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Research report

Tolerance to LSD and DOB induced shaking behaviour: Differential adaptations of frontocortical 5-HT_{2A} and glutamate receptor binding sites

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HIGHLIGHTS

- LSD and DOB induce a ketanserin sensitive increase in shaking behaviour.
- LSD and DOB induced shaking behaviour is undermined by tolerance development.
- Tolerance to DOB correlates with reduced frontocortical 5-HT_{2A} binding sites.
- Tolerance to LSD does not correlate with frontocortical 5-HT_{2A} binding sites.
- Tolerance to LSD correlates with reduced frontocortical glutamate binding sites.

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ABSTRACT

Serotonergic hallucinogens, such as lysergic acid diethylamide (LSD) and dimethoxy-bromoamphetamine (DOB), provoke stereotype-like shaking behaviour in rodents, which is hypothesised to engage frontocortical glutamate receptor activation secondary to serotonin2A (5-HT_{2A}) related glutamate release. Challenging this hypothesis, we here investigate whether tolerance to LSD and DOB correlates with frontocortical adaptations of 5-HT_{2A} and/or overall-glutamate binding sites. LSD and DOB (0.025 and 0.25 mg/kg, i.p.) induce a ketanserin-sensitive (0.5 mg/kg, i.p., 30-min pretreatment) increase in shaking behaviour (including head twitches and wet dog shakes), which with repeated application (7× in 4 ds) is undermined by tolerance. Tolerance to DOB, as indexed by DOB-sensitive [^{3}H]spiroperidol and DOB induced [^{35}S]GTP-gamma-S binding, is accompanied by a frontocortical decrease in 5-HT_{2A} binding sites and 5-HT_2 signalling, respectively; glutamate-sensitive [^{3}H]glutamate binding sites, in contrast, remain unchanged. As to LSD, 5-HT_2 signalling and 5-HT_{2A} binding, respectively, are not or only marginally affected, yet [^{3}H]glutamate binding is significantly decreased. Correlation analysis interrelates tolerance to DOB to the reduced 5-HT_{2A} (r = .80) as well as the unchanged [^{3}H]glutamate binding sites (r = .84); tolerance to LSD, as opposed, shares variance with the reduction in [^{3}H]glutamate binding sites only (r = .86). Given that DOB and LSD both induce tolerance, one correlating with 5-HT_{2A}, the other with glutamate receptor adaptations, it might be inferred that tolerance can arise at either level. That is, if a hallucinogen (like LSD in our study) fails to induce 5-HT_{2A} (down-)regulation, glutamate receptors (activated postsynaptic to 5-HT_{2A} related glutamate release) might instead adapt and thus prevent further overstimulation of the cortex.

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1. Introduction

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Serotonergic hallucinogens, such as lysergic acid diethylamide (LSD) or dimethoxy-bromoamphetamine (DOB) share structural elements with serotonin (5-hydroxytryptamine [5-HT]) [1,2], a neurotransmitter involved in mood (repetitive) gross motor output, vascular tonus, and thermoregulation. Although their structural resemblance to 5-HT renders most hallucinogens prone to bind to diverse 5-HT receptors [3], activation of the 5-HT_{2A} subtype is considered the key mechanism for their human *psychedelic* effect to occur [4,5]. In animals, hallucinogens evoke a variety of stereotype-like motor outputs, including head twitches, wet dog shakes, ear scratches, limb flicking, or backward walking [6]. As *head twitches* in mice and *wet dog shakes* in rats have a very similar pharmacology, with the latter most probably reflecting a more generalised version of the former [7,8], we consider both phenomena analogous, and subsume them under the term *shaking behaviour* [compare 9, 10]. Shaking behaviour is one of the most widely accepted and well-scrutinised model of central hallucinogenic activity [11,12]. It mirrors the human psychedelic effect in its three most important characteristics: It is primarily related to the activation of 5-HT_{2A} receptors [13,14]; it is induced by representatives of the two main groups of serotonergic hallucinogens, the indole- and phenylalkylamines [15–17]; and it rapidly develops tolerance [18,19]. Given its significance for the basic understanding of the human psychedelic effect, the neurophysiological correlates of the hallucinogen induced shaking behaviour are of high interest. In parallel to human research [20,21], and for the following main reasons, the current literature largely focuses on the frontal cortex as a primary correlate: (1) The (frontal) cortex is the region of the brain, where 5-HT_{2A} receptors are most abundantly expressed, notably on cortical output cells (i.e. layer V pyramidal cells) [22,23]. (2) When locally applied into the frontal cortex, hallucinogens evoke shaking behaviour sensitive to systemic 5-HT_{2A} antagonist application [24]. (3) In 5-HT_{2A} knock-out mice, shaking behaviour can be rescued with the expression of 5-HT_{2A} receptors selectively restored to the cortex [16]. Based on the electrophysiological properties of the frontocortical 5-HT_{2A} receptors, shaking behaviour most probably engages a glutamatergic mechanism [25]. In slice preparations of frontocortical layer V pyramidal cells, 5-HT_{2A} receptors increase the frequency of spontaneous excitatory postsynaptic currents/potentials (EPSCs/EPSPs) [26]. As this increase can be counteracted by AMPA receptor blockage or by metabotropic glutamate receptor type $_{2/3}$ ($mGlu_{2/3}$) activation, it is assumed to be accounted for by a 5-HT_{2A} related glutamate release onto AMPA receptors [27,28]; $mGlu_{2/3}$ receptors, in this model, interfere presynaptically with the glutamate release [27] and/or (postsynaptically) with the 5-HT_{2A} signalling [29]. Intriguingly, shaking behaviour has likewise been shown to be sensitive to pharmacological AMPA and $mGlu_{2/3}$ receptor manipulations. Similar to the EPSCs/EPSPs in the pyramidal cells, it can be inhibited by AMPA antagonists [28,30] and $mGlu_{2/3}$ agonists [29,31], but enhanced by $mGlu_{2/3}$ antagonists [32].

In the current work, we address the tolerance phenomenon characteristic for repeated hallucinogen application [for a review see 5, 33, 34]. Tolerance to hallucinogen induced shaking behaviour has often been associated with a downregulation of frontocortical 5-$HT_{2(A)}$ receptors [35–39]. However, mathematical correlations for this receptor-behaviour association, apart from one study on antagonist related upregulation of both parameters [40], have not been presented. Also, concomittant adaptations of the (downstream) glutamatergic system are largely obscure. Thus, assuming – as indicated by the above listed evidence – that shaking behaviour primarily relates to $mGlu_{2/3}$-sensitive glutamate release downstream of frontocortical 5-HT_{2A} activity, we here investigate whether behavioural tolerance to LSD and DOB co-occurs with adaptations of 5-HT_2 and $mGlu_{2/3}$ signalling, or of 5-HT_{2A} and/or overall-glutamate binding sites of the frontal cortex. To characterise the relationship between neurochemistry and behaviour more closely, we in addition probe the results by correlation analysis.

2. Methods and materials

2.1. Animals and housing

For all experiments, male *Sprague Dawley* rats (MolTac: SD, Taconic Denmark) (av. 10 weeks, av. 380 g) were used. They were housed in groups of five animals per cage, and held under controlled laboratory conditions (temperature 20 ± 2 °C, air humidity 55–60%, light/dark cycle 12:12 [light on at 6 a.m.]) with standard food pellets (ssniff SM/R/NH, 10 mm; ssniff Spezialdiäten GmbH, Soest, Germany) and tap water ad libitum. All experiments performed comply with the regulations of the *National Act on the Use of Experimental Animals* (Germany), as approved by the *Tierschutzkommission* Sachsen-Anhalt.

2.2. Behavioural experiments

2.2.1. Treatment

LSD tartrate, DOB hydrochloride (both from THC Pharm, Frankfurt am Main, Germany), and ketanserin tartrate (Biozol, Eching, Germany) were dissolved in isotonic saline, and applied into the peritoneum (i.p.) (10 ml/kg). Adequate dosing was determined by dose–response curves (LSD and DOB), or extrapolated from literature (ketanserin: 0.5 or 1.0 mg/kg, 30 min before agonist) [e.g. 17]. For *tolerance* experiments, both hallucinogens were applied seven times over four consecutive days. Every morning before observation (at ∼10 a.m.), a low dose was given (0.025 mg/kg LSD vs. 0.25 mg/kg DOB); in the evening of days 1–3 (at ∼10 p.m.), an additional high dose (0.25 mg/kg LSD vs. 0.75 mg/kg DOB) followed. Control animals were treated alike but received pure saline.

To estimate whether psychological habituation to the experimental setting might contribute to tolerance development, a fourth group of rats experienced a four days habituation phase before the above mentioned LSD treatment began. In this phase, the rats received daily saline injections, were put into the experimental cages, and observed as if they were in the actual LSD experiment.

2.2.2. Shaking behaviour

Shaking behaviour was defined as brisk rotational movement of the head (with or without propagation to shoulders and trunk [wet dog shakes vs. head twitches]) around the long axis of the rat's body. For 30 min, starting right after agonist application, the occurrence of shaking behaviour was continuously registered by a trained observer, and validated via digital camera recordings. For dose–response curve experiments, rats were observed individually, i.e. one animal per cage (acryl cylinder: 19 cm Ø, 23 cm H). For antagonist and tolerance experiments, rats were observed in larger Plexiglas cages (36 cm L × 38 cm H × 20 cm W), with groups of 2–3 animals per cage. To avoid grooming related shaking behaviour, no sawdust bedding was provided. For general habituation, all animals were repeatedly exposed to the experimenter, and put into the room of experimentation a few days beforehand.

2.3. Neurochemical experiments

2.3.1. 5-HT_{2A} and glutamate receptor binding

Twenty hours after the last treatment, rats were decapitated and frontal cortices were dissected. With slight modifications, receptor binding assays were performed as earlier described [41,42]. Tissue was homogenised, pelleted by centrifugation (10 min,

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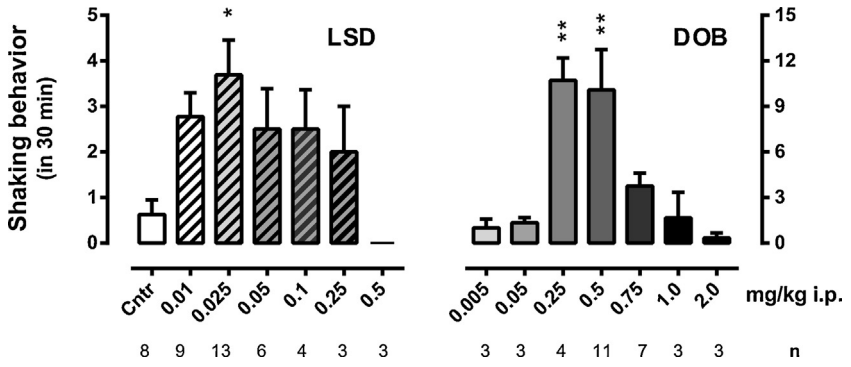

Fig. 1. Dose–response curves for LSD (left) and DOB (right) induced shaking behaviour in SD rats (as observed separated from one another [one animal per cage]). Note that LSD is more potent than DOB but less efficient. Mean + SEM. Comparison to control (Cntr), * $p < .05$, ** $p < .01$.

50,000 × *g*, 4 °C), and resuspended in assay buffer (5-HT_{2A}: 50 mM Tris–HCl with 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 8.0; glutamate: 50 mM Tris–HCI with $CaCl_2$, pH 7.4). Aliquots containing 175–200 μg protein were incubated at 37 °C with either [^{3}H]spiroperidol (0.25 nM, 30 min) (specific activity: 800 GBq/mM [Perkin-Elmer, MA, USA]), or [^{3}H]glutamate (50 nM, 40 min) (specific activity: 1.43 Tbq/mM [Perkin-Elmer, Massachusetts, USA]). D-Butaclamol (50 nM) was used as a mask to prevent [^{3}H]spiroperidol binding to D_2 receptors. The membrane fraction was collected on GF/A glass-fibre filters, washed with buffer, and a taken for liquid scintillation counting in a toluene-containing solvent. Specific binding was calculated by subtracting non-specific binding (radioligand in presence of different concentrations [1 nM to 100 μM range] of unlabelled DOB [5-HT_{2A}] and glutamate, respectively) from total binding (obtained with radioligand alone), and expressed as relative potencies (fold change over control).

2.3.2. 5-HT_2 and $mGlu_{2/3}$ receptor induced [^{35}S]GTP-gamma-S binding

For measurement of 5-HT_2 and $mGlu_{2/3}$ coupling to G-proteins [modified from 41, 43], crude synaptic membrane pellets were resuspended in assay buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, pH 7.4). Aliquots containing 15–20 μg protein were incubated with 3 μM GDP and 0.05 nM [^{35}S]GTP-gamma-S (specific activity: 46.3 TBq/mM [Perkin-Elmer, MA, USA]) in the presence and absence of DOB or LY354740 (10 μM) (THC Pharm, Frankfurt am Main, resp. Biozol, Eching, Germany). Incubation was terminated by rapid filtration, filters were rinsed in washing buffer (50 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM EGTA, pH 7.4), and taken for liquid scintillation counting of bound radioactivity. Total [^{35}S]GTP-gamma-S binding was corrected for unspecific binding (in presence of 10 μM unlabelled GTP-gamma-S), and expressed as Emax of agonist stimulation (fold change over control).

All determinations were performed at least in duplicate.

2.4. Statistical analysis

A two-factor ANOVA with repeated measures on one factor (mixed model) was conducted to assess main effects and interaction of *day* (the repeated measure factor) and *treatment* in tolerance development, and followed by pairwise contrast analysis. The data from the dose–response, individual vs. group, antagonist, and neurochemical experiments were analysed using nonparametric Kruskal–Wallis test with Dunn's multiple post hoc comparisons, or Mann–Whitney *U*-testing (as a priori planned). Relationships between behavioural and binding parameters were probed by product-moment correlations. Calculations were carried out by *SPSS* and *GraphPad Prism* software. Statistical significance was assumed if the null hypothesis could be rejected at .05 probability level.

3. Results

3.1. Behavioural experiments

3.1.1. Dose–response curves for LSD and DOB induced shaking behaviour

In SD rats (observed separated from each other), both serotonergic hallucinogens, LSD (0.025 mg/kg, i.p.) and DOB (0.25 and 0.5 mg/kg, i.p.) induce significant shaking behaviour (Fig. 1). LSD is about 10 times more potent than DOB, however, its maximal effect is much lower (mean ± SEM in 30 min: 3.69 ± 0.76 [0.025 LSD]; 10.71 ± 1.47 [0.25 DOB] and 10.09 ± 2.64 [0.5 DOB]) (Dunn's multiple comparison, $p < .05$, .01 and .01, respectively) (Fig. 1). Dose–response curves appear inversely *U*-shaped; at higher doses, flat body posture and/or backward walking increasingly displace shaking behaviour.

3.1.2. Effect of littermate presence on LSD and DOB induced shaking behaviour

Given that rats hardly respond to LSD when observed separated from their littermates (Fig. 1), all subsequent observations were performed with groups of 2–3 animals per cage. As shown in Fig. 2A, shaking behaviour evoked by LSD (0.025 mg/kg, i.p.) and DOB (0.25 mg/kg, i.p.) significantly increases when familiar

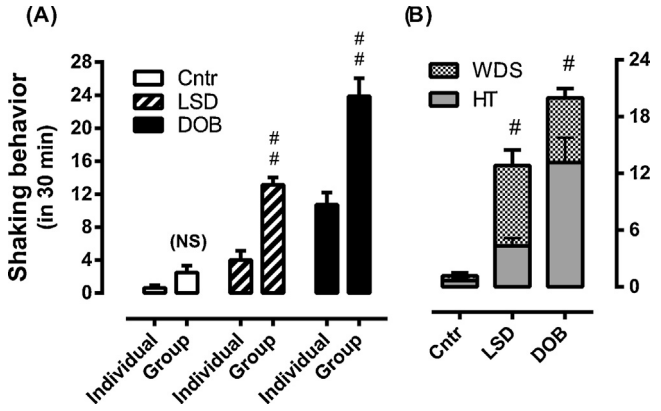

Fig. 2. (A) LSD (0.025 mg/kg, i.p.) and DOB (0.25 mg/kg, i.p.) induced shaking behaviour, as observed individually (single rat per cage) or in groups (2–3 rats per cage). Note that SD rats more reliably respond to serotonergic hallucinogens when littermates are around ($n = 7–8$). (B) Composition of LSD and DOB induced shaking behaviour (as observed in groups). Note that wet dog shakes (WDS) prevail for LSD, and head twitches (HT) for DOB ($n = 6–8$). Mean + SEM. Comparison to *individual* condition, ## $p < .01$, (NS) $p < .10$ (trend) (A); comparison WDS vs. HT, # $p < .05$ (B).

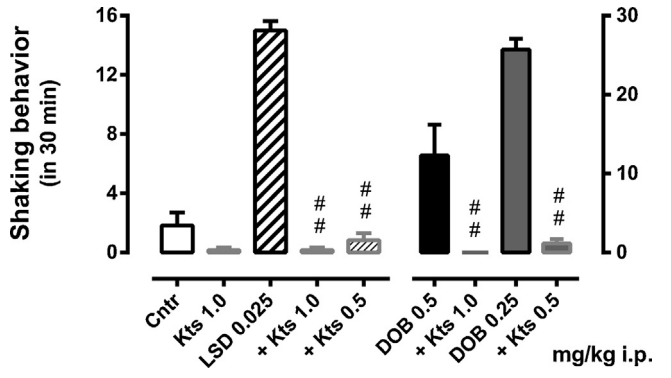

Fig. 3. Effect of the 5-HT_{2A} antagonist *ketanserin* (Kts) (0.5 or 1.0 mg/kg, i.p., 30-min pretreatment) on spontaneous, LSD (0.025 mg/kg, i.p.) (left) and DOB (0.25 or 0.5 mg/kg, i.p.) (right) induced shaking behaviour in SD rats (as observed in groups of 2–3 animals per cage) (n = 6–7). Note that ketanserin (+Kts) completely blocks the shaking behaviour by both hallucinogens. Mean + SEM. Comparison to agonist (without Kts pretreatment), ## p < .01.

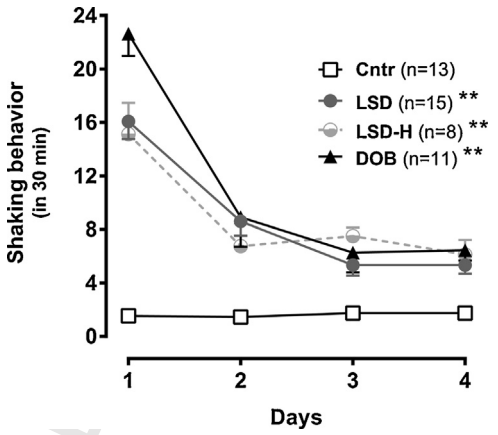

Fig. 4. Tolerance to LSD (grey circle) and DOB (black triangle) induced shaking behaviour in SD rats (as observed in groups of 2–3 animals per cage), with a total of seven applications over four consecutive days. Note that tolerance to LSD is hardly affected by a four days habituation to daily (saline) injections and observations (LSD-H) (half-filled light-grey circle, dotted line). Mean ± SEM. Repeated measures ANOVA, contrast to control (Cntr) (unfilled square), ** p < .01.

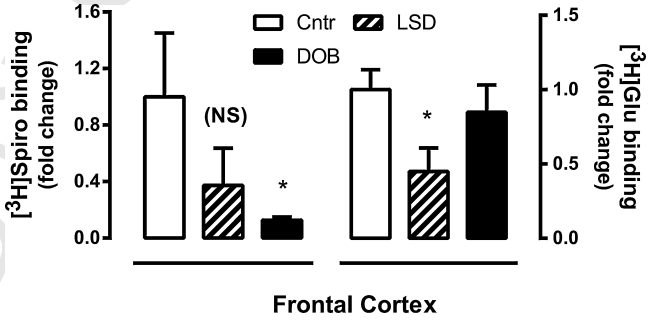

Fig. 5. Effect of repeated LSD and DOB treatment (7× in 4 ds, i.p.) on DOB-sensitive [^{3}H]spiroperidol (left) and glutamate-sensitive [^{3}H]glutamate binding (right) to crude membranes of the frontal cortex of SD rats (n = 5–6). Note that LSD reduces glutamate binding significantly, but 5-HT_{2A} binding as a trend only; for DOB treated animals, 5-HT_{2A} but not glutamate binding is significantly reduced (specific binding, fold change over control). Mean + SEM. Comparison to control (Cntr), * p < .05, (NS) p < .10 (trend).

littermates are present (group), as compared to rats observed separated from each other (individual) (u = 0.0, p < .01 [LSD]; u = 2.5, p < .01 [DOB]). As a trend, the same holds true for the control animals (u = 14, p = .057).

3.1.3. Composition of LSD and DOB induced shaking behaviour and effect of ketanserin

Shaking behaviour comprises head twitches (HT) *and* wet dog shakes (WDS). LSD induces more wet dog shakes than head twitches (u = 5.5, p = .026), for DOB it is reverse (u = 9.5, p = .028) (Fig. 2B). Ketanserin (Kts) (0.5 or 1.0 mg/kg, i.p., 30 min before agonist), a selective 5-HT_{2A} antagonist, blocks the overall shaking behaviour of both hallucinogens (u = 0.0, p < .01 [0.025 LSD ± 1.0 Kts], [0.025 LSD ± 0.5 Kts], and [0.25 DOB ± 0.5 Kts]; u = 3.5, p < .01 [0.5 DOB ± 1.0 Kts]) (Fig. 3).

3.1.4. Effect of repeated LSD and DOB application on shaking behaviour

The omnibus F-test revealed significant main effects for both factors, day ($F_{[2.54,\ 109.39]}$ = 77.99, p < .01) and treatment ($F_{[3,43]}$ = 31.38, p < .01), and a significant day × treatment interaction ($F_{[7.63,\ 109.39]}$ = 13.45, p < .01). Results were further probed by a priori specified contrasts for groups of interest. As depicted in Fig. 4, the LSD and DOB induced shaking behaviour significantly decreases over time (from 16.07 ± 1.31 to 5.33 ± 0.64 [LSD], and 22.64 ± 1.66 to 6.45 ± 0.76 [DOB] [mean ± SEM]), whereas the control behaviour remains constant ($F_{[1,26]}$ = 74.25, p < .01 [control vs. LSD]; $F_{[1,22]}$ = 63.92, p < .01 [DOB vs. control]). The decrease in responsiveness to LSD is not significantly altered by a four days habituation to injection and observation (from 15.13 ± 2.34 to 6.13 ± 1.08 [LSD-H]) ($F_{[1,21]}$ = 1.59, p = .22 [LSD vs. LSD-H]) ($F_{[1,19]}$ = 21.94, p < .01 [control vs. LSD-H]).

3.2. Neurochemical experiments

3.2.1. Effect of repeated LSD and DOB application on frontocortical 5-HT_{2A} and glutamate receptor binding sites

As shown in Fig. 5, repeated DOB treatment significantly reduces DOB-sensitive [^{3}H]spiroperidol binding to membranes of the frontal cortex (u = 5.5, p = .02), with glutamate-sensitive [^{3}H]glutamate binding being unaffected (u = 12, p = .19). In contrast, repeated LSD treatment significantly reduces frontocortical [^{3}H]glutamate binding (u = 4, p = .02), with [^{3}H]spiroperidol binding being decreased as a trend only (u = 9, p = .08).

3.2.2. Effect of repeated LSD and DOB application on frontocortical 5-HT_2 and $mGlu_{2/3}$ receptor signalling

After repeated DOB, but not LSD treatment, there is a significant decrease in DOB induced [^{35}S]GTP-gamma-S binding to frontocortical membranes (u = 4, p = .02 [DOB]; u = 10, p = .34 [LSD]) (Fig. 6). The LY354740 induced [^{35}S]GTP-gamma-S binding, on the other

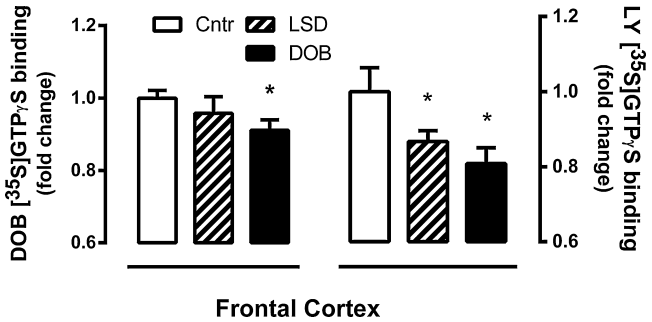

Fig. 6. Effect of repeated LSD and DOB treatment (7× in 4 d, i.p.) on DOB (left) and LY354740 (right) induced [^{35}S]GTP-gamma-S binding to crude membranes of the frontal cortex of SD rats (n = 4–6). Note that signalling of $mGlu_{2/3}$ receptors is reduced by both hallucinogens, 5-HT_2 signalling only by DOB (specific binding, fold change over control). Mean + SEM. Comparison to control (Cntr), * p < .05.

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hand, is significantly reduced following both, DOB ($u = 3$, $p = .01$) and LSD ($u = 5$, $p = .04$) (Fig. 6).

3.3. Relationship between the behavioural and neurochemical adaptations induced by repeated LSD and DOB application

For the DOB tolerant animals, both 5-HT_{2A} and [^{3}H]glutamate binding highly correlates with the number of shaking behaviour shown on the last day of repeated DOB treatment ($r = .80$, $p = .049$; $r = .84$, $p = .035$). For the LSD tolerant animals, on the other hand, such a correlation can only be found for [^{3}H]glutamate binding ($r = .86$, $p = .03$ [glutamate]; $r = .41$, $p = .24$ [5-HT_{2A}]). LY354740 induced [^{35}S]GTP-gamma-S binding and shaking behaviour negatively correlate for rats tolerant to DOB ($r = -.98$, $p = .001$) but not for rats tolerant to LSD ($r = .30$, $p = .27$). DOB induced [^{35}S]GTP-gamma-S binding, as opposed, does not share significant variance with tolerance to either hallucinogen ($r = -.25$, $p = .37$ [DOB]; $r = .55$, $p = .22$ [LSD]).

4. Discussion

Referring to the idea that hallucinogen induced shaking behaviour engages frontocortical 5-HT_{2A}-glutamate interaction, we here investigate whether tolerance to LSD and DOB correlates with adaptations of the local 5-HT_{2A} and/or overall-glutamate binding sites.

In line with published results for the SD strain [44,45], we show that LSD and DOB significantly increase shaking behaviour in doses around 0.025 and 0.25 mg/kg i.p., respectively (Fig. 1). LSD is about 10 times more potent than DOB, which matches their 5-HT_{2A} affinities [46] and human potencies [5]. That the frequency of the LSD induced shaking behaviour, as opposed, is much lower than the one seen with DOB, might be due to its lower intrinsic activity at 5-HT_{2A} [47] and/or counterregulation via 5-HT_{1A} [48,49]. As the individual caging seemed to intimidate the rats, often they were tense and immobile during observation, all further experiments were performed with group, instead of individual, caging. In the presence of familiar littermates, shaking behaviour – as unmasked from tension – more reliably occurs (Fig. 2A). Differentiating shaking behaviour into its components, we show that LSD and DOB induce head twitches *and* wet dog shakes (Fig. 2B). That DOB prefers the former and LSD the latter might reflect functional selectivity at 5-HT_{2A} [50] and/or modulations by non5-HT_{2A} receptors [3]. Given that the 5-HT_{2A} antagonist ketanserin blocks either component (Fig. 3), however, subsuming both as *shaking behaviour* seems justified.

In humans, tolerance to the psychedelic effect of LSD – given once a day – occurs in as little as three days [33,34]. Although described anecdotally only, similar might hold true for DOB, too [51]. In animals, tolerance to hallucinogens inconsistently manifests varying across different behaviours, species, and regimens [for an overview see 33, 34]. As to shaking behaviour in SD rats, a once-per-day regimen is *not* sufficient for tolerance to manifest (data not shown [see 34]) [compare for DOI 52]. Only with the application of a second (high) dose each day, shaking behaviour significantly decreases (Fig. 4). In literature, tolerance to LSD induced shaking behaviour has been described for cats and macaques and–as challenged by DOI or endogenous serotonin–for rabbits [34]. Tolerance to DOB induced shaking behaviour, although not specifically addressed in a paper before, partially occurred in the context of a multiple-weeks-application study on drug discrimination [53]. As to LSD induced (shaking) behaviour, pharmacokinetic adaptations seem not contribute to tolerance development [54,55]; behavioural habituation to the experimental setting, as indicated by our data, might also play a rather subordinate role (Fig. 4, LSD vs. LSD-H). Assuming tolerance to LSD (and DOB), instead, to be primarily a pharmacodynamic phenomenon, our data from the radioligand binding assay reveal important features. Repeated DOB application – as measured by DOB-sensitive [^{3}H]spiroperidol and DOB induced [^{35}S]GTP-gamma-S binding – leads to a significant reduction in frontocortical 5-HT_{2A} binding sites [compare 56] and 5-HT_2 signalling, respectively (Fig. 5 and 6). The reduction in 5-HT_{2A} binding sites correlates well with tolerance to DOB ($r = .80$); the reduced 5-HT_2 signalling – possibly due to non5-HT_{2A} receptors confounding the high-concentration Emax – does not. Glutamate-sensitive [^{3}H]glutamate binding sites are not affected by DOB, yet their status (in addition to the 5-HT_{2A} reduction) appears to be implicated in tolerance to the drug ($r = .84$). As to repeated LSD application, frontocortical 5-HT_{2A} binding sites are reduced as a trend, too ($p < .1$) (Fig. 5); 5-HT_2 signalling, however, is not affected (Fig. 6) and neither parameter correlates with tolerance to LSD. In contrast to its little (and unsystematic) effect on 5-$HT_{2(A)}$ receptors, LSD unlike DOB (and although it does not have any affinity for glutamate receptors [3]) significantly reduces frontocortical [^{3}H]glutamate binding sites (Fig. 5); this reduction, in addition, shares variance with tolerance to the drug ($r = .86$) (Fig. 5). Assuming, as outlined in the introduction, that shaking behaviour engages frontocortical glutamate receptor activation secondary to 5-HT_{2A} related glutamate release, the differential receptor adaptations noted for DOB and LSD, respectively, implicate that tolerance to serotonergic hallucinogens can arise at either level. That is, if a hallucinogen (like LSD in our study) for some reason fails to (down-)regulate 5-HT_{2A} receptors, glutamate receptors might instead adapt, and thus prevent cortical overstimulation (brought on by unabated 5-HT_{2A} related glutamate release). Why LSD in our study (unlike DOB) fails to (down-)regulate frontocortical 5-$HT_{2(A)}$ parameters, whereas in former studies it did not [34], is unclear. It might be suggested that there are different temporal phases in tolerance development that – depending on the structure of a hallucinogen, the dose, and regimen – differentially involve (complementary) adaptations of either 5-HT_{2A} and/or (downstream) glutamate receptors. Future research, evaluating the receptor status at multiple time points, might provide further insight.

Seemingly in accordance with the above suggested implication of glutamatergic adaptations for tolerance development, LSD and DOB (despite having no affinity [3]) also reduce frontocortical $mGlu_{2/3}$ signalling (Fig. 6) [compare for DOB 53]. The desensitisation might be a homologous adaptation to the hallucinogen induced excess in synaptic glutamate [57,58] and/or heterologously achieved by a direct interaction between 5-HT_{2A} and $mGlu_2$ signalling [29]. For DOB, the $mGlu_{2/3}$ desensitisation is negatively correlated with tolerance ($r = -.98$), which fits the fact that $mGlu_2$ receptors primarily suppress DOB induced shaking behaviour [59]. For LSD, as opposed, although its shaking behaviour is likewise sensitive to $mGlu_2$ receptors [29], there is no such correlation. Taken at face value, the given correlation coefficients suggest that $mGlu_{2/3}$ desensitisation – despite at first sight in line with the idea that glutamatergic adaptations play a role in hallucinogen tolerance – (in the case of LSD) does not seem to further or (in the case of DOB) even seems to counteract its development. Since our binding analysis did not differentiate $mGlu_2$ and $mGlu_3$ signalling, and the correlation coefficients accordingly cannot be disentangled as to the individual subtypes, either, such an appreciation of the coefficients needs to be regarded preliminary, though.

In toto, our data imply that tolerance to shaking behaviour, as induced by repeated application of serotonergic hallucinogens, might not always be a matter of mere 5-HT_{2A} regulation, but could also involve (complementary) adaptations of (downstream) glutamate receptors. Future research, along these lines, might screen for adaptations of AMPA or $_{(NR2B-)}$ NMDA receptors [60,61] and/or differentiate $mGlu_2$ and $mGlu_3$ receptors. Also, with regard to the local

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restriction of our binding analysis, future research might screen for 5-HT_{2A}-glutamate adaptations outside the frontal cortex [mind 62, 63]. Given the high conservedness of shaking behaviour [64,65], for instance, adaptations in more archaic areas such as the diencephalon, the brain stem, or the spinal cord could be promising candidates.

Conflict of interest

There are no conflicts of interest to declare.

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