International Journal of Drug Safety and Discovery

Judith Eschbach, et al, Int J Drug Disc 2017, 1: 1
1: 004

Research

# An Efficient Test Battery for Rapid Screening of Extrapyramidal Side-Effects of Phenothiazine Analogues

**Judith Eschbach[1*], Rachel Amouroux[1], Jean-Christophe Peter[2], Sylviane Muller[2,3,4] and Pierre Eftekhari[1]**

[1] *Inoviem Scientific, Bioparc 3, 67400 Illkirch-Graffenstaden, France*

[2] *Laboratory of Excellence Medalis (ANR-10-LABX0034_MEDALIS), Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France*

[3] *CNRS, Immunopathology and therapeutic chemistry, Institut de Biologie Moléculaire et Cellulaire, UPR 3572, 67000 Strasbourg, France*

[4] *University of Strasbourg Institute for Advanced Study (USIAS), 67000 Strasbourg, France*

## Abstract

Extrapyramidal symptoms (EPS) are undesired side-effects of antipsychotic treatments which include tremors, akathisia and Parkinsonism. The new generation of antipsychotics are expected to induce less EPS, however, these EPS still occurred in patients. The aim of this study was to optimize and implement a novel and robust protocol to evaluate the apparition of EPS and rank the intensity of those symptoms while screening new phenothiazine analogues. This method is based on the phenotypical assessment of two mouse strains during a battery of motor and sensorimotor tests after the injection of clozapine, the lead compound of atypical antipsychotics. In preclinical studies, these symptoms are challenging to identify since only the catalepsy test can predict the occurrence of EPS in rodents. We have identified mouse strains for translational drug development based on a simple and rapid battery of tests using drug-induced EPS such as locomotor activity and catalepsy. In sensorimotor tasks, clozapine treatment (5 and 10 mg/kg) in C57BL/6 induced a rapid impairment in string agility and deficits in beam-walking test, while it had very mild effect on MRL/lpr mice. Based on these results, the use of both mouse strains combined with beam-walking test and string agility test represents a suitable and sensitive in vivo screening method to evaluate the occurrence of antipsychotic-induced EPS for the development of new phenothiazine analogues.

**Keywords:** Clozapine; MRL/Lpr; Extrapyramidal Symptoms; Beam-Walking Test; String Agility

**Abbreviations:** APMs-Antipsychotic Medications; Clz-Clozapine; Clz-NO-Clozapine N-Oxide; CNS-Central Nervous System; EPS-Extrapyramidal Symptoms; Lpr-Lymphoproliferation; MRL/lpr-MRL/Mp-Fas$^{lpr}$/Fas$^{lpr}$ ; MRL-Murphy Roths Large; N-DMClz-N-Desmethyl Clozapine

## Introduction

Preclinical studies for the development of antipsychotic medications (APMs) are complex and challenging due to the lack of appropriate and predictive models for multifactorial psychiatric disorders that recapitulate all the disease features. A large panel of therapies has been developed over the years. The first typical APMs, found by serendipity in 1949, owe their efficacy through their ability to modulate the dopamine-mediated neurotransmission [1]. However, those treatments induce pronounced disabling side effects including extrapyramidal symptoms (EPS) [2; 3], which are characterized by movement disorders such as parkinsonian syndrome, dystonia and akathisia [4]. Long-term APMs treatment can further lead to tardive dyskinesia, which are involuntary contractions of both orofacial muscles and muscles involved in limb and trunk movement [5-7]. A second generation of APMs, based on the modulation of different receptors, has been developed to overcome these unwanted side effects. However, even if the latter were designed to lower the risk of developing EPS, the appearance of such disabling conditions was still common in patients treated for psychosis, leading the scientists to question the overall gain of the second-generation treatment [8; 9]. In particular, the

***Corresponding author: :** Judith Eschbach, Inoviem Scientific, Bioparc 3, 850 Bd Sébastien Brant, 67400 Illkirch, France, Tel: +339 72 58 68 75; E-mail : j.eschbach@inoviem.com

**Sub Date**: September 7th, 2017, **Acc Date:** September 21st, 2017, **Pub Date:** September 21st, 2017.

**Citation:** Judith Eschbach, Rachel Amouroux, Jean-Christophe Peter, Sylviane Muller and Pierre Eftekhari (2017) An Efficient Test Battery for Rapid Screening of Extrapyramidal Side-Effects of Phenothiazine Analogues. Int J Drug Disc 1: 004.

assessment of drug-induced adverse effects is often disregarded even though they constitute a real burden for the patients. One of the reasons is that no straightforward and comprehensive tests have been designed to screen those parameters during the preclinical development of new APMs. Indeed, several preclinical tests have been developed to predict the efficacy of antipsychotic drugs (such as the conditioned avoidance response, stereotypies, antagonism of amphetamine induced-hyperlocomotion and disruption of the prepulse inhibition of the startle response...) [10,11], however, only a few were developed to evaluate and predict the side effects of antipsychotics. To date, only the catalepsy test is used to predict EPS [12-14]. In rodents, catalepsy is defined by an immobile posture, and an inability to reposition itself from an unusual position. Nevertheless, the catalepsy test presents some disadvantages, indeed it has been reported that small procedural differences in the realization of the catalepsy test can result in important differences in results [12].

In order to detect the liability of new phenothiazine analogues to promote EPS, the aim of the present study was to establish a robust protocol to selectively and sensitively measure the phenothiazine-induced extrapyramidal effects by using a battery of behavioral tests. This battery of tests aimed at completing the characterization of phenothiazine drugs on their abilities to promote EPS, by using several motor and sensorimotor tests.

To test our model, we used clozapine, which remains the lead compound of the phenothiazine class and the gold standard since it demonstrated clinical superiority in the treatment of schizophrenia and reduced suicidality in comparison to typical antipsychotics [15] and other molecules from the same pharmacological class [16,17]. Clozapine is generally used as a last resort since it shows increased efficiency in resistant schizophrenia [18], indicating a superior efficacy. However, clozapine is not considered as a first line drug due to several life-threatening side effects including an increased risk of agranulocytosis [19,20], myocarditis and cardiomyopathy [21] and metabolic disorders such as weight gain and diabetes [22]. Concerning the neuropsychiatric side-effects due to clozapine administration, somnolence, dizziness, tremors, sleep disruptions and seizures have also been reported [23]. In the present study, we investigated the effects of clozapine and its two major metabolites N-desmethyl clozapine (N-DMClz) and clozapine N-oxide (Clz-NO) on their ability to promote EPS in two different mouse strains. We explored drug-mediated effects (such as locomotor suppression and catalepsy) in different motor tasks in C57BL/6 and in the MRL/Mp-Fas$^{lpr}$/Fas$^{lpr}$ (MRL/lpr) inbreed mice. MRL/lpr mice are one of the best established spontaneous model of neuropsychiatric systemic lupus erythematosus [24] with central nervous system (CNS) deficiency [25-27]. From the behavioral point of view, MRL/lpr mice showed defect in prepulse inhibition response [28], a measure of sensorimotor gating that is also altered in schizophrenic patients [29-31]. Furthermore, MRL/lpr mice spent less time exploring unfamiliar conspecifics [32], a behavior reminiscent to those observed in mouse model of schizophrenia [33-35]. Our results show profound differences in the behavioral responses to clozapine between the two mouse strains, which mimic drug-induced EPS in patients. Consequently, we propose the implementation of this protocol as a sensitive and reliable in vivo screening method to evaluate the risks of phenothiazine drugs-induced EPS, which can be used as a framework for the clinical development of new APMs.

## Materials and Methods

### Drugs Administration

Twelve 8 week-old MRL/lpr and 12 C57BL/6J (all males) were bred and maintained at the Institut de biologie moléculaire et cellulaire animal facility (Strasbourg, France). Mice were maintained in controlled temperature room (25 °C) with a 12 hour-light/dark cycle and were provided with food and water ad libitum. Both group of mice were housed in the same animal room. All experiments were performed on the same cohort of mice and a wash-out period of 3 days was observed between each injection. Mice were injected either with clozapine (8-Chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo[b,e]-[1,4] diazepine), Clz-NO or N-DMClz (Figure 1) (Enzo life science, Lyon, France). Compounds were dissolved in 0.1 M HCl and pH balanced in phosphate-buffered saline pH 7.4 (PBS) and were prepared freshly prior intraperitoneal (i.p.) injection (50 µL). These protocols were approved by the local and national ethical committees (Protocol ID: 2015031813376314(APAFiS#349)).

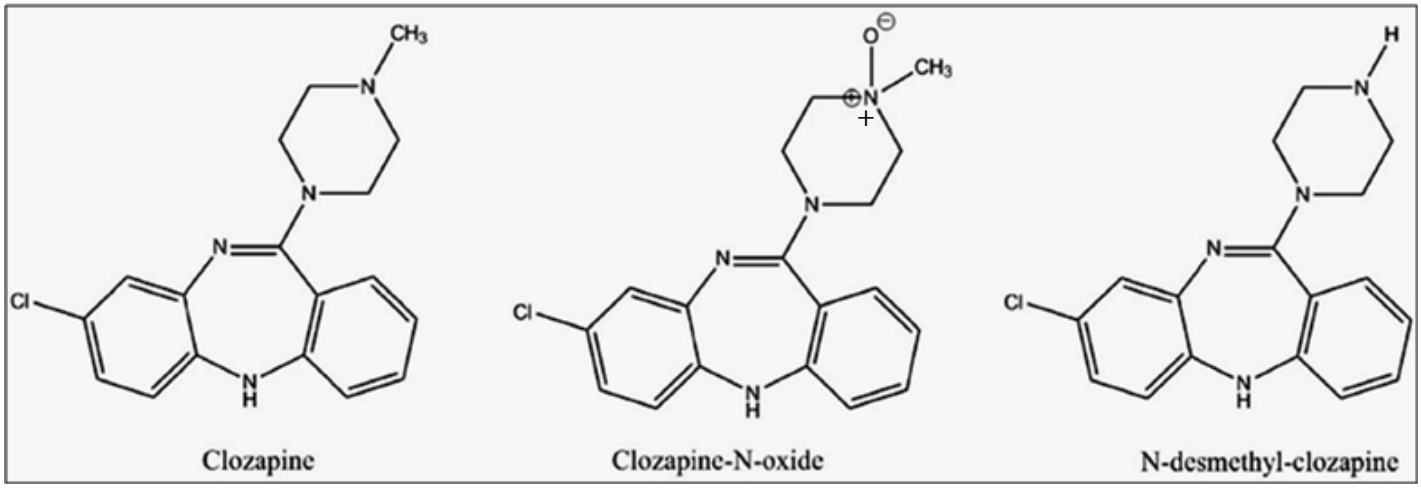


**Figure 1:** Structure of Clozapine (A) and its two major metabolites Clozapine-NO (B) and N-Desmethyl-clozapine (C).

### Catalepsy Test

Catalepsy test was performed as described before [12]. Briefly, catalepsy was evaluated by gently elevated the forepaws of a mouse (2.5 cm-height above the table). The catalepsy time was defined as the time for the mice to move all the four paws. The test was performed for a maximal duration of 20 s. Catalepsy was measured 15, 30, 60 and 75 min post drug administration. All animals were videotaped and the time spent in the cataleptic position was confirmed a posteriori by a manually operated timer by another experimenter in a blind manner.

### Beam-Walking Test

The beam-walking test was used to evaluate motor coordination and balance [36]. Briefly, we evaluated the ability of mice to cross a narrow beam to reach an elevated enclosed safety platform. The beam apparatus consists of 80 cm beam with a flat surface of 2 cm-width, and the enclosed safety platform was placed 40 cm above the table in order to allow an inclination of 25°. Mice were placed onto one end of a beam. Animals received three days of training before testing, all trainings were performed without injection and crossing beam was encouraged by pushing them slowly from behind with gloved fingers. On the first day of training, mice were placed for 1 min in the enclosed safety platform, and then animal were trained to cross 3 times the beam starting from the upper half of the beam and then another 3 times from the end of the beam. The mice were allowed to rest for 10 min in their home cages between training sessions. However, when they do stall, sniff or look around without proceeding forward, the investigator encouraged the mouse to continue moving forward by poking, or pushing it from behind with gloved fingers. Once mice reached the platform, they were allowed to rest for 1 min in the enclosed platform. Training was continued for 2 additional days, which correspond to the number of trials necessary to the mice to traverse the entire beam successfully without stopping.

Once the mice were trained, a baseline measure of performance was obtained prior to treatment (pretest condition). Mice were allowed up to 60 s to cross the beam and each trial was measured twice. The time to cross the beam was recorded for each trial and a rating system, ranging between 0 and 5, was used to evaluate the ability of mice to cross the beam (0 = no move – fall, 1 = 1$^{st}$ quarter crossed, 2 = half of the distance crossed, 3 = three quarters crossed, 4 = all the beam crossed, 5 = mouse on the platform). The time to cross and the score was evaluated pre-injection and 15, 30, 60 and 75 min post-drug administration. Analysis of each measure was based on the mean scores of the two trials and animals were videotaped. Time and score evaluation were confirmed a posteriori by a manually operated timer by another experimenter in a blind manner.

### String Agility Test

String agility test was performed to assess forepaw grip capacity and agility [37]. Mice were placed in the center of a 50 cm-long string suspended approximately 33 cm above a padded surface between two platforms. Mice were allowed to grip the string (diameter 0.25 cm) with only their forepaws and then released for a maximum of 60 s. Prior testing, animals received three days of training, consisting in placing mice on the platform for 1 min before receiving two consecutive trials. On the test day, a baseline measure of performance was obtained prior to treatment (pretest condition). A rating system, ranging between 0 and 5, was employed to assess string agility for a single 60 s trial (0 = animal unable to remain on string, 1 = hangs by two forepaws, 2 = attempts to climb onto string, 3 = two forepaws and one or both hind paws around string, 4 = four paws and tail around string, with lateral movement, 5 = escape to the platform). The string agility test was performed before (pretest condition) and at 15, 30, 60 and 75 min following drug administration. As C57BL/6 mice under clozapine treatment showed a severe defect in motor coordination, we further detected the time spent on the string till falling giving the maximum of 60 s for the mice that reached the platform during the trial. All animals were videotaped. Time and score evaluation were confirmed a posteriori by a manually operated timer by another experimenter in a blind manner.

### Statistical Analyses

Gaussian distribution of all data was tested with Shapiro-Wilk test. As normality could not be assumed, nonparametric testing was used. Means at different time points were compared using the nonparametric Friedman test. Comparison between dose 5 and 10 mg/kg for each time point treatment was tested using the Wilcoxon matched-pairs signed-rank test which allows the comparison between two matched groups. As normality is not assumed, standard two-way ANOVA tests could not be applied. All statistical analyses were performed using PRISM version 6.0a software (GraphPad, San Diego, USA).

## Result

### Increased Catalepsy Time in Response to Clozapine in C57bl/6 Mice

Catalepsy evaluation in response to antipsychotics remains a suitable well-known parameter in rodent for detecting compounds with EPS liability in humans [13]. The dose of 5 mg/kg of clozapine induced a moderate but significant phenotypical response in C57BL/6 mice whereas the 10 mg/kg-dose was sufficient to promote a pronounced cataleptic effect from the 45-min time point treatment (Figure 2a). In MRL/lpr, a slight albeit significant dose-dependent effect was observed although the time spent in the immobile position never reached more than 10 s (Figure 2b). Thus, C57BL/6 mice were sensitive to the effect of 10 mg/kg-dose of clozapine (catalepsy state was reached), whereas MRL/lpr mice were less responsive.

### Decreased Motor Performances in Response to Clozapine in C57bl/6 Mice but not Mrl/Lpr Mice

In order to complete our characterization on clozapine-induced behavioral effects, we next turned to evaluate the motor performance of mice after the administration of clozapine. Indeed, combination of the beam-walking test and string agility test provide a useful approach for the characterization of fine motor deficits in rodents [38]. C57BL/6 mice treated with 5 mg/kg of clozapine showed difficulty in crossing the beam compared to their motor performance before drug administration (referred to the pretest condition in the bar graph). This was shown by the increased time spent on the beam as well as by the significant decreased distance covered (score) (Figure 3a-b). Nonetheless, MRL/lpr mice were insensitive to the 5mg/kg dose of clozapine (Figure 3c-d). MRL/lpr mice were able to walk along the beam, and no difference was seen when compared to performance before drug administration. When mice were injected with 10 mg/kg clozapine,

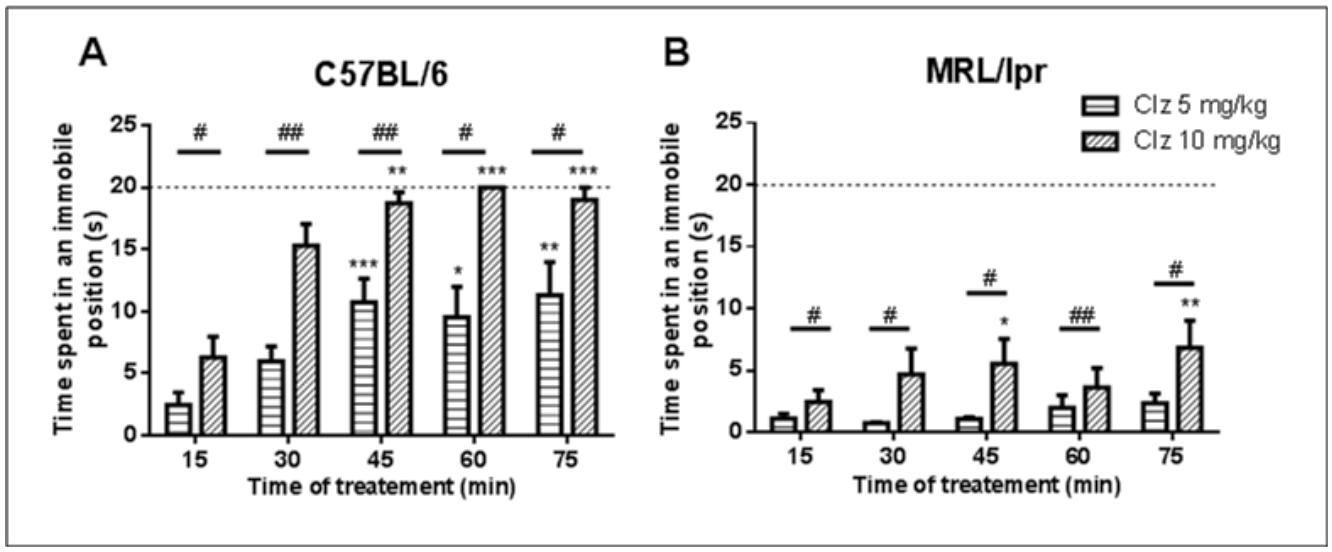


**Figure 2:** Cataleptic response to clozapine (Clz) in C57BL/6 (A) and MRL/lpr (B) mice. Mice were injected with 5 mg/kg or 10 mg/kg of clozapine. Catalepsy time (expressed in s) was determined during 75 min at 15-min intervals. The catalepsy test was interrupted when mice remained immobile for longer than 20 s (dotted line). Data represent mean values (+ SEM). Significance of differences from the earliest time point of treatment using the Friedman test * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs the earliest time point of treatment. Dose comparisons for each time point treatment were performed using the Wilcoxon test # $p < 0.05$, ## $p < 0.01$.

C57BL/6 mice were already unable to cross the beam after 15 min of treatment and stayed in the same position during the task (Figure 3b). These mice displayed ventral recumbence meaning that their thorax and abdomen were flattened against the upper surface of the beam and no displacement was possible. By contrast, motor performance of MRL/lpr following 10 mg/kg clozapine injection was unchanged when compared to their motor performance in the basal condition (Figure 3d). Friedman test demonstrated a significant effect of clozapine from 15 min of treatment in the C57BL/6 strain, but not in MRL/lpr mice. Furthermore, comparison of doses 5 and 10 mg/kg using the Wilcoxon test in C57BL/6 mice revealed a significant dose-dependent effect in the beam-walking test. These results indicate that clozapine impaired motor phenotype in a dose-dependent manner only in C57BL/6 mice, but not in MRL/lpr.

Motor coordination was also tested on those mice before and after clozapine administration using the string agility test, which measures the ability of mice to hold on a string using their forelimbs, and to catch the string with hindlimbs in order to join the escape platform. Each trial lasted 60 s, and in case of fall, the latency to fall was measured. Following 5 mg/kg of clozapine, C57BL/6 mice were able to hang on the string and attempt to climb, but these mice were unable to reach the platform and half of the mice fell from the string as shown by the score (Figure 4a). A decreased latency to fall was detected 45 min after clozapine injection in the C57BL/6 mice (Figure 4b). By contrast, MRL/lpr mice were able to perform readily the task, their ability to escape through the platform was not changed by the administration of clozapine (Figure 4c). Ten mg/kg of clozapine severely impaired motor coordination in C57BL/6 mice 15 min after clozapine administration. C57BL/6 mice were unable to remain suspended more than 20 s on the string, while a dose of 10 mg/kg barely affected motor coordination in MRL/lpr mice at 45 min of treatment as compared to motor performance before clozapine administration (pretest condition in the bar graph) (Figure 4d). While clozapine affected strongly motor coordination in C57BL/6 mice, only minor defects were seen in MRL/lpr mice. Indeed, evaluation of the latency of MRL/lpr mice to reach the escape platform revealed that the 5 mg/kg dose of clozapine significantly impaired the latency to success at 30 min of treatment in the string agility task (Figure 5). Additionally, a higher dose of clozapine (10 mg/kg) also significantly affects the latency to success (Figure 5). We conclude that the EPS induced by the administration of clozapine have to be evaluated using the latency to success in MRL/lpr mice and the latency to fall in C57BL/6 mice in the beam-walking test, and that clozapine strongly impaired motor activity and coordination in C57BL/6, while a lesser degree of motor activity impairment was observed in MRL/lpr mice following clozapine administration.

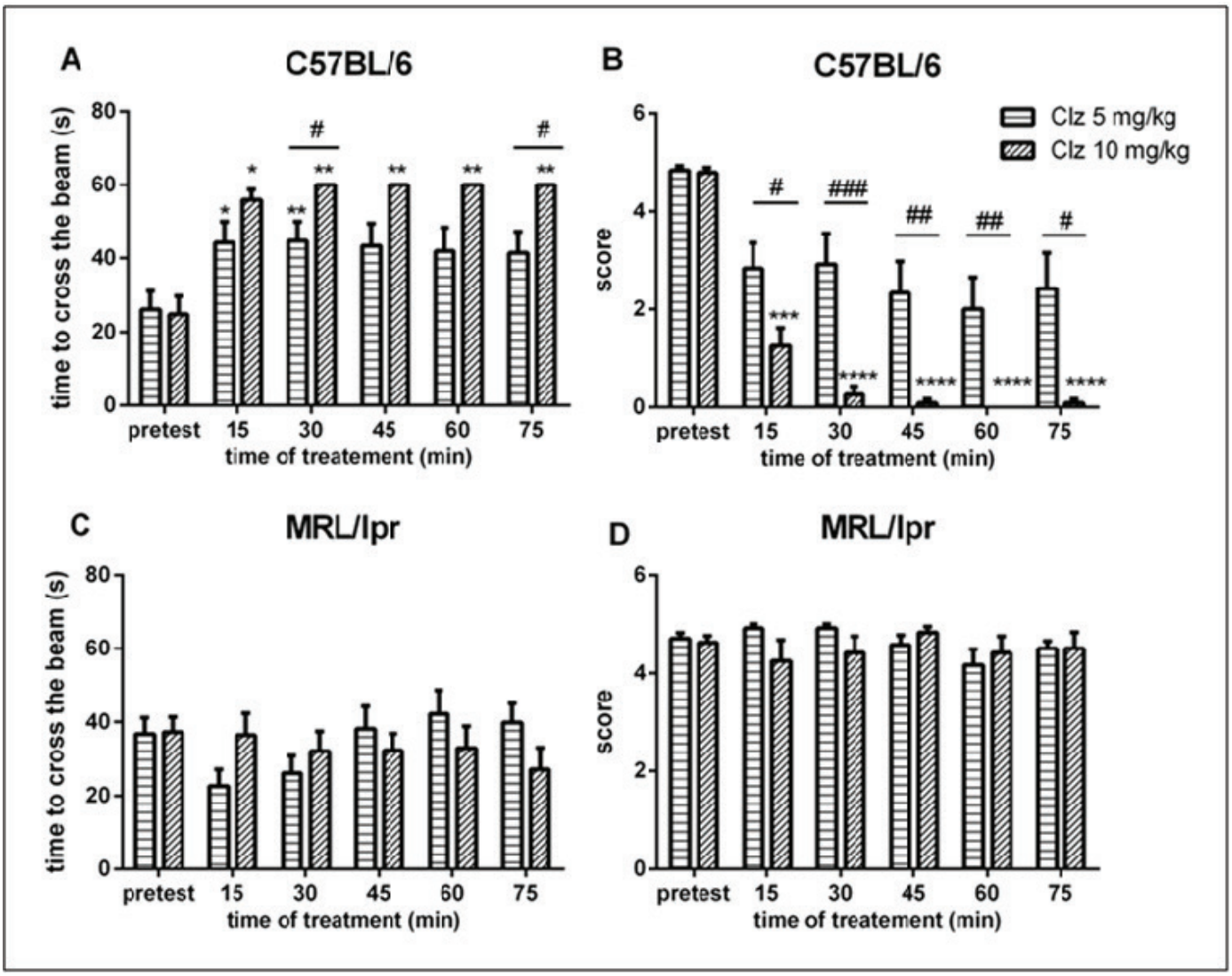


**Figure 3:** Motor performances in C57BL/6 (A-B) and MRL/lpr (C-D) mice before and after clozapine (Clz) injection. Pretest condition referred to the behavior response before injection. After 3 days of training, mice were injected with 5 or 10 mg/kg of clozapine and the beam-walking test was performed before and after clozapine injection at 15-min intervals during 75 min. The test was interrupted when mice remained on the beam for longer than 60 s. Time (s) to cross the beam before and after injection of 5 or 10 mg/kg of clozapine by C57BL/6 (A) or MRL/lpr (C) mice. Distance covered by C57BL/6 (B) or MRL/lpr (D) mice on the beam (graded by score) before and after injection of 5 or 10 mg/kg of clozapine. Data represent mean values (+ SEM). Statistical analysis using the Friedman test revealed that clozapine significantly increased the time spent on the beam and decreased the distance score of C57BL/6 mice. Wilcoxon test revealed that this effect was dose-dependent in C57BL/6, but not of MRL/lpr mice. Significance of differences from the pretest using the Friedman test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.001$ vs the pretest. Dose comparisons for each time point treatment were performed using the Wilcoxon test. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$.

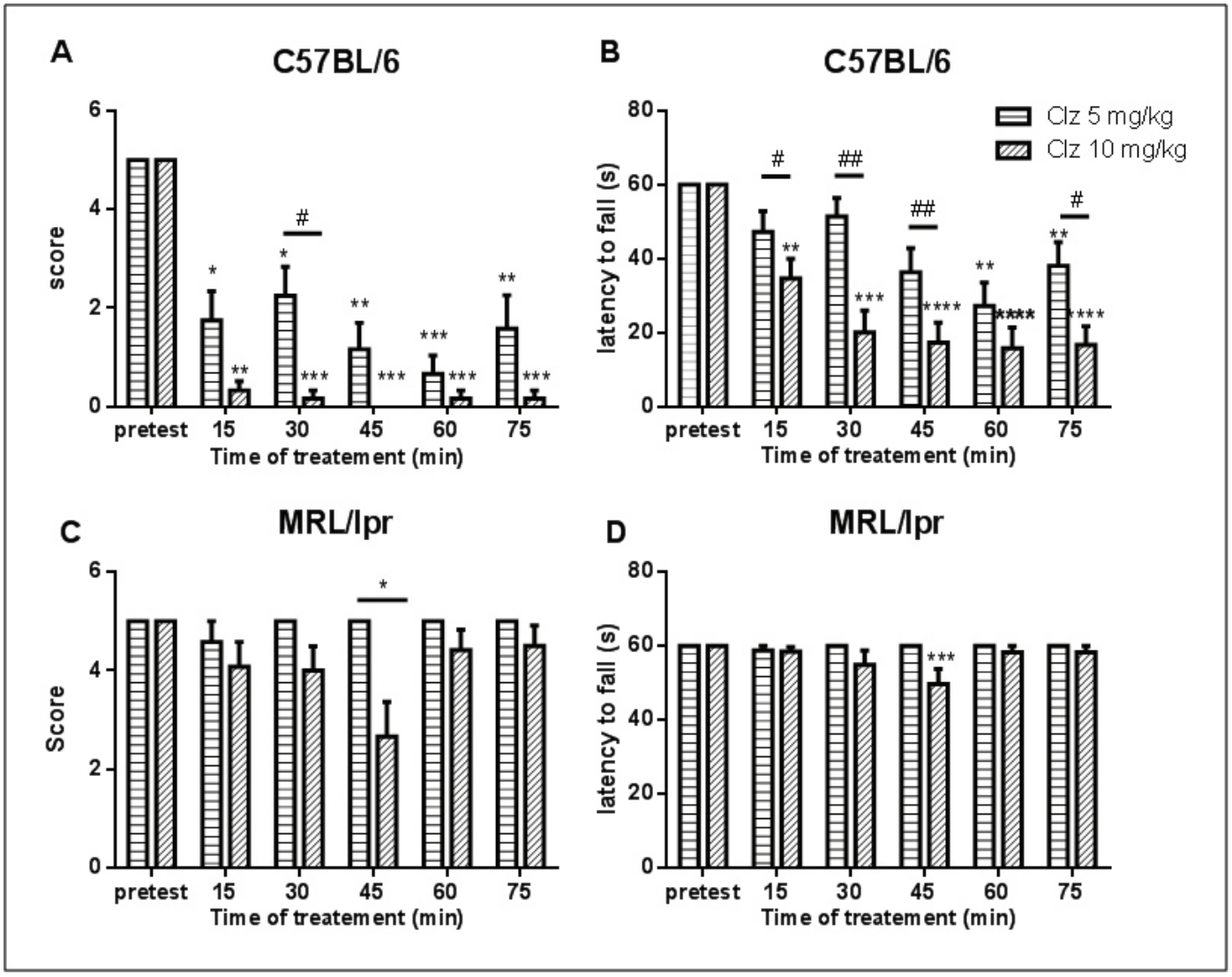


Figure 4: String agility in C57BL/6 (A-B) and MRL/lpr (C-D) mice and before and after clozapine (Clz) injection. Pretest condition referred to the behavioral response before injection. After 3 days of training, mice were injected with 5 or 10 mg/kg of clozapine and the string agility test was performed before and after clozapine injection at 15-min intervals during 75 min. String agility score of C57BL/6 (A) and MRL/lpr (C) mice before and after injection of 5 or 10 mg/kg of clozapine. Latency to fall in the string agility test for C57BL/6 (B) and MRL/lpr (D) mice before and after injection of 5 or 10 mg/kg of clozapine. Data represent mean values (+ SEM). Significance of differences from the pretest mice using the Friedman test * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ vs the pretest condition. Dose comparisons for each time point treatment were performed using the Wilcoxon test # $p < 0.05$; ## $p < 0.01$.

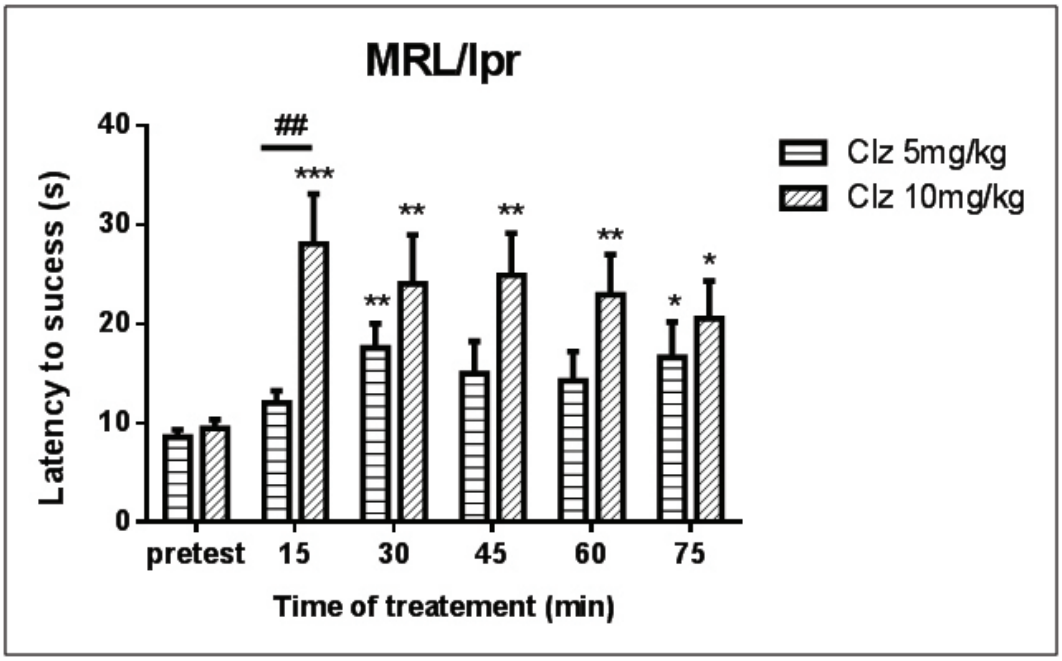


**Figure 5:** Clozapine (Clz) reduces the latency to reach the platform in the string agility test. MRL/lpr mice were injected with 5 or 10 mg/kg of clozapine and the string agility test was performed before and after clozapine injection at 15-min intervals during 75 min. Pretest condition referred to the behavioral response before injection. Data represent mean values (+ SEM). Significance of differences from the untreated mice using Friedman test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs the pretest comparison. Dose comparisons for each time point treatment were performed using the Wilcoxon test. ## $p < 0.01$.

6.3 Absence of Motor Impairment Following N-Desmethylclozapine and Clozapine N-Oxide Administration in C57bl/6 and Mrl/Lpr Mice

Clozapine is metabolized in N-desmethylclozapine (N-DMClz) and clozapine N-oxide (Clz-NO) before being excreted. Previous work pointed out a correlation between clozapine and N-DMClz plasma concentration and therapeutic response. Clozapine and N-DMClz plasma concentrations were significantly higher in responders [39, 40]. To determine whether clozapine pure metabolites are able to affect motor activity and coordination similarly to clozapine, we injected N-DMClz or Clz-NO in both strains at 5 and 10 mg/kg doses. No significant effects were seen (data not shown). Tables 1 and 2 summarize the results of the effects of both metabolites (dose 15 mg/kg) in the beam-walking test in the string agility test. Both metabolites failed to show a significant effect in both tests as compared to motor performance before clozapine metabolites administration (pretest condition). Furthermore, clozapine metabolites were unable to induce catalepsy in both strains even with a higher dose (Tables 1, 2). Altogether, our data show that clozapine but not its metabolites impairs motor performance as shown by the beam-walking and string agility tests in a dose-dependent manner in C57BL/6 mice, and to a lesser extend in MRL/lpr mice.

**Table 1** Effects of NO-Clz and N-DMClz (dose 15 mg/kg) on motor and agility performance (time to cross the beam or to execute the string agility test in s and score) in C57BL/6. Data represent mean values (+/- SEM).

| | | | pretest | 15 min | 30 min | 45 min | 60 min | 75 min |
|---|---|---|---|---|---|---|---|---|
| NO-Clz | Beam walking | time (s) | 23.6 ± 5.3 | 31.4 ± 7.5 | 30.8 ± 6.7 | 29.4 ± 6.4 | 21,0 ± 4.9 | 24.5 ± 6 |
| | | score | 4.8 ± 0.1 | 4.8 ± 0.2 | 4.5 ± 0.2 | 4.8 ± 0.1 | 5,0 ± 0 | 4.8 ± 0.1 |
| | string aglity | time (s) | 6.9 ± 0.5 | 9.8 ± 1.4 | 8.3 ± 1.1 | 8.6 ± 1.2 | 8.5 ± 1 | 7.1 ± 0.7 |
| | | score | 5,0 ± 0 | 5,0 ± 0 | 5,0 ± 0 | 5,0 ± 0 | 5,0 ± 0 | 5,0 ± 0 |
| N-DMClz | Beam walking | time (s) | 22.1 ± 1.6 | 23.9 ± 5.6 | 21.8 ± 6.3 | 28.4 ± 7.5 | 21.6 ± 6.6 | 23.5 ± 7.9 |
| | | score | 5,0 ± 0 | 5,0 ± 0 | 5,0 ± 0 | 5,0 ± 0 | 5,0 ± 0 | 4.8 ± 0.2 |
| | string aglity | time (s) | 7.3 ± 0.7 | 7,0 ± 1.6 | 8.6 ± 0.6 | 7.3 ± 0.6 | 6.2 ± 0.6 | 6.6 ± 0.7 |
| | | score | 5,0 ± 0 | 5,0 ± 0 | 5,0 ± 0 | 5,0 ± 0 | 5,0 ± 0 | 5,0 ± 0 |

**Table 2** Effect of Clz-NO and N-DMClz (dose 15 mg/kg) on motor and agility performance (Time to cross the beam or to execute the string agility test in s and score) in MRL/lpr. Data represent mean values (+/- SEM).

| | | | pretest | 15 min | 30 min | 45 min | 60 min | 75 min |
|---|---|---|---|---|---|---|---|---|
| NO-Clz | Beam walking | time (s) | 42.9 ± 4.8 | 42.4 ± 5.9 | 44.9 ± 5.5 | 47.1 ± 5.6 | 39.9 ± 0 | 47.5 ± 5.1 |
| | | score | 4.4 ± 0.1 | 4.3 ± 0.2 | 4.4 ± 0.2 | 4.4 ± 0.2 | 4.6 ± 0 | 4.5 ± 0.2 |
| | string aglity | time (s) | 7.2 ± 0.8 | 7.7 ± 0.7 | 7.8 ± 0.7 | 5.6 ± 0.6 | 5.2 ± 1 | 6,0 ± 0.4 |
| | | score | 5,0 ± 0 | 5,0 ± 0 | 5,0 ± 0 | 5,0 ± 0 | 5,0 ± 0 | 5,0 ± 0 |
| N-DMClz | Beam walking | time (s) | 33.6 ± 8.5 | 60,0 ± 0 | 46.3 ± 6.2 | 40,0 ± 9.6 | 47.7 ± 6.6 | 45.9 ± 9.2 |
| | | score | 4.8 ± 0.2 | 4.3 ± 0.2 | 4.3 ± 0.2 | 4.3 ± 0.2 | 4.5 ± 0 | 4.3 ± 0.2 |
| | string aglity | time (s) | 6.5 ± 1 | 5.5 ± 1 | 5.2 ± 0.4 | 6.2 ± 1 | 6,0 ± 0.6 | 6.7 ± 1.3 |
| | | score | 5,0 ± 0 | 5,0 ± 0 | 4.2 ± 0.8 | 5,0 ± 0 | 5,0 ± 0 | 5,0 ± 0 |

## Discussion

The preclinical development of antipsychotic medications (APMs) is complex since several parameters have to be considered such as antipsychotic activity, side effects and more specifically the risk of developing extrapyramidal symptoms (EPS). Indeed, EPS are strongly correlated with D2 receptor occupancy [41] and are characterized by parkinsonism, akathisia and dystonia [42-44]. The first generation of antipsychotics, such as haloperidol, are strong dopamine D2 receptor antagonists, known to promote severe EPS in patients [45]. Second generation of APMs, developed in order to overcome these unwanted EPS, are slightly more efficient in terms of efficacy and reduced EPS in comparison to typical APMs. However, parkinsonism and akathisia still occur in 10% of patients treated with atypical APMs while tardive dyskinesia occurs in up to 20% of the case [7]. Although clozapine treatment is associated with the fewer risks of EPS, longer exposure still induces tardive dyskinesia [46,47]. It is consequently an urgent clinical priority to develop molecules, which induce fewer side effects and so intrinsically to implement assays that help to predict the drug-induced EPS in patients. In this sense and in order to screen new antipsychotic molecules for their EPS activity, we developed a robust method and identified mouse models that met several criteria: face validity (efficiency to predict the effect to observe), reliability (in term of reproducibility) and sensitivity.

To date, only the catalepsy test is used to evaluate the APMs-mediated EPS. Several declinations of this test have been proposed such as the bar fix test or the wood block test (which consist on elevating the forepaws of a mouse and measuring the time necessary for the mouse to retract itself from this unusual position [12]). Another way to perform this catalepsy test is to use to paw test which use a special platform with four holes. Both forelimbs and hindlimbs are lowered through the holes and the retraction time is measured [48]. Finally, catalepsy can also be evaluated using the four corks test, where the animal is placed on four corks, one paw per cork, and the time that the front paws remained on the corks is recorded [49]. However, the two last ways to perform the catalepsy test are used in rats rather than in mice, and several limits have been reported. Indeed, mice positioning, height of forepaws elevation, repeat testing could result in important differences in results [12]. Thus, we decided to complete the characterization of clozapine-mediated EPS using psychomotor tests which evaluate motor abilities and coordination [38]. In parallel, we used the beam walking and string agility tests [38,50] to closely and finely monitor the motor ability of our mouse models after injection of clozapine. Although this molecule has been commercialized on the basis of the reduction of drug-induced EPS, we overall observed the appearance of a pronounced dose-dependent cataleptic state, a motor deficiency and an impediment for the realization of basic tasks in C57BL/6 mice. Surprisingly, only a slight effect was observed in the MRL/lpr strain (as shown by the increased latency to perform both tests). Interestingly the antipodal effects of clozapine injection in the two strains might reflect the panel of responses observed in APMs-treated patients in term of latency and intensity of the undesired symptoms. Consequently, the range of phenotypical responses in these two strains gives us a reliable in vivo screening method to evaluate the risk of drug-induced EPS for the clinical development of new compound. Indeed, in our model, the ideal analog of APMs would have a longer latency for inducing EPS and intensity of these effects would be reduced in both strains.

As EPS occur in patients after a week of treatment, it would be interesting to test this protocol in mice chronically treated with APMs. However, several studies reported that catalepsy durations after acute treatment of atypical APMs were longer than under a chronic treatment [51]. Furthermore, it has been suggested that acute treatment in animal can predict efficiently the effects of antipsychotics in clinic [52]. This is in line with the quick screening method we developed, where we tested only acute treatment of clozapine in mice.

To establish our protocol, we used two mouse strains: C57BL/6, widely used for psychomotor studies and MRL/lpr. MRL/lpr mice have been extensively characterized as a spontaneous model of neuropsychiatric systemic lupus erythematosus [24] with central nervous system deficiency [25-27]. However, the disease severity and progression are highly accelerated in females as compared to males [53-55]. Furthermore, male did not present such pronounced immunologic features as in female mice. On the phenotypic level, female MRL/lpr mice showed a lower locomotor activity while male did not show any motor deficit in openfield test [53, 54]. Thus, intrinsic motor impairment can be precluded in psychomotor tests. For those reasons, we have decided to include this particular strain in our tests and observe their phenotypical response towards the injection of clozapine. As it is discussed more in details below, the MRL/lpr strain developed very mild drug-induced EPS as the C57BL/6 did. Although the purpose of this study was not to characterize why such discrepancies exist between the two strains, we took the advantage of observing those

antipodal responses to define the phenotypical limits to our protocol.

In terms of sensitivity, we showed that the beam walking and the string agility tests were more sensitive than the catalepsy test to show directly the effects of clozapine-induced motor impairment. More specifically, we highlighted slight although significant effect of clozapine on MRL/lpr when measuring the latency to success whereas almost no difference was observed when measuring the latency to fall or when we score the overall success of the test. We conclude that the combination of catalepsy and motor tests in one hand, and the precise measurement of the latency to reach the platform especially for the MRL/lpr strain in the string agility test on the other hand, are very sensitive parameters suitable to characterize the phenotype induced by phenothiazine drugs. Thus, the motor characterization completed the observations obtained with the catalepsy test in both strains, and constitute a reliable, sensitive and valid method to characterize phenothiazine analogue-mediated motor defects.

In addition, we used the proposed protocol to test the effects of clozapine metabolites (N-DMClz and Clz-NO; Fig. 1) [56,57]. Indeed, former studies suggested that N-DMClz was an active metabolite due to its affinity to target several receptors (similar to clozapine) and may contribute to the therapeutic efficacy [58]. Here, in our model, equal or higher doses (up to 15 mg/kg) of both metabolites failed to show any motor impairment or catalepsy. Although we cannot state on their potential antipsychotic clinical activity, our results demonstrate that both metabolites are not responsible for EPS appearance, and that the molecular structure of clozapine is crucial to promote its effects.

## Conclusion

Altogether, the present study provides evidence that this screening protocol comprising both catalepsy and motor evaluation using beam walking and string agility tests is a suitable and powerful combination for a precise prediction of EPS induction during the clinical development of new therapy for schizophrenia. The next challenging step would be to develop in the same way a straightforward, robust and accurate protocol in order to screen the new phenothiazine analogues for their antipsychotics activity.

## Acknowledgements

This research was supported by funds from Alsace Innovation (France). We thank Sarah Welly, François Louis and Lucile Girardot for their excellent technical support and Isabel Bazle and Akkiz Bekel for their careful reading of the manuscript. We also sincerely thank Hélène Jeltsch-David for her helpful comments and discussions. We also thank the animal facility from IBMC and Chronobiotron animal facility (Strasbourg) for their technical assistance. We declare that all animal experiments were performed under the supervision of authorized investigators and followed current EU regulations.

## References

1. Kapur S (2003) Psychosis as a state of aberrant salience a framework linking biology, phenomenology, and pharmacology in schizophrenia. Am J Psychiatry 160(1): 13-23.
2. Van Os J, Kapur S (2009) Schizophrenia. Lancet 374(9690): 635-645.
3. Ellenbroek BA (2012) Psychopharmacological treatment of schizophrenia: what do we have, and what could we get? Neuropharmacology 62(3): 1371-1380.
4. Ega MF, Chrapusta SJ, Karoum F, Wyatt RJ (1996) The effects of acute and chronic haloperidol treatment on dopamine release mediated by the medial forebrain bundle in the striatum and nucleus accumbens. Neuropsychopharmacology 14(3): 211-223.
5. Mathews M, Gratz S, Adetunji B, George V, Mathews M, et al. (2005) Antipsychotic-induced movement disorders: evaluation and treatment. Psychiatry (Edgmont) 2(3): 36-41.
6. Shin HW, Chung SJ (2012) Drug-induced parkinsonism. J Clin Neurol 8(1): 15-21.
7. Caroff SN, Hurford I, Lybrand J, Campbell EC (2011) Movement disorders induced by antipsychotic drugs: implications of the CATIE schizophrenia trial. Neurol Clin 29(1): 127-148 viii.
8. Leucht S, Corves C, Arbter D, Engel RR, Li C et al. (2009) Second-generation versus first-generation antipsychotic drugs for schizophrenia: a meta-analysis. Lancet 373(9657): 31-41.
9. Weiden PJ (2008) Antipsychotic-induced movement disorders - forgotten but not gone. Acta Psychiatr Scand 117(6): 401-402.
10. Gobira PH, Ropke J, Aguiar DC, Crippa JA, Moreira FA, et al. (2013) Animal models for predicting the efficacy and side effects of antipsychotic drugs. Rev Bras Psiquiatr 35 Suppl 2: S132-139.
11. Jones TS, Holland EC (2011) Animal models for glioma drug discovery. Expert Opin Drug Discov 6(12): 1271-1283.
12. Sanberg PR, Bunsey MD, Giordano M, Norman AB (1988) The catalepsy test: its ups and downs. Behav Neurosci 102(5): 748-759.
13. Hoffman DC, Donovan H (1995) Catalepsy as a rodent model for detecting antipsychotic drugs with extrapyramidal side effect liability. Psychopharmacology (Berl) 120(2): 128-133.
14. Sanberg PR (1980) Haloperidol-induced catalepsy is mediated by postsynaptic dopamine receptors. Nature 284(5755): 472-473.
15. Kane J, Honigfeld G, Singer J, Meltzer H (1988) Clozapine for the treatment-resistant schizophrenic. A double-blind comparison with chlorpromazine. Arch Gen Psychiatry 45(9): 789-796.
16. Woodward ND, Purdon SE, Meltzer HY, Zald DH (2005) A meta-analysis of neuropsychological change to clozapine, olanzapine, quetiapine, and risperidone in schizophrenia. Int J Neuropsychopharmacol 8(3): 457-472.
17. McEvoy JP, Lieberman JA, Stroup TS, Davis SM, Meltzer HY, et al. (2006) Effectiveness of clozapine versus olanzapine, quetiapine, and risperidone in patients with chronic schizophrenia who did not respond to prior atypical antipsychotic treatment. Am J Psychiatry 163(4): 600-610.
18. Lieberman JA, Phillips M, Gu H, Stroup S, Zhang P, et al. (2003) Atypical and conventional antipsychotic drugs in treatment-naive first-episode schizophrenia: a 52-week randomized trial of clozapine vs chlorpromazine. Neuropsychopharmacology 28(5): 995-1003.
19. Lieberman JA, Johns CA, Kane JM, Rai K, Pisciotta AV, et al. (1988)

Clozapine-induced agranulocytosis: non-cross-reactivity with other psychotropic drugs. J Clin Psychiatry 49(7): 271-277.

20. Alvir JM, Lieberman JA, Safferman AZ, Schwimmer JL, Schaaf JA, et al. (1993) Clozapine-induced agranulocytosis. Incidence and risk factors in the United States. N Engl J Med 329(3): 162-167.
21. Michelsen JW, Meyer JM (2007) Cardiovascular effects of antipsychotics. Expert Rev Neurother 7(7): 829-839.
22. Gardner DM, Baldessarini RJ, Waraich P (2005) Modern antipsychotic drugs: a critical overview. CMAJ 172(13): 1703-1711.
23. Mustafa FA (2013) Schizophrenia past clozapine: what works? J Clin Psychopharmacol 33(1): 63-68.
24. Jeltsch-David H, Muller S (2014) Neuropsychiatric systemic lupus erythematosus and cognitive dysfunction: the MRL-lpr mouse strain as a model. Autoimmun Rev 13(9): 963-973.
25. Denenberg VH, Sherman GF, Rosen GD, Morrison L, Behan PO, et al. (1992) A behavior profile of the MRL/Mp lpr/lpr mouse and its association with hydrocephalus. Brain Behav Immun 6(1): 40-49.
26. Sakic B, Szechtman H, Denburg JA, Gorny G, Kolb B, et al. (1998) Progressive atrophy of pyramidal neuron dendrites in autoimmune MRL-lpr mice. J Neuroimmunol 87(1-2): 162-170.
27. Sled JG, Spring S, van Eede M, Lerch JP, Ullal S, et al. (2009) Time course and nature of brain atrophy in the MRL mouse model of central nervous system lupus. Arthritis Rheum 60(6): 1764-1774.
28. Petitto JM, Huang Z, Lo J, Beck RD, Rinker C, et al. (2002) Relationship between the development of autoimmunity and sensorimotor gating in MRL-lpr mice with reduced IL-2 production. Neurosci Lett 328(3): 304-308.
29. Braff DL, Grillon C, Geyer MA (1992) Gating and habituation of the startle reflex in schizophrenic patients. Arch Gen Psychiatry 49(3): 206-215.
30. Swerdlow NR, Braff DL, Taaid N, Geyer MA (1994) Assessing the validity of an animal model of deficient sensorimotor gating in schizophrenic patients. Arch Gen Psychiatry 51(2): 139-154.
31. Perry W, Viglione D Jr, Braff D (1992) The Ego Impairment Index and schizophrenia: a validation study. J Pers Assess 59(1): 165-175.
32. Kapadia M, Stanojcic M, Earls AM, Pulapaka S, Lee J, et al. (2012) Altered olfactory function in the MRL model of CNS lupus. Behav Brain Res 234(2): 303-311.
33. Corbett R, Hartman H, Kerman LL, Woods AT, Strupczewski JT, et al. (1993) Effects of atypical antipsychotic agents on social behavior in rodents. Pharmacol Biochem Behav 45(1): 9-17.
34. Li C, Tang Y, Yang J, Zhang X, Liu Y, et al. (2016) Sub-chronic Antipsychotic Drug Administration Reverses the Expression of Neuregulin 1 and ErbB4 in a Cultured MK801-Induced Mouse Primary Hippocampal Neuron or a Neurodevelopmental Schizophrenia Model. Neurochem Res 41(8): 2049-2064.
35. Vazquez-Borsetti P, Pena E, Rico C, Noto M, Miller N, et al. (2016) Perinatal Asphyxia Reduces the Number of Reelin Neurons in the Prelimbic Cortex and Deteriorates Social Interaction in Rats. Dev Neurosci 38(4): 241-250
36. Carter RJ, Morton J, Dunnett SB (2001) Motor coordination and balance in rodents. Curr Protoc Neurosci Chapter 8: Unit 8. 12.
37. Miquel J, Blasco M (1978) A simple technique for evaluation of vitality loss in aging mice, by testing their muscular coordination and vigor. Exp Gerontol 13: 389-396.
38. Brooks SP, Dunnett SB (2009) Tests to assess motor phenotype in mice: a user's guide. Nat Rev Neurosci 10(7): 519-529.
39. Perry PJ, Miller DD, Arndt SV, Cadoret RJ (1991) Clozapine and norclozapine plasma concentrations and clinical response of treatment-refractory schizophrenic patients. Am J Psychiatry 148(2): 231-235.
40. Miller DD, Fleming F, Holman TL, Perry PJ (1994) Plasma clozapine concentrations as a predictor of clinical response: a follow-up study. J Clin Psychiatry 55 Suppl B: 117-121.
41. Kapur S, Remington G, Zipursky RB, Wilson AA, Houle S (1995) The D2 dopamine receptor occupancy of risperidone and its relationship to extrapyramidal symptoms: a PET study. Life Sci 57(10): PL103-107.
42. Casey DE (1996) Extrapyramidal syndromes and new antipsychotic drugs: findings in patients and non-human primate models. Br J Psychiatry Suppl: 32-39.
43. Casey DE (1991) Neuroleptic drug-induced extrapyramidal syndromes and tardive dyskinesia. Schizophr Res 4(2): 109-120.
44. Casey DE, Keepers GA (1988) Neuroleptic side effects: acute extrapyramidal syndromes and tardive dyskinesia. Psychopharmacol Ser 5: 74-93.
45. Tran PV, Dellva MA, Tollefson GD, Beasley CM Jr, Potvin JH, et al. (1997) Extrapyramidal symptoms and tolerability of olanzapine versus haloperidol in the acute treatment of schizophrenia. J Clin Psychiatry 58(5): 205-211.
46. Modestin J, Stephan PL, Erni T, Umari T (2000) Prevalence of extrapyramidal syndromes in psychiatric inpatients and the relationship of clozapine treatment to tardive dyskinesia. Schizophr Res 42(3): 223-230.
47. Casey DE (1998) Effects of clozapine therapy in schizophrenic individuals at risk for tardive dyskinesia. J Clin Psychiatry 59 Suppl 3: 31-37.
48. Ellenbroek BA, Peeters BW, Honig WM, Cools AR (1987) The paw test: a behavioural paradigm for differentiating between classical and atypical neuroleptic drugs. Psychopharmacology (Berl) 93(3): 343-348.\
49. Morelli M, Di Chiara G (1985) Catalepsy induced by SCH 23390 in rats. Eur J Pharmacol 117(2): 179-185.
50. Crawley JN (1999) Behavioral phenotyping of transgenic and

knockout mice: experimental design and evaluation of general health, sensory functions, motor abilities, and specific behavioral tests. Brain Res 835(1): 18-26.

51. Nakai S, Hirose T, Uwahodo Y, Imaoka T, Okazaki H, et al. (2003) Diminished catalepsy and dopamine metabolism distinguish aripiprazole from haloperidol or risperidone. Eur J Pharmacol 472(1-2): 89-97.

52. Andersen MP, Pouzet B (2001) Effects of acute versus chronic treatment with typical or atypical antipsychotics on d-amphetamine-induced sensorimotor gating deficits in rats. Psychopharmacology (Berl) 156(2-3): 291-304.

53. Gao HX, Sanders E, Tieng AT, Putterman C (2010) Sex and autoantibody titers determine the development of neuropsychiatric manifestations in lupus-prone mice. J Neuroimmunol 229(1-2): 112-122.

54. Gao HX, Campbell SR, Cui MH, Zong P, Hee-Hwang J, et al. (2009) Depression is an early disease manifestation in lupus-prone MRL/lpr mice. J Neuroimmunol 207(1-2): 45-56.

55. Cohen MG, Pollard KM, Schrieber L (1988) Relationship of age and sex to autoantibody expression in MRL-+/+ and MRL-lpr/lpr mice: demonstration of an association between the expression of antibodies to histones, denatured DNA and Sm in MRL-+/+ mice. Clin Exp Immunol 72(1): 50-54.

56. Jann MW, Grimsley SR, Gray EC, Chang WH (1993) Pharmacokinetics and pharmacodynamics of clozapine. Clin Pharmacokinet 24(2): 161-176.

57. Baldessarini RJ, Frankenburg FR (1991) Clozapine. A novel antipsychotic agent. N Engl J Med 324(11): 746-754.

58. Li Z, Huang M, Ichikawa J, Dai J, Meltzer HY (2005) N-desmethylclozapine, a major metabolite of clozapine, increases cortical acetylcholine and dopamine release in vivo via stimulation of M1 muscarinic receptors. Neuropsychopharmacology 30(11): 1986-1995.