Synaptic Plasticity and Neuromodulation at the Distal Apical Dendrites Of CA1 Pyramidal Cells In Vivo

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Graduate Program in Neuroscience
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ABSTRACT
Long-term potentiation at the distal apical dendrites of CA1 pyramidal cells. This study investigated whether a theta burst stimulation (TBS) tetanus could induce long-term potentiation (LTP) at the distal apical dendrites via the temporoammonic (TA) pathway and the nucleus reuniens (RE)-CA1 pathway. Neuromodulation by dopamine (DA) and acetylcholine (ACh) during LTP in the TA synapse was also investigated. Extracellular potentials were recorded in hippocampal CA1 of urethane-anaesthetized mice. LTP was induced in the TA and RE-CA1 synapses. DA and ACh were also found to have neuromodulatory roles in LTP of the TA-CA1 synapse. Mice deficient in their ability to produce vesicular ACh transporter showed a reduced ability for LTP. Co-stimulation of the ventral tegmental area (VTA) during the TA tetanus did not affect LTP; DA antagonist haloperidol injected prior to the TA tetanus also did not affect LTP. However, co-stimulation of the TA pathway and VTA after haloperidol injection reduced LTP. Therefore, LTP may be induced in vivo at the distal apical dendrites, and this plasticity at the TA synapse is modulated by DA and ACh.

Keywords: synaptic plasticity; hippocampus; memory formation; distal apical dendrites; CA1; mice; current source density
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<tbody>
<tr>
<td>-</td>
<td>negative</td>
</tr>
<tr>
<td>+</td>
<td>positive</td>
</tr>
<tr>
<td>αCAMKII</td>
<td>alpha calcium/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AEP</td>
<td>average evoked potential</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APV</td>
<td>2-amino-5-phosphatepentanoic acid</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>calcium</td>
</tr>
<tr>
<td>CSD</td>
<td>current source density</td>
</tr>
<tr>
<td>DG</td>
<td>dentate gyrus</td>
</tr>
<tr>
<td>EC</td>
<td>entorhinal cortex</td>
</tr>
<tr>
<td>fEPSP</td>
<td>field excitatory post synaptic potential</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-Aminobutyric acid</td>
</tr>
<tr>
<td>HET</td>
<td>heterozygous (genotype)</td>
</tr>
<tr>
<td>HFS</td>
<td>high frequency stimulation</td>
</tr>
<tr>
<td>K&lt;sup&gt;+ &lt;/sup&gt;</td>
<td>potassium</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>magnesium</td>
</tr>
<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptor</td>
</tr>
<tr>
<td>MPP</td>
<td>medial perforant path</td>
</tr>
<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt;</td>
<td>sodium</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>OLM</td>
<td>oriens lacunosum-moleculare</td>
</tr>
<tr>
<td>PFC</td>
<td>prefrontal cortex</td>
</tr>
<tr>
<td>PPF</td>
<td>paired pulse facilitation</td>
</tr>
<tr>
<td>RADI</td>
<td>radiatum- and dentate-innervating (cells)</td>
</tr>
<tr>
<td>RE</td>
<td>nucleus reuniens</td>
</tr>
<tr>
<td>SLM</td>
<td>stratum lacunosum moleculare</td>
</tr>
<tr>
<td>TA</td>
<td>temporoammonic</td>
</tr>
<tr>
<td>TBS</td>
<td>theta burst stimulation</td>
</tr>
<tr>
<td>VAChT</td>
<td>vesicular acetylcholine transporter</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
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LITERATURE REVIEW

General introduction

The world is a jumble of predictable and unpredictable aspects: the seasons rotate with regularity, but one winter may be drastically different from the next; while we settle into patterns and familiarity, events like natural disasters can force us to change our ways of life. Thus, it is through memory and learning that we are able to navigate our way through life. In the stable aspects of our life, memory allows for better decision-making based on our past actions. With changes in our environment or simply the desire to improve an aspect of our life, it is through learning that we can adapt by developing new skills and patterns of action. Without the ability to form memories and learn, our ability to carry out more complex cognitive tasks would be greatly impaired. But unlike other body systems that directly produce a physical effect we can observe, memories and learning are intangible concepts that must be indirectly measured, whether by memory tasks or electrical and magnetic recordings. Despite these difficulties, neuroscience has made great strides in advancing our understanding of how memories are formed in the brain. But there is still much we do not know regarding the mechanisms of the brain, which impairs our ability to treat patients who show memory loss due to disease or accidents. By studying the brain’s mechanisms in memory, we may one day bridge the gap between brain activity and visible measures of memory and cognition.
Hippocampal anatomy

Located in the medial temporal lobe of the brain, the hippocampus has a curved and complex shape across mammals. The hippocampus formation consists of the hippocampus proper, the dentate gyrus, and the subiculum.

The hippocampus may be divided into layers based on function and anatomy. The subiculum has the stratum pyramidale and the dendritic stratum moleculare. The stratum oriens layer is between the alveus and stratum pyramidale. Below the stratum pyramidale is the stratum radiatum; below the stratum radiatum is the stratum lacunosum moleculare (SLM; Fig. 1B). Below the SLM is the dentate gyrus, separated by the hippocampal sulcus. Finally, the stratum moleculare of the dentate gyrus is located between the hippocampus sulcus and stratum granulosum, or the location of granule cells soma.

There are several inputs to the hippocampus. The trisynaptic circuit, which projects from the entorhinal cortex (EC) through the perforant path to the dentate gyrus (DG) to the CA3 via mossy fibres to the CA1 via Schaffer collateral synapse has been widely studied at the cellular and physiological level. Other inputs include a direct projection from the entorhinal cortex to the CA1, known as the perforant path or the temporammonic (TA) synapse, and also traversing through the perforant path, as well as a direct projection from the nucleus reuniens (RE) to the CA1 (Fig. 1A). The CA3 synapses on the basal and proximal apical dendrites, whereas the perforant pathway and the RE pathway project to the distal apical dendrites in the SLM (Dolleman-Van der Weel et al, 1997).

The RE is part of the ventral midline thalamus, as the largest midline thalamic nuclei, and is currently believed to be the primary if not major source of thalamic input to the hippocampus (Bokor et al, 2002). It receives projections from the medial prefrontal cortex (mPFC), an area
that receives hippocampal input but does not have any direct projections back (McKenna & Vertes, 2004). Anatomical studies indicate that the mPFC projects to the RE, and cells within the RE that receive these projections themselves project to the hippocampus (Vertes et al, 2007; Varela et al, 2014). The RE can also influence activity in the PFC (Saalmann, 2014). These studies, along with functional studies discussed later, implicate the RE as an important site where information may travel from the mPFC to the hippocampus. The RE is glutamatergic and projects to both the distal apical dendrites and to interneurons in the SLM, as well as to the entorhinal cortex and to the subiculum, with different cells within the RE selectively innervating different areas (Dolleman-Van Der Weel & Witter, 1996; Bokor et al, 2002).

The principal cells of the hippocampus are the pyramidal cells in the CA3 and CA1 and granule cells in the dentate gyrus. These cells are excitatory and use glutamate for neurotransmission (White et al, 1977; Colbert & Levy, 1992). Hippocampal pyramidal cells have a triangular cell body for which they are named after; the cell bodies are located in the stratum pyramidale layer. Basal dendrites extend from the soma into the stratum oriens, while apical dendrites extend into the stratum radiatum (proximal apical dendrites) and the SLM (distal apical dendrites).

To modulate the excitatory pyramidal cells, interneurons use the inhibitory neurotransmitter gamma-aminobutric acid (GABA) (Freund & Buzsáki, 1996). There are many types of interneurons that can be classified based on characteristics such as shape and location, and different types of interneurons innervate specific areas. In the SLM are several types of interneurons including: apical dendrite innervating cells, basket cells, perforant path-associated interneurons, Schaffer collateral pathway-associated neurons, radiatum- and dentate-innervating (RADI) cells, oriens lacunosum-moleculare (OLM) cells, and neurogliaform cells (Capogna, 2011; Leão et al, 2012). To date, the majority of work on these interneurons has been on the
neurogliaform cells, which gates information between the entorhinal cortex and the CA1 in the SLM and can be blocked by a GABA$_A$ receptor antagonist but not by a GABA$_B$ receptor antagonist. This corresponds with findings that GABA$_B$ agonists reduce the response evoked by stimulation of the Schaffer collaterals, but do not reduce the response evoked by stimulation of the perforant path (Colbert & Levy, 1992). These interneurons also fire in vivo at theta frequency, and thus may contribute to the generation of theta rhythm in the hippocampus (Capogna, 2011). OLM cells have also been found to inhibit distal dendrites of CA1 pyramidal cells while disinhibiting proximal dendrites (Leão et al, 2012).

In addition to being modulated differently, there are developmental differences between the pathways. The temporoammonic pathway shows a development increase in short-term facilitation from juvenile to young adult rats, which is the opposite pattern found in the Schaffer collaterals (Speed & Dobrunz, 2009). Thus, despite both originating from the entorhinal cortex and synapsing onto CA1 pyramidal cells, they appear to develop separately: and as discussed in the next section, they also show functional differences.
Figure 1. Schematic diagram of hippocampal connections. (A) Coronal section of a rat hippocampus depicting major subregions and circuitry. Arrows show direction of information propagation. Information enters the entorhinal cortex from neocortex and can project directly to the CA1 or through a trisynaptic circuit by first synapsing on the DG which then projects to the CA3 via mossy fibers (MF) and from the CA3 to the CA1 via the Schaffer collateral pathway. There is also a projection from the nucleus reuniens (RE) to the CA1 region. Information can then travel from the CA1 to the entorhinal cortex. (B) Organization of different layers in CA1 of the pyramidal cells. Basal dendrites are in the stratum oriens, proximal apical dendrites are in the stratum radiatum, and distal apical dendrites are located in the stratum lacunosum moleculare (original illustration by (Megias et al., 2001)).
**Hippocampal function**

Modern research on the hippocampus’ function has largely focused on spatial memory, episodic memory, and schizophrenia. The study of spatial memory has been primarily on hippocampal place cells, which are cells that respond when in a specific place in an environment; i.e. they are location-specific (O’Keefe, 1979). Lesions to the hippocampus result in reduced performance on the Morris water maze, a test of spatial memory (Morris, 1984). Imaging studies in humans have also found activation in the hippocampus during recall of spatial contexts (Burgess *et al.*, 2001).

The hippocampus also plays a role in episodic memory, or memory of experienced events. This has been demonstrated very vividly in patients with lesions to the hippocampus, such as Henry Molaison, best known as ‘Patient H.M.’. H.M. had his hippocampus surgically destroyed in an attempt to relieve his epileptic seizures; after surgery, he became unable to form new episodic memories. H.M. and other similar patients who have had their hippocampus destroyed through accidents or disease have been studied. While patients such as H.M. were unable to form new episodic memories, they were able to show learning in hand-eye coordination tasks (Milner, 1962). Despite improving on the task to the point they became very proficient at it, they claimed to have no memory of ever doing the task in the past. Although it is widely agreed that the hippocampus plays some role in episodic memory, its exact role – whether it is involved in encoding, consolidation, and/or retrieval - remains under debate (Squire & Wixted, 2011).

Research of the hippocampus and its role in memory has benefited greatly from patients like H.M., but they do not provide much insight into the cellular mechanisms of the hippocampus. Invasive experimental protocols help to understand how the hippocampus carries out its functions. Animals commonly used in hippocampal research include rats and mice, and similar results have been found across both species. The use of mice allows for genetic manipulations
that are often more precise than the manipulations used in rats – e.g. a genetic knockout or knockdown versus the use of drugs or lesions – and current research suggests that the function of the hippocampus is similar across mice, rats, and humans. By studying animals using protocols that cannot be done on humans, we may unravel the mechanisms of these pathways.

For years it was believed that hippocampal function was primarily due to the trisynaptic circuit. However, there is a growing body of evidence that the other inputs to the CA1 described previously may be just as important functionally. An imaging study in primates undergoing a variety of cognitive tasks found strong activation in the SLM of CA1, and very low activity in the CA3: indicating that on the tasks given to the subjects, the trisynaptic pathway was used considerably less than the direct pathways (Sybirska et al, 2000). Brun and colleagues found that a whole hippocampal lesion in rats led reduced performance on the Morris water maze, but a CA3 lesion did not show the same decline (Brun et al, 2002). In mice, transgenically inhibiting the trisynaptic pathway led to the same result: mutant mice were still able to learn the Morris water maze task (Nakashiba et al, 2008). The question then becomes: what pathways are involved, if not the trisynaptic pathway?

Physiological studies focusing on the direct projection from the entorhinal cortex to CA1 suggest that it has an important functional role. Blocking the direct temporoammonic pathway results in significant disruption of place cell firing in CA1, spatial working memory, and encoding during fear conditioning (Brun et al, 2008; Cutsuridis et al, 2010). While these studies show that the temporoammonic pathway appears to have its own functions and is capable of carrying out these functions even in the absence of the Schaffer collaterals, an electrophysiological study found that when the two pathways were paired together, long-term potentiation could be induced in the Schaffer collaterals (Dudman et al, 2007). The pairing would only induce the potentiation when
the temporoammonic stimulation preceded the Schaffer collateral stimulation by 20 ms, but not in reverse. This would suggest that physiologically, these two pathways may work together.

As previously stated, the RE receives projections from the mPFC and projects to the hippocampus: thus, the RE is believed to play a role in hippocampal tasks such as spatial and working memory. Learning the Morris water maze leads to increased neural activity in the RE, and inactivation of the RE affects performance on memory consolidation (Davoodi et al., 2011; Loureiro et al., 2012). A study by Ito and colleagues (2015) suggests that the mPFC-RE-CA1 circuit is important for spatial planning: being able to move to a goal destination based on different trajectory choices (Ito et al., 2015).

Other studies have found evidence of alternative possible roles of the RE. An impaired RE has been found to lead to overgeneralization during the acquisition phase of a fear conditioning paradigm, suggesting that the RE is important for acquiring specific memories (Xu & Südhof, 2013). Some studies have found that RE inactivation did not affect performance on the Morris water maze, but instead leads to impaired cognitive flexibility, or the ability to change strategies when the learned strategy is not working for the current problem (Dolleman-van der Weel et al., 2009; Cholvin et al., 2013). Also in contrast to findings that inactivation of the RE leads to memory impairments, high frequency stimulation and optogenetic activation have also been found to impair performance on working memory tasks (Eleore et al., 2011; Duan et al., 2015). Notably, in some studies the RE lesion refers to a lesion of both the RE and the nearby rhomboid nucleus, which may cause conflicting results. Overall, evidence suggests that the RE does play an important role in regulating information it transfers between the PFC and hippocampus, though its precise roles and mechanisms remain unclear.
The hippocampus has also been implicated in the study of schizophrenia. Schizophrenia is associated with abnormal hippocampal structure as well as irregular corticohippocampal interactions (Fukuzako et al., 1995; Heckers et al., 1998). Hippocampal slices in post-mortem brain tissue of schizophrenia patients have been found to have decreased levels of synaptic proteins, especially complexin II which is primarily found in excitatory synapses (Harrison & Eastwood, 1998; Sawada et al., 2003) as well as in vesicular glutamate transporter 1 which is also selectively expressed by glutamatergic neurons (Harrison & Eastwood, 2003). While schizophrenia is associated with dopamine (DA) hyperactivity, studies suggest that rather than a deficit in the DA system itself, the problem lies in other systems driving the DA system to be abnormal (Grace, 2000, 2012). At least one system involved may be glutamatergic receptors in the hippocampus: in addition to abnormalities in the hippocampus found in schizophrenia patients, NMDA receptor antagonists have also been shown to induce schizophrenic-like symptoms (Wang et al., 2001).

**Extracellular field potentials**

Electrophysiological studies of the hippocampus may be done in vitro using slice preparations or in vivo. While in vitro studies allow for more control over factors such as accurate electrode placement, they require severing connections to areas outside of the prepared slice. In vivo preparations reduce damage to the brain, as the only damage done is by the insertion of the electrodes. Recording probes inserted into the extracellular space measure field potentials. Field potentials do not only detect local signals, because local current sources and sinks generate potentials distributed across a conductive medium (brain), an effect called volume conduction. Therefore, field potentials are indirect measures of electrical activity that may be produced by a
single or multiple neurons. In the hippocampus, pyramidal cells are lined up in parallel, and synchronous activation during an evoked potential will summate field potentials from individual neurons to produce a large signal that can be recorded.

Current flows in a closed loop in a neuron, effectively setting up a dipole field. The opening of AMPA ion channels at the dendrites allows cations (net positive charges) to flow into a pyramidal cell. Where current (positive charge) flows from the extracellular medium to the intracellular medium, there is a current sink. The current flows in a closed loop to maintain electrical neutrality and flows from positive to negative potential; a current source is the local volume where current flows from the intracellular to extracellular medium (Leung, 2011). Thus, an active current sink during excitation exists when current flows into the cell, at a site of relative membrane depolarization, and a current source represents current flowing out of the cell, at a site of relative membrane hyperpolarization. Excitation of distal apical dendritic synapses generates a distal dipole field, where a current sink is formed at the distal dendrites and a current source is formed at the proximal apical dendrites (Fig. 2; Leung et al, 1995).
Figure 2. Evoked field potential during excitation of the distal apical dendrites, generating a current sink at the distal dendrites and a current source at the proximal apical dendrites (originally illustrated by Leung, 2011).
Synaptic Plasticity

Long-term potentiation

Theories of how the brain stores memories have been made for decades, with early ideas including the suggestion that there is a specific brain area for memories and learning. Eventually, Hebb and Konorski suggested that the synapse between two neurons is strengthened if they activate at the same time, and this strengthened synapse is the neural basis of memory. Early studies found that in accordance with this theory, high frequency stimulation trains to the hippocampus result in persistent increase in synaptic transmission. This effect came to be known as long-term potentiation (LTP), and continues to be main paradigm for studying learning and memory at the cellular level. LTP was first reported at the dentate gyrus of the hippocampus, and has since been studied in other parts of the hippocampus and elsewhere in the brain. Our understanding of LTP has advanced greatly since it was first discovered, with entire chapters and books devoted to the topic, but much still remains to be investigated (Bliss et al, 2007).

LTP has several basic properties: temporal persistence, cooperativity, associativity, and input-specificity. LTP persists over time: studies in vivo have found it to last for several days. Cooperativity indicates that there is a threshold that must be met to induce LTP: tetanic protocols that are too weak will not induce LTP, and various parameters may be changed to change the strength of the tetanus. Associativity refers to how a weak input can be potentiated if it is activated at the same time with a strong tetanus delivered to a separate but convergent input (Wigström & Gustafsson, 1986). Finally, LTP is input-specific: other pathways that are not activated in the tetanus are not potentiated. LTP’s temporal persistence and input-specificity make it a logical neural correlate to learning, as memories also last over long periods of time and
are specific and separate from one another. The inability to induce hippocampal LTP has been shown to impair acquisition and retention of spatial learning (Morris et al., 1986).

LTP can be induced using a variety of protocols. Early experiments used high-frequency stimulation (HFS) trains, often using 50 or more pulses at 100 Hz. In recent years, other tetanus protocols have been developed. One is the theta-burst stimulation (TBS), which uses several short bursts delivered at 5 Hz. Compared to high-frequency stimulation trains, these protocols are more similar to the firing pattern found in the hippocampus under physiological conditions (Fox & Ranck, 1975; Connors & Gutnick, 1990). Bursts at the theta frequency have been found to be effective at inducing LTP in the Schaffer collateral synapse (Leung & Shen, 1995).

During excitatory transmission in the hippocampus, glutamate is released from presynaptic vesicles, which then binds to postsynaptic AMPA and NMDA receptors. Activation of AMPA receptors allows for Na\(^+\) ions to move into the cell, which contributes to the excitatory postsynaptic potential (EPSP). In contrast, NMDA receptors are primarily involved in synaptic plasticity. At rest, NMDA receptors’ ion channels are blocked by Mg\(^{2+}\): but at sufficient depolarization, Mg\(^{2+}\) dissociates and Na\(^+\) and Ca\(^{2+}\) are allowed to flow into the cell. For NMDA receptors to be activated, glutamate must bind and there must be sufficient membrane depolarization. NMDA receptors play an important role in LTP, as application of an NMDA receptor antagonist (2-amino-phosphonpropionic acid, or APV) entirely blocks LTP at most hippocampal synapses and deletion of the NMDAR1 gene also leads to impaired LTP in the CA1 (Collingridge et al., 1983; Zalutsky & Nicoll, 1990; Hanse & Gustafsson, 1992; Tsien et al., 1996; Bliss et al., 2007). While NMDA alone can induce short-term potentiation, neither NMDA nor glutamate can induce long-term potentiation, which would suggest that LTP is dependent at least partly on presynaptic mechanisms (Collingridge et al., 1983; Bliss et al., 2007). NMDA receptor-
dependent LTP is also modulated by GABA, which inhibits NMDA receptor activation. It has been shown that endocannabinoids released from pyramidal cells can depress GABA inhibition which then facilitates LTP (Bliss et al., 2007). As previously mentioned, NMDA receptor antagonists have been found to induce schizophrenia-like symptoms. Further, impairments in NMDA receptor-dependent LTP was found in the post-weaning social isolation model of schizophrenia in the CA1-subiculum projection, and the schizophrenia-susceptibility gene neuregulin-1 was found to stimulate the internalization of GluR1-containing AMPA receptors, attenuating NMDAR-depending LTP (Roberts & Greene, 2003; Kwon et al., 2005).

Some forms of LTP do not depend on NMDA receptors, and these may be mediated by voltage-gated Ca$^{2+}$ channels (Johnston & Wu, 1995). Ca$^{2+}$ appears to be essential for LTP: blocking the refilling of Ca$^{2+}$ in intracellular stores or the increase of postsynaptic Ca$^{2+}$ blocks LTP induction, and increasing postsynaptic Ca$^{2+}$ can mimic LTP (Lynch et al., 1983; Malenka et al., 1988; Harvey & Collingridge, 1992). In addition to NMDA receptors, Ca$^{2+}$ may also be released by activation of metabotropic glutamate receptors (mGluRs): application of a selective mGluR antagonist was found to have no effect on basal synaptic transmission but completely inhibited LTP induction while having no effect on short-term potentiation (Bashir et al., 1993). The downstream signaling effects of Ca$^{2+}$ are mediated by $\alpha$-calcium/calmodulin-dependent protein kinase II ($\alpha$CaMKII): it is believed to phosphorylate AMPA receptor subunit GluR1, which leads to an increase in the single-channel conductance of homomeric GluR1 AMPA receptors, which induces LTP. Inhibiting or deleting CaMKII or GluR1 prevents LTP from being induced, whereas increasing the number of AMPA receptors on the postsynaptic membrane leads to LTP expression (Benke et al., 1998; Soderling & Derkach, 2000; Malinow & Malenka, 2002; Song & Huganir, 2002; Malenka & Bear, 2004). While phosphorylation of $\alpha$CaMKII for at least on hour
is associated with LTP induction, there is still no direct evidence that it must remain
constitutively active to maintain LTP. LTP is believed to also involve or be modulated by
multiple other kinases including protein kinase A, though the role of these kinases are not yet
well understood (Bliss et al, 2007).

If the EPSP increases and is maintained for at least 30 to 60 minutes, it is considered LTP.
Different types of LTP have been defined on the basis of its temporal persistence: LTP1, LTP2,
and LTP3 (Abraham and Otani, 1991). LTP1 requires activation of NMDA receptors and
ryanodine receptor-mediated Ca\textsuperscript{2+} release; however, it does not involve protein synthesis, and
thus decays quickly. LTP2 is also NMDA receptor-independent, but depends on protein
synthesis: however, it does not affect gene transcription. Finally, LTP3 has a voltage-gated Ca\textsuperscript{2+}
channel-sensitive component but is not dependent on store-mediated Ca\textsuperscript{2+} release, and it does
involve changes in gene transcription and translation, and thus is the most temporally robust.
Therefore, the temporal persistence of LTP depends on different cellular sources of Ca\textsuperscript{2+}
(Raymond, 2007).

In the temporoammonic synapse, LTP has been induced in vitro using a HFS protocol and a TBS
protocol. Application of the NMDA receptor antagonist 2-amino-5-phosphoneopentanoic acid
(APV) significantly reduces LTP, and co-application of APV and the voltage-gated Ca\textsuperscript{2+} channel
antagonist nifedipine completely prevents LTP. The GABA\textsubscript{A} receptor antagonist bicuculline did
not affect LTP, while the GABA\textsubscript{B} receptor antagonist CGP inhibited LTP; when both were co-
applied, LTP was enhanced (Remondes & Schuman, 2003). When the HFS tetanus was used,
potentiation lasted for at least eight hours and was found to be sensitive to protein synthesis
inhibition: application of anisomycin reduced late-phase LTP (Remondes & Schuman, 2002,
2003). These studies indicate that all three types of LTP described above can be found in the
temporoammonic synapse in an in vitro preparation. LTP has also been found in vivo in behaving rats (Aksoy-Aksel & Manahan-Vaughan, 2013, 2015; Gonzalez et al, 2016). To our knowledge, LTP has not been shown at the RE-CA1 synapse. An in vivo study found a HFS to not induce LTP, though it did prevent acquisition in an object discrimination task (Eleore et al, 2011). Although there has been growing evidence for its functional role, electrophysiological studies of this synapse are still lacking.

**Paired pulse facilitation**

Paired pulse facilitation (PPF) is an effect induced by two pulses applied within a short interval, where the response following the second pulse is larger. This is attributed to residual calcium still in the synapse when the second pulse is applied, resulting in enhanced Ca$^{2+}$ concentration and increase neurotransmitter release. Measures of residual calcium in the presynaptic neuron positively correlate with the degree of PPF seen (Wu & Saggau, 1994), and a study has also shown Mg$^{2+}$ concentrations to affect PPF (Creager et al, 1980). While this facilitation is believed to be at least primarily due to presynaptic mechanisms, there is also evidence of a postsynaptic component (Bagal et al, 2005). PPF may be seen as a kind of short-term synaptic plasticity, and some suggest that a similar mechanism may occur during LTP.

However, the relationship between LTP and PPF is not yet fully understood. Although Wu & Saggau (1994) found a positive correlation between residual calcium and the degree of PPF in individual recordings of the stratum radiatum-CA1 synapse, they did not detect a persistent increase in calcium after LTP was induced. Similarly, Manabe and colleagues (1993) did not find an change in PPF after inducing LTP in the Schaffer collaterals, and subsequently suggested that LTP is due to postsynaptic changes (Manabe et al, 1993). In contrast, another study of the same region found that LTP tends to correlate with decreased PPF (Schulz et al, 1994). While a
consensus has yet to be reached on whether LTP does tend to induce a reduced PPF, it provides a method of indirectly examining the possibility that LTP is partially due to an increase in Ca\textsuperscript{2+} release.

**Neuromodulation**

Much like synaptic plasticity, neuromodulation in the hippocampus has been studied primarily in the trisynaptic circuit. The main neurotransmitter in the CA1 region is glutamate, but previous studies have found that other neurotransmitters play key roles in modulating synaptic transmission and LTP.

One neurotransmitter that has been found to modulate the trisynaptic pathway is dopamine (DA). The hippocampus receives dopaminergic innervation from the ventral tegmental area (VTA); the VTA projects strongly to the subiculum, hilus, and SLM. D1, D2, and D5 receptor families have been found in the hippocampus (Goldsmith & Joyce, 1994). However, another study has argued that these dopaminergic inputs project more to the basal dendrites and somewhat to the proximal apical, with very few projections to the distal apical dendrites (Rosen et al, 2015).

Electrophysiological studies have found that DA has a facilitative effect on LTP at the basal and apical dendrites (Otmakhova & Lisman, 1996; Lisman & Grace, 2005; Navakkode et al, 2012) and is necessary to stimulate protein synthesis for late LTP to be sustained (Barco et al, 2002). Consistent with these findings are animal studies showing DA agonists injected into the CA1 can enhance memory (Bernabeu et al, 1997) and DA antagonists applied to the hippocampus impairs performance on memory (Umegaki et al, 2001; Morris et al, 2003).

However, there are few electrophysiological studies examining the role of DA in direct hippocampal projections. There is conflicting evidence about whether DA plays a role or not:
Goldsmith and Joyce (1994) found a high concentration of DA receptors in the SLM, and an in vitro study found that when DA is applied to slices, the response decreases in a dose-dependent manner while increasing paired pulse facilitation at an interpulse delay of 50 ms (Otmakhova & Lisman, 1999). However, Rosen and colleagues (2015) found that the basal and proximal apical dendrites have more DA synapses, and when they compared exogenously applied DA to optogenetic stimulation of the VTA to release DA they found different effects. While exogenously applied DA affected the temporoammonic path response with little effect on the Schaffer collateral response, optogenetic stimulation modulated the Schaffer collateral response with no effect on the temporoammonic path response. It is important to investigate this further, considering the evidence that the hippocampus plays a role in the pathology of schizophrenia. Due to the conflicting results from exogenous DA application versus endogenous DA release in the TA pathway, further studies examining endogenous DA’s role in this pathway are needed.

Similarly, acetylcholine (ACh) has been found to have a role in the hippocampus. The hippocampus receives cholinergic projections from the medial septum, and animals infused with ACh antagonists demonstrated impaired spatial encoding (Blokland et al, 1992; Hasselmo & Wyble, 1997), while lesions blocking cholinergic innervation of the EC can also lead to impaired performance on memory tasks (Turchi et al, 2005). Endogenous ACh acting at M2 muscarinic receptors have also been linked to improved working memory (Daniel & Dohanich, 2001). Notably, selective cholinergic lesions of the medial septum do not lead to the same level of impairment as whole medial septum lesions; as the medial septum also sends GABAergic projections, medial septum lesions may lead to decreases in both ACh and GABA (Pang & Nocera, 1999). Electrophysiological studies have shown that ACh facilitates LTP at the basal dendrites of CA1 pyramidal cells (Blitzer et al, 1990; Leung et al, 2003; Ovsepian et al, 2004;
Doralp & Leung, 2008). Similarly, a complete vesicular ACh transporter (VACht) knockdown in the basal forebrain impairs LTP at the proximal apical dendrites (Al-Onaizi et al., 2016).

However, its role at the distal apical dendrites is still not fully understood. At the SLM, application of an ACh agonist was found to suppress responses – though to a lesser extent compared to the stratum radiatum – and this effect was blocked when a muscarinic antagonist was applied (Hasselmo & Schnell, 1994). An in vitro study found that nicotine suppressed LTP in the TA synapse, and this effect was attenuated when α2 nicotinic ACh receptors are knocked out (Nakauchi et al., 2007). This effect may be due to the fact that OLM cells, the interneurons that act to inhibit the distal dendrites, are very sensitive to nicotine and thus are causing this inhibition (Leão et al., 2012). These studies indicate that both muscarinic and nicotinic receptors are present in the SLM, and that muscarinic receptors, but not nicotinic receptors, behave similarly in this layer compared to the stratum radiatum.

There is also evidence that DA and ACh interact with one another in the hippocampus. Both DA and ACh appear to have facilitative effects on synaptic plasticity in other hippocampal pathways. Further, in the previously described study by Umegaki and colleagues (2001), the D2 agonist injection was found to stimulate ACh release, and the ACh release could be blocked by infusion of a D2 receptor antagonist. Thus, it is possible that D2 receptors may play their facilitative role by increasing ACh release.

**Rationale and aims**

There is considerable anatomical and functional evidence implicating the importance of the temporoammonic pathway and the direct projection from the RE to the hippocampus. However, there have been few electrophysiological studies investigating these synapses. Thus, we wanted
to conduct experiments that would provide more insight into the properties of these synapses to add to the literature of these pathways and of hippocampal function as a whole.

Aim 1: To see if synaptic plasticity can be induced at the temporoammonic synapse and the RE-CA1 synapse.

Remondes and Schuman (2002, 2003) have demonstrated LTP at the temporoammonic synapse in rat hippocampal slices in vitro. More recent work reported LTP in the behaving rat, based on recording from a single electrode in CA1 (Aksoy-Akel et al., 2013, 2015; Gonzalez et al., 2016). Excitatory potentials in CA1 are not isolated by recording field potentials at a single recording site, because of volume conduction from other areas activated by TA stimulation. There has been no definitive study of synaptic plasticity of the TA to CA1 synapse in mice in vivo. We are not aware of reports of LTP at the nucleus reuniens to CA1 synapse, although there is literature that optogenetic HFS stimulation of the RE resulted in reduced cognitive performance (Duan et al., 2015). Therefore, we sought to study synaptic plasticity by recording excitatory sinks (current source density) from CA1 using multichannel recordings in urethane-anesthetized mice. A TBS tetanus protocol was applied to either the TA or RE pathway.

Aim 2: To determine the neuromodulatory effects of DA and ACh in the temporoammonic synapse.

Otmakhova and Lisman (1998) found that application of DA in hippocampal slices selectively suppressed the temporoammonic (distal apical dendritic) response, without affecting the Schaffer collateral (mid apical dendritic) response. This indicates that DA plays an important modulatory role at the temporoammonic synapse, also supported by the relatively higher concentration of DA receptors at the SLM. However, conflicting evidence was found by Rosen and colleagues
(2015) with the use of endogenously released DA. While DA has been found to generally increase LTP at the basal and apical dendrites, this has not been tested at the distal apical dendrites. In contrast, ACh decreased Schaffer collateral excitatory transmission without affecting the distal dendritic or temporoammonic response (Hasselmo et al., 1994; Leung and Peloquin, 2010). ACh is generally found to have a facilitative effect on LTP at the basal and proximal apical dendrites through muscarinic receptors. Nakauchi and colleagues (2007) have found that nicotinic receptor activation suppresses LTP activation. Studies examining ACh effect on distal dendritic LTP have not been conducted.

Aim 3: To examine the change in paired pulse facilitation at the temporoammonic synapse after the induction of LTP.

Even at the well-studied basal and proximal apical dendrites, there is still conflicting evidence of whether PPF declines after LTP is induced. This reduction would implicate a presynaptic component to LTP - that the PPF is reduced due to more Ca$^{2+}$ being released from the presynaptic neuron. In Otmakhova and Lisman’s study (1998), they found dopamine application increased PPF at the temporoammonic synapse. No studies to date have examined changes in PPF following LTP in the temporoammonic synapse.
METHODS

Animals

Adult male mice weighing 25 – 40 g were used. Wild-type mice, as well as genetically modified mice significantly deficient in the vesicular acetylcholine transporter (VACht), were used. Mice in the latter group had a heterozygous targeted deletion of the VACht gene, resulting in a 56% decrease in VACht protein expression (Prado et al, 2006). As the knockdown was in the VACht gene, this was expected to show the effects of a reduction in ACh only, as compared to a lesion that could target neurons that release Ach but may also release other neurotransmitters. The mutant mice were backcrossed with C57BL/6J animals to produce the heterozygous genotype. Both wildtype and mutant mice came from the Prado laboratory, and included a combination of pure wildtype and cre− genotype mice.

Mice were housed in standard caged in a temperature-regulated environment with a 12:12 hour light/dark cycle, with lights on at 7 am. Animals had ad libitum access to food and water. Experiments were conducted during the day (10 am – 10 pm). Mice were housed in animal headquarters for a minimum of 3 days prior to surgery.

Electrode Implantation

Electrodes

Stimulating electrodes were stainless steel wire, 0.005 inches in diameter and insulated with Teflon except at tips. Silicon recording probes were purchased from NeuroNexus, Ann Arbor, MI: the probes had 16 recording sites spaced 50 μm apart on a vertical shank (Model #: a1x16-5mm-177-CM16LP) (Hutchison et al, 2009).
**Surgery**

Mice were anaesthetized with incremental doses of 10% urethane anaesthesia (1.25 g/kg solution i.p. and supplemented as needed) (Hutchison et al, 2009). This was followed by administration of atropine methyl nitrate (7.5 mg/kg, i.p.) to block excess salivation. A rectal thermometer monitoring system was used to maintain body temperature between 35 – 37°C throughout the experiment. The skull was exposed and small skull holes were drilled based on coordinates relative to bregma. The 16-channel recording probe was lowered into the CA1 region of the hippocampus at the coordinate (P3.2, L2.8). The stimulating electrodes were placed to stimulate the medial perforant pathway (P4.1,L2.3), the nucleus reuniens (P0.8, L0), and the ventral tegmental area (P3.1,L0.5). An illustration of the placement of the recording probe and the electrodes stimulating the hippocampal pathways is shown in Figure 3. The exact electrode depth varied between animals and was based on the profile of evoked potentials monitored during electrode implantation. The recording electrode was lowered to ~2.1mm to record from both the proximal and distal apical dendrites of CA1 pyramidal cells. The MPP electrode was lowered to ~1.2mm, the RE electrode was lowered to ~3.8mm, and the VTA electrode was lowered to ~4.1mm. Final electrode depths were selected by optimized response based on electrophysiological criteria. Two screws were then secured onto the skull surface about the frontal cortex and cerebellum to serve as stimulus ground and recording ground respectively.

Upon completion of surgical preparation, a 1-hour intermission was given before recording to optimize the response.
Figure 3. A coronal section of the mouse hippocampus illustrating the position of recording and stimulating electrodes of hippocampal pathways. Stimulation of the angular bundle activated the medial perforant pathway to the distal apical dendrites. Stimulation of the nucleus reuniens also activated the distal apical dendrites. The 16-channel recording probe was inserted to record responses from the stratum radiatum to stratum lacunosum moleculare of CA1 to the dentate gyrus.
Experimental paradigm

Signals from the 16-channel recording probe were amplified 200-1000x by a TDT (Tucker-Davis Tech, TDT) headstage, a 16-channel Medusa preamplifier and fed by optic wires to TDT digital processors (RA16 Base Station). Signals were digitized at 6.1-24.4 kHz by TDT real-time processors and custom-made software by our lab. Stimulus pulses (0.2 ms duration) were delivered through a photo-isolated stimulation unit (PSIU6, AstroMed/Grass Instrument).

Baseline evoked responses were monitored at 1.5 – 2 times threshold intensity at a sampling rate of 24.4 kHz with 4096 samples. Averaged evoked potentials (AEPs) (N=4 sweeps) were acquired. Sampling time points occurred every 5 minutes. Current source density (CSD), or the local measure of the current source and current sink, was calculated from the recorded evoked potentials. As synaptic currents spread in the extracellular medium, the field potential does not show a local current. This effect, known as volume conduction, is removed by CSD. A one-dimensional CSD was calculated from the field potential and identified the macroscopic locations of the current sources and sinks (Fig. 4). CSD(z,t) as a function of depth z and time t was calculated by a second-order differencing formula:

\[ \text{CSD}(z,t) = \frac{\sigma(2\Phi(z,t) - \Phi(z + \Delta z, t) - \Phi(z-\Delta z,t))}{(\Delta z)^2} \]

Where \( \Phi(z,t) \) is the potential at depth z and time t, \( \Delta z \) is the spacing between adjacent electrodes on the 16-channel probe (50 \( \mu \)m). The conductivity \( \sigma \) was assumed to be constant. The CSDs were reported in the unit V/mm\(^2\). Figure 4 shows representative AEPs following stimulation to the MPP (Fig. 4A) and stimulation to the nucleus reuniens (Fig. 4C), as well as their respective CSD profiles (Fig. 4B and 4D respectively).

The slopes of the excitatory sinks in the distal apical dendrites were quantified from the CSDs (Fig. 5). The maximal slope value was taken as the estimate of the slope. After a stable baseline
was obtained for 30 minutes (which was defined as the coefficient of variation (SEM/mean) of the sink slopes < 0.10), the experiment would proceed. In experiments requiring a haloperidol injection, this injection was given immediately after a stable baseline had been obtained (1 mg/kg, i.p.). After ten minutes, another baseline measure was taken to ensure the response was still stable; if the coefficient of variation remained below 0.10, the tetanus was applied. For experiments that did not use haloperidol, the tetanus was applied immediately after a 30 minute stable baseline had been obtained.

The tetanus was a theta-burst stimulation delivered to either the MPP or RE, consisting of 10 trains of 10 bursts, with each burst consisting of 10 pulses at 100 Hz; bursts were delivered at 5 Hz, and trains every 20 s, delivered to the pathway of interest. For experiments with MPP and VTA co-stimulation, the tetanus parameters remained the same for the MPP, while VTA was co-stimulated at the first four pulses of each burst. The TA to CA1 response was used to determine the depth of the VTA stimulating electrode: the TA electrode was stimulating to induce a population spike, and the VTA was stimulated four times prior to the TA stimulation. When the VTA stimulation was seen to enhance the population spike of the TA response at 50-100 μA, the electrode was not lowered any further. The stimulation intensity used was 2-5 times threshold intensity; the intensity used for the VTA during the co-tetanus was 1-2 times the intensity found to enhance the population spike of the TA response (50-150 μA). Post-tetanic responses were recorded for 120 minutes at the initial intensity used to monitor baseline and were normalized by the baseline average. In preliminary experiments, five trains at 1.5 to 2 times threshold were used, but did not induce consistent potentiation. Increasing the number of trains was much more effective at inducing potentiation, and higher intensities also generally lead to more potentiation.
Three consecutive time points were averaged for the baseline and post-tetanus responses to reduce variability. The analysis was conducted on these averaged normalized data, involving every five minute time points of the baseline (-25 to -10 minutes) and post-tetanus responses (5 to 115 minutes). The PPF ratio was calculated from the maximal slope of the second response divided by the maximal slope of the first response. These ratios were also averaged in three consecutive time points in the same manner described above.
Figure 4. Averaged evoked potentials (AEPs: A and C) and current source density (CSD: B and D) transients in CA1 from representative mice following medial performant path excitation (A and B) or nucleus reuniens excitation (C and D). Potentials were recorded by a 16-channel electrode silicon probe with 50 µm interval between electrodes. Depths are indicated by the schematic CA1 pyramidal cell (Spruston, 2008). (A) AEPs (average of 4 sweeps) following stimulation of the angular bundle at 60 µA (2 times threshold). (B) CSD profile derived from the AEPs shown in (A). Stimulation of the angular bundle generated a current sink (negative deflection) in the CA1 distal apical dendrites. (C) Distal dendritic response as results of stimulating the nucleus reuniens at 400 µA (2 times threshold). (D) CSD profiles generated from AEPs shown in (C). Stimulation of the nucleus reuniens generated a current sink at the distal apical dendrites.
Figure 5. Slope measurements taken from CSD analyzed averaged evoked potentials. The slope of the excitatory sink was calculated at 1 ms intervals for the whole duration of the rising phase of the excitatory sink (Y1 to Y2) and the value of the maximal magnitude of the slope was taken as the estimate of the slope. The filled circle indicates the stimulus artifact.
Confirmation of Electrode Location

Perfusion

At the end of the experiment, the sites of the stimulating electrodes were lesioned by passing a 0.5 mA current for a duration of 0.5 s, three times with an interval of 10 s between pulses. The mouse was then intracardially perfused with 50 ml of saline followed by 25 ml of 4% formaldehyde solution. The brain was removed from the cranium and placed in 4% formaldehyde solution for a minimum of 24 hours prior to sectioning.

Histology and Staining

Brains were frozen on the cryostat and sliced into 40 μm thick coronal sections. Brain slices were mounted onto slides later stained with thionin. The locations of the stimulating and recording electrodes were identified and confirmed using a light microscope (Fig. 6).

Inclusion Criteria

The criteria for experiment inclusion in analysis were consistent across all experiments. If baseline recordings of the largest sink slope were unstable, baseline recording was run longer until 30 minutes of stable baseline was obtained. Further, if the recording electrode shifted such that the maximal sink moved more than one channel away, the data was not included in the group analysis.

Analysis and statistics

For experiments assessing LTP, repeated measures analysis of variance (ANOVA) was used for statistical analysis of this normalized averaged data at different times. If a significant main or interaction effect was found, Bonferroni-corrected post-hoc tests were applied. A p < 0.05 was
considered statistically significant. Comparisons were made between the TBS to MPP group (control) to the other tetanus protocols involving the temporoammonic synapse (MPP TBS with VTA stimulation, MPP TBS with VTA stimulation with haloperidol injection, MPP TBS with haloperidol injection). A comparison was also done between the TBS to the MPP group and the HET mice which received the same tetanus protocol. Experiments involving the RE did not have another control group and thus are compared only to their baseline responses.

Changes in PPF were first analyzed using a two-way mixed factor ANOVA (time x group), comparing the 30-minute baseline and an average of the 70 minutes following the tetanus within each group. To assess the relationship between synaptic plasticity and PPF, each averaged LTP value was regressed against its corresponding PPF value at its time point. Only post-tetanic time points were used and grouped by the tetanic protocol used. Separate analyses were run for each group.
Figure 6. Coronal slices indicating electrode location. Locations were based on coordinates from an atlas (Franklin & Paxinos, 1997). Schematic slices on the left illustrate stimulating and recording electrodes and examples of corresponding thionin stained sections on right. Lesions made through the stimulating electrode tip is marked with an arrow. A: Placement of stimulating electrode at the angular bundle (P4.1, L2.3). The ventral coordinates varied across animals and averaged 1.8mm below the skull surface. (B) Placement of the stimulating electrode of the nucleus reuniens (P0.8, L0). The ventral coordinates varied across animals and averaged 4.0mm below the skull surface. (C) Placement of the stimulating electrode at the ventral tegmental area (VTA) (P3.1, L0.5). The ventral coordinates varied across animals and averaged 4.5mm below the skull surface. (D) Placement of the recording probe at the CA1 (P3.2, L2.8). The ventral coordinates varied across animals and averaged 2.1mm below the skull surface. Drawings originally by Franklin and Paxinos (1997).
RESULTS

Baseline distal apical response during TA-CA1 stimulation

The threshold of the temporoammonic response was typically between 30 to 50 μA, and so the stimulus intensity used for monitoring averaged from 60 to 100 μA (1.5 to 2x threshold). Input-output curves were taken to ensure that the stimulus intensity used for monitoring was below the maximal response. The intensity used for the tetanic stimulation ranged from 50 to 150 μA, or 2 to 5x threshold. The intensity chosen for the tetanus (50 to 150 μA) was always below the intensity required to induce a maximal response.

Stimulation of the temporoammonic pathway generally induced a pattern of a sink at the distal apical dendrites and a corresponding source in the proximal apical dendrites, appearing in the AEP and CSD profiles as dorsal to the sink. In some cases, there was also a source ventral to the sink. At high intensities, a later response could be invoked, but these intensities were well above the intensity used for monitoring. Tetanus intensities were also chosen to be lower than what was required to invoke a mixed response (Supplementary Fig. 1). These parameters were consistent across all experiments of the temporoammonic pathway so that the groups could be compared against one another.

Theta burst stimulation to the TA-CA1 pathway

A TBS protocol, of 10 sweeps each with 10 theta-frequency bursts, was applied to induce LTP of the TA-CA1 synapse in vivo. A representative experiment illustrates LTP of the TA to CA1 distal dendritic excitatory sink following the TBS protocol (Figure 7). The size of the largest excitatory sink increased compared to the baseline (Fig. 7A). When plotted over time, there was considerable variation in the response at a single time point, which was reduced by running
The spatial profile of CSD shows a localized excitatory sink at the distal apical dendrites (located in the SLM), accompanied by source at stratum radiatum, and this profile does not shift throughout the experiment (Fig. 7C).

**Synaptic plasticity and the effect of dopamine**

The same TBS protocol was applied to different groups of animals intended to investigate the effects of endogenous dopamine release on LTP of the TA-CA1 synapse. Representative experiments are shown for each group: the MPP and VTA co-tetanus (Fig. 8), the MPP TBS after haloperidol was injected (Fig. 9), and the MPP and VTA co-tetanus after a haloperidol injection (Fig. 10). These experiments had similar profiles to the MPP TBS experiment and the profiles did not shift during the experiment. Variation of the response at one time point was smoothed by using running averages of three consecutive time points.

The time course of the normalized LTP of the four groups are shown in Fig. 11. A two-way mixed ANOVA (time x group) was used to compare among 4 groups (MPP TBS (N=11), MPP TBS+HP (haloperidol injection given 10 minutes before TBS applied to MPP pathway; N=4), MPP+VTA TBS (VTA and MPP tetanized simultaneously; N=7). MPP+VTA TBS + HP (haloperidol injection given 10 minutes before TBS applied to the VTA and MPP; N=4). There was a significant group effect (F(3,22) = 3.732, p = 0.0262), time effect (F(26, 572) = 4.430, p < 0.0001), and group x time interaction effect (F(78, 572) = 1.675, p = 0.0006) (Fig. 10). Post-hoc tests comparing the MPP TBS (mean (M)=1.290, SEM=0.0303) only group to the other three groups only found a significant difference with the MPP+VTA with haloperidol group (M=0.8831, SEM=0.0216) (p < 0.05). Comparisons to the MPP+VTA (M=1.330, SEM=0.0310) and the MPP TBS with haloperidol (M=1.378, SEM=0.0409) were not significant (p > 0.99).

Post-hoc tests comparing post-TBS time points with the baseline (-10 min) within each group
found significant differences across time within all groups excluding the MPP+VTA with haloperidol group. For the MPP TBS group, a significant increase in response was found from 5-70 minutes post-tetanus; for the MPP TBS with haloperidol group, a significant increase in response was found from 5-20 minutes post-tetanus, and in the MPP+VTA TBS group, a significant increase in response was found at 25-30, 55, and 75-85 minutes post-tetanus.

In conclusion, LTP lasting for at least 70 minutes was induced in vivo in the TA-CA1 synapse using a TBS protocol. This potentiation was not affected when MPP and VTA were co-tetanized or when MPP was tetanized with pretreatment with haloperidol; however, when MPP and VTA were co-tetanized after haloperidol pretreatment, potentiation at the TA-CA1 synapse was suppressed.

*Synaptic plasticity and the effect in HET mice*

A representative experiment of a TBS protocol delivered to MPP of a VACtT-HET mouse is shown in Figure 12. Comparable to previously shown experiments, MPP evoked an excitatory sink at the distal apical dendrites and a source dorsal to the sink in the proximal apical dendrites. In this particular experiment, a transient potentiation of the sink was found following TBS of the MPP, but it declined to baseline after 40 minutes, which was considerably shorter than the 70 minutes of LTP found in wild-type mice.

Group data confirmed that the TBS-induced potentiation at the MPP to CA1 distal dendritic excitation in VACtT-HET mice (N=5) was different from that in wildtype mice (N=11) (Fig. 13). A two-way mixed ANOVA showed a significant difference between groups (F(1, 14) = 15.31, p = 0.0016), a significant effect over time (F(26, 365) = 3.779, p < 0.0001), and a significant group x time interaction (F(26, 364)=4.067; p < 0.0001). Post-hoc tests comparing time within each
group found a significant difference over time in the wildtype mice from 5-70 minutes post-tetanus, but no change over time in the heterozygous mice.

Therefore, the ability of the TA-CA1 synapse to potentiate in response to a TBS protocol was decreased in mice heterozygous for VACHT. In the few HET mice that were able to potentiate, it did not last beyond 40 minutes, and in other mice there was no potentiation at all.
Figure 7. Data from a representative experiment in which TBS was applied to the temporoammonic pathway at time zero. (A) Current source density (CSD) temporal traces from 5 adjacent channels (9 to 13) are shown, with maximal sink at channel 12 (presumed distal apical dendrite). CSD at 2, 30, 60, and 120 minutes post-TBS (black traces) are overlaid with the CSD during baseline (red traces). The circle indicates the stimulus artifact. (B) The slope of the distal apical dendritic sink (channel 12) was analyzed and normalized by the average baseline sink slope. Three consecutive time points were then averaged to reduce variation. (C) CSD profiles, at a time the sink reached half-maximum at channel 12, shown during baseline (an average of 6 AEPs), and at 2, 30, 60, and 120 minutes post-tetanus.
Figure 8. Data from a representative experiment in which a TBS tetanus was applied to the temporoammonic pathway and ventral tegmental area. (A) Current source density (CSD) temporal traces from 5 adjacent channels (6 to 10) are shown, with maximal sink at channel 9 (presumed distal apical dendrite). CSD at 2, 30, 60, and 120 minutes post-TBS (black traces) are overlaid with the CSD during baseline (red traces). The circle indicates the stimulus artifact. (B) The slope of the distal apical dendritic sink (channel 9) was analyzed and normalized by the average baseline sink slope. Three consecutive time points were then averaged to reduce variation. (C) CSD profiles, at a time the sink reached half-maximum at channel 9, shown during baseline (an average of 6 AEPs), and at 2, 30, 60, and 120 minutes post-tetanus.
Figure 9. Data from a representative experiment in which a haloperidol was injected before a TBS tetanus was applied to the temporoammonic pathway. (A) Current source density (CSD) temporal traces from 5 adjacent channels (10 to 14) are shown, with maximal sink at channel 11 (presumed distal apical dendrite). CSD at 2, 30, 60, and 120 minutes post-TBS (black traces) are overlaid with the CSD during baseline (red traces). The circle indicates the stimulus artifact. (B) The slope of the distal apical dendritic sink (channel 13) was analyzed and normalized by the average baseline sink slope. Three consecutive time points were then averaged to reduce variation. (C) CSD profiles, at a time the sink reached half-maximum at channel 13, shown during baseline (an average of 6 AEPs), and at 2, 30, 60, and 120 minutes post-tetanus.
Figure 10. Data from a representative experiment in which haloperidol was injected prior to a TBS tetanus applied to the temporoammonic pathway and the ventral tegmental area. (A) Current source density (CSD) temporal traces from 5 adjacent channels (8 to 12) are shown, with maximal sink at channel 11 (presumed distal apical dendrite). CSD at 2, 30, 60, and 120 minutes post-TBS (black traces) are overlaid with the CSD during baseline (red traces). The circle indicates the stimulus artifact. (B) The slope of the distal apical dendritic sink (channel 11) was analyzed and normalized by the average baseline sink slope. Three consecutive time points were then averaged to reduce variation. (C) CSD profiles, at a time the sink reached half-maximum at channel 11, shown during baseline (an average of 6 AEPs), and at 2, 30, 60, and 120 minutes post-tetanus.
Figure 11. Mean excitatory sink slope with SEM (y-axis) over time (x-axis) evoked in CA1 by MPP stimulation, before and after TBS. MPP TBS: TBS was applied to MPP pathway only (N=11). MPP TBS+HP: haloperidol injection given 10 minutes before TBS applied to MPP pathway (N=4). MPP+VTA TBS: VTA and MPP tetanized simultaneously (N=7). MPP+VTA TBS + HP: haloperidol injection given 10 minutes before TBS applied to VTA and MPP simultaneously (N=4). A two-way mixed ANOVA found significant group, time, and interaction effects. Post-hoc tests comparing post-TBS sink slopes within each group relative to the last baseline point (-10 min) found significant difference for the MPP TBS group (at 5-70 minutes), MPP TBS with haloperidol group (at 5-20 minutes) and MPP+VTA TBS group (at 20-90 minutes and 115 minutes), but no significant difference at any time for the MPP+VTA TBS with haloperidol group. Post-hoc tests analyzing the group differences found that the MPP TBS group was only significantly different from the MPP+VTA TBS+HP group.
Figure 12. Data from a representative experiment in which a TBS tetanus was applied to the temporoammonic pathway in a VACHT-HET mouse. (A) Current source density (CSD) temporal traces from 5 adjacent channels (7 to 11) are shown, with maximal sink at channel 10 (presumed distal apical dendrite). CSD at 2, 30, 60, and 120 minutes post-TBS (black traces) are overlaid with the CSD during baseline (red traces). The circle indicates the stimulus artifact. (B) The slope of the distal apical dendritic sink (channel 10) was analyzed and normalized by the average baseline sink slope. Three consecutive time points were then averaged to reduce variation. (C) CSD profiles, at a time the sink reached half-maximum at channel 10, shown during baseline (an average of 6 AEPs), and at 2, 30, 60, and 120 minutes post-tetanus.
Figure 13. Mice heterozygous for the VACHT gene (HET) showed lower long-term potentiation of the MPP to CA1 distal dendritic sink than wildtype mice. The mean plus SEM of the distal dendritic sink slope with SEM (y-axis) was plotted with time (x-axis). MPP TBS: TBS delivered to the MPP in wildtype mice (N=11). MPP TBS-HET: TBS delivered to the MPP in HET mice (N=5). A two-way mixed ANOVA found a significant interaction (p < 0.0001) as well as significant main effects for time (p < 0.0001) and between groups (p = 0.0016). Post-hoc tests comparing time within each group relative to the last baseline point (-10 min) found significant differences over time for wildtype mice (from 5-70 minutes), but no significant differences within the HET mice.
Synaptic plasticity and paired pulse facilitation in the temporoammonic pathway

A two-way mixed ANOVA (time x group) was used to compare across 5 groups (MPP TBS (N=4), MPP TBS+HP (haloperidol injection given 10 minutes before TBS applied to MPP pathway; N=4); MPP+VTA TBS (VTA and MPP tetanized simultaneously; N=7); MPP+VTA TBS + HP (haloperidol injection given 10 minutes before TBS applied to the VTA and MPP; N=4); MPP-HET (TBS applied to MPP pathway in VChT-HET mice; N=5)). The average PPF of the baseline (30 minutes) was compared to the average PPF of 70 minutes following the tetanus, as the first 70 minutes (Fig. 14). There was a significant time effect (F(1,19)=12.93, p=0.0019), but no significant group effect (F(4,19)=1.049, p=0.4086) and no significant interaction (F(4,19)=1.559, p=0.2257). Therefore, across groups there was typically a decline in PPF after a tetanus.

Linear regression analyses were conducted to determine if there is a significant relationship between the degree of LTP with the degree of PPF (Fig. 15). Data from all mice that underwent the same tetanus were pooled together into one plot. The normalized LTP value was paired with its PPF value, and pairs were divided into one of two periods based on time: when an average group potentiation was found, or after average potentiation declined to baseline. For the knockdown mice, average potentiation was found at 5-30 post-tetanus, and no potentiation was found at 35-115 minutes post tetanus. For all other groups, as average potentiation was found at 5-70 minutes post-tetanus, and no potentiation at 75-115 minutes post-tetanus. A significant linear correlation was found for most groups in both time periods: the MPP TBS group (5-70 minutes: \( R^2=0.5877, p<0.0001; \) 75-115 minutes: \( R^2=0.2798, p=0.0009 \)), the MPP+VTA TBS group (5-70 minutes: \( R^2=0.09875, p=0.0016; \) 75-115 minutes: \( R^2=0.2033, p=0.0002 \)), the MPP+VTA TBS with HP group (5-70 minutes: \( R^2=0.4616; p<0.0001; \) 75-115 minutes:
$R^2=0.3915, p<0.0001$), and the MPP TBS in HET mice group (5-30 minutes: $R^2=0.4014; p=0.0002$, 35-115 minutes: $R^2=0.2322, p<0.0001$). The correlation in the MPP TBS with HP group was not found to be significantly different from zero (5-70 minutes: $R^2=0.06191, p=0.0552$; 75-115 minutes: $R^2=0.07559, p=0.0860$). The MPP+VTA TBS group was the only group that had a positive slope in both time periods (5-70 minutes: 0.1773; 75-115 minutes: 0.2540), while all other groups had negative slopes (MPP TBS: -0.9531, -0.4667; MPPTBS+HP: -0.2241, -0.1126; MPP+VTA TBS+HP: -0.8982, -0.7521; MPPTBS in HET: -0.3084, -0.4089).

In summary, in the MPP TBS group and the MPP TBS with HP group, both of which demonstrated similar levels of potentiation, also tended to show decreases in PPF when the response was potentiated. The MPP+VTA TBS with HP group and the MPP TBS in the HET mice group did not show significant potentiation, but also demonstrate a tendency to have decreased PPF when the response did increase post-tetanus. Finally, the MPP+VTA TBS group, despite having similar levels of potentiation to the MPP TBS and MPP TBS with HP group, did not demonstrate a decrease in PPF ratio with LTP; instead, it showed the reverse pattern of an increase of PPF ratio with LTP.
Figure 14. The mean PPF ratio tended to decrease from baseline (black column: average of 30 minutes before tetanus) to after the tetanus (grey column: average of 70 minutes immediately after tetanus) across all groups. A two-way mixed-factor ANOVA (time x group) found a significant effect over time (p = 0.0019) but no significant effect between groups (p = 0.4086) and no significant interaction effect (p = 0.2257).
Figure 15. Linear regression analyses of normalized LTP (x-axis) against its corresponding PPF ratio (y-axis) at all post-tetanic time points, grouped by tetanus protocol and divided by time. Blue dots were acquired during the period when average potentiation was found in a group, while red dots were acquired after potentiation subsided. MPP TBS: TBS protocol applied to MPP pathway only. MPP TBS+HP: haloperidol injection given 10 minutes before TBS applied to MPP pathway. MPP+VTA TBS: VTA and MPP tetanized simultaneously. MPP+VTA TBS + HP: haloperidol injection given 10 minutes before TBS applied to the VTA and MPP. MPP TBS-HET: TBS delivered to the MPP in HET mice. All groups showed significant correlations, but only the MPP+VTA TBS group had a positive slope; all other groups showed negative slopes.
Baseline distal apical response during RE-CA1 stimulation

The threshold needed to induce a response in the RE-CA1 synapse was typically 200 – 500 μA; therefore, the intensity used to monitor was usually 1 to 1.5 times threshold, of 300 – 500 μA. The tetanus intensity used was 1 to 2 times threshold, or 400 to 500 μA. Input-output curves were taken up to 600 μA to check that a larger response could be induced compared to 500 μA, but for monitoring and the tetanus the maximum intensity used was 500 μA.

Similar to the temporoammonic synapse, stimulation of the RE led to a sink at the distal apical dendrites and a dorsal source in the proximal apical dendrites. Mixed responses were not found when the RE was stimulated, which corresponds with lack of an anatomical projection from the RE to the dentate gyrus.

Theta burst stimulation to the RE-CA1 pathway

The TBS protocol was able to induce LTP in the RE-CA1 synapse. A representative experiment is shown in Figure 16. Compared to baseline, the amplitude of the sink and its corresponding source were larger at 30 and 60 minutes post-tetanus (Fig. 16A). Similar to the temporoammonic pathway experiments, there was considerable variation in the response from one time point to the next, and so we averaged three consecutive time points with the middle time point representing the average (Fig. 16B). Finally, sink profile analyses were conducted to ensure the largest sink and source did not shift channels throughout the experiment (Fig. 16C).

A one-way repeated measures ANOVA was used to analyze the effect of the tetanus over 120 minutes (Fig. 17). There was a significant effect over time (p = 0.0181), and so post-hoc tests were conducted to determine which points were significantly different from baseline (by comparing to the last baseline time point, -10 min). Post-hoc tests found that the baseline was
significantly different from the normalized response at 10-35, 45, 65, and 75 minutes post-tetanus.
Data from a representative experiment in which a TBS tetanus was applied to the RE-CA1 pathway. (A) Current source density (CSD) temporal traces from 5 adjacent channels (5 to 9) are shown, with maximal sink at channel 8 (presumed distal apical dendrite). CSD at 2, 30, 60, and 120 minutes post-TBS (black traces) are overlaid with the CSD during baseline (red traces). The circle indicates the stimulus artifact. (B) The slope of the distal apical dendritic sink (channel 8) was analyzed and normalized by the average baseline sink slope. Three consecutive time points were then averaged to reduce variation. (C) CSD profiles, at a time the sink reached half-maximum at channel 8, shown during baseline (an average of 6 AEPs), and at 2, 30, 60, and 120 minutes post-tetanus.
Figure 17. Theta burst stimulation tetanus to the RE induced LTP for up to 75 minutes. Mean excitatory sink slope with SEM (x-axis) over time (y-axis) in the RE-CA1 synapse before and after a TBS to the RE (N=9). A one-way repeated measures ANOVA found a significant difference over time (p = 0.0181). Bonferroni-correct post-hoc tests found significant differences between baseline (-10) and 10-35 minutes, as well as at 45, 65, and 75 minutes post-tetanus.
DISCUSSION

Synaptic plasticity at the distal apical dendrites

This study found that a theta burst stimulation protocol to the temporoammonic pathway or to the RE-CA1 synapse induces LTP in the synapse that receives the tetanic stimulation in urethane-anaesthetized mice. These findings are comparable to Remondes and Schuman’s (2003) findings in rat hippocampal slices and those by Gonzalez and colleagues (2016), though our study has found slightly lower levels of potentiation that do not last as long: they found greater potentiation that remained significantly high for several hours, while we saw an eventual decline to baseline approximately around 70 minutes. This may be attributed to the difference in animal models used. Urethane-anesthetized mice are expected to show stronger interneuronal inhibition in the hippocampus than isolated hippocampal slices from rats used by Remondes and Schuman (2003). We used slightly lower stimulus intensities (on average, 60 – 150 μA when stimulating and tetanizing the temporoammonic pathway), compared to Remondes and Schuman’s 150 – 250 μA). We found that even a tetanus at twice threshold – typically approximately 60 – 80 μA – induced a potentiated response, albeit typically lower than when higher intensities were used. Regardless, we have shown that it is possible to record a response from the temporoammonic pathway and induce LTP in this pathway in a mouse model. The ability to use mice provides opportunities to use genetically modified lines, as we did.

Our study also gave an original report of potentiation in the RE-CA1 synapse in response to TBS, as previous research has shown no effect of tetanic stimulation (a repeated high-frequency stimulation protocol) to this synapse (Eleore et al, 2011). It may be that the tetanic stimulation used in the previous paper was not sufficient to induce LTP: though they used a high-frequency tetanus protocol, it consisted of a total of 600 pulses while our protocol used 1000 pulses. The
intensities used were comparable (they used 0.4 – 0.9 mA, while we used 0.4 – 0.5 mA). There is also the possibility that the difference is due to their use of awake mice compared to our urethane-anaesthetized mice, and further studies will need to investigate whether a stronger tetanus is able to induce LTP in this synapse in awake animals.

**Neuromodulation in the temporoammonic pathway**

We investigated the role of VTA stimulation and haloperidol injection on LTP in the temporoammonic synapse. We did not find VTA stimulation to have any facilitative or inhibitory effect on LTP, nor did haloperidol injection prior to the tetanic stimulation affected the degree of potentiation. However, when haloperidol was injected and the VTA was stimulated, there was an obvious lack of potentiation, even at high intensities.

There are a few theories that may explain these results. It is important to note that the VTA is heterogeneous: while the majority of its neurons release DA, it also has glutamatergic and GABAergic neurons (Margolis et al., 2006; Nair-Roberts et al., 2008). In rats, non-dopaminergic neurons have been found to project to the hippocampus (Swanson, 1982). Therefore, one possibility is that when the VTA is stimulated, DA may have a facilitative effect that is blocked by the co-activation of inhibitory GABA neurons, but when the VTA is stimulated and haloperidol is injected, the DA is blocked but GABA is still activated, resulting in inhibition of the synapse’s ability to potentiate.

Another possibility is that different DA receptors may have opposing roles at this synapse. Haloperidol primarily blocks D2 receptors, but D1 receptors have also been implicated in the temporoammonic pathway (Otmakhova & Lisman, 1999; Ito & Schuman, 2012). Previous studies have found that when selective DA receptor antagonists were applied with DA, there was
a slight recovery of the temporoammonic response but not back to pre-DA levels. A combination of D1 and D2 receptor antagonists also did not fully recover the response. A full recovery was only found when clozapine was applied (Otmakhova & Lisman, 1999; Rosen et al, 2015).

Clozapine binds to a variety of receptors, including serotonin, D1, D2, and D4 (Van Tol et al, 1991). It is also possible that the DA antagonists can prevent DA action, but not recover DA action. Rosen and colleagues (2015) investigated the role of DA, though they used optogenetics to endogenously release DA from the VTA. When they compared their results from their optogenetic experiments to results from slices that had DA applied exogenously, they found opposing results: exogenous DA impaired the temporoammonic response, similar to Otmakhova and Lisman (1999), while having no effect on the SC-CA1 synapse, but endogenously released DA did not affect the temporoammonic response while affecting the SC-CA1 response. They also found that D4 receptor activation inhibited the SC-CA1 synapse by increasing excitation of the SC-interneuron synapse while D1 receptor activation enhanced excitatory transmission, and it is possible that a similar effect may occur at the temporoammonic synapse during synaptic plasticity. Therefore, one theory may be that when DA is released from the VTA to the CA1, it is mediated by at least these three receptor subtypes which have opposing effects to cancel one another out, with D2 receptors having a facilitative effect and D4 receptors having an inhibitory effect.

We also investigated the role of ACh in this pathway using genetically modified mice that have reduce ACh release due to impairments in the VACHT gene. In mice that had a complete knockdown of VACHT in the basal forebrain, there was also impaired synaptic plasticity at the proximal apical dendrites (Al-Onaizi et al, 2016), showing further evidence of the importance of the VACHT gene in hippocampal synaptic plasticity. Notably, even in experiments where the
response did potentiate, it always declined in approximately 30 minutes, which is much earlier than the minimum 70 minutes found in wildtype mice. It is important to note that the mice used were born with a deficiency in their ability to form VACht mRNA and proteins; therefore, adaptive changes or compensatory mechanisms that may have developed during growth could be causing the lack of LTP or reduced cholinergic tone as opposed to the reduced VACht itself. The VACht-HET mice have been shown to have reduced social memory compared to wildtype mice, and this deficiency is rescued by injecting the acetylcholinesterase (AChE) inhibitor galantamine (Prado et al, 2006). However, we have not tested whether LTP can be rescued in a similar fashion. Another possible confounding factor is that the wildtype mice used to compare to the VACht-HET mice were not all littermates. While no differences could be detected between littermates and non-littermates, further experiments could be done with littermate controls to ensure these findings are consistent.

Thus, we have shown that both DA and ACh play neuromodulatory roles at the temporoammonic pathway. Endogenous DA release following VTA stimulation does not appear to lead to any overall facilitative or inhibitory effect on synaptic plasticity, but our preliminary evidence suggests opposing roles by different subtypes of DA receptors or non-DA neurons. A genetic reduction in ACh release dampens the temporoammonic synapse’s ability to potentiate. Umegaki and colleagues (2001) found that D2 receptors may have a facilitative effect by inducing ACh release. While we have not tested this, a logical next step would be to test DA release and related manipulations in these VACht-modified mice, to determine if stimulating DA release could facilitate stronger potentiation.
**Paired pulse facilitation in the temporoammonic pathway**

The mechanism behind paired pulse facilitation has generally believed to be presynaptic residual calcium that has not yet been cleared by the time a second pulse is delivered, which results in an increase in the amount of neurotransmitter released in comparison to the previous pulse. Based on this theory, some studies have examined PPF before and after the induction of LTP as a way of examining whether LTP is due to presynaptic or postsynaptic changes: changes in PPF – typically attenuation – with LTP has been associated with presynaptic changes, while a lack of change in PPF with LTP has been linked to postsynaptic mechanisms driving LTP. However, Wang and Kelly (1997) have shown that PPF can be regulated postsynaptically at the Schaffer collaterals. CAMKII has been discussed in the introduction to be important in the induction of LTP by increasing the single-channel conductance of postsynaptic AMPA receptors: Wang and Kelly found that postsynaptic injections of CAMKII inhibitors in a potentiated response (that also showed reduced PPF ratio compared to baseline) led to a reduced response and reversed the PPF attenuation. Similarly, when calcineurin – a downstream protein that is activated through of NMDA receptors, associated with long-term depression – is inhibited, the Schaffer collaterals display synaptic potentiation and PPF attenuation. These findings indicate that PPF may be modulated postsynaptically, and thus changes in PPF do not necessarily need to come from presynaptic changes. A reduced PPF ratio following LTP may be attributed at least partially to a postsynaptic change such as AMPA receptor desensitization (Wang & Kelly, 1996).

Some studies have found that with the PPF ratio does not change when LTP has been induced (McNaughton, 1982; Manabe et al., 1993) while others have found a decline in the PPF ratio following synaptic potentiation (Schulz et al., 1994; Wang & Kelly, 1997). Our experiments
aligned with the latter group: we found that following a tetanic stimulation that induced synaptic potentiation in the temporoammonic synapse, the PPF ratio tended to decline in a proportional manner to the amount of potentiation. Current research suggests that this effect may indicate a presynaptic mechanism for potentiation in this pathway, such as through increased probability of neurotransmitter release from the presynaptic neuron following the first pulse, resulting in an attenuated increase following the second pulse. However, PPF can also be affected through postsynaptic manipulations, so further studies are needed to ascertain whether presynaptic changes have truly occurred as a result of this potentiation.

There was also evidence to suggest that DA may facilitate neurotransmitter release, as VTA stimulation did not lead to a decline in the PPF ratio even though LTP was found: in fact, the potentiated response correlated with an increase in the PPF ratio within this group. This aligns with Otmakhova and Lisman’s (1999) findings that DA results in higher PPF ratio when applied to slices. As Rosen and colleagues (2015) showed, in vitro application and in vivo modulation can sometimes lead to opposite results: therefore, we believe that our use of VTA stimulation provides stronger evidence of DA’s neuromodulatory role in synaptic plasticity at the temporoammonic pathway. While we have not proven that VTA stimulation led to DA release, this effect was abolished when haloperidol was applied ten minutes before VTA stimulation, which indicates that D2 receptors are involved in this facilitation. This also corresponds with the hypothesis that D2 receptors play a facilitative role.

ACh-deficient mice also showed a decline in PPF when the response potentiated, suggesting that this effect is not directly affected by ACh. While the VACHT-HET mice did appear to have a slightly lower baseline PPF ratio compared to the other groups, we did not find this to be significantly different from the other groups. It is possible that DA, through D2 receptors, affects
PPF by releasing ACh, though further studies are needed to test this hypothesis. Our experiments suggest that ACh on its own does not affect the PPF.

**Conclusion**

This study provides evidence of in vivo synaptic plasticity to two direct inputs to the CA1, the temporoammonic EC-CA1 synapse and the nucleus reuniens-CA1 synapse, and LTP induced by a theta burst stimulation protocol lasted approximately 70 minutes. Thus, these synapses are able to potentiate in a whole brain even in the presence of inhibitory interneurons. Further, we have shown neuromodulatory effects in the temporoammonic pathway by DA and ACh, and suggest mechanisms by which this neuromodulation may occur based on our findings and previous studies. Different DA receptor subtypes may have different roles, such as D2 having a facilitative role and ordinarily being balanced out by D1 receptors that have an inhibitory effect. ACh is also shown to be important for synaptic plasticity, as reduced ACh in a genetic model shows less potentiation than wildtype mice. Our analysis of paired pulse facilitation before and after tetanic stimulation also implicate presynaptic changes when LTP is induced in the temporoammonic synapse, and these presynaptic changes may be affected by DA. In conclusion, we have shown synaptic plasticity in both synapses in vivo. We also provide new evidence of how synaptic plasticity in the temporoammonic synapse is modulated by other neurotransmitters and that at least part of this plasticity may be due to presynaptic changes.
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APPENDIX

Ethical Approval for Animal Use

PI Name: Leung, Stan (lai-wo)

AUP Title: Neural plasticity of the forebrain

Official Notification of AUS Approval: A MODIFICATION to Animal Use Protocol 2010-261 has been approved.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura

on behalf of the Animal Use Subcommittee
Supplementary Figures

**Figure 1.** A CSD of a temporoammonic response at 150 μA. The red circle indicates the TA response, seen at lower intensities; at 150 μA, a later response can be seen ventral to the TA response (blue circle) as well as a small response at the basal dendrites (green circle). Original illustration of a CA1 pyramidal cell by Spruston (2008).
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