Effect of SSRI discontinuation on anxiety-like behaviours in mice

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Abstract

Background: Abrupt cessation of therapy with a selective serotonin reuptake inhibitor (SSRI) is often associated with a discontinuation syndrome, typified by numerous disabling symptoms including elevated anxiety, which is of unknown cause.

Aim: Here, the effect of SSRI discontinuation on anxiety-like behaviour was investigated in mice.

Methods: Mice were treated repeatedly with paroxetine, citalopram or saline using a 3-arm experimental design comprising saline, continued SSRI and discontinued SSRI. Mice were assessed over 5 days after SSRI discontinuation using the elevated plus maze (EPM) and other anxiety tests.

Results: In an exploratory experiment mice discontinued (2 days) from paroxetine (12 days) showed evidence of increased anxiety on the EPM, although this effect was observed in male and not female mice. Follow-up studies confirmed the EPM findings in male mice discontinued (2 days) from paroxetine (12 or 28 days) or citalopram (12 days) compared to saline controls. Continued treatment with paroxetine was also anxiogenic on the EPM but this was not the case for citalopram. Paroxetine exposure for more than a week was required to elicit evidence of a discontinuation response, which did not obviously strengthen with increased frequency or duration of dose. Finally, SSRI discontinuation effects were not resolvable from continued treatment in other anxiety tests applied between 3 and 5 days post-discontinuation.

Conclusion: Overall, the current study provides evidence for a short-lasting increase in anxiety-like behaviour in mice following SSRI discontinuation, and offers a means for the investigation of the neurobiological mechanisms involved.

Key words: 5-HT, SSRI, discontinuation, anxiety, paroxetine, citalopram

Introduction

Selective serotonin (5-hydroxytryptamine; 5-HT) reuptake inhibitors (SSRIs) are currently the first-line pharmacological treatment for major depression and anxiety disorder. Despite their generally improved tolerability and side-effect profile compared to older antidepressants (Cipriani et al., 2018), the abrupt cessation of a course of treatment with SSRIs, like other antidepressants, is often associated with a disabling discontinuation syndrome (Haddad, 1997). Typical SSRI discontinuation symptoms include heightened anxiety, insomnia, nausea, dizziness, irritability and increases in suicidal thoughts (Delgado, 2006; Warner et al., 2006; Horowitz and Taylor, 2019). SSRI discontinuation is considered distinct from depression relapse in that symptoms appear within days of discontinuation, whereas relapse typically takes several weeks to manifest. Moreover, discontinuation symptoms are often different to those evident during the depressive episode, and include the emergence of somatic symptoms (Delgado, 2006). Problematic effects of SSRI discontinuation are not specific to depressed patients in that they are also reported in other patient groups including those with seasonal affective disorder, social anxiety, and panic disorder (Lader et al., 2004; Montgomery et al., 2005; Black et al., 1993).

SSRI discontinuation has recently risen to prominence following reports that the syndrome may be more common, disabling and longer-lasting that previously recognised (APPG, 2018; Davies and Read, 2019). Despite its clinical importance, there are few systematic investigations of SSRI discontinuation and its mechanism remains unknown. As an example of the problem, we are aware of just two preclinical studies that directly address the behavioural effects of SSRI discontinuation. One study reported enhanced acoustic startle response in rats 2 days after discontinuation from repeated treatment with citalopram (Bosker et al., 2010). Another study reported increased locomotor behaviour 4 h after the first “missed dose” of repeated fluoxetine in rats, and this effect dissipated within 4 days (Bjork et al., 1998). In addition, a small number of studies report no change in anxiety or locomotor behaviour in rats 1-3 weeks after the last dose of

repeated SSRI administration (Elizalde et al., 2008; Popa et al., 2010; Bouet et al., 2012; Strekalova et al., 2013). Taken together, these limited preclinical findings are suggestive of a short-lasting behavioural change associated with SSRI discontinuation in rodents, but this requires further systematic investigation.

It is noteworthy that previous preclinical studies have identified behavioural correlates of withdrawal from a wide variety of drugs including opiates, alcohol, and psychostimulants (Emmett-Oglesby et al., 1990; Vuong et al., 2010; El Hage et al., 2012; Perez and De Biasi, 2015). Much of this work has centred on behavioural readouts of anxiety which is a core feature of many drug withdrawal states (note that the term 'withdrawal' as opposed to 'discontinuation' is commonly used for drugs associated with compulsive drug seeking behaviour, which is not the case for SSRIs or other antidepressants). Such work led to novel insights into drug withdrawal mechanisms and aided the identification of treatment strategies, as exemplified by the use of clonidine to manage anxiety and other symptoms of opiate withdrawal (Gowing et al., 2002; Kosten and Baxter, 2019).

The present study investigated the effect of SSRI discontinuation on the behaviour of mice, with a focus on anxiety which is a core symptom of SSRI discontinuation in patients. Moreover, both anxiety and fear are well know to be modulated by pharmacological and genetic manipulations of the 5-HT transporter (Handley and McBlane, 1993; Line et al., 2011; Barkus et al., 2014; Lima et al., 2019). Paroxetine was selected for detailed investigation since discontinuation from this SSRI is considered particularly problematic in patients (Fava et al., 2015), likely due in part to its short half-life (Price et al., 1996). Paroxetine treatment duration and frequency were varied in an attempt to optimise conditions to detect discontinuation effects. Also, citalopram was added for comparison due to its greater selectivity for the 5-HT transporter than paroxetine, and its short half-life in rodents (Fredricson Overø, 1982). Anxiety-like behaviours were measured using a battery of tests including the elevated plus maze (EPM), which has proven sensitive in

detecting the withdrawal states of other drugs (Emmett-Oglesby et al., 1990; Vuong et al., 2010; El Hage et al., 2012; Perez and De Biasi, 2015), and is highly sensitive to manipulations of the 5-HT system (Briley et al., 1990; Handley and McBlane, 1993; Ohmura et al., 2020).

Materials & Methods

Animals

Adult mice (C57BL/6J, 8-10 weeks, Charles River) were housed (21°C on a 12 h light/dark cycle; lights off 19:00 to 7:00) with littermates in open-top cages (3-6 mice per cage) lined with sawdust bedding, for at least 1 week before the start of treatment. Food and water were available *ad libitum*. Cardboard sizzle nests were used for cage enrichment, and mice were handled using a cardboard tunnel to minimize stress associated with repeated injections (Gouveia and Hurst, 2019). Experiments followed the principles of the ARRIVE guidelines and were conducted according to the U.K. Animals (Scientific Procedures) Act of 1986 with appropriate personal and project licence coverage.

Drugs and reagents

Paroxetine hydrochloride (Abcam, ab120069; Apexbio, B2252-APE) and citalopram hydrobromide (Abcam, ab120133) were dissolved in saline (1 and 2 mg/ml, respectively). Drug dose (10 mg/kg s.c.) was chosen on the basis of both the half-life of paroxetine (6.3 h) and citalopram (1.5 h) in rodents (Kreilgaard et al., 2008) (Fredricson Overø, 1982), and previous studies reporting antidepressant actions of repeated administration of these drugs in mice (Elizalde et al., 2008). Treatment frequency (once- or twice-daily) and duration (7-28 days) were varied in an attempt to optimise the detection of discontinuation effects.

Experimental design

Mice were allocated to 1 of 3 experimental groups by stratified randomisation: i) saline group: saline + saline continuation; ii) continuation group: SSRI + SSRI continuation; iii) discontinuation group: SSRI + saline continuation. Mice were weighed daily beginning 2 days before the start of the experiment to establish a baseline weight, and habituate mice to handling. Mice received once- or twice-daily injections

of paroxetine, or the equivalent volume of saline, for 7, 12 or 28 days, then treatment was either continued (saline and continuation groups) or swapped to saline injections (discontinuation group) for a further 5 days during which behavioural tests were conducted. The effect of twice-daily injections of citalopram or saline for 12 days was also tested using a similar experimental design. Testing commenced either 18 h (once-daily) or 6 h (twice-daily) after the last injection. The 5 day discontinuation period was based on previous rodent experiments reporting discontinuation effects (Bosker et al., 2010; Trouvin et al., 1993), and aimed to capture the timeframe when paroxetine and citalopram fall to undetectable levels in the blood plasma (Benmansour et al., 1999; Cremers et al., 2000). An initial exploratory investigation of paroxetine used mixed sex groups (6 males, 6 females per group) and revealed sexually dymorphic effects. Consequently, all follow up experiments utilised male mice. Details of this and all other experiments are summarised in *Table 1*.

Behavioural tests

Mice were habituated to a holding room (i.e. not their home cage room) for at least 1 h before testing and on the 3 days preceding behavioural testing. All testing was conducted in the light phase (10:00 – 17:00 h) by an observer blind to treatment. Other than the changes in anxiety measures reported herein, no overt behavioural or somatic effects to continued/discontinued SSRI treatment were observed. Both a novelty-induced hyponeophagia test (Line et al., 2011) and novelty-supressed feeding test (Santarelli et al., 2003) were conducted on day 3 following discontinuation from 12- and 28-day once-daily paroxetine treatment, but results were confounded by an effect of test order, and hence data were excluded from the study.

Elevated Plus Maze (EPM). The EPM model assesses approach-avoidance behaviour, relying on the conflict between the innate aversion of open, elevated spaces and the exploratory drive in mice (Komada et al., 2008). EPM experiments were carried out as previously described for studies examining the role of

the 5-HT transporter in unconditioned anxiety (Line et al., 2011). The EPM (50 cm off the floor) was placed in a dimly-lit room and comprised 2 open arms (35 x 6 cm) perpendicular to 2 closed arms (35 x 6 cm, 20 cm walls), with a central region (6 x 6 cm). Mice were initially placed facing the walls at the far end of the closed arm, and movement was automatically tracked for 300 s (ANY-maze software, Stoelting Co.).

Locomotor activity (LMA). Given the potential confound of changes in locomotion on anxiety measures, previous studies highlight the value of assessing locomotion in separate low anxiety environments (Lister, 1990). Here, spontaneous LMA was assessed in a dimly-lit room as previously described (Line et al., 2011). Mice were placed individually into an unfamiliar plastic cage (42 x 22 x 20 cm) lined with sawdust and covered with a perforated plexiglass lid. LMA was monitored by horizontal and vertical infrared beams, and the number of beam breaks was automatically recorded in 5 min bins for 60 min (Photobeam Frame software, San Diego Instruments).

Aversive open field (AOF). The open field test assesses exploration and behaviour in an anxiogenic environment (Prut & Belzung, 2003). Mice were placed in a brightly-lit, white cylindrical chamber (30 cm radius) and movement was automatically tracked for 600 s (ANY-maze software). The centre zone was designated as a central circle of 10 cm radius.

Light/dark box (LDB). The LDB assesses approach-avoidance conflict in rodents based on their innate fear of brightly-lit places (Bourin & Hascoet, 2003). The LDB arena (Pritchett et al., 2015) was inside a sound-attenuating cubicle and consisted of a dark, enclosed compartment (21 cm long x 16 cm high x 16 cm wide) separated by a small doorway from a brightly-lit, open compartment (46.5 cm long x 21 cm high x 21 cm wide). Mice were placed in a corner of the dark compartment and their movement was automatically tracked for 600 s by horizontal infrared beams (activity monitor software, Med Associates Inc).

Fear conditioning. Assessment of fear learning was performed as described previously (Line et al., 2014) using conditioning chambers (Med Associates Med Associates Inc., USA) with a floor of metal bars connected to a scrambled-shock generator (controlled by Med-PC IV software programme). In brief, mice were placed individually in a covered plastic box for 30 min before each session. Two different conditioning contexts were used, each associated with a specific scent and distinctive wall layout (black and white striped walls, lavender essential oil *versus* grey walls, sandalwood essential oil). Mice were trained in one context and tested in the other (context was counterbalanced across training and test days). On the training day, after a 180 s acclimatisation period, mice received 2 tone-shock pairings, each consisting of a 30 s tone (72 dB, 2900 Hz) paired with a shock (0.5 ms, 0.3 mA) delivered in the final 0.5 s of the tone (180 s inter-trial interval). On the test day, 24 h later, mice received the same tone presentations but did not receive the shock. Freezing was determined from video recordings (analysed by NIH ImageJ with a customised script) and defined as <0.07% pixel change in two consecutive frames (1 frame per second). Pre-tone freezing was calculated as % freezing in the 30 s before the tone, and freezing to tone was calculated as the % freezing for 30 s of tone. The change in freezing levels (ΔFreezing) was then calculated [freezing to tone – pre-tone freezing] and presented as average ΔFreezing per day.

Statistical analysis

D'Agostino-Pearson's test for normality was applied to all data sets. If data were normally distributed, then one-way ANOVA was used with Fisher's Least Significant Difference (LSD) to compare treatments. If data were not normally distributed, Kruskal-Wallis with Fisher's LSD was used. Sex was assessed as a co-factor using univariate analysis, and Kruskal-Wallis was used for small sample sizes ($n \leq 6$ per group). Data were analysed mainly using GraphPad Prism (v8) but occasionally using IBM SPSS Statistics (v24) (univariate analysis). Data are presented as mean ± standard error of the mean (SEM) values. $P<0.05$ was considered statistically significant.

Results

Effect of discontinuation from 12 days of once-daily paroxetine

An exploratory experiment (6 males, 6 females per group) tested the effect of discontinuation from 12 days of once-daily paroxetine. On day 2 following discontinuation, mice showed increased anxiety on the EPM (*Suppl. Fig. 1A-D*), but this effect was apparent in males and not females, and was also present in the continuous treatment group. Thus, statistical analysis of time spent in the open arms revealed a significant sex effect ($F_{(1,33)}$=14.01, p=0.0008) and a treatment x sex interaction ($F_{(2,29)}$=5.987, p=0.0067). Subsequent analyses were then conducted on male and female mice. Male mice discontinued from paroxetine spent less time in the open arms (treatment effect: H(2)=9.789, p=0.0027; *Suppl. Fig. 1A,* post-hoc p-values in figures legends), had fewer open arms entries (treatment effect: H(2)=11.55, p=0.0005; *Suppl. Fig. 1B*), increased latency for open arm entry (treatment effect: H(2)=6.327, p=0.0360; *Suppl. Fig. 1C*), and reduced distance travelled (treatment effect: H(2)=10.96, p=0.0009; *Suppl. Fig. 1D*). In contrast, female mice continuing paroxetine had a decreased latency to enter the open arms (treatment effect: H(2)=7.879, p=0.0119; *Suppl. Fig. 1C*), but there were no significant differences between the discontinued mice and saline controls on any parameter (*Suppl. Fig. 1A-D*). On day 4, discontinued male and female mice showed no change in LMA and AOF tests (*Suppl. Table 1*). On day 5, male discontinued mice showed evidence of increased anxiety on the LDB in terms of spending less time in the light zone (H(2)=8.667, p=0.0069; *Suppl. Fig. 1E*) with decreased distance travelled (H(2)=10.15, p=0.0021; *Suppl. Fig. 1F*). Male mice continuing paroxetine also showed evidence of increased anxiety and decreased distance travelled on the LDB (*Suppl. Fig. 1E-F*). In contrast, female mice discontinued from paroxetine actually spent more time in the light zone (H(2)=6.538, p=0.0304; *Suppl. Fig. 1E*). Overall, results of this exploratory experiment suggest that discontinuation from paroxetine was anxiogenic in mice but this effect was not readily distingishable from continuous treatment and the effect was apparent in males and not females.

To increase the power of this exploratory study, EPM measurements were repeated in a separate group of male mice (n=8/group additional mice) and the two sets of data from male mice were combined (n=14/group total). The pooled data confirmed that following 2 days discontinuation from 12 days of once-daily paroxetine, male mice showed evidence of increased anxiety on the EPM. Thus, discontinued mice spent less time in the open arms (treatment effect: $F_{(2,38)}$=6.830, p=0.0029; post-hoc p=0.0011 vs saline; *Fig. 1A*), had fewer open arms entries (treatment effect: $F_{(2,38)}$=13.94, p<0.0001; post-hoc p<0.0001 vs saline, p=0.0349 vs continuation; *Fig. 1B*), increased latency for open arm entry (treatment effect: $F_{(2,38)}$=7.790, p=0.0015; post-hoc p=0.0004 vs saline, p=0.0201 vs continuation; *Fig. 1C*), and reduced distance travelled (treatment effect: $F_{(2,38)}$=17.96, p<0.0001; post-hoc p<0.0001 vs saline, p=0.0006 vs continuation; *Fig. 1D*).

Although responses of mice discontinued from paroxetine were significantly different from mice on continued paroxetine on various performance measures on the EPM, the latter group also showed increased anxiety on several EPM parameters compared to saline controls (time in open arms: post-hoc p=0.0101 vs saline; entries to open arms: post-hoc p=0.0046 vs saline; distance travelled: post-hoc p=0.0494 vs saline*; Fig. 1*).

Effect of discontinuation from 28 days of once-daily paroxetine

To test whether increased duration of paroxetine treatment would produce a stronger discontinuation effect, male mice were treated once-daily with paroxetine for 28 days. On the EPM, mice discontinued for 2 days spent less time in the open arms (treatment effect: $F_{(2,29)}$=6.649, p=0.0042; post-hoc, p=0.0011 vs saline, p=0.0495 vs continuation; *Fig. 2A*), had reduced open arm entries (treatment effect: $F_{(2,29)}$=11.19, p=0.0002; post-hoc p<0.0001 vs saline; *Fig. 2B*) with increased latency for open arm entry (treatment effect: H(2)=13.38, p=0.0012; post-hoc p=0.0021 vs saline; *Fig. 2C*) and reduced distance travelled (treatment

effect: $F_{(2,29)}$=15.73, p<0.0001; post-hoc p<0.0001 vs saline; *Fig. 2D*). Again, mice on continued paroxetine also showed evidence of increased anxiety on the EPM compared to saline controls (entries to open arms: post-hoc p=0.0016 vs saline; latency to open arm entry: post-hoc p=0.0011 vs saline; distance travelled: post-hoc p=0.0004 vs saline; *Fig. 2*).

On day 4, discontinued mice had reduced activity on the LMA test compared to saline controls, as did mice on continuous treatment ($F_{(2,32)}$=3.872, p=0.032, *Suppl. Table 2*), but there was no effect of any treatment on performance on the AOF compared to saline controls (*Suppl. Table 2*). On day 5, discontinued mice spent less time in the light zone of the LDB ($F_{(2,29)}$=7.468, p=0.0024; post-hoc p=0.0014 vs saline; *Fig. 2E*) and had decreased distanced travelled ($F_{(2,29)}$=9.782, p=0.0006; post-hoc p=0.0006 vs saline; *Fig. 2F*), as did mice on continuous paroxetine to a similar extent (time in light zone: post-hoc p=0.0045 vs saline; distance travelled: post-hoc p=0.0007 vs saline; *Fig. 2*).

Overall, these results add further evidence that discontinuation from paroxetine generates an anxiety-like effect although whether this is a greater and longer lasting effect than 12 day paroxetine treatment is confounded by evidence of reduced locomotor activity detected in separate experiments (see Discussion).

Effect of discontinuation from 7 days of once-daily paroxetine

To investigate the required duration of treatment with paroxetine to produce discontinuation effects, male mice received once-daily paroxetine injections for 7 days. On day 2 following treatment cessation, discontinued mice did not perform differently on the EPM compared to saline controls or mice on continuous paroxetine in terms of time spent in the open arms ($F_{(2,33)}$=0.1256, p=0.8824, *Fig. 3A*), open arm entries ($F_{(2,33)}$=0.6936, p=0.5069; *Fig. 3B*), latency to first enter the open arms (H(2)=1.610, p=0.4471; *Fig.*

3C) and distance travelled ($F_{(2,33)}$=1.262, p=0.2964; *Fig. 3D*), although it is also worth noting that the overall levels of anxiety in this cohort of mice appeared to be higher than for other experiments in this study.

These results suggested that a paroxetine treatment duration of 7 days is not sufficient to induce discontinuation effects.

Effect of discontinuation from 12 days of twice-daily paroxetine.

To test whether increasing the frequency of paroxetine treatment (and hence increasing daily dose) would produce a more pronounced discontinuation effect, mice were treated twice-daily with paroxetine for 12 days. This experiment also tested the possibility that mice continuously treated with paroxetine may have been experiencing discontinuation effects, in that up to this point all 'continuous' treatment groups were tested 18 h after the last injection. Here mice were tested 6 h after the last injection so that plasma levels of paroxetine likely remained high. This experiment also examined the possibility that discontinuation from paroxetine would affect fear-learning, which is reported to be sensitive to manipulations of the 5-HT transporter (e.g. (Lima et al., 2019)).

On day 2 following treatment cessation, discontinued mice showed evidence of increased anxiety on the EPM in terms of reduced time on the open arms ($F_{(2,33)}$=10.83, p=0.0002; post-hoc p<0.0001 vs saline; *Fig. 4A*), reduced open arm entries ($F_{(2,33)}$=3.482, p=0.0424; post-hoc p=0.0141 vs saline; *Fig. 4B*) and reduced distance travelled ($F_{(2,33)}$=7.469, p=0.0021; post-hoc p=0.0008 vs saline, p=0.0086 vs continuation; *Fig. 4D*), albeit with unchanged latency for open arm entry ($F_{(2,33)}$=2.174, p=0.3373; *Fig 4C*). Again, mice maintained on paroxetine also showed evidence of increased anxiety on the EPM compared to saline controls (time in open arms: post-hoc p=0.0070 vs saline; *Fig. 4A*).

On day 3, neither discontinued mice nor mice receiving continued paroxetine were different from saline controls in terms of either LMA measurements ($F_{(2,33)}$=0.073, p=0.929) or the time spent in the centre zone of the AOF (*Suppl. Table 3*).

On days 4 and 5 following cessation of paroxetine treatment, mice were assessed for fear conditioning (training on day 4, testing on day 5). All mice exhibited increased freezing with the onset of the conditioned stimulus. However, neither discontinued mice nor mice on continued treatment were different from saline controls in terms of freezing to tone on the training day or in their response to the conditioned tone during recall on the test day (*Suppl. Table 3*).

These results add further evidence that mice discontinued from paroxetine show evidence of increased anxiety on the EPM, but that increased treatment frequency (and thereby dose) did not lead to a more clear-cut and longer-lasting discontinuation effect. Mice on continued paroxetine also showed increased anxiety on the EPM, even when tested 6 h after the last injection.

Effect of discontinuation from 12 days of twice-daily citalopram

A final experiment was carried out to determine whether evidence of increased anxiety was also observed in response to discontinuation from 12 days of twice-daily treatment with another SSRI, citalopram. On day 2 following treatment cessation, discontinued mice showed modest evidence of increased anxiety on the EPM in terms of increased latency to enter the open arms (treatment effect: H(2)=7.220, p=0.0221; post-hoc p=0.0078 vs saline; *Fig. 5C*) and a trend effect for reduced number of open arm entries ($F_{(2,51)}$=1.751, p=0.1839; post-hoc p=0.0862 vs continuation; *Fig. 5B*) although time spent in the open arms ($F_{(2,51)}$=0.5349; 0.5890; *Fig. 5A*) and distance travelled ($F_{(2,51)}$=1.949, p=0.1529; *Fig. 5D*) were not different between groups. Unlike mice treated continuously with paroxetine, mice on continuous citalopram (tested

6 h after the last injection) did not show signs of increased anxiety on the EPM compared to saline controls (*Fig. 5*).

Mice discontinued from citalopram were not different from either saline controls or mice maintained on citalopram on the LMA and AOF tests (day 3 of discontinuation), nor on any parameter measured during the training (day 4) or recall (day 5) stages of the fear conditioning paradigm (*Suppl. Table 4*).

These results indicated that discontinuation from citalopram is mildly anxiogenic in male mice.

Discussion

Abrupt cessation of a therapeutic course of treatment with an SSRI is often associated with a discontinuation syndrome comprising unpleasant centrally and peripherally-mediated changes, which are of unknown cause. Here, experiments determined the effect of SSRI discontinuation in mice with a focus on paroxetine which is particularly problematic in patients, and anxiety which is a core discontinuation symptom. The data provide evidence for the rapid emergence of an anxiety-like effect of SSRI discontinuation in mice. Specifically, 2 days following discontinuation from repeated administration of paroxetine or citalopram, mice showed evidence of increased anxiety on various EPM readouts compared to mice maintained on drug and saline controls. Attempts to optimise treatment protocols found that paroxetine exposure for more than a week was required to elicit an anxiogenic response to discontinuation, and this response did not obviously strengthen with increasing duration or dose of treatment. Moreover, the anxiogenic effect of paroxetine discontinuation was sexually-dymorphic in that, at least under the present conditions, the response was detected in male and not female mice. Finally, data from our other anxiety tests suggested that the heightened anxiety response in the paroxetine discontinuation group may have persisted for up to 5 days. However, this effect was relative to saline controls and not mice maintained on drug (specifically paroxetine), which themselves exhibited elevated anxiety throughout the period of behavioural testing. Overall, data from these experiments indicate the emergence of increased anxiety in male mice within 2 days of SSRI discontinuation although the experiments were unable to resolve the duration of this effect.

The EPM is widely used to assess anxiety levels in rodents, and has been previously used to detect increased anxiety evoked by discontinuation of other drugs including psychostimulants and alcohol, as well as for detecting changes in 5-HT function (see Introduction). The majority of these studies prioritise two EPM indices of anxiety: open arm entries and time spent on the open arms, which are closely correlated parameters (Lister, 1990). Here, one or other of these parameters were found to be decreased in discontinued

mice compared to those maintained on drug. Detection of anxiety-like behaviours on the EPM can be confounded by non-specific changes in locomotion (Lister, 1990; Handley and McBlane, 1993; Weiss et al., 1998), and in the present study SSRI discontinuation was often associated with reduced distance travelled on the EPM which may have impacted on open arm parameters. Analysis of covariance can be used to help determine the influence of distance travelled on EPM performance but the impact of this analysis is limited since, as pointed out elsewhere (Lister, 1990). Thus, in the context of an anxiogenic environment, a decrease in distance travelled would be consistent with an increase in anxiety reflecting the behavioural inhibition component of the anxiety response (Gray, 1982; McNaughton and Gray, 2000). Moreover, we carried out separate locomotor assessments in a low anxiety, home cage-like environment as previously recommended (Lister, 1990) and found little evidence that mice undergoing SSRI discontinuation had low spontaneous locomotor activity (with the exception of the 28 day paroxetine). Thus, decreased activity on the EPM appeared to be context-specific, and plausibly arose from increased anxiety levels due to exposure to a novel, anxiogenic environment rather than decreased locomotor activity *per se*. Nevertheless, without further investigation we cannot exclude the possibility that the elevated anxiety measures in discontinued mice is not genuine anxiety but instead linked to other unpleasant experiences of discontinuation including somatic changes that feature in the clinical syndrome. This is particularly the case for discontinuation from 28 day paroxetine where the confound of reduced locomotion in separate assessments suggests the emergence of a syndrome which may be driven by hypolocomotion.

The evidence of increased anxiety-like behaviour within 2 days of discontinuation from paroxetine and citalopram is consistent with the short half-lives of these drugs in rodents (6.3 h and 1.5 h, respectively) (Kreilgaard et al., 2008; Fredricson Overø, 1982), and an abrupt fall in brain levels of the drug once drug administration has stopped. Moreover, a rapid appearance of an anxiogenic effect would be consistent with earlier evidence of heightened startle responsivity in rats 2 days after discontinuation from repeated

treatment with citalopram (~24 mg/kg per day for 15 days) (Bosker et al., 2010). Our finding also fits with the clinical picture that SSRIs like paroxetine which have a short half-life in humans are particularly problematic in terms of discontinuation effects which can onset within days of treatment cessation (Rosenbaum et al., 1998; Michelson et al., 2000; Fava et al., 2015). Although plasma levels of paroxetine and citalopram were not measured in the present study, previous studies report that administration of paroxetine or citalopram to rodents at the doses used here, produces plasma drugs levels broadly in the therapeutic range that fall quickly once administration ceases. Thus, rat plasma levels of citalopram (121 ng/ml) were reported to fall around 8-fold within 24 h of stopping administration (24 mg/kg per day for 15 days) and were at the limits of detection at 48 h (Bosker et al., 2010). Also, rat plasma levels of paroxetine (411 ng/ml) fell by a similar degree 48 h after cessation of paroxetine (10 mg/kg for 21 days) and were undetectable after 96 h of washout (Benmansour et al., 1999).

A clinical feature of SSRI discontinuation is that in some patients the effect can endure over periods of several weeks or even months (Fava et al., 2007; Davies and Read, 2019). Since animals show an adaptive response following repeated testing on the EPM test and other anxiety tests (either due to habituation or even changes from one of anxiety to one of acquired fear (File, 2001)), the present study applied a battery of different anxiety tests to determine whether effects of SSRI discontinuation persist, and at the same time determine the generality of our findings across assays with different sensorimotor and motivational demands, but results were somewhat inconclusive. For example, whilst mice discontinued from paroxetine (eg. once-daily for 12 days) showed evidence of increased anxiety in the LDB on day 5 compared to saline controls, mice receiving continuous paroxetine also demonstrated increased anxiety in this test. These data potentially accord with a short-lasting anxiogenic effect of SSRI discontinuation in mice that may dissipate after 48 h. However, previous experiments found an enhanced acoustic startle response in rats discontinued from citalopram compared to those still receiving treatment, that persisted for at least 7 days after the last

dose (Bosker et al., 2010). The current data do not preclude the persistence of discontinuation effects (see also above). For example, it is possible that even the use of a battery of tests over successive days obscured the detection of genuine discontinuation versus continuation effects due to prior test exposure.

Here, the anxiety response to paroxetine discontinuation was detected after 12 and 28 days (but see above) but not after 7 days of continuous drug treatment, suggesting the involvement of a neuroadaptive mechanism. This timing accords with findings in clinical studies that taking an SSRI for a week is not sufficient to produce discontinuation symptoms (Yonkers et al., 2015), and other data from randomised control trials showing that discontinuation symptoms can emerge after several weeks of SSRI treatment (Baldwin et al., 2006). The nature of the neuroadaptive responses underpinning SSRI discontinuation is unknown. Although much is established regarding how the 5-HT systems adapts during a course of SSRI administration (Sharp, 2013; Artigas, 2013), remarkably few studies have investigated how 5-HT transmission changes when chronic SSRI administration is immediately stopped. Two earlier studies reported decreased 5-HT synthesis and metabolism in post-mortem rat brain tissue for up to 3 days following withdrawal from citalopram and fluoxetine (Trouvin et al., 1993; Bosker et al., 2010). On the other hand, SSRI discontinuation likely leaves 5-HT neurons in a state of reduced 5-HT_{1A} autoreceptor sensitivity (desensitisation), which theoretically would push the 5-HT system in the opposite direction and increase 5-HT neuron firing and 5-HT release (Richardson-Jones et al., 2010). Indeed, Trouvin and colleagues (1993) reported a paradoxical overshoot in brain 5-HT metabolite levels 7-21 days following discontinuation from fluoxetine. This is potentially more in line with the numerous lines of animal research which have shown that increased anxiety is often associated with increased 5-HT (Briley et al., 1990; Handley and McBlane, 1993; Ohmura et al., 2020), although it should be noted that the timing of this 5-HT overshoot is out of keeping with the current findings.

Neuroadaptive changes in certain postsynaptic 5-HT receptors as well as the 5-HT transporter itself are also reported following genetic knockout or pharmacological inhibition of the 5-HT transporter (Jennings et al., 2012; Benmansour et al., 1999) and might rebound during discontinuation. Thus, contrary to earlier speculations of either a hyper- or hyposerotonergic state underpinning the symptoms of SSRI discontinuation syndrome (Blier and Tremblay, 2006; Renoir, 2013), a potentially more complex and dynamic picture emerges of decreased synaptic availability of 5-HT, elevated 5-HT cell firing, altered postsynaptic 5-HT receptors, and overall destabilised (and dysfunctional) 5-HT signalling and related 5-HT-induced changes in neural plasticity. Although as a drug class SSRIs are pharmacologically selective, 5-HT has multiple interactions with other transmitter systems so it is also entirely plausible that these contribute to the effects of SSRI discontinuation. The key role of noradrenaline in the effects of opiate withdrawal (eg. Kosten and Baxter, 2019) illustrates this point. Finally, numerous studies have reported enhanced neuroplasticity and structural changes following repeated SSRI treatment which are thought to be responsible for their therapeutic effects (Castrén and Hen, 2013; Harmer et al., 2017). However, it is unlikely that these changes reverse immediately following SSRI discontinuation and thus it improbable that they are responsible for the acute behavioural effects detected here. Nonethless, the lack of data means this possibility cannot currently be excluded.

Our initial exploratory study did not detect an effect of discontinuation from paroxetine (once-daily, 12 days) in female mice. Further investigation of this finding is required, however there are many sexual dymorphisms reported in mice, some of which offer a plausible explanation for the male-female difference found here. For example, it is reported that female mice metabolise fluoxetine more effectively than males through increased activity of cytochrome P_{450} enzymes (Hodes et al., 2010). Since paroxetine is also metabolised by these enzymes, in the present study the paroxetine dosing regimen may not have been sufficiently high for female mice. Also, evidence of sexual dymorphism in the 5-HT system is reported for

mice (Renoir et al., 2011), in particular female mice were found to exhibit reduced 5-HT transporter mRNA compared to males. If this difference translated to the female mice used here, it would account for their reduced sensitivity to paroxetine. The stage of the estrous cycle of mice was also found to influence the behavioural response to fluoxetine treatment, with mice in the diestrus phase of the cycle being less sensitive the drug (Yohn et al., 2020). Furthermore, we cannot exclude the possibility that these differences reflect different home cage experiences of male and female mice, particularly in terms of aggressive behaviours and social dominance hierarchies. Nonetheless, there is evidence that men are more likely to report discontinuation symptoms following SSRI cessation (Coupland et al., 1996), so the current findings may reflect a the greater propensity of males to experience SSRI discontinuatuion syndrome rather than pitfalls of the preclinical model.

Finally, it is interesting to note that in the present experiments, mice *maintained* on paroxetine showed evidence of increased anxiety on the EPM and LDB. One possible explanation is that discontinuation effects are occurring even in these animals because in some experiments mice were tested 18 h after the last injection of paroxetine (see *Table 1*) when drug levels are likely to have fallen. However, since mice tested 6 h after the last injection still showed evidence of increased anxiety, discontinuation is not the likely cause. Another explanation is that increased anxiety is due to continuing blockade of the 5-HT transporter, and the consequent increase in synaptic 5-HT. In support of this idea, previous studies with mice reported that continuous treatment with certain SSRIs is anxiogenic on the EPM (Oh et al., 2009; Venzala et al., 2012; Turcotte-Cardin et al., 2019). Also, ourselves and others have reported that 5-HT transporter knockout mice show heightened anxiety on the EPM and other anxiety tests (Ansorge et al., 2004; Line et al., 2011). However, other findings argue against this position. Firstly, the high anxiety phenotype of the 5-HT transporter knockout mice may in part involve a neurodevelopmental mechanism (Ansorge et al., 2004). Secondly, previous studies on the effect of repeated administration of paroxetine to mice on EPM

performance do not consistently detect increased anxiety (Goeldner et al., 2005; Elizalde et al., 2008; Thoeringer et al., 2010; Guilloux et al., 2011), with mouse strain difference being one potential confound (Jin et al., 2017). Thirdly, the current study found that mice continuously treated with citalopram (twice-daily for 12 days) did not show an anxiogenic response on the EPM. Thus, on balance available data suggest that the anxiogenic effect of continuous paroxetine detected here is not easily explained by either discontinuation itself or a simple 5-HT-related mechanism. Alternative explanations include a role for other pharmacological effects of paroxetine (muscarinic receptor antagonism, noradrenaline reuptake blockade).

In conclusion, the present study reports evidence of increased anxiety-like behaviour in mice within 48 h of discontinuation from a course of SSRI treatment, that likely involves a neuroadaptive mechanism. This effect correlates with findings of increased anxiety in patients within days of stopping a course of SSRI therapy, and may therefore provide a useful model to aid the investigation of the neurobiological mechanisms underpinning of SSRI discontinuation.

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Declaration of Interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Table

Table 1

Experimental design. Elevated plus maze, EPM; locomotor activity, LMA; aversive open field, AOF; light/dark box, LDB; fear conditioning, FC. Time since last injection of SSRI for Continuation (CON) and Discontinuation (DIS) groups, DIS receive saline injections on discontinuation days. *Exploratory experiment, †data were pooled with the exploratory experiment, ††data excluded from the study (see Methods for details).

Drug	Dosing period duration (days)	Injection schedule	Time since last injection (h)	Group sex and size	Anxiety tests (day of test post discontinuation)
Paroxetine*	12	Once-daily	CON: 18 h DIS: 48 - 120 h	Males n=6 Females n=6	EPM (day 2); ††NIH, ††NSF (day 3); LMA, AOF (day 4); LDB (day 5)
Paroxetine†	12	Once-daily	CON: 18 h DIS: 48	Males n=8	EPM (day 2)
Paroxetine	28	Once-daily	CON: 18 h DIS: 48 – 120 h	Males n=12	EPM (day 2); ††NSF, ††NIH (day 3); LMA, AOF (day 4); LDB (day 5)
Paroxetine	7	Once-daily	CON: 18 h DIS: 48 h	Males n=12	EPM (day 2)
Paroxetine	12	Twice-daily	CON: 6 h DIS: 48 – 120 h	Males n=12	EPM (day 2); LMA, AOF (day 3); FC (day 4-5)
Citalopram	12	Twice-daily	CON: 6 h DIS: 48 – 120 h	Males n=10-18	EPM (day 2); LMA, AOF (day 3); FC (day 4-5)

Figure legends

Figure 1

Performance on the EPM (300 s) of male mice discontinued from 12 days of once-daily paroxetine. Panels represent time spent in open arms (A), entries to open arms (B), latency to enter the open arms (C) and distance travelled (D). SAL, Saline (n=14); CON, Continuation (n=13, one mouse excluded due to loss of data); DIS, Discontinuation (n=14). Mean ± SEM values are shown with individual values indicated by dots. One-way ANOVA followed by post-hoc Fisher's LSD. * $p<0.05$, ** $p<0.01$.

Figure 2

Performance on the EPM (300 s) and LDB (600 s) of male mice discontinued from 28 days of once-daily paroxetine. Panels represent time spent in open arms (A), entries to open arms (B), latency to enter the open arms (C) and distance travelled (D) on the EPM as well as time spent in the light zone (F) and distance travelled (G) on the LDB. SAL, Saline (n=10-11); CON, Continuation (n=11); DIS, Discontinuation (n=10-11). Mean ± SEM values are shown with individual values indicated by dots. One-way ANOVA followed by post-hoc Fisher's LSD (A-B, D-F), Kruskal-Wallis followed by post-hoc Fisher's LSD (C). * $p<0.05$, ** $p<0.01$.

Figure 3

Performance on the EPM (300 s) of male mice discontinued from 7 days of once-daily paroxetine. Panels represent time spent in open arms (A), entries to open arms (B), latency to enter the open arms (C) and distance travelled (D). SAL, Saline (males n=12); CON, Continuation (males n=12); DIS, Discontinuation (males n=12). Mean ± SEM values are shown with individual values indicated by dots. One-way ANOVA followed by post-hoc Fisher's LSD (A-B, D), Kruskal-Wallis followed by post-hoc Fisher's LSD (C).

Figure 4

Performance on the EPM (300 s) of male mice discontinued from 12 days of twice-daily paroxetine. Panels represent time spent in open arms (A), entries to open arms (B), latency to enter the open arms (C) and distance travelled (D). SAL, Saline (n=12); CON, Continuation (n=12); DIS, Discontinuation (n=12). Mean ± SEM values are shown with individual values indicated by dots. One-way ANOVA followed by post-hoc Fisher's LSD (A-B, D), Kruskal-Wallis followed by post-hoc Fisher's LSD (C). * $p<0.05$, ** $p<0.01$.

Figure 5

Performance on the EPM (300 s) of male mice discontinued from 12 days of twice-daily citalopram. Panels represent time spent in open arms (A), entries to open arms (B), latency to enter the open arms (C) and distance travelled (D). SAL, Saline (n=18); CON, Continuation (n=18); DIS, Discontinuation (n=18). Mean ± SEM values are shown with individual values indicated by dots. One-way ANOVA followed by post-hoc Fisher's LSD (A-B, D), Kruskal-Wallis followed by post-hoc Fisher's LSD (C). * $p<0.05$, ** $p<0.01$.

Figure 1

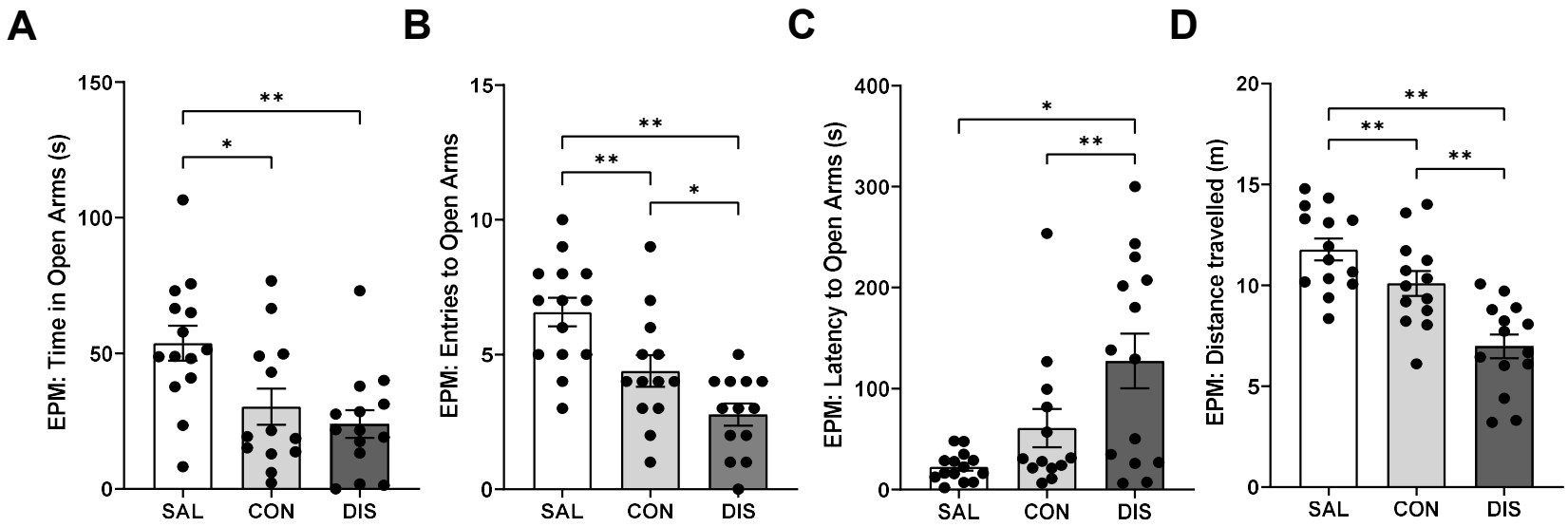

A
B
C
D
EPM: Time in Open Arms (s)
EPM: Entries to Open Arms
EPM: Latency to Open Arms (s)
EPM: Distance travelled (m)
SAL
CON
DIS
*
**

Figure 2

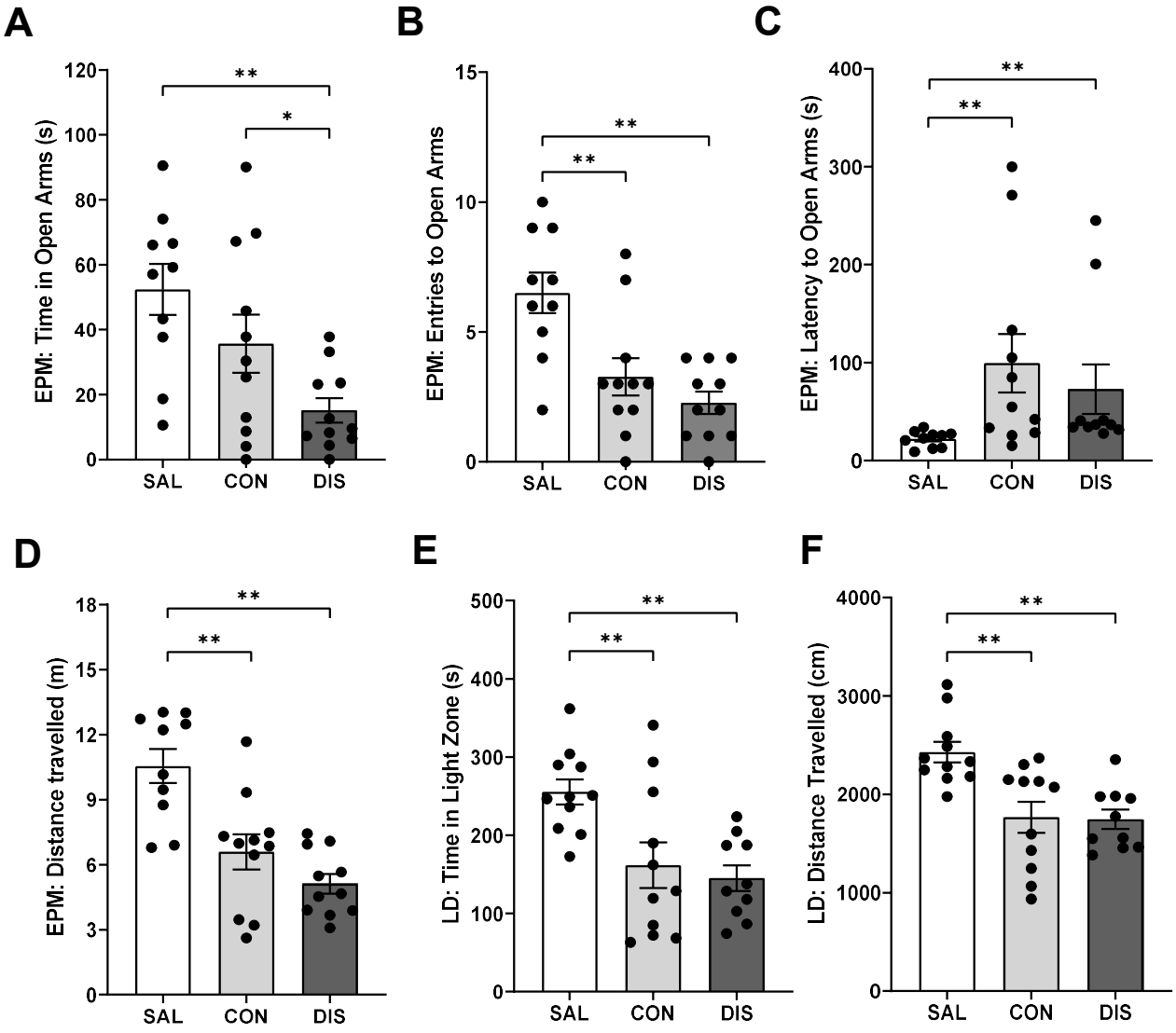

A
EPM: Time in Open Arms (s)
120
100
80
60
40
20
0
**
*
SAL
CON
DIS
B
EPM: Entries to Open Arms
15
10
5
0
**
**
SAL
CON
DIS
C
EPM: Latency to Open Arms (s)
400
300
200
100
0
**
**
SAL
CON
DIS
D
EPM: Distance travelled (m)
18
15
12
9
6
3
0
**
**
SAL
CON
DIS
E
LD: Time in Light Zone (s)
500
400
300
200
100
0
**
**
SAL
CON
DIS
F
LD: Distance Travelled (cm)
4000
3000
2000
1000
0
**
**
SAL
CON
DIS

Figure 3

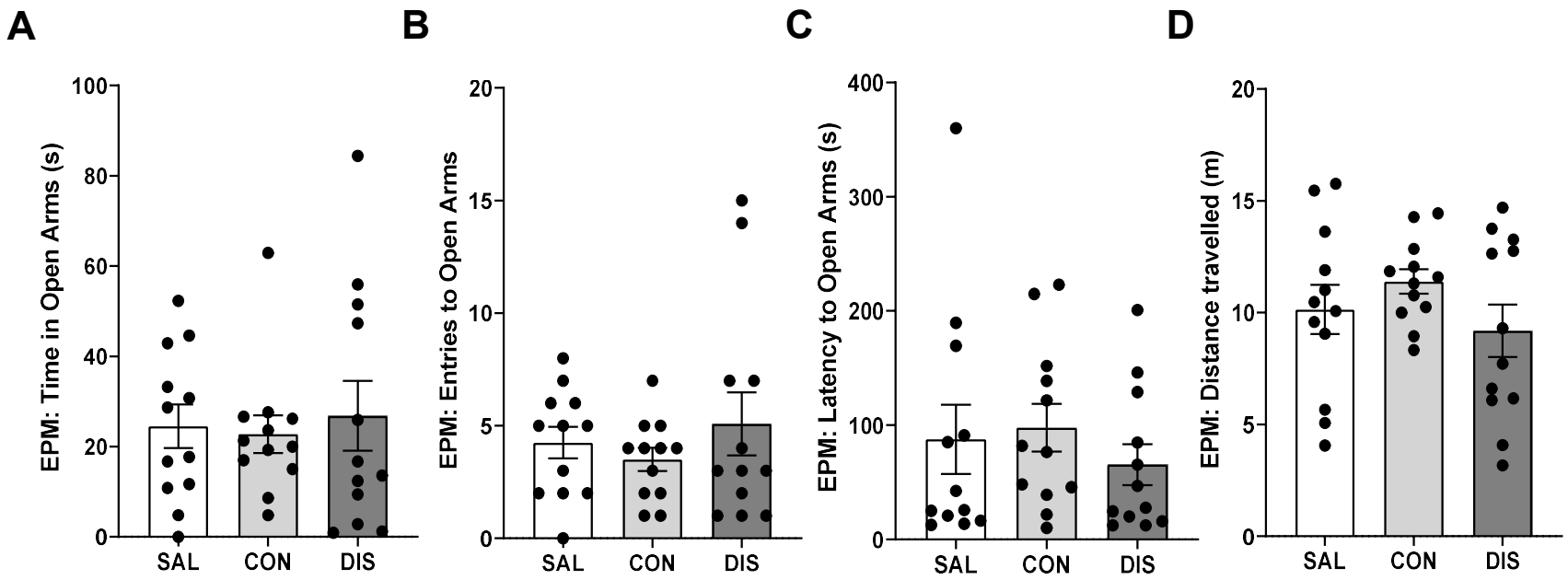
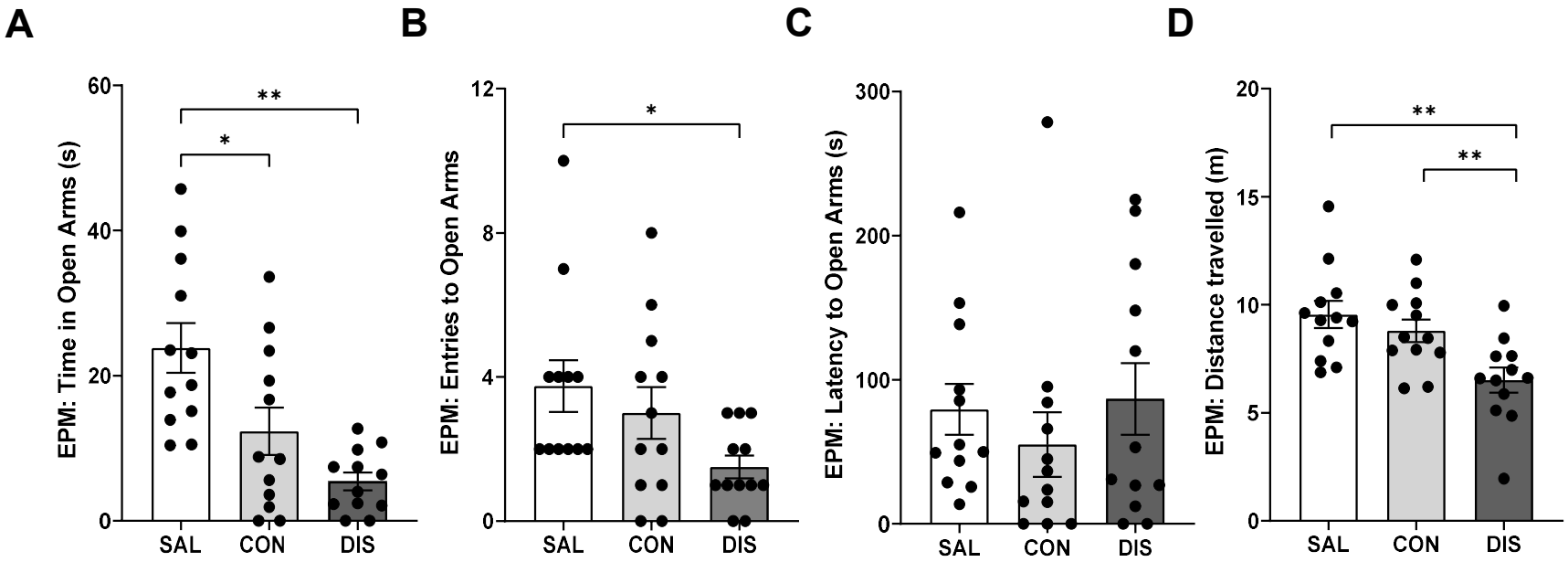

A
B
C
D
EPM: Time in Open Arms (s)
EPM: Entries to Open Arms
EPM: Latency to Open Arms (s)
EPM: Distance travelled (m)
SAL
CON
DIS

Figure 4


A
B
C
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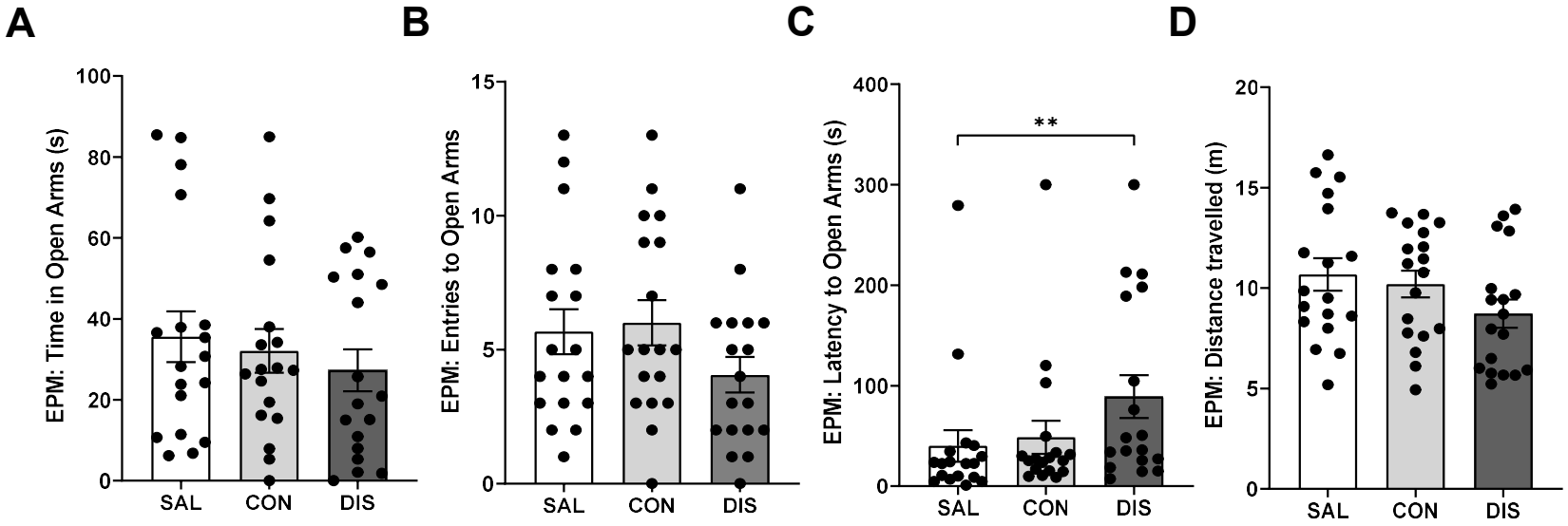
EPM: Time in Open Arms (s)
EPM: Entries to Open Arms
EPM: Latency to Open Arms (s)
EPM: Distance travelled (m)
SAL
CON
DIS
*
**

Figure 5


A
B
C
D
EPM: Time in Open Arms (s)
EPM: Entries to Open Arms
EPM: Latency to Open Arms (s)
EPM: Distance travelled (m)
SAL
CON
DIS
**