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Optogenetic studies of nicotinic contributions to cholinergic signaling in the central nervous system

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Abstract

Molecular manipulations and targeted pharmacological studies provide a compelling picture of which nicotinic receptor subtypes are where in the central nervous system (CNS) and what happens if one activates or deletes them. However, understanding the physiological contribution of nicotinic receptors to endogenous acetylcholine (ACh) signaling in the CNS has proven a more difficult problem to solve. In this review, we provide a synopsis of the literature on the use of optogenetic approaches to control the excitability of cholinergic neurons and to examine the role of CNS nicotinic ACh receptors (nAChRs). As is often the case, this relatively new technology has answered some questions and raised others. Overall, we believe that optogenetic manipulation of cholinergic excitability in combination with some rigorous pharmacology will ultimately advance our understanding of the many functions of nAChRs in the brain.

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Keywords

corelease; endogenous acetylcholine; nicotinic receptor; optogenetic; synaptic transmission and modulation

Introduction

Acetylcholine (ACh) was introduced into the scientific literature by Otto Loewi (1921) as ‘vagusstuff’ – a soluble factor that, when collected from one beating heart and transferred to another, changed the heart rate and force of contraction. Nicotinic ACh receptors (nAChRs) were described by Sir John Langley (1905), when he painted a nicotine solution onto sympathetic ganglia in the early 1900s. ACh and the nAChRs it can activate continued to lead the way in the development of the field of receptor biology as the muscle-type nAChR subunits were the first neurotransmitter receptor family to be molecularly cloned, sequenced (Sumikawa et al., 1982), and then functionally dissected by analyzing the biophysical consequences of mutating specific amino acids (Akabas et al., 1992; Karlin, 2002). Without doubt, ACh and neuromuscular junction (NMJ) nAChRs showed great promise as the iconic neurotransmitter system that would lead the way to understanding how synapses work. However, if one examines the literature on nAChRs in the central nervous system (CNS) rather than at the NMJ, the functional roles of ACh, in general, and nAChRs, in particular, in circuits and behavior remain controversial.

ACh release

There is still considerable controversy over how much, how long, and precisely where ACh is released relative to target nAChRs. Most investigators of CNS cholinergic circuits agree that the point-to-point, close apposition synapses typical of the NMJ are relatively rare in the CNS (e.g., see Picciotto et al., 2012 for recent review). The nature of ACh release in the CNS has been referred to as ‘volume transmission’ – a loosely defined concept that has been soundly and repeatedly challenged by Sarter et al. (2009, 2014). The term volume transmission – in the sense of a temporally coordinated and broadly distributed release of ACh throughout an entire brain region – seems unlikely. Recent studies of Zaborszky (2002) emphasize that the organization of basal forebrain cholinergic neurons include specific subsets of cholinergic neurons that project to distinct cortical fields, an organization that likely subserves selective release of ACh in discrete cortical domains. The concentrations of ACh achieved by phasic bursting of cholinergic neurons may be sufficient to influence a somewhat broader range of targets than in the immediate area of transmitter release. In sum, the concept of phasic release, as delineated by Sarter et al. (2009), provides a more accurate assessment of the ACh release process in the CNS as one that is longer in duration and perhaps mechanistically distinct from ‘classic’ fast exocytosis (reviewed in Parikh and Sarter, 2008; Sarter et al., 2009; Hasselmo and Sarter, 2011).

Nicotinic synapses and cholinergic nuclei

In the CNS, ACh release sites that are in close proximity to target nicotinic receptor pools characteristic of classical synapses have been difficult to demonstrate (but see Jones and Yakel, 1997; Alkondon et al., 1998; Frazier et al., 1998; Jones et al., 1999; Huh and Fuhrer,

2002; Klein and Yakel, 2006; Dunant et al., 2010). ACh released from ‘en passant’ cholinergic axons appears to interact with receptors localized on presynaptic terminals (i.e., axo-axonic synapses) and in preterminal zones as well as on perisynaptic or more traditional postsynaptic sites along dendrites (see Picciotto et al., 2012 for recent review and references therein).

Further complexity derives from the fact that cholinergic nuclei in the basal forebrain – including the diagonal band of Broca (DBB), medial septum (MS), and the nucleus basalis of Meynert (NBM) that contain the bulk of cholinergic projection neurons to neocortex, hippocampus, and amygdala – are heterogeneous populations of cholinergic neurons intermixed with a significant complement of neurons that are ‘not’ cholinergic (Henny and Jones, 2008). Add to that the highly branched nature of the cholinergic projections within their terminal fields, the common sites of projection of the cholinergic and noncholinergic neurons from the basal forebrain nuclei, and the increasing evidence for corelease of ACh with other fast transmitters and peptides (Eckenstein and Baughman, 1984; Richardson and Brown, 1987; Allen et al., 2006; Ren et al., 2011; Sun et al., 2013), and what emerges is a highly complex modulatory system whose understanding requires new tools for selective activation and/or inactivation of cholinergic inputs *in vivo*.

What we knew about nicotinic contributions to cholinergic circuits before optogenetics

Before assessing the potential for optogenetic approaches to advance our understanding of how nicotinic receptors contribute to the effects of cholinergic signaling in the CNS, we summarize the preoptogenetic literature that guides our current knowledge of cholinergic signaling in the modulation of CNS circuits and behavior. The combination of approaches afforded by the battery of increasingly specific pharmacological tools with methods for selective elimination of CNS cholinergic neurons has provided a solid foundation of research upon which we can build to better understand the physiology of cholinergic circuits in the CNS.

Extensive cholinergic projections and a panoply of AChRs

There are two classes of cholinergic neurons in the CNS, both of which elaborate dense axonal arbors – cholinergic projection neurons and cholinergic interneurons (CINs). Cholinergic projections from the brainstem nuclei of the pedunculopontine and laterodorsal tegmental areas and from the basal forebrain cholinergic nuclei contribute to terminal fields within select subcortical and cortical regions of the frontal, temporal, and parietal lobes (Mesulam, 1995 ; Zaborszky, 2002). CINs in the striatum (dorsal and ventral) are renowned for their dense projections and broad (but complex) roles in movement and motivation. In addition, there is a small number of CINs that are scattered through cortical regions, although the numbers differ between species (e.g., von Engelhardt et al., 2007).

ACh interacts with a wide variety of both ionotropic receptors (nicotinic; nAChRs) and metabotropic receptors (muscarinic, mAChRs). The nAChRs are cation selective, some with greater calcium permeability than others, and all subject to some degree of agonist-

dependent desensitization (Clementi et al., 2000; Picciotto et al., 2002; Lester et al., 2004; Giniatullin et al., 2005; Jensen et al., 2005; Gay and Yakel, 2007; Arias, 2010; Mineur and Picciotto, 2010; Yakel, 2010, 2013; Miwa et al., 2011). By judiciously targeting nAChRs to high impedance (hence high impact) locales, they can exert influence on synaptic excitability much greater than expected for receptors that are far less abundant than the ionotropic glutamate and γ -aminobutyric acid (GABA) receptors in the CNS (McGehee and Role, 1996 ; Wonnacott, 1997 ; Girod et al., 1999; MacDermott et al., 1999; Dani, 2001; Wonnacott et al., 2006; Dani and Bertrand, 2007; McKay et al., 2007; Exley and Cragg, 2008; Marchi and Grilli, 2010). Likewise, the metabotropic members of the AChR family exert considerable influence on synaptic excitability: different types of mAChRs abound to confer cholinergic activity at presynaptic and postsynaptic sites and their activation is amplified through G protein-coupled, second messenger cascades. In general, activation of M1, M3, and M5 mAChRs, which are linked to $G_{aq/11}$ -containing heterotrimeric G proteins, increases excitability by a variety of mechanisms including closing K^+ channels and promoting Ca^{2+} influx (e.g., Brown, 2010; Giessel and Sabatini, 2010; Drever et al., 2011), whereas activation of M2 and M4 mAChRs, negative regulators of adenylate cyclase, is typically inhibitory, increasing K^+ conductance, decreasing excitability, and/ or decreasing transmitter release (e.g., Higley et al., 2009; see Wess, 2003 for review).

Given the plethora of both nicotinic and muscarinic receptors with which to interact, there is considerable opportunity for ACh to differentially influence circuit excitability.

Pharmacological approaches to selective activation and inhibition of specific nicotinic-type receptors and genetic deletion of nAChR subunits have helped define the many possible types of nicotinic receptor-mediated responses to released ACh (e.g., Picciotto et al., 2001 ; Wess, 2003; Taly et al., 2009 ; Changeux, 2010). Likewise, selective deletion of specific populations of cholinergic neurons, through 192-IgG saporin targeting of p75 (Wiley, 1996 ; Wenk, 1997 ; Wrenn and Wiley, 1998), expressed by many but not all basal forebrain cholinergic neurons, and toxins targeted against ACh transporters (e.g., AF64A) (Sandberg et al., 1984; Schliebs et al., 1996; Gonzalez-Reyes et al., 2012), has revealed sets of behaviors for which specific cholinergic circuits are required. There is no doubt that cholinergic signaling in the CNS is important to a wide variety of behaviors ranging from cued attention to motivation, encoding both positive and negative salience to aspects of learning and memory and to the extinction of memories (Picciotto et al., 2002, 2008 ; Semenova et al., 2012; Paolone et al., 2013; Soll et al., 2013).

But even with all of the sophisticated pharmacological and genetic tools to manipulate specific classes of AChRs and the ability to eliminate cholinergic neurons with toxins, we have not yet been able to answer the question of ‘what is the mechanism(s) by which cholinergic neurons contribute to behavior X?’ There is no simple rule to define the effects that release of ACh in any given brain region may have on a nearby cholinergic circuit. The net effects will depend on the locus and concentration of ACh released; the extent of diffusion versus degradation of ACh by the highly efficient acetylcholinesterase (AChE); and the specific receptor populations, their numbers and their spatial disposition at presynaptic, postsynaptic, and perisynaptic sites. When a system is as complicated as this, it is often the development of new experimental approaches combined with established techniques that proves to be the key to interrogating the system at higher resolution.

The now decade-old introduction of optogenetic technology has, indeed, been particularly helpful in dissecting cholinergic signaling, as it provides the opportunity to rapidly, selectively, and reversibly manipulate the excitation pattern of specific sets of cholinergic neurons. Although optogenetics certainly does not resolve all of the challenges delineated above, in many cases, it ‘has’ offered important confirmations and extensions of prior work. Perhaps most important, optogenetic manipulation of cholinergic neurons in different brain regions has revealed new mechanisms and compelled us to face new complexities (see Surmeier and Graybiel, 2012 for perspective). The current review addresses what we have learned, how we have had to expand our thinking about the direct and modulatory effects of ACh in the CNS, and delineates some old and new questions that remain to be answered.

Optogenetics, a very brief primer

The engineering of genes encoding light-sensitive ion channels [e.g., channelrhodopsin (ChR2) or halorhodopsin (NpHR) and variants] from a number of simple organisms allows efficient expression and axonal targeting of these proteins in mammalian neurons (Zhang et al., 2007a,b, 2011; Deisseroth, 2011; Fenno et al., 2011; Madisen et al., 2012). Delivering these modified genes in viral vectors that restrict expression to specific populations of cells, typically as a result of constructing the viral vectors so that expression of the light-sensitive channel is dependent on Cre recombinase, allows highly selective, light-stimulated changes in excitability of these neurons (Fenno et al., 2011; Tye and Deisseroth, 2012). To be experimentally useful, these recombinant channels need to have minimal basal effects (i.e., light-independent) on membrane properties and cell viability, and they need to respond to light trains approximating typical neuronal firing rates. In their early studies, Deisseroth and colleagues addressed many of these issues, demonstrating that ChR2 does not significantly change basal electrical properties of hippocampal neurons (Boyden et al., 2005). In addition, ChR2 did not compromise cell health; even after exposing ChR2-expressing neurons to blue light, the membrane integrity (membrane resistance and resting potential, propidium iodide uptake), and basal electrical properties (spike count elicited from somatic current injection) were similar to nonexposed ChR2⁺ neurons (Boyden et al., 2005). Kalmbach et al. (2012) have addressed the effect of expressing ChR2 selectively in cholinergic neurons following infection with recombinant adeno-associated viruses (AAVs); no adverse effects on general neuronal health, electrical properties, or glial activation were seen as long as injection volumes were kept below 500 nl. To achieve temporal precision of fast biological information processing, optogenetics must work on a millisecond timescale to allow modulation of the precise activity pattern; indeed, as discussed below (see ‘Modulation/indirect effects of ACh on transmission in hippocampus and striatum’ and ‘Optogenetic studies of cholinergic modulation in cortex’), the stimulation paradigm used can significantly affect the results obtained. Temporal and spatial resolution of temporally focused laser pulses allows manipulation of neuronal activity (Andrasfalvy et al., 2010). Given that the fastest mechanical shutter can open and close at about 40 Hz, a light-emitting diode (LED) or laser may be the best choice of light source when higher-frequency stimulation is required. As an early optogenetic tool, ChR2 is sufficient for the formation of functional channels and exciting transfected neurons, but its rapid inactivation property limits the generation of high-fidelity action potentials over 30 Hz (Boyden et al., 2005; Wang et al., 2007). The response to subsequent light stimulation declines significantly after

the initial response (Cruikshank et al., 2010). To solve this problem, Lin et al. (2009) developed new ChR variants: ChEF, a chimera between ChR2-1 and ChR2-2 with additional point mutations introduced around the retinal-binding pocket, has reduced inactivation kinetics. ChIEF, in which mutation of isoleucine 170 of ChEF to valine, enhances the rate of channel closure after stimulation, allows more precise temporal control of depolarization, and permits activation of trains of action potentials in response to high-frequency (50 Hz) light stimulation (Lin et al., 2009; for review of ChR variant kinetics, see Lin, 2011; Yizhar et al., 2011).

Another caveat to using ChR2 and its derivatives is that these are nonspecific cation channels, allowing both Na^+ and Ca^{2+} to enter stimulated cells (Nagel et al., 2003). To address the potential contribution of ChR2 carried calcium, Oertner and colleagues used two-photon calcium imaging to demonstrate that although calcium transients evoked by light were significantly larger than those evoked by brief somatic current injection, the additional calcium influx during light stimulation was mainly due to increased activation of voltage-gated calcium channels (VGCCs) (Zhang and Oertner, 2007; Schoenenberger et al., 2011). The additional Ca^{2+} influx, whether via ChR2 or VGCCs, could change the presynaptic transmitter release probability and/or postsynaptic plasticity – factors that should be kept in mind when interpreting results using ChR2 and its variants.

One of the most powerful applications of optogenetic approaches is the ability to stimulate or inhibit transmitter release *in vivo* and then assay effects on specific behaviors. *In vivo* stimulation of ChR-expressing cells is typically achieved by transmission of light through a fiber optic, ranging from 100 to 400 μm core diameter, either implanted directly or inserted via a guide cannula (identical to a standard drug delivery cannula) (Zhang et al., 2010 ; Anikeeva et al., 2012). In either case, the implants are similar in size and shape to other commonly implanted devices such as *in vivo* recording electrodes and microdialysis probes and are expected to cause similar levels of tissue damage. Advantages of directly implanting a fiber optic include lower susceptibility to infection and smaller implant diameter (causing less damage to brain tissue). Advantages of using a guide cannula include higher efficiency and reproducibility of light transmission, the capability to deliver drugs to the target area, and lower cost. Light sources are most often a laser or LED coupled to an optical fiber. High-powered lasers can easily produce 10–15 mW of light at the fiber tip, an appropriate range for *in vivo* photostimulation (Zhang et al., 2010). Current LEDs are capable of producing sufficient wattage for stimulation and can be mounted directly on the head of an animal but cannot achieve the power levels that a laser can. Both LEDs and lasers deliver temporally precise (μs timescale) pulses of light. ChR2 and its variants have a peak excitation wavelength of approximately 450 nm, and a 473 nm laser or LED is typically used for stimulation. A recently engineered red-shifted ChR variant, ReaChR, can be excited with 590– 630 nm wavelength (Lin et al., 2013). NpHR and its variants have a peak excitation wavelength of about 570 nm, and a 594 nm laser or LED is typically used for stimulation.

Several studies have examined how light is scattered and absorbed in brain tissue. Generally, light intensity drops to 90% of its initial power from 500 to 1000 μm from the fiber tip, allowing precise and predictable spatial control of the stimulation (Aravanis et al., 2007;

Yizhar et al., 2011). Even greater spatial control can be achieved by modifying the fiber tip, for example, by adding a 'shield' on one side (Tye et al., 2011) or grinding the tip to create a diffuser lens. More advanced models of the power and distribution of light from a fiber tip in brain tissue have been developed (Bernstein et al., 2008).

Another cautionary note for studies dealing with genetically modified mice or rats: not all gene targeting is benign. This point has been poignantly addressed in a recent report demonstrating significant baseline effects on both synaptic transmission and behavior in mouse lines carrying cholinergic locus transgenes (Kolisnyk et al., 2013). Clearly, any combination of optogenetics and targeted gene manipulations must be considered in the context of extensive controls.

Expected results: optogenetic studies demonstrating direct transmission and modulatory effects of ACh

Unraveling the contribution of nAChRs to the fine-tuning and long-term plasticity of cholinergic circuits in the CNS is a complex but important task. In the CNS, ACh can interact with nAChRs and exert both direct and indirect or neuromodulatory effects on presynaptic and/or postsynaptic excitability (Figure 1A and 1B) (Picciotto et al., 2012; Sarter et al., 2014). The CNS effects of ACh include altering presynaptic release probability of an array of fast synaptic transmitters (e.g., GABA and glutamate) or modulators [e.g., dopamine (DA), serotonin, and ACh itself] as well as modulating the firing rates of specific neurons. With optogenetic techniques, some established ideas have been reinforced while, at the same time, previously unknown, circuit mechanisms have been revealed.

ACh as a direct transmitter via nAChRs in the CNS

Direct postsynaptic nicotinic currents were found in hippocampal interneurons by electrical stimulation of cholinergic inputs more than a decade ago (e.g., Alkondon et al., 1998; Jones et al., 1999; Huh and Fuhrer, 2002; Klein and Yakel, 2006; Dunant et al., 2010). More recent studies of transgenic models of green fluorescent protein (GFP)-expressing cholinergic neurons have also convincingly demonstrated direct synaptic transmission of cholinergic inputs to pyramidal neurons in the hippocampus (Grybko et al., 2011).

Recent optogenetic studies of cholinergic circuits in hippocampus both affirm prior findings and extend our current understanding. By combining a variety of genetically modified mouse lines that allow selective Cre-dependent expression in cholinergic neurons with AAV-based delivery of ChR2 (or NpHR/Arch), investigators use light to selectively regulate cholinergic neuron excitability and locally control ACh release in cholinergic terminal fields in select brain regions. For example, light stimulation of septal-hippocampal projections from cholinergic neurons expressing ChR2 affirmed prior findings of direct nicotinic activation of GABAergic interneurons in CA1 of hippocampus and allowed pharmacological demonstration that $\alpha 4\beta 2^*$ nicotinic receptors were the primary mediators of these direct nicotinic, excitatory postsynaptic potentials (EPSPs) (Bell et al., 2011).

A similar study, with somewhat different conclusions, has emerged from Alger and colleagues (Nagode et al., 2011). These authors show that optogenetic stimulation of

MS/DBB cholinergic neurons induced rhythmic bursts of perisomatic GABAergic inhibitory postsynaptic currents (IPSCs) in CA1 pyramidal neurons (Nagode et al., 2011). These IPSC bursts greatly outlasted the light stimulation and were, for the most part, mediated by mAChRs. It should also be noted that, in this study, the optogenetic stimulation of ACh release alone was not sufficient to elicit the rhythmic bursting; the addition of cholinesterase inhibitors and 4-AP (a K⁺ channel antagonist) was required to reliably generate the light-evoked IPSCs. In addition, Nagode et al. (2011) demonstrated that light-evoked stimulation of interneuron activity could synchronize pyramidal neuron firing as seen in prior studies using electrical stimulation. A more recent study from the same group suggested that optogenetic release of endogenous ACh from septal afferents induces rhythmic, θ -frequency IPSCs in CA1 pyramidal neurons, and these rhythmic IPSCs are equally sensitive to type 1 cannabinoid receptor (CB1R) and m-opioid receptor (MOR) activation (Nagode et al., 2014).

Perhaps the most comprehensive study, integrating both electrical and optogenetic stimulation of cholinergic inputs to hippocampal circuits, is the recent work of Yakel and colleagues. These studies provide evidence that both the direct activation of GABAergic interneurons in hippocampal stratum oriens and the indirect modulation of pyramidal neurons in CA1 by ACh are essential to cholinergic timing-dependent synaptic plasticity (Berg, 2011; Gu and Yakel, 2011; Gu et al., 2012).

The potential role of nAChR-mediated signaling in cholinergic circuits in cortex has also been reexamined with combined optogenetic stimulation and electrophysiological recording. Using mice in which ChR2 was selectively expressed in basal forebrain cholinergic neurons, Arroyo et al. (2012) found that cholinergic fibers made direct contact with a subset of GABAergic interneurons in sensorimotor cortex. Photostimulation of ACh fibers evoked direct cholinergic EPSPs in all classes of layer 1 interneurons (L1) as well as in layer 2/3 (L2/3) late-spiking interneurons and L2/3 ChAT-expressing bipolar interneurons (Arroyo et al., 2012). The same optogenetic stimulation protocol did not affect the L2/3 fast-spiking interneurons (Arroyo et al., 2012). The cortical cholinergic postsynaptic response had two components: a large fast component, presumably mediated by $\alpha 7$ -containing nAChRs ($\alpha 7^*$, where the * indicates that the subunits that comprise the receptor include, but may not be limited to, the indicated subunit) that was blocked by methyllyl-caconitine (MLA) and a small, slow component that was blocked by antagonists of $\beta 2^*$ nAChRs [e.g., dihydro- β -erythroidine (DHbE)] (Arroyo et al., 2012). L1 and L2/3 late-spiking cells exhibited both a fast and a slow component, while L2/3 CINs had only the slow component of synaptic cholinergic input (Arroyo et al., 2012).

Perhaps the most enlightening piece provided by these optogenetic studies of the role of cholinergic afferents from basal forebrain to somatosensory cortex is in the identification of the loci and mechanisms of direct effects of ACh in the context of circuit output. It seems quite clear that, in somatosensory cortex, $\beta 2^*$ nAChRs elicit action potentials in L1 GABAergic interneurons (direct) (Arroyo et al., 2012). This in turn results in a delayed and prolonged, di-synaptic, inhibitory barrage in L2/3 glutamatergic pyramidal neurons (Arroyo et al., 2012). Using optogenetic stimulation of CINs, direct cholinergic transmission was also demonstrated to control specific GABAergic circuits by regulating spiny projection

neuron firing in neostriatum (English et al., 2012). Optogenetic activation of neostriatal CINs elicited direct nicotinic EPSPs and triggered action potential firing in neuropeptide Y (NPY)-expressing neurogliaform (NGF) interneurons. The optogenetic elicited action potentials were blocked by DHbE, consistent with $\beta 2$ -containing nAChRs mediating the effect.

It should also be noted that many optogenetic studies have further emphasized the role of muscarinic receptors mediating the actions of endogenously released ACh, which, although not the focus of this review, is an important component of cholinergic signaling in the CNS. Stimulation of cholinergic MS/DBB causes direct depolarizing, hyperpolarizing, and biphasic muscarinic responses in hippocampal CA1 interneurons (Bell et al., 2013). These investigators also reported that the depolarizing response required more intense light stimulation (a train of 120 light pulses at 20 Hz) of the cholinergic terminal fields than was required for hyperpolarization (20 pulses at 20 Hz). Likewise, elevating extracellular ACh with an AChE inhibitor had a larger effect on the depolarizing responses versus hyperpolarizing responses. Using pharmacological tools, these investigators delineated that M4 receptors mediated the hyperpolarizing responses by activating inwardly rectifying potassium channels. Activation of M4 receptors potentiated the amplitude of the hyperpolarization and significantly altered biphasic interneuron firing patterns. The receptor mediating the depolarizing response is less well defined, as it was unaffected by M1, M4, or M5 receptor modulators (Bell et al., 2013). The inhibition of the cholinergic depolarization by atropine, although consistent with a muscarinic receptor mechanism, does not exclude a nAChR-mediated component, as atropine is known to have off-target effects on certain nAChR subtypes (Zwart and Vijverberg, 1997).

Overall, optogenetic studies in hippocampus (Bell et al., 2011; Gu and Yakel, 2011; Nagode et al., 2011; Gu et al., 2012), cortex (Letzkus et al., 2011; Arroyo et al., 2012), and striatum (Witten et al., 2010; Cachepe et al., 2012; English et al., 2012; Threlfell et al., 2012) corroborate prior findings and provide additional strong evidence for nicotinic cholinergic transmission in the CNS. In general, it appears that the most common targets for such direct effects of ACh are GABAergic interneurons in hippocampal (Bell et al., 2011, 2013; Nagode et al., 2011), striatal (English et al., 2012), and neocortical (Letzkus et al., 2011; Arroyo et al., 2012) regions. The direct cholinergic transmission to GABAergic interneurons is mediated by $\beta 2^*$ nAChRs (Arroyo et al., 2012) and, most likely, mAChRs (Bell et al., 2013) depending on the brain region. Although optogenetic activation of ACh release and consequent activation of $\alpha 7^*$ nAChRs can elicit large, fast postsynaptic responses, the long slow depolarizations mediated by $\beta 2^*$ nAChRs, and in some cases mAChRs, predominate in the cholinergic activation of GABAergic interneurons (Arroyo et al., 2012; Bell et al., 2013). The net effect of optogenetic activation of cholinergic input to the GABAergic neurons is to elicit bursts of inhibitory activity that in turn synchronizes circuit activity (Nagode et al., 2011, 2014; English et al., 2012; Bell et al., 2013).

Modulation/indirect effects of ACh on transmission in hippocampus and striatum

The preoptogenetic literature on ACh effects in the CNS documents the many ways in which ACh can modulate neuronal excitability and regulate transmitter release (Wonnacott, 1997;

Girod et al., 1999; MacDermott et al., 1999; Dani, 2001; Wonnacott et al., 2006; Dani and Bertrand, 2007 ; McKay et al., 2007 ; Exley and Cragg, 2008; Brown, 2010; Marchi and Grilli, 2010; Drever et al., 2011; Picciotto et al., 2012). Both major classes of ACh receptor – nAChRs and mAChRs – have been implicated as mediators of modulatory effects of ACh on circuit excitability.

The concept of a ‘modulator’ has been defined in a number of ways. We will use it here (as in Picciotto et al., 2012) to refer to indirect actions of ACh, that is, circumstances under which ACh release and interaction with AChRs precipitates a cascade of events leading to alterations in presynaptic activity (i.e., increased or decreased release of fast transmitters such as GABA and glutamate as well as changes in the release of other modulatory transmitters such as DA or serotonin). Electrophysiological and pharmacological studies in the preoptogenetic era demonstrated that a prominent effect of ACh is to modulate transmitter release. Activation of presynaptic (axon terminal) nicotinic receptors can enhance glutamatergic transmission in prefrontal cortex (Vidal and Changeux, 1993), hippocampus (Gray et al., 1996), habenula (McGehee et al., 1995), ventral tegmental area (VTA) (Mansvelder and McGehee, 2000), and amygdala (Jiang and Role, 2008). Likewise, action potential-independent [tetrodotoxin (TTX)-resistant] modulation of GABA transmission has been demonstrated in lateral hypothalamus (Jo et al., 2005), VTA (Mansvelder and McGehee, 2000 ; Yang et al., 2011), spinal cord (Liu et al., 2011), and ventral striatum (Britt and McGehee, 2008). The effects of ACh on action potential-independent DA release, especially in striatum, has been long been debated and is perhaps finally resolved by recent optogenetic studies of Cragg and Cheer and their colleagues (Cachope et al., 2012; Surmeier and Graybiel, 2012; Threlfell et al., 2012).

The activation of presynaptic mAChRs has been implicated in the modulation of release of the same array of transmitters in a similar set of brain regions, often in a direction opposite to that elicited by nAChR activation (Brown, 2010; Drever et al., 2011; Picciotto et al., 2012). The net effects of mAChR activation in the CNS include postsynaptic excitation, postsynaptic inhibition, and presynaptic (auto) inhibition (Brown, 2010; Drever et al., 2011). Presynaptic inhibition typically results from the activation of M2 or M4, $G_{i/o}$ -linked receptors that inhibit VGCCs. Presynaptic mAChRs have been shown to inhibit the release of glutamate, GABA, and ACh (Sugita et al., 1991 ; Rawls et al., 1999 ; Li et al., 2007). M2 receptors are present on medial septal hippocampal cholinergic terminals, where they can significantly inhibit ACh release, thereby introducing a negative feedback loop following activation of septal-hippocampal cholinergic circuits (Zhang et al., 2002; Li et al., 2007; Brown, 2010; Drever et al., 2011).

mAChR activation can either enhance the excitability of inhibitory GABAergic interneurons (as above) or decrease inhibition by decreasing the synaptic release of GABA (Sugita et al., 1991) depending on the brain region and experimental configuration. There is no general rule as to the effects of mAChR activation in a given brain region. As such, one might expect that the selective activation of endogenous ACh release using optogenetics would help to sort out the contributions of specific mAChRs in a particular circuit.

What has optogenetics added to our understanding of cholinergic modulation? Using electrophysiology, optogenetic, and pharmacologic manipulations, Yakel's group has again made an important set of contributions to deciphering the role of cholinergic signaling in hippocampus. In addition to the direct effects of endogenous ACh on GABAergic interneurons, these investigators found that septal cholinergic induction of hippocampal synaptic plasticity is timing dependent and that this effect of timing involved both direct and modulatory effects of ACh (Berg, 2011; Gu and Yakel, 2011; Gu et al., 2012). These phenomena were first observed with electrical stimulation of the stratum oriens – a major cholinergic terminal field. Using selective optogenetic stimulation of the cholinergic fibers in stratum oriens, the authors replicated the results precisely (Gu and Yakel, 2011). Using optogenetics and MS/ hippocampal slice coculture, the same group determined that both presynaptic and postsynaptic cholinergic activities contribute to the timing dependence of cholinergic hippocampal synaptic plasticity (Gu and Yakel, 2011).

The dorsal and ventral striatum remain unmatched as sites for major controversies on how and what cholinergic signaling does to circuit activity. In the striatum, the CINs comprise only 1–5% of the total neuronal population (depending on the species), but the cholinergic projections branch and ramify throughout both dorsal and ventral areas resulting in a dense network of ACh release sites (Zhou et al., 2002; Britt and McGehee, 2008; Threlfell et al., 2010; Threlfell and Cragg, 2011).

The CINs or the tonically active neurons (TANs) are known for their characteristic 2–8 Hz spontaneous action potential firing. In monkey behavior studies, the CINs display a pause in their tonic firing activity after a conditioned stimulus, which becomes salient when it links to reward (Aosaki et al., 1994; Morris et al., 2004; Joshua et al., 2008). A similar pause in firing of the CINs has also been observed in rats (Schulz et al., 2011) and mice (Ding et al., 2010; Straub et al., 2014). The mechanisms underlying the pause in firing of the CINs might be suppression from midbrain DA neurons (Morris et al., 2004; Chuhma et al., 2014; Straub et al., 2014), inhibition from VTA GABA neurons (Brown et al., 2012), or glutamatergic excitation from thalamus-generated intrinsic afterhyperpolarization (Schulz et al., 2011). ACh and DA are well known for their complementary role in maintaining balance on signal integration in the striatum. Recent work emphasizes the intricate interactions of cholinergic and dopaminergic signaling in processing of saliency in striatum (e.g., see Cragg, 2006; Surmeier and Graybiel, 2012; Schulz and Reynolds, 2013; Straub et al., 2014).

Prior to the development of methods for the selective identification of these neurons and their terminals, selective stimulation of cholinergic projections was difficult. Because of the sparse distribution of cholinergic neurons among noncholinergic neurons and the dense networks of mixed terminal fields, extracellular electrical stimulation in the dorsal and ventral striatum evokes release of a mixture of transmitters. Even with direct electrical stimulation of individual striatal cholinergic neurons, the results obtained have been confusing. Can optogenetics help us dissect the contributions of endogenous ACh release to activity in ventral and dorsal striatum?

One might argue that the most important measure of the contribution of ACh release to striatal output would be obtained by selectively stimulating versus inhibiting striatal

cholinergic neurons and measuring the effects on animal behavior. Deisseroth and colleagues (Witten et al., 2010) provided this ‘bottom-line’-type analysis of cholinergic signaling in ventral striatum, demonstrating that optogenetic inhibition of CINs diminished reward-related behavior in a cocaine conditioned place preference task. Furthermore, their analysis of mechanism demonstrated that optogenetic excitation of CINs [by activation of ChR2, selectively expressed in choline acetyltransferase (ChAT)-positive neurons] inhibited 80% of medium spiny neurons (MSNs) but excited 20% of MSNs. Conversely, direct inhibition of CINs in striatum using the inhibitory optogenetic probe (eNpHR3.0) while recording from MSNs revealed that suppression of ACh release resulted in a net increase in firing of about 75% of MSN and decreased activity in the remaining population (Witten et al., 2010). These findings are largely as expected based on the prior literature, but they add an additional dimension. Deisseroth and colleagues demonstrated that selective activation of ventral striatal cholinergic neurons directly affected cocaine-related behaviors and that the effect of cholinergic circuits on cocaine-related behavior was mediated by direct and/ or indirect regulation of the activity of MSNs. Neither the contribution of muscarinic versus nicotinic pathways nor the role of direct ACh actions versus indirect modulatory effects was addressed in this study. Subsequent studies have focused more on the mechanisms underlying the contribution of ACh to striatal circuits.

As discussed above, there is increasingly strong evidence for direct, AChR-mediated transmission to GABAergic neurons in several brain regions, including the striatum (Koo and Tepper, 2002; Khiroug et al., 2003). Combining ChAT-Cre mice, vectors allowing Cre-dependent expression of optogenetic probes in striatal CINs, and voltammetry assays of DA release, Cheer and colleagues showed that optogenetic stimulation of nucleus accumbens (nAcc) CINs was sufficient to elicit DA release both in slices and *in vivo* (Cachope et al., 2012). Optical stimulation in trains and in the presence of muscarinic antagonists elicited significant increases in DA release that were mediated by activation of $\beta 2^*$ nAChRs (Cachope et al., 2012). A portion of this nAChR-activated DA release was blocked by AMPA receptor antagonists. In other words, optogenetic activation of nAcc CINs by trains of stimulation increases local DA release in accumbens but, according to Cachope et al. (2012), this is due in part to direct actions of ACh via nicotinic receptors on DA terminals and in part to indirect actions mediated by glutamate. What is less clear is whether the GluR-mediated component of DA release arises from ACh modulation of glutamate release, which subsequently increases DA release, or if it is due to corelease of glutamate with ACh from the CINs in striatum (as discussed in “Optogenetic activation of ‘cholinergic neurons’ reveals corelease and other less expected findings”).

Perhaps the most compelling evidence for a presynaptic (axo-axonic) mechanism of cholinergic modulation in striatum is provided by studies comparing electrical and optogenetic stimulation of the same CINs combined with electrophysiological and DA release assays by voltammetry (Threlfell et al., 2012). Cragg and colleagues showed that optogenetically synchronized activity in multiple CINs elicited robust release of striatal DA that was independent of activation of DA projection neurons. In contrast, single flash activation of cholinergic neurons failed to evoke detectable DA release, even when multiple action potentials were fired by the cholinergic neuron (Threlfell et al., 2012). This report,

although similar in approach to the work of Cheer and colleagues combining optogenetic activation of cholinergic neurons and voltammetric measures of DA release, requires a new view of the role of ACh in the striatum: ACh may play a primary role in controlling DA release in the striatum, interacting as the authors say ‘through a privileged relationship’ with DA terminals to short-circuit action potential activity in the DA projection neurons and directly cause DA release (Threlfell et al., 2012).

Optogenetic studies of cholinergic modulation in cortex

The major cholinergic afferents to neocortex are from NBM. Kalmbach et al. (2012) delivered selective optogenetic stimulation to NBM cholinergic axons in neocortex and found that light-evoked release of ACh desynchronized the local field potential (LFP). These effects of optogenetic stimulation of cholinergic input to cortex could be blocked by mixed atropine and mecamylamine, consistent with contributions of both mAChRs and nAChRs (Kalmbach et al., 2012). A striking result, which differs from findings with electrical stimulation of the NBM, is that this optogenetic stimulation protocol applied in the neocortical terminal fields evoked only a few seconds, rather than tens of seconds, of desynchronization (Kalmbach et al., 2012).

Optogenetic activation of cholinergic neurons in the horizontal limb of the DBB, which send their fibers to the main olfactory bulb, inhibited the spontaneous firing activity of all major olfactory bulb cell types (Ma and Luo, 2012). The inhibition was significant with 10 Hz light stimulation and was further enhanced at higher frequencies. In view of the differences between the frequency-sensitive effects in the olfactory bulb and the frequency-independent effects on DA release in the striatum (Threlfell et al., 2012), the mechanisms by which cholinergic modulation controls excitability in these two areas is likely very different.

Another particularly comprehensive study of the effects of cholinergic stimulation by Dan and colleagues revealed that optogenetic activation of the cholinergic neurons in the basal forebrain or of their axon terminals in the V1 terminal field improved performance of a visual discrimination task (Pinto et al., 2013). Blue light was delivered to the basal forebrain to activate cholinergic neurons, and both LFP and spiking activity from all layers in the V1 of awake mice were recorded. Optogenetic stimulation reliably desynchronized cortical LFP by reducing the power at low frequencies and increasing the power at high frequencies. This effect occurred rapidly after optogenetic stimulation and returned to baseline after laser offset. This is in contrast to electrical stimulation of the basal forebrain, in which the desynchronization lasts for 5–20 s. The authors then tested whether optogenetic stimulation improves visual perception with different task difficulty by adjusting the contrast; optogenetic activation of basal forebrain cholinergic neurons improved performance across all contrasts tested. Direct optogenetic activation of the cholinergic axon terminals in V1 significantly reduced the low-frequency LFP power and improved performance in the discrimination task. However, V1 stimulation improved the performance at 20% and 40% contrasts but not at 100% contrast, suggesting that the effect of basal forebrain activation may consist of a V1-mediated perceptual improvement at low contrasts and a nonperceptual component through other pathways at high contrast. They found that activation of basal fore-brain cholinergic neurons not only increased V1 firing rates at 20%, 40%, and 100%

contrast tested but also increased the spontaneous firing rate (i.e., 0% contrast), thus causing a shift in the baseline of contrast response, similar to the effect of local application of an ACh agonist. The authors also selectively inactivated basal forebrain cholinergic neurons in ChAT-ARCH and ChAT-HALO mice. Optogenetic inactivation synchronized V1 LFP, reduced the spontaneous firing rate at the single neuron level in V1, and impaired behavioral performance.

A recent minireview (Arroyo et al., 2014) discusses the cell-type specificity of nicotinic receptor expression, synaptic mechanisms mediating direct cholinergic transmission, and functional role of nicotinic receptor activation as revealed by optogenetic studies in the cortex.

In summary, optogenetic studies of cholinergic circuits have affirmed and significantly extended our knowledge of the modulatory effects of ACh via both nAChRs and mAChRs in several brain areas. Given the rapid rate of receptor desensitization and the unknown concentrations of ACh in and around synapses under both basal and stimulated conditions, it has been difficult to assess the physiological effects of endogenous ACh by application of exogenous AChR agonists and antagonists (Surmeier and Graybiel, 2012), a limitation circumvented using optogenetic approaches. Likewise, the ability to dissect the difference between activation of one versus coordinate activation of multiple cholinergic neurons has revealed new, previously unappreciated roles for action potential-independent modulation of DA release in intact striatum (Surmeier and Graybiel, 2012; Threlfell et al., 2012).

Optogenetic activation of ‘cholinergic neurons’ reveals corelease and other less expected findings

The use of genetically modified mice and optogenetic techniques has certainly facilitated the dissection of some of the physiological functions of ACh, but it has also uncovered new mechanistic complexities. A major finding that compels us all to think more broadly is the mounting evidence for corelease of a variety of neurotransmitters from cholinergic neurons. The concept that single classes of neurons corelease multiple ‘fast’ neurotransmitters is long established (Figure 1C) (Bartfai et al., 1988; El Mestikawy et al., 2011; Hnasko and Edwards, 2012). Indeed, there are many prior examples including ACh corelease with GABA (O’Malley et al., 1992; Lee et al., 2010), glutamate (Docherty et al., 1987; Manns et al., 2001; Allen et al., 2006; Huh et al., 2008; Guzman et al., 2011), ATP (Richardson and Brown, 1987), vasoactive intestinal polypeptide (VIP) (Eckstein and Baughman, 1984), or norepinephrine (Benardo, 1991). Despite this literature, many questions remain, not the least of which is the physiological importance of corelease *in vivo*. Thus, we knew that a wide range of neurons could release more than one neurotransmitter, and we had some information on how and whether release was coordinately or differentially regulated. The combination of optogenetic approaches, genetic manipulations (e.g., selective deletion of vesicular transporters or neurotransmitter receptors), and pharmacology now allow us to probe the relative importance of ACh alone versus ACh plus coreleased transmitters to circuit activity and in the regulation of behavior.

ACh and GABA are coreleased in the retina where they mediate motion sensitivity-direction selectivity (Lee et al., 2010). Using paired recordings between starburst amacrine cells and 'on-off' direction-selective ganglion cells, Zhou and colleagues demonstrated a differential contribution of cholinergic and GABAergic inputs to ganglion cell light responses (Lee et al., 2010). Of particular interest, the extent of ACh versus GABA release from starburst amacrine cells was differentially dependent on intracellular calcium concentration and the pattern of stimulation. Thus, ACh release required higher extracellular calcium and repetitive stimulation and GABA release required lower extracellular calcium and was less sensitive to repetitive stimulation (Lee et al., 2010). The authors suggested that ACh and GABA were released from different vesicle populations. Altogether, these studies revealed the differential roles of coreleased neurotransmitters and began to uncover the functional implications of cotransmission. This example, in which optogenetic stimulation is combined with the ability to do paired recordings, provides evidence for segregation of the two transmitters, presumably into different terminals, and raises important cautions about studies claiming coreleased transmitters. These paired recording studies between identified cholinergic neurons and a specific set of ganglion cells in retina underscore the potential challenges that subsequent optogenetic studies encounter. It seems that a range of stimulation paradigms might be required to fully interrogate the locations and relative contributions of multiple, 'coreleased' transmitters to circuit activity.

Striatal CINs represent another example where there is evidence for possible corelease of ACh and a second transmitter, in this case, glutamate. Striatal CINs express both the vesicular ACh transporter (VACHT) and the vesicular glutamate transporter, vGlut3 (Nickerson Poulin et al., 2006; Gras et al., 2008). Targeted deletion of VACHT (using a D2R-Cre driver) allowed Guzman et al. (2011) to probe the differential role of ACh and glutamate release from striatal CINs in the regulation of motor and reward-related behaviors. The investigators found that spontaneous locomotion and cocaine-induced hyperactivity, which were previously thought to be dependent on ACh release in striatum, were essentially intact following deletion of striatal VACHT (Guzman et al., 2011). If indeed these behaviors are significantly influenced by the function of the striatal CINs, the authors argue that they are likely regulated by local glutamate, rather than ACh, release (Guzman et al., 2011). In contrast, cholinergic tone per se was important for regulating expression and sensitivity of DA receptors (D1R and D2R) in MSNs and of locomotor activity in response to dopaminergic agonists (Guzman et al., 2011).

Guzman et al. (2011) did not attempt to identify specific targets of striatal ACh or glutamate using electrophysiological approaches. This has been assessed using optogenetic techniques in a series of recent studies (Higley et al., 2011; Ren et al., 2011; Cachope et al., 2012; English et al., 2012; Threlfell et al., 2012). In the first, Higley et al. (2011) optogenetically targeted and stimulated striatal CINs while recording postsynaptic responses in MSNs. The resultant profiles of MSN activity were consistent with direct, glutamatergic activation. Optical stimulation of CINs generated EPSPs in nearby MSNs that were sensitive to 10 μ M NBQX and 10 μ M CPP, ionotropic glutamatergic receptor blockers, and were absent in slices from mice lacking VGLUT3. The surprising finding was the lack of light-evoked ACh-induced EPSPs; all EPSPs were resistant to both nicotinic and muscarinic receptor blockers. The authors argued that cholinergic neurons directly release glutamate in an activity-

dependent manner, and they found little to no evidence for ACh release from the 'cholinergic' interneurons (Guzman et al., 2011). This lack of ACh effect on MSNs following light stimulation is at odds with other studies discussed below, which document nicotinic inhibition of MSNs (English et al., 2012). Determining whether ACh and/or glutamate are actually released from the striatal interneurons with different stimulation protocols and whether individual vesicles actually contain both glutamate and ACh awaits higher-resolution approaches.

A recent study by Cheer and colleagues demonstrates potential collaboration between striatal ACh and glutamate in the modulation of DA release in nAcc (Cachope et al., 2012). Through a combination of optogenetic techniques that selectively target and stimulate CINs in the nAcc, electrophysiology and fast-scan cyclic voltammetry, the authors demonstrated that endogenous ACh enhances DA release and that this effect was mediated by nicotinic (nAChRs; specifically $\beta 2$ -containing nAChRs), mAChRs, and AMPA receptors. The nAChR-mediated DA release in this study was consistent with that reported by Threfell et al. (2012). Optical stimulation of CINs evoked excitatory postsynaptic currents (EPSCs) on MSNs that were attenuated by AMPA receptor blocker, thus confirming that CINs were driving AMPA receptor-mediated activation of MSNs (Cachope et al., 2012). Performing similar studies in anesthetized mice, Cachope et al. (2012) demonstrated that increased DA release following selective optical stimulation of nAcc cholinergic neurons also occurs *in vivo*.

Although some of the discrepancy in the above findings could result from dorsal versus ventral striatal heterogeneity, English et al. (2012) also found evidence for ACh release from dorsal/neostriatal CINs. This group found that activation of striatal CINs with optogenetic tools recruited a very distinctive set of GABAergic circuits; CINs, acting via nAChRs, activated NPY-expressing NGF interneurons, which in turn contributed to the slow GABAergic inhibition of the projection neurons. The authors suggested that the slow time course of the nAChR-mediated EPSPs in NPY interneurons facilitates the integration of synaptic input during semisynchronous activation of CINs (English et al., 2012). Koos and Tepper (2002) also demonstrated that the pause-excitation activity pattern of CINs regulates the firing of spiny projection neurons both *in vitro* and *in vivo*. Optogenetically evoked IPSCs have another fast component, whose source has not been identified (English et al., 2012). A recent optogenetic study on striatal CINs by Kreitzer and colleagues revealed a very interesting mechanism. They find that these inhibitory responses are action potential independent and mediated by nicotinic receptors. Striatal fast-spiking GABAergic interneuron ablation has no effect on them, but these cholinergically driven IPSCs were greatly reduced after release from striatal DA terminals was blocked or terminals were destroyed. Their results delineate a mechanism that optogenetic stimulation of striatal CINs can drive GABA release from DA terminals (Nelson et al., 2014).

Further optogenetic evidence that cholinergic neurons corelease ACh with other neurotransmitters is provided by studies of the medial habenula to interpeduncular nucleus neuron synapses (IPN) (Ren et al., 2011). Ren et al. optically stimulated cholinergic axonal terminals from the medial habenula in the IPN. Light stimulation of medial habenula axons elicited two distinct responses: a single 5 ms light pulse elicited DNQX-sensitive EPSCs that

were unaffected by 5 μ M mecamylamine and 50 μ M hexamethonium. However, sustained or higher-frequency optical stimulation evoked slow inward currents that were sensitive to nicotinic antagonists. The authors concluded that ACh and glutamate are coreleased from adult medial habenula cholinergic neurons and act through distinct modes of transmission, volume transmission for ACh (although phasic might be a better term as discussed in 'ACh release') and direct transmission for glutamate, respectively. These results further underscore the need to evaluate corelease of transmitters from cholinergic neurons under a range of stimulation paradigms.

Conclusions and future directions: what have optogenetic studies of cholinergic circuits given us?

With the optogenetic era of cholinergic circuit dissection well underway, it is time to ask what additional information and novel perspectives this approach has given us. Overall, it seems clear that this new technology has both confirmed and extended our understanding of cholinergic circuits per se and has enhanced our repertoire of approaches to analyzing the contributions of cholinergic signaling to behavior. Optogenetic manipulation of cholinergic neuron excitability and the ability to tune the level of endogenous ACh release in a selective and quantitatively definable manner is certainly a powerful addition to the armamentarium of techniques available for assaying cholinergic circuit function.

The biggest surprises have been in the findings of what transmitter(s) instead of, or in addition to, ACh actually gets released when a ChAT-expressing neuron is depolarized. Some studies using optogenetic stimulation of neurons that are genetically cholinergic have come to the conclusion that the predominant postsynaptic effects observed are due to glutamate rather than ACh per se (Higley et al., 2011). However, the work of Ren et al. (2011) looked more deeply into the problem. They examined the stimulus dependence of glutamate versus ACh release based on the efficacy of specific receptor antagonists and found that postsynaptic actions via glutamate receptors were detected with brief duration light pulses, akin to those used in Higley et al. (2011), whereas the AChR-mediated actions were only seen when higher-frequency stimulation was used. In view of (a) complementary findings that TTX-independent, ACh-elicited DA release requires coordinate activation of striatal cholinergic neurons (Threlfell et al., 2012) and (b) the proposal that phasic firing may be optimal for ACh release (Sarter et al., 2009), it seems that the appropriate conclusion is that the stimulation parameters for effective release of ACh may be more stringent than those required for glutamatergic transmission, at least in striatum. Of particular interest from these and other more circumspect analyses of cholinergic signaling in striatum is that the *in vivo* effects of ACh may be quite different in dorsal versus ventral striatum (Threlfell and Cragg, 2011) and involve significant contributions of nAChRs as well as mAChRs.

Results from studies using optogenetics to probe cholinergic effects in hippocampus, cortex, and amygdala have largely confirmed and, in some cases, greatly extended our understanding of the role of cholinergic signaling in these circuits. In hippocampus, in particular, the combination of targeted optogenetics with careful receptor pharmacology is allowing better parsing of the distinct contributions of particular subtypes of both nAChRs and mAChRs to the endogenous effects of ACh (Bell et al., 2011; Gu and Yakel, 2011; Gu

et al., 2012). Using these striatal and septal-hippocampal optogenetic studies as proof-of-principle, the time is now ripe to extend these approaches to other critical circuits where pharmacology and electrophysiology have implicated ACh as an important regulator of behavior (feeding behaviors and metabolism, sleep, emotional learning, etc.) (Mark et al., 2011; Platt and Riedel, 2011; Avena and Rada, 2012; Kenney et al., 2012; Vanini et al., 2012; Zoli and Picciotto, 2012; Guzman et al., 2013; Lima et al., 2013).

It is certainly true that huge strides were made in understanding cholinergic signaling in studies that predate the optogenetic analyses, including compelling evidence that differing patterns of tonic versus phasic ACh release play different roles in cortical cholinergic signaling and in specific behaviors (reviewed by Parikh and Sarter, 2008; Hasselmo and Sarter, 2011). These approaches will be enhanced as more basic information about cholinergic signaling is obtained, including use of more sensitive and less damaging probes for measuring ACh release following light stimulation and deeper understanding of ‘appropriate’ light stimulation paradigms that accurately reflect the normal firing rates and patterns of cholinergic neurons. No doubt, much needs to be done to address these and other issues, but the promise of combined optogenetic stimulation and *in vivo* recording in awake behaving animals is clear. It looks like work in the optogenetic era of cholinergic circuit dissection has just begun to get really interesting.

In short, over the past decade, the use of targeted optogenetics in cholinergic circuits has helped clarify our understanding of aspects of cholinergic signaling that had been proposed but unproven for as long as 30 years, confirming and extending our understanding of ACh contributions to circuits and behaviors. In toto, these initial optogenetic forays can be seen as a proof-of-principle that optogenetic approaches can be brought to bear on cholinergic function in the CNS. The generation of new tools that improve the flexibility, diversity, and precision of light-stimulated regulation, of membrane excitability and second messenger signaling is progressing rapidly. As such we can only conclude that the avenues opened by this technology will help us reach an as yet unanticipated depth of understanding of the importance, complexity, and subtlety of cholinergic signaling in complex behaviors.

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Figure 1. Multiple configurations for cholinergic and cholinergic synapses have been proposed

(A) The classic type of cholinergic synapse where direct ('wired') transmission is mediated by an ACh containing presynaptic input contacting a postsynaptic nicotinic and/or muscarinic receptor rich site. Hints of the real complexity, even at such a classic cholinergic synapse, are illustrated by inclusion of nicotinic and/or muscarinic autoreceptors. (B) Indirect or modulatory effects of ACh are illustrated with a cartoon of an axo-axonic interaction. An 'en passant' type cholinergic axon may release ACh in the vicinity of a presynaptic terminal that releases glutamate, GABA, DA, serotonin, or peptides. The net effect of the interaction of released ACh with presynaptic nAChRs and or mAChRs will depend on receptor type and location relative to the release machinery of the recipient axon terminal. Both activation and inhibition of release by ACh at axo-axonic synapses have been reported. (C) ACh corelease (and costorage?) has been proposed in numerous brain regions and highlighted by optogenetic studies. ACh may be coreleased from the same terminals and perhaps even costored with other 'fast' transmitters such as glutamate and GABA.