# Optimization And Pharmacological Validation Of A Set Shifting Procedure For Assessing Executive Function In Rats

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## Introduction

Deficit in executive functions is one of the core component of cognitive impairments in neurological and psychiatric diseases such as Parkinson's disease or schizophrenia [1, 2, 3]. For decades, extra-dimensional set-shifting tests have been used for assessing behavioral flexibility and executive functions both in humans and animals [4, 5, 6]. The extra-dimensional set shifting in a cross-maze was described by Ragozzino et al. 1999 [7] and Floresco et al. 2006 [8] to assess executive functions in the rat.

Strategy set-shifting tasks have been conducted on either a cross-maze or operant chambers. On the maze, rats are initially trained to make a 90° right turn to receive food reinforcement. A visual cue is randomly placed in one of the choice arms on each trial, but do not reliably predict the food location. During the set-shift, the rat is now required to use a visual-cue discrimination strategy, entering the arm with the visual cue, requiring either a right or left turn. Thus, the rat must shift from the old strategy and approach the previously irrelevant cue in order to obtain reinforcement. The capacity of rats to shift strategy is considered as a measure of behavioral flexibility, and is assessed by the number of trials and the number and type of errors made to learn the rule.

The current work aims at optimizing Floresco et al. (2006) set –shifting protocol for standardized drug testing [8]. The following parameters were modulated: caloric restriction, reward preference, length of daily session, definition of turn bias. The new protocol has then been used to assess behavioral flexibility in rats with pre-frontal hypo function induced by sub-chronic phencyclidine (PCP) administration (2mg/kg bid for 7days followed by 7 days wash out). Reversal of PCP-induced deficit have been demonstrated with tolcapone, a brain penetrant COMT inhibitor shown to increase dopamine turnover and function in prefrontal cortex.

## Material and method

**Animal:** Male Lister Hooded rats (Harlan, France) were group housed with *ad libitum* access to water and food until beginning of caloric restriction, in a temperature and humidity controlled animal facility (12h : 12h light:dark cycle). Rats were caloric restricted to 48% of their daily food intake in order to reach 85% of their initial body weight after about 2 weeks (Figure 1). All animal procedures were conducted in strict adherence to the European Union Directive 2010/63/EU and were approved by UCB ethical committee. Behavioral experiments were carried out between 9:00 and 16:00, in a sound attenuated and air-regulated experimental room.

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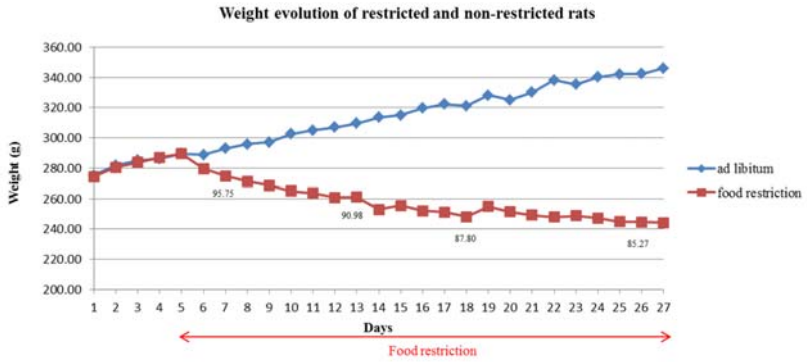


Figure 1: Daily body weight of restricted (n=10, red curve) and ad libitum fed (n=2, blue curve) rats. Numbers under red curve indicated weight, in percentage, compared to initial weight.

**Preference for palatable food reward:** In an attempt to suppress caloric restriction, 12 *ad libitum* fed rats were given the choice between 6 different palatable food during a 30 min trial repeated for 4 consecutive days. The palatable foods were laying in equal amount (equivalent to 2 sugar pills,~90 mg) on a home cage floor devoid of sawdust: sugar pills (BioServ), chocolate cereal (Kellogg's coco pops), chocolate pearls (Jacques Pearls), crunch peanuts (Delhaize), petal corn (De Halm), wheat cereal (Kellogg's all bran). The sequence of food tasting and the time spent to consume each reward were measured. The first two trials were considered as habituations to new food. Food preference was assessed during the last two trials by the sequence at which rat fully ate each reward. Accordingly, a score from 6 to 1 was attributed to each palatable reward for each rat, 6 being the food first eaten. Data are presented in terms of mean ± sem. Food preference was analyzed with a mixed design ANOVA with the subjects as a random factor and the "palatable food" as a fixed factor.

**Cross maze task:** The test was preceded by 3 individual habituations to food reward run as the habituations to palatable food preference described above. The maze was a white PVC cross-maze, in which one arm was closed to form a T-maze, the start arm being the foot of the T (Figure 2). A sliding door defined a start box in the bottom of the starting arm. The close arm randomly changed between trials to avoid extra-maze spatial reference cueing leading to food, and one arm was never used as starting arm. Before rule learning, rats were habituated to the maze in a series of 10 successive trials (two 3 min trials followed by eight 1min trials), during which they were allowed free maze exploration, rewards being located at the end of each arm for the 4 last trials. Preferred turn side was defined during these 10 trials as the first turn choice of each trial. Number of left and right turns defined a turn bias when 8 or more first turn were done toward the same side. Habituation sessions were followed by a series of 60 min daily rule learning sessions during which visual cues were randomly placed on the walls of one arm (black-and-white stripes). Each session comprised a series of rule learning trials spaced by 45s stay in the star box and lasting for max 2 min. The first rule leading to reward was egocentric (e.g. figure 2A: right turn, independently of visual cue location). Rule was considered as learnt after 10 successful consecutive trials and two successful probe tests. Probe test was a trial starting from the arm never used before. The day following completion of first rule learning, a shift in rule was applied, reward location being cued by a visual cue (Figure 2B). Number of errors and number of trials to learn each rule were recorded. Error types during the second rule learning were further analyzed to decipher learning strategy. Succession of errors were gathered by bins of four errors, not considering successful trials. A bin was defined as perseverative errors when rat applied 3 or 4 times the egocentric rules, which indicated that rat did not shift strategy (Figure 2C). Bins were defined as regressive when rats made less than three perseverative errors, and indicated a beginning of a shift in strategy. Finally, bin was defined as never-re-inforced when rats adopted neither the egocentric nor visual rule to choose the turn side. Statistical analysis on number of trials and number of errors to criteria and types of errors was performed with the Mann Whitney test, data are expressed as mean ± sem.

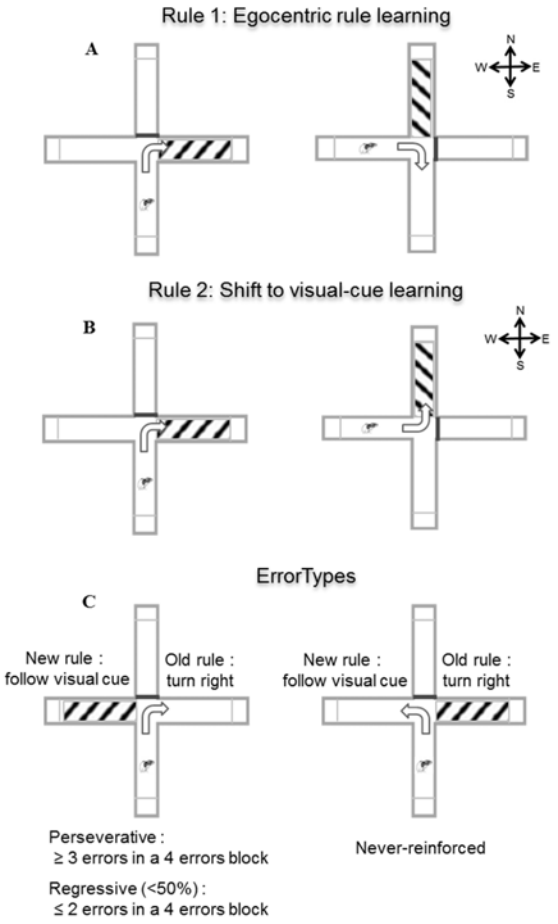


Figure 2: Example of egocentric (A – right turn) and visual (B) rule to be learnt in the set-shifting task. (Arrows represents the correct choice to receive reward). (C) Illustration of error types committed during the shift to rule 2.

**Drug administration:** Sub-chronic PCP treated rats received intra-peritoneal administration of 2 mg/kg PCP (National Measurement Institute, Australia) twice a day (every 12h) for 7 days in a volume of 5 mL/kg body weight. PCP was dissolved in sterile phosphate-buffered saline (PBS). PCP administration was followed by a 7-day washout period during which rats were daily handled, weighted, and habituated to reward. Control rats received PBS only.

Tolcapone (free base, UCB chemistry department) was suspended in methylcellulose/tween 80/antifoam vehicle (1%/0.1%/0.1%) and intra-peritoneally administered at a dose of 30 mg/kg, 40 minutes prior to each session of rule 2, in a volume of 5mL/kg body weight. Fresh solutions were prepared daily.

## Results

The first aim of this work was to optimize Floresco's protocol to decrease duration and to standardize it for compatibility with an industrial drug testing setting. Habituation sessions in the cross maze were merged to reduce their number from 7 to 1, reward being only located at the end of the arm. Session and trial duration was fixed at 60 min and maximum 2 min, respectively. Identification of turn bias, which is the natural tendency to repeatedly turn right or left, was performed during the 10 trials of habituation to the maze. Protocol optimization also attempted to

replace caloric restriction by palatable reward and assessed the robustness of turn bias described in the original protocol.

Analysis of food preference showed that rats developed preference for sweet rewards, which obtained highest preference scores [$F(5,55) = 18.3$, $p<0.0001$] (Figure 3). The preferred reward during the second tasting trial was sugar pills (5.17 ±0.30), closely followed by coco pops (4.25 ± 0.22), crunch peanuts (4.25 ± 0.48) and black chocolate pearls (4.00 ± 0.37). All bran and petal corn obtained lowest scores (1.83 ± 0.24 and 1.50 ± 0.20). Data from the first tasting trial were similar to second tasting trial (data not shown). Sugar pills and coco pops were tested as rewards in a set shifting test with *ad libitum* fed rats. 75% of rats failed to fulfill learning criteria for the first rule and stopped searching for reward after about 155.0 ± 30.8 trials. Time to reach reward was much longer than for caloric restricted rats (21.3 s ± 5.9 vs 5.5 s ± 0.6) and it increased with number of trials. Consequently to these poor performances of *ad libitum* fed rats, all subsequent experiments were done with rats under caloric restriction.

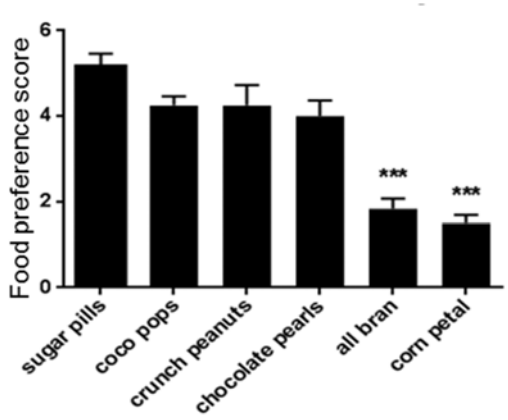


Figure 3: Food preference score evaluated in *ad libitum* fed rats using 6 palatable food rewards. Statistics: mean ± sem, n=12 (***) = $p<0.001$, compared to sugar pills.

Power analysis was done on turn side to define threshold for turn bias (Figure 4A). In sessions with 7 trials, turn bias toward one side could be defined with a $p < 0.05$ when rats made 7 turns on the same side (left or right). In sessions with 10 trials, turn bias with a $p < 0.05$ could be defined when rats made 8 or more turns on the same side. Analysis of natural turn bias in Lister Hooded rats were done on 27 rats using a 10 trial session (Figure 4B). 23 rats made less than 8 out of 10 turns toward the same side indicating that about 85% of rats made random choice for their turn side. Only 4 rats (15%) showed consistent bias toward right or left turn. Those rats were excluded from further testing in order to avoid bias during the egocentric rule learning.

A

| # of trials | # of turn toward same side | p value |
|---|---|---|
| 7 | 4 | 0.273 |
| 7 | 5 | 0.164 |
| 7 | 6 | 0.055 |
| 7 | 7 | 0.008 |
| 10 | 7 | 0.117 |
| 10 | 8 | 0.044 |
| 10 | 9 | 0.010 |
| 10 | 10 | 0.001 |

B

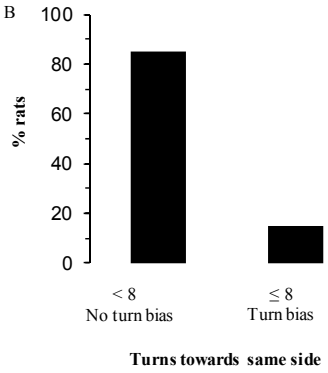


Figure 4: (A) Power analysis defining number of turn toward the same side required to define a turn bias in a 7 and 10 trial session. (B) Percentage of rats (n=27) presenting a turn bias defined as at least 8 turns toward the same side out of 10 trials

Pharmacological validation of the above optimized set shifting procedure was done using 3 treatments groups: rats treated with repeat vehicle (n=10) were compared to rats treated with sub-chronic PCP receiving vehicule (n=13) or 30mg/kg tolcapone (n=13), administered i.p. , 40 min before each session of second rule learning.

Vehicle treated rat learnt the first egocentric rule in 70.7 ± 8.4 trials and made 19.2 ± 2.7 errors. After learning the first rule, vehicle treated rats were able to shift to the rule based on visual cue; they required 91.9 ± 9.7 trials and made 35.5 ± 6.1 errors for shifting.

Sub-chronic PCP-treated rats learnt the first rule as fast as their controls. They required similar number of trials to reach criterion: 85.18 ± 12.46 trials ($U=45$, $p>0.05$). The number of errors to reach learning criterion was 31.0 ± 5.9 and was not significantly different than vehicle-treated group ($U=36.5$, $p>0.05$). PCP treated rats thus showed normal capacity to learn an egocentric rule. In contrast, PCP-treated rats performed significantly more errors (58.5 ± 7.1; $U=22$, $p<0.05$) and required significantly more trials (159.5 ± 10.8; $U=12$, $p<0.01$) to shift to the visual cue; indicating an impairment in their capacity to shift from egocentric rule to visual rule.

Sub-chronic PCP-treated rats receiving 30 mg/kg tolcapone learnt this second rule faster than the PCP-treated rats receiving vehicle. They required 95.9 ± 8.6 trials and made 39.3 ± 5.6 errors to acquire the second rule. The number of trials and number of errors required to learn the second rule was not statistically different than the vehicle treated rats (trials: $U=53$, $p>0.05$, and errors: $U=50$, $p>0.05$), indicating that 30 mg/kg tolcapone completely alleviated the PCP-induced impairment in set shifting. This is the first time that the brain penetrant COMT inhibitor tolcapone is shown to counteract sub-chronic PCP-induced deficit in extra-dimensional set shifting, a recognized paradigm to evaluate behavioral flexibility.

Detailed analysis of error sub-types demonstrated that all groups mostly made perseverative errors, which is repeatedly applying first rule during second rule learning (Figure 6). Other error types, regressive or never reinforced were marginal. Sub-chronic PCP treated rats made significantly more perseverative errors than other groups ($U=32.5$, $p<0.05$ compared to vehicle group ; $U=55.5$, $p=0.14$ compared to PCP + Tolcapone group) while regressive or never reinforced errors were similar ($p>0.05$).

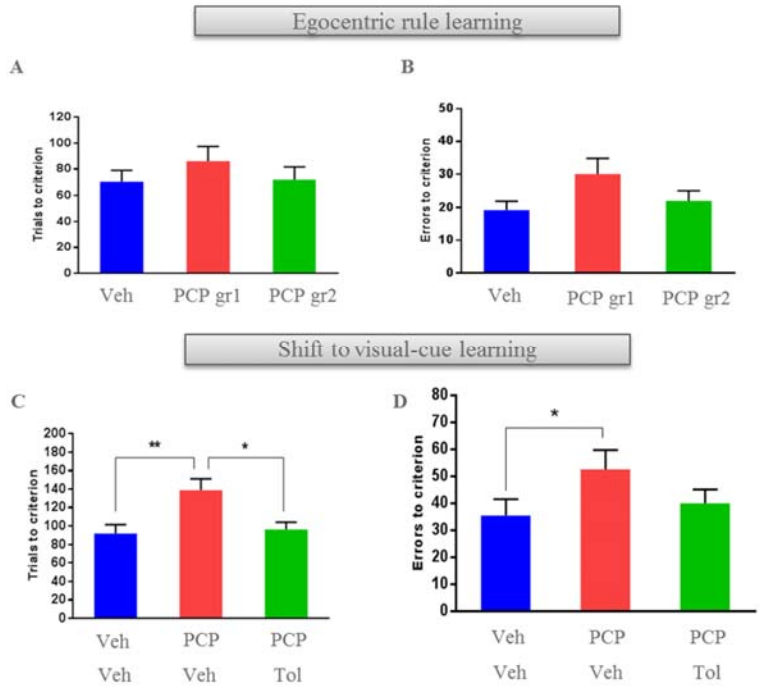


Figure 5: Detrimental effect of sub-chronic PCP treatment on set-shifting and reversal by acute tolcapone administration.(A) Number of trials (A &C) and number of errors (B & D) to reach learning criteria during the egocentric rule 1 (A-B) and visuo-spatial rule 2 (C-D). Blue columns : vehicle only treated rat, red columns: sub-chronic PCP treated rats (2 mg/kg) receiving vehicle before rule 2 learning; green columns: sub-chronic PCP treated rats (2 mg/kg) receiving 30 mg/tolcapone before rule 2 learning. Mean-sem, (*) = $p<0.05$ and (**) = $p<0.01$.

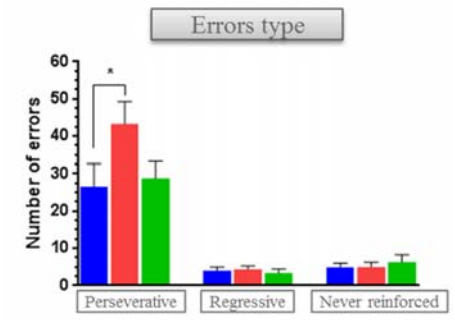


Figure 6: Number of perseverative, regressive and never-reinforced errors committed during the set-shift.

## Discussion

The current study aimed at optimizing a procedure assessing executive functions in rodent in order to test new drugs having the potential to counteract cognitive deficits observed in neurological disease such as PD or schizophrenia. The extra-dimensional set shifting task in a cross maze described by Ragozzino et al.(1999) [7] and Floresco et al. (2006) [8] has been modified and pharmacologically validated in a model of subchronic PCP administration. Protocol duration has been significantly reduced by decreasing number of habituations, and by coupling the habituations with identification of turn bias. Criterion to define turn bias has been validated by statistical power analysis. Analysis of turn side in absence of rule learning revealed no particular turn bias in most rats, which led us to introduce an exclusion criterion for the 15% of rats presenting a turn bias.

The attempt to replace caloric restriction by palatable food revealed clear preference for sweet food. Unfortunately, use of preferred food as reward for *ad libitum* fed rats did not seemingly generate enough motivation to complete first rule learning. Consequently, caloric restriction was kept as a mean to motivate rats for learning the task. Further testing would be required to assess whether coupling preferred food either with very low caloric restriction, e.g.; reaching 95-95% initial body weight, or with overnight food deprivation in *ad libitum* fed rats would provide enough motivation for completing the task.

The methodology to compute error types (perseverative vs regressive or never re-enforced) was modified compared to Floresco's protocol to better reflect number of perseverative errors committed during shift to second rule. The current methodology analyzed the succession of errors by bins of 4 errors (excluding successful trials) rather than bins of 4 trials including successful trials. Using such methodology a succession of 2 perseverative errors (P) followed by two successful trials (S) would be classified as regressive bin while the errors are all perseverative. For instance, the sequence of 8 trials [P-P-S-S]-[P-P-S-S] would be counted as 4 regressive errors with Floresco's methodology and as 4 perseveratives errors with the current methodology [PPPP]. As a consequence, Floresco's methodology underestimated bins of perseverative errors (table 1). The current methodology better reflects the increase in perseverative errors committed by PCP-treated rats while Floresco 's methodology did not. In contrast, Floresco methodology is more sensitive to detect the appearance of regressive error bins, which is the time when the animal start shifting strategy.

Table 1 : Comparison of methodologies for classification of errors type between protocol described by Ragozzino et al. (1999) and Floresco et al. (2006), and the optimized protocol.

| **Treatment** | **Perserverative** | | **Regressive** | | **Never-reinforced** | |
|---|---|---|---|---|---|---|
| *Methodologies* | *Troudet* | *Floresco* | *Troudet* | *Floresco* | *Troudet* | *Floresco* |
| Veh | 26.2 | 7.5 | 4 | 21.3 | 4.9 | 4.9 |
| PCP + veh | 41.4 | 9.9 | 4.4 | 35.7 | 5.1 | 5 |
| PCP + tolcapone | 26.6 | 8.9 | 3.1 | 20.9 | 5.8 | 5.9 |

The extra-dimentional set-shifting test is a relevant translational test; adapted from tests currently used in clinical studies to assess executive functions in humans, such as the Wisconsin Card Sorting Test (WCST). Impairments of cognitive flexibility have been observed in schizophrenic patients in this test. Repeated administration of moderate doses of PCP (2 to 2.8 mg/kg) in rats led to a significant decrease in release and use of basal dopamine in the prefrontal cortex, which was subsequently correlated with impaired delayed alternation task [9, 10]. In this study, the optimized protocol was validated in the sub-chronic PCP model of memory deficit. PCP disrupted the shift in strategy to reach the reward without affecting first rule learning. PCP treated rats increased number of trial to criterion and made significantly more perseverative errors to shift to the second rule. PCP-induced deficit was

alleviated by 30mg/kg tolcapone administration 40 min before each session of second rule learning. Beside protocol validation, these data provided the first evidence for an efficacy of COMT inhibitor on sub-chronic PCP induced set shifting deficit.

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