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Cholinergic control of perirhinal cortex and recognition memory circuits

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Abstract of the Dissertation

Phenotypical effects of *MeCP2* deletion from cholinergic neurons on the recognition memory circuit

by

Elizabeth C. Ballinger

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Acetylcholine is an important neurotransmitter in the brain that plays a vital role in various aspects of cognition, including attention, spatial memory, and emotional memory. The role of cholinergic signaling in recognition memory, however, is less well understood. Recognition memory is a type of declarative memory and an important aspect of human cognition that is dependent on the perirhinal cortex. Cholinergic signaling within the perirhinal cortex appears to be especially important, as blockade of this signaling either pharmacologically or with selective lesions impairs recognition memory performance. Recognition is believed to be encoded by reductions in response of the perirhinal cortex upon exposure to previously experienced stimuli. This "reduction response" is dependent upon cholinergic signaling and thus cholinergic mediated long term depression is theorized to underlie recognition encoding. However, the effect of endogenous acetylcholine release in the perirhinal cortex has not previously been investigated. Here I probe for the first time the consequences of endogenous acetylcholine release in the PRH. I have also investigated the circuit level disruptions that may underlie recognition memory impairment in an animal model of intellectual disability. I have shown that the rate of reduction responses in the perirhinal cortex induced by ACh is the same as the rate of reduction responses induced by familiarity. Additionally, I have shown that when *MeCP2* is deleted from cholinergic neurons, perirhinal cortex firing is impaired not only in its response to cholinergic input, but also in firing variability at baseline. Therefore cholinergic signaling in the perirhinal cortex is likely important both at baseline and after stimulation. Acetylcholine therefore may act over multiple time scales and via multiple mechanisms to subserve recognition memory.

To Josh and Nick, who have inspired this journey and to my mom and dad

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List of Abbreviations

ACh	Acetylcholine
ChAT	Choline – acetyl transferase
vAChT	Vesicular acetylcholine transporter
AChE	Acetylcholinesterase
NBM	Nucleus Basalis of Meynert/Magnocellularis
PRH	Perirhinal cortex
nAChR	Nicotinic Acetylcholine receptor
mAChR	Muscarinic Acetylcholine receptor
SI	Substantia Innominata
MEC	Medial Entorhinal Cortex
ChT	Choline transporter
MS	Medial Septum
DB	Diagonal Band of Broca
BFCN	Basal forebrain cholinergic neurons
PFC	Prefrontal Cortex
PNS	Peripheral nervous system
CNS	Central nervous system
GFP	Green fluorescent protein
LTP	Long term potentiation
LTD	Long term depression
LFP	Local field potential
FF	Fano Factor
FR	Firing rate

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2012- present **Graduate Research Assistant,** Laboratory of Dr. Lorna Role, Department of Neurobiology and Behavior, Stony Brook University, Stony Brook, NY

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2009-2010 **Junior Specialist,** Department of Pediatrics and the M.I.N.D. Institute, University of California, Davis School of Medicine, Sacramento, CA

- collected pre-pulse inhibition data on as a potential clinical outcome measure for clinical trials in patients with Fragile X Syndrome and for a study of Fragile X Associated Tremor and Ataxia Syndrome
- behavioral coding and data analysis for all participating sites in the clinical trial that collected pre-pulse inhibition data
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2008-2009 **Junior Specialist,** Department of Psychiatry and Behavioral Sciences and the M.I.N.D. Institute, University of California, Davis School of Medicine, Sacramento, CA

- collection of electrocardiography, electromyography and galvanic skin response from participants with various developmental disabilities

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2007-2008 **Research Assistant,** M.I.N.D. Institute, University of California, Davis School of Medicine, Sacramento, CA

- laboratory focused on physiological measures of autonomic function and their relationship with measures of behavior and anxiety among individuals with typical development, Fragile X Syndrome, and other developmental disorders
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PUBLICATIONS

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Composed the majority of the text of this review article and created 1 of the 4 figures.

Jiang, L., Kundu, S., Lederman, J.D., López-Hernández, G.Y., **Ballinger, E.C.,** Wang, S., Talmage, D.A., Role, L.W. (2016) Cholinergic signaling controls conditioned-fear behaviors and enhances plasticity of cortical-amygdala circuits. *Neuron, 90(5), 1057-1070.*

Contributed imaging data, helped in manuscript preparation.

Ballinger, E., Cordeiro, L., Chavez, A., Hagerman, R. and Hessl, D. (2014) Emotion Potentiated Startle in Fragile X Syndrome. *Journal of Autism and Developmental Disorders, 44(10),* 2536-46.

Collected behavioral and physiological data, coded behavioral data, assisted in fine tuning behavioral analysis, scored behavioral questionnaires, conducted statistical analysis, prepared manuscript.

Schneider, A., **Ballinger, E.,** Cordeiro, L., Chavez, A., Olichney, J., Niese, A., Hagerman, R., Hessl, D. (2012) Prepulse Inhibition in patients with Fragile X related Tremor and Ataxia. *Neurobiology of Aging, 33(6),* 1045-53.

Collected physiological and behavioral data, coded behavioral data and scored behavioral questionnaires.

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Collected and scored behavioral questionnaires, helped with manuscript preparation.

Yuhas, J., Cordeiro, L., Tassone, F., **Ballinger, E.,** Schneider, A., Campbell, A., Ornitz, E., Long, J., Hagerman, R. and Hessl, D. (2011) Brief Report: Sensorimotor Gating in Idiopathic Autism and Autism Associated with Fragile X Syndrome. *Journal of Autism and Developmental Disorders, 41(2),* 248-53.

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PRESENTATIONS

2016 **Ballinger, E.,** Schaaf, C., Talmage, D., Zoghbi, H. and Role, L. Alterations in the Novel Object Recognition Circuit Following *MeCP2* Deletion from Cholinergic Neurons. Poster presentation: 46th Annual Society for Neuroscience Meeting, San Diego, CA.

2015 **Ballinger, E.,** Schaaf, C., Talmage, D., Zoghbi, H. and Role, L. Alterations in the Novel Object Recognition Circuit Following *MeCP2* Deletion from Cholinergic Neurons. Poster presentation: 45th Annual Society for Neuroscience Meeting, Chicago, IL.

2014 **Ballinger, E.,** Schaaf, C., Talmage, D., Zoghbi, H. and Role, L. Phenotypic Effects of *MeCP2* Deletion from Cholinergic Neurons. Podium presentation: 44th Annual Society for Neuroscience Meeting, Washington, D.C.

2010 **Ballinger, E.,** Cordeiro, L., Chavez, A., Campbell, A., Ollison, M., Abucayan, F. and Hessl, D. A Biobehavioral Probe of Amygdalar Activation in Fragile X Syndrome. Podium presentation: 12th Annual International Fragile X Conference, Detroit, MI.

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2009 Yuhas, J., Cordeiro, L., Schneider, A., **Ballinger, E.,** Campbell, A., Ornitz, E., Long, J., Hagerman, R. and Hessl, D. Prepulse Inhibition: Its Expanded Use in Assessing Autism Caused by Fragile X Syndrome Versus Idiopathic Autism. Poster presentation: 8th Annual International Meeting for Autism Research, Chicago, IL.

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- Gave a brief demonstration of *in vivo* electrophysiology data collection
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Spring 2013 **Teaching Assistant,** Principles of Neurobiology, Stony Brook University, Stony Brook, NY

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Chapter 1:

Basal forebrain cholinergic circuits and signaling in cognition

INTRODUCTION

Although it was originally discovered at the neuro-muscular junction, acetylcholine (ACh) is also synthesized and released in the brain and is a key neurotransmitter in the central nervous system (CNS). Cholinergic signaling appears to be especially important in mediating cognition, as decreases in cholinergic signaling in the brain are associated with Alzheimer's Disease and Parkinson's Disease with dementia, two catastrophic diseases characterized by dramatic cognitive impairment (for review see:(Ballinger et al., 2016). Even in non-diseased individuals, enhancing cholinergic signaling ameliorates low cognitive performance. Boosting cholinergic signaling enhances attention and memory performance both in the context of naturally occurring poor performance and after experimentally induced impairments (Bubser et al., 2014; Callahan et al., 2014; Galloway et al., 2014; Karamihalev et al., 2014; Knott et al., 2014; Knott et al., 2015; Niemegeers et al., 2014; Paolone et al., 2013; Reches et al., 2013; Rezvani et al., 2012; Timmermann et al., 2012; Vieira-Brock et al., 2015). Reches et al identified two distinct activation patterns associated with memory in healthy volunteers: one obtained at baseline and a separate, distinct pattern typified by more frontal activation obtained after enhancement of the cholinergic signal by blocking ACh breakdown with donepezil treatment (Reches et al., 2013). Participants whose pattern at baseline was most similar to the typical donepezil pattern actually performed the best on a memory task (and showed the least improvement with donepezil), while those whose baseline pattern was the most dissimilar to the final donepezil pattern showed great improvement on the task after donepezil treatment (Reches et al., 2013). This suggests that there is an optimal concentration of ACh for cognitive performance and that naturally occurring poor performers owe their phenotype to low ACh levels. It seems that correction of these low levels with cholinergic enhancement can rescue the poor behavioral performance.

Here I review recent insights into how ACh might exert these effects. This includes advances in understanding of how ACh signals in the brain and the mechanisms by which it might mediate or modulate cognitive performance.

1.1 CHOLINERGIC NEURONS AND CHOLINERGIC SIGNALING MECHANISMS IN THE CNS

1.1a.Basics of cholinergic signaling

The rate limiting step in synthesis of ACh is catalyzed by the enzyme Choline Acetyl Transferase (ChAT). ACh is then packaged into vesicles by the vesicular Acetylcholine Transporter (vAChT) and released. After release and binding to post synaptic receptors, its actions are stopped by hydrolysis by Acetylcholinesterase (AChE). The resultant choline is then taken up by the presynaptic terminal using the choline transporter (ChT).

Upon release, ACh exerts its effects by binding to a variety of receptors. These receptors are broadly classified as two types: nicotinic or muscarinic, based on their binding capacity for nicotine or muscarine, respectively. Generally, nicotinic receptors are ligand gated ionotropic receptors that rapidly open in response to ligand binding and are permeable to cations. Muscarinic receptors, on the other hand, are G protein coupled metabotropic receptors with slower effects. Nicotinic receptors are either heteromeric or homomeric pentamers composed of 5 subunits. There are many different kinds of subunits: termed α2-10 and β2-4 (Dani and Bertrand, 2007). The most common nicotinic receptors expressed in the brain are α7*and α4β2* (where the * indicates "containing", (Gotti et al., 2006). Muscarinic receptors are of the types M1-M5. M1, M3 and M5 interact with G_q proteins while M2 and M4 interact with G_i proteins (Thiele, 2013). All 5 are expressed in the brain.

1.1b. Functional organization of cholinergic neurons & their projections

1.1b.i. Overview of cholinergic neurons & projections

The vast majority of cholinergic input to cortical and subcortical structures engaged in cognition arises from distal projection neurons whose cell bodies reside in the basal forebrain (**Fig 1.1A**). The basal forebrain cholinergic projection neurons elaborate highly extensive, multiply branched inputs to neocortex, archeocortex and other subcortical structures (Woolf, 1991). The cell bodies of the basal forebrain cholinergic neurons are interspersed with non-cholinergic neurons and distributed in a series of nuclei, including the medial septal (MS) nucleus, the diagonal band (DB) nuclei – with vertical and horizontal domains - the preoptic nucleus, the nucleus basalis (NBM), and the substantia innominata (SI; **Fig 1.1** & Woolf, 1991). In primates, the cholinergic nuclear groups are referred to somewhat differently: Ch1 = MS, Ch2 = vertical limb of the Diagonal Band of Broca (DBB), Ch3 = horizontal limb of DBB, Ch4 = the basal magnocellular complex that

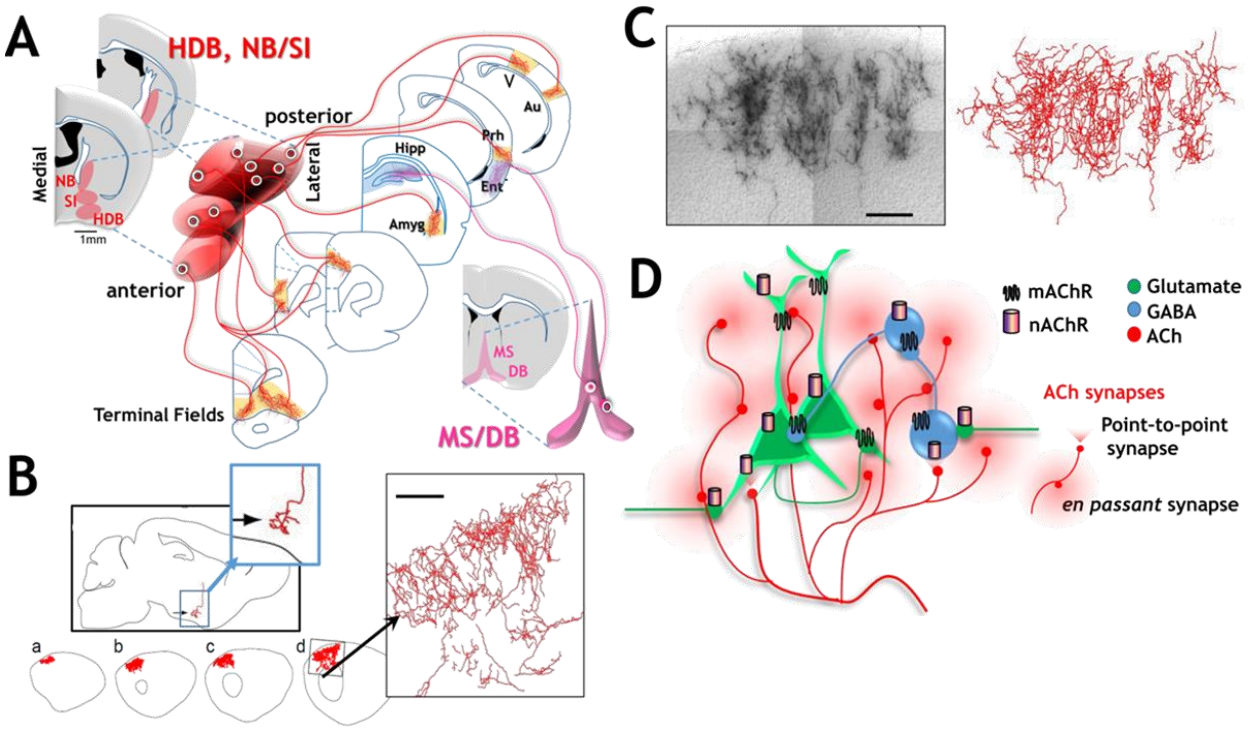


Figure 1.1: Functionally modular projection patterns, exotic axonal morphologies and diverse ACh release-receptor interactions contribute to complex spatio-temporal dynamics of ACh signaling by basal forebrain cholinergic neurons (see text and Reviews by Munoz & Rudy, 2014; Picciotto et al., 2012; Sarter, 2016; Zaborzsky et al., 2015). **A. Schematic of projection patterns of basal forebrain cholinergic neurons.** Left hand side: schematic of coronal sections indicating the approximate anterior to posterior and medial to lateral distribution of the HDB (horizontal limb of the diagonal band) and NB/SI (Nucleus Basalis/ Substantia innominata). Anterior medial BFCNs within these nuclear groups project to medial frontal cortical targets whereas posterior located cholinergic neurons project to more posterior targets such as the BLA and perirhinal cortex. Right hand side: Medial septal (MS) and vertical limb of the diagonal band (VDB) neurons provide cholinergic input to the hippocampus and entorhinal cortex. **B.** and **C.** Axonal morphology of fully reconstructed basal forebrain cholinergic neurons and the extensive terminal arborization formed in cortex. Adapted with permission from Wu et al., 2014 https://creativecommons.org/licenses/by/3.0/. **D.** Schematic representation of both point-to-point (focused, triangular) and *en passant* (broad circular) mechanisms by which ACh is released in the CNS, thereby effecting both glutamatergic (green) and GABAergic (blue) neuronal excitability. Such release profiles may correspond to the more rapid and transient responses and the slower, longer lasting modulatory effects of ACh, respectively (see text for discussion). Also shown are representative distributions of both muscarinic (depicted as 7 TM squiggles) and nicotinic (represented as single tubes) AChRs at pre, post and peri synaptic sites. Both mAChR and nAChR subtypes at each of these locations contribute to the direct and indirect mechanisms by which ACh can alter synaptic excitability (see text for discussion).

includes the SI, the Nucleus Basalis of Meynert (NBM), the magnocellular preoptic nucleus and the ventral pallidum (Mesulam et al., 1983).

1.1b.ii. Projection fields of Basal Forebrain Cholinergic Neurons

The MS and DB form a functional cluster that sends cholinergic projections primarily to the hippocampus, parahippocampus olfactory bulb and midline cortical structures (Knox and Keller, 2015). In contrast, the NB (or NBM) and SI provide the majority of cholinergic projections to neocortex and to the amygdala (Woolf, 1991). Each of these nuclei is heterogeneous in phenotype: the cholinergic neurons are intermingled with neurons of distinct transmitter and peptide content (Gritti et al., 2006; Mufson et al., 2006).

Although the concept that there is a rough topographical organization of the basal forebrain has been discussed since the 1980's (Mesulam et al., 1983; Saper, 1984; Zaborszky et al., 1999) the cholinergic system has more typically been viewed as both spatially and functionally "diffuse" (Saper, 1984, Woolf, 1991). Recent studies paint a somewhat different picture by combining a rich array of immunological and genetic techniques to efficiently label neurons expressing choline acetyltransferase (ChAT), the biosynthetic enzyme for acetylcholine (ACh) and the vesicular ACh transporter (VAChT). These genetic approaches have been united with anterograde, retrograde and transynaptic labels to optimize the comprehensive mapping of cholinergic cell bodies and their projection fields (although see Yi et al. 2015 for a consideration of the caveats associated with purely transgenic probes). Functional connectivity is further explored using photoactivatable, genetically targeted probes that permit selective excitation or inhibition of ChAT+ neurons down to select branches of their terminal arbors (**Fig 1.1A**; (Jiang et al., 2016; Unal et al., 2015). Alternatively, intentionally sparse genetic labeling has been used to provide unprecedented insight into the morphology of single BFCNs, revealing that these neurons have a considerably more extensive and complex axonal architecture than previously appreciated (**Fig 1.1B, C**; (Wu et al., 2014).

Overall, these new studies support the idea that the connectivity of cholinergic projection neurons to their cortical targets has sufficient specificity to subserve functionally and spatially selective signaling. Perhaps the most important generalization is the predominant role of a *functionally* based topographical organization of the BFCNs as suggested by Zaborsky & colleagues.

The projections from basal forebrain cholinergic neurons to frontal cortical targets are the most completely described (Bloem et al., 2014; Chandler and Waterhouse, 2012; Chandler et al., 2013). Medial frontal cortex receives projections from more medial and anterior located cholinergic neurons within the basal forebrain nuclear groups. The dorsal regions of prefrontal cortical areas receive projections from medially located NB/SI and DB neurons, whereas more ventral regions of prefrontal cortex receive projections from more laterally located BFCNs (**Fig 1.1A** & Bloem et al., 2014). More rostrally positioned BFCNs

appear to project to both superficial and deep layers of frontal cortex, while more caudally placed BFCNs preferentially project to deep layers of cortex (Bloem et al., 2014).

Lateral portions of cortical and subcortical areas, for the most part, receive projections from more lateral and more posterior located cholinergic neurons (**Fig 1.1A** & Kondo and Zaborszky, 2016; Zaborszky et al., 2015a; Zaborszky et al., 2015b). There are at least two exceptions to this general topography: the hippocampus and the entorhinal cortex (in contrast to the adjacent perirhinal/ectorhinal cortices) receive the majority of their cholinergic input from the MS/VDB cholinergic groups (Kondo and Zaborszky, 2016; Woolf, 1991).

Simultaneous retrograde BF labeling from multiple cortical regions reveals several organizing principles. First, although there is overlap within the basal forebrain of cholinergic neurons that project to non-adjacent cortical areas, the degree of overlap is dependent upon the interconnectivity of the cortical projection areas in question. These findings suggest a level of organization of the BFCN neurons that might facilitate coordinate control of functionally linked, although spatially distinct, cortical projection areas (**Fig 1.1A**; Zaborszky et al., 2015a; Zaborszky et al., 2015b). Second, axons from very distinct BF areas can innervate immediately adjacent cortical regions, such as the perirhinal (NB/SI) and entorhinal cortices (MS/vDB). Whether this latter example indicates a hierarchy of coordinate functional modulation or is the result of developmental differences between archeo and neocortex remains to be seen.

1.1b.iii. Axonal morphology of basal forebrain cholinergic neurons

Axons from individual cholinergic neurons form collaterals that innervate multiple cortical regions that are functionally related (Chandler and Waterhouse, 2012; Chandler et al., 2013; Chavez and Zaborszky, 2016), but do not appear to have collaterals to functionally distinct regions of neocortex (e.g. A1 vs V1; (Kim et al., 2016).Recent experiments employing retrograde labeling of BFCNs neurons demonstrate that as many as 20% of labeled NBM/SI neurons have axons that terminate in 3 *different* prefrontal domains, including the medial PFC, anterior cingulate cortex *and* the orbitofrontal cortex (Chandler and Waterhouse, 2012; Chandler et al., 2013).

The ability of single BFCN neurons to target large territories of cortex clearly requires extensive collateral formation. Just how elaborate individual cholinergic axon arbors can be was demonstrated - rather elegantly - by sparse genetic labeling (Wu et al., 2014); and see **Fig 1.1B & 1.1C**, taken from Wu et al., 2014 with permission). Based on their measurements the calculated average length of a single axon of a basal forebrain cholinergic neuron in a mouse brain, including all of its terminal branches, is ~30 cm. Calculations based on human post mortem data are consistent with single cholinergic axons of >100 meters! Clearly the BF cholinergic neurons have the capacity to modulate activity

across multiple cortical areas or columns within specific cortices – but likely at an enormous metabolic cost. Indeed, metabolic demand to maintain function over the lifespan has been raised as a possible basis for the sensitivity of the BF cholinergic system in neurodegenerative disorders (Wu et al., 2014).

1.1b.iv. Summary

Taken together the more recent mapping and morphological studies of BFCNs demonstrate that (a) these cholinergic projection neurons can be extremely elaborate in both the extent of axonal arbors and the number of axonal branches, (b) there is topographic, rather than diffuse, organization of BFCNs and their target fields along anterior to posterior, ventral to dorsal and medial to lateral axes (**Fig 1.1**; Kim et al., 2016), (c) in frontal cortex some cholinergic projection neurons are "dedicated" i.e. there are BFCNs that project to a single region of orbitofrontal, anterior cingulate or prefrontal cortex, but the majority of BFCNs project to more than one frontal domain. These multiply projecting BFCNs appear to innervate functionally connected structures and might mediate co-ordinate cholinergic signaling in behaviorally related targets. Consistent with this idea, BFCN input to operationally distinct areas of sensory cortex are segregated from one another along the anterior posterior axis of BFCN (Kim et al., 2016). Overall, the concept of cholinergic signaling occurring in functional modules has received robust support in this new era of brain mapping.

An area of study that has received relatively little scrutiny with the modern mapping techniques is the architecture of cholinergic projections to deeper structures, such as the hippocampus and amygdala. Given that the basal lateral amygdala (BLA) appears to receive the densest cholinergic innervation of any structure besides the striatum, and cholinergic input to the BLA exerts an important influence on acquisition and retention of emotional memories (Knox, 2016), detailed single neuron mapping of the cholinergic neurons that project to the amygdala should be very revealing.

1.1c. Multiple temporal & spatial profiles of ACh release & ACh actions.

1.1c.i. Overview of ACh release mechanisms

In the peripheral nervous system (PNS), ACh is typically released in excess and in close apposition to the post synaptic target. Despite rapid hydrolysis by acetylcholinesterase (AChE), sufficient ACh survives the transynaptic journey to elicit fast and robust signaling via activation of muscarinic and/or nicotinic receptors in a highly temporally & spatially restricted manner. In the CNS, cholinergic signaling is also initiated when ACh is released and is mediated by interaction with ACh receptors on target cells. This is where the similarities between PNS and CNS cholinergic signaling appear to end. ACh release in the brain has classically been conceptualized as slow and tonic (Descarries et al., 1997). This idea of a "volume" mode of transmission was supported by early views that the cholinergic

system was anatomically diffuse (an idea that is increasingly being challenged, as per above), and early microdialysis experiments - with spatially and temporally limited probes – that documented ambient levels of ACh in the micromolar range tonically present in brain tissue (Descarries et al., 1997). This notion has now been substantially revised (Sarter & colleagues; see (Sarter et al., 2014).

1.1c.ii. Temporal profiles of ACh release: transient vs tonic

Our understanding of the spatiotemporal profiles of ACh release from basal forebrain cholinergic neurons has been transformed by the generation of new, high resolution stimulation and recording techniques. Based on more rapid assays for ACh release and on new approaches for selective activation of cholinergic neurons and specific BFCN terminal fields, we now know that ACh release and downstream signaling can be faster and more focal than previously appreciated (Munoz and Rudy, 2014; Sarter and Kim, 2015). The discovery of relatively fast increases (over seconds, rather than minutes) in ACh release was made possible by the development of choline oxidase coated microelectrodes that detect the choline formed following breakdown of ACh (Parikh et al., 2004). These microelectrodes are highly selective and offer more acute temporal and spatial resolution of ACh release.

Using electrochemical detection, Sarter & colleagues have convincingly demonstrated the existence of cholinergic "transients" - relatively rapid spikes of ACh - that begin within 200-500 msec of a behaviorally relevant stimulus and last several seconds (Sarter et al., 2014). By combining optogenetic stimulation of BFCN cell bodies or cholinergic terminal fields in mPFC with electrochemical detection, Gritton et al (2016) detected ACh release within 100 msec of the light stimulus. A recent study by Nelson & Mooney (2016) demonstrates even faster kinetics of cholinergic signaling: they report direct BFCN to pyramidal neuron (nicotinic) EPSCs with ~10 msec delay from optogenetic activation of terminal fields to evoked postsynaptic responses.

Despite the growing evidence for more temporally precise ACh release and cholinergic signaling, there is still strong support from experiments that support a slower and spatially broader change in ACh concentration. Runfeldt et al (2014) demonstrate profound effects of these slower, more extensive changes in ACh on microcircuit properties in the cortex that may be important for detection and encoding of information. Likewise, the time course of ACh responses following optogenetic stimulation of BF cholinergic projection neurons vs their terminal fields in BLA are consistent with relatively rapid effects of released ACh (detection within <20-100 msec), and yet appear to be entirely "modulatory" in nature, changing BLA principal neuron firing patterns and/or the efficacy of transmission at cortical-BLA synapses over long time scales(Jiang et al., 2016; Unal et al., 2015).

Distinct cellular phenotypes of cholinergic neurons may provide the cellular basis for two different modes of signaling: fast and focal and slow and paracrine. Zaborszky and colleagues have performed detailed electrophysiological characterization of the cholinergic neurons in the NBM and identified two populations: a more excitable, early firing population that show spike frequency adaptation and a less excitable, late firing population that could maintain low frequency tonic firing (Unal et al., 2012). Overall, it appears that *both* more rapid/transient as well as less temporally and spatially focal modes of ACh release play important roles in different aspects of information processing (Picciotto et al., 2012; Sarter and Kim, 2015).

It is not yet clear whether cholinergic transients mediating relatively rapid responses are fundamentally different from the signaling that underlies longer lasting, neuromodulatory changes in circuit dynamics by ACh. The term "neuromodulation" has had variable definitions associated with "changes in state of a neuron or a group of neurons that alters the response to subsequent stimulation" (Picciotto et al., 2012). Neuromodulation by ACh has been shown to include changes in release probability, shifts in firing patterns or altered excitability through shifts in the input/output relationship (Picciotto et al., 2012). The spatial and temporal dynamics of ACh signaling underlying cholinergic modulation may vary depending on the system and/or brain region.

1.1c.iii. Summary

The cumulative evidence on ACh release appears to support multiple temporal modalities and spatial domains. In addition to tonic, low levels of ACh release, temporally and spatially discrete release of ACh may play a vital and specific role in mediating cognitive processes. In view of the potentially vast "synaptic space" covered by the axonal terminal arbors of single cholinergic neurons (Wu et al., 2014 ; **Fig 1.1**), the onset and duration of co-ordinate ACh effects may be very broad, despite focal release at each synaptic bouton. Alternatively, one might propose mechanisms for selective activation of a subset of boutons along a single BFCN terminal arbor. An interesting avenue for future research is how differential expression of acetylcholinesterase may influence the various temporal profiles of ACh release and signaling. Resolution of the details of the temporal and spatial dynamics of ACh signaling are within reach with new technologies for focal, selective stimulation and monitoring of resultant Ca signals within specific subcellular compartments.

New sites and mechanisms of cholinergic signaling have come to the fore, adding to the already rich diversity of means by which ACh can contribute to the mediation and modulation of activity in neural networks. Overall, recent findings converge on the idea that both mAChRs and nAChRs are distributed to contribute to multiple modes of ACh signaling. The real challenge lies with discerning which of the myriad signaling mechanisms available are actually employed when ACh is released *in vivo*. The latter challenge has been

significantly advanced with the advent of selective stimulation and recording techniques that allow the assessment of endogenously released ACh.

There are increasing numbers of reports consistent with cholinergic transmission being mediated by fast synaptic release and consequent activation of postsynaptic nicotinic and/or muscarinic AChRs (e.g. Nelson and Mooney, 2016). On the other hand, the paucity of morphological evidence for point-to-point cholinergic contacts and the prolonged temporal profile of ACh effects are consistent with more modulatory actions (Mechawar et al., 2002; Umbriaco et al., 1995; Umbriaco et al., 1994). The latter include examples of long lasting modulatory changes in excitability due to presynaptic changes mediated by nicotinic and/or muscarinic AChRs. Recent optogenetic studies further demonstrate that ACh release from BFCNs can elicit LTP and STD in hippocampus (Gu and Yakel, 2011) and LTP at cortical- BLA synapses (Jiang et al., 2016). Likewise, durable changes in post synaptic excitability are also documented that may engage AChRs coupled to G protein signaling and/or changes in Ca signaling networks.

Although the multiplicity of potential mechanisms and the complexity of cholinergic signaling may be inconvenient to study, the data are, in fact, consistent with multiple temporal and spatial mechanisms by which ACh can interact with its cognate receptors *in vivo*. We can now combine the increased resolution afforded by significant technological advances, in both selective cholinergic stimulation and release assays, with advancing methodologies for electrophysiological and imaging based recording in awake, behaving animals. These combinatorial approaches to examine cholinergic signaling dynamics *in vivo* bring us closer to physiologically and behaviorally relevant answers to the question of HOW cholinergic signaling influences the excitability of specific circuits and networks to alter cognitive processing.

1.2. CHOLINERGIC SIGNALING AND CIRCUITS INVOLVED IN COGNITION: ATTENTION AND MEMORY

A wealth of prior physiological, lesion, pharmacological and genetic studies converge on the idea that ACh is involved in cognitive processes, including attention and memory (Hasselmo and Sarter, 2011; Micheau and Marighetto, 2011; Picciotto et al., 2012). Here I will briefly review the evidence that acetylcholine signaling is involved in specific aspects of cognitive processing, focusing on recent work on circuit mechanisms underlying cognition and beginning with the best studied cognitive domain with respect to ACh: attention.

1.2a. Cholinergic signaling and circuits in attention

1.2a.i. Overview of the role of ACh in mediating attention

Pharmacological exposure to exogenous substances sparked early work into the potential involvement of AChR signaling in attention. Nicotine has been widely reported to improve performance in specific attention tasks and exposure to nicotine during development can lead to lasting impairments in attention performance and in the brain areas thought to mediate attention (Bloem et al., 2014; Jung et al., 2016). Studies of polymorphisms, mutations, and deletions of various cholinergic genes further link alterations in cholinergic signaling to modified attention performance (Sarter et al., 2016a; Sarter et al., 2016b). Overall, the last 5 years have greatly sharpened our understanding of how cholinergic circuits co-ordinate with both prefrontal and sensory cortices to shape behavior in response to attentional tasks.

Attention has been conceptualized as consisting of two separate processing streams: goal or cue driven attention is termed "top down" while sensory driven attention is termed "bottom up" (Katsuki and Constantinidis, 2014; Sarter et al., 2016b). Essentially, top down attention may be thought of as voluntary, or "feed-back" driven - whereby incoming sensory information is modulated by higher cortical areas. In contrast, bottom up attention is thought of as involuntary, or "feedforward" - whereby sensory information is fed forward and up to the cortex (Katsuki and Constantinidis, 2014; Sarter et al., 2016b). Here I will focus on cholinergic circuit mechanisms of "top down" aspects of attention, as it is the best studied and most pertinent to cognitive performance.

1.2a.ii. Cholinergic modulation of prefrontal cortex related to attention

The prefrontal cortex (PFC) is an integral node in circuits underlying attention, exerting top down control over sensory cortical areas to enhance detection of task relevant cues. The PFC is also a significant target of cholinergic modulation: the organization of cholinergic inputs to the PFC from the NBM and the DB has now been mapped in detail (**Fig 1.2**; Bloem et al., 2014, Chandler and Waterhouse, 2012, Chandler et al., 2013).

Sarter and his colleagues have used their enzyme selective microelectrodes to document the presence of transient (subsecond to seconds) increases in ACh in the PFC in response to attention task related cues (Howe et al., 2013; Parikh et al., 2007). Based on the speed and relatively short lived duration of the choline transients, they propose that, at least in PFC, ACh mediates, rather than modulates, cue detection and cue triggered changes in goal oriented behavior (Howe et al., 2013, Parikh et al., 2007). In other words, the authors suggest that ACh release in the PFC is the neurological correlate of cue detection that signals to the animal the presence of a behaviorally relevant cue. Gritton et al. (2016) demonstrated that enhancing the cue associated cholinergic transient with optogenetic stimulation improved cue detection and that stimulation of a cholinergic transient during a non-cued trial led to a "false positive" behavioral response from the animal, presumably due to a mistaken detection of a cue. Blocking the ACh transients with optogenetic inhibition of BFCNs caused the animals to "miss" many of the cues, consistent with the idea that ACh

release is a requisite signal for cue detection (Gritton et al., 2016). As such, all of the pertinent arguments are now in place: the relevant PFC circuits are cholinoceptive, ACh is released in the PFC during execution of goal-directed attention tasks and the release of ACh is both necessary and sufficient to mediate task related cue detection.

Together these data support the idea that ACh signaling in the PFC signals cue detection to the animal via transient cholinergic release/signaling.

1.2a.iii. Cholinergic modulation of sensory cortex related to attention

In sensory cortices, the successful detection of behaviorally relevant stimuli during attention tasks is accompanied by 2 electrophysiological hallmarks: increased firing by task

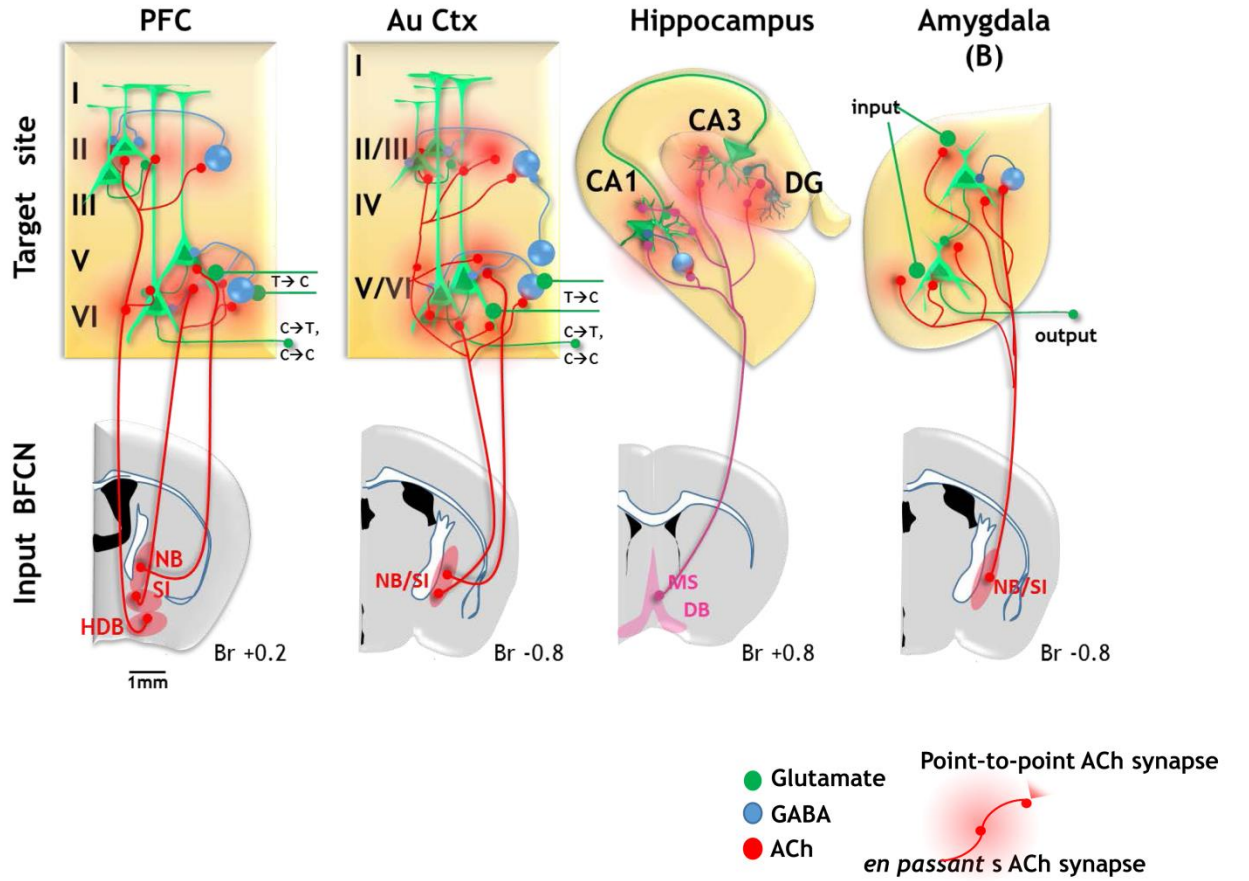


Figure 1.2: Schematic Representation of Cholinergic Inputs and Signaling in Cortical, Hippocampal and Amygdala circuits. (see text) from Bloem et al., 2014.; Kim et al., 2016; Nelson & Mooney, 2016; Jiang et al., 2016; Munoz & Rudy, 2014; Gu & Yakel, 2011; Cheng & Yakel, 2016. Numerous studies now converge on specific mechanisms of ACh release and profiles of AChR activation in different brain areas. Each schematic represents a summary of recent studies of the cholinergic projection neurons (below) and consequent signaling effects of ACh in local circuit activity in (above; left to right) the Prefrontal Cortex (PFC), Auditory cortex (Au Ctx), hippocampus and (basal) amygdala.

relevant sensory cortical neurons, and decorrelation of intra cortical noise, or decorrelation of the variance of sensory cortical neuron population responses to a given stimulus. This decorrelation can be measured as a reduction in power of low frequency LFP, which is called "desychronization" of the LFP. During the actual performance of a behavioral task, decorrelation increases the response reliability of a given sensory cortex neuron to the appropriate stimuli by reducing the influence of variability of other sensory cortex neurons within the population and rendering the firing of the neuron of interest more independent (Cohen and Maunsell, 2009; Mitchell et al., 2009). Decorrelation of intra cortical noise is the vital electrophysiological phenomenon that mediates attention-related improvements in detection of relevant sensory information (Cohen and Maunsell, 2009). Stimulation of cholinergic signaling from the basal forebrain to sensory cortex (S1, A1 or V1) has now been definitively demonstrated to mediate decorrelation of neuronal activity (Chen et al., 2015; Goard and Dan, 2009; Kalmbach et al., 2012; Kalmbach and Waters, 2014; Kim et al., 2016; Pinto et al., 2013). Chen et al. (2015) identified the micro-circuitry that is likely responsible for the cholinergic control of desynchronization during visual attention tasks. Optogenetic stimulation of cholinergic signaling in V1 elicits IPSCs in PV+ interneurons and in pyramidal neurons receiving input from SOM+ interneurons. The cholinergic stimulation of SOM+ interneuron activity appears to be both necessary and sufficient to mediate desynchronization (Chen et al., 2015). Optogenetic inhibition of cholinergic neurons in the basal forebrain leads to increased synchronization of cortical neurons and decreased response reliability of cortical sensory neurons, again consistent with the idea that cholinergic signaling is both necessary and sufficient for mediating cortical correlates of attention (Pinto et al., 2013). ACh application in sensory cortices also improves the ability of the cortex to discriminate between stimuli and increases modularity of microcircuit activity by modulating the response to thalamic inputs, thereby seeming to facilitate the processing of specific stimuli (Runfeldt et al., 2014; Thiele et al., 2012). However, at least in auditory cortex, ACh actually seems to broaden tuning curves (Nelson and Mooney, 2016).

1.2a.vi. Summary

Recent studies delineate the multiple contributions of basal forebrain cholinergic signaling to the synaptic and circuit mechanisms engaged in attentional processing in PFC and sensory cortex (**Fig 1.2**). Although we don't yet know the mechanisms underlying the integration of these cortical processes of attention at the level of network interactions, the central role of BF cholinergic signaling in cue detection and attentional processing is becoming clear. **Figure 1.3** presents a schematic of potential cholinergic interactions with networks engaged in selective attention. In this schematic, task relevant information would be "filtered" by PFC and integrated with modality specific encoding in sensory cortices via the cholinergic basal forebrain relay. Cholinergic modulation of circuits within the sensory cortices induces decorrelation of intracortical noise, which contributes to increasing the signal to noise ratio and facilitating response reliability to behaviorally relevant stimuli. If

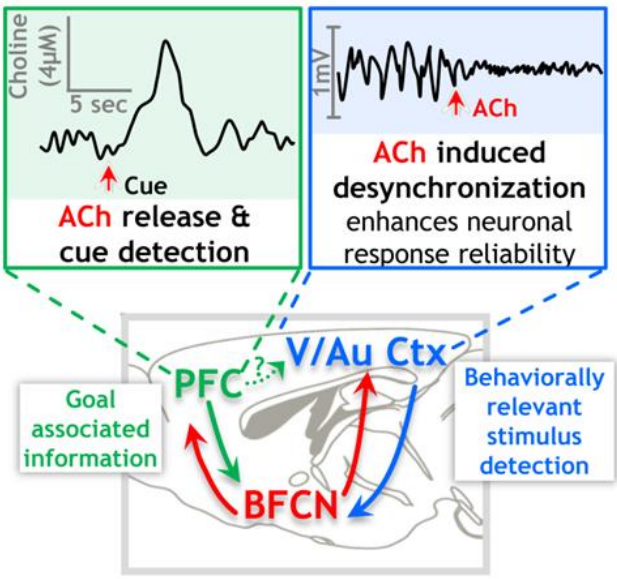


Figure 1.3: Schematic of BFCN interaction with attention related circuitry. Task oriented information from the PFC is transmitted to the basal forebrain, which signals to sensory cortex where cholinergic signaling causes decorrelation (Chen et al., 2015; Goard & Dan, 2009; Kalmbach et al., 2012; Kalmbach & Waters, 2014; Pinto et al., 2013; Runfeldt et al., 2014; Thiele et al., 2012; Kim et al., 2016) and enhances response reliability (Cohen & Maunsell, 2009; Mitchell et al. 2009). Once a task relevant stimulus is detected in the sensory cortex, cholinergic signaling from the basal forebrain to the PFC is stimulated and transient ACh release within the PFC signals cue detection (Sarter et al., 2016a; Parikh et al., 2007; Howe et al., 2013).

the PFC exerts top down control over sensory cortices during attentional performance, then the attention related activity changes observed in sensory areas may be due, at least in part, to indirect PFC signaling via cholinergic basal forebrain relays (Nguyen et al., 2015). Detection of a behaviorally relevant cue is then communicated back to the PFC by the sensory cortex, via circuitry including the cholinergic basal forebrain which triggers a transient release of ACh in the PFC, signaling cue detection to the animal.

There are, of course, several unresolved issues with this conceptual framework. In particular, the pathway(s) by which proposed PFC-stimulation influences sensory cortical activity remains to be directly demonstrated. In fact, the top down effects of PFC on visual cortex are only partially blocked by lesion of the cholinergic basal forebrain relay (Nguyen et al., 2015). In addition, although the enhanced signal to noise ratio in V1 is known to be mediated by cholinergic mechanisms of decorrelation, the mechanisms by which cholinergic signaling elicits increased excitability in V1 are less clear. Recent work by Nelson and Mooney (2016) in auditory cortex reveals that stimulating BF cholinergic input increased both excitation and inhibition via fast synaptic activation of nAChRs and that the net effect of BFCN input to auditory cortex is to broaden the bandwidth of individual neurons while restricting the dynamic range of the response strength by enhancing weak responses to non-preferred tones and attenuating strong responses to preferred tones among auditory cortex neurons.

More work is needed to resolve the contribution of cholinergic signaling to both the enhanced signal to noise ratio and to the increased excitability. Recent studies stressing the importance of M1 type receptors in mediating top down attention (Gould et al., 2015) and the considerable literature on AChR-mediated synaptic enhancement in PFC (Poorthuis et

al., 2013) will be important to pursue to better understand how cholinergic signaling participates in PFC/sensory cortical interactions.

Finally, it should be noted that I have focused on cholinergic mechanisms of "top down" attention. Nicotine administration enhances ERP indices of both top down and bottom up attention and cholinergic mediated decorrelation of neuronal activity in V1 increases response reliability of neurons under both goal oriented, trained paradigms as well as during observation of naturalistic movies (Goard and Dan, 2009; Knott et al., 2014; Pinto et al., 2013). Enhancing cholinergic tone may therefore increase signal to noise ratios and facilitate stimulus detection whether or not performance is goal-oriented. Clearly more work is needed to determine the contribution of basal forebrain cholinergic inputs in the modulation of bottom up attention. Genetic manipulation of different cholinergic receptors appears to differentially influence performance on top down vs bottom up attention, with M1 mAChRs and α7* nAChRs being particularly important for top down, while α4* nAChRs are implicated in both attentional streams (Gould et al., 2015; Guillem et al., 2011; Hyde et al., 2016).

1.2b. Cholinergic signaling & circuits related to memory

1.2b.i. Overview of ACh in mediating memory

Memory is perhaps the most complex of cognitive functions, engaging a multiplicity of brain regions and a vast array of circuit and synaptic mechanisms for the initial acquisition, short and long term storage, recall, and/or extinction of a single memory. The following discussion is limited to potential contributions of acetylcholine to memory encoded in only two brain regions. Specifically, I discuss recent work on the role of cholinergic signaling in spatial memory, which heavily engages the hippocampus, and in emotionally salient memories, with a focus on studies in the amygdala (Burgess et al., 2002; Janak and Tye, 2015).

1.2b.ii. Basal Forebrain Cholinergic regulation of Hippocampal circuits related to memory

Cholinergic signaling from the MS and DB to the hippocampus is certainly important for formation of spatial memories: ACh has consistently been shown by microdialysis to be elevated in the hippocampus during performance of various memory tasks (Mitsushima et al., 2013; Roland et al., 2014; Stanley et al., 2012), and numerous studies have probed the effects of exogenous – and more recently endogenous - ACh on hippocampal plasticity and performance in spatial memory tasks (Cobb and Davies, 2005; Kutlu and Gould, 2016). Blockade of mAChR signaling locally in the hippocampus impairs memory (Carli et al., 1997; Wallenstein and Vago, 2001). The potential role for nAChRs-mediated signaling in spatial memory and context-dependent conditioning is more complex with some recent studies showing nAChRs upregulated in the hippocampus following spatial memory task

acquisition (Kutlu and Gould, 2016; Shanmugasundaram et al., 2015; Subramaniyan et al., 2014), while other studies show that nicotine administration can variably enhance, depress or have little effect on short vs long term hippocampal based memories (Gould et al., 2015; Kutlu and Gould, 2016).

1.2b.ii.a. Cellular and Synaptic Mechanisms of ACh in the Hippocampus

Hippocampal circuits are renowned for their susceptibility to activity dependent synaptic plasticity, commonly considered a cellular substrate of memory. Recent work has demonstrated that cholinergic signaling, and specifically signaling via α7* nAChRs and M1-type mAChRs, plays an important role in long term potentiation (LTP) and plasticity at hippocampal synapses, providing a potential cellular level mechanism by which ACh may mediate memory (**Fig 1.2**; Cheng and Yakel, 2015; Gu et al., 2012; Gu and Yakel, 2011). Exogenously applied or endogenously released ACh induces significant changes in synaptic plasticity in the hippocampus in a manner that is precisely controlled by the timing between the activation of cholinergic signaling and the activation of glutamatergic inputs to CA1 (Gu et al., 2012; Gu and Yakel, 2011). Changing the temporal relationship between optogenetic stimulation of cholinergic signaling and electrical stimulation of the CA3 → CA1 input, switches the type of synaptic plasticity induced from LTP to short term depression (STD), indicating that temporally specific cholinergic signaling is extremely important in determining how information in the hippocampus is encoded (Gu et al., 2012). Cholinergic stimulation at 100 ms before CA3 → CA1 input elicited an LTP that was entirely dependent on α7* nAChRs, whereas if cholinergic stimulation occurred 10 ms *after* the CA3 → CA1 stimulation, the LTP that was elicited was blocked (only) by muscarinic antagonists (Gu and Yakel, 2011).

Nicotinic AChR-mediated LTP in hippocampus depends on α7* nAChR expression in *both* the pre and post synaptic neurons and is accompanied by long lasting increases in calcium signals in both CA3 and CA1 neurons (Gu et al., 2012). Cholinergic-facilitated-STD was accompanied by decreases in calcium currents (Gu et al., 2012). Taken together, these data are consistent with the idea that endogenous cholinergic signaling modulates CA3 → CA1 synaptic plasticity in the hippocampus via α7* nAChR triggered calcium signals (Gu et al., 2012) as well as via M1 mAChRs, presumably by inactivating SK channels (Buchanan et al., 2010; Gu and Yakel, 2011). α7* nAChR selective agonists have also been shown to potentiate transmission and strengthen synapses in the hippocampus independent of presynaptic activity (Cheng and Yakel, 2014). Last, but not least, recent reports describe an entirely new cellular mechanism for hippocampal LTP by M1-type mAChR enhancement of axonal excitability (Martinello et al., 2015).

Nicotinic AChR-dependent strengthening of hippocampal synapses can be mediated by stabilizing GluA1 receptors on dendritic spines, an effect which is dependent upon α7* nAChR expression at hippocampal synapses (Halff et al., 2014). Galvez et al. (2016)

studied the stabilization, rather than induction, of LTP and showed that that stabilization of LTP within the hippocampus is dependent upon cholinergic signaling, and specifically upon α7* nAChR signaling as well. Lesion of hippocampal cholinergic input or blockade of nAChRs rendered previously potentiated synapses vulnerable to "depotentiation" by low frequency stimulation. This effect was mediated by increased stabilization of f-actin and dendritic spines and independent of effects on AMPAR internalization. It therefore seems that nAChR signaling is critically important for both inducing and maintaining synaptic plasticity in the hippocampus. On the other hand, mAChR blockade prevents learning induced increases in AMPA/NMDA receptor ratio in the hippocampus, indicating an important role for mAChRs in mediating hippocampal synaptic plasticity (Mitsushima et al., 2013).

1.2b.ii.b. Circuit and Network Mechanisms of ACh in the Hippocampus

At the network level, the balance between gamma and theta band oscillations in hippocampal activity has been shown to be important for learning and memory (Duzel et al., 2010; Hasselmo and Stern, 2014). Cholinergic signaling can both induce these oscillations, and modulate their strength, perhaps providing an integrated electrophysiological substrate by which cholinergic signaling improves memory (Dannenberg et al., 2015; Douchamps et al., 2013; Lu and Henderson, 2010; Newman et al., 2013; Zhang et al., 2015).

Theta band oscillations are proposed to mediate the balance between encoding and retrieval of memory, with encoding occurring at theta peak and retrieval at theta trough (Hasselmo, 2014; Kunec et al., 2005). The separation between encoding and retrieval is considered vital for formation of accurate associations (Easton et al., 2012; Hasselmo and Stern, 2014; Kunec et al., 2005). In support of an essential role for cholinergic signaling in facilitating encoding, muscarinic blockade has been shown both to shift CA1 pyramidal cell firing towards theta trough (thus away from the encoding peak) during exploration of a novel environment and to impair the encoding of an experimental environment (Douchamps et al., 2013; Newman et al., 2014). Theta oscillations in CA3 are partially dependent on α7* nAChR signaling and theta oscillations in CA1 are completely abolished by knockout of the α7 gene (Lu and Henderson, 2010). Taken together, these studies indicate that both nAChR and mAChR signaling are important for induction and maintenance of theta oscillations as well as for memory encoding. Recent work has also shown that stimulation of cholinergic neurons in the MS led both to signaling via a direct, cholinergic basal forebrain - hippocampal projection and to the recruitment of an indirect, cholinergic to GABAergic basal forebrain to hippocampal pathway. The two pathways worked synergistically to maximize hippocampal firing synchrony with theta oscillations (Dannenberg et al., 2015).

Gamma oscillations, on the other hand, are thought to be an index of gating of information flow through the hippocampus: high frequency CA1 gamma is associated with gamma phase locking between CA1 and medial entorhinal cortex (MEC) and low frequency

CA1 gamma is associated with gamma locking between CA3 and CA1 (Colgin et al., 2009). As the MEC provides the majority of information input to CA1, and CA3 is a critical area for information storage, gamma oscillations may be another mechanism to separate encoding from retrieval. Systemic cholinergic blockade reduces theta - gamma locking in the MEC, and reduces encoding of an enclosure (Douchamps et al., 2013). Gamma oscillations in CA3 are dependent upon α4β2 nAChRs (Zhang et al., 2015).

1.2b.iii. Basal Forebrain Cholinergic regulation of Amygdala circuits related to memory

In contrast to hippocampal-dependent spatial memory, the consolidation of emotionally salient memories is mediated in large part by the amygdala, a subcortical limbic structure that receives a dense cholinergic projection (Janak and Tye, 2015; Woolf, 1991). Cholinergic signaling specifically within the amygdala is vital for encoding emotionally salient memories (**Fig 1.2**; Knox, 2016). Optogenetic stimulation of cholinergic signaling in the amygdala strengthens emotionally salient memories, and optogenetic inhibition weakens them: both nAChRs and mAChRs appear to be involved (Jiang et al., 2016). Stimulation of cholinergic signaling in the amygdala can induce LTP under the same conditions that strengthen memory retention *in vivo*, perhaps providing a mechanism by which ACh mediates the formation of emotional memories (Jiang et al., 2016). These findings are consistent with other work demonstrating that cholinergic signaling via α7* nAChRs is necessary to induce activity dependent LTP in amygdala - paralleling results from the hippocampus showing that some forms of cholinergic LTP were uniquely dependent on α7* nAChR receptor expression (Gu et al., 2012; Jiang et al., 2013).

Other recent studies have emphasized the importance of inhibitory effects of cholinergic signaling in the amygdala, perhaps pertaining to spike timing dependent LTD (Gu and Yakel, 2011; Unal et al., 2015). Unal et al. (2015) report that optogenetic stimulation of cholinergic signaling in the amygdala has a state dependent, and largely inhibitory, effect on pyramidal cell firing. They also showed that the effects of endogenous ACh release by optogenetics contrasted with pharmacological stimulation of cholinergic receptors, which resulted in long lasting depolarization of pyramidal cells.

Taken together the optogenetic studies of cholinergic signaling in the amygdala and hippocampal circuits highlight a few key points. First, it appears that the effects of endogenous ACh release may not be adequately modeled by the application of exogenous agonists. Second, cholinergic signaling is exquisitely time sensitive and temporally specific: subtle differences in ACh stimulation paradigms can yield very different circuit effects. As such it is especially important that going forward we focus on defining stimulation paradigms for the examination of cholinergic signaling that are as behaviorally and physiologically relevant as possible. Such an approach will facilitate moving the field from

demonstrating what ACh *can* do to what ACh *does* do in subserving cognitive task performance.

1.2b. iv. Summary

The most consistent finding in circuit level studies of memory is that endogenous release of ACh, likely acting via both nicotinic and muscarinic AChRs, plays an important role in the induction of LTP, a synaptic substrate of memory. In the amygdala this effect appears to mediate the retention of emotional memories. In the hippocampus cholinergic signaling both facilitates LTP and modulates cognition associated oscillatory activity. Theta rhythm phase can both modulate the likelihood that LTP is induced and determine whether stimulation will induce synaptic potentiation or depression (Hasselmo and Stern, 2014). Oscillations within the hippocampus seem to signal separation of encoding from retrieval processes, a distinction that is essential for memory as the status of these oscillations at the beginning of a behavioral task predicts learning success (Backus et al., 2016). Overall, recent work reinforces the idea that specific patterns of cholinergic signaling in memory related brain regions plays important roles in state dependent optimization of learning and memory. Another exciting new avenue for exploration in the modulation of hippocampal based memory not discussed above is the influence of cholinergic signaling on newborn hippocampal neurons.

Many of the recent studies that have demonstrated a central role of cholinergic signaling in both attentional and memory related circuits and behaviors have been made possible by the advent of optogenetics for the selective stimulation of BF cholinergic neurons and their terminal fields (Jiang et al., 2014; Luchicchi et al., 2014). An additional benefit of the technology is its illumination (pun intended) of the extent to which many transmitters – including ACh - are co-stored and, under some stimulation conditions, may be co-released with other transmitters (e.g. GABA, glutamate(Granger et al., 2016). Of course such issues are readily addressed by combining a bit of modern pharmacology with the optogenetics, as has been done in the reports summarized here on the role of endogenous ACh signaling in attention and learning.

1.3. CORTICAL MEMORY: A FRONTIER OF COGNITIVE RESEARCH

An emerging frontier for the investigation of cholinergic mechanisms of cognition is the study of cortical memory. There are several memory tasks that critically engage cholinergic and neocortical circuits: one of the best studied is recognition memory (Brown and Banks, 2015). ACh seems to be vital to this aspect of cognition, as systemic impairment of cholinergic signaling either via lesion, pharmacological, or genetic knock down impairs recognition memory (De Jaeger et al., 2013). However, the mechanisms by which cholinergic signaling participates in this aspect of cognition are not well understood.

1.3a. The perirhinal cortex

Recognition memory is thought be mediated by a part of the brain known as the perirhinal cortex (PRH; for review see Banks et al., 2012; Brown and Banks, 2015). The perirhinal cortex is an allocortex that is evolutionarily and developmentally between the archeocortex of the Hippocampus and the neocortex. It is laminated but does not contain a prominent layer 4 (Beaudin et al., 2013; Ranganath and Ritchey, 2012). It is also an associative cortex that is ideally positioned within the brain to serve as a mediator between neocortex and hippocampus (Kealy and Commins, 2011). Indeed, it seems to serve a gating function between neocortical information and the hippocampus (de Curtis and Pare, 2004). It has extensive connections to subcortical and neocortical structures (Kealy and Commins, 2011; Ranganath and Ritchey, 2012). It receives input from many sensory cortices, and has prominent reciprocal connections with the temporal association area and the amygdala (Kealy and Commins, 2011; Ranganath and Ritchey, 2012).

1.3b. Perirhinal cortex cholinergic signaling in recognition memory

Cholinergic signaling within the PRH *per se* seems vital to performance of its cognitive functions, as cholinergic lesion or blockade selectively within the perirhinal cortex impairs recognition memory (Brown and Banks, 2015; Dere et al., 2007; Jacklin et al., 2015). However the mechanism by which cholinergic signaling mediates this form of memory is not well understood.

The aim of this thesis is to elucidate the role of PRH cholinergic signaling in recognition memory by investigating the effects of endogenous ACh release in the PRH. Additionally I will probe the electrophysiological and molecular deficits that underlie a recognition memory deficit in an animal model of cognitive impairment to better understand the mechanisms by which ACh encodes recognition memory.

Chapter 2:

Cholinergic mechanisms of recognition memory encoding in perirhinal cortex

INTRODUCTION

Recognition memory is a form of declarative memory that is disrupted in many animal models of human intellectual disability (Dodart et al., 2000; Dodart et al., 1999; Polydoro et al., 2009; Stearns et al., 2007; Ventura et al., 2004). It therefore appears to be an important aspect of human cognition, however the mechanisms by which it is encoded are not completely understood. Lesion studies have shown that recognition, especially of physical objects or visual stimuli, is mediated by the perirhinal cortex (PRH; for review see Brown and Banks, 2015; Banks et al., 2012; Ranganath and Ritchey, 2012). Mapping of neuronal activity in rats has shown that the number of cells activated in the PRH is modulated by the relative familiarity or novelty of stimuli that they encounter (for review see (Aggleton et al., 2012);(Albasser et al., 2010; Warburton et al., 2003; Zhu et al., 1995). Specifically, the population of PRH cells activated by exposure to a familiar object is reduced when compared to a novel object. *In vivo* recordings in rats and primates have shown that individual cells within the PRH that exhibit an initial robust increase in firing rate when exposed to a novel visual stimulus have an attenuated response when they are re-exposed to the same, now familiar, stimulus (Brown et al., 1987; von Linstow Roloff et al., 2016; Zhu and Brown, 1995). This reduction in excitability of specific "familiarity-sensitive" neurons within the PRH is thought to signal familiarity and encode recognition (Brown and Aggleton, 2001).

"Familiarity-sensitive" neurons that exhibit modulated firing in response to familiar as compared to novel objects have also been identified in the basal forebrain, which sends a cholinergic projection to the PRH (Kondo and Zaborszky, 2016; Wilson and Rolls, 1990; Woolf, 1991). Indeed, cholinergic signaling within the PRH seems to be vital to encoding recognition memory, as lesion of the basal forebrain or of cholinergic projections selectively in the PRH

impairs recognition memory performance (Nimmrich et al., 2008; Okada et al., 2015; Turchi et al., 2005; Winters and Bussey, 2005). More reversible cholinergic impairments that pharmacologically block cholinergic signaling in the PRH also impair recognition memory and have further clarified that both nicotinic and muscarinic receptors play a role (Abe and Iwasaki, 2001; Bartko et al., 2014; Tinsley et al., 2011; Warburton et al., 2003; Winters et al., 2006). Cholinergic blockade also prevents the familiarity-induced reduction of PRH population activity that is thought to encode recognition (Warburton et al., 2003). The reduction-response of familiarity-sensitive PRH neurons is thought to be mediated by long term depression (LTD), and e*x vivo* electrophysiological recordings in acute brain slices have shown that long term depression in the PRH is dependent on cholinergic signaling (Banks et al., 2012; Bogacz and Brown, 2003; Brown and Banks, 2015; Massey et al., 2001; Warburton et al., 2003). This supports the idea that acetylcholine-mediated LTD may underlie the reduction in activity that encodes recognition. However, the effect of endogenous acetylcholine (ACh) release in the PRH *in vivo* is not known. Similarly, the mechanism by which recognition memory is encoded in the PRH in mice has not been studied. As recognition memory is commonly used in mice to screen new pharmacological agents for cognitive efficacy (for example: Hill et al., 2017; Iwuagwu et al., 2017; Feuerbach et al., 2015; Bertaina-Anglade et al., 2006) and to phenotype mouse models of human disease (Silverman et al., 2010; also see Stearn et al., 2007; Polydoro et al., 2009; Ventura et al., 2004; Dodart et al., 1999; Dodart et al., 2000) it is imperative to fully understand the encoding mechanisms underlying this type of memory in this species.

Here I have conducted the first quantification of PRH cell activation after exposure to novel and familiar objects in mice. Further, I have investigated the effect of release of endogenous ACh, a crucial neurotransmitter for cognition, and specifically for recognition memory, in the PRH. My results will enhance understanding of the mechanisms of encoding of this integral aspect of cognition.

METHODS

Animals

For brain activity mapping experiments transgenic mice expressing a destabilized GFP under control of the *c fos* promoter maintained on a C57 background (*c-fos*-GFP; Jackson Lab stock #018306) were crossed with mice on a 129/C57 F1 background. Male mice from the F1 generation of this cross were used for experiments. For electrophysiological experiments transgenic mice expressing Cre recombinase under control of the *ChAT* promoter (*ChAT*-Cre; Jackson Lab stock #006410) maintained on a C57 background were crossed with mice maintained on a 129 background. Male mice from the F1 and F1b generations of this cross were used for experiments. All mice were maintained on a reversed 12 hour light cycle and allowed food and water *ad libitum*. Mice were either pair or group housed when possible. No singly housed mice were used for behavioral experiments.

Recognition memory training

Mice were aged to 15 weeks and then underwent recognition memory testing (Bevins and Besheer, 2006). The behavioral apparatus consisted of a dark room and table on which were placed two 25x40cm rectangular cages painted matte white with IR transparent lids. One cage was used as a staging arena and the other cage was used as the behavioral arena. A small plastic platform was used to transfer mice from their home cage to the behavioral apparatus. On day 1 mice were handled by the experimenter and habituated to the transfer platform. On days 2 and 3 mice were placed in the staging arena and then the behavior arena for 5 minutes each. On day 4 two identical objects were placed in the behavioral arena and mice were placed in the staging arena for 5 minutes and then in the behavioral arena with the two identical objects for 5 minutes. On day 5 one of the objects was replaced and mice in the "novel object" group were placed in the staging arena for 5 minutes and then the behavior arena with the two mismatched objects for 5 minutes. On day 5 mice in the "familiar object" group were re-exposed to the matched pair of objects from day 4. On days 4 and 5 mice in the "arena control" group were placed in the staging arena for 5 minutes and in the empty behavioral arena for 5 minutes. Behavior on days 4 and 5 was recorded with an IR sensitive video camera.

Behavioral coding

Behavior was coded offline using JWatcher and time spent exploring each object was quantified. The following behaviors were considered "exploration": whisking the object, biting the object, touching the object, nose oriented towards and within 2cm of the object.

Sample preparation for light and confocal microscopy

Between 150 and 180 minutes following entrance into the behavioral arena on day 5 mice were anesthetized with a 9:1 mixture of ketamine and xylazine and transcardially perfused with 1x PBS followed by 4% PFA. Brains were removed and post fixed overnight in 4% PFA before being sucrose equilibrated and frozen in OCT. Brains were then cryosectioned (Leica Biosystems Inc., Buffalo Grove, IL) at 50 micron thickness. Brain slices were blocked and permeabilized for 30 minutes in 1x PBS with 5% donkey serum and 0.1% Triton X100 and then incubated for 90 minutes in a 1:200 dilution (in blocking/permeabilization solution) of the NeuroTrace blue fluorescent Nissl stain (Thermo Fisher Scientific, Waltham, MA) followed by 3 five minute washes in 1x PBS. Slices were then dehydrated for 5 minutes in 70% ethanol and then incubated in the Autofluorescence Eliminator Reagent (EMD Millipore, Billerica, MA) for 5 minutes, followed by 3 one minute washes in 70% ethanol. Slices were then rehydrated with 1x PBS and coverslipped with DAPI Fluoromount-G (Southern Biotech, Birmingham, AL).

Imaging

Nissl staining of brain slices was imaged on a stereoscope (Zeiss, Oberkochen, Germany). *C fos* GFP and DAPI staining were imaged on a confocal microscope (Olympus, Center Valley, PA) with a 40x objective at a z step of 5 microns.

Quantifying cells activated by behavior

Images of the Nissl and *c fos* GFP staining for each brain slice were aligned using the Align Image plugin for Image J. Nissl staining was used to histologically identify the perirhinal cortex and its constituent layers as per Beaudin et al., 2013. Cells were considered c -fos positive if they stained both for GFP and DAPI. Cells were counted using the Cell Counter plugin for ImageJ. Analyzed brain slices were at intervals of 400 microns from each other and spanned the entire anterior-posterior extent of the PRH (approx. Bregma -1.34 to Bregma -4.04).

Viral injection

Mice were aged to at least 11 weeks and were then anesthetized with isoflurane and mounted on a stereotaxic frame (Kopf Instruments) with a heated stage. An incision in the scalp was made and a small hole was drilled in the skull above the left NBM (coordinates from Bregma: A/P: -0.7mm M/L: 1.7mm Z: -4.0mm). 0.5 µL of either AAV9-Ef1a-DIO-ChETA-eYFP or AAV9-CAG-DIO-oChIEF-tdTomato was injected using a micro syringe (Hamilton, Reno, NV). Mice were given Ketorolac (6mg/kg) as needed for 2-3 days following surgery.

Electrophysiological recording

At least 3 weeks after viral injection mice were anesthetized with isoflurane and placed on a surgical stereotax (Kopf instruments) with a heated stage. Craniotomies over the left perirhinal cortex and the left NBM were performed. A 1 MΩ parylene-C insulated tungsten electrode (AM systems, Sequim, WA) and a 200 µm optical fiber (Thorlabs, Newton, NJ) coupled to a 473nm laser (Shanghai Dream Lasers Technology) were positioned into the NBM while a tungsten electrode of either 1 MΩ or 5 MΩ (AM systems, Sequim, WA) was positioned into the posterior PRH (coordinates from Bregma: A/P: -3.25mm, z: -3.35mm to -3.85mm, M/L: from temporal ridge: -200 to +500 microns). Extracellular recordings from both sites were pre amplified by separate head stages and then fed to an amplifier.

Signals were acquired at a sampling rate of 40KHz and band pass filtered at 100-1000Hz by an amplifier (AM systems, Sequim, WA) before being passed through a Humbug Noise Eliminator (AM systems, Sequim, WA) and fed through a Tektronix TDS 2014B oscilloscope to a CED 1401 data board to the computer where they were visualized and collected using Spike 2 software (CED, Cambridge, UK). Laser stimuli used for optical stimulation of cholinergic neurons consisted of 20 laser pulses of 1ms duration delivered at a frequency of 10Hz.

Relocalization of recording site

At the end of each recording session an electrolytic lesion was created by passing 100μA of current for 45 seconds to facilitate relocalization of the recording site. The mouse was then perfused and brain slices obtained as above.

For a subset of mice, slices containing the perirhinal cortex were stained using NeuroTrace (Thermo Fisher Scientific, Waltham, MA) blue fluorescent Nissl stain as above. For all mice slices containing the NBM and the PRH were imaged on a stereoscope (Zeiss, Oberkochen, Germany) to visualize the recording sites and confirm viral expression.

Electrophysiological data analysis

Extracellular recordings were sorted offline using the Offline Sorter (Plexon Inc., Dallas, TX). Features of the waveforms were extracted and individual units were demarcated by manually identifying clusters of waveforms in a 2 dimensional feature space of spike properties (Gray et al., 1995). The quality of each sort was rated according to isolation distance between clusters. Only recordings of high sort quality, with less than 5% overlap with other clusters, were used for further analysis. Units with firing rates lower than 0.05Hz were excluded from further analysis.

Statistics

Single unit recordings were then further analyzed using Matlab. To determine if a unit exhibited a change in firing pattern as a result of optogenetic stimulation a permutation test of the F statistic with 10,000 permutations was used to compare inter spike intervals occurring in the 140s immediately before and after stimulation. To identify delayed responders and to characterize the length of individual responses the same analysis was performed comparing the 140s immediately before stimulation and a sliding 140s window following stimulation. Each slide step was 70s. A response was detected if the resultant *p* value from these permutation tests was less than 0.05. A unit was considered to exhibit a "laser locked" response if a response was detected in the first window following stimulation. A unit was considered to exhibit a "delayed" response if a response was detected at any point in the first 560s following stimulation.

Waveform properties of each unit were calculated from the average waveform using the Field Trip toolbox for Matlab (Oostenveld et al., 2011).

Variability of firing was measured using the Fano Factor (FF=variance/mean) of inter spike intervals. Percent change in firing rate and Fano Factor were calculated using the following formula:

$[(FF \text{ or } FR)_{baseline}-(FF \text{ or } FR)_{response\ onset}]/(FF \text{ or } FR)_{baseline}$

Baseline firing rate and variability of firing were compared using the Kruskal Wallis test.

K means cluster analysis was conducted using Matlab.

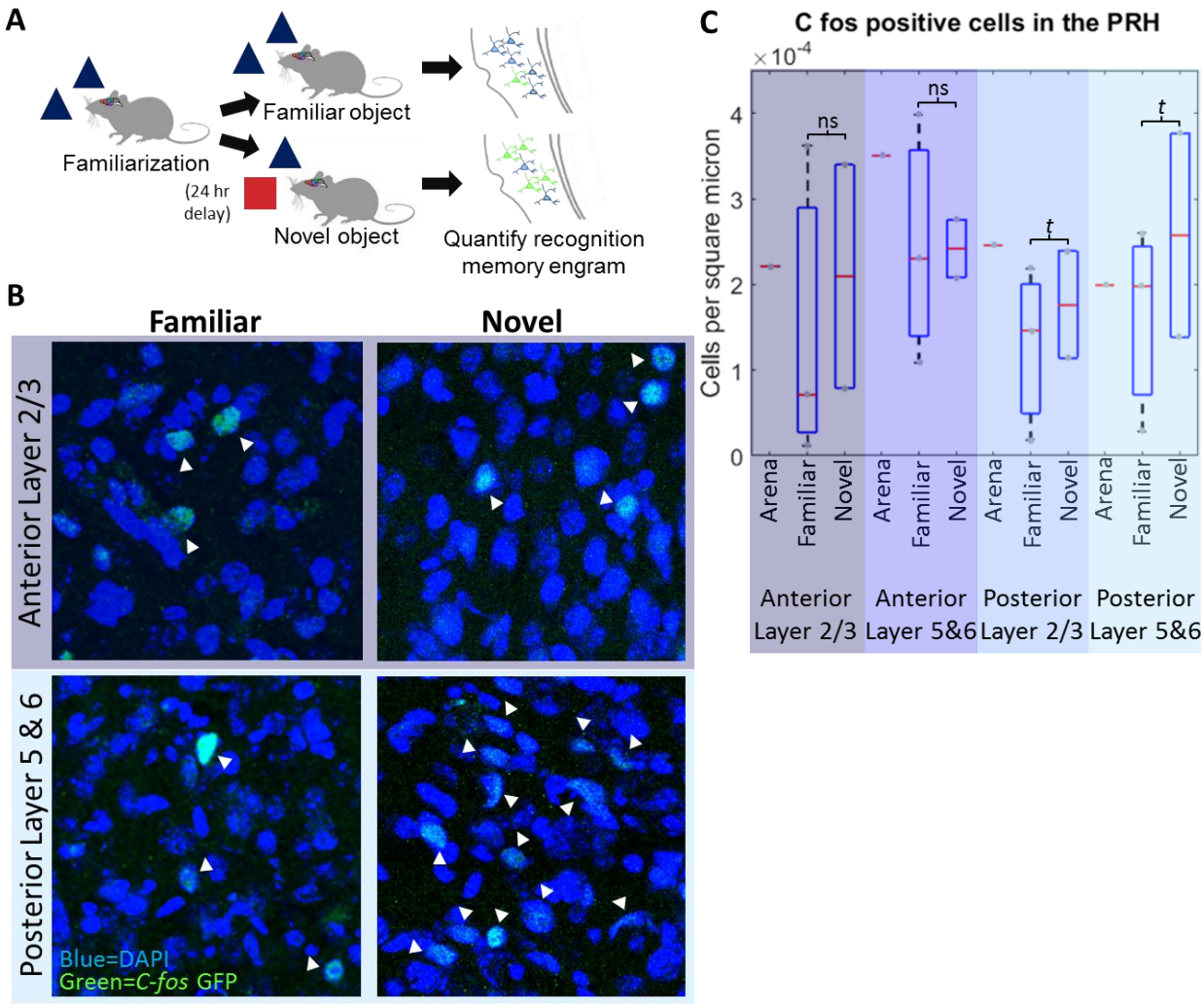


Figure 2.1: Mapping recognition memory related neural activity. A: Diagram of experimental procedure: c-fos GFP mice are first familiarized to a pair of identical objects. Following a 24 hour delay a subset of the mice are re-exposed to the pair of identical familiar objects and a subset of mice are exposed to a novel object. The mice are then sacrificed and the active neurons in the PRH are quantified. **B:** Representative images showing fluorescent labeling of activated neurons in layer 2/3 of the anterior portion of the PRH and from layers 5 & 6 of the posterior portion of the PRH from mice who were exposed to the pair of familiar objects and from mice who were exposed to the novel object. **C:** Quantification of cells activated by exposure to novel or familiar objects separated by position within the PRH. Neural activity in layers 5 & 6 of the posterior PRH showed the greatest degree of behavioral modulation. (Anterior layer 2/3: Kruskal Wallis, $\chi^2(2)=0.43$, $p=0.81$; Anterior layer 5&6: Kruskal Wallis, $\chi^2(2)=0.81$, $p=0.67$; Posterior layer 2/3: Kruskal Wallis, $\chi^2(2)=2.38$, $p=0.30$; Posterior layer 5&6: Kruskal Wallis, $\chi^2(2)=0.43$, $p=0.81$) ns = not statistically significant, *t* = trending

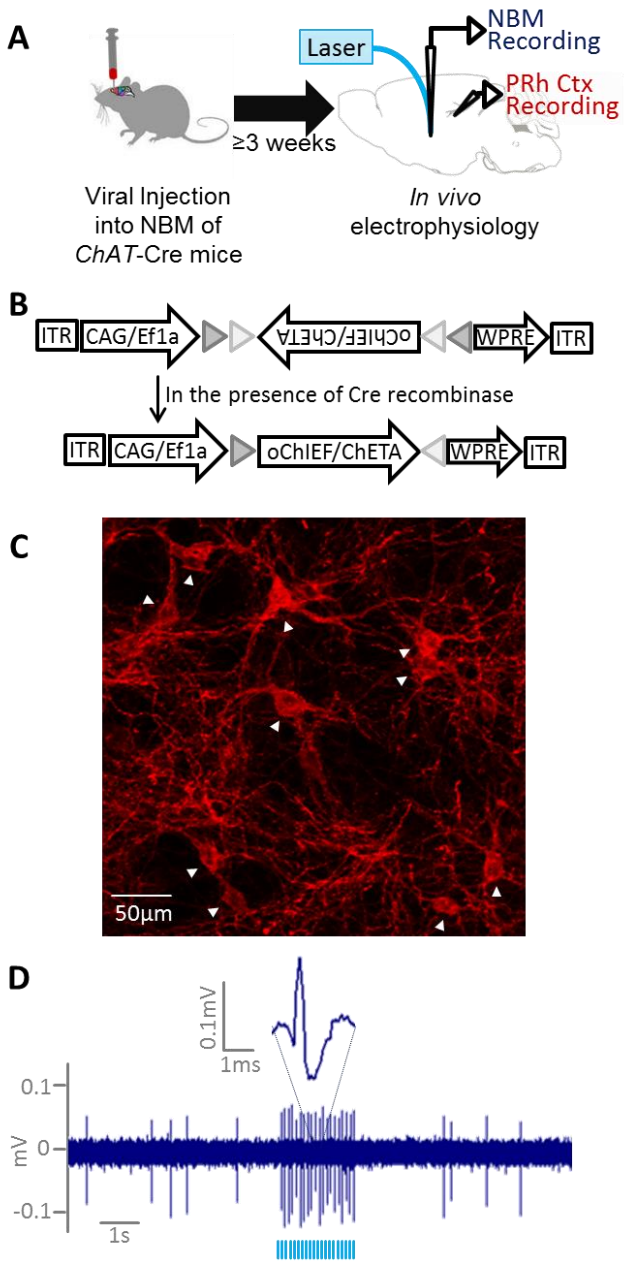


Figure 2.2: Strategy for optogenetic stimulation of cholinergic neurons in the NBM. A: Diagram of the experimental protocol: a viral vector encoding a light activated excitatory ion channel is injected into the NBM of a mouse expressing Cre recombinase under control of the *ChAT* promoter. *In vivo* recordings from the NBM and the Perirhinal Cortex are later collected. **B:**. The viral vector is of a flip excision switch design such that it will be expressed only in the presence of Cre recombinase. **C:** Representative viral labeling of cholinergic neurons (indicated by arrowheads) in the NBM of a *ChAT* Cre mouse. **D:** Sample *in vivo* recording from the NBM with laser evoked action potentials is shown. The timing of laser pulses in the NBM are indicated by light blue hash marks.

RESULTS

Mapping recognition memory related neural activity

PRH activity patterns associated with recognition memory have been studied in great detail in rats, however little is known about these patterns in mice. In order to investigate how and where recognition memory is encoded in mice, I used a transgenic mouse line in which activated cells were labeled with GFP (*c-fos* GFP). After being familiarized to an object, a subset of these mice were re-exposed to the familiar object and a subset were exposed to a novel object. I then quantified the number of cells in the PRH that was activated by each of these experiences **(fig 2.1A**). As seen in **figure 2.1C** neural activity was modulated by behavior in posterior portions of the PRH only, where the number of cells activated by interaction with a novel object was higher than the number of cells activated by interaction with a familiar object. This behavior dependent difference in activity was most pronounced in layers 5 & 6 of posterior PRH and was less pronounced in anterior portions of the PRH (**fig 2.1B&C**).

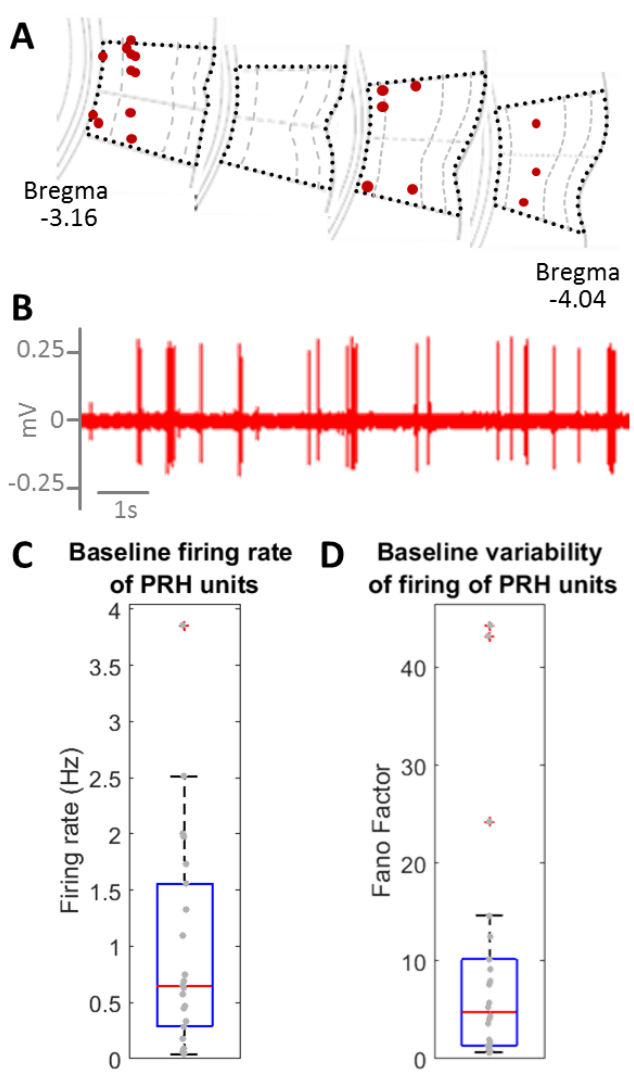


Figure 2.3: Perirhinal cortex units have low firing rates and high variability of firing. A: Schematic of recording sites within the PRH. Recordings were collected from layers 5 & 6 in the posterior portion of the PRH. **B:** Representative raw data from a PRH unit. **C:** Box plot of baseline firing rates for all recorded PRH units (mean: 0.97Hz ± 0.97). **D:** Box plot of baseline Fano Factor of all recorded PRH units (mean: 9.36 ± 12.50).

Optogenetic stimulation of NBM cholinergic neurons

As ACh is known to be vital for successful encoding of recognition memory, I next asked what endogenous ACh actually does in areas important for encoding, namely layers 5 & 6 of the posterior PRH. The mouse PRH is not thought to contain intrinsic cholinergic neurons. It instead receives the majority of its cholinergic innervation from projection neurons in the NBM, with some contribution from the Diagonal Band of Broca (Kondo and Zaborszky, 2016; Woolf, 1991). In order to study the effect of endogenous ACh release in the PRH I used an optogenetic approach to selectively stimulate cholinergic neurons in the NBM (**fig 2.2A**; Zhang et al., 2010). I injected a viral vector encoding the inverted open reading frame of a light activated ion channel into the NBM of mice expressing Cre recombinase in cholinergic neurons (**fig 2.2A&B**). The viral vector was designed such that it would be expressed only in the presence of Cre (**fig 2.2B&C**). Thus I was able to selectively target cholinergic neurons and evoke action potentials from them using laser light (**fig 2.2C&D**).

PRH recording

Concurrent with optogenetic stimulation of NBM cholinergic neurons, I collected extracellular recordings from PRH units (**fig 2.2A, 2.3B**). Recordings were from layers 5 & 6 of the posterior section of the PRH, as this is the region that appears to be particularly relevant for recognition memory encoding (**fig 2.3A**). These units exhibited a low firing rate (n=22, mean: 0.97Hz ± 0.97; **fig 2.3C**) and high variability of firing, as measured by the Fano Factor (n=22, mean: 9.36 ±12.50; **fig 2.3D**).

PRH response to optogenetic stimulation of cholinergic input

Upon stimulation of cholinergic input from the NBM to the PRH, a subset of PRH units exhibited a change in variance of inter spike interval as shown in **figure 2.4B**. Responsive units exhibited a change in variance following optical stimulation that was greater than 95% of 10,000 random permutations of the data (**fig 2.4B**). Non responsive units exhibited a change in variance following optical stimulation that was not greater than that generated by random permutations of the data (**fig 2.4A**). 59.1% (13/22) of PRH units exhibited a response to optical stimulation of cholinergic input while 40.9% (9/22) exhibited no detectable response. 22.7% (5/22) of PRH units exhibited a response that was time locked to optical stimulation of cholinergic input while 36.3% (8/22) of PRH units exhibited a delayed response (**fig 2.4C&D**).

Characterization of the PRH response to stimulation of cholinergic input

To determine how ACh encodes recognition memory in the PRH, I next asked what effect ACh signaling had on responsive PRH neurons. Almost all responsive units exhibited an increase in firing rate (10/13) and a decrease in variability of firing (9/13) in response to optical stimulation of cholinergic input (**fig 2.5A&B**).

To determine over what time frame cholinergic induced changes in PRH firing might support recognition memory encoding, I measured the duration of the observed responses. PRH responses to stimulation of cholinergic input varied in length: the shortest detected response lasted only 70s while the longest detected response lasted over 20 minutes (**fig 2.5C**). The average length of sustained response was 309.2s (SD: 335.6s, n=13). Laser locked responses tended to be longer lasting (mean: 518s±465.9s; range: 70s-1120s) than delayed responses (mean: 201.3s±114.9s; range: 70s-420s; **fig 2.S1**).

Comparing properties of non responsive vs responsive units

I next asked if baseline characteristics of a PRH unit predicted whether it would respond to stimulation of cholinergic input or not. At baseline, units that exhibited a laser locked response to optical stimulation of cholinergic input had the highest firing rates (mean: 1.63Hz±1.61), followed by units that exhibited delayed responses (mean: 1.08Hz±0.57) and finally units with no detectable response (mean: 0.50Hz±0.60), although these differences were not statistically significant (Kruskal Wallis, $\chi^2(2)=4.61$, p=0.0998; **fig 2.6A**).

There was a significant difference between responder groups on baseline variability of firing as measured by the Fano Factor: Kruskal Wallis, $\chi^2(2)=6.75$, $p=0.03$. Post hoc comparisons revealed that non responsive units had a significantly higher mean rank (mean rank:

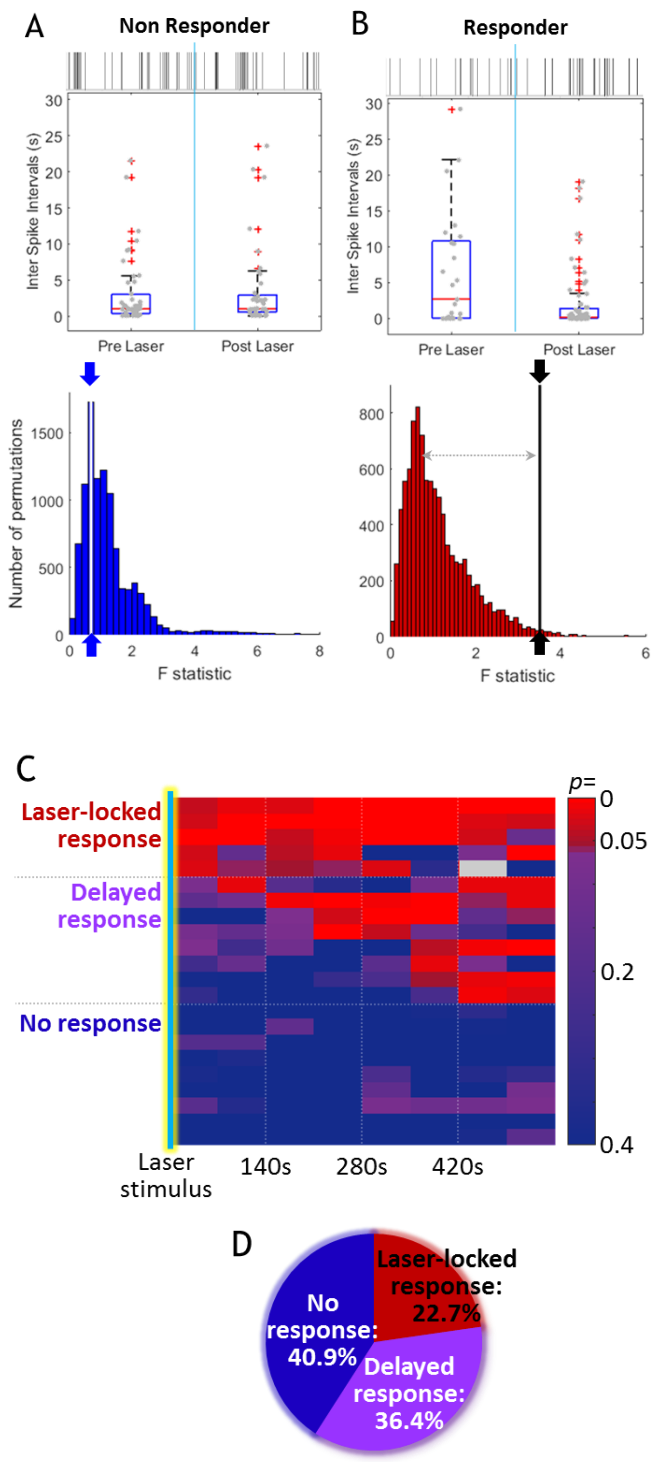


Figure 2.4: **PRH unit responses to stimulation of cholinergic input. A,B:** Raster plot, (top) box plot of inter spike intervals (ISI, middle), and distribution of the F statistic generated by randomly shuffling ISI's 10,000 times (bottom) for a representative unit exhibiting no response to stimulation of cholinergic input (**A**) and a representative unit exhibiting a response to stimulation of cholinergic input (**B**). The timing of optical stimulation is represented by a vertical blue bar. The F statistic value for the non-shuffled, laser centered data is indicated by a vertical line and arrows. A response was detected when the experimental value was greater than 95% of the randomly generated values (gray dotted line). **C:** Heat map of *p* values of permutation F test obtained for each PRH unit as a function of time since optical stimulation. **D:** Pie chart showing the percentage of PRH units that exhibit responses time locked to laser stimulus, the percentage of units exhibiting delayed responses to laser stimulation of cholinergic input and the percentage of units exhibiting no response to stimulation of cholinergic input.

15.8; mean FF: 15.98±16.14) than units with a delayed response (mean rank: 8; mean FF: 3.12±2.95). Units with a laser locked response (mean rank: 9.8; mean FF: 7.45±10.19) were not significantly different from either of the other groups (**fig 2.6B**).

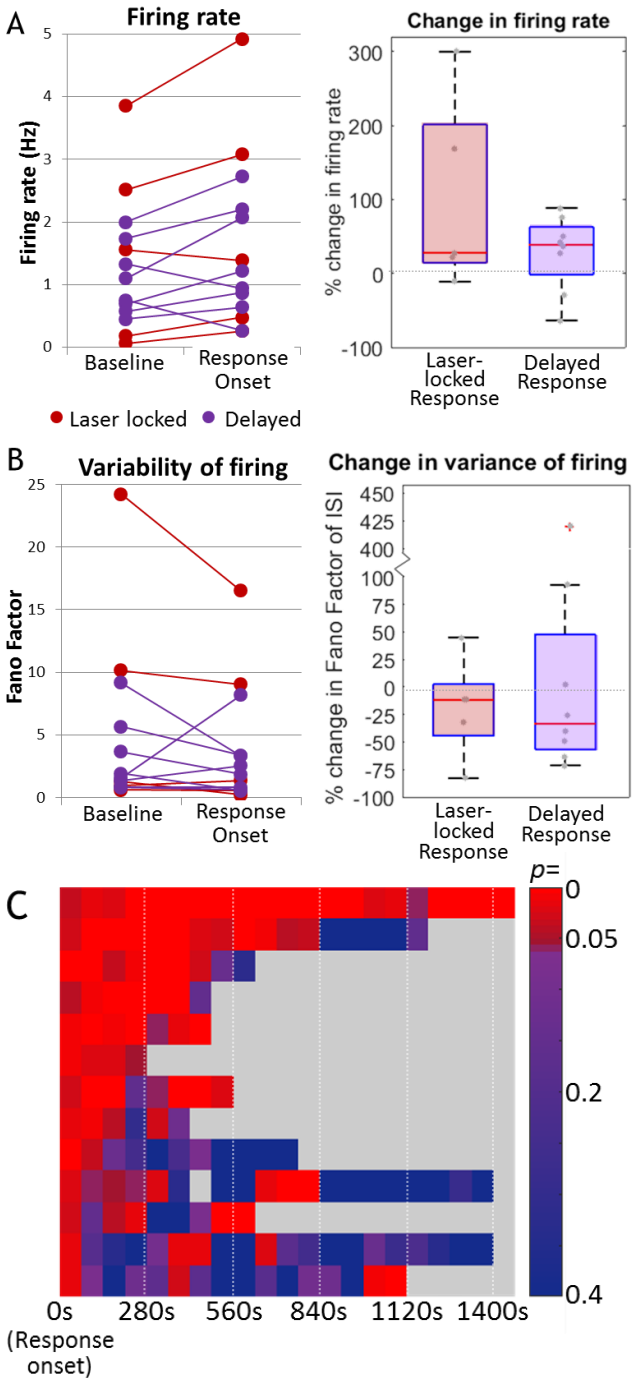


Figure 2.5: PRH units increase firing rate and decrease firing variability in response to stimulation of cholinergic input. A: Line plot of firing rate obtained for each responsive unit at baseline and during the first analysis bin in which the unit showed a significant response to cholinergic stimulation (left) and box plot of the percent change in firing rate (right). The vast majority of responsive units exhibit an increase in firing rate. **B:** Line plot of Fano Factor obtained for each responsive unit at baseline and during the first analysis bin in which the unit showed a significant response to cholinergic stimulation (left) and box plot of the percent change in Fano Factor (right). The vast majority of responsive units exhibit a decrease in variability of firing. **C:** Heat map of *p* values of Permutation F test obtained for each responsive unit aligned to the onset of the response to demonstrate duration of the response. Responses ranged from a length of 70s to 1,120s (mean: 309.17s ± 335.65).

I next asked if PRH units that responded to stimulation of cholinergic input were interneurons or pyramidal neurons. In *in vivo* extracellular recording, putative interneurons may be identified and separated from putative pyramidal neurons based on their short waveforms and high firing rates (Mitchell 2007). Three of the units with laser locked responses had very short wavelengths and relatively high firing rates, consistent with the profile of an interneuron (**fig 2.6C**). However this was not identified as an independent cluster by K means cluster analysis (**fig 2.S2**) and all of the other responders had waveform lengths similar to non responsive units.

Finally I asked if units that responded to stimulation of cholinergic input were unique in terms of their anatomical location within the PRH. As seen in **figure 2.6D**, responsive units were distributed throughout the posterior portion of the PRH. 8 responders were located in Brodmans area 36 (dorsal PRH) and 5 responders were located in Brodmans area 35 (ventral PRH).

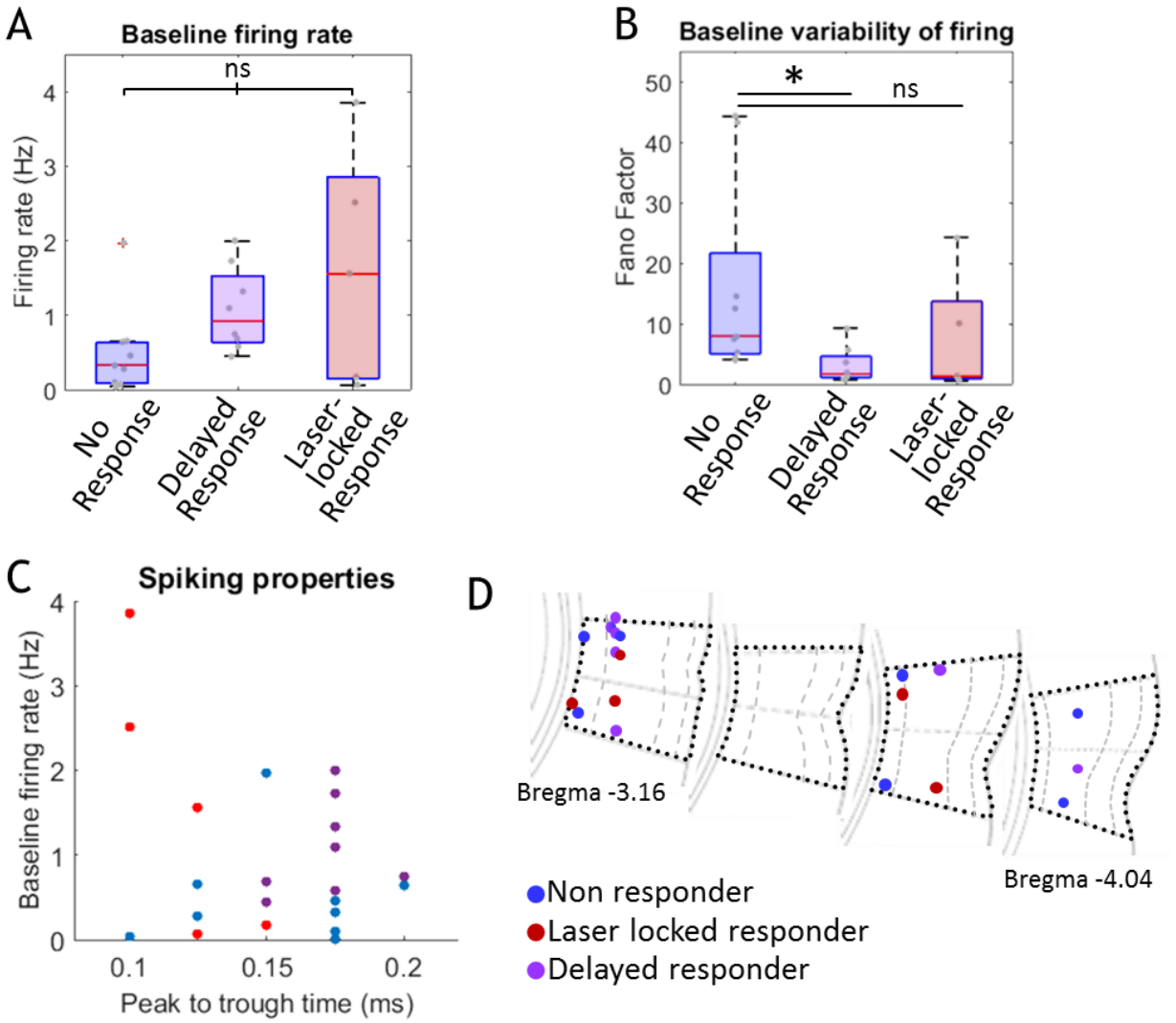
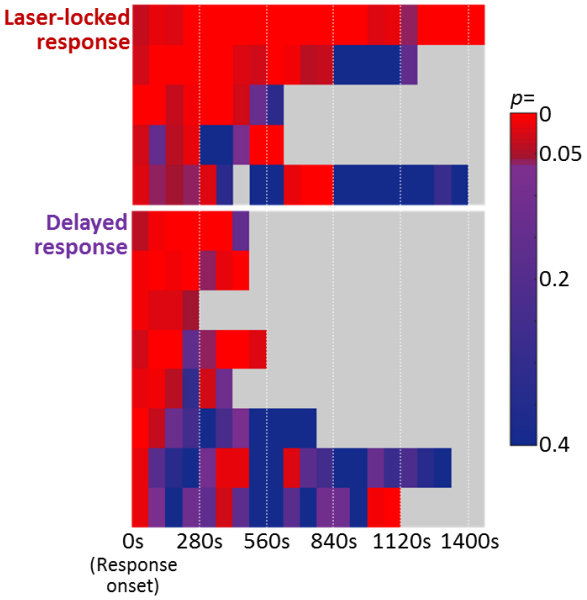


Figure 2.6: Characterization of responsive vs non responsive units. A: Box plot of baseline firing rates for non responsive units, units with a delayed response and units with a laser locked response. Units with laser locked responses had the highest firing rates at baseline (mean: 1.63Hz ± 1.61), followed by units with delayed responses (mean: 1.08Hz ± 0.57) and finally non responsive units had the lowest firing rates (mean: 0.50Hz ± 0.60). However, these differences were not statistically significant (Kruskal Wallis, $\chi^2(2)=4.61$, $p=0.0998$). **B:** There was a significant difference between groups on baseline variability of firing as measured by Fano Factor (Kruskal Wallis, $\chi^2(2)=6.75$, $p=0.03$). Post hoc comparisons revealed that non responsive units had a significantly higher mean rank (mean rank: 15.8) than units with a delayed response (mean rank: 8). Units with a laser locked response (mean rank: 9.8) were not significantly different from either of the other groups. **C:** Scatter plot of baseline firing rate as a function of peak to trough time did not reveal any significant clusters. **D:** Diagram of the anatomical locations of PRH units with no, delayed or laser locked responses to stimulation of cholinergic input.

2.S1



2.S2

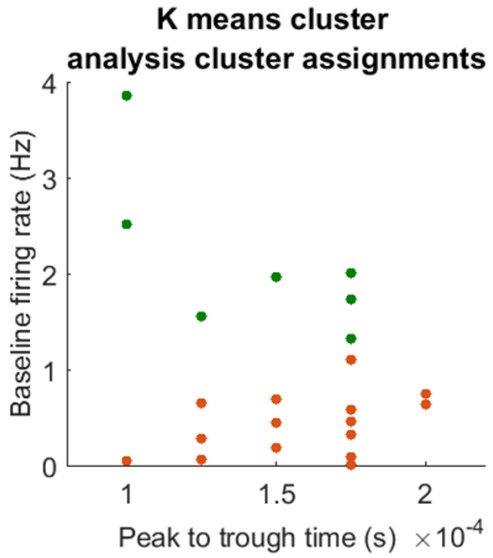


Figure 2.S1: A heat map showing the duration of PRH unit responses to stimulation of cholinergic input. Laser locked responses tended to be longer lasting than delayed responses. **2.S2:** Results of K means cluster analysis on the plot of spiking properties. Cluster assignments were not changed by the addition of a third spiking parameter, the area of the AHP (data not shown).

DISCUSSION

Here I have completed the first investigation of the effects of release of endogenous ACh in the PRH, as well as demonstrated for the first time PRH activity changes induced by recognition memory in mice. I showed that neural activity in the PRH is modulated by behavior largely in the posterior portions of the PRH and especially in layers 5 & 6. This is consistent with prior evidence from rats demonstrating significant increases in c-fos positive cell counts in response to novel objects in caudal portions of the PRH, but not in rostral or mid portions (Albasser et al., 2010). Furthermore, experiments in which cholinergic blockers are delivered to the PRH and impair recognition memory routinely target the posterior PRH (Bartko et al., 2014; Tinsley et al., 2011; Warburton et al., 2003; Winters et al., 2006).

Given the above result I focused my investigation into the effect of endogenous ACh release on the posterior portion of PRH. Here I found that approximately 60% of PRH units exhibit a detectable response to stimulation of cholinergic input from the NBM. This response rate is actually quite high, given the anatomical challenge presented by the NBM to PRH projection. Although the NBM is now known to exhibit broad topographical organization, it is a dispersed nucleus that covers a large anatomical area, the entirety of which cannot be stimulated by a single illumination. Further, cholinergic neurons are sparse within the NBM, comprising only 15-25% of the total neuronal population. Of these, only about 25-30% actually project to the PRH and these neurons are distributed over almost the entire anterior posterior extent of the basal forebrain (Kondo and Zaborszky, 2016;

Winters and Bussey, 2005). Given these anatomical obstacles, this high response rate may be reflective of another recently appreciated aspect of cholinergic anatomy, which is that each individual cholinergic neuron has an extraordinarily expansive axonal arbor and large cortical areas are innervated by very few cholinergic neurons (Wu et al., 2014; for review see Ballinger et al., 2016). Indeed, at times multiple functionally related cortical areas are innervated by a single cholinergic neuron (Wu et al., 2014).

I have shown that the response of PRH units to stimulation of cholinergic input is characterized by a change in variance of firing, and that responsive units typically showed a decrease in the Fano Factor with an accompanying increase in firing rate. This is somewhat unexpected as familiarity is thought to be encoded in the PRH by a decrease in activity. One possible explanation for this finding is that ACh facilitates familiarity encoding by recruiting a microcircuit whose overall effect is to decrease excitability of the PRH, but whose effect on individual neurons within the circuit is to increase firing. Consistent with this idea is the fact that several of the responsive neurons which I have documented here have spiking profiles consistent with the expected profile of inhibitory interneurons (Mitchell et al., 2007). Indeed responsive units in general had higher firing rates than non responsive units, which is again consistent with the profile of an inhibitory interneuron, although even the highest firing rates seen here are lower than would be considered typical of an interneuron (Mitchell et al., 2007). The concept of microcircuit recruitment may also explain the fact that cholinergic signaling in the PRH elicits both time locked and delayed responses. Delayed responses may represent neurons located relatively downstream in the microcircuitry. Further investigation of larger populations of PRH units will be necessary to determine if the majority of responsive neurons are interneurons or pyramidal neurons. It would also be very interesting to evaluate endogenous release of ACh in the PRH via electrophysiological recording in brain slices, where further dissection of the microcircuitry can be performed. Post hoc gene expression analyses could be used to ascertain cell types of responding cells and monosynaptic vs multisynaptic effects of ACh release could be dissected.

As novelty is believed to be processed in the PRH as an increase in firing rate, the fact that ACh predominantly increases excitability may alternatively be interpreted as meaning that ACh release in the PRH signals novelty, rather than leading to encoding of familiarity. If this was the case then administration of cholinergic antagonists prior to exposure to a novel object would be expected to impair or attenuate the animal's response to the novel object. However, many behavioral studies in rats and primates have shown that blockade of cholinergic signaling impairs recognition memory only when administered during familiarization/training and not when administered during memory testing/exposure to novel objects (Aigner et al., 1991; Browning et al., 2010; Gutierrez et al., 2004; Jacklin et al., 2015; Warburton et al., 2003; Winters et al., 2007; Winters et al., 2006). Thus ACh seems to be vital for acquisition of recognition, but not for signaling the presence of a novel stimulus.

Although the majority of responsive units that I recorded showed an increase in firing rate, 13.6% (3/22) of the units that I recorded showed a response to cholinergic stimulation but had *decreases* in firing rate. Interestingly, previous *in vivo* recordings in rats have demonstrated that the incidence of familiarity-sensitive neurons in the PRH is from 13-16% (von Linstow Roloff et al., 2016; Zhu and Brown, 1995). Thus it is possible that a proportion of PRH neurons that encode recognition do indeed respond to endogenous cholinergic release with a reduction in excitability. These results are consistent with the idea that ACh signaling in the PRH may facilitate encoding of familiarity by inducing an overall decrease in excitability of the PRH, possibly via microcircuit recruitment.

The PRH unit responses that I have documented here are both short (~70s) and extremely long lasting (>20min). Such long lasting responses are in keeping with previous documentation of cholinergic responses: *ex vivo* electrophysiological recordings from acute brain slices have shown amygdalar responses to optogenetic stimulation of cholinergic input can last over 30min (Jiang et al., 2016). Furthermore, *in vivo* optically evoked cholinergic responses in the amygdala can last for more than 60 minutes (Kundu et al., unpublished data).

The presence of both relatively short and long lasting effects may represent a difference in the type of receptors involved in these responses. Unfortunately, not much is known about the specific cholinergic receptors expressed in the PRH and their relative locations. A few studies in acute brain slices have demonstrated electrophysiological effects in layer 2/3 of the PRH that are M1, M2, M3 and M4 dependent (Massey et al., 2001; Navaroli et al., 2012; Warburton et al., 2003). Studies combining pharmacology and behavior suggest important roles for both muscarinic and nicotinic signaling in the PRH (Abe and Iwasaki, 2001; Aigner et al., 1991; Callahan et al., 2014; Jacklin et al., 2015; Nikiforuk et al., 2015; Tinsley et al., 2011; van Goethem et al., 2015; Warburton et al., 2003; Winters et al., 2006). Interestingly one study has shown that muscarinic blockade selectively impairs recognition memory performance at long intervals (24hrs) while nicotinic blockade selectively impairs memory at short intervals (15min) (Tinsley et al., 2011). Signaling via these different receptors may therefore subserve different aspects of recognition memory encoding. Further research combining *in vivo* recordings and optogetic stimulation of cholinergic neurons with pharmacology is needed to determine whether different types of cholinergic responses are differentially dependent on varying receptor types.

Finally as these recordings were done *in vivo* and I stimulated the cholinergic cell bodies within the NBM, which project broadly to the cortical mantle, the responses I have recorded may represent secondary or integrated effects of cholinergic release in other brain areas which in turn project to the PRH. A vital next step to understanding these results is targeted stimulation of endogenous cholinergic release locally in the PRH.

CONCLUSION

Recognition memory in the mouse is primarily encoded by decreased excitability in layers 5&6 of the caudal portion of the PRH. Units in this area respond to stimulation of cholinergic input with both increased and decreased firing rates. ACh may therefore reduce PRH excitability and encode familiarity via microcircuit recruitment in the PRH.

Chapter 3:

Phenotypic effects of *MeCP2* deletion from cholinergic neurons

INTRODUCTION

Rett Syndrome is an Autism-associated disorder that affects 1 in 10,000 girls and is caused by mutations in a gene known as *MeCP2*, which encodes the transcription regulator Methyl CpG Binding Protein 2 (Amir et al., 1999; Hagberg et al., 1983; Lewis et al., 1992; Neul et al., 2010; Rett, 1966). The disorder is characterized by severe cognitive impairment and is a common cause of intellectual disability among girls (Hagberg et al., 1983; Neul et al., 2010; Rett, 1966). Its most striking diagnostic characteristic is the presence of developmental regression that starts around 18 months and includes the loss of previously learned cognitive and motor abilities (Hagberg et al., 1983; Neul et al., 2010; Rett, 1966). Given the profound intellectual disability associated with Rett Syndrome, and the vital role of acetylcholine (ACh) in cognitive processes, the role of ACh in mediating or mitigating aspects of the disorder is an exciting area of translation research. Indeed, post mortem immunohistochemical studies of the brain from individuals with Rett Syndrome have shown profound cholinergic deficits: there are reduced numbers of Choline acetyl transferase (ChAT) positive cells in the basal forebrain, reduced ChAT and vAChT activity and reduced cholinergic receptor expression (Kitt et al., 1990; Wenk, 1997; Wenk and Hauss-Wegrzyniak, 1999; Wenk and Mobley, 1996; Yasui et al., 2011). Animal models of Rett Syndrome have shown reductions in both ACh and ChAT, dramatically attenuated cholinergic currents in electrophysiolgical experiments and altered cholinergic receptor expression profiles (Oginsky et al., 2014; Ricceri et al., 2011; Ward et al., 2009) although see also (Zhou et al., 2017). Finally, cholinergic marker reductions as evaluated by SPECT imaging *in vivo* have been correlated with increased clinical severity in patients with Rett Syndrome, indicating that the cholinergic system and its function are important mediators of clinical severity and may prove a promising target for treatment (Brašić et al., 2012).

To evaluate in more detail the contribution of the cholinergic system to the Rett Syndome phenotype, a mouse model has been generated in which *MeCP2* is selectively deleted from

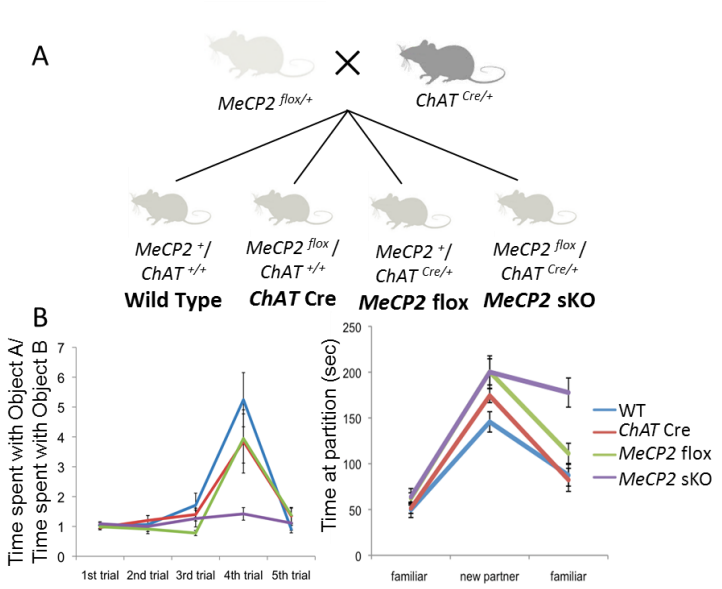


Figure 3.1: *MeCP2* cholinergic selective knock out mice are impaired in recognition memory of either an object or a conspecific A: *MeCP2* flox mice are crossed with *ChAT* Cre mice to generate *MeCP2* selective knock out mice (*MeCP2* sKO) and all genetic controls. **B:** Only the *MeCP2* sKO mice were impaired on novel object recognition and the partition test as compared with WT (and all other genetic controls). On the partition test, *MeCP2* sKO mice were impaired selectively in their ability to recognize a familiar mouse, and not on interaction with a novel mouse. Adapted from Schaaf and Zoghbi.

Test	Phenotype	Result
Novel Object Recognition	Recognition Memory	impaired
Partition test	Familiar Conspecific Recognition	impaired
Rotarod	Motor learning	normal
Morris Water Maze	Spatial Learning	normal
Fear Conditioning	Context/Cue Learning	normal

Table 3.1: Cognitive phenotype of mice in whom *MeCP2* is selectively deleted from cholinergic neurons. Adapted from Schaaf and Zoghbi.

cholinergic neurons only (**fig 3.1A**). This targeted deletion approach has previously been applied to the GABAergic and aminergic systems and has provided an added level of resolution in investigation of the significance of these neurotransmitter systems to Rett Syndrome and their relative potential as treatment targets (Chao et al., 2010; Samaco et al., 2009). Cognitive-behavioral phenotyping of mice with selective cholinergic *MeCP2* knock out, such as that performed by Schaaf and Zoghbi (table 3.1), has revealed that the mice are selectively impaired on tasks involving recognition memory, a form of declarative memory that may map onto cognitive deficits seen in individuals with Rett syndrome (**fig 3.1B**; (Zhang et al., 2016). However the circuit level deficits underlying this impairment are unknown.

As discussed, recognition memory is mediated by an area of the brain known as the perirhinal cortex (PRH; for recent review see Brown and Banks, 2015; Banks et al., 2012; Ranganath and Ritchey, 2012). Neural correlates of this form of memory are found in the form of individual neurons in the PRH which reduce their firing rate and show a reduction in responsiveness in the presence of a familiar – as opposed to novel – stimulus (for recent review see Brown and Banks, 2015; Banks et al., 2012; but also see von Linstow Roloff et al., 2016). This reduction-

response signals recognition to the animal. Cholinergic signaling within the PRH is particularly important to the establishment of recognition memory. Pharmacological experiments using cholinergic receptor blockers delivered either systemically or locally to the PRH during learning consistently impair recognition memory performance (Bartko et al., 2014; Tinsley et al., 2011; Warburton et al., 2003; Winters et al., 2006). Although the mouse PRH is not classically thought to contain any intrinsic cholinergic neurons, it receives cholinergic projections from the basal forebrain, primarily from a nucleus called the Nucleus Basalis Magnocellularis (NBM; (Kondo and Zaborszky, 2016; Woolf, 1991). Selective lesion of these cholinergic projections within the PRH also impairs subsequent recognition memory (Turchi et al., 2005; Winters and Bussey, 2005). Neurons which exhibit differential response to novel versus familiar stimuli have also been found in the basal forebrain and lesion of the NBM itself significantly impairs recognition memory (Okada et al., 2015; Wilson and Rolls, 1990). It is therefore this NBM-PRH cholinergic circuit that seems to be vital to recognition memory and vulnerable in the context of *MeCP2* deletion from cholinergic neurons. However, the molecular and electrophysiological changes in this circuit that may underlie the behavioral impairment have not yet been investigated.

Here I probe the neuroanatomical and electrophysiological consequences of *MeCP2* deletion from cholinergic neurons on the recognition memory circuit. My results will help further understanding of the cholinergic contribution to Rett syndrome, as well as the significance of *MeCP2* expression in the cholinergic system.

METHODS

Animals

For electrophysiological experiments transgenic male mice expressing Cre recombinase under control of the *ChAT* promoter (*ChAT*-Cre; Jackson Lab stock #006410) maintained on a C57 background were crossed with female mice expressing a floxed *MeCP2* allele (*MeCP2* flox; Jackson Lab stock #007177) maintained on a 129 background. Male mice from the F1 and F1b generations of this cross were used for experiments.

For brain activity mapping experiments transgenic male mice expressing a destabilized GFP under control of the *c-fos* promoter maintained on a C57 background (*c-fos* GFP; Jackson Lab stock #018306) were crossed with female mice from the F1 generation of the above cross (*ChAT*-Cre x *MeCP2* flox on a 129/C57 F1 background). Males from the F1 generation of this cross were used for experiments. For stereology and terminal field quantification transgenic male mice expressing a tauGFP fusion protein under control of the *ChAT* promoter (*ChAT* GFP, gift from gift from S.Vijayaraghavan, see (Grybko et al., 2011) maintained on a C57 background were again crossed with female mice from the F1 generation of the above cross (*ChAT*-Cre x *MeCP2* flox on a 129/C57 F1 background). Male mice from the F1 generation of this cross were used for experiments. All mice were maintained on a reversed 12 hour light cycle and allowed

food and water *ad libitum*. Mice were either pair or group housed when possible. No singly housed mice were used for behavioral experiments.

Sample preparation for light and confocal microscopy

Mice were anesthetized with a 9:1 mixture of ketamine and xylazine and transcardially perfused with 1x PBS followed by 4% PFA. Brains were removed and post fixed overnight in 4% PFA before being sucrose equilibrated and frozen in OCT. Brains were then cryosectioned (Leica Biosystems Inc., Buffalo Grove, IL) at 50 micron thickness.

Immunohistochemistry

For Nissl staining brain slices were blocked and permeabilized for 30 minutes in 1x PBS with 5% donkey serum and 0.1% triton X and then incubated for 90 minutes in a 1:200 dilution (in blocking/permeabilization solution) of the NeuroTrace blue fluorescent Nissl stain (Thermo Fisher Scientific, Waltham, MA) followed by three 5 minute washes in 1x PBS. All steps were performed at room temperature.

For NeuN staining, slices were stained free floating. They were first blocked and permeabilized for 1 hour at room temperature in 1x TBS with 7.5% donkey serum and 0.3% Triton X. Slices were then incubated in a 1:500 dilution (in TBS-TX) of 1° mouse anti-NeuN antibody (cat#MAB377, EMD Millipore, Billerica, MA) overnight at 4° on a shaker followed by four 10 minute washes in 1x TBS. Finally the slices were incubated in a 1:500 dilution of 2° donkey anti-mouse 594 antibody (cat#A-21203, Life Technologies, Carlsbad, CA) at room temperature for 4 hours then washed twice in 1x TBS for 10 minutes each.

To minimize autofluorescent signals, slices were dehydrated for 5 minutes in 70% ethanol and then incubated in the Autofluorescence Eliminator Reagent (EMD Millipore, Billerica, MA) for 5 minutes, followed by 3 one minute washes in 70% ethanol. Slices were then rehydrated with 1x PBS and coverslipped with DAPI Fluoromount-G (Southern Biotech, Birmingham, AL). All steps were performed at room temperature.

Recognition memory training

C fos GFP mice were aged to 15 weeks and then underwent recognition memory testing (Bevins and Besheer, 2006). The behavioral apparatus consisted of a dark room and table on which were placed two 25x40cm rectangular cages painted matte white with IR transparent lids. One cage was used as a staging arena and the other cage was used as the behavioral arena. A small plastic platform was used to transfer mice from their home cage to the behavioral apparatus. On day 1 mice were handled by the experimenter and habituated to the transfer platform. On days 2 and 3 mice were placed in the staging arena and then the behavior arena for 5 minutes each. On day 4 two identical objects were placed in the behavioral arena and mice were placed in the staging arena for 5 minutes and then in the behavioral arena with the two

identical objects for 5 minutes. On day 5 one of the objects was replaced and mice in the "novel object" group were placed in the staging arena for 5 minutes and then the behavior arena with the two mismatched objects for 5 minutes. On day 5 mice in the "familiar object" group were re-exposed to the matched pair of objects from day 4. On days 4 and 5 mice in the "arena control" group were placed in the staging arena for 5 minutes and in the empty behavioral arena for 5 minutes. Behavior on days 4 and 5 was recorded with an IR sensitive video camera.

Behavioral coding

Behavior was coded offline using JWatcher and time spent exploring each object was quantified. The following behaviors were considered "exploration": whisking the object, biting the object, touching the object, nose oriented towards and within 2cm of the object.

Quantifying cells activated by behavior

On day 5 of behavioral testing, between 150 and 180 minutes following entrance into the behavioral arena, *c-fos* GFP mice were perfused and coronal brain slices were prepared, stained for Nissl, and treated for autofluorescence as above. Nissl staining of brain slices was then imaged on a stereoscope (Zeiss, Oberkochen, Germany) while GFP and DAPI were imaged on a confocal microscope (Olympus, Center Valley, PA) with a 40x objective at a Z step of 5 microns.

Images of the Nissl and GFP/DAPI staining for each brain slice were aligned using the Align Image plugin for Image J. Nissl staining was used to histologically identify the perirhinal cortex and its constituent layers as per Beaudin et al., 2013. Cells were considered c -fos positive if they were labeled both by GFP and DAPI. Cells were counted using the Cell Counter plugin for ImageJ. Analyzed brain slices were at intervals of 400 microns from each other and encompassed the posterior half of the PRH (approx. Bregma -3.16 to Bregma -4.04).

Stereological analysis of the NBM

ChAT GFP mice were allowed to age to 14 weeks and then underwent perfusion and coronal brain slices were prepared and stained for NeuN as above. GFP+ and NeuN+ cell counts were then acquired using the Optical Fractionator work flow of Stereo Investigator (MBF Bioscience, Williston, VT) and an epifluorescent microscope. The NBM was delineated using anatomical landmarks in bright field with a 5x objective and 100µmx100µm dissectors were randomly placed to cover 50% of the NBM. Guard zones of 4µm at the top and bottom of the slice were used. Counts were performed using a 20x objective.

Terminal field quantification

ChAT GFP mice were allowed to age to 14 weeks and then underwent perfusion and coronal brain slices were prepared, stained for Nissl, and treated for autofluorescence as above. GFP-labeled terminal fields in the PRH were imaged at 40x using the NanoZoomer S60

(Hamamatsu, Japan). Nissl staining was imaged using a stereoscope (Zeiss, Oberkochen, Germany). The coronal slice corresponding to Bregma -3.16 was then analyzed.

Images of the Nissl and GFP labeling were aligned using the Align Image plugin for Image J and Nissl staining was used to delineate the PRH as per Beaudin et al., 2013. As the ventral and medial borders of the PRH are the most reliably identified, a 200µmx200µm ROI was placed approximately 100µm lateral and dorsal to the point where these two borders meet. Fiber density within this ROI was then quantified as in Sathyanesan et al., 2012. In short, each Nanozoomer image was transformed into a 32 bit gray scale image and processed using the Hessian feature extraction available in the FeatureJ plugin for Image J. Absolute eigenvalue comparison was performed using the largest eigenvalue of the Hessian tensor and a smoothing scale of 0.5. Line scans of length 200µm were then collected within the ROI defined above. 3 line scans oriented perpendicular to the external capsule were collected and 3 line scans oriented parallel to the external capsule were collected. The line scans were spaced 100µm from each other. A final line scan was collected from within the external capsule to quantify autofluorescence within the slice.

Line scans were then further analyzed using Matlab. Each line scan was first background adjusted using the msbackadj command with a window size and step size of 10µm and with peak heights preserved. Fiber crossings were then detected as peaks in fluorescence within the background adjusted line scan using the mspeaks command. The mean pixel intensity of the line scan from the external capsule was used as a threshold for peak detection. The number of peaks per micron for each line scan was then calculated and averaged between the 3 perpendicular line scans and the 3 parallel line scans from each slice to yield the average fiber density in the perpendicular (D_{Pr}) and parallel (D_{Pl}) orientations respectively. Total fiber density was calculated using the following formula (from Sathyanesan et al., 2012):

$$D_v = \frac{D_{Pr} * D_{Pl}}{z * \cos(45)} * 10^6 fibers/100\mu m^3$$

Where z is the section thickness (50) and the factor cos(45) adjusts for the random orientation of fibers within the tissue (Sathyanesan et al., 2012).

Viral injection

Mice for optogenetic experiments (ie *ChAT* Cre mice ± *MeCP2* flox) were aged to at least 11 weeks and were then anesthetized with isoflurane and mounted on a stereotaxic frame (Kopf Instruments) with a heated stage. An incision in the scalp was made and a small hole was drilled in the skull above the left NBM (coordinates from Bregma: A/P: -0.7mm M/L: 1.7mm Z: -4.0mm). 0.5 µL of either AAV9-Ef1a-DIO-ChETA-eYFP or AAV9-CAG-DIO-oChIEF-tdTomato was injected using a micro syringe (Hamilton, Reno, NV). Mice were given Ketorolac (6mg/kg) as needed for 2-3 days following surgery. Mice were allowed to recover and the virus to express for at least 3 weeks before being used for electrophysiolgical recording.

Donepezil pump implantation

A subset of *MeCP2* sKO mice underwent viral injection as above at 12 weeks and simultaneously underwent subcutaneous implantation of a mini osmotic pump (Alzet model 2006, Cupertino, CA). After the virus was injected, hemostats were lubricated with saline and then passed through the original incision and used to open the subcutaneous space by separating the skin from the subcutaneous fascia. The pump was then implanted in this space and the mouse was allowed to recover as above for 2 weeks before being used for electrophysiological recording. Pumps delivered donepezil HCl (Biotang Inc, Lexington, MA) in saline at a dose of 0.3mg/kg/day.

Electrophysiological recording

Mice for electrophysiological experiments were aged to at least 13 weeks and were then anesthetized with isoflurane and placed on a surgical stereotax (Kopf instruments) with a heated stage. A craniotomy over the left perirhinal cortex was performed and a tungsten electrode of either 1 MΩ or 5 MΩ (AM systems, Sequim, WA) was positioned into the posterior PRH (coordinates from Bregma: A/P: -3.25mm, z: -3.35mm to -3.85mm, M/L: from temporal ridge: -200 to +500 microns). Extracellular recordings were pre amplified with a head stage and then fed to an amplifier (AM systems, Sequim, WA). Mice for optogenetic experiments (ie *ChAT* Cre mice ± *MeCP2* flox) received an additional craniotomy over the left NBM through which a 1 MΩ parylene-C insulated tungsten electrode (AM systems, Sequim, WA) and a 200 µm optical fiber (Thorlabs, Newton, NJ) coupled to a 473nm laser (Shanghai Dream Lasers Technology, Shanghai, China) were positioned into the NBM.

Signals were acquired at a sampling rate of 40KHz and band pass filtered at 100-1000Hz by an amplifier (AM systems, Sequim, WA) before being passed through a Humbug Noise Eliminator (AM systems, Sequim, WA) and fed through a Tektronix TDS 2014B oscilloscope to a CED 1401 data board to the computer where they were visualized and collected using Spike 2 software (CED, Cambridge, UK). Laser stimuli used for optical stimulation of cholinergic neurons consisted of 20 laser pulses of 1ms duration delivered at a frequency of 10Hz.

Relocalization of recording site

At the end of each recording session an electrolytic lesion was created by passing 100µA of current for 45 seconds through the recording electrode to facilitate relocalization of the recording site. The mouse was then perfused and brain slices obtained as above.

For a subset of mice, slices containing the perirhinal cortex were stained using NeuroTrace (Thermo Fisher Scientific, Waltham, MA) blue fluorescent Nissl stain as above. For all mice slices containing the PRH were imaged on a stereoscope (Zeiss, Oberkochen, Germany). For mice used for optogenetic experiments slices containing the NBM were imaged to confirm viral expression.

Electrophysiological data analysis

Extracellular recordings were sorted offline using the Offline Sorter (Plexon Inc., Dallas, TX). Features of the waveforms were extracted and individual units were demarcated by manually identifying clusters of waveforms in a 2 dimensional feature space of spike properties (Gray et al., 1995). The quality of each sort was rated according to isolation distance between clusters within the recording. Only recordings of high sort quality, with less than 5% overlap with other clusters, were used for further analysis. Units with firing rates lower than 0.05Hz were excluded from further analysis.

Single unit recordings were then further analyzed using Matlab. To compare baseline variability of firing between genotypes, 300s of baseline recording was binned over varying time intervals and the number of spikes occurring in each bin was calculated. The Fano Factor (FF=variance/mean) of these spike counts was computed and plotted. The bins used were (in sec): [.01, .02, .05, .1, .2, .5, 1, 2, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100].

Response to optogenetic simulation was evaluated using a permutation test of the F statistic with 10,000 permutations comparing inter spike intervals occurring within the 140s immediately before and after stimulation. To identify delayed responders the same analysis was performed comparing the 140s immediately before stimulation and a sliding 140s window following stimulation. Each slide step was 70s. A response was detected if the resultant *p* value from these permutation tests was less than 0.05. A unit was considered to exhibit a "laser locked" response if a response was detected in the first window following stimulation. A unit was considered to exhibit a "delayed" response if a response was detected at any later window within the first 560s following stimulation. If no responses were detected within the first 560s following stimulation, the unit was considered to have no response.

Statistics

Response rates to optogenetic stimulation were compared between groups using a Chi square test of homogeneity: Cressie-Read power divergence statistic method as this method is modestly superior to the traditional Pearson method for small tables (Rudas, 1986; Thorvaldsen et al., 2010). All other comparisons were performed with the Kruskal Wallis test. All comparisons were performed in Matlab.

RESULTS

Quantification of cholinergic neuroanatomy

As cholinergic cell loss has been documented in the basal forebrain of individuals with Rett syndrome (Kitt et al., 1990; Wenk and Hauss-Wegrzyniak, 1999) and neuronal fiber loss has been documented in other mouse models of Rett syndrome, I first asked if the

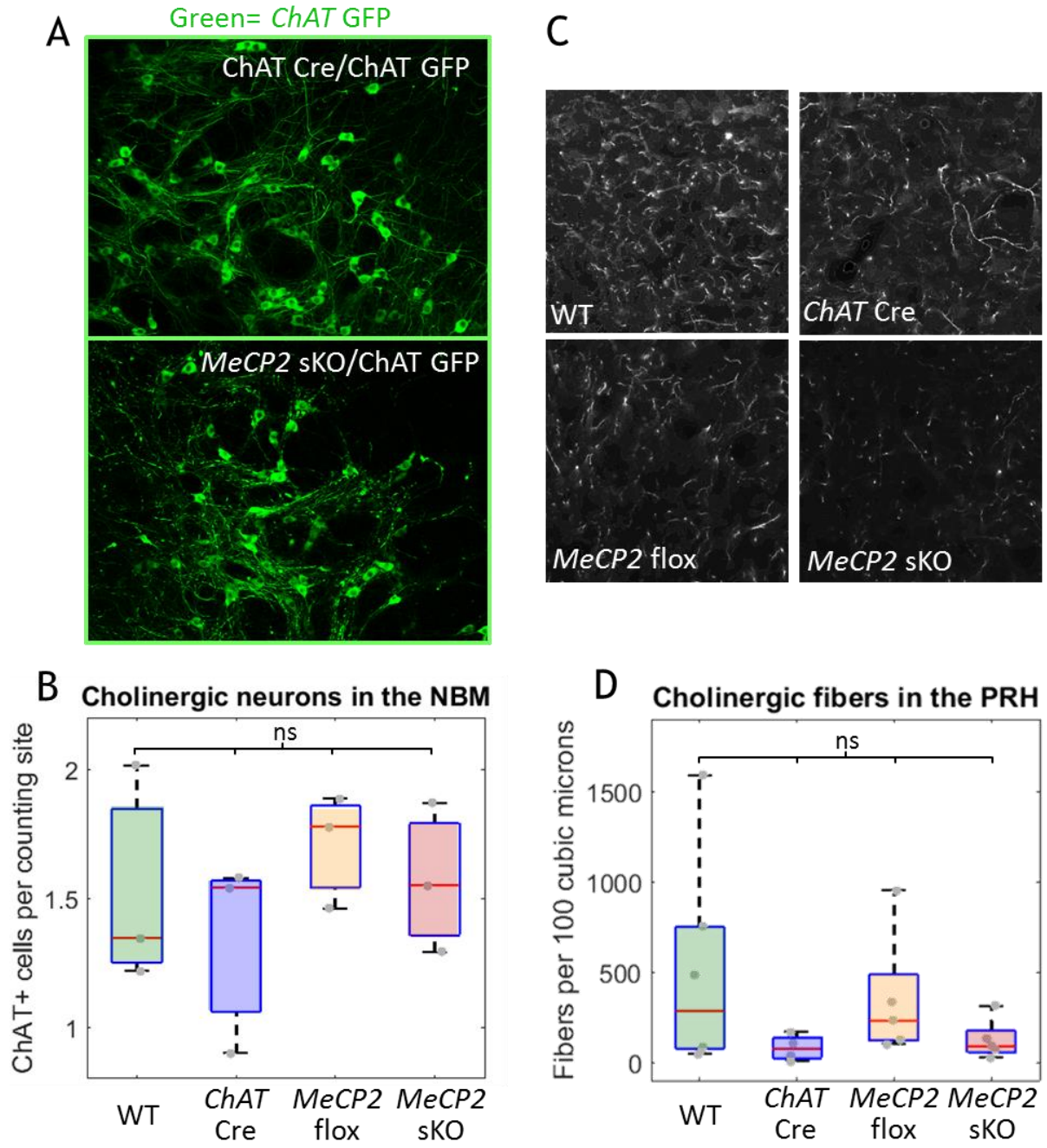


Figure 3.2: Cholinergic neuroanatomy is not affected by deletion of *MeCP2*. A: Representative images of the NBM from *MeCP2* sKO and control mice in which cholinergic neurons and their processes are genetically labeled by a tauGFP fusion protein under control of the *ChAT* promoter (*ChAT* GFP). **B:** Quantification of cholinergic neuron density in the NBM shows that it is not changed in *MeCP2* sKO (Kruskal Wallis, $\chi^2(3)=1.36$, $p=0.72$). **C:** Representative images of genetically labeled cholinergic fibers in the PRH. **D:** Quantification of cholinergic fiber density in the PRH. Although WT mice had a higher fiber density than all other genotypes, this difference did not reach statistical significance (Kruskal Wallis, $\chi^2(3)=4.18$, $p=0.24$).

cholinergic neuroanatomy subserving the recognition memory circuit was damaged by deletion of *MeCP2* from cholinergic neurons.

Figure 3.2A shows representative images of cholinergic neurons in the NBM of a control mouse and an *MeCP2* sKO mouse. Quantification of these neurons revealed no difference in cholinergic cell density with in the NBM between genotypes (**fig 3.2B**; Kruskal Wallis, $\chi^2(3)=1.36$, $p=0.72$). Because neuronal density within the NBM is highly heterogeneous, the proportion of total neurons within the NBM that were cholinergic was also quantified. This measure also indicated that there was no difference between genotypes (**fig 3.S1**; Kruskal Wallis, $\chi^2(3)=1.5$, $p=0.68$).

Figure 3.2C shows representative images of cholinergic fibers within the PRH. WT mice had denser innervation of the PRH (mean: 505.9±601.6 fibers/100μm^3) than all of the other genotypes (*ChAT* Cre mean: 80.7±71.4 fibers/100μm^3; *MeCP2* flox mean: 349.3±350.4 fibers/100μm^3; *MeCP2* sKO mean: 126.4±111.7 fibers/100μm^3). However these differences were not statistically significant although this may be due to the insufficient n value (Kruskal Wallis, $\chi^2(3)=4.18$, $p=0.24$).

Electrophysiological recording of the PRH

The denser cholinergic innervation of the PRH seen in WT mice above is not sufficient to explain the behavioral impairment seen in *MeCP2* sKO mice, as *ChAT* Cre and *MeCP2* flox mice, in whom recognition memory is intact (**fig 3.1B**), also show reduced PRH cholinergic fiber density. I therefore asked if there was a functional change in PRH firing patterns as a result of deletion of *MeCP2* from cholinergic neurons that might explain the behavioral impairment. In order to investigate this, I collected extracellular recordings *in vivo* from the PRH of anesthetized mice.

Recordings were collected from layers 5 & 6 of the posterior PRH (**fig 3.3A**). **Figure 3.3B** shows example extracellular recordings obtained *in vivo* from anesthetized mice. PRH units from control mice (WT, *ChAT* Cre and *MeCP2* flox) have a highly variable firing pattern. However, PRH units from *MeCP2* sKO mice exhibit a very regular and rhythmic firing pattern with little variability (**fig 3.3B**). **Figure 3.3C** shows how a measure of variability of firing, the Fano Factor, changes as a function of the time bin with which it is calculated for each genotype. Teich et al. (1997) showed that this measure will continuously diverge from zero for spike trains which contain long time scale oscillations and intercorrelations. The measure will continuously decrease for spike trains with very consistent rhythmicity and regular firing and will hover near zero for spike trains that fire at random (Teich et al., 1997). **Figure 3.3C** shows this measure continuously diverges from zero for all 3 genetic controls, indicating the presence of patterns over long periods of time. However this measure decreases over many time bins and hovers closer to zero for *MeCP2* sKO mice, indicating a loss of these patterns.

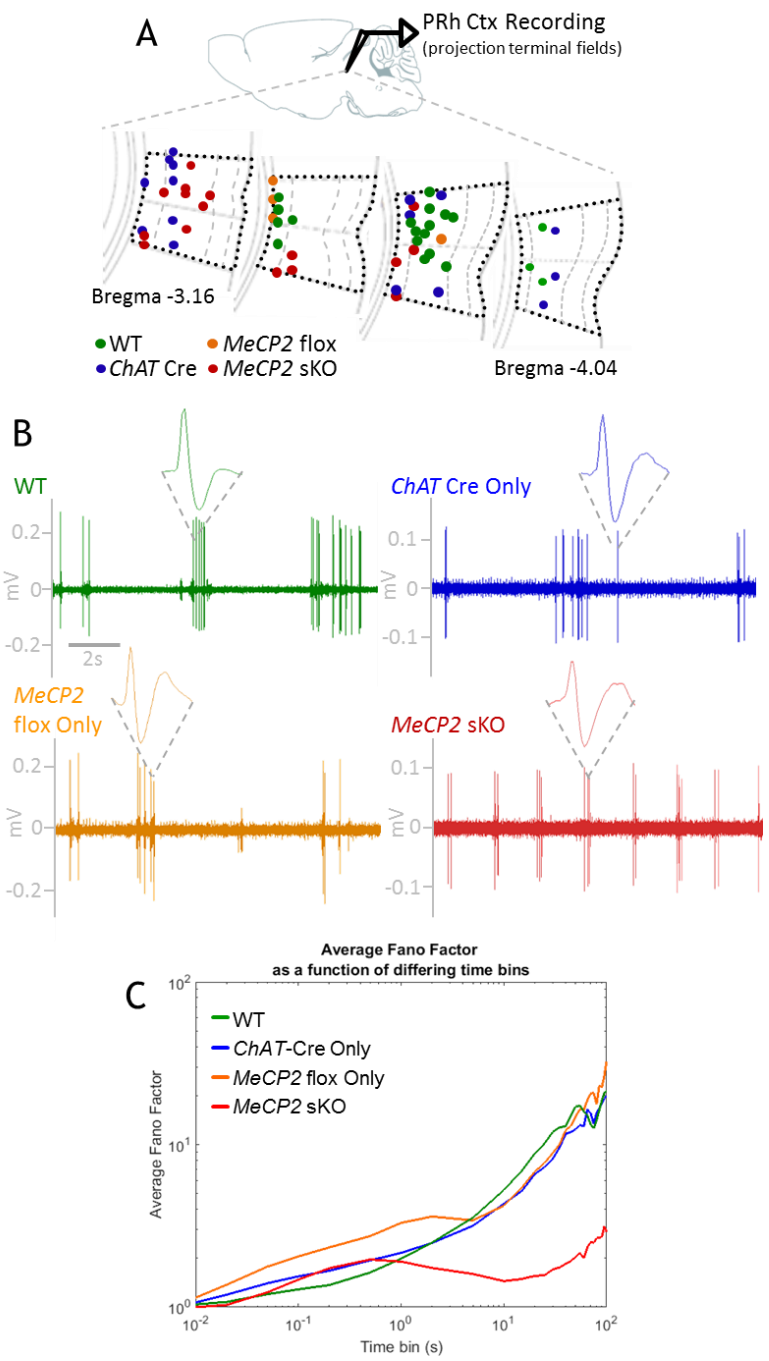


Figure 3.3: Neuronal firing in the PRH is highly variable, and this variability is lost in *MeCP2* sKO mice. A: *In vivo* recordings were collected from layers 5 and 6 of the Perirhinal Cortex. **B:** Representative recordings show the highly variable baseline firing in controls that is lost in *MeCP2* sKO. **C:** Average fano factor for each group calculated at differing time bins. Control values continuously diverge from 0, indicating the presence of long time scale oscillations, while *MeCP2* sKO values decrease over many bin values, indicating the presence of very rhythmic and non variable firing (as per Teich et al., 1997; WT n=24 units from 6 mice, *ChAT* Cre n=22 units from 11 mice, *MeCP2* flox n=6 units from 3 mice, *MeCP2* sKO n=18 units from 7 mice).

Optogenetic stimulation of cholinergic input to the PRH

The above results indicate that *MeCP2* deletion from cholinergic neurons has an important functional effect on PRH firing at baseline. I next asked if it would have an effect on the response of the PRH to stimulation of endogenous acetylcholine release.

Figure 3.4A shows the experimental strategy for stimulating endogenous ACh release in the PRH. *ChAT* Cre and *MeCP2* sKO mice received injections of a viral vector encoding a light activated excitatory ion channel into the NBM as this nucleus provides the majority of the cholinergic innervation of the PRH (Kondo and Zaborszky, 2016; Woolf, 1991). The vector was a flip excision switch design such that it was expressed only in the presence of Cre recombinase and therefore was selectively expressed in cholinergic neurons (**fig 3.4B**). **Figure 3.4C** shows example viral labeling of cholinergic neurons in the NBM of a *ChAT* Cre mouse (top) and an *MeCP2* sKO mouse (bottom). **Figure 3.4D** shows representative optically evoked action potentials from the NBM of a *ChAT* Cre mouse (blue, top) and an *MeCP2* sKO mouse (red, bottom).

Figure 3.5A shows data from a representative control PRH unit and its response to stimulation of cholinergic input. **Figure 3.5A** (middle) shows a box plot of ISI's obtained before and after laser stimulation. There is a change in variance of ISI, as measured by the F statistic ($variance_{pre}/variance_{post}$) as a result of laser stimulation. This change in variance was significantly greater than when the data was randomly shuffled (**fig 3.5A**, bottom). **Figure 3.5B** shows data from a representative *MeCP2* sKO PRH that did not respond to optical stimulation of cholinergic input.

Figure 3.5C shows a heat map of response for each PRH unit as a function of time since laser stimulation for control mice (left) and *MeCP2* sKO mice (right). While 22.7% (5/22) of control PRH units exhibited a response that was time locked to the laser stimulation, no *MeCP2* sKO PRH units did. The proportion of units exhibiting delayed responses to laser stimulation was similar between control and *MeCP2* sKO mice (*ChAT* Cre: 36.4%, 8/22; *MeCP2* sKO: 33.3%, 6/18). In contrast, the proportion of units with no detectable response to optical stimulation was higher in *MeCP2* sKO mice (66.7%, 12/18) than controls (40.9%, 9/22). However these differences were not statistically significant (Chi square test for homogeneity, CR(2)=5.68, p=0.058). This data is summarized in **fig 3.5C** (bottom) and **fig 3.5D**.

Effect of donepezil on MeCP2 sKO electrophysiology

The recognition memory impairment of *MeCP2* sKO mice has previously been shown to be rescued by treatment with donepezil, an acetylcholinesterase inhibitor (**fig 3.6A**). I therefore asked if the electrophysiological deficits that I have documented above could be similarly rescued by treatment with donepezil.

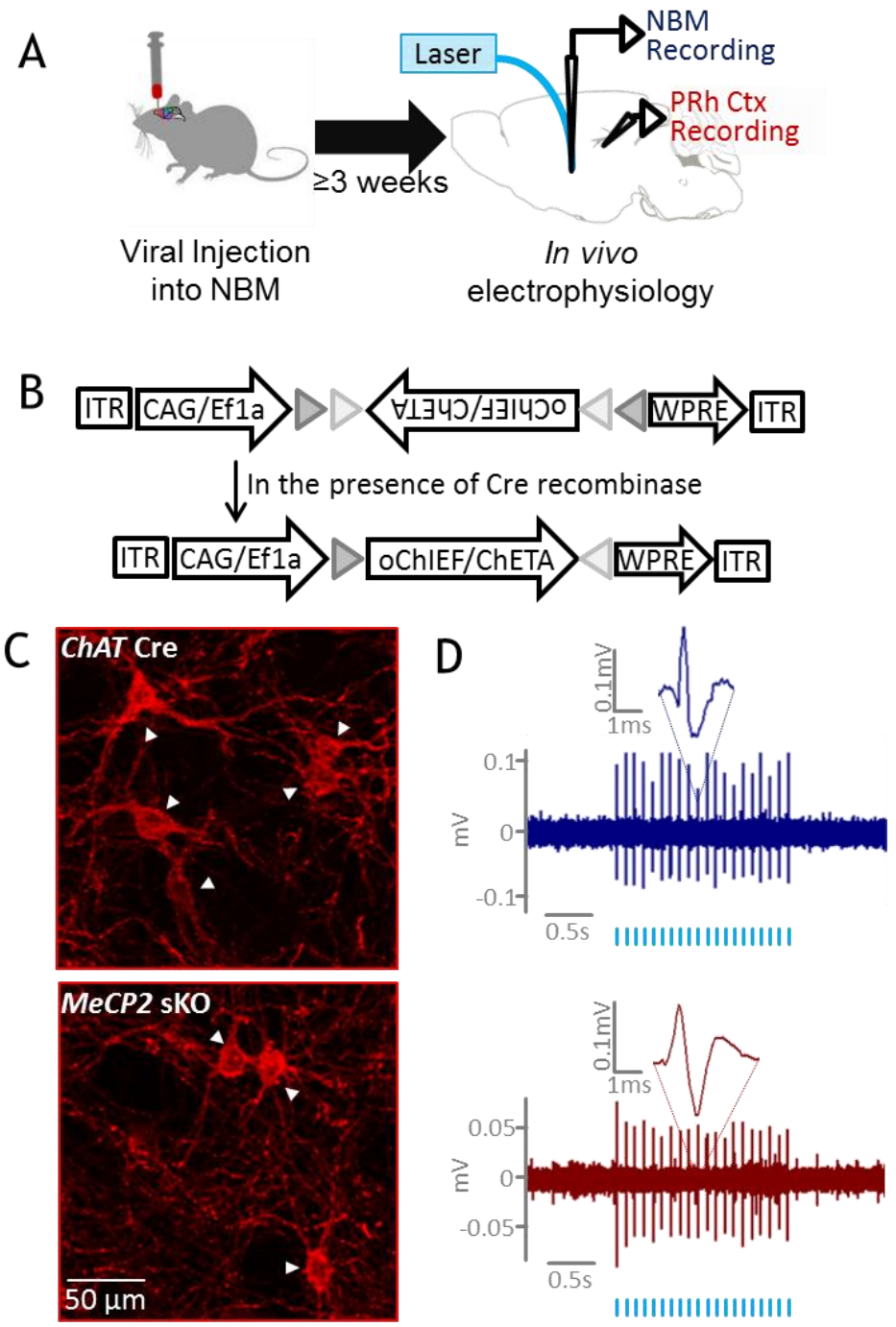


Figure 3.4: Strategy for optogenetic stimulation of cholinergic neurons. A: Schematic of the experimental paradigm. **B:** A viral vector encoding an optically activated excitatory ion channel is injected into the NBM. The viral vector is of a flip excision switch design such that it will be expressed only in the presence of Cre recombinase. **C:** Representative images of virally labeled cholinergic neurons (white arrowheads) from a control mouse (blue, top) and an *MeCP2* sKO mouse (red, bottom). **D:** Representative optically evoked action potentials in the NBM of a control mouse (top) and an *MeCP2* sKO mouse (bottom). The timing of laser pulses delivered into the NBM is indicated by light blue hash marks.

Figure 3.6B shows a representative extracellular recoding obtained from the PRH in vivo from an anesthetized *MeCP2* sKO mouse that had been treated with systemic donepezil for at least 2 weeks prior to recording (*MeCP2* sKO + Dpz). The recording shows a highly variable firing pattern. **Figure 3.6C** shows how the Fano Factor varies as a function of time bin. Values for *MeCP2* sKO + Dpz mice continuously diverge from zero, similar to control mice. This indicates that long time scale oscillations and pattern have been restored.

I next tested whether the PRH response to stimulation of cholinergic input was similarly restored. **Figure 3.6D** shows a representative PRH unit from an *MeCP2* sKO + Dpz mouse in which the PRH cholinergic input has been optically stimulated. The box plot of ISI's (left) obtained before and after stimulation shows a change in variance of ISI. This change is significantly greater than that generated when the data is randomly shuffled (right, grey arrow). **Figure 3.6E** shows a heat map of responses of MeCP2 sKO + DPz PRH units as a function of time since laser stimulation. Laser locked response rate is partially rescued to 16.7% (2/12). The proportion of units with delayed responses is slightly higher than in control or *MeCP2* sKO mice (*MeCP2* sKO + Dpz: 41.7%, 5/12) while the proportion of units with no detectable response is rescued to 41.7% (5/12). However, the difference in proportions between *MeCP2* sKO and *MeCP2* sKO + Dpz mice is not statistically significant (Chi square test for homogeneity, CR(2)=3.97, p=0.137).

Quantifying PRH activation by exposure to familiar vs novel objects

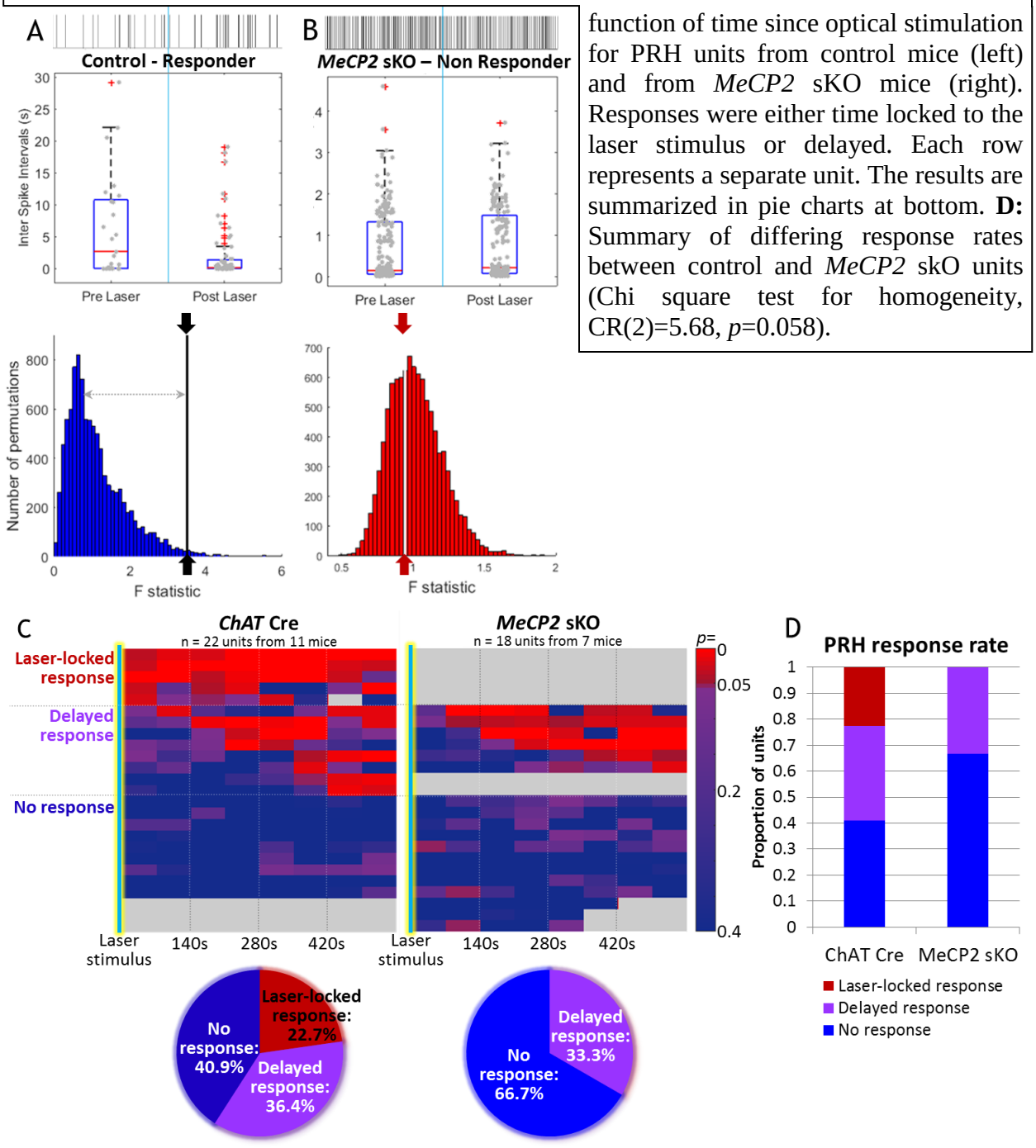
Given the above electrophysiological deficits and the dependence of recognition memory upon cholinergic signaling in the PRH (Tinsley et al., 2011) I next asked if encoding of novel object recognition in the PRH was impaired by *MeCP2* deletion from cholinergic neurons.

Figure 3.7A shows the experimental strategy for evaluating recognition memory encoding in the PRH. *C fos* GFP mice, in whom activated cells are genetically tagged with GFP, were exposed to either a novel or familiar object and the response of cells in layers 5 & 6 of the posterior PRH was quantified. **Figure 3.7B** shows representative images of the PRH from a control (top) and an *MeCP2* sKO (bottom) mouse exposed to either a familiar (left) or a novel (right) object. More PRH cells were activated by a novel object than a familiar object in both control and *MeCP2* sKO mice (**fig 3.7B&C**). In fact, the difference between number of cells activated by a novel as compared to a familiar object was even greater in *MeCP2* sKO mice than it was in controls (**fig 3.7C**). Although this difference did not reach statistical significance in either group (Control: Kruskal Wallis, $\chi^2(2)=0.43$, p=0.81, *MeCP2* sKO: Kruskal Wallis, $\chi^2(2)=4.5$, p=0.11).

DISCUSSION

Here I have performed the first investigation into effects of cholinergic *MeCP2* deletion on the recognition memory circuit. I demonstrated that PRH firing is altered by *MeCP2* deletion from cholinergic neurons both at baseline and after stimulation of cholinergic input.

Figure 3.5: PRH response to stimulation of endogenous cholinergic signaling is impaired in *MeCP2* sKO. A,B: Sample data from a Perirhinal Cortex unit in a control mouse that responded to stimulation of cholinergic input (**A**) and a Perirhinal cortex unit from an *MeCP2* sKO mouse that did not respond to stimulation of cholinergic input (**B**). (top) Representative raster plot of spikes. Vertical light blue bar indicates timing of optical stimulation. (middle) Box plot of inter spike intervals. (bottom) Distribution of F statistic of ISI's generated by shuffling the ISI's 10,000 times. The experimental (laser centered) F value is indicated by a vertical line and arrowheads. A response was detected when the experimental value was greater than 95% of the randomly generated values (gray dotted line). **C:** Heat map of *p* values as a function of time since optical stimulation for PRH units from control mice (left) and from *MeCP2* sKO mice (right). Responses were either time locked to the laser stimulus or delayed. Each row represents a separate unit. The results are summarized in pie charts at bottom. **D:** Summary of differing response rates between control and *MeCP2* skO units (Chi square test for homogeneity, CR(2)=5.68, *p*=0.058).



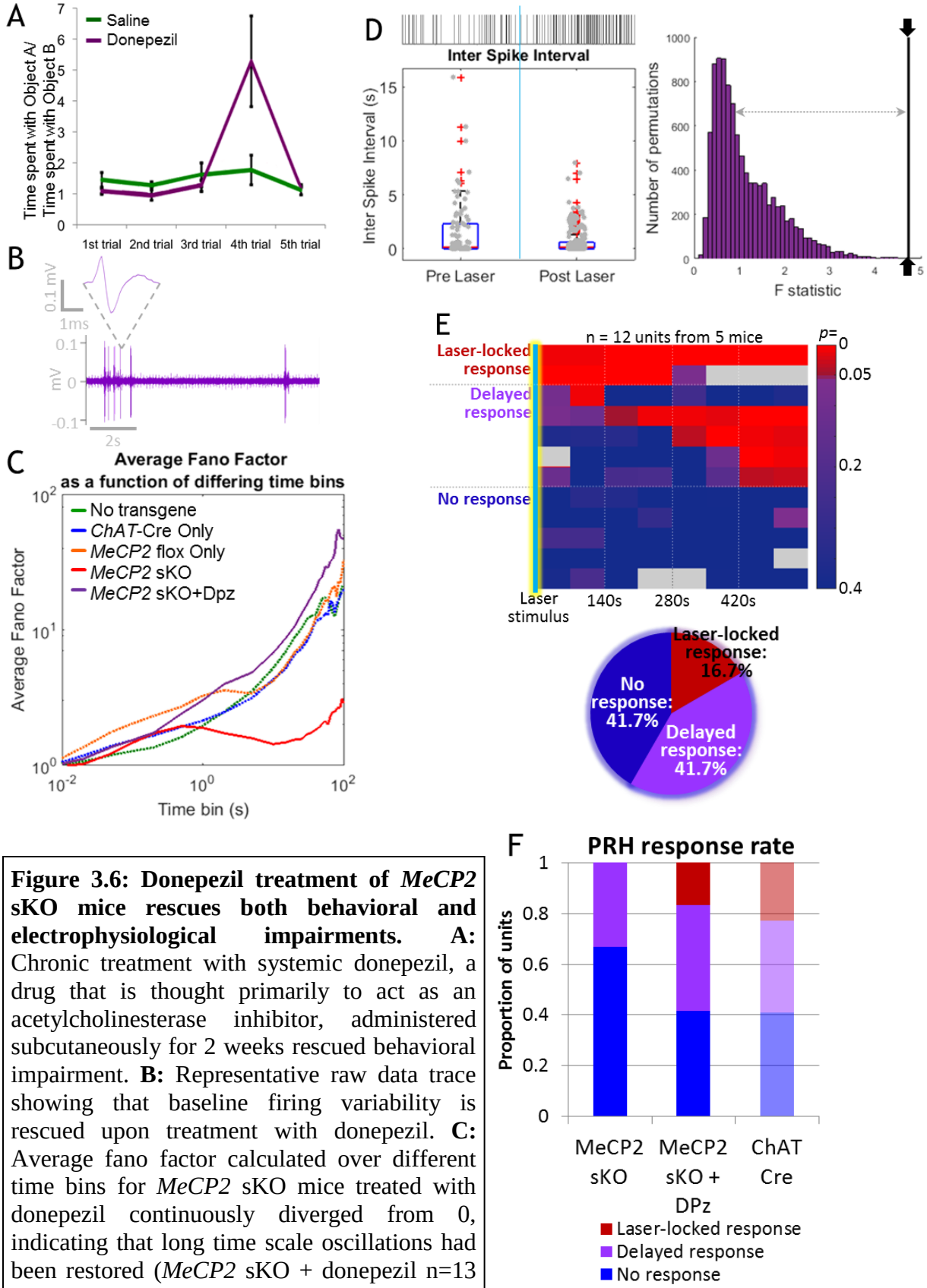


Figure 3.6: Donepezil treatment of *MeCP2* sKO mice rescues both behavioral and electrophysiological impairments. A: Chronic treatment with systemic donepezil, a drug that is thought primarily to act as an acetylcholinesterase inhibitor, administered subcutaneously for 2 weeks rescued behavioral impairment. **B:** Representative raw data trace showing that baseline firing variability is rescued upon treatment with donepezil. **C:** Average fano factor calculated over different time bins for *MeCP2* sKO mice treated with donepezil continuously diverged from 0, indicating that long time scale oscillations had been restored (*MeCP2* sKO + donepezil n=13 units from 5 mice).

Figure 3.6: D: Sample response to optogenetic stimulation in an *MeCP2* sKO mouse after treatment with donepezil. At top is shown a raster plot of action potentials before and after stimulation of cholinergic input (indicated by light blue vertical bar). In the middle is shown a box plot of inter spike intervals obtained before and after optical stimulation of cholinergic neurons in the NBM. At right is shown a distribution of the F statistitic of ISI's generated by shuffling the data 10,000 times. The experimental (laser centered) F statistic is indicated by a vertical line and arrowheads. The experimental value is larger than 95% of the randomly generated values (gray dotted line), indicating that this unit responded to stimulation of cholinergic input. **E:** Heat map of *p* values as a function of time since optical stimulation. Responses were either time locked to the laser stimulus or delayed and are summarized in the pie chart at bottom. **F:** Summary of response types. Laser locked responses were restored in *MeCP2* sKO mice treated with donepezil. (Chi square test for homogeneity, CR(2)=3.97, p=0.137)

Therefore the impairment of cholinergic signaling appears to result in multiple effects over differing time scales. This is not so surprising as ACh is thought to exert its effects via both more tonic, volume type transmission as well as via time locked, transient signals (for review see Ballinger et al., 2016). The degree to which these two different modes of signaling contribute to different or overlapping cognitive functions is not well understood. The observation here that there are effects of a genetic cholinergic lesion both at baseline and after stimulation of cholinergic input suggests that cholinergic transmission via both of these signaling mechanisms is vital in the PRH.

At baseline, the effect of impairing cholinergic signaling via *MeCP2* deletion is to reduce variability of firing. This may represent a loss of dynamic range over which individual neurons can encode. The function of more tonic, volume type cholinergic transmission in the PRH may therefore be to increase this dynamic range. Loss of biological variability is frequently documented in mouse models of human psychiatric disorders and may represent a common mechanism for cognitive impairment. At stimulation of cholinergic input, the effect of *MeCP2* sKO is to impair the response of the projection target. This may represent reduced functional connectivity of the NBM-PRH circuit.

If the electrophysiological deficits that I have documented here are indeed necessary for disruption of recognition memory performance, then I would expect that treatment with donepezil would rescue the electrophysiological impairments, as this treatment has been shown to rescue the recognition memory impairment of *MeCP2* sKO mice. This is in fact the case, consistent with the idea that these electrophysiological impairments play a role in disrupting recognition memory. Additionally, although cholinergic neurons are known to synthesize many different neurotransmitters (Allen et al., 2006; Saunders et al., 2015; Tkatch et al., 1998), the fact that donepezil rescues these impairments demonstrates an essential role of ACh *per se*.

The behavioral and electrophysiological impairments discussed above don't appear to be due to changes in cholinergic neuroanatomy. I found no decrease in the number of cholinergic cells in the NBM. This is in contrast to post mortem studies from individuals with Rett

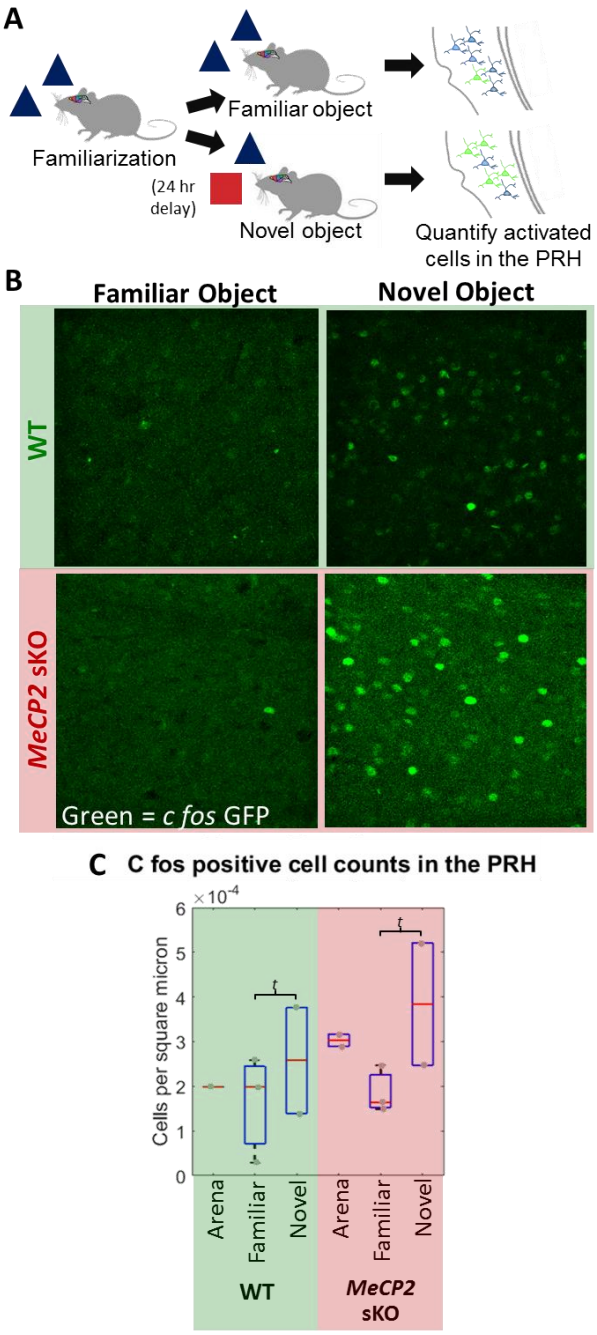


Figure 3.7: Behavioral modulation of PRH activity is intact in *MeCP2* sKO mice A: Diagram of the experimental paradigm. **B:** Sample labeling of activated cells in layers 5 & 6 of the posterior PRH of control and *MeCP2* sKO mice after exposure to a novel or a familiar object. Activated cells are labeled green. **C:** In both control and *MeCP2* sKO mice more cells are activated in the PRH after exposure to a novel object than to a familiar object. This difference is even greater in *MeCP2* sKO mice than in control mice. However, this difference does not reach statistical significance in either group (WT: Kruskal Wallis, $\chi^2(2)=0.43$, $p=0.81$, *MeCP2* sKO: Kruskal Wallis, $\chi^2(2)=4.5$, $p=0.11$). *t* = trending

syndrome, which showed a loss of cholinergic cells in the basal forebrain (Kitt et al., 1990; Wenk, 1997; Wenk and Hauss-Wegrzyniak, 1999). This suggests that cholinergic cell death is not a cell autonomous effect of *MeCP2* deletion and may be a consequence of dysfunction of surrounding cells, such as glia, or cells to which cholinergic cells project and from which they receive survival signals.

Although *ChAT* Cre, *MeCP2* flox and *MeCP2* skO all demonstrated decreases in cholinergic PRH innervation, this does not appear to explain the behavioral and electrophysiological results as only *MeCP2* sKO mice demonstrate impaired behavior, decreased variability of firing and altered PRH cholinergic responsivity. It is therefore possible that cholinergic neurons in *ChAT* Cre and *MeCP2* flox mice have the capacity to compensate for reduced fiber density. Such compensatory mechanisms may be disabled or impaired by *MeCP2* deletion. It is also possible that preserved fiber density is not critical for behavioral performance or cholinergic modulation of the PRH.

Given that the electrophysiological and behavioral phenotypes seem to be independent of observable changes in cholinergic neuroanatomy it is possible that they are a result of changes in cholinergic gene expression. This seems particularly likely given the role of *MeCP2* as a transcriptional regulator (Chahrour et al., 2008). Indeed, *MeCP2* sKO mice have been shown to

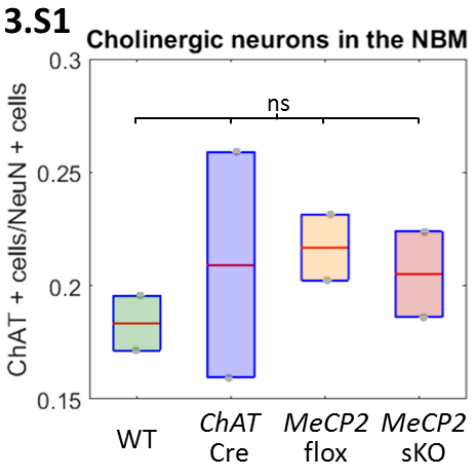


Figure 3.S1: There was no difference in the proportion of NBM neurons that were cholinergic (Kruskal Wallis, $\chi^2(3)=1.5$, $p=0.68$).

have reduced *ChAT* expression in both whole brain extracts and isolations of the basal forebrain (Schaaf and Zoghbi, 2013; Zhang et al., 2016). Future studies combining anatomical, genetic, electrophysiological and/or behavioral measurements in the same mice may be able to dissect out the relative contributions of each of these lesions to the behavioral impairment.

Finally, the finding that recognition memory encoding in the PRH is actually intact, or perhaps even enhanced, in the face of the behavioral deficits in *MeCP2* sKO mice is a bit counterintuitive. This suggests an uncoupling of PRH activity from behavioral output. Interestingly, this has been documented previously: Miller et al showed that monkeys performing a recognition memory task showed the expected decrease in response of inferior temporal cortex units when exposed to previously viewed stimuli. Administration of systemic scopolamine to these animals impaired recognition memory performance but *did not* alter this reduction-response (Miller and Desimone, 1993). ACh is anatomically positioned to function as a "binder" that couples functionally related cortical areas by modulating them coordinately (Zaborszky et al., 2015a). Thus the cholinergic lesion here may represent an uncoupling of PRH from other functionally related areas. Apergis-Schoute et al. (2007) showed that the PRH has long range GABAergic projections to the entorhinal cortex, and that these projections are inhibited presynaptically by M2 receptors. This suggests that a major role of ACh in the PRH may be to facilitate information flow through the PRH to the entorhinal cortex and perhaps to other areas. Although the entorhinal cortex *per se* is not traditionally thought to be necessary for recognition memory performance, this may be a common mechanism for cholinergic control of PRH output. Indeed, the finding of reduced responsivity of PRH units to stimulation of cholinergic input in *MeCP2* sKO mice certainly indicates that the functional integrity of the cholinergic NBM-PRH circuit is compromised. This uncoupling not only leads to impaired behavioral performance but also may lead to reduced feedback from downstream areas and thus to enhancement of the PRH response to novel object exposure, as I have shown. Further experiments are needed to map the brain areas to which the PRH is functionally bound during recognition memory in control and *MeCP2* sKO mice.

CONCLUSION

MeCP2 deletion from cholinergic neurons leads to reduced variability of PRH firing and reduced PRH responsivity to stimulation of cholinergic input. This is likely due to changes in cholinergic gene expression and may underlie the recognition memory impairment seen in *MeCP2* sKO mice. Cholinergic gene expression therefore may prove a promising target for treatment of cognitive deficits in individuals with Rett Syndrome.

Chapter 4:

General discussion and future experiments

GENERAL DISCUSSION

The aim of this thesis was to investigate how ACh encodes recognition memory in the PRH by investigating the effects of endogenous ACh release in the PRH and the consequences of a genetic cholinergic lesion known to impair recognition memory. Although the importance of cholinergic signaling in the PRH for establishment of recognition memory is well known, the actual effect of endogenous cholinergic release has not been previously investigated. Most research documenting the importance of cholinergic PRH signaling has been conducted using pharmacological blockade or physical lesion of cholinergic signaling, and has thus inferred the role of ACh by observing the consequence of its absence. However, the function of ACh in the PRH has not previously been studied directly. Existing literature has documented the loss of recognition memory performance, the loss of differential activation of the PRH upon exposure to familiar vs novel stimuli and the loss of LTD within the PRH upon impairment of cholinergic signaling. These lines of evidence have therefore converged on the hypothesis that during encounter with a novel object, cholinergic signaling induces LTD, thereby attenuating the response elicited by re-exposure to this now familiar object and this reduction-response is the signal of recognition.

My work has shown that endogenous ACh release elicits reduction-responses in the PRH at about the same rate as familiarization to a stimulus. Thus it seems likely that the effect of endogenous cholinergic-mediated LTD may indeed underlie familiarity-induced reduction-responses. However, I have found that the more common effect of endogenous ACh release *in vivo* in the PRH is actually to increase excitability (**fig 1.4A**). A vital mechanism of cholinergic encoding of familiarity may therefore be to recruit microcircuitry whose overall effect is inhibitory, but whose effect on individual, probable GABAergic, neurons is excitatory.

I have also found that when cholinergic signaling is impaired via a genetic lesion, in this case *MeCP2* deletion from cholinergic neurons and reduced expression of *ChAT*, not only is the PRH response to cholinergic stimulation impaired, but baseline PRH firing is also altered. It therefore seems likely that ACh has dual effects in the PRH, one exerted at baseline, and one exerted upon stimulation of input. These differing effects occur over different time scales and may represent an example of the temporal diversity over which ACh can exert its effects, which has only recently come to be appreciated (for review see Ballinger et al., 2016). The main effect of tonic, volume type transmission in the PRH seems to be to maintain baseline levels of variability and dynamic range over which the PRH neurons can respond and encode. The effect of transient, stimulated increases in ACh in the PRH is predominantly to increase firing rate and decrease variability, but perhaps to decrease firing in familiarity sensitive neurons. Thus the dominant effects of ACh over these differing time scales are antagonistic: the predominant effect of ACh is to increase variability at baseline, and yet decrease variability upon stimulation. This perhaps indicates recruitment of different types of receptors, with varying affinities and propensities for desensitization to ACh. It's also possible that the initiation of these effects is spatially segregated within the PRH. Tonic, volume type transmission may be mediated by different cholinergic cells within the NBM than temporally transient transmission, and these neurons may project to different areas of the PRH (Unal et al., 2012). More detailed anatomical study of the PRH, including mapping of cholinergic receptor distribution and of individual cholinergic projections, is necessary to tease apart these possibilities.

As the effects of ACh removal from *MeCP2* sKO mice is to decrease variability of PRH firing at baseline, and the predominant effect of ACh stimulation is also to decrease variability, the *primary* effect of *MeCP2* deletion from cholinergic neurons may actually be solely to impair baseline maintenance of variability. The lack of responsiveness of *MeCP2* sKO units would thus be interpreted as a "flooring" effect of their firing variability. Future experiments that employ new enzymatic ACh detectors can determine if ACh release is impaired at baseline, after stimulation, or both, in *MeCP2* sKO mice (Parikh et al., 2004). This information could prove vital towards understanding the dependence of cholinergic neurons on *MeCP2* expression, as this may be an element of phenotypic variability between cholinergic neurons.

Mapping of PRH activity in response to novel and familiar objects in *MeCP2* sKO mice indicates that encoding of recognition memory is intact in these mice, although behavioral performance is not. Thus the electrophysiological effects of genetic cholinergic impairment discussed above may be more important for facilitating PRH output than for encoding *per se*. ACh is anatomically poised to act as a binder that coordinates activity between functionally relevant areas, however the areas from which PRH may have become uncoupled in *MeCP2* sKO mice are unknown (Zaborszky et al., 2015a). Under control conditions, performance of a recognition task increases functional connectivity of the PRH with the entorhinal cortex and hippocampus (Albasser et al., 2010; McLelland et al., 2014; Staresina et al., 2012). However, lesion studies have demonstrated that recognition memory encoding is not dependent upon either

of these areas. This suggests that during recognition under normal circumstances, many different memory areas are preferentially recruited, including PRH, entorhinal cortex and hippocampus. However, recruitment of this complete circuit is not strictly necessary for recognition memory performance, and when entorhinal cortex and/or hippocampus are damaged and unavailable, the animal may recruit a secondary or perhaps partial circuit to ensure successful performance of the task. This secondary, compensatory circuit must be unavailable to *MeCP2* sKO mice, who cannot successfully perform the task in spite of successful encoding in the PRH. Further research is necessary to identify the components of this putative secondary circuit, perhaps via functional mapping of activated areas during recognition memory performance in animals with entorhinal cortex and/or hippocampal lesions.

FUTURE EXPERIMENTS

The great advantage of the approach used here, which involved stimulation of cholinergic cell bodies and the recording of their effects in a distal projection target, is that it allows the study of integrity of an entire circuit, from cell body to terminal field. However, stimulation of cholinergic neurons likely stimulates many terminal fields, some of which are likely connected to the PRH, thus the effects documented here cannot truly be attributed to cholinergic signaling in the PRH *per se*. Future studies that examine the effect of targeted stimulation of cholinergic fibers in the PRH *per se* are necessary to confirm these findings.

Additionally, release of ACh in the PRH during recognition memory has never actually been demonstrated. A crucial next step to understanding the role of ACh in encoding recognition memory is to combine recognition memory experiments with measurement of ACh release in the PRH using enzyme based amperometric microelectrodes such as those developed by Sarter and colleagues (Parikh et al., 2004).

Information gathered from these experiments, combined with the data presented here, will help lead to a comprehensive understanding of cholinergic mechanisms underlying recognition memory encoding.

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Appendix: Data locations

Item: Log of electrophysiological recordings

Location: on *in vivo* computer (Florian)
C:/Users/Florian/Documents/Liz/analysis/analysis log.xlsx

Data: list of all electrophysiological recordings by date including the following information:

- animal ID #
- whether recording was successful
- whether NBM AP's were observed
- whether recordings relocalized to PRH or elsewhere
- whether viral expression was observed
- when optogentic stimulation was delivered
- which viral lot was injected
- whether data has been sorted and analyzed
- quality of sort of each unit/recording (5-10 scale, 5/M = unsortable multiunit recording, 6 = definite unit but not separable from noise, 7 = unit with some contamination from noise, 8, 9, 10 = units with distinct clouds)

Item: raw electrophysiological data

Location: On *in vivo* computer (Florian) C:/Users/Florian/Documents/Liz

Data: folders organized by date, one folder for each day of recording. Each folder includes:

- Raw electrophysiology data files (.smr and .s2r)
- Spike sorted data (.plx and .nex files; some spike sorted data may be found on the analysis computer)
- Log sheet for recording session including:
    - stereotax coordinates for each recording collected
    - notes on each recording
    - time of optogentic stimulus
    - optogenetic stimulus protocol
    - mouse ID #
    - mouse DOB
    - viral injection information (date of injection, viral vector lot)

Item: Mouse log

Location: on desk computer (zsraine): C:/Users/zsraine/Documents/Liz/Mice.xlsx

Data: list of all mice used including the following information:

- ID#
- DOB
- Strain
- Genotype (additional genotype information can be found in my bright green genotyping binder in bottom drawer of my desk, DNA can be found in molecular side -20° freezer by Mala/Liz bench)
- Experiment done
- Date used for experiment
- Date injected
- Sex

Item: Images

Location: on desk computer: C:/Users/zsraine/Documents/Liz/Images

Item: Samples for imaging

Location: in Role lab -80° freezer:

- unsliced brains in racks labeled "Liz" and "Rotation"
- Sliced brains in black slide boxes to the right of these racks and in 3 (?) colored slide boxes on top of these racks

Item: tissue from microdissections (PFC and NAcc in aCSF; MS, NBM and striatum in RNAlater) and previously isolated RNA

Location: in Role lab -80° freezer in racks labeled "Liz" and "Rotation"