

**LSP5-2157 a new inhibitor of vesicular glutamate transporters**

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**Abstract**

Vesicular glutamate transporters (VGLUT1-3) mediate the uptake of glutamate into synaptic vesicles. VGLUTs are pivotal actors of excitatory transmission and therefore of all brain functions. Their implication in various pathologies such as Parkinson and Alzheimer disease, epilepsy, schizophrenia, anxiety, addiction or deafness has been clearly documented. Despite their functional importance, the pharmacology of VGLUTs is particularly underdeveloped and limited at non-specific dyes such as Trypan Blue, Rose Bengal or Brilliant Yellow type. Here we report the design and the conception of new potent analogs based on Trypan Blue scaffold. Our best compound, named LSP5-2157, has an EC<sub>50</sub> of 50nM on glutamate vesicular uptake. Using a 3D homology model, we determined putative binding subdomains of VGLUT1 that bind LSP5-2157. To better estimate the specificity and potency of LSP5-2157, we investigated its ability to block glutamatergic transmission in autaptic hippocampal cells. Neither ionotropic glutamate receptors nor GABAergic transmission or transmission machinery such as Ca<sup>2+</sup> channels were affected by application of LSP5-2157. Low doses of LSP5-2157 (2μM) reversibly reduce glutamatergic neurotransmission in hippocampal autapses. LSP5-2157 had a low and depressing effect on synaptic efficacy in hippocampal slice. Furthermore, LSP5-2157 (2μM) had no effect on NMDA-R- mediated fEPSP. This compound was able to reduce synaptic plasticity induced by 3 trains of 100Hz. However, LSP5-2157 had no effect on plasticity induced by theta burst, which suggests that the readily releasable pool of glutamate was not affected by the drug. Finally, we showed that LSP5-2157 had the capacity to inhibit VGLUT3-dependent auditory synaptic transmission in the guinea pig cochlea. In this model, LSP5-2157 abolished the compound action potential of auditory nerve at high concentration showing the limitation of LSP5-2157 permeation in an *in-vivo* model. Therefore, the new ligand LSP5-2157 has a high affinity for VGLUTs and shows some permeability in isolated neuron, tissue preparations or *in vivo* in the auditory system. These findings open the way to the use of VGLUTs inhibitors to assess glutamatergic functions *in vitro* and *in vivo*.

## Introduction

Glutamate (Glu) the major excitatory neurotransmitter in the Central Nervous System (CNS) is involved in a vast majority of neurophysiological functions as well as neuropathologies. Before its exocytotic release, Glu is accumulated in synaptic vesicles by vesicular glutamate transporters (VGLUTs). The three isoforms of VGLUTs (VGLUT1-3) share a high degree of sequence homology (more than 75%) but have almost complementary brain distributions (El Mestikawy et al., 2011). VGLUTs are pivotal functional and anatomical markers of glutamatergic transmission. Various studies suggest the involvement of VGLUTs in human neurological conditions such as Parkinson's disease, Alzheimer disease or epilepsy (Kashani et al., 2007; Kashani et al., 2008; Kirvell et al., 2006; van der Hel et al., 2009) as well as psychiatric disorders (Eastwood and Harrison, 2010; Oni-Orisan et al., 2008; Sakae et al., 2015; Uezato et al., 2009). VGLUTs were suggested as potential target for the treatment of temporal lobe epilepsy (Van Liefferinge et al., 2013). Despite their key role in excitatory transmission the pharmacology of VGLUTs is notably under-developed. Pharmacological tools are therefore crucially needed to better understand the functional implication of VGLUT1-3 in normal and pathological conditions. Additionally, these compounds could be developed as radiomarkers to follow the fate of glutamatergic terminals in human pathologies of the CNS.

Few chemical compounds efficiently target this important family of glutamate transporters. Presently, three major types of VGLUT's inhibitors have been identified (Thompson et al., 2005) : glutamate-like inhibitors (Thompson et al., 2005), substituted quinolines (Bartlett et al., 1998; Carrigan et al., 2002) and dyes (Kehrl et al., 2017; Ozkan and Ueda, 1998; Roseth et al., 1995, 1998; Tamura et al., 2014). Dyes have  $K_i$  in the 20nM-10 $\mu$ M range, substituted quinolines (DCQ) in the 40-300 $\mu$ M range and glutamate analogs with  $IC_{50}>230\mu$ M. Glutamate-like inhibitors interact with glutamate receptors and transporters (Thompson et al., 2005). Dyes display higher affinities but a lower selectivity than quinolines (Shigeri et al., 2004). We previously reported that the potency of Rose Bengal was equally effective on monoamine vesicular accumulation and on VGLUTs (Pietrancosta et al., 2010). On the other hand, DCQ derivatives remain potential candidates for this pharmacological purpose. However, Carrigan et al. have shown their limit due to their moderate affinities (Carrigan et al., 2002; Laras et al., 2012).

Trypan Blue or Evans Blue are well known universal cellular dyes that lack selectivity and chemical variations of these dyes are not easily accessible. Furthermore, these compounds have the ability to inhibit glutamate vesicular accumulation as well as excitatory synaptic transmission (Neale et al., 2014).

In the present study, we synthesized and characterized derivative of Trypan Blue. We thoroughly evaluated the capacity of one new compound (named LSP5-2157) to selectively inhibit VGLUT activity as well as glutamatergic synaptic transmission in hippocampal autapses and slices. In addition, we evaluated the ability of LSP5-2157 to modulate auditory input *in vivo* in the guinea pig cochlea. This study establishes that LSP5-2157 has a high affinity/high selectivity for VGLUTS and furthermore is partially membrane permeant.

## Material and methods

### *Synthesis*

Compounds were synthesized according to procedure already described (Favre-Besse et al., 2014) with minor modifications. (Supplementary Methods)

### *Glutamate vesicular uptake*

Vesicular Glutamate uptake was assayed as previously described (Kish and Ueda, 1989; Naito and Ueda, 1985; Pietrancosta et al., 2010) with minor modifications. Aliquotes of crude rat synaptic vesicles obtained by differential centrifugation were diluted in a solution containing 4 mM HEPES-KOH, pH 7.4 and 0.32M sucrose. Test compounds were dissolved in 1% Ethanol, 1% DMSO (pH was adjusted at 7.4 with KOH 5%) to initial concentrations between 2mM and 200µM. Compounds were then diluted at final concentrations between 200 µM to 0.02 µM in a solution containing 10 mM HEPES-KOH, pH 7.4, 0.32 M sucrose, 4 mM KCl, 4 mM MgSO<sub>4</sub> (named TPU). Test compounds were preincubated in a solution containing 3 M ATP-Mg, 1mM L-aspartic acid, 100 µM Glutamate and 0.4 µM [<sup>3</sup>H]Glutamate (1.547TBq/mmol). After 4 min at 37 °C, the uptake reaction was initiated by addition of rat synaptic vesicles. This mixture was incubated 10 min at 37 °C before of ice-cold TPU (3.5 mL) were added and rapidly filtered through membrane filter (0.45µM, Millipore MF reference HAWP02500) using a manifold under vacuum. Filters were washed five times with 3.5 mL of TPU and placed in scintillation vials with scintillation cocktail (optiphase hisafe 3, reference 1200-437, Perkin Elmer). Radioactivity bound to the filters was measured in a Beckman LS 6500 scintillation spectrophotometer. Values obtained in the absence of ATP-Mg or in the presence of Carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 50 µM Sigma reference C2759) were used to determine non-specific uptake activity.

### *Patch-clamp recordings on hippocampal autapses*

#### Cell culture

Astrocyte microislands and autaptic neuronal cultures from hippocampi of C57BL/6 mice were prepared as previously described (Arancillo et al., 2013). In brief, astrocytes were derived from mouse P1 cortices and plated onto substrate 7 days before neurons were plated. Neurons were isolated from P0 mouse hippocampi and plated with a density of 3000 neurons per 35 mm well. For recording, only the islands containing a single neuron were selected.

#### Electrophysiological recordings of autapses

Electrophysiological recordings of neurons took place from 13-16 days *in vitro* (DIV). Whole cell recordings were conducted with a Multiclamp 700B amplifier (Molecular Devices) under the control of a Digidata 1440A Digitizer (Molecular Devices) and pCLAMP software (RRID:rid\_000085, Molecular Devices). Cells were voltage clamped at -70 mV. EPSCs were evoked by depolarizing the cell to 0 mV for 2ms. Data were sampled at 10 kHz and Bessel filtered at 3 kHz. Series resistance was generally <10 MOhms and only cells with < 15 MOhm resistance were included. Series resistance was compensated by at least 70%. For all recordings, the internal solution contained: 135 mM KCl, 17.8 mM HEPES, 1mM EGTA, 4.6 mM MgCl<sub>2</sub>, 4 mM NaATP, 0.3 mM NaGTP, 12 mM creatine phosphate and 50 U/mL phosphocreatine kinase. The external solution contained: 140 mM NaCl, 2.4 mM KCl, 10 mM HEPES, 10 mM glucose, 4 mM MgCl<sub>2</sub>, and 2 mM CaCl<sub>2</sub>, and a pH of 7.3 and an osmolarity of 300 mOsm. In the LSP5-2157-incubated groups, cells were recorded in the same external solution with a concentration of 2  $\mu$ M LSP5-2157.

#### LSPS incubation and wash-in

For all LSP5-2157 incubation experiments, cells were incubated in 2  $\mu$ M LSP5-2157 in Neurobasal A (NBA) cell culture medium plus B27 (Invitrogen, Life Technologies, Carlsbad, California), 50IU/ml penicillin and 50  $\mu$ g/ml streptomycin at 37 °C for two hours (media was the same composition as the original cell culture media). Cells in the LSP group were then recorded using external solution containing 2  $\mu$ M LSP5-2157. Cells in the washout group were incubated in 2  $\mu$ M LSP5-2157 for two hours and moved to a well of fresh NBA medium for at least another two hours before recording in external solution without drug.

In wash-in experiments and kainate experiments, baseline measurements were taken in the extracellular solution described above, before acutely applying 2  $\mu$ M LSP5-2157 in extracellular solution. In kainate experiments, kainate (10  $\mu$ M) was applied for 2 seconds at three separate times throughout the recording period, as described in the scheme (Figure 3a); during baseline, during LSP5-2157 application (3 minutes post-wash in) and after LSP5-2157 washout (3 minutes after LSP5-2157 application was terminated). Solutions were exchanged with a time constant of 20-30 ms using the fast-flow application system (Rosenmund et al., 1995).

In all LSP5-2157 wash-in experiments the baseline EPSC amplitude, paired-pulse ratio (PPR), and mEPSC characteristics were measured in external solution, immediately followed by a 30 second kynurenic acid application (3 mM). LSP5-2157 was then applied in three-minute intervals, alternating with 30 seconds of kynurenic acid application for a total of six minutes.

#### *Electrophysiological recordings of hippocampal slices*

### Ex vivo hippocampal slices preparation

Mice (C57/Bl6, 3-5 months old) were anesthetized with halothane and decapitated. The brain was rapidly removed from the skull and placed in a chilled (0-3°C) artificial cerebrospinal fluid (ACSF) containing (mM) NaCl 124, KCl 3.5, MgSO<sub>4</sub> 1.5, CaCl<sub>2</sub> 2.5, NaHCO<sub>3</sub> 26.2, NaH<sub>2</sub>PO<sub>4</sub> 1.2, glucose 11. Transverse slices (300-400 µm thick) were cut using a vibratome and placed in a holding chamber (at 27°C) containing the ACSF solution, at least one hour before recording. Each slice was individually transferred to a submersion-type recording chamber and submerged with ACSF continuously superfused and equilibrated with 95% O<sub>2</sub>, 5% CO<sub>2</sub>.

### Patch-clamp recordings

Electrophysiological recordings of CA1 pyramidal neurons were performed in acute hippocampal slices (Androuin *Acta Neuropathologica*, 2018, 135:839-854.). Whole cell recordings were conducted with the cell voltage clamped at -60 mV for both the measurement of evoked AMPA current and spontaneous activity. For all recordings, the internal solution contained: 135 mM CsCH<sub>3</sub>SO<sub>4</sub>, 6 mM CsCl, 10 mM HEPES, 1 mM EGTA, 2 mM MgCl<sub>2</sub>, 5 mM QX-314, 4 mM ATP, pH 7.3 adjusted with CsOH 1N; 300 mOsm. Electrophysiological data were acquired and analyzed using WinLTP (Anderson and Collingridge, 2005; [www.winltp.com](http://www.winltp.com)). For mEPSC frequency and amplitude analysis, events were detected by template in Spike 6.

### Extracellular recordings

Recordings were obtained from the apical dendritic layers of the hippocampal CA1 area, using glass micropipettes filled with 2M NaCl and with a resistance of 2-6 MΩ. Fiber volleys (FV) and field excitatory postsynaptic potentials (fEPSPs), mostly resulting from the activation of AMPA receptors, were evoked by electrical stimulation of Schaeffer collaterals afferent to CA1 and commissural fibers in the stratum radiatum.

To construct Input/Output (I/O) curves the averaged slopes of three successive FV and fEPSPs were measured using WinLTP software (Anderson and Collingridge, 2001) and the fEPSP/FV ratio was plotted against the stimulus intensity (from 100 to 300 µA). To investigate isolated NMDA-R-mediated fEPSPs (fNMDA), slices were perfused with low-Mg<sup>2+</sup> (0.1 mM) aCSF supplemented with the AMPA/kainate receptor antagonist 2,3- dioxo-6-nitro-1,2,3,4-tetrahydrobenzoquinoxaline-7-sulfonamide (NBQX, 10 µM).

For the pharmacological studies, drugs were superfused through the bath for at least 15 min before, and during the whole recording. For each pharmacological study, experiments under control conditions were systematically carried out on the same day. Drugs used in this study include LSP5-2157, (2 µM), the NMDA receptor antagonist D-APV (D-2-amino-5-phosphonovalerate, 80 µM, Tocris, Illkirch, France).



Paired-pulse facilitation (PPF) of synaptic transmission was induced by paired-pulse stimulation with inter-stimulus intervals (ISIs) of 40 ms. PPF was quantified by normalizing the slope of the second response by the slope of the first one.

Two kind of electrically-induced long-term potentiation (LTP) were evoked. A strong, saturating LTP consisting in 3x100 Hz (3x 100 pulses, 1s, spaced of 20 sec), and a lower stimulation, the theta-burst stimulation mimicking the natural stimulation at the theta frequency arising from the medial septum to the hippocampus, consisting of five trains of four 100 Hz pulses each, separated by 200 ms and delivered at the test intensity. The sequence was repeated three times with an interburst interval of 10s. After applying stimulations, testing with a single pulse was resumed for 60 min to determine the level of LTP. Drug were applied on a different population of slices than control since we cannot induce the plasticity two times in a row on the same slice, but slices used came from the same animal for the control LTP and the LTP in the presence of LSP5-2157.

#### Data analysis of autapses and hippocampal slices recordings

For hippocampal autapses studies data were analyzed using Axograph X (Axograph Scientific, Berkeley, California), Excel (Microsoft, Redmond, Washington), and GraphPad Prism (GraphPad Software, San Diego, California). For mEPSC frequency and amplitude, traces were filtered to 1 kHz and events were detected by template in Axograph. To eliminate noise contamination of real events, “false” events detected in the presence of an AMPAR or GABAR antagonist (kynurenic acid or bicuculline, respectively) were subtracted from the events detected in control recording solution. For frequency subtraction:  $\text{frequency}_{\text{real}} = \text{frequency}_{\text{total}} - \text{frequency}_{\text{inh}}$ . For amplitude subtraction:  $\text{amplitude}_{\text{real}} = ((\text{amplitude}_{\text{total}} * \text{frequency}_{\text{total}}) - (\text{amplitude}_{\text{inh}} * \text{frequency}_{\text{inh}})) / \text{frequency}_{\text{real}}$ . Statistics were determined by Student's t test (two groups), one-way ANOVA with Tukey post hoc test (more than two groups), or two-way ANOVA with Bonferoni post hoc test (groups with two independent variables). Cells with excessive noise contamination (>20% observed frequency present in antagonist) were excluded from mEPSC or mIPSC analysis.

For LTP analysis, average values of fEPSPs slope were calculated for 10 minutes between the 50<sup>th</sup> and 60<sup>th</sup> minute after the end of the conditioning stimulation and expressed as a percentage of the baseline response (% baseline)  $\pm$  SEM. Statistical significance was assessed using one-factor analysis of variance (ANOVA) or ANOVAs for repeated measures data. For patch-clamp recordings in hippocampal slices, data were reported as mean  $\pm$  SEM. Significance was calculated using multivariate analyses of variance (ANOVA) followed by post hoc (paired) t test.

*In vivo recording of cochlear and compound action potential of the auditory nerve  
guinea pig*

Adult pigmented guinea pigs (250–300g) were purchased from Janvier Laboratories (Le Genest Saint Isle). Guinea pigs were housed in facilities accredited by the French Ministry of Agriculture and Forestry (B-34 172 36 - March 11, 2010). Experiments were carried out in accordance with both the European Communities Council Directive of 24 November 1986 (86/609/EEC) and French Ethical Committee: (agreements C75-05-18), regarding the care and use of animals for experimental procedures. All efforts were made to minimize the number and suffering of animals used.

Drug preparation

Artificial perilymph solution (AP) consisted of the following (in mM): 137 NaCl; 5 KCl; 2 CaCl<sub>2</sub>; 1 MgCl<sub>2</sub>; 1 NaHCO<sub>3</sub>; 11 glucose; pH 7.4; osmolarity: 304 ± 4.3 mOsm/kg. Before each experiment, LSP5-2157 was prepared in AP to a final concentration of 0, 1, 3, 10, 30, 100, 1000 or 2000 µM.

Surgery and perilymphatic perfusion technique

The guinea pigs were anaesthetized with an intraperitoneal injection of urethane (1.4 g/kg; Sigma). Supplementary doses of urethane were administered as required to maintain deep anaesthesia. A tracheotomy was performed, the electrocardiogram was monitored and the central temperature regulated at 38 ± 1 °C by a thermally regulated heating blanket. The pinna and external meatus were resected to ensure good close-field acoustic stimulation. The method used to monitor the effect of multiple perilymphatic perfusions on sound-evoked potentials was performed as previously described (Puel et al., 1995). Briefly, the cochlea was exposed ventrally and two holes (0.2 mm in diameter) were gently drilled in the scala vestibuli and scala tympani of the cochlear basal turn. Cumulative perfusions were administered through the hole in the scala tympani at 2.5 µL/min using a glass pipette coupled to a syringe pump (Syringe Pump Model 11; Harvard Apparatus, Holliston, MA, USA). The perfused solutions flowed out of the cochlea through the hole in the scala vestibule. All perfusions were performed at room temperature, and lasted 10 min.

Functional assessments

*Cochlear and compound action potential of the auditory nerve*

Acoustic stimulation were generated by a NI PXI-4461 signal generator (National Instruments) consisting of 10 ms tone bursts with a 1 ms rise and fall time delivered at a rate of 10/s. Tone bursts were passed through a programmable attenuator and delivered to the animal by a JBL 075 loudspeaker (James B. Lansing Sound, Los Angeles, CA, USA) in a calibrated free field, positioned at 10 cm from the tested ear. Cochlear compound action potentials (CAPs) evoked by a 8 kHz tone burst were recorded from a silver electrode placed on a third hole made in the scala vestibule of the basal turn. The electrophysiological signal was amplified (32000) by a Grass P511 differential amplifier and digitalized (50 kHz sampling rate, with a 12-bit dynamic range and 1024 samples per burst), averaged 256 times, and stored on a computer (Dell Dimensions, Austin, TX, USA). The stored potentials were then digitally filtered with a low-pass filter (cutoff frequency 3.5 kHz) to display the CAP of the auditory nerve and summing potential (SP) generated by the inner hair cells. A narrow band filter (6-10 kHz) was used to extract the cochlear microphonic generated by the outer hair cells (OHCs). Amplitude-intensity relationships were obtained by varying tone burst levels from 0 to 100 dB SPL, in 5 dB steps.

The CAP, SP and CM thresholds were defined as the level of decibels SPL needed to elicit a measurable response ranged from 2 to 5  $\mu$ V.

## RESULTS

### Effect of VGLUTs inhibitors on glutamate vesicular accumulation

Compounds **1** to **8** were synthesized and characterized as described in the supplementary Material and Methods

As shown in Figure 1a and b, thirteen analogs of Trypan Blue were able to dose-dependently inhibit ATP-dependent [<sup>3</sup>H]-Glutamate vesicular accumulation. Sigmoidal curves were generated for all compounds allowing the determination of their Efficiency Concentration 50 (EC<sub>50</sub>, Figure. 1a). In Figure 1b compounds were listed by order of potency of their EC<sub>50</sub>. LSP5-2157 demonstrated an EC<sub>50</sub> of 56nM whereas Trypan Blue, the reference compound, blocked [<sup>3</sup>H]-Glutamate uptake with an EC<sub>50</sub> of 100nM (Figure 1a, b). Therefore, LSP5-2157 was more efficient than TB at inhibiting VGLUTs activity.

To efficiently interact with VGLUTs, compounds should bear two hydrophilic motifs separated by a hydrophobic linker (Kehrl et al., 2017). Previous work has shown that a shorter molecule bearing only one hydrophobic and one hydrophilic counterpart led to drastic loss of inhibitory activity (Carrigan et al., 2002; Favre-Besse et al., 2014). All competitive inhibitors with submicromolar activities conserved this pattern of features (i.e. Trypan Blue, Evans Blue or Brilliant Yellow). Indeed, comparing structures of Evans Blue and Trypan Blue, highlight the variability of hydrophilic naphthyl moieties but comparison between EB (EC<sub>50</sub> = 100nM) and Chicago Sky Blue (EC<sub>50</sub> = 0.3-3μM, not shown) confirmed the potentiality of modifying the hydrophobic linker (Fig. 1b). We also tested the linker substituent in order to optimize its interactions. As expected, biaryl modifications had a high impact on activity and new compounds inhibit glutamate VGLUTs uptake in a range of IC<sub>50</sub> from 56 to 1000 nM (Figure 1a).

To better understand the ligand/VGLUT interaction, we generated a VGLUT1 homology model based on appropriated template (Glycerol-3-Phosphate transporter, pdb code = 1PW4; (Almqvist et al., 2007); supplementary Methods). The homology model built in cytosol open conformation revealed a large binding pore with three successive binding regions: a hydrophilic – a hydrophobic and another hydrophilic layers. The first layers composed by various polar residues of the end of Transmembrane Helix 4 (TM4) and TM10 (T199-H204 and N435-D440) and those of TM5 and those at the beginning of TM11 (E215-S223 and S453–N454) represent a Low Affinity Hydrophilic Layer (LAHL) (in red in Figure 1c). Residues of LAHL are in direct contact with the cytoplasm therefore the strength of interaction between putative ligands should remain limited. The second layer is mainly hydrophobic and formed with hydrophobic amino acid within TM1 (F86), TM2 (F167- F137), TM5 (Y228), TM10 (I431- F434) and TM11 (L459) (the Hydrophobic Middle Layer (HBML) in green in Figure 1c). This region is only partially exposed to solvent and could form stronger

interactions with ligands. Finally, the third domain appeared particularly interesting since it is formed by non-solvent exposed hydrophilic residues represented the interaction with the higher potential (the High Affinity Hydrophilic Layer (HAHL) in pink in Figure 1c). In particular charged residues such as R93 (TM1), H133 (TM2), R189 (TM4) or D196 (TM4) could form very stable interactions (ionic bonds) with ligands.

To validate this putative 3D organization of VGLUT, we performed docking experiments with previously described shorter blue dyes analogs (Favre-Besse et al., 2014) and the newly synthesized full blue dye analogs (Supplementary Methods). As expected, polar ligands did not access to the second hydrophilic layer and bound only the solvent exposed layer leading to a low inhibitory potency (Figure 1a, family III, Figure 1c). More hydrophobic compounds of family II could bind the hydrophilic exposed layer as well as the middle hydrophobic region of VGLUT leading to a relatively better inhibitory activity than shorter analogs (Figure 1a and d). Interestingly, as for glutamate, small polar ligands such as short Trypan Blue Analogs or Xanthurenic acid or other quinolone derivatives have low affinity for VGLUT (Carrigan et al., 2002).

To obtain a potent inhibition, we generated series of 7 compounds with various domains based on Trypan Blue structure (Family I). We also design compounds with a hydrophilic polar head, a hydrophobic core and another polar moiety to respectively interact with the LAHL, the HBML and the HAHL domains of the VGLUTs. Docking experiment indicated a better potency of LSP5-2157 since it could form ionic bonds with R93 and H133 in the HAHL (Figure 1f-h). Ortho substitution of the biaryl linker decreases the inhibitory potency. Indeed, whereas LSP5-2157 ( $R^1 = R^2 = H$ ) is the most potent VGLUT inhibitor, LSP9-2106 (7) ( $R^1 = R^2 = CH_3$ ) or LSP9-1148 (4) were inactive. This may be interpreted by a narrow HBML or internal steric hindrance that induces an unfavorable conformation of the compound.

In summary, in our conditions, the new compound LSP5-2157 was the more efficient to inhibit vesicular glutamate uptake.

### **LSP5-2157 reduces evoked and spontaneous release at hippocampal autapses.**

We then investigated whether LSP5-2157 inhibits glutamate release. Primary autaptic cultures of hippocampal neurons were prepared and recorded using whole-cell voltage clamp. After incubation for two hours in the presence of LSP5-2157 (2 $\mu$ M), we observed a marked reduction in the amplitude of the excitatory postsynaptic current (EPSC). This inhibition of EPSC was partially recovered when neurons were subsequently washed in medium for two additional hours (Figure 2a and b).

To determine kinetic of action of LSP5-2157, the drug (2  $\mu$ M) was acutely applied using fast-flow application in the recording chamber. The perfused neuron was stimulated with paired action potentials (25 ms interstimulus interval) every five seconds and evoked and miniature

EPSCs were recorded. A dramatic decrease in EPSC amplitude was observed throughout LSP5-2157 application. Within the first two minutes of drug application, the average EPSC amplitude dropped by approximately 50% ( $p < 0.001$ , 2-way ANOVA, Figure 2c). After 6 minutes of LSP5-2157 perfusion the amplitude reached approximately 10% of baseline ( $p < 0.001$ , 2 way ANOVA, Figure 2c).

Paired-Pulse ratio (PPR) is a mechanism reflecting changes in  $\text{Ca}^{2+}$ -dependent presynaptic glutamate release. It is calculated by measuring the increase in the response following application of a double stimulation separated by 40 ms. PPR was not different after 6 minutes in the presence or absence of LSP5-2157 thus suggesting that the drug did not interfere with presynaptic calcium current (Fig 2d).

We then recorded spontaneously released miniature EPSCs (mEPSCs) on autaptic preparations with only minimal stimulation (10 action potential of stimulation to establish baseline parameters rather than an action potential every 5 seconds for 6 minutes as in previous experiments). Spontaneous mEPSCs recorded from cells treated with LSP5-2157 showed a marked decrease in frequency (90% reduction,  $p < 0.001$ , 2 way ANOVA) and amplitude (34% reduction,  $p < 0.001$ , 2 way ANOVA) (Figure 2 e-g). Therefore, the inhibitory effect of LSP5-2157 appeared to be independent from stimulation. The magnitude of inhibition of mEPSC frequency was stronger than that of mEPSC amplitude. Altogether, these observations suggest that the presence of LSP5-2157 prevents the re-filling of SVs, therefore leading to the presence of empty vesicles and consequently, to a decreased frequency of mEPSC (Figure 2h). The limited effect of the inhibitor on the amplitude of mEPSC could be explained by the fact that some vesicles are only partially filled (not shown in the model Figure 2h).

Blue Evans was previously reported to inhibit kainate-evoked current by AMPA receptors (Keller et al., 1993; Price and Raymond, 1996). We therefore investigated whether the decreased mEPSC amplitude could be due to a direct effect of LSP5-2157 onto postsynaptic glutamate receptors. To assess this possibility, we measured the response to an AMPAR agonist, kainate (10  $\mu\text{M}$ ), before, during, and after the wash-in of LSP5-2157 (as shown in Figure 2i). There was no significant difference between the amplitude of the steady state current evoked by kainate application with relation to the presence or absence of LSP5-2157 (Figure 2j), although the EPSC decreased dramatically with LSP5-2157 wash-in (Figure 2k). Notably, the EPSC amplitude of LSP5-2157 exposed cells did not recover during the three-minute wash-out period although the kainate-evoked current remained stable (figure 2 l, m). The stability of the kainate response in the presence of LSP5-2157 suggests that the decrease in mEPSC amplitude was not related to inhibition of post-synaptic AMPA receptors. We then explored if LSP5-2157 could directly interfere with GABAergic transmission. LSP5-2157 (2  $\mu\text{M}$ ; 2 hrs) was unable to inhibit evoked inhibitory postsynaptic current (IPSC) of

GABAergic striatal autapses compared to control conditions (Figure 2n-r). Consistent with the lack of change in IPSC amplitude (Figure 2o), there was also no change in paired-pulse ratio (Figure 2p), or miniature IPSC frequency or amplitude (Figure 2q and r) following incubation with LSP5-2157. Taken together these results suggest that LSP5-2157 does not interact with ionotropic glutamate or GABA receptors.

### **LSP5-2157 inhibits synaptic and network glutamatergic transmission on hippocampal slices.**

The action of LSP5-2157 was then tested on an integrated neuronal system: hippocampal slices from adult (8 weeks) male C57Bl6 mice. To characterize the effects of LSP5-2157 on basic properties of spontaneous glutamatergic transmission, we first measured miniature EPSC (mEPSC) in CA1 pyramidal neurons (Figure 3a). The mEPSC frequency decreased by 25% after 30 to 45 min of LSP5-2157 application (2  $\mu$ M) (n=10 cells in 7 mice, p=0,001; Figure 3a, b). The mEPSC amplitude during the application of LSP5-2157 also exhibited a significant 15% decrease (n=10, p<0.05, Fig 3a, c). Therefore, LSP5-2157 significantly decreased spontaneous activity of glutamatergic neurons in hippocampal slices as expected for a VGLUT inhibition.

Input/output (I/O) curves of AMPA-mediated fEPSP were then constructed before and 20 min after application of LSP5-2157. We observe a significant decrease in the fEPSP slope suggesting a depressant effect of LSP5-2157 on AMPA receptors (n=20, paired t-test, p<0.001) (Figure 3d top). However, afferent fibers volleys (FV), representing the course of action potentials on axons coming from hippocampal CA3 area were also depressed by the drug, in the same magnitude than fEPSP. Besides, the ratio fEPSP slope/FV slope was unchanged by the drug (n=20, paired student t-test, p=0. 5) (Fig 3d bottom). This data suggests that LSP5-2157 had a low and nonspecific depressing effect on synaptic efficacy. Furthermore, LSP5-2157 (2 $\mu$ M) had no effect on NMDA-R- mediated fEPSP (fNMDA) (n=8, p=0.45, paired student t-test) (Fig. 3e). These data suggest that the low and global depressing effect of LSP5-2157 on synaptic efficacy in hippocampal slices is not due to its activity on AMPA/Kainate or NMDA receptors but on VGLUT action.

PPF was not statistically altered by LSP5-2157 (facilitation of  $142.4 \pm 4.9\%$  before and  $144.7 \pm 7.2\%$  30 min after LSP5-2157 application) (Fig 3f). This observation suggests that as in autapse, LSP5-2157 has no effect on  $\text{Ca}^{2+}$ -dependent presynaptic glutamate release.

We then inspected the effect of LSP5-2157 on hippocampal synaptic plasticity. The plasticity induced by Theta-Burst Stimulation (TBS) protocol was not statistically altered by application of LSP5-2157 ( $119.8 \pm 4.9\%$  in controls, n=11 slices in 10 mice and  $109.7 \pm 6.0\%$  in the presence of 2  $\mu$ M LSP5-2157, n=10 slices) (F(1,18)=1.55, p=0.22, ns) (Fig 3g). This suggests that the readily releasable pool of glutamate was not affected by LSP5-2157.

When synapses were strongly activated by a 3x100Hz protocol, the resulting LTP was statistically impaired by LSP5-2157 ( $131.6 \pm 9.8\%$  in controls,  $n=12$  slices in 11 mice, vs  $100.1 \pm 4.0\%$  in the presence of LSP5-2157,  $n=10$  slices in 9 mice) ( $F(1,20)=7.9$ ,  $p=0.01$ ) (Fig 3h). These findings on synaptic plasticity suggest that LSP5-2157 is more effective when the glutamatergic synapse is strongly stimulated.

### **LSP5-2157 inhibits compound action potential of the auditory nerve**

The inner hair cells of the cochlea use glutamate as a neurotransmitter. In inner hair cells synaptic vesicles are filled with glutamate by the atypical vesicular glutamate transporter type 3 (VGLUT3) (Ruel et al., 2008; Seal et al., 2008). The capacity of LSP5-2157 to inhibit synaptic transmission was evaluated in guinea pig cochlea *in vivo*.

The initial perfusion of artificial perilymph did not induce significant changes in Compound Action Potential (CAP) amplitude and its, N1 latency, CM or SP (data no shown). The drug effects were therefore compared with the cochlear potentials recorded after the artificial perilymph perfusion. Whatever the intensity of sound stimulation, LSP5-2157 reduced the CAP amplitude and increased its latency measured at the first negative wave N1 in a dose-dependent manner (Figure 4a). The first significant ( $p<0.05$ ) effects on CAP amplitude occurred at 1 mM LSP5-2157. In contrast, no changes in CM and SP (Figure 4b) were observed at all tested doses. The CAP amplitude and the N1 latency partially returned to control values after 2 hours rinsing LSP5-2157 out of the cochlea with artificial perilymph (data no shown). We averaged the CAP amplitudes across the intensities (100–40 dB) to give an overall average CAP amplitude change for each concentration of drug tested. The mean amplitudes were then expressed in percent, the AP control being 100%. The dose-responses were fitted to a curve using a non-linear least-square logistic equation (Figure 4e). LSP5-2157 had a visible effect at 300 $\mu$ M and its calculated IC<sub>50</sub> was 0.93 mM. Therefore LSP5-2157 inhibits glutamatergic transmission *in vivo* in the cochlea.



## Discussion

In the present study, we described new VGLUTs inhibitors. Our best compound, LSP5-2157, is structurally analogous to Trypan Blue and was used to investigate its mode of action upon glutamatergic neurotransmission from *in vitro*, to *ex vivo* and to *in vivo* integrated level. Its activity was evaluated not only in vesicular uptake of [<sup>3</sup>H]glutamate but also upon glutamatergic transmission in autaptic hippocampal neurons and hippocampal slices. In addition, we show for the first time that this VGLUT inhibitor can be of interest to inhibit audition.

VGLUTs belong to the SLC17 superfamily and little is known concerning their 3D structure (Almqvist et al., 2007, Pietrancosta, 2012). The 3D homology model build in cytosol open conformation reveals a large binding pore with three successive binding regions: a hydrophilic layer, a hydrophobic layer and another hydrophilic layer. According to this model, the features required for potent inhibition are: two polar moiety linked by a hydrophobic scaffold as observed with known competitive ligands (Pietrancosta et al., 2010; Tamura et al., 2014). Interestingly, as glutamate, small polar ligands such as short Trypan Blue analogs or Xanthurenic acid or other quinolone have low affinity for VGLUT (Carrigan et al., 2002). The structural comparison of a potent ligand confirmed that VGLUTs have a large degree of tolerance for the polar scaffold as illustrated by the potency of Brilliant Yellow, Trypan Blue, Evans Blue or LSP5-2157. Nevertheless, the high polarity required for potent inhibition induced a low membrane permeation as observed in *ex vivo* experiments with hippocampal slices. Indeed, concentrations used to inhibit the amplitude of fEPSPs in the CA1 region of the hippocampus with Brilliant Yellow or Chicago Sky Blue are respectively of 200μM and 300μM (Neale et al., 2014).

LSP5-2157 in addition to efficiently inhibit glutamate vesicular accumulation also demonstrated the capacity to decrease excitatory transmission in hippocampal autapses and slices at relatively low concentrations. Our results are well in line with previous finding showing that VGLUTs inhibitors (xanthurenic acid, Chicago Sky Blue, Congo Red and Rose Bengal) also blocked glutamatergic transmission of prefrontal and hippocampal neurons at high doses (Neale et al., 2014; Neale et al., 2013). One simple explanation for this inhibition could be that decreasing the vesicular content in glutamate and/or the number of glutamate-filled vesicles impacts on glutamatergic release and hence on synaptic transmission. Previous studies found that submicromolar concentrations of Evans Blue are able to inhibit Kainate or glutamate-evoked current in human embryonic kidney 293 (HEK) cells or *Xenopus Laevis* oocytes (Keller et al., 1993; Price and Raymond, 1996). Evans Blue is an analog of Trypan Blue and of LSP5-2157. However, in our conditions, LSP5-2157 was not able to reduce Kainate-induced current on hippocampal autapses thus putatively ruling out a

direct action of the compound on postsynaptic glutamate receptor. Furthermore, LSP5-2157 application had no effect GABAergic striatal autapses. Another alternative explanation could be an indirect inhibition of glutamatergic current through metabotropic Glu receptors (mGLUR). Indeed, mGluR modulator such as LY354740 efficiently block fEPSC in the hippocampus (Neale et al., 2013). However, LSP5-2157 neither activates nor inhibits mGluR activity in HEK transfected cells at active concentrations. (Supplementary table 1 and 2) Taken together our data suggest that in the concentration range used for these experiments LSP5-2157 acts preferentially on VGLUTs. However, further experiments will be necessary to fully establish this point. In addition to its effect on basal electrophysiological properties, LSP5-2157 was able to disturb the hippocampal network in *ex vivo* slices. Synaptic plasticity induced by theta burst stimulation was not depressed by application of LSP5-2157 suggesting that the readily releasable pool of synaptic vesicles was not preferentially affected by LSP5-2157. In contrast, LTP induced by a saturating stimulation (3x100Hz) was statistically lower in the presence of LSP5-2157. This suggests that LSP5-2157 is more effective when glutamatergic synapses are strongly solicited. Taken together these observations could imply that in an active terminal, LSP5-2157 act differently on various synaptic pools. Identifying which pools are more vulnerable to LSP5-2157 will be the subject of future investigations.

Finally, LSP5-2157 was shown to be efficient in physiological condition in the cochlea to inhibit auditory transmission. This promising finding open the way to *in vivo* usage of VGLUTs inhibitors. Therefore, VGLUTs ligands modulate glutamatergic neurotransmission in various preparation as illustrated here. This could represent a crucial opening for the therapeutic strategy targeting glutamatergic systems. However, to inhibit VGLUTs on synaptic vesicles LSP5-2157 has a 50nM EC<sub>50</sub>,  $\approx$  1 $\mu$ M on isolated neurons, 1-10 $\mu$ M on hippocampal slices and 1mM in the intact cochlea. These data demonstrate that the efficacy of VGLUTs inhibitors to penetrate in complex tissues should be improved if we wish to use these compounds to target VGLUTs *in vivo*.

## Figure legends

### Figure 1 Pharmacology of new VGLUTs Inhibitors.

Capacity of compounds to block VGLUTs glutamate uptake. IC<sub>50</sub> was only determined for compounds with more than 50% of inhibition at a 1  $\mu$ M cut off. **(a)** Table of inhibitory activity of new compounds and related analogs described in Favre-Besse et al. **(b)** Inhibition curves of newly synthesized compounds and their relative shorter analogs **(c-e)** Docking of LSP5-2157 into a homology model of VGLUT1 shows that compound can interact with the hydrophobic layer (green residues) and the two hydrophilic layers (red residues). The binding mode of compounds determines their ability to inhibit glutamate uptake triggered by VGLUT. **(f)** Trypan Blue analog, LSP5-2157 fully enters into the VGLUT pore and can interact with residues of the different layers. **(g)** Structure of LSP5-2157 **(h)** LSP5-157 can interact with residues in High Affinity Hydrophilic Layer (HAHL) such as H360 and R93 while smaller molecules remain at the VGLUT surface.

### Figure 2 LSP5-2157 and hippocampal autapses.

**(a)** Representative traces of hippocampal neurons incubated in 2  $\mu$ M LSP5 for 2 hours (Blue), in NBA for at least 4 hours (black), and “washout” neurons incubated in 2  $\mu$ M LSP5 for 2 hours and then in NBA for at least 2 hours (Red). **(b)** Average EPSC amplitude for each of the three treatments, normalized to average amplitude in NBA. EPSC amplitude ( $p < 0.05$ , 1-way ANOVA with Tukey Multiple comparison). The normalized results shown are from a single culture of hippocampal neurons, though inhibition and partial washout were stably demonstrated across multiple cultures. (NBA,  $n = 8$ , LSP5,  $n = 13$ , washout,  $n = 11$ ). **(c)** Within-cell wash in of LSP5-2157. Autaptic neurons on microdots were stimulated with paired-pulses (25 ms ISI) at 0.2 Hz throughout the protocol. An average of EPSCs recorded during the first minute in control external solution was considered the baseline. After baseline measurements, LSP5-2157 (2  $\mu$ M) was applied to the inhibited group for 6 minutes while cells were stimulated with paired-pulses at 0.2 Hz. Kynurenate was applied for a 30 seconds interval after 3 minutes to allow for the subtraction of noise from the mEPSC analysis. In the control group, cells were stimulated in control external solution, in the inhibited group, cells were washed with external solution containing LSP5-2157 (2  $\mu$ M) immediately following baseline for the next 6 minutes. Data points are binned averages by minute normalized to the baseline average amplitude for that cell. (LSP5-2157,  $n = 8$ ; control,  $n = 8$ ;  $p < 0.001$  Bonferroni post-tests in 2 way ANOVA with LSP5-2157 as the treatment group; ANOVA was significant for treatment, time, and interaction). **(d)** Normalized PPR after min 6 of wash-in with either control external solution or external solution with 2  $\mu$ M LSP5-2157. **(e)** Sample mEPSC traces from the same cell at baseline (upper), or during minute 6 of wash-in with 2

$\mu\text{M}$  LSP5-2157 (lower). **(f, g)** Normalized mEPSC frequency (f) and amplitude (g) of events detected during minute 6 of wash-in of either control solution or LSP5-2157. Frequencies and amplitudes shown are corrected for noise contamination by subtraction of the amplitude and/or frequency of events detected during application of kynurenic acid (LSP5-2157,  $n=6$ ; control,  $n=5$ ) ( $p<0.001$  2-way ANOVA with Bonferroni post-test). Results shown are from two different cultures, all normalized within cell. **(h)** Vesicular model explaining the results obtained in e-g.

**(i)** Schematic of kainate application experiment. **(j)** Bar graph of steady-state kainate amplitudes during baseline, LSP5-2157, and washout periods. **(k)** Normalized EPSC in parallel with the kainate application and LSP5 wash in and wash-out. **(l)** Sample kainate application traces from the same cell. **(m)** sample EPSC traces during kainate application experiment. **(n)** Example recording of IPSCs evoked in striatal GABAergic neurons incubated in either fresh NBA medium (black) or NBA medium with 2  $\mu\text{M}$  LSPS (gray). **(o)** Average IPSC amplitudes recorded from striatal neurons incubated in NBA ( $n = 18$ ) or LSPS ( $n = 19$ ). **(p)** Cells were stimulated with a pair of pulses (ISI 100 ms), and the resulting average paired-pulse ratios (IPSC2/IPSC1) are shown ( $n = 18$ ) and LSPS ( $n = 14$ ) Cells that consistently had no detectable first response in a paired-pulse protocol were excluded from paired pulse ratio calculations. **(q)** Average mIPSC amplitude (bicuculine subtracted; LSPS,  $n = 16$ ; NBA,  $n = 17$ ) **(r)** Average frequency of mIPSC events (bicuculine subtracted; LSPS,  $n = 19$ , NBA,  $n = 17$ ). Cells with excessive noise were excluded from all mIPSC evaluation, and cells with a frequency of 0 Hz were included in the mIPSC frequency results but excluded from mIPSC amplitude results. Results shown are from the recordings of two cultures.

### Figure 3 Effect of LSP5-2157 hippocampal slices

**(a)** Representative traces of mEPSC recordings before (top) and after (bottom) application of LSP5-2157 (2  $\mu\text{M}$ ,  $n=10$ ). LSP5-2157 decreased frequency **(b)** and amplitude **(c)** of mEPSC. **(d)** LSP5-2157 (2 $\mu\text{M}$ ) depressed the fEPSP slopes by 15 to 28% depending on stimulus intensities (top). FV slopes were also depressed by the drug, so the ratio fEPSP slope/FV slope was comparable (bottom). **(e)** (Top) Sample traces of evoked NMDA-R-mediated fEPSP (fNMDA) recorded in control condition, in presence of LSP5-2157 (2  $\mu\text{M}$ ,  $n=8$ ) or after addition of the NMDAR antagonist APV (20  $\mu\text{M}$ ) at different stimulus intensities. (Bottom), Mean curves of the ratio fNMDA slope /fiber volley slope as a function of stimulus intensities in control conditions or in the presence of LSP5-2157 ( $n=8$ ). They are not statistically modified by application of LSP5-2157 (Paired Student  $t$ -test,  $p=0.45$ ). **(f)** PPF of synaptic transmission induced by paired-pulse stimulation with inter-stimulus intervals of 40 ms was unchanged by LSP5-2157. **(g)** The plasticity induced by Theta-Burst Stimulation (TBS) was not statistically changed by application of LSP5-2157 (2  $\mu\text{M}$ ) suggesting that the readily

releasable pool of glutamate was not affected by the drug (Control, n=11, LSP5-2157, n=10). **(h)** In contrast, a 3x100Hz LTP was markedly reduced by LSP5-2157. For **g** and **h**, LSP5-2157 was applied 15 min before stimulation and throughout recordings. \*\*p< 0.01 repetitive measure ANOVA.

**Figure 4: Effect of LSP5-2157 on cochlear and auditory nerve potentials.**

**(a)** CAP amplitude, **(b)** N1 latency, **(c)** CM and **(d)** SP amplitude evoked by 8 kHz tone-bursts. Graphs are obtained after the perfusion of artificial perilymph containing 0  $\mu$ M (gray circles), 1 $\mu$ M (green circles), 10 $\mu$ M (blue circles), 300 $\mu$ M (rose circles), 1000 $\mu$ M (red circles) and 2000 $\mu$ M (black circles) of LSP5-2157. All points represent mean  $\pm$  sem values calculated from seven animals. **(e)** Dose–response curves obtained with LSP5-2157. Dose–responses curves were fitted to a curve using a non-linear least-square logistic equation. The IC50 value was 930  $\mu$ M.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**Authors Contributions:** LEM, MAH performed experiments and analyzed data. MAH, CR designed experiments. OP, MAK, MK, BP, SM, JW, FCFB, YL, HOB, PD, NP performed experiments. FA, JLP, BG, JE, PD, SD, SEM, NP designed the experiments. NP, PD and SEM wrote the paper with the help of LEM, MAH, FA, JPP, JLP, BG, JE, CR and SD.

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