5-2020

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An Undergraduate Thesis Submitted in Partial Fulfillment
of the Requirements for the
University Honors Scholars Program
Honors College
and the
Honors-in-Discipline Biology Program
College of Arts and Sciences
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Abstract

The adenosine system has become a promising target for the treatment of schizophrenia due to its unique relationship with dopamine D_2 receptors. Dopamine D_2 receptors display heightened sensitivity in schizophrenia, and inhibition of these receptors has been shown to alleviate some of the psychotic symptoms of the disorder. Inhibition of adenosine A(2A) receptors has been shown to decrease dopamine D_2 receptor sensitivity, making this receptor a potential target for treatment of the disorder. This effect occurs because adenosine A(2A) receptors form a mutually inhibitory heterodimeric complex with dopamine D_2 receptors. The present study looked at the effects of an adenosine agonist on prepulse inhibition (PPI) and cyclic-AMP response binding element protein (CREB) concentrations in the nucleus accumbens (NAc) using a rodent model of schizophrenia (NQ model) that presents with increased D_2 receptor sensitivity. Results showed that the A(2A) agonist was effective in improving PPI in NQ-treated animals. The agonist was also effective in reducing increased CREB concentrations in the NAc of NQ-treated animals to control levels. The effectiveness of the agonist suggests that the adenosine system may be a viable target for the treatment of some of the psychotic symptoms associated with schizophrenia.

Introduction

According to the National Institute of Mental Health, schizophrenia is a neurological disorder affecting approximately 1% of the population and involves both positive and negative symptoms, as well as cognitive deficits (NIMH, 2018). Positive symptoms of the disorder can include hallucinations and delusions, whereas the negative symptoms involve emotional disruptions, including anhedonia, lack of will, and reduced speech. Lastly, cognitive deficits associated with the disorder can include impaired working memory, reduced mental speed, and reduced reaction times (Lesh et al., 2011). Antipsychotic medications are usually prescribed to individuals diagnosed with schizophrenia to alleviate some of the described symptoms, but the current medications have been shown to have only moderate efficacy and significant harmful side effects (Rummel-Kluge, 2010; Leucht, 2013), with nearly 75% of individuals quitting treatment within a year (Gray, 2010).

Inhibition of dopamine D_2 receptors to decrease dopamine signaling is the mechanism used by current antipsychotics to treat the symptoms of schizophrenia. Heightened dopamine D_2 receptor sensitivity is an important underlying mechanism in the pathology of schizophrenia (McCutcheon et al., 2020). Therefore, inhibition of dopamine D_2 receptors has been shown to alleviate some of the psychotic symptoms of the disorder. This is the mechanism shared by all current antipsychotic medications, but the harmful side effects associated with these medications call for a better alternative.

The adenosine system has emerged as a possible target for treatment of the disorder, specifically the psychotic symptoms (Boison et al., 2012). Adenosine is a neuromodulator that regulates the release of dopamine and glutamate. Past research has shown that stimulating the adenosine A(2A) receptor (agonist) reduces the sensitivity of dopamine D_2 receptors. This effect is hypothesized to occur because the adenosine A(2A) receptor forms a heterodimer complex with dopamine D_2 receptors (Borroto-Escuela and Fuxe, 2017), resulting in a mutually inhibitory interaction between the two receptors when the adenosine A(2A) receptor is stimulated (Filip et al., 2012; Díaz-Cabiale, 2001). Due to this interaction, an adenosine A(2A) agonist has the potential to decrease dopamine D_2 receptor sensitivity and potentially alleviate some of the psychotic symptoms associated with schizophrenia (Domenici et al., 2019).

The goal of the present study was to investigate the effectiveness of an adenosine A(2A) agonist (CGS 21680) in alleviating auditory sensorimotor gating deficits, a behavioral hallmark of schizophrenia, using the neonatal quinpirole rodent model of schizophrenia. Analysis of the efficacy of the agonist is crucial in determining whether an adenosine A(2A) agonist can be therapeutic and potentially provide an alternative to the current antipsychotic medications in use. The neonatal quinpirole model was a model developed in Dr. Richard Kostrzewa's laboratory

(Kostrzewa, 2012). Increased dopamine D_2 receptor sensitivity is increased through neonatal treatment with quinpirole, a dopamine D_2-like agonist, and is independent of a change in receptor number. Increased dopamine D_2 receptor sensitivity has been shown to play a major role in schizophrenia symptomology (Abi-Dargham et al., 2000). This model has been validated through a number of studies and is consistent with many aspects of schizophrenia pathology (Brown et al., 2012 and 2018; Peterson et al., 2017).

Important to this study, NQ treatments have been shown to produce auditory sensorimotor gating deficits using prepulse inhibition (Thacker et al., 2006; Maple et al., 2015). Auditory sensorimotor gating is the regulation of the transmission of auditory information to the motor system, and prepulse inhibition (PPI) of startle is a measure of sensorimotor gating in which a weak (decibel; dB) auditory stimulus is delivered 250 milliseconds (ms) before a strong, intense startling stimulus. The presentation of the weak stimulus 250 milliseconds (ms) before the startling stimulus predicts the startle response, and the animal learns to inhibit the startle response. The mechanism underlying prepulse inhibition (PPI) is the "gating" or filtering of the auditory information received before transmission to the motor system. PPI is a good quantitative measure of behavioral and neurological deficits associated with schizophrenia, as well as the effects of therapeutic drug treatments in the NQ model because deficits in auditory sensorimotor gating are related to dopamine D_2 receptor activation, as well as positive and negative symptoms directly associated with schizophrenia and cognitive deficits (Braff et al., 1995; Swerdlow et al., 2006).

The underlying neural circuitry of auditory sensorimotor gating has been mapped and involves a number of prominent forebrain structures, including, and key to this study, the nucleus accumbens (Brown et al., 2018; Kumari and Sharma, 2002). For this reason, cyclic-AMP

response element-binding protein (CREB) levels in the nucleus accumbens were analyzed. CREB is a neurobiological protein downstream of glial cell-line derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF). Both GDNF and BDNF are neurotrophic factors shown to be important in neural plasticity, as well as the behavioral responses in the NQ model, and are important in neural and behavioral functioning in individuals diagnosed with schizophrenia (Peterson et al., 2017; Brown et al., 2018). Reports have shown that CREB is an important regulator of both BDNF and GDNF and has also been linked directly to behavioral deficits observed in schizophrenia (Wang et al., 2018). Importantly, research has shown that chronic quinpirole treatment to adult rats results increased CREB levels in the nucleus accumbens (Culm et al., 2004), which is associated with behavioral deficits associated with emotional regulation.

The overall hypothesis is that the adenosine agonist CGS 21680 will be successful in alleviating sensorimotor gating deficits in the neonatal quinpirole rat model. This will be achieved through the agonist decreasing the sensitivity of the dopamine D_2 receptor through the $A(2A)/D_2$ heteromer. It is also hypothesized that neonatal treatment with quinpirole by itself will result in significant sensorimotor gating deficits, along with significant increases of levels of CREB in the nucleus accumbens. Lastly, it is hypothesized that treatment with the adenosine agonist will reduce CREB levels in the rats neonatally treated with quinpirole to control levels.

Methods

Subjects: A total of 71 Sprague-Dawley rats were used as subjects in this experiment. Both male and female rats were used as subjects, and one subject per litter was assigned per treatment condition to control for within litter variance. The animals were housed socially in groups of two

or three in climate-controlled housing under a 12-hour light/dark cycle, with food available ad libitum. The housing conditions and procedures were compliant with regulations of both East Tennessee State University Animal Care and Use Committee and the National Institutes of Health Guide for the Care and Use of Animals.

Neonatal Drug Treatment: The day of birth was counted as postnatal day (P)0. The day after birth, subjects were administered a single intraperitoneal (IP) injection daily of either 0.9% saline (NS) or 1 mg/kg quinpirole (NQ). This treatment continued from P1-21. Subjects were weighed daily, and the treatment dose administered was 1% of their body weight. From P21-44 there were no drug treatments administered, and the subjects were moved to social housing (2-4 per cage).

PPI Methods: On P44, behavioral testing began. All animals were tested in a behavioral apparatus specialized for PPI behavioral analysis (Kinder Scientific, Inc., Poway, CA). Different groups of NS or NQ-treated rats were IP administered one of three treatments: saline, saline + 0.09 mg/kg CGS 21680, or saline + 0.03 mg/kg CGS 21680. 15 minutes after the injection, the subjects were tested for three consecutive days using PPI. For PPI testing, rats were randomly assigned and administered three different trial types. The trial types included *pulse* trials, *prepulse* trials, and *no stimulus* trials. A *pulse* trial is a 120 dB startle pulse administered alone. A *prepulse* trial is an auditory stimulus that is 3, 6, or 12 dB above the 70 dB background noise (73, 76, and 82 dB). A *no stimulus* trial is a trial in which no stimulus is given.

The testing apparatus was a stainless-steel dome attached to a platform mounted on a stainless-steel ellipse in a sound-attenuating chamber. On each daily session, the subjects were placed into the dome and given a 5-minute habituation period with only the background noise

(70 dB white noise) presented. All animals underwent 25 randomized trials which included 5 *pulse*, 5 *no stimulus*, and 15 *prepulse* trials (5 trials of each 73, 76, and 82dB). The subject's behavioral response was recorded during a 250 ms window immediately after the stimulus was presented and was measured in Newtons (N).

CREB Analysis: On P50, approximately 24 h after the locomotor activity test, brain tissue was harvested and the NAc was dissected away and placed into a pre-weighed 1.5 ml Eppendorf vial. Tissue was homogenized using a Fisher Scientific (Atlanta, GA) Dismembrator 5000. Tissue was in a RIPA buffer that contained protease and phosphatase inhibitors to protect the sample (P0044, P5726, P8340 and phenylmethylsulfonyl fluoride). Tissue was centrifuged twice at 4$_{o}$C. CREB was analyzed using an ELISA kit from MyBioSource.com (San Diego, CA, USA; Cat. No. MBS2098054), and the protocol was closely followed. In brief, the standard provided was serially diluted from 20ng/ml to 0.312 ng/ml and run in duplicate. Homogenized samples were also run in duplicate across the entire 96-well plate. After the standards and samples were applied to the plate, the plate was incubated for 1 hour at 37$_{o}$C. Liquid was removed from each well, and 100µl of detection reagent A was applied—diluted 100-fold with the provided diluent in the kit. After another 1-hour incubation at 37$_{o}$C, the plate was washed three times, and 100µl of detection reagent B was applied, also diluted 100-fold with the diluent provided. After a 30 min incubation at 37$_{o}$C, the plate was washed five times using the wash buffer provided in the kit. Ninety microliters of the substrate solution, which was 3'3'5'5' tetramethylbenzadine (TMB), was applied to the plate. After a 20-min incubation at 37$_{o}$C, the stop solution was applied and the plate was read immediately at 450 nm on a BioTek Plate Reader elx800 (BioTek, Winooski, VT). The data provided was calculated according to the standard curve and converted

to picograms of CREB/mg weight of the tissue.

Results

PPI behavioral test: The data obtained from PPI testing was analyzed using ANOVA, and the Newman-Keuls post hoc test was used to analyze any significant interactions. Percent of PPI is shown as a function of the decibel of the prepulse administered in **Figure 1**. A four-way ANOVA did not reveal any significant main effects or interactions involving sex as a variable. Thus, this factor was dropped and a three-way ANOVA with neonatal drug treatment, adolescent drug treatment and decibel level as factors revealed significant main effects of decibel level $F(2,130)=578.12$, $p<.001$ and neonatal drug treatment $F(1,65)=5.29$, $p<.024$, significant two-way interactions of neonatal drug treatment x adolescent drug treatment $F(2,65)=7.55$, $p<.001$) and adolescent drug treatment x decibel level $F(4,130)=6.28$, $p<.001$, as well as a significant three-way interaction of neonatal drug treatment x adolescent drug treatment x decibel level $F(4,130)=3.44$, $p<.01$ As seen in **Figure 1**, the Q-S treatment group (neonatal quinpirole, adolescent saline) showed significant deficits in 73 dB and 76 dB trials. These deficits confirmed that the NQ model was effective in modeling the sensorimotor gating deficits that are a hallmark of schizophrenia and are consistent with previous work (Thacker et al., 2006; Maple et al., 2015). The Q-0.03 CGS and Q-0.09 CGS groups' prepulse inhibition levels were elevated to control levels at both 73 and 76 dB. In addition, S-0.03 CGS and S-0.09 CGS groups' PPI levels were also equal to control levels (Group S-S). This indicates that both doses of the CGS 21680 worked to significantly improve PPI to control levels at 73 dB and 76 dB trials in NQ animals and did not result in PPI deficits in controls administered the drug.

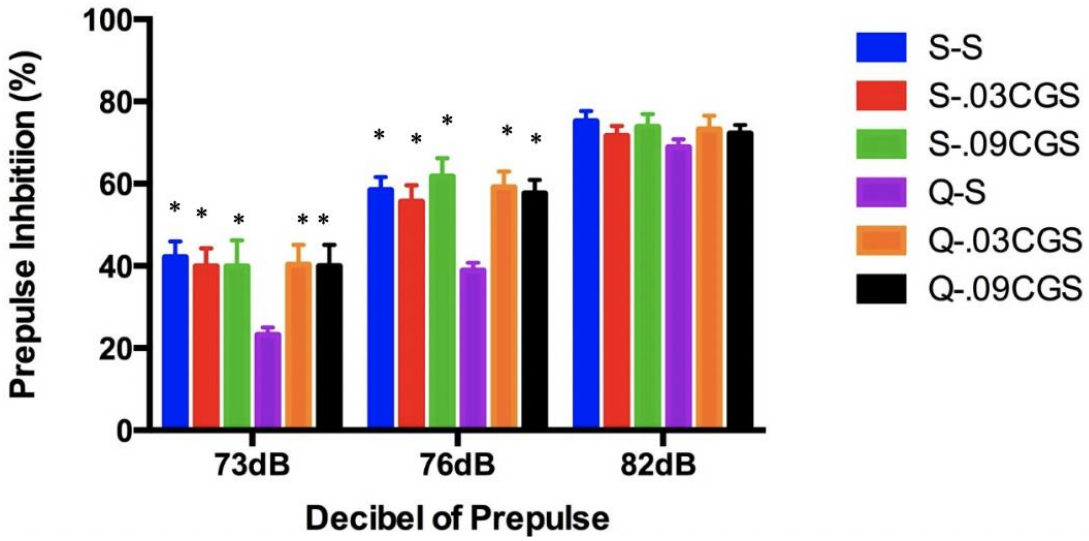

Figure 1. Percent prepulse inhibition is shown as a function of decibel of the prepulse administered. The colored key indicates the different treatment groups. All significant interactions are shown in the graph. * indicates a significant interaction ($p < 0.05$).

CREB Analysis: The data obtained from the CREB analysis was analyzed using ANOVA, and the Newman-Keuls post hoc test was used to analyze any significant interactions. The concentration of CREB in picograms per milligram tissue (pg/mg) is shown as a function of the treatment group. A two-way ANOVA with neonatal and adolescent drug treatment as factors revealed significant main effects of neonatal drug treatment $F(1,61)=6.71$, $p<.01$, adolescent drug treatment $F(2,61)=8.44$, $p<.001$, and a significant two-way interaction of neonatal drug treatment x adolescent drug treatment $F(2,66)=5.35$, $p<.007$. As seen in **Figure 2**, the Quinpirole-Saline (Q-S) treatment group demonstrated significantly elevated accumbal CREB protein levels. Q-0.03 CGS and Q-0.09 CGS groups' accumbal CREB concentrations were decreased to control levels, and neither dose of CGS 21680 affected CREB in the NAc. These results indicate that both doses of the adenosine agonist were successful in returning accumbal CREB concentrations to control levels.

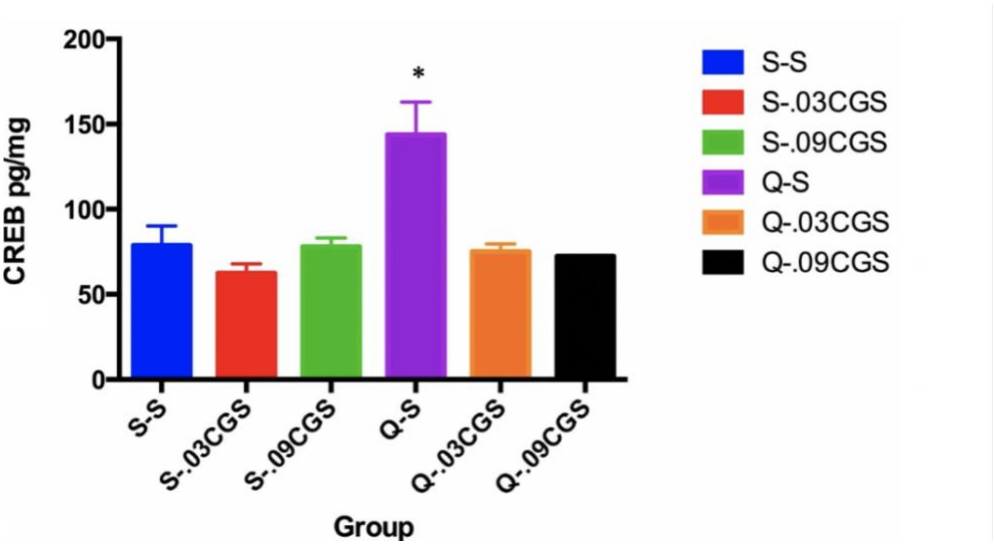

Figure 2. CREB concentration in picograms per milligram (pg/mg) is shown as a function of adolescent and neonatal drug treatment. The colored key indicates the different treatment groups. All significant interactions are shown in the graph. * indicates a significant interaction ($p < 0.05$).

Discussion

Results of this study demonstrated that the NQ model is effective in modeling the sensorimotor gating deficits found in schizophrenia (Grillon et al., 1992). In addition, NQ treatment increased CREB protein in the nucleus accumbens, which is consistent with past work that has shown that stimulation of the dopamine D_2 receptor results in an increase in accumbal CREB. This may be related to emotional symptoms that are demonstrated during the negative symptoms of schizophrenia (Culm et al., 2004; Kring and Moran, 2008). The study also demonstrated that both doses of the adenosine A(2A) agonist CGS 21680 (0.03 and 0.09 mg/kg) were effective in alleviating sensorimotor gating deficits in the NQ model to control levels. Importantly, both doses had no significant effects in controls, which demonstrates that the action of this agonist was effective to alleviate PPI deficits in NQ treated rats, but not result in deficits in controls. There were no deficits in NQ treated rats on the 82dB trials, suggesting that sensorimotor gating deficits are most prevalent on prepulse trials that are more difficult to

associate, because these lower dB trials are closer in level to the white noise baseline. Finally, the agonist lowered CREB levels in the NAc of NQ treated rats to control levels. Overall, the results confirmed our original hypothesis that CGS 21680 would be effective in improving PPI in the NQ model as well as alleviating increases in accumbal CREB.

Prepulse inhibition deficits are a hallmark of schizophrenia, as well as numerous other brain disorders (Swerdlow et al., 2016) and have been suggested as a biomarker for the disorder (Walters and Owen, 2007). PPI deficits associated with the disorder have been hypothesized to be related to impaired cognitive function known to be present in schizophrenia. PPI is a reflection of the ability to regulate the amount and relevancy of sensory information being processed at a particular time, and, since PPI is impaired in SZ, it is thought that these deficits may be the cause of the sensory information overload associated with the disorder (Cadenhead et al., 1997). PPI is a measure of the ability to filter relevant sensory information, and measurements of PPI have shown that sensorimotor gating is impaired in patients diagnosed with schizophrenia (Braff et al., 1992). Sensorimotor gating impairment in rodent models is attributed to increased dopamine in the NAc (Braff and Geyer, 1990). This may be a reason why antipsychotics that inhibit the dopamine D_2 receptor in rodents are effective in reducing PPI deficits (Geyer et al., 2001). Since adenosine A(2A) agonists also act to indirectly inhibit the dopamine D_2 receptor, they should also be effective in reducing PPI deficits. This hypothesis was supported by our results which showed that both doses of the agonist were effective in reducing PPI deficits. The validity of the use of the NQ model in measuring PPI deficits related to SZ has been confirmed in several labs (Maple et al., 2015; Wan and Swerdlow, 1993).

Sex differences in symptomology are present in human schizophrenia (Abel et al., 2010), as well as with rodent models of schizophrenia (Hill, 2016). There is conflicting evidence,

however, to whether there are sex differences associated with PPI in rodent schizophrenia models. Some work has provided evidence of sex differences in PPI in rats (Monte et al., 2017), whereas other research has found no sex differences between male and females (Moran et al., 2016; Gogos et al., 2017). In past work, we did not find sex differences in PPI (Maple et al., 2015), and likewise, we did not discover sex differences in PPI in the present study.

The results from the CREB analysis in the NAc complemented the behavioral data, supporting the PPI results. First, results showed that the NQ-saline group demonstrated a robust increase in levels of CREB in the nucleus accumbens. Second, these results also supported our initial hypothesis that treatment with the agonist would lower CREB to control levels in the CGS treatment groups.

Abnormalities in the expression of CREB in the nucleus accumbens have been found in schizophrenia, with research suggesting a link between CREB expression and the pathophysiology of the brain's emotional system in schizophrenia (Wang et al., 2018). A reduction of CREB signaling pathways have been shown to assist in the recovery of sensorimotor gating deficits (Culm et al., 2004). Abnormalities in CREB expression have also been implicated in reduction of emotional reactivity. Elevated CREB levels in the NAc have been shown to be correlated with the lack of sensitivity to emotional and stress stimuli in rodents (Barrot et al., 2002), consistent with the negative symptomology of schizophrenia. Increases in CREB in the NAc have also been shown to be associated with depressive-like effects, drug tolerance, and drug dependence (Carlezon et al., 2005). It is well known that patients suffering from schizophrenia demonstrate vulnerability for drug addiction, and epidemiology studies have shown dramatic increases of cigarette smoking, cannabis use, and alcoholism in this population (Batel, 2000; Reiger et al., 1990). In the current study, the CREB analysis showed that both

treatment dosages of CGS 21680 resulted in significantly lowered CREB concentrations in the NAc which suggests that an agonist of the adenosine A(2A) receptor may be effective in improving emotional reactivity, as well as stimulating recovery of sensorimotor gating deficits.

Limitations. The generalizability of the results is limited by the sample size and the use of a rodent model. With a sample size of 71 and 6 treatment groups, there was a relatively small N, with approximately 12 animals (6 males, 6 females) per group. The statistical power, however, was still high enough to conduct statistical analyses with confidence. The conclusions of this research are also constrained by the use of a rodent model of schizophrenia. A rodent model cannot encapsulate all of the aspects of human schizophrenia. For example, we cannot model paranoia or other positive symptoms present in schizophrenia. Therefore, it is beyond the scope of this study to make any grand conclusions regarding the efficacy of an adenosine agonist as a treatment for human schizophrenia, although it is known that adenosine A(2A) agonists are currently in clinical trial and, although some have failed, more recent work has shown some promising results (Jacobson et al, 2019). The NQ model, however, does encapsulate the neural and behavioral hallmarks of schizophrenia relevant to this study, including sensorimotor gating impairment, increased CREB in the NAc, and increased dopamine D2 receptor sensitivity (see Brown et al, 2012, for review). Modeling of these neural and behavioral hallmarks allows for analysis of the underlying mechanisms that cause these abnormalities, as well as possible treatments. The results of this study cannot comment on the efficacy of an adenosine agonist for the treatment of human schizophrenia, but it can determine the efficacy of an adenosine agonist in a rodent schizophrenia model. Success in a rodent model will call for further research in a broader scope.

Conclusions. This research was aimed to analyze the effectiveness of the use of an adenosine A(2A) receptor agonist to relieve sensorimotor gating impairment in an NQ model of schizophrenia. Based on the results of this study, it can be concluded that both doses of CGS 21680, the adenosine A(2A) agonist, were effective in alleviating PPI deficits in NQ-treated rats. Both doses of the agonist were also effective in reducing the increase of CREB protein in the NAc produced by NQ treatment. These results support the possibility that the adenosine A(2A) receptor poses as a viable target for the treatment of some of the psychotic symptoms associated with schizophrenia. Current antipsychotics used for the treatment of schizophrenia have only limited efficacy and many unwanted side effects (Rummel-Kluge, 2010; Leucht, 2013). In addition, some antipsychotics have also been shown to further elevate PPI deficits (Geyer et al., 2001). The adenosine system, however, appears to be an effective treatment in rodent schizophrenia models, with limited side effects. The agonist was effective in reducing PPI deficits and CREB concentrations in the NAc, both of which are associated with the improvement of schizophrenia symptoms. Future work should focus on determining whether chronic treatment with an adenosine A(2A) agonist is viable. Studies regarding chronic adenosine agonist treatment would be helpful in determining optimal dosage and conditions to maximize effectiveness while minimizing side effects. Chemical and pharmacological strategies to improve the current delivery and inhibition mechanisms of the adenosine A(2A) agonist should also be developed to optimize treatments. Results of this study confirm that the use of an adenosine agonist on the adenosine A(2A) receptor is effective in reducing PPI deficits and CREB concentrations in the NAc in a rodent schizophrenia model, adding further compelling evidence that future research should explore the use of an adenosine agonist as an antipsychotic treatment for schizophrenia.

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