Mediation of Rat's Social Dominance by Medial Prefrontal Cortex

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Abstract

Social dominance in animals predicts competitive success and access to desirable resources. Dominant animals tend to monopolise food and forage more effectively than subordinate group members. At the neuronal level, a region commonly associated with dominance-related behaviours is the medial prefrontal cortex (mPFC). Mice studies demonstrated that manipulating mPFC neurons in-vivo shifts dominance rank in the hierarchy. However, there are limited studies on rats involving the effects of in-vivo mPFC manipulation. Our study applied chemogenetic methods to investigate the role of mPFC neurons in the social dominance of male and female rats. Rats were tested in individual and group competitions to account for dominance behaviour in different interactions. In individual competitions, mPFC inhibition led to a delayed decrease in male dominance behaviour yet an instantaneous decrease in female dominance behaviour. These changes did not affect dominance rank. In group competitions, the effects of mPFC inhibition were variable. Our findings suggest that mPFC activity is likely one component in a multivariate mechanism that mediates rats' social dominance.

Introduction

Animals are known to establish social hierarchies with varying complexity depending on species. More importantly, rank in the hierarchy determines access to resources and mating opportunities within an animal group. Dominant animals tend to monopolise food and forage more effectively than subordinate group members who subsequently adjust their competitive efforts (Li et al., 2022). Social ranks can be determined by several factors including size, gender, and personality traits (Ferreira-Fernandes & Peca, 2022). In simple species, where social hierarchy relies heavily on physical contest, dominant animals are typically larger and less timid males. More complex species, such as humans and non-human primates, have more complex rules in their social hierarchies (Ferreira-Fernandes & Peca, 2022).

At the neuronal level, dominance-related behaviours of animals are associated with the medial prefrontal cortex (mPFC) (Holson, 1986; Uylings et al., 2003; Wang et al., 2011; Zhou et al., 2017). Rats with mPFC lesions are lower in social rank and express more timid behaviours than intact controls (Holson, 1986). Manipulations of mPFC neurons in mice cause instantaneous changes in competitive successes and effortful behaviours (Zhou et al., 2017). Findings in animal studies are compatible with patient studies and functional neuroimaging in humans. The prefrontal cortical region is attributed to social information processing and social behaviours (Blair & Cipolotti, 2000; Mah et al., 2004; Zink et al., 2008; Chiao, 2010). These findings suggests that the mPFC is essential for assessing social context in the environment and producing appropriate behaviour. However, there is a lack of study in the literature comparing potential functional differences between sex. Social impairments implicated in neuropsychiatric disorders, such as Autism Spectrum Disorder, commonly exhibit sex differences (Ochoa et al., 2012; Werling & Geschwind, 2013; Li et al., 2016). It is unclear if there are sex-specific functional differences in relevant regions, including the mPFC, that may contribute to this symptomatic variability. To better understand the mPFC's role in the social dominance of both sexes, we investigated the effects of inhibiting mPFC neurons in male and female rats.

In the wild, rats may compete dyadically with conspecifics or in groups. A comprehensive study on rats' social dominance should consider dominance behaviour in different competitive interactions. Two behavioural paradigms were adopted in this study to observe social dominance in individual and group competition. The tube test is commonly used in the literature to determine hierarchy in rodents due to its simplicity (Zhou et al., 2018). It involves a narrow tube where a pair of rats meet at the centre and attempt to advance by pushing their forcing their counterpart to retreat. Whichever rat is successful is declared the winner of the trial. Winner rats are likely to be more dominant and higher ranked in the hierarchy (Zhou et al., 2018). On the other hand, the sucrose competition is a relatively novel test designed to observe behaviours in a group setting. Rats compete to occupy a desirable reward for as long as possible. To achieve this, a dominant rat would remove a preexisting occupant while resisting attempts by other rats to prevent itself from being displaced. As such, dominance behaviour in this paradigm is defined by the total time spent occupying the bottle containing sucrose solution. In both paradigms, social dominance has two components: dominance behaviour and dominance rank. Dominance behaviours are operationally defined by a metric in each paradigm: David's Score measures dominance behaviour in the tube test, total time spent occupying the sucrose bottle measures dominance behaviour in the sucrose competition. Dominance rank is derived from the degree of dominance behaviour expressed relative to other group members. Male and female rats are ranked daily to identify their hierarchy and detect any changes.

This study uses a chemogenetic tool known as Designer Receptors Exclusively Activated by Designer Drugs (DREADD), which are a class of G-coupled protein receptors artificially engineered to bind with synthetic ligands. DREADDs lack an endogenous ligand to activate them but are sensitive to the inert drug clozapine N-oxide (CNO) (Smith et al., 2016). DREADD's reversible and highly specific nature is ideal for behavioural studies involving in vivo manipulations. A common vector used to express DREADD on neuronal membranes is known as an adeno-associated virus (AAV). Thus, to apply chemogenetics in this study, manipulated rats are injected with AAV in the mPFC region. Once DREADD expression is achieved in two weeks, DREADD agonist CNO can be injected intraperitoneally to inhibit mPFC neurons on demand. Effects of CNO are observable 15-20 minutes after injection and are expected to last no longer than 9 hours (Zhou et al., 2017; MacLaren et al., 2016; Jendryka et al., 2019). Finally, we hypothesise that the targeted inhibition of mPFC neurons reduces dominance behaviour in all competitions and decreases dominance rank in the hierarchy.

Methods

Animals. All procedures were approved by the University of Illinois Urbana-Champaign's IACUC. All experiments were performed on wild-type Long Evans male (n = 4) and female rats (n = 4) ages 1 to 3 months. Animals were bred from a lineage of rats received from Charles River Laboratories. Animals were assigned into experimental groups based on sex and housed together in large (480 x 375 x 210 mm) cages. Animals were allowed to freely interact with their group members at least 3 days before experimentation. Animals were tail- marked with Sharpie permanent markers and remarked every week. Rats were maintained on a 12:12 light-dark cycle (6am to 6pm) with food and water provided ad libitum. Experiments were conducted during the light phase of the cycle and bodyweights were recorded daily.

Tube Test. The apparatus was made up of a one-metre clear acrylic tube with chambers connected on both ends. A slot was cut out at the centre of the tube to insert a divider. The diameter of the tube was large enough to allow a rat to pass through from one end to another, but not sufficient for two rats to pass each other. Tube with incresaing diameters were used over time to accommodate the growing rats. Before testing began, each rat was acclimated to the apparatus by ensuring that they were comfortable entering the tube. Acclimation was considered unsuccessful and repeated the next day if rats failed to complete 10 tube-crossings in 15 minutes. During testing, a rat was placed in each chamber and the divider was removed when both rats met in the middle of the tube. The trial ended when a rat was forced to retreat out of the tube. The rat that successfully displaced its counterpart from the tube was declared the winner. Trials were video-recorded and arranged in a round-robin format to ensure every possible pairing was tested each day. Rats were also randomly assigned to the chambers to control for side bias. The apparatus was wiped down with 70% isopropyl alcohol between each trial. The dominance behaviour of each rat in their respective group was calculated daily using a metric known as David's Score (DS). DS accounts for cumulative wins and losses. Rats were ranked daily according to their DS to identify the hierarchy of the group. Tube tests were conducted for five consecutive days in the first week to allow rats to establish a stable hierarchy before surgery. Later, two weeks of tube testing were conducted with 5mg/kg CNO and 10mg/kg CNO manipulation respectively. On the weeks of CNO manipulation, CNO was administered on Day 2 and Day 4.

Sucrose Competition. Animals in a group competed for access to a bottle containing 10% sucrose solution in an open field arena. The arena (1 x 1 x 0.5 m) was built with 16 polyethylene panels and a bottle holder installed onto one of the panels. The unique panel was also modified to include a cylindrical extension surrounding the bottle tip and acrylic panels (72 x 305 mm) on both sides of the cylindrical extension serving as barriers. These modifications were made to only allow the head of one animal to reach the bottle tip. This designed required a competitor to forcefully displace the existing occupant to gain access to the sucrose reward. We performed one day of acclimation by allowing 25 minutes for rats to explore the arena as a group and learn the reward. The arena was wiped down with 70% isopropyl alcohol before the next group of rats was acclimated. All rats were food restricted approximately 15 hours before acclimation or testing to increase salience of sucrose reward. During testing, rats were placed in the arena as a group for 25 minutes and the session was recorded using a camera installed above the arena. The floor of the arena was wiped down with 70% isopropyl alcohol before the next group of rats was tested. Dominance behaviour was measured by the amount of time each rat spent occupying the sucrose bottle. Each rat was subsequently ranked to identify the groups' hierarchy. Sucrose competition took place the week following the completion of the tube test. After one day of acclimation, testing was conducted for five consecutive days with 10mg/kg CNO was administered on Day 2 and Day 4.

Viral Injection. Before selecting rats for viral injection, we ran a week of tube testing to identify the social hierarchy of each group. By the end of the week, rank-1 and rank-2 rats of each group (n = 4) were selected to receive AAV injection to enable local inhibition of mPFC neurons. Animals were anaesthetised with 3-5% isoflurane via inhalation followed by intraperitoneal (IP) injection of a ketamine-xylazine mixture. The mixture contained 3.25 mL of 100mg/mL ketamine, 1.65 mL of 20mg/mL xylazine, and 10mL saline solution. The head of the anaesthetised rat was fixed on a Kopf stereotaxic frame, followed by bilateral craniotomies lateral of the sagittal suture and anterior of bregma. A syringe with a needle connected to a syringe pump was slowly lowered to the stereotaxic coordinates relative to bregma: AP: +3.0 mm, ML: +/- 0.6 mm, DV: -3.3 mm to target mPFC. 2000 nL of AAV8-CaMKIIa-hM4D(Gi)- mCherry was injected bilaterally at a rate of 5 nL/s. Sham surgeries were performed on rank-3 and rank-4 rats of the groups. The protocols for anaesthesia and stereotaxic surgery were replicated with saline solution held

Chemogenetic Manipulation. One animal was selected from each group to receive CNO injections based on their rank in the hierarchy. Two criteria for selection were: (1) animal must have received AAV injection during surgery and (2) animal must be ranked in the upper half of the hierarchy (i.e., rank-1 and rank-2). On days of manipulation, rats with AAV received a CNO injection 30 minutes before testing while others received a saline injection. All injections were administered via IP 30 minutes before testing. CNO injections were prepared by dissolving 5 or 10 mg of CNO in 100 µL of dimethylsulfoxide (DMSO) and then diluted in 900 µL of saline.

Bodyweight-Dominance Correlational Analysis.
The correlation between bodyweight and social dominance is calculated using Pearson product-moment correlation coefficient, r. This dimensionless index ranges from -1.0 to 1.0 and measures the extent of a linear relationship between two data sets. Correlational analysis for the tube test compared the daily bodyweight of each rat against their DS and dominance rank for each day. Similarly, correlational analysis for the sucrose competition compared daily bodyweight of each rat against their total time spent occupying the sucrose bottle and dominance rank for each day. A total of eight correlational analyses were conducted, correlations with r above +/- 0.7 were regarded as significant.

Results

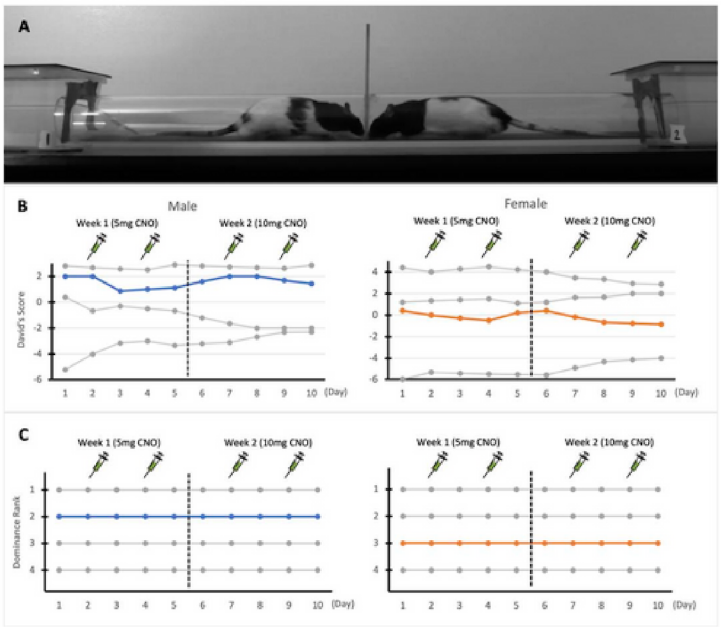

Fig. 1. Effects of mPFC inhibition on dominance in tube test. **(A)** Setup of a tube test trial. **(B)** David Scores of rank-2 male rat and rank-3 female rat after receiving 5mg/kg CNO injection in Week 1 and 10mg/kg CNO injection in Week 2. **(C)** Dominance in the tube test ranked by David's Score of each rat.

One group of male rats and one group of female rats were tube tested. Fig. 1B illustrates the effects of 5mg/kg and 10mg/kg CNO on the David Score (DS) of both sexes. On the first week, 5mg/kg CNO had no immediate effect on male DS on Day 2, but male DS decreased on Day 3. Although male DS increased slightly following 5mg/kg CNO on Day 4, it remained below baseline (DS = 2.0) since the initial decrease on Day 3. Male DS restored close to baseline over the 10mg/kg. There were no changes in male DS after the first CNO injection on Day 7. Male DS decreased slightly after the second CNO injection on Day 9 and continued to decrease on Day 10.

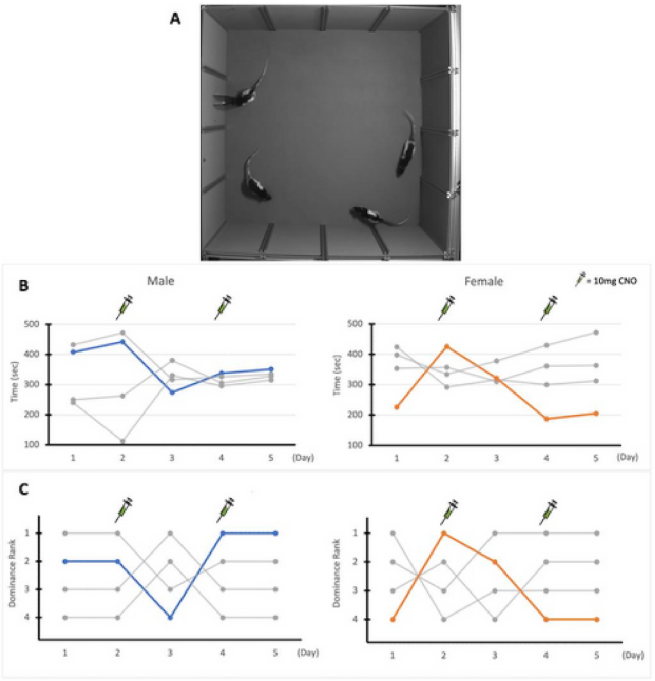

Fig. 2. Effects of mPFC inhibition on dominance in sucrose competition. (A) Setup of the sucrose competition. **(B)** Total time spent occupying sucrose bottle by rank-2 male rat and rank-4 female rat after 10mg/kg CNO injection. **(C)** Dominance in the sucrose competition ranked by the total time spent occupying the sucrose reward by each rat.

In females, DS decreased after administering 5mg/kg CNO injection on Day 2 and continued to decrease steadily over subsequent days. Female DS was lowest in that week following the second 5mg/kg CNO injection on Day 4. Similar to the male rats, DS restored to near baseline levels (DS = 0.4) over the weekend between Week 1 and Week 2 of tube testing. On the second week of tube test, female DS exhibited a similar pattern to Week 1 where DS decreased steadily after the first CNO treatment. Here, DS was the lowest on the last day of Week 2. Collectively, 5mg/kg and 10mg/kg CNO did not affect the dominance rank of the male and female rat (Fig. 1C).

Fig. 2 illustrates the effects of 10mg/kg CNO injection during one week of sucrose competition. In Fig. 2B, male's total time spent occupying the sucrose bottle increased immediately following 10mg/kg CNO injection on Day 2 and Day 4. However, there was a sharp decline in total time on Day 3 before a slight rebound on Day 4. It was noted that there was a large deviation between the total time of rank-1, rank-2 rats and rank-3, rank-4 rats on the first two days of sucrose competition. This deviation diminished after Day 2 and the total time of all males became close in proximity on Days 4 and 5. In terms of dominance rank, the CNO- injected male experienced a downward shift in rank the day after the first CNO injection. This did not occur after the second CNO as the hierarchy remained unchanged on Day 5. At the same time, female's total time also increased upon receiving 10mg/kg CNO injection on Day 2, though this was not

observed on Day 4. Total time gradually returned towards baseline (226 seconds) after the initial increase on Day 2. It was noted that the female's total time on Day 3 was very close to that of rank-3 and rank-4 female rats. As for dominance rank, 10mg/kg CNO injection induced an instantaneous upward shift in female dominance rank from rank-4 to rank-1. The dominance rank later shifted downwards on Day 3 and returned to rank-4 on Day 4 after second CNO injection. Like its male counterpart, the female hierarchy remained unchanged on Day 5.

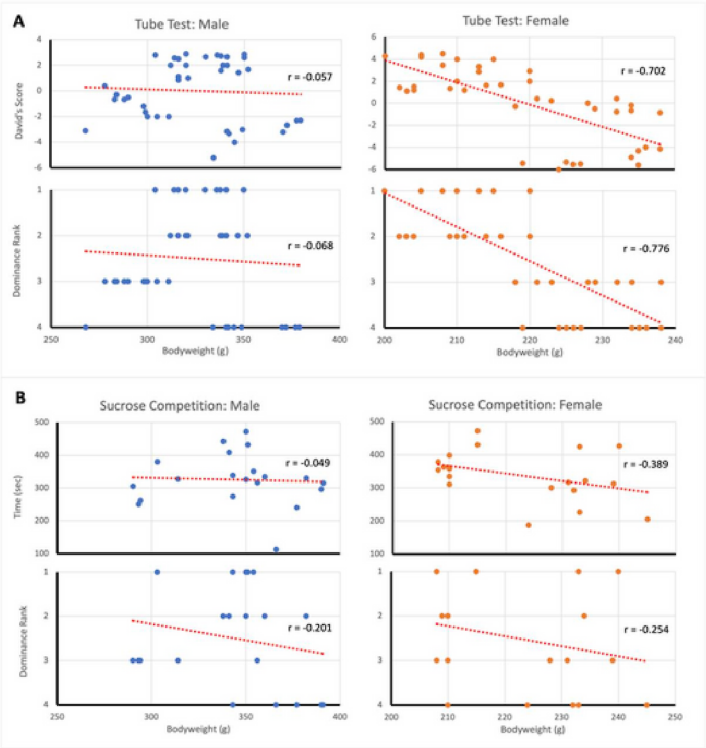

Fig. 3. Correlational analysis between bodyweight and dominance. (A) Relationship between bodyweight and measures of dominance in the tube test. **(B)** Relationship between bodyweight and measures of dominance in the sucrose competition.

The relationship between bodyweight and dominance was analysed using the Pearson correlational coefficient (Fig. 3). During 10 days of tube testing, bodyweight was not correlated to DS (*r*DS = -0.057) or the dominance rank (*r*Rank = -0.068) of male rats. However, there was a negative correlation between female bodyweight and both measures of dominance (*r*DS = -0.702 and rRank = -0.776; Fig. 3A) in the test tube. In the sucrose competition, there were also no correlation between male bodyweight and DS (rDS = -0.049) or dominance rank (rRank = -0.201). In females, there was no correlation between bodyweight and both measures of dominance in the sucrose competition (rDS = -0.389 and rRank = -0.254; Fig. 3B).

Discussion

The tube test was intended to study rat's social dominance in an individual competition. At the start of each testing week, male rats did not show instantaneous change in dominance behaviour in response to the first CNO injection (Fig. 1B). But we observed a decrease in dominance behaviour 24 hours later. The absence of immediate effects was likely due to animals behaving based on past competitive successes. If a subordinate rat has consistently lost against a dominant rat, the subordinate rat may expect to lose again. This win history may prompt the subordinate rat to initiate fewer pushes and offer less resistance against push attempts (Zhou et al., 2017). Thus, a dominant rat that was recently given CNO may still be able to win the initial bouts easily although it no longer expressed usual levels of dominance-related behaviours. This idea is supported by the observations made during video reviews of the tube test. However, when rats are returned to their home cage, the CNO-injected rat and saline-injected rats are able to interact over an extended period. Then, the effects of CNO on the dominant rat becomes apparent to its group members and the social hierarchy is affected. This may explain the delayed decrease in the male's dominance behaviour following the first CNO injection in Week 1 and Week 2. Past CNO studies with mice found that the behavioural effects of CNO is known to last between six to nine hours (Alexander et al, 2009; Zhou et al., 2017). This suggests that the delayed changes in dominance behaviour that occurred nine hours after CNO injection was not caused directly by the CNO's inhibition of mPFC neurons.

In contrast, CNO injection caused an instantaneous decrease in the dominance behaviour of the female rat (Fig. 1B). Unlike males, female rat hierarchies are less linear and more susceptible to external factors (Williamson et al., 2019; Varholick et al., 2019). Studies observed markedly less strict hierarchies in female rats and mice (Fulenwider, 2022). In our study, the effects of CNO readily influenced the interactions of the CNO-injected female with other group members and led to changes in the outcomes of the tube test. Although mPFC inhibition via CNO was insufficient to shift ranks in the female hierarchy (Fig. 1C), a decrease in David's Score (DS) indicated losses in trials where wins were expected. DS is a measurement that accounts for past results by comparing both animals' proportion of wins and losses (Gammell et al., 2003). An animal with high win proportions is 'expected' to win, DS will shift more significantly upon an upset. The DS of the rank-3 female rat decreased over both days of CNO which was accompanied by a similar trend on Week 2 (Fig. 1B). This suggests that mPFC inhibition attenuated dominance behaviour in female rats.

CNO dosage increased from 5mg/kg to 10mg/kg on the second week of tube test (Fig. 1B, C) to increase the salience of its effect, if any. Ultimately, mPFC inhibiton did not affect the social hierarchy of male and female rats in our sample. Although studies in the literature found that manipulations of the mPFC consistently shifted dominance ranking in the hierarchy, the manipulations were performed on mice (Wang et al., 2011; Zhou et al., 2017; Li et al., 2022). Mice are simpler species where social hierarchy is largely determined by physical contest. In such cases, changes in dominance behaviour caused would shift ranking within the hierarchy more readily. However, rats are more socially tolerant and less hierarchical (Schweinfurth, 2020). Our results suggest rat's social hierarchy may be more complex. The dominance rank of rats may be mediated by other factors in the environment in which the inhibition of mPFC neurons alone may not be significant. This might be a result of their

naturalistic behaviour in the wild, where rats are found to live in large groups (Schweinfurth, 2020). Hence, it is necessary to consider social dominance in a group setting to assess the effects of mPFC inhibition by CNO.

In group competitions, the effects of mPFC inhibition on social dominance were highly variable. Male dominance behaviour decreased 24 hours after the first CNO injection, but the same effect was not observed after the second CNO administration (Fig. 2B). At first glance, the decrease in dominance behaviour on Day 3 may resemble tube test results. But the saline- injected rank-1 rat also saw a significant decrease in dominance behaviour on Day 3 (Fig. 2B). Moreover, rank-1 rat behaved similarly to the CNO-injected rat throughout the testing period. Given the limited testing period of the sucrose competition, no comparison can be made to better explain these outcomes. The male results on Day 3 could not be definitively ascribed to the effects of mPFC inhibition. The behaviour observed in rank-3 and rank-4 rats via video review implies that both rats had not learned the reward. Both rats had tendencies to explore the arena and showed less interest in competing for the reward. This allowed rank-1 and rank-2 rats to dominate the sucrose competition on the first two days of testing. This rationale is supported by the large deviation in total time spent occupying the bottle between the learned and unlearned group (Fig. 2B). Furthermore, the competition narrowed beginning on Day 3 and remained as such in the following days. This suggests that the laggard rats had acquired the reward on Day 3 and began to compete for the reward from then on. For this reason, the results of Days 2 and 3 could not be compared to those of Days 4 and 5. It is unclear how the effects of mPFC inhibition contributed to results on Day 3.

As for females, there was a significant increase in dominance behaviour and dominance rank on the day of CNO. However, this was a single occurrence that did not repeat upon the second CNO injection. Both measures of social dominance steadily returned to baseline over the next three days. Here, CNO led to an increase in dominance behaviour and improvement in dominance rank on Day 2 only (Fig. 2). This isolated occurrence was likely the result of factors unrelated to mPFC inhibition as we identified hunger level as a possible cause. When food restriction was imposed the evening prior to testing on Day 2, food may have been removed when the rank-4 rat was not fully satiated. At the start of testing the following day, the rat would have been especially hungry and highly motivated to consume a gustatory reward. This did not occur again on other days of testing. We also note that upon receiving CNO on Day 1, the rat was already at the bottom of the hierarchy with considerably low dominance behaviour as shown by the difference in total time at the bottle (Fig. 2B). This demonstrates a floor effect where the effects of mPFC inhibition cannot be measured accurately due to a lower limit. The lowest- ranked rat is unable to fall further in dominance rank, the ability to observe changes in dominance behaviour is also limited. In this case, any manipulation introduced to the rat has two possible outcomes: (1) social dominance remains unchanged, or (2) increased social dominance.

The rank-4 female rat was selected for manipulation despite the limitations of the floor effect because it was previously selected for manipulation during the tube test as well. To control for the long-term effects of mPFC inhibition, the rat was selected again for manipulation in sucrose competition.

Social hierarchies emerge whenever there is competition between individuals for important resources (Williamson et al., 2019). The more intense the competition, the more likely that a highly linear social hierarchy will develop within the group. In mammals including rodents, males often form highly linear social hierarchies through high intra-sexual competition. Female rodents, on the other hand, form hierarchies that are less linear, steep, and despotic—in some cases even non-existent (Varholick et al., 2019; Williamson et al., 2019; Fulenwider, 2022). To test the findings in our study, a correlation analysis was conducted between the bodyweight and social dominance of our rats (Fig. 3). Overall, no relationship was found between bodyweight and either measure of social dominance, except for the female tube test results (Fig. 3A). This finding aligns with other rodent studies in the literature that generally found no association between bodyweight and dominance rank (Lindzey et al., 1961; Berdoy et al., 1995; So et al., 2015; Williamson et al., 2016; Williamson et al., 2019). Other intrinsic and extrinsic factors have been proposed as determinants of social hierarchy in rodents (Berdoy et al., 1995; Fulenwider, 2022). Intrinsic factors are inherent physical and mental attributes, such as antagonistic behaviours; whereas extrinsic factors originate from the environment, such as past competitive successes (Zhou et al., 2018). Nevertheless, an interesting finding in this correlational analysis was the negative correlation between bodyweight and female dominance in the tube test. This can be attributed to the design of the tube test. Video review of the tube test suggested that although larger rats may have better resistance against push attempts, smaller rats could force their counterpart to retreat by scratching or headbutting. This indicates that the tube test does not necessarily favour big, heavy rats since there are alternative methods for rats to express dominance-related behaviours.

The sample size used in this study served as the primary limitation. Without any comparisons to make, the interpretations of our findings had to consider occurrences by probability. A larger sample size for both male and female groups would have allowed inferences to account for probability. There were also limitations found within the design of our study. Firstly, the habituation for sucrose competition was inadequate in which testing began before all rats in the group acquired the reward. This affected the male results, and possibly female results, in addition to the limited number of sucrose competitions that were run. Future studies involving the sucrose competition should ensure that the acclimation process is continued until all rats display a noticeable interest towards the reward. Subsequently, the timeframe for sucrose competition was limited to five days with one day between the first and second manipulation. This prevented the possibility of observing behavioural trends over the weekend when manipulation was absent. A two-week

timeframe for the sucrose competition would also allow a result comparison between Weeks 1 and 2. Future directions of this study should expand to include mPFC excitation in both sexes for comparison with similar experiments done in mice studies. Although the tube test has been established over many decades, the sucrose competition is a novel paradigm that more closely resembles natural foraging behaviour in rats. The sucrose competition is a useful behavioural paradigm that should complement existing paradigms in the literature. Adopting more than two paradigms in a behavioural study is an effective method to overcome the limitations posed by each paradigm.

In conclusion, we found that the inhibition of mPFC neurons via chemogenetics decreased dominance behaviour in individual competition. The effect was delayed in male rats but instantaneous in female rats due to the dynamic nature of female hierarchies. However, mPFC inhibition was insufficient to induce a downward shift in the dominance rank. Increased inhibition of the mPFC neurons did not alter the effects on social dominance. In group competitions, the role of mPFC in social dominance was unclear. Correlation analysis found that bodyweight was generally not associated with social dominance, except for a negative correlation in female individual competition. This negative correlation was attributed to the nature of the competition which was less reliant on size. Collectively, our findings suggest a degree of complexity in the social dominance and hierarchy of rats. mPFC neurons may be recruited as a component in a multivariate mechanism that mediates rats' social dominance

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