Chemogenetic inhibition of striatal cholinergic neurons on hallucination-like perception

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Abstract

Psychotic disorders pose a significant global health burden with high incidence and relapse rates and the development of treatments has stagnated for decades, in part due to the lack of animal models that effectively recapitulate the defining symptoms of psychosis, hallucinations and delusions. The cholinergic hypothesis remains one the prevailing theories of the underlying aetiology of psychotic disorders, however, a causal relationship between cholinergic activity and a defining symptom of psychosis, hallucinations, has yet to be established in animal models. We hypothesised that the chemogenetic inhibition of striatal cholinergic neuron activity could produce a quantifiable change in hallucination-like perception, using a task developed by Schmack et al. (2021). Mice (n=5) were trained to perform the task with high accuracy, maintained throughout baseline and experimental periods. Within-subject differences in false alarm rates and high confidence false alarm (HALIP) rates were analysed as behavioural readouts of hallucination-like perception. We were unable to confirm our hypothesis, as our results showed no statistically significant differences in false alarm rates, HALIP rates or HALIP time investments compared to control. Our results may suggest that hallucination-like perception is not mediated by striatal cholinergic activity alone. The complexity of cholinergic modulation of striatal circuitry and the proposed mechanisms of cholinergic drugs mechanisms underlying their psychotogenic/antipsychotic effects point towards a conflicting role of cholinergic signalling in psychosis-like behaviour, so further studies that manipulate in-vivo activity in a more temporally-precise and spatially-specific manner, with concurrent recordings, are needed to establish causality and pave the way for novel treatments.

Introduction

Psychotic disorders such as schizophrenia represent a major source of global disease burden, imposing significant social and economic costs. The incidence rate has not seen improvement for at least half a century (Kirkbride et al., 2012) and the relapse rate remains around 80% (Robinson et al., 1999; Taylor et al., 2019;). Across that same period, advancements in the pharmacological treatment of psychotic disorders have been minimal because the development of mechanistic models of the underlying pathology has long stagnated.

Most pharmacological treatments for psychosis are dopamine antagonists, in line with the long-standing dopamine hypothesis of schizophrenia; another prevailing theory of the underlying aetiology of schizophrenia is the cholinergic hypothesis. Muscarinic antagonists such as scopolamine are well-known to induce psychosis-like states with hallucinations such as delirium, while muscarinic agonists and positive allosteric modulators have been implicated since as early as the 1950s (Pfieffer and Jenney, 1957) in attenuating psychotic symptoms in schizophrenia patients, gaining traction for pharmacological development in the 1980s with another serendipitous observation (Bodick et al., 1997) of antipsychotic effects with xanomeline, a muscarinic M1/M4 agonist. However, despite demonstrating rapid and robust antipsychotic effects in a small clinical trial (Shekhar et al., 2008), substantial peripheral cholinergic-related adverse events precluded xanomeline's development, until recent trials of a novel coformulation strategy in KarXT - combining it with a peripherally restricted muscarinic antagonist - yielded robust results, which, if approved, would represent the first novel pharmacological approach to treating psychotic disorders in several decades. These findings implicate decreased cholinergic transmission in psychosis.

Despite a sparse distribution - comprising only ~1% of striatal neurons - striatal cholinergic interneurons (ChIs) are one of the principal sources of acetylcholine in the brain (Bolam et al., 1984) and crucially, appear to play a central role in the neuromodulation of local striatal circuitry, which is long thought to be dysfunctional in schizophrenia. There appears to be a complex bidirectional relationship between dopaminergic and cholinergic transmission in the striatum (Chantranupong et al., 2023, Matiyahu et al., 2023, Krok et al., 2023), with pauses in ChI activity linked to

phasic dopamine (Straub et al., 2014; Chuhma et al., 2014). Activity in striatal cholinergic interneurons has been linked to behavioural flexibility, reward and modulating prediction error signals (Bennet et al., 2000; Apicella et al,. 2007; Ding et al., 2010; Bradfield et al., 2013). However, the causal role of striatal cholinergic transmission in the perceptual aberrations characteristic of psychosis has yet to be elucidated.

The tail of the dorsal striatum (or auditory striatum) plays a role in the integration of sensory processing (Xiong et al., 2015; Guo et al., 2018) and shows unique input-output connectivity (Jiang and Kim, 2018; Hunnicut et al., 2016), suggesting that the tail of striatum could be a canditate region mediating the link between cholinergic signaling and hallucinations in psychosis.

The defining symptoms of psychosis, hallucinations and delusions, are subjective experiences and as such, prove challenging to quantify, model and study, particularly in non-human animals - for whom there is no way to directly report perceptual phenomena - and thus, the development of mechanistic models of the underlying pathology has long stagnated. As a result of these inherent challenges, research has depended on indirect behavioural measures (e.g. pre-pulse inhibition and amphetamine-induced hyperactivity) and a range of models (drug-induced, lesion, genetic and developmental) with limited face validity, capable of capturing only objective behavioural (e.g. locomotor or attentional) or neurological changes, which although may often correlate with psychosis, provide no insight into the actual perceptual experience. As a consequence, these behavioural measures and models offer relatively poor construct and predictive validity, resulting in limited translational value for psychosis treatments and more broadly, significantly constrains the range of possible approaches for interrogating the relationship between neuronal circuitry and psychosis-relevant experiences.

Inspired by the phenomenology of hallucinations and building on advances in measuring decision confidence in rodents (Kepecs et al., 2008), Schmack et al. (2021) developed a behavioural approach in the HALIP task that facilitates quantification of these experiences with refined face validity. Here, we therefore applied the HALIP task to test whether the inhibition of striatal cholinergic neurons

would result in a change in the HALIP rate, which could serve as evidence for a casual role of striatal cholinergic transmission in hallucination-like perception.

Materials and Methods

Animals

In order for our chemogenetic manipulation to specifically target cholinergic neurons, we used transgenic ChAT-Cre C57BL/6J mice (3 females and 3 males, with one female experiencing a complication from surgery that led to culling before data collection). By the end of data collection, mice were 30-35 weeks old. Mice were kept in a 12-hour inverted light-dark cycle, with all training and procedures carried out during the dark cycle. Food was provided ad-libitum in the cages and behavioural setup, but water could only be consumed as reward during the behavioural sessions, with animals generally consuming 1-1.4ml during a 1.5-hour session, run once per day, 5 days a week. On non-experimental days, animals were provided with 2% citric acid water as a way of preventing dehydration, while also maintaining their motivation to attain water to ensure task engagement. Weights of all mice were measured weekly.

Surgery and Viral Injection

To allow for the chemogenetic inhibition of cholinergic neurons in the striatum, the ChAT-Cre mice were anaesthetised and placed into the stereotaxic frame and injected bilaterally (a total of 6 points of injection with the following coordinates: +-3.3 ML, -1.35AP with two depths: -2.8 and -2.5DV +- 3.35ML, -1.7AP, -2.65DV) with multiple injections along the anterior-posterior axis to ensure adequate spread of expression with a viral construct, AAV8- hSyn-DIO-hM4Di-mCherry, to express DREADD (hM4Di) and the flourescent protein mCherry, only in the injected region. The surgical injections of the viral vector were modified to with multiple injections along the anterior-posterior axis to ensure adequate spread of hM4Di expression in the tail of striatum. Surgeries were performed by a PhD student in the lab, Aiste Viduolyte and a surgical technician, Sophie Wood. Mice recovered for up to a week before being placed back on water restriction and resuming training.

Deschloroclozapine preparation and administration

In order to stimulate specifically the Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), such as the hM4Di receptor in our study, a ligand selective for the receptor is chosen. Deschloroclozapine (DCZ) is a relatively novel DREADD ligand that is brain-penetrable displays superior receptor selectivity, affinity and chemogenetic receptor occupancy compared to CNO (Nagai et al., 2020, Nentwig et al., 2022) which also has the added issue of reverse-metabolism to clozapine, a potent antipsychotic drug. At doses as low as 1ug per kg, DCZ is capable of rapidly producing effects in-vivo (observed as changes in fluorescence signals) reaching their peak at around 10 minutes following intraperitoneal injection, plateauing for at least 150 mins before returning to baseline levels 4 hours post injection (Nagai et al., 2020). However, no effects were observed on food intake suppression in DREADD-expressing mice at this dose, where they were observed at 0.1mg/kg. On the other hand, at 5 mg/kg DCZ affects sleep-quality and quantity in DREADD-expressing mice but not in those lacking DREADD expression, suggesting that there are not significant off-target behavioural effects at this dose (Ferrari et al., 2022). Taken together, a dose of 1mg/kg was chosen as it is capable of producing robust in-vivo and behavioural effects, while ensuring that minimal off-target effects are present. Given the rapid onset of effects, DCZ was administered at 1mg/kg by intraperitoneal injection immediately before the start of the behavioural session. Water-soluble DCZ (HB9126 - HelloBio) was diluted with saline to a 1mg/kg concentration for a 0.05mL injection in 30g mouse, aliquoted and stored at -20 degrees Celsius, and then immediately after thawing, diluted with saline to the appropriate dose according to the mouse's weight (in order to before injecting 0.05mL of the solution in all mice with a 0.5mL BD microfine 30G syringe.

Behavioural setup (Schmack et al., 2021)

All mice were trained and tested on the HALIP task in a behavioural rig setup which comprised a small box with a centre port and two lateral ports, each with LEDs for visual signals corresponding to stages of the task, infrared photodiodes for detecting port entry/exit, and metal spouts for water reward delivery. Water was delivered through tubing connected from gravity-fed syringes to the metal spouts, controlled by

solenoid valves. The water delivery system calibration was tested, and recalibrated if necessary, before and throughout behavioural training. Auditory stimuli were delivered using speakers either side of the box and were calibrated prior to behavioural training to ensure standardised volume levels. Task parameters relating to auditory stimulus presentation and reward delivery were controlled from a Windows PC connected to a behavioural controller Bpod (Bpod State Machine r2.0; Bpod Hifi Module HD, Sanworks) with custom software written in MATLAB.

HALIP task (Schmack et al., 2021)

HALIPs were quantified in an auditory detection task with time-investment based confidence reports (Fig. 2). During the entire duration of the experiment, constant auditory white background noise (55dB SPL) was played. Mice self-initiated a trial by poking into the centre port, where a light stimulus would be presented. On signal trials, the centre port light cue was accompanied by a simultaneous upsweep tone signal (10 – 15 kHz) with varying volume between 55 dB SPL and 90dB SPL on top of the background noise. 50% of the trials were no-signal trials, in which the centre port light cue was not accompanied by any additional signal. Signal and no-signal trials were randomly interleaved in equal proportions. After stimulus delivery, mice withdrew from the centre port and poked into the left or right choice port to receive reward. Reward was available at one (e.g. the left) port on signal trials and at the other (e.g., the right) port on no-signal trials. The stimulus–reward mapping was pseudorandomized across animals. For correct choices, reward was delivered after a variable delay between 0.5s and 30s (with an exponential distribution with a decay constant of 1s, resulting in a relatively constant level of reward expectancy). In 15% of correct choices, rewards were omitted, allowing us to measure the time the mice were willing to invest in correct trials. For incorrect choices, no reward and no feedback were delivered thereby enabling us to measure time investment at each incorrect trial. HALIPs were classified as false alarms (trials during which no signal was played but the animal reported hearing a signal) where the animal waited a long time for the reward (with time investment above the median time investment of correct catch trials).

Histological Analysis

All processing of brain tissue and histological analysis was performed by Aiste Viduolyte, the PhD student in the lab guiding this project. Mice were perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. Brains were then removed and kept in 4% PFA, then PBS for long-term storage. Brains were then sliced into sections of 50µm thickness with a vibratome.

After transferral to a 6-well plate and rinsing, the brain slices were blocked with a blocking buffer (5% BSA, TritonX-100 PBS), before adding primary antibodies (chicken anti-mCherry, ab205402, rabbit anti-VAChT, 139-103-SY) and incubating. The slices were then washed in PBS before adding 1:500 secondary antibodies (goat anti-chicken antibody, A11041, goat anti-rabbit antibody, ab150077). The slices were then washed again before being mounted with DAPI Flouromount-G. The brain Slices were then imaged under the microscope AxioImager Apotome2.

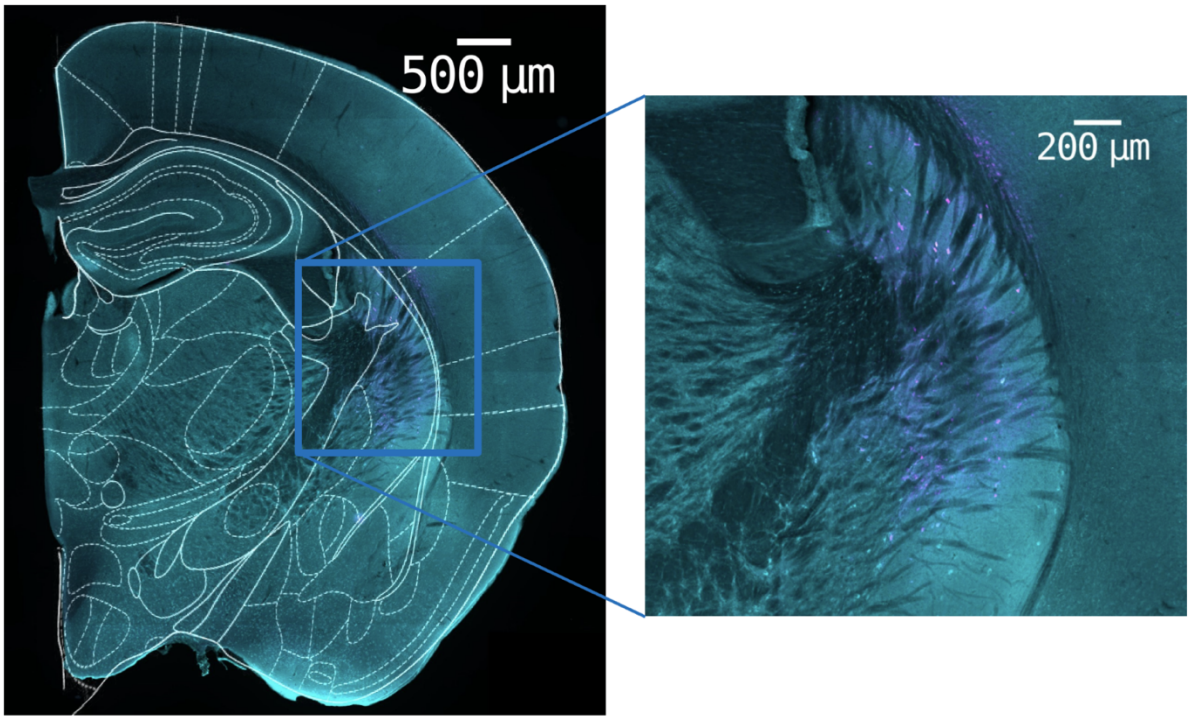

Figure 1. *Histology results of brain slice from one animal confirming the expression of the hM4Di receptor-mCherry construct (magenta) in the tail of striatum and colocalisation with vesicular acetylcholine transporter (VAChT) labelling (teal), a*

marker for cholinergic neurons, overlayed with Mouse Brain Atlas (Paxinos and Franklin, 2001).

Experimental design and analysis

The sample size was primarily limited by time and rig set-up constraints on the number of animals that could be trained simultaneously, as well as the culling of one mice prior to data collection due to surgical complications. A second cohort of two mice, started months after the first, is currently underway. The planned final sample (n=8) size is based on the effect sizes observed in previously published data from comparable optogenetics experiments (Schmack et al. 2021). We used the effect size for high-confidence false alarms (d=1.20) to calculate required group sizes to achieve a statistical power of 0.8 (beta=0.2) with an alpha of 0.05. Sample size calculations were performed in Matlab, using the routine sampsizpwr.

DCZ and saline eppendorfs used for injections were blinded and the injection schedule was pseudo-randomised with blocks of two. The low sample size also precluded the inclusion of more rigorous controls (such as a control group of mice injected with a viral construct lacking the gene for the hM4Di receptor) however, the off-target effects of hM4Di receptor expression and/or DCZ administration are likely to be relatively minimal. No sex or age differences were observed in the baseline data.

Behavioural data analysis was performed using custom MATLAB code and statistical analyses were performed with repeated- measures ANOVA (using the ranova, ttest2 and multcompare functions), with a significance threshold of p=0.05. Sessions with accuracy below 60% were excluded from data analysis to ensure the quality of the data and time investments below two seconds were also excluded from analyses of confidence as they may reflect insufficient engagement rather than very low confidence.

Results

Mice trained on the HALIP task to a high level of performance

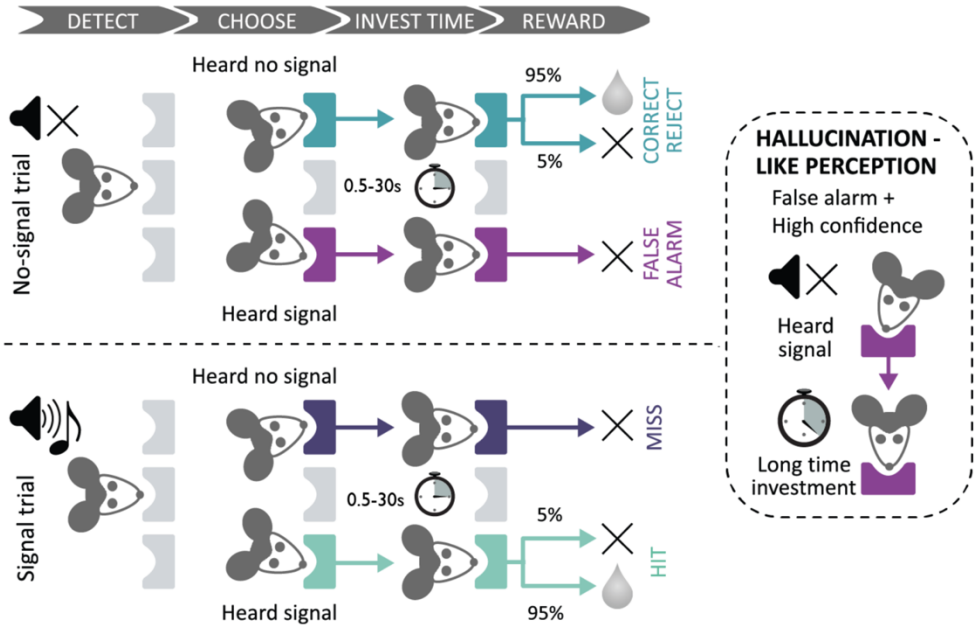

Figure 2. Schematic of the HALIP task *(taken from Schmack et al., 2021). Water-restricted mice were presented with tone signals with graded levels of volume (50-85dB), interleaved across trials, embedded in a constant background of white-noise (50dB). Mice then reported the perception of a signal/no-signal by poking into one choice port if they perceived a signal and the other choice port if they did not perceive a signal. Correct choices are rewarded with water, which is delivered after a variable and unpredictable delay of 0.5-30s. The time duration that mice were willing to invest for a reward provides a quantitative measure of decision confidence. 5% (or in this study, 15%) of trials are catch trials, in which water is never delivered - this allows for the quantification of an animals time investments on correct trials. A hallucination-like perception (HALIP) can be defined as a false alarm trial (where the mouse reports perceiving a signal on a no-signal trial) with a long time investment (classified as those above the median time investment on catch trials), indicating that this false percept was experienced with high confidence. (Adapted from Schmack et al., 2021)*

Across a period of 14 -19 weeks, 5 animals were trained on the HALIP task (Fig. 2), rewarded for correctly discriminating between the signal tone of varying volumes and background noise at a static volume. With training, all mice saw an increase in the

proportion of correct trials and a decrease in false alarm rates (Fig. 3A). Generally, the false alarm rates held stable throughout the baseline data collection period (ranging from 4-7 days). The psychometric curves (Fig. 3B) of all animals during baseline data collection show an increase in accuracy with the signal-noise ratio of the auditory signal stimulus, with animals exhibiting saturation (generally >90%) on the loudest signal trials (85-90dB depending on the animal), while around 10% of executed trials were reported as false alarms. Taken together, these results show that mice could be successfully trained to report whether they perceived a signal or not. Given the high saturation on the loudest trials, these false alarms should reflect a perception of a signal on no-signal trials.

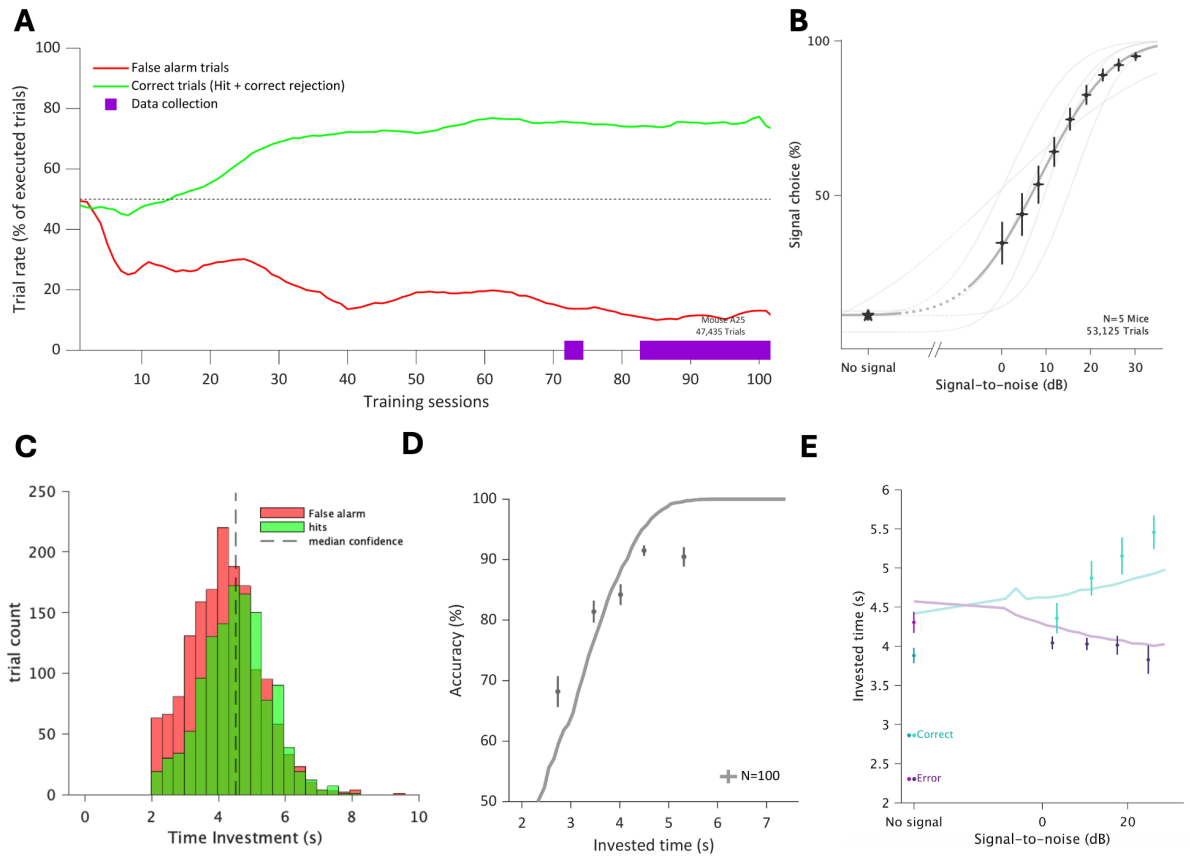

Figure 3: *Baseline behavioural outcomes of the HALIP task.* ***A)*** *Correct trial rate (green, signal and no-signal trials) and false alarm rate (red, no-signal trials only) throughout the length of training and data collection (purple) in an example mouse. An increase in correct trials and a decrease in false alarm rates across training indicates that mice improved their accuracy in the task over time. Both correct trial*

and false alarm rates remained relatively stable throughout baseline data collection. ***B)*** *Average psychometric curve for all animals (black line) overlaid on each animal's individual psychometric curves (grey lines, n=5). Signal choice percentage is fit to a psychometric function. Black dots represent the mean signal choice percentage with error bars. Roughly 10% of no-signal trials were reported as false alarms, while >90% accuracy was achieved on the loudest signal trials.* ***C)*** *Histogram of distribution of time investments in hit (green) and false alarm (red) trials in an example mouse.* ***D)*** *Statistical decision confidence as predicted by model (grey line) and observed time investment (mean ± SD across sessions) in an example mouse.* ***E)*** *Vevaiometric curves for correct and error trials showing predicted confidence (teal and purple lines) and observed time investments (mean ± SD across sessions) in an example mouse.*

We also assessed the distributions of time investments on correct and hit trials and compared time investment behaviour with a model of statistical decision confidence. Building on work from Kepecs et al (2008), decision confidence can be operationalised as the time investment of an animal waiting for a reward. The median values of time investments on correct catch trials per animal were therefore used as a threshold to classify trials as either high confidence trials (above the median) or low confidence trials (below the median). Thus, false alarm trials with time investments above the median were classified as high confidence false alarms and constitute HALIPs. The significant overlap of time investment distributions between false alarm trials and hit trials (Fig. 3C) suggests that the perceived confidence of false alarms with high time investments (HALIPs) was comparable to the confidence on correct trials. The time investments were then quantitatively explained by statistical decision confidence. Statistical decision confidence is defined as the probability of being correct for a given choice. The observed psychometric choice behaviour was fitted to compute statistical confidence probabilities, which were then transformed using quantile normalisation into predicted time investments. Time investments roughly followed the predicted statistical confidence such that time investments were calibrated to accuracy (Figure 3D) and were higher after correct rather than incorrect choices (Figure 3E). However, a closer fit between observed confidence and predicted statistical confidence could only be attained by adding

significant noise to the model and even then, the fitting was still worse compared to previous cohorts trained on the HALIP task, such as in Schmack (2021). It's possible that the relatively longer time taken to train the animals before baseline data collection could have affected this. Despite this, overall, the analysis indicates that observed time investments on the HALIP task could be used as a behavioural readout of confidence.

Chemogenetic inhibition of striatal cholinergic neurons did not affect performance on the HALIP task

After establishing that the mice perform reliably on the HALIP task, we investigated whether chemogenetic inhibition of striatal cholinergic neurons would affect HALIP task performance and behaviour. Based on the evidence for the cholinergic hypothesis and the well-documented importance of the striatum in psychosis, we hypothesised that inhibition of striatal cholinergic neurons would result in an increase in the high confidence false alarm (HALIP) rate, a behavioural readout for hallucination-like perception. In order to test this, all animals (n=6) underwent stereotaxic surgery to express the hM4Di receptor specifically in ChAT+ neurons via a bilateral injection of [virus] in the tail of striatum. Post-mortem histological investigations appear to confirm hM4Di expression in the tail of striatum with colocalization on cholinergic neurons (Fig. 1). Deschloroclozapine (DCZ) (1 mg/kg) and saline, administered via intraperitoneal injection, were pseudo-randomised (between 12 DCZ injections and 12 saline injections in blocks of 2, blinded) for each animal (n=5) and given immediately before the start of each experimental session.

There was no significant difference in the psychometric choice behaviour observed between DCZ and control sessions, with animals generally displaying >90% signal choice on the loudest signal trials (Figure 4A). There was also no significant difference in accuracy between DCZ and control sessions (Fig. 4B; DCZ n=5, 80.6% ± 5.3%; control n=5, 80.9% ± 5.1%, $F(1,4)=0.8$, $p=0.432$) indicating that chemogenetic inhibition of cholinergic neurons did not interfere with the animals' ability to perform the task.

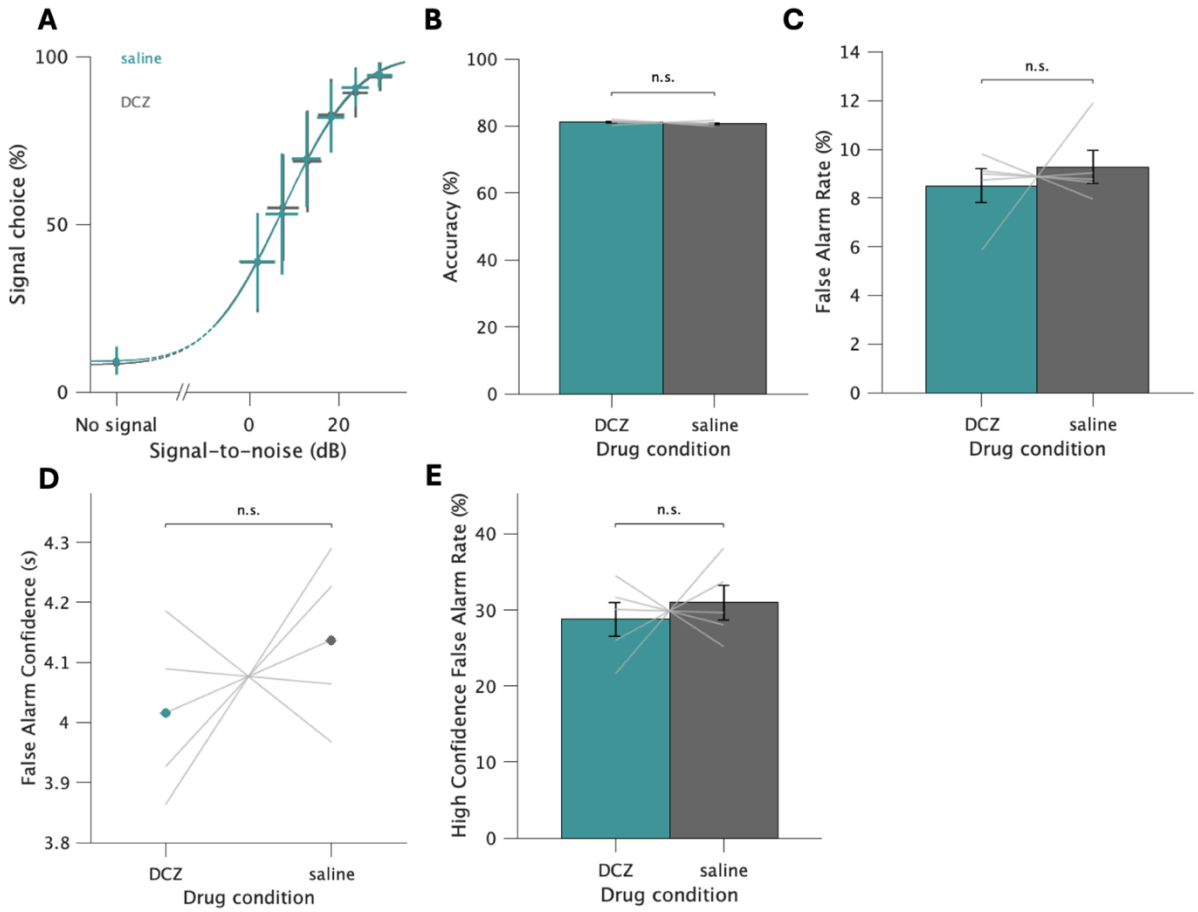

Figure 4: Effect of chemogenetic inhibition of striatal cholinergic interneurons on HALIP performance A) *Average psychometric curves of animals administered with saline and DCZ. Markers represent mean signal choice (%) for a given signal-to-noise ratio (dB), with error bars representing the standard deviation from the mean on both axes. Psychometric choice behaviour was not affected by the administration of DCZ.* ***B)*** *Average accuracy of animals administered with saline and DCZ. Administration of DCZ produced no significant difference in accuracy.* ***C)*** *False alarm rate between saline and DCZ sessions. No significant difference in false alarm rates were observed between saline and DCZ sessions.* ***D)*** *Average time investment (s) of correct trials between control and DCZ sessions; no significant difference was observed. Markers represent group averages for DCZ and saline.* ***E)*** *High confidence false alarm (HALIP) rates between saline and DCZ sessions; no significant difference in HALIP rates were observed.* ***A-E****: saline data represented in grey (n=5) and DCZ data represented in teal (n=5).* ***B,C,E:*** *bars represent mean*

value across each outcome (saline or DCZ); error bars represent SEM around the mean; subject-matching lines between subject means, normalised around the grand mean of all subjects with the Cousineau method (2005).

Chemogenetic inhibition of striatal cholinergic neurons did not alter measures of hallucination-like perception

In order to investigate the effect of chemogenetic inhibition of striatal cholinergic neurons on hallucination-like perception, we compared the false alarm rate and high confidence false alarm (HALIP) rate between the DCZ and control sessions. The results did not reveal any statistically significant differences in either false alarm rate (Fig. 4C; DCZ n=5, 9.3% ± 1.8%; control n=5, 9.9% ± 1.8%, $F(1,4)=0.3$, $p=0.609$) or HALIP rate (Fig. 4E; DCZ n=5, 5.1% ± 0.12%; control n=5, 4.9% ± 0.15%, $F(1,4)=0.2$, $p=0.655$). We also assessed the effect of DCZ administration on mean time investments on false alarm trials and found no statistical difference between the two groups of sessions (Fig. 4D; DCZ n=5, 4.0s ± 0.24s; control n=5, 4.0s ± 0.71s, $F(1,4)=1.1$, $p=0.350$). Curiously, we observed a clear outlier among the subjects in both false alarm rate (Fig 4C) which shows a significant difference in the opposite direction to the rest. This animal was a female and of a relatively low weight; however, the other female in the cohort, of an even lower weight, did not show any significant difference between DCZ and control sessions, so it is unlikely to be a dose-dependent effect. This subject did not exhibit a significant difference in the overall accuracy between the two drug conditions, so the effect appears to be specific to false alarms and not an overall impairment of task performance. Although the subject shows a difference in the mean invested time on false alarm trials (Fig 4D), this difference did not translate into a difference in the mean high confidence false alarm (HALIP) rate (Fig 4E). There are no obvious biases in the injection dose, procedure or drug (DCZ/saline) that would have plausibly occurred in this animal compared to the rest of the cohort. Overall, these results indicate that DCZ administration did not produce any differences in either false alarm or HALIP rate compared to control.

Limitations of methods:

In this study, we used a chemogenetic approach in the context of the HALIP task to study the effect of inhibition of striatal cholinergic neurons on hallucination-like perception. Neuroscience has undoubtedly been revolutionised following the introduction of optogenetics and chemogenetics, providing researchers with a powerful tool to manipulate in-vivo activity. However, chemogenetics is inherently limited in temporal precision, constrained by the administration of DREADDs, their pharmacokinetics, diffusion and clearance and their subsequent binding and the relatively slow downstream cellular response to the activation of the metabotropic receptor (in this study, the modified human muscarinic M4 receptor), as opposed to ionotropic light-sensitive proteins in optogenetics, whose activation can be precisely timed with light stimulation. However, chemogenetics is less invasive than optogenetics, which requires an implanted optical cannula. DREADDs such as CNO are prone to producing off-target effects. We chose to use DCZ for its superiority over CNO in documented off-target effects, potency and capacity to cross the blood brain barrier. Off-target effects are a possibility, but according to the literature, they are unlikely to be present at the dose used (1mg/kg). We injected a virus vector (AAV8-hSyn-DIO-hM4Di-mCherry) containing the gene for the hM4Di receptor and for the fluorescent protein mCherry into ChAT-Cre mice. There are reports of significant changes in vesicular acetylcholine transporter expression and amplified cholinergic tone, as well as behavioural deficits in Chat-Cre mice (Kolisnyk et al., 2013; Critenden et al., 2014), however, baseline behaviour in the ChAT-Cre mice in this study was quantitatively similar to behaviour previously observed in wild-type mice (Schmack et al., 2021). This suggests that there were no significant behavioural effects of the ChAT-Cre line. Despite proving invaluable as a powerful tool for precise spatial and temporal control of genetic manipulations, the Cre-lox system can present several challenges. The Cre-recombinase enzyme can occasionally exhibit off-target activity at similar but undesired DNA sequences, which may affect behaviour and training on the HALIP task. Additionally, incomplete recombination can result in inadequate and inconsistent expression of the target gene across a neuronal population, which could potentially limit the power and reliability of inhibition achieved with DCZ administration. Although we were able to qualitatively verify expression of the chemogenetic construct in cholinergic neurons

in one mouse (Fig. 1), quantitative expression levels might vary and be insufficient in some of the animals. Overall, the inhibitory power of chemogenetics paired with Cre-lox can be inadequate and we cannot confirm whether our manipulation resulted in a corresponding inhibition of neuronal activity, as we only measured and evaluated the behavioural readouts of HALIP task performance.

The HALIP task, developed by Schmack et al (2021) allows for the quantification of hallucination-like perception. The HALIP task design is inherently low-throughput and relatively training-intensive. As a result, sample sizes can be relatively low; in this study, a cohort of only 6 mice were trained daily on the task for a period of over 4 months. However, the task design allows for repeated-measures experimental designs and the acute and reversible effects of DCZ allowed us to collect data for 24 sessions in total per animal (12 DCZ, 12 control/saline), constrained by regulations that set limits on the number of intraperitoneal injections. A common criticism raised against the HALIP task is the potential for attentional or locomotor effects to contribute to the behavioural measures of task performance (such effects might predispose mice to spend more or less time in the ports, which could be read as a change in confidence). However, signal and no-signal ports are randomised between animals and time investments can be evaluated across both ports to rule out these effects - in the absence of perceptual differences, such effects should not be side-biased. The HALIP task paradigm relies on the assumption that time investment can be used as a reliable behavioural readout of the animal’s confidence in their decision. Here, time investments across correct and incorrect trials can be compared to a model of statistical decision confidence to assess whether the observed time investments are statistically appropriate for their predicted confidence computed from an individual's psychometric choice behaviour. Finally, arguably the most significant limitation of the HALIP task is that the extent to which the neurobiological mechanisms underlying changes in high-confidence false alarm (HALIP) rates are shared with the spontaneous hallucinations experienced in acute psychosis is unclear. However, the comparable phenomenological features between high confidence false alarms and the perceived confidence of spontaneous hallucinations indicate that some mechanisms are likely to be shared and notably, HALIPs correlated with self-reported proneness to hallucinations in humans.

Discussion

In this study, we investigated the effect of chemogenetic inhibition of striatal cholinergic neurons on hallucination-like perception in mice, quantified by a task developed by Schmack et al (2021). The mice learned and performed the task to a high degree of accuracy, which was sustained throughout baseline data collection and the experimental period, allowing us to analyse within-subject differences in the false alarm rates and high confidence false alarm (HALIP) rates as a behavioural readout of hallucination-like perception. Our results revealed no statistically significant differences in false alarm rate or HALIP rate between the sessions where mice were administered with DCZ or control (saline). Our failure to detect a significant effect of chemogenetic inhibition on the HALIP rate might indicate that hallucination-like perception is not mediated by striatal cholinergic neuron hypofunction alone or that a decrease in cholinergic signalling in the targeted population in the tail of striatum is insufficient to produce a psychosis-like phenotype.

While chemogenetics provides several advantages over alternative techniques, there are a number of possible reasons why our approach did not appear to translate into a behavioural effect. Although an alternative approach such as optogenetics would provide the temporal precision suitable for trial-wise manipulation, chemogenetic approaches offered us the ability to manipulate in-vivo activity for the entire duration of the session in a relatively steady, uniform manner with only a single systemic drug administration and is comparatively less invasive in freely behaving mice (without the requirement for the implantation of an optical cannula). The administered dose of DCZ (1mg/kg) was on the higher end of doses used in the literature that saw robust in-vivo and behavioural effects (see *Limitations of Methods*), therefore it is unlikely that the dose was a limiting factor. Importantly, we did not take concurrent recordings of the effect of the chemogenetic inactivation on striatal cholinergic neuron activity nor on acetylcholine levels in the striatum, so we cannot definitively confirm whether a decrease in neuronal activity occurred. Despite this, even if we assume adequate expression of the hM4Di receptor and the resulting inhibition of neuronal activity, the possibility remains that some proportion of cholinergic neurons within or nearby to

the injected area may have compensated for this localised and sustained decrease in cholinergic activity. Despite accounting for only a miniscule fraction (1-3%) of all striatal cell types, their extensive axonal arborization makes them one of the main sources of acetylcholine in the brain (Bolam et al., 1984). It is thought that these numerous axonal varicosities generate an extrasynaptic volume-transmitted signal as opposed to mediating rapid synaptic transmission (Descarries et al., 1997). This volume transmission, extending over relatively large areas from a single cholinergic interneuron, may have rendered our targeted, regionally-specific inhibition redundant. In future, an approach with greater inhibitory power or over a larger area of the striatum may be necessary to observe a robust effect on behaviour.

Striatal cholinergic interneurons' role in modulating striatal circuitry is incredibly complex and as such, hallucination-like perception may not be mediated by changes in overall cholinergic signalling in the striatum, but instead a pathway- or input-specific mechanism. In this study, we expressed the inhibitory chemogenetic receptor hM4Di specifically in choline acetyltransferase (ChAT) positive neurons and the expression was localised to the tail of striatum. We did not undertake further histological analysis to characterise the neuronal population that expressed hM4Di, but we can be relatively confident that this ChAT+ population was comprised of two classes of cholinergic neuron: striatal cholinergic interneurons (ChIs) and cholinergic afferents projecting from the PPN and LDT (Dautan et al., 2014; von Engelhardt et al., 2007). Although the precise relative contribution of each class to the set of ChAT+ in the striatum is not known, the large majority are likely to be ChIs, which constitute the main source of acetylcholine in the striatum (von Engelhardt et al., 2007). The effect of chemogenetic inhibition of striatal ChIs is likely to be varied and wide-ranging. In addition to acetylcholine, striatal ChIs have been shown to co-release GABA and glutamate (Lozovaya et al., 2018, Higley et al., 2011). There is also growing evidence for a bidirectional, tightly coupled relationship between striatal ChI pauses and striatal dopamine dynamics (Chantranupong et al., 2023, Matiyahu et al., 2023, Krok et al., 2023). This presents an incredibly complex picture of inputs/outputs and inhibitory/excitatory effects of cholinergic manipulation in the striatum, involving various neurotransmitter systems. It might be speculated that, as opposed to striatal hypocholinergia per se, the role of aberrant cholinergic signalling

in the striatum, if any, may instead be situated in a particular pathway or the differential potentiation of ChI inputs (Zhang et al., 2018).

Despite the wealth of evidence supporting a central role of striatal ChIs in behaviour (Mallet et al., 2019), the absence of an effect of striatal ChI inhibition in the HALIP task can be reconciled with previous literature and the importance of temporality in the effect of striatal ChI activity should be stressed. Most of the literature studying the importance of ChIs in behaviour have focused on its wide-ranging roles in reward, learning and locomotion (Apicella et al., 2007; Ding et al., 2010; Bradfield et al., 2013). In contrast, the HALIP task paradigm allows for the isolation of quantifiable differences in auditory perception and perceptual confidence from other behavioural (e.g. attentional and locomotor) effects (see Limitations of Methods). It's not clear how the tonic, steady inhibition we presume occurred in our study would affect the firing and pause properties of ChIs. Given the ChI's characteristic pause firing in response to salient stimuli (Asaoki et al., 1994; Apicella et al., 1997) as well as to pulses in striatal dopamine, which may encode prediction errors, and to thalamostriatal inputs (Ding et al., 2010), the temporal nature of ChI activity may be crucial. Therefore, an approach with improved temporal precision, such as optogenetics, would be valuable in interrogating the role of striatal ChIs in hallucination-like perception.

Given the dearth of alternative animal models that recapitulate psychosis with any semblance of face validity, the psychotogenic and antipsychotic effects of muscarinic drugs in humans (which constitute key evidence for the cholinergic hypothesis) can offer potential mechanisms for the role of cholinergic signalling in hallucination-like perception. In contrast to a decrease in striatal cholinergic neuron activity and a corresponding striatal hypocholinergia, there is evidence that the psychotogenic effects of muscarinic antagonists such as scopolamine may be mediated primarily by inhibition of muscarinic autoreceptors (most likely M2/M4) on cholinergic projections from the PPT and LDT - providing the principal cholinergic input to key areas such as the VTA and SN, which comprise the classical psychosis circuit (Birsch et al., 2014) - resulting in increased dopamine efflux in the striatum (Chapman et al., 1997,

Yeomans,, 1995; Ichikawa et al 2002). Meanwhile, the antipsychotic effects of xanomeline - currently the most promising novel non-dopaminergic drug in clinical trials for acute psychosis - are believed to be mediated primarily via the M4 receptor (Wolley et al., 2009; Dencker et al., 2011). In addition to their expression on the mesopontine cholinergic afferents, M4 receptors are highly expressed in the striatum, where they have been found to co-localise primarily on D1 MSNs, where M4 activation may indirectly modulate striatal dopamine release (Jeon et al 2010, Tzavara et al 2004). Finally, M4 activation on striatal cholinergic interneurons decreases subsequent acetylcholine release, resulting in a depression of the nAChR-dependent mechanism of DA release in the striatum (Threlfell et al., 2012; Brimblecombe et al., 2018). Taken together, as opposed to a non-specific striatal hypocholinergia, the role of cholinergic signalling in psychosis-like behaviour might instead lie in the modulation of striatal dopaminergic signalling by mesopontine cholinergic afferents or a complex, temporally specific weighting of the inputs governing striatal ChI pause firing in response to salient stimuli.

In summary, our results reveal that chemogenetic inhibition of striatal cholinergic neurons did not produce any change in the false alarm or HALIP rate and as such, psychosis-like behaviour might not be mediated by striatal cholinergic neuron activity alone. However, we did not measure neuronal activity or acetylcholine levels so cannot confirm whether the chemogenetic inhibition had a significant effect. The integration of multiple inputs by striatal ChIs to produce temporally specific activity in relation to perceptual stimuli and the importance of striatal modulation by extrinsic cholinergic afferents necessitates further studies which interrogate striatal circuitry in the context of this novel behavioural paradigm, some of which will soon be undertaken in the Schmack lab. In future, in the context of the HALIP task, the effect of optogenetic excitation or injection of muscarinic antagonists (e.g. scopolamine) in mesopontine cholinergic afferents with concurrent recordings of ChI burst-pause firing activity - in addition to recordings of striatal acetylcholine and dopamine dynamics with fiber photometry - could also be carried out, with a potential follow-up experiment of rescuing baseline behaviour with an antipsychotic. Building on the findings of our study, research along these lines may uncover a complex role of

cholinergic transmission in hallucination-like perception that could lay the foundations for novel treatments of psychotic disorders.

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