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Characterizing Non-Hallucinogenic Psychedelics Beyond the Head Twitch Response: Phenotypic Fingerprinting of Lisuride and LSD

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ABSTRACT (190 words)

The head-twitch response (HTR) is widely used as a preclinical assay for the hallucinogenic potential of compounds, with its utility based on $5HT_{2A}R$ activation. Here, we examine how the polypharmacological agonists lisuride and lysergic acid diethylamide (LSD) influence behavioral and physiological outcomes in mice to test whether the HTR reliably reflects overall psychoactivity. Lisuride (0.5 mg/kg) elicited no HTR yet impaired locomotion and coordination, evoked a pronounced stress response, disrupted cognitive function, and markedly reduced prefrontal cortex (PFC) electroencephalogram (EEG) amplitude across several frequency bands. In contrast, LSD (0.1 mg/kg) produced a robust $5HT_{2A}R$-dependent HTR but had minimal impact on locomotion, coordination, stress responsivity, cognitive function, or PFC EEG power. None of these other behavioral or physiological effects of lisuride or LSD were mediated by $5\text{-}HT_2A$ receptors, as pretreatment with the selective antagonist MDL 100907 did not alter outcomes. These results reveal a striking dissociation: the compound that evoked no HTR produced broad behavioral, physiological, and cortical disruptions, while the compound that elicited robust HTRs had little effect. Our findings demonstrate that the HTR alone is not sufficient to identify psychoactivity and is best used in conjunction with other endpoints.

INTRODUCTION (470 words)

Psychedelic compounds have re-emerged as potential therapeutics for psychiatric disorders resistant to conventional treatments. Clinical and preclinical evidence suggests benefits in major depressive disorder[1-4], alcohol use disorder[5], smoking cessation[6], and other conditions. Yet, the mechanisms underlying these effects remain unclear. The profound alterations in consciousness and perception that define psychedelics represent a clinical, practical, and economic barrier to widespread therapeutic application. Because of these effects, psychedelic-assisted therapy, as currently practiced, requires significant time, cost, and supervision[7,8]. These subjective effects are mediated by serotonin 2A receptor ($5HT_{2A}R$) activation[9]. Whether therapeutic benefit requires subjective effects or $5HT_{2A}R$ activation remains unresolved.

For purposes of this paper, we define drugs that produce the human subjective response as 'hallucinogenic,' and consider them a specific subset of compounds that are psychoactive, i.e. drugs that affect the brain and produce changes in perception, mood, consciousness, cognition, or behavior.

Considerable effort is underway to develop compounds that activate $5HT_{2A}Rs$ yet produce minimal hallucinogenic or other psychoactive effects[10,11]. In humans, subjective responses can be measured directly, but preclinical models rely on indirect proxies of psychoactivity. In rodents, the head twitch response (HTR) is widely used because the potency of compounds to elicit HTRs is strongly correlated with their potency for eliciting human subjective effects[12] and activating $5HT_{2A}R$-mediated signaling via the Gq pathway[20], and both are blocked by $5HT_{2A}R$ antagonists. While the HTR is thus an excellent indicator of $5HT_{2A}R$-dependent hallucinogenic potential, it has limitations and does not rule out other forms of psychoactivity. Some compounds, such as fenfluramine, induce HTRs in mice but are non-hallucinogenic in humans[13-14]. Conversely, other compounds, such as ibogaine[15] , cause strong subjective effects in humans but are not $5HT_{2A}R$ agonists and do not elicit HTRs. Putative non-hallucinogenic compounds like fenfluramine and lisuride are also indistinguishable from lysergic acid diethylamide (LSD) and $5HT_{2A}R$-specific agonists in drug discrimination assays[17-19]. These findings raise a key question—does the absence of HTRs provide sufficient evidence that a compound is not hallucinogenic or psychoactive?

We addressed this question by comparing LSD and lisuride in mice using a broad set of behavioral and physiological assays. Lisuride is a potent partial $5HT_{2A}R$ agonist that does not induce HTRs at commonly used doses[20,21] and is widely considered non-hallucinogenic[22,23] (but see: [24]), leading to its frequent use as a control compound[e.g. 25]. We hypothesized that LSD's psychoactivity would impair behavior in mice, but not lisuride. Contrary to expectation, lisuride impaired locomotion and coordination, induced a strong stress response, impaired cognition, and decreased prefrontal cortex (PFC)

electroencephalogram (EEG) amplitude. In contrast, LSD at the dose eliciting a maximal HTR had minimal impact across assays. Thus, although lisuride does not elicit HTRs, it alters behavior and brain activity, demonstrating that it is strongly psychoactive. These results show that HTR cannot be used alone to identify reliably non-psychoactive compounds and underscore the need for broader phenotyping approaches.

MATERIALS AND METHODS

Animals

All procedures were approved by the University of Colorado Anschutz Medical Campus IACUC and conformed to NIH guidelines. Adult male and female C57BL/6J mice (Jackson Laboratory, Strain #: 000664) were used. Behavioral and physiological assays were performed during the light phase.

Drugs

Lisuride maleate (Tocris) was used at 0.5 mg/kg based on prior reports of antidepressant-like effects at this concentration[26]. LSD (NIDA Drug Supply Program) was used at 0.1 mg/kg, corresponding to peak HTR response[21]. LSD also has antidepressant-like actions at comparable doses[27,28]. For $5HT_{2A}R$ block, 0.5 mg/kg MDL 100907 (MDL, Tocris) was injected 15 minutes before lisuride or LSD; control animals received two saline injections 15 min apart. All drugs were delivered intraperitoneally (i.p.) in sterile saline (0.9%). Both drugs have comparable pharmacokinetic profiles in rodents, reaching peak brain concentrations in ca. 15 min, and declining with a half-life of ca. 1 hour[29,30].

Behavioral assays

Behavioral data was collected using EthoVision XT software (Noldus Information Technology BV, software version 17) connected to an ELP 2.0 Megapixel USB Camera (ELP-USBFH01M-SFV).

Head Twitch Response. To validate dosing, we recorded HTRs using high-speed video (Basler ACE2) for 30 min immediately following drug injection. MDL's ability to block LSD-induced HTRs was also tested[21]. Counts were scored post hoc in 5-min bins by a blinded observer (OC).

Open Field. Locomotor activity was assessed in a 44 × 44 × 25 cm arena. Mice were injected with saline or antagonist, allowed to explore for 30 min, then injected again (saline, lisuride, LSD, or antagonist) and monitored for another 30 min.

Rotarod. Motor coordination was measured on a rotating rod (3.5 cm diameter) accelerating from 4 to 27 rpm over 180 sec. After injections, mice were placed on the rod at various time intervals and latency to fall was recorded (max 240 sec).

Stress Response. To measure hypothalamic-pituitary axis (HPA) axis activity, tail blood (10 µL) was collected immediately before and at 15, 60, and 120 min after saline, lisuride, or LSD injection. Plasma corticosterone was measured by ^{125}I-corticosterone radioimmunoassay (MP Biomedicals, catalog no. 07120103), as previously[31,32].

Puzzle Box. Cognition was tested in a two-chamber puzzle box[33]. Mice moved from a brightly lit chamber to a preferred dark chamber (**Figure 5A**) across nine trials of increasing difficulty, as indicated. Identical trials separated by 20 min or 24 h assessed short- and long-term memory, while shifts in barrier configuration assessed problem solving. On day 3, mice were injected with saline/antagonist followed by saline, lisuride, or LSD before trials 7–9 (**Figure 5B**). Based on locomotion data, lisuride groups began trial 7 one-hour post-injection; LSD groups began 15 min after. Animals that did not complete trials 1-6 within 180 seconds were excluded from data analyses.

EEG Recording

Wireless transmitters (DSI ETA-F10) were implanted under isoflurane anesthesia. Leads were affixed to skull screws above the PFC (+1.7 AP, +1.5 ML) and cerebellum (–6.5 AP, 0 ML) for differential recording[34]. After recovering for 7 days, a 20 min baseline recording period was followed by MDL or saline injection. Lisuride or LSD were injected 15 minutes later and EEG was recorded for 40 min. Data were clipped to –10 to +40 min relative to drug, artifact-cleaned, and band-pass filtered into delta–gamma bands. As described in detail in below, data were normalized to mean power for the 5 min prior to lisuride or LSD injection. We also assessed bursting (IQR variability), spectral entropy, and Lempel-Ziv complexity. Analyses used bandpass-filtered (1–100 Hz) EEG data in sliding windows. Increases in IQR indicated greater synchronous bursting; entropy reflects the distribution of spectral power; and LZ-complexity assays signal predictability. All EEG analyses were performed in MATLAB (MathWorks, R2023b) using EEGLAB[35].

Experimental Design and Statistical Analysis

Statistical analyses for all behavioral tasks, corticosterone assays, and EEG power analyses were performed with *Statistical Package for the Social Sciences* (SPSS, v28). All analyses were performed with experimenters blinded to drug treatment.

Animals were randomly assigned to treatment groups, and both sexes were included in approximately equal numbers unless otherwise noted. Sample sizes were informed by prior published studies using similar assays and endpoints and were sufficient to detect medium-to-large effect sizes (α = 0.05, power = 0.8). No animals were excluded except in the puzzle box experiments, where pre-established behavioral criteria were not met.

Full statistical outcomes (animal numbers, F-values, degrees of freedom, corrected p-values) are provided in the Results and Figure Legends.

RESULTS

Head Twitch Response

We first examined the effects of lisuride and LSD on the HTR. LSD evoked a robust HTR, with peak HTRs occurring 10 minutes after LSD administration, but lisuride did not, consistent with previous observations[21] (**Figure 1A,B**, n = 3 for all drug conditions for each sex). MDL pre-administration prevented LSD-induced HTRs. A two-way repeated measures ANOVA with sex and drug as between factors revealed a significant main effect of time and a significant time*drug interaction, indicating that the number of HTRs evoked was dependent on which drug was delivered. Post-hoc analyses showed that significant increases in head twitch frequency were elicited by LSD in both sexes, but not by saline or LSD+MDL. This dose of MDL was thus sufficient to fully antagonize activation of $5HT_{2A}$R-dependent head twitches by this dose of LSD, as reported previously[21]. There were no time*sex or time*sex*drug interactions, indicating that there were no sex-specific effects of these drugs.

Locomotion

We next compared the effects of lisuride and LSD on locomotion in an open field arena (**Figure 2**). We observed significant main effects of both sex and drug, but no sex*drug interaction, indicating that the drug combinations impaired locomotion similarly in females and males.

Lisuride significantly decreased distance traveled (n = 8 females, 8 males) compared to saline (n = 9 females, 8 males, $p < 0.001$), as reported previously[26], whereas LSD had no effect (n = 13 females, 13 males, p = 1). Female and male animals traveled similar distances after injections of saline. Mice injected with lisuride also traveled significantly less compared to saline injected controls when $5HT_{2A}$Rs were blocked by prior MDL administration (n = 12 females, 11 males, $p < 0.001$). Injection of LSD after prior administration of MDL resulted in a significant decrease in locomotion compared to saline or LSD alone (n = 13 females, 13 males, $p < 0.001$).

Rotarod performance

Decreased locomotion in the open field could reflect a voluntary decision to remain immobile or result from an involuntary effect on psychomotor function. We therefore tested motor performance on the rotarod (**Figure 3**). There was a main effect of time, and all three interactions were significant (time*sex, time*drug, time*sex*drug), indicating that there were sex-specific effects of the drugs. All conditions and sexes had five animals, except for MDL+LSD which had only four males.

Lisuride significantly impaired the ability of both females and males to stay on the rod, compared to animals who were injected with saline ($p < 0.001$). Conversely, LSD had no effect on either sex ($p = 1$). When $5HT_{2A}$Rs were blocked by MDL before lisuride administration, performance remained significantly impaired compared to saline ($p < 0.001$), lisuride ($p = 0.047$), LSD ($p < 0.001$), and MDL+LSD ($p < 0.001$). When MDL was applied before LSD injection, there was no change from saline ($p = 0.66$) or LSD alone ($p = 1$), and performance remained significantly better than lisuride ($p < 0.001$) and MDL+lisuride ($p < 0.001$). We conclude that lisuride's effects on locomotion result, at least in part, from impairment in psychomotor function.

HPA axis activation

We hypothesized that that LSD would produce measurable increase in corticosterone secretion, as it does in humans[39], presumably because of its powerful subjective effects, but not lisuride. A two-way repeated measures ANOVA showed a main effect of time, and a significant time*drug interaction, but there were no effects of sex (time*sex and time*drug*sex), indicating that the drugs had a significant and equal impact in both sexes.

Contrary to our prediction, both females and males had an elevation in plasma corticosterone after lisuride injection ($n = 4$ females, 4 males; $p < 0.001$; **Figure 4**). The elevation of was apparent within 15 min of injection and peaked at 60min post-injection. Peak levels were comparable to those induced by 15 min of restraint stress[31,32]. In contrast, LSD-evoked corticosterone levels were not different from levels seen in saline controls ($n = 7$ females, 7 males; $p = 0.56$).

Puzzle box performance

We hypothesized that a drug-induced hallucinatory alteration of perception would impair the cognitive ability of mice, and therefore predicted that LSD, but not lisuride would impair performance in the puzzle box task. Mice were trained over two days (trials 1-6). Drugs were administered on day 3 before trial 7 (**Figure 5B**). LSD was administered 15 min prior to trial 7, corresponding to the peak of the HTR. Because of the locomotor effects of lisuride, we waited 60 min after injection before starting trial 7.

Two-way repeated measures ANOVA were performed with data from both sexes and trials 6-9. We found a main effect of trial and a significant trial*drug interaction, indicating that the drugs had a significant impact on cognitive performance. However, there were no significant interactions involving sex indicating that males and females performed the tasks in a similar amount of time and that the drug combinations similarly impacted cognitive performance in both sexes.

Contrary to our prediction, lisuride ($n = 12$ females, 11 males) significantly worsened puzzle box performance compared to saline ($n = 10$ females, 9 males; $p < 0.001$), whereas LSD had no significant effect ($n = 8$ females, 11 males; $p = 1$; **Figure 5C-F**).

Prior administration of MDL had no significant effect on lisuride's impairment of performance ($p = 1$), which remained significantly worse than saline alone ($p < 0.001$). In contrast, application of MDL followed by LSD (n = 7 females, 7 males) resulted in performance that was significantly worse than either saline ($p < 0.001$) or LSD alone ($p = 0.002$).

To quantify the specific effects of drug administration on recall, we subtracted performance in a given trial from performance in the subsequent trial such that positive and negative values indicate improved and impaired performance, respectively. We used the time differences between trials 6 and 7 to quantify drug effects on overnight recall and trials 8 and 9 to quantify effects on short-term recall. Drug effects on problem solving were quantified from the time difference between trials 7 to 8.

For overnight recall (**Supplemental Figure 1A,D**), a two-way ANOVA found a main effect of drug but no effect of sex. Lisuride significantly impaired overnight recall compared to saline ($p = 0.012$) and LSD ($p < 0.001$) in females and males. As with the other behavioral assays, LSD had no effect compared to saline ($p = 1$). Prior application of MDL significantly worsened performance for both lisuride ($p = 0.002$) and LSD ($p = 0.012$). Mice given MDL prior to LSD had significantly worse performance than mice given LSD after saline ($p < 0.001$).

For problem solving (**Supplemental Figure 1B,E**), a two-way ANOVA revealed a main effect of drug and no main effect of sex. Overall, lisuride significantly impaired performance compared to saline ($p < 0.001$), and LSD had no effect ($p = 0.074$). Administration of MDL did not significantly change the effects of either lisuride or LSD compared to when they were administered after saline (lisuride $p = 1$, LSD $p = 0.071$).

Differing from most of our other assays, we found that problem solving had a significant drug*sex interaction. To examine this interaction, we ran one-way ANOVAs for each drug combination with sex as a between factor, and MDL+LSD was the only significant result. This is likely because males had an average time of 232 seconds to complete the task whereas females' average time was 82 seconds. Similarly, males also had a slower average time to complete the task after receiving LSD alone, and both overall puzzle box performance and overnight recall were impaired when $5HT_{2A}$Rs were blocked.

For short-term recall (**Supplemental Figure 1C,F**), a two-way ANOVA revealed a significant main effect of drug but no main effect of sex or a sex*drug interaction. Lisuride caused no significant difference in short-term recall performance compared to saline ($p = 0.14$), whereas LSD produced a small but significant improvement in performance compared to saline ($p = 0.044$). Neither lisuride nor LSD had any significant effect on performance when injected after MDL administration ($p>0.05$).

Taken as a whole, these behavioral and physiological results demonstrate a significant impact of lisuride on locomotion, balance, psychomotor coordination, and cognition in a $5HT_{2A}R$-independent manner. Surprisingly, LSD had relatively little effect in any of these behavioral assays. Additionally, lisuride, but not LSD, induced a significant stress response.

EEG activity

As a physiological measure of drug effects on brain activity, we recorded the EEG over the PFC before and after acute lisuride or LSD administration, with and without MDL. After recording for a 60-minute baseline period during acclimation, we injected saline or MDL, followed 15 minutes later by either lisuride or 0.1 mg/kg LSD injection (lisuride, 3F/4M; low dose LSD, 3F/4M). We then recorded for a further 40 minutes. We also recorded responses to injection of saline alone or injection of LSD at 1 mg/kg (saline, 5F/4M; high dose LSD, 5F/4M).

We first asked whether the injections of saline or MDL had an impact on EEG power. We compared power in the last 10 minutes of baseline (pre-injection) to the first 10 minutes after saline or MDL injection. We looked at all frequency bands and calculated average power in 60 second bins. A four-way repeated measures ANOVA with sex, drug, frequency band, and pre- or post-injection as between factors revealed a main effect of time ($F_{(9,2250)} = 9.07$, $p < 0.001$, $\eta p^2 = 0.035$), and significant time*injection ($F_{(9,2250)} = 2.28$, $p = 0.043$, $\eta p^2 = 0.009$), time*freq band ($F_{(9,2250)} = 1.68$, $p = 0.029$, $\eta p^2 = 0.026$), and time*drug ($F_{(9,2250)} = 0.66$, $p = 0.66$, $\eta p^2 = 0.003$) interactions. There was no significant time*sex ($F_{(9,2250)} = 0.64$, $p = 0.55$, $\eta p^2 = 0.004$), time*drug*sex ($F_{(9,2250)} = 1.6$, $p = 0.16$, $\eta p^2 = 0.006$), time*sex*injection ($F_{(9,2250)} = 1.54$, $p = 0.17$, $\eta p^2 = 0.006$), time*drug*injection ($F_{(9,2250)} = 1.17$, $p = 0.322$, $\eta p^2 = 0.005$), time*drug*freq band ($F_{(36,2250)} = 0.414$, $p = 0.99$, $\eta p^2 = 0.007$) or time*sex*freq band ($F_{(36,2250)} = 0.421$, $p = 0.99$, $\eta p^2 = 0.007$) interactions. Taken together, there were significant effects of injection on EEG power, but no effects of saline or MDL, and the effects (or lack thereof) were consistent between sexes.

Based on these analyses, we normalized all EEG recordings to the average power of the 5 minutes before the second injection (i.e. lisuride or 0.1 mg/kg LSD) to account for injection effects. We then calculated average normalized power in 5-minute bins for the 40 minutes after lisuride or LSD injection for all frequency bands, starting at the time of injection.

We ran a three-way repeated measures ANOVA with sex, drug and frequency band as between factors, with power data divided into averaged 5-minute bins for the duration of the 40-minute recording period. We found a main effect of time and significant time*drug, time*freq band, time*sex*drug, and time*drug*freq band interactions, suggesting that

drugs were having sex- and frequency band-specific effects. Post hoc analyses show that there was a significant difference between lisuride and LSD ($p < 0.001$), but no difference between LSD and MDL+LSD ($p = 1$) or lisuride and MDL+lisuride ($p = 1$).

Spectrograms and power analyses showed that lisuride decreased power in lower frequency bands, but 0.1 mg/kg LSD did not (**Figure 6**). Samples of EEG recordings in the theta and alpha frequency bands are illustrated in **Supplemental Figure 2**. The differential effects of lisuride and LSD on PFC EEG signals were particularly robust in the delta, theta, and alpha frequency bands, with lisuride causing a ca. 40-50% decrease in power (**Figure 6E-I**). These effects were comparable in males and females, with the exception of the decrease in theta power, which was only present in females. Lisuride had no effects on power in the beta band. All effects of lisuride peaked around 20 min after injection and were recovering by 40 min post injection.

LSD, in contrast, had no specific significant effects on power in these frequency bands. The failure to detect significant effects of LSD was not due to an inadequate dose, as injection of LSD at 1 mg/kg also failed to produce significant changes in PFC EEG power.

Pretreatment with MDL did not prevent any of these effects on EEG power produced by lisuride or alter the effects of LSD on EEG power (**Figure 6C-G**).

Lisuride and LSD produced modest, transient increases power in the gamma frequency band (**Figure 6I)** in both males and females under all conditions. These changes may be related to the IP injection procedures, since they were also observed after saline injection, although the effects of LSD at 1 mg/kg LSD appeared greater than those of LSD at 0.1 mg/kg and the LSD-induced changes in gamma power were eliminated by MDL.

Psychedelic drug responses in humans are accompanied by changes in EEG complexity, bursting, and entropy. We detected no significant changes in response to lisuride or LSD, with or without MDL, in our data, however (**Supplemental Figure 3**).

When taken together, these results show that lisuride decreases EEG power in the PFC in the low- to mid-range frequency bands, with effects being generally more pronounced in females than in males, whereas LSD has little effect in either sex. MDL did not have a strong effect on responses to either drug, suggesting that the lisuride effects observed are not $5HT_{2A}$R-mediated. These results are broadly consistent with our behavioral results.

Effects of blocking $5HT_{2A}$Rs with MDL

The role of $5HT_{2A}$Rs in normal brain function is not well established. We therefore asked whether administration of MDL alone might have any effects in our behavioral assays. MDL was given 15 minutes before a sterile saline injection to control for number of injections. In the open field (**Supplemental Figure 4A**), distance traveled after MDL

injection decreased significantly compared to saline controls (n = 9 female saline, n = 8 male saline, n = 12 MDL+saline for both females and males; p = 0.002). In contrast, there were no significant differences in rotarod performance between MDL and saline injection (p = 0.245, n = 5 for both females and males; data not shown). There was a significant drug*sex interaction, but the post-hoc one-way ANOVA with sex as a between factor was not significant ($F_{(1,43)}$ = 2.0, p = 0.165, ηp^2 = 0.44). Administration of MDL alone did not significantly influence overall puzzle box performance compared to saline (**Supplemental Figure 4B**; n = 5 females, 5 males; p = 0.48).

When examining the individual cognitive modalities parsed by the puzzle box, both overnight recall and problem solving were significantly impaired after MDL administration (**Supplemental Figure 4C,D**; p < 0.05 for both), although shorter-term recall was not impacted (**Supplemental Figure 4E**; p = 0.14). However, the mean overnight recall performances were similar between saline (female n = 10, male n = 9) and MDL (female n = 5, male, n = 5), with almost no variability in the saline group – perhaps accounting for the significant difference. There were also no sex specific effects of MDL on problem solving performance. As with open field, this suggests that MDL is not a neutral antagonist and is possibly having its own impact on memory and cognition.

Discussion

The present study reveals a clear dissociation between the utility of the HTR in mice as an indicator of $5HT_{2A}R$ agonists with potential hallucinogenic activity in humans and the identification of compounds with acute psychoactivity across diverse behavioral and physiological assays. We were surprised to find that administration of LSD at a dose that elicits a robust HTR (0.1 mg/kg)[20,21] did not produce measurable changes in locomotion, rotarod, or cognitive performance; trigger a stress response; or alter EEG activity over the prefrontal cortex. We were also surprised that lisuride, widely used as a non-hallucinogenic 5-$HT_{2A}R$ agonist because it does not elicit HTRs, significantly altered all behavioral and physiological measures. No striking sex differences were observed. These findings emphasize that HTR magnitude is not a sufficient proxy for identifying psychoactive compounds. A limitation of our study is that we did not examine outcomes across a full range of doses, so it is possible that effects might be seen with different doses.

Effects of Lisuride and LSD on Behavior

We compared the acute effects of lisuride and LSD at fixed doses of 0.5 mg/kg and 0.1 mg/kg because of their ability to induce an antidepressant-like response[26] and a maximal HTR[20,21] at those doses, respectively. Lisuride is known to reduce locomotor activity, a finding we confirm here[26,36]. In mice, locomotion is suppressed at doses from 0.01 – 10mg/kg, with maximal inhibition observed at 0.1-0.5 mg/kg[36]. We observed a ca. 80%

decrease in distance traveled at 0.5 mg/kg, consistent with previous findings[26]. LSD is also reported to produce a dose-dependent effect on locomotion, with no effect at 0.06 mg/kg[37] and increased activity at 0.3 mg/kg[38]. At our intermediate dose of 0.1 mg/kg, we confirmed that LSD produced no locomotor changes, despite the robust HTR. We extend these findings by reporting that lisuride, but not LSD, acutely impaired psychomotor coordination on the rotarod, whereas a dose of LSD that produced a robust HTR had no effect on rotarod performance. This effect of lisuride suggests that the decrease in locomotion in the open field may reflect not just a lack of voluntary exploratory movement, but also impaired motor capability.

The puzzle box is a validated and ethologically relevant assay of executive function, memory, and problem solving in rodents[33]. We observed that lisuride disrupted performance through impairment of short-term (20 min) and overnight memory recall, as well as solving novel problems to accomplish the escape task. Although the puzzle box behaviors require motor coordination and thus may have been affected by lisuride's actions on locomotion, we waited 60 min after lisuride injection before testing in the puzzle box, a time in which ability to stay on the rotarod has partially recovered. Given our expectation that LSD is strongly psychoactive in mice, it is surprising that it had no detectable influence on these relatively cognitively demanding behavioral tasks. This is particularly true considering that the mice performed their initial task 15 min after LSD injection, a time at which mice displayed a peak HTR.

We conclude that neither a lack of HTRs nor maximal HTRs are predictive of functional disruption of behavior.

Effects of Lisuride and LSD on Corticosterone Responses

Mice are highly stress reactive, and we therefore predicted that LSD-induced psychoactivity would provoke a robust activation of the HPA axis. Indeed, LSD acutely increases plasma cortisol levels in humans[39]. Psilocin, the active metabolite of psilocybin, produces immediate early gene activation in the paraventricular nucleus of rats[40], suggesting a potential direct action on neurons expressing corticotrophin releasing factor. We observed that lisuride, but not this dose of LSD, significantly increased corticosterone levels in mice. The peak levels of plasma corticosterone after lisuride administration (250-300 ng/ml) were greater than those produced with 15 min of restraint (150 ng/ml)[31,32]. Our findings demonstrate that the impact of psychedelics in mice is not limited to neural and behavioral endpoints but also encompasses endocrine responses. It is possible that negative feelings engendered by LSD's powerful subjective effects, such as anxiety[41], trigger stress responses in humans that are absent in animals, whereas lisuride's immobilizing actions may be perceived as stressful to a mouse.

Effects of Lisuride and LSD on Prefrontal Cortical Activity

The psychoactive properties of lisuride were accompanied by electrophysiological changes. We provide the first description of lisuride's effect on cortical EEG activity, and report decreased power in the PFC across several frequency bands. This pattern of response is generally consistent with previous studies of serotonergic psychedelics in which the power of lower frequency oscillations decreases in a compound-specific manner. For example, the $5HT_{2A}$R-selective agonist DOI produces a decrease in the power of delta frequency oscillations <4 Hz in the rat PFC[42], whereas the pan-serotonergic agonist psilocybin decreases delta, theta, and alpha power in the mouse cingulate cortex[43]. We found that lisuride also reduced power in the delta, theta and alpha frequency bands. Lisuride was reported previously to reduce local field potential oscillations in the nucleus accumbens, as well[44]. Lisuride's effects were not altered by MDL co-administration, indicating that they are not mediated by $5HT_{2A}$Rs.

LSD at 0.1 mg/kg, in contrast, produced no significant effects on any EEG frequency band in the absence or presence of MDL. An effect of LSD on PFC EEG power in mice was not revealed with a 10-fold higher dose (1 mg/kg). The lack of effects is at first surprising, given evidence that LSD reduces delta, theta, and alpha power and reorganizes large-scale cortical networks in humans[45], and psilocybin produces a convergent suppression of low frequency cortical activity[46]. Consistent with our results, Inserra et al. also reported that LSD at 0.1 mg/kg produced no detectable changes in infralimbic PFC local field potentials in mice[47]. We also observed no change in PFC signal complexity after lisuride or LSD. The overall increase in human EEG and MEG signal complexity after LSD appears to be less pronounced over the PFC than more posterior cortical regions[48,49], providing a testable potential explanation for the discrepancy.

Role of 5-HT_{2A} Receptors

LSD and lisuride are full and partial antagonists of $5HT_{2A}$ receptors, respectively[50]. $5HT_{2A}$Rs are broadly expressed in cortical and subcortical brain regions[51,52]. Indeed, we observed that administration of the $5HT_{2A}$R antagonist MDL100907 at 0.5 mg/kg blocked the HTR elicited by 0.1 mg/kg LSD, confirming its efficacy at this concentration[21]. In contrast, none of the actions of lisuride on locomotion, performance on the rotarod and in the puzzle box, or EEG activity were blocked by MDL. It has also been observed previously that MDL does not block the effects of lisuride on pre-pulse inhibition[53]. The receptor underlying these effects remains unidentified. Lisuride is also an agonist at $5HT_{1A}$ and dopamine D2 receptors[54] and some of lisuride's actions are blocked by $5HT_{1A}$[22,23] and dopaminergic antagonists[26], and should therefore be tested against these effects in future studies. We did not test whether MDL had any action of lisuride-induced HPA axis activation.

Although LSD alone had little or no action on locomotion or puzzle box performance, we observed that administration of LSD in the presence of MDL did affect both, producing a lisuride-like impairment. In addition to $5HT_{2A}$Rs, LSD is an agonist of many serotonin and

dopamine receptors[54]. Interactions between $5HT_{1A}$Rs and $5HT_{2A}$Rs have been reported previously. For example, 5-HT_{1A} antagonists reveal HTRs in response to high concentrations of LSD[55]. It is possible that a similar interaction may underlie the effects we observed here.

We conclude that additional approaches to the HTR are needed to guide preclinical dose selection for 5-HT_{2A}R agonists, such dose-receptor occupancy curves[56,57]. For example, the doses of lisuride that are used for treating Parkinsons Disease produce only low levels of $5HT_{2A}$R occupancy, accounting for the lack of hallucinations, whereas higher doses produce greater occupancy and are hallucinogenic. These findings highlight the need for incorporating measures beyond behavioral proxies—such as pharmacokinetics, molecular biomarkers, and circuit-level physiology—into dose selection.

Our findings underscore the challenges of preclinical identification of non-hallucinogenic and non-psychoactive compounds and the need for multimodal preclinical assessment. Compounds that are psychoactive, but non-hallucinogenic might still hamper widespread clinical use. Certainly, the lack of HTRs is insufficient by itself for determining psychoactivity. Lisuride, while a useful comparator, engages non-$5HT_{2A}$Rs to produce robust behavioral, physiological, and electrographic effects. Taken together with older data reporting evidence of powerful hallucinatory responses to lisuride[24,58], the lack of HTR in rodents produced by lisuride does not indicate that it is not psychoactive in humans, but rather that it is used on humans at a dose that is too low to produce sufficient 5-HT_{2A}R activation to produce hallucinations[21]. The MDL-insensitive actions effects of lisuride on psychomotor and memory/recall functions in the puzzle box indicate the power of other receptor systems to exert psychoactive actions beyond the $5HT_{2A}$R. Systematically testing of a range of doses in several behavioral and physiological assays is critical for the rational development of novel non-psychoactive ligands with therapeutic potential.

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Author Contributions. JLK, performed experiments, analyzed data, prepared figures, drafted manuscript; DPE, JW, JC, CBP, performed experiments; CO, analyzed data; ST, conceived experiments, provided funding, edited manuscript.

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Competing Interests: SMT is listed as an inventor on patents filed by the University of Maryland Baltimore concerning treatment of psychiatric disease with psychedelics, serves on the Scientific Advisory Board of Terran Biosciences, and is a co-founder of ProNovo Therapeutics. The other authors report no conflicts.

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Figure Legends

Figure 1. The puzzle box and head twitch responses. (A) Illustration of the brightly lit and dark chambers of the arena, with the tunnel and door connecting them. (B) Timeline of the puzzle box training and testing protocol. The door and tunnel are progressively obstructed in succeeding trials, as shown, making escape to the dark chamber more difficult. There are 3 trials each day, separated by 20 min. Drugs were injected at the start of day 3. Comparison of adjacent trials 6 and 7, 7 and 8, and 8 and 9 allows analysis of drug effects on different cognitive processes (overnight recall, problem solving, short-term recall, respectively). Head twitch responses in female (C) and male (D) mice induced by injection of 0.5 mg/kg lisuride (closed blue triangles), 0.1 mg/kg LSD (closed red triangles), and LSD after MDL pretreatment (open red inverted triangles). Data is presented in 5-minute bins with the average total number of HTRs that occurred in that bin on the Y axis. A 2-way repeated measures ANOVA revealed a main effect of time ($F_{(6,72)}$ = 36.54, $p < 0.001$, $\eta p^2 = 0.75$) and a significant time*drug interaction ($F_{(6,72)}$ = 36.18, $p < 0.001$, $\eta p^2 = 0.86$), indicating that the number of HTRs evoked was impacted by which drugs mice were given. There was no time*sex ($F_{(6,48)}$ = 1.24, p = 0.31, ηp^2 = 0.094) or time*sex*drug interactions ($F_{(6,48)}$ = 1.27, p = 0.31, ηp^2 = 0.174). Error bars are SEM. ***, p<0.001.

Figure 2: Effects of lisuride and LSD on locomotion in an open field. Total distance traveled (m) over 30 min is shown for female (A) and male (B) mice for all drug combinations. A two-way ANOVA revealed main effects of sex (F(1,141) = 4.37, p = 0.04, ηp2 = 0.03) and drug (F(5,141) = 49.83, p < 0.001, ηp2 =0.64), but there was no sex*drug interaction (F(1,141) = 4.37, p =0.04, ηp2 = 0.03), suggesting that the various drug combinations impacted sexes to similar extents. Post-hoc analyses showed that distance traveled was significantly less for lisuride, MDL+lisuride and MDL+LSD compared to mice injected with saline or LSD. LSD had no effect on total distance traveled compared to saline (p = 1). Error bars are SEM. **, p<0.005, ***, p<0.001.

Figure 3: Effects of lisuride and LSD on rotarod performance. Latency to fall off the rotarod is plotted for female (A) and male (B) mice at various times after drug injection for all drug combinations. A two-way repeated measures ANOVA revealed a main effect of time ($F_{(4,142)}$ = 3.68, p = 0.007, ηp^2 = 0.074), and all three interactions were significant (time*sex $F_{(4,128)}$ = 3.23, p = 0.023, ηp^2 = 0.066, time*drug $F_{(20,128)}$ = 4.26, $p < 0.001$, ηp^2 = 0.316, time*sex*drug $F_{(12,128)}$ = 1.77, p = 0.043, ηp^2 = 0.161). Post-hoc analyses showed that lisuride and MDL+lisuride both significantly impaired performance compared to saline (p<0.001), whereas LSD had no effect on performance (p = 1). The only drug that had a sex-specific effect was saline (post hoc t-test, $p < 0.001$). Error bars are SEM. **, p<0.01, ***, p<0.001.

Figure 4: Effects of lisuride and LSD on HPA axis activation. Plasma corticosterone levels are plotted for female (A) and male (B) mice at various times after saline (black), lisuride (blue), or LSD (red) injection. A two-way repeated measures ANOVA revealed a main effect of time (($F_{(3,69)}$ = 45.88, $p < 0.001$, $\eta p^2 = 0.67$) and a significant time*drug interaction ($F_{(6,69)}$ = 12.08, $p < 0.001$, $\eta p^2 = 0.512$), but there was no effect of sex (time*sex ($F_{(3,69)}$ = 1.85, p = 0.168, $\eta p^2 = 0.075$), time*sex*drug ($F_{(6,69)}$ = 1.25, p = 0.305, ηp^2 = 0.098)). Post-hoc tests showed that lisuride evoked significantly greater corticosterone levels compared to both saline and LSD (P < 0.001 for both), but LSD had no effect compared to saline (p = 0.56). Error bars are SEM. ***, p<0.001.

Figure 5: Effects of lisuride and LSD on puzzle box performance. Latency for mice to enter the dark chamber is plotted for female (A, C) and male (B, D) mice in trials 6-9 after lisuride (A, B; blue) or LSD (C, D; red) injection. Performance of saline-injected rats is duplicated in both rows (black). Effects of prior administration of MDL are shown with dashed lines. A two-way repeated measures ANOVA revealed a significant main effect of trial ($F_{(3,261)}$ = 81.24, $p < 0.001$, $\eta p^2 = 0.48$) and a significant trial*drug interaction ($F_{(15,261)}$ = 6.6, $p < 0.001$, $\eta p^2 = 0.28$), but there were no effects of sex (trial*sex $F_{(3,261)}$ = 1.4, p = 0.24, $\eta p^2 = 0.016$, trial*sex*drug $F_{(3,261)}$ = 1.27, p = 0.23, $\eta p^2 = 0.068$). Post-hoc analyses showed that lisuride and MDL+lisuride significantly impaired performance compared to saline (p < 0.001) and LSD (p < 0.001). LSD did not have a significant effect compared to saline (p = 1), but MDL+LSD significantly impaired performance compared to both saline (p < 0.001) and LSD (p = 0.002). Error bars are SEM. ***, p<0.001

Figure 6: Effects of saline, lisuride, and LSD on EEG power in the prefrontal cortex. Representative spectrograms are shown with white bars indicating the time of injection of saline (A), saline followed by lisuride (B), saline followed by LSD at 0.1 mg/kg (C), and LSD at 1 mg/kg (D). (E-I) PFC EEG power is shown normalized to the end of the baseline recording period for standard frequency bands and all drug combinations. Male and female responses have been pooled (saline, 5F/4M; lisuride, 3F/4M; low dose LSD, 3F/4M; high dose LSD, 5F/4M). A three-way ANOVA reported a main effect of time ($F_{(8,688)}$ = 5.04, $p < 0.001$, $\eta p^2 = 0.055$), and significant time*drug ($F_{(24,688)}$ = 3.79, $p < 0.001$, ηp^2 = 0.117), time*freq band ($F_{(32,688)}$ = 7.13, $p < 0.001$, $\eta p^2 = 0.25$), time*sex*drug ($F_{(24,688)}$ = 2.99, $p < 0.001$, $\eta p^2 = 0.094$), and time*drug*freq band ($F_{(96,688)}$ = 2.41, $p < 0.001$, ηp^2 = 0.25) interactions. Post hoc analyses show that there was a significant difference between lisuride and low dose LSD (p < 0.001), but no differences between low dose LSD +/- MDL (p = 1) or lisuride +/- MDL (p = 1). Error bars are SEM.

Figure 1

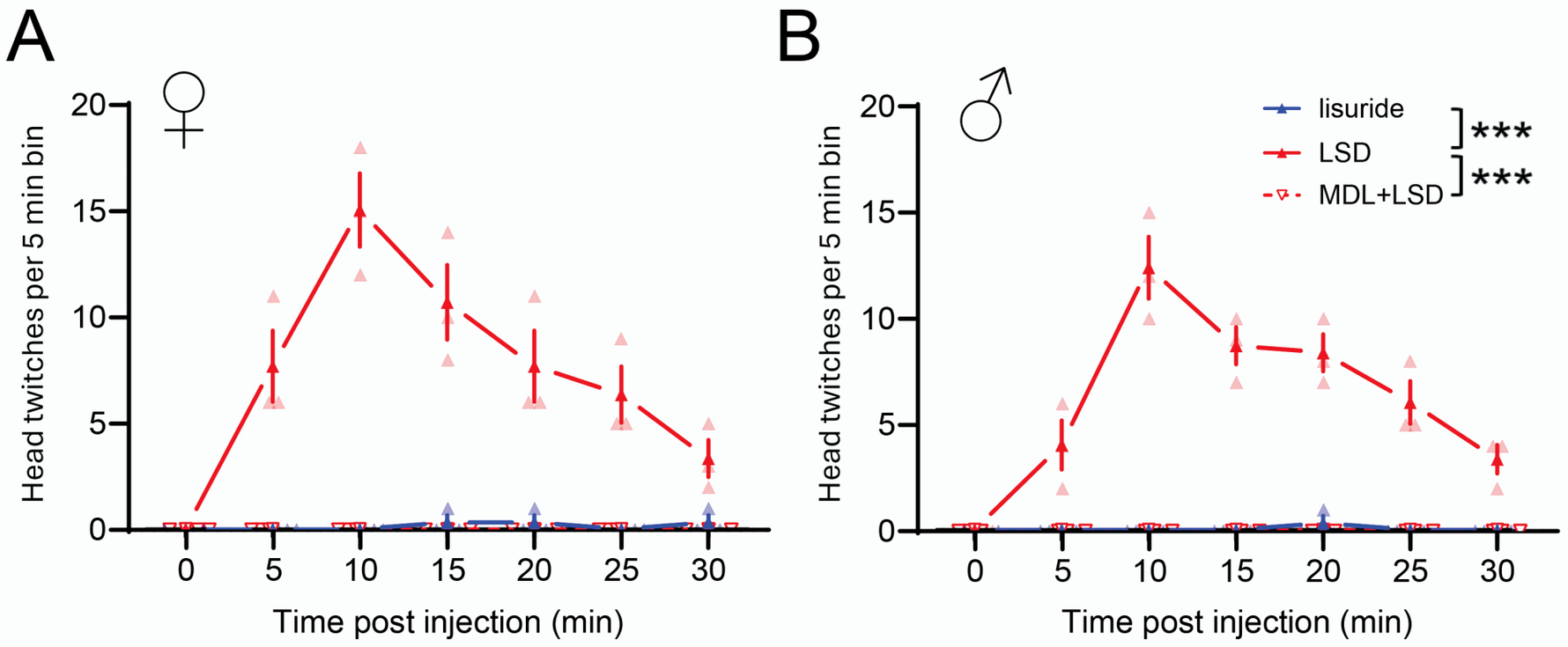

A
B
♀
♂
Head twitches per 5 min bin
Time post injection (min)
0
5
10
15
20
25
30
lisuride
LSD
MDL+LSD
***
***

Figure 2

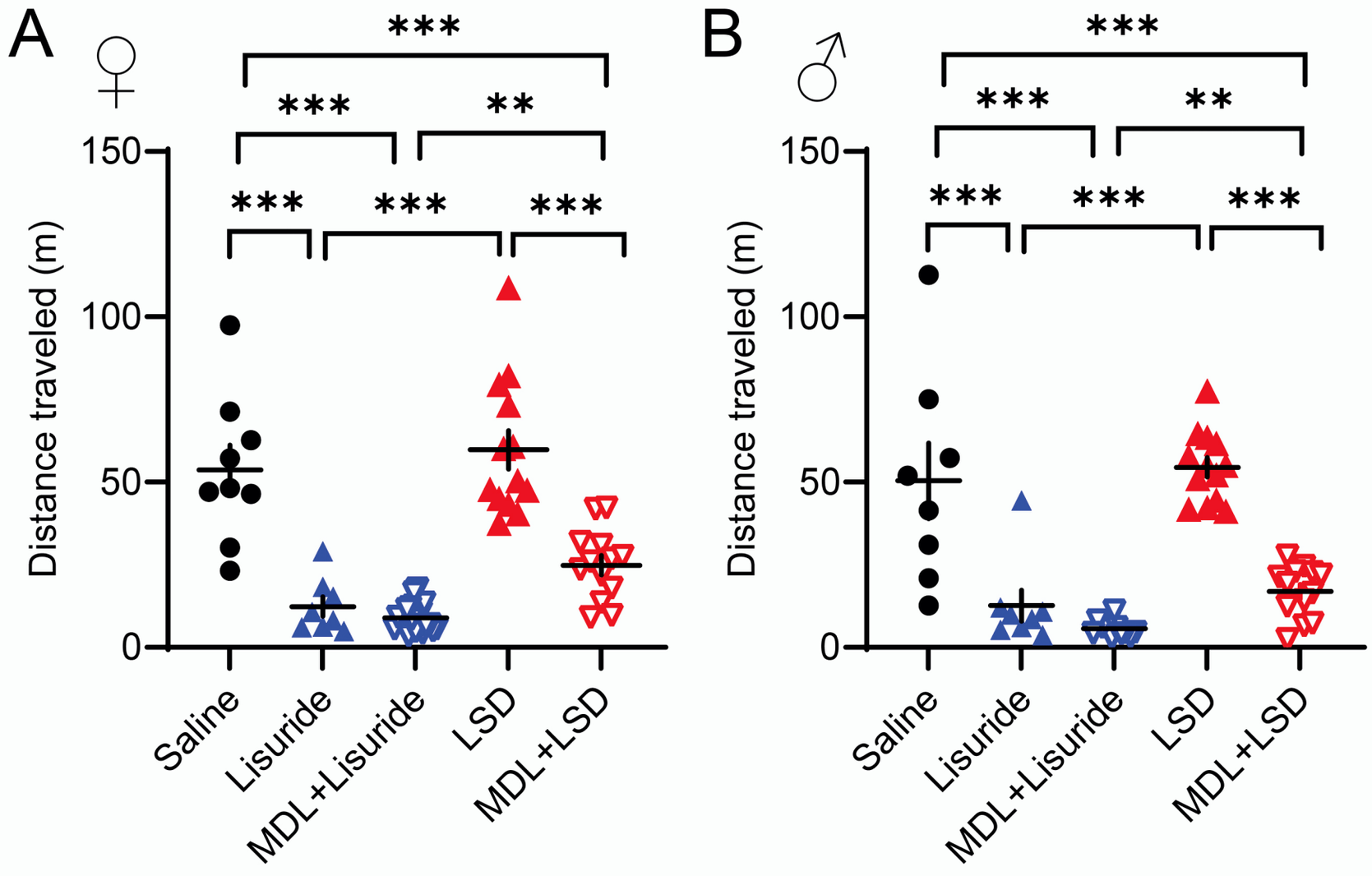

A
♀
B
♂
***
**
***
***
***
***
***
***
150
100
50
0
Distance traveled (m)
Saline
Lisuride
MDL+Lisuride
LSD
MDL+LSD

Figure 3

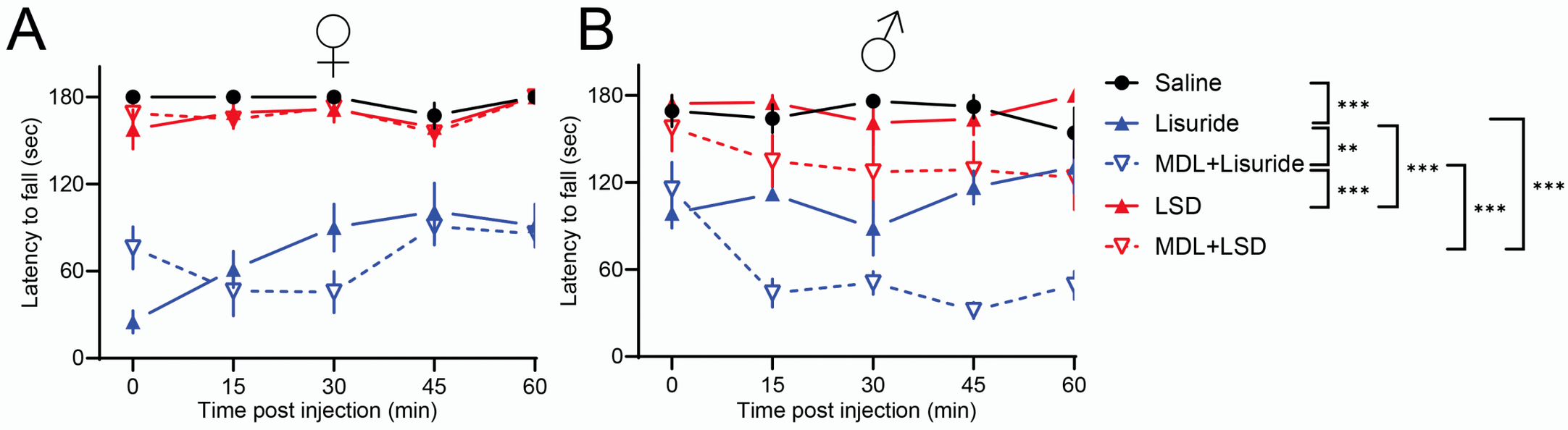

A
♀
B
♂
Latency to fall (sec)
180
120
60
0
0
15
30
45
60
Time post injection (min)
Saline
Lisuride
MDL+Lisuride
LSD
MDL+LSD
***
**
***
***
***
***

Figure 4

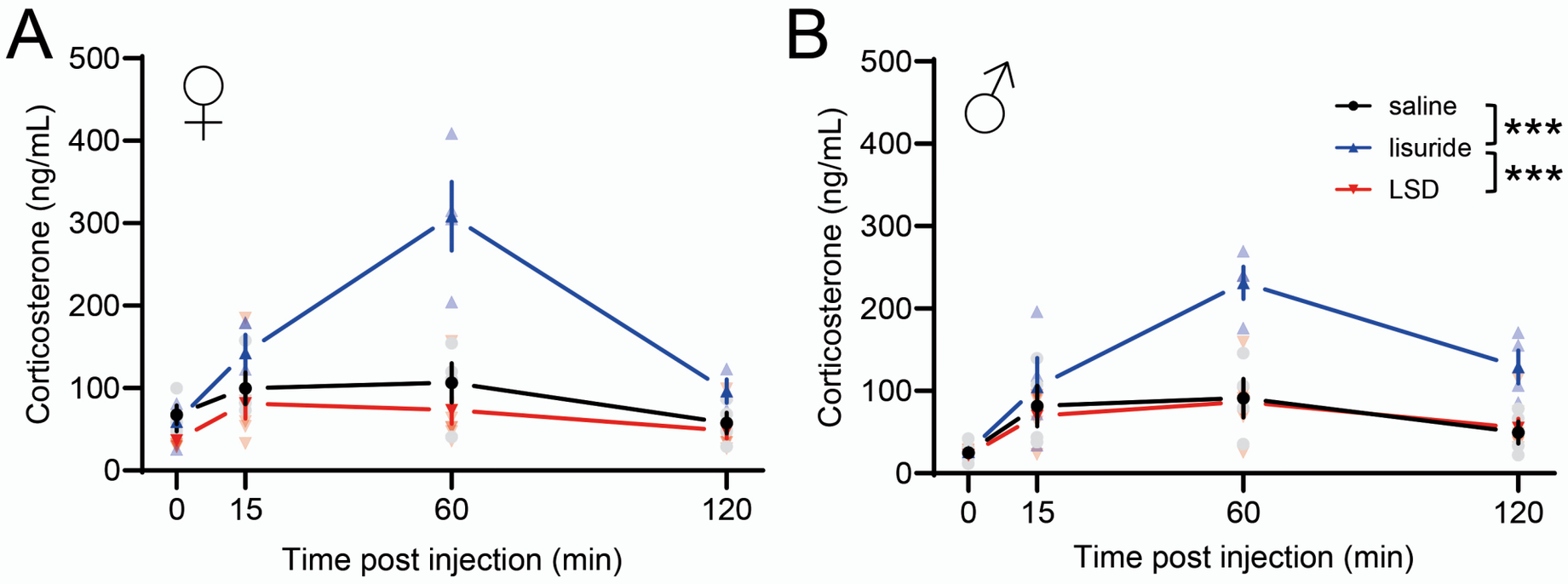

A
♀
Corticosterone (ng/mL)
500
400
300
200
100
0
0
15
60
120
Time post injection (min)
B
♂
saline
lisuride
LSD
***
***
Corticosterone (ng/mL)
500
400
300
200
100
0
0
15
60
120
Time post injection (min)

Figure 5

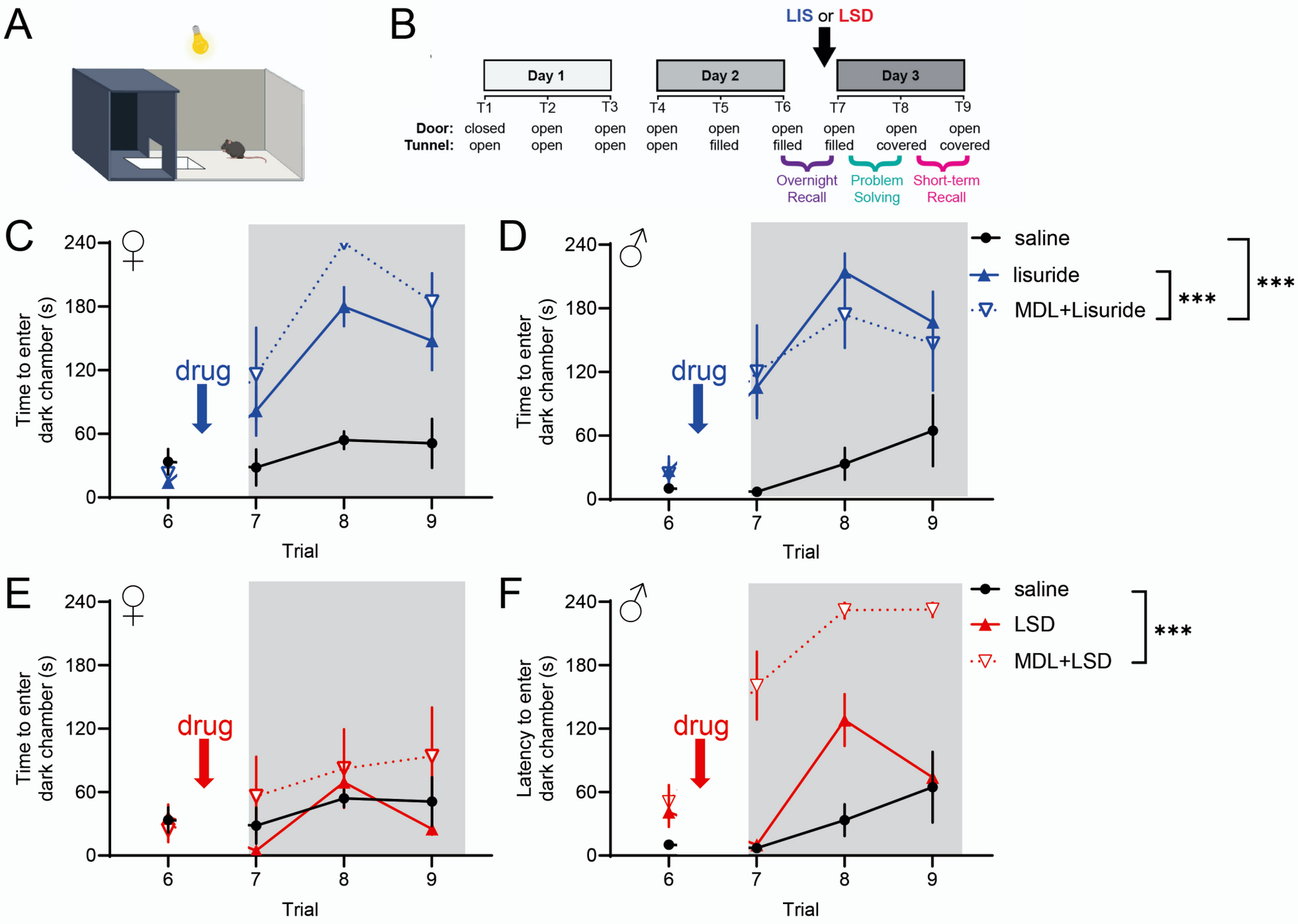
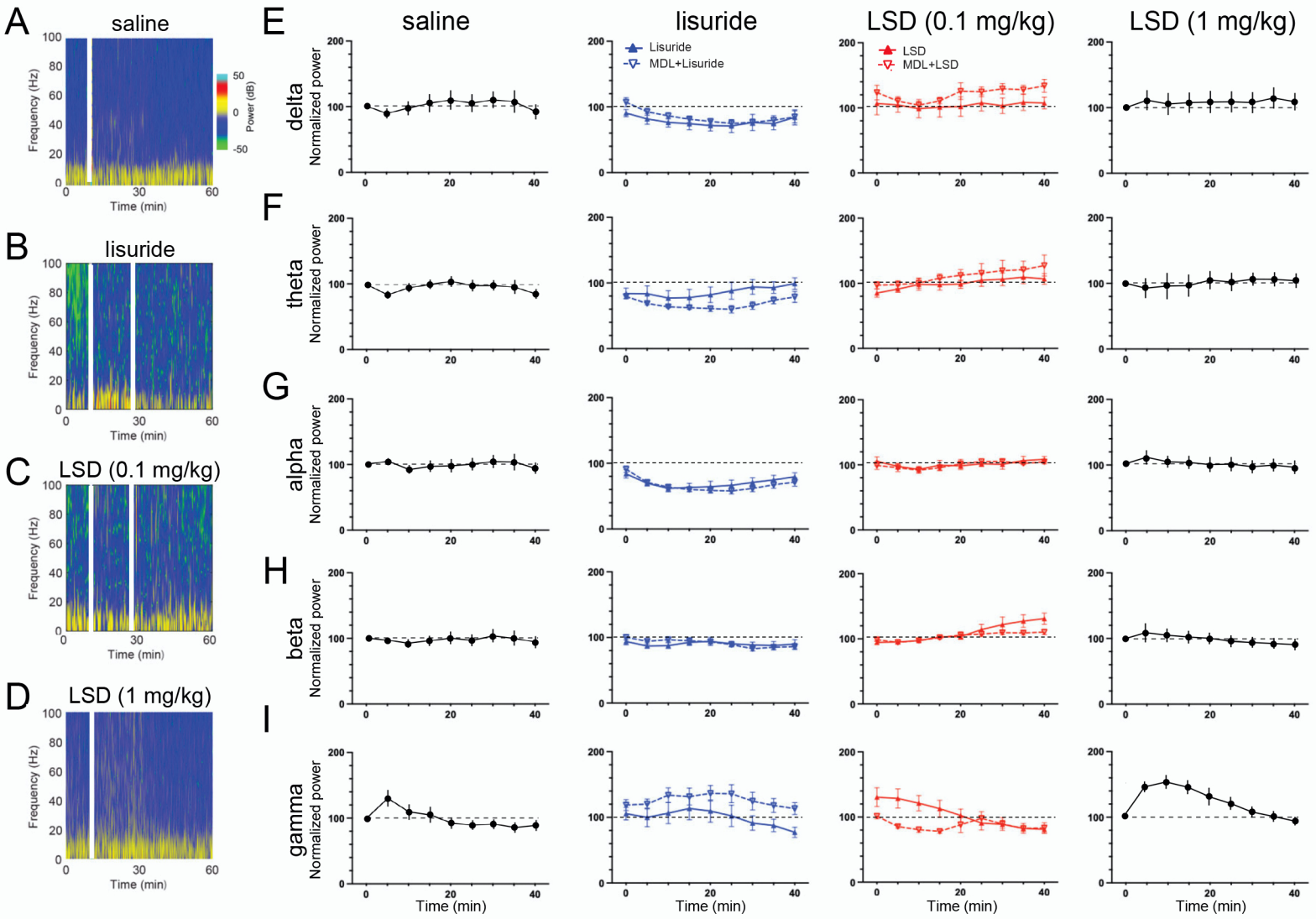

A
B
LIS or LSD
Day 1
Day 2
Day 3
T1 T2 T3 T4 T5 T6 T7 T8 T9
Door: closed open open open open open open open open
Tunnel: open open open open filled filled filled covered covered
Overnight Recall
Problem Solving
Short-term Recall
C
D
E
F
Time to enter dark chamber (s)
Latency to enter dark chamber (s)
drug
Trial
saline
lisuride
MDL+Lisuride
LSD
MDL+LSD
***

Figure 6


A
saline
B
lisuride
C
LSD (0.1 mg/kg)
D
LSD (1 mg/kg)
Frequency (Hz)
Time (min)
Power (dB)
E
saline
lisuride
LSD (0.1 mg/kg)
LSD (1 mg/kg)
Lisuride
MDL+Lisuride
LSD
MDL+LSD
delta
theta
alpha
beta
gamma
Normalized power
Time (min)