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Original article

The Study of Apomorphine Effects and Heterogeneity in the Medial Prefrontal Cortex on the Dopaminergic Behaviors of Rats

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Abstracts

Objective(s)
While the nucleus accumbens and the striatum have received much attention regarding their roles in stereotyped behaviors, the role of the medial prefrontal cortex (mPFC) has not been investigated to the same degree. Few studies have reported the role of the mPFC in dopaminergic induction of locomotor hyperactivity. The mPFC is a heterogeneous area (the anterior cingulated, prelimbic, and the infralimbic) with particular inputs and outputs to subcortical regions that may have different effects on stereotyped behaviors. In this work, apomorphine, a non-specific dopamine agonist, was microinjected into the three different subregions of the mPFC for induction of stereotyped behaviors to show the role of the three subareas of the mPFC on behaviors and its heterogeneity.
Materials and Methods
Cannulas implanted in the infralimbic, the prelimbic or the anterior cingulated areas of the mPFC. Apomorphine microinjected at five doses and then behaviors recorded.
Results
There were significant differences among three areas. The rats receiving apomorphine in the anterior cingulated showed less sniffing and climbing but more chewing behaviors. Yawning observed more significantly in the rats given apomorphine in the prelimbic area. The rats getting apomorphine in the infralimbic of the mPFC showed more climbing behavior.
Conclusion
It was indicated that manipulation of the dopaminergic system in mPFC alters behaviors and with regard to this, there may be heterogeneity among its three subregions.

Keywords: Apomorphine, Chewing, Climbing, Heterogeneity, Prefrontal cortex, Sniffing

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Introduction

Schizophrenia is one of the most important and prevalent cases of psychosis that affects about one percent of people (1, 2). The pathological changes in the prefrontal cortex are involved in schizophrenia and negative symptoms of the disease (3-7). Disturbances in the medial prefrontal cortex (mPFC) functions may also be involved in some other psychiatric diseases (6-9).

There are some animal models for the study of neuronal mechanisms of schizophrenia and screening of antipsychotic drugs (5, 7, 8, 10-3). Each model studies some aspects of psychosis. One of the most important models is stereotyped behaviors induced by dopamine agonists such as apomorphine in rodents. Motor stereotypes are characterized as repetitive, continuous movements with little variability, topologically invariant and apparently purposeless behaviors (14-16). Stereotyped behaviors have been observed in several neuropsychiatric disorders (14, 15).

The mPFC is one of the regions of the prefrontal cortex, a medially located cortical region, which constitutes the major portion of the medial wall of the hemisphere anterior and dorsal to the genu of the corpus callosum (9). The mPFC is involved in higher cognitive functions (7-9, 17).

The mPFC is not a homogenous structure (7, 9, 18, 19) and can be subdivided into at least three subareas: the infralimbic (IL), the prelimbic (PL) and the anterior cingulated (ACd), each of which has different intrinsic organization, functions and distinct afferent-efferent connections (7, 20).

It has been reported that distinct areas of the brain have different effects on stereotyped behaviors and could have different responses to psychostimulants. In most studies, the roles of the striatum and the nucleus accumbens (NAC) in stereotyped behaviors have been defined, but the exact relation between the mPFC and stereotyped behaviors has not been explained.

Because of the importance of the mPFC in the pathogenesis of schizophrenia, the pathophysiologic models have focused on the cortical regulation and its dysfunction on subcortical dopamine (DA) neurotransmission (21). To investigate the involvement of dopaminergic innervation of the mPFC on stereotyped behaviors, we studied the behavioral effects of microinjection of apomorphine as a nonselective DA agonist on three subareas of the mPFC.

Materials and Methods

Ethics

The protocol used in this study approved by the Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran.

Animals

Locally bred male Sprague Dawley rats provided by Razi Institute of Shiraz. The rats had free access to food and water and their weights were 275-400 g.

Materials

Apomorphine (Sigma, Germany) was prepared in 0.1% ascorbic acid (Merck, Germany), diethyl ether (Merck, Germany), formaldehyde (Merck, Germany), Ketamine (Rotexmedica, Germany) and Xylazine (Alfasan, Netherlands).

Experimental design

The rats divided into three groups; the infralimbic (IL), prelimbic (PL) and the anterior cingulated (ACd). Each group divided further into seven subgroups: sham, control, and five doses of apomorphine (0.005, 0.01, 0.05, 5 μg/0.5μl and 20 μg/1μl). There were seven rats in each subgroup. Sham groups used as the control of microinjection conditions, since they did not receive any drug. Control groups only received 0.1% ascorbic acid solution, 0.5μl/rat as vehicle of apomorphine. The selected doses used in a pilot study.

Surgery

The rats anesthetized by intraperitoneally injection of ketamine (60 mg/kg) + xylazine (8 mg/kg) and mounted in a stereotaxic frame (Stoetling, Wood Dale, IL, USA). Guide cannulas implanted in each of three subareas of the mPFC: the anterior cingulate (AP, +2.7; L, +0.4; V,-2) or the prelimbic (AP, +3.2; L,

+0.7; V, - 4.0) or the infralimbic (AP, +3.2; L, +0.7; V, - 5.4) (22). Sterile obturator with the same length as the guide cannula inserted into the guide.

Microinjection and recording of Behaviors

Behavior recording carried out one week after the surgery. On the experiment day, the rats habituated to an experimental cage. It (50×50×50 cm) made of transparent Plexiglas and its walls lined with 4 cm^2 (2×2) wire mesh. The height of the wire mesh was 10 cm higher than that of the cage. The rats gently handled while the injection needle inserted inside the cannula one mm beyond the tip of the guide cannula. Using a syringe pump (Harvard Apparatus, USA), microinjection **was** performed for one minute at a constant speed, and 0.5 µl of the drug solution or vehicle (1.0 µl for dose 20 µg) delivered into the brain. After the end of the injection, the needle was left in the guide cannula for three additional min.

The rats put in the cage individually and during 30 min the time spent on each behavior recorded for 5 min episodes (23). Behaviors included chewing, sniffing, climbing, taffy pulling, grooming, rearing, gnawing and licking. Also, during 30 min the frequency of yawning recorded. The behaviors noted between 9 AM and 4 PM in a silent environment, under red light and the temperature set at 20-25 °C. In addition, to video record the behaviors experiments, two persons filmed the behaviors independently. The results did not reveal any significant differences.

Statistical analysis

The analysis of data accomplished by SPSS 11.5. The total behavioral time analyzed by two and one way ANOVA followed by LSD (Least Significance Difference) test. Two- way repeated measure ANOVA using time as a repeated factor used for time-course data appropriately. Then one-way Kruskal Wallis ANOVA followed by Mann-Whitney U test used for multiple comparisons. The significance level set at $P<0.05$.

Histological verification

At the end of each test, the rats sacrificed with an overdose of diethyl ether. Their brains removed and stored in formalin for several days. Then, they embedded in paraffin and serial sections provided coronally by a microtome (Leitz, Germany). The photo of each slide took by a stereomicroscope (Blue Light, the USA). The position of cannula tracing compared with the rat brain atlas (22). The data related to the rats with cannula placements outside of the brain regions of interest were not used. The samples of cannula traces of the subregions are shown in Figure 1.

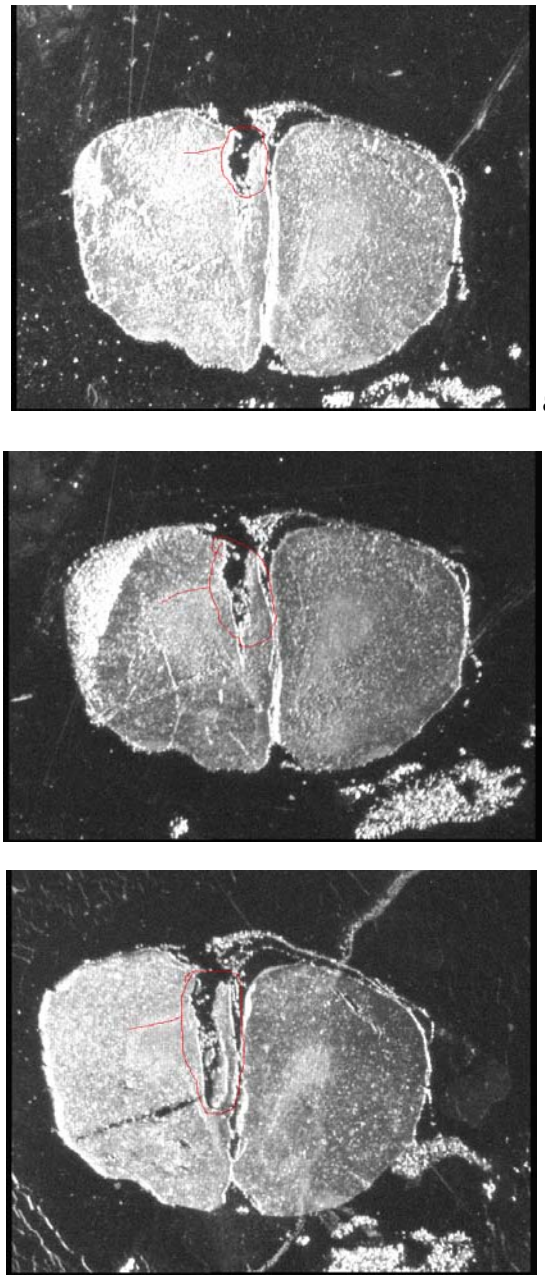

Figure 1. Stereomicroscopic photo of coronal section at the level of the anterior cingulated (a), the prelimbic (b) and the infralimbic(c). Arrowheads indicate cannula tracing in each subterritories of the medial prefrontal cortex.

Results

Total behavioral time data

Figure 2 shows the mean±SEM of the total time spent on climbing during 30 min for the three regions. Two-way ANOVA showed that the factors of region and dose were not significant. The mean±SEM of the total time spent on chewing behavior during 30 min is shown in Figure 3.

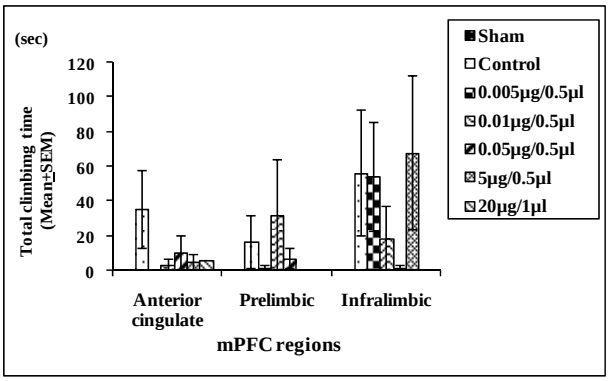

Figure 2. The effects of APO microinjection on total climbing time in three regions of the mPFC during 30 min. Each bar represents the mean of total climbing time ± SEM (SEM≤100%).
Control group: Ascorbic acid 0.1% as apomorphine vehicle
APO: Apomorphine (0.005-20 µg/0.5-1µl) immediately before recording
n=7 in each group, Statistical test: Two-way ANOVA

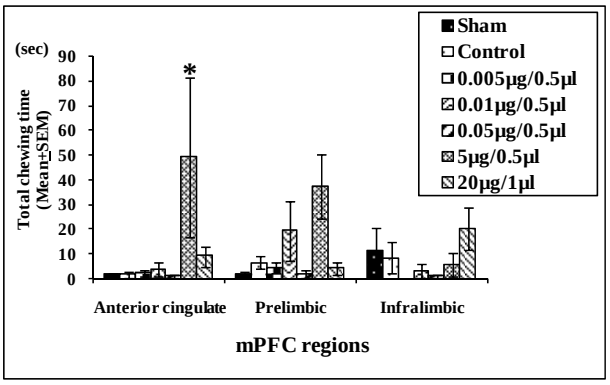

Figure 3. The effects of APO microinjection on total chewing time in three regions of the mPFC during 30 min. Each bar represents the mean of total chewing time ± SEM (SEM≤85%).
Control group: Ascorbic acid 0.1% as apomorphine vehicle
APO: Apomorphine (0.005-20 µg/0.5-1µl) immediately before recording
*: Significant to same dose in the infralimbic
n=7 in each group $P<0.05$, Statistical test: Two and one-way ANOVA followed by LSD test

Two-ways ANOVA showed that factor of region was not significant, but factor of dose was (dose: F (6,126) =2.135; $P<0.05$). Then, one-way ANOVA followed by LSD test showed that apomorphine injection in the ACd, significantly increased chewing in dose of 5 µg/0.5 µl as compared to the control group, ($P<0.05$). Also, there was a significant increase of the total chewing time for the dose of 5 µg /0.5 µl in the ACd area in comparison with that of the same dose in the IL area ($P<0.01$).

Figure 4 shows the mean±SEM of the total yawning frequency. Two-ways ANOVA showed that the factor of region was not important, but the factor of dose was (dose: F (6,126) =2.784; $P<0.05$). Then, one way-ANOVA followed by LSD test showed that apomorphine injection in the PL area increased yawning in dose of 5 µg/0.5 µl compared to the control group ($P<0.05$). Also, considerable increase of the total yawning frequency for this dose was observed in the PL area as compared to that of the same dose in the IL area ($P<0.05$).

There was not any remarkable difference in the other behaviors of taffy pulling, grooming, licking and rearing at the level of subregions of the mPFC and at the level of doses (data not shown). Behavior of gnawing was not observed in the rats. There was not any notable difference between the sham groups.

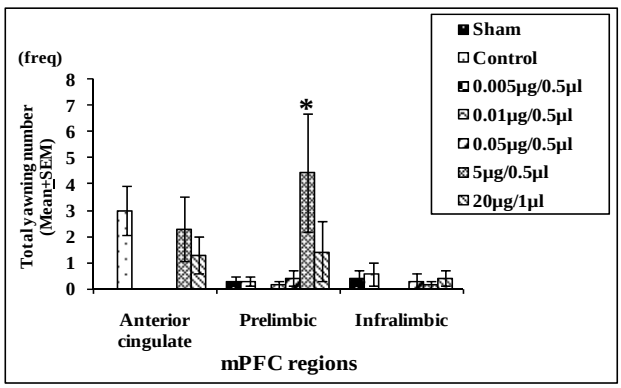

Figure 4. The effects of APO microinjection on total yawning frequency in three regions of the mPFC during 30 min. Each bar represents the mean of total yawning frequency±SEM (SEM≤100%).
Control group: Ascorbic acid 0.1% as apomorphine vehicle.
APO: Apomorphine (0.005-20 µg/0.5-1µl) immediately before recording
*: Significant to same dose in the infralimbic
n=7 in each group $P<0.05$, Statistical test: Two and one-way ANOVA followed by LSD test

Time-course data

Time-course graphs show the sum of times spent on each behavior in 5 min episodes, during 30 min. In Figure 5, time-course graph

of sniffing for the ACd area is shown. Two-ways repeated measure ANOVA using time as a repeated measure factor showed that factors of dose and time were significant (time: F (5, 630)=167.469, $P<0.01$). Then, one-way Kruskal Wallis ANOVA followed by Mann-Whitney U test showed that in the ACd area, at the first 5 min episode, the rats receiving apomorphine 5 µg/0.5 µl spent significantly less time on sniffing as compared to the control group, (χ^2 (6)= 14.451; $P<0.05$).

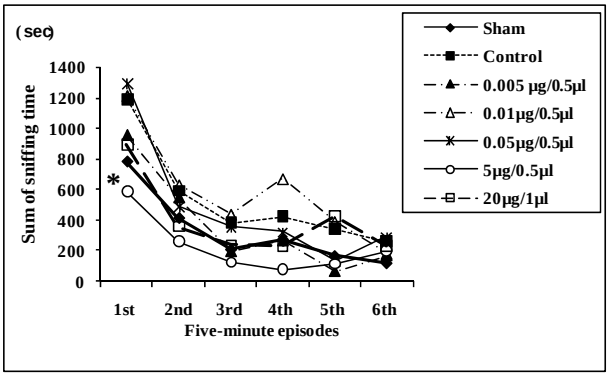

Figure 5. The effects of APO microinjection on sum of sniffing time in the anterior cingulated in each 5 min episode. Each line represents time-course graphs of sum of sniffing time for each dose.
Control group: Ascorbic acid 0.1% as apomorphine vehicle
APO: Apomorphine (0.005-20 µg/0.5-1µl) immediately before recording
*: Significant to the control group; n=7 in each group, $P<0.05$
Statistical test: Two-way repeated measure ANOVA, then one-way Kruskal Wallis ANOVA followed by Mann-Whitney U test

In Figure 6, time-course graph of climbing for the ACd area is shown. Two-ways repeated measure ANOVA showed that the factor time was important (time: F (5, 630)=22.988; $P<0.05$). Then, one-way Kruskal Wallis test followed by Mann-Whitney U test revealed that in the first 5 min rats receiving apomorphine 0.005, 0.01 and 5 µg/0.5 µl spent considerably less time on climbing, in comparison with the control group, (χ^2 (6)=16.989; $P<0.05$).

Figure 7 shows time-course graph of chewing for the ACd area. Two-ways repeated measure ANOVA showed that the effect of time was significant (time: F (5, 630) =2.356; $P<0.05$). One-way Kruskal Wallis test followed by Mann-Whitney U test showed that in the third 5 min episode the rats receiving apomorphine 5 µg/0.5 µl spent remarkably more time on chewing, as compared to the control group, (χ^2 (6)=18.734; $P<0.05$). There was not any obvious difference between the 5 min episodes and doses in each subregion of the mPFC, for other behaviors (data not shown).

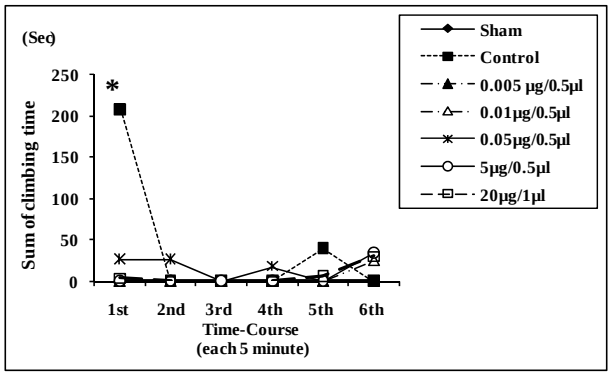

Figure 6. The effects of APO microinjection on sum of climbing time in the anterior cingulated in each five min episode. Each line represents time-course graphs of sum of climbing time for each dose.
Control group: Ascorbic acid 0.1% as apomorphine vehicle
APO: Apomorphine (0.005-20 µg/0.5-1 µl) immediately before recording
*: Significant to the control group; n=7 in each group, $P<0.05$
Statistical test: Two-way repeated measure ANOVA, then one-way Kruskal Wallis ANOVA followed by Mann-Whitney U test

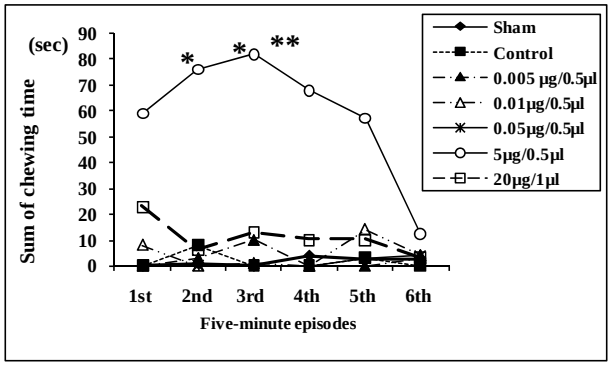

Figure 7. The effects of APO microinjection on sum of chewing time in the anterior cingulated in each five min episode. Each line represents time-course graphs of sum of chewing time for each dose.
Control group: Ascorbic acid 0.1% as apomorphine vehicle
APO: Apomorphine (0.005-20 µg/0.5-1µl) immediately before recording
*: Significant to the sham group; **: Significant to the control group; n=7 in each group, $P<0.05$
Statistical test: Two-way repeated measure ANOVA, then one-way Kruskal Wallis ANOVA followed by Mann-Whitney U test

Discussion

In this study, the effects of microinjection of apomorphine on three areas of the mPFC were investigated. Results showed that manipulation of dopaminergic system in the mPFC alters behaviors and there may be heterogeneity

among the three subareas of the mPFC regarding the apomorphine effects on behaviors of climbing, chewing, sniffing and yawning.

DA acts as a neuromodulator through action on different processes depending on its concentration, DAergic innervation density, its depth and location in the pyramidal cells of mesocortical system. Also, the effect of DA may depend on the level of activity in the local PFC network and DA might have opposite effects at high and low activity levels (19, 24-26). Low concentrations of DA enhance and high DA levels decrease the neuronal excitability of the mPFC. There is a critical and optimal concentration of DA required for modulating normal cortex-dependent behavioral processes (7, 24-27). Therefore, the effects of DA agonists in the mPFC are complex and not predictable easily.

In several studies sniffing has been related to the nigrostriatal DAergic system (11, 28) or mesolimbic system (29, 30) and also, clozapine which has the most effect on the mPFC and the least effect on the striatum, does not have significant effect on decreasing of the systemic apomorphine-induced stereotyped behavior of sniffing (31, 32). Then, mesocortical system may not have a direct effect on this behavior. However, the mPFC has the ability to modulate stereotyped behaviors and motor response to psychostimulants through its control over the functions of the subcortical areas (33). The present study showed that microinjection of apomorphine into the ACd area had overall more effect on sniffing than that of other subareas of mPFC (data not shown). Efferent pathways from the ACd compared to other subregions of the mPFC, have more projection to the dorsal striatum, nigrostriatal system and dorsal aspects of the NAC than to the core or shell of the NAC and mesolimbic system (7, 9, 20). Therefore, these reports are consistent with the present results that apomorphine microinjection into the ACd had more effect on sniffing, as a behavior related to the nigrostriatal DAergic system.

There are reports that attribute climbing to DAergic system of the mesolimbic or NAC (34-36). In a study it has been reported that apomorphine (0.001-10 μg) failed to cause any inhibition of spontaneous climbing when injected into the cortex areas such as the anteromedial, supragenual or suprarhinal cortex, although facilitation was recorded at the highest doses injected, 10 μg/1μl (134-166% of control values) (36). Also, it has been reported that atypical antipsychotic drugs did not antagonize apomorphine-induced sniffing, but could antagonize apomorphine-induced climbing in rodents (37). Because, the preferential effect of atypical antipsychotic drugs on DA release is in the mPFC, therefore, it was proposed that the medial prefrontal cortex may have an effect on apomorphine-induced or spontaneous climbing directly or through its relation with the subcortical systems such as the NAC. When apomorphine was injected through subcutaneous or intra NAC, its effect on dose-response curve of spontaneous climbing behavior was U-shaped (34-36). The result of these studies showed that apomorphine had inhibitory effects on spontaneous climbing when was injected into the NAC of mice at low doses, 0.25-0.5 μg/1 μl. But at a higher dose 4 μg/1 μl, apomorphine had a restoration effect on spontaneous climbing behavior. In the present study, we also, observed a U-shaped pattern of climbing after microinjection of apomorphine in to the IL area (Figure 2). Apomorphine decreased 66% and 96% spontaneous climbing at doses 0.01 and 0.05 μg/0.5 μl, respectively and then it restored climbing at dose of 5 μg/0.5 μl compared to the control value. These effects did not reach to a significant value. At a higher dose of apomorphine (20 μg/1 μl), it may have nonspecific effects. Because, maximal stimulation of the dopamine receptors is required to elicit the climbing behavior (38). This observation may imply that the most effect of apomorphine on modulation of climbing was in the IL with more innervation of DA system than other subregions of the mPFC (7, 9, 19, 20, 24, 26, 39). Also, at the present study, the effect of apomorphine injected into the mPFC of rats

on climbing was started at a lower dose (0.005 μg/0.5 μl) than that has been reported for the NAC of mice (0.25 μg/1 μl) (35). Therefore, the IL area of the mPFC may have a more sensitivity to apomorphine, regarding climbing than that of the NAC. It should be mentioned that the dose-response curve of apomorphine in mice and rats are the same (15, 16, 19, 30, 34, 36). However, further study should be done by microinjection of apomorphine into the both areas of the mPFC and NAC of rats in one experiment. The effect of the IL on climbing may be an indirect effect. Thus, the IL has more projections to the mesolimbic system than superficial parts of the mPFC like the ACd (7, 9, 20, 40, 41). However, the main limitation of this study might be the climbing behavior. So, the rats usually show this behavior less than mice. Therefore, more rats might be studied. The doses used in this work were chosen by the previous pilot study.

Another finding in this work was an increase in chewing in dose of 5 μg/0.5 μl of apomorphine injected into the ACd area (Figures 3 and 7). When a relatively small fraction of the total DA receptor pool is activated by lower doses of DA agonists, mainly hyperlocomotion and sniffing might be elicited, whereas higher doses result in reduced locomotor activity and sniffing with concomitant increase in oral stereotypes (including licking, chewing and/or biting) (30). The present study is consistent with this work, which an increase in chewing was observed with a decrease in sniffing in dose of 5 μg/0.5 μl in the ACd area (Figures 3, 5 and 7). This works also showed that apomorphine at dose of 5 μg/0.5 μl injected only in the ACd increased stereotyped chewing (that lasted more than 5 min and continued for 30 min) as compared to the control group, significantly. Moreover, significant increase of total chewing time was observed for the dose of 5 μg/0.5μl in the ACd area compared to that of the same dose in the IL area (Figures 3 and 7). These results can be explained considering reports that show chewing behavior like other oral stereotyped behaviors relates to the striatum (28, 29). So, the ACd compared to other subregions of the mPFC has more relation to the dorsal striatum system (7, 9, 20).

Apomorphine at dose of 5 μg/0.5 μl increased yawning (Figure 4). The increase of DA transmission in the mPFC especially in the ventral or deeper part of the mPFC including the PL and IL areas has an inhibitory effect on the mesolimbic DA system (5, 7, 9, 12, 19, 42, 43). Increase of dopaminergic activity in the mPFC has an inhibitory control on hyperfunction or hyperresponsiveness of the mesolimbic DA system (3, 7, 10, 21, 24-26, 39, 42-44). Also, locomotor activity and hyperactivity of animals were correlated directly with the time course of changes in the amount of released DA in the NAC (28, 29, 36) and the mesocortical system (31, 32). Therefore, because of coincidence of motor inhibition and yawning (45-47), increase of DA transmission in the PL for example at higher dose of apomorphine leads to the appearance of yawning and has a depressant effect on locomotor activity or hyperactivity of animals. In the present study, the observed yawning for the PL at dose of 5 μg /0.5 μl was more than that of the same dose for the IL area. The PL and IL areas do not have the same targets in subcortical systems, efferents of the PL project to the NAC core and for the IL project to the NAC shell (7, 9, 20, 41, 48) as the PL area projects heavily to autonomic centers in the pons and medulla (9). Moreover, the NAC core preferentially interacts with motor output structures of the basal ganglia and control the expression of dopaminergic receptor-mediated unconditioned motor behaviors such as yawning (46). However, yawning has been related to the activity of the oxytocinergic neurons of paraventricular nucleus in the hypothalamus projecting into the brain stem areas such as pons and medulla oblongata (49). Therefore, more effect of the PL compared to the IL on yawning could be explained by its more connection with locomotor activity centers and brain stem areas than that of the IL area.

Conclusion

Manipulations of dopaminergic system in the mPFC altered behaviors and there may be heterogeneity among its three subregions in this regard. Behaviors with nigrostriatal origin such as chewing observed more after microinjection of apomorphine in the superficial parts of the mPFC such as the ACd that has more connection with dopaminergic transmission of nigrostriatal system. Apomorphine had more effect on climbing and yawning when injected into the IL and PL areas, respectively. Probably, yawning and climbing have a mesocortical origin, or the mPFC could have effects on their control through its connection with the subcortical area. It seems that behaviors such as yawning and climbing can be used as stereotyped models for better screening of atypical antipsychotic drugs.

To understand more about the role of mPFC on rat behaviors, to serve as better animal models of mPFC related disorders such as schizophrenia, the effects of DA antagonists and other agonists on the mPFC are required to be investigated as well.

It is proposed to do a more narrow range of apomorphine in the next study. Moreover, at present, it is not clear which DA receptor subtype is involved in the effects of microinjected apomorphine on the mPFC. This subject has been studied as extension of the present work by using the clozapine as a DA D_4 antagonist (data in preparation).

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