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Reversal of Clozapine Effects on Working Memory in Rats with Fimbria–Fornix Lesions

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Clozapine is an effective antipsychotic drug, but its effects on cognitive function are unclear. Previously, we found that clozapine caused a working memory deficit, which was reversed by nicotine. Hippocampal systems are important in determining clozapine effect on memory. In the current study, the memory effects of clozapine and nicotine administration were determined in rats with lesions of the fimbria–fornix, a fiber bundle which carries cholinergic and other projections between the septum and the hippocampus. Female Sprague–Dawley rats were trained on a win-shift procedure in the radial-arm maze, in which each arm entry was rewarded once per session. Then, 13 rats received bilateral knife-cut lesions of the fimbria–fornix, while 14 rats underwent sham surgery. The rats were tested after subcutaneous injections with combinations of clozapine (0 and 1.25 mg/kg) and nicotine (0, 0.2, and 0.4 mg/kg). In sham-operated rats, clozapine caused a significant ($P<0.005$) working memory impairment. Fimbria–fornix lesions also caused a significant ($P<0.05$) memory impairment. Interestingly, clozapine had the opposite effect on working memory in the lesioned *vs* sham-operated rats. In contrast to its effects in controls, clozapine (1.25 mg/kg) significantly ($P<0.05$) attenuated the working memory deficit caused by fimbria–fornix lesions. Nicotine (0.2 mg/kg) did not quite significantly improve memory in lesioned rats. The effects of clozapine and nicotine were not additive in the lesioned rats. This study demonstrates the efficacy of clozapine in improving working memory in fimbria–fornix-lesioned rats, whereas it causes impairments in intact rats. Therapeutic treatment with clozapine in people with malfunctions of the hippocampus such as seen in schizophrenia may improve cognitive performance, whereas the same doses of clozapine may impair memory in individuals without hippocampal malfunction.
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INTRODUCTION

Cognitive impairment in schizophrenia is pronounced and includes deficits in attention and memory function (Cornblatt and Keilp, 1994; Gallhofer *et al*, 1996; Sharma and Mockler, 1998; Stip, 1996; Tollefson, 1996). Such impairments appear to be a core feature of schizophrenia (Weinberger and Gallhofer, 1997), but are exacerbated by classic antipsychotic drugs (Gallhofer *et al*, 1996; Levin *et al*, 1996; Stip, 1996). The cognitive effects of atypical antipsychotics such as clozapine may hold promise for improvements in cognitive function; however, the current data regarding clozapine effects on cognitive function are mixed (Hoff *et al*, 1996; Tollefson, 1996). Some studies of people with schizophrenia have shown clozapine-induced cognitive improvements in attentional tasks (Gallhofer *et al*, 1996; Hagger *et al*, 1993; Lee *et al*, 1999; Manschreck *et al*, 1999; McGurk, 1999; Meltzer and McGurk, 1999; Sharma and Mockler, 1998). Other clinical studies, however, have demonstrated clozapine-induced memory deficits (Goldberg *et al*, 1993; Goldberg and Weinberger, 1996). Furthermore, rodent studies have shown clozapine-induced memory impairments in intact rats (Addy and Levin, 2002; Skarsfeldt, 1996).

In discussing memory performance in the schizophrenic population, one must consider the effects of nicotine and antipsychotic co-administration, since a great majority of these individuals smoke cigarettes and thus self-administer nicotine (Herran *et al*, 2000; Hughes *et al*, 1986). They may be self-medicating to combat cognitive impairments that result both from schizophrenia itself and from antipsychotic drug treatment. We have found that people with schizophrenia have significant nicotine-induced improvements in attentional performance when nicotine is administered via skin patch treatment (Levin *et al*, 1996). Nicotine treatment also significantly reduced impairments in memory and

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information-processing speed caused by haloperidol, a typical antipsychotic drug (Levin *et al*, 1996).

Chronic and acute nicotine administration improves working memory performance in rats tested on a radial-arm maze (Bancroft and Levin, 2000; Levin and Simon, 1998). These nicotine-induced improvements are blocked by the nicotinic antagonist mecamylamine (Levin and Simon, 1998). Nicotine acts directly by stimulating nicotinic acetylcholine receptors and also promotes the release of downstream neurotransmitters including dopamine and serotonin (Wonnacott *et al*, 1989). These nicotinic receptors are located in a variety of brain regions including the prefrontal cortex and the hippocampus, both of which are important for memory function (Goldman-Rakic, 1996; Jarrard, 1993; Laroche *et al*, 2000). We have shown that nicotinic receptors in the hippocampus are important for memory performance as local hippocampal infusion of nicotinic receptor antagonists significantly impairs memory function (Arthur and Levin, 2002; Bancroft and Levin, 2000; Bettany and Levin, 2001; Felix and Levin, 1997; Kim and Levin, 1996; Levin *et al*, 2002).

Hippocampal involvement in memory function has also been demonstrated through lesions of the fimbria–fornix, which normally relays hippocampal output to the septum. Fimbria–fornix lesions have been repeatedly shown to cause pronounced memory impairments (Levin *et al*, 1999a; Balse *et al*, 1999; Levin *et al*, 1993). These fimbria–fornix lesion-induced impairments can be reversed by chronic nicotine infusion (Levin *et al*, 1993). Ibotenic acid lesions of the hippocampus have been shown to block chronic nicotine-induced memory improvement (Levin *et al*, 1999b). Therefore, in fimbria–fornix-lesioned animals, nicotine may be acting at intact postsynaptic receptors, which are damaged in ibotenic-acid-lesioned animals. Hippocampal lesions can be used to address the possible hippocampal involvement of nicotine and the effects of atypical antipsychotics on memory performance.

The aim of the current study was to examine hippocampal involvement in the effects of nicotine and clozapine, an atypical antipsychotic, on working memory performance in rats tested on the radial-arm maze. Previous work demonstrated clozapine-induced impairments that were reversed by nicotine coadministration (Addy and Levin, 2002). In the current work, we examined the possibility of hippocampal involvement in such mechanisms by comparing nicotine and clozapine effects on memory performance in rats with and without fimbria–fornix lesions.

METHODS

Subjects

A total of 27 female Sprague–Dawley rats were used in this experiment. The rats were housed three per cage and were on a reverse 12 h light/12 h dark cycle. The subjects were given *ad lib* feeding for 1 week and then kept at approximately 85% of free-feeding levels. Testing took place during the dark cycle. The rats were fed daily after testing. This study was conducted in compliance with the Guide for the Care and Use of Laboratory Animals and approval of the Duke University Animal Care and Use Committee.

Radial-Arm Maze

Cognitive tests were performed using a black, wooden eight-arm radial maze. The maze was elevated 30 cm off the ground with a central platform 35 cm in diameter and eight arms each $10 \times 80\,cm^2$. Each arm contained a food cup, at its terminal end, which was baited during testing with 1/2 piece of Kellogg's Froot Loops® cereal. Then a 30-cm opaque ring was placed on the central platform and a rat was placed inside the ring for 10 s. Following this interval, the ring was removed and timing began. The rat was allowed to run on the maze until all eight arms were entered or until 300 s had passed. An arm entry was recorded when all four of the animal's legs had crossed the threshold of the arm. Choice accuracy was measured by entries to repeat, which was the number of arms entered until a repeat entry was made in the same arm. Latency was recorded as the total time divided by the number of entries. After 18 sessions of training that brought the rats to asymptotic levels of choice accuracy, the rats were divided into two groups matched for choice accuracy so that rats of comparable choice accuracy were put into two different surgery groups.

Fimbria–Fornix Lesions

Fimbria–fornix lesions were performed on approximately half of the rats, while the other rats received sham lesion treatment. The rats were anesthetized with an i.p. injection of ketamine (75 mg/kg) and medetomidine (0.3 mg/kg). The rats were then secured in the stereotaxic instrument. The rat's body temperature was maintained by an electric blanket. The bite bar was elevated at the head at an angle 5 mm above the intra-aural line. An incision was made extending from just behind the eyes to the front of the ears, exposing bregma. After bregma was marked, the targeted area for lesioning was determined using the measurements A/P −0.06 mm, M/L +1.5 to +4.0 mm. The skull was then drilled between these two markers to provide a cavity for the knife-cut lesion. Then the blade was lowered into the brain at one end of the cavity using the measurement D/V −4.5 mm from the dura. To lesion the fimbria–fornix, the knife was moved from one end of the cavity to the other and then lifted out of the skull. The procedure was then repeated on the opposite side of the brain. After both sides had been lesioned, the incision was sutured and the animal was revived with an i.p. injection of antisedan (0.3 mg/kg). Rats in the control sham-lesioned group underwent identical surgical procedures, except that the knife blade was not lowered. After the end of the study, the rats were killed and the brains were examined using histological sectioning and microscopic examination of the extent of the knife-cut lesion tract. It was documented that that the lesions were correctly placed for transecting the fimbria–fornix.

Drug Administration

Starting one week after surgery, each rat received acute injections (SC, injection volume 1 ml/kg) of the drug combinations 20 min prior to testing on the radial-arm maze. Drugs were administered every other day to allow for washout. Clozapine (Research Biochemical International)

and nicotine ditartrate (Sigma) were dissolved in saline, which served as the vehicle, and were injected in combined mixtures. Clozapine HCl was injected at doses of 0 or 1.25 mg/kg, while nicotine was injected at doses of 0, 0.2 or 0.4 mg/kg. The solutions were acidified with 0.1 M HCl to enhance solubility and 0.1 M NaOH was used to rebalance the pH to a target of 7. The doses were calculated as a function of the salt. The six resulting drug combinations were administered in a repeated-measure counterbalanced order.

Data Analysis

The choice accuracy and response latency measures were assessed by a mixed between- and within-subjects design ANOVA. The between subjects factors were lesion (sham lesion *vs* fimbria–fornix lesion) and the cohorts of rats (each of the three cohorts included sham-lesioned and lesioned rats). The total *N* was 27 (14 sham and 13 lesioned). The within-subjects factors were nicotine and clozapine administration. A *p*-value of less than 0.05 (two-tailed) was considered significant. Planned comparisons were made between the sham lesion + vehicle condition and the fimbria–fornix lesion + vehicle condition, as well as with the sham lesion + each of the drug treatments and between the fimbria–fornix lesion + vehicle condition and each of the drug treatment conditions within the lesioned groups.

RESULTS

Choice Accuracy

There was a significant clozapine × lesion interaction ($F(1,21) = 8.76$, $p < 0.01$). As shown in Figure 1, clozapine had opposite effects on working memory performance in rats with sham lesions and those with fimbria–fornix lesions. With the sham-operated rats, there was a significant ($F(1,11) = 10.36$, $p < 0.01$) clozapine-induced memory deficit. With 1.25 mg/kg clozapine, the rats averaged 4.81 ± 0.31 entries to repeat, while without clozapine they averaged 5.93 ± 0.37 entries to repeat. Nicotine cotreatment was not seen to alter this effect in this study. The opposite effect was seen in the rats with fimbria–fornix lesions; 1.25 mg/kg clozapine given alone significantly ($F(1,10) = 7.58$, $p < 0.025$) improved the working memory performance from 4.31 ± 0.28 with saline injections to 5.54 ± 0.37 with 1.25 mg/kg clozapine. Nicotine cotreatment did not potentiate this improvement. Rather, nicotine cotreatment reduced the beneficial effect of clozapine such that with the 0.4 mg/kg nicotine dose and 1.25 mg/kg clozapine the choice accuracy performance was only 4.94 ± 0.33 entries to repeat.

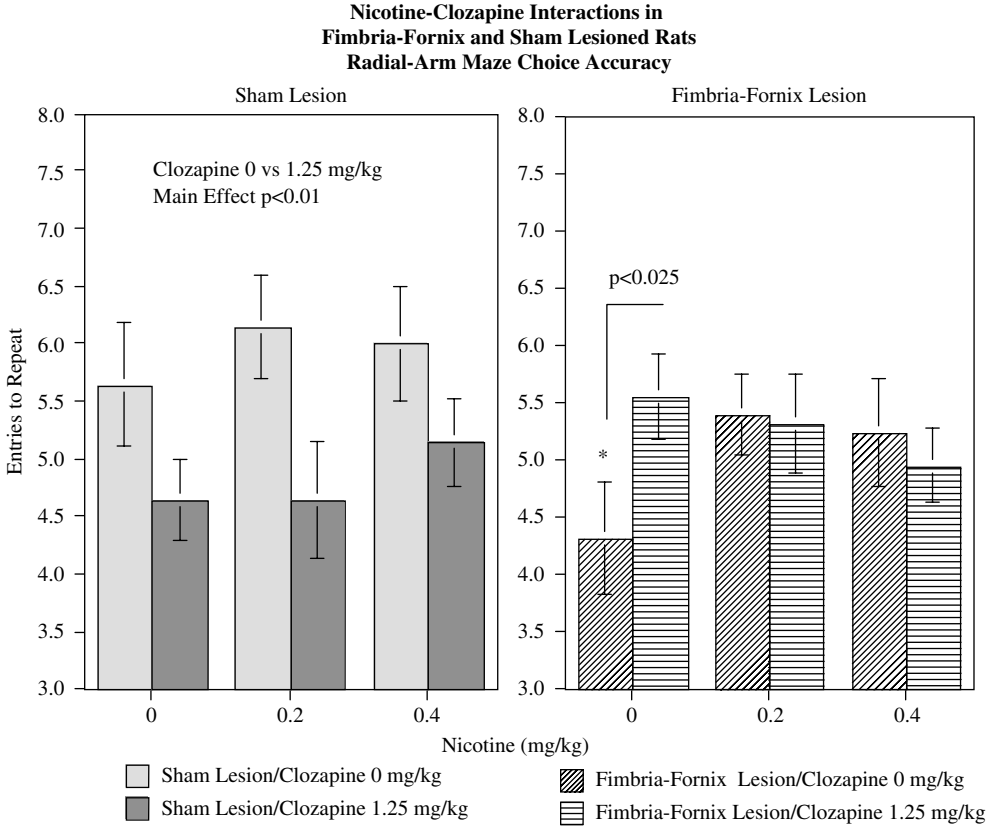

Figure 1 Choice accuracy (entries to repeat) with nicotine and clozapine treatment in rats with fimbria–fornix and sham lesions (mean ± SEM).

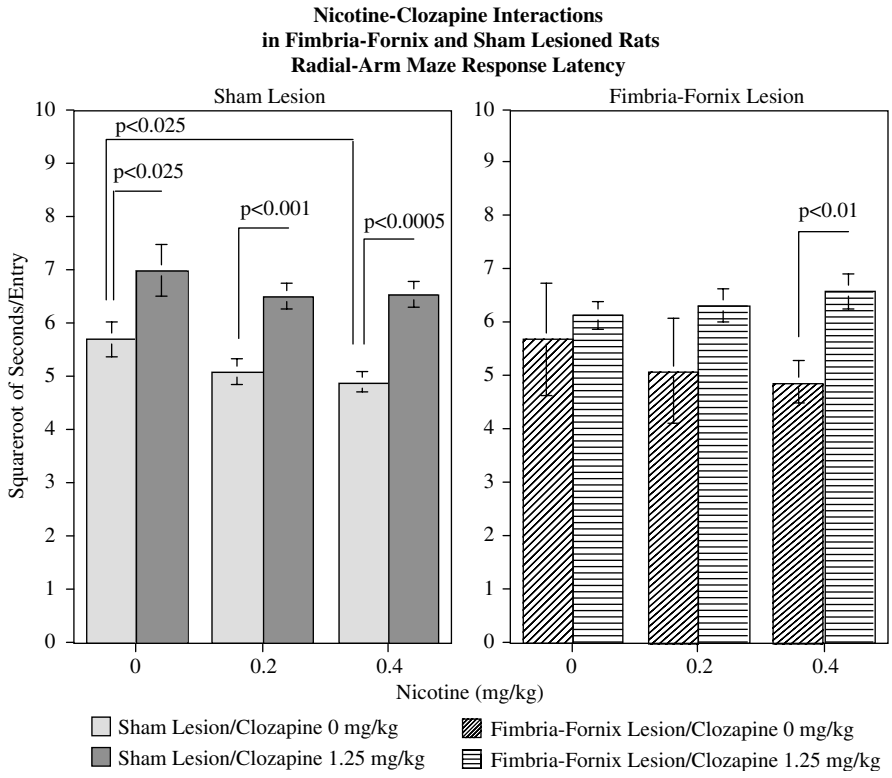

Figure 2 Choice latency (seconds per entry) with nicotine and clozapine treatment in rats with fimbria–fornix and sham lesions (mean ± SEM).

Response Latency

There was a significant ($F(1,21) = 6.62$, $p < 0.025$) clozapine × lesion interaction with response latency. The sham-operated rats had a significant ($F(1,11) = 41.89$, $p < 0.001$) clozapine-induced slowing of response from 28.2 s per entry without clozapine to 45.9 s per entry with 1.25 mg/kg of clozapine. As shown in Figure 2, clozapine caused significant increases in latency in the sham-operated group when administered alone ($p < 0.025$) or in conjunction with 0.2 mg/kg ($p < 0.001$) or 0.4 mg/kg of nicotine ($p < 0.0005$). The higher nicotine dose also was seen to significantly ($p < 0.025$) lower the response latency in the sham-operated group. This nicotine effect on latency was clearly significant, but was modest in extent. In contrast, in the rats with fimbria–fornix lesions, the only significant clozapine increase in latency was seen when clozapine was given together with 0.4 mg/kg of nicotine ($p < 0.01$), and no nicotine effects on response latency were seen.

DISCUSSION

The results illustrate the differential memory effects of clozapine in fimbria–fornix-lesioned *vs* nonlesioned rats. Clozapine impaired memory in nonlesioned rats, whereas it significantly attenuated the memory impairment caused by fimbria–fornix lesions. Acute nicotine administration did not significantly interact with these effects, though there was a higher average memory performance level with nicotine treatment in the lesioned animals. The clozapine-induced memory impairment in intact rats replicated our earlier study showing this effect in the radial-arm maze (Addy and Levin, 2002), as well as a complementary finding showing attentional impairment in rats given clozapine, in the same dose range (Rezvani *et al*, 2004). The fimbria–fornix lesion-induced working memory impairment also replicated earlier findings (Levin *et al*, 1999a, 1993). The most important finding of the current study was that not only was the clozapine-induced memory impairment eliminated by the fimbria–fornix lesion, but also that clozapine treatment reversed the memory impairment caused by this lesion.

Clozapine Effects at Diverse Receptors

Clozapine has antagonistic effects at a variety of transmitter receptors important for memory function, including dopaminergic, serotonergic and muscarinic acetylcholinergic receptors. All of these actions may underlie the differential effects of clozapine in rats with and without fimbria–fornix lesions.

One of the most critical effects of clozapine with regard to memory function may be its antagonism of muscarinic acetylcholinergic receptors (Michal *et al*, 1999). Muscarinic receptors, particularly those in the hippocampus (Kim and Levin, 1996; Ohno *et al*, 1994), are critically important for working memory function. The fimbria–fornix lesion interrupts the cholinergic innervation of the hippocampus from the septum. The reversal of clozapine effects on memory in rats with fimbria–fornix lesions may have been due to this interruption of the cholinergic innervation of hippocampal muscarinic receptors, obviating the muscarinic antagonist effects of clozapine in the hippocampus.

Other receptor subtypes are also implicated in the mechanism of clozapine-induced modulation of memory performance. Like many of the typical antipsychotic medications, clozapine acts as an antagonist at the D_2 dopaminergic receptor (Burns, 2001). In contrast to typical antipsychotics, clozapine also blocks D_4 receptors and 5-HT_2 serotonergic receptors (Burns, 2001; Matsubara *et al*, 1993). Both D_2 and D_4 ligands have been shown to significantly affect memory performance (Bernaerts and Tirelli, 2003; Levin, 1997; Levin *et al*, 1996; McGurk *et al*, 1988). In previous work, we demonstrated that antagonism of D_2 receptors in the ventral hippocampus by infusions of the D_2 antagonist raclopride, causes working memory impairments in rats (Wilkerson and Levin, 1999). Umegaki *et al* (2001) have also demonstrated T-maze memory performance deficits after hippocampal infusion of D_2 antagonists. D_4 receptor antagonist effects of clozapine may contribute the differential effects seen in the fimbria–fornix-lesioned and sham-operated rats in the current study. Zhang *et al* (2004) found that a selective D_4 receptor antagonist impaired working memory performance in rats performing at high levels of accuracy, but improved working memory performance in rats with poor baseline performance levels. This is similar to the finding in the current study.

The serotonergic system has also been implicated in memory function. Several studies have demonstrated that either serotonin depletion or antagonism of serotonergic receptors leads to memory deficits, particularly in working memory (Cassaday *et al*, 2003; Lieben *et al*, 2004; Pache *et al*, 2003; Park *et al*, 1994; Porter *et al*, 2003).

Serotonergic receptors within the hippocampus, primarily $5HT_1$ subtype receptors, have also been shown to modulate memory performance (Buhot, 1997). Stimulation of 5-HT_{1A} or 5-HT_{1B} serotonergic receptors in the hippocampus impairs memory performance (Buhot *et al*, 1995; Warburton *et al*, 1997). Blockade of 5-HT_{1A} receptors in the hippocampus, in contrast, has been shown to attenuate scopolamine-induced impairments in memory performance (Carli *et al*, 1997; Ohno and Watanabe, 1996). Clozapine has agonist effects at 5-HT_{1A} receptors (Chou *et al*, 2003) that may partially underlie its memory impairing effects in normal animals. Its antagonistic effects at 5-HT_2 receptors (Burns, 2001) may also contribute to this effect as well. Thus, we suggest clozapine activity in the hippocampus contributes to decreased working memory performance primarily through muscarinic cholinergic blockade, although dopaminergic antagonism and complex serotonergic effects may contribute as well.

Lesions of the Fimbria–Fornix

When the fimbria–fornix was lesioned, clozapine administration led to a working memory improvement, illustrating that clozapine can still modulate memory performance in the absence of the septo-hippocampal projection. We propose that the clozapine-induced improvement in working memory is due to clozapine activity in other regions of the brain outside the hippocampus. The prefrontal cortex, in particular, has been shown to be important for working memory performance (Goldman-Rakic, 1996). Thus, in the absence of the septohippocampal projection, clozapine effects in the prefrontal cortex could exert a larger influence on working memory and produce the clozapine-induced working memory improvement in lesioned animals. In support of this mechanism, clozapine has been shown to increase acetylcholine release in the hippocampus and prefrontal cortex, possibly due to presynaptic blockade of muscarinic acetylcholine receptors (Parada *et al*, 1997; Shirazi-Southall *et al*, 2002). In the prefrontal cortex, this clozapine-induced increase in acetylcholine is suggested to be related to 5-HT receptor interactions (Ichikawa *et al*, 2002). Furthermore, this acetylcholine increase in the cortex has been observed during a working memory task (Hironaka *et al*, 2001). In rodent studies, aged mice show a decrease in cortical acetylcholine, which is accompanied by an increase in working memory errors (Ikegami, 1994). In monkeys, increases in cortical acetylcholine accompany working memory improvements (Tsukada *et al*, 2004). Given the literature and our results, we suggest that the clozapine-induced memory performance improvement in the fimbria–fornix-lesioned animals may result from clozapine-induced increases in cortical acetylcholine.

Acute nicotine treatment like acute clozapine showed an effect of reducing the fimbria–fornix lesion-induced memory impairment. However, coadministration of nicotine with clozapine in the lesion group did not further reduce the lesion-induced memory impairment. If anything, nicotine may have attenuated the beneficial effects of clozapine. Curiously, in the current study, nicotine did not attenuate the clozapine-induced memory impaired in the sham-operated group. Previously, in unoperated rats, we found that 0.4 mg/kg of nicotine effectively attenuated the memory impairment caused by 1.25 mg/kg of clozapine (Addy and Levin, 2002). In the current study, we did not find this effect. There was a slight increase in accuracy with 0.4 mg/kg nicotine given to sham-operated rats with 1.25 mg/kg of clozapine, but this was not nearly significant. There may have been some carryover from the surgical procedure and anesthesia that attenuated the effect.

The fimbria–fornix lesions were used as a test of the role of septohippocampal connections in the effect of clozapine and nicotine. Hippocampal innervation, particularly cholinergic innervation, has been long known to be critically important for working memory function. Further studies will characterize the role of other areas in these effects of nicotine and clozapine on memory function. We felt that it was important to first characterize the effects of acute clozapine. Later studies will address the effects of chronic clozapine, which will provide a closer approximation to the clinical situation.

Summary

The current results show that the fimbria–fornix connections are important for the actions of clozapine on memory function. Clozapine impairs memory function in intact subjects. This effect appears to depend on the septohippocampal connections. Fimbria–fornix lesions not only blocked the clozapine-induced memory impairment, clozapine reversed the fimbria–fornix lesion-induced memory impairment to control levels of performance. This finding both adds to the basic understanding of the mechanisms of memory function and to the understanding of the clinical

effects of clozapine on memory. In patients with schizophrenia who are given clozapine, the extent of hippocampal dysfunction may be critical in determining clozapine effects on cognition. Clozapine may be useful in attenuating cognitive impairment in people with hippocampal dysfunction, but may be contraindicated in those with intact hippocampal functioning because of cognitive impairment. Assessment of the regional integrity of brain function may be critical for determining optimal therapeutic drug treatment.

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