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Dr. Paul Greengard was the initial PI. He passed away on April 13, 2019. A transfer PI request to Dr. Marc Flajolet was submitted and approved.

14. ABSTRACT

See next page.

15. SUBJECT TERMS

Antidepressants, ketamine, mGluR5 antagonists, TRAP assay, P11, Major Depression, Parkinson's disease

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Depression is one of the most prominent psychiatric symptoms in Parkinson's Disease (PD), affecting 30% to 50% of the patients, and causes additional impairment in daily activities in PD patients. In the United States, depression is a common psychiatric disease with an estimated lifetime incidence of over12% in men and over 20% in women, accounting for a high socio-economic burden as well as for a major loss of quality-adjusted life year.

Currently, the antidepressant drugs called SSRIs (selective serotonin reuptake inhibitors), which mainly increase the availability of the brain chemicals serotonin and norepinephrine, present significant limitations; 1) only half of patients are responding (improved symptoms) to the currently available treatment, 2) it takes a long time (few weeks to several months) to observe the beneficial effect of the treatment. Thus, there is a great need for the development of next generation treatments with rapid onset of action and higher efficacy for patients resistant to SSRIs.

Recently, another type of chemical called glutamate has been shown to be better suited for a rapid therapeutic response, representing an alternative strategy for the treatment of depression. Two essential components responsible for glutamate regulation are cellular receptors called NMDA and mGluR5 receptors. Although much effort has been made, little is known about the molecular and cellular mechanisms of the actions of these antidepressant drugs (e.g. ketamine and MTEP) targeting these receptors.

Our laboratory has identified a component, p11, as an important factor mediating depression-like states and antidepressant responses of SSRIs. Our team has recently demonstrated that not all nerve cells are involved in SSRIs effects and identified specific nerve cell types responsible for the therapeutic effect. In preliminary studies, we have also linked p11 to the therapeutic responses to compounds acting on NMDA and mGgluR5 receptors.

To uncover novel metabolic pathways involved for depression, we propose to study the relationship between p11 cell type specificity and the beneficial action of ketamine and MTEP in mice. To do this, we will: 1) genetically modify mice to specifically remove p11 in the cell types previously identified (critical for the antidepressant actions); 2) determine which cell types are involved in the action of the compounds acting on NMDA and mGluR5 receptors; and 3) look for the common p11-dependent regulators responsible for these responses. With this knowledge, we will be able to provide cellular and molecular framework of p11 in the therapeutic actions of all three classes of antidepressant drugs. Altogether, this work will help us identify novel molecular mechanisms and propose novel therapeutic strategies for the treatment of depression.

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1. INTRODUCTION

In the United States, depression is a common psychiatric disease, with an estimated lifetime incidence of over 12% in men and over 20% in women (1, 2, 3). Currently available antidepressant drugs, such as selective-serotonin reuptake inhibitors (SSRIs) have limitations, including a high percentage of treatment resistance and a slow onset of response. Therefore, there is a need for the development of novel treatments that eliminate these limitations. p11 (the protein product of the S100a gene) is an important factor that mediates depression-like states and antidepressant responses to SSRIs (4). Our laboratory has shown that p11 is involved not only in the therapeutic response to *chronic* SSRI treatment, but in the *acute* treatment response of rapid acting classes of antidepressants, such as NMDA antagonists and mGluR5 antagonists, as well (5, 6). Thus, our hypothesis is that there must be p11-dependent common downstream molecules critical for the responses to all three antidepressants. The overarching goal of our research project is to identify these common molecular signatures regulated by p11 for the antidepressant actions of SSRIs, NMDA antagonists (e.g. ketamine), and mGluR5 antagonists (e.g. MTEP). Identifying common downstream molecules critical for the responses to all three types of antidepressants could yield a new therapeutic strategy for the treatment of depression. This is important in the context of currently used antidepressants and their limitations as mentioned above. Using translating ribosome affinity purification (TRAP) technology, we have previously shown induction of serotonin receptor subtype 4 expression in response to chronic SSRI in specific cell-types (i.e. pyramidal neurons) expressing p11 in the cortex (7). To determine whether similar downstream molecules are involved in the response to the fast-acting antidepressants (ketamine, MTEP)(8,9,10), we first needed to identify the p11 cell-types that are responsible for the response to these antidepressants. These activities constituted the majority of the work completed during Year 1, where we determined p11 in cholinergic (ChAT), but not glutamatergic cortical, parvalbumin-positive or cholecystokinin-positive GABAergic, neurons are required for the antidepressant-like behavioral response to ketamine. In Year 2, we were able to expand upon this finding to study the ketamine metabolite, hydroxynorketamine (HNK)(11). Based upon the findings from Year 1, we were also able to spend a considerable amount of time in Year 2 doing molecular profiling to compare common molecular signatures regulated by p11 for the actions of SSRIs vs. the fast-acting antidepressants.

In Year 3, due to the events related to Dr. Greengard (Original PI) passing, we have accomplished only part of the proposed work. During the PI transition period, and also because of our new ACURO submission, we were not able to perform experiments involving animals. To compensate for these delays, we requested and obtained a one-year extension at no cost. The other aspects of the work have been mostly in line with our original timing as described in the last three quarterly reports corresponding to this reporting period. These activities are summarized below. Taken together, the activities completed so far represent critical steps to eventually develop highly targeted drugs that improve therapies for depression with fewer side effects. Over the last year, a manuscript has also been prepared, submitted, revised and accepted for publication at *Journal of Neuroscience* (12).

2. KEYWORDS

Major depression, antidepressants, p11, ketamine, MTEP, mGluR5, glutamate, SSRI, ChAT, novelty suppressed feeding, forced swim test, TRAP

3. OVERALL PROJECT SUMMARY

Below is a brief summary of the progress which occurred during the Year 3 reporting period, followed by a presentation of the results. Year 3 activities were centered on Aim 4:

Specific Aim 4: We will determine whether the common molecular signatures of p11 revealed by the TRAP assay mediate the antidepressant actions of SSRI, ketamine, and MTEP.	***Timeline (Months)***
4a. *Generation of Cre-dependent adeno-associated virus (AAV) vectors expressing the candidate genes revealed by Aim 3:* *- Construction of AAV vectors expressing the candidate genes* *- Checking the efficiency of the AAV vectors in the cell culture system* *- Packaging of the AAV vectors for in vivo*	24-31
4b. *Behavioral assessment in animals with stereotactic injection of AAV vectors expressing the genes* *- Stereotactic administration of AAV vectors into Cre-driver lines* *- Behavioral tests (NSF test and FST)*	32-36

The third objective of this project was to search for common molecular signatures of p11 critical for the responses to both SSRIs, and fast-acting antidepressants (ketamine, and MTEP) (Specific Aim 3). The first step toward achieving this objective was to generate triple transgenic and control transgenic mouse lines for subsequent use in a TRAP assay, which we completed in Year 2. The subsequent steps consisted in performing a molecular profiling of these mice after treatment with a fast-acting antidepressant (ketamine, MTEP) and in comparing the molecular profiles to search for common molecular signatures of p11 responsible for the effects of SSRIs, ketamine and/or MTEP. Some of this work has been included in a manuscript that has been prepared during Year 2 and submitted for publication during Year 3. Briefly, we performed dose-response MTEP experiments in order to identify efficacious doses to test in the chosen/appropriate cell-type specific p11 knockout mice (i.e. ChAT-p11 KO mice). These optimization steps were performed for both FST and NSF tests. Considering the large range of effective doses used and the breeding scheme that we are using, it was important to calibrate these experiments on novel cohorts of mice in order to ensure an appropriate basal response. In addition, there are not too many studies reporting

the effects of MTEP in mice on latency to feed in the NSF test. These findings identified an effective dose of MTEP (10 mg/kg) for the FST assay, specifically in ChAT p11-KO mice. The lack of an effect of MTEP on latency to feed in the NSF test may suggest that this particular test is not sensitive to the effects of this compound. This work has been now partially published in the *Journal of Neuroscience* in June 2019 (12).

The fourth and last objective of this proposal (Specific Aim 4a, b) is to determine whether the common molecular signatures identified by the TRAP assay are relevant for p11-dependent depression-like phenotypes and mediate some of the antidepressant actions of SSRIs, especially fast-acting antidepressants. The method proposed consists in injecting stereotaxically AAV viral vectors into the brains of mice to over-express the candidate genes and measure behaviorally the impact of the over-expression.

In preparation for Specific Aim 4 which commenced as scheduled the first month of Year 3, *in-vivo* control experiments consisting of viral injections were performed at the end of Year 2 in order to calibrate technical conditions. We have previously described the preparatory work performed which included testing 3 different AAV subtypes and behaviorally profiling two control conditions in order to select candidate genes, design AAV vectors, testing *in-vitro* efficiency and generate viral stocks for *in-vivo* studies. We also behaviorally profiled two control conditions (Sham lesion versus AAV-GFP over-expression) and compared how independent cohorts of animals would perform in an open field arena to evaluate global locomotor activity. We conducted this experiment in 3 cohorts to determine the stability of the effect of sham and AAV-GFP lesions on activity behavior. These experiments were crucial to determine the number of animals to be used for our final experiments.

The p11-mouse model, as well as control wild-type mice, were bred to generate cohorts of mice large enough to perform behavioral profiling (N>10 for each of 2-3 groups per cohort). They were characterized behaviorally using an open field test in order to remove potential outliers and to balance animals based on global locomotor activity.

All viral stocks specific to the candidate genes used are new and therefore it is crucial to characterize systematically each viral stock for their viral titer and cellular toxicity. Viral titers are typically obtained using molecular technologies that do not take into account the viral particles viability. As a consequence, the efficacy of infection of a given viral stock can vary largely even though the genomic viral titer is known. Prior to injecting a new viral stock to mice, a toxicity test in mammalian cultured cells has been performed. Only cleared viral stocks were then inject 3-5 animals to assess *in vivo* toxicity by injecting the viral stock in the same experimental conditions (concentration, volume, injection speed) than the ones used later on for behavioral characterization.

During the last reporting period, such experiments were performed for viral stocks corresponding to five candidate genes. These correspond to the first five putative candidate genes RAIPD1-5 (for Rapid-Acting Antidepressants induced p11-dependent genes) that were chosen for further evaluation. Importantly, two are coding for uncharacterized, orphan G-protein coupled receptors (GPCRs) also known as GPR85 and GPR161; such receptors represent particularly attractive candidates due to their great therapeutic potential.

As mentioned above, for each of these five candidate genes, the full-length cDNA was cloned and verified by DNA-sequencing, then sub-cloned into an AAV over-expressing vector and the AAV viral stocks were produced, purified and validated.

AAV viral injections were performed on two cohorts to initiate the behavioral impact of the over-expression of RAIPD1 (GPR85) and RAIPD2 (GPR161). Viability post-surgery was excellent, and no obvious toxicity was reported. The post-surgery mice were placed in an open field arena; no major difference was observed (vertical and horizontal activities) which was to be expected. Once post-surgery validation has been performed, the mice were then coded and balanced into testing groups based on the virus they were injected with (control vs. RAIPDs); the experimenter remains unaware of the coding system during the entire process. A typical results obtained for Open Field (OF) analysis has been presented previously in Year3-Q3 report (Figure 1).

The work to further evaluate basic cognitive abilities of these mice and depression-related tests (novelty suppressed feeding and forced swim tests) is well advanced and will resume pending the ACURO approval. Briefly, so far, five mouse groups were subjected to depression-like behavioral testing. Between 6 and 12 mice were analyzed per group. A representative example of typical results obtained for Forced Swim Test (FST) has been presented previously in Year3-Q3 report (Figure 2) in which three groups were compared (AAV-GFP vs. AAV-RAIPD1 vs. AAV-RAIPD2). No significant difference was found between the groups in this experiment. Similar experiments were carried out for Novelty Suppressed Feeding (NSF) test. The cohort size could still be increased at this point and we will consider adding more animals to the groups being studied when a trend is detected. AAV viral injections have also been performed on another cohort using an AAV viral stock corresponding to RAIPD3. Viability post-surgery was excellent, and no obvious toxicity was reported. The general motor activity did not lead to the identification of outlier. The behavioral testing related to depression has been initiated.

Due to the recent publication of a study demonstrating that a ketamine metabolite, hydroxynorketamine (HNK), represents a better compound than ketamine itself, we then expanded upon this finding by doing a more comprehensive investigation using the ketamine metabolite. As a result we examined the effects of HNK in the NSF and FST behavioral assays in WT control mice. Male control mice were intra-peritoneally injected with Vehicle (saline) or HNK (10 mg/kg) 24 hours before FST and NSF. A total of 46 mice (Saline: n=24; HNK: n=22) were tested in FST. In a separate cohort, a total of 30 mice (Saline: n=16; HNK: n=14) were tested in NSF. As previously reported, we found that HNK decreased FST immobility and decreased the latency to feed in the NSF test.

Next, we evaluated the effect of HNK in p11 KO mice in order to determine whether an antidepressant-like response would be absent in the absence of p11. Closer examination of the FST results in the WT mice indicated that the effect of HNK on immobility was modest. That is, 22-24 mice were necessary in order to observe a significant ($p=0.05$) effect of HNK. This modest effect size, combined with the fact that the effects of HNK on latency to bite in

the NSF were slightly confounded by increased food consumption during the control test, caused us to re-examine using HNK. For these reasons, we paused the use of HNK for behavioral studies in the p11 ChAT KO lines.

We recently received animal protocol approval from IACUC to initiate a study in which mice will be exposed to anti-depressants for a longer time. Typically, mice are treated chronically for 14-21 days. We believe that this does not reflect well patient treatment exposure as patients are usually treated for much longer time periods, even relatively to the mouse lifespan. We will compare the 21 days treatment typically used for SSRIs with the new condition (up to 6 months of anti-depressant provided in the drinking water). We submitted documentation of this IACUC-approved amendment to ACURO and it is currently under review.

Furthermore, based on the magnitude of differential gene expression observed so far in our BAC-TRAP experiments, it is plausible that BAC-TRAP results obtained after longer chronic exposure might help improve the robustness of our candidate list. Our plan remains to wait for ACURO approval, and then initiate the work.

4. KEY RESEARCH ACCOMPLISHMENTS

Some of this work has been published in the *Journal of Neuroscience* in June 2019 (12). A PDF of the published study is presented in annex. A summary of all key research accomplishments emanating from the research project thus far are summarized below:

- We completed the colony work to generate the triple transgenic and control transgenic lines necessary for the TRAP assay. The Cre-dependent TRAP line was obtained, genotyped, amplified and was crossed with the cell-type specific p11 KO mice (i.e., the ChAT-p11 KO mice).
- After validation of the libraries' quality, significant progress was made generating and sequencing 6 cDNA libraries.
- The molecular data sets obtained using next generation sequencing have been analyzed.

- MTEP *in vivo* pharmacology (dose response) was completed behaviorally in naïve control animals in order to identify efficacious doses to test in the appropriate cell-type specific p11 knockout mice (i.e. ChAT-p11 KO mice). It was critical to calibrate these experiments to ensure an appropriate basal response and importantly to determine the number of mice per group necessary to achieve statistical significance.
- We determined an effective dose of MTEP (10 mg/kg) for the FST behavioral assay in ChAT p11-KO mice, to use in subsequent experiments.
- The effect of MTEP on latency to feed in the NSF test has been evaluated twice. These results coupled with a consistent trend of MTEP to induce confounding changes in appetite/food consumption raised two caveats: 1) test sensitivity and 2) feeding confounds when considering future experiments with the ChAT-p11 KO mice.

- HNK is a ketamine metabolite that has been recently presented as being more suitable than ketamine itself because it does not interfere with NMDA signaling and does not present side effects typically associated with interfering with NMDA currents.

- HNK synthesis protocol was qualitatively and quantitatively optimized in-house.
- We generated sufficient quantities of HNK to allow us to evaluate the behavior effects of HNK in control animals.
- We determined that, although HNK had an antidepressant-like behavioral effect in control animals, its effect size was very small (FST) and confounded by nonspecific effects of HNK on appetite and/or food consumption (NSF).

- We designed, generated and validated molecular constructs (cDNAs, expression constructs) corresponding to the 5 chosen candidate genes.
- We designed, produced and tested AAV viral tools corresponding to 5 candidate genes for Aim 4.
- We conducted experiments in 3 cohorts of mice to determine
- The stability of the effect of sham and AAV-GFP lesions after stereotactic surgeries has been determined on 3 cohorts of mice using the open field test to evaluate motor activity.
- The viral stocks corresponding to five candidate genes were produced and tested in cells for efficacy and toxicity.
- Several stereotactic injection experiments were performed for these five viral stocks and the mice were characterized behaviorally for their general motor activity, and also for depressive-like behaviors.
- Two cohorts of mice were used to initiate the behavioral impact of the over-expression of RAIPD1 (GPR85) and RAIPD2 (GPR161).
- Once post-surgery validation has been performed, the mice were then coded and balanced into testing groups and tested in the Open Field test.
- Five mouse groups were subjected to depression-like behavioral testing. Between 6 and 12 mice were analyzed per group. Forced Swim Test (FST) has been presented previously in which three groups were compared (AAV-GFP vs. AAV-RAIPD1 vs. AAV-RAIPD2).
- AAV viral injections have also been performed on another cohort using an AAV viral stock corresponding to RAIPD3. The general motor activity did not lead to the identification of outlier.

- IACUC approval was granted to perform a study in which mice will be exposed to anti-depressants for a longer time. We submitted documentation of this IACUC-approved amendment to ACURO and it is currently under review.

5. CONCLUSION

Identifying neuronal cell types responsible for the therapeutic response to fast-acting classes of antidepressants (such as ketamine, HNK and MTEP) have important medical, military and scientific implications. Indeed, little is known about the mechanisms underlying these fast-acting anti-depressants and we are convinced that identifying the neuronal cell types involved represent the first step towards identifying specific downstream molecules critical for this response. The identification of such molecules is necessary to develop highly

targeted drugs. This better targeted strategy should lead to improved treatments for the symptoms of major depression while presenting fewer side effects.

To reach these long-term goals, the steps delineated in the proposal for the third year were mostly successfully completed. We are sad to report that Dr. Greengard passed away on April 13th 2019. The present award has been successfully transferred to Dr. Marc Flajolet. The progress of the work has been slightly affected during the change in PI. To compensate for that we have successfully requested a one-year extension at no cost. Regarding the experimental procedures we did not encounter significant problems and we do not anticipate problems in the coming months.

Based upon our findings that p11 in cholinergic (ChAT), but not glutamatergic cortical, parvalbumin-positive or cholecystokinin-positive GABAergic, neurons are required for the antidepressant-like behavioral response to ketamine, we expanded upon these results to study the behavioral effects of the ketamine metabolite, hydroxynorketamine (HNK).

We also evaluated MTEP dose-response behavioral experiments in naïve cohorts of control mice to inform our decision to pursue MTEP in ChAT-p11 KO.

We completed the generation of the triple transgenic (floxed TRAP/floxed p11/CRE) (using ChAT-p11 KO line) and control transgenic (floxed TRAP/Cre) lines for the TRAP assay.

We performed the TRAP assays on mice after treatment with ketamine or MTEP and analyzed the molecular profiles to search for common molecular signatures of p11 responsible for the effects of SSRIs, ketamine, and MTEP. Completing such goals kept us on track for Specific Aim 3.

Furthermore, by testing multiple AAV subtypes, behaviorally profiling two control conditions, generating AAV tools and viral stocks, injecting stereotactically AAV viral stocks for five candidate genes and performing depression-related behavioral testing kept us on track for Specific Aim 4 in Year 3. Some behavioral characterization for two of the candidate genes will be performed in the upcoming months.

In conclusion, with the knowledge obtained thus far and the knowledge to be obtained next year, we feel we will be able to provide a cellular and molecular framework of p11 in the therapeutic actions of all three classes of antidepressant drugs. Altogether, this work will help us identify novel molecular mechanisms and propose novel therapeutic strategies for the treatment of depression.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

Several of the control and exploratory experiments performed for this proposal, such as MTEP calibration and optimization in the context mGluR5 signaling, were also useful for another ongoing project in the laboratory. While this project is not entirely relevant for the present proposal in regard to the disease mechanism, we thought it would be appropriate to mention it in this publication anyway considering that it did help the recently submitted study from a

practical and methodological point of view. We have attached a PDF version of the manuscript published (See Appendix).

7. INVENTIONS, PATENTS AND LICENSES

Nothing to report

8. REPORTABLE OUTCOMES

- Identification of the ideal dose of MTEP (10 mg/kg) for use *in vivo* in FST test; Identification of MTEP ineffectiveness in NSF test.
- Generation of triple transgenic and control transgenic lines necessary for the TRAP assay. Generation of several mouse cohorts.
- Optimized protocol for the chemical synthesis of HNK, a ketamine derivative that does not affect NMDA current. Characterization of the effectiveness of HNK *in vivo*.
- Generation of six cDNA libraries and next generation sequencing after validation of the libraries' quality.
- Organization and analysis of the molecular data sets obtained after next generation sequencing.
- Identification of five candidate genes that could participate or explain how p11 mediates the therapeutic responses to rapid-acting antidepressants.
- Generation and validation of molecular and viral tools for the five above mentioned candidate genes.

9. OTHER ACHIEVEMENTS

- Based on the results obtained early on, and considering the results obtained with MTEP, mGluR5 KO control mice were clearly needed. Thus, in addition to the 4 cell-type specific p11 KO mouse lines generated earlier, we have also generated control mouse lines where mGluR5 has been KO specifically in the same cell types relevant for this program. We previously also established breeding and genotyping protocols for these mouse lines, and we performed basic biochemical and behavioral characterization. In Year 2 (Q1), using the open field, we have been able to characterize locomotor activity, and to some extent basic anxiety measures, in the ChAT-mGluR5, EMX-mGluR5, and PV-mGluR5 KO mice. No confounds were found behaviorally and we were able to validate biochemically the functionality of these mouse lines. Since then, we are happy to report that we have continued to make good progress with these mouse lines. Specifically, when comparing in our pilot experiments the new batch of HNK (that we produced internally) with ketamine and MK-801, another NMDA modulator (non-competitive NMDA antagonist), we discovered that mGluR5 has an important role in mediating NMDA effects specifically in the cholinergic neurons (ChAT-mGluR5 mouse line).

- We received IACUC protocol approval for an amendment to initiate a study in which mice will be exposed to anti-depressant for a longer time. Typically, mice are treated chronically for 14-21 days. We believe that this does not reflect well patient treatment exposure as patients are usually treated for much longer time periods. We will compare the 21 days

treatment typically used for SSRIs with the new condition (up to 6 months of anti-depressant provided in the drinking water).

- We submitted this proposal for ACURO approval and it is currently under review.

10. - PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Paul Greengard
Project Role:	Project Director
Researcher Identifier (e.g. ORCID ID):	0000-0002-4437-0893
Nearest person month worked:	1.2 calendar months
Contribution to Project:	Dr. Greengard has supervised the project and met regularly with Dr. Flajolet to review and analyze the data. Due to Dr. Greengard's passing on April 13th, 2019, Dr. Flajolet will serve as PI on this project.

Name:	Marc Flajolet
Project Role:	Research Associate Professor
Researcher Identifier (e.g. ORCID ID):	0000-0002-0518-7666
Nearest person month worked:	3.0 calendar months
Contribution to Project:	Dr. Flajolet is the new PI. He oversees all the work and he has provided comments and discussion about molecular profiling, tool generation and for animal production/animal protocol. This transfer PI request was approved on July 11, 2019.

Name:	Junghee Jin
Project Role:	Postdoctoral Associate
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person month worked:	4.2 calendar months
Contribution to Project:	Dr. Jin has provided comments and discussion about animal use protocol and animal production.

Name:	Jodi Gresack
Project Role:	Senior Research Associate
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person month worked:	2.4 calendar months
Contribution to Project:	Dr. Gresack oversees behavior testing (including the design) of the animals and provides instructions to all personnel regarding the training for animal behavioral tests. Dr. Gresack has left the project as of May 2019. Her departure from the project will not affect the remaining Statement of Work.

Name:	Clark Pitcher
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person month worked:	6.0 calendar months

Contribution to Project:	Mr. Pitcher has worked on animal maintenance (breeding, weaning, tail clipping) including genotyping until July 1st, 2019. He is replaced by Mr. Evan Cater.
Name:	Evan Cater
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person month worked:	6.0 calendar months
Contribution to Project:	Mr. Cater has worked on animal maintenance (breeding, weaning, tail clipping) including genotyping since July 1st, 2019. He also assists Dr. Jin with the maintenance of behavior equipment as described in the approved protocol.
Name:	Claire Song
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person month worked:	6.0 calendar months
Contribution to Project:	Ms. Song has worked on animal maintenance (breeding, weaning, tail clipping) including genotyping. Ms. Song has left the project as of June 2019. This change will not affect the remaining Statement of Work.
Name:	Evan Nair
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person month worked:	2.4 calendar months
Contribution to Project:	Mr. Nair assisted Dr. Gresack with the maintenance of behavior equipment as described in the approved protocol. Mr. Nair has left the University as of May 4, 2019. Mr. Cater will continue this work with Dr. Jin. This change will not affect the remaining Statement of Work.

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12. APPENDIX

A manuscript published in *'Journal of Neuroscience'* entitled "*Cholinergic neurons of the medial septum are crucial for sensorimotor gating*" and including some of the pharmacological results presented earlier is attached.

Quad Chart.

Neurobiology of Disease

Cholinergic Neurons of the Medial Septum Are Crucial for Sensorimotor Gating

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Hypofunction of NMDA receptors has been considered a possible cause for the pathophysiology of schizophrenia. More recently, indirect ways to regulate NMDA that would be less disruptive have been proposed and metabotropic glutamate receptor subtype 5 (mGluR5) represents one such candidate. To characterize the cell populations involved, we demonstrated here that knock-out (KO) of mGluR5 in cholinergic, but not glutamatergic or parvalbumin (PV)-positive GABAergic, neurons reduced prepulse inhibition of the startle response (PPI) and enhanced sensitivity to MK801-induced locomotor activity. Inhibition of cholinergic neurons in the medial septum by DREADD (designer receptors exclusively activated by designer drugs) resulted in reduced PPI further demonstrating the importance of these neurons in sensorimotor gating. Volume imaging and quantification were used to compare PV and cholinergic cell distribution, density, and total cell counts in the different cell-type-specific KO lines. Electrophysiological studies showed reduced NMDA receptor-mediated currents in cholinergic neurons of the medial septum in mGluR5 KO mice. These results obtained from male and female mice indicate that cholinergic neurons in the medial septum represent a key cell type involved in sensorimotor gating and are relevant to pathologies associated with disrupted sensorimotor gating such as schizophrenia.

Key words: cholinergic; gating; mGluR5; NMDA; sensorimotor

Significance Statement

The mechanistic complexity underlying psychiatric disorders remains a major challenge that is hindering the drug discovery process. Here, we generated genetically modified mouse lines to better characterize the involvement of the receptor mGluR5 in the fine-tuning of NMDA receptors, specifically in the context of sensorimotor gating. We evaluated the importance of knocking-out mGluR5 in three different cell types in two brain regions and performed different sets of experiments including behavioral testing and electrophysiological recordings. We demonstrated that cholinergic neurons in the medial septum represent a key cell-type involved in sensorimotor gating. We are proposing that pathologies associated with disrupted sensorimotor gating, such as with schizophrenia, could benefit from further evaluating strategies to modulate specifically cholinergic neurons in the medial septum.

Introduction

Hypofunction of ionotropic NMDA receptors has been hypothesized to underlie for the pathophysiology of schizophrenia (Snyder and Gao, 2013; Jadi et al., 2016). Previous reports demonstrated that administration of NMDA receptor antagonists, such as ketamine and phencyclidine, induced schizophrenia symptoms in human subjects (Bubeníková-Valesová et al., 2008; Driesen et al., 2013). In animals, administration of a noncompetitive NMDA receptor antagonist, MK801, leads to schizophrenia-like behaviors, including disruption of prepulse inhibition of the startle response (PPI), social interaction deficit, and enhanced locomotor activity (Gururajan et al., 2010). Therefore, small molecular agents capable of increasing NMDA receptor function have been considered as a therapeutic option for the treatment of schizophrenia. However, direct activation of NMDA receptors triggers rapid Ca^{2+} influx that can lead to excitotoxic neuronal death. Indirect potentiation of NMDA receptor signaling could represent another approach for the development of antipsychotic drugs.

Metabotropic glutamate receptor subtype 5 (mGluR5) is closely associated, both physically and physiologically, with NMDA receptors (Conn et al., 2009; Lindsley and Stauffer, 2013).

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Global genetic deletion of mGluR5 of modification of its cell-surface expression triggers behaviors categorized as schizophrenia-like phenotypes, such as decreased PPI and increased sensitivity to locomotor hyperactivity induced by NMDA receptor antagonists (Kinney et al., 2003; Brody et al., 2004; Wang et al., 2009). Consistent with the study of global mGluR5 knock-out (KO) mice, mGluR5 antagonists potentiate the psychotomimetic effects of NMDA receptor antagonists in animals (Henry et al., 2002). In the CNS, mGluR5 is predominantly expressed in glutamatergic, GABAergic, and cholinergic neurons (Pisani et al., 2001; López-Bendito et al., 2002; Wu et al., 2004; Sun et al., 2009). For all these reasons, mGluR5 has emerged as a potential target for the treatment of schizophrenia (Lindsley et al., 2004; Kinney et al., 2005; Stefani and Moghaddam, 2010; Rook et al., 2015; Maksymetz et al., 2017).

Disruption of sensorimotor gating, a measure of which is PPI of startle, contributes to cognitive disorganization and represents an important symptom in schizophrenia. PPI deficits remain mostly unaffected by most antipsychotics (Geyer, 2006). The relationship between mGluR5 function in different cell types and the pathophysiology of disrupted sensorimotor gating remains largely unknown. In general, the cell-type specificity associated with sensorimotor gating deficiencies is not well characterized. In this study, we started by generating three cell-type-specific mGluR5 KO mouse lines to address this question and further confirm, using DREADD (designer receptors exclusively activated by designer drugs) technology, the nature and location of the neurons involved. Conditional KO of mGluR5 specifically in cholinergic neurons reduced PPI and enhanced sensitivity to MK801-induced activity. Along these lines, interference with cholinergic neurons in the medial septum using DREADD also lead reduced PPI validating the role of these neurons in sensorimotor gating. Furthermore, using mGluR5 KO mice, electrophysiology recordings of cholinergic neurons of the medial septum showed reduced NMDA receptor-mediated currents. All together, these results indicate that cholinergic neurons in the medial septum are important for sensorimotor gating and that mGluR5 mediates some of these effects.

Materials and Methods

Animals. All animal procedures were approved by the Rockefeller University Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health guidelines. The floxed mGluR5 mouse line (generous gift from Dr. A. Contractor, Northwesten University; Xu et al., 2009, 2014; Lee et al., 2015) were crossed with EMX1-cre (stock 005628, The Jackson Laboratory; Gorski et al., 2002), parvalbumin (PV)-cre (stock 008069, The Jackson Laboratory; Hippenmeyer et al., 2005), and choline acetyl transferase (ChAT)-cre (stock 006410, The Jackson Laboratory; Rossi et al., 2011) mouse lines to delete mGluR5 in glutamatergic, PV-positive GABAergic, and cholinergic neurons, respectively. The ChAT-cre mouse line (stock 006410, The Jackson Laboratory) was used for DREADD-mediated manipulation of cholinergic neurons.

For each line, enough animals of the same age were generated, taking advantage of *in vitro* fertilization and embryo transfer techniques (Transgenic Facility, Rockefeller University), and housed four per cage with a 12 h light/dark cycle and *ad libitum* access to food and water was provided. The 10- to 16-week-old male mice were used for behavioral testing and assigned to different experimental groups based on their genotype. Other experiments were performed on males and females.

PPI. Startle was measured using a San Diego Instruments SR-Lab Startle Response System as described previously (Wang et al., 2009). Mice were placed into nonrestrictive Plexiglas cylinders resting on a Plexiglas platform. High-frequency speakers (33 cm above the cylinders) produced all acoustic stimuli. Piezoelectric accelerometers mounted under the cylinders transduced movements of the mice, which were digitized and stored by an interface and computer assembly. Beginning at startle stimulus onset, 65 consecutive 1 ms readings were recorded to obtain the amplitude of the mouse's startle response. The house light remained on throughout all testing sessions. For the acoustic startle sessions, the intertrial interval between stimulus presentations averaged 15 s (range: 7–23 s). A 65 dB background was presented continuously throughout the session. Startle pulses were 40 ms in duration, prepulses were 20 ms in duration, and prepulses preceded the pulse by 100 ms (onset–onset). The Plexiglas holders were wiped clean and allowed to dry between runs.

The acoustic startle sessions consisted of three blocks. Sessions began with a 5 min acclimation period followed by delivery of five startle pulses (120 dB). This block allowed startle to reach a stable level before specific testing blocks. The next block tested response threshold and included four each of five different acoustic stimulus intensities: 80, 90, 100, 110, and 120 dB (data not shown) presented in a pseudorandom order. The third block consisted of 42 trials including 12 startle pulses (120 dB) and 10 each of 3 different prepulse trials (i.e., 68, 71 and 77 dB preceding a 120 dB pulse). We focused only on the 77 dB prepulse trial because we did not see a difference between the three cell-type-specific mGluR5 KO and WT groups at 68 dB and 71 dB. PPI was calculated as follows using the trials in the third block: $100 - ([(\text{average startle of the prepulse + pulse trials})/\text{average startle in the pulse alone trial}] \times 100)$. In all experiments, the average startle magnitude over the record window (i.e., 65 ms) was used for all data analysis. For AAV-injected animals, clozapine-*N*-oxide (CNO; 1 mg/kg, i.p.; Sigma-Aldrich) was given 1 h before the test.

MK801-induced locomotor activity. MK801-induced locomotor activity was measured in open-field arenas ($42 \times 42 \times 22.5$ cm) as described before (Wang et al., 2009). Automated Accuscan software was used to calculate total distance traveled. Mice were habituated to the chamber for 30 min before MK801 injection (0.3 mg/kg, i.p.; Sigma-Aldrich). Their activity was monitored for 1 h immediately after injection. For AAV-injected animals, CNO (1 mg/kg, i.p.; Sigma-Aldrich) was given 1 h before the test.

Stereotaxic viral injection. Cre-dependent AAV8-hSyn-DIO-mCherry and AAV8-hSyn-DIO-hM4D(Gi)-mCherry were obtained from the Vector Core Facilities of University of North Carolina (Chapel Hill, NC). Mice were anesthetized with 2.5% avertin (250 mg/kg, i.p.) and placed onto a stereotaxic device (Leica). AAV vectors (1 μl for medial septum, 0.8 μl per side for nucleus accumbens) were microinjected using a Micro4 injector (World Precision Instruments) into medial septum (coordinates AP/DV/ML = 0.98/−4.34/0.00 mm) or nucleus accumbens (coordinates AP/DV/ML = 1.18/−4.67/±1.10 mm). After surgery, animals received ibuprofen in drinking water (50 mg/kg/d) for 3 d and were allowed to recover for at least 2 weeks before behavioral assessments.

Immunohistochemistry. Animals were deeply anesthetized and transcardially perfused with PBS, followed by 4% paraformaldehyde (PFA) in PBS. Brains were postfixed in 4% PFA overnight at 4°C, and then cryoprotected using 30% sucrose for at least 24 h, followed by freezing in OCT medium (Sakura Finetek). A cryostat was used to collect coronal sections of 40 μm thickness. Free-floating sections were washed in PBS and incubated in blocking buffer (0.5% Triton X-100, 5% normal goat serum or 5% normal donkey serum, in PBS) at room temperature for 1 h. Sections were then incubated with the primary antibodies diluted in the blocking buffer at 4°C overnight. Primary antibodies: anti-ChAT (goat polyclonal, EMD Millipore; 1:250), anti-VGLUT2 (guinea pig polyclonal, Synaptic Systems; 1:250), anti-parvalbumin (mouse monoclonal, Swant; 1:1000), anti-mGluR5 (rabbit monoclonal, Abcam; 1:500). After incubation, sections were washed in PBS three times and incubated with AlexaFluor-conjugated secondary antibodies (Invitrogen). After secondary incubation, sections were washed in PBS three times and mounted on glass slides with DAPI (Life Technologies). Confocal images were obtained on a Zeiss LSM 710 confocal imaging system (Carl Zeiss).

Microscopy. AAV vector-injected mice were deeply anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg, i.p.) and transcardially perfused with 4% PFA. Brains were rapidly removed and further fixed in 4% PFA overnight. Brains were cryoprotected with 30% (w/v) sucrose solution, sectioned (50 μm), and mounted on glass slides. Confocal images were acquired on a Zeiss LSM 710 confocal imaging system (Carl Zeiss)

using a 10×/0.3 objective lens. Images were analyzed using the Zen 2010 software (Carl Zeiss).

Volume imaging and quantification. Volume staining was performed using the bulk tissue labeling and clearing method, iDISCO, as described previously (Renier et al., 2014; Liebmann et al., 2016). Mice were first perfused with 4% PFA and postfixed overnight at 4°C, followed by washing with PBS and coronal sectioning into 3 mm blocks centered on the respective regions-of-interest. Tissue blocks were then gradually dehydrated in methanol, bleached and rehydrated in PBS. After a 0.3 M glycine wash and serum blocking, each tissue block was incubated both with rabbit anti-PV (Swant PV27; 1:100) and goat anti-choline acetyltransferase (Millipore ab144p; 1:50) for 4 d. Unbound antibodies were washed for 2 d in PBS containing heparin with repeated solution change before incubation with donkey anti-rabbit AlexaFluor 647 and donkey anti-goat AlexaFluor 568 for 4 d, each at 20 μg/ml. Excess secondary antibody was washed as previously described and tissue was cleared using methanol, dichloromethane and dibenzylether.

Volume imaging was performed on a LaVision light sheet microscope with a 4× objective in the Bio-Imaging Resource Center at The Rockefeller University. For optimal axial resolution, images were taken every 3 μm with horizontal focus applied at 10 positions across the sample. Automated cell counting was performed with Imaris object detection, with filter settings adjusted for background intensity and minimum cell size according to the respective region. For the dentate gyrus and prefrontal cortex, analysis was restricted to 1 mm tissue thickness. The entire medial septum and all of the nucleus accumbens contained within the slice were counted. ChAT staining in the dentate gyrus and prefrontal cortex was almost exclusively from axons, therefore cells were not counted.

Slice preparation. Mice were deeply anesthetized using carbon dioxide and decapitated. The brain was removed quickly and placed into the ice-cold NMDG solution containing the following (in mM): 93 NMDG (*N*-methyl-D-glucamine, supports neurons in brain slice preparations), 93 HCl, 10 $MgSO_4$, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 0.5 $CaCl_2$, 20 HEPES, 25 glucose, 5 Na-ascorbate, 3 Na-pyruvate, and 2 Thiourea, pH 7.4, 300 mOsm. Coronal slices (300 μm) were cut with a vibratome (VP1000S; Leica Microsystems) and incubated at 37°C for 15 min in NMDG solution. Then brain slices were transferred to ACSF containing the following (in mM): 130 NaCl, 26 $NaHCO_3$, 1 $CaCl_2$, 2 $MgCl_2$, 2.5 KCl, 1.25 NaH_2PO_4, 25 glucose, pH 7.4, 300 mOsm, bubbled with 95% O_2 and 5% CO_2, and kept at room temperature (22–24°C) for 2 h before recordings.

Electrophysiological recordings. Whole-cell voltage-clamp recording was performed in cholinergic projection neurons in the medial septum slices as reported previously (Cheng et al., 2014). Mouse slices were positioned in a perfusion chamber attached to the fixed stage of an upright microscope (Olympus) and submerged in continuously flowing oxygenated ACSF. The firing rate of cholinergic neurons in the medial septum/diagonal band of Broca (MSDB) was recorded by using cell-attached patch-clamp. The patch electrodes were filled with internal solution (in mM: 126 K-gluconate, 10 KCl, 2 $MgSO_4$ 0.1 BAPTA, 10 HEPES, 4 ATP, 0.3 GTP, and 10 phosphocreatine, pH 7.3, 290 mOsm). To examine the regulation by DREADD on neuronal activity, cholinergic neurons were recorded 10 min as baseline and then CNO (1 μM) was bath applied to the brain slices. Bicuculline (20 μM) and CNQX (20 μM) were added in NMDAR mediated EPSC recordings. Bicuculline (20 μM) and TTX (5 μM) were added in mEPSC recordings. Patch electrodes contained the following internal solution (in mM): 130 Cs-methanesulfonate, 10 CsCl, 4 NaCl, 10 HEPES, 1 $MgCl_2$, 5 EGTA, 2 QX-314 (Na^+ channel blocker), 12 phosphocreatine, 5 MgATP, 0.2 Na_3GTP, pH 7.2–7.3, 265–270 mOsm. Medial septal cholinergic neurons were visualized with a 40× water-immersion lens and recorded with the MultiClamp 700B amplifier (Molecular Devices). For NMDAR-EPSC, the cell (clamped at −70 mV) was depolarized to +60 mV for 3 s before stimulation to fully relieve the voltage-dependent Mg^{2+} block. Evoked EPSCs were generated with a series of pulses with different stimulation intensities (120–150 μA) from a stimulation isolation unit controlled by a S48 pulse generator (Grass Technologies). A bipolar stimulating electrode (FHC) was placed ~80 μm from the neuron under recording. For mEPSC recordings, the membrane potential was held at −70 mV.

Data analyses were performed with Clampfit (Molecular Devices), Mini Analysis Program (Synaptosoft), and GraphPad Prism 6 (GraphPad Software). Amplitude of the NMDAR-EPSC was subjected to two-way ANOVA followed by *post hoc* Bonferroni tests. Statistical comparisons of the mEPSC amplitude and frequency were made by using unpaired Student's *t* test.

Statistical analysis. Statistical tests were performed by two-tailed unpaired *t* test or ANOVA using Prism 6 software (GraphPad). Statistical significance was set at $p \leq 0.05$. All behavioral data are representative of at least two experiments using different cohorts of animals.

Results

To identify key neuronal populations involved in sensorimotor gating, relevant for the pathophysiology of psychiatric disorders such as schizophrenia, in the context of mGluR5 signaling, we used three cell-type-specific mGluR5 KO mouse lines and analyzed their sensorimotor gating properties. We generated the three conditional mouse lines by crossing existing and well characterized mouse lines. Briefly, floxed mGluR5 mice (Xu et al., 2009, 2014; Lee et al., 2015) were crossed with three different Cre lines (1) empty spiracles homeobox 1′ (EMX)-Cre (Gorski et al., 2002; Lee et al., 2015), (2) PV-positive-Cre (Hippenmeyer et al., 2005; Lee et al., 2015), and (3) ChAT-Cre (Rossi et al., 2011) to delete mGluR5 in glutamatergic, PV-positive GABAergic, and cholinergic neurons, respectively. For each mouse line, immunohistochemical labeling studies were performed to ensure that the KO of mGluR5 was effective. Several anti-mGluR5 antibodies were tested and the one producing the least background was chosen (see Materials and Methods for details). Representative images are shown in Figure 1*A–C*. First, we ensured that baselines corresponding to basic behaviors such as locomotor activity in the open field were not altered. Next, we evaluated PPI in three cell-type-specific mGluR5 KO mouse lines according to standard protocols (Wang et al., 2009). A significant PPI deficit was observed in ChAT-mGluR5 KO mice compared with wild-type (WT) littermates (unpaired two-tailed *t* test, $t = 2.274$, $p = 0.0341$; $n = 10$; $^*p < 0.05$; Fig. 1*D*). In contrast, EMX-mGluR5 and PV-mGluR5 KO mice showed normal PPI at a prepulse intensity of 77 dB compared with WT littermates (Fig. 1*E*,*F*; $n = 9$ and $n = 12$, respectively). There was no significant difference in the startle response to 120 dB pulses within the PPI block between the three mGluR5 KO and WT groups. MK801-induced locomotor activity in cell-type-specific mGluR5 KO mice was then assessed. After MK801 treatment, ChAT-mGluR5 KO mice showed a significant enhanced sensitivity to MK801-induced locomotor activity (two-way ANOVA for the interaction between genotype and block; $F_{(17,252)} = 2.442$; $n = 8$; $^*p < 0.05$ and $^{***}p = 0.0015$, two-way ANOVA followed by the Bonferroni *post hoc* test; Fig. 1*G*). In contrast, there was no difference in locomotor activity in either EMX- or PV-mGluR5 KO mice compared with their WT littermates (Fig. 1*H*,*I*; $n = 11$ and $n = 8$, respectively).

These results suggest that the KO of mGluR5 specifically in cholinergic neurons recapitulates phenotypic characteristics observed in global mGluR5 KO mice, and highlight cholinergic neurons as a component of the circuitry involved in sensorimotor gating.

The cholinergic system consists of two main cell types: (1) cholinergic interneurons in the striatum and (2) projection neurons from the pedunculopontine/lateral tegmental areas and the basal forebrain complex including medial septum (MSDB) (Scarr et al., 2013). Several lines of evidence suggest that mGluR5 is involved in the excitability of cholinergic interneurons in the

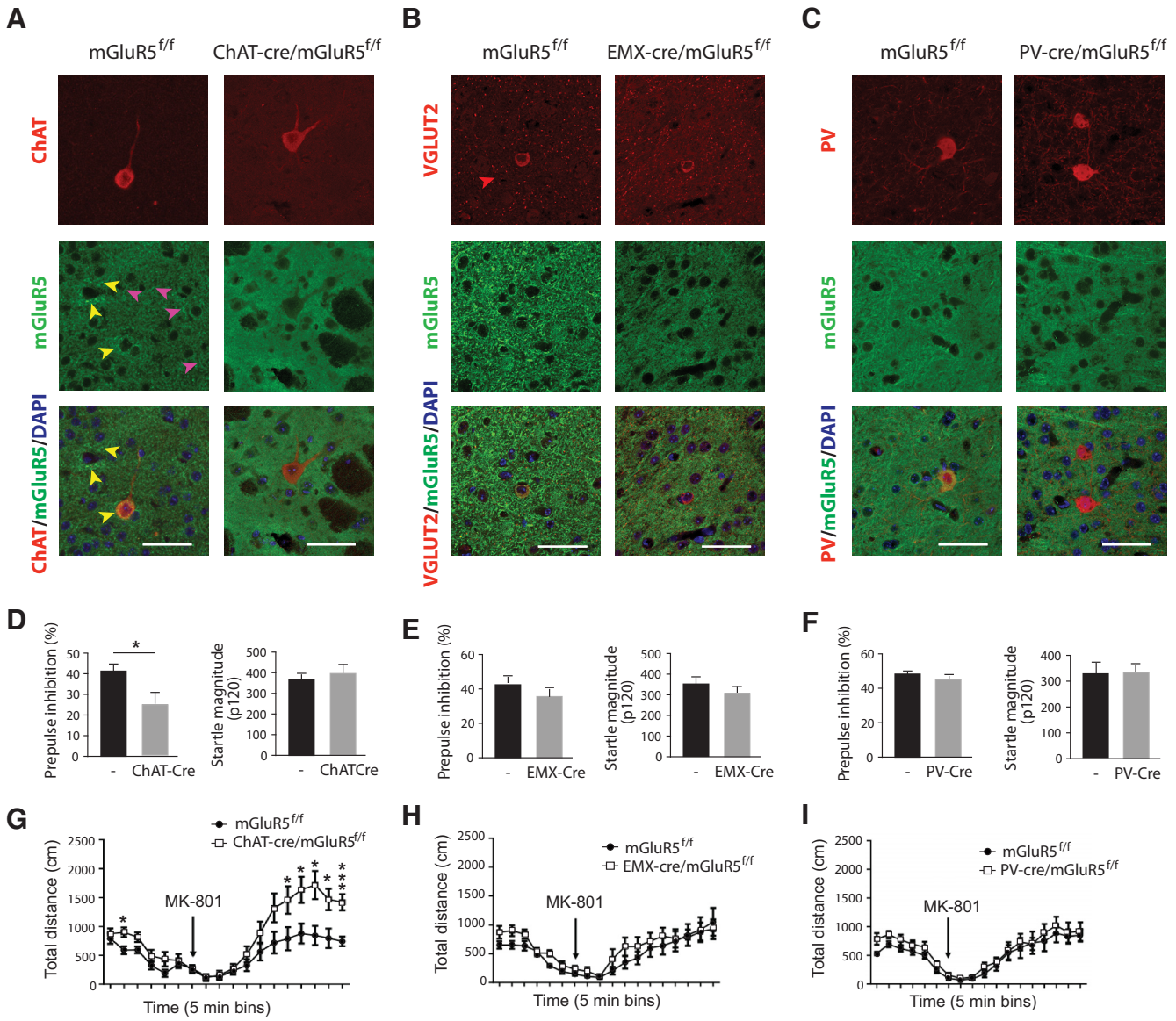

Figure 1. Analysis of prepulse inhibition of the startle response and locomotor response to MK801 in ChAT, EMX, and PV cell-type-specific mGluR5 KO mouse lines. ***A–C***, Representative images after immunohistochemical stainings of ChAT, VGLUT2 (EMX marker), PV, and mGluR5 in WT and conditional KO neurons. Scale bars, 50 μm. Arrowheads indicate some mGluR5-positive cells (yellow) and some fibrous processes (pink). Prepulse inhibition of the startle response and average startle magnitudes at 120 dB were measured in ChAT-mGluR5 (***D***), EMX-mGluR5 (***E***), and PV-mGluR5 (***F***) KO mice. The results obtained for each KO line are compared with their cre(-) mGluR5$^{f/f}$ littermates. MK801-induced locomotor activity was analyzed for ChAT-mGluR5 (***G***), EMX-mGluR5 (***H***), and PV-mGluR5 (***I***) KO mice. Mice were injected with MK801 (0.3 mg/kg, i.p.) at the indicated time (arrow) and total distance traveled was measured using the open-field paradigm. Bar graphs are presented as mean values ± SEM. *$p < 0.05$. ***$p = 0.0015$.

striatum and septohippocampal cholinergic projection neurons originating from the MSDB (Wu et al., 2004; Bonsi et al., 2005).

We hypothesized that inhibition of cholinergic neurons in the nucleus accumbens or the MSDB might be required for the behavioral differences observed in ChAT-mGluR5 KO mice. Therefore, an inhibitory DREADD, where a Gi coupled G-protein coupled receptor (GPCR) can be activated by a ligand that does not target other endogenous GPCRs (Urban and Roth, 2015), was used to identify the type(s) of cholinergic neurons responsible for mGluR5-mediated behaviors. We found that inhibition of cholinergic neurons in the nucleus accumbens of ChAT-cre mice after stereotaxic injection of cre-dependent AAV-Gi-mCherry (Fig. 2*A*) resulted in normal PPI and startle reactivity compared with the control AAV-mCherry-injected group (Fig. 2*C*; $n = 8$). We also targeted the septohippocampal cholinergic projection in the MSDB, a major cholinergic input to the hippocampus. Interestingly, mice subjected to stereotaxic injection of AAV-Gi-mCherry into the MSDB (Fig. 2*D*) displayed disrupted PPI compared with the control AAV-mCherry-injected group (Fig. 2*F*, left graph). There was no significant difference in the startle response to 120 dB pulses between control AAV-mCherry and AAV-Gi-mCherry groups (unpaired two-tailed t test; F; $t = 2.564$, **$p = 0.0058$; $n = 9$; Fig. 2*F*, right graph). Images obtained after IHC and confocal microscopy analysis of cre-dependent control AAV-mCherry (CTRL) or AAV-Gi-mCherry (Gi)-infected neurons did not show significant differences (Fig. 2 *B*, *E*). Next, electrophysiological recordings were performed to assess the neuronal modulation by DREADD on the cholinergic neurons in the MSDB. Control AAV-mCherry (CTRL) had no effect on the firing rate of cholinergic neurons in the absence or pres-

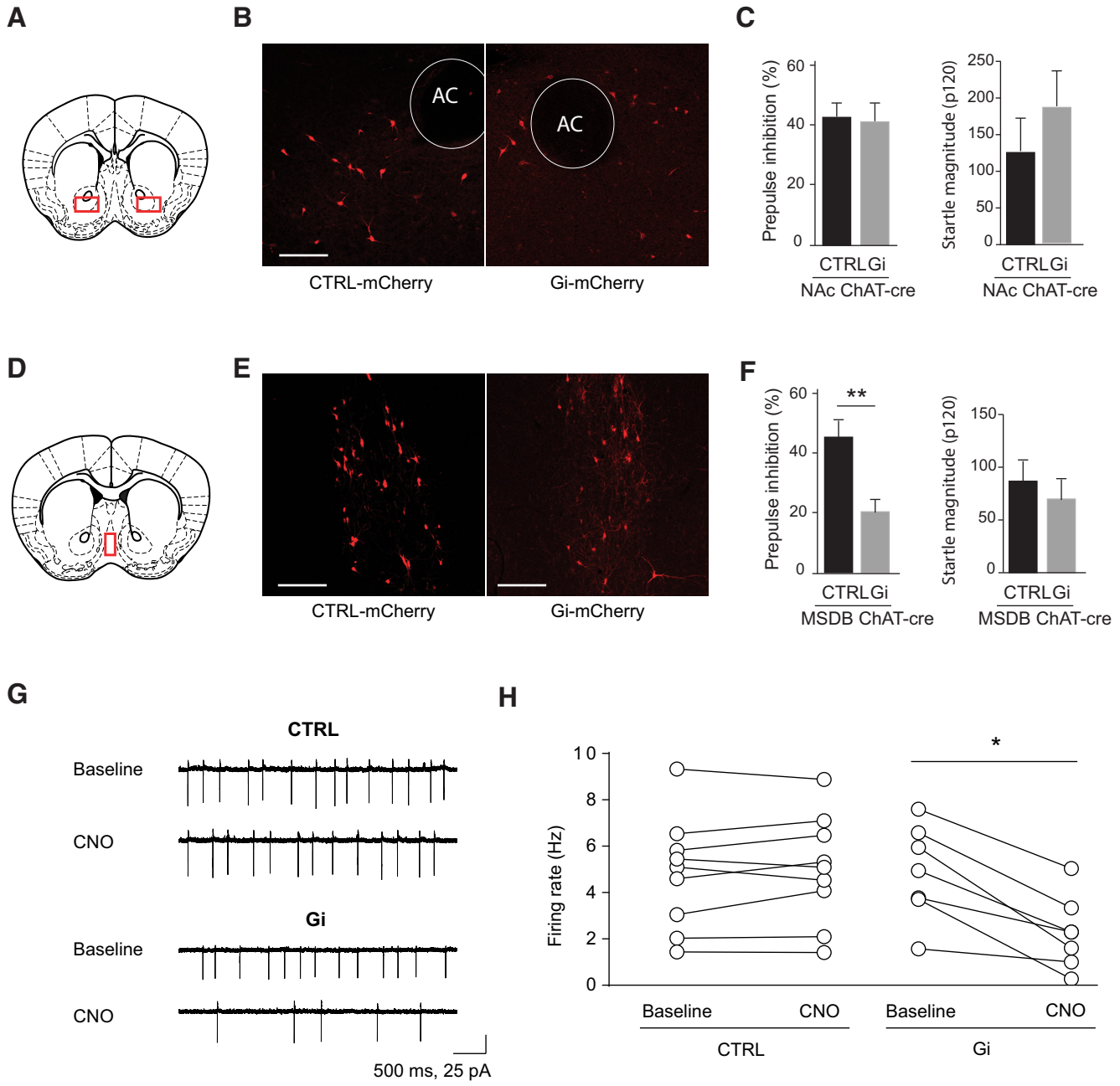

Figure 2. Chemogenetic reduction of medial septum ChAT cell activity. Selective reduction of cholinergic neuron activity was obtained by injecting cre-dependent DREADD AAV viruses to the nucleus accumbens (NAc; ***A–C***) or MSDB (***D–F***) of ChAT-cre mice. ***A***, Schematic view of a coronal mouse brain section indicating the NAc injection zone (red rectangles). ***B***, Representative images obtained after IHC and confocal microscopy analysis of cre-dependent control AAV-mCherry (CTRL) or AAV-Gi-mCherry (Gi)-infected neurons in the NAc of ChAT-cre mice. AC: anterior commissure. Scale bar, 200 μm. ***C***, Prepulse inhibition of the startle response and average startle magnitudes at 120 dB. ***D***, Schematic view of a coronal mouse brain section indicating the MSDB injection zone (red rectangle). ***E***, Representative images obtained after IHC and confocal microscopy analysis of cre-dependent control AAV-mCherry (CTR) or AAV-Gi-mCherry (Gi)-infected neurons in the MSDB of ChAT-cre mice. Scale bar, 200 μm. ***F***, PPI of the startle response and average startle magnitudes at 120 dB. ***G***, ***H***, Representative traces (***G***) and summary graph (***H***) showing the firing rate of cholinergic neurons in the MSDB expressing Control AAV-mCherry (CTRL) or AAV-Gi-mCherry (Gi). CNO was applied to activate DREADD. Bar graphs are presented as mean values ± SEM. *$p < 0.05$. **$p = 0.0058$.

ence of the DREADD agonist, CNO. Expression of AAV-Gi-mCherry itself did not alter the basal activity of cholinergic neurons. Importantly, CNO activation of the Gi DREADD significantly reduced the firing rate of cholinergic neurons in the MSDB (paired two-tailed t test; H; $t = 3.75$, $^*p = 0.033$; $n = 3$ mice per group; Fig. 2*G*,*H*). These results suggest that septohippocampal cholinergic projection neurons in the MSDB could be one of the cell types involved in the circuitry relevant for behaviors involving sensorimotor gating.

To ensure that general characteristics of the brain regions studied were not altered in the mouse lines used, quantification of PV and ChAT cells in multiple brain regions were performed using volume imaging. Representative iDISCO visualization of optical sections from the indicated brain regions stained for parvalbumin (cyan) and acetylcholine esterase (magenta) did not show significant differences in the medial septum (Fig. 3*A*). The quantification for the entire medial septum is shown in Figure 3*C*. Similar quantifications were performed for the other regions studied (Fig. 3*B*).

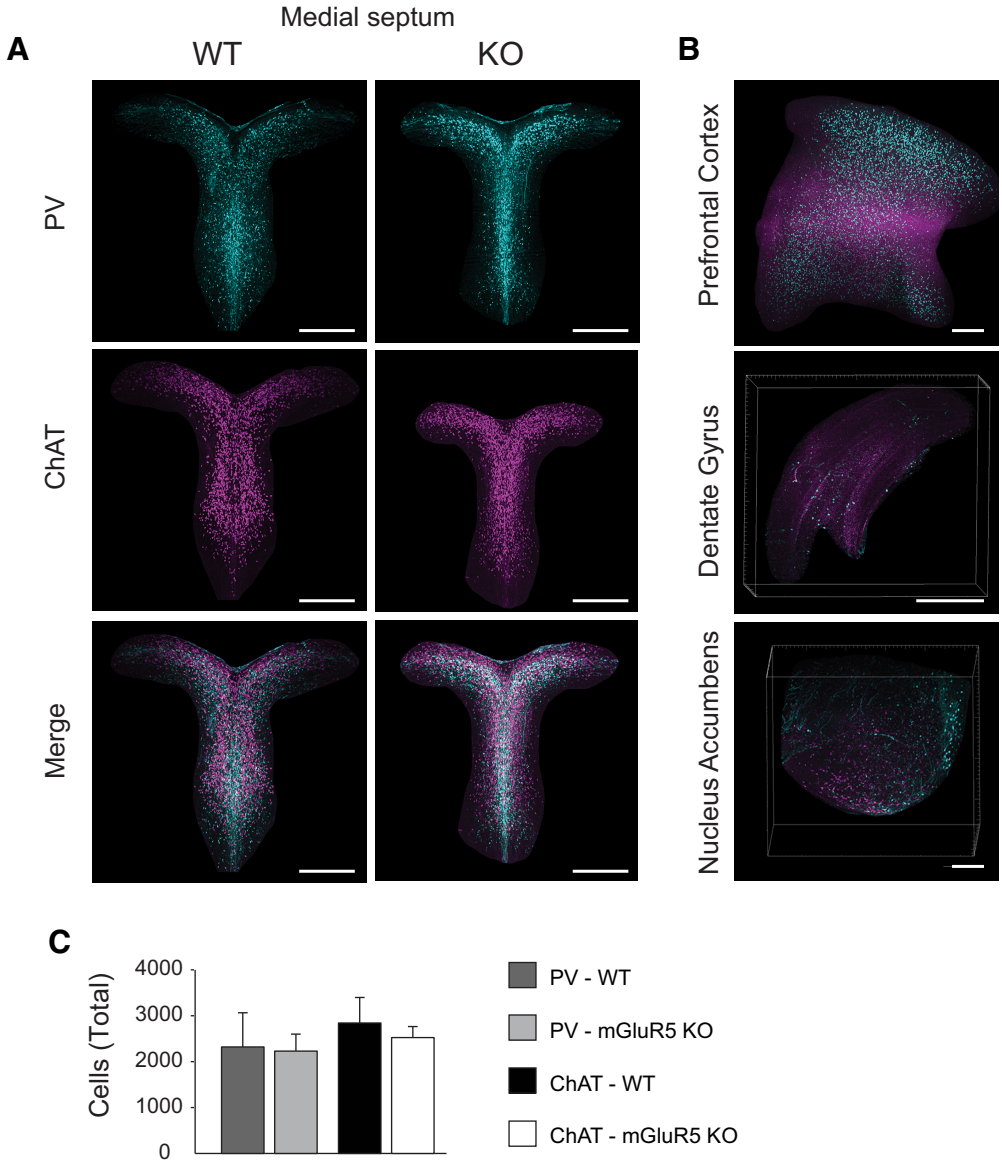

Figure 3. Quantification of PV and ChAT cells in multiple brain regions using volume imaging. ***A***, Representative iDISCO visualization of optical sections from medial septum stained for PV (cyan) and ChAT (magenta) for both WT and KO animals. ***B***, Representative iDISCO visualization of optical sections from three different brain regions costained for PV (cyan) and ChAT (magenta). Prefrontal cortex and dentate gyrus were restricted to 1 mm optical sections. ***C***, Total cell counts of PV-positive and ChAT cells in the entire medial septum from volume images. Each group included four animals; both hemispheres were used. Scale bars, 500 μm.

Next, we examined the NMDAR function in cholinergic projection neurons in the medial septum of WT and mGluR5 KO mice. NMDAR-mediated EPSCs (NMDAR-EPSCs) induced by a series of stimulus intensities were recorded. As shown in Figure 4*A* and *B*, NMDAR-EPSC was markedly reduced in cholinergic neurons in mGluR5 KO mice (55–60% decrease, WT: $n = 5$ cells/3 mice, mGluR5 KO: $n = 6$ cells/3 mice). Two-way ANOVA revealed a significant main effect of genotype and stimulation intensity ($F_{(1,44)} = 30.3$, $^*p < 0.01$) and stimulation intensity ($F_{(3,44)} = 9.8$, $^*p < 0.01$). Furthermore, we examined the AMPAR function and synaptic glutamate release by recording mEPSC in cholinergic neurons. The amplitude and frequency of mEPSC were relatively small in the cholinergic cells and were not remarkably altered in mGluR5-KO mice [Fig. 4*C–G*; amplitude (pA), WT: 12.6 ± 1.1, $n = 5$ cells/3 mice, mGluR5 KO: 15.2 ± 0.7, $n = 6$ cells/3 mice; frequency (Hz), WT: 0.63 ± 0.11, mGluR5 KO: 0.57 ± 0.04, $p > 0.05$]. Together, these results suggest that the function of NMDARs is reduced in medial septal cholinergic neurons in mGluR5 KO mice.

Discussion

Several *In vivo* studies focusing on mGluR5 (Kinney et al., 2003; Brody et al., 2004; Wang et al., 2009, 2018) raised the possibility that mGluR5 plays a role in the pathophysiology of schizophrenia. Yet most studies report little or no change of mGluR5 mRNA expression or protein levels in postmortem samples from schizophrenic patients in the prefrontal cortex, striatum, and hippocampus (Volk et al., 2010; Corti et al., 2011; Matosin and Newell, 2013). Assuming that the quality of these postmortem samples was satisfactory, this observation can be explained by various factors. First, GPCRs undergo various modes of regulation that do not involve necessarily transcriptional and translational modifications (e.g., cell-surface expression, allosteric modulation,

phosphorylation, recycling). Second, GPCRs, including mGluR5, often have more than one variant/isoform. Third, closely related receptors (e.g., mGluR1) might contribute partially to some of the physiological differences described (Brody et al., 2003; Gupta et al., 2005; Pietraszek et al., 2007). Finally, the studies performed with patients on postmortem samples lack the anatomical precision that would be required to analyze specific cell types (e.g., ChAT interneurons). Without this level of specificity, a discreet specific differential expression might be diluted out and overlooked.

Here, we have shown that EMX-mGluR5 KO mice displayed normal PPI and normal response to MK801-induced locomotor activity, suggesting that mGluR5 in excitatory glutamatergic neurons, in the cortex and hippocampus, is not crucial for the behavioral differences mentioned earlier. This might be one explanation why most studies performed on postmortem tissues from schizophrenic patients did not observe significant mGluR5 expression level changes in these regions. However, based on the data presented here, we believe that studying mGluR5 in the context of schizophrenia by focusing on the MSDB region might be helpful in identifying procognitive cotreatments.

Intriguingly, an increased sensitivity to MK-801 after mGluR5 KO in cholinergic neurons was observed. According to the simplest model, the loss of mGluR5 should impair NMDA receptors and, considering the mode of action of MK-801, this should translate into a decreased sensitivity to MK-801. Several hypotheses could explain these results but the two main ones are as follows: (1) mGluR5 loss leads to a different NMDAR outcome, either because the signaling pathways (e.g., adaptor proteins, receptor dimers), feedback loops and the receptors' dynamic are different in this cell type, and/or because the channel properties are modified (e.g., decrease in channel opening probability or channel number); (2) MK-801 through its non-NMDA targets might allow for compensatory mechanisms and complicate the mGluR5/NMDAR model. Because of the physical absence of mGluR5 in our system we believe that it is more likely that the absence of synaptic surface co-clustering between mGluR5 and NMDAR would affect functional channel number (Aloisi et al., 2017), rather than the opening probability. Regarding MK-801 non-NMDAR known and unknown molecular targets (e.g., dopamine and se-

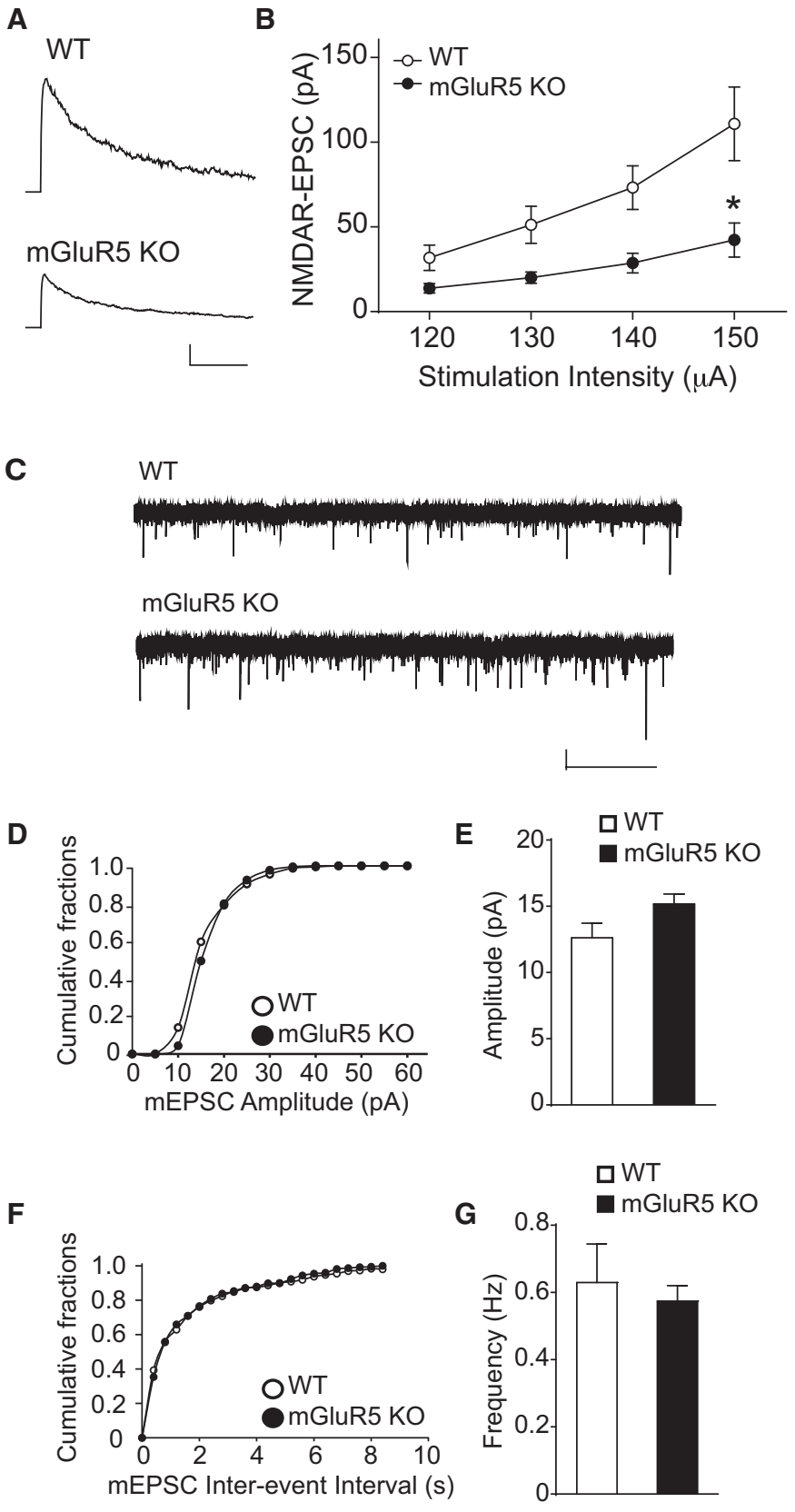

Figure 4. Glutamatergic neurotransmission in cholinergic neurons in the medial septum. ***A***, Representative NMDAR-EPSC traces at 150 μA stimulation. Scale bars: 20 pA, 100 ms. ***B***, Input/output curves (mean ± SEM) of NMDAR-EPSC in response to a series of stimulation intensities in medial septal cholinergic neurons from WT and mGluR5 KO mice. ***C***, Representative traces of mEPSC. Scale bars: 5 pA, 5 s. ***D***, ***F***, Cumulative plots of the distribution of mEPSC amplitude (***D***) and frequency (***F***) in WT and mGluR5 KO mice. ***E***, ***G***, Bar graphs (mean ± SEM) showing the amplitude (***E***) and frequency (***G***) of mEPSC. *$p < 0.05$.

rotonin receptors and transporters, nicotine acetylcholine α7 receptors; Ramoa et al., 1990; Clarke and Reuben, 1995; Iravani et al., 1999; Seeman et al., 2005) more work will be needed to elucidate these alternative pathways and their possible contribution. Finally, one can imagine a higher level of specificity or a greater diversity of interneurons as it was recently demonstrated for the dorsal striatum (Muñoz-Manchado et al., 2018).

In contrast to PPI, we found that selective inhibition of cholinergic neurons by AAV-Gi-mCherry in the MSDB did not affect MK801-induced locomotor activity (data not shown). The absence of an effect on locomotor activity after injection in the MSDB suggests that other circuits such as the brainstem pedunculopontine/laterodorsal tegmental nuclei may be involved in MK801-induced locomotor activity (Mori et al., 2016). Interestingly, MSDB has been recently involved in locomotion speed-correlated input through glutamatergic synaptic integration and glutamatergic projections toward the medial entorhinal cortex (Justus et al., 2017). This could represent alternative options to consider for future work.

Dysfunction of the cholinergic system has been associated with the pathophysiology of schizophrenia. Postmortem and neuroimaging studies suggest that cholinergic receptor signaling is reduced in the cortex and subcortical regions (e.g., hippocampus and striatum) in individuals with schizophrenia (Crook et al., 2000; Dean et al., 2002; Raedler et al., 2003). As a result, targeting the cholinergic system has been a promising strategy to more effectively treat cognitive deficits for quite some time now. Here, we provide the evidence that dysfunction of cholinergic neurons in the MSDB specifically lead to behavioral deficits related to sensorimotor gating, further supporting the relevance of the cholinergic system for treating psychiatric conditions involving perturbances of the sensory gating system such as schizophrenia.

Although our results presented here are clearly indicating a role of ChAT-neurons, the results regarding the PV-positive GABAergic neurons and the glutamatergic neurons might require further validation. Based on the literature, one might have expected that PV-positive interneurons could also have a role in the described phenomena (Barnes et al., 2015; Bygrave et al., 2016). Discrepancies may be attributable to the animal models used including differences in their genetic background and their purity (Paylor and Crawley, 1997). The three mouse lines used in the present study had the same genetic background and were backcrossed similarly.

So far, positive allosteric modulators of mGluR5 have shown promise in preclinical studies using animal models of schizophrenia (Lindsley and Stauffer, 2013; Matosin et al., 2017). Although positive allosteric modulators of mGluR5 have not yet reached FDA approval, it is anticipated that mGluR5-based therapeutics might provide a viable alternative option for the treatment of schizophrenia, alone or in combination (Lindsley et al., 2004; Kinney et al., 2005; Stefani and Moghaddam, 2010; Rook et al., 2015; Maksymetz et al., 2017). Because deficits in PPI are rather common in a large but specific subset of psychiatric diseases, without being able to distinguish diagnostic overlap from comorbidities (Geyer, 2006), targeting the "component" PPI rather than the entire disease, might be beneficial for schizophrenia and for any pathology involving gating disturbances.

Conclusion

The present study using conditional mGluR5 KO mice demonstrates that a specific neuronal cell type, the cholinergic neurons of the medial septum, seems important for the development of phenotypes related to psychiatric disorders, for sensorimotor gating specifically. We believe that identifying the precise cell type(s) involved in pathological behaviors is crucial to better understand specific components involved in complex diseases and disorders, and ultimately to design targeted therapeutic strategies for these components rather than the entire syndromes.

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A role for p11 in the therapeutic response to rapidly acting antidepressants

Log: PD150037 / Task: **Specific Aim 4a-b**
Award: W81XWH-16-1-0681 / Y3 Annual Report

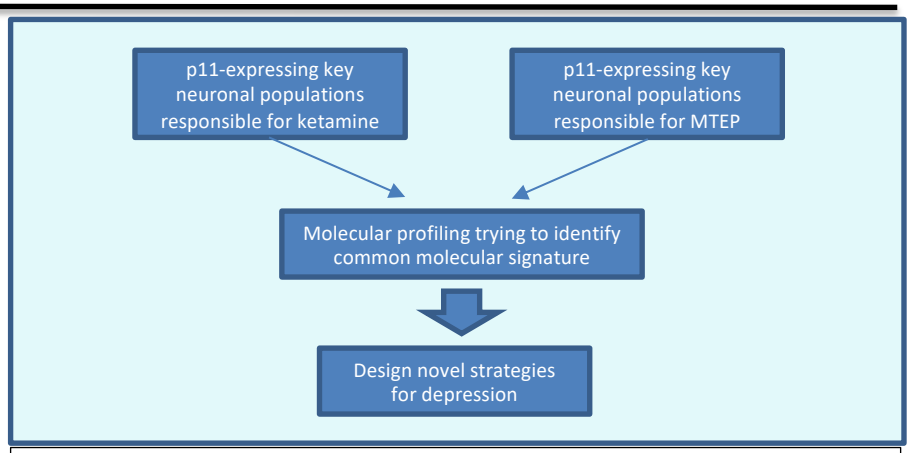
PI: Marc Flajolet **Org:** The Rockefeller University **Award Amount:** $2,542,500

Study/Product Aim(s)

- We will identify p11-expressing key neuronal populations responsible for the antidepressant action of ketamine.
- We will identify p11-expressing key neuronal populations responsible for the antidepressant action of MTEP.
- We will search common molecular signatures of p11 crucial for the antidepressant responses to SSRI, ketamine/HNK, and MTEP.
- We will determine whether the common molecular signatures of p11 revealed by the TRAP assay mediate the antidepressant actions of SSRI, ketamine, and MTEP.

Approach

We will generate a variety of cell-type specific p11 knockout mice and examine the behavioral responses to ketamine and MTEP. Once the cell-type(s) are identified in terms of pharmacological responses, in a search for molecular mechanisms, we will employ the TRAP assay to find common downstream regulators of p11 mediating the therapeutic effects of both ketamine and MTEP. For the most promising candidates obtained from the TRAP assay, we will generate cre-dependent AAV vectors expressing candidate genes and inject these AAV vectors into specific cre-lines to check the behavioral responses to ketamine and MTEP.



Accomplishments: 1) Injected stereo-tactically several mouse groups for behavioral characterization following over-expression of RAIPD1-3; 2) Performed behavioral characterization for general motor activity and depression-like phenotype for RAIPD1-2. 3) Designed, produced and tested 2 AAV viral stocks for candidate genes RAIPD4-5; 4) Analysis of TRAP results and molecular confirmation.

Timeline and Cost

Activities CY	16	17	18	19
Animal use protocol submission, Animal breeding setup, Behavioral tests				
TRAP assay, RNA sequencing, DATA analysis				
AAV vector construction, Stereotaxic experiment, Behavioral tests				
Estimated Budget ($K)	**$211**	**$847.5**	**$847.5**	**$636.5**

Updated: October 8, 2019

Goals/Milestones (Example)

CY16 Goal – Animal Breeding setup

☑ Set up for generation of cell-type specific p11 knockout mice

CY17 Goals – Colony expansion & behavioral tests

☑ Generation of cell-type specific p11 knockout mice

☑ Identify p11-expressing key neuronal populations in response to ketamine & MTEP

CY18 Goal – TRAP analysis

☑ Molecular profiling of animals treated with antidepressants

CY19 Goal – Stereotaxic experiment & behavioral tests

☑ Validation of the genes responsible for all three antidepressants

Comments/Challenges/Issues/Concerns

- Change in PI.
- One year extension at no cost.
- ACURO modification under review.

Budget Expenditure to Date

Projected Expenditure: $2,542,500
Actual Expenditure: $2,472,725