Supporting information

Qualification of LSP1-2111 as a Brain Penetrant Group III Metabotropic Glutamate Receptor Orthosteric Agonist

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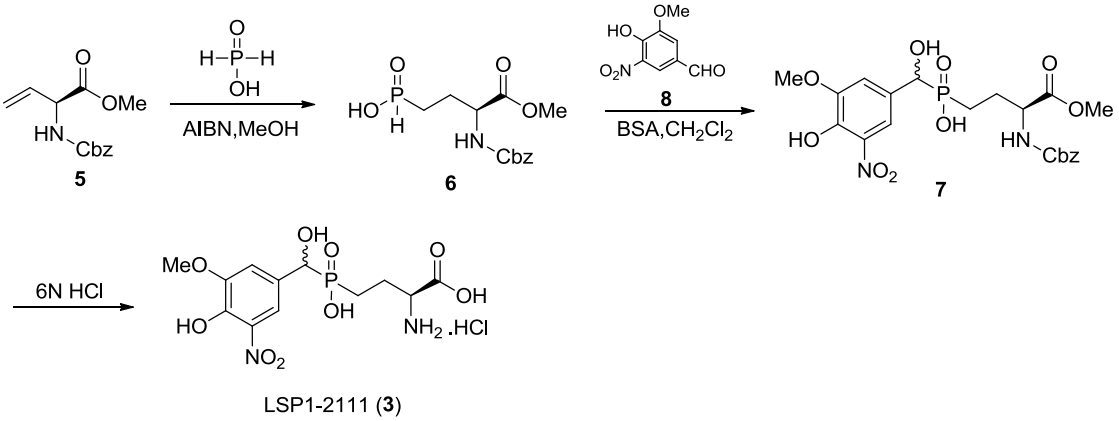
1. Synthesis and characterization of LSP1-2111

LSP1-2111 was synthesized according to Scheme S1, as reported in Selvam C. *et al.* (*J. Med. Chem.* **2010**, *53*, 2797-813):



Scheme S1. Synthetic sequence for the preparation of LSP1-2111.

Step 1. Procedure for the preparation of (*S*)-3-(((benzyloxy)carbonyl)amino)-4-methoxy-4-oxobutyl)phosphinic acid (**6**). To a solution of compound **5** (5 g, 20 mmol) and hypophosphorous acid (13.2 g, 100 mmol) in MeOH (50mL) was added α,α'-azobisisobutyronitrile (AIBN, 162 mg, 1mmol) under N_2 atmosphere. The resulting mixture was heated to 80°C for 6 h. After cooling to room temperature, the solvent was removed. The residue was diluted with water and extracted with EtOAc (3 × 20 mL). The combined organic layers were dried over Na_2SO_4 and concentrated. The residue was purified by preparative HPLC to give compound **6** (4.0 g, yield: 63.2%). **^{1}H NMR** (CD_3OD, 400MHz) δ7.25-7.17 (m, 5H), 4.99 (s, 2H), 4.18-4.03 (m, 1H), 3.62 (s, 3H), 2.08-1.95 (m, 1H), 1.84-1.67 (m, 3H).

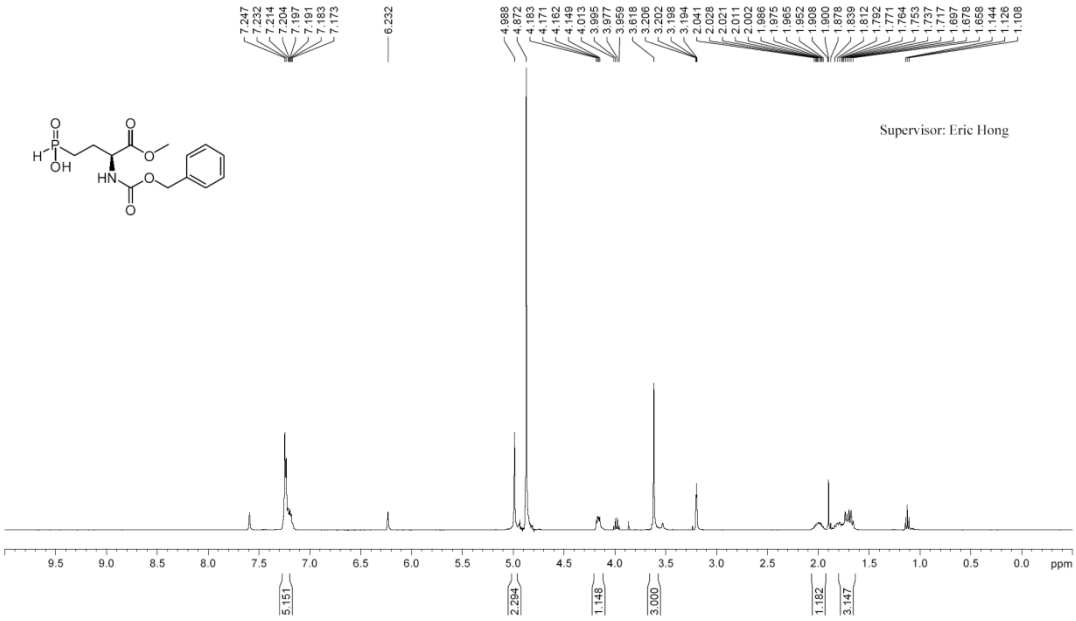


Chart S1. H-1 NMR spectrum of compound 6.

Step 2. Procedure for the preparation of ((*S*)-3-(((benzyloxy)carbonyl)amino)-4-methoxy-4-oxobutyl)(hydroxy(4-hydroxy-3-methoxy-5-nitrophenyl)methyl)phosphinic acid (**7**).

To a mixture of compound **6** (9 g, 28.6 mmol) and compound **8** (16.9 g, 85.7 mmol) in CH_2Cl_2 (300 mL) was added drop-wise N,O-bis(trimethylsilyl)acetamide (51.3 mL, 200.2 mmol) at 0°C under N_2 atmosphere. The mixture was warmed to room temperature and stirred overnight. Then, 1N HCl (200 mL) was added at 0°C. The resulting mixture was extracted with EtOAc (50 mL×3). The combined organic layers were concentrated and the residue was recrystallized from EtOAc/petroleum ether to give compound **7** (9 g, yield: 61.5%). **^{1}H NMR** (CD_3OD 400MHz) δ7.57 (s, 1H), 7.30-7.10 (m, 6H), 4.96 (s, 2H), 4.75-4.70 (m, 1H), 4.19-4.05 (m, 1H), 3.80 (s, 3H), 3.58 (s, 3H), 2.11-1.90 (m, 1H), 1.85-1.65 (m, 3H). The compound was obtained as a 1:1 mixture of diastereoisomer benzylic alcohols.

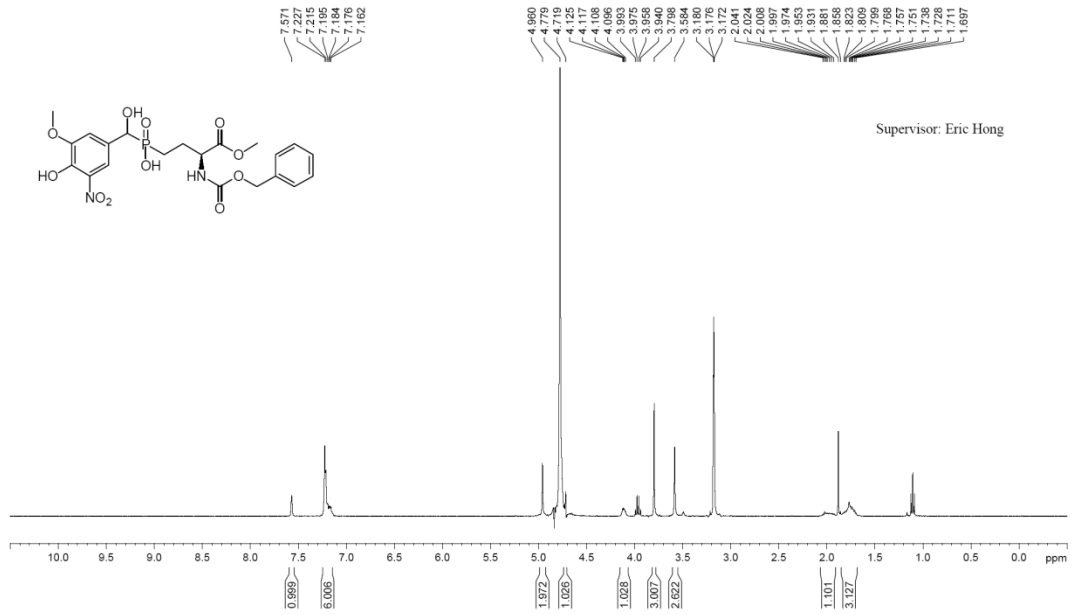


Chart S2. H-1 NMR spectrum of compound 7.

Step 3. Procedure for the preparation of (2*S*)-2-amino-4-(hydroxy(hydroxy(4-hydroxy-3-methoxy-5-nitrophenyl)methyl)phosphoryl)butanoic acid (LSP1-2111, **3**).

A mixture of compound **7** (10 g, 20 mmol) in 6N HCl (50 mL) was heated to 110°C overnight. After cooling to room temperature, the solvent was removed. The residue was purified by preparative HPLC (HCl as buffer) to yield compound **3** (2.74 g, yield: 35.1%). **^{1}H NMR** (DMSO-d_6 400MHz) δ8.50 (s, 3H), 7.49 (s, 1H), 7.32 (s, 1H), 4.85 (d, J= 8.0 Hz, 1H), 4.09-3.95 (m, 1H), 3.86 (s, 3H), 2.20-1.60 (m, 4H). The compound was obtained as a 1:1 mixture of diastereoisomer benzylic alcohols.

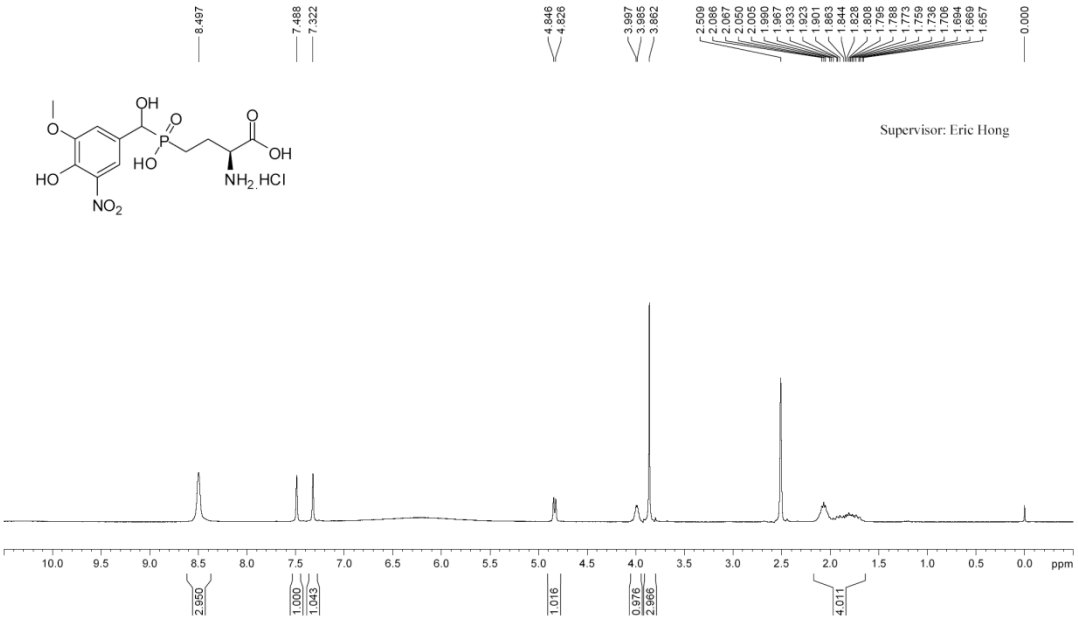
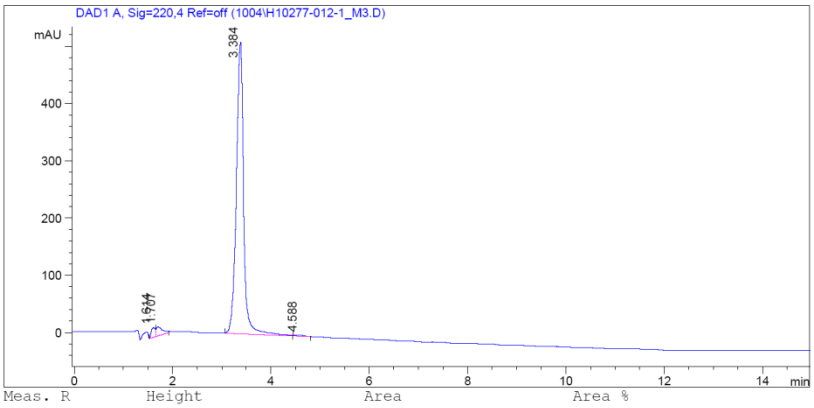


Chart S3. H-1 NMR spectrum of compound 3.

HPLC analysis

Instrument: Shimadzu LC-20AB, Column: Atlantis HILIC Silica150*4.6mm, 5um, Mobile Phase: 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B), using the elution gradient 90%-60% (solvent B) over 10 minutes and holding at 60% for 5 minutes at a flow rate of 1.5 ml/minutes; RetentionTime:3.384min; Wavelength:220nm.

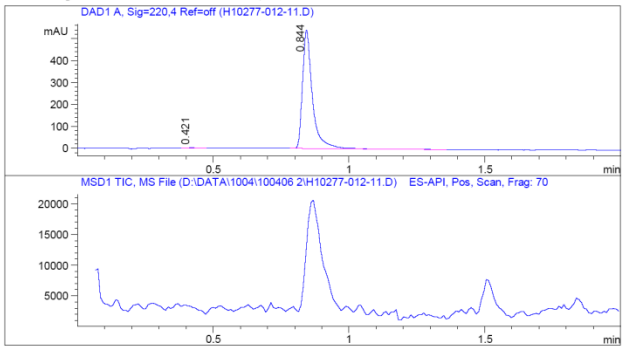


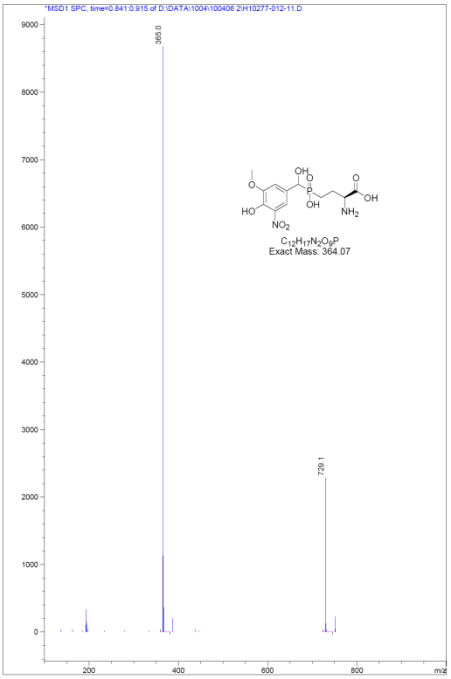
Meas. R	Height	Area	Area %
1.614	17.040	89.823	1.639
1.707	16.177	144.227	2.632
3.384	509.687	5226.618	95.382
4.588	1.719	18.974	0.346
Total:	544.624	5479.642	100.000

Chart S4. HPLC trace of compound 3.

LC-MS analysis

A:Shim-Pack XR-ODS 3.0*30mm,SN:80531084
B:XBrige Shield RP18 2.1*50mm,SN:01113830313306





Report

Signal 1 : DAD1 A, Sig=220,4 Ref=off

#	Meas. Ret.	Height	Width	Area	Area %
1	0.421	3.724	0.026	6.511	0.474
2	0.844	538.445	0.038	1367.346	99.526

Chart S5. LC trace and mass spectrum obtained during the HPLC analysis of compound 3.

2. Time course for brain and plasma exposures of LSP1-2111 administered *via* IP route in rat

Preliminary studies exploring plasma and brain exposure of LSP1-2111 were conducted using IP administration in order to recapitulate work previously reported in the literature. However, our work (see graph S1) indicated that the exposures obtained using the IP route of administration, while roughly equivalent in magnitude and the rate of disappearance, were significantly more variable than what was determined in subsequent studies using the SC route (as shown in manuscript). Thus, for subsequent rat studies exploring CNS disposition of LSP1-2111, the SC route of administration was used.

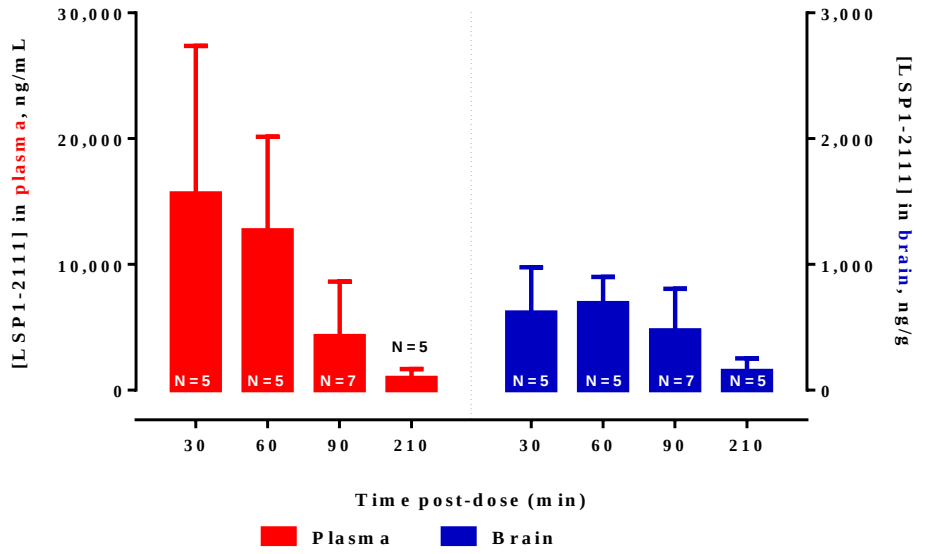


Figure S1. Time course of plasma and brain exposure profiles of LSP1-2111 (10 mg/kg dose) in rat following IP administration. Magnitude of the error bars (SD) showing a significantly higher variability than the results from SC administration studies shown in the manuscript.

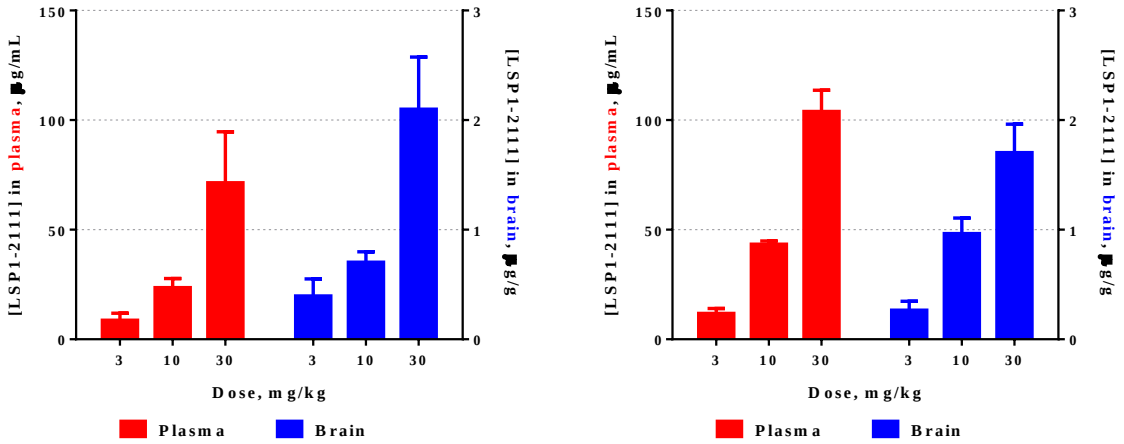


Figure S2. Plasma and brain exposures at 10 min (left) and 20 min (right) post-dosing of LSP1-2111 in rat following IP administration at 3, 10 and 30 mg/kg doses (N=4 in each group).

3. Tables with figures corresponding to microdialysis studies

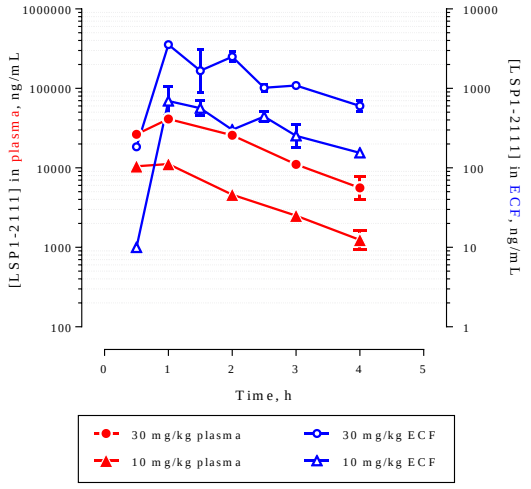


Figure 1. Rat plasma and ECF dialysate concentration time profile of LSP1-2111 following subcutaneous (SC) administration at 10 and 30 mg/kg. The ECF concentration values have been corrected for recovery loss based on the 96% *in vivo* probe recovery under the same flow rate.[23]

Table S1. Plasma and ECF concentrations in the microdialysis studies corresponding to Figure 1.

Time (h)	Dose			
	10 mg/kg		30 mg/kg	
	Plasma (µg/mL)	ECF (µg/mL)	Plasma (µg/mL)	ECF (µg/mL)
0.5	10.5	0.01	26.5	0.18
1	11.2	0.70	41.4	3.5
1.5	---	0.56	---	1.7
2	4.6	0.30	25.7	2.5
2.5	---	0.44	---	1.0
3	2.5	0.25	11.1	1.1
4	1.2	0.16	5.6	0.6

4. Numeric data for Figure 2

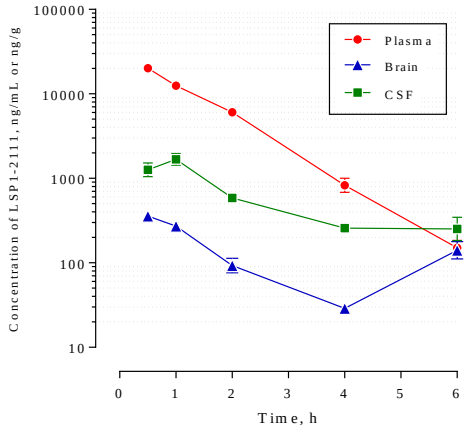


Figure 2. Plasma, brain and CSF concentration time profile study of LSP1-2111 upon subcutaneous (SC) administration at 10 mg/kg. Standard error bars larger than marker size are only shown.

Table S2. Plasma, brain and CSF concentrations corresponding to Figure 2.

Time (h)	Plasma (µg/mL)	Brain (µg/g)	CSF (µg/mL)
0.5	20.1	0.36	1.3
1	12.5	0.27	1.7
2	6.0	0.09	0.59
4	0.83	0.03	0.26
6	0.15	0.14	0.25

5. Numeric data for Figure 3

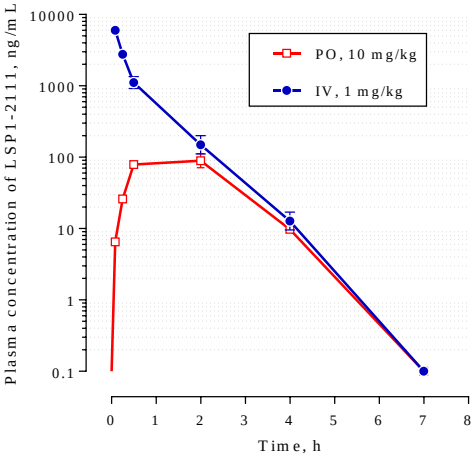


Figure 3. Plasma concentration time profile of LSP1-2111 after oral administration in SD rats (N=2). Standard error bars larger than marker size are only shown.

Table S3. Plasma concentrations in the pharmacokinetic study shown in Figure 3.

Time (h)	IV (1 mg/kg) Plasma (μg/mL)	PO (10 mg/kg) Plasma (ng/mL)
0.083	6.0	7
0.25	2.8	26
0.5	1.1	79
2	0.15	90
4	0.013	10
7	0.001	0.1

6. Quantitative microdialysis studies

Experiments used male SD rats. Animals were housed in a temperature-controlled vivarium with free access to food and water. All procedures with the animals were approved by the local IACUC. Each animal to be used for microdialysis experiments was placed into a stereotaxic frame and guide cannulas were implanted into the medial prefrontal cortex. Animals were allowed to recover for 5-7 days. On the day of the experiment, MetaQuant (BrainLink, The Netherlands) microdialysis probes (polyarylethersulphone (PAES) membrane, 4 mm length) were inserted through the cannulas, connected to the pump and allowed to stabilize for 2 h. *In vitro* probe recovery for MetaQuant probes for LSP1-2111 was 96 ± 4.0%. The dialysis probes were perfused with 0.2% (w/v) bovine serum albumin (BSA) dissolved into a filtered Ringer buffer containing 147 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, and 1.2 mM $MgCl_2$. Ultra-slow dialysis was performed using flow rate of 0.2 μL/min. Dialysate was diluted with the incoming flow of 1.8 μL/min, resulting in total flow rate of 2 μL/min. 30-minute samples were collected for 4 h in chilled glass tubes using refrigerated fraction collector and then analyzed using a quantitative LC/MS/MS method. Ultra-slow dialysis raises the extraction efficiency of the probe. Ultra-slow perfusion with a flow rate of 0.2 μL/min allows the annulus fluid to approach the equilibration with the adjacent tissue at the outflow and result in 100% recovery rate in vivo. In our study we achieved 96% *in vivo* recovery and all values were adjusted using this factor. This approach has been widely used in preclinical and clinical applications.

7. Bioanalysis of LSP1-2111 in tissue samples

Frozen rat brains were weighed and homogenized in 3× (weight/volume) of brain homogenization buffer consisting of 50% water, 30% 2-propanol and 20% DMSO. A 150 μL internal standard solution (Lu AA34745, a compound from an internal project, in 80% and 20% acetonitrile and DMSO respectively) was added to 50 μL homogenized brain/plasma sample. The samples were mixed and centrifuged. The supernatant was injected directly into the Thermo Finnigan Quantum Ultra LC/MS/MS system for analysis. A standard curve (0-4000 ng/mL) was generated in rat brain homogenates to de-

termine the concentrations of LSP1-2111 in the brain. A standard curve (0-1000 ng/mL) was generated in rat plasma to determine the LSP1-2111 concentration in plasma samples (Tables S1 and S2, supplementary material).

8. HPLC and mass spectrometry conditions for the bioanalysis of compound 3 in plasma, brain, CSF and ECF.

Table S4. HPLC experimental conditions for the analysis of compound 3.

HPLC Experimental Conditions	
System	Spark-Holland HPLC
Eluting Column	Atlantis T3 2.1×30mm 3µ (Waters Corp)
Pump Conditions	
Injection volume	10 µL
Mobile Phase A	0.1 % formic acid in water
Mobile Phase B	0.01% formic acid in acetonitrile

Step	**Time(min)**	**mL/min**	**% A**	**% B**
1	00:10	0.2	95	5
2	00:12	0.3	95	5
3	01:40	0.3	50	50
4	01:50	0.2	5	95
5	02:00	0.2	5	95
6	04:00	0.2	5	95
7	04:10	0.5	95	5
8	04:35	0.5	95	5
9	04:55	0.3	95	5
10	05:00	0.2	95	5

Table S5 Mass spectrometry conditions for the analysis of compound 3.

Mass Spectrometer Experimental Conditions	
System	Thermo Finnigan TSQ Quantum Ultra LC/MS/MS
Scan mode	SRM
Acquisition type	Centroid
Ionization mode	Heated Electrospray (positive)
Source Conditions	
Spray voltage	3000 V
Vaporizer Temperature	300°C
Sheath gas pressure	10
Ion Sweep Gas	0
Aux gas pressure	5
Capillary temperature	325 °C
Collision gas pressure	1.0 mTorr
MS1 Res	0.70
MS3 Res	0.70

Compound	**SRM Transition**	**Tube Lens**	**Collision Energy**
LSP1-2111	365.25 → 56	95	21
	365.25 → 122	95	19
	365.25 → 301	95	19
	365.25 → 347	95	14
Lu AA34745*	552.32 → 203	130	50

*Lu AA34745 = *N*-[3-(1-{3-[2,2-bis(4-fluorophenyl)acetylamino]propyl}(4-piperidyl))-4-fluorophenyl]-2-methylpropanamide

9. Studies reported in the literature using LSP1-2111 in preclinical models.

Table S6. Doses of LSP1-2111 tested in preclinical models (as reported in the referenced literature).

Reference	Species	Dosing conditions*	Model
3; Beurrier *et al.*	Rat	1, **15** and 25 mg/kg, IP	Haloperidol-induced catalepsy
8; Lopez *et al.*	Mouse	**30** mg/kg, IP	6-OHDA unilateral lesions (sub-chronic)
9; Wierońska *et al.*	Mouse	**2** and **5** mg/kg, IP	Elevated plus-maze test
	Mouse	0.75, **2** and **5** mg/kg, IP	Stress-induced hyperthermia
	Mouse	1, 2.5, 5 and 10 mg/kg, IP	Tail suspension test
	Mouse	1 and 5 mg/kg, IP	Forced swim test
10; Wierońska *et al.*	Mouse	5 mg/kg, IP	Locomotor activity of habituated mice
	Mouse	5 mg/kg, IP	Spontaneous locomotor activity
	Mouse	0.3, **1**, **2**, and **5** mg/kg, IP	MK-801-induced hyperactivity
	Mouse	0.3, **1**, **2**, and **5** mg/kg, IP	Amphetamine-induced hyperactivity
	Mouse	0.3, **1**, **2**, and **5** mg/kg, IP	Head twitch test
11; Wierońska *et al.*	Rat	0.5, **2**, and **5** mg/kg, IP	MK-801-induced deficits in social interaction
	Rat	0.5, 2, and **5** mg/kg, IP	Novel object recognition
	Mouse	0.3, **5** mg/kg, IP	MK-801-induced hyperactivity
	Mouse	0.3, **5** mg/kg, IP	Amphetamine-induced hyperactivity
	Mouse	0.3, **5** mg/kg, IP	Head twitch test
12; Davis *et al.*	Mouse	**2.5**, **5** and 7.5 mg/kg, IP	Acquisition and extinction of cued fear

**Doses in red are efficacious in the corresponding tests.*