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LSP2-9166, an orthosteric mGlu4 and mGlu7 receptor agonist, reduces cocaine self-administration under a progressive ratio schedule in rats

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Abbreviations

Amy: amygdala ; CPP: conditioned place preference ; DA: dopamine; DS: dorsal striatum ; G-protein coupled metabotropic glutamate: mGlu; HPC: hippocampus; LTP: long-term potentiation; PFC: prefrontal cortex; PR: progressive ratio; NAc: Nucleus Accumbens; SA: self-administration; VTA: ventral tegmental area

ABSTRACT

Cocaine addiction is a serious health issue in Western countries. Despite the regular increase in cocaine consumption across the population, there is no specific treatment for cocaine addiction. Critical roles for glutamate neurotransmission in the rewarding effects of psychostimulants as well as relapse have been suggested and accumulating evidence indicates that targeting mGlu group III receptors could represent a promising strategy to develop therapeutic compounds to treat addiction. In this context, the aim of our study was to examine the effect of LSP2-9166, a mGlu4/mGlu7 receptor orthosteric agonist, on the motivation for cocaine intake. We used an intravenous self-administration paradigm in male Wistar rats as a reliable model of voluntary drug intake. We first evaluated the direct impact of cocaine on *Grm4* and *Grm7* gene expression. Voluntary cocaine intake under a fixed ratio schedule of injections induced an increase of both mGlu4 and mGlu7 receptor transcripts in nucleus accumbens and hippocampus. We then evaluated the ability of LSP2-9166 to affect cocaine self-administration under a progressive ratio schedule of reinforcement. We found that this compound inhibits the motivation to obtain the drug, although it induced a hypolocomotor effect which could biais motivation index. Our findings demonstrate that mGlu group III receptors represent new targets for decreasing motivation to self-administer cocaine.

Introduction

Cocaine addiction is a chronic brain disease characterised by a high level of motivation for the drug and relapse in cocaine users [1]. Given the multifaceted health issue that results from this, including mortality and morbidity, research aimed at identifying the neurobiological mechanisms underlying cocaine's rewarding effects may lead to a better understanding of the processes involved in addiction and potential therapeutic strategies. It is well established that the dopamine (DA) neurotransmission system, particularly the mesolimbic pathway connecting the ventral tegmental area (VTA) to the nucleus accumbens (NAc), is crucial for the motivational component of behavior. This is classically measured in rodents using a progressive ratio (PR) schedule of cocaine self-administration (SA) [2]. In addition, emerging evidence points to a major role for glutamate in the progressive elaboration of cocaine-seeking behaviors [3] and glutamate levels are modified during relapse [4]. Glutamate acts on fast-acting ligand-gated ion channels, the ionotropic receptors, and slow-acting G-protein coupled metabotropic glutamate (mGlu) receptors. Three groups of mGlu receptors exist in the brain. Group I receptors (mGlu1/5 receptors) were the first to be demonstrated playing a role in drug dependence [5]. However, mnesic processes were also altered by these compounds [6]. Also, activation of group II mGlu receptors (mGlu2/3) reduces both seeking and taking behavior towards distinct drugs of abuse in rodents, through normalization of an altered glutamatergic neurotransmission induced by drug exposure [4]. However, activation of mGlu2/3 produced reduced food-seeking behaviors [7] and a decrease in cognitive performance [8, 9]. Among receptors of group III, mGlu6 and mGlu8 receptors lack precise behavioral function, whereas mGlu4/7 receptors negatively control glutamate transmission and have been recently proposed as targets for several neurodegenerative and neuropsychiatric conditions [10, 11].

The mGlu4 and mGlu7 receptors are expressed in the basal ganglia and structures of the reward circuit [12, 13]. L-AP4, an agonist of group III mGlu receptors, inhibits Glu and/or GABA release in key structures of the basal ganglia, such as striatum and globus pallidus [14, 15], therefore blocking cocaine-induced hyperlocomotion in rats [16]. Interestingly, a selective mGlu4 agonist, LSP1-2111, reduced the expression of cocaine sensitization [17]. A selective allosteric agonist of mGlu7 receptors, AMN082 [18], reduced cocaine SA [19] and altered both alcohol preference and intake [20]. Side-effects of this compound were observed, with a reduction of sucrose intake [21], and decreased locomotor activity at high doses [22].

Anti-depressive and anxyolitic effects were also observed and proposed to be linked to a rapid internalization process induced by AMN082 [23, 24].

The design of an orthosteric brain permeant subtype-selective ligand has facilitated new investigations. Indeed, LSP2-9166, a mGlu4/7 receptor orthosteric agonist [25], represents the most powerful orthosteric compound synthezised so far. Remarkably, it was recently shown to inhibit both morphine-induced place preference and reinstatement after extinction in mice [26], and ethanol intake in rats [27]. To complete this work on drug abuse, we first examined brain transcript levels for both mGlu4 and mGlu7 receptors following voluntary cocaine intake. We then examined whether the LSP2-9166 could modulate cocaine motivation in an intravenous (i.v.) SA procedure in rats.

Materials and methods

Chemical compound

LSP2-9166 was prepared according to an improved synthesis [25]. Briefly, **Figure 1** illustrates these improvements from recently reported preparation of derivatives [28, 29] to avoid L-AP4 formation, a side product with selective and potent group III mGluR agonist activity. We first improved a straightforward synthesis of the *N*-Boc-(S)-vinylglycine-*O*-*tert*-butyl ester from Boc-Asp-O*t*Bu in two steps without intermediate purification affording a scale-up [30, 31]. Purity was checked by NMR (^{31}P), with no detectable L-AP4, and confirmed before use by a pharmacological approach (ARPEGE Platform, Montpellier https://www.igf.cnrs.fr/index.php/fr/research-fr/facility-fr). Final deprotection under milder conditions led to the final product LSP2-9166 in higher yield, with a high purity.

Subjects

Male Wistar rats (Janvier Labs, France), aged five weeks and weighting 150-175 g upon arrival, were housed in standard home cages (5 rats/cage), under a reversed 12h light/dark cycle (lights on at 7pm), with water and food *ad libitum*. All procedures involving animal care were conducted in compliance with current laws and policies, validated by a Comité d’Ethique en Expérimentation Animale (CREMEAS) and authorised by the Ministère de l’Enseignement Supérieur, de la Rechercher et de l’Innovation (APAFIS#7050-2016093016163350). Eighteen rats were used for the molecular study and 69 for the SA procedure and locomotor activity.

Pharmacological treatment

Cocaine hydrochloride (Cooper, France) solution was adjusted with 0.9% NaCl to infuse an i.v. dose of 0.33 or 0.5 mg/kg/injection for SA and 1.5 mg/kg for locomotor activity. LSP2-9166 was first diluted into water at 20 mg/ml, pH was adjusted to 7.4 and final concentration was set at 2 or 5 mg/ml using phosphate-buffered-saline (PBS). LSP2-9166 (2 or 5 mg/kg, i.v.) or vehicle (PBS) was administered 15 min before each SA session.

Surgery and cocaine operant self-administration

All rats went through an i.v. catheterization procedure for operant SA, performed as described in detail previously [32]. Rats were anesthetized by an intraperitoneal injection (2 ml/kg) of a mixture containing ketamine (Imalgene 1000, 90 mg/kg, Centravet) and xylazine (Rompun, 10 mg/kg; Centravet) to perform surgical implantation of a chronic indwelling catheter in the right jugular vein. Catheters were flushed daily with heparin (100 U/mL) and ampicillin (50 mg/mL) to prevent clotting and infection. Animals were then housed individually. Following a 7-day recovery period, rats were submitted to behavioral experiments conducted during the dark period. Each operant chamber was equipped with two 2.5 cm-diameter holes, 4 cm above the floor. Holes were selected as active (delivering cocaine), and inactive (without programmed consequence), and counterbalanced across groups. When the required number of active nose-pokes was reached, cocaine 60 µL was delivered [32]. For molecular studies, rats followed a fixed ratio (FR) 1 schedule of reinforcement (cocaine, 0.33 mg/kg/injection, 2h/session/10d) concurrently with saline control rats. For the pharmacological study, rats were first trained for 2 h daily sessions under FR1 (3d) and FR5 (6d) and then submitted to a PR schedule, in which the number of nose-pokes required to earn an injection escalated within the session. Rats were trained for 6 consecutive daily sessions before the pharmacological treatment with LSP2-9166 or vehicle (5d). Each session lasted for 5h or until animals did not achieve the ratio for delivery of an injection within 1h. The breaking point to extinguish SA behavior was determined in each animal.

Horizontal locomotor activity

Locomotor activity was measured in individual dedicated home-cages with two infrared light beams. Numbers of longitudinal crossings were counted in 5 min bins. Three groups of rats implanted with catheters were processed (vehicle/NaCl; vehicle /Cocaine;

LSP/Cocaine). Rats were first habituated to the actimetry cages and injections (vehicle) (3d). Following habituation to the cages (1h), rats received either LSP2-9166 (5 mg/kg, i.v.) or vehicle, and locomotor activity was recorded for 15 min. Then rats received cocaine (1.5 mg/kg, iv) or NaCl 0.9%, and activity was recorded for 1h.

Brain sample dissection and quantitative real-time PCR

Animals were given an overdose of pentobarbital (182 mg/kg, i.p.), 24h following the last SA session, brains were removed and sliced (1-mm coronal brain matrix, Harvard apparatus, MA, USA). Structures of interest were collected [33] (see **Figure 2**). Samples were frozen on dry ice and kept at -80°C. Total RNA was extracted using Ribozol (VWR, France). Reverse transcription was performed on 750 ng of total RNA, with iScript (iScript™ cDNA Synthesis Kit, Biorad, France). Real-time PCR was performed in triplicate using a CFX96 Touch™ apparatus and Sso Advanced™ Universal SYBR Green supermix (Biorad, France). Thermal cycling parameters were 30sec at 95°C followed by 40 cycles of 5sec at 95°C and 45sec at 60°C. Primer sequences: Rplp0 Fw CTGCCCGAGCCGGTGCCATC, Rv TTCAATGGTACCTCTGGAG ; *Grm4*, Fw TCCAGGACCAACGGACACTT Rv ACGTGACCATCAGCAGCATG ; *Grm7* Fw AGACACAGAAGGGAACGCCT, Rv TCGGTTCTCATTGGGCCTCT. Expression levels were normalized to *rplp0* housekeeping gene levels and compared between saline and treated samples using the $2^{-\Delta\Delta Ct}$ method [34].

Statistical analysis

All results are expressed as mean ± sem. Data from qPCR were analyzed using unpaired student's t-test. Data from SA experiments were analyzed using repeated measures ANOVAs with group as between-subject factor and treatment and session as within-subject factors. Data from locomotor activity experiments were analyzed using ANOVA with group as between-subject factor and bin as within-subject factor. The ANOVAs were followed by a Tukey HSD posthoc test when required. Significance was set at $p \leq 0.05$ (Statistica v13).

Results

Effect of cocaine on mGlu4 and mGlu7 receptor gene expression

We first examined whether voluntary cocaine intake would modulate mGlu4 and mGlu7 receptor gene expression in reward-related brain structures. Following 10 days of cocaine SA (2h/d/FR1), rats displayed a stable level of cocaine intake (>100

injections/session) and directed more than 90% of the nose-pokes onto the active hole (data not shown). Rats receiving saline did not differentiate between holes (37.8±1.34% in the active hole). Microdissected brain samples were processed to measure gene expression levels by qPCR (**Figure 2**). Both mGlu4 and mGlu7 transcripts were detectable, with a higher expression (> 2-fold) of mGlu7 compared to mGlu4 in NAc, PFC and HPC, as previously described [12, 13] (data not shown). Expression of both transcripts were significantly higher in cocaine- compared to saline-treated animals in NAc (**Figure 2B**) (*Grm4* p=0.0011; *Grm7* p=0.0005) and HPC (**Figure 2D**) (*Grm4* p=0.0023; *Grm7* p=0.0047). A significant decrease was observed for mGlu4 in Amy (**Figure 2E**) (*Grm4* p=0.0024) while no regulations were observed for mGlu7 in Amy and for both receptors in PFC (**Figure 2A**) and DS (**Figure 2C**).

Effect of LSP-9166 on cocaine motivation

We performed cocaine SA under a PR schedule of reinforcement (see methods). Treatment with LSP2-9166 (0, 2 or 5 mg/kg i.v.) began 15 min before each session, for 5 days. The choice of the doses was based on preliminary experiments and literature [26, 27, 35]. Several SA sessions were necessary for the rats to display stable behavior, the ANOVA analysis showing no statistical difference between sessions 5 and 6 [$F(1, 38)=5.36$, p=0.97] (**Figure 3A**). Therefore, statistical analysis was performed on data from the last two sessions of each treatment (sessions 5/6 and sessions 10/11). The ANOVA (treatment, group, session) revealed a significant treatment effect [$F(1, 38)=6.97$, p=0.012] and a significant treatment x group interaction [$F(2, 38)=3.66$, p=0.035)], without significant effect of the sessions nor interaction between session and the other factors. Tukey HSD posthoc test showed that rats receiving vehicle or LSP2-9166 2 mg/kg did not display any behavioral changes in comparison to baseline (vehicle, p=0.97 and LSP2-9166 2 mg/kg p=0.99). In contrast, administration of LSP2-9166 5 mg/kg resulted in a significant reduction in the breaking point (p=0.004). We noted similar results for the active nose-pokes (treatment x group interaction, $F(2,38)=5$, p=0.012), with significant difference between baseline and LSP2-9166 5 mg/kg (p<0.001). A global decrease was observed for the inactive nose-poke (significant treatment effect, $F(1,38)=5$, p=0.016) but without any significant group effect (p=0.69), suggesting a motivational rather than a motor effect of the compound (**Figure 3B**).

Effect of LSP-9166 on locomotor activity

We then evaluated the effect of LSP2-9166 (5 mg/kg) on spontaneous locomotor activity (first injection) and on cocaine-induced hyperlocomotion (second injection) (**Figure 3C**).

Following the first injection (bin 60-75), the ANOVA revealed a significant interaction (bin x treatment, $F(6, 72) = 2.24$, $p=0.049$). Locomotor activity increased only in vehicle/NaCl and vehicle/Cocaine groups (bin 60-65, $p<0.0002$; LSP/Cocaine, $p=0.75$) (Tukey HSD test). No significant differences were observed between the 3 groups for all the bins (60-75). This indicates that LSP2-9166 reduced spontaneous activity-induced by the injection. For the second injection (bin 75-135) a significant interaction (bin x group $F(24, 288) = 3.03$, $p= 0.000006$) was observed. The Tukey HSD posthoc analysis showed a significant increase of locomotor activity in all three groups compared to their baseline (bin 75-80 $p<0.0004$). A significant difference was observed at bin 80 between vehicle/Cocaine and the two other groups ($p<0.002$), suggesting a reduction of cocaine-induced locomotor activity in presence of LSP2-9166. No significant differences were observed between the 3 groups for the other bins of the analyzed period. These results indicated a global hypolocomotor activity induced by LSP2-9166.

Discussion

In this study, we show that voluntary cocaine intake induces an increase of mGlu4 and mGlu7 receptor transcripts in NAc and HPC. The orthosteric agonist LSP2-9166 inhibits the motivation to obtain the drug in a PR schedule of reinforcement, although this ligand induces a hypolocomotor effect. An increasing interest has emerged in the addiction field directed to mGlu receptors, which are well known to regulate glutamatergic neurotransmission [36, 37]. Group III mGlu receptor ligands were developped, with LSP1-2111 [29], an agonist for mGlu4, and AMN802 [18], acting as the first allosteric mGlu7 agonist. Recently, the orthosteric LSP2-9166 efficaciously decreased expression and reinstatement of a morphine CPP in mice [26] as well as ethanol consumption, motivation for ethanol and reacquisition of ethanol SA following abstinence in rats [27]. Our hypothesis that such modulatory effects may be expanded to cocaine behavioral responses has been tested here and we confirm a reduced motivation for drug taking in an operant paradigm.

Our results on locomotor activity have to be taken into account in the interpretation of the effects of LSP2-9166 on the motivation for cocaine. We observed a decreased spontaneous locomotor activity following a single injection of LSP2-9166 and a reduction in cocaine-induced hyperlocomotion. Interestingly, hypolocomotor effect of acute LSP2-9166 was previously reported in rats [27], at high doses in mice [26], with a small transient inactive state resembling catalepsy [35], with no effect on motor coordination in this latter study. Also

no difference was detected on circular locomotor activity or rotarod test following systemic injection of LSP2-9166 (10 mg/kg ip,14 d) [35]. In addition, it did not impact other behaviors like sucrose intake or spatial memory [26, 27], suggesting a moderate locomotor impact on behavior. Moreover, mice deficient for mGlu4 receptor showed a higher basal locomotor activity [38], and mGlu7 receptor knockout mice presented a significant reduction of amphetamine-induced hyperlocomotor activity [39], compared with wild-type animals. Also, activation of mGlu7 receptors by AMN802 decreased activity in rodents [21, 22] whereas no effect on locomotor activity was reported following LSP1-2111, a mGlu4 receptor orthostetic agonist, used at a lower dose [17]. In addition, anxiety may play a role in the effect of LSP2-9166 on cocaine-induced behavior, as it is known that a higher level of anxiety may decrease the motivation for cocaine in a PR paradigm [40]. Both anxiogenic and anxiolytic effects of LSP2-9166 have been described, at a high dose in mice [26], and following chronic LSP2-9166 treatment in rats [35], respectively. Altogether, such effect may depend on the dose and the species under study and we can not exclude any impact in our paradigm.

We show here that voluntary cocaine intake increased both *Grm4* and *Grm7* in NAc and HPC, whereas a decrease was observed for *Grm4* in the Amy. These regulations may play a direct role on motivation (NAc) counterbalanced by an increase in anxiety (Amy). Such adaptations may participate to a global homeostatic regulation following cocaine intake, that involves complex neuronal circuitry.These regulations are region-specific as no modification were detected in the PFC or DS. Also, they were observed following 10 d of cocaine intake, 24 h after the last drug exposure, suggesting that they do not result from short term effects of cocaine. It would be of crucial interest to evaluate whether these regulations are long-lasting, reversible and whether they occur at distinct stages of addiction (withdrawal, seeking events), to highlight changes that could specifically mediate active drug seeking. No data are yet available on the regulation of these receptors by other drugs of abuse, at the protein level.

In our conditions, the breaking point under a PR schedule was reduced, indicating that the LSP2-9166 compound was able to reduce the motivational properties of cocaine. Our results are in accordance with previous publications showing that AMN082 reduces cocaine intake under a PR schedule of reinforcement [19]. Nevertheless, the neurobiological mechanisms involved in these effects are still not clear. The LSP2-9166 reduced cocaine reward-related effects probably through attenuation, via group III mGlu receptors activation, of the hyperglutamatergic state. Indeed, LSP2-9166 was demonstrated to be highly potent at mGlu4 (EC_{50} = 0.06 μM) and mGlu7 (EC_{50} = 2.2 μM) [25] receptors. By increasing the

activity of mGlu4/7 located in presynaptic terminals of glutamatergic afferents coming from the PFC, Amy and/or HPC, LSP2-9166 may lower glutamate release. Consequently, this would decrease the strength of goal-directed behavior to obtain the drug. Another hypothesis would be that the effects of LSP2-9166 are mediated by a reduction of GABA release on NAc neuronal targets, such as ventral pallidum and/or pars reticulata of the substantia nigra. Indeed, activation of group III mGlu receptors is able to weaken GABA neurotransmission, for instance in globus pallidum [14]. Future electrophysiological or neurochemical studies will investigate these effects on cocaine-induced glutamate and/or GABA neurotransmission.

The LSP2-9166 ligand also blocked morphine CPP expression and reinstatement after extinction [26]. Noticeably, it was more efficient in Hajasova et al.'s study since effects were already observed at lower doses (0.5 mg/kg, ip). The explanations for such discrepancies are still unclear and may be related to differences in species, behavioral paradigms, and/or the nature of the drug studied. Another explanation could be that mGlu receptors form heterodimers that may strongly modify the ligand affinity, as very recently described for mGlu2/7 receptors [28], or their constitutive activity, for mGlu4/7 [28, 41]. Such molecular interactions could occur between mGlu4/7 in specific reward-related brain structures, in a distinct manner depending on the drugs of abuse, and thus participate to the different effective doses observed between the studies.

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Figure Captions

Figure 1. Optimization of LSP2-9166 synthesis. Reagents and conditions: (i) $NH_4H_2PO_2$ (2.5 eq), Et_3B (1 eq), O_2, MeOH, RT, 30 min, 2 times; (ii) *tert*-butyl 2-(4-formyl-2-(trifluoromethoxy)phenoxy) acetate (1 eq), *N,O*-bis(trimethylsilyl) acetamide (BSA) (4 eq), degassed CH_2Cl_2, RT, 24 h; (iii) a) 3 M aqueous HCl, THF, RT, 48 h; b) C-18 flash chromatography. Bu: butyl; Boc; *tert*-butoxycarbonyl; BSA: *N,O*-bis(trimethylsilyl)acetamide; eq: equivalent; RT: room temperature.

Figure 2. Cocaine effect on mGlu group III receptor gene expression. Transcript levels for *Grm4* and *Grm7* genes were examined by qPCR in PFC (**A,** 7-8/group), NAc (**B,** 7-9/group), DS (**C,** 6-8/group), HPC (**D,** 5-10/group) and Amy (**E,** 6-8/group) following cocaine SA. Bars represent mean ± SEM fold change vs saline group for each receptor. $^{**}p < 0.01$; $^{***}p < 0.001$. Samples were dissected with large (3 mm) or small (1.9 mm) punchers as indicated.

Figure 3. Effect of LSP2-9166 on cocaine-self administration and locomotor activity. Effect of LSP2-9166 (2 and 5 mg/kg i.v., 15 min before the operant session) on cocaine SA in a progressive ratio (PR) schedule of reinforcement. Number of injections (**A**) and number of nose-pokes (**B**) are presented across all sessions. Results are expressed as mean ± SEM for each group (vehicle, n=18; LSP2-9166, 2mg/kg, n=8; LSP2-9166, 5mg/kg, n=15). $^{**}p < 0.01$, baseline (S5/S6) vs treatment (S10/S11) of LSP2-9166 5mg/kg. Locomotor activity (**C**) was recorded following habituation (1h), i.v. injection of LSP2-9166 (5 mg/kg, iv) or vehicle (bin 60-75), and then cocaine (1.5 mg/kg) or NaCl 0.9% (bin 75-135); (vehicle/NaCl n=8; vehicle/cocaine n=10; LSP2-9166/cocaine n=9). $^{***}p<0.001$ vehicle/cocaine vs LSP/cocaine at bin 80.

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Author's contribution

PR, JZ, KB conceived the study and wrote the paper. PR, DN, CQ, DF performed the experiments. PR, DN, RB, KB analyzed the data and designed the figures. FA and IMT designed and synthesized the mGlu compound. All authors approved the final version of the manuscript.