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Effects of Xanomeline on Attention in Rats

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Abstract

The cholinergic system plays a large role in regulating attentional processing. Diseases such as Alzheimer's Disease are known to degrade cholinergic neurons and deactivate cholinergic M1 receptors. Dysfunction in the cholinergic system results in a wide range of cognitive deficits, including a decrease in attention. The cholinergic system has been a focus of drug research to help modulate acetylcholine levels in order to relieve AD symptoms. Xanomeline is a drug previously used in this endeavor that works via agonism of acetylcholine M1 and M4 receptors. Cognitive improvements of Xanomeline administration in AD are thought to be due to agonism of M1, a receptor that is widely expressed in the cortex. The present study investigated the effects of Xanomeline administration on attention to determine if M1/M4 agonism could improve sustained attention. Results of this experiment revealed that M1/M4 agonism by Xanomeline did not improve attention as predicted, in fact some measures of sustained attention reflected poorer performance. The measures that were affected suggest that agonism of M4 may have a significant impact on motivation and movement during attentional testing, and M4 agonism may have obscured any improvements on attention by agonism of M1. This study may support recent evidence that Xanomeline administration in vivo produces greater activation of M4 than M1. Future studies investigating the role of M1 in attention should focus on drugs that are more selective for the M1 receptor.

Effects of Xanomeline on Attention in Rats

Attention is a complex executive function that includes the ability to sustain focus over time, the ability to focus on some environmental stimuli while filtering out extraneous information, and the ability to shift the attentional set (Bushnell & Strupp, 2009). Sustained attention requires vigilance and constant focus on the surrounding environment, resulting in a high cognitive load (Sarter et al., 2001).

The prefrontal cortex (PFC) is heavily implicated in a wide range of tasks involving executive function (Euston et al., 2012). Specifically in rodents, the medial prefrontal cortex (mPFC) is responsible for attention, including sustained attention (Totah et al., 2009). Innervation of the mPFC by cholinergic neurons plays a critical role in its control of attention, and makes the cholinergic system a good target for drug therapies to increase attention (Bloem et al., 2014). Additionally, in Alzheimer's Disease, there is cholinergic cell loss throughout the brain that results in a loss of attention (Sharma et al., 2019). A drug that alters cholinergic functioning would provide a potential treatment in offsetting symptoms of the disease.

Acetylcholine and Attention

Acetylcholine (ACh) is an amine neurotransmitter that has neuromodulatory effects throughout the CNS, including modulations in neuronal excitability and presynaptic neurotransmitter release (Picciotto et al., 2012). Cholinergic neurons in the CNS mainly originate from the basal forebrain cholinergic system (BFCS), and have widespread cortical projections, including to the mPFC (Bloem et al., 2014). ACh plays many different roles in regard to attention depending on the brain region it is acting in, as well as the type of receptor (Klinkenberg et al., 2011). Thus, the projections from the BFCS can have effects on different aspects of attention. In particular, ACh is involved in orienting, selecting, and top-down

attentional processing (Klinkenberg et al., 2011). Top-down attentional processing includes tasks where prior knowledge influences the filtering of relevant stimuli to direct attentional focus to where it is known that important signals will appear (Sarter et al., 2001). For instance, in the case of a visual sustained attention task, ACh modulates the top-down attentional processing that focuses rodent attention on the light stimulus being presented.

Acetylcholine levels are associated on multiple dimensions to the ability to sustain attention (Decker & Duncan, 2020). On a large scale, cortical ACh levels are increased during the awake stage, and decrease during slow-wave-sleep and periods of rest (Jones, 2005). These changes follow observed capacities for sustained attention throughout the day, as both cortical ACh levels and sustained attention are higher in the morning and decline as the day progresses (Decker & Duncan, 2020; Riley et al., 2017). On a smaller scale, during tasks with high attentional demand, there is a large increase in ACh levels in the mPFC (Kozak et al., 2006). During a visual sustained attention task, cortical ACh levels have been shown to increase compared to baseline tasks with no attentional demand (Arnold et al., 2002; Himmelheber et al., 2000). Additionally, pharmacological inhibition of cholinergic function causes deficits in the maintenance of selective attention (Furey et al., 2008). Altogether, considerable evidence associates increased ACh with increased sustained attention.

Muscarinic Receptors and Attention

Acetylcholine has two classes of receptors that are expressed in the CNS, nicotinic and muscarinic. Investigation of ACh receptor subtypes has revealed that blockage of muscarinic receptors causes attentional deficits, whereas blockage of nicotinic receptors does not (Ruotsalainen et al., 2000). Additionally, a more recent investigation determined that the blockage of muscarinic receptors specifically impairs maintenance of selective attention (Furey

et al., 2008). These findings indicate that cholinergic projections onto neurons containing muscarinic receptors, likely in the mPFC, are important in sustained attention. However, the effects of muscarinic receptor activation are not limited to the mPFC, as changes in V1-mediated sustained attention have also been shown (Herrero et al., 2008). Additionally, changes in muscarinic ACh activity in the PFC has been implicated in modulating attention in other brain areas, such as the posterior parietal cortex (Nelson et al., 2005). Sustained attention relies on muscarinic receptor activation in the PFC and may act through activation of the mPFC, or through modulation in other areas.

Most previous studies investigating the role of ACh in attention have used the non-subtype selective muscarinic antagonist scopolamine to determine the role of nicotinic vs muscarinic receptor subtypes (McQuail & Burk, 2006). While this has been useful in determining the roles of each receptor type, it does not give important information to the specific subtype involved. Additionally, limited research has been conducted that uses antagonists that are more specific to muscarinic receptor subtype (Robinson et al., 2012). Another approach, employed in the present experiment, is to use drugs that act as agonists at more specific muscarinic receptors.

M1 Receptors

Muscarinic receptors are metabotropic ACh receptors that are present throughout the CNS, and have different cellular actions depending on the subtype. M1, M3 and M5 are each expressed postsynaptically and are coupled to a G_q protein that provides excitation via activation of phospholipase C, leading to an intracellular cascade that activates the release of Ca^{2+} (Thiele, 2013). The M2 and M4 subtypes are mostly expressed presynaptically and are coupled to a G_i protein that modifies ACh release via inhibition of adenylyl cyclase (Thiele, 2013). The

muscarinic receptor subtypes are expressed differentially throughout the brain, with M1 being the dominant receptor, making up 50-60% of all muscarinic subtypes (Bertrand & Wallace, 2020). Expression of the M1 receptor is seen in almost all areas of the forebrain, including the mPFC (Jiang et al., 2014). The M1 receptor has been implicated in many cognitive functions, including sustained attention. Pharmacological blockage of the M1 receptor resulted in a decrease in sustained attention in rodents (Robinson et al., 2012). Additionally, a change in M1 receptor function in the frontal cortex in AD has been linked to cognitive decline. In AD, M1 receptor densities remain constant, however pathogenic amyloid-beta formation results in a physical uncoupling of the G_q protein from the M1 receptor, leaving it inactive (Tsang et al., 2006). This makes the M1 receptor a particularly interesting drug target for potential AD treatments.

Xanomeline

Xanomeline is an orthosteric M1 and M4 agonist that first used in the late 1990s as a potential drug in the treatment of Alzheimer's Disease (AD). It was used as a potential treatment for AD to restore the loss in cholinergic function that occurs in disease progression. In AD, there is a general loss of cholinergic neurons, as well as a decline in M1 signaling due to decoupling of the G-protein (Francis et al., 1999). A few studies revealed that Xanomeline treatment in humans was able to enhance cognitive function and decrease psychosis related symptoms of AD (Bodick et al., 1997; Bymaster et al., 1997). However, around half of the participants discontinued use of Xanomeline due to side effects that are commonly seen in cholinomimetic treatments, such as gastrointestinal problems and hyposalivation (Mirza et al., 2003). Recently, interest around Xanomeline has increased, and a treatment for schizophrenia involving Xanomeline is currently in Phase 2 clinical trial (Brannan et al., 2020). This group demonstrated that Xanomeline in

combination with Tospium, a peripheral muscarinic receptor antagonist, has increased Xanomeline's tolerability while maintaining therapeutic benefits.

Despite these problematic effects in humans, Xanomeline remains a useful research tool to investigate the role of the M1 and M4 receptors. Xanomeline is a small and lipophilic molecule, making it capable of crossing the blood-brain barrier and producing effects in the CNS (Sauerberg et al., 1992). Xanomeline shows preferential binding to M1 and M4 receptor subtypes, and has a particularly strong binding affinity to M1 and binds in a wash-resistant manner (Broadley & Kelly, 2001). The exact mechanism for the persistent binding of Xanomeline to M1 is unknown, but is thought to be due to binding to multiple sites on the M1 receptor (Christopoulos et al., 1999; Jakubik et al., 2006). Due to the dominance of M1 in cortical areas, it is thought that cognitive improvements shown in Xanomeline treatment are due to agonism at the M1 receptor (Bender et al., 2017).

Focus in Xanomeline studies has mainly been on the M1 receptor, however, Xanomeline also binds with some affinity to the M4 receptor. In fact, recent in vivo investigations suggest that Xanomeline has potent effects on the M4 receptor in low doses (Thorn et al., 2019). Unlike M1, activation of the M4 receptor is inhibitory, and decreases presynaptic ACh release. M4 has low expression throughout the cortex and is mainly expressed in the striatum. The striatum has approximately equal expression of M1 and M4, but the ratio between M4 and M1 in the striatum is much higher than in other areas (Thiele, 2013). Thus, activation of the M4 receptor produces a decrease in ACh release in the striatum, which is thought to be the mechanism by which Xanomeline produces anti-psychotic like effects (Woolley et al., 2009).

Attentional Measures

There are multiple ways by which attention can be measured depending on the specific type of attention being characterized. To measure sustained attention, two main paradigms are used – the five choice serial reaction task and the sustained-attention task (Bushnell & Strupp, 2009). The five choice serial reaction task involves rodents maintaining attention on five spaced out openings and responding to a light stimulus by poking their head through the correct opening (Bushnell & Strupp, 2009). The sustained-attention attention task is non-spatial, has signal and non-signal trials, and provides more measures of sustained-attention. The sustained-attention task may be considered a purer measure of attention because it does not engage the rat's natural tendency to respond based upon spatial information. The sustained-attention task requires differential responses for when there is presence vs absence of a signal, and measures correct responses and rejections, misses, and omissions (Zajo et al., 2016). These measurements allow for an in-depth characterization and comparison of attention in experimental models. In this task, rodents are trained to pay attention to the presence of a light stimulus within an operant testing chamber, and then respond by pressing a lever to report the presence or absence of the light. Attention is measured for signal and non-signal trials over a period of 126 trials and requires attentional focus for reward presentation. After training, drugs can be administered to assess changes in sustained attention.

Current Study

Previous studies have identified that in humans, one of the effects of Xanomeline is an increase in attention (Bymaster et al., 1997). The exact effects of Xanomeline and the M1/M4 receptors on attention however have not been directly investigated. In this study, the sustained-attention task was used to assess rodent attention with exposure to Xanomeline or saline

injection. The goal is to show that M1 agonism by Xanomeline administration increases attentional capabilities.

Methods

Subjects

A total of six Sprague-Dawley rats were used in this experiment. Experimental protocol was approved by the Institutional Animal Care and Use Committee at the College of William & Mary. Subjects were individually housed in the temperature and humidity controlled environment and kept on a 14/10-hour light/dark schedule. Food was available ad-libitum throughout the experiment, and water was restricted to 10 minutes per day in addition to water received as a reward during testing.

Testing Apparatus

Rats were tested and trained in one of six operant testing chambers (Med Associates Inc.) that were located within a sound-attenuating box. Each chamber consisted of two retractable levers, a water port with retractable water dipper (0.01 mL) between the levers, and three panel lights. One panel light is located above each lever, and one above the water port, but during testing only the middle light above the water port was used. Additionally, there is a house light that illuminates the testing chamber on the opposite panel.

Behavioral Training

Training the rats to perform the sustained-attention task occurred in three stages. The first stage involved shaping the rats to press a lever. During shaping, the levers were extended at all times, and the press of either lever resulted in a water reward via the water dipper rising for two seconds. In order to minimize bias for either lever, if one lever was pressed five times in a row, the water reward would not be presented until the opposite lever was pressed. After rats had

successfully pressed a lever and received the water reward 120 times for three sessions, the next stage of training began.

After the rats were trained to press a lever, they were trained to discriminate between a signal and non-signal. The signal stimulus was a one second illumination of the panel light, and a non-signal was no light illumination. In this stage, each trial begins with the levers retracted, then after the stimulus is presented, the levers extend. For half of the rats, pressing the right lever after signal stimulus presentation resulted in the water reward being presented and this was recorded as a hit. A miss was recorded when the rat pressed the wrong lever in response to the signal stimulus. In order to account for any side bias, the other half of the rats were trained such that pressing the left lever after signal stimulus produced a water reward. After a non-signal, rats that pressed the opposite of their reward lever were presented with the water reward, which was recorded as a correct rejection. If the reward lever was pressed during a non-signal trial, this was recorded as a false alarm. Any incorrect response was followed by a correction trial that repeated the previous trial, but only the correct lever extended after stimulus presentation. An omission was recorded if there was no lever press after the lever was extended for three seconds. Intertrial interval (ITI), the time between trials, was 12 seconds. Rats were trained in this stage until they reached a threshold of 70% hit rate and 70% correct rejection rate for three consecutive sessions.

The final stage of training involved the use of different signal durations, and there were no correction trials. During this stage of training, the goal was to increase attentional demand, which is done in a few different ways. First, the signal stimulus was presented for either 500, 100, or 25 ms, and the durations were randomly varied. Also, the ITI was decreased to 9 ± 3 seconds. This stage was conducted for 126 trials per session, half with signal and half with non-signal trials. Once subjects reached levels of 70% hits (for 500 ms signal), 70% correct

rejections, and had less than 20 omissions for three testing sessions, the subject was considered trained to move on to drug administration and testing.

Drug Administration and Testing

Xanomeline was prepared by dissolving Xanomeline oxalate (Abcam) in saline to reach a concentration of 3.0 mg/mL. Xanomeline solution was stored in a -40 °C freezer when not in use and was administered no more than three days after preparation. Drug administration of vehicle and drug was performed on each subject after training criteria were met. On the first day of injection, half of the rats received two intraperitoneal (i.p.) injections of saline (totaling 2 mL/kg, equal to drug volume), and half received two i.p. injections of Xanomeline (totaling 6 mg/kg). Testing was then conducted approximately 10 minutes after injection, as Xanomeline has a short half-life of 32 minutes (Bender et al., 2017). The second injection day occurred three days after the first injection day, with at least one session of drug-free testing between administrations. The same procedure occurred for the second day of injections, but the injection groups switched.

The testing that occurred after injection was a modified version of the sustained-attention task the subjects had been trained on. One day prior to the first injection day, rats were exposed to this version of the task such that it was not a novel experience in combination with drug injection. The testing session included three distinct blocks of equal lengths (42 trials each). The first block of the testing session was the normal sustained-attention task, and the house light on during the duration of the task. The second block is considered the distractor block, it had the same stimulus presentation and signal duration; however, the house light was functioning as a distractor, and would flash on and off every 0.5 seconds. This block is particularly attentionally taxing, as the flashing house light is disorienting and distracting. The third block functions as a recovery block, and the task is back to the same settings as the first block. In this version of the

sustained-attention task, a baseline response can be measured, then response during distraction is measured, followed by a measurement of recovery from distraction.

Measurements and Statistics

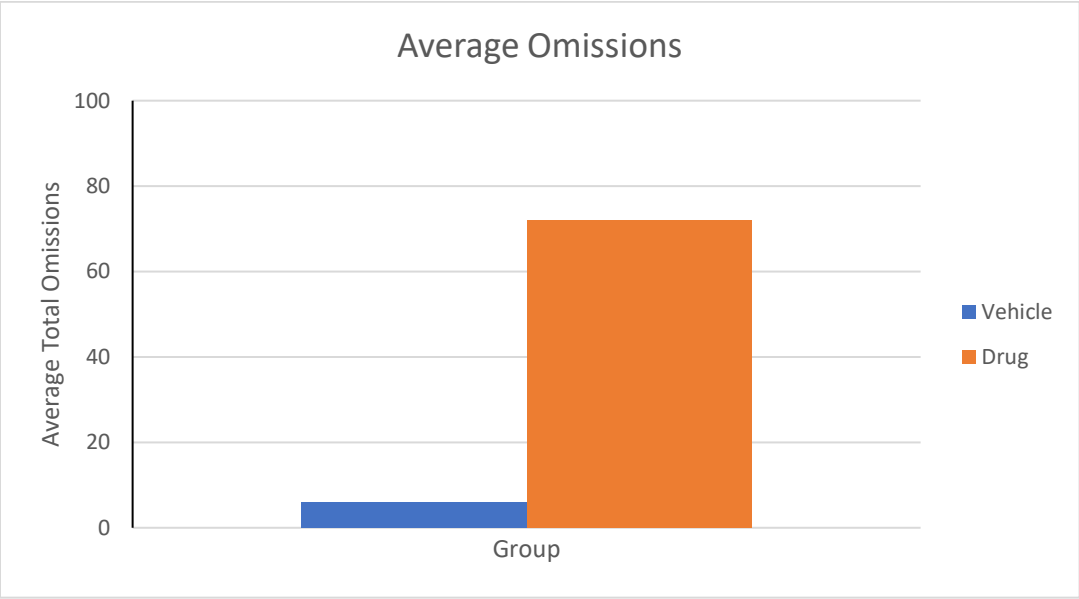
For each block of the testing session, the number of hits and misses for each signal duration, the number of correct rejections, and the number of omissions were recorded for each block. Repeated-measures analyses of variance (ANOVAs) were conducted using factors of drug (saline or Xanomeline) and block (block 1, block 2, block 3), in combination with signal duration for hits, correct rejections, or omissions. Significant interactions were further investigated using paired samples t-test. All analyses used an alpha level of 0.05 to determine statistical significance.

Results

A drug x block ANOVA for correct rejections yielded no significant main effect of drug or of the drug x block interaction ($F(1,5) = 0.348$, $p = 0.581$). The results of the two-way repeated measures ANOVA revealed a significant main effect of drug on omissions ($F(1,5) = 9.337$, $p = 0.028$). Xanomeline significantly increased omissions compared with saline administration (Fig. 1). The number of omissions were similar across each block within drug groups.

Figure 1

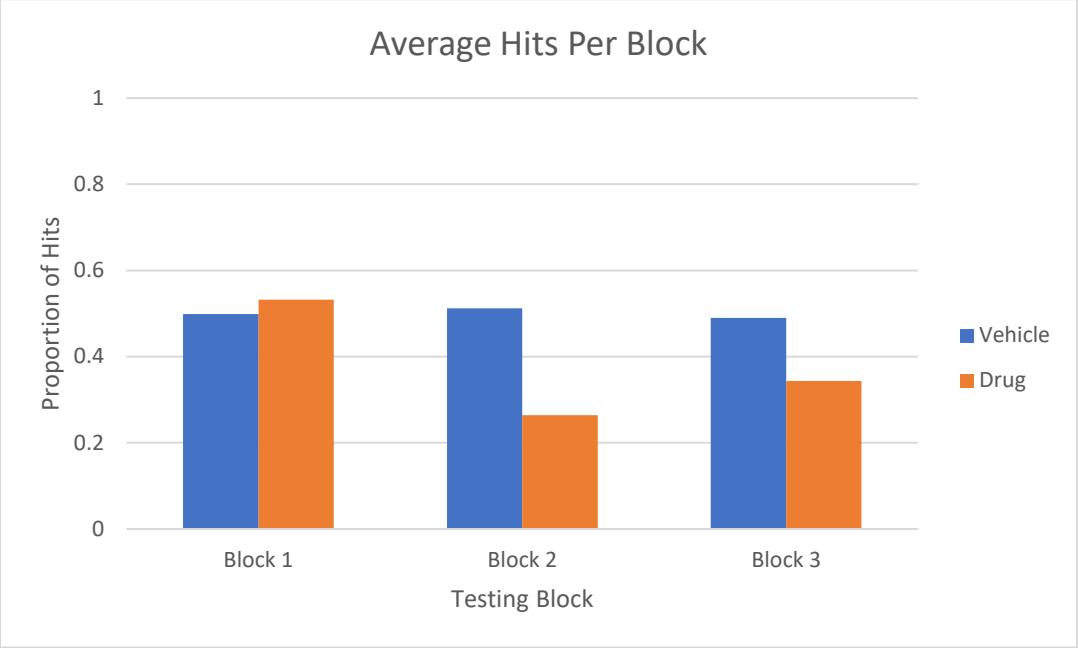
Xanomeline administration increases omissions



Next, a repeated measures ANOVA with the factors of drug, block, and signal duration was conducted for hits. The main effect of drug was not significant, however the drug × block ($F(2,10) = 2.920$, $p = 0.111$) and the drug × signal duration ($F(2,10) = 4.436$, $p = 0.063$) interactions trended toward significance. Further analysis was performed using paired samples t-tests, comparing drug and saline signal detection accuracy (hits) during each block and for each signal duration. Results of the paired samples t-test showed that the mean difference of vehicle block 2 versus drug block 2 was statistically significant ($t(5)= 5.01$, $p = .004$). Average proportion of hits per block for each drug group are shown in Fig. 2, with the y-axis showing the average proportion of hits to hits and misses.

Figure 2

Average proportion of hits per testing block



Discussion

Attentional deficits from dysfunction of the cholinergic system are prevalent in diseases such as AD and schizophrenia. Currently, there are limited viable treatment options to enhance the functioning of the cholinergic system. Xanomeline is a M1 and M4 agonist that was used in the treatment of these diseases; however, its use was discontinued after intolerable adverse effects. Investigations are currently underway to determine if Xanomeline in combination with a peripheral muscarinic antagonist can produce similar therapeutic effects while minimizing adverse effects. However, the effects of Xanomeline on attention have not been explicitly studied. Determining the effects of Xanomeline on sustained attention will help to further characterize the roles of M1 and M4 receptors in attention.

In this study, we used i.p. Xanomeline administration prior to sustained-attention testing to determine the impacts the drug has on attention. Previously, the literature has characterized

Xanomeline's action on the M1 receptor to be responsible for improvement of cognition, and action on the M4 receptor results in antipsychotic effects (Bender et al., 2017). This finding, in combination with the fact that in vitro, Xanomeline activates M1 154 times more than M4 (Heinrich et al., 2009), lead us to predict that Xanomeline would help improve attention via activation of M1. Interestingly, Xanomeline did not increase attention as predicted, and in fact it significantly decreased some measures in the sustained-attention task. This effect is likely due to activation of M4, which regulates ACh release in the striatum, an area involved in movement and reward processing. Agonism of the M4 receptor was likely involved in the decrease of attention because the specific measures that were decreased reflect a decrease in movement and/or reward capabilities.

The lack of significant change regarding correct rejections and hits during blocks 1 and 3 suggests that the subjects still knew the rules of the task. However, during drug trials subjects omitted significantly more often for each testing block, indicating a decrease in attention, reward motivation, or movement. Additionally, during block 2 when the distractor was presented, hits significantly decreased during drug trials compared to vehicle, indicating a decrease in attention with high attentional demand. Overall, results indicate that Xanomeline impaired the ability of the subject to respond and decreased attention during the attentionally-taxing block 2 but did not impair the knowledge of the rules or attention during normal cognitive load.

Since there has not been a study that explicitly investigates the impact of Xanomeline on attention, the results of this study do not contradict existing literature. However, the results are surprising given findings from previous studies that highlighted an increase in cognitive capabilities with Xanomeline administration. The decrease of Xanomeline in this one specific aspect of cognition may have to do with the fact that M4 receptors are also activated, which may

not cause a problem for other cognitive tasks but is important in attention. The recent finding that low doses of Xanomeline in vitro may have more activation of M4 over M1 supports this reasoning (Thorn et al., 2019). The decrease in attentional measures in this task was likely due to M4 agonism because that would decrease motivation and movement, and any agonism of the M1 receptor was not enough to overcome these deficits.

There are some limiting factors to this study, including a small sample size that may have obscured some significant findings. Different results may also have been found if this study were conducted using subjects with cholinergic neuron loss, as Xanomeline intervention may have provided enough muscarinic agonism to rescue attentional deficits. Another limitation is that only one administration of one dosage was used, so no conclusions can be drawn about effects after chronic administration or dose-dependent effect. Future studies regarding Xanomeline and attention should try to use larger doses as that may have differential effects on which receptor is activated. A larger dose is more likely to impact M1 (Thorn et al., 2019), thus may allow for beneficial attentional effects to be seen.

Future work regarding M1/M4 activity in attention should be conducted, as cholinergic loss is relevant in diseases such as AD and schizophrenia. It would be interesting to see how Xanomeline impacts attention in a model of one of these diseases to see if that has a different change in attention. Since there is a recent rise in interest regarding Xanomeline in combination with a peripheral muscarinic antagonist, future studies may consider investigating any attentional differences in this combination versus Xanomeline alone. Future work should also include investigations into other drugs that have more selective M1 agonism to compensate for the loss of M1 activity in AD.

The findings of this study provide evidence for the importance of M1 and M4 activity in attention. Xanomeline agonism of M1 and M4 decrease the ability to respond during the sustained-attention task and decrease response accuracy during high-demand portions of the task. This is likely due to the activation of the M4 receptor that decreases motivation and movement that obscures any increase of attention that agonism of M1 may produce. More research should be done to see if this effect still occurs in disease models to determine if Xanomeline or new M1 agonists could serve as a useful treatment for diseases such as AD and schizophrenia.

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