired pulse facilitation (PPF), both of which are electrophysiological correlates of LTP at T-LA synapses (McKernan and Shinnick-Gallagher, 1997). In the same year, enhanced synaptic transmission within CA1 hippocampal neurons was demonstrated following trace eyeblink conditioning (Power et al., 1997). Subsequently, LTP-like changes were also demonstrated in the EC-LA pathway (Schroeder and Shinnick-Gallagher, 2005). Taken together, these results were the first demonstrations that learning is accompanied LTP-like changes in the underlying neuronal circuit strengthening the link between LTP and long-term memory storage.

Less direct evidence for the link between LTP and long-term memory comes from studies indicating an occlusion of LTP induction following learning (Tsvetkov et al., 2002; Schroeder and Shinnick-Gallagher, 2004, 2005; Whitlock et al., 2006). The occlusion of LTP-induction has been interpreted as a proof that learning induces LTP-like changes which reduces the capacity of future synaptic plasticity as the synaptic strength is saturated

(or close to saturation). Such occlusion of LTP induction has been demonstrated at the EC-LA synaptic pathway following fear conditioning (Tsvetkov et al., 2002; Schroeder and Shinnick-Gallagher, 2004, 2005) and can last for up to 10 days (Schroeder and Shinnick-Gallagher, 2005). Subsequently, occlusion of LTP induction was also demonstrated in hippocampal CA1 neurons following inhibitory avoidance (Whitlock et al., 2006). Thus, the magnitude of LTP induction can be affected by prior learning.

In addition to occlusion of LTP, learning can also affect LTP-induction in other ways. For example, in addition to demonstrating LTP occlusion at EC-LA synapses Shinnick-Gallagher et al. also demonstrated that a LFS (low frequency stimulation) is capable of inducing LTP in the EC-LA pathway of fear conditioned but not control animals (Schroeder and Shinnick-Gallagher, 2004). In addition, we have recently demonstrated that following trace fear conditioning, good learners show facilitated LTP induction at the hippocampus CA1 synapses (Song et al., 2012). Taken together, these data indicate that not only is learning accompanied by synaptic plasticity but learning can also impact the capacity for future synaptic plasticity. That said, the reasons for post-learning occlusion of LTP in some cases and LTP facilitation in others are still unclear.

These issues are especially relevant to the current proposal. Given the premise that memories are encoded as synaptic changes, long-lasting occlusion of synaptic plasticity following learning may indicate a reduced capacity of learning. Contrary to this, we have discussed evidence that learning-related changes in intrinsic excitability facilitate new memory formation (Kuo et al., 2006; Zelcer et al., 2006; Cohen-Matsliah et al., 2009). This facilitation of learning can be explained by reduced threshold for synaptic plasticity following an increase in neuronal intrinsic excitability. In fact, a situation where learning

leads to occlusion as well as facilitation of LTP can be envisioned. Given that learning-related synaptic plasticity only occurs at a small percentage of synapses within a neuron, it is possible that these synapses are unable to undergo further potentiation. This would explain why the overall capacity for potentiation of synaptic transmission is reduced. However, it is also possible that learning-induced intrinsic plasticity reduces the threshold for synaptic plasticity, but these changes can only be evident at the synapses that were previously unmodified. As a result, the threshold for LTP is reduced but the magnitude of LTP induction following a saturating induction protocol is also reduced. Long term, homeostatic plasticity can ensure that the overall synaptic and intrinsic properties of the neurons remain stable.

Experimental support for this hypothesis comes from the fact that most studies demonstrating LTP occlusion use such saturating protocols (Schroeder and Shinnick-Gallagher, 2004, 2005; Hong et al., 2012). Furthermore, those studies demonstrating facilitation of LTP induction use either a sub-threshold or non-saturating protocol (Schroeder and Shinnick-Gallagher, 2004; Song et al., 2012).

Mechanism of learning-related LTP in LA. Many different induction protocols have been used to induce LTP at the auditory inputs to LA neurons. These include the EC-LA and T-LA pathways (as discussed in the previous section). The general theme that emerges is that variations in the LTP induction protocol also lead to variations in molecular mechanisms underlying LTP induction (Bauer et al., 2002).

LTP at the T-LA pathway following learning is accompanied by a decrease in the paired pulse ratio, an indication that an increase in the transmitter release probabilities at the presynaptic end is involved (McKernan and Shinnick-Gallagher, 1997). In addition to

presynaptic mechanisms, postsynaptic mechanisms that affect synaptic transmission have also been demonstrated (Rumpel et al., 2005). For example, Malinow and colleagues elegantly demonstrated that fear conditioning leads to an increase in GluR1 AMPA receptor insertion at the T-LA synapses. Using viral transfection of GluR1 transgenes, they demonstrated that 36 % of the LA neurons undergo synaptic plasticity. Furthermore, using a dominant negative form of this transgene, they were able to demonstrate that blocking such plasticity in as few as 10- 20% of LA neurons can impair recall of the fear memory (Rumpel et al., 2005). These data indicate that synaptic plasticity is critical for fear memory formation and the changes that allow memory formation only occur in a subset of neurons — these neurons may constitute the *memory engram*.

PROPOSED EXPERIMENTS

Our current experiments aim to take advantage of the well-established and critical role of LA in fear memory formation to determine the mechanism by which intrinsic excitability relates to synaptic plasticity. Intrinsic plasticity has been demonstrated in the hippocampus and cortical structures following learning (Moyer et al., 1996; Saar et al., 1998; Santini et al., 2008; Song et al., 2012). Although important for memory formation, the exact role of these structures in the memory of the relevant tasks is unclear. In contrast, LA is likely the structure where fear memories are stored (Han et al., 2009). More importantly, neural correlates of synaptic (but not intrinsic) plasticity within LA have been extensively studied (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997; Rumpel et al., 2005).

We have reviewed evidence that intrinsic plasticity can modulate the strength as well as the allocation of memories. Genetic and pharmacological manipulation of intrinsic excitability can also enhance synaptic plasticity. However, it is unknown whether behaviorally-induced increases in intrinsic excitability can impact synaptic plasticity. Preliminary data from our lab demonstrates that fear conditioning results in enhanced intrinsic excitability within a subset of LA neurons. The proportions of neurons that display intrinsic plasticity (~ 30%) are similar to those that are believed to constitute the memory engram as evident from measures of synaptic plasticity, immediate early gene expression, and CREB overexpression and ablation studies (Rumpel et al., 2005; Han et al., 2007; Han et al., 2009; Zhou et al., 2009). The implications of such learning-induced intrinsic plasticity in LA neurons are still unclear.

It is likely that A) enhanced intrinsic excitability is downstream of similar mechanisms that result in storage of long-term memories and thus, would be evident in the same neurons undergoing synaptic plasticity. B) Since enhanced intrinsic excitability facilitates LTP induction *in vitro*, LA neurons that display higher intrinsic excitability following fear conditioning should display greater LTP induction. If this hypothesis were true, it would suggest that neurons undergoing learning-induced enhanced excitability are capable of undergoing greater LTP induction (induced behaviorally or through *in vitro* stimulation) and may be more likely to participate in future memory formation.

Aim 1. Determine the time course of learning-related changes in intrinsic plasticity.

Fear conditioning causes time-dependent changes in intrinsic excitability measures (the AHP and spike frequency adaptation) within LA neurons (see preliminary data). Based on previously published data that learning-induced changes in intrinsic excitability are transient in hippocampal and piriform cortex neurons (Moyer et al., 1996; Zelcer et al., 2006; Cohen-Matsliah et al., 2009), the working hypothesis for this aim is that learning-related intrinsic plasticity within LA neurons is transient and will last for days or weeks.

Aim 2. Determine whether learning induces LTP and intrinsic plasticity within the same LA neurons.

Fear conditioning requires LTP by insertion of GluR1 containing AMPARs in one-third LA neurons (Rumpel et al., 2005). Based on the published and preliminary data that learning-induced intrinsic plasticity is also evident in a similar proportion of neurons and shares many of the same underlying pathways, our working hypothesis is that increased intrinsic excitability and learning-related LTP occurs in overlapping populations.

Measurement of intrinsic excitability and AMPA-EPSCs from the same neurons would determine whether intrinsic plasticity is limited to the fear memory encoding neurons.

SIGNIFICANCE OF THE PROPOSED EXPERIMENTS

Successful completion of this work would determine whether learning-related intrinsic plasticity occurs in the memory encoding neurons. In addition, it would determine whether behaviorally-induced intrinsic plasticity could enhance subsequent plasticity. These findings would allow determination of mechanisms that underlie learning-induced facilitation of learning. Ultimately, these data can guide efforts that involve modulation of memory formation including 1) preventive and therapeutic approaches combating cognitive decline during aging, and 2) strengthening of exposure based therapies to ameliorate maladaptive memories such as posttraumatic stress disorder.

PRELIMINARY DATA

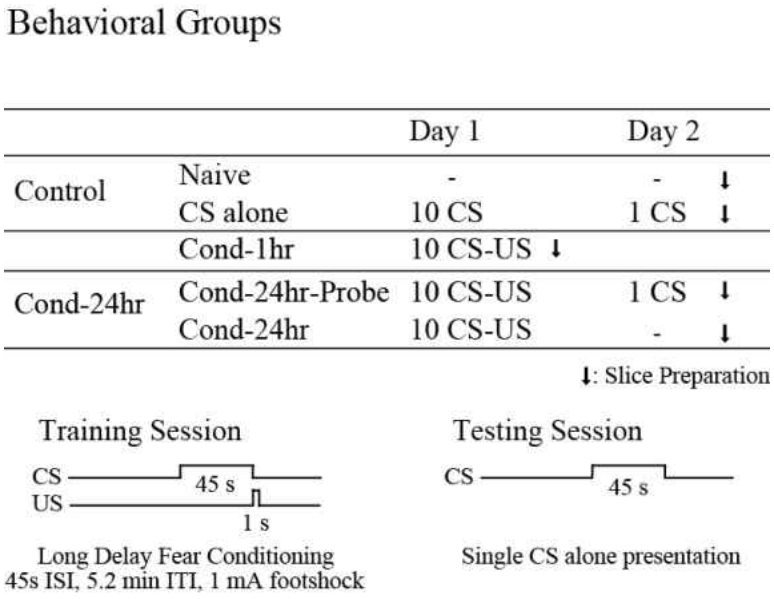
We investigated whether learning causes modulation of intrinsic excitability of LA neurons using fear conditioning, an amygdala-dependent task. To assess any time-dependent effects of fear learning on intrinsic excitability, acute brain slices were collected either immediately or 24 hr following fear conditioning, and intracellular recordings were obtained from LA neurons. Our data suggest that fear learning enhances intrinsic excitability in a subset of LA neurons in a time-dependent manner.

Rats were trained on an auditory fear conditioning paradigm. Specifically, we used a long-delay fear conditioning paradigm as we have previously demonstrated that this training protocol leads to high levels of cued fear but very low levels of background context fear (Detert et al., 2008). This allowed us to study the effects of cued fear while minimizing the confounding effects of context fear learning. Additionally, a subset of rats was tested the following day to ensure fear memory recall was consistent with that previously observed (see *Figure 3 for the experimental setup*).

To assess memory, the percentage of time spent freezing during the baseline and the CS presentations for the training and testing sessions was measured. During behavioral training (day 1), rats from the two conditioned groups (Cond-1hr and Cond-24hr; probed and non-probed rats) showed comparable acquisition of fear conditioning while the levels of freezing for CS-alone rats remained low (see *Figure 4a*). Average percent freezing for Cond-1hr and Cond-24hr were not significantly different ($p = .36$). An ANOVA and post hoc analysis confirmed that while baseline and CS trial 1 freezing were not significantly

Figure 3. Experimental design used to study learning-related changes in the amygdala. *Behavioral groups.* Rats were divided into five groups: two control groups [naïve (N = 12) and CS-alone (N = 4)] and three experimental groups [Cond-1hr (N = 8), Cond-24hr-Probe (N = 9), and Cond-24hr (N = 3)] that were trained using a long-delay fear conditioning protocol. Adapted from Sehgal, Ehlers & Moyer, 2014.

Figure 3


Behavioral Groups
Day 1
Day 2
Control
Naive
-
-
CS alone
10 CS
1 CS
Cond-1hr
10 CS-US
Cond-24hr
Cond-24hr-Probe
10 CS-US
1 CS
Cond-24hr
10 CS-US
-
: Slice Preparation
Training Session
CS
US
45 s
1 s
Long Delay Fear Conditioning
45s ISI, 5.2 min ITI, 1 mA footshock
Testing Session
CS
45 s
Single CS alone presentation

different between groups, CS-alone rats froze significantly less than Cond-1hr and Cond-24hr rats for trials 2-7 (all p-values < 0.05). Thus, analysis of percent freezing during training session indicates both Cond-1hr and Cond-24hr groups acquired fear conditioning but the CS-alone group did not.

Behavioral testing

Analyses of percent time freezing during probe test on day 2 revealed that both CS-alone and Cond-24hr-Probe rats froze little during baseline (see *Figure 4b*). While CS-alone rats expressed little freezing during CS presentation (0.59 ± 0.59%), Cond-24hr-Probe rats froze significantly more during the CS probe (72.84 ± 7.74%) as compared to baseline [$t(3) = -1.0$, $p = .39$ and $t(8) = -9.3$, $p < 0.001$ respectively]. Furthermore, the time spent freezing during CS probe was significantly greater for the Cond-24hr-Probe rats than the CS-alone rats [$t(8.09) = -9.3$, $p < 0.001$]. These data indicate that conditioned but not CS-alone rats display robust learning-specific fear memory to the CS presentation.

No significant differences were found between the naive and CS-alone groups on any measure of intrinsic excitability. Thus, the data from these 2 groups were combined into a Control group. Similarly, the Cond-24hr and Cond-24hr-Probe rats did not differ significantly during acquisition of fear conditioning as well as on any measure of intrinsic excitability. Thus, data from Cond-24hr and Cond-24hr-Probe rats were combined and represented as the Cond-24hr group.

Figure 4. Rats readily acquire long-delay fear conditioning. *a. Training*. During the fear conditioning session on day 1, CS-alone rats (N = 4) froze significantly less than Cond-1hr (N= 8) and Cond-24hr (N = 12) rats. The Cond-1hr and Cond-24hr rats froze at comparable levels. The Cond-24hr group includes both probed and not probed rats. The high average freezing for 10^{th} trial for CS alone group (43.75 %) is likely immobility due to time in the chamber (~1 hr) as the average freezing for the 45 s period preceding the 10^{th} CS presentation was also relatively high (47.19 %) *b. Testing*. During the probe test on day 2, Cond-24hr-Probe rats (N = 9) froze significantly more than CS-alone rats (N = 4). Baseline freezing was low for both CS-alone and Cond-24hr-Probe rats. Asterisk (*) indicates $p < 0.05$ relative to baseline freezing. Abbreviations: B, baseline freezing; CS, freezing during CS presentation. Adapted from Sehgal, Ehlers & Moyer, 2014.

Figure 4

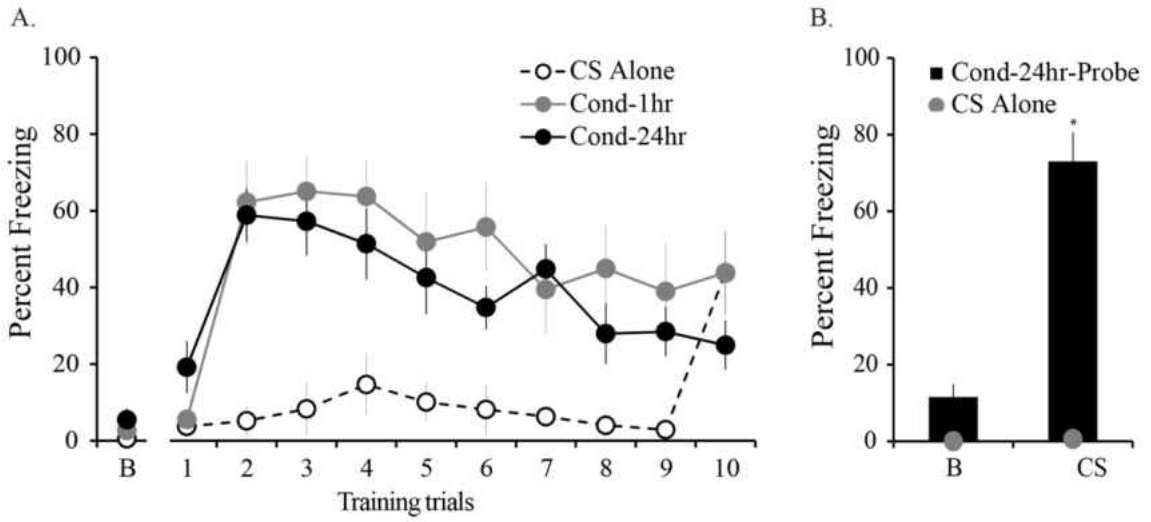
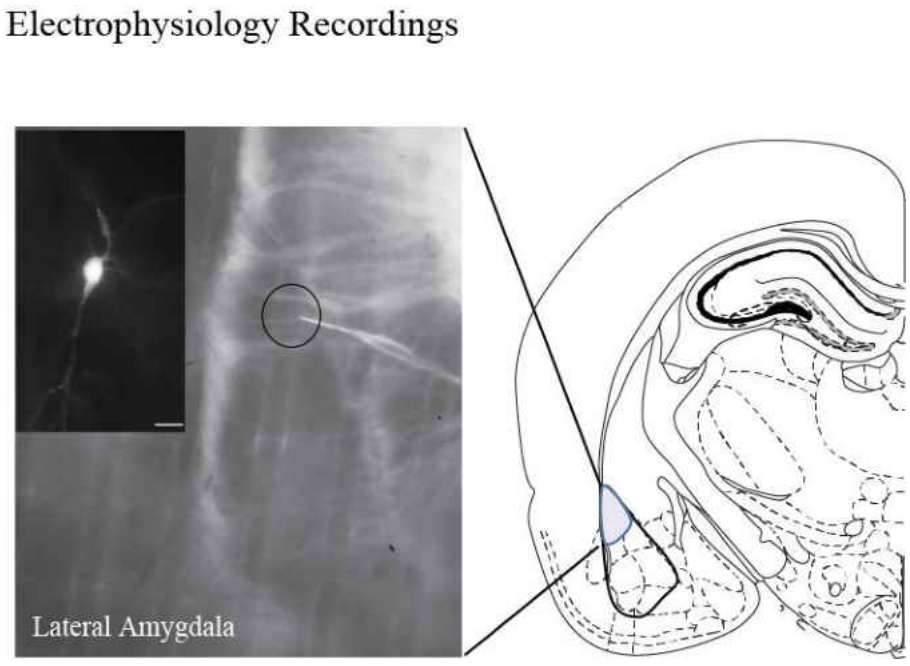

A.
Percent Freezing
100
80
60
40
20
0
B
1
2
3
4
5
6
7
8
9
10
Training trials
CS Alone
Cond-1hr
Cond-24hr
B.
Percent Freezing
100
80
60
40
20
0
B
CS
Cond-24hr-Probe
CS Alone
*

Figure 5. Electrophysiological Recordings. Right panel is a schematic of a typical coronal brain slice showing the location of the lateral amygdala (LA). Left panel is a photomicrograph of a brain slice showing the location of a typical recording electrode (inset is a neurobiotin-filled LA pyramidal neuron; scale 40 μm). Adapted from Sehgal, Ehlers & Moyer, 2014.

Figure 5



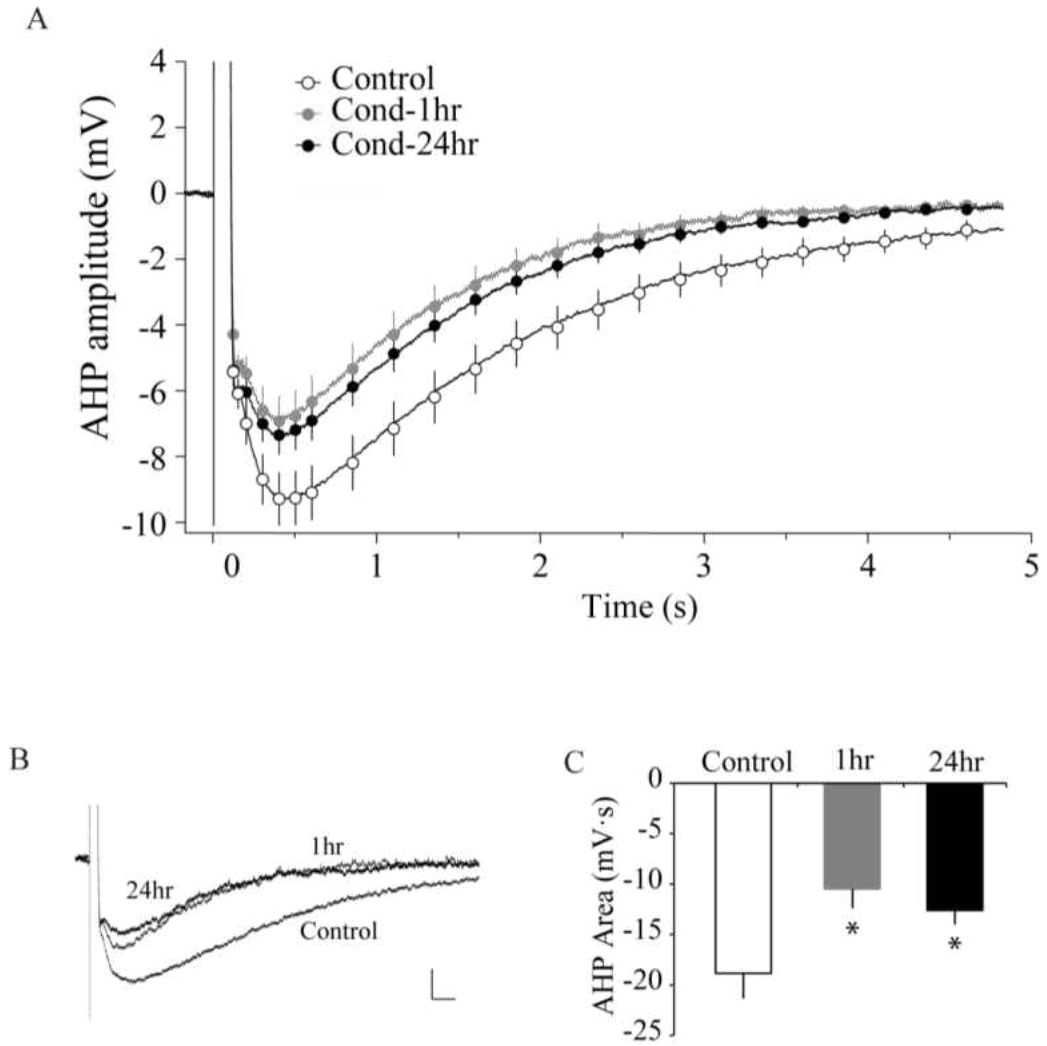
Fear conditioning reduces postburst afterhyperpolarization

The postburst AHP is a measure of intrinsic excitability and is mainly composed of multiple calcium-dependent potassium currents that make up the fast, medium and slow AHP (Madison and Nicoll, 1984; Storm, 1990). Intracellular recordings from LA neurons revealed that the AHP amplitude was significantly different between groups from 0.5s to 4s after current offset (all values, $p < 0.05$; except $p = 0.053$ at 3.5s; see *Figure 6a and 6b*). Post hoc comparisons revealed that the AHP amplitude was significantly reduced in LA neurons from both the Cond-1hr as well as the Cond-24hr groups relative to the control rats (all values $p < 0.05$). Similar group effects on postburst AHP area were also observed [$F(2, 66) = 4.1$, $p < 0.05$; see *Figure 6c* and *Table 1*]. Post hoc comparisons confirmed that area of the postburst AHP is significantly reduced in LA neurons from the Cond-1hr and the Cond-24hr rats in comparison to Control rats ($p < 0.05$). The sAHP in LA neurons corresponds to the period of several hundred milliseconds to up to 6s following current offset (Faber and Sah, 2002). These results indicate that fear conditioning leads to a reduction in the sAHP within 1 hr of fear conditioning in LA neurons, and that these changes persist for up to 24 hr.

In addition to the sAHP, fast and medium AHP currents also contribute to neuronal excitability. In LA neurons, medium AHP is evident during the period of tens to few hundred milliseconds following action potential discharge (Faber and Sah, 2002). Although a strong trend towards reduced medium AHP in neurons from Cond-1hr and Cond-24hr groups relative to Control was observed, these changes did not reach statistical significance ($p < 0.1$, 200-500ms following current offset; see *Figure 6a*). We also measured the fast AHP amplitude following the first AP during the AHP measurement and

Figure 6. Long-delay fear conditioning reduces the postburst AHP in lateral amygdala pyramidal neurons. *a. Time course of the postburst AHP amplitude.* Neurons from Cond-1hr (n = 13) and Cond-24hr rats (n = 28) had a significantly smaller AHP relative to Control rats (n = 28) at all points between 0.5 and 4.0 s following current offset. *b. Representative traces of the postburst AHP.* Voltage sweeps illustrate LA pyramidal neurons from fear conditioned rats have smaller AHPs compared to control rats (scale: 1 mV, 100 ms). C: Area of the postburst AHP. Bar graphs illustrate that the AHP area is significantly smaller in neurons from Cond-1hr and Cond-24hr compared to control rats. Asterisk (*) indicates $p < 0.05$ relative to LA neurons from Control rats. Adapted from Sehgal, Ehlers & Moyer, 2014.

Figure 6


A
Control
Cond-1hr
Cond-24hr
AHP amplitude (mV)
Time (s)
B
1hr
24hr
Control
C
Control
1hr
24hr
AHP Area (mV·s)
*
*

found no significant changes in fast AHP amplitude between groups [$F(2, 66) = 2.37$, $p = 0.1$, *see Table 1*]. Thus, fear conditioning reduces sAHP but not fast AHP and may lead to a reduction in medium AHP as well.

Fear conditioning reduces spike frequency adaptation

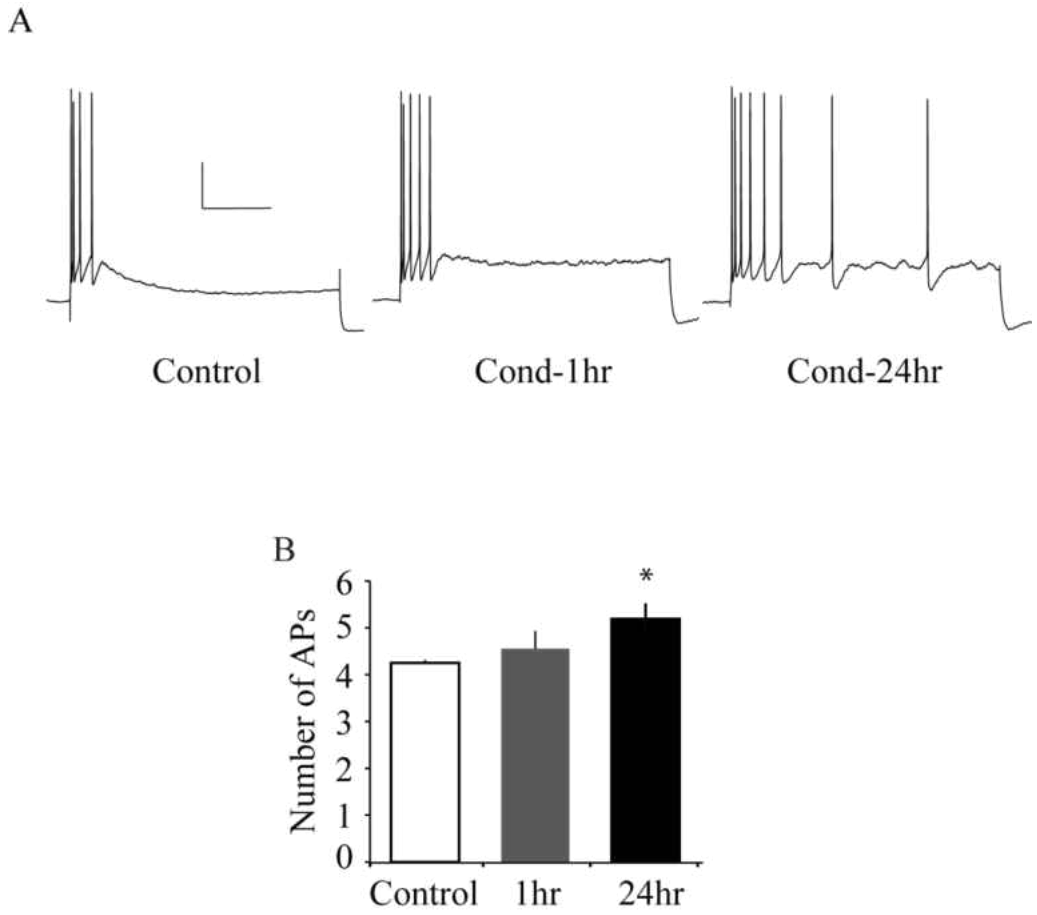
Changes in the postburst AHP are often accompanied by changes in spike frequency adaptation (Madison and Nicoll, 1984; Faber et al., 2001). To quantify spike frequency adaptation, the current used for the postburst AHP measurement was extended over a 1s duration and the number of action potentials (APs) elicited were counted. A one-way ANOVA revealed a significant effect of group for spike frequency adaptation [$F(2, 66) = 3.8$, $p < 0.05$; see *Figure 7* and *Table 1*]. Post hoc tests indicated that LA neurons from Cond-24hr rats fired significantly more APs than those from Control rats ($p < 0.01$). Surprisingly, the number of APs elicited in LA neurons from Cond-1hr rats did not differ significantly when compared with those from Control or Cond-24hr rats. These data indicate that fear conditioning-induced reductions in spike frequency adaptation of LA neurons are time-dependent and follow a different time course than sAHP changes.

Relationship between postburst AHP and spike frequency adaptation

The time course over which learning-related changes in spike frequency adaptation emerge is slower than that of the postburst AHP (compare *Figures 6 and 7*), suggesting dissociation between the two measures of intrinsic excitability. To further explore the relationship between these two measures of intrinsic excitability, the AHP amplitude was plotted as a function of the number of APs elicited during a long current injection (see *Figure 8*). For neurons from Control and Cond-1hr rats, no significant correlation was

Figure 7. Long-delay fear conditioning reduces spike frequency adaptation in a time-dependent manner. *a. Representative traces illustrating spike frequency adaptation in response to a prolonged current injection.* Note that LA pyramidal neurons from Control rats (n = 28) but not Cond-24hr rats (n = 28) display remarkable spike frequency adaptation. Scale: 20 mV, 0.2s. *b. Average number of action potentials (APs) during prolonged current injection.* LA pyramidal neurons from Cond-24hr rats fire significantly more APs than those from Control rats. Neurons from Cond-1hr rats (n = 13) are not significantly different from any other group. Asterisk (*) indicates $p < 0.05$ relative to LA neurons from Control rats. Adapted from Sehgal, Ehlers & Moyer, 2014.

Figure 7

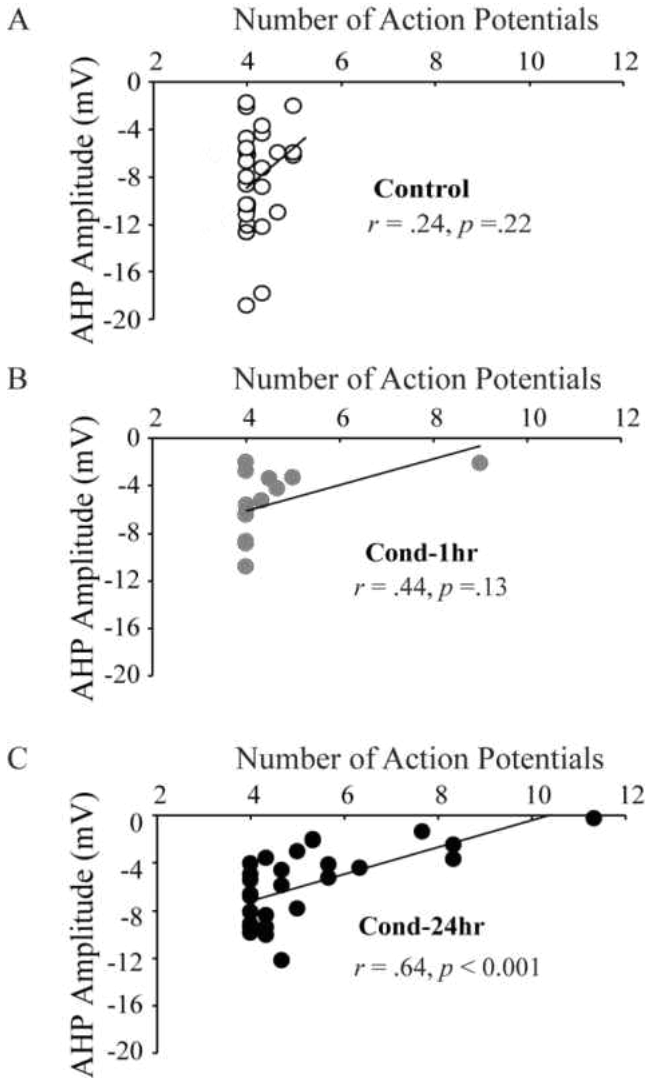

A
Control
Cond-1hr
Cond-24hr
B
Number of APs
0
1
2
3
4
5
6
*
Control
1hr
24hr

observed between the AHP amplitudes (at any time point after current offset) and the number of APs elicited (all p-values > 0.05; *Figure 8a and 8b*). This lack of correlation is expected as the sAHP amplitude influences AP firing towards the later part of the neuronal response to a prolonged current injection (Faber et al., 2005) and LA neurons from Control and Cond-1hr rats displayed remarkable spike frequency adaptation and rarely fired during the latter part of the current injection. In contrast, for neurons from the Cond-24hr rats the AHP amplitude was significantly correlated with the number of APs elicited (for AHP amplitude 0.08s – 3.75s after current offset; all p-values < 0.05, see *Figure* 8c). Similar results were found for area of the postburst AHP, with no significant correlation for neurons from Control [r = .232, p = 0.22] and Cond-1hr rats [r = .363, p = 0.13] but a significant correlation emerged at 24 hr [r = .644, p < 0.001]. These data indicate that although the postburst AHP is not correlated with spike frequency adaptation under control conditions and immediately post-conditioning, a significant correlation emerged 24 hr post-conditioning.

Factors other than the sAHP can modulate spike frequency adaptation. For LA neurons, spike frequency adaptation is controlled by both sI_{AHP} (voltage-insensitive calcium-dependent potassium currents that contribute to the sAHP) and I_D (voltage-dependent potassium currents) (Faber et al., 2005). To determine whether a modulation of I_D underlies the observed reduction in spike frequency adaptation, we measured the initial spike frequencies and interspike intervals (ISIs), a characteristic dependent on I_D in LA neurons (Faber et al., 2005). A one-way ANOVA revealed no significant effect of fear conditioning on ISIs for the three pairs of APs [$F(2, 66)$ = 1.1, 1.2, 1.3 for 1st, 2nd and 3rd pair of APs respectively, all p-values > 0.05]. Similar results were observed for the spike

Figure 8. Fear conditioning modulates the relationship between the sAHP and spike frequency adaptation. The postburst AHP amplitude (measured at 750 ms following current offset) is not correlated with number of action potentials fired during a prolonged current injection for control (n = 28, a) and Cond-1hr (n = 13, b) rats. In contrast, AHP amplitude is significantly correlated with number of action potential for Cond-24hr rats (n = 28, c). Asterisk (*) indicates $p < 0.05$. Adapted from Sehgal, Ehlers & Moyer, 2014

Figure 8


A
Number of Action Potentials
AHP Amplitude (mV)
2
4
6
8
10
12
0
-4
-8
-12
-16
-20
Control
r = .24, p =.22
B
Number of Action Potentials
AHP Amplitude (mV)
Cond-1hr
r = .44, p =.13
C
Number of Action Potentials
AHP Amplitude (mV)
Cond-24hr
r = .64, p < 0.001

frequencies as well (data not presented). Thus, it is unlikely that a reduction in I_D was responsible for the learning-related changes in spike frequency adaptation.

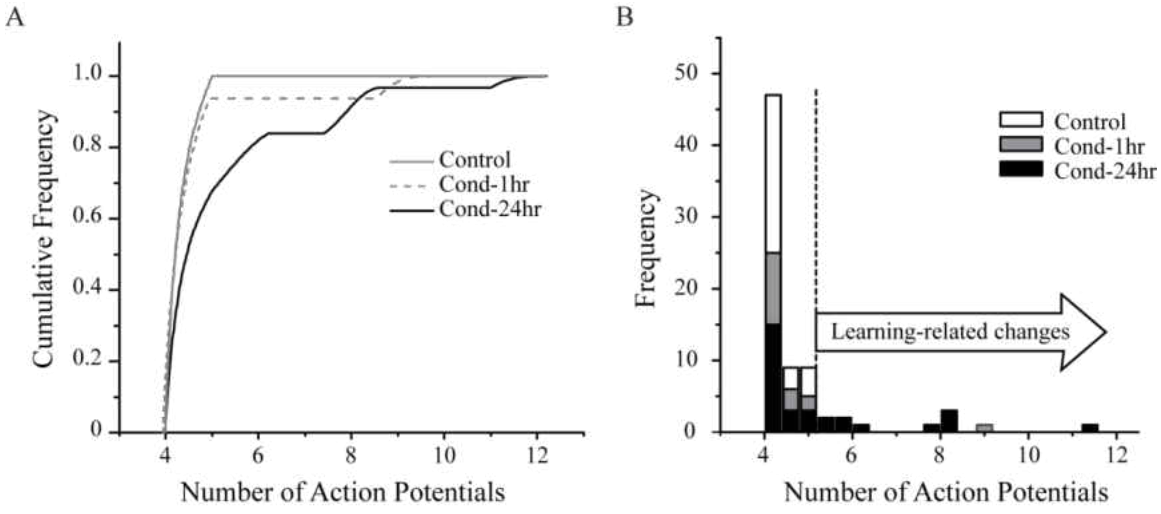
Dissociation between the sAHP and spike frequency adaptation has also been observed in other structures, such as the hippocampus (Moyer et al., 1996) and basolateral amygdala (Motanis et al., 2012). For example, using trace eye-blink conditioning in rabbits, a significant reduction has been observed in the sAHP as well as spike frequency adaptation of hippocampal neurons as early as 1 hr following acquisition. While spike frequency adaptation begins to revert to baseline levels by the third day after learning, the sAHP changes last until the seventh day (Moyer et al., 1996). Thus, the dissociation between the sAHP and spike frequency adaptation changes observed in the current study is consistent with that from previous studies.

Fear conditioning enhances intrinsic excitability in a subset of LA neurons

We next examined whether the enhancement in the intrinsic excitability occurs in all LA neurons or whether such changes are present in only a subset of neurons. The cumulative frequency distribution of the number of APs elicited from LA neurons indicates that the curve for Cond-24hr rats is shifted to the right relative to that from Control or Cond-1hr rats (see *Figure 9a*). Significantly, the rightward shift in Cond-24hr rats is only evident for a part of the curve indicating only a subset of LA neurons change as a result of conditioning. LA neurons from Control rats displayed remarkable spike frequency adaptation with none of the recorded neurons firing more than 5 APs (e.g. see *Figures 7* and *9a*). To quantify a learning-related change in spike frequency adaptation, any neuron firing more APs than the most excitable Control neuron (5 APs) was defined as having changed. Using this criterion (AP firing), we find that 8% of neurons from Cond-1hr rats

Figure 9. Fear conditioning enhances intrinsic excitability in a subset of lateral amygdala neurons. a. Cumulative frequency distribution of "number of action potentials (APs)" for LA neurons. Number of APs elicited in LA neurons from Cond-24hr rats (n = 28) is shifted to the right relative to neurons from Control (n = 28) or Cond-1hr (n = 13) rats. The rightward shift in Cond-24hr rats is only evident for a part of the curve indicating only a subset of LA neurons change as a result of conditioning. b. Frequency histogram for number of APs. Approximately 32% of the LA neurons (9/28) have enhanced intrinsic excitability (i.e., those that fire more APs than the most excitable LA neuron from a Control rat) 24 hr following fear conditioning. For both graphs, dashed line indicates the maximum number of APs fired by a LA neuron from a Control rat. Adapted from Sehgal, Ehlers & Moyer, 2014

Figure 9


A
Cumulative Frequency
1.0
0.8
0.6
0.4
0.2
0
4
6
8
10
12
Number of Action Potentials
Control
Cond-1hr
Cond-24hr
B
Frequency
50
40
30
20
10
0
4
6
8
10
12
Number of Action Potentials
Control
Cond-1hr
Cond-24hr
Learning-related changes

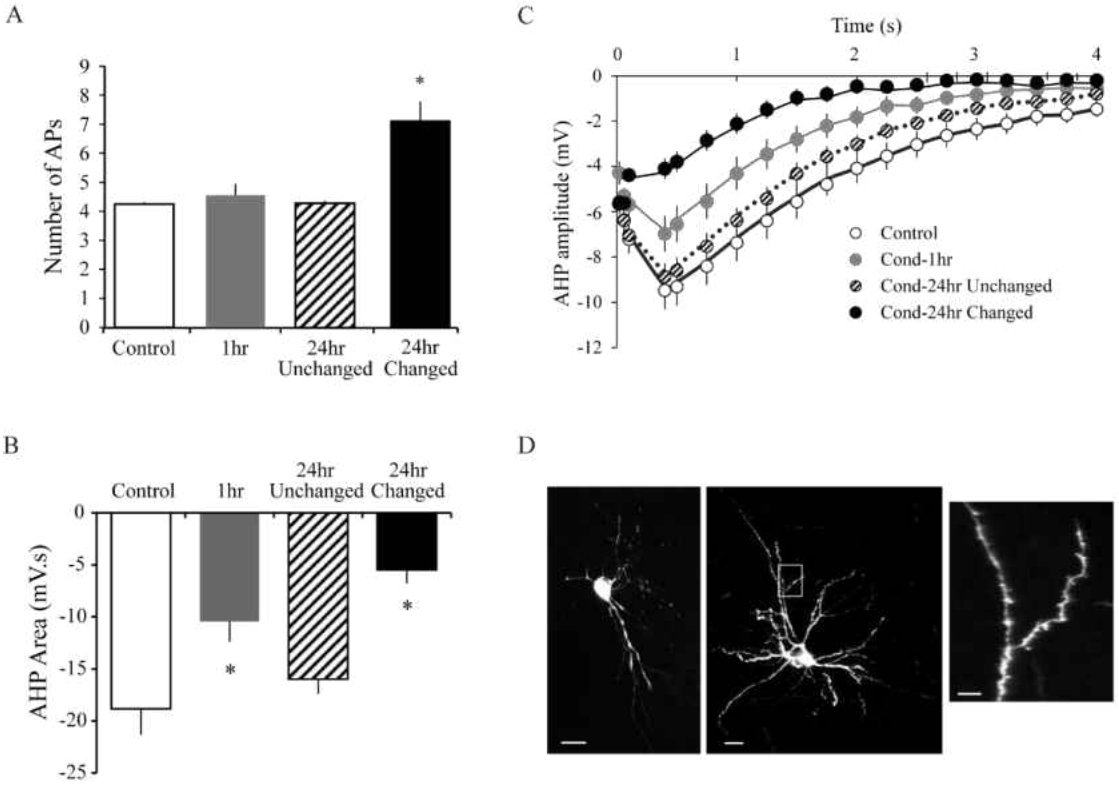
(1 of 13) and 32% of neurons from Cond-24hr rats (9 of 28) displayed a learning-related change (see *Figure 9b*). Hence, fear conditioning enhances intrinsic excitability, but only in a subset of LA neurons.

To confirm whether the reduction in spike frequency adaptation was a result of fear conditioning and not sampling error, we performed a shuffling analysis on the data from the spike frequency adaptation experiment. Briefly, data (number of APs fired) from all the recorded LA neurons were shuffled and then randomly assigned to the 3 groups (group sizes were the same as the actual experiment). The number of cells that met the criterion for learning-related changes (more than 5 APs) was quantified for each group. For 1000 such iterations, none of the runs resulted in our observed learning-related changes ($p <$ .001). Thus, it is extremely unlikely that such a distribution would result from sampling error alone and indicates that a subset of LA neurons underwent a time-dependent learning-specific enhancement of intrinsic excitability.

Next, we confirmed whether learning-related changes in the AHP were also restricted to a subset of neurons. We divided LA neurons from Cond-24hr rats into Cond-24hr "changed" or "unchanged" based on the above mentioned criteria. As expected, spike frequency adaptation was significantly reduced in Cond-24hr changed neurons relative to neurons from Control, Cond-1hr and Cond-24hr unchanged groups [$F(3, 65) = 22.0$, $p < 0.001$; post hocs $p < 0.001$; see *Figure 10A*]. More importantly, the size of the AHP was also selectively reduced in Cond-24hr changed neurons relative to Cond-24hr unchanged groups. The AHP amplitude (from 0.09s to 4s after current offset) was significantly reduced in Cond-24hr changed (all p values < 0.05) but not Cond-24hr unchanged group

Figure 10. Learning-related changes in lateral amygdala at 24 hr after fear conditioning are exclusively observed in a subset of neurons. a. Spike frequency adaptation in LA neurons is reduced only in neurons that were defined as "changed" (n = 9). Notice that LA neurons classified as "unchanged" (n = 19) were virtually identical with those from control animals (n = 28). b. The AHP amplitude (from 0.09-4.0s following current offset) was reduced in *Cond-24hr changed* but not *Cond-24hr unchanged* neurons relative to Control group. c. Area of the postburst AHP is also significantly smaller for *Cond-24hr changed* but not *Cond-24hr unchanged* neurons relative to neurons from the Control group. D: The Cond-24hr changed neurons are LA pyramidal neurons. In addition to electrophysiological criteria, representative neurobiotin cell fills from Cond-24hr changed neurons confirm that these are LA projection neurons because 1) they either have a prominent apical dendrite and a characteristic pyramidal morphology; left panel or 2) numerous spines on the dendrites; right panel. Inset: a higher magnification image of a dendrite from the area in the white box in the right panel. Scale, left panel: 25 μm; right panel: 25 μm; inset: 10 μm. Asterisk (*) indicates $p < 0.05$. Adapted from Sehgal, Ehlers & Moyer, 2014

Figure 10


A
Number of APs
Control
1hr
24hr Unchanged
24hr Changed
B
AHP Area (mV.s)
Control
1hr
24hr Unchanged
24hr Changed
C
Time (s)
AHP amplitude (mV)
Control
Cond-1hr
Cond-24hr Unchanged
Cond-24hr Changed
D

(all p values > 0.08) relative to Controls (see *Figure 10B*). Notice that the time period of 0.09- 4s comprises both medium as well as sAHP. Hence, both medium and sAHP are reduced in Cond-24hr changed neurons relative to Controls. Similarly, AHP area was also reduced in Cond-24hr changed but not the Cond-24hr unchanged group relative to Controls [$F(3, 65) = 5.4$, $p < 0.01$; post hocs $p < 0.001$ and $p = 0.326$ respectively; see *Figure 10c*]. In contrast to medium and sAHP, and as previously reported (see *Table 1*), the fast AHP was unaltered between groups [$F(3,65) = 2.25$, $p = 0.09$]. Therefore, spike frequency adaptation as well as medium and sAHP reduction was restricted to a subset (~32%) of LA neurons at 24hr post-conditioning.

The above data suggest that the Cond-24hr changed neurons represent a distinct subset of LA neurons within the Cond-24hr rats. In order to confirm that Cond-24hr neurons only differed in their firing properties and did not represent a distinct neuronal subtype (e.g. LA interneurons) we used electrophysiological and morphological criteria. Using electrophysiological parameters such as spike frequency adaptation, input resistance, spike half width and fast AHP, we were able to confirm that Cond-24 changed neurons (as well as other recorded neurons) were LA pyramidal neurons (see *Tables 1, 2* and Methods). Furthermore, we obtained complete neurobiotin cell fills from 5 of the Cond-24hr changed neurons (see *Figure 10d*). These cells were confirmed as pyramidal neurons either due to the presence of large soma (~ 300 μm^2), prominent apical dendrite or numerous dendritic spines (Faber et al., 2001; Sosulina et al., 2006; Sosulina et al., 2010). Lastly, the shuffling analysis described above supports the idea that such a distribution of spike frequency adaptation as observed in neurons from Cond-24hr rats could not have resulted from biased sampling of a different neuronal type in Cond-24hr rats. Thus, the

electrophysiological and morphological analyses confirmed that the all recorded neurons including Cond-24hr changed neurons represent LA pyramidal neurons.

Basic membrane and AP properties

A change in spike frequency adaptation can result from a change in basic membrane properties. Passive membrane properties like input resistance (R_N) were not significantly different between groups [$F(3, 65) = 0.538$, $p = 0.66$; see *Table 2*]. A one-way ANOVA indicated a significant effect of group on resting membrane potential [(RMP), $F(3, 65) = 3.08$, $p < 0.05$]. However, post hoc tests indicated only a small and non-significant decrease in RMP of neurons from Cond-1hr group relative to those from Controls ($p = 0.06$). Additionally, the current injection amplitude used to perform the AHP and spike frequency adaptation studies also did not differ between groups [$F(3, 65) = 0.85$, $p = .47$]. The threshold for AP initiation and rheobase were also unaltered as a result of fear conditioning [$F(3, 65) = 0.49$, $p = .69$ and $F(3, 65) = 0.03$, $p = .758$ respectively]. Taken together, these data suggest that the changes in spike frequency adaptation in Cond-24hr changed neurons were not a result of changes in basic membrane properties.

In addition to number of APs, properties of a single AP can also contribute to neuronal excitability. Analysis of the first AP evoked during the AHP measurements revealed that AP amplitude increased following fear conditioning [$F(3, 65) = 2.66$, $p = 0.055$]. Post hoc tests revealed that AP amplitude was significantly higher in LA neurons from Cond-1hr as well as Cond-24hr changed neurons relative to those from control rats ($p < 0.05$). In contrast, AP half-width remained unchanged following fear conditioning [$F(3, 65) = 0.34$, $p = 0.8$]. The increased AP amplitude could result from increased Na^+

and Ca^{2+} conductances and lead to enhanced synaptic gain as well facilitation of synaptic plasticity (Varela et al. 2012).

Table 1. Summary of learning-related changes on intrinsic excitability of LA neurons

Group (no. of cells)	Postburst AHP			Spike Frequency Adaptation	
	Amplitude (mV)	Area (mV·s)	fAHP (mV)	Number of APs	% of cells changed
Control (28)	-8.07 ± 0.81	-18.8 ± 2.5	-4.7 ± 0.5	4.25 ± 0.07	-
Cond-1hr (13)	-5.33 ± 0.77 *	-10.5 ± 1.9 *	-5.3 ± 0.1	4.60 ± 0.38 *	8% (1/13)
Cond-24hr (28)	-5.88 ± 0.59 *	-12.6 ± 1.4 *	-6.3 ± 0.7	5.19 ± 0.33 *	32% (9/28)

Data are presented as the mean ± SE. AHP amplitude is measured at 750ms following current offset. Abbreviations: AHP, Afterhyperpolarization; fAHP, fast AHP; APs, Action potentials. *$p < 0.05$ as compared with control.

Table 2. Effects of fear conditioning on intrinsic properties of LA neurons

				AP Characteristics		
Group (no. of cells)	V_m (mV)	R_N (MΩ)	Rheo (pA)	AP_{thresh} (mV)	AP_{amp} (mV)	AP_{width} (ms)
Control (28)	-73.93 ± 1.1	47.7 ± 3.0	324.1 ± 27.6	-51.33 ± 0.89	83.47 ± 0.93	1.22 ± 0.03
Cond-1hr (13)	-70.27 ± 1.9[&]	47.8 ± 4.4	319.2 ± 32.9	-49.83 ± 0.95	86.69 ± 0.91*	1.20 ± 0.04
Cond-24hr (28)	-74.62 ± 1.0	52.0 ± 2.6	319.6 ± 27.6	-50.71 ± 0.46	86.05 ± 0.76*	1.18 ± 0.02
Cond-24hr Unchanged (19)	-73.19 ± 5.0	51.0 ± 3.1	339.5 ± 32.8	-50.73 ± 0.84	85.52 ± 0.74	1.19 ± 0.06
Cond-24hr Changed (9)	-77.65 ± 5.3	54.3 ± 4.8	277.8 ± 50.8	-50.78 ± 0.44	87.17 ± 1.81*	1.20 ± 0.02

Data are presented as the mean ± SE. Abbreviations: V_m, resting membrane potential; R_N, input resistance; Rheo, rheobase; AP_{thresh}, action potential threshold; AP_{amp}, action potential amplitude; AP_{width}, action potential width. *$p < 0.05$ as compared with control; [&]$p < 0.06$ as compared with Control. Neurons from the Cond-24hr rats were separated based on the number of APs fired during a long current injection (changed: more than 5APs and unchanged: 5 or less APs).

MATERIALS AND METHODS

Aim 1. Determine the time course of learning-related changes in intrinsic plasticity.

Subjects and behavioral training. Adult male Sprague Dawley rats (~ 3 months) were individually housed in clear plastic cages. Rats were maintained on a 14 hour light/10 hour dark cycle (lights on at 7am) with unlimited access to both water and standard laboratory rat chow (Harlan Laboratories). All procedures were conducted in accordance with the University of Wisconsin-Milwaukee animal care and use committee (ACUC) and NIH guidelines.

Apparatus. *Fear conditioning chambers.* Fear conditioning was conducted in an apparatus previously described (Song et al., 2012; Sehgal et al., 2014). Briefly, Plexiglas and stainless steel chambers (30.5 X 25.4 X 30.5 cm; Coulbourn Instruments, Whitehall, PA) with a standard grid floor consisting of 26 parallel steel rods (5 mm diameter and 6 mm spacing) and located in a sound-attenuating box were used. The floor of the chamber was connected to a precision adjustable shock generator (Coulbourn Instruments) for delivery of a scrambled footshock, the unconditioned stimulus (US). A ventilation fan produced a constant background noise of about 58 dB (measured by a sound level meter, Realistic, A scale; model #33-2050, Fort Worth, TX) inside the sound attenuating box. The chamber was illuminated by a miniature incandescent white lamp (28V, type 1819) and was wiped with a 5% ammonium hydroxide solution prior to each training session to provide a distinct olfactory cue. During training, the room lights were left on (illumination 20.9 lux) for the entire session.

Testing chambers. An additional Plexiglas chamber served as a novel context for the auditory cue test. This chamber was located within a separate sound-attenuating box

located in the same room. The test chamber was physically different from the training chamber in that it was a hexagonal chamber, the floor was black-painted Plexiglas, and it was illuminated with an infrared light. In addition, the tray below the test chamber floor contained clean bedding and the test chamber was wiped with 2% acetic acid prior to each test session to provide a different olfactory stimulus from that used during training. The room lights were turned off (illumination 0.2 lux) for the entire testing session.

Behavioral groups. *Training.* Rats were randomly divided into seven groups (*see Table 3*). On day 0, the 4 experimental groups received one 10-trial session of auditory long-delay fear conditioning using a 45 s CS (conditioned stimulus, 80 dB white noise) followed by a brief footshock US (unconditioned stimulus; 1 s; 1 mA), and a 5.2 min intertrial interval (ITI). We have previously demonstrated that this long-delay fear conditioning protocol results in robust freezing in response to the CS but low levels of freezing to the training context (Detert et al., 2008). Thus, this particular fear conditioning protocol allows us to determine the effect of auditory fear learning on lateral amygdala intrinsic plasticity while minimizing any confounding effects of context fear conditioning. To determine the time course of fear conditioning-related intrinsic plasticity, brain slices from the Cond-1hr, Cond-24hr, Cond-4d and Cond-10d rats were obtained either 1hr, 24hr, 4d or 10d following fear conditioning, respectively. The time points of 1 hr and 24 hr post-conditioning were chosen based on preliminary data. The time point of 4 day post-conditioning was chosen in accordance with previous studies that demonstrate that learning-related intrinsic plasticity within amygdala starts to return to naïve levels by the third day after learning (Motanis et al., 2012). Lastly, the time point of 10 days was chosen because learning related intrinsic plasticity starts to return to baseline within hippocampal

Table 3. Behavioral groups for Aim 1

Group	***Day 0***	***Day 1***	***Day 4***	***Day 10***
Naïve	↓			
CS-Alone	10 CS	CS↓		
Immediate shock	10 US	CS↓		
Cond-1hr	10(CS-US) ↓			
Cond-24hr	10(CS-US)	CS↓		
Cond-4d	10(CS-US)		CS↓	
Cond-10d	10(CS-US)			CS↓

↓: indicates the brain slice preparation, orange and blue shading represents the conditioning and training context respectively

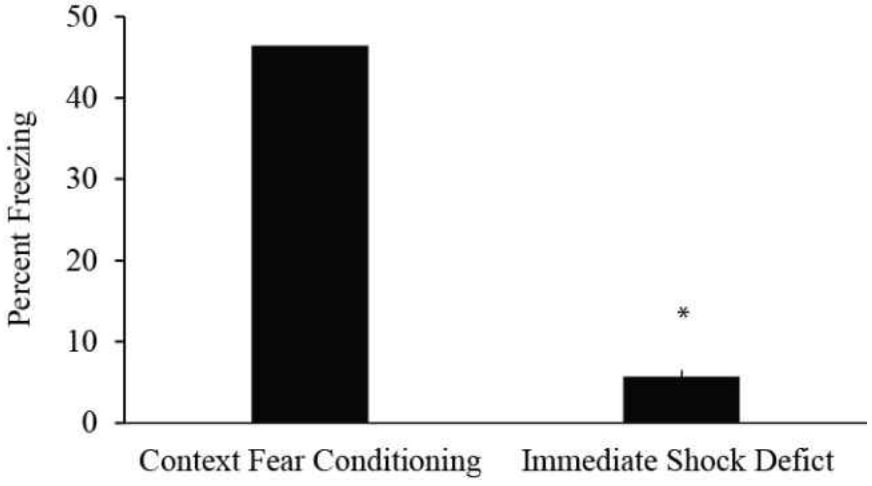
neurons after a week (Moyer et al., 1996). The three Control groups were experimentally naive rats (never handled, Naive), or rats that received unpaired CS (CS-alone) or US (US-alone) trails. The CS-alone group received 10 CS presentations separated by a 5.2 min ITI on day 0 and served as a control for CS presentation. The rats from the US-alone group were placed in the training chamber where they received 10 continuous US presentations (1 sec, 1 mA each) immediately afterwards and then rapidly removed from the chamber. Such US presentations in the absence of context exploration or encoding lead to a failure to associate the training chamber with US presentations and has been described as immediate shock deficit paradigm (Fanselow, 1986). This US-alone control group allows us to determine any effect US presentations alone (in the absence of learning) might have on LA intrinsic excitability.

To assess memory retention, rats in the CS-alone, Cond-24hr, Cond-4d and Cond-10d group were given a probe test in a novel context immediately before slice preparation. Any conditioned animals that displayed less than 50% freezing during CS presentation were separated into a *Cond-poor learners* group. The immediate shock group was tested in the conditioning chamber (instead of a novel chamber) to assess context-shock association. Pilot data demonstrate (see *Figure 11*) that relative to context fear conditioned rats (10X, 1s, 1mA US, 5.2min ITI) rats in the immediate shock group display less freezing to the training context 24hrs later.

Analyses of behavioral data. The training sessions were recorded using a remote CCTV video camera (model #WV-BP334; Panasonic Corp., China) mounted to the top of each behavioral chamber. The video data were fed to a PC running FreezeFrame 2.04. Freezing was defined as the absence of all movement except that required for respiration

Figure 11. Immediate shock deficit prevents freezing to the training context one day later. Immediate shock rats (N=3) received a 10 s 1 mA shock immediately after being placed in the training chamber and were returned to the home cage after shock termination. Context fear conditioning involved 10 shocks, 1 s, 1 mA at 5.2 min ITI. Pilot data demonstrated that rats in the immediate shock group spent significantly less time freezing during a 2 min stimulus free period than the rats that were context fear conditioned.

Figure 11


50
40
30
20
10
0
Percent Freezing
*
Context Fear Conditioning
Immediate Shock Defict

(Blanchard and Blanchard, 1969) and a 1 sec bout of immobility was scored as freezing using FreezeView 2.04 (Actimetrics Software, Coulbourn Instruments).

Slice preparation. Brain slices were prepared by an individual blind to the training condition. Rats were deeply anesthetized with isoflurane and decapitated. The brain was quickly removed and placed in ice-cold oxygenated (95% O_2/5% CO_2) aCSF (composition in mM: 124 NaCl, 2.8 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 20 dextrose). The brain was then blocked and 400 μm-thick coronal brain slices were cut in aCSF at ~ 0°C using a vibrating microtome (VT1200, Leica). Only slices that were located between 1.88 and 3.30 mm posterior to bregma, thus containing lateral amygdala, were used (Paxinos and Watson, 1998). Slices were then transferred to a holding chamber (Moyer and Brown, 1998) containing oxygenated aCSF at 30°C and allowed to recover for 30 min. The slices were then kept at room temperature (21-23°C) for an additional 30 min before any electrophysiological recordings began.

Electrophysiological recordings. For electrophysiological measurements, slices were transferred as needed to the recording chamber, where they were perfused with oxygenated aCSF at 32°C. The slices were held in place using nylon net stretched within a U-shaped platinum wire. Visually-guided whole cell patch clamp recordings were made using infrared differential interference contrast optics. All recordings were obtained using a MultiClamp 700B amplifier system (Molecular Devices, Union City, CA). Experiments were controlled by PClamp 10 software running on a PC, and the data were acquired using the Digidata 1440A acquisition system. All recording electrodes (3-8 MΩ) were pulled from thin-walled capillary glass (A-M Systems, Carlsborg, WA) using a Sutter Instruments P97 puller. The patch pipettes were filled with internal solution containing (in mM) 110

K-gluconate, 20 KCl, 10 Di-Tris-P-Creatine, 10 HEPES, 2 $MgCl_2$, 2 Na_2ATP, 0.3 Na_2GTP, 0.2% Biocytin, with a pH of 7.3 and osmolarity of 290 mOsm. Only cells with a stable, uncorrected resting membrane potential (RMP) between -50 to -85 mV, overshooting action potentials, and an input resistance (R_N) > 100 MΩ were used. *Figure 5* shows a photograph of a coronal slice containing lateral amygdala with typical recording site noted.

Intrinsic Excitability. To minimize the influence of voltage-dependent changes on membrane conductances, all cells were studied at a membrane potential near -60 mV (using constant current injection under current clamp mode). To study intrinsic excitability, WCRs were conducted under current clamp using the following protocol:

(1) Voltage–current (V-I) relations were obtained using 400 ms current steps (range -50 pA to rheobase) and by plotting the plateau voltage deflection against current amplitude. Neuronal input resistance (R_N) was determined from the slope of the linear fit of that portion of the V-I plot where the voltage sweeps did not exhibit sags or active conductance.

(2) Intrinsic excitability measurements were obtained using 1s current steps (range 0 to 500 pA) and by plotting the number of action potentials fired against current amplitude.

(3) The postburst afterhyperpolarization (AHP; 3X, at 20 s intervals) was evoked using a 100 ms depolarizing current injection just sufficient to elicit a burst of three action potentials. The same current injection does not always lead to the exact same neuronal response, and sometimes the current pulse used results in more than or less than 3 action potentials, usually 2 or 4. In such a case, AHP measurements were continued till 3 sweeps with 3 APs each were obtained from a

neuron. Both the amplitude (at various time points) and peak AHP were measured. Fast AHP was measured by subtracting the action potential threshold from the peak of the afterhyperpolarization following the first evoked action potential (Faber and Sah, 2002; Santini et al., 2008). The medium AHP was measured as the AHP amplitude from few tens to 500 ms following current offset (Faber and Sah, 2002). The sAHP was measured 500 ms and onwards following the current offset. Action potential (AP) characteristics were analyzed from the first AP evoked during the AHP measurements. AP amplitude (AP_{amp}) was measured relative to the threshold and AP width (AP_{width}) was measured at one-half the AP_{amp}.

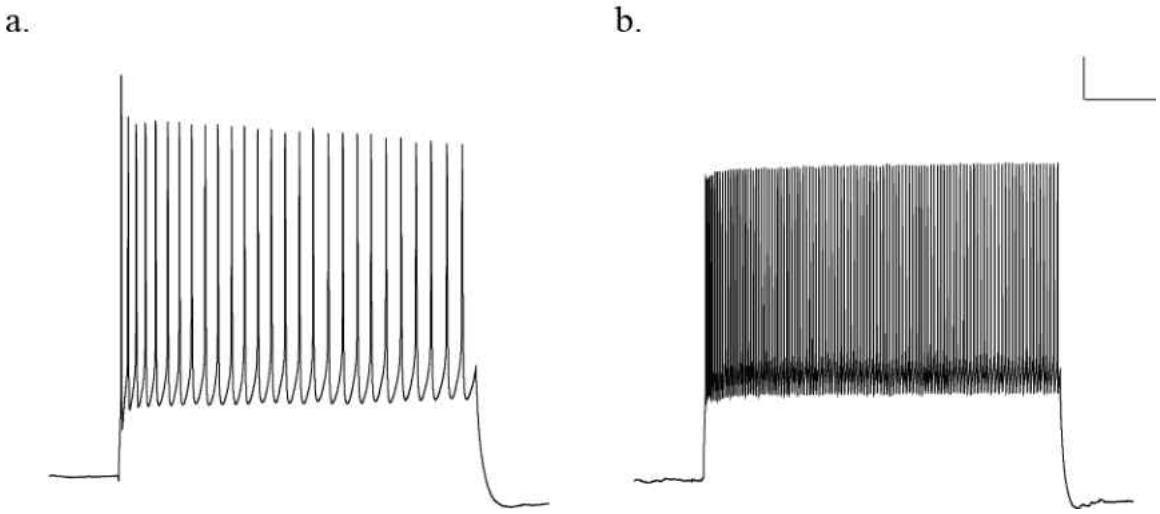
(3) Spike frequency adaptation (accommodation; 3X, at 30 sec intervals) was studied using a 1 s depolarizing current injection of the same stimulus intensity used to study the AHP. For each sweep, the number of action potentials elicited were counted.

(4) Resting membrane potential (RMP) was calculated as the difference between mean membrane potential during the first minute immediately after obtaining whole cell configuration and after withdrawing the electrode from the neuron.

Characterization of LA pyramidal neurons. We used electrophysiological criteria to confirm that all electrophysiological recordings were obtained from LA pyramidal neurons (spike frequency adaptation, input resistance, spike half width and fast AHP). For example, most LA interneurons display very little spike frequency adaptation (*see Figure 12*) and fire at high frequencies following large somatic depolarization (~100Hz). Data from LA interneurons were excluded from the analysis.

Figure 12. Representative waveforms demonstrating the difference in firing properties of LA excitatory neurons (*a*) and interneurons (*b*) following large somatic current injections (500 pA). Interneurons show little spike frequency adaptation and fire at high frequencies (< 100 Hz) relative to excitatory neurons that display spike frequency adaptation, attenuation of AP amplitude and a pronounced AHP.

Figure 12

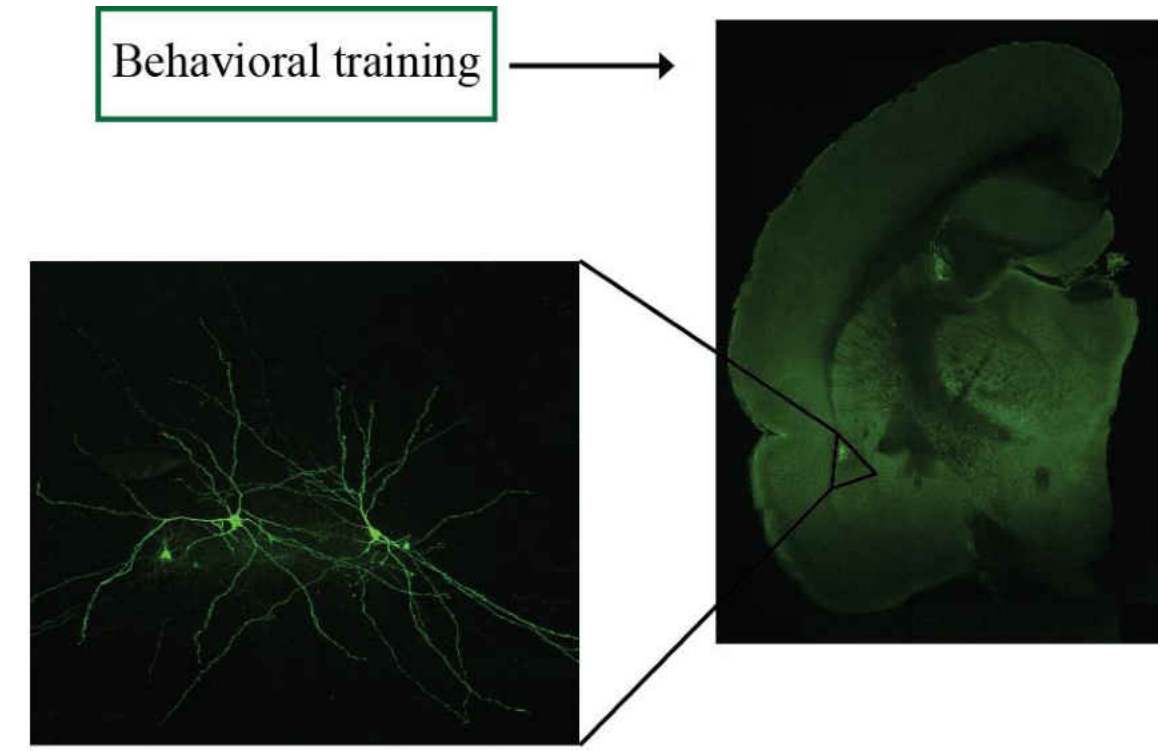


Biocytin staining. Neurons were filled with biocytin to confirm the position and identity of pyramidal cells in LA. After obtaining electrophysiological measurements, slices were fixed in 10% neutral-buffered formalin at 4 °C for 1 to 7 days before further processing. To visualize LA neurons, slices were incubated in 3% H_2O_2/10% methanol for 45 min, washed with PBS for 10 min (3X), followed by 0.25% Triton X-100/ 2% BSA for 60 min. The slices were then incubated with 1:500 streptavidin Alexa Fluor 488 (Invitrogen) for 120 min in the dark, and washed with PBS for 10 min (3X). They were mounted onto slides, coverslipped with Ultra Cruz Mounting Medium (Santa Cruz Biotechnology, Santa Cruz, CA), and sealed with nail polish. The neurons were viewed and photographed using a fluorescence microscope (BX51WI, Olympus) at 20X or Olympus Fluoview FV1200 confocal system. Neurons were classified as pyramidal when either a prominent apical dendrite, large soma or dendritic spines were detected. Representative biocytin-filled LA pyramidal neurons are shown in *Figure 13*.

Statistical Analyses. The overall treatment effects were examined using either a repeated-measures ANOVA, one-way ANOVA or t-tests using SPSS 13.0 (SPSS, Chicago, IL). A repeated-measures ANOVA was used to compare freezing levels across training trials and the AHP across time for each group of rats. A Fisher's PLSD test was used for post hoc comparisons following significant main effects ($\alpha = 0.05$), unless otherwise noted.

Figure 13. Patch clamp electrophysiological recordings. Following behavioral training, lateral amygdala containing coronal slices were made and patch clamp electrophysiology recording were performed. Right panel is a photomicrograph of a typical coronal brain slice showing the location of the lateral amygdala (LA). Inset in the left panel are a pair of biocytin-filled LA pyramidal neuron.

Figure 13


Behavioral training
Patch-Clamp electrophysiology
Lateral amygdala neurons

Aim 2. Determine whether learning induces LTP and intrinsic plasticity within the same LA neurons.

Subjects and behavioral training. (same as that for Aim 1)

Apparatus. (same as that for Aim 1)

Behavioral groups. *Training.* Rats were randomly divided into five groups (see *Table 4*). On day 0, the 2 experimental groups (Cond-1hr and Cond-24hr) were fear conditioned as described for Aim 1. These two time points were chosen as the learning-related changes in AHP and spike frequency adaptation were first witnessed at 1 hr and 24 hr respectively (see preliminary data). To determine whether learning induces LTP and intrinsic plasticity within the same LA neurons, brain slices from the Cond-1hr and Cond-24hr rats were obtained 1 hr or 24 hr following fear conditioning, respectively. The three Control groups were treated identical to that described for Aim1.

Analyses of behavioral data. (same as that for Aim 1)

Slice preparation. (same as that for Aim 1)

Electrophysiological recordings. Visually guided whole cell patch clamp recordings were obtained from LA pyramidal neurons as described for Aim 1 with a few variations. All measurements were performed in ACSF containing picrotoxin (100 μm) to obtain pure excitatory responses.

After obtaining measures of intrinsic excitability under current-clamp mode (see Aim 1), measures of synaptic plasticity were obtained.

Table 4. Behavioral groups for Aim 2

Group	Day 0	Day 1	Day 4
Naïve	↓		
CS-Alone	10 CS	CS ↓	
Immediate shock	10 US	CS ↓	
Cond-1hr	10(CS-US) ↓		
Cond-1d	10(CS-US)	CS ↓	
Cond-4d	10(CS-US)		CS ↓

↓: indicates the brain slice preparation, orange and blue shading represents the conditioning and training context respectively

Synaptic plasticity measurements:

1) Under voltage-clamp configuration, baseline synaptic transmission was measured by obtaining an input-output (I/O) curve. I/O curves were generated by presynaptic stimulation (100 μs duration) to the thalamic fibers traversing the LA using a concentric bipolar microelectrode (FHC, Brunswick, ME). Varying stimulation intensities (from EPSC threshold to EPSC spike generation) were plotted against the resultant peak EPSC amplitude and EPSC slope. To ensure that evoked excitatory postsynaptic currents (EPSCs) were monosynaptic the following criteria were used: a) the latency of the response, on average 3-4 ms (Clem and Huganir, 2010) b) response latencies remained constant across stimuli (Weisskopf et al., 1999; Clem and Huganir, 2010) c) only the early part the EPSC/EPSP slope was measured (Bauer et al., 2002), and d) only the lowest stimulation intensities were used, unless otherwise noted (Weisskopf et al., 1999).

2) Paired pulse ratios (PPR) were obtained to determine any changes in presynaptic release probabilities. Pairs of EPSCs were generated by two temporally close presynaptic stimulations ranging from 25 to 150 ms apart at 0.1Hz. Stimulation intensities were kept to a minimum so as to generate a reliable EPSC (greater than 50 pA) without recruiting polysynaptic responses or spiking. PPR were calculated as the ratio of the peak of the second EPSC to the first EPSC (McKernan and Shinnick-Gallagher, 1997).

3) AMPA:NMDA ratios were obtained to determine any increase in AMPA receptor mediated transmission, an electrophysiological signature of LTP. Evoked EPSCs were recorded by gradually increasing the holding potential from -70 mV

to -30 mV. AMPA:NMDA ratios were calculated as the ratio of peak current at -70 mV to the current at peak, 40ms, 80ms, 100 ms after stimulus onset at -30 mV.

4) Rectification index of the AMPA receptor-mediated excitatory postsynaptic currents (EPSCs) was obtained to determine the contribution of GluR1 containing (or GluR2 lacking) receptors. Rectification index was calculated as the ratio of the linear fit of the I-V plot (for AMPA currents) for negative holding potentials (-70 mV to 0 mV) to that at the positive holding potentials (0 mV to +50 mV) (Clem and Huganir, 2010).

$$\text{Rectification Index} = \frac{\text{Slope of I/V plot at negative holding potential}}{\text{Slope of I/V plot at positive holding potential}}$$

For a subset of experiments, rectification index was calculated using pharmacologically isolated AMPA or NMDA receptor mediated EPSCs by adding D, L APV (100 μm) or DNQX (100 μm) to the ACSF to block NMDA receptor mediated currents. To verify the accuracy of voltage clamp under the above mentioned recording conditions, a subset of recordings were made using internal solution containing (in mM) 145 CsCl, 10 HEPES, 0.5 EGTA, 5 QX-314, 5 MgATP, 0.2 GTP, 0.2% Biocytin, with a pH of 7.3 and osmolarity of 290 mOsmol.

Statistical Analyses. The main effects and post hoc comparisons were analyzed similar to that for Aim 1. Additionally, Pearson's and Spearman's correlation coefficients were calculated to determine whether post-training intrinsic excitability predicts learning-induced LTP. All data were expressed as mean ± SEM. Statistical analyses were performed on cells.

AIM 1

RESULTS

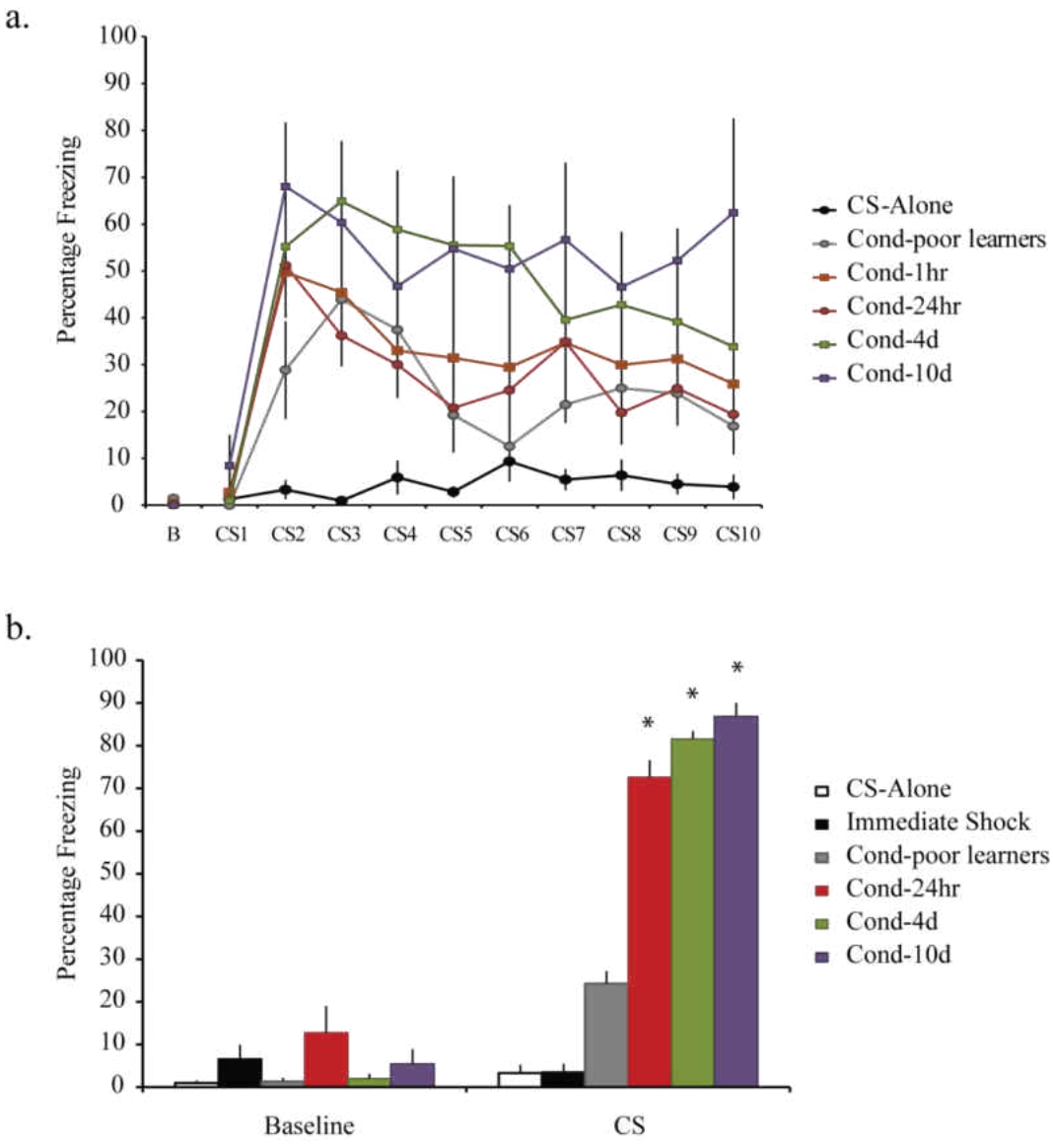
Behavioral results

Using sharp intracellular recordings, we have previously demonstrated that long-delay fear conditioning results in enhanced intrinsic excitability within a subset of lateral amygdala neurons. These changes were present up to 24hr following fear conditioning, the longest time point tested in this study. In order to determine how long intrinsic excitability changes last, in the current study we obtained electrophysiological recording from LA neurons from animals fear conditioned 1 hr, 1 day, 4 days, or 10 days prior.

Similar to the previous study, we used a long-delay fear conditioning paradigm to determine the time course of intrinsic plasticity within lateral amygdala. To assess fear learning, we used percentage of time freezing as our dependent variable. During the conditioning session, rats from all groups displayed low levels of baseline freezing (0.41 ± 0.14 %) with no significant differences observed between groups [$F(5, 31) = 1.8$, $p = 0.13$; see *Figure 14*]. Following the baseline period, rats in all the conditioning groups displayed comparable levels of increased freezing while the control group, CS-alone, that received CS presentations in the absence of US displayed low levels of freezing (see *Figure 14a).* A repeated measures ANOVA revealed a significant effect of training trial [$F(5.9, 181.4) = 16.2$, $p < 0.001$; Greenhouse-Geisser corrected] as well as group [$F(5, 31) = 4.7$, $p < 0.005$]. Pairwise comparisons demonstrated that the CS-alone group froze less than all other groups except Cond-poor learners during CS presentations (all p values < 0.05). While all other groups displayed higher freezing with training trial ($p < 0.05$), the CS-alone

Figure 14. Long-delay fear conditioning is readily acquired and lasts up to 10 days *a. Training*. During the fear conditioning session on day 1, CS-alone rats (N = 5) froze significantly less than conditioned rats in Cond-1hr (N= 8), Cond-24hr (N = 10), Cond-4d (N = 6) and Cond-10d (N = 4) rats. *b. Testing*. During the probe test, the immediate shock rats displayed low levels of freezing to the training context (not significantly different from the baseline freezing for all other groups in a novel context). During CS presentation, rats in the conditioned groups (Cond-24hr, Cond-4d and Cond-10d) displayed significantly higher freezing than CS-alone, immediate shock and Cond-poor learners. Asterisk (*) indicates $p < 0.05$ relative to Cond-poor learners group. Abbreviations: CS, freezing during CS presentation.

Figure 14


a.
Percentage Freezing
100
90
80
70
60
50
40
30
20
10
0
B
CS1
CS2
CS3
CS4
CS5
CS6
CS7
CS8
CS9
CS10
CS-Alone
Cond-poor learners
Cond-1hr
Cond-24hr
Cond-4d
Cond-10d
b.
Percentage Freezing
100
90
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70
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20
10
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*
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Baseline
CS
CS-Alone
Immediate Shock
Cond-poor learners
Cond-24hr
Cond-4d
Cond-10d

group did not display a significant increase in CS freezing over the course of conditioning. These data indicate that all animals that received paired presentations of CS and US display successful acquisition of fear learning relative to control animals receiving CS presentations alone.

To obtain an index of auditory fear learning, all animals (other than the immediate shock group) were tested in a novel context prior to electrophysiological recordings. Table 3 indicates the groups and the days at which animals were tested and electrophysiological recordings were made. Similar to the training session, the average baseline freezing was low across groups (6.0 ± 2.1 %, see *Figure 14b*) and there were no significant differences across groups [$F(5, 30) = 1.07$, $p = 0.40$]. Specifically, the immediate shock (IS) group which was tested in the original training context to assess lack of context-US association also displayed low baseline or context freezing (6.6 ± 3.32 %). Therefore, as expected, the IS rats did not form a fearful association between the training context and US presentation and are thus an appropriate control for shock presentation in the absence of learning.

Following the baseline period, rats in the fear conditioned groups (Cond 24hr, Cond 4d, and Cond 10d) displayed robust freezing to the CS presentation. Some rats displayed low levels of fear to the CS presentation (less than 50%) and were separated into a Cond-poor learners group. A one-way ANOVA revealed a significant effect of group on CS freezing [$F(5, 28) = 117.7$, $p < 0.001$]. Post hoc tests indicated that the control groups (CS-alone and immediate shock) as well as Cond-poor learners displayed significantly lower CS freezing than all the conditioned groups ($p < 0.001$) with no significant differences between the control and conditioned groups themselves. Thus, the rats in the conditioned

groups displayed robust fear memory up to 10 days following fear conditioning while the rats exposed to CS or US presentations alone displayed no fear learning.

Fear conditioning enhances intrinsic excitability in a transient manner

To determine the time course of fear learning-related intrinsic excitability changes, we used patch clamp whole cell recordings to assess LA neuronal excitability. LA neurons were injected with 1s long current pulses ranging from 0-500 pA and the number of APs elicited were counted (*see Figure 15a and 16*). A repeated-measures ANOVA indicated a significant effect of current [$F(1.83, 606.7) = 1984.4$, $p < 0.001$; Greenhouse-Geisser corrected], behavioral condition [$F(7, 332) = 5.3$, $p < 0.001$] as well as a group by current interaction [$F(12.8, 606.7) = 5.589$, $p < 0.001$; Greenhouse-Geisser corrected] on the number of APs elicited. Post hocs revealed that LA neurons from Cond-1hr, Cond-24hr, Cond-4d as well as IS group fired significantly more APs relative to naïve rats ($p < 0.05$). The number of APs fired did not differ between naïve, CS-alone, Cond-poor learners and Cond-10d groups. Follow-up one way ANOVAs indicated a significant effect of behavioral condition on number of APs fired for current injections ranging from 100-500 pA (all p values < 0.05). Post hoc comparisons confirmed that relative to LA neurons from naïve rats, LA neurons from Cond-1hr, Cond-24hr, Cond-4d and IS group fired more APs following a 300-500 pA current injection. These data demonstrate that successful acquisition of fear conditioning leads to a transient increase in LA neuronal excitability that returns to naïve levels 10 days following fear conditioning. These changes in LA excitability were not observed in rats that failed to acquire fear conditioning (Cond-poor learners). However, rats in the IS group also display increased LA neuronal excitability

Figure 15. Fear conditioning enhances intrinsic excitability of LA neurons. *a.* LA neurons from Cond-1hr, Cond-24hr, Cond-4d and immediate shock group rats fired significantly more action potential than those from naïve rats (300-500 pA; $p < 0.05$). LA neurons from CS-alone, Cond-poor learners and Cond-10d group did not differ from LA neurons from naïve rats. *b.* Maximum number of action potentials fired for current injections ranging from 0-500 pA were significantly increased in LA neurons from Cond-1hr, Cond-24hr, Cond-4d and immediate shock group rats but not for LA neurons from CS-alone, Cond-poor learners and Cond-10d group. Asterisk (*) indicates $p < 0.05$ relative to LA neurons from naïve rats. Abbreviations: IS, immediate shock.

Figure 15

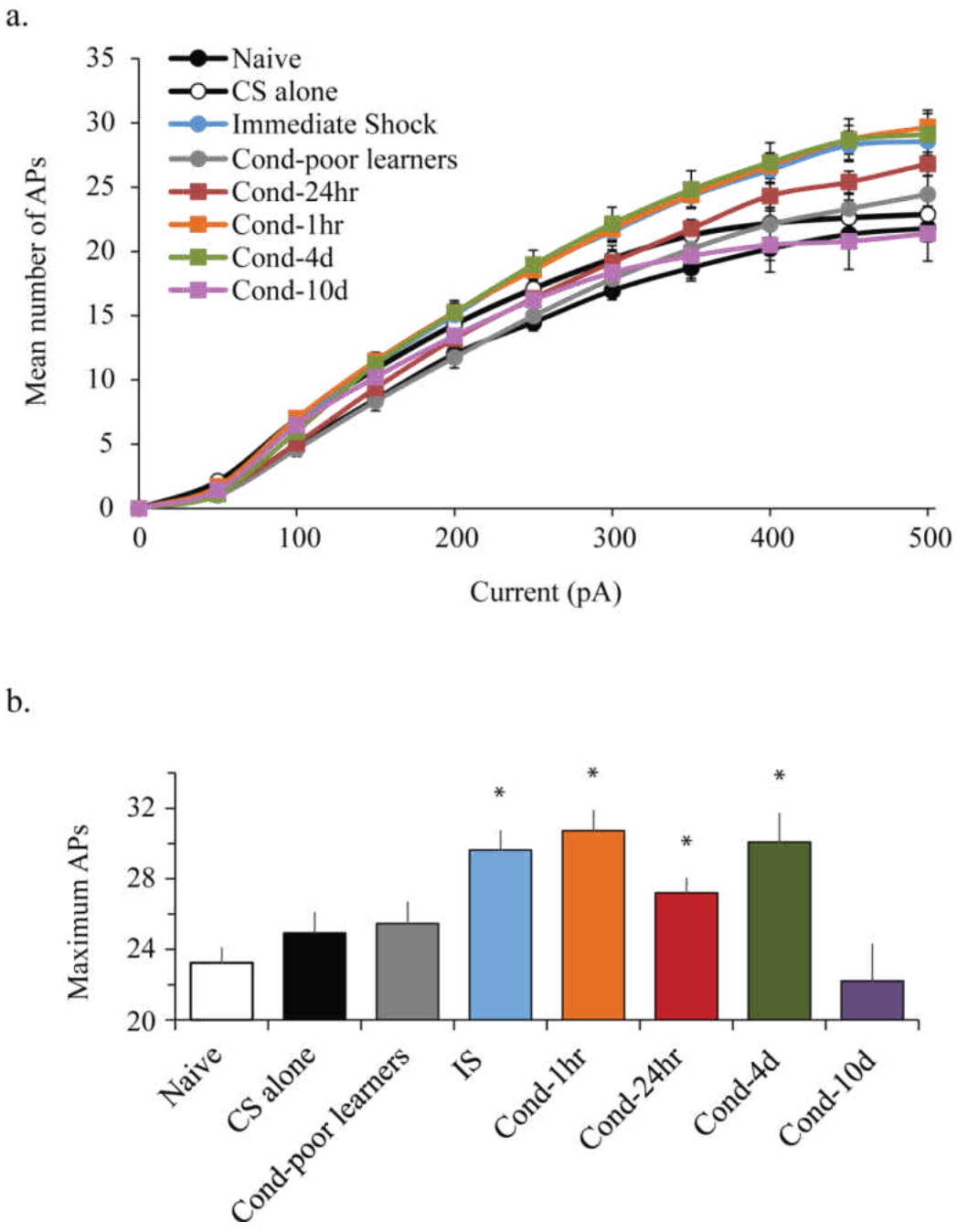
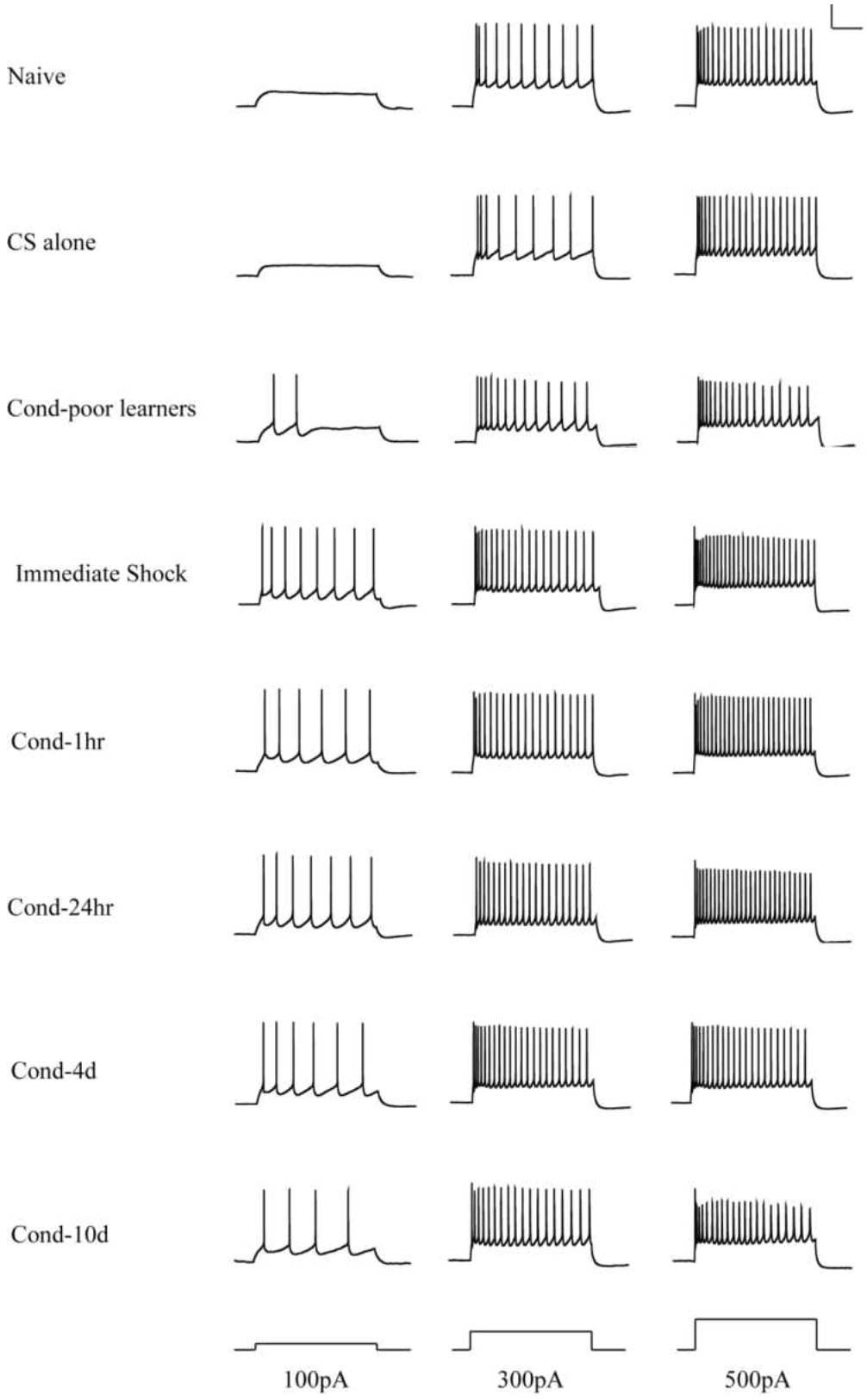

a.
Naive
CS alone
Immediate Shock
Cond-poor learners
Cond-24hr
Cond-1hr
Cond-4d
Cond-10d
Mean number of APs
Current (pA)
0
5
10
15
20
25
30
35
0
100
200
300
400
500
b.
Maximum APs
20
24
28
32
*
*
*
*
Naive
CS alone
Cond-poor learners
IS
Cond-1hr
Cond-24hr
Cond-4d
Cond-10d

Figure 16. LA neurons from conditioned rats fire more action potentials. Representative waveforms of action potentials fired following 100, 300 and 500 pA somatic current injections for LA neurons from various behavioral groups. Scale: 30 mV, 0.25 s.

Figure 16


Naive
CS alone
Cond-poor learners
Immediate Shock
Cond-1hr
Cond-24hr
Cond-4d
Cond-10d
100pA
300pA
500pA

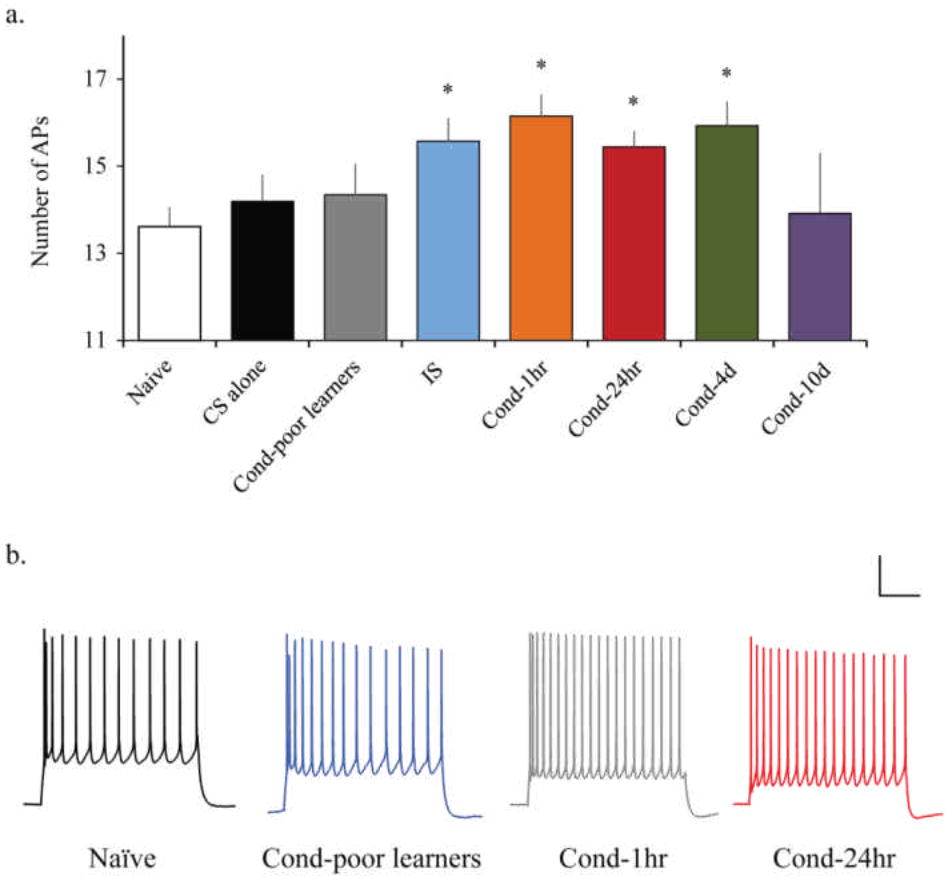
indicating that shock presentation alone could lead to alterations in LA intrinsic excitability.

Although neuronal response to a stepwise increase in somatic depolarization is widely used as measure of neuronal excitability, it suffers from certain drawbacks. Most important among these is the dependence of neuronal firing on subtle changes in input resistance, and in certain cases the cessation of AP firing following very high somatic depolarization. To obtain additional indices of intrinsic excitability, we also measured the maximum number of APs fired during 0-500 pA current injections (see *Figure 15b*) and spike frequency adaptation (see *Figure 17*). Once more, a one-way ANOVA revealed a significant effect of behavioral group on maximum number of APs elicited [$F(7, 335) = 6.95$, $p < 0.001$]. Post hoc tests confirmed that relative to LA neurons from naïve rats, those from IS, Cond-1hr. Cond-24hr and Cond-4d rats had significantly greater number of maximum APs (all p values < 0.05). No significant differences were found between naïve and other control groups (i.e., CS-alone and Cond-poor learners) as well as Cond-10d group. These data confirm our earlier observation of a transient learning-dependent increase in intrinsic excitability within LA neurons of conditioned animals and a learning-independent increase in IS animals.

To obtain a measure of spike frequency adaptation, a current injection sufficient to elicit 3 APs in the first 100 ms was injected over a period of 1 s and the number of APs elicited were counted (*see Figure 17*). Again, a one-way ANOVA demonstrated a significant effect of behavioral condition on the number of APs elicited [$F(7, 321) = 3.5$, $p < 0.001$]. Post hoc tests confirmed that LA neurons from Cond-1hr, Cond-24hr, Cond-4d and IS rats fire significantly more APs relative to LA neurons from naïve rats. As before,

Figure 17. Fear conditioning reduces spike frequency adaptation within LA neurons. *a.* A current injection sufficient to elicit 3 APs within 100 ms was extended over a 1 s period and number of action potentials elicited were counted. LA neurons from Cond-1hr, Cond-24hr, Cond-4d and immediate shock group rats fired significantly more action potentials than those from naïve rats (300-500 pA; $p < 0.05$). No significant differences were found among naïve, CS-alone, Cond-poor learners and Cond-10d group. *b.* Representative waveforms demonstrating reduced spike frequency adaptation in LA neurons from Cond-1hr and Cond-24hr relative to that from naïve and Cond-poor learners. Asterisk (*) indicates $p < 0.05$ relative to LA neurons from naïve rats. Abbreviations: IS, immediate shock. Scale: 15 mV, 0.25 s

Figure 17


a.
Number of APs
17
15
13
11
*
*
*
*
Naive
CS alone
Cond-poor learners
IS
Cond-1hr
Cond-24hr
Cond-4d
Cond-10d
b.
Naïve
Cond-poor learners
Cond-1hr
Cond-24hr

no significant differences were found between the number of APs elicited from LA neurons from Cond-poor learners, CS-alone and Cond-10d relative to naïve rats. These data demonstrate that 1) rats that successfully acquire fear conditioning display increased LA neuronal excitability relative to rats that don't learn, 2) these changes in intrinsic excitability are transient, last up to 4 days but are back to naïve levels by 10 days and 3) LA intrinsic excitability may also increase following shock presentation alone.

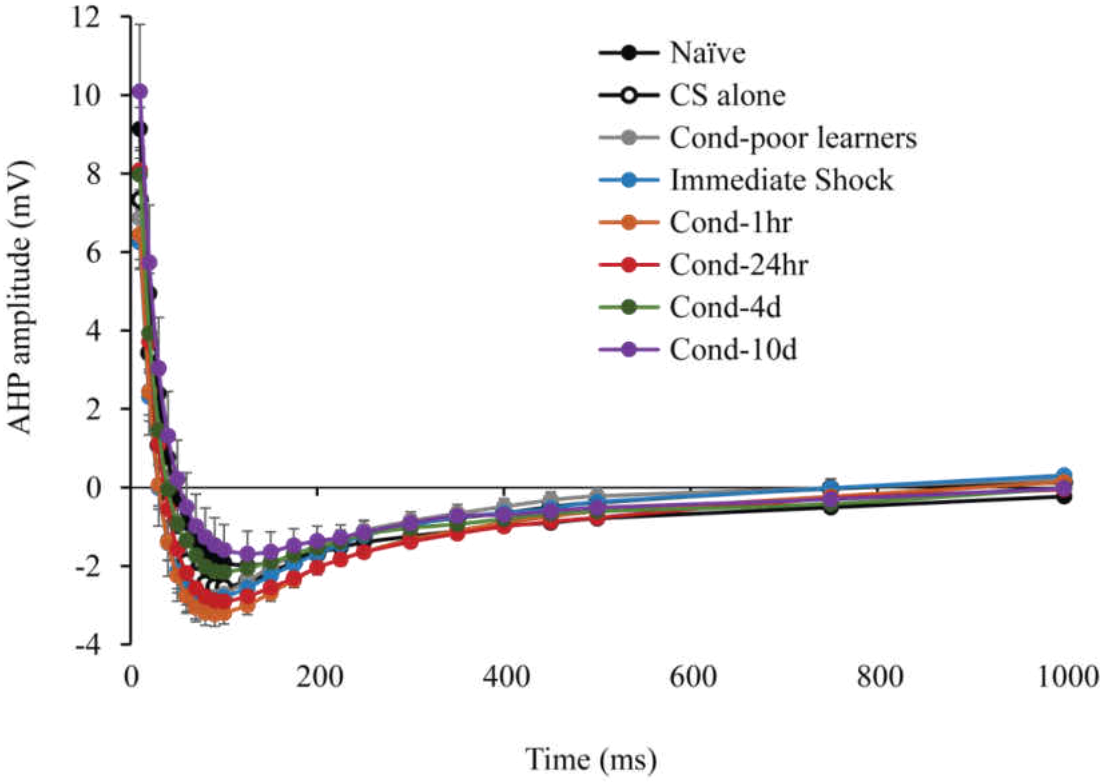
Learning-related changes in post-burst afterhyperpolarization

Changes in neuronal excitability are often accompanied by a change in post burst afterhyperpolarization. To measure afterhyperpolarization, LA neurons were injected with a somatic current just sufficient to elicit 3 action potentials within 100 ms (*see Figure 18*). The AHP amplitude was measured at several time points following the offset of current injection (50 ms – 1 s). A repeated-measures ANOVA indicated a significant effect of time [$F(1.2, 368.5) = 92.5$, $p < 0.001$; Greenhouse-Geisser corrected], group by time interaction [$F(8.4, 368.5) = 3.8$, $p < 0.05$; Greenhouse-Geisser corrected] and a strong trend towards an effect of group [$F(7, 307) = 1.83$, $p = 0.08$]. To establish the time points at which AHP amplitude was significantly different between groups, a one-way ANOVA was performed for AHP amplitudes at various time points. AHP amplitude was significantly different between groups from 50 ms to 150 ms after current offset (all p values < 0.05). Post hoc comparisons revealed that the amplitude for this early component (90 ms-125 ms) of AHP was significantly increased in LA neurons from Cond-1hr, Cond-24hr as well as IS group rats in comparison to naïve rats (all values $p < 0.05$). In addition, peak AHP amplitude was also significantly altered as a function of group [$F(7,307) = 2.81$ $p < 0.01$]. Post hocs confirmed that relative to LA neurons from naïve rats, those from IS and Cond-1hr had

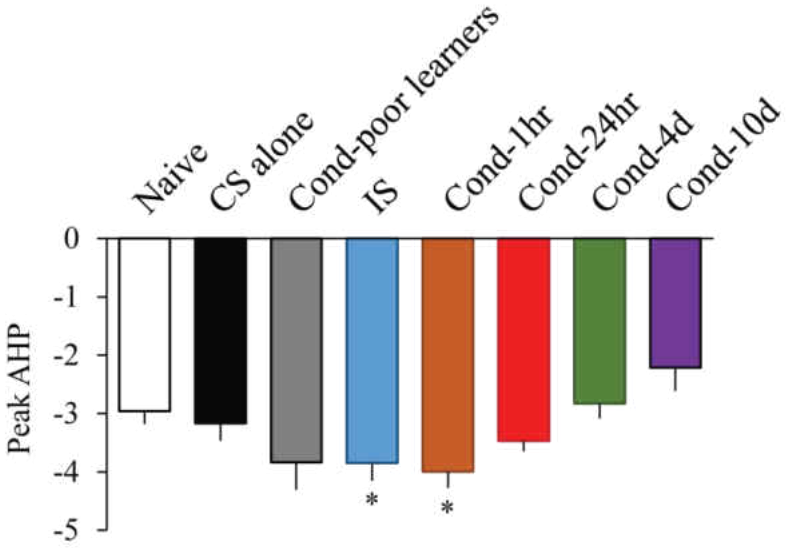
Figure 18. Peak AHP is increased despite enhanced intrinsic excitability of LA neurons. *a*. A current injection sufficient to elicit 3 APs within 100 ms was extended was used to measure post burst AHP. LA neurons from Cond-1hr, Cond-24hr, Cond-4d and immediate shock group rats fired significantly more action potentials than those from naïve rats (300-500 pA; $p < 0.05$). No significant differences were found among naïve, CS-alone, Cond-poor learners and Cond-10d group. *b.* Representative waveforms demonstrating reduced spike frequency adaptation in LA neurons from Cond-1hr and Cond-24hr relative to that from naïve and Cond-poor learners. Asterisk (*) indicates $p < 0.05$ relative to LA neurons from naïve rats. Abbreviations: IS, immediate shock.

Figure 18

a.


AHP amplitude (mV)
12
10
8
6
4
2
0
-2
-4
0
200
400
600
800
1000
Time (ms)
Naïve
CS alone
Cond-poor learners
Immediate Shock
Cond-1hr
Cond-24hr
Cond-4d
Cond-10d

b.


Naive
CS alone
Cond-poor learners
IS
Cond-1hr
Cond-24hr
Cond-4d
Cond-10d
Peak AHP
0
-1
-2
-3
-4
-5
*
*

a significantly greater peak AHP amplitude (p value < 0.05). These results indicate that fear conditioning leads to a paradoxical increase in the early AHP amplitude within 1hr of fear conditioning in LA neurons, and that these changes persist for up to 24hrs. In addition, peak postburst AHP is also increased within LA neurons from IS group.

Learning-related enhancement of intrinsic excitability is usually accompanied by a decrease in AHP. We found an opposite effect for the early component of the postburst AHP – an increase in peak and early postburst AHP (< 150 ms). It is possible that this early phase of the AHP amplitude is contaminated/confounded with the repolarization phase of the action potential. To support this, we found that there were non-significant changes in the time at which the peak AHP amplitude was reached for various groups [$F(7,307) = 1.65$ $p = 0.12$]. Planned comparisons revealed that relative to LA neurons from naïve rats, AHP amplitude peaked faster for LA neurons from Cond-poor learners, Cond-1hr, Cond-24hr and IS groups (all p values < 0.05 except for IS group; $p < 0.08$). Thus, it is likely that our observed changes in the early component of AHP are artifacts of changes in AP repolarization.

It is noteworthy that the above mentioned changes in AHP amplitude reflect a very early component of AHP and do not constitute AHP currents commonly classified as medium or slow AHP (Faber and Sah, 2002). Within amygdala neurons, action potential firing early on during the current onset (early inter-spike intervals) as well as during the latter part of the current injection (spike frequency adaptation) are largely controlled by medium and slow AHP amplitude (Faber and Sah, 2002, 2005). We did not find any learning-related changes in these components of AHP. This is in contrast to our previous data demonstrating robust reductions in AHP amplitude immediately as well as 24hrs

following fear conditioning (see Figure 6 and Sehgal et al., 2014). The discrepancies in these studies is likely due to the recording methods used. Sharp microelectrode recordings result in more physiological measures of AHP due to little to no dilution of intracellular signaling components. On the other hand, AHP measures obtained during patch clamp whole cell recording vary in terms of amplitude as well as stability depending upon the internal solution used within the recording pipette (Kaczorowski et al., 2007). We used potassium gluconate based internal solution which results in AHPs that are much smaller (albeit more stable) than those obtained with a perforated patch setup. It is likely that we are not able to detect learning-related slow AHP changes due to a possible floor effect. Despite these limitations, we chose to use a potassium gluconate based internal solution to ensure long-term stability of our recordings (especially for experiments in Aim 2).

Passive membrane and action potential properties

Changes in neuronal excitability can also result from changes in certain passive membrane properties (see *Table 5*). A more depolarized resting membrane potential (RMP) would mean that it is easier for the neuron to reach threshold and fire action potentials. We controlled for any effect RMP differences would have on excitability by obtaining all our intrinsic excitability measurements at -60 mV. In addition, we did not observe any significant differences in RMP between groups [$F(7,336) = 1.0$, $p = 0.4$]. As previously mentioned, the input resistance of the neuron can affect the resulting depolarization following a somatic current injection. Like RMP, input resistance was also unchanged between various groups [$F(7, 336) = 0.367$, $p = 0.9$]. These data indicate that any changes in intrinsic excitability observed following behavioral training were not a result of changes in passive membrane properties.

Table 5. Effects of fear conditioning on passive membrane and AP properties of LA neurons

Group	n (N)	RMP (mV)	R_N (MΩ)	AP properties	
				Amplitude (mV)	Halfwidth (ms)
Naive	68 (14)	-61.5 ± 0.7	221.5 ± 6.7	66.8 ± 1.2	1.1 ± 0.02
CS-Alone	33 (5)	-62.1 ± 0.8	217.4 ± 10.9	69.6 ± 1.5	1.2 ± 0.02
Cond-poor learners	32 (4)	-61.7 ± 1.06	219.5 ± 15.8	68.4 ± 1.9	1.1 ± 0.04
IS	42 (5)	-59.7 ± 0.9	210.6 ± 10.3	69.2 ± 1.2	1.3 ± 0.33
Cond-1hr	58 (10)	-60.4 ± 0.9	214.9 ± 8.7	66.7 ± 1.6	1.1 ± 0.02
Cond-24hr	70 (10)	-60.3 ± 0.7	205.6 ± 7.5	67.3 ± 1.0	1.1 ± 0.02
Cond-4d	26 (6)	-59.6 ± 1.2	200.6 ± 9.7	67.3 ± 1.7	1.1 ± 0.03
Cond-10d	14 (4)	-62.8 ± 0.9	219.9 ± 15.0	68.6 ± 2.0	1.2 ± 0.08

Data are presented as the mean ± SE. AP properties were measured from threshold. Abbreviations: n, number of cells; N, number of animals; RMP, resting membrane potential; R_N, input resistance; AP, action potential; IS, immediate shock.

Action potential properties can also accompany changes in intrinsic excitability. We measured single AP properties and found no significant differences in AP amplitude [$F(7, 305) = 0.583$, $p = 0.8$] and AP halfwidth among different behavioral groups [$F(7, 305) = 0.502$, $p = 0.8$]. Therefore, action potential amplitude or duration was not changed as a result of fear conditioning.

Correlation with behavioral performance

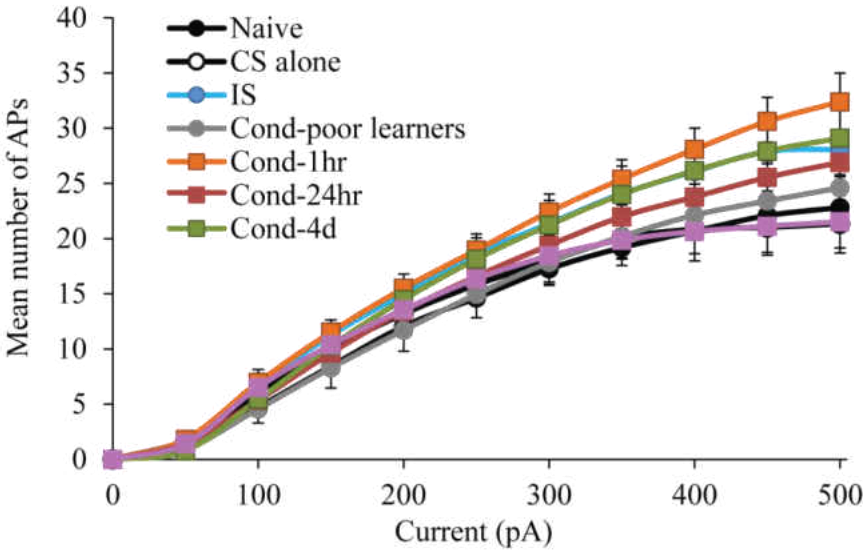
Thus far, all statistical analysis presented were performed on cells. In order to eliminate any sampling bias and in order to correlate behavioral performance with intrinsic excitability, we also performed the above statistical analysis on electrophysiological data averaged for each animal (*see Figure 19*). As before, we found that intrinsic excitability as measured by number of APs fired following somatic current injection (350-500 pA), maximum number of action potentials fired (for current injections ranging from 0-500 pA) as well as spike frequency adaptation was increased in Cond-1hr, Cond-24hr, Cond-4d as well as IS group relative to naïve (all p values < 0.05, except Cond-24hr p values < 0.1). Furthermore, these measures remained unchanged in CS-alone, Cond-poor learners and Cond-10d group. These data further validate our findings that LA amygdala neuronal excitability is increased following fear conditioning and returns to naïve levels by 10 days following fear conditioning.

To further explore the correlation between intrinsic excitability and behavioral performance, correlations between percent time spent freezing during the CS presentation and various measures of intrinsic excitability were analyzed. Despite significant differences between intrinsic excitability of LA neurons from Cond-poor learners and Cond-1hr, Cond-24hr and Cond-4d group, we found that behavioral performance was not

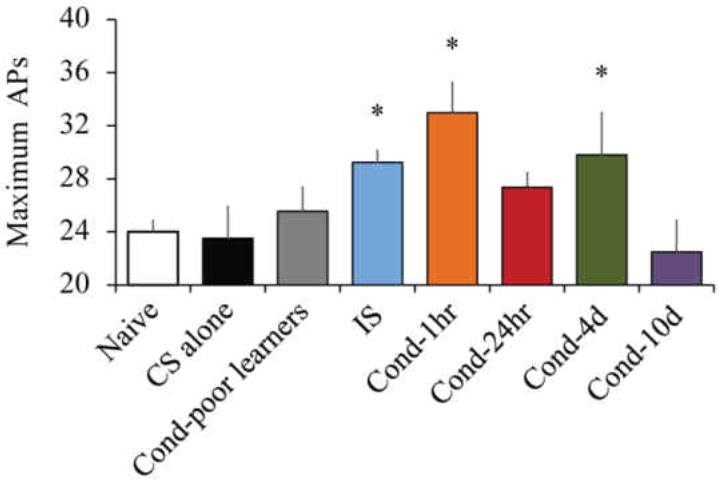
Figure 19. Fear conditioning enhances intrinsic excitability of LA neurons (data averaged per animal). *a.* LA neurons from Cond-1hr, Cond-4d and immediate shock group rats fired more action potential than those from naïve rats (300-500 pA; $p < 0.05$). LA neurons from CS-alone, Cond-poor learners and Cond-10d group did not differ from LA neurons from naïve rats. *b.* Maximum number of action potentials fired for current injections ranging from 0-500 pA were significantly increased in LA neurons from Cond-1hr, Cond-4d and immediate shock group rats but not for LA neurons from CS-alone, Cond-poor learners and Cond-10d group. Asterisk (*) indicates $p < 0.05$ relative to LA neurons from naïve rats. *c.* Spike frequency adaptation was significantly reduced for Cond-1hr, Cond-24hr, Cond-4d as well as IS group ($p < 0.05$). Abbreviations: IS, immediate shock.

Figure 19

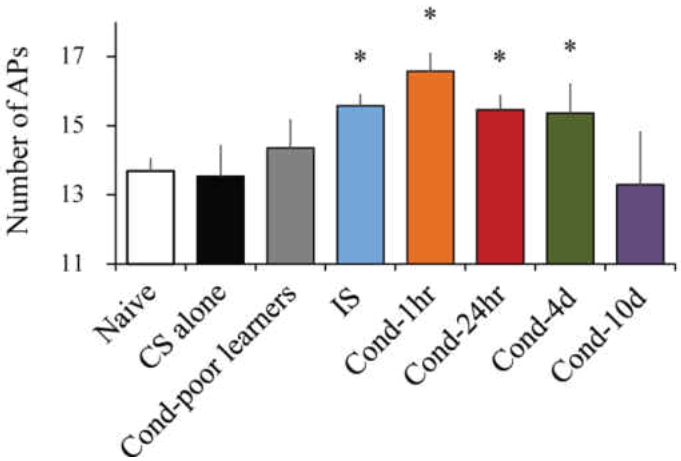
a.



b.



c.



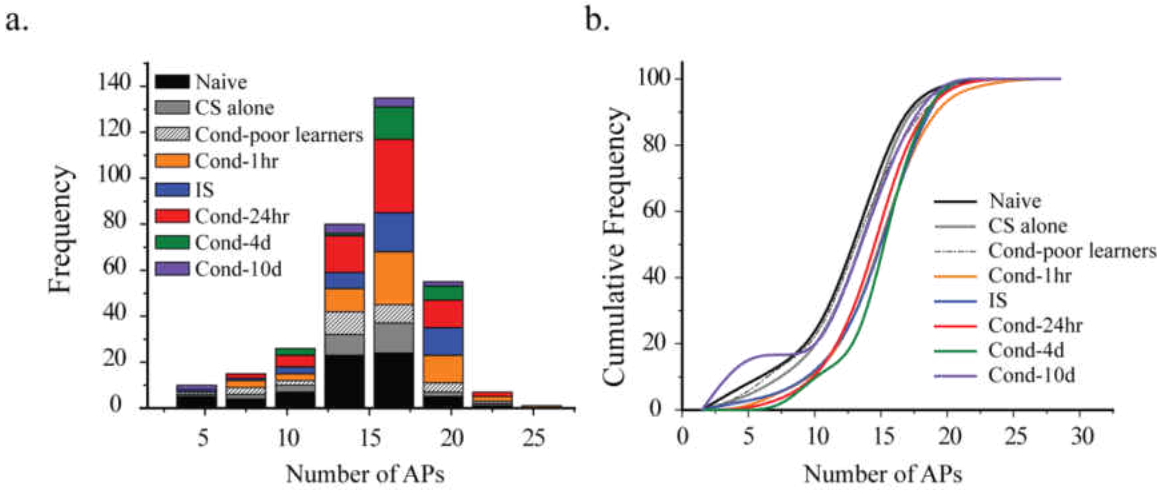
significantly correlated with measures intrinsic excitability (all *p values* > 0.05). This is likely due to the very narrow range of data for both percent time spent freezing as well as measures of intrinsic excitability in each of these groups.

Specificity of learning-related changes in excitability

To estimate the cellular heterogeneity in behaviorally-induced intrinsic excitability changes we plotted the cumulative frequency of number of action potentials fired during spike frequency adaptation for different groups (*see Figure 20a*). In all cases where a significant change in intrinsic excitability was observed, we found that the cumulative frequency plot was shifted smoothly to the right indicating an overall change in intrinsic excitability of neurons. A frequency histogram reveals a very similar pattern (see *Figure 20b*). This is in sharp contrast to the narrow AP firing range for LA neurons we observed in our previous study using sharp microelectrodes (*see Figure 9*). Specifically, LA neurons from naïve animals displayed marked spike frequency adaptation using sharp microelectrode recordings whereas following patch clamp recordings the range of spike frequency adaptation is much greater. These observations make it difficult and somewhat unreliable to identify the subset of neurons that are changed following learning using intrinsic excitability measurements alone (see results for Aim 2).

Figure 20. Distribution of fear conditioning related intrinsic plasticity. *a.* Frequency histogram for number of APs. Number of APs elicited in LA neurons during spike frequency adaptation from rats in various behavioral groups. *b.* Cumulative frequency distribution of number of action potentials (APs) for LA neurons. The smooth rightward shift evident for the curve representing IS, Cond-1hr, Cond-24hr and Cond-4d neurons relative to naïve indicating increased intrinsic excitability.

Figure 20


a.
b.
Frequency
Number of APs
Cumulative Frequency
Number of APs
Naive
CS alone
Cond-poor learners
Cond-1hr
IS
Cond-24hr
Cond-4d
Cond-10d

DISCUSSION

For the current aim, we sought to determine the time course of learning-related intrinsic plasticity within amygdala. We found that intrinsic excitability is increased immediately, and up to 4 days following fear conditioning. These changes are transient as the increase in excitability is no longer evident at 10 days following fear conditioning even though fear memory retrieval is unaffected. The increase in intrinsic excitability was also evident in the immediate shock group which received shock presentations for the same amount of time as the conditioned animals but displayed no evidence of learning the day after. Since conditioned animals that fail to acquire fear conditioning did not display intrinsic plasticity, we conclude that fear conditioning as well as shock presentations can enhance intrinsic excitability of lateral amygdala neurons.

Intrinsic plasticity is transient

As previously described, a majority of studies investigating the time course of intrinsic plasticity demonstrate that it is transient; lasting less than 7-14 days (Moyer et al., 1996; Saar et al., 1998; Zelcer et al., 2006; Motanis et al., 2012). However, a few exceptions have been described. For example, in cerebellum Purkinje neurons intrinsic excitability changes following eyeblink conditioning last for up to 1 month (Schreurs et al., 1998). It is possible that persistence of learning-related intrinsic plasticity is different between structures involved in regulating sensory input and behavioral output, such as cerebellum and amygdala, relative to structures like hippocampus that are involved in information processing.

Here, we find that learning-related changes in LA neuronal excitability were transient and were back to naïve levels by 10 days following fear conditioning. This is in

accordance with data from olfactory discrimination learning studies where changes in neuronal excitability within basolateral amygdala start to reverse by 3 days following learning (Motanis et al., 2012). Thus, learning-related amygdalar intrinsic plasticity, specifically within basolateral amygdala appears to be transient. The transient nature of such learning-related plasticity can be informative. At a basic level, it means that any effect of amygdala intrinsic plasticity on future plasticity, synaptic or behavioral is likely to be transient too.

On a broader level, the transient nature of learning-related intrinsic plasticity has been considered an argument against a memory encoding role of intrinsic plasticity. A similar argument has been used against learning induced excitability changes within hippocampus. A counter argument is that with time, hippocampus-dependent memory becomes hippocampus-independent and the transient nature of excitability simply parallels this transition. Until recently, it was assumed that structures like amygdala (as opposed to hippocampus) have a more permanent role in memory storage and hence the evidence of the memory trace should also be more permanent. These concepts drove some of the early work on amygdala dependent synaptic plasticity as well as my work on LA intrinsic plasticity. This assumption has been challenged very recently. Optogenetic inactivation of basolateral amygdala immediately but not 7 days following fear conditioning impairs fear memory retrieval (Do-Monte et al., 2015). These changes correspond to a shift in fear retrieval circuit from prelimbic cortex-basolateral amygdala-central amygdala to prelimbic cortex-paraventricular thalamus-central amygdala from recent (1d) to a remote time point (7d). Fear conditioning-related synaptic plasticity as measured by increased AMPA: NMDA ratio is no longer evident 7 days after conditioning (Clem and Huganir, 2010).

These data are consistent with the hypothesis that the failure to detect a memory trace in a particular structure may reflect a shift in retrieval circuit rather than a proof that original plastic change was not memory encoding.

Indeed there are few reports that demonstrate long-term synaptic changes in the relevant structures. If synaptic alterations are how memories are stored, then synaptic changes should be long lasting, a finding that is hard to come by. This raises the possibility that lack of evidence for long lasting changes may be a detectability issue. Irrespective of the permanence or detectability of memory traces, global excitability changes such as the ones measured in the current study remain an inefficient way to store information. It is more likely that memory storage involves a delicate balance of synaptic plasticity and more localized dendritic excitability changes that evolve over time as the memory circuits' shift. The more global intrinsic changes may act much like norepinephrine's role in memory modulation – non-specific and time-dependent.

Immediate shock deficit enhances intrinsic excitability

In order to control for the effect of US presentations on LA intrinsic excitability, we used an immediate shock group. The phenomenon of immediate shock deficit is based on the principle that if a rat is shocked immediately after being placed into the chamber (e.g. within 6s), the rat displays no fear of context on a subsequent day as there was no time for a context-shock association (Fanselow, 1986). It is possible to rescue this immediate shock deficit by pre-exposure to the context the day before shock presentation (Fanselow, 1990). This allows the rats to form a context representation, indicating that immediate shock deficit is a context processing deficit (but see Lattal and Abel, 2001; Landeira-Fernandez et al., 2006). In the current study, rats were presented with the same duration of

footshock as other conditioned groups (10s) immediately upon being placed in the conditioning chamber and removed from the chamber as soon as the shock presentation terminated.

We found that IS rats displayed little freezing upon exposure to the conditioning context the following day. Surprisingly, LA neurons from IS rats did show a robust increase in neuronal excitability. The lack of any another neurophysiological data using an IS group restricts our interpretation of these data to theoretical possibilities. We discuss several of these possibilities here. First, it is possible that our observed changes in neuronal excitability within LA neurons are due to shock presentation rather than learning itself. We think this is unlikely as the poor learners (animals that received conditioning but displayed little or no fear to the tone the following day) display no change in excitability relative to LA neurons from naïve rats. This demonstrates that learning, irrespective of stimulus presentation, is necessary for a reduction in excitability.

Furthermore, repeated footshock (US) presentations in the absence of learning are a rodent model for stress (Valenti et al. 2011) that can result in plasticity within LA neurons. Indeed, chronic but not acute stress leads to enhanced excitability of LA neurons (Rosenkranz et al. 2010). However, it is unlikely that our results on LA neuronal excitability are due to the effect of stress for several reasons. Most important among these are that a single session of fear conditioning or immediate shock presentations is akin to acute stress which does not lead to changes in LA excitability. Additionally, the degree of intrinsic plasticity following chronic stress (reduced RMP, increased input resistance, reduced sAHP and spike frequency adaptation) was more extensive than that seen in the current study. It is possible that within amygdala, learning-related intrinsic plasticity

facilitates future learning, whereas more extensive intrinsic plasticity following chronic stress may also contribute to emotional disorders like PTSD (Roozendaal et al. 2009).

Another possibility for excitability changes in IS animals is that even though IS rats do not associate conditioning context with shock presentation, animals do learn to associate other cues (e.g transport, handler etc.) with the shock. Evidence for this comes from a study from O'Reilly lab where rats received several days of preexposure to form an association between the transport cage and context A (Rudy et al., 2002). The rats then received immediate shock in another novel context B. On the subsequent day, the rats were either tested in chamber associated with transportation or the ones in which they were actually shocked. Rats displayed little freezing to context B where they were shocked indicating an immediate shock deficit and no evidence of learning. However, when the rats are tested in the context A, that was associated with the transport cage, rats displayed high levels of fear indicating that the shock was associated with the transport which was in turn associated with context A. Therefore, even if testing in the conditioning context indicates no evidence of learning, rats do form associations following an immediate shock paradigm that may not be readily observable. It is possible that such context learning is driving our observed changes in neuronal excitability.

Whether the neuronal excitability changes in IS group are due to learning or the stress/shock presentation, the problem of finding a good control for US presentation for fear conditioning studies still remains. The most popular control for US presentation, namely pseudo-conditioning (or explicitly unpaired CS and US presentations) leads to inhibitory conditioning to the CS, i.e animals receiving unpaired CS and US presentations encode CS as a safety signal (Rescorla and Lolordo 1965) and display distinct forms of

plasticity within amygdala (Amano et al. 2010). In addition, unpaired animals show higher context fear than those receiving paired CS-US presentations (Phillips and LeDoux 1994). These factors make pseudo-conditioning a poor control for studies investigating learning-related neurophysiological changes in amygdala.

Fear conditioning related intrinsic plasticity is learning specific

The long-delay fear conditioning paradigm used here produces robust fear learning. However, we found that a few rats tested at different time points (2 for Cond-24hr, 1 each of Cond-4d and Cond-10d) displayed low levels of fear to the CS presentation indicating poor retention of memory. In accordance with a wealth of data (extensively discussed earlier), we found that these rats also failed to modulate LA intrinsic excitability. It is also possible that these rats had much lower basal levels of LA neuronal intrinsic excitability preventing successful acquisition of fear learning. Regardless, once again we find that intrinsic excitability levels are a good index of learning making modulation of intrinsic excitability a good target for therapeutic interventions.

Learning-related intrinsic plasticity: acquisition or retrieval driven?

In the current study, a probe test was used to measure learning immediately before slices were made and intrinsic excitability was measured. This raises the possibility that the enhanced excitability of LA neurons observed in the Cond-24hr and Cond-4d group could be due to memory retrieval. We think that this is unlikely at least in the Cond-24hr group as our previous study demonstrated that increased intrinsic excitability was evident 24hr later whether or not animals were presented a probe test. This does not rule out any retrieval-related intrinsic plasticity but simply means that if intrinsic excitability is already increased due to the learning event, it may obscure identification of any retrieval-related

changes. Since we have not performed a similar control for Cond-4d group (with or without probe test), it is possible that some of the intrinsic excitability changes at this time point are due to memory retrieval. Either way, learning or retrieval-related changes in LA neurons are no longer evident 10d following fear conditioning. This time point also corresponds to the time when basolateral amygdala is no longer necessary for fear retrieval (Do-Monte et al., 2015).

AIM 2

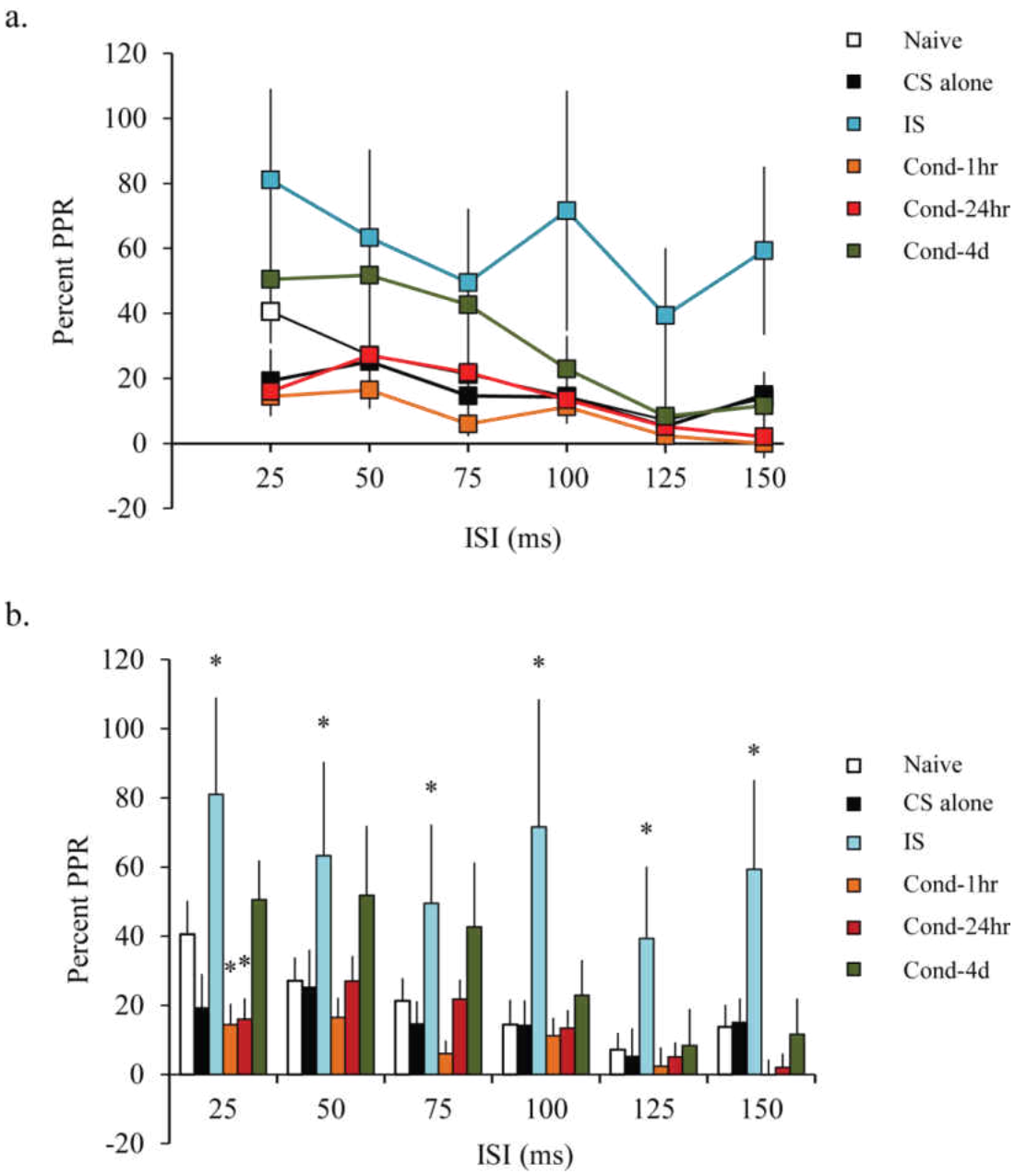
RESULTS

To investigate the relationship between fear learning related intrinsic and synaptic plasticity, we obtained measurements of synaptic strength as well as intrinsic excitability from the same neurons. Rats were fear conditioned on a long-delay fear conditioning paradigm as described for Aim1 (*see Table 4*). Control groups included naïve, CS-alone or IS rats treated same as that for Aim1. After obtaining intrinsic excitability measurements under current clamp mode (as described for Aim1), neurons were voltage clamped at -70 mV (unless mentioned otherwise) and measures of synaptic strength were obtained.

Fear learning related synaptic plasticity can be pre- or post-synaptic. A well-established measure of short term presynaptic plasticity is paired pulse ratio. To obtain a measure of paired pulse ratio, pairs of EPSCs were generated by temporally close presynaptic stimulation of thalamic fibers synapsing onto the LA neurons. Stimulation intensity was adjusted to obtain a reliable EPSC ranging from 50-150 pA. Baseline measurements were obtained for 1 min to establish that the EPSC amplitude remained stable. The interstimulus interval (ISI) varied from 25 ms to150 ms (see *Figure 21*). A repeated measures ANOVA revealed a significant effect of ISI [$F(4.0, 460.9) = 13.9$, $p < 0.001$; Greenhouse-Geisser corrected], group [$F(5, 116) = 3.62$, $p < 0.005$] as well as a group by time interaction [$F(19.9, 460.9) = 1.65$, $p < 0.05$; Greenhouse-Geisser corrected]. A follow-up one-way ANOVA demonstrated a significant effect of behavioral training on paired pulse ratio (PPR) at the shortest interstimulus interval (ISI), i.e. 25 ms [$F(5,121) = 4.5$, $p < 0.001$]. Post hoc tests revealed that while LA neurons from naïve rats displayed a facilitation of the second EPSC (paired pulse facilitation), this facilitation was significantly

Figure 21. Fear conditioning reduces paired pulse facilitation within thalamo-amygdala synapses. *a.* Line graph representing the percentage PPR as a function of increasing ISI (25 -150 ms) and behavioral condition. The PPR is reduced as the ISI between the presynaptic stimulation is increased. *b.* LA neurons from Cond-1hr and Cond-24hr display siginificantly lower PPR at 25 ms ISI relative to naïve LA neurons. The PPR for LA neurons from IS group is significantly is increased relative to LA neurons from naïve rats at all ISIs tested. LA neurons from CS-alone and Cond-4d were not significantly from naïve LA neurons. Asterisk (*) indicates $p < 0.05$ relative to LA neurons from naïve rats. Abbreviations: IS, immediate shock; PPR, paired pulse ratio.

Figure 21


a.
Percent PPR
120
100
80
60
40
20
0
-20
25
50
75
100
125
150
ISI (ms)
Naive
CS alone
IS
Cond-1hr
Cond-24hr
Cond-4d
b.
Percent PPR
120
100
80
60
40
20
0
-20
*
**
25
50
75
100
125
150
ISI (ms)
Naive
CS alone
IS
Cond-1hr
Cond-24hr
Cond-4d

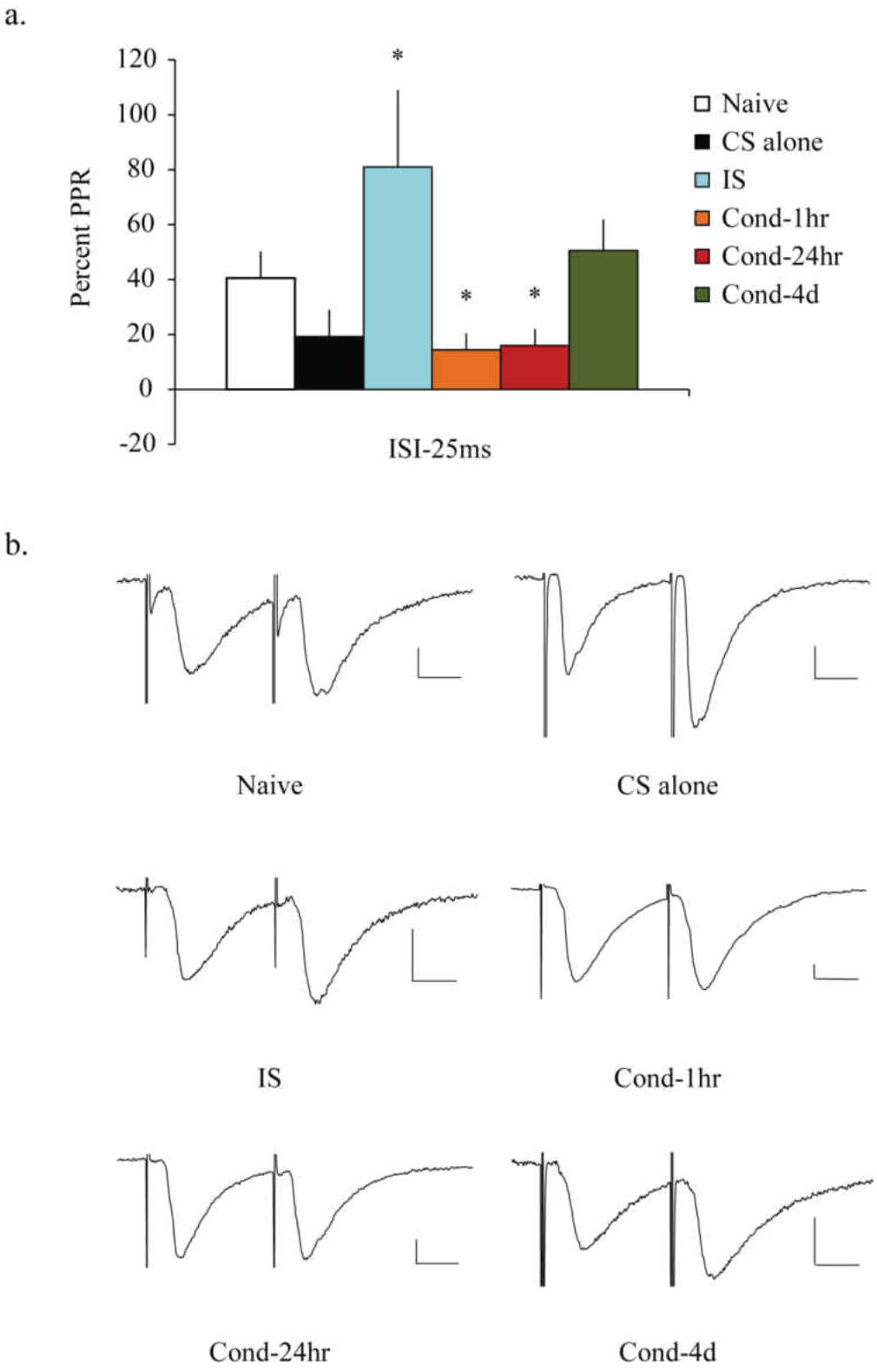
attenuated in LA neurons from Cond-1hr as well as Cond-24hr group (*see Figure 22*, $p < 0.05$). Interestingly, IS group was also significantly different from the naïve rats but in the opposite direction than that of conditioned animals ($p < 0.05$). The paired pulse ratio was significantly higher for LA neurons from IS rats than those from naïve rats. No significant differences were observed between naïve, CS-alone and Cond-4d rats. These data as well as various other published reports (e.g., McKernan and Shinnick-Gallagher, 1997) demonstrate that fear learning is accompanied by a reduction in paired pulse ratio, a well-accepted measure of presynaptic plasticity.

In addition to a change in paired pulse ratio at a short ISI of 25 ms, follow up ANOVAs demonstrated a significant effect or strong trend of behavioral group on PPR at various other time points (all p values < 0.08). Post hoc tests revealed that these effects were driven by a significant increase in PPR in LA neurons from IS rats relative to those from naïve rats ($p < 0.05$). Interestingly, in contrast to all other behavioral groups varying the interstimulus interval between the pairs of EPSCs had no significant effect on the PPR for IS group [repeated measure; $F(5, 50) = 1.822$, $p = 0.13$]. Therefore, the synaptic plasticity observed in the IS group is more robust and in the opposite direction to that observed in the conditioned animals.

In order to obtain additional indices of post synaptic plasticity we sought to measure AMPA: NMDA ratios as well as rectification index for thalamo-amygdala synapses. These measurements are technically challenging as they require obtaining reliable measure of intrinsic plasticity followed by synaptic measures while the cell is voltage clamped at potentials of +40 mV. Successful voltage clamp studies in the past have used potassium as well as sodium channel blockers, especially to ensure that distal dendrites are effectively

Figure 22. Fear conditioning reduces paired pulse facilitation within thalamo-amygdala synapses at short ISI. *a.* Relative to the PPR of LA neurons from naïve rats, Cond-1hr and Cond-24hr is display siginificantly lower PPR whereas the PPR for IS group is significantly is increased ($p < 0.05$). *b.* Representative waveforms displaying learning-related changes in PPR of LA neurons Scale 25 pA, 10 ms. Asterisk (*) indicates $p < 0.05$ relative to LA neurons from naïve rats. Abbreviations: IS, immediate shock; PPR, paired pulse ratio.

Figure 22

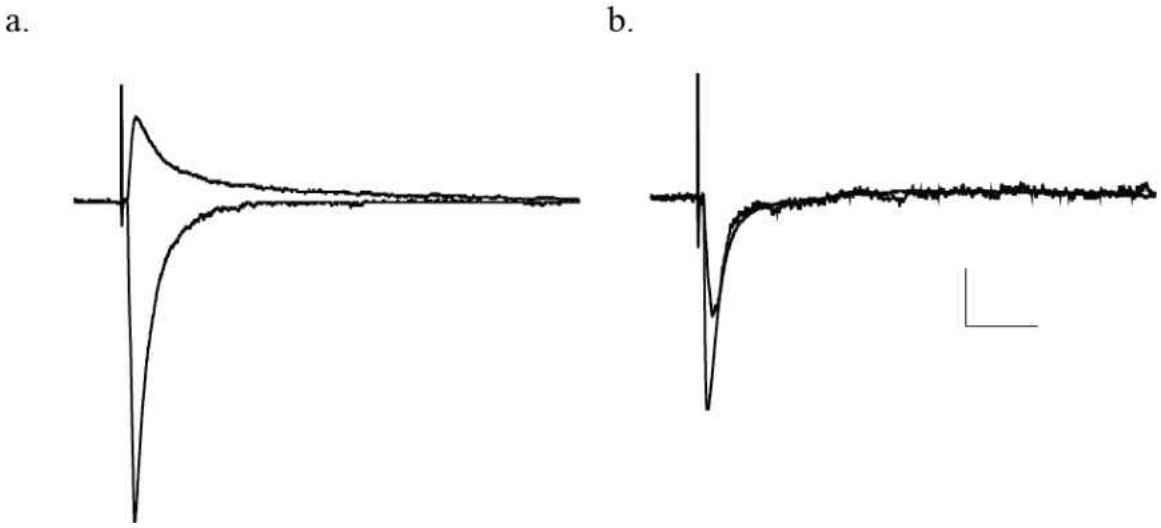

a.
120
100
80
60
40
20
0
-20
Percent PPR
*
*
*
ISI-25ms
Naive
CS alone
IS
Cond-1hr
Cond-24hr
Cond-4d
b.
Naive
CS alone
IS
Cond-1hr
Cond-24hr
Cond-4d

clamped (Clem and Huganir, 2010; Sepulveda-Orengo et al., 2013). Of course, blocking either potassium or sodium channels interferes with measuring intrinsic excitability. Therefore, we were not able to use previously reported methods by which such measurements have been obtained. We found that using the K-gluconate based internal solution in the recording pipette prevents the reversal of evoked synaptic current under voltage clamp conditions (*see Figure 23*). We were not able to obtain such reversal even when the neuron was depolarized at +40 mV. In contrast, using a Cesium based internal solution allowed us obtain evoked excitatory currents that reversed near 0 mV; this is very likely due to an ineffective voltage clamp (Williams and Mitchell, 2008). Thus, we were not able to correlate rectification index of the thalamo-amygdala synapses with intrinsic excitability.

We next attempted to obtain a ratio of AMPA to NMDA currents for LA neurons. Instead of attempting to obtain NMDA currents at +40 mV, we chose to measure NMDA currents at -30 mV, a voltage at which the Mg^{2+} block preventing NMDA receptors from opening should be removed and AMPA currents reduce linearly with the voltage. Thus, we obtained AMPA currents at -70 mV and NMDA currents at -30 mV. In a subset of experiments, we applied DNQX to the slices, to obtain pure NMDA currents at -30 mV. We found that a small late current remained at -30 mV in the presence of DNQX whereas EPSCs were completely abolished at -70 mV. However, the AMPA: NMDA ratios obtained by pharmacologically isolating the current and by voltage changes alone did not correlate. These data indicate to us that we were not able to obtain reliable NMDA currents using this method. It is possible that focal stimulation (less than 100um from soma) would

Figure 23. Reversal of evoked EPSCs depends on the composition of internal solution. *a.* Reversal and inward rectification of evoked EPSCs at +40 mV when a Cesium based internal solution is used. *b.* Evoked EPSCs are reduced but do not display reversal even at a holding potential of +40 mV when a K-gluconate based internal solution was used.

Figure 23



result in more reliable NMDA currents as the voltage is clamped more effectively this close to the soma (see Williams and Mitchell, 2008 for a discussion on the effect of internal solutions and dendritic distance on voltage clamp errors). This was not possible for the current study as we wanted to obtain an index of thalamo-amygdala synaptic plasticity.

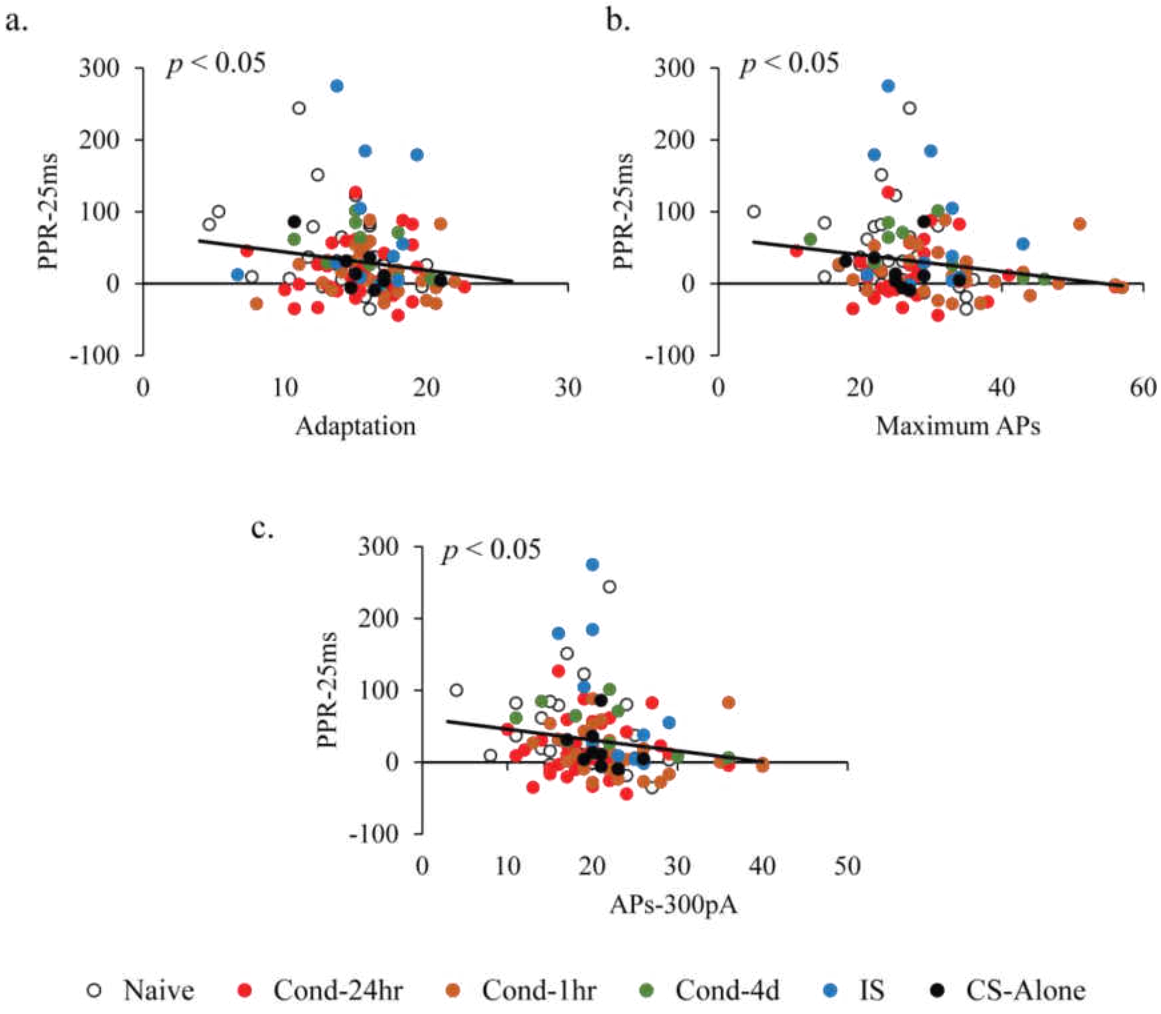
Correlation between synaptic and intrinsic plasticity

The primary aim of this experiment was to investigate whether learning-related intrinsic and synaptic plasticity are co-localized within the same neurons. In order determine this, we measured the relationship between PPR at 25 ms ISI (PPR-25) and various measures of intrinsic excitability (see *Figure 24*). PPR-25 was found to be significantly correlated with spike frequency adaptation, the number of APs elicited following current injections ranging from 50-500 pA as well as maximum number of APs elicited (Spearman's correlation; all $p < 0.05$). This correlation was not learning specific and was also observable in LA neurons from naïve animals ($r = -0.36$, $p < 0.05$). Thus, a significant correlation seems to exist between synaptic efficacy and intrinsic excitability.

To further analyze the relationship between these two forms of plasticity, we spilt the neurons from Cond-1hr and Cond-24hr group into high (unchanged) and low (changed) PPR based on the median PPR-25 value for these groups (*see Figure 25*). This is especially important as correlations are highly sensitive to outliers and non-normal distribution of data, and thus a median split allows us to better analyze the co-localization (or lack of it) of these two forms of plasticity. The neurons in the low PPR or changed groups should be the ones that display reduced PPR as a result of fear learning. As predicted, one-way ANOVA revealed a significant effect of group on PPR [$F(4, 91) = 9.4$, $p < 0.001$]. The PPR was significantly reduced for Cond-1hr changed as well as Cond-24hr changed

Figure 24. Intrinsic excitability measures are correlated with paired pulse ratio. PPR at 25 ms ISI was plotted as a function of intrinsic excitability measures. Spike frequency adaptation (*a)*, maximum number of action potentials fired for 0-500pA current injection (*b*) and number of action potentials fired following a 300pA current injection (*c*) are significantly correlated with PPR ($p < 0.05$). Asterisk (*) indicates $p < 0.05$. Abbreviations: AP, action potential; PPR, paired pulse ratio.

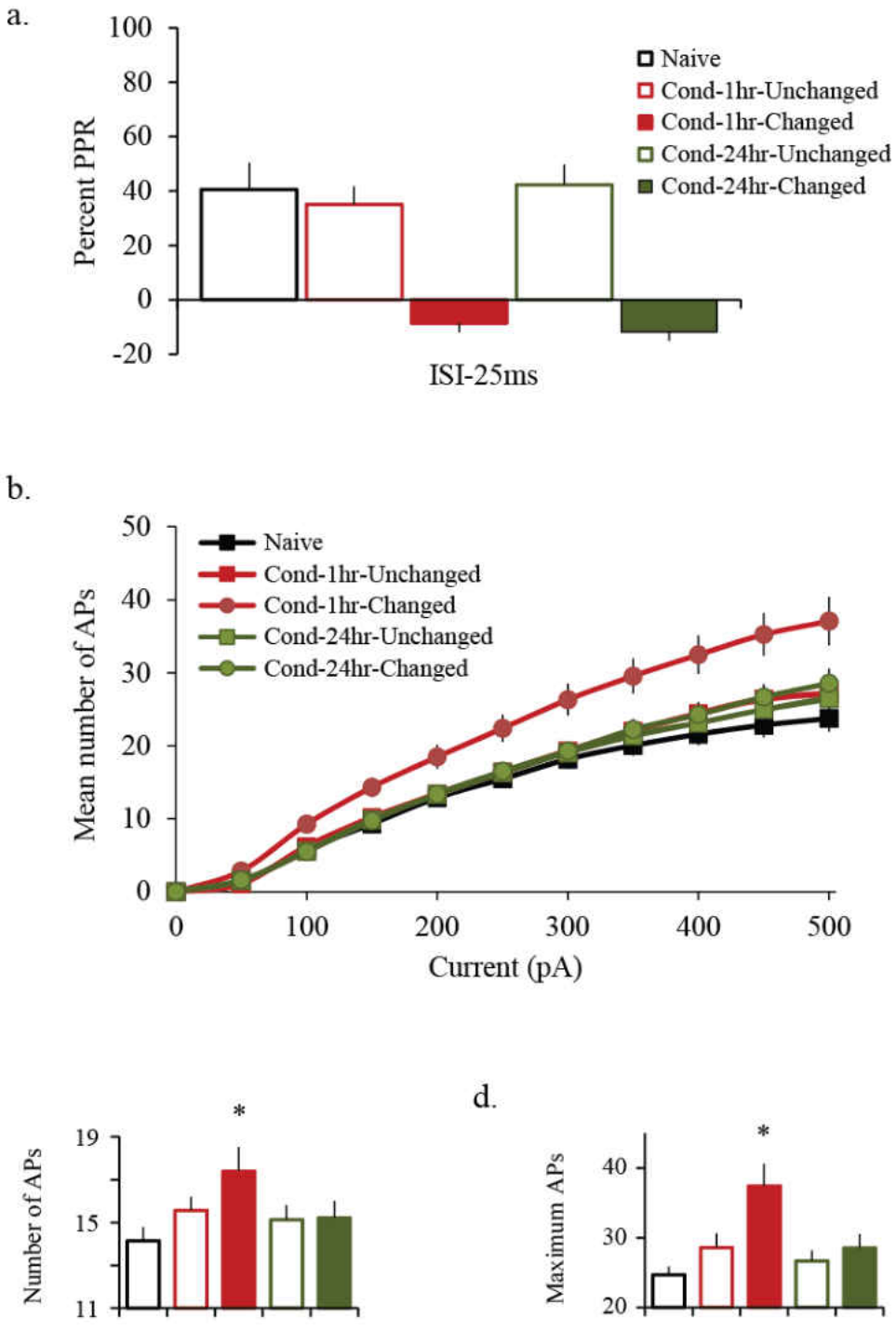
Figure 24


a.
b.
c.
$p < 0.05$
PPR-25ms
Adaptation
Maximum APs
APs-300pA
Naive
Cond-24hr
Cond-1hr
Cond-4d
IS
CS-Alone

neurons but not Cond-1hr unchanged and Cond-24hr unchanged neurons ($p < 0.001$). More importantly, we next analyzed the measures of intrinsic excitability based on these new groups. One-way ANOVA revealed that the number of APs elicited following a series of current pulses ranging from 0 – 500 pA were significantly different between groups. This effect was observed for current injections ranging from 100-500 pA (all p values < 0.05). Interestingly, these results were driven by a significant increase in AP firing in Cond-1hr changed neurons [$F(4, 91) = 6.4$, $p < 0.001$] relative to neurons from the naïve group. No significant differences were found between Cond-1hr unchanged, Cond-24hr changed, Cond-24hr unchanged and naïve groups. As before, relative to neurons from the naïve group, maximum number of APs elicited during these current steps were also significantly increased for Cond-1hr changed but not any other group ($p < 0.001$). A one-way ANOVA revealed a strong trend for a similar effect on spike frequency adaptation [$F(4, 91) = 2.26$, $p = 0.07$]. Post hoc tests confirmed that neurons from Cond-1hr changed group were more excitable relative to other groups ($p < 0.005$). These data demonstrate that learning-related potentiation of intrinsic excitability as well as synaptic efficacy is co-localized immediately following fear conditioning but not 24 hr following fear conditioning.

Figure 25. Intrinsic excitability changes are colocalized to the neurons undergoing synaptic plasticity immediately following fear conditioning. *a.* PPR is significantly reduced in Cond-1hr changed as Cond-24hr changed neurons relative naïve as well as Cond-1hr unchanged and Cond-24hr unchanged group. *b.* Number of APs elicited following 0-500pA current injection are increased in Cond-1hr changed group neurons relative to other groups. *C.* Spike frequency adaptation is significantly reduced in Cond-1hr changed but not in any other group relative to

Figure 25


a.
Percent PPR
100
80
60
40
20
0
-20
ISI-25ms
Naive
Cond-1hr-Unchanged
Cond-1hr-Changed
Cond-24hr-Unchanged
Cond-24hr-Changed
b.
Mean number of APs
50
40
30
20
10
0
0
100
200
300
400
500
Current (pA)
Naive
Cond-1hr-Unchanged
Cond-1hr-Changed
Cond-24hr-Unchanged
Cond-24hr-Changed
c.
Number of APs
19
15
11
*
d.
Maximum APs
40
30
20
*

DISCUSSION

The current experiment sought to measure learning-related synaptic and intrinsic plasticity changes within the same neurons. We found that fear learning results in reduced paired pulse ratio at the thalamo-amygdala synapses immediately as well as 24hr following fear conditioning. These changes were no longer evident 4 days following fear conditioning indicating the transient nature of such presynaptic plasticity. In addition, we found robust changes in paired pulse ratio in the immediate shock group but the direction of these changes were reversed. The IS group had a larger mean paired pulse ratio than naïve LA neurons. More importantly, in the LA neurons from conditioned animals these changes were co-localized with the intrinsic excitability changes immediately following fear conditioning. These data support our hypothesis that intrinsic and synaptic plasticity is colocalized within lateral amygdala neurons following fear conditioning.

Learning-related synaptic plasticity

It is widely believed that memory formation during auditory fear conditioning involves synaptic plasticity within LA neurons. Paired pulse ratio (PPR) is one such way to measure presynaptic plasticity. Here, pairs of presynaptic stimulation that were separated by a short interval (interstimulus interval - ISI) were used to generate a pair of evoked synaptic responses. The magnitude of the second response can either be facilitated (paired pulse facilitation) or reduced (paired pulse depression). Whether a synapse displays paired pulse facilitation or depression depends not only upon the ISI but also by the behavioral state (e.g., learning). Plasticity in the paired pulse ratio is an indication of short term presynaptic plasticity. Specifically, a switch from paired pulse facilitation to reduction (or

a decrease in PPR) indicates an increase in the release probability of the synapse indicating improved synaptic efficacy.

In LA neurons from naïve animals, a short ISI (e.g. 25ms) usually results in paired pulse facilitation (*see Figure 21*) (McKernan and Shinnick-Gallagher, 1997). As the ISI between presynaptic stimulation increases, the PPR is reduced. We demonstrate that long-delay fear conditioning is accompanied by a reduction in paired pulse ratio immediately as well as 24 hr following fear conditioning. These changes were no longer evident 4 days following fear conditioning and PPR was back to naïve levels at this time point. These data are in accordance with other reports that fear learning is accompanied by a reduction in PPR at the synapses carrying auditory information onto the LA neurons.

In addition to learning-related changes, we also found a robust increase in PPR from LA neurons from IS animals. This increase appears to be independent of the ISI as varying the ISI did not seem to affect the PPR. Therefore, shock presentation alone results in a decrease in release probability at the thalamo-amygdala synapses. As with the changes in intrinsic excitability, it is possible that these synaptic changes in the IS group could be either due to shock presentation or learning.

Co-localization between synaptic and intrinsic plasticity

In order to determine a relationship between intrinsic and synaptic plasticity, we looked for a correlation between the two forms of plasticity. We found that intrinsic excitability and PPR were negatively correlated i.e., a higher presynaptic release probability was associated with increased intrinsic excitability. Such a correlation was evident in LA neurons from naïve as well as Cond-1hr animals. Thus, synaptic efficacy

appears to contribute to intrinsic excitability levels under basal conditions as well as following learning.

We found that learning-related synaptic changes predicted intrinsic excitability changes. Specifically, neurons showing the most reduction in PPR immediately following conditioning were also the most excitable. This demonstrates that learning-related synaptic and intrinsic excitability is co-localized to the same neurons. Interestingly, although both synaptic and intrinsic plasticity were significantly altered 24hrs post conditioning, we did not observe a significant correlation between these forms of plasticity at this time point. These data indicate a time-dependent shift in the relationship between learning-related intrinsic and synaptic plasticity.

Taken together, the current experiments demonstrate that fear learning-related intrinsic and synaptic plasticity is transient and can be colocalized to the same neurons. Based on the current models of memory allocation, these data predict that future amygdala dependent memories should be colocalized to the same neuron undergoing intrinsic plasticity albeit in a time dependent manner.

FUTURE DIRECTIONS

In the current experiments, we demonstrate that fear learning related intrinsic and synaptic plasticity is co-localized to the same subset of neurons. These data can inform future work on determining the impact of such co-localization on future synaptic and behavioral plasticity. It is likely that learning-related increase in excitability would facilitate future synaptic plasticity in these neurons and ultimately result in behavioral plasticity or better learning. Mechanistically, it elucidates how memories that are related in time or context or information could be stored by overlapping yet distributed populations of neurons.

It is noteworthy that although intuitive, especially given the data presented earlier, few studies have demonstrated a direct effect of *learning-related intrinsic plasticity* on synaptic or behavioral plasticity. While overt changes in intrinsic excitability such as those mediated by viral transfections and pharmacological manipulations lead to dramatic effects on plasticity and learning, these mechanisms are unlikely to reflect the more subtle and tightly regulated changes in excitability that accompany learning. It is important to understand the distinction between the mechanisms and efficacy of endogenous intrinsic plasticity and that induced by experimental methods. For example, overexpression of CREB and immediate early gene Arc in subset of neurons has been demonstrated to bias memory allocation to these neurons (Han et al., 2007; Han et al., 2009; Zhou et al., 2009; Gouty-Colomer et al., 2015). Such increase in excitability is enough to drive memory of another task learned in close temporal proximity to the same neurons (Zhou et al., 2009), but learning is unable to increase neuronal excitability any further (Gouty-Colomer et al., 2015). Therefore, while it is clear that experimental manipulations are capable of driving

allocation and co-allocation of memories to a subset of neurons, it is unclear that learning-related changes can accomplish the same.

If intrinsic excitability does determine the allocation of future memories such that neurons with higher intrinsic excitability store the memory, additional mechanisms must exist that prevent neighboring neurons from undergoing similar changes so that size of the engram can be maintained. There is relatively little understanding regarding such mechanisms. It is possible that plasticity of inhibitory transmission and local synaptic connectivity (Kim et al., 2013) interact with neuronal excitability to regulate memory allocation. Future studies addressing the mechanisms neuronal competition would be instrumental in our understanding of information processing within the nervous system.

CONCLUSIONS

Understanding how memories are formed and modulated is of significant clinical relevance. According to the US Census Bureau, middle-aged and aged individuals will constitute 45 percent of the US population by the year 2050, drastically increasing the socio-economic impact of aging-related cognitive decline (U.S. Census Bureau, 2004). Such aging-related cognitive decline is well-documented for hippocampus- and PFC-dependent tasks (Burke and Barnes, 2006). Furthermore, these impairments can be rescued by manipulating intrinsic excitability for hippocampal (Deyo et al., 1989; Disterhoft and Oh, 2006; Moyer et al., 1992) as well as PFC-dependent learning (Wang et al., 2011). In addition to normal aging (Chang et al., 2005; Kaczorowski et al., 2012; Moyer et al., 2000; Moyer et al., 1992), rodent models of Alzheimer's disease also display aberrant intrinsic plasticity (Kaczorowski, Sametsky, Shah, Vassar, and Disterhoft, 2011). Modulation of intrinsic excitability could be an important factor in the search for neurobiological approaches to mitigate or prevent the onset of aging-related cognitive impairments and even rescue those deficits after they emerge.

Associative memories can predict aversive or appetitive stimuli. In some cases, such as PTSD, these memories are maladaptive and can lead to reoccurrence of the traumatic events (Mahan and Ressler, 2012). Such an abnormal fear response may arise as a result of metaplasticity, where some prior events lead to alterations in the intrinsic excitability of neurons within the fear circuit (Rosenkranz, Venheim, and Padival, 2010). In other cases, associative memories can provoke drug seeking as a result of presentation of cues previously associated with drug taking (Childress, McLellan, and O'Brien, 1986). By understanding the interplay between intrinsic excitability and behavioral plasticity, it

may be possible to develop neurobiologically based treatment strategies that when combined with exposure therapy causes extinction of these abnormal associations (Myers, Carlezon, and Davis, 2011). Strengthening this extinction learning by enhancing intrinsic excitability can provide treatment for pathological forms of memory.

Finally, the old saying, *an ounce of prevention is worth a pound of cure* is certainly relevant here. Understanding the fundamental mechanisms that underlie memory formation may influence our ability to maximize the beneficial effects of experience-dependent plasticity and facilitate development of treatment strategies aimed at improving our quality of life.

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Wierzynski CM, Lubenov EV, Gu M, Siapas AG (2009) State-dependent spike-timing relationships between hippocampal and prefrontal circuits during sleep. Neuron 61:587-596.

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Zhang W, Linden DJ (2003) The other side of the engram: experience-driven changes in neuronal intrinsic excitability. Nat Rev Neurosci 4:885-900.

Zhou Y, Won J, Karlsson MG, Zhou M, Rogerson T, Balaji J, Neve R, Poirazi P, Silva AJ (2009) CREB regulates excitability and the allocation of memory to subsets of neurons in the amygdala. Nat Neurosci 12:1438-1443.

Zovkic IB, Guzman-Karlsson MC, Sweatt JD (2013) Epigenetic regulation of memory formation and maintenance. Learn Mem 20:61-74.

MEGHA SEHGAL

EDUCATION

2009- present	PhD Candidate, University of Wisconsin-Milwaukee Advisor: James R. Moyer, Jr., PhD **GPA: 4:00**
2013	M.S., Psychology, 2013 University of Wisconsin-Milwaukee **GPA: 4:00**
2003-2006	B.Sc. (Honors), Microbiology, University of Delhi, Delhi, India **GPA: 3.79**

PUBLICATIONS

Sehgal, M., Ehlers, V.E. & Moyer, J. R., Jr. (*2014*). Fear conditioning enhances intrinsic excitability of lateral amygdala neurons. *Learning and Memory,* 21(3):161-70

Sehgal, M., Song, C., Ehlers, V.E. & Moyer, J. R., Jr. (*2013*). Learning to learn – intrinsic plasticity as a metaplasticity mechanism for memory formation. *Neurobiology of learning and memory,* 105: 186-199

Song, C., Detert, J. A., **Sehgal, M.**, and Moyer, J. R., Jr. (*2012)*. Effects of trace fear conditioning on synaptic and intrinsic plasticity in rat hippocampal CA1 neurons. *Journal of Neurophysiology*, 107 (12): 3397-408.

IN PROGRESS PUBLICATIONS

Sehgal, M., Detert, J.A., & Moyer, J. R., Jr. (*in preparation*). Aging reduces basal neuronal activation as measured by immediate early gene expression within medial prefrontal cortex

Sehgal, M., Detert, J.A., & Moyer, J. R., Jr. (*in preparation*). Early detection of region-specific changes in immediate early gene expression within hippocampus during normal aging

Sehgal, M., Bula T.B., Hummer B.H., Fulleylove-Krause B.K. & Moyer, J. R., Jr. (*in preparation*). Neural correlates of aging-related learning deficits in a distributed brain network.

INVITED TALKS AT INTERNATIONAL CONFERENCES

Selected to speak at the **Nanosymposium session**, 44, Neural circuitry underlying anxiety and processing of fear, **Society for Neuroscience**, 2014, Extended Amygdala Circuits and Behavior.

Selected to speak at the **Nanosymposium session**, 42, Neural circuitry underlying anxiety and processing of fear, **Society for Neuroscience**, 2012, Fear conditioning enhances intrinsic excitability of lateral amygdala neurons.

Selected to speak at the **Molecular and Cellular Cognition Society annual meeting**, 2012, Fear conditioning enhances intrinsic excitability of lateral amygdala neurons.

PUBLISHED ABSTRACTS PRESENTED AT INTERNATIONAL CONFERENCES

Sehgal, M., Bula, T. S., Fettinger N.B. & Moyer Jr., J. R. (2014) Neural circuitry underlying extinction of trace fear conditioning. *Society for Neuroscience Abstracts, 44, Program Number 293.03*

Reis, D.S., **Sehgal, M.**, Helmstetter, F.J. (2014) Activity dependent proteolysis in the amygdala modulates protein synthesis in the amygdala and dorsal hippocampus during consolidation of fear conditioning. *Society for Neuroscience Abstracts, 44, Program Number 90.22/RR1*

Sehgal, M., Bula, T. S., Hummer, B.H., Fulleylove-Krause, B.K., Velic, A.J. & Moyer Jr., J. R. (2014) Aging-related changes in the neural circuitry underlying extinction of trace fear conditioning. Presented at the *Molecular and Cellular Cognition Society*

Sehgal, M., Bula, T. S., Fettinger N.B. & Moyer Jr., J. R. (2014) Neural circuitry underlying extinction of trace fear conditioning. Presented at the *Annual Meeting of Pavlovian Society*

Reis, D.S., **Sehgal, M.**, Helmstetter, F.J. (2014) Activity dependent proteolysis in the amygdala modulates protein synthesis in the amygdala and dorsal hippocampus during consolidation of fear conditioning. Presented at the *Annual Meeting of Pavlovian Society*

Sehgal, M., Bula, T. S., Detert J. A. & Moyer Jr., J. R. (2013) Aging reduces basal neuronal activation as measured by immediate early gene expression within medial prefrontal cortex and hippocampus. Presented at the *Molecular and Cellular Cognition Society*

Moyer Jr., J. R., **Sehgal, M**., Detert, J. A. & Bula, T. S. (2013) Early detection of region-specific changes in immediate early gene expression within hippocampus during normal aging. *Society for Neuroscience Abstracts, 43, Program Number 579.12/LLL11*

Sehgal, M., Detert J. A., Bula, T. S. & Moyer Jr., J. R. (2013) Aging reduces basal neuronal activation as measured by immediate early gene expression within medial prefrontal cortex. *Society for Neuroscience Abstracts, 43, Program Number 579.11/LLL10*

Sehgal, M., Bula, T. S., Detert, J. A. & Moyer Jr., J. R. (2013) Aging reduces basal neuronal activation as measured by immediate early gene expression within medial prefrontal cortex and hippocampus. Presented at the *Annual Meeting of Pavlovian Society*

Sehgal, M., Ehlers, V.E., Moyer, J. R., Jr. (2013) Fear conditioning enhances intrinsic excitability in a subset of lateral amygdala neurons. Presented at the *Society for Neuroscience Milwaukee Area Chapter Meeting*

Bula, T.S., Aitken, J.C., **Sehgal, M.**, Moyer, J. R., Jr. (2013) Aging reduces immediate early gene expression in a region-specific manner. Presented at the *Society for Neuroscience Milwaukee Area Chapter Meeting*

Sehgal, M., Girgis, A.M., Moyer, J. R., Jr. (2012) Fear conditioning enhances intrinsic excitability of lateral amygdala neurons. Presented at the *Society for Neuroscience Abstracts, 42, Program Number 422.09*

Sehgal, M., Girgis, A.M., Moyer, J. R., Jr. (2012) Fear conditioning enhances intrinsic excitability of lateral amygdala neurons. Presented at the *Molecular and Cellular Cognition Society*

Sehgal, M., Girgis, A.M., Moyer, J. R., Jr. (2012) Fear conditioning enhances intrinsic excitability of lateral amygdala neurons. Presented at the *Annual Meeting of Pavlovian Society*

Sehgal, M., Detert, J.A., Girgis, A.M., Moyer, J. R., Jr. (2011) Aging-related Changes in Immediate Early Gene Expression in Rat Medial Prefrontal Cortex. Presented at the *Society for Neuroscience Abstracts, 41, Program Number 411.13*

Sehgal, M., Detert, J.A., Girgis, A.M., Moyer, J. R., Jr. (2011) Aging-related Changes in Immediate Early Gene Expression in Rat Medial Prefrontal Cortex and Hippocampus. Presented at the *Molecular and Cellular Cognition Society*

Sehgal, M., Detert, J.A., Lambrecht, T.L., Moyer, J. R., Jr. (2011) Aging-related Changes in Immediate Early Gene Expression in Rat Medial Prefrontal Cortex and Hippocampus. Presented at the *Annual Meeting of Pavlovian Society*

Song, C., Detert, J.A., **Sehgal, M.**, Moyer, J. R., Jr. (2011) Trace Fear Conditioning Enhances Synaptic and Intrinsic Plasticity in Rat Hippocampus. Presented at the *Society for Neuroscience Milwaukee Area Chapter Meeting, Program Number 38*

Song, C., Detert, J.A., **Sehgal, M**., Moyer, J. R., Jr. (2010) Trace fear conditioning enhances synaptic and intrinsic plasticity in rat hippocampus. Oral presentation in the Nanosymposium at *Society for Neuroscience Meeting, 40, Program Number 735.3*

Song, C., Detert, J.A., O'Hara, D.T., **Sehgal, M.**, Moyer, J. R., Jr. (2010) Enhancement of synaptic and intrinsic plasticity in hippocampus after trace fear conditioning. Presented at the *Society for Neuroscience Milwaukee Area Chapter Meeting*

Song, C., O'Hara, D. T., Detert, J. A., **Sehgal, M.,** Moyer, J. R., Jr. (2009) Learning-induced synaptic and intrinsic plasticity in hippocampus. *Society for Neuroscience Abstracts, 39, Program Number 319.19*

Sehgal, M., Krishna S. (2008) Role of Notch signaling in self renewal of mammalian neural stem cells. Presented at the *Model Organisms and Stem Cells in Development, Regeneration and Disease*.

AWARDS AND HONORS

2014	UWM-Department of Psychology Summer Research Fellowship
2014-2015	UWM Distinguished Dissertator Fellowship
2013-2014	Event grant for Association for graduate students in psychology (AGSIP) annual symposium
2013	Best presentation at the annual meeting of Association for graduate students in psychology (AGSIP), 2013
2012	Graduate Student Travel Award for Society for Neuroscience Conference, University of Wisconsin-Milwaukee
2013	Travel Grant for Graduate Student in Neuroscience
2012	Sigma-Xi Grants-in-Aid of Research Recipient
2012	Travel Grant for Graduate Student in Neuroscience
2012-2013	UWM Distinguished Graduate Student Fellowship
2011	Travel Grant for Graduate Student in Neuroscience
2011	Graduate Student Travel Award for Society for Neuroscience Conference, University of Wisconsin-Milwaukee
2010	Travel Grant for Graduate Student in Neuroscience
2010	Graduate Student Travel Award for Society for Neuroscience Conference, University of Wisconsin-Milwaukee
2010-2011	Chancellors Award, University of Wisconsin-Milwaukee
2009	Travel Grant for Graduate Student in Neuroscience
2009	Graduate Student Travel Award for Society for Neuroscience Conference, University of Wisconsin-Milwaukee
2009-2010	Chancellors Award, University of Wisconsin-Milwaukee
2008-2009	Chancellors Award, University of Wisconsin-Milwaukee

TEACHING EXPERIENCE

2013 Spring - 2014 Fall Semester	Physiological Psychology (Instructor)
2011 Spring - 2012 Spring Semester	Psychological Statistics (Teaching Assistant)
2009 Fall - 2010 Fall Semester	Physiological Psychology (Teaching Assistant)
2008 Fall - 2009 Spring Semester	Anatomy and Physiology (Lab)

POSITIONS HELD

President, Association for Graduate Students in Psychology, UW-Milwaukee, 2013-2014

Vice-President, Association for Graduate Students in Psychology, UW-Milwaukee, 2012-2013

Secretary, Graduate Students in Neuroscience, UW-Milwaukee, 2009-2014

PROFESSIONAL MEMBERSHIPS

Association for Graduate Students in Psychology, UW-Milwaukee

Graduate Students in Neuroscience, UW-Milwaukee

Molecular and Cellular Cognition society

Society for Neuroscience

The Milwaukee Chapter for the Society for Neuroscience

RESEARCH EXPERIENCE

2008-current Doctoral research, Advisor: Dr. James R. Moyer Jr.

2006-2008 **National Centre for Biological Sciences, Bangalore, India**

1. ***Advisor:*** Dr. Sumantra Chattarji
   Projects: *a. 'Effect of ovarian hormones on the effect of chronic immobilization stress in female rats'*

b. ***'****Long-term effects of acute immobilization stress on persistence of anxiety–like behavior and hippocampus BDNF levels in rats*'

2. ***Advisor:*** Dr. K. Vijayraghavan
   Project: '*Novel molecules involved in the development and maintenance of the motor neuron-muscle-tendon circuit in Drosophila*'
3. ***Advisor:*** Dr. Sudhir Krishna
   Projects: *a.* '*Differential gene expression profile of $CD133^+$ and $CD133^-$ cells in CaSki, a cervical cancer cell line*'
   b. '*Role of Notch signaling in self-renewal of mammalian neural stem cells*'

Summer, 2006 — *'Isolation and Characterization of Group A Streptococci from patient samples'* under the guidance of **Prof. A.K. Johri** at the 'School of Life Sciences', at **Jawaharlal Nehru University (JNU), New Delhi**. This **2 month** project entailed the collection of patient samples (throat swabs), isolation and identification of Group A *Streptococci* (GAS) from these samples, followed by serotyping to check for the most prevalent strains and mammalian cell invasion assays to check for their virulence.

Summer, 2004 — Voluntary work at the Microbiology lab of Din Dayal Upadhyay Hospital, New Delhi for a period of 2 weeks. During this period I performed diagnostic and antibiotic sensitivity tests on clinical samples for various infectious diseases.