STUDY PROTOCOL

REVISED Muscarinic receptor agonists and positive allosteric modulators in animal models of psychosis: protocol for a systematic review and meta-analysis

[version 2; peer review: 1 approved, 2 approved with reservations]
Previous title: Muscarinic receptor agonists in animal models of psychosis: protocol for a systematic review and meta-analysis

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Abstract

Background

Muscarinic receptor agonism and positive allosteric modulation is a promising mechanism of action for treating psychosis, not present in most D2R-blocking antipsychotics. Xanomeline, an M1/M4-preferring agonist, has shown efficacy in late-stage clinical trials, with more compounds being investigated. Therefore, we aim to synthesize evidence on the preclinical efficacy of muscarinic receptor agonists and positive allosteric modulators in animal models of psychosis to provide unique insights and evidence-based information to guide drug development.

Methods

We plan a systematic review and meta-analysis of *in vivo* animal studies comparing muscarinic receptor agonists or positive allosteric modulators with control conditions and existing D2R-blocking antipsychotics in animals subjected to any method that induces behavioural changes of relevance for psychosis. We will identify eligible studies by searching multiple electronic databases. At least two independent reviewers will conduct the study selection and data extraction using prespecified forms and assess the risk of bias with the SYRCLE's tool. Our primary outcomes include locomotor activity and prepulse inhibition measured with standardized mean differences. We will examine other behavioural readouts of relevance for psychosis as secondary outcomes, such as social interaction and cognitive function. We will synthesize the data using multi-level meta-analysis with a predefined random-effects structure, considering the non-independence of the data. In meta-regressions we will explore potential sources of heterogeneity from a predefined list of characteristics of the animal population, model, and intervention. We will assess the confidence in the evidence considering a self-developed instrument thatconsiders the internal and external validity of the evidence.

Protocol registration

PROSPERO-ID: CRD42024520914

Keywords

antipsychotic; schizophrenia; psychosis; muscarinic receptor; acetylcholine; meta-analysis

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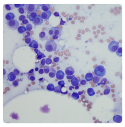
Any reports and responses or comments on the article can be found at the end of the article.



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REVISED **Amendments from Version 1**

To address the reviewers' comments, we more clearly mentioned that muscarinic receptor positive allosteric modulators will also be examined alongside agonists (e.g., mentioned in the title and throughout the manuscript), provided additional details about the methodology (e.g., clarifications about the comparison groups, and details about the exploration of heterogeneity especially in terms of the pharmacological and physiochemical properties of the drugs) and limitations (e.g., not examining cholinergic adverse events), and revised the search strategy to include terms for allosteric modulation (and updated the extended data accordingly).

Any further responses from the reviewers can be found at the end of the article

Introduction

Antipsychotic drugs that block the dopamine D2 receptor (D2R) have been the cornerstone of pharmacological treatment for schizophrenia for over 70 years.[1–3] These drugs have demonstrated efficacy in reducing symptoms of psychosis, particularly positive symptoms such as hallucinations and delusions,[1] yet approximately one-third of patients exhibit an inadequate response to these treatments.[4] This mechanism of action targets a downstream pathway of the aetiopathophysiology of psychosis, with limited efficacy on other core domains, such as negative symptoms like blunted affect and social withdrawal, as well as cognitive impairment.[5] Moreover, the risk-to-benefit ratio of antipsychotics is often challenged by their multiple side-effects associated with their receptor-binding profiles and frequently linked to off-target actions beyond D2R blockade.[1,2] Therefore, there has been a recognized need for more efficacious and tolerable medications for treating psychosis, but previous attempts to develop non-dopaminergic drugs have long been unsuccessful.[3,6]

Muscarinic acetylcholine receptor agonism and positive allosteric modulation has recently been recognized as promising mechanism of action in the treatment of psychosis that can target components of the pathophysiology underlying schizophrenia, a property not present in most D2R-blocking antipsychotics.[7–9] Xanomeline, a muscarinic M1/M4-preferring receptor agonist, has demonstrated improvements in symptoms of schizophrenia in early and late-stage randomized placebo-controlled controlled trials with medium-to-large effect sizes, with potential cholinergic adverse events mitigated by its combination with trospium, a peripheral muscarinic antagonist.[10–13] In September 2024, the U.S. Food and Drug Administration approved xanomeline combined with trospium for schizophrenia, marking it as the first antipsychotic targeting muscarinic receptors.[14] Emraclidine, a drug with a different mode of action, acting as a selective M4 positive allosteric modulator (PAM), has also shown promising findings in an early clinical trial,[15] and additional muscarinic agents (e.g., NBI-1117568)[16] are under development.

Several unanswered questions remain however, including the comparative efficacy of different muscarinic receptor agonists, their comparison with existing D2R-blocking antipsychotics, the roles of individual muscarinic receptor subtypes (M1-M5) and their effects on specific symptom domains, and the potential differences between orthosteric agonists and PAMs. For example, PAMs modulate receptor activity by binding to a site distinct from the natural ligand, offering theoretical advantages such as increased selectivity and safety over orthosteric agonists.[17,18] In this context, preclinical studies can provide early insights and inform further drug development. Their large number and limitations in terms of internal and external validity, however, make the translation of their findings challenging. For this reason, a critical synthesis of their evidence is required, but none exists.

Objectives

We therefore plan a systematic review and meta-analysis on the effects of muscarinic receptor agonists and PAMs in animal models of relevance for psychosis concerning behavioural and motor outcomes as compared to control conditions and existing D2R-blocking antipsychotics. We will also carefully assess the potential biases of these studies and evaluate the confidence in the evidence, ultimately providing evidence-based information to facilitate future drug development in schizophrenia.

Protocol

The protocol of the review is reported according to the PRISMA statement for protocols (PRISMA-P)[19] (see the checklist in the extended data), the guidelines from SYRCLE[20,21] and CAMARADES.[22] The protocol was registered with PROSPERO (ID: CRD42024520914) on 04.04.2024. The methodology of the protocol has been informed by our previous systematic review and meta-analysis conducted with the GALENOS project,[23] which examined trace-amine associated receptor 1 (TAAR1) agonists in animal models of psychosis.[24,25]

Study eligibility criteria and outcomes

Study design

We will include *in vivo* animal experimental studies examining any muscarinic receptor agonist or PAM versus inactive or active comparison groups in animal models of relevance for psychosis, as detailed below. There will be no restrictions on the inclusion criteria in terms of the randomization, blinding or other factors related to risk of bias, unit of allocation, duration of the study, publication status, year, country and language. We will exclude uncontrolled preclinical experiments, observational studies, and literature reviews.

Animal population and model induction

We will include animals that have undergone laboratory methods to induce psychosis-like behaviours and features. There are numerous models fitting this description, each with varying degrees of validity and unique strengths and weaknesses, but none are considered the gold standard.[26–33] Therefore, we will include the "classical" pharmacological models of psychosis and their behavioural readouts (see "Outcomes"), which have been widely utilized in drug discovery and possess some predictive validity, especially for positive symptoms, including the administration of psychostimulants (e.g., amphetamine, cocaine) or N-methyl-D-aspartate (NMDA) receptor antagonists (e.g., phencyclidine, ketamine, MK-801).[32,34,35] Other methods of induction will also be eligible and can include other pharmacological models, neurodevelopmental methods, lesion methods, genetic models, and combinations of different methods.[27–33] Such relatively broad inclusion criteria were utilized in our previous systematic review,[24,25] and any decision on the eligibility of the psychosis models will be made in consultation with experts in preclinical research (MRM, AB, FJ, FT, IM, SN, AdB, SH, NID, UT).

There will be no restriction on species, strain, age, and sex. Regarding genetic composition, we will include both wildtype animals and those that have undergone genetic interventions, if these interventions belong to eligible methods of induction (see above). Moreover, in the eligible studies, we will extract data from "naïve" animal cohorts (i.e., animals that have not undergone models of psychosis), and animal cohorts that have undergone both models of psychosis and muscarinic receptor antagonism via genetic (i.e, genetically modified animals with knockout of all or specific muscarinic receptors, either globally or in specific neuron populations)[36] or pharmacological manipulation (e.g., co-administration of selective or non-selective muscarinic receptor antagonists like scopolamine)[37] (see further details in "Comparison groups"). The eligible animal cohorts can be found in Table 1.

We will exclude animals that have undergone induction methods for other specific conditions (e.g., transgenic models of Alzheimer's disease,[38] the valproic acid-induced model of relevance for autism[39] and methods aiming to specifically model depressive-like behaviours, such as animal models of physical, social, or chronic mild stress).[40] We will also exclude *in vitro*, *ex vivo*, *in silico* studies and studies in humans. We will however consider extracting data from *ex vivo* measurements (e.g., Fos expression, autoradiography) following eligible *in vivo* experiments, potentially analysed in secondary publications (see "Outcomes").

Interventions

We will include any pharmacological agent acting as an agonist or positive allosteric modulator at any of the five subtypes of muscarinic acetylcholine M1-M5 receptors. There will be no restrictions on their receptor selectivity, pharmacological potency and efficacy (e.g., full or partial agonists), dose, timing of administration relative to the induction method, pharmacokinetic properties, or route of administration, provided the method is suitable for achieving effects in the central nervous system. These pharmacological agents can be administered individually or in combination with D2R-blocking antipsychotics or other medications, and combinations can be considered as distinct interventions. We will also consider data on the effects of muscarinic receptor agonists in the context of muscarinic receptor antagonism (see "Animal population and model induction") to evaluate whether the effects depend on the activation of muscarinic receptors or other mechanisms (Table 1).

We will exclude clozapine, an existing antipsychotic acting on multiple neurotransmitter receptor systems and also as muscarinic receptor partial agonist,[2,8] which will be considered as a control intervention (see "Comparison groups"). Clozapine's metabolite N-desmethylclozapine however, which has low affinity for dopamine receptors and acts as a muscarinic M1/M4 receptor agonist,[41] will be considered among the experimental interventions. Additionally, we will exclude pharmacological agents with different mechanisms of action (e.g., nicotinic acetylcholine receptor agonists, acetylcholinesterase inhibitors) and non-pharmacological interventions, including genetic interventions for muscarinic receptor overexpression.

Table 1. Eligible animal cohorts and planned comparisons.

Animal cohort	Interventions received by the animal cohort					Planned comparisons with the following comparison groups
	Model for psychosis	Muscarinic receptor agonists or PAMs	D2R-blocking antipsychotics	Vehicle only or no treatment	Muscarinic receptor antagonism	
Muscarinic receptor agonists or PAMs	Yes	Yes	No	No	No	Inactive and active control conditions
Combination of muscarinic receptor agonists or PAMs and D2R-blocking antipsychotics	Yes	Yes	Yes	No	No	Active control conditions
Inactive control condition	Yes	No	No	Yes	No	Not applicable (comparison group only)
Active control condition	Yes	No	Yes	No	No	Inactive control conditions (it will be used to inform the assessment of indirectness)
Muscarinic receptor agonists or PAMS in the context of muscarinic antagonism	Yes	Yes	No	No	Yes	Inactive control conditions with or without muscarinic receptor antagonism
Sham procedures	No	No	No	Yes	No	Not applicable (it will be used for the calculation of normalized mean differences)

We will aim to extract data from additional animal cohorts, if available, and consider additional potential comparisons if sufficient data are available.

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Comparison groups

We will include the following comparison groups: i) inactive control conditions, consisting of animals undergoing models of a method to induce psychosis-like behaviour and features and receiving vehicle (e.g., injection of saline) or no treatment, and ii) active control conditions, consisting of the aforementioned animal cohorts treated with D2R-blocking antipsychotics. D2R-blocking antipsychotics will be defined as those medications listed in the Anatomical Therapeutic Chemical (ATC) classification with a code of N05A, except for lithium (N05AN01, a mood stabilizer primarily used for the treatment of bipolar disorder), pimavanserin (N05AX17, a 5-HT2A antagonist indicated for Parkinson's disease psychosis but not schizophrenia), and any muscarinic receptor agonists approved by any regulatory body for schizophrenia (which will be considered as experimental interventions in this review, e.g., xanomeline combined with trospium[14]), with no further restrictions.

Table 1 outlines the planned comparisons, which include evaluating muscarinic receptor agonists or PAMs, alone or combined with D2R-blocking antipsychotics, against active and inactive control conditions in animal models of psychosis.

Additionally, we will examine the comparison between active and inactive control conditions to provide insights into the consistency of the effects of D2R-blocking antipsychotic in the included experiments and to inform the assessment of indirectness (see "Assessment of indirectness of the animal experiments").

We will also compare muscarinic receptor agonists or PAMs with inactive control conditions in contexts of genetic or pharmacological muscarinic receptor antagonism (as described in "Animal population and model induction") to determine if any observed differences result from muscarinic receptor activation. Separate analyses may be conducted for the antagonism of specific muscarinic receptor subtypes and/or within specific neuronal populations, if sufficient data are available.

We will also consider data in the eligible studies from sham procedures, consisting of "naïve" animal cohorts not subjected to methods of inducing psychosis-like behaviours or features and receiving either vehicle or no treatment, to calculate normalized mean differences in a sensitivity analysis (see "Effect sizes").

Outcome measures

There is no gold standard measure of preclinical antipsychotic efficacy due to the limited homology with the clinical symptoms of psychosis and the lack of an established biomarker.[6,28,29,32–35,42–44] Therefore, we aim to provide a comprehensive assessment of the effects of muscarinic receptor agonists by using data from a broad range of behavioral measures in various animal models of relevance for psychosis (see "Animal population and model induction"). This approach will facilitate the identification of strong clinical canditates.[27,28,33–35]

We will examine as co-primary outcomes the effects on i) locomotor activity and ii) prepulse inhibition of the acoustic startle reflex, as these have been widely used with some predictive validity to identify antipsychotic effects in animal models of relevance for psychosis (see "Animal population and model induction").[24,29,34] Furthermore, we will examine additional behavioral measures as secondary outcomes, including potential proxies for positive symptoms (e.g., stereotypies, hallucinatory-like percepts),[45] negative symptoms (e.g., lack of social interaction, operant-based motivational tasks),[34,46] depressive- (e.g., forced swim, tail suspension, sucrose preference tasks)[34,46] and anxiety-like behaviors (e.g., elevated plus maze), and cognitive function (e.g., tests recommended by the CNTRICS initiative).[33,42,44] The eligibility and any potential grouping of the behavioral measures will be evaluated in collaboration with experts in preclinical research prior to commencing data analysis, and any decisions will be documented.

We will exclude non-behavioural outcomes, such as histopathological and neurobiological measures due to the lack of an established biomarker,[6] and adverse events due to the inconsistent and scarce reporting in animal studies, as identified in our previous review.[24] Nonetheless, we will aim to extract their data, if available, and potentially analyse them in secondary publications.

Study identification

We will conduct searches in PubMed, MEDLINE via Ovid, Web of Science, EMBASE, PsychINFO, from database inception using keywords for psychosis, muscarinic agents, and animal filters,[47] similar to a previous review.[24,25] For muscarinic agents, we will use broad terms like muscarinic agonists or allosteric modulators and the names/synonyms of specific relevant agents identified from IUPHAR/BPS[48] and previous reviews.[7,49] There will be no restrictions such as on language or publication date. The search strategies will be developed in collaboration with information specialist (see

"Acknowledgment"). A draft search strategy in MEDLINE via Ovid can be found in the extended data, and the final search strategies will be reported according to the PRISMA statement for reporting literature searches (PRISMA-S).[50]

We plan additional searches to improve the coverage of the study identification and identify potentially unpublished studies:

1) We will aim to search in the Systematic Online Living Evidence Summaries for preclinical psychosis research (psychosis-SOLES),[51] which is a dedicated and continuously updated database utilizing machine learning and text mining algorithms.

2) We will search preclinical animal study registries (i.e., animalstudyregistry.org, https://preclinicaltrials.eu/), although pre-registration of animal studies has not been widely adopted.

3) We will aim to search preprint registries (i.e., medRxiv, bioRxiv), Google patents, specific journals of neuropsychopharmacology.

4) We will inspect reference lists of included studies, previous reviews,[7,8,49,52] and conference proceedings published within the last 20 years.

5) We will contact the first/corresponding author of included studies and pharmaceutical industries of muscarinic agents for additional studies and/or missing data in their studies. We will send emails with two follow-up reminders in case of no response.

Study selection

At least two independent reviewers will screen in the Systematic Review Facility (SyRF)[26] the de-duplicated records identified in the searches in two phases: i) title and abstract, and ii) full-text screening. Any discrepancy between the two reviewers will be resolved through discussion with a more senior reviewer. If not resolved by discussion, the full-text of the study will be acquired (if at the title/abstract level) or additional information from the original study authors will be obtained (if at the full-text level).

At the title/abstract screening, records will be excluded according to the following hierarchy: i) review articles, ii) not referring to *in vivo* animal study, iii) not referring to muscarinic receptors or agents acting on them.

At the full-text screening, records will be excluded from the review and/or meta-analysis according to the following hierarchy against the eligibility criteria: i) ineligible study design, ii) ineligible animals/population, iii) ineligible intervention, iv) ineligible comparison groups, v) ineligible outcome measure, vi) inadequate reporting of outcome data. If possible, we will consider the studies excluded in terms of their reported outcomes in the assessment of reporting bias (see "Data synthesis"). The study selection process and the reasons for exclusions at the full-text level will be reported in a flow diagram.[53]

Data extraction

At least two independent reviewers will conduct the data extraction in SyRF[26] using pre-specified data extraction forms that will be adapted from a previous systematic review.[24] Discrepancies between the two reviewers will be reconciled by a more senior reviewer, or if not possible, by contacting study authors for additional information.

We will extract data regarding study identification (e.g., author names, title, publication year), study design (e.g., risk of bias, reporting completeness), animal population and model induction (e.g., age, sex, species strain, body weight, characteristics of the induction method), experimental and control interventions (e.g., type, dose and timing of the administration of drugs), and outcome measures (e.g., exact name of the behavioural task, methods of measurement, quantitative data). We will seek information from various sources in the following order of priority: i) text and tables, ii) figures using WebPlotDigitizer version 4 (a free and opensource tool distributed under GNU Affero General Public License Version 3),[54] iii) contacting authors for missing information, and iv) employing imputation methods.

We will extract quantitative data for the outcomes anticipated to be reported as continuous measures. Endpoint and change scores will both be eligible and jointly synthesized.[55] When both are reported, we will prefer endpoint scores because they do not require baseline assessments and are expected to be the most frequently reported in the eligible animal experiments. We will extract the unit of measurement, mean, standard deviation and the number of participants that these

correspond to. We will apply a minus transformation whenever appropriate to harmonize the direction of effects across the extracted data (e.g., a higher score indicating a better outcome). Missing standard deviations will be derived by the following methods according to their order of priority: i) calculation from standard error, ii) estimation from test statistics (e.g., p-values, t-tests, median and ranges), iii) contacting the authors of the original studies for additional information, iv) imputed from the standard deviations of other studies (although this method has not been validated in animal studies that often have small sample sizes).[56,57] If the exact number of animals is not reported,[58] we will estimate it with available information (e.g., using the lower boundary of a range, if reported) or consider imputation methods. If dichotomous measures are reported, we will extract the number of animals with the event and the total number of animals analysed. We will exclude studies with imputed data in a sensitivity analysis (see "Sensitivity analysis").

For crossover trials, we will prefer data from the first phase to avoid carryover effects, but we will also use data from the entire phase by applying appropriate corrections considering the within-subject correlation.[59] These studies will be excluded in a sensitivity analysis (see "Sensitivity analysis").

We will use data from any reported time point, but preference will be given for the longest time point following multiple administrations of the intervention over an extended period. If outcomes are measured multiple times after a single administration, we will consider calculating the area under the curve.[25,60]

If multiple variations of the same outcome are reported, we will extract and jointly analyse them (see "Data synthesis").

Assessment of risk of bias and reporting completeness

Two independent reviewers will assess risk of bias using the SYRCLE's tool considering domains for selection, performance, detection, attrition, reporting and other biases.[61] We will assign an overall high risk of bias to a study if at least one domain in the SYRCLE's tool is assessed as having a high risk of bias. Since high-quality reporting is essential for assessing the risk of bias, two independent reviewers will also evaluate the completeness of reporting using a modified version of the ARRIVE Essential 10 checklist.[25,62] This is necessary because the reporting completeness of animal studies is often poor, frequently leading to unclear assessments of risk of bias. Any discrepancies between the two reviewers will be resolved through discussion with a more senior reviewer or by contacting the study authors for additional information.

Assessment of indirectness of animal experiments

We expect that the findings from various animal models of relevance for psychosis will have different degrees of applicability to clinical trial settings. However, there is no established method for assessing their indirectness in the context of a systematic review. Moreover, there is limited synthesized evidence on the validity of animal models of relevance for psychosis, and we expect substantial variability in the methods of modelling and measuring psychosis-like behaviours in animals.[28,29] This makes it challenging to set predefined criteria for assessing the indirectness of animal experiments in the context of psychosis.

Nevertheless, we will aim to use the extracted data to provide an experiment-level judgment of indirectness as "low risk", "high risk", or "some concerns", considering how closely the experiment reflects the clinical trial setting in terms of animal population, model induction, intervention, and outcome. This assessment will inform the evaluation of indirectness domain in the confidence in the evidence (see "Confidence in the evidence").

To achieve this, we will evaluate the validity in animal experiments and the applicability of the intervention (e.g., treatment over an extended period, initiation of treatment after model induction) based on previous frameworks and checklists.[24,63–67] Specifically, we will apply the framework of Belzung and Lemoine[67] to assess the following domains (and sub-domains) of validity in animal experiments, i.e., homological (species strain), pathogenic (ontopathogenic, triggering), mechanistic, face (ethological, biomarker) and predictive (induction, remission). This framework can offer a more refined and systematic approach compared to the traditional domains of construct, face and predictive validity, which have often been inconsistently applied in the literature.[28,67,68] However, the exact methods will be determined *a posteriori* in consultation with experts in preclinical research.

Data synthesis

Planned comparisons

Our main aim is to synthesize data for each outcome and for the comparisons described in Table-1. Meta-analysis will be conducted when there are at least two independent effect sizes for the same outcome, as in our previous systematic review.[24]

We will examine the data and if there is reasonable consistency across the comparisons, we will consider network meta-analysis to examining the comparative effects of the different muscarinic receptor agonists, various D2R-blocking antipsychotics, and inactive control conditions.[69]

Effect sizes

The main effect size will be the standardized mean difference (SMD) due to the varying measures and units of the behavioural outcome measures across the studies. We will also use normalized mean difference (NMD) in a sensitivity analysis.[58,70] If outcomes are reported as dichotomous, we will calculate odds ratios (ORs) and convert them into SMDs using the Hasselblad and Hedges method[71] to enable their combination with results of continuous measures.

In addition to the estimation of the average treatment effects, we also aim to conduct a meta-analysis of variation to estimate the inter-individual variability of the effects using the variability ratio (VR) or the coefficient of the variation ratio (CVR) in case a mean-variance relationship is expected.[70,72] This analysis will be conducted for the comparison of muscarinic receptor agonists to inactive control conditions.

Data synthesis approach

We will opt for synthesizing the data using multilevel meta-analytic models, which enable handling non-independent data.[70] We will use a predefined multilevel random-effects structure with nested levels, from higher to lower, for animal strain, study, and experiment, provided there are at least five distinct categories for at least one of the levels, as in our previous systematic review.[24] For non-independent sampling errors, we will estimate the within-study variance-covariance (VCV) matrix using any reported correlation in the original studies or assuming a correlation of 0.5 (see other assumed correlations in "Sensitivity analysis").[70] The restricted maximum likelihood (REML) method will be used to estimate the between-study variance (τ^2) and between-study VCV.[70,73] We will adjust the confidence intervals using t- or F-distributions with degrees of freedom appropriate for the multilevel model.[74]

To our knowledge, network meta-analysis has not been widely applied to the synthesis of animal experiments, and we anticipate several challenging issues, including the limited evidence for or against inconsistency, small sample sizes, and non-independent effect sizes.[65] We will examine whether the assumptions of a network meta-analysis can be fulfilled by comparing the distribution of potential effect modifiers across treatment comparisons and measuring incoherence using statistical tests.[75] Justifying these assumptions with preclinical data however, might be challenging. If a network meta-analysis is deemed feasible, we will extend the multilevel models and consider covariate-adjusted analysis (see "Exploration of heterogeneity"). The exact methodology will be defined *a posteriori*, and, if a network meta-analysis is justified, will be thoroughly reported in an amendment of the protocol before conducting it.

We will present the effect sizes with their 95% confidence intervals and prediction intervals.

Exploration of heterogeneity

We will quantify heterogeneity using the variance of the random effects with its components, and the 95% prediction intervals. We will explore potential sources of heterogeneity for each outcome through meta-regression or subgroup analyses, if sufficient data are available, considering the following tentative list of potential effect-modifiers: age, sex, species/strain, comorbidities, characteristics of the model of induction of psychosis-like behaviours (e.g., pharmacological or genetic, severity), type of muscarinic receptor agonists or PAMs, their pharmacological and physiochemical properties, and dose (details below), route of administration, duration of treatment, timing of the intervention (e.g., before or after model induction), co-treatments, characteristics of the study (e.g., publication year, reporting completeness, and risk of bias), and outcome measurements (e.g., different measures for locomotor activity). Additional variables will be considered if they are deemed relevant and there are sufficient data.

We will examine the pharmacological properties of muscarinic receptor agonists, considering mode of action (e.g., orthosteric agonism, positive allosteric modulation) and other key characteristics as sources of heterogeneity, using data primarily from established databases (e.g., IUPHAR/BPS)[48] and original publications that characterize these compounds. Specifically, we will assess potency (half-maximal effective concentration, EC50) and efficacy (maximal response, distinguishing between full and partial agonism) for orthosteric agonists, and modulation of acetylcholine potency and efficacy (cooperative factors α and β), intrinsic efficacy (τ_B), and affinity for the allosteric site (K_B) for PAMs.[17,76] These properties will be examined across various muscarinic receptor subtypes, with a preference for human receptors while considering potential cross-species differences, and across downstream signaling pathways (e.g., via $G\alpha_s$, $G\alpha_q$,

$G\alpha_{i/o}$ subunits, β-arrestin). Given the variability across assays and experimental settings, we will consider calculating a common standardized index, if possible, such as estimating differences in log((maximal response)/EC50) between the examined drugs and the natural ligand acetylcholine.[76] This approach may enable comparisons across compounds, receptor and signaling pathway selectivity, but summarizing the different properties into a single index may result in information loss, necessitating additional analyses. Moreover, the exact procedure cannot be predetermined due to anticipated heterogeneity in the data across the different compounds examined. Additionally, we will assess off-target actions by evaluating the mode of action and affinity of each compound toward other neurotransmitter receptors.

We will use the Central Nervous System Multiparameter Optimization Desirability algorithm[77,78] to examine the physicochemical properties of the drugs as source of heterogeneity. This algorithm calculates a composite desirability score based on six key properties, i.e., lipophilicity, distribution coefficient, molecular weight, topological polar surface area, number of hydrogen bond donors, and the most basic center, which aligns well with the pharmacokinetic attributes, blood-brain barrier permeability and safety.[77,78] Values for these properties will be estimated based on the chemical structure information using OPEn structure–activity/property Relationship App (OPERA) version 2.9.1 (a freely available, open-source provided distributed under MIT license).[79]

We will also aim to standardize doses across drugs to examine dose-effects relationships using the standardized dose as a covariate in a meta-regression. However, any potential standardization method cannot be predefined due to the pharmacological differences among various drugs[24] and differences across species/strains.[80]

Sensitivity analysis

The robustness of the findings for each outcome will be examined through sensitivity analysis by: i) excluding studies with an overall high risk of bias (see "Assessment of risk of bias and reporting completeness"), ii) using normalized mean difference (see "Effect Sizes"), iii) excluding studies with imputed data (e.g., standard deviations, number of animals) and crossover studies reporting data from the entire period, iv) excluding interventions examined in single studies due to potentially inflated effect estimates,[65] v) assuming correlations of 0.2 and 0.8 in estimating the within-study VCV,[70] and vi) using a robust variance estimation to obtain cluster-robust standard errors.[70,81]

Reporting bias and small-study effects

Publication and other non-reporting biases are highly prevalent in preclinical research, potentially having a substantial impact on the estimated efficacy.[82,83] We will aim to evaluate potential within- and across-study non-reporting biases by adapting the framework for assessing the risk of bias due to missing evidence (ROB-ME) in clinical trials, assigning ratings of "low risk", "high risk", or "some concerns".[84–86] We will include both published and unpublished studies, although the latter may be difficult to find due to the limited adoption of pre-registration protocols. We will evaluate potential reasons of non-reporting of outcome data in studies excluded due to ineligible outcome measures or inadequate reporting of outcome data, although this may be more challenging due to the poor reporting quality of animal studies (see "Assessment of risk of bias and reporting completeness"). Moreover, we will explore small-study effects for each outcome using contour-enhanced funnel plots and multilevel regression-based tests,[70] using the square root of the sample size, when there are sufficient data from at least 10 studies.

Confidence in the evidence

We will evaluate the confidence in the evidence for each of outcome using a modified version of the GRADE framework[63,64] taking into account the domains of risk of bias (and reporting completeness), indirectness, heterogeneity, imprecision, and reporting bias, similar to our previous systematic review.[24] Ultimately, we will aim to draw an overall conclusion on the preclinical efficacy of muscarinic receptor agonists by considering the evidence from the different behavioural domains.

Statistical software

Data analysis will be conducted in R statistical software[87] using the package *metafor*,[74] along with other appropriate packages for data cleaning, specific meta-analytic models, and visualization. We will report the complete list of packages used along with their versions in the publication of the results.

Dissemination of information

We plan to publish the systematic review and meta-analysis as open access in peer-reviewed journals, potentially resulting in multiple publications, and present the findings at conferences. Lay language summaries will be prepared and

disseminated with the help of patient and relative groups, such as BASTA (Bündnis für psychisch erkrankte Menschen) and ApK (Aktionsgemeinschaft der Angehörigen psychisch Kranker e.V.). We will also make the methods, data, and code publicly available in a GitHub repository.

Study status

As of the date of the first submission of this protocol on 18.08.2024, we have completed the preliminary searches and piloting of study selection process and started, but not completed, the full searches and the full screening of search results against the eligibility criteria. We have not yet started the data extraction, risk of bias and quality assessment, or data synthesis.

There were no changes from the original PROSPERO registration of the protocol, except for expanding the methods with additional details (also in response to the reviewer's comments),[88,89] revising the search strategy to include terms for allosteric modulation as suggested by a reviewer,[88] adding physicochemical properties of the compounds in the "Exploration of Heterogeneity" per another reviewer's comment,[89] and deciding not to search Scopus and CINAHL after consulting with the information specialist (which is not expected to affect the coverage of our search). Any additional deviations or modifications will be reported along with the findings and in updates to the PROSPERO registration.

Discussion

The planned systematic review and meta-analysis aims to evaluate the preclinical efficacy of muscarinic receptor agonists or positive allosteric modulators in animal models of relevance for psychosis. This will be achieved through a comprehensive search, advanced data synthesis methods, and a critical evaluation of potential risks of bias and confidence in the evidence. The systematic review has the potential to provide unique insights into important unanswered questions regarding muscarinic receptor agonism in the treatment of psychosis. It may also identify promising muscarinic agents or specific mechanisms of action, which could guide future drug development for schizophrenia.

Furthermore, conducting a systematic review to examine the preclinical efficacy of antipsychotics is a relatively novel approach. Only a few such reviews currently exist,[24,29,90] making this work particularly valuable. Therefore, our review has the potential to highlight the limitations of existing animal models of relevance for psychosis and address potential translational disconnects to improve the design of future preclinical research.[6,28,34]

We focused on muscarinic receptor agonism due to its potential to target the underlying pathophysiology of schizophrenia and the promising findings for agonists or PAMs of M1/M4 muscarinic receptors,[7–9] with xanomeline combined with trospium approved by the Food and Drug Administration in September 2024.[14] Muscarinic receptor agonism has also the most advanced data compared to other novel mechanisms of action being investigated in the treatment of psychosis.[9] For example, there were encouraging results from a phase II trial for ulotaront, a dual TAAR1 agonist and 5-HT_{1A} partial agonist.[91] A recent synthesis of evidence from early- and late-stage clinical trials, and animal studies however, suggested that TAAR1 agonists may be less efficacious compared to existing D2R-blocking antipsychotics, but additional data are required to draw more definitive conclusions and more drugs are under development.[24]

We anticipate limitations and challenges in conducting the review. There is no gold standard animal model for psychosis,[6,28,34] and cross-species differences in the muscarinic receptor system and the effects of their agonists or PAMs may exist.[8] Therefore, we will include all relevant animal models and behavioural readouts of preclinical efficacy, critically evaluating the confidence in the evidence to provide a comprehensive synthesis with potential translational relevance. We will not analyse neurobiological measures due to their heterogeneous use across studies and the lack of an established biomarker in schizophrenia,[6] but they could further elucidate the mechanisms of muscarinic receptor agonism and its role in regulating dopaminergic signalling and other underlying pathophysiological mechanisms.[8,52] Similarly, we will not examine cholinergic adverse events in animals, such as salivation, lacrimation and diarrhea, which may offer insights into the risk-benefit profile but could be mitigated in clinical settings with peripheral muscarinic receptor antagonists, as shown with the xanomeline-trospium combination.[10–13] We will aim to extract and potentially analyse these measures in secondary publications. Additionally, we will explore whether heterogeneity in behavioral effects may be explained by differences in drug interactions with muscarinic receptors. Given the limited availability and application of *in vivo* readouts for muscarinic agonism, we will use *in vitro* data. We will not conduct a systematic review of *in vitro* assays and pharmacokinetic studies to synthesize information about the pharmacological properties of the drugs, but instead will consider reliable sources and using standardized methods to assess these as sources of heterogeneity (see "Exploration of heterogeneity"). Finally, the methods for systematic reviews of animal studies in psychiatry are not well established and may present unique challenges.[92] Our interdisciplinary team, which includes methodologists, statisticians, and clinical and preclinical researchers, will address any potential issues to ensure a robust synthesis of preclinical evidence.

In conclusion, our planned systematic review and meta-analysis will be the first to examine the preclinical efficacy of muscarinic receptor agonists or positive allosteric modulators for schizophrenia, with the potential to provide evidence-based information to guide future preclinical and clinical research on this topic.

Ethics and consent

Not applicable.

Authors contributions

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Data availability

Underlying data

No data associated with this article.

Extended data

Zenodo: Protocol for meta-analysis on muscarinic receptor agonists in animal models of psychosis (ANIMUS-SR), https://doi.org/10.5281/zenodo.14534308.[93]

This project contains the following extended data:

- Draft search strategy in MEDLINE via Ovid.pdf
- PRISMA-P checklist.pdf

Reporting guidelines

Zenodo: PRISMA-P checklist for "Protocol for meta-analysis on muscarinic receptor agonists in animal models of psychosis (ANIMUS-SR)", https://doi.org/10.5281/zenodo.14534308.[93]

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Open Peer Review

Current Peer Review Status: ? ? ✓

Version 2

Reviewer Report 21 January 2025

https://doi.org/10.5256/f1000research.176234.r357863

✓ **Xiaonan Guo**
Zhejiang University, Hangzhou, Zhejiang, China

This paper is a comprehensive protocol studying muscarinic receptor agonists and positive allosteric modulators in animal models of psychosis. Generally, I think it will promote the clinical use and biological insight of the muscarinic receptor agonists and positive allosteric modulators in treating schizophrenia. The method is so perfect that no obvious limitations can be found. I only provide the minor suggestion to improve the article, which the authors could consider.

Since psychosis is strongly associated with dopamine function, it's better to introduce the influence of dopamine function when treating animal models of psychosis with muscarinic receptor agonists and positive allosteric modulators. Linking the muscarinic function with dopamine function will shed light on clinical translation of muscarinic agonist.

Is the rationale for, and objectives of, the study clearly described?
Yes

Is the study design appropriate for the research question?
Yes

Are sufficient details of the methods provided to allow replication by others?
Yes

Are the datasets clearly presented in a useable and accessible format?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Brain, metabolic, and genetic mechanism in psychiatric disorders (especially in BD).

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 24 October 2024

https://doi.org/10.5256/f1000research.170522.r325671

?

William Messer
Department of Medicinal and Biological Chemistry, College of Pharmacy and Pharmaceutical Sciences, Department of Pharmacology and Experimental Therapeutics,, University of Toledo, Toledo, OH, USA

The article outlines a protocol for a specific systematic review and meta-analysis examining the impact of muscarinic agonists (and allosteric modulators) in animal models of psychosis. As such, it is more a research proposal than a paper describing results.
The authors indicate that D_2 dopamine antagonists have been the cornerstone for schizophrenia therapy for the last 70 years, although atypical antipsychotics target other receptors, including 5-HT_2 receptors. Such compounds, including clozapine and olanzapine, are linked to a different set of adverse effects than classical dopamine antagonists including hypoglycemia and weight gain. Nevertheless, the topic is timely given the recent approval of xanomeline, in combination with trospium, for the treatment of schizophrenia.
A systematic review and a meta-analysis are proposed for examining the impact of muscarinic agonists and allosteric modulators in animal models of psychosis. The level of complexity is high with respect to the varying types of compounds (agonists vs. partial agonists, receptor subtype selectivity, binding affinity, etc.), which will make it difficult to accurately categorize compounds. For example, positive allosteric modulators often exhibit a range of effects that include modulation of acetylcholine potency and efficacy in addition to allosteric agonist activity. The degree to which such activities have been adequately assessed will likely vary from lab to lab and such data may not be readily available in the papers that address in vivo efficacy in animal models of psychosis. These concerns are addressed to some extent in the “Exploration of heterogeneity” section, but a more explicit discussion of how concepts of potency and efficacy, for example, will be handled is warranted. In addition to the sources of heterogeneity discussed, the physicochemical properties of compounds may impact the effectiveness of treatments; consideration of ADME properties of compounds could provide an important perspective. For example, multiparameter optimization values could help explain variability in responses for compounds with similar receptor activity profiles.
Beyond the pharmacological properties of the compounds under investigation, the impact of muscarinic receptor activation might vary significantly depending on the different compounds utilized to induce behavioral changes (e.g., NMDA antagonists vs. dopamine agonists) or the

behavioral readouts (e.g., pre-pulse inhibition of startle response vs. apomorphine induced climbing). The authors refer to some of the inherent difficulties in the "Assessment of indirectness of animal experiments" section, including concerns about the applicability of various animal models to clinical utility. In this sense, it might be helpful to examine the consistency of comparator compounds (e.g., D_2 and 5-HT_2 antagonists) that are used clinically in treatment of schizophrenia, and are often used as control compounds in studies of muscarinic agonists and other potential therapeutic compounds. This is perhaps alluded to in the "Data synthesis/planned comparisons" section, although additional details would be helpful.
An additional consideration is the presence or absence of adverse effects for the compounds under investigation. The clinical utility of muscarinic agonists has been limited by adverse effect profiles, which vary from compound to compound, but likely include both off-target (e.g., M_3 receptor-mediated) and on-target (i.e., M_1 or M_4 receptor-mediated) effects. Adverse effects such as salivation, lacrimation, diarrhea, or convulsions would provide important information regarding the *in vivo* pharmacological profile of compounds and the separation between beneficial and undesirable effects (i.e., the therapeutic window).
Overall, the topic is of timely interest and worth pursuing with some additional attention to the details to be evaluated.

Is the rationale for, and objectives of, the study clearly described?
Yes

Is the study design appropriate for the research question?
Yes

Are sufficient details of the methods provided to allow replication by others?
Partly

Are the datasets clearly presented in a useable and accessible format?
Not applicable

Competing Interests: I am an inventor on patents related to the use of muscarinic agonists and allosteric modulators for the treatment of neuropsychiatric disorders. I am also the founder and owner of Psyneurgy Pharmaceuticals LLC, a company focused on the development of treatments for neuropsychiatric disorders.

Reviewer Expertise: My research focuses on the design and development of selective agonists and allosteric modulators of muscarinic receptors with potential utility in the treatment of neuropsychiatric disorders.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 20 Dec 2024

Spyridon Siafis

#2.1: The article outlines a protocol for a specific systematic review and meta-analysis examining the impact of muscarinic agonists (and allosteric modulators) in animal models of psychosis. As such, it is more a research proposal than a paper describing results.

Our reply:

We would like to thank the reviewer for their constructive comments on our protocol, which have helped improve the clarity of the methods. This manuscript indeed corresponds to a protocol for a systematic review, and the results of the completed review will be published in a peer-reviewed journal, as outlined in the "Dissemination of information" section.

Please find our point-by-point responses to the comments below.

#2.2: The authors indicate that D_2 dopamine antagonists have been the cornerstone for schizophrenia therapy for the last 70 years, although atypical antipsychotics target other receptors, including 5-HT_2receptors. Such compounds, including clozapine and olanzapine, are linked to a different set of adverse effects than classical dopamine antagonists including hypoglycemia and weight gain. Nevertheless, the topic is timely given the recent approval of xanomeline, in combination with trospium, for the treatment of schizophrenia.

Our reply:

We agree with this point that although the primary mechanism of action for antipsychotics has historically been the blockade of dopamine D2 receptors, these drugs also interact with other neurotransmitter receptors, with adverse events often linked to their receptor-binding profiles. Accordingly, we have revised the introduction to incorporate this information and have also noted the recent approval of xanomeline-trospium, which had not been approved at the time of the initial manuscript submission.

Changes in the manuscript:

- We revised the introduction to mention the association of adverse events of antipsychotics with their receptor-binding profiles and off-target actions on other neurotransmitter receptors, as well as the approval of xanomeline-trospium for schizophrenia by the US FDA:

"Antipsychotic drugs that block the dopamine D2 receptor (D2R) have been the cornerstone of pharmacological treatment for schizophrenia for over 70 years. [1–3] .....Moreover, the risk-to-benefit ratio of antipsychotics is often challenged by their multiple side-effects *associated with their receptor-binding profiles and frequently linked to off-target actions beyond D2R blockade.*[1,2] .....
Xanomeline, a muscarinic M1/M4-preferring receptor agonist, has demonstrated improvements in symptoms of schizophrenia in early and late-stage randomized placebo-controlled controlled trials with medium-to-large effect sizes, with potential cholinergic adverse events mitigated by its combination with trospium, a peripheral muscarinic antagonist. [10–13] *In September 2024, the U.S. Food and Drug Administration approved*

xanomeline combined with trospium for schizophrenia, marking it as the first antipsychotic targeting muscarinic receptors. (1) "

#2.3: A systematic review and a meta-analysis are proposed for examining the impact of muscarinic agonists and allosteric modulators in animal models of psychosis. The level of complexity is high with respect to the varying types of compounds (agonists vs. partial agonists, receptor subtype selectivity, binding affinity, etc.), which will make it difficult to accurately categorize compounds. For example, positive allosteric modulators often exhibit a range of effects that include modulation of acetylcholine potency and efficacy in addition to allosteric agonist activity. The degree to which such activities have been adequately assessed will likely vary from lab to lab and such data may not be readily available in the papers that address in vivo efficacy in animal models of psychosis. These concerns are addressed to some extent in the "Exploration of heterogeneity" section, but a more explicit discussion of how concepts of potency and efficacy, for example, will be handled is warranted.

Our reply:

We would like to thank the reviewer for this insightful comment. We have now expanded the "Exploration of heterogeneity" section to elaborate on how these pharmacological properties will be examined, taking into account different modes of action and efforts to calculate indices that minimize potential variability and heterogeneity in measuring these properties (e.g., if possible, differences in the log((maximal efficacy/EC50)).(2) We will aim to use reliable sources for obtaining information about these properties (e.g., IUPHAR/BPS database)(3); however, we will not conduct a systematic review of studies to synthesize them, and we have noted this limitation in the "Discussion" section.

Changes in the manuscript:

- We expanded the methods in the "Exploration of heterogeneity" accordingly:

We will examine the pharmacological properties of muscarinic receptor agonists, considering mode of action (e.g., orthosteric agonism, positive allosteric modulation) and other key characteristics as sources of heterogeneity, using data primarily from established databases (e.g., IUPHAR/BPS) [43] and original publications that characterize these compounds. Specifically, we will assess potency (half-maximal effective concentration, EC50) and efficacy (maximal response, distinguishing between full and partial agonism) for orthosteric agonists, and modulation of acetylcholine potency and efficacy (cooperative factors α and β), intrinsic efficacy (τ_B), and affinity for the allosteric site (K_B) for PAMs.(2,4) These properties will be examined across various muscarinic receptor subtypes, with a preference for human receptors while considering potential cross-species differences, and across downstream signaling pathways (e.g., via $G\alpha_s$, $G\alpha_q$, $G\alpha_{i/o}$ subunits, β-arrestin). Given the variability across assays and experimental settings, we will consider calculating a common standardized index, if possible, such as estimating differences in log((maximal response)/EC50) between the examined drugs and the natural ligand acetylcholine. (2) This approach may enable comparisons across compounds, receptor and signaling pathway selectivity, but summarizing the different properties into a single index may result in information loss, necessitating additional analyses. Moreover, the exact procedure cannot be predetermined

due to anticipated heterogeneity in the data across the different compounds examined. Additionally, we will assess off-target actions by evaluating the mode of action and affinity of each compound toward other neurotransmitter receptors."

- We added to the limitations that a systematic review of in vitro assays and pharmacokinetic studies will not be conducted:

"... Additionally, we will explore whether heterogeneity in behavioral effects may be explained by differences in drug interactions with muscarinic receptors. Given the limited availability and application of in vivo readouts for muscarinic agonism, we will use in vitro data. We will not conduct a systematic review of in vitro assays and pharmacokinetic studies to synthesize information about the pharmacological properties of the drugs, but instead will aim to use reliable sources and standardized methods to assess these as sources of heterogeneity (see "Exploration of heterogeneity")..."

#2.4: In addition to the sources of heterogeneity discussed, the physicochemical properties of compounds may impact the effectiveness of treatments; consideration of ADME properties of compounds could provide an important perspective. For example, multiparameter optimization values could help explain variability in responses for compounds with similar receptor activity profiles.

Our reply:

Thank you for raising this important point. Similarly to the above, we expanded the "Exploration of heterogeneity" section to include physicochemical parameters and their integration with the Central Nervous System Multiparameter Optimization Desirability approach. (5,6)

Changes in the manuscript:

- We expanded accordingly the "Exploration of heterogeneity" section:

"... We will explore potential sources of heterogeneity for .... type of muscarinic receptor agonists or PAMs, their pharmacological and physiochemical properties, and dose (details below), ...
We will use the Central Nervous System Multiparameter Optimization Desirability algorithm (5,6) to examine the physicochemical properties of the drugs as source of heterogeneity. This algorithm calculates a composite desirability score based on six key properties, i.e., lipophilicity, distribution coefficient, molecular weight, topological polar surface area, number of hydrogen bond donors, and the most basic center, which aligns well with the pharmacokinetic attributes, blood-brain barrier permeability and safety. (5,6) Values for these properties will be estimated based on the chemical structure information using OPEn structure–activity/property Relationship App (OPERA) version 2.9.1 (a freely available, open-source provided distributed under MIT license) .(7)"

#2.5: Beyond the pharmacological properties of the compounds under investigation, the impact of muscarinic receptor activation might vary significantly depending on the different compounds utilized to induce behavioral changes (e.g., NMDA antagonists vs. dopamine agonists) or the behavioral readouts (e.g., pre-pulse

inhibition of startle response vs. apomorphine induced climbing). The authors refer to some of the inherent difficulties in the "Assessment of indirectness of animal experiments" section, including concerns about the applicability of various animal models to clinical utility. In this sense, it might be helpful to examine the consistency of comparator compounds (e.g., D_2 and 5-HT_2 antagonists) that are used clinically in treatment of schizophrenia, and are often used as control compounds in studies of muscarinic agonists and other potential therapeutic compounds. This is perhaps alluded to in the "Data synthesis/planned comparisons" section, although additional details would be helpful.

Our reply:

We agree with this point, and we have added a comparison of D2R-blocking antipsychotics with inactive control conditions (e.g., vehicle) to examine the consistency of their effects in the included experiments and to inform the "Assessment of indirectness of animal experiments". This is now described in the "Comparison groups"

Changes in the manuscript:

- We added this comparison in the "Comparison groups":

"Table 1 outlines the planned comparisons, ...
Additionally, we will examine the comparison between active and inactive control conditions to provide insights into the consistency of the effects of D2R-blocking antipsychotic in the included experiments and to inform the assessment of indirectness (see "Assessment of indirectness of the animal experiments")."

#2.6: An additional consideration is the presence or absence of adverse effects for the compounds under investigation. The clinical utility of muscarinic agonists has been limited by adverse effect profiles, which vary from compound to compound, but likely include both off-target (e.g., M_3receptor-mediated) and on-target (i.e., M_1 or M_4 receptor-mediated) effects. Adverse effects such as salivation, lacrimation, diarrhea, or convulsions would provide important information regarding the *in vivo* pharmacological profile of compounds and the separation between beneficial and undesirable effects (i.e., the therapeutic window).

Our reply:

Thank you for this point. We agree that examining adverse events is important for assessing the risk-to-benefit ratio of these drugs. However, we had chosen already from the start of our protocol to focus on behavioural outcomes and not include adverse events due to their sparse and inconsistent reporting in studies of animal models of psychosis (as also noted in our previous review on TAAR1 agonists)(8) and their examination in other study designs excluded from our review (e.g., using naïve animals not subjected to models of psychosis). Furthermore, cholinergic adverse events may potentially be mitigated in clinical settings through the combination of peripheral antagonists, as seen with xanomeline-trospium.(9)Nonetheless, we will aim to extract adverse event data if found in the included

studies and consider secondary publications where relevant. These considerations are addressed in the "Discussion" section under limitations.

Changes in the manuscript:

- We expanded the limitations in the "Discussion" section to include consideration of cholinergic adverse events, similar to the previously reported neurobiological measures:

"We will not analyse neurobiological measures due to their heterogeneous use across studies and the lack of an established biomarker in schizophrenia, [6] .. Similarly, we will not examine cholinergic adverse events in animals, such as salivation, lacrimation and diarrhea, which may offer insights into the risk-benefit profile but could be mitigated in clinical settings with peripheral muscarinic receptor antagonists, as shown with the xanomeline-trospium combination. [10 – 13] We will aim to extract and potentially analyse these measures in secondary publications."

#2.7: Overall, the topic is of timely interest and worth pursuing with some additional attention to the details to be evaluated.

Our reply:

Thank you once again for your overall endorsement and for the thoughtful comments that have substantially strengthened our protocol. In addition to the above-mentioned changes, we have also updated the "Study status" to reflect the changes during the revision:
"...There were no changes from the original PROSPERO registration of the protocol, except for expanding the methods with additional details *(also in response to the reviewer's comments),(10,11)revising the search strategy to include terms for allosteric modulation as suggested by a reviewer,(10)adding physicochemical properties of the compounds in the "Exploration of Heterogeneity" per another reviewer's comment, (11)* and deciding not to search Scopus and CINAHL after consulting with the information specialist (which is not expected to affect the coverage of our search)...."

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Competing Interests: Spyridon Siafis: None. Nobuyuki Nomura: N.N. has received manuscript fees from Sumitomo Pharma. Johannes Schneider-Thoma: None. Irene Bighelli: None. Alexandra Bannach-Brown: None. Fiona J. Ramage: None. Francesca Tinsdeall: None. Ioannis Mantas: None. Sameer Jauhar: SJ has received honoraria for educational talks given for Boehringer Ingelheim, Lundbeck, Janssen, and Sunovion, and has advised on antipsychotics to LB Pharmaceuticals. Sridhar Natesan: None. Anthony Vernon: ACV has received research support from UCB S.A and Bit.Bio (https://www.bit.bio/) and has received honoraria for seminars at GlaxoSmithKline. Andrea de Bartolomeis: AdB has received research support from Janssen, Lundbeck, and Otsuka and lecture fees for educational meeting from Chiesi, Lundbeck, Roche, Sunovion, Vitria, Recordati, Angelini and Takeda; he has served on advisory boards for Eli Lilly, Jansen, Lundbeck, Otsuka, Roche, and Takeda, Chiesi, Recordati, Angelini, Vitria. Sabine M. Hölter: None. Natascha I. Drude: None. Ulf Tölch: None. Wulf-Peter Hansen: None. Virginia Chiocchia: None. Oliver Howes: ODH has received investigator-initiated research funding from and/or participated in advisory/speaker meetings organised by Angellini, Autifony, Biogen, Boehringer-Ingelheim, Eli Lilly, Elysium, Heptares, Global Medical Education, Invicro, Jansenn, Karuna, Lundbeck, Merck, Neurocrine, Ontrack/ Pangea, Otsuka, Sunovion, Recordati, Roche, Rovi and Viatris/ Mylan. He was previously a part-time employee of Lundbeck A/v. Dr Howes has a patent for the use of dopaminergic imaging. Josef Priller: None. Georgia Salanti: None. Malcolm R. Macleod: None. Stefan Leucht: SL has received received honoraria for advising/consulting and/or for lectures and/or for educational material from Angelini, Boehringer Ingelheim, Eisai, Ekademia, GedeonRichter, Janssen, Karuna, Kynexis, Lundbeck, Medichem, Medscape, Mitsubishi, Otsuka, NovoNordisk, Recordati, Rovi, Teva.

Reviewer Report 11 October 2024

https://doi.org/10.5256/f1000research.170522.r325666

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Daniel Foster
Department of Pharmacology, Physiology and Neuroscience, University of South Carolina School of Medicine, Columbia, USA

The authors provide a study protocol to conduct a systematic review and meta-analyses to assess pharmacological targeting of muscarinic receptors in preclinical studies relevant to schizophrenia. This is an especially timely study that has the potential to summarize a vast preclinical literature and provide insights into the efficacy of targeting muscarinic receptors and possible sources of heterogeneity in these data sets that could arise due to drugs that have different selectivity for muscarinic receptor subtypes, or different modes of pharmacological activity. The choice of primary and secondary behaviors is well justified, and the exclusion of studies that only assess neurophysiological or histopathological measures is clearly stated.

One potential caveat of this study protocol is the use of the pharmacological term "agonist" throughout the protocol. In specific sentences, the authors acknowledge the potential role for allosteric modulators in mediating antipsychotic-like effects. For example, in the interventions section, the authors write: "We will include any pharmacological agent acting as an agonist or positive allosteric modulator at any of the five subtypes". They then state in the Exploration of Heterogeneity section that they will consider: "pharmacological characteristics (e.g., mode of action, selectivity for muscarinic or other receptors, potency, efficacy)". These statements are much appreciated, and these meta-analyses could provide important insights into how positive allosteric modulators and agonists differ in efficacy in preclinical studies across numerous symptom domains. Given the potential importance of understanding how allosteric modulators and agonists are similar or differ in their ability to modulate different preclinical assays relating to numerous schizophrenia symptom domains, it may be worth changing the title and clarifying the importance of this pharmacological distinction more clearly. The agonist vs allosteric modulator issue is also evident in the search terms included in the extended data where only "agonist" is used and "allosteric modulator" is absent. In these searches, numerous allosteric modulators are included by name. However, this approach has the potential to miss any allosteric modulators not included by name in the search terms. The importance of studying potential discrepancies between agonist and allosteric modulators is highlighted by ongoing clinical trials for both types of compounds, with allosteric modulators currently being investigated in the clinic by AbbieVie, Neumora, and Neurosterix, and muscarinic agonist therapy having been approved for Karuna/Bristol Myers Squibb and under clinical investigation by several other companies including Neurocrine, Anavex, and Maplight.

Another potential issue that warrants attention is the use of genetically modified knockout animals. Some of the most compelling evidence we have for determining which muscarinic receptor subtypes mediate specific behavioral effects comes from studies that combine selective

pharmacology tools in mice where the receptor has been deleted either globally or from specific populations of neurons. However, the current study design does not clearly outline how or if studies in these knockout animals will be evaluated.

The use of a similar methodology by the authors previously to assess TARR1 agonists in non-human studies is a strength, and the team assembled is well-suited to carry out this study. Overall, this study protocol is thoughtfully written and addresses important and timely questions with the potential to unveil critical insights into the ability of compounds targeting muscarinic receptors to modulate schizophrenia-relevant behaviors across numerous symptom domains in preclinical studies.

Is the rationale for, and objectives of, the study clearly described?

Yes

Is the study design appropriate for the research question?

Yes

Are sufficient details of the methods provided to allow replication by others?

Yes

Are the datasets clearly presented in a useable and accessible format?

Not applicable

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: I study muscarinic receptor modulation of preclinical physiology and behaviors relevant to schizophrenia.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 20 Dec 2024

Spyridon Siafis

#1.1: The authors provide a study protocol to conduct a systematic review and meta-analyses to assess pharmacological targeting of muscarinic receptors in preclinical studies relevant to schizophrenia. This is an especially timely study that has the potential to summarize a vast preclinical literature and provide insights into the efficacy of targeting muscarinic receptors and possible sources of heterogeneity in these data sets that could arise due to drugs that have different selectivity for muscarinic receptor subtypes, or different modes of pharmacological activity. The choice of primary and secondary behaviors is well justified, and the exclusion of studies that only assess neurophysiological or histopathological measures is clearly stated.

Our reply:

We would like to thank the reviewer for recognizing the relevance of our planned work, for the overall endorsement of our methods, as well as for the constructive comments that further improved our methodology.

Please find our point-by-point responses to the comments below.

#1.2 One potential caveat of this study protocol is the use of the pharmacological term "agonist" throughout the protocol. In specific sentences, the authors acknowledge the potential role for allosteric modulators in mediating antipsychotic-like effects. For example, in the interventions section, the authors write: "We will include any pharmacological agent acting as an agonist or positive allosteric modulator at any of the five subtypes". They then state in the Exploration of Heterogeneity section that they will consider: "pharmacological characteristics (e.g., mode of action, selectivity for muscarinic or other receptors, potency, efficacy)". These statements are much appreciated, and these meta-analyses could provide important insights into how positive allosteric modulators and agonists differ in efficacy in preclinical studies across numerous symptom domains. Given the potential importance of understanding how allosteric modulators and agonists are similar or differ in their ability to modulate different preclinical assays relating to numerous schizophrenia symptom domains, it may be worth changing the title and clarifying the importance of this pharmacological distinction more clearly.

Our reply:

We would like to thank the reviewer for raising this important point. We had already planned to examine both orthosteric receptor agonists and positive allosteric modulators, as well as their differences, which we had highlighted in several parts of the manuscript. However, for simplicity, we had generally used the term 'agonism' throughout the protocol. We agree with the reviewer on the importance of this distinction, and we have clarified this in response to the reviewer's comments.

Changes in the manuscript:

- We have revised the title to include 'positive allosteric modulation':

"Muscarinic receptor agonists *and positive allosteric modulators* in animal models of psychosis: protocol for a systematic review and meta-analysis."

- We have incorporated 'positive allosteric modulation' more frequently throughout the manuscript to emphasize this distinction.

- We have clarified its significance further in the introduction:

"...., and the potential differences between orthosteric agonists and PAMs. *For example, PAMs modulate receptor activity by binding to a site distinct from the natural ligand, offering theoretical advantages such as increased selectivity and safety over orthosteric agonists.(1,2)*".

- In relationship to the comments of another reviewer,(3) we have also expanded the "Exploration of heterogeneity" section by providing more details in the pharmacological properties considered for orthosteric agonists and positive allosteric modulators:

"We will examine the pharmacological properties of muscarinic receptor agonists, considering mode of action (e.g., orthosteric agonism, positive allosteric modulation) and other key characteristics as sources of heterogeneity, using data primarily from established databases (e.g., IUPHAR/BPS) [43] and original publications that characterize these compounds. Specifically, we will assess potency (half-maximal effective concentration, EC50) and efficacy (maximal response, distinguishing between full and partial agonism) for orthosteric agonists, and modulation of acetylcholine potency and efficacy (cooperative factors α and β), intrinsic efficacy (τ_B), and affinity for the allosteric site (K_B) for PAMs.(1,4) These properties will be examined across various muscarinic receptor subtypes, with a preference for human receptors while considering potential cross-species differences, and across downstream signaling pathways (e.g., via $G\alpha_s$, $G\alpha_q$, $G\alpha_{i/o}$ subunits, β-arrestin). Given the variability across assays and experimental settings, we will consider calculating a common standardized index, if possible, such as estimating differences in log((maximal response)/EC50) between the examined drugs and the natural ligand acetylcholine. (4)This approach may enable comparisons across compounds, receptor and signaling pathway selectivity, but summarizing the different properties into a single index may result in information loss, necessitating additional analyses. Moreover, the exact procedure cannot be predetermined due to anticipated heterogeneity in the data across the different compounds examined."

#1.3 The agonist vs allosteric modulator issue is also evident in the search terms included in the extended data where only "agonist" is used and "allosteric modulator" is absent. In these searches, numerous allosteric modulators are included by name. However, this approach has the potential to miss any allosteric modulators not included by name in the search terms. The importance of studying potential discrepancies between agonist and allosteric modulators is highlighted by ongoing clinical trials for both types of compounds, with allosteric modulators currently being investigated in the clinic by AbbieVie, Neumora, and Neurosterix, and muscarinic agonist therapy having been approved for Karuna/Bristol Myers Squibb and under clinical investigation by several other companies including Neurocrine, Anavex, and Maplight.

Our reply:

We would like to thank the reviewer for highlighting this potential caveat in our search strategy. In collaboration with Dr. Farhad Shokraneh, an experienced information specialist (see Acknowledgments in the manuscript), we have revised the search strategy across multiple electronic databases to include terms relevant to allosteric modulation, ensuring we capture any studies on this category of medications. As it was already noted in the methods section, we will also supplement our searches using the psychosis-SOLES,(5)a dedicated database of preclinical psychosis studies, allowing us to conduct broader searches without restricting terms to "agonism" or "allosteric modulation". These methods will enhance our search coverage and reduce the risk of missing relevant studies on positive allosteric modulators.

Changes in the manuscript:

- We have revised the methods to clarify that the search strategies will encompass both agonism and allosteric modulation:

'For muscarinic agents, we will use broad terms like muscarinic agonists *or allosteric modulators* and...'

- Additionally, we have updated the draft search strategy in MEDLINE via Ovid within the extended data to include terms for "allosteric modulation". See the revised extended data(https://doi.org/10.5281/zenodo.14534308):

"...#3. Muscarinic Agonists/ or Muscarine/ or (Muscarin or Muscarine or Muscarinic or M1 or M2 or M3 or M4 or M5 or Muscarinomimetic).ti,ab,tw.*
#4. Allosteric Regulation/ or Allosteric Site/ or (Alloster or PAM or PAMs or Modulat* or Agonis*).ti,ab,tw.*
#5. 3 and 4...."

#1.4: Another potential issue that warrants attention is the use of genetically modified knockout animals. Some of the most compelling evidence we have for determining which muscarinic receptor subtypes mediate specific behavioral effects comes from studies that combine selective pharmacology tools in mice where the receptor has been deleted either globally or from specific populations of neurons. However, the current study design does not clearly outline how or if studies in these knockout animals will be evaluated.

Our reply:

Thank you for this point. We agree that genetically modified knockout animals can be important tools for delineating the potential receptor mechanisms of pharmacological interventions. We had already planned to compare muscarinic receptor agonists or PAMs with inactive control conditions in the context of genetic or pharmacological muscarinic receptor antagonism to determine if any observed differences result from muscarinic receptor activation, as it had been outlined in Table 1 and "Animal Population and Model Induction". However, we agree that this was not clearly described, and we have therefore revised the methods accordingly.

Changes in the manuscript:

- We revised the "Animal Population and Model Induction" section to better describe the inclusion of animal cohorts with genetic knockouts of muscarinic receptors or those receiving pharmacological muscarinic antagonists:

"....Moreover, in the eligible studies, we will extract data from "naïve" animal cohorts (i.e., animals that have not undergone models of psychosis), and animal cohorts that have undergone both models of psychosis and muscarinic receptor antagonism via genetic *(i.e, genetically modified animals with knockout of all or specific muscarinic receptors, either globally or in specific neuron populations) (6)* or pharmacological manipulation *(e.g., co-administration of selective or non-selective muscarinic receptor antagonists like scopolamine) (7) (see further*

details in "Comparison groups")…."

- We expanded the "Comparison groups" to mention explicitly the comparison of muscarinic drugs versus inactive control conditions (e.g., vehicle) in animal models of psychosis in the context of muscarinic receptor antagonism:

"*…We will also compare muscarinic receptor agonists or PAMs with inactive control conditions in contexts of genetic or pharmacological muscarinic receptor antagonism (as described in "Animal population and model induction") to determine if any observed differences result from muscarinic receptor activation. Separate analyses may be conducted for the antagonism of specific muscarinic receptor subtypes and/or within specific neuronal populations, if sufficient data are available..*"

#1.5: The use of a similar methodology by the authors previously to assess TARR1 agonists in non-human studies is a strength, and the team assembled is well-suited to carry out this study. Overall, this study protocol is thoughtfully written and addresses important and timely questions with the potential to unveil critical insights into the ability of compounds targeting muscarinic receptors to modulate schizophrenia-relevant behaviors across numerous symptom domains in preclinical studies.

Our reply:

Thank you once again for your overall endorsement and for the thoughtful comments that have substantially strengthened our protocol. In addition to the above-mentioned changes, we have also updated the "Study status" to reflect the changes during the revision:
"…There were no changes from the original PROSPERO registration of the protocol, except for expanding the methods with additional details *(also in response to the reviewer's comments),(3,8)revising the search strategy to include terms for allosteric modulation as suggested by a reviewer,(8)adding physicochemical properties of the compounds in the "Exploration of Heterogeneity" per another reviewer's comment, (3)* and deciding not to search Scopus and CINAHL after consulting with the information specialist (which is not expected to affect the coverage of our search)…."

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