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Extracellular matrix plasticity and GABAergic inhibition of prefrontal cortex pyramidal cells facilitates relapse to heroin seeking

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Running title: ECM adaptation enhances vulnerability to heroin relapse

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Abstract

Successful treatment of drug addiction is hampered by high relapse rates during periods of abstinence. Neuroadaptation in the medial prefrontal cortex (mPFC) is thought to play a crucial role in vulnerability to relapse to drug seeking, but the molecular and cellular mechanisms remain largely unknown. To identify protein changes that contribute to relapse susceptibility, we investigated synaptic membrane fractions from the mPFC of rats that underwent 21 days of forced abstinence following heroin self-administration. Quantitative proteomics revealed that long-term abstinence from heroin self-administration was associated with reduced levels of extracellular matrix (ECM) proteins. After extinction of heroin self-administration, down-regulation of ECM proteins was also present in the mPFC, as well as nucleus accumbens (NAc), and these adaptations were partially restored following cue-induced reinstatement of heroin seeking. In the mPFC, these ECM proteins are condensed in perineuronal nets that exclusively surround GABAergic interneurons, indicating that ECM adaptation might alter activity of GABAergic interneurons. In support of this, we observed an increase in inhibitory GABAergic synaptic inputs received by mPFC pyramidal cells following re-exposure to heroin conditioned-cues. Recovering levels of ECM constituents by metalloproteinase inhibitor treatment (FN-439) prior to a reinstatement test attenuated subsequent heroin seeking, suggesting that the reduced synaptic ECM levels during heroin abstinence enhanced sensitivity to respond to heroin-conditioned cues. We provide evidence for a novel neuroadaptive mechanism, in which heroin self-administration-induced adaptation of the ECM increased relapse vulnerability, potentially by augmenting the responsivity of mPFC GABAergic interneurons to heroin-associated stimuli.

Introduction

Treatment of drug addiction is confounded by a high incidence of relapse to drug-taking during periods of drug abstinence. During drug intake, environmental stimuli become over time associated with the drugs' rewarding effects (O'Brien et al., 1986). Exposure to these drug-conditioned cues can provoke drug craving and subsequent relapse to drug use even after prolonged periods of abstinence (Wikler, 1973; O'Brien et al., 1986). During the last decade, it has become clear that exposure to drugs of abuse leads to long-lasting changes in neuronal circuitry that mediates processing of motivationally relevant stimuli (Robinson and Berridge, 1993; Kalivas and Volkow, 2005). However, the molecular mechanisms underlying these changes, as well as how they contribute to relapse to drug seeking remain largely unresolved.

With respect to opiate drugs, heroin administration leads to long-term alterations in gene expression in the NAc shell (Jacobs et al., 2002). At the cellular level, changes in neuronal connectivity and neuronal physiology have been observed following opiate exposure. For instance, morphine treatment leads to changes in the complexity of dendritic branching on cells in the neocortex and hippocampus during development (Hammer et al., 1989) and affects the number of synaptic spines and dendritic arborizations of neurons in the mPFC and NAc (Robinson and Kolb, 1999; Robinson et al., 2002). In addition to structural changes, opiate exposure results in a change in synapse physiology of neurons in the ventral tegmental area (Saal et al., 2003), hippocampus (Pu et al., 2002) and NAc (Dong et al., 2007), and leads to long-lasting changes in dopamine and acetylcholine release at nerve terminals in the striatum (Tjon Tien Ril et al., 1993; Tjon et al., 1994; Vanderschuren et al., 2001). Recently, we found that re-exposure to heroin conditioned cues results in acute synaptic depression of pyramidal neurons in the ventral mPFC (Van den Oever et al., 2008). Thus, addiction might be a pathological process that involves mechanisms of synaptic plasticity similar to those observed in neuronal models of

learning and memory (Nestler, 2002; Kelley, 2004; Hyman, 2005), arguing for a thorough analysis of long-lasting changes in synaptic function resulting from drug exposure.

The mPFC is a brain area that plays a pivotal role in relapse to opiate seeking during periods of drug abstinence (Schmidt et al., 2005; Koya et al., 2006; Rogers et al., 2008; Van den Oever et al., 2008). Drug-induced neuroadaptations in the mPFC might be intrinsic to the synapse, e.g. glutamate receptor-based plasticity, or extrinsic, e.g. by changing the extracellular matrix (ECM). In the last decade, the role of the ECM and more specifically the role of matrix molecules in perineuronal nets, both in neural development and synaptic plasticity, has attracted attention (Pizzorusso et al., 2002; Dityatev and Schachner, 2003). Studies on the role of the ECM in plasticity of the visual cortex (Pizzorusso et al., 2002; Pizzorusso et al., 2006) and hippocampal-dependent learning (Meighan et al., 2006) gave way to the intriguing prospect that the ECM contributes to various forms of persistent neuroadaptation (Wright and Harding, 2009), including those resulting from repeated drug exposure. In support of this, a study by Brown et al. suggests that alteration of ECM plasticity by inhibition of matrix metalloproteinase (MMP) activity reduces the conditioned rewarding effects of cocaine in a conditioned place preference model (Brown et al., 2007).

In the present study, we used a rat model of relapse to heroin seeking (Epstein et al., 2006), in which animals self-administer heroin in the presence of compound cues (Van den Oever et al., 2008). We show that heroin self-administration leads to long-term changes in synaptic levels of ECM constituents in the mPFC. Interference of the ECM by a broad spectrum metalloproteinase inhibitor FN-439 treatment reduced subsequent cue-induced reinstatement of heroin seeking, suggesting that adaptation of the ECM contributes to relapse susceptibility. Moreover, we show that these ECM proteins are condensed in perineuronal nets surrounding mPFC GABAergic interneurons and that cue-induced relapse is accompanied by an increase in spontaneous

inhibitory network activity. Together, these data may point towards ECM-mediated enhancement of GABAergic interneuron reactivity to heroin-associated stimuli.

Materials and Methods

Animals

Male Wistar rats (Harlan, Horst, The Netherlands) weighing 280-300 g on the day of arrival were single housed on a reversed 12-h light-dark cycle with standard food (Teklad, Harlan, Horst, The Netherlands) and water available *ad libitum*. All experiments were approved by the Animal Users Care Committee of the VU University.

Heroin self-administration, extinction and reinstatement

Animals:

Heroin self-administration rats and their saline self-administration controls were implanted with jugular vein catheters and allowed to recover from surgery for at least 1 week (Schmidt et al., 2005).

Self-administration phase: Rats were trained to self-administer heroin (100 µg/kg, diacetylmorphine-HCL, OPG, The Netherlands) in operant cages (Med Associates, USA) in once daily sessions (3 h) during the lights-off period for a total of 16 sessions (Schmidt et al., 2005). Nose-poking in the active (heroin-paired) hole resulted in a 2-s heroin infusion and the presentation of compound audiovisual cues. For details see **Supplementary Methods**.

Forced abstinence: After the final self-administration session rats were left undisturbed in their home-cages for 21 days. On the 21st day of heroin abstinence, rats were decapitated and brains were rapidly frozen using ice-cold (−50° C) isopentane and stored at −80° C until further use.

Extinction: Additional groups of animals underwent extinction sessions for 60 min once daily for 5 days a week (15 sessions) following heroin self-administration. During the extinction period, heroin was not available and cues were not presented. Extinction was considered successful when nose poking was less than ten nose pokes per session (extinction criterion), as was usually obtained after 10-11 sessions.

Reinstatement: Extinction trained rats were placed in the operant cages with discriminative cues turned on and nose poking resulting in the presentation of discrete audiovisual cues (but no heroin infusion) on an FR4 schedule. Control animals underwent an additional extinction session. For electrophysiological recordings and the FN-439 experiment, recording of nose-poke responses in the active and inactive hole was measured until 30 min after start of the test. For immunoblotting, rats of both cue and no-cue groups were decapitated immediately after the reinstatement/extinction session, and brains were frozen and stored until further use.

Tissue preparation, iTRAQ labeling and 2D LC-MS/MS:

The mPFC, NAc and dorsal striatum were dissected freehand at -20° C from 1 mm thick brain slices. Bregma coordinates were inferred from the Paxinos and Watson rat brain atlas: mPFC (3.7 to 2.7 mm) and NAc (2.1 to 1.1 mm). Subsequently, brain tissue of 3 animals was pooled, resulting in 2 independent pools per treatment (**Figure S1**).

Synaptic membrane preparation: The four samples were processed simultaneously and synaptic membranes were isolated as described previously (Van den Oever et al., 2008) (**Supplementary Methods**).

Protein digestion and iTRAQ labeling: Trypsin digestion of synaptic membrane proteins and iTRAQ labeling of peptides was performed as described in the iTRAQ reagent kit manual (Applied Biosystems, Foster City, CA; **Supplementary Methods**).

Two dimensional liquid chromatography (2DLC): The dried iTRAQ sample was separated on a polysulphoethyl A column (PolyLC), and subsequently on a C18 trap column and an analytical capillary C18 column. The eluent was mixed with MALDI-matrix, delivered at a flow rate of 1.5 $\mu\text{l}/\text{min}$ and robotically deposited off-line to the Applied Biosystems metal target every 15 s for a total of 192 spots (**Supplementary Methods**).

Mass spectrometry (MS/MS): MALDI plates were analyzed on a 4700 proteomics analyzer (Applied Biosystems, Foster City, CA). Peptide CID was performed at 1 kV with nitrogen collision gas. MS/MS spectra were collected from 5000 laser shots. Peptides with signal to noise ratio above 50 at the MS mode were selected for MS/MS, at a maximum of 30 MS/MS per spot. The precursor mass window was set at 180 relative resolution (FWHM).

MS data analysis and statistics:

Protein identification: MS/MS spectra were searched against the rat database (Swissprot and NCBI) using GPS Explorer (Applied Biosystems, Foster City, CA) and Mascot (MatrixScience). Next, a library was generated containing all annotated peptides with a confidence interval (C.I.) higher than 20%. Database redundancy and sequence redundancy were removed. 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 ≥ 3 peptides, of which at least one peptide had a C.I. $\geq 95\%$, 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/>).

Protein quantification: iTRAQ areas (m/z 114-117) were extracted from raw spectra and corrected for isotopic overlap using GPS explorer. To compensate for the possible variations in the starting amounts of the samples, the individual peak areas of each iTRAQ signature peak were \log_2 transformed to obtain a normal distribution and then normalized to the mean peak area

for each sample. Significant protein regulation was determined by comparing the average iTRAQ area of label 114 and 116 (saline groups) with the average iTRAQ area of label 115 and 117 (heroin groups) of all unique peptides that were annotated to a protein (\log_2 -scale; Student's *t*-test $p < 0.05$). In addition, the standard deviation (σ) was calculated from the heroin to saline ratios of all quantified proteins. Proteins with a regulation more than the average regulation $\pm \sigma$ were considered relevant for further validation by immunoblotting.

Immunoblotting:

For immunoblotting, mPFC, NAc and dorsal striatum synaptic membrane fractions and total protein were isolated from additional groups of animals that self-administered heroin or saline (n=9 pool of 3, abstinence and extinction experiments) as described above. For the FN-439 experiment, synaptosomal fractions were isolated from individual animals (n=6, no pooling). Protein pellets were subsequently treated with chondroitinase ABC (Sigma Aldrich, Zijdrecht, Netherlands) at 0.5 U/50 μ g protein for 90 min at 37° C. SDS-PAGE and immunoblotting were performed according to standard protocols (for details: **Supplementary Methods**) using the following primary antibodies: mouse anti-Tenascin-R (1:2,000; Dr. P. Pesheva, Bonn, Germany) and rabbit anti-brevican or guinea pig anti-brevican (respectively 1:10,000 and 1:2,000; Dr. C. Seidenbecher, Magdeburg, Germany).

Real-time quantitative PCR

Heroin-induced changes in transcript levels were analyzed from the mPFC of an independent group of rats (n=6-7) using Real-Time qPCR as described previously (Van den Oever et al., 2006). Transcript specific primers were designed for Bcan, Tnr and reference genes GAPDH and

NSE (see **Table S2**). For all statistical calculations, the absolute amount of normalized transcript of interest, $C * E^{-Ct} / NF$ ($C = 10^7$) was used.

Immunohistochemistry (IHC)

The labeling of perineuronal nets using WFA (marker for CSPG positive perineuronal nets; 5 μ g/mL, Sigma-Aldrich), ECM proteins, parvalbumin and CAMKII-alpha immunoreactive neurons was performed in naïve rats (n=3) according to standard protocols (for details; **Supplementary Methods**) using the following primary antibodies: rabbit anti-brevican (1:5,000), mouse anti-Tenascin-R (1:1,000), mouse anti-parvalbumin (1:1,000 Sigma-Aldrich) and mouse anti-CAMKII-alpha (1:200 Pierce antibodies).

Electrophysiology

Separate groups of rats (n=5 per group) trained to self-administer heroin or saline were used for recordings of IPSCs following both cue and no cue exposure tests. Immediately following a 30 min extinction or reinstatement test, rats were decapitated and brains were rapidly removed and put in ice-cold artificial cerebrospinal fluid (ACSF) which contained (in mM): NaCl 125; KCl 3; NaH_2PO_4 1.25; MgSO_4 3; CaCl_2 1; NaHCO_3 26; glucose 10; Osmolarity: 300 mOsm.

Coronal mPFC slices of 350 μ m thickness were prepared in sucrose-containing ACSF consisting of (in mM) KCl 3.5; CaCl_2 2.4; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.3; KH_2PO_4 1.2; Sucrose 215.5; NaHCO_3 26; D-glucose 10. Slices were stored in holding chambers containing normal ACSF consisting of (in mM): NaCl 125; KCl 3; NaH_2PO_4 1.25; MgSO_4 2; CaCl_2 1; NaHCO_3 26; glucose 10, bubbled with carbogen gas (95% O_2 / 5% CO_2). Pyramidal neurons and interneurons in layer II/III in the mPFC were visualized using either Hoffman modulation or differential interference contrast microscopy and whole-cell recordings from pyramidal neurons were made

using Multiclamp 700A amplifier (Axon Instruments, CA), digitized by the pClamp software (Axon), and later analyzed off-line using Synaptosoft (Delaware, GA) software. The pipette medium contained (in mM) Cs gluconate 120; CsCl 10; NaCl 8; MgATP 2; phosphocreatine 10; EGTA 0.2; HEPES 10; Tris GTP 0.3, QX 314-Cl 1. For detection of IPSCs, neurons were held at 0 mV potential. Statistical analysis was performed on individual neurons using 2-factor ANOVA with treatment (saline/heroin) and cue (cue/no cue) as between subject factors.

FN-439 intervention

Rats (n=7-9 per group) were implanted with jugular vein catheters as described above and for injection into the right lateral ventricle, a guide cannula (Plastics One 26-gauge) was stereotaxically placed against the dura with the coordinates anterior posterior -0.8 mm and lateral 1.3 mm relative to Bregma and thereafter securely fastened to the skull by acrylic dental cement. FN-439 (35 µg; Sigma Aldrich, Zwijndrecht, Netherlands) was injected 90 min before the first reinstatement test (Brown et al., 2007). The micro-injector (30-gauge) penetrated 3.7 mm below the dura and the compound was infused in a volume of 5 µl over 60 s. The injector was left in situ for another 120 s. For immunoblotting after reinstatement testing, rats were decapitated 3 hrs after the second reinstatement test and brains were rapidly frozen and stored until further use.

Results

iTRAQ-based proteomics: regulation of ECM proteins

We trained animals to self-administer heroin or saline for subsequent biochemical analysis as described above (**Fig. 1a**). Two-factor analysis (repeated measures) for responses in the active (heroin-paired) hole revealed a significant interaction for treatment ($F_{1,24} = 7.99$; $P < 0.009$),

session ($F_{15,360} = 12.59$; $P < 0.001$), and session x treatment ($F_{15,360} = 7.77$; $P = 0.001$). To identify relatively persistent heroin-induced changes in the synaptic proteome, the mPFC was dissected from rats 21 days after the final heroin self-administration session (**Fig. 1b**). Changes of a broad range of synaptic and synapse-associated proteins were quantified using isobaric Tags for Relative and Absolute Quantification (iTRAQ™) combined with two dimensional liquid chromatography and tandem mass spectrometry (2D LC-MS/MS), a powerful and reliable technology for quantification of neuronal proteins simultaneously from multiple samples (Hu et al., 2006; Li et al., 2007; Van den Oever et al., 2008). To isolate mPFC synaptic membrane fractions and to perform a duplicate measurement within a single iTRAQ experiment, 2 pools of heroin self-administration and 2 pools of saline self-administration (3 animals per pool) were generated (**Fig. S1**). In total 486 proteins were identified, with an average heroin to saline ratio of all quantified proteins of approximately zero (respectively -0.01 (\log_2)) and a σ of 0.13. Long-term forced abstinence from heroin self-administration resulted in significant ($P < 0.05$) and substantial regulation ($> \text{average} \pm \sigma$) of 27 proteins in mPFC that could be classified into proteins involved in cell adhesion and ECM proteins, signal transduction, neurotransmitter secretion, regulation of cell growth and metabolism (**Table 1**). In terms of magnitude of regulation, proteins of the ECM (Tenascin-R (Tnr), Brevican (Bcan) and Hyaluronan and proteoglycan link protein 1 (Hapl1)) stood out. Since these proteins have been reported to play an important role in neuronal plasticity but have not been described before in terms of regulation by drugs of abuse, they were selected for in depth studies and confirmation by immunoblotting.

Confirmation of regulation by immunoblotting and real-time quantitative PCR

Immunoblotting and real-time quantitative PCR (qPCR) were used to validate regulations found with iTRAQ-based proteomics at the protein and transcript level. In line with the observed down-

regulation of ECM proteins Bcan and Tnr by iTRAQ analysis, immunoblotting revealed a 49% decrease in the 160 Tnr isoform ($t_4 = 3.33$, $P = 0.03$) and a 41% decrease in the abundance of the Bcan 145 kDa isoform ($t_4 = 2.91$, $P = 0.04$) in mPFC synaptic fractions during long-term forced abstinence from heroin self-administration as determined in an independent group of rats (**Fig. 2a, b**). Synaptic membrane expression of Tnr 180 kDa was decreased in heroin animals, but did not reach significance ($t_4 = 2.43$, $P = 0.07$). Abundance of the membrane bound GPI anchored 120 kDa Bcan isoform (Seidenbecher et al., 1995) was unaltered. Levels of the corresponding proteins in total mPFC homogenate as well as transcript levels of Bcan and Tnr in mPFC did not differ after heroin self-administration (**Fig. 2b, c**), indicating that the observed down-regulation of these proteins occurred specifically at the synapse.

To gain more insight in the role of ECM proteins in extinction and reinstatement of heroin seeking, we determined whether expression of synaptic ECM proteins was also altered after 21 days of extinction from heroin self-administration and immediately after a cue-induced reinstatement test (30 min). Exposure to heroin conditioned cues induced robust heroin seeking (**Fig. 3**). We observed a significant difference for responses in the active hole between groups ($t_{16} = 3.25$, $P = 0.005$). Inactive nose-pokes were also significantly different between groups, but remained low (<10 responses; $t_{16} = 3.50$, $P = 0.003$). Similar to abstinence from heroin self-administration, One-way ANOVA with post-hoc analysis revealed a significant reduction in the level of Bcan 145 kDa (30%; $F_{2,6} = 16.2$; $P = 0.004$) after extinction of heroin self-administration and a similar (non-significant) trend for decrease in Tnr 180 and 160 kDa (~33%, $P > 0.05$) (**Fig. 4a**). Strikingly, we found that synaptic Bcan protein levels following exposure to heroin-conditioned cues were equivalent to the saline control group, indicating that cue-induced reinstatement of heroin seeking resulted in a rapid recovery of Bcan in the mPFC. Glutamatergic projections from the mPFC to the striatum play a critical role in reinstatement of drug seeking

(Kalivas et al., 2005; LaLumiere and Kalivas, 2008; Peters et al., 2009). Therefore, we determined whether Bcan and Tnr were also regulated in the NAc and dorsal striatum (**Fig 4b + c**). Indeed, levels of ECM proteins in synaptic membrane fractions were also reduced in the NAc, with a significant reduction in Tnr 180 kDa ($F_{2,6} = 46.7$; $P < 0.001$) and 160 kDa ($F_{2,6} = 40.3$; $P < 0.001$) abundance after extinction of heroin self-administration (~14% of control levels, $P < 0.05$). Similar to the mPFC, cue-induced reinstatement was paralleled by a significant increase in Tnr levels in the NAc, although the recovery was not complete (compared with saline control). We observed a trend (40% of control levels; $F_{2,6} = 4.161$; $P = 0.07$) for reduction in Bcan expression in the NAc that was also partially restored after cue exposure. Both Tnr and Bcan protein levels were unaltered in synaptic fractions of the dorsal striatum, indicating that the adaptation of ECM proteins is brain region specific.

Cellular localization of Tnr and Bcan in the mPFC

Previously, a direct interaction between Bcan and Tnr (Aspberg et al., 1997) and colocalization of Bcan and Tnr immunoreactivity in perineuronal nets in mid- and hindbrain was found (Hagihara et al., 1999). To establish that Bcan and Tnr colocalize in perineuronal nets around mPFC neurons, we performed a double-staining for both proteins on brain sections of naïve rats. Although Tnr and Bcan immunoreactivity could be observed at low levels throughout the entire frontal cortex, we observed an intense staining and complete overlap of Bcan and Tnr proteins condensed in perineuronal nets surrounding a subset of neurons within the mPFC (**Fig. 5a**). Moreover, PNN staining of Bcan colocalized with that of wisteria floribunda agglutinin (WFA; **Figure S2**), an ubiquitous marker for chondroitin sulfate proteoglycan positive perineuronal nets (Bruckner and Grosche, 2001; Pizzorusso et al., 2002).

Next, identification of the neuronal subtypes covered by perineuronal nets in mPFC was performed using WFA combined with the GABAergic interneuron marker parvalbumin (PV) and the excitatory neuron marker calmodulin-dependent kinase II alpha (CAMKII α). WFA-positive perineuronal nets mainly surround PV expressing interneurons in neocortex (Hartig et al., 1992), but some perineuronal nets envelope pyramidal neurons, the frequency of which differs between various regions of the cortex and even within subfields of cortical areas (Wegner et al., 2003; Alpar et al., 2006). We found that WFA positive perineuronal nets in infralimbic, prelimbic and anterior cingulate area of the mPFC always localized around the majority of PV expressing interneurons (**Fig 5b** and **Figure S2**). Colocalization with CAMKII-alpha was never observed (**Fig 5c**). This indicates that mPFC pyramidal neurons are not surrounded by perineuronal nets, and implies that the observed change in ECM proteins occurred specifically at GABAergic interneurons.

GABAergic transmission is increased after cue-induced reinstatement

The observation that ECM protein enriched around GABAergic interneurons in the mPFC are regulated after cessation of heroin self-administration and re-exposure to heroin-conditioned cues suggests that GABAergic interneuron activity may play a role in cue-induced reinstatement. This is supported by our previous observation that increasing GABAergic transmission in the mPFC enhances cue-induced reinstatement of heroin seeking behavior (Schmidt et al., 2005). Therefore, we investigated whether GABAergic synaptic transmission to mPFC pyramidal neurons was altered after extinction of heroin self-administration and immediately after a cue-induced reinstatement test. Animals with a history of saline self-administration served as controls. As PV-expressing interneurons are relatively sparse but target multiple pyramidal neurons in the mPFC (Couey et al., 2007), we recorded from mPFC pyramidal neurons. We monitored the spontaneous

inhibitory synaptic activity received by these neurons, which most likely reflects the activity of a number of inhibitory interneurons. Spontaneous inhibitory postsynaptic currents (IPSCs) were monitored in acute mPFC brain slices from rats that were exposed or not-exposed to heroin-conditioned cues (30 min). We found that the sIPSC frequency was almost doubled in animals re-exposed to heroin-conditioned cues (**Fig. 6**). A two-factor ANOVA with treatment (heroin/saline) and cue (cue/no cue) as between factors revealed a significant treatment effect ($F_{1,72} = 4.78$; $P = 0.03$), an effect of cue ($F_{1,72} = 5.74$, $P = 0.02$), and an interaction for treatment and cue ($F_{1,72} = 7.73$, $P = 0.01$). The frequency of sIPSCs did not differ between heroin self-administration animals that were not cue-exposed and saline control animals (**Fig. 6c**), indicating that specifically the presentation of heroin-conditioned cues strongly increased inhibition of mPFC pyramidal neurons. sIPSC amplitude and decay time constant were unchanged ($P > 0.05$) by heroin self-administration and cue presentation (**Fig. 6c**).

FN-439 attenuates cue-induced heroin seeking

Degradation and remodeling of the ECM is determined by the action of MMPs, a group of zinc-containing proteolytic enzymes and their endogenous antagonists, the tissue inhibitors of MMPs (Malemud, 2006). In order to show a direct relationship between the down-regulation of ECM proteins in the mPFC and NAc, and reinstatement of heroin seeking, we injected animals with the broad spectrum metalloproteinase inhibitor FN-439 (Otake et al., 1994), via intracerebral ventricular (ICV) microinjection 90 min before the first reinstatement test (Brown et al., 2007). A second reinstatement test was performed 24 hrs later. Presentation of cues in vehicle treated rats reliably reinstated responding in the active hole both on the first and the second test day, however, FN-439 treated animals showed an overall reduction in cue-induced reinstatement of heroin seeking (**Fig. 7a**). A repeated measures ANOVA with treatment as between-factor and test

as within-factor revealed a significant FN-439 treatment effect ($F_{1,31} = 5.85$; $P = 0.03$), and an effect of test ($F_{1,31} = 9.66$, $P = 0.01$), but no interaction between treatment and test. To determine the consequence of the FN-439 treatment on synaptic ECM protein levels, we analyzed Bcan and Tnr levels in mPFC and NAc synaptic membrane fractions 3 hours after the last reinstatement test (**Fig 7b-d**). Indeed, in FN-439 treated animals, Tnr 160 kDa (155%; $t_{10} = 2.33$, $P = 0.04$) and Bcan 145 kDa (185%; $t_{10} = 2.44$, $P = 0.03$) were present at significantly higher levels compared with vehicle-treated animals in the mPFC (**Fig 7c**) and to a smaller extent (non-significant) in the NAc (**Fig 7d**), indicating that restoring levels of ECM proteins prior to a reinstatement test reduced subsequent responding of animals to heroin-conditioned cues.

Discussion

The persistent nature of drug seeking behavior is thought to be caused by long-lasting alterations in neuronal circuitry involved in motivational and cognitive processing, such as the mPFC and striatum. Here, using an iTRAQ-based proteomics approach that allows changes in protein levels to be probed directly (Van den Oever et al., 2008), we show that heroin self-administration leads to persistent changes in protein levels in mPFC synaptic membrane fractions. Regulated proteins were classified into several functional groups, and the functional group of proteins with the most profound change in abundance after heroin self-administration contained several components of the ECM known to condense in perineuronal nets. The regulation of this set of proteins was subsequently confirmed in an independent experiment.

Perineuronal nets are net-like structures of the brain ECM that surround cell bodies, proximal dendrites and synapses of a subset of neurons in the CNS (Yamaguchi, 2000; Dityatev and Schachner, 2003). During development of the nervous system, the ECM is suggested to play a role in cell adhesion, cell migration and axon guidance (Bandtlow and Zimmermann, 2000;

Berardi et al., 2004), whereas in the adult brain ECM proteins condensed in perineuronal nets are suggested to maintain established neuronal connections (Murakami and Ohtsuka, 2003). Apart from a role in structural plasticity, perineuronal nets might act in the context of buffers for neurotransmitter release, electrical insulation and growth factor supply of synapses (Yamaguchi, 2000; Dityatev and Schachner, 2003). Degradation of perineuronal nets, by treatment with chondroitinase ABC, results in functional recovery of cortical plasticity (Pizzorusso et al., 2002; Pizzorusso et al., 2006) and promotes functional recovery of nervous system injury (Bradbury et al., 2002).

We found that long-term forced abstinence from heroin self-administration is associated with a decrease in the abundance of Bcan, Tnr and hyaluronan and proteoglycan link protein 1 (Hapln1), all constituents of perineuronal nets, locally at the synapse in the mPFC. Moreover, a similar change in protein levels was observed in synapses of the mPFC and NAc, but not dorsal striatum, following extinction of heroin self-administration. This suggests that long-term cessation of heroin self-administration is associated with adaptation of the ECM in the mPFC-NAc pathway, a neural circuit that plays a pivotal role in relapse to drug seeking (McFarland and Kalivas, 2001; Kalivas et al., 2005; Peters et al., 2008; Van den Oever et al., 2009). Bcan interacts with its C-type lectin domain with the ECM glycoprotein Tnr (Aspberg et al., 1997). LTP is impaired in mice that lack Bcan or Tnr, implicating a role of perineuronal nets in synaptic plasticity mechanisms (Saghatelian et al., 2001; Brakebusch et al., 2002). Link proteins, such as Hapln1, are thought to play an important role in formation and stability of perineuronal nets (Carulli et al., 2006). Bcan and Tnr immunoreactivity colocalizes in perineuronal nets in midbrain and hindbrain (Hagihara et al., 1999). Indeed, we observed that throughout the mPFC, Bcan and Tnr always colocalize in perineuronal nets, supporting the observed co-regulated decrease in expression after heroin self-administration and the possibility of interaction of these

proteins. Remarkably, we found that levels of Bcan rapidly increase following re-exposure to heroin-associated cues. The specific down-regulation of ECM proteins in synaptic membrane fractions, suggests that the expression level of these molecules is altered by the activity of locally acting MMPs (Wright and Harding, 2009). Furthermore, locally reduced MMP activity and extrasynaptic redistribution of ECM constituents may underlie the rapid nature of the recovery of ECM protein levels in synaptic fractions of the mPFC and NAc after exposure to heroin-conditioned cues.

In the mPFC, perineuronal nets are associated with PV-expressing GABAergic interneurons. PV-expressing neurons are fast-spiking inhibitory interneurons (Cauli et al., 1997; Kawaguchi and Kondo, 2002). Therefore, adaptation of ECM molecules may lead to an alteration in the structural stability or physiological properties of synapses at fast-spiking GABAergic interneurons. Interestingly, GABAergic interneurons in the neocortex exert powerful inhibitory control over the excitatory output of pyramidal neurons (Cowan et al., 1994; Constantinidis et al., 2002). Therefore, it is thought that GABAergic neurotransmission within the mPFC is an important regulator of inhibitory control over drug related behavior (McFarland et al., 2003; Steketee, 2005). Here, we found that cue-induced reinstatement of heroin seeking is associated with almost a doubling of the frequency of inhibitory inputs to mPFC pyramidal neurons, pointing to increased GABAergic interneuron activity upon cue exposure. At the same time, the decay time constant and amplitude of IPSCs were unchanged, arguing against a postsynaptic mechanism in mPFC pyramidal neurons. Increased GABAergic transmission upon exposure to heroin-conditioned cues is in line with our previous observation that infusion of GABA receptor agonists muscimol and baclofen in the prelimbic area enhances cue-induced reinstatement of heroin seeking (Schmidt et al., 2005). Similarly, cocaine-seeking assessed with the conditioned place preference model is accompanied by robust activation of GABAergic interneurons, as

opposed to pyramidal neurons, in the prelimbic area (Miller and Marshall, 2004). In addition, mPFC GABAergic transmission appears critically involved in cocaine sensitization (Steketee, 2005), and reinstatement of cocaine seeking (McFarland et al., 2003; Peters et al., 2007). When infused in the infralimbic area, GABA agonists augment cocaine seeking under extinction conditions by reducing glutamatergic output to the NAc shell (Peters et al., 2008). Although changes in ECM proteins were not analyzed at the subregional level within the mPFC, potentially, molecular changes in ECM constituents may account for enhanced responsivity of ventral mPFC (comprising the ventral prelimbic and infralimbic area) GABAergic interneurons to heroin-conditioned cues, thereby increasing inhibition of ventral mPFC pyramidal neurons during cue-induced reinstatement.

The final step in this study was to determine whether the observed ECM changes are indeed involved in vulnerability to relapse to heroin seeking. To that end, we counteracted the heroin self-administration-induced down-regulation of the ECM in mPFC and NAc by central application of the MMP inhibitor FN-439 (Otake et al., 1994) prior to a reinstatement test. Inhibition of MMP activity increased synaptic levels of Tnr and Bcan in mPFC (and to some extent in NAc) and attenuated cue-induced reinstatement of heroin seeking. Although inhibition of MMP activity in other brain regions than the mPFC and NAc may have contributed to this effect as well, it suggests that down-regulation of ECM proteins after cessation of heroin self-administration renders animals more sensitive to reinstatement by heroin-conditioned cues. The present observation is in line with the finding that inhibition of MMP activity impairs reinstatement of cocaine seeking in a conditioned place preference paradigm (Brown et al., 2007). However, the decrease in heroin seeking appears to be in contrast with the rapid up-regulation of Bcan in the mPFC and Tnr in the NAc that we observed following a reinstatement test. Assuming that the elevation in ECM protein abundance plays a role in cue-induced heroin

seeking, we speculate that this rapid increase in ECM proteins levels only affects heroin seeking (or is more effective) when initial ECM levels are low, such as observed during forced abstinence/extinction. Hence, recovering ECM protein levels by FN-439 treatment prior to a reinstatement test blunts the endogenous increase in ECM levels during reinstatement and may consequently reduce responding upon exposure to heroin-conditioned cues. A similar homeostatic mechanism involving extracellular glutamate levels in the NAc core is thought to underlie reinstatement of cocaine seeking (Kalivas, 2009). Alternatively, the acute up-regulation of ECM proteins may not be directly related to cue-induced heroin seeking, but may be a compensatory mechanism to counteract the increased inhibitory network activity during reinstatement. Future research should clarify whether this rapid accumulation of ECM proteins at synapses in the mPFC and NAc is long-lasting and how it contributes to extinction/reinstatement and GABAergic transmission.

Potentially, the modulation of ECM proteins condensed around GABAergic interneurons in the mPFC may represent a novel mechanistic element to augment GABAergic inhibition of pyramidal neuron activity during cue-induced relapse to heroin seeking. This, together with our previous observation that synaptic strength in ventral mPFC pyramidal neurons is reduced after exposure to heroin-conditioned cues (Van den Oever et al., 2008), suggests that excitatory output from the ventral mPFC is diminished. Consequently, response inhibition to drug-associated cues may be impaired (Peters et al., 2008; Van den Oever et al., 2009). Moreover, the observation that inhibition of MMP activity also impairs acquisition and reconsolidation of cocaine conditioned place preference (Brown et al., 2007), suggests that long-term adaptation of the ECM may play a more general role in the persistence of drug seeking behavior.

Concluding remarks

Here, we demonstrate that long-term cessation of heroin self-administration is associated with reduced levels of ECM proteins locally at the synapse in the mPFC and NAc. In the mPFC, these ECM proteins are condensed in perineuronal nets surrounding synapses at fast-spiking GABAergic interneurons, and cue-induced reinstatement is accompanied by an increase in GABAergic transmission. Our data suggest that ECM proteins are part of a mechanism that alters cue-conditioned responsivity of mPFC GABAergic interneurons and contributes to vulnerability to relapse to heroin seeking after prolonged cessation of heroin self-administration. A better understanding of these molecular mechanisms is of ultimate importance for the discovery of new entry points into enabling synaptic flexibility: i.e. providing plasticity and renewed learning at ‘static synapses’ under conditions of psychiatric disease, including drug addiction.

Disclosure

All authors report no biomedical financial interests or potential conflicts of interest.

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Tables

Acces. nr.	Protein ID	Gene Symbol	# unique peptides	Reg ± SEM (log ₂)	p-value	Ratio Her-Sal
Cell adhesion / Extracellular matrix						
P55068	Brevican core protein precursor	Bcan	5	-0.33 ± 0.12	0.0495	0.80
6981668	Tenascin R	Tnr	11	-0.33 ± 0.12	0.0195	0.80
P03994	Hyaluronan and proteoglycan link protein 1 precursor	Hapln1	8	-0.25 ± 0.08	0.0183	0.84
109468364	similar to Catenin delta-1	Ctnnd1	4	-0.28 ± 0.09	0.0442	0.82
20302073	Cadherin 13	Cdh13	4	0.18 ± 0.05	0.0314	1.13
Signal Transduction						
P10824	Guanine nucleotide-binding protein G(i), alpha-1 subunit	Gnai1	5	0.27 ± 0.08	0.0286	1.20
P68182	cAMP-dependent protein kinase, beta-catalytic subunit (PKA Cβ)	Prkacb	7	-0.28 ± 0.09	0.0248	0.82
16758606	Arg/Abl-interacting protein ArgBP2	Argbp2	8	0.17 ± 0.07	0.0432	1.12
P63322	Ras-related protein Ral-A	Rala	4	0.26 ± 0.07	0.0399	1.20
Q6NYB7	Ras-related protein Rab-1A	Rab1	9	0.15 ± 0.04	0.0033	1.11
P08050	Gap junction alpha-1 protein (Connexin 43)	Gjal	9	0.17 ± 0.06	0.0181	1.12
Neurotransmitter secretion						
13489067	N-ethylmaleimide sensitive fusion protein	Nsf	20	0.13 ± 0.04	0.0055	1.10
Q63537	Synapsin-2	Syn2	14	0.15 ± 0.07	0.0457	1.11
Regulation of cell growth						
P07936	Neuromodulin (Axonal membrane protein GAP-43)	Gap43	20	0.18 ± 0.07	0.0160	1.14
109504667	similar to G protein-regulated inducer of neurite outgrowth 1	Gprin1	24	0.14 ± 0.03	0.0000	1.10
Metabolism						
P47858	6-phosphofructokinase (Phosphofructokinase 1)	Pfkm	5	-0.19 ± 0.06	0.0275	0.88
P11951	Cytochrome c oxidase polypeptide VIc-2	Cox6c	7	-0.15 ± 0.03	0.0035	0.90
Q63362	NADH-ubiquinone oxidoreductase 13 kDa-B subunit	Ndufa5	5	-0.21 ± 0.04	0.0052	0.86
27663138	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6	Ndufa6	4	-0.23 ± 0.06	0.0252	0.85
109480482	similar to NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 12	Ndufa12	5	-0.30 ± 0.08	0.0216	0.81
55926203	Isocitrate dehydrogenase 3 (NAD ⁺) beta	Idh3B	6	0.28 ± 0.08	0.0147	1.22
P19234	NADH-ubiquinone oxidoreductase 24 kDa subunit	Ndufv2	6	-0.26 ± 0.07	0.0173	0.84
Other						

34854665	calcium channel beta-4 subunit	Cacnb4	3	0.52 ± 0.11	0.0423	1.44
37812499	Optic atrophy 1-like protein	Opa1	9	-0.19 ± 0.08	0.0463	0.88
P63018	Heat shock cognate 71 kDa protein	Hspa8	12	-0.18 ± 0.05	0.0062	0.89
Q9QZR6	Septin 9	Sept9	6	-0.16 ± 0.06	0.0363	0.89
109480904	similar to lymphocyte antigen 6 complex, locus H	Ly6h	4	0.22 ± 0.04	0.0142	1.16

Table 1: Heroin induced alteration of synaptic protein levels in mPFC. Proteins (indicated by name and gene symbol) that showed a significant change ($P < 0.05$) with a regulation (ratio; \log_2) that is more than the average $\pm \sigma$ classified based on their function (Gene Ontology). Accession numbers were derived from the rat SwissProt and NCBI database. Ratios are based on the average iTRAQ area abundance of two independent synaptic membrane fractions for each treatment.

Figure legends

Figure 1 Acquisition of heroin self-administration and mPFC dissection. (a). Heroin self-administering rats developed a stable preference for the active hole, whereas active hole responses remained low for saline self-administration rats (data not shown). Responding increased for heroin self-administering rats when the fixed ratio (FR) was doubled (FR1, FR2, FR4), indicative of a motivational drive to self-administer heroin. Inactive hole responses remained low for both heroin and saline rats during all sessions. Data represent mean \pm SEM of responses in active (heroin-paired) or inactive (unpaired) holes. (b) Approximate anatomical dissection limits of the mPFC (between bregma 3.7 to 2.7) are depicted by the hatched area.

Figure 2 Protein level changes of ECM proteins Bcan and Tnr confirmed by immunoblotting. (a) Synaptic protein (Syn) and total protein (Total) fractions were isolated from the mPFC of a group of animals (independent of iTRAQ experiment) and analyzed by immunoblotting using antibodies against Tnr and Bcan. Representative examples are shown. Levels of immuno-detected bands were normalized to proteins on a coomassie (coom) stained gel that was run in parallel. (b) Optical densities (blot, coomassie) were analyzed and set to the saline control. A significant decrease was detected in the level of Tnr 160 kDa isoform and Bcan 145 kDa isoform in synaptic fractions of the mPFC. Tnr and Bcan protein levels were unaltered in the total mPFC homogenate. (c) The mRNA level of Tnr and Bcan was unaltered in the mPFC during forced abstinence from heroin self-administration. All data are mean \pm SEM. * $P < 0.05$ (Student *t*-test).

Figure 3 Cue-induced reinstatement of heroin seeking. Exposure to the compound visual and auditory cue reinstated responding in the active (previously heroin-paired) hole. Bars represent mean number of nose pokes \pm SEM. * $P < 0.01$ (Students *t*-test) for between group difference of responses in active hole and within group difference of active and inactive responses.

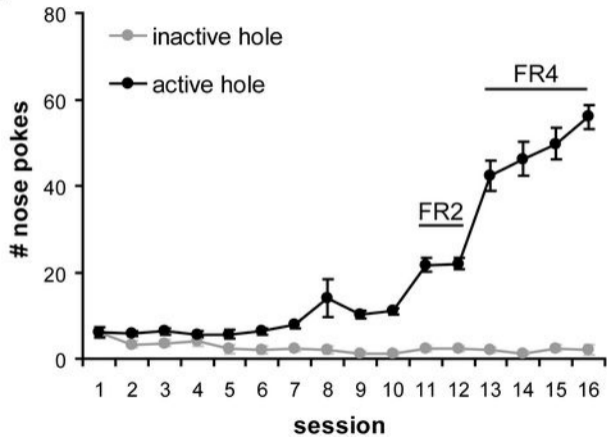
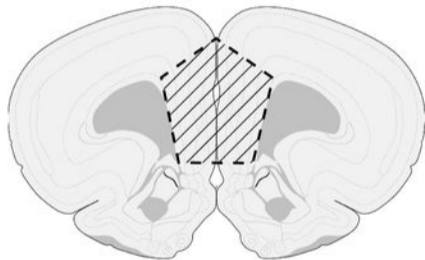
Figure 4 ECM proteins are also down-regulated after extinction of heroin self-administration. We compared levels of Bcan and Tnr proteins in synaptic fractions of the mPFC, NAc and dorsal striatum (dStriatum) between animals that underwent saline or heroin extinction, and heroin extinction animals that were re-exposed to heroin-conditioned cues (30 min). Representative examples are depicted on the left side. **(a)** We observed a significant reduction in the synaptic protein levels of Bcan (145 kDa; $P < 0.05$) and a similar (non-significant) trend for decrease in Tnr 180 and 160 kDa ($P > 0.05$) levels after extinction of heroin self-administration. The abundance of Bcan 145 kDa did not differ between saline animals and heroin animals that were cue-exposed. Similar to levels during abstinence from heroin self-administration, the level of the Bcan 120 kDa isoform was not altered. **(b)** In the NAc, the levels of Tnr 180 kDa and 160 kDa were significantly ($P < 0.001$) different, with post-hoc analysis revealing a significant difference between all groups. The level of Bcan 145 kDa followed a similar pattern but ANOVA analysis only reached near significance ($P = 0.074$). **(c)** Abundance of all Bcan and Tnr isoforms in synaptic fractions of the dorsal striatum was not significantly different. All data are mean \pm SEM.

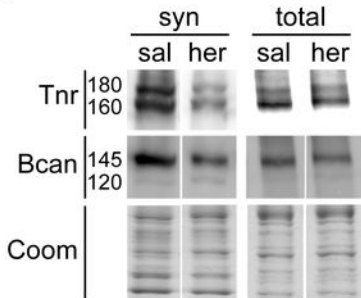
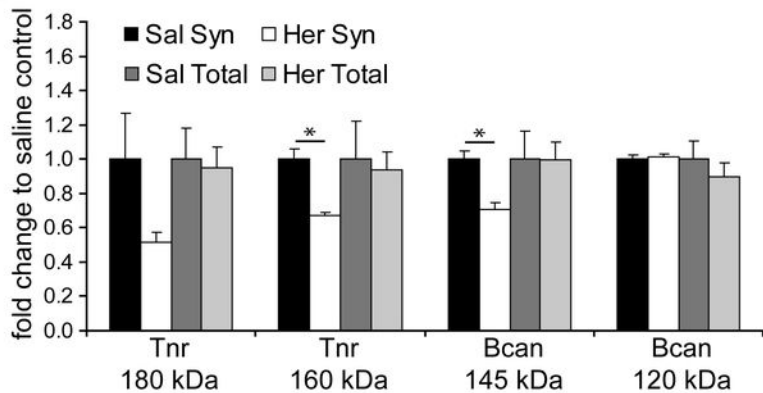
Figure 5 Immunohistochemical identification of ECM proteins in the mPFC. (a) Colocalization of Tnr and Bcan in perineuronal nets in the mPFC. Sections were double-stained using Tnr (green) and Bcan (red) specific antibodies and analyzed by confocal microscopy. Immunoreactivity of Tnr and Bcan is colocalized (merged image) in perineuronal nets (white arrows) surrounding a subset of neurons in the anterior cingulate, prelimbic and infralimbic area of the mPFC. **(b + c)** Biotinylated-WFA (green) was used to detect chondroitin sulfate proteoglycan-containing perineuronal nets in combination with a PV-specific antibody (red) or CAMKII α antibody (red). Throughout all areas of the mPFC, perineuronal nets were associated with the majority of PV-expressing interneurons **(b)**, but never with CAMKII-positive pyramidal neurons **(c)**. *Scale bar: 20 μ m.*

Figure 6 Cue-induced enhancement of GABAergic inhibition of mPFC pyramidal neurons.

(a) Example traces of whole-cell recordings from mPFC pyramidal neurons in control and heroin self-administration animals ($n = 5$ per group) that were re-exposed (cue) or not exposed (no cue) to the cues. Number of neurons in each group: Heroin cue, $n = 20$; Heroin no cue, $n = 20$; Saline cue, $n = 17$; Saline no cue, $n = 16$. (b) Average IPSC frequency in all neurons recorded during a 3-minute period (10 second bins). (c) Summary data and statistical analysis: average IPSC frequency was significantly higher in heroin animals upon cue re-exposure (* $P = 0.007$). IPSC amplitude and decay time constant remained unaltered ($P > 0.05$) upon cue re-exposure. Data represent mean \pm SEM.

Figure 7 FN-439 treatment attenuates cue-induced heroin seeking and increased ECM protein levels in the mPFC. (a) ICV injection of FN-439 attenuated cue-induced heroin seeking as compared to vehicle (aCSF; n = 7-9 per treatment). Nose-poking in the inactive hole was not altered by FN-439 treatment (data not shown). (b) Three hours after the last reinstatement test, synaptic fractions were isolated from the mPFC of these animals and analyzed with immunoblotting. (c) A significant increase was detected in the levels of Tnr 160 kDa and Bcan 145 kDa isoform in the mPFC. (d) Expression of Tnr and Bcan in the NAc was increased after FN-439 treatment, but did not reach significance ($P > 0.05$). * $P < 0.05$. Data represent mean \pm sem.

a**b**

a**b****c**