Chapter 5

Cue-induced nicotine seeking in rats is associated with SHPS-1 downregulation in the medial prefrontal cortex

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Abstract

Exposure to cues associated with drug taking is a major cause of relapse in humans. The medial prefrontal cortex (mPFC) and the insular cortex are two brain regions that have been strongly implicated in cue-induced relapse to smoking. To increase our understanding of the underlying molecular mechanisms in these areas, we analyzed synaptic protein regulation associated with cue-induced reinstatement after extinction or abstinence from intravenous nicotine self-administration using iTRAQ-based proteomics in rats. After extinction training and forced abstinence, respectively 3 and 51 proteins of 566 detected synaptic proteins were regulated in the mPFC by cue-induced reinstatement. No significant regulation of proteins was observed in the insula. We observed a downregulation of Src homology 2 domain-containing protein tyrosine phosphatase substrate-1 (SHPS-1), a transmembrane protein involved in intercellular communication, in the mPFC upon reinstatement after extinction, which was validated in an independent set of animals. These results confirm the involvement of the mPFC in cue-induced reinstatement of nicotine seeking, potentially entailing a molecular mechanism that involves SHPS-1.

Introduction

Relapse is a major hallmark of the persistent nature of drug addiction. Drug-associated cues have the ability to induce drug craving[255] and precipitate relapse in both humans and animals[209,256]. Therefore, it is of great interest to elucidate the neural mechanisms involved in cue-induced relapse.

In humans, craving for a cigarette is experimentally induced by presentation of smoking-related cues, such as images of cigarettes or smoking paraphernalia[205,257]. Cue-induced craving is associated with activation of the mesocorticolimbic system, including the PFC, and other brain areas, such as the insular cortex[25,30,258]. In a retrospective functional MRI study, smokers who relapsed during the experimental period had a higher degree of cue-induced activation of these two areas at the time of quitting compared with subjects remaining abstinent[259], suggesting a central role for the PFC and the insula in relapse to smoking.

Accordingly, brain damage to the insula, a brain area implicated in the monitoring of interoceptive states and conscious urges, has been linked to effortless disruption of smoking[260]. Subsequent animal studies have confirmed a role for the insula in addictive behavior, including conditioned place preference[45,261] and drug self-administration[262,263]. Of particular interest, reversible inactivation of the insula attenuated cue-induced reinstatement of nicotine seeking, but not food seeking[263].

Involvement of the human PFC and the rodent mPFC in cue-induced relapse is well established[27,156]. We recently demonstrated that pharmacological inactivation of the mPFC, by local infusion of the $GABA_A$ receptor agonist muscimol, attenuated reinstatement of nicotine seeking[170]. Moreover, an analysis of reinstatement-induced regulation of synaptic glutamate and GABA receptors revealed a $GABA_A$ receptor-mediated molecular mechanism controlling cue-induced reinstatement of nicotine seeking.

Novel high-content analysis techniques, such as quantitative proteomics, can be a useful tool for the identification of such acute plasticity mechanisms[264,265]. Whereas acute effects of drug-associated cues on molecular composition and physiological parameters of addiction circuitry have repeatedly been reported for cocaine and heroin[73,221,266], our knowledge of the molecular mechanisms underlying cue-induced relapse to nicotine seeking is still limited. In this study, we used iTRAQ-based proteomic analysis of synaptic membrane fractions of the mPFC and the insula to identify novel proteins involved in reinstatement of nicotine seeking. We found Src homology 2 domain-containing protein tyrosine phosphatase substrate-1 (SHPS-1), a transmembrane protein involved in intercellular communication, downregulated in the mPFC after cue-induced reinstatement of nicotine seeking.

Materials and Methods

Animals

Male Wistar rats (Harlan CPB, Horst, The Netherlands) weighing 300–375 g at the time of surgery were single housed in Macrolon cages with *ad libitum* food and water available. All experiments were conducted during the dark phase of a reversed 12 h light-dark cycle and approved

by the Animal Care Committee (DEC) of the VU University Amsterdam.

Drugs

(-)-Nicotine hydrogen tartrate salt (Sigma, St. Louis, MO, USA) was dissolved in sterile saline.

Nicotine self-administration and reinstatement

Nicotine self-administration and cue-induced reinstatement were performed as described previously[170]. Briefly, animals (*n*=64 for iTRAQ proteomics and *n*=36 for validation; see Figure 1 and 2) were equipped with an intravenous catheter that was connected to a syringe filled with saline or nicotine before each self-administration session. Nicotine was dissolved in sterile saline at 0.94 mg/mL in order for a rat weighing 350 g to receive 40 µg/kg free base nicotine per 42.52 µL infusion. The operant cage offered two holes; a response in the active hole resulted in a saline or nicotine infusion and simultaneous presentation of audio-visual cues whereas a response in the inactive hole was without consequences. Self-administration training consisted of 10 fixed ratio 1 (FR1), 2–3 FR2 and 4–5 FR4 sessions, each lasting 60 min. After the acquisition phase the animals were divided into two groups. The Abstinence group was left undisturbed in their home cage for approximately three weeks, whereas the other group received fourteen extinction sessions. During these 60-min sessions, active hole responses did not result in a drug infusion nor in presentation of the drug-associated cues. After this phase, both groups were again divided into two, with the No-cue group either staying in their home cage (Abstinence) or getting another extinction session of 30 min (Extinction), whereas both Cue groups received a 30-min reinstatement session. During the reinstatement session, the drug-associated cues were presented under an FR4 schedule, but no drug was infused. A repeated-measures ANOVA with test (extinction and reinstatement) as within-subjects factor and group (No-cue and Cue) as between-subjects factor was used to compare responding during the reinstatement session to responding during the first 30 min of the extinction session one day before the reinstatement session between the Extinction groups. To test for differences in reinstatement responding between the Extinction-no-cue, Extinction-cue and Abstinence-cue groups, a one-way ANOVA followed by a *post hoc* two-tailed Tukey honestly significant difference test was used.

Tissue dissection and synaptic membrane isolation

Immediately after the end of the last session, animals were decapitated and brains were rapidly frozen in 2-methylbutane (isopentane) and stored at –80 °C. Subsequently, at –18 °C, the mPFC and insular cortex were dissected between coordinates 2.28 and 4.20 mm Bregma and -0.48 and 4.20 mm Bregma, respectively[232] (see Figure 1). Synaptic membrane fractions were isolated as described previously[170]. In short, tissue was homogenized in homogenization buffer (0.32 M sucrose, 5mM HEPES, pH 7.4) with fresh protease inhibitors and 5% of the homogenate was collected to determine protein levels in total cell lysates. After centrifugation at 1000x *g* for 10 min, supernatant was loaded on top of a sucrose gradient (0.85 M and 1.2 M sucrose, 5 mM HEPES, pH 7.4) and centrifuged at 30,000x *g* for 2 h. The synaptosome fraction at the interface between 0.85 M and 1.2 M sucrose was concentrated by centrifugation at 25,000x *g* for

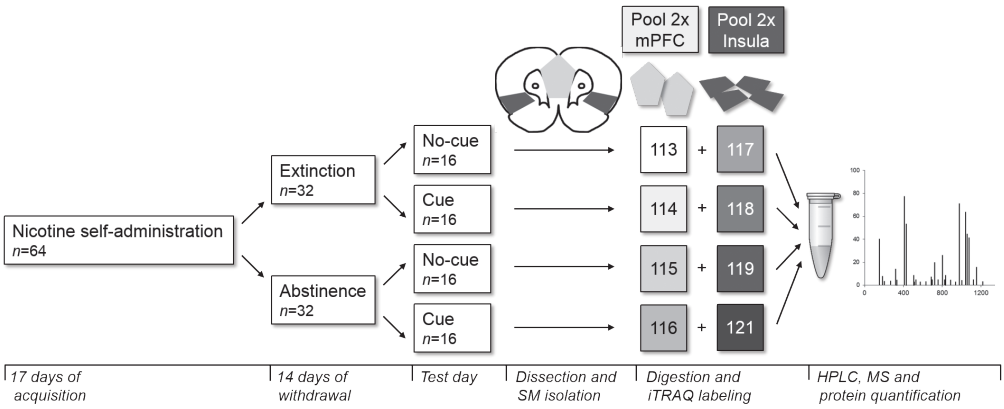

Figure 1. Experimental setup of the iTRAQ proteomics study. After acquisition of nicotine self-administration, half of the animals received extinction sessions, whereas the other half stayed in their home cage (Abstinence). Subsequently, animals were assigned to No-cue or Cue groups (n=16 per group). Medial prefrontal cortex (mPFC) and insula of two animals were pooled to obtain eight biological replicates for each experimental condition. After synaptic membrane (SM) isolation, protein digestion and iTRAQ labeling (labels 113–119 and 121 for the eight experimental conditions), peptides were pooled and analyzed (HPLC, high-performance liquid chromatography; MS, mass spectrometry; for details see Materials and Methods).

30 min and lysed in a hypotonic solution (5 mM HEPES, pH 7.4) with fresh protease inhibitors. Synaptic membranes were obtained by transferring this suspension to a second sucrose gradient and repeating the steps above. Protein concentrations were determined using a Bradford protein assay (Bio-Rad, Hercules, CA, USA).

Protein digestion and iTRAQ labeling

Protein samples were digested and labeled for iTRAQ analysis as described before[123,267]. Briefly, for each of eight experimental conditions (see Figure 1), 75 µg of protein was dissolved in Dissolution Buffer and Reducing Buffer (iTRAQ reagent kit; AB Sciex Pte. Ltd., Foster City, CA, USA) with 0.85% RapiGest (Waters Corporation, Milford, MA, USA), incubated for 1 h at 55 °C and mixed with Cysteine Blocking Buffer (iTRAQ reagent kit). Samples were vortexed, incubated for 10 min at RT and digested overnight with trypsin (sequencing grade; Promega, Fitchburg, WI, USA) at 37 °C. The next day, isopropanol and 1 U iTRAQ Reagent were added, samples were incubated for 2 h at RT and pooled. The pH was adjusted to 3.0–3.5 using 5% trifluoroacetic acid and samples were dried in a SpeedVac before liquid chromatography. In total, eight 8-plex experiments were performed to yield a total of eight biological replicates of each experimental condition.

Two-dimensional liquid chromatography and mass spectrometry

Liquid chromatography, mass spectrometry and data analysis were performed as before[123,267]. The dried samples were dissolved in loading buffer (20% acetonitrile, 10 mM KH_2PO_4, pH 2.9) and injected into a strong cation exchange column (polysulfoethyl A column; PolyLC Inc., Columbia, MD, USA). Peptides were eluted with a linear gradient of 0–500 mM KCl in loading

buffer over 25 min at a flow rate of 200 µL/min. Fractions were collected at 1-min intervals, dried in a SpeedVac, dissolved in 0.1% trifluoroacetic acid and delivered with a FAMOS autosampler at 30 µL/min to a reverse phase C18 trap column. After separation on an analytical capillary C18 column at 0.4 µL/min using the LC-Packing Ultimate system, peptides were separated using a linearly increasing acetonitrile concentration (6 to 45%) in 50 min, and to 90% in 1 min. Eluent fractions were mixed with matrix (a-cyano-hydroxycinnaminic acid in 50% acetonitril, 0.1% trifluoroacetic acid and 10 mM ammonium dicitrate) delivered at 1.5 µL/min and deposited to the AB Sciex metal target plate every 15 s for a total of 384 spots using a Probot (Dionex, Sunnyvale, CA, USA). Fractions were analyzed on an ABI 4800 proteomics analyzer (AB Sciex Pte. Ltd.). Peptide collision induced dissociation was performed at 2 kV; the collision gas was air. MS/MS spectra were each collected from 2500 laser shots. Peptides with a signal-to-noise ratio above 50 were selected for MS/MS analysis, with a maximum of 25 MS/MS per spot. The precursor mass window was set at 200 relative resolution (full width at half maximum).

Protein identification and quantification

As described in detail elsewhere[268], experiment-level MSMS spectra were annotated against a concatenated target-decoy database of the Uniprot rat reference proteome database (version 00/2012, 73654 rat entries) using Mascot Server software (version 2.3.01, Matrix Science, Boston, MA, USA). Database searches were performed with trypsin/P specificity allowing up to two missed cleavages. iTRAQ modifications on lysine residues and N-termini, and methylthio modifications on cysteine residues, were set as fixed modification. iTRAQ modifications on tyrosine residues, and oxidation of methionine residues, were allowed as variable modification. Mass tolerance was 200ppm for precursor ions and 0.4 Da for fragment ions. For each spectrum the best scoring peptide sequence was selected as spectrum annotation. Protein inference was performed on spectra from all experiments simultaneously using in-house modified IsoformResolver software[269] (software available on request), resulting in consistent protein assignment of peptides across experiments. False discovery rates (FDR) for peptide and protein identification were established using Mayu software[270]. FDR of protein identification were based on unique (assigned to a single globally inferred protein) spectra exclusively.

For protein quantification only unique spectra that were within 5% peptide identification FDR were used. In case of multiple spectra assigned to the same peptide sequence in an experiment, only the spectrum with the highest ions score was used for quantification. Spectra with very low iTRAQ reporter signals (maximum intensity of any of the reporter ions less than 100) were removed. Only proteins with a minimum of two unique peptides in each experiment that passed the above criteria and that were within 5% protein level FDR were considered for quantification. After correction for isotope impurities, iTRAQ reporter ions were log-transformed, error-corrected and normalized by variance stabilizing normalization implemented in the VSN r-package (performed on all spectra in each experiment)[271]. Normalized $\log_2$-transformed iTRAQ reporter intensities of each spectrum were centered to the average intensity of all iTRAQ reporter ions in the respective spectrum (i.e., 'standardized'). Sample-level pro-

tein abundance was determined by taking the average centered iTRAQ reporter intensities of the respective iTRAQ reporters of all spectra assigned to that protein. Statistical evaluation of protein abundance differences was performed with SAMR r-package[272]. Changes in synaptic membrane protein levels were considered to be significant when FDR $q<0.05$.

Functional group analysis

Overrepresentation of functional groups or pathways was evaluated using the Web-based gene set analysis toolkit (WebGestalt)[273,274] after functional annotation of proteins identified in the iTRAQ proteomics experiment. Uniprot accession numbers of proteins regulated in the mPFC / Abstinence group were uploaded and compared with a reference set containing all identified proteins. Gene Ontology (GO) analysis of functional groups and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of functional signaling pathways were performed using standard settings. The significance level was set to $p<0.05$.

Immunoblotting

To validate protein regulation identified in the iTRAQ experiment, an independent group of animals was trained (per condition $n=3$ for saline and $n=6$ for nicotine; see Figure 2) and mPFC synaptic membrane fractions were isolated. Protein samples were mixed with SDS sample buffer and heated for 5 min at 95 °C. After separation using 4–20% Criterion™ TGX Stain-Free™ precast polyacrylamide gels (Bio-Rad), proteins (2.5 µg per lane) were transferred to PVDF membrane. Membranes were blocked with 5% milk for 30 min and probed overnight with rabbit anti-SHPS-1 (1:20,000; Lifespan Biosciences Inc., Seattle, WA, USA) or rabbit anti-CamKIIβ (1:1,000; Abcam, Cambridge, UK). After incubation with alkaline phosphatase or horseradish peroxidase-conjugated secondary antibody (1:10,000; Dako, Glostrup, Denmark) for two h and ECF Substrate (GE Healthcare, Piscataway, NJ, USA) or Femto Chemiluminescent Substrate

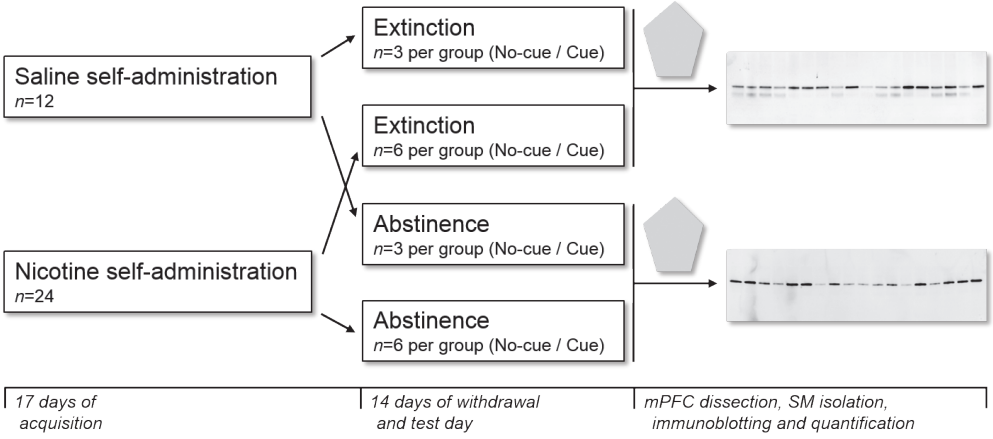

Figure 2. Experimental setup of the validation study. After acquisition of either saline or nicotine self-administration, animals were divided into Extinction / Abstinence and No-cue / Cue as before, yielding an $n=3$ for each saline group and an $n=6$ for each nicotine group. The synaptic membrane (SM) fraction of the mPFC was isolated for each individual animal and protein levels were determined using immunoblotting (for details see Materials and Methods).

(Thermo Scientific, Rockford, IL, USA) blots were scanned (FLA 5000 (Fujifilm, Tokyo, Japan) or Li-Cor Odyssey Fc (Westburg, Leusden, The Netherlands)) and analyzed with Quantity One (BioRad, Hercules, CA, USA) or Image Studio (Li-Cor, Lincoln, NE, USA). For visualization of total protein, gels were scanned using a Gel Doc EZ imager (BioRad) and analyzed with Image Lab (BioRad) to correct for input differences per sample. For statistical analysis, Saline-no-cue

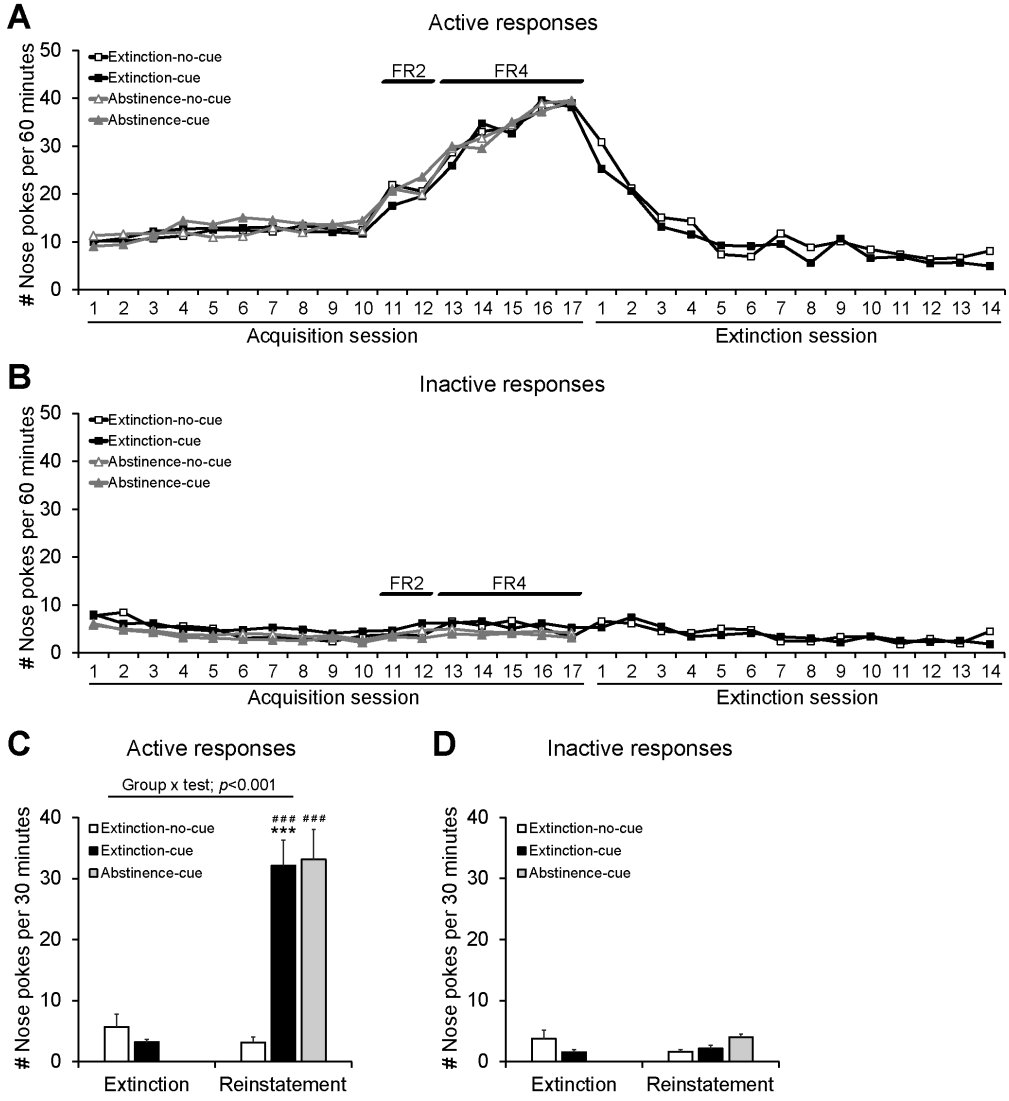

Figure 3. Levels of active and inactive hole responding during nicotine self-administration, extinction and reinstatement of animals used for the quantitative proteomics study. Active (**A**) and inactive (**B**) responses during the acquisition and extinction phase and active (**C**) and inactive (**D**) responses during the first 30 min of the last extinction session and the 30-min reinstatement test (all groups n=16). For extinction groups, a significant effect of group and test was found. Student's t-test $^{***}p<0.001$ versus Extinction; $^{###}p<0.001$ versus Reinstatement Extinction-no-cue. Note that during the extinction phase the Abstinence groups, and during reinstatement the Abstinence-no-cue group, stayed in their home cage and hence no data is presented.

and Saline-cue groups were combined (no significant difference between these two groups was found using a Student's *t*-test), and a one-way ANOVA followed by a post-hoc two-tailed Tukey HSD test was used to determine significant differences between groups.

Results

Nicotine self-administration

Rats were trained to self-administer nicotine (Figure 3A,B). After a period of either extinction or abstinence, two commonly used regimes for animals to abstain from drug taking that have distinct neural substrates[209,275,276], half of the animals underwent a cue-induced reinstatement session. The remaining animals received another extinction session or stayed in their home cage (Abstinence; Figure 3C,D). A repeated-measures ANOVA revealed a significant effect of test (Extinction versus Reinstatement; $F_{1,30}$=31.35; p<0.001) and group ($F_{1,30}$=30.52; p<0.001), and a group x test interaction ($F_{1,30}$=44.72; p<.001) for active hole responses of the extinction groups (Figure 3C). Furthermore, reinstatement responding of the cue groups deviated significantly from the Extinction-no-cue group (One-way ANOVA, $F_{2,47}$=20.52; p<0.001; Figure 3C). The number of active hole responses of the Abstinence-cue group during the 30-minute reinstatement test did not differ significantly from the number of responses during the first 30 minutes of the last FR4 acquisition session (average of 26 ± 1.6 responses during the last FR4 session and 33 ± 4.9 responses during the reinstatement test; Student's *t*-test; t_{15}=-1.50; p=0.155; data not shown). No significant changes in inactive hole responding were observed (Figure 3D).

Mass spectrometry and protein quantification

Being the predominant site of neural plasticity, synapses are subject to acute activity-dependent changes in molecular composition and physiology. To identify proteins regulated after cue-induced reinstatement of nicotine seeking, synaptic membrane fractions were isolated from the mPFC and the insula and subjected to quantitative proteomics analysis. Eight 8-plex iTRAQ proteomics experiments were performed, each comprising all eight experimental conditions (with variables "regime" (Extinction or Abstinence), "reinstatement" (No-cue or Cue) and "brain area" (mPFC or insula), see Figure 1). A total of 566 proteins were identified (Table 1) using the criteria described in the Materials and Methods section. We observed no

	mPFC		Insula	
	Extinction	Abstinence	Extinction	Abstinence
# proteins detected	566	566	566	566
# proteins SAM FDR q<0.05	3	51	0	0
# proteins overlap Ext / Abs	2		0	

Table 1. Number of proteins detected and regulated in the iTRAQ proteomics study. For each condition, significant differential expression between the No-cue and the Cue condition was determined. In the mPFC, two proteins were found regulated after both the extinction and the abstinence regime (see Table 2).

Protein ID	Gene symbol	Extinction			Abstinence		
		Log_2 change (cue / no cue)	SAM FDR *q*-value	t-test *p*-value	Log_2 change (cue / no cue)	SAM FDR *q*-value	t-test *p*-value
SV2B_RAT	Sv2b	-0.06	0.28	0.033	-0.26	0.00	0.000
EAA1_RAT	Slc1a3	0.07	1.00	0.172	0.24	0.00	0.000
RAB3C_RAT	Rab3c	-0.05	0.43	0.458	-0.22	0.00	0.000
CADM3_RAT	Cadm3	-0.09	0.28	0.026	-0.24	0.00	0.000
SC6A1_RAT	Slc6a1	-0.07	0.28	0.052	-0.27	0.00	0.000
EAA2_RAT	Slc1a2	-0.11	0.28	0.148	-0.30	0.00	0.000
SYN1_RAT	Syn1	-0.12	0.28	0.228	-0.13	0.00	0.000
NEUM_RAT	Gap43	-0.08	0.28	0.014	-0.26	0.00	0.000
SEPT5_RAT	Sept5	-0.04	0.43	0.319	-0.25	0.00	0.000
AT2B1_RAT	Atp2b1	-0.03	0.43	0.408	-0.12	0.00	0.000
B2RZ72_RAT	Arpc4	0.04	1.00	0.324	-0.16	0.00	0.001
F1M0J7_RAT	Prickle2	-0.06	0.28	0.017	-0.13	0.00	0.001
VISL1_RAT	Vsnl1	-0.11	0.28	0.063	-0.17	0.00	0.001
CX6C2_RAT	Cox6c2	-0.03	0.55	0.615	-0.27	0.00	0.001
S4A10_RAT	Slc4a10	-0.07	0.28	0.107	-0.16	0.00	0.002
NAC2_RAT	Slc8a2	-0.02	0.55	0.478	-0.14	0.00	0.002
CNTFR_RAT	Cntfr	-0.11	0.28	0.045	-0.22	0.00	0.002
STX1A_RAT	Stx1a	-0.04	0.43	0.439	-0.14	0.00	0.002
ACTN4_RAT	Actn4	0.00	1.00	0.916	-0.14	0.00	0.002
D3ZEI4_RAT	Hepacam	-0.05	0.43	0.259	-0.22	0.00	0.002
Q5XIJ3_RAT	Idh3g	-0.03	0.43	0.417	-0.15	0.00	0.003
SV2A_RAT	Sv2a	-0.04	0.43	0.159	-0.18	0.00	0.003
THY1_RAT	Thy1	-0.22	0.28	0.049	-0.32	0.00	0.003
SHPS1_RAT	Sirpa	-0.20	0.00	0.000	-0.20	0.00	0.004
CNTN4_RAT	Cntn4	-0.04	0.43	0.456	-0.21	0.00	0.008
D3ZS58_RAT	Ndufa2	-0.20	0.28	0.130	-0.19	0.00	0.008
GTR1_RAT	Slc2a1	-0.10	0.28	0.073	-0.37	0.00	0.010
Q4V7D9_RAT	Smpdl3b	-0.03	0.43	0.361	-0.14	0.00	0.011
GNAI2_RAT	Gnai2	0.03	1.00	0.423	-0.15	0.00	0.011
D3ZX01_RAT	Rps4y2	-0.11	0.28	0.037	-0.15	0.00	0.012
HBB1_RAT	Hbb	0.06	1.00	0.577	-0.35	0.00	0.012
HBB2_RAT	NA	0.06	1.00	0.617	-0.37	0.00	0.022
COX41_RAT	Cox4i1	-0.11	0.28	0.174	-0.20	0.00	0.022

D3ZFB6_RAT	Prrt2	-0.21	0.28	0.054	-0.17	0.00	0.024
DLG4_RAT	Dlg4	-0.02	0.55	0.546	-0.10	0.02	0.001
SEPT7_RAT	Sept7	0.00	1.00	0.802	-0.10	0.02	0.001
EF1A2_RAT	Eef1a2	-0.04	0.43	0.241	-0.13	0.02	0.008
NCAM1_RAT	Ncam1	-0.07	0.28	0.083	-0.14	0.02	0.014
F1M4K2_RAT	NA	-0.06	0.28	0.131	-0.13	0.02	0.015
D3ZZ21_RAT	Ndufb6	-0.09	0.28	0.174	-0.16	0.02	0.019
D3ZFY7_RAT	Psd3	-0.09	0.28	0.009	-0.16	0.02	0.030
D3ZFQ8_RAT	Cyc1	-0.10	0.28	0.217	-0.19	0.02	0.035
KCC2B_RAT	Camk2b	-0.17	0.00	0.002	-0.15	0.02	0.037
Q5PQZ9_RAT	Ndufc2	-0.19	0.28	0.092	-0.20	0.02	0.038
F1LUT4_RAT	Atp8a1	-0.05	0.28	0.127	-0.10	0.04	0.005
G3V881_RAT	Lingo1	0.00	1.00	0.878	-0.11	0.04	0.012
AP2S1_RAT	Ap2s1	-0.02	0.55	0.547	-0.12	0.04	0.013
RAC1_RAT	Rac1	-0.08	0.28	0.102	-0.12	0.04	0.015
STXB1_RAT	Stxbp1	-0.03	0.55	0.584	-0.12	0.04	0.025
CA2D3_RAT	Cacna2d3	-0.05	0.43	0.229	-0.12	0.04	0.027
HBA_RAT	Hba1	0.10	1.00	0.367	-0.22	0.04	0.090
D3ZC55_RAT	Hspa12a	-0.13	0.00	0.006	-0.05	0.37	0.237

Table 2. Synaptic proteins regulated in the mPFC by cue-induced reinstatement after extinction of, or abstinence from, nicotine self-administration. Proteins are indicated by UniProtKB/Swiss-Prot entry name and gene symbol and are listed if the false discovery rate was below 5% (q<0.05).

significant protein regulation in the insula. In contrast, significant differences were found in the mPFC. Whereas 3 proteins were regulated by cue-induced reinstatement after extinction, 51 proteins were regulated when the reinstatement test was performed after abstinence (Cue group compared with respective No-cue group, SAM FDR q<0.05). A detailed list of all regulated proteins is given in Table 2. Interestingly, expression levels of two proteins, SHPS-1 and CamKIIβ, were reduced by cue-induced reinstatement after both extinction and abstinence.

Analysis of functional groups

To evaluate overrepresentation of functional groups in the set of proteins regulated after abstinence in the mPFC, these proteins were analyzed using the Gene Ontology Analysis and KEGG Analysis functions of the Web-based gene set analysis toolkit (WebGestalt)[273,274]. No significant overrepresentation of functional groups was found.

Validation of protein regulation

To validate reinstatement-associated protein regulation, an independent group of animals was trained to self-administer nicotine (Figure 4 and [170]). A saline self-administration group was

included to distinguish reinstatement effects from long-term adaptations in proteins levels after nicotine self-administration. Protein samples of nicotine and saline extinction-cue and no-cue groups were derived from animals that were used in a previous study[170]. For Abstinence groups, a Student's *t*-test revealed that average active hole responding during the reinstatement test was significantly higher for animals that self-administered nicotine compared with animals that self-administered saline (t_7=-2.7; p=0.031; Figure 4A). No differences in inactive hole response rates were observed (Figure 4B).

Whereas no functional groups of proteins were overrepresented, we decided to evaluate protein levels of the two proteins regulated after both extinction and abstinence. For all animals, synaptic membrane fractions of the mPFC were obtained and probed with anti-SHPS-1 and anti-CaMKIIβ. No significant regulation was observed for CaMKIIβ in the Abstinence and the Extinction groups. However, a one-way ANOVA revealed a significant decrease of SHPS-1 protein levels in the Nicotine-cue-group after extinction, but not abstinence, compared with Saline and Nicotine-no-cue groups (64% relative to Nicotine-no-cue; $F_{2,15}$=5.59; p=0.015; Figure 5A). Comparison of SHPS-1 levels between the Saline and Nicotine-no-cue group revealed that this protein is not regulated after nicotine self-administration and extinction, but is

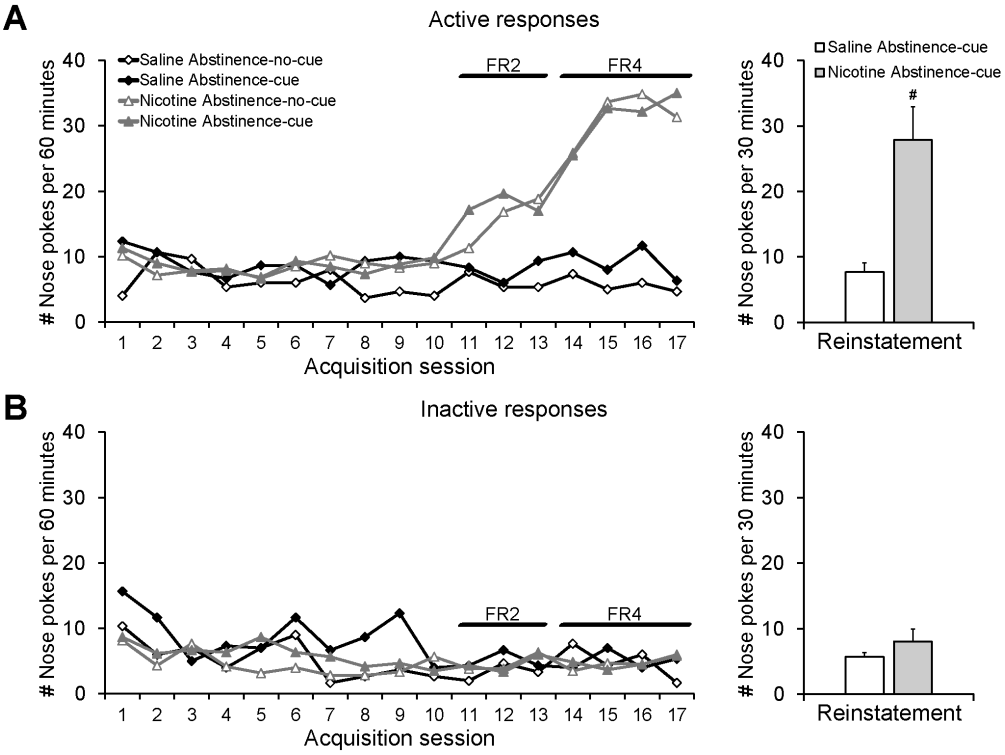

Figure 4. Active and inactive hole responses during acquisition of self-administration and reinstatement of the Abstinence group used for the validation study. (**A**) Active and (**B**) inactive responses during acquisition of saline or nicotine self-administration and reinstatement after a period of abstinence (n=3 for saline and n=6 for nicotine groups). $^{\#}p<0.05$ (Student's *t*-test, Nicotine-abstinence-cue versus Saline-abstinence-cue). Self-administration and reinstatement data of the Extinction group was published elsewhere[170].

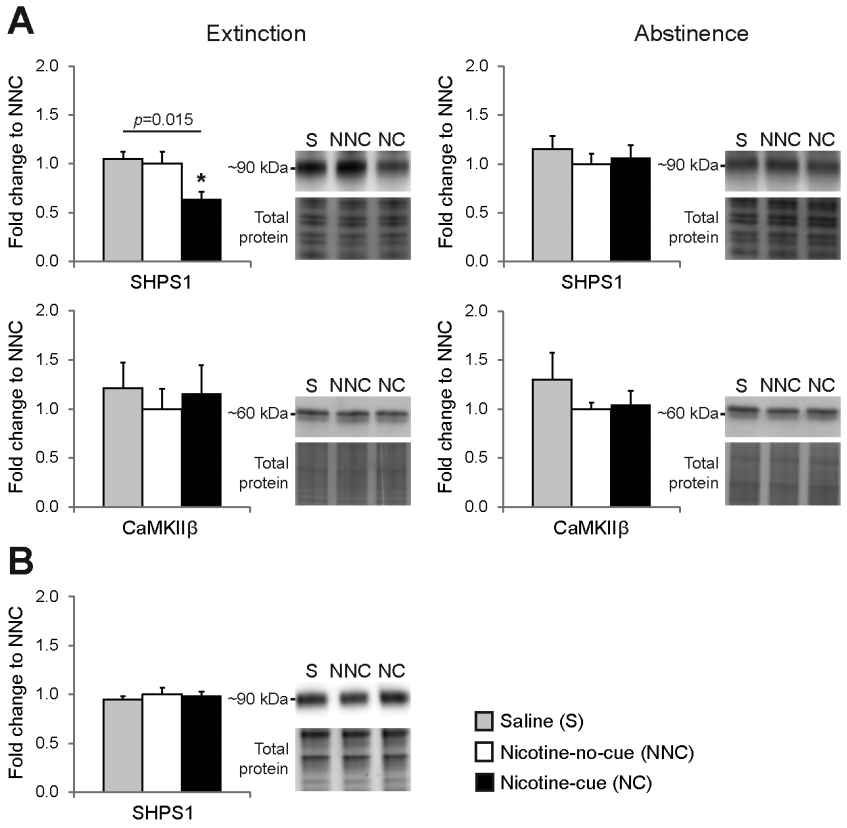

Figure 5. SHPS-1 is downregulated in mPFC synaptic membranes by cue-induced reinstatement after extinction of nicotine self-administration. (**A**) Average SHPS-1 and CaMKIIβ protein levels in mPFC synaptic membrane fractions of animals from Saline (S), Nicotine-no-cue (NCC) and Nicotine-cue (NC) groups (all groups n=6) after extinction of or abstinence from saline or nicotine self-administration were determined by immunoblotting. Protein levels were normalized for loading (total protein shown below immunodetected bands) and are expressed relative to the Nicotine-no-cue group. Statistical analysis using a one-way ANOVA revealed a significant regulation of SHPS-1 in the extinction group. *p<0.05 versus other groups. (**B**) After extinction, no differences were found in SHPS-1 protein levels in total cell lysates of the mPFC.

acutely regulated by cue-induced reinstatement. To test whether SHPS-1 is specifically regulated in synaptic membranes, protein levels in total cell lysates of the mPFC of the same animals were determined (Figure 5B). No significant regulation was found, suggesting that regulation of SHPS-1 occurs specifically at the synapse.

Discussion

Here, we exploited quantitative iTRAQ-based proteomics to identify proteins that are regulated after cue-induced reinstatement of nicotine seeking. Our results indicate that reinstatement-associated acute neuroplasticity occurs in the mPFC, whereas no protein regulation was observed in the insula. Reinstatement after forced abstinence is associated with more extensive changes in synaptic protein levels in the mPFC (51) than reinstatement after extinction (3) of nicotine self-administration (Table 2). Moreover, we show that SHPS-1, a transmembrane pro-

tein involved in intercellular communication, is downregulated specifically in mPFC synaptic membranes after cue-induced reinstatement in animals that underwent extinction.

This study identifies acute regulation of SHPS-1 to be associated with cue-induced reinstatement of nicotine seeking. SHPS-1 is a cell surface receptor that binds to the transmembrane protein CD47. It is most abundantly expressed in synapse-rich areas of the brain[277,278] and depends on CD47 for its localization in the synapse[279]. SHPS-1/CD47 signaling has been implicated in spine formation and synaptic plasticity[277]. In addition, using mutant SHPS-1 lacking most of the cytoplasmic domain, it has been shown that SHPS-1 signaling is involved in integrin-mediated cytoskeletal reorganization and cell motility[280]. The fact that CD47, also known as integrin-associated protein (IAP), has been recognized to functionally associate with the integrin β3 subunit[281], suggests that the interaction between SHPS-1 and CD47 may also mediate these functions. The finding that reinstatement is associated with downregulation of SHPS-1 in the mPFC suggests that altered SHPS-1/CD47 signaling has a role in nicotine relapse, possibly by shaping relapse-associated synaptic plasticity. Future research is needed to clarify the molecular consequences and the functional significance of SHPS-1 regulation for cue-induced relapse to nicotine seeking.

The observation that nicotine reinstatement is associated with acute neuroplasticity in the mPFC is in line with the pivotal role this area has in addictive behavior and more specifically in relapse. Studies in humans and animals that evaluated nicotine cue-associated activation of brain areas or neuronal activation implicated the involvement of the mPFC in nicotine relapse[205,206,216]. We showed that both the dorsal and the ventral part of the mPFC are part of the neural circuitry driving cue-induced reinstatement of nicotine seeking[170]. Reinstatement-associated GABAergic and glutamatergic plasticity in respectively the mPFC and nucleus accumbens (NAc), a subcortical area involved in reward and motivated behavior, has been shown to control cue-induced reinstatement of nicotine seeking[170,248]. These studies hint towards a central role of prefrontal glutamatergic projections to the NAc in relapse behavior, as has been suggested for other types of drugs of abuse[218].

An acute upregulation of synaptic $GABA_A$ receptor subunits α1 and γ2 was found associated with cue-induced reinstatement of nicotine seeking after extinction training in an earlier study[170]. These proteins were not identified as significantly regulated in this study. The γ2 subunit was not quantified as a result of unmet detection requirements (<2 peptides identified in 4 out of 8 iTRAQ experiments). The $GABA_A$ receptor subunit α1 was not regulated in our proteomics dataset ($\log_2$ change -0.005 ± 0.039). This difference might be due to the generation of false negatives that is virtually inherent to the analysis of large datasets. Despite careful setup of detection requirements and application of statistics to minimize the number of false positives and false negatives, it is still possible that regulation of the α1 subunit was not detected in our proteomics study. Furthermore, independent validation of a number of presynaptic proteins that were significantly downregulated in the mPFC after reinstatement and abstinence in the iTRAQ data (synapsin-1, syntaxin-1 and synaptic vesicle glycoprotein 2A and 2B; see Table 2), was not successful (data not shown). Similarly, no regulation was observed in the validation groups for SHPS-1 (Abstinence group) and CamKIIβ (both groups). This suggests the presence

of a considerable number of false positives in our dataset and underlines the necessity of independent validation of protein regulation found in large proteomics datasets.

While molecular changes in the NAc and the mPFC evoked by cue-induced reinstatement were recently identified[170,248], we observed no changes in the levels of any of the identified 566 proteins in the insula. It is possible that the insula is not subject to reinstatement-associated acute neuroplasticity, but the absence of detectable changes in protein expression may also be due to the following alternative explanations: first, both in humans and rodents, the insula is a heterogeneous brain region comprised of multiple subareas that have distinct anatomical and functional characteristics[33,282,283]. Use of the entire insula for the proteomics study might have obscured identification of protein regulation in a subarea of the insula by diluting the regulation. Second, in contrast to the mPFC, clear landmarks that mark the borders between the insula and neighboring regions are absent. This might have impeded an accurate dissection of this area and increased variability across the biological replicates used for this study. Third, pathways and mechanisms that are not represented in the set of identified proteins of the proteomics study might contribute to the regulation of cue-induced reinstatement of nicotine seeking in the insula. For example, it is known that nicotine self-administration depends on hypocretin transmission in the insula[262]. Hypocretin receptors were not identified by mass spectrometry analysis of insular synaptic membrane fractions, possibly due to confidence settings, detection limits of current technology or subcellular localization of the receptors. Fourth, any reinstatement-associated neuroplasticity outside of the synapse could not have been detected using the methods employed by us. The insula remains an interesting candidate for studies investigating neural mechanisms of relapse, however, it is important to take into account the characteristics of this area and adjust experimental design accordingly.

Extinction of drug self-administration is used as a method to isolate the effects of specific drug-related cues on relapse[276]. During extinction sessions, animals are exposed to the environment in which they previously self-administered a drug, but in the absence of discrete drug-associated cues. Subsequently, these cues can be employed to precipitate relapse (reinstatement)[209]. Alternatively, forced abstinence has been applied to the self-administration model to mimic the human withdrawal situation where drug use is discontinued in absence of drug-associated cues, both discrete and contextual[275]. With forced abstinence, animals remain in their home-cage and are not exposed to contextual and discrete drug-associated cues for a period of time. Unlike abstinence, extinction does not reflect a specific phase in the drug cycle of humans. Nonetheless, apart from being a method to specifically investigate the contribution of discrete cues to relapse, extinction training can be part of a behavioral therapy for human addicts to remain abstinent, as has been shown for cocaine in rats and heroin in rats and humans[284,285]. In these studies, rendering drug-associated memories labile by a short retrieval session allowed a subsequent extinction session to reduce future drug seeking and craving. It follows from the above that extinction and abstinence are distinct processes that are likely to affect distinct neural substrates. This underlines the importance of gaining better insight in the overlap, as well as the differences between these two regimes. In line with this, it has been shown that reinstatement of cocaine seeking after extinction and abstinence depend

on distinct neural circuitry[276]. To our knowledge, this is the first study that directly compares the consequences of extinction and abstinence on cue-induced reinstatement of nicotine seeking. Whereas only very modest protein regulation was observed during reinstatement after extinction of self-administration, reinstatement after a period of abstinence was associated with regulation of a substantial number of proteins (3 versus 51 significantly regulated proteins; Table 1). This might reflect an increased complexity of reinstatement-associated neuroplasticity due to the re-exposure to the drug context containing multiple drug-associated stimuli. After both regimes, reinstatement is associated with acute neuroplasticity in the mPFC, however, the observed differential regulation of synaptic proteins indicates that the neurobiological mechanism mediating relapse might be influenced by the specific withdrawal conditions of the addicted subject. This should be taken into consideration when the development and personal application of smoking cessation therapies are concerned.

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