Abstract—Cognitive flexibility, the ability to adjust behavior in response to new and unexpected conditions in the environment, is essential for adaptation to new challenges and survival. The cholinergic system is an important modulator of this complex behavior however, the exact cholinergic circuits involved in this modulation and the precise influence of acetylcholine (ACh) in the process is still not fully understood. Here we review the role of different cholinergic circuits in cognitive flexibility. Strong evidence indicates that cholinergic interneurons (CINs) from the dorsomedial striatum are essential for facilitating the establishment of a new selected strategy; an effect that seems to depend mainly on activation of muscarinic receptors. Cholinergic neurons from the nucleus basalis magnocellularis (nBM), which project to the prefrontal cortex, seem to modulate the initial inhibition of a previously learned strategy, however, this concept is still controversial. Additionally, some studies suggest that basal forebrain cholinergic neurons projecting to the hippocampus, basolateral amygdala, and posterior parietal cortex may also participate on the modulation of cognitive flexibility. We highlight the fact that when investigating effects of ACh on behavioral flexibility, or any other behavior, one has to keep in mind two important particularities of the cholinergic system: (1) Many cholinergic neurons in the brain co-release glutamate or GABA with ACh. Methodologies that rely on neuronal silencing or ablation lead to simultaneous elimination of both neurotransmitters, making interpretation of results complex. (2) The cholinergic gene locus has a unique organization, with the vesicular acetylcholine transporter (VAcChT) gene present within the intron between the first and second exons of the choline acetyltransferase (ChAT) gene. Thus, behavioral studies using transgenic animals generated with ChAT bacterial artificial chromosome (BAC) clones should be considered carefully, taking into consideration that these mice may over-express VAcChT and therefore, present a hypercholinergic tone that can be a confounder in behavioral studies.

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Key words: cholinergic, cognitive flexibility, striatum, frontal cortex, reversal learning, set-shifting.

INTRODUCTION

Cognitive flexibility, the ability to appropriately adjust one’s behavior in response to new and unexpected conditions in the environment, is essential for adaptation and survival. This complex behavior depends on effective engagement of different brain processes to identify salient changes in the surroundings, direct attention to changed elements, determine that a previous strategy is no longer appropriate, inhibit previous responses and establish a new strategy (Dajani and Uddin, 2015; Nilsson et al., 2015). Deficits in cognitive flexibility have been associated with diverse pathologies such as autism, schizophrenia, Parkinson’s disease, Alzheimer’s disease and attention deficit hyperactivity disorder (Daban et al., 2006; Schoenbaum et al.,...
It has long been recognized that the prefrontal cortex and the striatum, which are part of the cortico-basal-ganglia–thalamic circuit, work together to modulate cognitive flexibility and a wealth of data indicate that acetylcholine (ACh) transmission facilitates the process [for a review see (Ragozzino, 2003)]. For instance, systemic administration of cholinesterase inhibitors to boost cholinergic transmission improved reversal learning and attentional set-shifting deficits in old rats (Tait et al., 2013; Nikiforuk et al., 2015), in rat models of schizophrenia (Alexander et al., 2013), as well as in rats with immunolesion of basal forebrain cholinergic neurons (Cutuli et al., 2009). Conversely, mice with forebrain cholinergic signaling impairment, which includes the whole cortical mantle, hippocampus, amygdala and striatum showed consistent deficits in reversal learning assessed using the Morris Water Maze (MWM) (Marty et al., 2012; Al-Onaizi et al., 2016), as well as a severe reversal learning impairment in a visual discrimination task (Kolinsky et al., 2013a). Moreover, selective decrease of hippocampal cholinergic tone disturbs reversal learning in the MWM similarly to mice in which forebrain cholinergic tone is disrupted (Al-Onaizi et al., 2016).

Systemic administration of scopolamine, a muscarinic receptor antagonist, impaired set shifting and reversal learning in rats while having no effect on discrimination performance (Soffie and Lamberty, 1987; Chen et al., 2004). In addition, systemic injections of a selective M1 muscarinic agonist (CDD-0102A) had a significant effect on enhancing strategy switching under changing environmental demands in rats (Ragozzino et al., 2012).

Systemic administration of nicotinic agonists (Allison and Shoabi, 2013; Terry et al., 2016), as well as of α7-nAChR-positive allosteric modulators (Nikiforuk et al., 2015) also showed significant effect on enhancing strategy switching under changing environmental demands.

Because cholinergic innervation is widespread throughout the brain, including the entire cortical mantle and the striatum, and ACh can signal through a large variety of ionic and G-protein-coupled receptors that are present pre- and post-synaptically on distinct neuronal systems, it has been hard to determine the exact role of specific cholinergic circuits on the modulation of cognitive flexibility. However, in the last decade, cholinergic circuits and brain mechanisms underlying this behavior have been extensively studied. Here we will briefly describe important components of the cholinergic system as well as the location of main cholinergic nuclei and their projection to different areas of the brain. We will shortly describe how analyses of reversal learning in discrimination tasks and/or attentional set-shifting tasks are used to investigate behavioral flexibility. We will review biochemical, pharmacological and behavioral studies implicating cholinergic interneurons (CINs) from the striatum as well as basal forebrain cholinergic projections to the cortical mantle, amygdala and hippocampus in the modulation of cognitive flexibility. We will discuss evidence that CINs from the striatum facilitate exploration of new strategies when conditions change. We will also discuss a possible role for basal forebrain cholinergic projections on determining whether a previous strategy is no longer appropriate.

THE CHOLINERGIC SYSTEM IN THE BRAIN

ACh synthesis at the nerve terminal depends on the uptake of choline by the high-affinity choline transporter (CHT1) (Ribeiro et al., 2006) and on the enzyme choline acetyltransferase (ChAT); which catalyzes the acetylation of choline with acetyl-CoA (Fig. 1A); [see review on (Prado et al., 2002, 2013)]. The neurotransmitter is then loaded into synaptic vesicles by the vesicular acetylcholine transporter (VACHT) [see review on (Parsons, 2000; Prado et al., 2002, 2013)], a process dependent on the electrochemical gradient generated by a V-type proton ATPase (Parsons, 2000). Arrival of the nerve impulse to the terminal leads to membrane depolarization and consequent Ca2+ influx, which triggers vesicle fusion and release of ACh into the synaptic cleft. Released ACh binds to both pre- and postsynaptic muscarinic (mAChR) and nicotinic (nAChR) receptors. Half-life of ACh at the synaptic cleft is short as it is hydrolyzed within milliseconds by the enzyme acetylcholinesterase or butyrylcholinesterase (Prado et al., 2002; Zimmerman and Soreq, 2006).

Binding of ACh to pre- and postsynaptic receptors present on distinct neuronal systems can alter neuronal excitability, influence synaptic transmission, induce synaptic plasticity, and coordinate firing of groups of neurons [for excellent reviews see (Picciotto et al., 2012; Dineley et al., 2015; Soreq, 2015)] and as a result produce diverse consequences for brain activity. Cholinergic tone can also regulate long-term changes in target cells by modulating microRNA and RNA metabolism with consequences for gene expression and alternative splicing (Berson et al., 2012; Kolinsky et al., 2013a, 2016; Soreq, 2015), which has important consequences for cognitive function, neuronal resilience and Alzheimer’s disease pathology.

The brain is highly innervated by cholinergic neurons and ACh modulates numerous brain functions. Most cholinergic innervations originate from projection neurons that target distal areas. The basal forebrain (BF) complex for instance (Fig. 1C, D), which includes the nucleus basalis magnocellularis (nBM), the medial septum (MS), the nuclei of the diagonal band (nDB) and substantia innominata (SI), provides the major input to the whole cortical mantle, hippocampus, thalamus and amygdala (Mesulam et al., 1983). The pedunculopontine–laterodorsal tegmental complex (PPTg–LDT) innervates midbrain regions (Fig. 1E, F), the thalamus, the striatum and pontine targets (Hallanger and Wainer, 1988). The medial habenula (MHB) nuclei projects to the interpeduncular nucleus in the midbrain (Ren et al., 2011). In the striatum (Fig. 1B), most cholinergic innervations come from interneurons (Woolf and Butcher, 1981; Bolam et al., 1984) with some projections coming from PPT cholinergic neurons (Dautan et al., 2014). Striatal CINs (also named tonically active neurons or TANS) are
large aspiny neurons that are tonically active. Although CINs represent only a small fraction of the striatum total cell population (1–3% in rodent and up to 20% in primates), they have dense and extensive axonal arborization (Kawaguchi et al., 1995).

ACh receptors are classified into two subtypes: ligand-gated nicotinic receptors and metabotropic muscarinic receptors. Muscarinic ACh receptors are a subfamily of G-protein-coupled receptors that consists of five distinct subtypes named M1 to M5. These receptors can be divided into two classes: M1-class (M1, M3, M5), which couples to Gq/11 family and therefore activates phospholipase C isoforms; and M2-class (M2, M4) which preferentially signals through Gi/α family, inhibiting adenylyl cyclase [see review (Kruse et al., 2014)]. To note, muscarinic acetylcholine receptors are highly expressed on nerve terminals of major neurotransmitter systems that project to the striatum, on CINs as well as on medium spiny neurons (MSN), the most abundant (90–95%) striatal neuronal population and the sole output of the striatum [see review (Goldberg et al., 2012)]. Mainly three subtypes of muscarinic receptors are expressed in the striatum, M1, M2 and M4. The M2 receptor is expressed exclusively in CINs as a presynaptic autoreceptor (Bernard et al., 1992; Hersch et al., 1994), and therefore activation of M2 receptors in the striatum inhibits

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**Fig. 1.** The cholinergic system in the brain. (A) Schematic drawing of the cholinergic nerve terminal indicating main cholinergic pre-synaptic markers as well as nicotinic and muscarinic cholinergic receptors. VACHT: Vesicular acetylcholine transporter; ChAT: Choline acetyltransferase; CHT1: High affinity choline transporter; AChE: acetylcholinesterase; ACh: acetylcholine; Ch: choline. M: muscarinic receptor; N: nicotinic receptor. (B–F) High resolution map of brain cholinergic interneurons. Images built using data from the Allen mouse brain connectivity atlas [brain-map.org; available from: http://connectivity.brain-map.org (Oh et al., 2014)]. Images detail axonal projections labeled by viral tracers unilaterally injected on ChAT-IRES-Cre driver mouse line. (B) Striatal cholinergic interneurons. (C, D) Axonal projections of basal forebrain cholinergic neurons. Blue: viral tracer injected mainly on diagonal band nucleus (NDB), but also reached partially the substantia innominata (SI), and Magnocellular nucleus (nBM). Pink: viral tracer injection mainly on Substantia innominata and Magnocellular nucleus. Green: viral tracer injected into the medial septum (MS). (E, F) Pedunculopontine and laterodorsal tegmental nuclei. Red: viral tracer injected mainly on pedunculopontine nucleus (PPN). Yellow: viral tracer injected on pontine central gray (PCG) also reaching the laterodorsal tegmental (LDT). (C, E) Sagittal images. (D, F) top images.
the function of CINs (Calabresi et al., 1998; Ragozzino et al., 2009; Bradfield et al., 2013). Densities of the M4 receptors are highest in the striatum (Flynn et al., 1995). Interestingly, in the striatum, the M1 and M4 receptors are richly expressed by MSNs. Notably, while M1 receptors are expressed on both striatonigral and striatopallidal MSNs, M4 receptors are expressed predominantly on striatonigral MSNs (Harrison et al., 1996) where they exert a direct inhibitory control on dopamine D1-like receptor signaling (Onali and Olianas, 2002; Jeon et al., 2010). M1 muscarinic receptors are also highly abundant in the cortical mantle and hippocampus, while M2 receptors are only enriched in the occipital cortex and very low amounts of M4 receptors are present in the cortical mantle and hippocampus (Levey et al., 1991; Flynn et al., 1995). M1 receptors are mainly localized to cell bodies and neurites, consistent with its role as a major postsynaptic muscarinic receptor (Levey et al., 1991).

Nicotinic acetylcholine receptors (nAChR) are pentameric ligand-gated cation channels composed of varying combinations of different α and β subunits (see review in Picciotto et al., 2000; Dineley et al., 2015). Seven subtypes of α subunits (α2–α7, α9 and α10) and 3 subtypes of β subunits (β2–β4) are expressed in the mammalian brain. These subunits assemble with different stoichiometry to form nAChRs with different properties, including distinct cationic permeability, agonist affinity and desensitization properties (Dani and Bertrand, 2007). Homomeric α7 nAChRs and heteromeric α4β2– (the asterisk indicates the possible presence of other subunits in the complex) containing nAChRs are widely distributed throughout the brain. In the striatum, the major nAChR subtypes are α4β2– and α6β2– while α7 nAChRs are present at a much lower density (Clarke et al., 1985). In the cortical mantle and hippocampus, α7 nAChRs and α4β2– predominate (Dani and Bertrand, 2007).

CHOLINERGIC NEURONS CAN CO-RELEASE GLUTAMATE OR GABA

Although for a long time the dogma that one neuron stores and releases only one type of classical neurotransmitter has prevailed, accumulating evidence indicates that a vast number of exceptions exist (for recent reviews see El Mestikawy et al., 2011; Hnasko and Edwards, 2012; Munster-Wandowski et al., 2016). To note, many cholinergic neurons in the brain co-release glutamate or GABA with ACh (Nishimaru et al., 2005; Lee et al., 2010; Higley et al., 2011; Ren et al., 2011; Saunders et al., 2015b). Of importance for cognitive flexibility, cholinergic neurons from basal forebrain co-release GABA with ACh (Saunders et al., 2015a) and CINs co-release ACh and glutamate (Gras et al., 2008b; Higley et al., 2011; Sakae et al., 2015; Gangarossa et al., 2016).

In addition to VAChT, striatal CINs also express the vesicular glutamate transporter 3 (VGLUT3) in synaptic vesicles (Fremeau et al., 2002; Gras et al., 2002; Takamori et al., 2002); which give them the ability to co-release ACh and glutamate (Higley et al., 2011). VGLUT3 expression in CINs seems to be important for the modulation of vesicular loading of ACh (Gras et al., 2008a; Nelson et al., 2014). Importantly, based on behavioral studies using mice with null expression of VGLUT3 (Gras et al., 2008b; Divito et al., 2015; Sakae et al., 2015), and mice with selective elimination of VAChT from striatal CINs (Guzman et al., 2011, 2013), it has been suggested that glutamate released from CINs can also modulate striatal behavioral functions. In fact, some behaviors previously thought to be modulated by ACh are now suggested to be modulated by CIN-released glutamate (Guzman et al., 2011; Prado et al., 2013; Divito et al., 2015; Sakae et al., 2015; Gangarossa et al., 2016).

Cholinergic neurons from basal forebrain co-express the GABA synthetic enzyme GAD65 and the vesicular GABA transporter, and have been shown to co-release GABA with ACh (Saunders et al., 2015a,b). Although the physiological role of ACh/GABA co-release by basal forebrain cholinergic neurons has not yet been determined, it is very likely that GABA released from these neurons influence behavioral outputs. Thus, when investigating the influence of ACh on behavior, it is important to have in mind that elimination of cholinergic neurons results in decreased release of both neurotransmitters simultaneously, complicating the interpretation of behavioral analysis. The same is true for methodologies that silence cholinergic neurons, such as optogenetics (Tye and Deisseroth, 2012) or chemogenetics (Roth, 2016).

MEASURING COGNITIVE FLEXIBILITY

Comparing results from different studies investigating cognitive flexibility is not an easy task due to the multitude of ways it has been tested and analyzed in the literature. In this review, we have focused on animal studies that used reversal learning in discrimination tasks and/or attentional set-shifting tasks, the two commonly used behavioral paradigms used in laboratories to investigate cognitive flexibility (Nilsson et al., 2015). Before being tested on reversal learning, animals go through the acquisition phase, where they are trained to discriminate between two stimuli that often involve a single perceptual dimension (ex. visual, olfactory, sensory). Responses to the correct stimulus are rewarded (CS+) while responses to the incorrect stimulus are non-rewarded (CS−). Sessions are repeated until animals learn the initial CS+ versus CS− discrimination (Fig. 2A-top for a touchscreen pairwise visual discrimination task). For reversal learning the contingencies are reversed, that is, the incorrect stimulus at acquisition become correct and is rewarded and vice versa (Fig. 2A-bottom). For successful reversal learning, animals need to learn to inhibit responding to the previously correct stimulus (response inhibition stage) and then learn the new association (stimulus-reward learning stage). Comparison of number of sessions or number of trials taken to successfully learn the new association between control and test animals is used to indicate the presence or absence of reversal learning deficits. Incorporation of error pattern analysis allows a better understanding of the underlying process. Incorrect responding to previously...
Learn to press a lever based on lever location, in this case left-side lever is correct (CS+ rewarded) and right side lever is the incorrect (CS−, non-rewarded), while a light stimulus is randomly presented above either the left or right lever. In the set-shifting phase, animals have to pay attention to the light stimulus. That is, a visual cue strategy is required in which the correct lever (CS+, rewarded) is indicated by an illuminated light while the incorrect lever (CS−, non-rewarded) stays non-illuminated.

**STRIATUM CHOLINERGIC SYSTEM AND COGNITIVE FLEXIBILITY**

Initial evidence for the involvement of striatal ACh on the modulation of cognitive flexibility came from electrophysiological recordings in monkeys performing conditioned behavioral tasks (Apicella et al., 1991; Ravel et al., 2001). These studies showed that TANS become more responsive (show a phasic depression of the tonic firing, followed by transient increase in discharge rate) when there is a change in task contingencies. Corroborating these findings, in vivo microdialysis studies showed dynamic changes in extracellular ACh levels in the rat dorsomedial striatum when animals were performing the reversal-learning phase of a place discrimination task (Ragozzino and Choi, 2004; Ragozzino et al., 2009). ACh stayed at basal levels during the initial acquisition phase of the task. During reversal learning, ACh levels increased specifically in DMS but not in dorsolateral striatum (DLS) and decreased gradually as rats improved their performance using the new place strategy (Ragozzino and Choi, 2004).

What is therefore, the exact role of DMS CINs in behavioral flexibility? Increasing evidences support the hypothesis that ACh in the DMS facilitates the establishment of a new selected strategy (Ragozzino et al., 2002a,b; Ragozzino, 2003; Palencia and Ragozzino, 2004; Ragozzino and Choi, 2004; Tzavos et al., 2004; Palencia and Ragozzino, 2006; McCool et al., 2008; Ragozzino et al., 2009; Brown et al., 2010; Bradfield et al., 2013; Matamales et al., 2016). For instance, bilateral infusion of the nonspecific muscarinic antagonist scopolamine (Ragozzino et al., 2002a), or M1-selective muscarinic antagonists (pirenzepine or muscarinic-toxin 7; (Tzavos et al., 2004; McCool et al., 2008) into the DMS during acquisition phase of a response discrimination test did not influence learning, supporting the notion that influencing cholinergic function in the DMS does not affect the initial acquisition of place associations. When infused during reversal learning, on the other hand, these drugs led to impairment in the rat ability to learn the new contingency. Scopolamine- or M1-antagonist-infused rats did not show change in perseverative errors; but showed deficits in regressive errors, suggesting deficits in the ability to maintain a new correct choice pattern (Ragozzino et al., 2002a; Tzavos et al., 2004).

Studies using inactivation or disconnection of thalamic afferents to DMS (Brown et al., 2010; Bradfield et al., 2013) further support a role for ACh in the DMS in facilitat-

**Fig. 2.** Behavioral tasks commonly used to measure cognitive flexibility. (A) Pair-wise visual discrimination and reversal task. Illustration of shape stimulus pair used in the automated touchscreen visual discrimination and reversal task. In the acquisition phase animals are trained to discriminate between two visual stimuli. Responses to the correct stimulus are rewarded (CS+) while responses to the incorrect stimulus are non-rewarded (CS−). Sessions are repeated until animals learn the initial CS+ versus CS− discrimination (top). For reversal learning the contingencies are reversed, that is, the incorrect stimulus at acquisition become correct and is rewarded and vice versa (bottom). (B) Attentional set-shifting task. This test involves at least two superimposed perceptual dimensions, in this example a lever pressing response strategy and a visual cue strategy. In the acquisition phase animals have to differentiate between a response strategy and a visual cue strategy. For the response strategy, animals learn to press a lever based on lever location, in this case left-side lever is correct (CS+, rewarded) and right side lever is the incorrect (CS−, non-rewarded), while a light stimulus is randomly presented above either the left or right lever (top). In the set-shifting phase, animals have to pay attention to the light stimulus. That is, a visual cue strategy is required in which the correct lever (CS+, rewarded) is indicated by an illuminated light while the incorrect lever (CS−, non-rewarded) stays non-illuminated (bottom).
ing the establishment of a new selected strategy. Parafascicular thalamic fibers that innervate CINs in the DMS modulate firing rate and intrinsic activity of these neurons (Bradfield et al., 2013), with consequent effect on ACh efflux (Brown et al., 2010). Disconnection of these thalamic fibers to the DMS has the effect of blocking behaviorally induced increase of ACh output in DMS, and has been shown to impair strategy switching in a place task (Brown et al., 2010) as well as in a contingency reversal task (Bradfield et al., 2013). Rats with parafascicular thalamic inactivation exhibited selective increase in regressive errors in the reversal place task, suggesting difficulty to maintain a new correct choice pattern (Brown et al., 2010). Additionally, ACh in the DMS has been shown to facilitate the establishment of a new selected strategy possibly by reducing interference between new and existing learning (Bradfield et al., 2013). Food deprived rats with parafascicular lesion or sham operated controls could quickly learn to press two levers on random ratio schedules of reinforcement, one delivering food pellets and the other a sucrose solution, and increased the number of lever pressing as the ratio requirement increased. When one or the other outcome was devaluated, by giving the rat unrestricted access to either the pellets or sucrose for 1hr prior to the extinction test, both lesioned rats and sham operated reduced the number of lever pressing on the devalued-lever relative to the non-devaluated lever (Bradfield et al., 2013). These results indicate that rats with parafascicular lesion were able to encode specific lever press-outcome associations during the learning task phase. However, when the contingency for one of the levers was changed and pressing it would no longer deliver the reward, lesioned rats showed deficits to adjust to the change in contingency. While sham operated rats decreased the number of times they pressed the non-rewarding lever, lesioned rats kept pressing both levers equally (Bradfield et al., 2013). To further test if lesioned rats showed deficits to adjust to the change in contingency, the authors retrained the rats on the initial contingencies and then reversed the relationship between the actions-outcomes, i.e., the lever that initially delivered sucrose was made to deliver pellets and vice versa. When both sham and lesioned groups were performing similarly on these new action-outcome contingencies, the authors tested whether the rats were able to encode the new action-outcome contingency using the same outcome devaluation test used after initial learning. After reversal of contingencies, however, lesioned rats were unable to choose appropriately when one of the two outcomes was devalued, and pressed both levers equally, while sham-operated rats reduced pressing the lever most recently associated with the devaluated outcome. These elegant experiments support the idea that impaired activation of cholinergic neurons in the DMS result not only in failure to encode the new learning, but also in inability to express either the old or the new learning (Bradfield et al., 2013). Interestingly, a recent study demonstrated that aging leads to deficits in activation of cholinergic DMS neurons by parafascicular fibers, which affects the capacity to encode new learning in mice (Matamales et al., 2016).

As indicated above, bilateral infusions of scopolamine as well as different M1-selective muscarinic antagonists into DMS lead to deficits in reversal learning of a place association task in rats, which are characterized by inability to maintain a new correct choice pattern (Ragozzino et al., 2002a; Tzavos et al., 2004). These results point to an important role for DMS muscarinic signaling in mediating ACh influence on behavioral flexibility. Additionally, these studies suggest that ACh acting on DMS M1 muscarinic cholinergic receptors facilitates learning during a change in task contingency. It is important to keep in mind that other cholinergic receptors present in this region also influence the final behavioral outcome. On that note, inhibition of CINs by bilateral injections of oxotremorine (muscarinic receptor agonist with very high affinity for M2 muscarinic receptors) into the DMS prior to reversal learning blocked increase in ACh output and impaired reversal learning, an effect that was reversed when oxotremorine was injected in combination with AF-DX-116, a M2 receptor antagonist (Ragozzino et al., 2009). Also, injections of oxotremorine in the DMS replicate the effect of disconnection of parafascicular fibers to the DMS on reversal learning (Bradfield et al., 2013). Interestingly, even though M4 muscarinic receptors are highly expressed in the striatum (Flynn et al., 1995), bilateral injections of M4-selective muscarinic agonists into the DMS did not affect place acquisition or reversal learning (McCool et al., 2008). Likewise, DMS nicotinic receptors do not seem to be important for the modulation of cognitive flexibility as bilateral injections of mecamylamine, a nicotinic receptor antagonist, did not affect acquisition or reversal learning (Tzavos et al., 2004).

A recent study using anti-ChAT IgG-saporin toxin to lesion CINs from DMS in rats also supports a role for DMS ACh in cognitive flexibility (Aoki et al., 2015). However, in contrast to the several studies described above, DMS cholinergic deficits in these immunotoxin lesioned rats did not cause deficits in reversal learning. Also contrasting was the observation that immuno-lesioned rats showed deficits in set-shifting paradigms expressed as increased perseverative errors (Aoki et al., 2015), which suggests that DMS ACh affects the ability to inhibit the old strategy. One possible explanation for these discrepancies is that the anti-ChAT IgG-saporin toxin strategy used in the study may not have been able to efficiently lesion cholinergic cells. As a quantitative assessment of neuronal loss or determination of ACh release was not presented by Aoki et al. (2015), it is not possible to clearly determine the extent of the lesions. However, the study describing anti-ChAT IgG-saporin toxin as a tool to lesion CINs has shown that the toxin effectively depletes around 50% of CINs (Laplanite et al., 2011). In fact, it is surprising that anti-ChAT antibodies, which presumably do not have access to intraneuronal ChAT, direct the saporin toxin to CINs.

Another plausible explanation for the discrepancies lays on the fact that CIN show ACh/glutamate co-transmission (see above). The discrepancies observed in the study conducted by Aoki et al. (2015) may be due to the fact that ablation of CINs affects release of both
ACh and glutamate. Investigation of cognitive flexibility in mice with selective deletion of VACHT or VGLUT3 in the striatum will be necessary to determine the contribution of CIN-glutamate release on the process.

Interestingly, another recent study using selective elimination of DMS CINs in rats to investigate cognitive flexibility (Okada et al., 2014) showed results that were the opposite of what was previously observed on the pharmacological, electrophysiological and microdialysis studies described above, and also contradict the immunotoxin ablation study by (Aoki et al., 2015). Okada et al. (2014) data suggested that selective elimination of DMS CINs enhances reversal and extinction learning of a place discrimination task. They also tested for contribution of muscarinic receptors on the behavior using gene-specific silencing of M4 or M1 muscarinic receptors by lentiviral expression of short hairpin RNA (shRNA) and showed that improvement in strategy switching was dependent on M4 muscarinic receptors and independent of M1 receptors (Okada et al., 2014). The same concerns raised above for the interpretation of behavioral data in studies using immunotoxins ablation of CINs in the striatum of mice are also valid here. Another confounder lays on the fact that ablation of CINs done by Okada et al. (2014) relied on using transgenic rats containing the ChAT bacterial artificial chromosome (BAC). Selective elimination of CINs from rat dorsal-medial striatum in this study was done in transgenic rats expressing the human interleukin-2 receptor a-subunit (IL-2Ra) under the control of the ChAT promoter. These rats were generated using a ChAT BAC clone that carries also the VACHT gene, embedded within the intron between the first and second exons of the ChAT gene (Bejanin et al., 1994; Erickson et al., 1994; Roghani et al., 1994). Although the authors do not present a detailed characterization of these transgenic rats, indicating for instance how many copies of the ChAT BAC are present and the protein expression levels for VACHT, it is reasonable to assume that, similar to transgenic mice containing the ChAT BAC (Nagy and Aubert, 2012, Nagy and Aubert, 2015; Kolisnyk et al., 2013b), the transgenic rats used by Okada et al. (2014) present a hypercholinergic phenotype. To note we previously reported (Kolisnyk et al., 2013b) that transgenic mice expressing channelrhodopsin-2 (ChR2) protein under the control of the ChAT promotor (ChAT–ChR2–EYFP) carry 50 copies of VACHT gene, which leads to overexpression of functional VACHT and consequently increased cholinergic tone. ChAT–ChR2–EYFP mice have marked improvement in motor endurance, however, they present severe deficits in attention, working memory and spatial memory (Kolisnyk et al., 2013b). ChAT–ChR2–EYFP mice also show exacerbating drug-induced stereotypic behaviors (Crittenden et al., 2014). Other mice carrying the ChAT BAC, and therefore extra copies of VACHT, have also been shown to present behavioral abnormalities (Nagy and Aubert, 2012, 2013, 2015). Importantly, these results show that increased VACHT expression alters cholinergic tone making information processing fundamentally different in ChAT-BAC mutant mice versus wild-type mice. It is important to note that, although less likely, other factors such as difference in experimental schedules or the use of permanent lesion of cholinergic neurons instead of transient inactivation could also contribute for the discrepant results observed by Aoki et al. (2015) and (Okada et al., 2014). Although most of the studies investigating the involvement of striatal ACh on the modulation of cognitive flexibility focus on the DMS, some reports implicate also the ventral striatum in the modulation of strategy switching (Setlow et al., 2003; Floresco et al., 2006; Aoki et al., 2015). It has been suggested that the role of CINs from DMS and ventral striatum in set-shifting is dissociable and depends on the behavioral context (Aoki et al., 2015). CIN from the ventral striatum, which receive direct inputs from the hippocampus (Kelley and Domesick, 1982; Ito et al., 2008), may facilitate change to a new selected strategy when the subject is presented with a novel (previously unknown) stimulus. Conversely, CIN from DMS may facilitate change to a new selected strategy when subject has to choose between previously known stimuli (Aoki et al., 2015). Importantly, these results further support a role for CIN in suppressing the use of an old strategy and promoting exploration of a new rule. Further studies will be necessary to explore between these different possibilities.

**PREFRONTAL CORTEX ACH AND MODULATION OF COGNITIVE FLEXIBILITY**

Although a number of studies indicate that prefrontal cortex ACh is important for the modulation of cognitive flexibility the exact role of cholinergic signaling in this process is still controversial. Evidence suggesting that manipulations of prefrontal cortex ACh selectively affect behavioral flexibility came initially from studies examining the effects of non-specific excitotoxic lesions of the nucleus basalis of Meynert (nbM) in marmosets (Roberts et al., 1990). Marmosets that had been previously trained on a visual discrimination task prior to excitotoxic lesions of the basal forebrain had little or no effect on initial acquisition of the discriminations but showed impairment in reversal learning over a series of reversals, characterized by an increase in perseverative errors on the first reversal and a failure to improve performance over successive reversals (Roberts et al., 1990, 1992). When tested on intra- and extra-dimensional attentional set-shifting, using visual discrimination stimuli composed of two abstract dimensions (lines superimposed on shapes) (Roberts et al., 1992), marmosets with bilateral lesions of the nbM did not show impaired ability to switch attention from one dimension to the other (extra-dimensional set-shifting). However, they showed facilitated performance of a well-learned contingency when novel stimuli of the irrelevant dimension were introduced. These results indicated that bilateral excitotoxic lesions of the basal forebrain in the marmoset influenced cognitive flexibility by impairing learning of stimulus-reward (or stimulus–response) associations (Roberts et al., 1990). Although the lesions were shown to reduce ChAT activity by 50% in the frontal cortex, they potentially damaged also non-cholinergic neurons in the nbM, therefore the results could not be irrefutably attributed to ACh.
Additional experiments have given support to a role of prefrontal ACh on cognitive flexibility. Marmosets that were transplanted with ACh-rich tissue into the necocortex three weeks after bilateral excitotoxic nbM lesions did not show reversal learning deficits on a visual discrimination task (Ridley et al., 1994). Also, marmosets that received bilateral injections of IgG saporin into the nbM (to produce selective lesions of cholinergic neurons), and were treated with a dose scopolamine (muscarinic antagonist) which did not impair performance in control animals showed deficits on reversal learning of perceptually easy visual discrimination tasks (Fine et al., 1997). In rats, selective IgG saporin cholinergic lesion of the nbM spared acquisition of an operant discrimination task, but caused impairment in reversal learning, especially on the first reversal, where lesioned rats showed increased perseveration (Cabrera et al., 2006). Taken together, these studies suggest that prefrontal cortex cholinergic signaling is critical for the initial inhibition of a previously learned strategy.

However, there have been reports suggesting that ablation of cholinergic neurons from the rat nbM does not interfere with reversal learning (McGaughy et al., 2008; Tait and Brown, 2008). IgG-saporin infusions into the rat nbM did not impair performance in a task that used odor/texture discriminations to measure acquisition, reversal-learning as well as attentional set-shifting (McGaughy et al., 2008; Tait and Brown, 2008). These contrasting findings might result from the fact that the immunotoxin lesions do not fully deplete the cholinergic neurons. That is, it is possible that only nbM lesions that lead to substantial loss of cholinergic innervations in the cortex significantly impair reversal learning (Fine et al., 1997). Another alternative explanation is that only lesions that spread from the nbM to other basal forebrain cholinergic nuclei interfere with reversal learning, as they would encompass the prefrontal cortex as well as other brain areas. The hippocampus (Al-Onaizi et al., 2016), basolateral amygdala (Schoenbaum et al., 2003; Stalnaker et al., 2007), and posterior parietal cortex (Fox et al., 2003) are additional areas that could be possibly affected. They all receive extensive cholinergic innervations and have been shown to influence cognitive flexibility. Mice with impaired cholinergic signaling in the hippocampus, due to genetic elimination of VACHT from the medial septum cholinergic neurons, showed relatively preserved acquisition of spatial memory whereas reversal spatial memory was significantly impaired (Al-Onaizi et al., 2016), suggesting a role for hippocampal ACh in spatial cognitive flexibility. Also, rats with neurotoxic lesions of the posterior parietal cortex have been shown to present selective impairment on the extradimensional shift phase of an attentional set-shifting paradigm (Fox et al., 2003). Additionally, increasing evidence suggests that the prefrontal cortex facilitates cognitive flexibility by regulating associative encoding in the amygdala (Stalnaker et al., 2007, 2009).

Nicotinic receptors seem to be important modulators of the ACh effect on behavior flexibility in the cortex. Recent studies indicate that IP injections of nicotinic receptor agonists such as nicotine (Allison and Shoaib, 2013) and varenicline (Terry et al., 2016) enhance cognitive flexibility. Nicotinic receptors from prefrontal cortex appear to modulate these effects on cognitive flexibility (Allison and Shoaib, 2013). α7 nicotinic receptor signaling seems to be an important mediator of these effects as PNU-120596, a α7 nicotinic receptor agonist, as well as different positive allosteric modulators of α7 nicotinic receptors effectively facilitate shifting of response patterns (Nikiforuk et al., 2015). Whether prefrontal cortex muscarinic receptors also play a role in cognitive flexibility is not clear. Intraperitoneal injections of the M1 muscarinic agonist CDD-0102A were shown to facilitate the change between a place and visual discrimination (Ragozzino et al., 2012). On the other hand, M1 knockout mice showed increased perseveration in attentional tasks, but normal reversal learning in a visual discrimination task (Bartko et al., 2011). However, in both studies the effects on cognitive flexibility involve changes in M1 receptors in multiple brain areas. As previously described, M1 muscarinic receptors are abundant in the prefrontal cortex and striatum (Levey et al., 1991), the two brain areas critical for the modulation of cognitive flexibility.

Thus, much more needs to be done to clearly determine the role of cortical ACh on the modulation of cognitive flexibility. Selective elimination of ACh release from cholinergic neurons by AAV-Cre viral injections of VACHt-floxed mice (Martins-Silva et al., 2011; Al-Onaizi et al., 2016) or Chat-floxed mice (Misgeld et al., 2002; Patel et al., 2012) will be of great value, as it represents a refined approach for causing cholinergic deficit on defined population of neurons within the basal forebrain nuclei. It will be interesting to use this approach to further investigate the role of cholinergic signaling in posterior parietal cortex and/or amygdala on reversal learning.

**PEDUNCULOPONTINE CHOLINERGIC NEURONS AND COGNITIVE FLEXIBILITY**

The pedunculopontine tegmental nucleus (PPT) has been suggested to modulate behavioral flexibility by facilitating a shift to a new choice pattern when there is a change in reward contingencies (Taylor et al., 2004; Maclaren et al., 2013; Syed et al., 2016), particularly by increasing sensitivity to positive reward outcomes (Hong and Hikosaka, 2014; Syed et al., 2016). However, a recent study suggests that ACh transmission may not be essential for this modulation, as selective depletion of cholinergic neurons in the PPT does not interfere with acquisition of a fixed-ratio schedule or a switch to a variable-ratio schedule (MacLaren et al., 2016). Further studies will be necessary to clearly determine the involvement of PPT cholinergic neurons in cognitive flexibility, particularly on facilitating the reliable execution of a new choice pattern under conditions in which outcomes are uncertain.

**SUMMARY AND CONCLUSIONS**

What can we conclude from all these studies? It seems quite convincing that dorsal-medial striatum ACh signaling is essential for facilitating the establishment of a new selected strategy; an effect that seems to depend mainly on binding of the neurotransmitter to muscarinic receptors. It might be possible that, depending on the
context, cholinergic signaling in the ventral striatum also participates on the establishment of a new selected strategy. Prefrontal cortex ACh seems to modulate the initial inhibition of a previously learned strategy; however, this concept is still controversial. Additionally, ACh presence on brain areas such as hippocampus (Al-Onaizi et al., 2016), basolateral amygdala (Schoenbaum et al., 2003; Stalnaker et al., 2007), and posterior parietal cortex (Fox et al., 2003) may also participate on the modulation of cognitive flexibility.

When investigating effects of ACh on behavioral flexibility, or any other behavior, one has to keep in mind important particularities observed in the cholinergic system: (1) Many cholinergic neurons in the brain show co-transmission, including cholinergic neurons from basal forebrain, which co-release GABA with ACh (Saunders et al., 2015a) and CINs that co-release ACh and glutamate (Gras et al., 2008b; Higley et al., 2011; Sakae et al., 2015; Gangarossa et al., 2016). Methodologies that rely on elimination or silencing of cholinergic neurons will inhibit both neurotransmitters simultaneously, complicating the interpretation of behavioral analysis. Fortunately the availability of genetically modified mice, which allow for specific targeting of components of cholinergic, glutamatergic and GABAergic machinery, permits restricting the release of a single neurotransmitter from these neurons. Combining these different genetic models with pharmacological, opto- and chemo-genetics approaches will allow separating the function of these co-released neurotransmitters in different behaviors, including cognitive flexibility. (2) The cholinergic gene locus has a unique organization (Eiden, 1998). Transgenic mice generated using the full ChAT genomic sequence carry also the VACHT gene, which leads to overexpression of VACHT protein and consequent increase in cholinergic tone (Nagy and Aubert, 2012; Kolinsky et al., 2013b). Importantly, increased cholinergic tone interferes considerably with cognition (Kolisnyk et al., 2013b; Crittenden et al., 2014). Thus, in case these animals are used in behavioral analysis, results obtained should be considered carefully taking in consideration the hypercholinergic phenotype.

In conclusion, there is unquestionable evidence that ACh is important for the modulation of cognitive flexibility however, more needs to be done to clearly identify all the cholinergic circuits involved in this modulation and unravel the exact role of ACh in the process. Addition of genetically modified mice, in which the cholinergic machinery can be spatially and temporally targeted, as well as optogenetics and chemogenetics to modulate neuronal activation, to the portfolio of tools available to investigate behavior should allow progress in separating the function of the different cholinergic circuits.

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