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Chronic Underactivity of Medial Frontal Cortical β 2-Containing Nicotinic Receptors Increases Clozapine-Induced Working Memory Impairment in Female Rats

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Abstract

Nicotinic receptor decreases in the frontal cortex and hippocampus are important mediators of cognitive impairment in both schizophrenia and Alzheimer's disease. Drug treatments for these diseases should take into account the impacts of compromised brain function on drug response. This study investigated the impact of compromised nicotinic receptor activity in the frontal cortex in rats on memory function. Since both Alzheimer's disease and schizophrenia can involve psychosis, antipsychotic drugs are often given. The impacts of antipsychotic drugs on cognitive function have been found to be quite variable. It is the hypothesis of this and previous studies that the cognitive effects of antipsychotic drugs on cognitive function depend on the integrity of brain systems involved in cognition. Previously in studies of the hippocampus, we found that chronic inhibition of β 2-containing nicotinic receptors with dihydro- β -erythrodine (DH β E) impaired working memory and that this effect was attenuated by the antipsychotic drug clozapine. In contrast, chronic hippocampal α 7 nicotinic receptor blockade with methyllycaconitine (MLA) potentiated the clozapine-induced memory impairment which is seen in rats without compromised nicotinic receptor activity. The current study determined medial frontal cortical α 7 and β 2-containing nicotinic receptor involvement in memory and the interactions with antipsychotic drug therapy with clozapine. Chronic DH β E and MLA infusion effects and interactions with systemic clozapine were assessed in female rats tested for memory on the radial-arm maze. Antipsychotic drug interactions with chronic systemic nicotine were investigated because nicotinic procognitive treatment has been proposed. The same local infusion DH β E dose that impaired memory with hippocampal infusion did not impair memory when infused in the medial frontal cortex. Frontal DH β E infusion potentiated clozapine-induced memory impairment, whereas previously the memory impairment caused by hippocampal DH β E infusion was attenuated by clozapine. Frontal cortical MLA infusions at a dose that previously was found to potentiate the clozapine-induced memory impairment with hippocampal infusion had no significant effect when infused into the medial frontal cortex. The location and subtype of nicotinic receptor underactivity are critical determinates for clozapine effects on memory. Patients with hippocampal β 2-containing nicotinic receptor loss may be well treated with clozapine therapy, while those with frontal cortical β 2-containing receptor loss may potentiate the memory impairment caused by clozapine.

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Keywords

Clozapine; Frontal Cortex; $\alpha 7$ Nicotinic Receptors; $\alpha 4\beta 2$ Nicotinic Receptors; Memory

Introduction

Nicotinic acetylcholine receptors have been shown in a wide variety of studies in rodents, monkeys and humans to play crucial roles in memory function (Levin et al. 2006). Nicotinic receptor expression decreases seen in Alzheimer's disease (Kellar and Wonnacott 1990; London et al. 1989; Perry et al. 1987; Schröder et al. 1991) and schizophrenia (Durany et al. 2000; Guan et al. 1999), which are both diseases with memory impairment (Baddeley et al. 1991; Tollefson 1996). Nicotinic receptors are located in areas of the brain known to be critical substrates for memory processing including the limbic system structures such as the hippocampus as well as the frontal cortex (Levin et al. 2006). The specific roles of nicotinic receptor subtypes in each of these areas with memory function are now in the process of being discovered.

Nicotinic receptor losses in the hippocampus and frontal cortex are likely to underlie the cognitive decline in Alzheimer's disease (Kendziorra et al. 2006; London et al. 1989; Schröder et al. 1991). The location of the greater amount of nicotinic receptor loss may be a critical determinant of the nature of the cognitive deficits and the nature of therapeutic drug response. With schizophrenia there are also substantial cognitive declines (Tollefson 1996) as well as decreased nicotinic receptor number in these areas (Mihailescu and Drucker-Colin 2000; Leonard et al. 2002; Martin et al. 2004).

The importance of nicotinic receptors in particular brain regions for cognitive function can be discovered with local infusions of antagonists. The majority of work has been done with the hippocampus, since it has been shown to be critically involved in memory function (Becker et al. 1980; DeLong 1992; Izquierdo et al. 1993). Local infusions of the nonspecific nicotinic receptor blocker mecamylamine into the ventral hippocampus has been found to cause working memory impairment (Levin et al. 2002; Ohno et al. 1993). Acute local infusions of either the dihydro- β -erythrodine (DH β E) a $\beta 2$ -containing nicotinic receptor antagonist (Alkondon and Albuquerque 1995) or methyllycaconitine (MLA) an $\alpha 7$ nicotinic antagonist (Alkondon et al. 1992) into the ventral or dorsal hippocampus caused memory impairment (Bancroft and Levin 2000; Bettany and Levin 2001; Levin et al. 2002; Nott and Levin 2006). Nicotinic receptors in the medial frontal cortex are also important for working memory function as shown by the memory impairing effects of acute local infusions of $\alpha 7$ and $\alpha 4\beta 2$ antagonists (Chan et al. 2007; Granon et al. 1995).

Chronic underactivity of nicotinic $\alpha 7$ and $\beta 2$ -containing nicotinic receptor subtypes in particular brain regions schizophrenia and Alzheimer's disease (Lawrence and Sahakian 1998; Mihailescu and Drucker-Colin 2000) can be modeled by chronic local infusion of selective nicotinic antagonists. This is the tact that we have taken with our previous studies in which we found that four-week long infusions of DH β E into the ventral hippocampus continued to cause significant memory impairment in rats (Arthur and Levin 2002; Pocivavsek et al. 2006). In the current study we extended this investigation into the medial frontal cortex.

When considering cognitive function in syndromes with psychosis it is important to understand interactions of nicotinic receptor systems with the functional effects of antipsychotic drugs. Psychosis is a hallmark of schizophrenia. Therefore, the impact of antipsychotic drugs in the face of regional nicotinic receptor loss is important to understand. Often overlooked is the frequent appearance of delusions and psychosis with Alzheimer's disease often treated with

antipsychotic drugs including clozapine (Stoppe et al. 1999). Given that antipsychotic drugs are often given to patients with Alzheimer's disease as well as those with schizophrenia, as with schizophrenia it is important to determine the impact of antipsychotic drugs in the face of regional nicotinic receptor loss in schizophrenia and Alzheimer's disease.

We have found that in rats without lesions or receptor blockade that clozapine causes impairments in working memory and attention, while in impaired rats clozapine can help reverse cognitive deficits (Addy and Levin 2002; Addy et al. 2005; Rezvani et al. 2004; Rezvani et al. 2008). The varying patterns of nicotinic receptor loss may play a role. Chronic underactivity of $\beta 2$ -containing nicotinic receptors in the ventral hippocampus, achieved with chronic local infusions of DH β E, caused a memory impairment that was significantly attenuated by systemic clozapine administration. In contrast, chronic underactivity of $\alpha 7$ -containing nicotinic receptors in the ventral hippocampus, achieved with chronic local infusions of MLA potentiated the amnesic effects of clozapine (Pocivavsek et al. 2006). Thus, the specific subtype of nicotinic receptor loss in the hippocampus made considerable impact on the nature of the cognitive effects of clozapine treatment.

The current study was conducted to determine the roles medial frontal cortical $\alpha 7$ and $\alpha 4\beta 2$ nicotinic receptors play in working and reference memory. The interactive effects of clozapine on memory in presence of normal and decreased frontal cortical nicotinic $\alpha 7$ and $\beta 2$ -containing nicotinic receptor activity was assessed to identify its differential utility in conditions with deficits of nicotinic receptors in the frontal cortex. The interaction of chronic systemic nicotine with these effects was studied because the great majority of people with schizophrenia smoke cigarettes (Hughes et al. 1986).

Methods

Subjects

Adult female Sprague-Dawley rats were used in the present study (N=72). Female rats were used because of the history of using females in the series of studies (Pocivavsek et al. 2006) so that the results could be directly compared. Females have been used in this series of studies because with chronic studies they keep a relatively constant weight better than males, with the use of potentially sedative drugs they are more likely to continue to perform on the radial-arm maze and the fact that the majority of people with Alzheimer's disease are female (Miech et al. 2002). We did not artificially disrupt the estrus cycle of the rats by removing the ovaries or administering hormones. Rather the chronic study involved repeated testing spanning many estrus cycles with average data analyzed. The rats were maintained on a reverse 12-hour on: 12-hour off light cycle, with all testing conducted during the dark phase. This was done because rats sleep more during the light phase and testing then would introduce greater disruption of their normal sleep cycle. They were housed in metal cages with wood shavings, three rats per cage pre-cannulation and one rat per cage post-cannulation. They had *ad lib* access to water and were fed approximately 15-20 g/day of Purina rat chow daily following testing. This feeding schedule was kept throughout the shaping, training and experimental periods. Prior to testing, each rat was handled for two sessions of 5-10 minutes to provide habituation to handling by the experimenter. During this handling period, twelve pieces of bait, half-pieces of the sweetened cereal Kellogg's Froot Loops[®] (Kellogg, Battle Creek, MI, USA), were provided per animal per day in the housing cages.

Radial-Arm Maze

Testing was conducted on an 8-arm radial maze constructed of wood and painted black. The maze was elevated 30 cm from the floor, the central platform was 50 cm in diameter, and each of the radial arms measured 10 cm $\times$ 60 cm. The food cup was located 2 cm from the end of

each arm. The maze was located in a room with ample stable extramaze visual cues. Rats were shaped for two sessions. Each session consisted of confining the rat to the central platform within the open-topped cylindrical arena in the center of the maze. Each of the arms was baited with a half of a Froot Loop®. The rat was allowed to freely navigate the maze until all pieces had been consumed or 5 minutes had elapsed. The procedure for running the rats on the maze was standardized for the duration of both the training and drug testing phases. An entry was scored whenever all four paws crossed the entrance of the arm. The choice accuracy was indexed by the number of correct entries until the first error (entries to repeat). The response latency was indexed by the number of seconds per arm entry. The square root transformation of the latency scores was made to reduce the typical skewed variance of this measure.

Cannulation

Following at least 18 training sessions during which no drugs were administered, the rats were cannulated in the medial frontal cortex. The target coordinates from bregma used for the medial frontal cortex were: AP +3.2, ML $\pm$ 0.5, DV -1.5 according to the rat brain atlas of Pellegrino (Pellegrino et al. 1979). Rats were anaesthetized with an IP injection of 75 mg/kg of ketamine combined with 15 mg/kg of Domitor (medetomidine HCl). The rat was placed on an electric heating pad to maintain body temperature and secured in a stereotaxic instrument (David Kopf instruments, Tujunga, CA, USA) with ear and bite bars that held the head elevated 5 mm above the intra-aural line. Fur was shaved from the rat's head and the skull was exposed with a lengthwise incision. Coordinates were measured from bregma, and appropriate cannulae holes were drilled into the skull. Posterior to these holes, four screws were fixed and tied together with wire to anchor the protective cranioplastic cap that would be built. Steel 22-gauge guide cannulae were lowered to the aforementioned coordinates and cranioplastic cement was applied to secure them and cover the opened area, forming a protective cap. Dummy cannulae were placed in the guide cannulae to prevent infection or blockage. After completion of the surgery, 15 mg/kg of Antisedan (atipamezole HCl) was administered intraperitoneal (IP) to revive the animal. The rats were allowed to rest for a full week after surgery. One to two post-surgery trials were then performed to ensure that the rats did not possess significant surgery-related deficits and two trials with infusions of artificial cerebrospinal fluid (aCSF) were conducted to adjust them to the infusion procedure.

Drug Administration

There were six nicotinic drug conditions comprised of three chronic local medial frontal cortical infusions: aCSF infusion, 83 μ g/side/day of MLA or 100 μ g/side/day of DH β E crossed with two chronic systemic nicotine ditartrate infusions levels (0 or 5 mg/kg/day calculated as of the base weight). The group sizes are listed below.

Group Sizes for Nicotinic Drug Treatment Conditions			
		Chronic Systemic Nicotine (mg/kg, sc)	
		0	5
Frontal			
Cortical	aCSF	14	16
Nicotinic	MLA	10	12
Antagonist	DH β E	10	10

These drugs were infused with osmotic minipumps (Alzet Model 2ML4, infusion rate 2.5 μ l/hr) for the subcutaneous (SC) nicotine infusion and two Alzet Model 2004 pumps for the bilateral local infusions, infusion rate 0.25 μ l/hr for each pump). The pumps delivering systemic nicotine administration were implanted in a subcutaneous pocket made under the skin between

the scapulae. The pumps delivering local infusions of nicotinic antagonists were connected to the infusion cannulae via PE tubing and implanted subcutaneously behind the neck of the rats. Saline was the vehicle for the systemic drug administration and aCSF was the vehicle for the local infusions. The vehicles were used for the control infusions. The local infusions of nicotine into the medial frontal cortex were included to model the effects of chronic nicotinic receptor loss in this area. Chronic systemic nicotine was included in the design because the large majority of people with schizophrenia smoke tobacco and we wanted to determine possible interactions of this drug taking with local nicotinic receptor antagonist infusion and systemic clozapine effects on memory. The infusions lasted four weeks. For the first week of infusion after surgery the rats were given time for recovery and thus were not tested. During the following three weeks the rats were tested three times/week with s.c. injections of clozapine (0, 1.25 and 2.5 mg/kg in a volume of 1 ml/kg) being given in a counterbalanced order. Clozapine was included as a benchmark atypical antipsychotic drug quite effective in the treatment of schizophrenia. The doses used have been previously shown in our studies to span the threshold from being near threshold to being clearly effective in causing memory impairments in the radial-arm maze without causing substantial sedation (Addy and Levin 2002; Addy et al. 2005; Pocivavsek et al. 2006). Saline was the vehicle and served as the control injection. Injections were administered 20 minutes before testing on the radial-arm maze.

Histology

Following the completion of all drug treatments, the rats were killed and the cannula placements were determined. To sacrifice the rats, they were anaesthetized with sodium pentobarbital. Chicago sky-blue dye diluted in saline (1 μ l/side) was then infused to highlight the area of drug infusion. Then, the rats were deeply anesthetized with pentobarbital, killed by exsanguination and perfused with a 9% phosphate buffered saline solution followed by a 4% formaldehyde solution. The brain was removed and stored in 4% formaldehyde. The brain was later frozen on dry ice and sliced with a cryostat in 20 micron sections. Histological slides were made and examined to confirm appropriate cannula placement. Only those subjects with both cannula placements within the target area (Fig. 1) were included in the data analysis.

Statistics

Analysis of variance (ANOVA) was performed with working memory errors, reference memory errors and latency dependent measures. Between subjects factors were local medial frontal cortical nicotinic antagonist infusion and systemic nicotine administration. The within subjects factor was acute clozapine injection. Data across weeks of testing was averaged for analysis. Planned comparisons were made between the treated groups and controls. A cut-off of $p < 0.05$ (two-tailed) was used as the threshold for statistical significance.

Results

Choice Accuracy

The effects of systemic clozapine injections and local frontal cortical nicotinic antagonist infusions on working memory performance are shown in figure 2. There was a significant main effect of clozapine ($F(2,132)=16.27$, $p < 0.0001$) lowering choice accuracy. Both the 1.25 mg/kg ($F(1,132)=6.69$, $p < 0.025$) and the 2.5 mg/kg ($F(1,132)=32.45$, $p < 0.0001$) clozapine doses significantly impaired choice accuracy (Clozapine 0 mg/kg= 6.00 ± 0.17 , Clozapine 1.25 mg/kg= 5.55 ± 0.18 and Clozapine 2.5 mg/kg= 5.07 ± 0.16 entries to repeat). None of the other main effects were significant. The subjects receiving the chronic nicotine treatment with no other treatment had slightly higher mean accuracy scores than subject receiving the saline vehicle (saline= 5.82 ± 0.44 , nicotine= 6.07 ± 0.40 entries to repeat), but there was no significant nicotine effect or interaction. The figures show data averaged across the nicotine factor, but both nicotine-treated rats and their controls were included in all analyses. There was a significant

clozapine $\times$ nicotinic antagonist interaction ($F(4,132)=2.53$, $p<0.05$) indicating a differential effect of the nicotinic antagonist treatment in conjunction with the clozapine treatments. Tests of the simple main effects of nicotinic antagonist at each of the clozapine doses showed that DH β E significantly ($F(1,132)=5.72$, $p<0.025$) potentiated the memory impairment caused by the 2.5 mg/kg clozapine dose. Figure 2 shows the results of the nicotinic $\times$ clozapine interaction averaged across systemic nicotine administration. This effect was clearly evident even though the DH β E infusions into the frontal cortex did not affect choice accuracy when clozapine was not co-administered. In contrast, the MLA treatment was not found to cause any significant effect on choice accuracy either with or without clozapine. Figure 3 shows the complete array of mean accuracy data in the different treatment groups including acute clozapine injections, chronic local infusions of nicotinic antagonists and chronic systemic nicotine infusion. After withdrawal, no significant effects of previous exposure to nicotinic antagonists or nicotine were seen. There continued to be a significant working memory impairment caused by continued acute injections of clozapine. The 2.5 mg/kg clozapine dose (5.29 ± 0.37 entries to repeat) caused a significant ($p<0.025$) decrease in accuracy relative to control (6.19 ± 0.34 entries to repeat). The 1.25 mg/kg clozapine dose (5.94 ± 0.38 entries to repeat) was not significantly different from control.

Response Latency

Clozapine caused a significant main effect ($F(2,132)=36.03$, $p<0.0001$) on response latency. Both the 1.25 mg/kg ($F(1,132)=38.14$, $p<0.0001$) and the 2.5 mg/kg ($F(1,132)=66.12$, $p<0.0001$) clozapine doses caused a significant increase in response latency (Clozapine 0 mg/kg = 5.56 ± 0.15 , Clozapine 1.25 mg/kg = 6.84 ± 0.21 and Clozapine 2.5 mg/kg = 7.28 ± 0.22 square root of seconds per entry) As shown in figure 4 this slowing was not different in the aCSF, MLA and DH β E infused groups. None of the other main effects or interactions were significant with regard to response latency. The figure includes all data including subjects receiving nicotine or vehicle. Since nicotine treatment did not cause a significant effect the graph shows the data averaged across the nicotine factor. The nicotinic antagonist infusions also did not have a significant effect on response latency. The figure shows the breakdown of these groups to facilitate comparison with the nicotinic antagonist interactions with clozapine on choice accuracy (Fig. 2), which were significant.

Discussion

Chronic blockade of β_2 -containing nicotinic receptors of the medial frontal cortex significantly increased the amnesic effects of systemic clozapine administration. The effect of a challenge systemic dose of 2.5 mg/kg of clozapine caused significantly greater memory impairment in rats chronically administered DH β E an $\alpha_4\beta_2$ antagonist into the medial frontal cortex compared to the effect of the same systemic clozapine dose given to rat infused with the vehicle aCSF into the medial frontal cortex. The current study replicated the consistent finding of memory impairment caused by clozapine administration in intact subjects (Addy and Levin 2002; Addy et al. 2005; Pocivavsek et al. 2006). We have also found clozapine to significantly impair attentional performance in intact subjects (Rezvani et al. 2006; Rezvani and Levin 2004). Clozapine effects on cognition depend on the integrity of the neural systems underlying cognitive function. Clozapine improves memory performance caused by hippocampal dysfunction caused either by lesions of the fimbria-fornix (Addy and Levin 2002) or chronic local infusions of the $\alpha_4\beta_2$ nicotinic antagonist DH β E into the ventral hippocampus (Pocivavsek et al. 2006). Both of these interventions caused significant memory impairment, which was attenuated by clozapine. The current study extended investigation into the role of frontal cortical nicotinic receptor interactions with clozapine effects on memory. In contrast to the findings in the hippocampus, DH β E infused into the medial frontal cortex at the same dose as previously in the hippocampus did not cause significant working memory impairment

on the radial-arm maze. Also, in contrast to the results in the hippocampus chronic DH β E infusion in the medial frontal cortex significantly potentiated the memory impairment caused by clozapine.

No drug is completely specific to a particular receptor. Certainly clozapine is known to bind to a variety of receptors (Seeman 2002). The nicotinic antagonists used MLA and DH β E may have some cross-reactivity with regard to different nicotinic receptor subtypes, but their differential effects seen on memory function supports the contention that their critical pharmacodynamic effects were mediated through distinct mechanisms.

The potentiation of the clozapine-induced memory impairment by medial frontal cortical DH β E infusion was specific to the choice accuracy effects of clozapine inasmuch as the sedating effect of clozapine was not affected by the frontal cortical DH β E infusion. This is similar to our earlier work with hippocampal infusions of nicotinic antagonists in which DH β E infusions significantly impacted clozapine effects on memory performance but not response latency (Pocivavsek et al. 2006).

MLA chronically infused into the medial frontal cortex did not significantly potentiate the memory impairment caused by clozapine as it did when infused into the ventral hippocampus (Pocivavsek et al. 2006). The differential effects of the same MLA doses infused into the ventral hippocampus and into the medial frontal cortex point to differential involvement of $\alpha 7$ receptors in these areas with regard to cognitive function. Like all drugs MLA and DH β E have effects in addition to their primary actions on specific receptor subtypes. At high local concentrations there could be some cross-reactivity of these drugs to other nicotinic receptors subtypes. However this possible effect did not appear to compromise the differential effects of these two antagonists. Their primary effects blocking $\alpha 7$ and $\beta 2$ -containing nicotinic receptors respectively appear to predominately underlie provide their differential behavioral effects.

In this study nicotine did not cause a significant improvement in working memory performance. This is also similar to our earlier work with hippocampal infusions of nicotinic antagonists in which chronic systemic nicotine did not cause a significant improvement in memory performance despite a modest increase in mean choice accuracy scores (Pocivavsek et al. 2006). It may be the case that the full effectiveness of chronic nicotine for improving memory performance is attenuated by the stereotaxic neurosurgery involved in placement of chronic infusion cannulae. Previously, in a variety of studies we have found that in rats that did not have brain implants of infusion cannulae that this same dose of chronic nicotine infusion caused a significant improvement in memory performance on the radial-arm maze (for review see (Levin et al. 2006))

This study adds to the evidence that nicotinic receptor underactivity in different areas of the brain play different roles in the neural substrate of memory function. Chronic blockade of $\beta 2$ -containing nicotinic receptors in the ventral hippocampus significantly impairs memory function while the same dose in the medial frontal cortex has no discernible effect. Importantly, nicotinic $\beta 2$ -containing receptor underactivity in these two areas also had opposite interactions with the antipsychotic drug clozapine with regard to memory function. The effect of clozapine on memory was significantly reversed by $\beta 2$ -containing nicotinic receptor underactivity in the ventral hippocampus while clozapine induced amnesic effects were significantly exacerbated by $\beta 2$ -containing nicotinic receptor underactivity in the medial frontal cortex. Patients with hippocampal $\beta 2$ -containing nicotinic receptor loss may be well treated with clozapine therapy, while those with frontal cortical $\beta 2$ -containing receptor loss may potentiate the memory impairment caused by clozapine.

Acknowledgments

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Abbreviation List

ANOVA	Analysis of variance
aCSF	Artificial cerebrospinal fluid
DHβE	Dihydro- β -erythrodine
IP	Intraperitoneal
MLA	Methyllycaconitine
SC	Subcutaneous

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A.

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B.

Figure 1.

A. Area of infusion cannula location within the medial frontal cortex (Pellegrino et al. 1979).

B. Photomicrograph of the infusion track for chronic local infusion in the medial frontal cortex.

Chronic Medial Frontal Nicotinic Antagonist Infusion and Acute Clozapine Effects on Memory in the Radial-Arm Maze

Figure 2.

Interactions of medial frontal cortex infusions of MLA and DHβE with clozapine averaged across chronic nicotine treatment and working memory function (entries to repeat) in the 8-arm radial maze (mean ± standard error of the mean).

Chronic Medial Frontal Nicotinic Antagonist Infusion Acute Clozapine and Chronic Nicotine Effects on Memory in the Radial-Arm Maze

Figure 3.

Interactions of medial frontal cortex infusions of MLA and DHβE with clozapine and chronic nicotine treatment and working memory function (entries to repeat) in the 8-arm radial maze (mean ± standard error of the mean).

Chronic Medial Frontal Nicotinic Antagonist Infusion and Acute Clozapine Effects on Response Latency in the Radial-Arm Maze

Figure 4.

Interactions between chronic medial frontal cortex infusions of MLA and DHβE with clozapine and response latency (seconds per arm entry) in the 8-arm radial maze (mean $\pm$ standard error of the mean).