Prefrontal cortex extracellular matrix plasticity and conditioned inhibition of pyramidal cells facilitates relapse to heroin seeking

Supplementary Materials and Methods, Tables and Figures

Heroin SA, abstinence, extinction and reinstatement tests

Acquisition: Heroin self-administering rats were implanted with jugular vein catheters and allowed to recover from surgery for one week. Rats were trained to self-administer heroin (100 μg/kg, diacetylmorphine-HCL, OPG, The Netherlands) in 15 daily 3 h sessions (Schmidt ED et al., 2005). Acquisition of heroin SA in standard operant chambers (Med Associates) started with a fixed ratio 1 (FR1, number of nose-pokes to obtain one infusion) schedule of reinforcement for 10 sessions. A house light and a red cue light above the active nose poke hole were turned on at the beginning of each session and indicated availability of the drug. Nose pokes in the active hole resulted in a 2-s heroin infusion simultaneously switched with a 2-s audio cue (Sonalert 2900 Hz Tone Module, Med Associates) and a yellow cue light placed inside the active hole. This event was followed by a time-out period of 15 s, during which nose pokes were ineffective and the house light and the red cue light were turned off. When rats developed a stable preference for the active hole, response requirement was increased to a FR2 (2 sessions) followed by a FR4 (3 sessions) schedule of reinforcement. Successful acquisition was acquired when nose-poking in the active (heroin-paired) hole increased with each increase in the FR schedule. The criterion for successful acquisition was defined as the amount of nose pokes in the active hole being fourfold more than in the inactive hole, and there being subsequent increases in nose pokes with each increase in the FR schedule. Subsequently, rats were divided into two groups and underwent either abstinence for a period

of 21 days (home cage) or 15 once daily extinction sessions (1 h) spanning a drug abstinence time frame of 21 days (Schmidt ED *et al.*, 2005).

Extinction: Extinction sessions were conducted for 60 min once daily for 5 days a week (15 sessions). During the extinction period, heroin was not available and the cues previously associated with heroin (the house light and red cue light) and that associated with the actual heroin infusions (compound audiovisual cues) were not presented. Extinction was considered successful when nose poking was less than ten nose pokes per session (extinction criterion), as was obtained after 10-11 sessions.

Reinstatement: On the test day, extinction trained rats were placed in the operant cages with the house light and red cue light turned on and nose poking resulted in the presentation of the discrete compound audiovisual cues (but no heroin infusion) on an FR4 schedule. Control animals underwent an additional extinction session. For the proteomics experiments, recording of nose-poke responses in the active and inactive hole was measured until 30 min after start of the test.

Synaptic membrane isolation:

Pools of dissected brain tissue were homogenized in ice-cold 0.32 M sucrose buffer at pH 7.4 and centrifuged at 1,000 x *g* for 10 min. Supernatant was loaded on top of a discontinuous sucrose gradient consisting of 0.85 M and 1.2 M sucrose. After centrifugation for 2 h at 110,000 x *g*, the synaptosomal fraction at the interface of 0.85 M and 1.2 M sucrose was collected and lysed in hypotonic solution. The resulting synaptic membranes were recovered by centrifugation using the discontinuous sucrose gradient as described above. The synaptic membrane fraction was collected from the 0.85 M/1.2 M interphase and protein concentrations were determined using a Bradford assay (Bio-Rad). For each sample, 100 µg

of protein was used for iTRAQ^{TM} labeling. Finally, synaptic membrane fractions were dried in a speedvac overnight (ON).

Protein digestion and iTRAQ labeling:

Synaptic membranes were resuspended in 28 µl of dissolution buffer and 2 µl cleavage reagent (iTRAQ reagent kit, with 0.85% RapiGest (Waters, Milford, MA) to solubilize synaptic membranes. After incubation for 1 h, 1 µl of cys blocking buffer (Applied Biosystems) was added and vortexed for 20 min. Next, 10 µl of trypsin (Promega, Leiden, Netherlands) dissolved in water was added and incubated ON at 37° C. The trypsinized peptides were then tagged with iTRAQ reagents dissolved in 80 µl ethanol. Peptides from the saline groups were labeled with iTRAQ reagents 114 and 116 and heroine groups with iTRAQ reagents 115 and 117. After incubation for 3 h the four samples were pooled and acidified with 10% TFA to pH 2.5-3.0. After 1 h, the final sample was centrifuged and the supernatant was dried in a SpeedVac overnight.

Two-dimensional liquid chromatography (2D LC):

The dried iTRAQ sample was dissolved in 300 µl of loading buffer (20% acetonitrile, 10 mM KH_2PO_4, pH 2.9) and loaded into a polysulphoethyl A column (PolyLC). Peptides were eluted with a linear gradient of 0-500 mM KCl in 20% acetonitrile, 10 mM KH_2PO_4, pH 2.9, over 25 min at a flow rate of 50 µl/min. Fractions were collected at 1-min intervals. In the second dimensional liquid chromatography separation, peptides were delivered with a FAMOS autosampler at 30 µl/min to a C18 trap column (1 mm x 300 µM i.d. column) and separated on an analytical capillary C18 column (150 mm x 100 µm i.d. column) at 500 nl/min using the LC-Packing Ultimate system. Peptides were separated using a linear increase in concentration of acetonitrile from 5-50% in 30 min, and up to 100% in 5 min. The eluent

was mixed with matrix (7 mg α-cyano-hydroxycinnaminic acid in 1 ml 50% acetonitrile, 0.1% TFA, 10 mM dicitrate ammonium) delivered at a flow rate of 1.5 µl/min and deposited off-line to the Applied Biosystems metal target every 15 s for a total of 192 spots using a robot (Dionex, Sunnyvale, CA).

MS data analysis and statistics:

Protein identification: MS/MS spectra were searched against the rat database (Swissprot and NCBI) using GPS Explorer (Applied Biosystems) and Mascot (MatrixScience; http://www.matrixscience.com) with trypsin specificity and fixed iTRAQ modification at lysine residues and the N-termini of peptides. Mass tolerance was 100 ppm for precursor ions and 0.5 Da for fragment ions; one missed cleavage was allowed. Next, a library was generated containing all peptides that were annotated with the Swissprot rat database and a confidence interval (C.I.) higher than 20%. If a spectrum could not be annotated using Swissprot database, a second Mascot search was performed in the larger but more redundant NCBI database. Next, database redundancy (multiple accession numbers for a single protein) and sequence redundancy (peptide sequences annotated to multiple proteins) were removed. First, database redundancy was removed through clustering of full-length protein NCBI sequences that shared more than 90% similarity over 85% of the sequence length with SwissProt sequences. Then, all peptides were matched against the generated protein clusters. Peptides matched to more than one protein represent common protein motifs and were excluded for protein identification and quantification. Hence, quantification was performed only on those peptides that were annotated to a single protein, and are referred to as 'unique peptides'. Only proteins with a minimum of 3 peptides, of which at least one peptide was identified with a C.I. higher than 95%, were selected for quantification and statistical analysis. Gene Ontology

(GO) protein functional classification was performed with the Gene Ontology Tree Machine (GOTM, http://bioinfo.vanderbilt.edu/gotm/).

Immunoblotting:

Proteins were resolved in *Laemmli* sample buffer. Separation of proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 8% gels under reducing conditions and transfer of proteins onto PVDF membrane was performed according to standard protocols. Blots were incubated at 4° C ON with primary antibodies: rabbit anti-brevican (1:10,000; Dr. C. Seidenbecher, Magdeburg, Germany), guinea pig anti-brevican (1:5,000; Dr. C. Seidenbecher, Magdeburg, Germany), or mouse anti-Tenascin-R (1:2,000; Dr. P. Pesheva, Bonn, Germany). Immunodetection was performed using the ECF western blotting detection system (GE Healthcare, Diegem, Belgium) and blots were scanned with the FLA-5000 (Fuji Photo Film Corp.). Relative amounts of immunoreactivity were quantified using the Quantity One software package (Bio-Rad, Hercules, CA).

Immunohistochemistry:

Rats were perfused with 4% paraformaldehyde in 125 mM phospate buffer, pH 7.4. Series of frontal sections were cut at 35 µm with a freezing microtome and stored in cryoprotectant. Sections were incubated ON at 4° C with biotinylated-WFA (5 µg/mL, Sigma-Aldrich) and one or more of the following primary antibodies: rabbit anti-brevican (1:5000), mouse anti-Tenascin-R (1:1,000), mouse anti-parvalbumin (1:1,000 Sigma-Aldrich), mouse anti-CAMKII alpha (1:200 Pierce antibodies). Subsequently, sections were incubated with streptavidin-conjugated to the fluorescent Cy2 and one or more of the following secondary antibodies: donkey anti-mouse conjugated to Cy3 and/or goat anti-rabbit conjugated to Cy5

(all 1:400, Jackson Immunoresearch Laboratories Inc., Suffolk, UK). Colocalization of proteins was analyzed by confocal microscopy (LSM 510, Zeiss, Sliedrecht, Netherlands).

References:

Schmidt ED, Voorn P, Binnekade R, Schoffelmeer AN, De Vries TJ (2005) Differential involvement of the prelimbic cortex and striatum in conditioned heroin and sucrose seeking following long-term extinction. Eur J Neurosci 22: 2347-2356.

Supplementary Figures

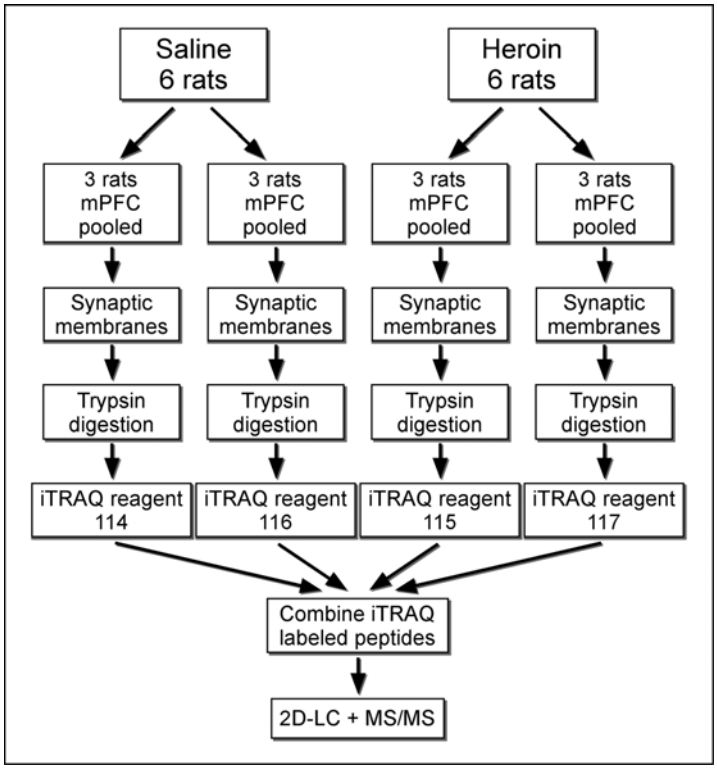

Figure S1: Schematic view of the iTRAQ proteomics experimental design.

First, dissected mPFCs from heroin and saline animals were divided into two independent pools. Subsequently, synaptic membranes were isolated, proteins were digested with trypsin and peptides were labeled with iTRAQ reagents. After labeling, peptides from the 4 different pools were combined and analyzed by 2D LC-MS/MS.

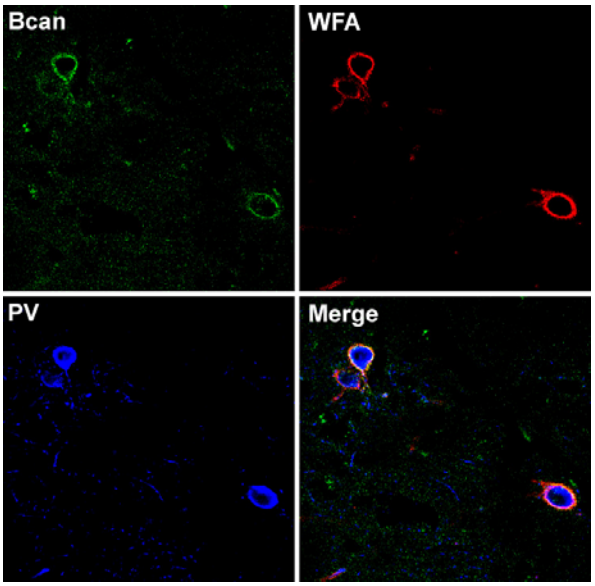

Figure S2: Colocalization of Bcan, WFA and PV in the mPFC.

Triple staining for Bcan, WFA (PNN marker) and PV (fast-spiking GABAergic interneuron marker, parvalbumin) revealed that Bcan immunoreactivity colocalized with WFA and PV in the mPFC.

Supplementary Tables

	mPFC	
	Peptides	**Proteins**
Measured	15711	
Annotation: - > 20% CI	9506	1964
Data analysis (alignments) - database redundancy - sequence redundancy	6212	1824
Inclusion criteria - ≥ 3 peptides per protein - Confidence > 95%	4588	486
Regulation criteria - $p<0.05$ (*t*-test) - Reg. > average ± σ	224	27

Table S1: Number of peptides and proteins included at each stage of data analysis for the iTRAQ-based proteomics experiments in the mPFC. After database searching, approximately 60% of the measured peptides were annotated. The subsequent reduction in number of peptide and protein annotations by data analysis and inclusion criteria was necessary to improve reproducibility of protein identification and quantification (see Materials and Methods)

Gene name	Genbank	Forward primer	Reverse primer
Reference genes			
GAPDH	NM_017008	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGA
NSE	NM_139325	ACGTGGTTCCATTTCAAGATGAC	CGAGCTTCAGTTAGTGCACCAA
ECM genes			
Brevican (Bcan)	NM_012916	CCTCCCTTGTGCCTTTTGTTC	TGACTATTCTGGTCCCCAGAGGT
Tenascin R (Tnr)	NM_013045	CAGAGGTGAGCGCTTTCAAAG	GGAAACCGAGTCCCAGAGATTT

Table S2: Transcript specific primer sequences used for RT-qPCR.