Journal of Sciences, Islamic Republic of Iran 26(3): 205 - 212 (2015) http://jsciences.ut.ac.ir
University of Tehran, ISSN 1016-1104

Knocking Down the DRD2 by shRNA Expressing Plasmids in the Nucleus Accumbens Prevented the Disrupting Effect of Apomorphine on Prepulse Inhibition in Rat

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Received: 7 August 2015 / Revised: 19 September 2015 / Accepted: 4 October 2015

Abstract

Prepulse Inhibition (PPI), the objective measure of sensorimotor gating disturbance has being widely used in animal models of schizophrenia. Dopaminergic direct and indirect agonists impair PPI. However, the profile of dopaminergic receptors involved in PPI impairment by dopamine agonists is not clear. By injecting shRNA expressing plasmids against dopamine D2 receptor genes (DRD2) in the nucleus accumbens, here, we studied the effect of apomorphine on PPI in D2 down-regulated rats. Seventy two adult Wistar rats assigned randomly in nine groups, each received coding (250 and 500 ng/µl) or noncoding shRNA expressing plasmids against DRD2 in the nucleus accumbens, with or without apomorphine (0.5 mg/kg, S.C., 72 hours after treatment with plasmids). Auditory startle response and PPI were measured after apomorphine injection. Real time RT-PCR was used to measure DRD2 expression. Results showed that apomorphine significantly decreased PPI in noncoding plasmid treated rats; While, PPI did not impaired in rats pretreated with 250 and 500 ng/µl shRNA expressing plasmids. Accordingly, the expression of DRD2 mRNA in the nucleus accumbens showed 72-78% decrease in expressing plasmid treated rats. Additionally, treatment with expressing plasmids had no effect on basal PPI and/or auditory startle response. Taken together, our results demonstrated that DRD2 silencing in the nucleus accumbens can prevent PPI impairment by apomorphine. These observations suggest application of molecular techniques such as the use of shRNA against DRD2s in studies of schizophrenia pathophysiology and development of new treatments in schizophrenia.

Keywords: shRNA; DRD2; Nucleus Accumbens; Schizophrenia; Prepulse Inhibition.

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Introduction

Schizophrenia, a very complex mental disorder with a prevalence of about one percent in population [1], is characterized by positive symptoms (i.e. hallucination, delusion and abnormal perception), negative symptoms (i.e. flattened mood, apathy, and social withdrawn), and cognitive abnormalities [2]. It has been shown that perception, cognition, and emotional domains of the brain function are affected in schizophrenia. However, the underlying pathophysiological mechanisms and involved circuits have not been precisely understood in schizophrenia. Disturbances in different neurotransmittersand neuromodulators of the brain have been reported in schizophrenia; including dopaminergic, serotonergic, noradrenergic, cholinergic, glutamatergic and gabaergic systems [3]. Dopamine is the most studied neurotransmitter that has shown to play a pivotal role in the pathophysiology of schizophrenia. It has been reported that decreased activity of dopaminergic system in the prefrontal cortex results in cognitive and negative symptoms, while hyperactivity of the mesostriatal dopaminergic system is responsible for positive and psychotic symptoms of schizophrenia [3].

Various animal models have being used to study the pathophysiology of schizophrenia and to develop new antipsychotics; including pharmacological, developmental, and genetic models [4,5]. Prepulse inhibition (PPI), the operational measure of sensorimotor gating, have being widely used in schizophrenia studies for more than two decades [6]. Briefly, the ability of a weak non-startling stimulus (prepulse) to reduce the response to a startling stimulus (pulse) usually referred to as PPI. It has frequently reported that PPI is impaired in schizophrenic patients and also some of their unaffected relatives [7-11]. However, PPI impairment has also been reported in some other neuropsychiatric disorders. PPI impairment was observed in most animal models of schizophrenia; indicating the advantage of this reliable measurement in cross species studies of schizophrenia, with acceptable predictive and constructive validities[4]. Traditionally, each pharmacologic agent with the ability to reduce PPI impairment in animal models of schizophrenia has been referred to as a potential antipsychotic [12]. In this regard apomorphine, a nonspecific agonist of dopamine receptors, impairs PPI [13]; and agents with the ability to reverse disruptive effect of apomorphine on PPI have been considered as potential antipsychotics [12].

Dopamine has a pivotal role in prepulse inhibition [6, 12-15]. Direct dopamine agonists such as apomorphine and quinpirole, as well as indirect agonists like amphetamine decrease PPI [6,13]. Further studies revealed that ventral striatum, including nucleus accumbens, is the main action site of dopaminergic agonist to modulate PPI [16]. It has been also reported that the activation of both D1 and D2 dopamine receptors impair PPI in rodents [17,18]. In pharmacologic studies, D2 receptors is suggested as the main target for PPI disrupting effects of nonspecific dopamine agonists like apomorphine [6]; While dopamine D1 receptors has been proposed by another studies as well [17-20]. However, precise mechanism and the type of receptors involved in dopamine mediated impairment of PPI have not been clearly understood.

It has shown that transfection of rat neuroblastoma cell line (B65) with shRNA expressing plasmids decreased the expression of D2 receptor gene(DRD2) efficiently[21]. shRNAs are short length double stranded RNA molecules, which bind specifically to a target mRNA leading to its degradation [22, 23]. Here, we used shRNA expressing plasmids against DRD2 in the nucleus accumbens of rat, aiming to study its effects on PPI impairment by apomorphine.

Materials and Methods

1. Animals

Seventy two adult male Wistar rats were used in this study (225-278 gr.). Rats were obtained from Pasture Institute (Tehran, Iran). Animals were housed in clear polycarbonated cages (three per cage), with sawdust bedding and free access to food and water *ad libitum*, and were kept in 12-h reversed light/dark cycles (lights off from 07:00 to 19:00 pm). Behavioral tests were carried out during the light phase between 09:00 and 14:00. Animals handled individually for 5 minutes within three days of arrival and one week after surgery. Study protocols were approved by the ethical committee of Iran Universities of Medical Sciences. All efforts were made to reduce the suffering and pain of animals, and also the number of animals used in this study.

2. Experimental design

One week after arrival in laboratory, animals were assigned randomly in nine groups: control group with no intervention; two experimental groups treated by vehicle (0.1 % ascorbic acid, 1ml/kg, S.C.) or apomorphine (Apo, 0.5 mg/kg, S.C.); three experimental groups treated by non-coding shRNA or scramble (Non-shRNA) or shRNA expressing plasmids (250 and 500 ng/µl) against DRD2 (shRNA 250 and shRNA 500) in the nucleus accumbens bilaterally; and three experimental groups treated by

non-expressing or expressing plasmid in addition to the apomorphine (0.5 mg/kg, S.C.), 72h after plasmid injection (Non-shRNA+Apo, shRNA250+Apo and shRNA500+Apo).

3. Production of ShRNA expressing plasmids

Plasmids expressing shRNA against DRD2 and non-coding shRNA were produced as our previous study [21]. Briefly, shRNA encoding complementary DNA sequences were designed using online ambion software. Oligonucleotides were hybridized together and cloned into pSilencer TM4.1-CMVneo plasmid vectors (Ambion, USA). Efficiency of plasmids for knocking down the DRD2 gene expression was assessed using quantitative real-time PCR on stable cell line produced by integration of recombinant plasmids into their genome. Our previous study showed that expression level of DRD2 mRNA in B65 neuroblastoma cell line which transfected with αDRD2 shRNA expressing plasmid reduced to 40% of scramble bearing cells [21].

4. Stereotaxic surgery and microinjection of plasmids

In order to injecting the plasmids into the nucleus accumbens in shRNA treated groups stereotaxic surgery was performed. In brief, rats anesthetized by ketamine and Xylazine (80 mg/kg and 6 mg/kg, I.P., respectively), were mounted on stereotaxic apparatus (BorjSanat, Iran). The scalp and superficial tissues were removed after makingan incision on the scalp, afterwards two small holes were made bilaterally in the skull, and 24-gauge guide cannulas were implanted 1mm above the nucleus accumbens. Cannulas were occluded with stylet for keeping them open during recovery period (7 days). Nucleus accumbens coordinates were determined according to the Paxinos and Watson atlas[24] as following, and refined empirically in pilot experiments: The anterior-posterior (AP): 1.4 mm; lateral (L): 1.2 mm; and dorsal-ventral (DV): 7.8 mm from skull. Guide cannulas were fixed with dental cement.

After recovery, rats were microinjected by one of the scramble (non-coding shRNA), shRNA 250 ng/µl or shRNA 500 ng/µl (0.2 µL each side) through the mounted cannulas. The dose of plasmids and the time of behavioral experiments were chosen based on our previous study [21]. The plasmids were microinjected in one minute, and injection needles remained for an additional minute in the site for complete delivery of solution. All injection needles and tubes were autoclaved before the procedure.

5. Auditory startle response and prepulse inhibition tests

Auditory startle response and prepulse inhibition (PPI) were measured using single startle chamber (SR-LAB, San Diego Instruments, San Diego, CA) according to our previous study [25]. In brief, this system consist of a ventilated sound proof enclosure with a computer controlled speaker mounted 24 centimeter above a plexiglas cylinder (8.2 cm in diameter) that restrains the rat while allowing limited movements. A piezoelectric stabilimeter under the cylinder precisely recorded and transduced the motion of rat within the cylinder. Stimulus delivery was controlled by the SR-LAB software and interface assembly that also digitized, rectified and recorded stabilimeter readings, with one hundred 1-ms readings collected in the beginning of stimulus onset. Startle amplitude was defined as the average of 100 readings. By using predefined blocks of trials, we were able to record the startle response of rat to auditory stimuli. The auditory stimuli were consisted of blocks of randomly assigned trials. Three different trials were used in each block; PULSE trials (120 dB noise bursts, 40ms), PREPULSE-PULS trials (20ms noise bursts 3, 6 and 12 dB over 68 dB background, followed 100ms by a 120 dBPULSE) and NONSTIM trials (stabilimeter recordings obtained when no stimulus was presented). The inter-trial times were between 9 to 21 seconds.

Rats were transferred to the test room and kept in their cages individually, 72h after plasmid microinjection. One hour later, rats were put in the startle chamber and tests were started. In the apomorphine treated groups, rats were injected subcutaneously with apomorphine (0.5 mg/kg, Sigma-Aldrich, USA) or vehicle (0.1% ascorbic acid, Sigma-Aldrich, USA), then immediately transferred to the startle chamber. The test sessions were consisted of 5 min, 68 dB background acclimation period followed by 60 presentations of acoustic stimuli to measure acoustic startle and PPI. The 52 acoustic trials were consisted of: twenty-two PULSE trials; ten presentations of each prepulse intensity (71, 74, and 80 dB) 100 ms prior to a 40ms presentation of a 120dB broad band pulse (PREPULSE-PULSE trials); and finally 8 NOSTIM trials. All trial types were presented several times in a pseudo-random order. Four of the PULSE trials, not included in the calculation of PPI values, were presented at the beginning of the test session to achieve a relatively stable level of startle reactivity for the remainder of the session. The other four PULSE trials which were also not included in the calculation of PPI values were presented at the end of the test session to assess startle habituation. Thus, the middle 14 PULSE

trials were used to calculate PPI. Between the two mentioned groups of four PULSE trials, the remaining trials were divided into two blocks in which all the trial types were equally represented[26]. Auditory startle response was calculated from the mean of PULSE (120 dB) trials. Percent PPI for each of the three prepulse intensities was calculated according to the following formula: PPI=100-{[(startle response for PREPULSE-PULSE trial)/ (startle response for PULSE trial)]-100}.

6. Tissue sampling from the nucleus accumbens

After startle tests, animals were decapitated and the brains were removed and rinsed in the ice cold DEPC treated Milli Q water to remove any surface blood. Brains were placed on the cold metal plate and were cut bi-half into right and left hemispheres. Then, two cut plains were made from medial side of each hemisphere, the first one in front of genue of corpus callosum and the other in front of fornix[27]. The slice between these two cut plains which contained striatum and nucleus accumbens were used for punching nucleus accumbens. A micro-punch with diameter of 1 mm was used to punch the region 0.5 mm lateral to midline and 1 mm dorsal to the floor of the brain. The nucleus accumbens specimens were put in liquid nitrogen and stored at -80°C for RNA extraction.

7. RNA extraction and quantitative real-time RT-PCR

Tissue samples were homogenized using repeated snap freezing (using liquid nitrogen) and squishing. Total RNA was extracted from homogenized tissue samples using RNeasy mini kit (Qiagen, Germany, Cat.no: 74104) as the recommended protocol. Quality and quantity of RNA samples were evaluated by agarose gel electrophoresis and nanodrop 2000, respectively. 1 µg of RNA was used for cDNA synthesis by Quantitect reverse transcriptase kit (Qiagen, Germany, Cat.no: 205313). Quantitative real-time PCR reaction was performed using SYBR® Premix Ex Taq (TAKARA), without ROX dye, on a Q-rotor detection system (TaKaRa Clontech, USA, Cat.no: RR820A) programmed by following thermal cycling conditions: an initial Taq enzyme activation step for 5min at 95 °C followed by 40 cycles including a denaturation step for 10s at 95 C ° and a combined annealing/extension step for 30s at 60 °C. Primer sequences were the same as our previous study[21]. Gene expression changes were calculated by delta-delta CT method and hypoxanthine-guanine phosphoribosyl-transferase (HPRT) gene was considered as the normalizer.

8. Statistical analysis

The average of PPI in all three prepulse intensities was used as the mean of prepulse inhibition (% PPI). Separate one way ANOVA tests were used for comparison of % PPI, auditory startle response, and expression of DRD2 in the nucleus accumbensof experimental groups. When a significant effect of factors was detected (p<0.05), *Tukeypost-hoc* analysis was performed to evaluate significant differences. SPSS ver.16 was used to analyze data.

Results

1. The effect of apomorphine on PPI

Results of one way ANOVA revealed a significant effect of treatment with apomorphine on %PPI in male Wistar rats [F(2,19) = 5.40, p=0.014]; Tukey*post-hoc* analysis showed a significant decrease of %PPI in apomorphine group compared to control group(12.11± 4.11 vs. 38.42± 4.99, p= 0.01) [Fig. 1]. Results of separate ANOVA test showed no significant effect of treatment with apomorphine or vehicle [F(2,19) = 0.936, p= 0.41] (Fig. 1).

2. The effect of treatment with shRNA expressing plasmids against DRD2 in the nucleus accumbens on PPI impairment induced by apomorphine

The percentage of PPI after apomorphine injection in groups treated with coding or non-coding plasmids were compared to the control group. The results of ANOVA revealed a significant effect of treatment on the PPI

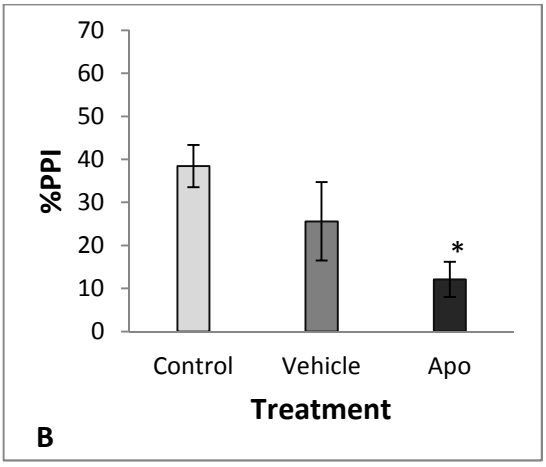

Figure 1. The effect of apomorphine on PPI. Figure shows the effect of subcutaneous injection of 0.5mg/kg apomorphine (Apo) or vehicle (0.1%ascorbic acid) on auditory startle response [A], and mean of prepulse inhibition (%PPI) [B], in male Wistar rats. Data represented as mean±SEM. [*: p<0.05 compared to control group].

[F(4,31)= 3.56, *p*= 0.017]. Tukey*post-hoc* analysis showed that % PPI in groups without plasmid treatment (Apo: 12.11± 4.11) and groups treated with non-coding shRNA expressing plasmid plus apomorphine (Non-shRNA+Apo: 13.16± 6.8) were lower than control group (38.42± 4.9) [*p*= 0.017 and 0.031, respectively]; while %PPI in the shRNA expressing plasmid groups (shRNA250+Apo: 20.87± 6.7 and shRNA500+Apo: 18.52± 6.2) were not differ from the control group (*p* = 0.194 and 0.199, respectively) [Fig. 2]. Results of separate ANOVA test showed no significant effect of treatments [F(4,31) = 0.430, *p*= 0.78] (Fig. 2).

3. The effect of treatment with shRNA expressing plasmids against DRD2 in the nucleus accumbens on basal PPI

To assess the potential effect of treatment with shRNA expressing plasmids against DRD2 in the nucleus accumbens on basal PPI, the mean of PPI in three separate groups (Non-shRNA, shRNA250, and shRNA500) were compared to the control group. Results of one way ANOVA showed no significant effect of treatment with plasmid on the basal PPI [F(3,23) = 0.60,*p*=0.621] (Fig. 3). The results of another ANOVA also showed no significant effect of treatments on auditorystartle response [F(3,23) = 1.51,*p*=0.237] (Fig. 3).

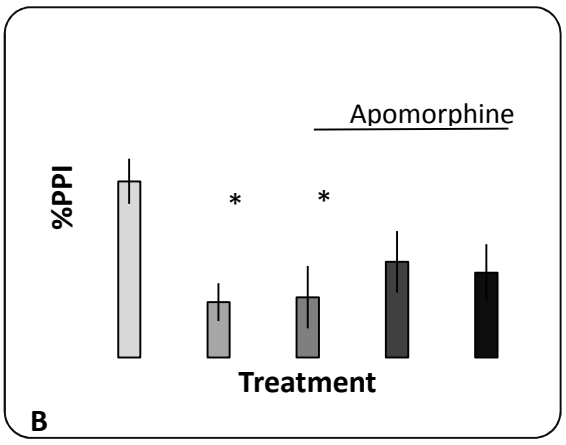

Figure 2. The effect of treatment with shRNA expressing plasmids against DRD2 in the nucleus accumbens on PPI impairment induced by apomorphine.Figure represents the significant effect of apomorphineon mean of PPI (% PPI) in the groups not receivingshRNA expressing plasmids (Apo and Non-shRNA+Apo); while PPI levels in groups treated with shRNA expressing plasmids against DRD2 (shRNA250+Apo and shRNA500+Apo) were the same as the control group (B). Treatments have not any significant effect on auditory startle responses (A).Data represented as mean±SEM. [*: *p*<0.05 compare to control group].

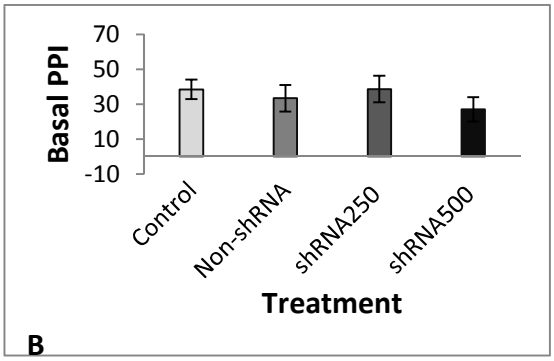

Figure 3. The effect of treatment with shRNA expressing plasmids against DRD2 in the nucleus accumbens on auditory startle response (A) and basal PPI (B). Data represented as mean±SEM.

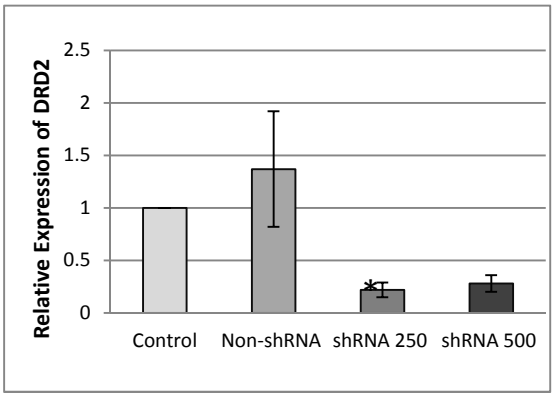

Figure 4. The effect of treatment with shRNA expressing plasmids on the expression of DRD2 in the nucleus accumbens.Figure shows the significant effect of treatment with plasmids on the relative expression of DRD2 in the nucleus accumbens. As shown, the microinjection of 250 and 500 ng/µl of shRNA expressing plasmids reduced the relative expression of DRD2 in the nucleus accumbens compared to control group (78 and 72 percent, respectively). However, the difference was significant just in the shRNA250 group [*: *p*<0.05 compare to the control group].

4. The effect of treatment with shRNA expressing plasmidson the expression of DRD2 in the nucleus accumbens

To confirm the effect of plasmids on the expression of DRD2 in the nucleus accumbens, real-time PCR measurement of DRD2 mRNA was carried out on specimens punched from the nucleus accumbens of plasmids treated rats. All samples were analyzed in triplicate, and out of range samples were excluded. The one way ANOVA revealed a significant effect of treatment with shRNA expressing plasmids on the

expression of DRD2 [F(3,21) = 5.86,p=0.004]. Results of Tukey*post-hoc* analysis showed 77 and 72 percent reductions in the relative expression of DRD2 in the nucleus accumbens of groups treated with shRNA 250 and 500ng/µl, respectively, compared to the control group (p=0.04 and 0.121, respectively) [Fig. 4].

Discussion

The results of this study showed that apomorphine, 0.5 mg per kg, decreased the prepulse inhibition in adult male Wistar rat. This disrupting effect of apomorphine was significantly diminished by knocking down of dopamine D2 receptors in the nucleus accumbens following injection of shRNA expressing plasmids against these receptors. Our findings also revealed that silencing of DRD2 in the nucleus accumbens does not have any effects on basal PPI and auditory startle responses in rat.

Apomorphine, a direct agonist of dopamine receptors [20], binds to D1 and D2 receptors with different affinities. Low doses of apomorphine stimulate D2 receptors and produce behaviors relevant to stimulation of these receptors in rodents [28]. However, the role of D1 receptors in impairment of PPI, following treatment with direct dopamine agonists such as apomorphine is unclear [20]. Application of advanced molecular techniques such as knocking down the specific receptors by using naked shRNA or shRNA expressing plasmids could further clarify the role of these receptors in specific behaviors. Our results showed that the impairment of PPI following apomorphine effectively prevented by injection of shRNA expressing plasmid against DRD2 in the nucleus accumbens; which may indicate principal role of D2 receptors in this phenomenon. These finding support the previous reports that state disrupting effect of apomorphine on PPI is mainly exerted by dopamine D2 receptors [18, 19, 29, 30]. Our results also revealed that decreasing the D2 receptors in the nucleus accumbens has no effect on the auditory startle response. This may suggest that the observed effects of our intervention on PPI dos not mediated by modification of startle response.

Few studies have reported that dopamine D2 receptor antagonists increase the basal PPI in rats [18, 31], while others have not shown this effect[6]. However, the findings of present study indicated that silencing DRD2of nucleus accumbens has not significant effect on basal PPI. Nevertheless, it should be mentioned that the reported effects of systemic D2 antagonists on basal PPI, in addition to other parameters like strain, stimulus parameters or/and experimental conditions [6] may also be related to an antagonistic effect on D2 receptors in other sites rather than nucleus accumbens[32]. Moreover, consistent with other studies [16, 30, 33-35] our results indicated that dopamine D2 receptors of nucleus accumbens are not involved in the amount of basal PPI following treatment with dopamine agonists; although, these receptors might play the main role in the disrupted PPI following heightened dopamine in the nucleus accumbens.

Traditionally, dopamine D2 receptors antagonists were prescribed for treating schizophrenia. However, severe side effects of these drugs such as dystonia; parkinsonism; and tardive dyskinesia, due to the binding of these agents to their corresponding receptors in the sites rather than ventral striatum, has limited their use. In some circumstances schizophrenic patients have been discouraged to continue these medications and have withdrawn from the treatment with these drugs [34, 36]. Introducing newer second-generation antipsychotics with better effects on cognitive and negative symptoms of schizophrenia and fewer side effects bring new hopes in the treatment of these patients. The second generation antipsychotics have not only an antagonistic activity on D2 receptors, but also affect the serotonergic and noradrenergic receptors [37]. However, it has been reported that D2 receptor antagonistic activity of these drugs is necessary to therapeutic effects of second generation antipsychotics [36]. It seems that the final destination of complex and various pathophysiologic mechanisms of schizophrenia is the heightened dopaminergic activity in the nucleus accumbens via D2 receptors [38]. Theoretically, it seems reducing the activity of dopamine via D2 receptors in the nucleus accumbens selectively would be a preferred strategy in the treatment of schizophrenia. The findings of this preliminary study suggest the use of shRNA or shRNA expressing plasmids against DRD2s, as a new molecular technique, to knocking downof these receptorsin nucleus accumbens, at least in animal studies.

Application of shRNA for treating neurologic disorders has been started recently [39-41]. However, there are some considerations regarding the use of shRNA, including cell specific targeting; use of naked shRNA or vector mediated delivery of shRNA expressing plasmids; utilization of viral or non-viral vectors; and conjugation with lipofectamine in aim to better impregnation in target tissues[42, 43]. These results showed that using non-viral plasmids conjugated with lipofectamine is effective to transfect nucleus accumbenscells, with consequent 72-78 percent reduction in DRD2s expression. Although, here we studied the effects of plasmids on PPI and gene expression 72 hours after injection, based on findings of our previous study, the time course and also the area of

transfection are tow limitations of this study.

In conclusion, findings of this study showed that the use of shRNA expressing plasmids in the nucleus accumbens to knocking down dopamine D2 receptors prevented the sensorimotor gating disturbance induced by apomorphine in rats. Additionally, reduction of DRD2s expression in the nucleus accumbenshas not considerable effect on basal PPI and auditory startle responses. Furthermore, applying plasmids conjugated with lipofectamine is suggested as a reliable method to down-regulation of target molecules in the nucleus accumbens. These observations also suggest application of molecular techniques such as the use of shRNA against DRD2s in studies of schizophrenia pathophysiology to development of new treatments in schizophrenia.

Acknowledgment

This research has been supported by the Iran National Science Foundation (INSF), grant no.83088. There is no actual or potential conflict of interest regarding this article. Some data from this project were presented at Schizophrenia International Research Society Conference (SIRS) 2012, Florence, Italy as a poster.

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