LSD's effects are differentially modulated in arrestin knockout mice

Running title: β -arrestin 1 and 2 modulation of LSD-evoked responses

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ABSTRACT

Recent evidence suggests that psychedelic drugs can exert beneficial effects on anxiety, depression, and ethanol and nicotine abuse in humans. However, the hallucinogenic side-effects of psychedelics often preclude their clinical use. Lysergic acid diethylamide (LSD) is a prototypical hallucinogen and its psychedelic actions are exerted through the 5-HT_{2A} serotonin receptor (5-HT_{2A}R). 5-HT_{2A}R activation stimulates Gq- and β -arrestin- (β Arr) mediated signaling. To separate effects of these signaling modes, we have used β Arr1 and β Arr2 mice. We find that LSD stimulates motor activities to similar extents in WT and β Arr1-KO mice, with non-significant effects in β Arr2-KOs. LSD robustly stimulates many surrogates of psychedelic drug actions including head twitches, grooming, retrograde walking, and nose poking in WT and β Arr1-KO animals. In contrast, LSD only slightly stimulates head twitches in β Arr2-KO mice, without effects on retrograde walking or nose poking. The 5-HT_{2A}R antagonist MDL100907 (MDL) blocks these LSD effects. LSD also disrupts prepulse inhibition (PPI) in WT and β Arr1-KOs; PPI is unaffected in β Arr2-KOs. MDL restores PPI in WT mice, but this antagonist is without effect and haloperidol is required in β Arr1-KOs. LSD produces a biphasic body-temperature response in WT mice, a monophasic response in β Arr1-KOs, and is without effect in β Arr2 mutants. Both MDL and the 5-HT_{1A}R antagonist, WAY 100635 (WAY), block the effects of LSD on body temperatures in WT mice, whereas WAY is effective in β Arr1-KOs. Collectively, these results reveal that LSD produces diverse behavioral effects through β Arr1 and β Arr2, and that LSD's psychedelic drug-like actions appear to require β Arr2.

INTRODUCTION

Lysergic acid diethylamide (LSD) is a prototypical psychedelic drug and is recognized as one of the most potent drugs in this class [1]. LSD was synthesized by Albert Hofmann in 1938, who later discovered its hallucinogenic properties [1-2]. LSD alters sensation, perception, thought, mood, sense of time and space, and consciousness of self in humans [1,3]. Since LSD-induced states appear to bear many similarities to early acute phases of psychosis and because serotonin (5-HT) and LSD both contain an indolamine moiety, Woolley and Shaw [4] proposed that aberrant 5-HT levels in brain could produce mental disturbances that included psychosis. This suggestion gave rise to the 5-HT hypothesis for schizophrenia and stimulated researchers to examine LSD responses in the hopes they could gain a better understanding of the basis for schizophrenia. However, this research was largely curtailed when LSD was classified as a DEA Schedule I drug in the 1960's. Recent research has revealed that LSD has medicinal value in treating cluster headaches [5], anxiety and depressive disorders in life-threatening conditions when combined with psychotherapy [6], and that it may have potential for studying human consciousness and substance abuse [7-8].

As noted above, LSD shares structural similarities to 5-HT [Nichols, 2016]. Thus, it is not surprising that LSD binds to all thirteen of the 5-HT G protein coupled receptors (GPCRs) [9-11]. Besides 5-HT receptors, LSD activates a number of other biogenic amine GPCRs [9] and this polypharmacy may contribute to LSD's wide ranging activities. One activity in particular regarding LSD has been its hallucinogenic actions. This activity is ascribed to stimulation of the 5-HT 2A receptor (5-HT_{2A}R) since in drug discrimination studies, discrimination potency is highly correlated with hallucinogenic potency in humans [12]. The same psychedelics produce head twitches in mice and, as a result, this response has been proposed as a proxy for hallucinations in humans [13], even though non-psychedelic drugs like 5-hydroxytryptophan induce robust head-twitch responses [14]. Hallucinogen-induced head twitches in rodents are blocked by the highly selective 5-HT_{2A}R antagonist MDL100907 [15-17] and are absent in *htr2A* knockout (KO) mice

[18-19]. In addition, recent human studies have shown the hallucinogenic actions of LSD are blocked with the 5-HT_{2A}R preferring antagonist ketanserin [20]. Thus, the hallucinogenic effects of LSD appear to be mediated through the 5-HT_{2A}R [21].

The 5-HT_{2A}R is a member of the rhodopsin family of GPCRs that is coupled to G_q protein and β -arrestin (β Arr) mediated signaling [22-25]. Recent experiments have found the 5-HT_{2A}R preferentially activates G_q family members, with moderate activity at G_z, and minimal detectable activity at other G_i, G_{12/13}, and G_s-family members [26]. However, the 5-HT_{2A}R binds to both β Arr1 and β Arr2 proteins *in vitro* and is complexed with β Arr1 and β Arr2 in cortical neurons *in vivo* [25]. While most GPCR agonists, like 5-HT, activate both G protein and β Arr modes of signaling, it has become established that ligand binding can activate the G protein-dependent signaling pathway while serving to activate or inhibit a G protein-independent pathway through β Arr. Hence, a given ligand can act as an agonist at one pathway while inhibiting the other pathway or it can serve various combinations of these actions. This property has been termed functional selectivity or biased signaling [27-29] and ligands have been developed to exploit these signaling features [c.f., 30]. Although LSD activates G protein signaling at many GPCRs [11], this psychedelic stimulates β Arr-mediated responses at most tested biogenic amine GPCRs [9]. Interestingly, LSD displays β Arr-biased signaling at the 5-HT_{2A}R [10-11,26]. Most 5-HT_{2A}R-containing neurons express both β Arr1 and β Arr2 [25] and mice have been generated that have *Arrb1* or *Arrb2* deleted [31-32]. The purpose of the present investigations was to determine whether LSD would produce behavioral and physiological effects that were differential among the wild-type (WT), β Arr1-KO, and β Arr2-KO mice.

MATERIALS AND METHODS

Details of the materials and methods can be found in the Supplementary Information.

Subjects

Adult male and female WT and β Arr1-KO, and WT and β Arr2-KO mice were used in these experiments [1-2]. All studies were conducted with an approved protocol from the Duke University Institutional Animal Care and Use Committee.

Open field activity

Motor activities were assessed over 120 min in an open field (Omnitech Electronics, Columbus, OH) illuminated at 180 lux [3].

Head twitch, grooming, and retrograde walking

These behaviors were filmed during the assessment of motor activity in the open field.

Nose-poking responses

Nose-pokes were monitored in a mouse 5-choice serial reaction-time apparatus (Med Associates Inc., St. Albans, VT) [4].

Prepulse inhibition (PPI)

PPI of the acoustic startle response was conducted using SR-LAB chambers (San Diego Instruments, San Diego, CA) as reported [3].

Regulation of body temperature

Baseline body temperatures were taken (Physitemp Instruments LLC, Clifton, NY) in the absence of the vehicle or antagonist. Immediately afterwards mice were injected with vehicle or different

doses of MDL or WAY. Fifteen min later (time 0) their body temperatures were taken and they were administered vehicle or LSD. Subsequently, body temperatures were taken at 15, 30, 60, 120, 180, and 240 min.

Radioligand binding and immunohistochemistry of 5-HT_{2A}R

Radioligand binding experiments on mouse brains were conducted as previously detailed using [³H]-ketanserin as the radioligand [5]. The 5-HT_{2A}R immunofluorescence/microscopy was performed as described [35] with a previously validated 5-HT_{2A}R-specific antibody [36].

Statistics

All statistical analyses were performed with IBM SPSS Statistics 27 programs (IBM, Chicago, IL).

RESULTS

Effects of Arrb1 or Arrb2 deletion on LSD-stimulated motor responses

LSD stimulates, inhibits, produces biphasic effects on motor activities in rats [18,37-43]. The β Arr1 and β Arr2 mice were used to determine whether disruption of either of these gene products could modify responses to LSD and to test whether 5-HT_{2A}R antagonism differentially could block the effects of LSD. Locomotor, rearing, and stereotypical activities were monitored in the β Arr1 and β Arr2 mice at 5-min intervals over the 120 min test with the β Arr1 and β Arr2 mice (Supplementary Figs. S1-S2; Supplementary Tables S1-S2). When cumulative baseline locomotion was examined in β Arr1 mice, activity was not differentiated by genotype or the pre-assigned treatment condition (Supplementary Table S3; Supplementary Table S4). By contrast, overall cumulative baseline rearing activities were lower in groups that were to receive 0.1 or 0.5 mg/kg MDL with LSD compared to mice that were to be given LSD or the vehicle (p -values $\leq$ 0.016). For stereotypy in β Arr1 animals, overall cumulative baseline activities were lower in groups that were to receive 0.1 or 0.5 MDL with LSD than the vehicle control (p -values $\leq$ 0.005); stereotypy in the group to be injected with 0.1 mg/kg MDL with LSD was lower also than the group to be given LSD (p =0.002). In comparison to the in β Arr1 mice, baseline cumulative locomotor, rearing, and stereotypical activities in the β Arr2 animals were not distinguished by genotype or treatment (Supplementary Table S5; Supplementary Table S6)

When cumulative LSD-stimulated motor activities were examined in the β Arr1 mice, no genotype effects were found. This psychedelic stimulated locomotion relative to the groups given 0.5 mg/kg MDL alone or the vehicle (p -values $\leq$ 0.001) (Fig. 1a; Supplementary Table S4). Importantly, both 0.1 and 0.5 mg/kg MDL blocked the locomotor-stimulating effects of LSD (p -values $\leq$ 0.001). When cumulative rearing activities were examined, these responses were found to be lower in groups given 0.5 mg/kg MDL alone or administered 0.1 or 0.5 mg/kg MDL with LSD (p -values $\leq$ 0.028) (Fig. 1c). An assessment of cumulative stereotypical activities in the β Arr1 animals revealed responses in the MDL group, as well as in the 0.1 and 0.5 mg/kg MDL plus LSD

groups to be significantly decreased relative to the LSD-treated mice (p -values ≤ 0.027) (Fig. 1e). Hence, MDL normalized the LSD-induced hyperactivity in the β Arr1 animals.

Effects of LSD in the β Arr2-KO mice were quite different from those of its WT controls (Supplementary Table S6). LSD was more potent in stimulating cumulative locomotor and rearing activities in the WT than in β Arr2-KO mice (p -values < 0.001); Fig. 1b,d); no genotype differences were observed for cumulative stereotypical activities (Fig. 1f). When locomotion was analyzed for WT animals, the LSD-stimulated responses were higher than those for all other treatment groups (p -values < 0.001) (Fig. 1b). Hence, all three doses of MDL were efficacious in suppressing the LSD-induced hyperlocomotion. Although LSD increased locomotor activity in β Arr2-KO mice, it was not significantly different from any other treatment group. In WT mice rearing activities were increased with LSD over that for the MDL and vehicle controls (p -values < 0.001) (Fig. 1d). Although rearing was higher in WT mice given 0.05 mg/kg MDL with LSD than in the MDL and vehicle controls (p -values ≤ 0.029), the 0.1 and 0.5 mg/kg doses of MDL reduced the LSD-stimulated rearing activity (p -values ≤ 0.001) to the levels of these controls. By comparison, LSD was without effect in the β Arr2-KO mice. An examination of stereotypy failed to detect any significant genotype effects (Fig. 1f). Nonetheless, treatment effects were evident overall with LSD stimulating stereotypical activities over that of the vehicle and MDL controls (p -values ≤ 0.013); however, when 0.5 mg/kg MDL was given with LSD the psychedelic effects were abrogated ($p=0.003$). Collectively, these results indicate that LSD stimulates motor responses to similar extents in the β Arr1 and β Arr2 WT mice and in β Arr1-KO animals, and that the 5-HT_{2A}R antagonist MDL blocks these LSD-stimulated activities. By striking comparison, LSD exerts minimal effects on these same responses in the β Arr2-KO mice where none of these activities were significantly increased above that of the vehicle or MDL controls.

LSD and MDL 100907 effects on additional LSD-stimulated behaviors

LSD affects a number of behaviors in mice [13,18,44-48] that include, as a minimum, head twitches, grooming, and retrograde walking. In addition, LSD produces changes in hole-poking behavior in rats [41-42,45-50]. Despite an absence of genotype effects with β Arr1 mice for head twitches, grooming, retrograde walking, and nose-poking, overall treatment effects were evident (Supplementary Table S7). Relative to the MDL and vehicle controls, LSD stimulated head twitches in WT and β Arr1-KO mice (p -values<0.001) (Fig. 2a). Both 0.1 and 0.5 mg/kg MDL blocked these LSD effects by restoring the numbers of head twitches to those of controls. A similar effect was observed for grooming where LSD augmented this response over that of groups administered the vehicle or 0.5 mg/kg MDL alone (p -values<0.001) (Fig. 2c). When 0.1 or 0.5 mg/kg MDL was given with LSD, both doses of the 5-HT_{2A}R antagonist suppressed the LSD stimulatory effect (p -values<0.001) to that of the controls.

Besides grooming, LSD was efficacious in potentiating retrograde walking in the β Arr1 mice compared to the vehicle and MDL controls (p -values<0.001) (Fig. 2e) (Supplementary Table S7). While 0.1 and 0.5 mg/kg MDL depressed this LSD-mediated response (p -values<0.001), the lower dose of MDL was less efficacious than the higher dose in normalizing this behavior (p =0.010). When nose poking behaviors were examined, LSD was observed to increase this response overall relative to the vehicle and MDL controls (p -values<0.001) (Fig. 2g). Both doses of MDL suppressed the LSD stimulation of nose-poking behaviors (p -values<0.001).

In contradistinction to the β Arr1 mice, genotype differences were found between the β Arr2 animals (Supplementary Table S8). The numbers of head twitches were significantly increased in the WT than in β Arr2-KO mice treated with LSD alone or 0.05 mg/kg MDL with LSD (p -values<0.001) (Fig. 2b). In WT mice head twitches were augmented by LSD and by 0.05 or 0.1 mg/kg MDL plus LSD relative to the MDL and vehicle controls (p -values<0.001). While 0.05 mg/kg MDL was ineffective in reducing this LSD-mediated behavior, both the 0.1 and 0.5 mg/kg doses significantly suppressed this response (p -values≤0.002) – with the higher MDL dose being the more efficacious (p <0.001). The LSD and 0.05 or 0.1 mg/kg MDL plus LSD treatments also

increased head twitch behaviors in β Arr2-KO animals compared to their MDL and vehicle controls and the 0.5 mg/kg MDL plus LSD treatment (p -values $\leq$ 0.023). Only 0.5 mg/kg MDL was sufficient to depress this LSD-initiated response (p =0.019) to the levels of the controls.

For grooming, the durations of grooming were higher in the WT than in the corresponding β Arr2-KO groups administered LSD alone, 0.05 mg/kg MDL plus LSD, or 0.5 mg/kg MDL with LSD (p -values $\leq$ 0.016) (Fig. 2d) (Supplementary Table S8). In WT mice LSD augmented grooming relative to the MDL and vehicle controls (p <0.001). While 0.05 mg/kg MDL failed to block the LSD effect, both the 0.1 and 0.5 mg/kg doses were efficacious (p -values<0.001) in returning responses to control levels. In β Arr2-KO animals, LSD failed to increase the duration of grooming responses relative to the MDL and vehicle controls. Nevertheless, grooming was enhanced in the group administered 0.05 mg/kg MDL plus LSD relative to all groups (p -values $\leq$ 0.013), except those given LSD alone.

Aside from disturbing grooming, LSD also induced retrograde walking (Supplementary Table S8). Here, genotype effects were observed in mice that received LSD or 0.05 mg/kg MDL with LSD (p -values $\leq$ 0.040) (Fig. 2f). In WT mice LSD potentiated the incidences of retrograde walking compared to the MDL and vehicle controls (p <0.001). Although 0.05 mg/kg MDL was ineffective in decreasing this LSD-stimulated behavior, both the 0.1 and 0.5 mg/kg doses suppressed this response (p <0.001). By contrast, LSD was without significant effect on retrograde walking in the β Arr2-KO animals. Differential nose-poking responses were observed between the β Arr2 mice (Fig. 2h). Here, LSD stimulated nose-poking behaviors in WT mice relative to all other groups (p <0.001). All doses of MDL suppressed LSD-stimulated nose poking in WT mice (p -values $\leq$ 0.007) to the MDL and vehicle controls. By comparison, nose-poking in β Arr2-KO animals was not distinguished by treatment condition.

Since LSD can induce a fragmentation of consciousness [3], we examined grooming in detail since it has a chained organization of responses in rodents [51]. Inspection of the video-recordings confirmed that all genotypes engaged in a normal sequence of grooming beginning

with the face, progressing down the body, and ending at the feet or tail (Movie 1). When LSD was administered, the sequence of grooming in the WT and β Arr1-KO mice became abbreviated, non-sequential, and/or restricted to one area of the body (Movies 2-3). By sharp comparison, the grooming sequence was complete and rarely perturbed in the β Arr2-KO animals (Movie 4). When MDL alone was injected, the organization of grooming was intact in the WT and β Arr1-KO mice (Movie 5). With MDL the β Arr2-KO animals might pause in the grooming bout and/or display twitching of neck and back muscles but they would finish the sequence (Movie 6). The patterns of grooming among the genotypes with MDL plus LSD were divergent. In WT mice, when MDL was given with LSD, the organization of grooming was restored (Movie 7). When the β Arr1 mutants received the same treatment, they began the grooming sequence, engaged in focal grooming of a part of the body, and then completed the sequence (Movie 8). When this same drug combination was administered to β Arr2-KO mice, they usually began the sequence appropriately, but at some mid- or later-point they would become focused on one area; however, they usually completed the grooming sequence (Movie 9).

In summary, responses to LSD across these LSD-stimulated behaviors were usually similar between the β Arr1 genotypes and MDL reduced these responses to levels of the controls. Importantly, the β Arr2 mice responded quite differently than the other genotypes. Responses to LSD were significantly higher overall in WT than in β Arr2-KO animals. LSD did not significantly increase grooming, retrograde walking, or nose poking behaviors in these mutants. Notably, LSD disrupted the sequences of grooming in WT mice from both strains and in the β Arr1-KO mice; the β Arr2-KO animals were unaffected. Nonetheless, divergent responses to MDL alone or MDL plus LSD were observed among the genotypes.

LSD and MDL 100907 effects on prepulse inhibition

LSD disrupts PPI in both rats and humans [44,52] and the response can be restored with a 5-HT_{2A}R antagonist [44,53]. β Arr1 mice were pre-treated with the vehicle or with 0.1 or 0.5 mg/kg

MDL. Subsequently, they were administered the vehicle or 0.3 mg/kg LSD and tested in PPI. No significant genotype or treatment effects were observed for null activity or in response to the 120 dB startle stimulus (Supplementary Fig. S3a-b; Supplementary Table S9). In contrast, LSD depressed PPI in both β Arr1 genotypes (p -values $\leq$ 0.002) relative to the MDL and vehicle controls (Fig. 3a). When WT mice were administered either dose of the 5-HT_{2A}R antagonist with LSD, it restored PPI to control levels. In stark contrast, neither dose of the antagonist was effective in blocking the LSD effects in the β Arr1-KO mice. Notably, PPI in these two groups was significantly lower than that in similarly treated WT animals (p -values $\leq$ 0.018).

Since haloperidol can normalize PPI in mouse models [33], we tested whether this antipsychotic drug could normalize the LSD-disrupted PPI in the β Arr1-KO mice (Supplementary Table S10). Overall null activity was higher in the 0.1 mg/kg haloperidol plus LSD group than in mice treated with haloperidol alone or the vehicle control (p -values $\leq$ 0.009) (Supplementary Fig. S2c). An assessment of startle activity revealed this activity was lower overall in the WT relative to the β Arr1-KO mice (p =0.028) (Supplementary Fig. S2d). For PPI, overall responses were reduced in the β Arr1-KO compared to the WT animals (p =0.008; Fig. 3b). Treatment effects were noted where LSD suppressed PPI relative to all other treatment conditions (p -values $\leq$ 0.002). Hence, haloperidol normalized the LSD-disrupted PPI in both β Arr1 genotypes.

PPI responses in the β Arr2 mice were also examined. Overall null activity was decreased in the combined 0.1 mg/kg MDL plus LSD group compared to the vehicle control and the LSD group (p -values $\leq$ 0.003) (Supplementary Fig. S2e; Supplementary Table S11). No significant differences were detected for startle activity (Supplementary Fig. S2f). Nevertheless, striking genotype differences were evident for PPI (Fig. 3c). Here, responses to LSD and to the 0.05 MDL plus LSD treatment were reduced in WT relative to the β Arr2-KO mice (p -values $\leq$ 0.001). In WT animals, LSD suppressed PPI compared to the MDL and vehicle controls (p =0.001). The 0.05 mg/kg dose of MDL was insufficient to restore the LSD-disrupted PPI relative to the vehicle and MDL controls, whereas with 0.1 mg/kg MDL normalization occurred. By dramatic comparison,

LSD was completely without effect in the β Arr2-KO mice. Collectively, these findings show that LSD disrupts PPI in both genotypes of the β Arr1 mice and in the β Arr2 WT animals. MDL restores PPI in both WT strains, whereby haloperidol is required to normalize it in β Arr1-KO mice. By contrast, PPI in β Arr2-KO mice is unaffected by this psychedelic.

LSD and MDL 100907 effects on temperature regulation

LSD is reported to increase and to decrease body temperatures in rodents [54] and both the 5-HT_{2A} and the 5-HT_{1A} receptors are known to mediate changes in temperature [55-56]. Baseline body temperatures were not significantly different between the genotypes (β Arr1: WT 37.6 ± 0.05 °F, KO 37.6 ± 0.10 °F; β Arr2: WT 37.7 ± 0.10 °F, KO 37.8 ± 0.10 °F). Immediately afterwards mice were injected with the vehicle or different doses of MDL or WAY. Fifteen min later (time 0) body temperatures were taken and the mice were administered the vehicle or LSD. Responses to LSD were followed over 4 hours in all genotypes. Initial analyses were run with the vehicle, and MDL or WAY controls and LSD. Subsequent analyses included the vehicle, LSD, and MDL or WAY with LSD.

For β Arr1 mice, genotype differences were detected with the vehicle, MDL, and LSD (Supplementary Table S12). Under vehicle conditions, body temperatures were higher in WT mice at 0 and 120 to 240 min compared to the β Arr1-KO animals (p -values ≤ 0.011) (Fig. 4a-b). With MDL, temperatures in WT animals were lower at 0 min, but were higher at 180 and 240 min than in the mutants (p -values ≤ 0.026). LSD produced a biphasic response in WT animals, but only a monophasic effect in mutants with genotype differences at 30, 120, 180, and 240 min (p -values ≤ 0.046). Within WT animals, LSD reduced body temperatures at 15 and 30 min, but increased them at 60, 120, and 180 min relative to vehicle (p -values ≤ 0.008). By comparison, in β Arr1-KO animals LSD only enhanced temperatures at 60 through 240 min (p -values ≤ 0.011). Although MDL decreased body temperatures in WT mice at 0, 15, and 30 min (p -values ≤ 0.019), it exerted no effects on the mutants at these times.

Since LSD produced such dramatic effects in body temperatures between the β Arr1 genotypes, different doses of MDL were administered with LSD in an attempt to normalize their temperatures (Supplementary Table S12). No genotype differences were obtained when 0.25 mg/kg MDL was given with LSD (Fig. 4c-d). Responses to 0.5 mg/kg MDL with LSD, however, appeared as higher temperatures in WT animals at 0, 30, 120, 180, and 240 min (p -values $\leq$ 0.021) and lower temperatures at 30 min than in the mutants (p <0.001). In WT mice, 0.025 mg/kg MDL normalized their LSD-perturbed body temperatures (Fig. 4c). By comparison, this low dose was ineffective in β Arr1-KO animals, whereas 0.5 mg/kg MDL was required to normalize their body temperatures to the vehicle control (Fig. 4d).

Because body temperatures in β Arr1-KO mice were not fully restored with the 5-HT_{2A}R antagonist, we examined whether a 5-HT_{1A}R antagonist could block LSD's effects (Supplementary Table S12). Temperature responses to 0.25 mg/kg WAY 100635 (WAY) were significantly higher in WT than mutants at 30 and 60 min (p -values $\leq$ 0.012) (Fig. 4e-f). The LSD-induced responses in WT mice were lower at 30 min but higher from 120 to 240 min than in the β Arr1-KO animals (p -values $\leq$ 0.036). In WT mice, body temperatures were lower in WAY-treated mice than in those given LSD at 15 to 180 min (p -values $\leq$ 0.052) (Fig. 4e). By comparison, temperatures in β Arr1-KO mice given WAY were higher than the LSD group at 0 min (p =0.028), but were lower from 60 min to 180 min (p -values $\leq$ 0.002) (Fig. 4f). Apart from these differences, body temperatures to WAY across time in β Arr1-KO mice were different from those of the vehicle group only at 0 and 240 min (p -values $\leq$ 0.046), whereas no significant differences across time were observed for WT mice.

To determine whether WAY exerted any effects on LSD responses, 0.1 or 0.25 mg/kg WAY was administered with this psychedelic (Supplementary Table S12). Although in WT mice both doses of WAY normalized the LSD-induced changes in body temperatures to the vehicle control, 0.25 mg/kg WAY appeared to be the more efficacious (Fig. 4g). While there were some variabilities across time in mutants with the 0.1 and 0.25 mg/kg doses, their LSD-disturbed

temperatures were restored to levels that were statistically indistinguishable from the vehicle—with the exception of 0.1 mg/kg WAY at 120 min ($p=0.011$) (Fig. 4h). Together, 0.25 mg/kg MDL and 0.25 mg/kg WAY were most efficacious in WT mice, while 0.5 mg/kg MDL was partially effective at later times and 0.25 mg/kg WAY was most proficient in the mutants.

Effects of LSD on temperature regulation were examined also in the β Arr2 mice (Supplementary Table S13). Responses to the vehicle or 0.5 mg/kg MDL were undifferentiated by genotype (Fig. 5a-b). By comparison, the LSD-induced changes in body temperatures were lower at 15 min ($p=0.001$) and higher at 60, 120, and 180 min in WT than in the β Arr2-KO mice (p -values ≤ 0.019). In WT animals, LSD produced biphasic changes in temperatures at 30, 120, and 180 min relative to vehicle (p -values ≤ 0.032) (Fig. 5a). By contrast, no significant LSD effects were observed in β Arr2-KO mice at any time-point (Fig. 5b). Temperature responses to MDL in mutants, however, were decreased relative to vehicle at 15 and 60 min (p -values ≤ 0.013). When 0.25 mg/kg MDL was given with LSD, no differential genotype effects were noted (Fig. 5c-d). Nonetheless, body temperatures in WT mice administered 0.5 mg/kg MDL plus LSD were higher than in the mutants (p -values ≤ 0.032). This same dose also produced deviations from the vehicle control in WT animals at 15 and 30 min (p -values ≤ 0.035) and in β Arr2-KO mice at 15, 60, and 120 min (p -values ≤ 0.035). Regardless, in WT animals 0.025 mg/kg MDL normalized the LSD-induced changes in body temperature across time (Fig. 5c).

Effects of WAY were evaluated next in the β Arr2 mice (Supplementary Table S13). Genotype differences in response to the 0.5 mg/kg WAY control were observed at 0, 60, and 180 min (p -values ≤ 0.023) (Fig. 5e-f). Body temperatures were modulated by LSD in WT relative to β Arr2-KO animals at 15, 60, 120, and 180 min (p -values ≤ 0.017). Within WT mice, temperatures were enhanced with WAY at 0 min relative to the vehicle control ($p=0.021$) (Fig. 5e). In contrast, responses to WAY were not evident in β Arr2-KO animals (Fig. 5f). When 0.25 or 0.5 mg/kg WAY was administered with LSD, genotype differences were found at 15, 30, and 240 min with the lower dose (p -values ≤ 0.048) and at 240 min with the higher dose ($p=0.024$) (Fig. 5g-h). For WT

mice, 0.25 or 0.5 mg/kg WAY restored the LSD-disturbed body temperatures to levels that were not significantly different from the vehicle control (Fig. 5g). In summary, the low doses of MDL and WAY were both efficacious in reverting the LSD-induced changes to normal in WT animals. By comparison, LSD was without effect in β Arr2-KO mice.

Effects of Arrb1 or Arrb2 deletion on 5-HT2AR expression

Finally, we examined whether deletion of *Arrb* could alter 5-HT2AR expression by radioligand binding with brains from WT and β Arr1-KO, and WT and β Arr2-KO littermates. When [³H]-ketanserin affinity binding was examined, it was found to be significantly higher in β Arr2-KO samples than in β Arr1 WT and KO brains (p -values $\leq$ 0.039) (Supplementary Fig. S4a). By contrast, the numbers of 5-HT2AR binding sites were higher in brain extracts from β Arr2-KO mice than from β Arr1 WT and KO animals and the β Arr2 WT mice (p -values $\leq$ 0.034); no significant differences were observed among the latter three genotypes (Supplementary Fig. S4b). We examined also 5-HT2AR immunofluorescence in β Arr1 and β Arr2 brain sections (Supplementary Fig. S4c-f). Here, we detected no apparent alterations in the relative receptor distributions among the genotypes. Together, these results are consistent with the hypothesis that neither *Arrb1* nor *Arrb2* genetic deletion decreases 5-HT2A receptor expression. Nonetheless, *Arrb2* disruption leads to increased expression of 5-HT2ARs in brain.

DISCUSSION

In the present study, we analyzed whether global deletion of *Arrb1* or *Arrb2* modifies responses to LSD in the mutants relative to their respective WT controls. In the open field 0.3 mg/kg LSD stimulated motor activities in the β Arr1 and β Arr2 mice. While these activities were observed in WT animals from both β Arr strains and in the β Arr1-KO mice. LSD failed to modify significantly rearing or stereotypical activities in WT and β Arr1-KO mice. By comparison, LSD stimulated locomotor and rearing activities in β Arr2 WT animals, while LSD was without effect in the β Arr2-KO mice. Together, these results indicate that LSD-induced motor activities are regulated primarily through β Arr2-mediated signaling. In this regard, β Arr2 has been reported to play a similar role in morphine-stimulated hyperlocomotion [57] and amphetamine-stimulated locomotor and rearing activities in β Arr2 mice [58].

In our experiments, LSD augmented motor activities in β Arr1 and β Arr2 WT mice and in β Arr1-KO animals. By contrast, in rats this hallucinogen has been reported to inhibit ambulation and rearing [40], to stimulate locomotor activities [37,43], to induce divergent effects on motor responses [38], or to produces biphasic (i.e., inhibitory, then stimulatory) effects [39,41-42]. In addition, a sex effect in rats has been observed with LSD [46]. In our experiments with mice, we failed to discern LSD effects attributable to sex. The inhibitory effects of LSD in rats were seen very soon after placement or entry into the open field. In our open field experiments, we did not observe any inhibition of responses with 0.3 mg/kg LSD; only stimulatory effects were evident. This absence of LSD-induced inhibitory effects in our studies could be attributed to differences in the species tested, the dose of LSD used, the test environment and apparatus, and/or the test procedure. For instance, in humans LSD's behavioral effects are well-known to be context specific [1-3] and the 30 min habituation to the novel environment prior to LSD injection may have reduced emotionality in our mice, such that only the stimulatory effects of LSD were evident.

To determine whether the motor-stimulating effects of LSD were due to 5-HT_{2A}R activation, MDL was used as an antagonist. When administered alone, 0.5 mg/kg MDL exerted

no effects on motor performance in either β Arr mouse strain. Parenthetically, MDL has been found also to be without effect on spontaneous motor activity in rats [59]. Nonetheless, 0.1 and 0.5 mg/kg MDL blocked the motor-stimulating effects of LSD in the β Arr1 and β Arr2 WT mice and in the β Arr1-KO animals. A similar effect has been attributed to MDL's effects on LSD-stimulated motor activities in rats [43]. Hence, at the doses tested in our experiments, the present results indicate that the LSD-induced hyperactivity in the β Arr mice is promoted through the 5-HT_{2A}R.

Besides motor activity, we examined the effects of LSD on head twitch, grooming, retrograde walking, and nose-poking behaviors. LSD and other psychedelics are well-known to stimulate head-twitch responses in mice [18, 45,48] and this response has been proposed as a proxy for hallucinations in humans [13]. Compared to the vehicle, LSD stimulated head-twitch responses to similar extents in WT and β Arr1-KO mice. This psychedelic also activated head twitches in β Arr2 animals; however, responses in WT controls were significantly more robust than in β Arr2-KO mice. It should be emphasized that these results were surprising since the numbers of 5-HT_{2A}R binding sites were increased in brains of the β Arr2-KO animals relative to the other genotypes and because head twitches are mediated through the this receptor [18]. Regardless, in both β Arr1 and β Arr2 mice, MDL reduced the numbers of head twitches to levels of the vehicle controls. These findings are consistent not only with the known action of MDL on blocking head-twitch responses to various hallucinogens [15-17], but also to the inability of LSD and other psychedelics to induce this response in the *htr2A* homozygous mutant mice [18-19,45].

Aside from head twitches in rodents, LSD stimulates grooming behaviors in cats [60] and it can stimulate or inhibit grooming in mice [46-47]. In our investigations, LSD augmented grooming in β Arr1 and β Arr2 WT mice, and in β Arr1-KO animals. By comparison, LSD failed to prolong grooming in β Arr2-KO mice beyond that of vehicle controls. In all cases, 0.1 and 0.5 mg/kg MDL returned the LSD-stimulated grooming to levels indistinguishable from the controls. Thus, antagonism of the 5-HT_{2A}R was sufficient to restore LSD-induced grooming to baseline levels.

Effects of LSD were examined also on the organization of grooming. Under vehicle treatment, all mice displayed similar patterns of grooming that began with the face, progressed to the flanks, and ended with the feet or tail. LSD disorganized this sequence of events such that in the WT and β Arr1-KO mice, grooming was often restricted to the flanks, feet, or tail, was non-sequential, or was fragmented. By comparison, grooming in the β Arr2-KO mice was largely unaffected by LSD. MDL did not alter grooming in the WT and β Arr1-KO mice, whereas it prolonged grooming and promoted twitching of the neck and back muscles in the β Arr2 mutants. This antagonist blocked the LSD-disrupting effects on grooming in WT mice and it mostly restored the organization of grooming in β Arr1-KO animals. The MDL-LSD combination in β Arr2-KO animals produced some disturbances, but the mice typically completed the grooming sequence. Together, these results suggest that additional receptor systems may be involved in these LSD-induced grooming responses. It should be emphasized that LSD perturbs grooming also in rats [43] and cats [60].

LSD effects on retrograde walking and nose-poking responses were examined in the β Arr mice. Retrograde walking was one of the first LSD-elicited responses identified in mice [442]. In the behavioral pattern monitor, LSD decreased and then increased or maintained the frequencies of head-poking behaviors on a hole-board [41-42,49-50]. Instead of head-poking on a hole-board, we have used the 5-choice serial reaction-time apparatus to evaluate effects of LSD on nose-poking behavior. LSD stimulated retrograde walking and nose-poking behaviors in WT animals from both β Arr strains as well as in the β Arr1-KO mice. However, LSD promoted neither response in β Arr2-KO animals. Nevertheless, in the other genotypes MDL restored retrograde walking and nose-poking to the levels of vehicle controls. Hence, this 5-HT_{2A}R antagonist normalized these LSD-stimulated responses.

LSD-induced states share many similarities with the early acute phases of psychosis [3]. PPI is abnormal in individuals diagnosed with schizophrenia [61] and LSD disrupts PPI in rats [43,46,53]. In β Arr1 mice LSD impaired PPI in both genotypes without affecting startle or null

activities. Both 0.1 and 0.5 mg/kg MDL restored LSD-disrupted PPI, but only in the WT mice; an effect consistent with the action of the 5-HT_{2A}R antagonist MDL11,939 in rats [53]. By comparison, β Arr1-KO animals were unresponsive to MDL. Since LSD activates human dopamine D₂ receptors [9,62], we used haloperidol as a D₂ antagonist. At a dose of 0.1 mg/kg, haloperidol restored the LSD-disrupted PPI in β Arr1-KO mice. Parenthetically, both 0.1 and 0.2 mg/kg haloperidol failed to rescue PPI in rats given 0.1 mg/kg LSD (s.c.) [43]; the possible reasons for this discrepancy in mice versus rats are unclear. When β Arr2 mice were tested, LSD was observed to impair PPI only in WT mice. Notably, β Arr2-KO mice were completely unresponsive to this psychedelic. As with β Arr1 WT animals, 0.5 mg/kg MDL normalized the LSD-disrupted PPI in β Arr2 WT animals. Thus, the LSD effects on PPI in the β Arr mice are complex, with restoration of PPI with MDL in both strains of WT mice, normalization of PPI with haloperidol in β Arr1-KO animals, and without any discernable effect in the β Arr2-KO subjects.

Aside from behavioral studies, LSD effects on body temperatures were evaluated. Parenthetically, LSD serves as an agonist at both 5-HT_{1A}Rs and 5-HT_{2A}Rs [10-11], and stimulation of either receptor can modify body temperatures in rats [63]. LSD is reported to increase or have no effects on temperatures in mice and to increase or decrease temperatures in rats [54]. We found LSD to alter body temperatures in a biphasic fashion in β Arr1 and β Arr2 WT mice, in a monophasic manner in β Arr1-KO animals, and to be without effect in β Arr2-KO subjects. Both MDL and WAY normalized these LSD effects in both WT strains and in β Arr1-KO mice. Neither antagonist appeared to be more efficacious than the other. This finding should not be surprising since functional interactions have been described between the 5-HT_{1A}Rs and 5-HT_{2A}Rs in regulating body temperatures [61]. Although we did not test it, 5-HT_{2C}R antagonists are known to participate in this process. Nevertheless, it appears that antagonism of 5-HT_{1A}Rs and 5-HT_{2A}Rs is sufficient to normalize the LSD-induced changes in body temperatures in β Arr1 and β Arr2 mice.

LSD and other psychedelics are well-known for their hallucinogenic actions [1] and these responses have been attributed to 5-HT_{2A}R agonism [12]. We observed LSD to stimulate motor activity, head twitches, grooming, retrograde walking, and nose-poking in the β Arr1 and β Arr2 WT mice and in β Arr1-KO animals. LSD also disrupted PPI and produced diverse changes in body temperatures in these same mice. The LSD-elicited responses in β Arr2-KO mice were either significantly attenuated or completely absent. In conditions where LSD produced changes in behavior, these alterations were blocked with the 5-HT_{2A}R antagonist MDL. While these results suggest that the 5-HT_{2A}R is an essential component for all these responses, it should be recalled that LSD exerts a plethora of actions at many GPCRs [9-11] and, aside from head twitch, other responses are inconsistently affected by hallucinogens [18]. Hence, it is likely that LSD's effects on the 5-HT_{2A}R are involved in a cascade of many GPCR-signaling events mediating these varied responses.

Like other GPCRs, agonist actions at the 5-HT_{2A}R can lead to G protein-dependent and –independent signaling, the latter involves β Arr [23-25]. While both β Arr1 and β Arr2 are ubiquitously expressed in adult rodent brain, expression of β Arr2 mRNA is much higher than that for β Arr1 – except in selected brain areas [64]. Thus, it may not be surprising that the LSD-elicited responses were less disturbed in the β Arr2-KO than in the β Arr1-KO mice, because the latter is still retained intact β Arr2-mediated signaling. In this regard, it was especially intriguing that LSD-induced head twitch responses were much more robust in the β Arr1 and β Arr2 WT mice and in the β Arr1-KO animals, than in the β Arr2 mutants. Our results with LSD indicate that β Arr2 may be essential for the expression of hallucinogenic-like actions. Hence, it may be possible to develop novel functionally-selective ligands that preferentially signal through G protein at the 5-HT_{2A}R and avoid the hallucinogenic effects modulated through β Arr.

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The authors have no conflicts. BLR is listed as an inventor on a submitted patent related to 5-HT2AR agonists. In the past 12 months, Dr Roth has received compensation as a consultant from Third Rock Ventures, Google Ventures, Kalliope, Inc, Arena Pharmaceuticals, and Otsuka Pharmaceuticals. In the past 12 months the University of North Carolina at Chapel Hill has licensed technology from Dr. Roth's lab to Astellis Pharmaceuticals and Exscientia. The work was supported by NIDA R37-DA045657 and DARPA [Grant No. DARPA-5822 (HR001119S0092-FP-FP-002)]. Preliminary experiments from Drs. WCW and BLR were supported by U19-MH082441. The views, opinions, and/or findings contained in this material are those of the authors and should not be interpreted as representing the official views, policies, or endorsement of the Department of Defense or the U.S. Government.

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AUTHOR CONTRIBUTIONS

Mr. Vineet Nadkarni conducted initial open field studies and some ethological experiments with the β Arr2 mice and helped to statistically analyze these preliminary data. Mr. Christopher Means conducted the behavioral experiments with the β Arr1 and β Arr2 mice and organized the data. Dr. Ramona M. Rodriguiz oversaw the experiments, conducted the temperature regulation studies, statistically analyzed the data, and graphed the results. Dr. Yi-Ting Chiu conducted the

radioligand binding and immunohistochemical investigations with the β Arr1 and β Arr2 mice. Drs. Wetsel and Roth conceived the experiments, proposed the experimental designs, and wrote the manuscript.

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FIGURE LEGENDS

Fig. 1 Effects of LSD and MDL100907 on cumulative motor activities in the β -arrestin 1 and β -arrestin 2 mice.

Mice were administered the vehicle or different doses of MDL100907 (MDL) and placed into the open field for 30 min. They were removed injected with the vehicle or 0.3 mg/kg LSD and immediately returned to the test arena for 90 min. The cumulative baseline results (0-30 min) are depicted in Supplementary Tables S3 and S5. The statistics for the cumulative LSD-stimulated (31-120 min) motor activities are presented in in Supplementary Tables S4 and S6. **a** Locomotor activities in WT and β Arr1-KO mice. **b** Locomotor activities in WT and β Arr2-KO subjects. **c** Rearing activities in β Arr1 animals. **d** Rearing activities in β Arr2 subjects. **e** Stereotypical activities in β Arr1 mice. **f** Stereotypical activities in β Arr2 animals. N = 8-17 mice/group. * p <0.05, WT vs. KO; # p <0.05, vs. LSD overall (β Arr1 all motor activities; β Arr2 for stereotypy) or within genotype (β Arr2 for locomotion and rearing); † p <0.05, vs. 0.05 mg/kg LSD + LSD.

Fig. 2 Effects of LSD and MDL100907 on behavioral responses in the β -arrestin 1 and β -arrestin 2 mice.

A description of the experimental design is presented in the legend for Figure 1. The head twitch, grooming, and retrograde walking results represent the first 30 min after injection of LSD. Nose poking was examined in a 5-choice serial reaction time apparatus (no rewards) with a similar time-course for vehicle and MDL injections (as in the open field) followed by administration of the vehicle and LSD. The statistics for the β Arr1 and β Arr2 responses can be found in Supplementary Tables S7 and S8, respectively. **a** Head twitches in WT and β Arr1-KO mice. **b** Head twitches in WT and β Arr2-KO animals. **c** Duration of grooming in β Arr1 animals. **d** Duration of grooming in β Arr2 subjects. **e** Incidences of retrograde walking in β Arr1 subjects. **f** Incidences of retrograde walking in β Arr2 mice. **g** Nose poking in WT and β Arr1-KO mice. **h** Nose poking in WT and β Arr2-KO animals. N = 8-17 mice/group for head twitch, grooming, and retrograde walking; N =

9-15 mice/group for nose-poking. * $p < 0.05$, WT vs. KO; # $p < 0.05$, vs. LSD overall (β Arr1) or within genotype (β Arr2); † $p < 0.05$, vs. 0.05 mg/kg MDL + LSD; ‡ $p < 0.05$, vs. 0.1 mg/kg MDL + LSD overall (β Arr1) or within genotype (β Arr2).

Fig. 3 Effects of LSD, MDL100907, and haloperidol on prepulse inhibition in β -arrestin 1 and β -arrestin 2 mice.

Mice were injected with MDL100907, haloperidol, or the vehicle and administered subsequently the vehicle or LSD prior to testing PPI. The statistics for the β Arr1 and β Arr2 responses are located in Supplementary Tables S9-S11. **a** PPI in WT and β Arr1-KO mice treated with MDL or LSD. **b** PPI in WT and β Arr1-KO mice treated with haloperidol or LSD. **c** PPI in WT and β Arr2-KO mice treated with MDL or LSD. N = 8-12 mice/group. * $p < 0.05$, WT vs. KO; # $p < 0.05$, vs. LSD overall (β Arr1 with haloperidol) within genotype (β Arr1 and β Arr2 with MDL); † $p < 0.05$, vs. 0.05 mg/kg MDL + LSD within genotype; ‡ $p < 0.05$, vs. 0.1 mg/kg MDL + LSD within genotype; ^ $p < 0.05$, vs. 0.5 MDL + LSD within genotype.

Fig. 4 Effects of LSD, MDL100907, and WAY 100635 on body temperature in β -arrestin 1 mice.

Mice were injected with the vehicle, MDL, or WAY and 15 min later they received the vehicle or LSD and were examined. The statistics for the β Arr1 responses are found in Supplementary Table S12. **a** Body temperatures in WT mice treated with the vehicle, 0.5 mg/kg MDL, or LSD. **b** Temperatures in β Arr1-KO animals given similar treatments. **c** Body temperatures in WT mice given the vehicle, LSD, or 0.25 or 0.5 mg/kg MDL plus LSD. **d** Temperatures in similarly treated β Arr1-KO animals. **e** Body temperatures in WT mice administered the vehicle, LSD, or 0.25 mg/kg WAY. **f** Temperatures in β Arr1-KO animals that received the same treatments. **g** Body temperatures in WT mice that were given the vehicle, LSD, or 0.1 or 0.25 mg/kg WAY with LSD. **h** Temperatures in β Arr1-KO animals subjected to the same treatments. N = 9-12 mice/group.

^A $p < 0.05$, WT vs. KO for vehicle; ^B $p < 0.05$, WT vs. KO for low dose MDL or WAY; ^C $p < 0.05$, WT vs. KO for LSD; ^D $p < 0.05$, WT vs. KO for low dose MDL or WAY with LSD; ^E $p < 0.05$, WT vs. KO for high dose MDL or WAY with LSD; ^F $p < 0.05$, vehicle vs. MDL or WAY alone within genotype; ^G $p < 0.05$, vehicle vs. LSD within genotype; ^H $p < 0.05$, vehicle vs. low dose MDL or WAY with LSD within genotype; ^I $p < 0.05$, vehicle vs. high dose MDL or WAY with LSD within genotype; ^J $p < 0.05$, MDL or WAY alone vs. LSD within genotype; ^K $p < 0.05$, LSD vs. low dose MDL or WAY with LSD within genotype; ^L $p < 0.05$, LSD vs. high dose MDL or WAY with LSD within genotype; ^M $p < 0.05$, low dose MDL or WAY with LSD vs. high dose MDL or WAY with LSD within genotype.

Fig. 5 Effects of LSD, MDL100907, and WAY 100635 on body temperature in β -arrestin 2 mice.

The statistics for the β Arr2 responses are located in Supplementary Table S13. **a** Body temperatures in WT mice treated with the vehicle, 0.5 mg/kg MDL, or LSD. **b** Temperatures in β Arr2-KO animals given similar treatments. **c** Body temperatures in WT mice given the vehicle, LSD, or 0.25 or 0.5 mg/kg MDL plus LSD. **d** Temperatures in similarly treated and β Arr2-KO animals. **e** Body temperatures in WT mice administered the vehicle, LSD, or 0.25 mg/kg WAY. **f** Temperatures in β Arr2-KO animals that received the same treatments. **g** Body temperatures in WT mice that were given the vehicle, LSD, or 0.1 or 0.25 mg/kg WAY with LSD. **h** Temperatures in β Arr2-KO animals subjected to the same treatments. N = 8-14 mice/group. ^B $p < 0.05$, WT vs. KO for low dose MDL or WAY; ^C $p < 0.05$, WT vs. KO for LSD; ^D $p < 0.05$, WT vs. KO for low dose MDL or WAY with LSD; ^E $p < 0.05$, WT vs. KO for high dose MDL or WAY with LSD; ^F $p < 0.05$, vehicle vs. MDL or WAY alone within genotype; ^G $p < 0.05$, vehicle vs. LSD within genotype; ^H $p < 0.05$, vehicle vs. low dose MDL or WAY with LSD within genotype; ^I $p < 0.05$, vehicle vs. high dose MDL or WAY with LSD within genotype; ^J $p < 0.05$, MDL or WAY alone vs. LSD within genotype; ^K $p < 0.05$, LSD vs. low dose MDL or WAY with LSD within genotype; ^L $p < 0.05$, LSD vs. high dose MDL or

WAY with LSD within genotype; ^M $p < 0.05$, low dose MDL or WAY with LSD vs. high dose MDL or
WAY with LSD within genotype.