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Behaviorally timid rats respond differentially to conventional and atypical neuroleptics

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Abstract

An inhibited temperament can be manifested as simple shyness or as social phobia and is perhaps related to the extreme social dysfunction often accompanying schizophrenia. Here, we present a methodology for selecting subjects and testing changes in social attraction in an animal model of behavioral timidity. In Experiment 1, randomly selected female rats were chronically administered either vehicle only, the conventional neuroleptic haloperidol (0.1 mg/kg) or atypical drugs sulpiride (65 mg/kg) or clozapine (18 mg/kg). The animals were tested over 3 weeks for changes in attraction to a social stimulus. Findings revealed a statistically significant decrease in social investigation in the haloperidol treated animals compared to controls but no significant differences among the other groups. Experiment 2 employed pretests to select behaviorally timid (BT) animals. Only female rats having little initial attraction to unfamiliar non-social and social stimuli were chosen to serve as subjects for the experiment using the same drug exposure regiments and behavioral measures used in experiment 1. Results with pre-selected BT animals indicated that clozapine treated animals significantly increased social investigation whereas chronic exposure to either sulpiride or haloperidol groups did not increase social investigation. Indeed, haloperidol appears to have magnified avoidance of social contact. That there were minimal differences between drug groups on a measure of non-social general activity points to the beneficial increases in investigation from clozapine being specific to social inhibition. Conclusions are that timidity may involve aspects of the serotonergic system uniquely influenced by clozapine, and the animal model of the second experiment may prove useful for studies of the biological underpinnings of behavioral timidity.

Keywords: Clozapine; Sulpiride; Haloperidol; Serotonin; Shyness; Inhibited temperament; Animal model

1. Introduction

In most social groups, there is a subset of individuals who tend to be reticent to approach unfamiliar conspecifics or objects. These individuals have attracted the attention of a notable variety of researchers. Field biologists have expressed concerns that data from feral animals in their natural habitats may be skewed by observing only the decidedly non-timid individuals (Reale et al., 2000; Wilson et al., 1994). Personality psychologists and psychiatrists have observed that an inhibited temperament can manifest itself as simple shyness or social phobia. There also is evidence of a link between shyness and the extreme social dysfunction that is a hallmark of the negative symptomology of schizophrenia (Henderson and Zimbardo, 1998; Goldberg and Schmidt, 2001).

Neuroscientists have focused on biological mechanisms underlying behavioral inhibition in both humans and animal models. An example is the recent search to identify a specific gene locus, likely the serotonin transporter promoter gene, involved in the shyness of some second graders (Arbelle et al., 2003). Children classified as possessing either an inhibited or an uninhibited temperament as two-year olds have been followed into early adulthood (Schwartz et al., 2003a,b). Neuroimaging of the shy individuals, now adults, demonstrated an overactive amyg-

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dalar response to novelty compared to their uninhibited counterparts.

The merits of studies of shy individuals are that timidity may be an early marker for later psychopathology (Freedman, 2002). A vulnerability model (Schmidt and Fox, 1999) suggests that early biological and behavioral aspects of shyness are linked to sensitivity in the forebrain limbic system producing a dysfunction in an individual's ability to regulate social stress. Extreme social inhibition in childhood may be an indicator of poor adult mental health (Lewine et al., 1978; Reznick et al., 1992). The relation of social inhibition to schizophrenia is of particular interest (Dickerson et al., 2000; Goldberg and Schmidt, 2001). Conventional antischizophrenic drugs antagonize dopamine, alleviate primarily positive symptoms and have a higher risk of extrapyramidal side-effects (EPS). The conventional drugs also have historically been less effective in treating the negative symptoms, including the social deficits, of the disease. Atypical antipsychotic drugs with greater neurotransmitter specificity and lower EPS risks (Owens and Risch, 1998; Strange, 2001), especially those targeting serotonergic pathways, have been more effective in relieving asociality in schizophrenia (Meltzer and Fatemi, 1998; Tandon and Jibson, 2003).

Here, we used female rats exposed chronically either to a conventional dopamine antagonist neuroleptic (haloperidol), an atypical drug (sulpiride) that is a more highly specific antagonist of dopamine, or an atypical medication (clozapine) targeting serotonergic pathways. Our interest was to evaluate the effectiveness of the drugs in modifying social investigation of an unfamiliar conspecific. The first of two experiments used females chosen randomly from the animal colony. The working hypothesis suggested by findings in the literature (Corbett et al., 1993) was that clozapine, but not haloperidol or sulpiride, would increase investigation of another female rat.

2. Experiment 1

2.1. Methods

2.1.1. Subjects

Female Long–Evans rats, 5–11 months old, from the animal colony maintained at the University of Missouri—St. Louis served as subjects ($N=40$) for the experiment. In addition, a pool of ovariectomized adult females ($N=25$) of the same ages and strain served as social stimuli. Beginning one month prior to the experiment, all animals were housed individually in hanging wire cages (26 cm × 36 cm × 25 cm). Water and Purina Lab Chow were available ad libitum to the rats throughout the experiment. Lighting was maintained on a 12 h light–dark cycle. Room temperature (20–22 °C) and relative humidity (55 ± 5%) were controlled automatically. Animals were tested during the dark phase of the cycle. Animals used in these experiments were maintained in accordance with the guidelines of the Committee of Care and Use of Laboratory Animals, National Research Council and were approved by the campus animal care and use committee.

2.1.2. Materials

Glass terraria (73 cm × 30 cm × 42 cm) were used to test social investigation. Also, small mesh wire cages measuring 20 cm × 16 cm × 24 cm were used to house the social stimulus. The small cages were placed inside the terrarium, one on each side, and a stimulus animal was randomly placed in one of the cages.

2.1.3. Drugs

Drugs were purchased from Sigma Chemical Company (St. Louis, MO). Animals received haloperidol (0.1 mg/kg), clozapine (18 mg/kg), sulpiride (65 mg/kg), or saline only vehicle. These doses were chosen based on receptor binding data (Motohashi et al., 1992; Csernansky et al., 1993). The logic was to use equivalency of receptor occupancy, specifically occupancy of 50% of the D2 receptors in the nucleus accumbens and corpus striatum, to determine the drug dosages employed. Drugs were injected subcutaneously (SC) in a volume of 1 ml/kg of body weight. Clozapine and haloperidol were prepared by being dissolved in a minimal volume of acetic acid, diluted with saline and neutralized with small quantities of 10 N NaOH to pH 7. Sulpiride was solubilized in a small amount of ethanol and diluted with saline.

2.2. Procedures

2.2.1. Experimental design

Female rats were randomly assigned to one of four groups ($n=10$ per group) to be administered haloperidol, sulpiride, clozapine, or vehicle only. Animals were injected with drug six days a week, for a total of three weeks, and behaviorally observed in a terrarium each week.

2.2.2. Experimental procedures

To assess interest in another animal, we adapted methods from our prior research that had examined interest in an opposite-sex conspecific (Taylor et al., 1991). Here, the method was used to assess social interest of a same-sex conspecific. One of two small mesh wire cages remained empty and was positioned on one side of the terrarium. An ovariectomized (OVX) stimulus female was placed in the other small cage located on the opposite side. Rendering the stimulus animal inaccessible allowed for social exposure via all sensory modalities but without direct social interactions that could confound multiple testing of subject animals. OVX females were used to minimize changing odors with the estrous cycle that could have provided a confounding source of attractant or repellant odors to the female subjects.

Animals were tested for social investigation of an OVX conspecific three times, once per week during drug

administration. The experimental female was introduced into the terrarium for a 10 min session. Time spent in close proximity to the OVX stimulus animal, defined as the nose of the subject within 2 cm of the cage, was recorded (Taylor et al., 1983).

All tests were conducted by experimenters who were blind to the drug treatments. Injections were given 30 min before a test session.

2.2.3. Statistical analysis

Data analyzed in Experiment 1 were time, in seconds, spent investigating the stimulus female during each session. These social investigation scores were analyzed by a 4×3 repeated measures factorial analysis of variance (ANOVA) with main factor of drug (clozapine, haloperidol, sulpiride, or vehicle) and weeks as the repeated factor. Means and standard errors were calculated. All analyses were performed with the SPSS statistical program for Macintosh computers. With a statistically significant interaction, simple main effects were planned for further analyses. Tukey's HSD method was used for post hoc comparisons. Probability value for all analyses was $p < 0.05$.

2.3. Results and discussion

Social investigation scores for Experiment 1 are presented in Fig. 1. The factorial ANOVA on investigation times revealed a significant main effect for drug exposure, $F(3,36) = 4.15$, $p < 0.05$, and a significant interaction between drug exposure and week of treatment, $F(2,72) = 2.307$, $p < 0.05$. Consequently, simple main effects were examined first to evaluate between group differences at each week and second to assess within group changes over weeks. Results revealed no significant differences among groups at any week. Simple main effects within groups indicated only the haloperidol treated group changed significantly over weeks, $F(2,72) = 4.45$, $p < 0.05$. Post hoc comparisons with Tukey's HSD test indicated that the haloperidol treated animals were less social between week 1 and week 3.

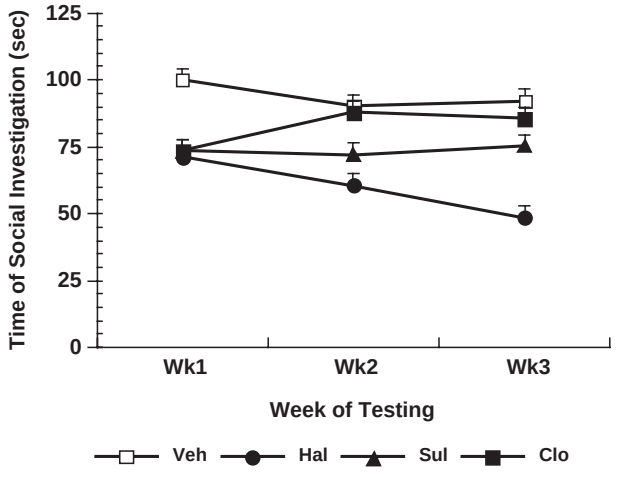

Fig. 1. Changes over weeks in investigation times of a social stimulus (an inaccessible ovariectomized female) by unoperated female rats selected at random from the animal colony and chronically exposure to clozapine (Cloz), haloperidol (Hal), sulpiride (Sul) or saline (Veh). Values are means and standard errors of the mean.

Results from Experiment 1 indicated that clozapine failed to increase social investigation in randomly selected female rats compared to controls. Indeed, the social investigation scores did not change for any of the groups over weeks of drug exposure, except for the decrease in the investigation scores of the haloperidol group after 3 weeks of injections. To more closely represent a sample with an inhibited temperament, Experiment 2 was conducted using only animals selected during pretests before drug administrations began.

3. Experiment 2

3.1. Methods

3.1.1. Subjects

An original group ($N = 227$) of experimentally naïve female Long–Evans rats were pretested individually in a non-social (open field) paradigm and a social (investigation of conspecific odors) paradigm. Based on criteria for behavioral timidity, subjects were selected for the experiment ($N = 32$) and housed and maintained as described in Experiment 1.

3.1.2. Materials

The same drugs, terraria and other materials used in the first experiment were also used in Experiment 2. In addition, Petri dishes containing clean or soiled bedding were used for the social odor investigation pretest. An open field apparatus was a platform 90 cm × 120 cm onto which 15 cm squares were drawn for quantifying locomotor activity (Taylor et al., 1996).

3.2. Procedures

3.2.1. Experimental design

Animals meeting the criteria for behavioral timidity were selected as subjects and randomly assigned to one of four treatment conditions ($n = 8$ per group) to be administered daily doses of clozapine (18 mg/kg), haloperidol (0.1 mg/kg), sulpiride (65 mg/kg), or saline vehicle. Subject animals were SC injected six days a week, over three weeks. Each subject was tested a total of 8 times, $4\times$ in the social investigation paradigm and $4\times$ in the open field paradigm.

3.2.2. Pretests for selecting timid animals

Behavioral ecologists and other biologists use the words shy and bold unapologetically to describe timidity of non-

human animals in, mostly, non-social situations (Dingemanse et al., 2002; Reale et al., 2000; Wilson et al., 1994). Psychologists and psychiatrists use shyness mostly in reference to humans and mainly to describe inhibition in social settings (Arbelle et al., 2003; Goldberg and Schmidt, 2001). We assessed reticence to approach both non-social and social stimuli and use the term behaviorally timid (BT) to describe the most reticent animals.

The selection of BT animals was designed to take advantage of the wide individual variation among rats to approach and investigate an unfamiliar object or conspecific (Barnett and Cowan, 1976). Each animal from a large group ($N=227$) of adult females was given a pair of tests of timidity separated at least by two days. One was a non-social test situation and the other was a social test. The logic was to ensure stability of behavioral timidity over both social and non-social settings. Tests were conducted in a dimly lit room.

The open field test served as the non-social measure, with BT defined as an animal reluctant to explore the apparatus. The rat was placed in the open field and allowed to move about freely for 5 min. Number of squares crossed were recorded.

The stimulus for the social test was provided by soiled bedding from an unfamiliar, ovariectomized female rat (Sawyer et al., 1984). Employing odor rather than another animal as a social stimulus was designed to avoid the potential confound of defining social timidity with the same measure (presence of another animal) used in the subsequent experimental tests.

A Petri dish filled with soiled bedding from an unfamiliar animal was placed near the rear wall of one side of the terrarium. On the opposite side of the terrarium was a Petri dish filled with clean bedding. The rat was introduced into the middle of the terrarium, and time investigating the soiled bedding was recorded during the 10 min session.

Animals were rank ordered according to number of squares crossed in the non-social tests, and again for time spent investigating the soiled bedding. Animals scoring in the bottom 25% on *both* measures were characterized as BT rats and were chosen for Experiment 2.

3.2.3. Experimental testing

BT females ($N=32$) serving as subjects were tested in the same social investigation paradigm used in Experiment 1. That is, the animal was injected and 30 min later was placed in a terrarium in which an OVX female had been caged on one side. Time spent in close proximity to the stimulus animal was recorded. Each rat was tested a total of four times, once before drug treatments began and once a week during the three weeks of drug exposure. Animals were also tested, on a different day each week, in the open field apparatus. All tests were conducted by experimenters who were blind to the drug treatment of the animal.

3.2.4. Statistical analysis

The primary measures in Experiment 2 were time spent investigating the stimulus female and numbers of squares crossed in the open field. These social investigation scores and open field data were analyzed by 4×4 repeated measures factorial ANOVAs with main factor of drug (clozapine, haloperidol, sulpiride, or vehicle) and week (pre-drug week, and 3 weeks of drug exposure) as a repeated factor. Means and standard errors were calculated for each measure. All analyses were performed with the SPSS statistical program for Macintosh computers. With a statistically significant interaction, simple main effects were planned for further analyses, and Tukey's HSD tests were used for post hoc comparisons. In addition, a Pearson's product–moment correlation was calculated for the pretest data from social (seconds investigating the odor of another animal) and non-social (number of squares crossed) settings. Probability value for all analyses was $p<0.05$.

3.3. Results and discussion

Social investigation scores for the BT rats of Experiment 2 are presented in Fig. 2. The main effect of groups was statistically significant, $F(3,28)=6.05$, $p<0.05$, as was the interaction between groups and weeks, $F(3,84)=6.191$, $p<0.05$.

Simple main effects revealed a statistically significant value between groups at week 2 and week 3, $F(3,84)=14.34$ and 14.01, respectively, $p<0.05$. Pairwise post hoc assessments indicated that the clozapine treated animals had higher social investigation scores than the other three groups at week 2. At week 3, the clozapine treated group remained more social than either the sulpiride or haloperidol treated groups, however, the difference between the clozapine

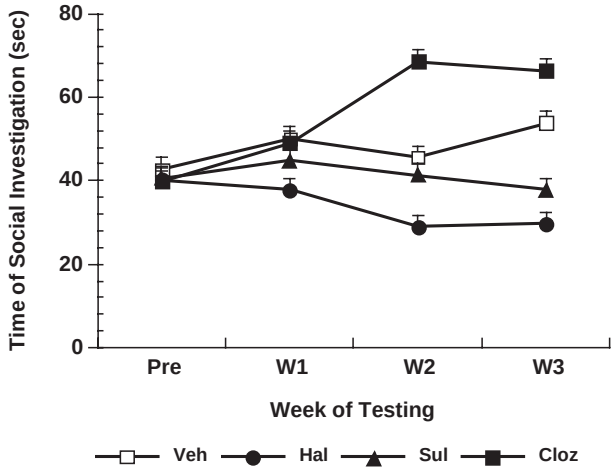

Fig. 2. Changes over weeks in investigation times of a social stimulus (an inaccessible ovariectomized female) by unoperated female rats identified in pretests as being behaviorally timid animals and chronically exposure to clozapine (Cloz), haloperidol (Hal), sulpiride (Sul) or saline (Veh). Values are means and SEM.

treated animals and controls did not achieve statistical significance.

Simple main effects analyzed over weeks for each group indicated no statistically reliable changes for the controls or sulpiride treated animals. However, the values for both the clozapine and haloperidol treated groups were statistically significant, $F(3,84)=2.64$ and 15.74, respectively, both $p<0.05$. Post hoc comparisons within those two groups revealed notably different patterns of change over weeks. Social investigation scores of the haloperidol treated group decreased between pretest and week 3 of drug treatments. Investigation scores of clozapine treated animals, on the other hand, increased between pretest and week 2, and that increase continued into week 3.

Results of the ANOVA on the open field data, presented in Fig. 3, indicated a statistically reliable difference for the main effect of weeks, $F(3,84)=3.477$, $p<0.05$. Neither the main effect for groups nor for the interaction between groups and weeks were statistically significant. Post hoc analyses of weeks demonstrated that only at week 1 were there reliable differences. Specifically, the control animals were more active in the open field than the other groups. There were no activity differences within or between drug-treated groups. The conclusion is that the differences in social investigation observed in the clozapine and haloperidol treated groups were not simply a result of drug related changes in activity level.

Finally, pretest data for the animals selected as subjects were examined to determine the relation of non-social activity and social investigation. As expected, the correlation coefficient, $r=0.647$, $p<0.01$, was statistically significant. That is, there was a high, positive correlation of timidity of individual animals in the social and the non-social pretest settings.

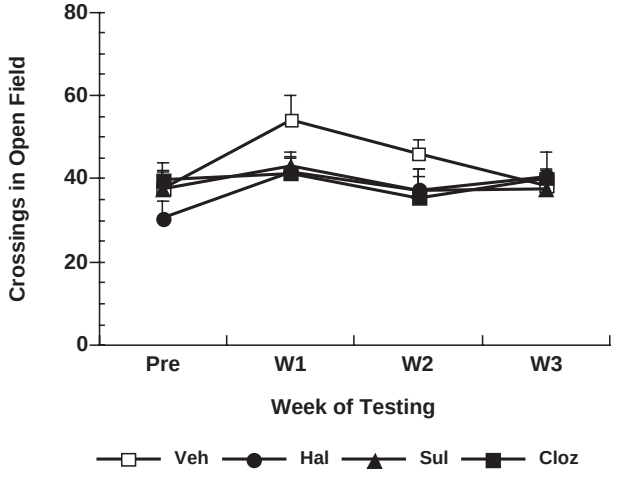

Fig. 3. Locomotor activity in the open field paradigm, measured by numbers of squares crossed, by behaviorally timid female rats chronically exposed to clozapine (Cloz), haloperidol (Hal), sulpiride (Sul) or saline (Veh). Values are means and SEM.

4. General discussion

The findings of Experiment 2 confirmed our working hypotheses. Chronic serotonin antagonism with clozapine, but not dopaminergic antagonism, increased the social investigation by female rats of a conspecific (Corbett et al., 1993). However, this increased level of sociality with clozapine was observed only when BT animals were employed as subjects. Prior to drug exposure, animals in Experiment 2 were selected as presenting as behaviorally timid in tests with unfamiliar social and non-social stimuli.

The logic of using only timid animals was suggested by the results of Experiment 1 that randomly selected animals administered clozapine, haloperidol or sulpiride showed no signs of increased social investigation. Demonstrating a change in social investigation, we reasoned, may require that the animal have an initial tendency to avoid contact with unfamiliar stimuli akin to the asociality characteristic of many schizophrenics (Hendrie et al., 2003; Weiss and Kilts, 1998).

Our paradigm for selecting BT rats was influenced by the classic work of Kagan (Kagan et al., 1988; Woodward et al., 2001) and others (Schmidt and Fox, 1999) who have extensively studied the biological basis of inhibited temperaments in children. One example is the importance of selecting a sample that accurately represents an extreme of a behavior by using multiple behavioral measures to unambiguously identify timid individuals (Kagan et al., 2002). Only animals showing a stable tendency across social and non-social situations met our criterion of timidity in rats. Females were designated as BT only if they scored in the bottom quartile of a large group of over 200 animals in the tendency to approach and investigate unfamiliar stimuli in both social and non-social pretests.

Between group comparisons indicated that BT animals treated with clozapine showed significantly more social interest at week 2 than the other groups, with continued greater social interaction scores in week 3 than haloperidol or sulpiride treated animals (Qiao et al., 2001).

Of particular note was the immediacy of the behavioral changes in clozapine animals. Within group comparisons indicated increased investigation of the stimulus animal after only one week of treatment with the serotonin antagonist. Social interest under clozapine remained high for the remainder of the experiment. Controls also showed a modest increase in social investigation between pretest and the final week of testing. This change likely reflects familiarity with the paradigm and procedures by originally timid animals.

Haloperidol treated animals, on the other hand, significantly decreased their social investigation. Findings in the psychiatric literature suggest a similar conclusion. There is a report of increased social phobia in school children treated with haloperidol for Tourette's syndrome (Mikkelsen et al.,

1981), and decreased dopamine activity is closely associated with schizoid/avoidant behaviors (Blum et al., 1997). The suggestion is that suppression of DA activity in the mesolimbic dopamine brain reward pathways is associated with detached behaviors.

Our results and the findings from other animal (Gao and Cutler, 1992; Lightowler et al., 1994) and human (Arbelle et al., 2003; van Ameringen et al., 2000) studies point to serotonergic system involvement in avoidance of social contact. There are likely to be different underlying causal factors for a shy temperament, behavioral inhibition, social phobia and the asociality of schizophrenics. Nonetheless, there may be similarities in the physiological basis, behavioral expression, and changes with drug treatment in both physiology and behavior. Drugs that target the serotonergic system have been effective in relieving the negative symptoms of schizophrenia (Meltzer et al., 1991), as well as social anxiety in non-schizophrenic patients (Liebowitz et al., 2002; Tancer and Uhde, 1997).

It would seem intuitive that anxiety and behavioral inhibition are closely linked. Timid animals almost certainly exhibit anxiety in approaching a conspecific. However, anxiety is not a reliable predictor of timidity (Heiser et al., 2003; Manuck et al., 2003), and anxious animals may seek social contact to reduce their fear and anxiety (Taylor, 1981). It is nonetheless a reasonable hypothesis to suggest that typical and atypical antipsychotic drugs may differ in their influences on social anxiety and thus timidity. Different anxiolytic profiles for haloperidol and clozapine are commonly reported in the literature with animal models (Dazzi et al., 2004; Millan et al., 1999; Rex et al., 1998; cf. Boulay et al., 2004; Shannon et al., 1999). It is possible that the basis of the increased social investigation by the clozapine animals we observed is the capacity of serotonin antagonists to reduce anxiety. A mechanism suggested recently is that clozapine elevates cortical levels of allopregnanolone, a neurosteroid that is a potent modulator of the GABA receptor (Marx et al., 2003).

The role of serotonin in social behavior has forged a new avenue for the development of animal models of inhibited temperament (Kennett et al., 1997). There is evidence in the literature for a relation of high serotonin levels and social timidity in non-human animals (Corbett et al., 1993; Manuck et al., 2003). Still, our data do not rule out other possible transmitter involvements. For example, animal data suggest conventional and atypical neuroleptics may differentially influence the glutamatergic (Pietraszek et al., 2002) and GABAergic systems (Nechmad et al., 2003), and both systems appear involved in the pathophysiology of schizophrenia.

The conclusion is that it is unlikely that a simple relationship exists between timidity and serotonin or any other single neurotransmitter system. Nonetheless, we believe the methods and animal model presented here could prove to be a useful animal model for investigating the biological underpinnings of BT, as well as for the testing of new pharmaceutical agents on social dysfunction.

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