Cocaine-Induced Expression Changes of Axon Guidance Molecules in the Adult Rat Brain

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Running title: *"Cocaine-induced Plasticity and Regulation of Axon Guidance Molecules"*

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*KEY WORDS: **COCAINE, DRUGS OF ABUSE, SEMAPHORIN, EPH, EPHRIN, PLASTICITY, AXON GUIDANCE, DNA-MICROARRAY, QUANTITATIVE REAL-TIME PCR***

ABSTRACT

Administration of drugs of abuse induces strong molecular adaptations and plasticity within the mesolimbic dopamine (DA) system, a pathway essential for reward-seeking behavior. Little is known about the specific targets involved in this neuroadaptation process, but there are indications that cocaine and other drugs of abuse share the ability to alter the morphology of neuronal dendrites and spines, the primary site of excitatory synapses in the brain. Axon guidance molecules, the very molecular cues that regulate the formation of axon-target connections during development, may mediate these alterations. To test this hypothesis, we investigated mRNA expression changes of 39 axon guidance molecules, including 17 Semaphorins, 12 Ephs, 8 Ephrins and 2 neuropilins in the mesolimbic dopamine system of cocaine-treated animals under different paradigms by mean of DNA-Microarray and quantitative real-time PCR. In all cases, strong changes in gene expression are observed, yielding to up or down-regulation of these axon guidance molecules. Our data suggest that cocaine treatment induces activation of a complex program of synaptic rearrangements, which may partly recapitulate the plastic changes occurring during development, and may underlie the important neuroplastic adaptations that occur in the reward- and memory-related brain centers following drug action. We conclude that in some brain regions, exposure to psychomotor-stimulant drugs produce expression changes in axon guidance molecules, which may contribute to cognitive deficits associated with drug abuse.

INTRODUCTION

Repeated exposure to drugs of abuse induces complex rewiring of neural circuitry that results in the behaviors associated with addiction (Chao and Nestler, 2004, Nestler 2000, Berke et al., 1998). Molecular adaptations following acute and chronic drug administration involve the mesolimbic dopamine (DA) system as the primary site affected in drug dependence and underlies strong plasticity in response to the drug (Schultz, 1998). Addictive drugs (cocaine and amphetamines), depressants (ethanol) and opiate narcotics (heroin and morphine) are very powerful reinforcers and produce their rewarding effects of euphoria or pleasure through an interaction with the mesolimbic DA system (Nestler, 1997). Within the tegmental area, interaction between neurotrophic factors or cytokines and drugs of abuse has been well established (Berhow et al., 1996; Nestler et al., 1996). CNTF mimics some of the long-term effects of cocaine and opiates in the tegmentum through its GP130 receptor (Ip and Yancopoulos, 1996), which triggers the JAK-STAT pathway. There is also evidence for drug-regulated alterations of glial cells in the tegmentum (O'Callaghan and Miller, 1994). Major known neuronal adaptations include intracellular down-stream changes, such as decreases in Giα and Goα levels, changes in the cAMP pathway (Nestler et al., 1990), increases in tyrosine hydroxylase levels, and altered expression of transcription factors (ΔfosB) (Nestler et al., 1999, Moratalla et al, 1996, Moratalla et al., 1993), neurofilaments and phosphoproteins (α-internexin, NF-66) (Sklair-Tavron 1996, Beitner-Johnson, 1992). Perturbation in the BDNF signaling cascade, yielding an increase of extracellular signal regulated kinase (ERK) and tyrosine hydroxylase induction has also been reported (Sklair-Tavron 1996; Berhow et al., 1995).

However alterations at synapses - a key process in drug-related plasticity - may be guided by the expression of axon guidance molecules (Grünwald, 2004, Gosh, 2002, Goldenschwegge et al., 2002, Atwal et al., 2004, Pasquale, 2000). Semaphorins might significantly contribute to the maintenance and stability of neuronal networks and play important roles in the regeneration, or failure thereof, of

neuronal connections (De Wit and Verhaagen, 2003). Eph receptors are involved in synaptic plasticity (Grünwald et al, 2001, Henderson et al, 2001) and several Eph molecules are upregulated after injury (Willson et al, 2002, Battaglia et al, 2003, Knöll et al, 2001). Nevertheless little is known about cell surface molecules involved in neuroadaptations after drug intake. Strong induction of a surface tetraspanin protein, CD81, has been described (Michna et al., 2001, Halladay et al., 2000, Brenz-Verca et al., 2001, Bahi et al., 2004). Also specific axon guidance cues, such as EphB1 and ephrin-B2, are expressed after challenges with psychomotor stimulants, (Zhou, 1998; Yue et al., 1999). These two complementary cues guide neurons during the development of the central nervous system for the establishment of topographic projections in the mesolimbic dopaminergic pathway (Yue et al., 1999), suggesting that the very cues that attract, repulse or hem axon growth cones on their way to their synaptic targets, are re-expressed in the adult brain after a drug challenge to the CNS. Alterations at synapses might therefore be guided by the very molecular cues that formed the projection-target connections during development. Based on this suggestion, we further tested this hypothesis on a large number of axon guidance molecules, namely the semaphorins and their cognate receptors, the neuropilins, and the Eph receptor tyrosine kinases and their cognate ligands, the Ephrins.

The semaphorins are among the best-studied axonal guidance molecules and appear to play key roles in these cellular events (Bagri et Tessier-Lavigne, 2002, Atwal et al., 2004, Püschel et al., 2002, Wit and Verhaagen, 2003). To date, more than 20 different semaphorins have been identified. They fall into eight groups on the basis of domain organization and species of origin, but all semaphorins contain a conserved, 500-amino-acid-length "Sema" domain at their amino-terminus (Püschel et al, 2002). This domain is also present in the plexin (Plex) family (Püschel et al, 2002). The other family of axon guidance molecules investigated in this study, the Eph receptors and ephrin ligands, have provided important insights into the molecular mechanisms underlying cellular interactions regulating axon guidance, cell migration, and morphogenesis (Flanagan and Vanderhagen, 1998). Interactions between ephrins and Ephs require

cell-cell contact, thereby raising the intriguing possibility that in some developmental contexts, signaling is bidirectional (Osterfield et al., 2003).

We investigated the expression of these guidance cues in the mesolimbic dopamine system of cocaine-treated animals under various paradigms by means of DNA-Microarrays and quantitative real-time PCR. Drastic and complex changes in expression observed upon cocaine administration are discussed.

EXPERIMENTAL PROCEDURES

1. Animal Handling

Subjects used in this experiment were male Wistar rats weighing 225-250 g (BRL, Fillingsdorf, Switzerland). All animal experiments were carried out in accordance with the guidelines and regulations for Animal Experimentation, BAG, Bern, Switzerland. The animals were housed in trios in clear plastic cages with wire grid lids. Access to food and water was unrestricted. The animals were kept in the animal facility maintained on a 12-h light: 12-h dark cycle (lights off at 19:00 h).

Drug administration Protocols*:** Four different protocols were used in this study, depicted in Figure 1. These protocols represent different paradigms of drug administration, based on established methodologies and allow for good evaluation of drug-induced expression changes and pattern correlation among the various candidates. These included *(a)* an ***acute treatment, consisting of a single dose of cocaine (15 mg/kg, i.p.); *(b)* a ***binge treatment*** consisting of four high doses (30 mg/kg i.p.) at two hours interval within a single day *(c)* a ***chronic treatment***, consisting of a daily dose of cocaine (15 mg/kg i.p. over 15 days); and *(d)* a ***sensitization protocol*** subdivided into four test groups. After sacrifice, four brain regions were dissected out (Hippocampus, Nucleus Accumbens, Striatum and Ventral Tegmental Area), the total RNA was extracted, amplified, labeled and hybridized onto microarrays. The remaining RNA was reverse transcribed for quantitative real-time PCR.

Acute Paradigm: rats (n=6) were i.p. injected once with 15 mg/kg cocaine-HCl (Sigma Chemical Co., Buchs, Switzerland). Control animals received 0.9% saline i.p. injection. All the animals were sacrificed 24 h after the last injection by decapitation

Binge Paradigm: rats (n=6) were i.p. injected with 30 mg/kg cocaine-HCl (Sigma Chemical Co., Buchs, Switzerland) every 2 hours for 4 injections. Control animals received 0.9% saline i.p. injection under the same schedule. All the animals were sacrificed 24 h after the last injection by decapitation

Chronic Paradigm: rats (n=6) were daily i.p. injected with 15 mg/kg cocaine-HCl (Sigma Chemical Co., Buchs, Switzerland) for a period

of 15 days. Control animals received 0.9% saline i.p. injection under the same schedule. All the animals were sacrificed 24 hours after the last injection by decapitation

Sensitization Paradigm (see Figure 1 for samples identification): Four groups (n=6 per group) were used. Groups **S1** and **S2** represent control groups, treated with saline during an initial period, when groups **S3** and **S4** received cocaine over five days of sensitization. After a short withdrawal period (3 days, groups **S1** and **S3**) or a long withdrawal period (14 days, groups **S2** and **S4**), animals were challenged and sacrificed 24 h later

S1 and S2 groups: rats were treated ("sensitized") with daily i.p. injections of 0.9% saline during 5 days. On the 3rd day (**group S1**) or on the 14th day (**group S2**) after the last administration ("sensitization"), half of the groups were challenged with a single i.p. injection of 0.9% saline and the other half with a single i.p. injection of 15 mg/kg cocaine-HCl (Sigma Chemical Co., Buchs, Switzerland). Groups were sacrificed by decapitation 24 h later, on the 4th and the 15th days for groups **S1** and **S2**, respectively.

For the S3 and S4 groups: rats were sensitized with daily i.p. injections of 15 mg/kg cocaine-HCl (Sigma Chemical Co., Buchs, Switzerland) during 5 days. On the 3rd day (**group S3**) or on the 14th day (**group S4**) after the last sensitization, half of the groups were challenged with a single i.p. injection of 0.9% saline and the other half with a single i.p. injection of 15 mg/kg cocaine-HCl (Sigma Chemical Co.). Groups were sacrificed by decapitation 24 h later, on the 4th and the 15th days for groups **S3** and **S4**, respectively.

2. RNA Preparation and Microarray Hybridization

RNA Extraction: After sacrifice, brain regions were dissected out (Hippocampus, Nucleus Accumbens, Striatum and Ventral Tegmental Area), and the total RNA was extracted. In all cases brain regions were pooled before preparing RNA, since previous control experiments with unpooled samples had shown no statistical differences compared to pooled samples.

Total RNA was isolated using RiboPure Kit (Ambion, UK) according to the manufacturer's protocol. Briefly, 1 ml of RNAwiz was added to 50 mg of tissue, homogenized with a Polytron homogenizer, vortexed

for 30 s and incubated at room temperature for 5 min. Residual protein was removed by the addition of 0.2 ml of chloroform, mixing for 30 s, incubation at room temperature for 5 min, vortexing for further 30 s, and centrifugation for 15 min at 12000 g and room temperature. The aqueous phase was precipitated with 0.5 ml of absolute ethanol, filtered over a column and washed by centrifugation. The total RNA was then eluted with 0.1 ml of DEPC-treated water, incubated at 60 °C for 10 min and stored at –80 °C.

RNA Amplification: This was performed according to Kacharmina et al. (1999). First Strand Synthesis was performed in 0.2 ml RNAse-free PCR tubes as follows: 2 μg of total RNA was added to 9 μl of 0.6M Trehalose in DEPC water and 1 μg Eberwine oligo-dT/T7 primer (5'AAA CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG CGC T15-3'). These components were mixed well by pipetting and heated at 65°C for 10 minutes, then kept on ice. To the mixture 4 μl of 5X First Strand Buffer (Invitrogen, Basel, Switzerland) was added, followed by 2 μl 0.1M DTT, 10 U RNAsin (Invitrogen, Basel, Switzerland), 1 μl 10 mM dNTP mix (Promega, Wallisellen, Switzerland), 1 μl linear acrylamide (0.1 mg/ml), 1 μl 200 U/μl Superscript II RNaseH-Reverse Transcriptase (Invitrogen, Basel, Switzerland). The mixture was incubated in a thermocycler at: 37°C, 5 min; 45° C, 5 min; then 25 cycles alternating between 60°C, 2 min and 55°C, 2 min. Tubes were kept on ice, while adding second strand components. Second Strand Synthesis was followed by adding 106 μl DEPC water, 15 μl Second Strand Buffer [200 mM Tris pH 6.9, 900 mM potassium chloride, 46 mM magnesium chloride, 1.5 mM Nicotine Adenine Dinucleotide (Calbiochem, Nottingham, UK), 100 mM ammonum sulfate, 3 μl 10 mM dNTP mix (Promega, Wallisellen, Switzerland), 10 U E. coli DNA Ligase (Bioconcept, Allschwill, Switzerland), 30 U E. coli DNA Polymerase I-holoenzyme (Bioconcept, Allschwill, Switzerland), 2 U RNase H (Invitrogen, Basel, Switzerland). The mixture was incubated at 16°C for 4 hours. The reaction was stopped with 7.5 μl 1M NaOH/2mM EDTA with incubating at 65°C for 10 minutes. The double-stranded DNA was extracted once with Phenol:chloroform:isoamyl (25:24:1). 150 μl of organics were added directly to PCR tubes, mixed and centrifuged

for 5 min at 15'000 g at room temperature. The aqueous phase was transferred to Rnase-free 1.5   l Eppendorf tube and DNA was precipitated with 70 μl of 7.5 M ammonium acetate (in DEPC-water, 0.2 micron filtered) and 1 ml absolute ethanol (-20°C). Tubes were vortexed and centrifuged immediately for 20 min at 15'000 g at room temperature. The pellet was washed once with 100 μl absolute ethanol and resuspended in 10 μl of DEPC-water. *In vitro* transcription was then performed with the Ambion T7 Megascript Kit (Ambion, UK), according to the manufacturer's protocol.

Probe Preparation and Hybridisation: Each aRNA sample (one from control sample and one from treated sample) was reverse-transcribed in the presence of Cy3-dCTP (control samples) or Cy5-dCTP (treated samples) (Amersham Pharmacia Biotech, Dübendorf, Switzerland). Each reaction was performed in a 30 μl volume containing 2 μg of aRNA, 2 μg of oligo(dT) 21-mer, 500 μM each of dATP, dGTP, and dTTP, 200 μM dCTP, 100 μM Cy3-dCTP or Cy5-dCTP, 30 units of RNase inhibitor, 10   M DTT, and 400 units of SuperScriptII reverse transcriptase (Invitrogen, Basel, Switzerland) in SuperScript buffer. After incubation at 42°C for 3 hours, the sample tubes containing Cy3 and Cy5 labeling were pooled and treated with 2.65 μl of 25 mM EDTA and 3.3 μl of 1 M NaOH for 10 min at 65°C to degrade the RNA. After the addition of 3.3 μl of 1 M HCl and 5 μl of 1 M Tris-HCl, pH 6.8, labeled single stranded DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of absolute ethanol, and the pellet was washed with 80% ethanol, dried, and resuspended in 10 μl of hybridization solution containing 3X SSC, 0.2% SDS, and 0.02% yeast tRNA (Invitrogen, Basel, Switzerland). Probes were purified by Millipore (Volketswil, Switzerland) Ultrafree-MC filters.

Hybridization Reaction and Microarray Analysis: Before hybridization, the probe solution was boiled for 1 min and rapidly applied to the microarray under a cover slip. Slides were placed in hybridization chambers, and 20 μl of 3X SSC was placed inside each chamber before sealing. Slides were incubated for 14 to 16 hours in a water bath at 64°C and washed sequentially in the following solutions: 2X SSC, 0.1% SDS twice for 5 min, 0.2X SSC twice for 1 min, and 0.1X SSC twice for 1 min. Slides were dried by centrifugation at

900 g for 2 min before scanning.

Microarrays were scanned with a scanning laser microscope Affymetrix® 428™ Array Scanner; (Affymetrix, INC., Santa Clara US). Separate images were acquired for each fluorochrome at a resolution of 10 μm per pixel. To normalize the two channels with respect to signal intensity, photomultiplier and laser power settings were adjusted such that the signal ratio of the control genes was as close to 1.0 as possible. The average fluorescence intensity for each fluorochrome and for each gene was determined by using Jaguar 2.0 software (Affymetrix, INC., Santa Clara US). Background fluorescence was calculated as the median fluorescence signal of non-target pixels around each gene spot. Induction or repression of a gene was defined as a minimum 1.5-fold change in its transcript level.

Statistical Evaluation of Microarray data: Signal intensities of spots corresponding to a given candidate from three independent tissue samples, with mean standard deviation, were plotted as cocaine-treated versus saline-treated probes. Quantification was presented as the ratio between experiments (Cy5-labeled samples) versus control (Cy3-labeled samples) changes in expression at mRNA level, after background substraction. The raw data were initially analyzed using Jaguar 2.0 (Affymetrix, INC., Santa Clara US), which calculates normalized expression levels and generates ratios of experimental (Cy5) versus control (Cy3) signals. Then data sets from comparison files were imported into Excel (Microsoft) for further analysis and p-values calculations. A stringent cut-off was used for significance: genes were considered to be down regulated if the ratio of experimental vs. control was ≤2.0 (with p<0.01) and up-regulated if the ratio was ≥2 (with p<0.01). Significance was calculated using a t-test (McClung and Nestler, 2003).

3. Microarray Design

Selected Gene Candidates (see Table 1): Microarray design was performed according to published methods (Shalon et al., 1996). 20 control oligonucleotides were included consisting of 10 negative controls (oligonucleotides randomly generated with no expression within neither the mouse nor the rat genomes) and 10 positive controls: β-Actin (NM_031144), Transcription factor E2α (X17500),

Lactate dehydrogenase A (NM_017025), Hsp70-1 (NM_031971), Nuclear pore glycoprotein 62 (M62992), Ribophorin 20814 (NM_031698), Ribosomal protein L5 (NM_031099) Ribosomal protein S9 (NM_031108, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (NM_017008) and Polyubiquitin (D16554). The means of values of the negative controls were considered as background and subtracted from all other data. Besides these positive and negative control oligonucleotides, the microarray included oligonucleotides corresponding to the 39 axon guidance gene candidates selected for this study, which are listed in Table 1. These were 17 members of the Semaphorin family, 2 members of their corresponding receptors (Neuropilin 1 and Neuropilin 2), 12 members of the Eph Receptor Tyrosine Kinase (7 members of the EphA family and 5 members of the EphB family) and 8 members of their corresponding Ephrin ligands (5 Ephrin-A and 3 Ephrin-B). Candidates, including gene ID and oligonucleotide sequences and BLAST reference are listed in **Table1**. _Note:_ Oligonucleotides sequences of positive and negative controls are patented (QIAGEN™, Basel, Switzerland) and are not listed.

Specific oligonucleotides were designed (*see below*) and sythetized with 5′-amino modification. Oligonucleotides were resuspended in 1.5 M Betaine, 3X SSC to a final concentration of 40 μM and then arrayed (8 spots/oligo) onto QMT Aldehyde Slides (Quantifoil, Germany) by using a high precision OmniGrid Accent™ Microarrayer (GeneMachines, San Carlos, CA) equipped with four printing tips. Each candidate was spotted eight fold, providing hybridization replicates on the same slide. After printing, slides were allowed to dry, and unbound DNA was removed with 0.2% SDS and double-distilled water; covalently bound DNA was denatured for 2 min in boiling water. Free aldehydes were reduced by soaking slides for 5 min in 68 mM Sodium Borohydride (dissolved in PBS containing 25% ethanol). Several washing steps were performed with 0.2% SDS and double-distilled H_2O; then slides were dried by centrifugation at 500g for 5 min and stored at room temperature for further hybridizations.

Probe design and selection rules (QIAGEN™, Basel, Switzerland):

For each selected gene candidate, several 70mer probes were designed. Each probe was sequence optimized using BLAST (Basic Local Alignment Search Tool) for nucleotide sequence to minimize cross hybridization in the microarray experiments. The 70mers were also *Tm*-normalized to 78°C (±5°C) for a more stringent hybridization at higher temperatures. Where 70mers could not be designed within the specified *Tm* range, shorter- or longer-length oligos were substituted to maintain the stringent *Tm* conditions. In addition, each 70mer was flanked by a 5' C6 amino linker. Designed oligo were chosen within 1000 bases from the 3' end of the gene sequence such as excluding contiguous single nucleotide base repeats, poly(N) tracts longer than 10 bases or hairpin structures with a stem-length longer than 9 bases. A normalized score was assigned to each oligo, based on the number of repeats, and with ≤70% identity to all other genes. Using BLAST, each oligo was aligned against all 59,018 representative sequences in Rat UniGene Build Rn 90 and 62,758 representative sequences in Rat UniGene Build Rn 108. Using the alignment with the candidate oligo versus the highest scoring non-self gene, a BLAST percent identity score was computed, defined as the sequence that yielded the most matched bases in an alignment.

This BLAST percent identity was also referred to as cross-hybridization identity of the oligo. This calculated cross-hybridization identity was dependent on the size of the sequence database used to BLAST against oligo sequence, and the use of either gapped or no-gap alignment method. Furthermore, oligos could not have greater than 20 contiguous bases common to any other gene. Once oligo candidates were designed, satisfying all selection rules mentioned above, each oligo was ranked based on BLAST percent identity and one final oligo for each gene was selected with minimum percent identity or cross hybridization similarity.

Real-Time PCR and mRNA Quantification
Total RNA was isolated from different brain regions using RiboPure Kit (Ambion, UK), according to the manufacturer's protocol including a RNase-free DNase step, and stored at –80°C. RNA were quantified by spectrophotometry. Integrity of the RNA was verified by agarose gel electrophoresis and visualized with ethidium bromide staining. First strand cDNA was synthesized using 1µg of total RNA and Oligo(dT) primers with the M-MLV reverse transcription kit (Invitrogen, Basel, Switzerland) in a total volume of 15 µl .

Real-time PCR was performed to confirm Microarray results. Primer sets were designed to amplify 100- to 300-bp products, using PRIMER3 software

(http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

To quantify expression of cDNAs the real-time PCR iCycler (BioRad, Reinach, Switzerland) was used. For PCR, 5µl cDNA preparation, 0.5µM of forward and reverse primers and 10µl of IQ SYBR Green Supermix (Biorad, Reinach, Switzerland) in a total volume of 20µl were applied. The following PCR program was performed: 3 min at 95°C (initial denaturation); 20°C/sec temperature transition rate up to 95°C for 30 sec, 45 sec 62°C, repeated for 40 times (amplification). The PCR reaction was evaluated by melting curve analysis following the manufacturer's instructions and checking the PCR products on 2% agarose gel.

The PCR cycle number at which each assay target reached the threshold detection line was determined (Ct value). cDNA samples were assayed on at least 3 dilutions to check for assay reliability

using duplicate assay on each dilution. The ΔCt for each candidate was calculated as ΔCt = [Ct (candidate) – Ct (GAPDH or β-Actin)]. The relative abundance of each target in each protocol can be calculated as the ratio between treated and untreated samples (Mühlbauer et al., 2004).

GAPDH and β-Actin were used as endogenous controls for normalization. No difference was observed between these two genes for all quantifications (data not shown).

Statistical Analysis: Statistical significance was evaluated by the two-tailed Student's t-test. P<0.05 was considered statistically significant.

RESULTS

The aim of this study was to investigate plasticity changes induced by drug administration. Preliminary investigations in our lab, based on differential display with semaphorin-targeted primers, identified several axon guidance molecules as suitable candidates involved in drug-induced neuroadaptations (Widmer, 2000). Based on these findings a microarray was designed, containing 39 guidance cues together with 10 positive and 10 negative control genes (Table 1), with a view of evaluating expression changes in four different brain regions under various paradigms of drug treatment (Figure 1). The results are summarized in Figures 2 and 3. As shown in Figure 2 (upper panel), positive controls displayed no change in expression in the brain areas under investigation, whatever the drug administration protocol: in all cases, the expression ratios of cocaine-treated versus saline-treated animals were almost equal to 1.0, with <8.5% variation. Data were statistically highly coherent in all brain regions and under any tested condition. Each data represents the average of eight different hybridization slots from three independent tissue samples. Slightly higher variations were observed in the CPu of acute-treated animals or in chronically treated animals; but such variations were always within very small limits and were supported with very significant statistical correlation for all these control candidates. From these data, a 1.5-fold change in gene expression can be considered as significant. However only two-fold changes or higher will be discussed here.

**Drug-induced expression changes of Semaphorin:** Expression changes of semaphorins are depicted in Figure 2.

In the hippocampus, six Semaphorins displayed high (2.5 to 8-fold) induction, namely Sema3A, Sema3B, Sema3E, Sema6A, Sema4C and Sema5A. Changes were highest in groups S4 (animals sensitized with cocaine and challenged after long term-withdrawal) or after chronic treatment. Between 8 and 4-fold up-regulation were observed with these candidates, depending upon regimen, with Sema5A>Sema4C=Sema3E>Sema3B. By contrast Sema3C, Sema6C and Sema4G were strongly down regulated (8- to 4-fold, with Sema4G>Sema6C>Sema3C), mostly after acute treatment. A number of other Semaphorins also displayed expression changes upon

cocaine, but to a lesser degree. Correspondingly, neuropilin-1 and –2, which are receptors for soluble semaphorins (Sema3 family members), displayed 2.5 - 4.5 fold up-regulation in this brain region, and the effect was strongest in S4 and chronic groups.

In the Nucleus Accumbens strong down-regulation was observed in many cases, particularly in acute groups. Sema3A, Sema3E, Sema3F, Sema4G, Sema5B and Sema7A displayed >3.5-fold down-regulation after acute treatment and significant down-regulation in all other groups, chronic groups being least affected. Strong up-regulation was observed with most other candidates, particularly after chronic treatment, where >8-fold changes were observed (Sema3B>Sema4C=Sema4B>Sema6B>Sema3C=Sema4A>Sema4F).

Two candidates, Sema5A and Sema6A were little affected by cocaine in this region in all protocols. Receptors for soluble Semaphorins, Neuropilin-1 and Neuropilin-2, were also induced in this brain region under all conditions, mainly in chronic groups (ca 5-fold induction).

In the Caudate Putamen, changes were less important. However, some candidates displayed high expression changes: Sema4C was highly up regulated, about 10-fold in S4 groups, 7-fold in chronic groups and 5.5-fold in binge groups. Other candidates displaying strong changes included Sema4A, Sema4B and Sema3E (5-6-fold over expressed in S4 groups, >4-fold in chronic and binge groups) and Sema3F (>4-fold up-regulated in chronic and S4 groups). In contrast Sema4D was about 8-fold down-regulated in acute groups and Sema3A, Sema5B, Sema7A and particularly Sema6C were down regulated (4- to 6-fold, depending upon regimen). Neuropilins were up regulated 3- to 5-fold in all groups.

In the Ventral Tegmental Area, greatest changes were observed with most members of the Sema3, Sema4 and Sema7 and mainly in either acute or in S4 groups. Strong up-regulation was observed generally, but Sema3E and Sema4G were down regulated. In S4 groups, up to >6-fold up-regulation was found for Sema3A, Sema3C, Sema4B, and Sema4C, and about 10-fold up-regulation for Sema4D, sema4F and Sema5A. Acute treatment has little effects in expression changes for these candidates, but binge and chronic treatments induce 3-5 fold up-regulation. Down-regulation in this region was highest in acute

treatments and involved a few candidates: Sema3E (6-fold in acute and S4 groups), Sema4G (7-fold in acute groups), Sema5B and Sema7A (4-fold in acute groups). Sema4A was poorly affected, except in acute treatment (4-fold down-regulation).

In all regions, one candidate (Sema6C) displayed strong down-regulation under all protocols whereas Sema4B, Sema4C and neuropilins were up regulated in each region and under all conditions. Furthermore most candidates (except Sema3A, and Sema3F) displayed similar patterns of expression changes both in the Nucleus Accumbens and in the Ventral Tegmental Area the major dopaminergic regions included in this study.

Clearly, the observed expression changes are very consistent, the changes of a single candidate in a given region being very similar, independent of the treatment, with only relative intensities being affected. All the changes observed from microarray data could be confirmed by real-time PCR (Figure 4) and the same pattern of changes was observed in all cases. Figure 4 displays a comparison of the data from qRT-PCR and microarray for major conditions, i.e. where greatest changes in expression were observed (about 15% of the conditions tested by microarray): in all cases both sets of data matched perfectly. Table 3 displays the statistical analysis of the candidates modified by cocaine on microarray and whose expression change has been confirmed by qRT-PCR. Changes observed in the Nucleus Accumbens on chronically treated animals are very significant for Sema3B, Sema4B, Sema4C and to a lesser extend for Sema3E and NP-1. In the Ventral Tegmental Area most significant changes are observed on chronocally treated animals for Sema3C, and Sema5A, whereas changes observed for Sema3C, Sema3E, Sema3f, Sema4G, Sema5A, Sema5B and NP-1 are highly significant in S4 groups. NP-1 and Sema3E display very significant changes in all regions under most conditions.

Drug-induced expression changes of Eph's and Ephrins: Neuroadaptations after drug treatment also strongly affected expression of Eph's receptor tyrosine kinase and their ligands, as shown in figures 3. Changes are very significant, although less intense for these families of axon guidance molecules.

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In the Hippocampus, all Eph and Ephrins were slightly up regulated. Only in S4 groups and sometimes in acute groups were >2-fold up-regulations observed for EphA's. For EphB's, changes are >2-fold in acute groups as well as in S4 groups. Greatest changes were observed with Eph-B1, EphB4 and EphB2. The corresponding ligands, Ephrins, were also up regulated in all cases, but Ephrin-A's displayed little changes (≤2-fold) except in S4 groups (2-4 fold up-regulation). Ephrin-B's, by contrast, were more affected by regimen: S4 and S3 groups displayed ca. 6- and 4-fold up-regulation, respectively while in acute and chronic groups gene expression increased 3-4 fold.

In the Nucleus Accumbens, Eph-A are little affected and >2-fold changes were observed only after chronic treatments or sometimes in acute groups (see Eph-A5 and Eph-A8). Their ligands Ephrin-A's displayed comparable adaptation, greatest changes being observed after chronic treatments (about 4-fold up-regulation in all cases). Eph-B's displayed more important up-regulation: again greatest induction pattern were found after chronic treatments (4-6 fold up-regulation), but acute and binge treatments also strongly affected Eph-B2, Eph-B3 and Eph-B4 (3-5 fold up-regulation). Furthermore, S3 and S4 groups were also strongly affected (4-6 fold up-regulation). Greatest changes were observed with Eph-B4 in all protocols (4-8 fold up-regulation). The corresponding Ephrin-B's ligands were also induced after drug administration under all protocols, mostly after chronic treatment (4-6 fold up-regulation).

In the Caudate Putamen expression changes of Eph-A and Ephrin-A were more complex. Three Eph-A1, EphA3 and Eph-A5 were down regulated together with their ligands Ephrin-A1 and Ephrin-A3; this effect was most important (6-7 fold down-regulation) after acute treatments for Eph-A1, Eph-A3 and the two Ephrins. By contrast, the other Eph-A's like the other Ephrin-A's were rather over expressed (≤2-fold in most cases, but slightly >2-fold in S4 groups). For Eph-B's and Ephrin-B's up-regulation was observed in all cases; this effect was most important for Eph-B4 (4-6 fold up-regulation) and to a lesser extent for Ephrin-B2 and Ephrin-B3.

In the Ventral Tegmental Area, no great changes were observed. Only S4 groups displayed >2-fold changes in Eph-A expression while Ephrin-A's were not much affected either. Ephrin-A1 and Ephrin-A3

were 3-fold down regulated after acute treatment. For Eph-B3, Eph-B4 and Eph-B6 also expression was not much changed in this region. By contrast, the other Eph-B's and their corresponding Ephrins displayed stronger induction: with Eph-B1 and Eph-B2, 3-fold up-regulation after chronic and binge treatments, and 6-fold up-regulation in S4 groups were observed, which matched with 2-4 fold up-regulation and 4-6 fold up-regulation under the same regimen, respectively, of Ephrin-B1 and Ephrin-B2.

Expression changes observed on microarrays for Eph's and Ephrins have been confirmed by means of qRT-PCR. Figure 4 displays the comparison of microarray data and qRT-PCR. For simplicity only the most characteristic expression changes of Eph's and Ephrins in the four brain regions are displayed (representing 14% of the conditions tested by microarray). As shown in Figure 4, qRT-PCR fully corroborated the changes observed in microarray data, in all cases, without exception, and in most cases the values of the both sets of data matched perfectly. Statistical analysis of changes observed by these candidates (Table 3) shows robust changes in the Caudate Putamen under acute treatment for EphA1, EphA2 and their partner ligands EphrinA1 and EphrinA3. Under chronic treatment, most robust changes were observed with EphB's in the Nucleus Accumbens and in the Hippocampus.

DISCUSSION

A gene profiling of a number of axon guidance molecule transcripts in several protocols of drug administration has been undertaken in this study, with a view to determine their involvement in drug-induced neuroadaptations. These plasticity-related secreted or cell surface molecules are developmentally regulated cues that play a major function during development and direct the projection of the extending axon growth cone to its synaptic target, enabling proper formation of neuronal synapses. A role in the adult brain, however, has been poorly described in a few cases only (De Wit and Verhaagen, 2003). For example, specific re-expression of EphB1 and ephrinB2 was observed in the adult, following a drug challenge (Yue et al., 1999), a finding consistent with the notion that long-term, molecular adaptations of neurons following exposure to drugs of abuse underlie a complex rewiring of neural circuitry that results in the behaviors associated with addiction (Bardo, 1998, Wang and McGinty, 1997, Nestler and Aghajanian, 1997, Robinson and Berridge, 1993). Based on this observation, a gene profiling of two classes of axon guidance molecules, the semaphorins and Ephs/ephrins, was investigated in the present study in different protocols of drug administration.

Drug-induced Plasticity involves Expression Changes of a Combination of Semaphorins and related Receptors: Semaphorins are a large family of secreted, GPI-linked or transmembrane proteins that may bind to neuropilins (secreted Sema-3) and/or plexins and function as chemorepellents or inhibitors of growth cones (Yu and Bargmann, 2001, Skoliora et al, 1998). The function of Semaphorins in the adult brain is poorly described, but a role in regeneration of injured nerve fibres is well documented (De Wit and Verhaagen, 2003). During development, different semaphorins are generally co-expressed and interact in synergistic ways, innervation specificity being achieved through the combined action of multiple guidance cues (Steup et al., 1999, Skaliora et al., 1998). For example, during development most striatal and cortical interneurons arise from the basal telencephalon, whose migration is controlled by chemorepulsive signal composed mainly of semaphorin 3A and semaphorin 3F (Marin et al., 2001) and

migrating interneurons expressing neuropilins are directed to the cortex, while those lacking them go to the striatum. Recent studies have revealed extensive regulation of these cues and their receptors as well as combinatorial mechanisms that integrate information from different families of guidance cues (Yu and Bargmann, 2001). The combinatorial and simultaneous input of multiple guidance cues, i.e. the relative balance of attractive (growth-promoting) and repulsive (growth-inhibiting) forces, provides synapse specificity by allowing axons to select various subsets of nerve cells as their targets.

Our study suggests similar synergistic mechanisms in the adult brain after drug administration: drug-induced plasticity involves the regulation of several semaphorins and their related neuropilin receptors. All members of the five classes of semaphorins included in this study display strong expression changes (>6-fold, except Sema6A and Sema6B) in at least one condition or region, highest changes affecting Sema3A, Sema3B, Sema4B, Sema4C, Sema4F and Sema5A (for nomenclature, see Semaphorin Nomenclature Committee, 1999).

Drug-induced neuroadaptations is known to primarily affect the mesolimbic dopaminergic pathway (Robinson and Berridge, 1993, Nestler, 2000). In this pathway, cocaine induced largest expression changes of many semaphorins, mainly Sema3A, Sema3B, Sema3F, Sema4B, and Sema4C in the Nucleus Accumbens, and Sema3C, Sema3E, Sema4D, Sema4F, Sema4G, Sema5A, Sema5B and Sema7A in the Ventral Tegmental Area.

Sema3 are secreted proteins and act generally as repellents of different classes of axons and their regulation in the adult brain has been described in some disorders. Aberrant expression or signalling of secreted Semaphorins might be of significance in altered structural plasticity in neurodegenerative disorders such as Alzheimer (Good et al, 2002). Sema3A is the most potent repellent and repels axons that are responsive to NGF (Messersmith et al, 2001), but this depends upon the age of the animal (Puschel et al., 1996). Sema3A and its ligand Neuropilin-1 are temporarily down-regulated in the hippocampus in epileptic rat brain, correlating with robust mossy fiber sprouting into the molecular layer, a layer normally devoted of mossy fibers. Kainate-induced status epilepticus also induces a

persistent down-regulation of Sema3C and Sema3F (Barnes et al., 2003). We found Sema3F to be strongly down regulated in the Nucleus Accumbens after acute treatment and up regulated in other regions, particularly in the Ventral Tegmental Area and the Hippocampus in chronic or S4 groups. This candidate may suppress NGF-induced activation of PI3-kinase-Akt and dampen neutrophin signalling, a novel mechanism of semaphorin-mediated growth cone collapse, providing an intracellular mechanism for cross talk between positive and negative axon growth cues (Atwal et al., 2003).

Sema4C is a transmembrane semaphorin that binds to proteins involved in neurite outgrowth; its strong up-regulation (8-fold) in chronic groups in the Nucleus Accumbens, Hippocampus and striatum may regulate drug-induced plasticity in these areas. Sema4F, which is strongly up regulated after cocaine in the Ventral Tegmental Area, interacts with PSD-95, a scaffold protein which concentrates ion channels, adhesion molecules and neurotransmitter receptors at the post-synaptic membrane (Sheng, 2001). Sema4D associates with cytosolic kinases and binds to proteins involved in neurite outgrowth and cytoskeletal reorganization. These interactions regulate actin dynamics and neurotransmitter receptor clustering, transport and docking of synaptic vesicles and thus modulate spine plasticity (De Wit and Verhaagen, 2003).

Sema5A is a transmembrane protein with seven thrombospondin repeats. It acts as a permissive cue for growing axons (Adams et al., 1996, Neugebauer et al., 1991, Simmons et al, 1998), although a recent report shows that its expression by oligodendrocyte lineage cells contributes to the glial cues that inhibit CNS regeneration. (Goldberg et al., 2004). Sema5A may be responsible for some of the features of the Cri-du-chat syndrome of mental retardation since the gene covers at least 10 percent of the deleted region (Simmons et al., 1998, Inagaki et al., 1995). It is strongly up-regulated (8-fold) in chronic groups in the Hippocampus and to a lesser extend in the Ventral Tegmental Area, but it is down regulated in the striatum.

Sema6C, which is strongly down regulated in all brain regions and in all conditions, is highly expressed in the lateral ventricle, the striatum, the midbrain, the pons/midbrain junction, and the choroid plexus (Qu et al., 2002). Finally, Sema7A, a

glycosylphosphatidylinositol-linked semaphorin strongly down-regulated in the Ventral Tegmental Area after drug treatment, displays highest expression in the nervous system and in the adult brain (Sato and Takahashi, 1998, Xu et al., 1998, Lange et al., 1998) and promotes axon growth through integrin receptors (Pasterkamp et al, 2003).

From our study, one is surprised that such a large variety of semaphorins and related receptors display expression changes after drug treatment. On the other hand, splice variants of semaphorin RNA transcripts also are important for regulating plasticity in neurons (Daoud et al., 1999) and nonfunctional isoforms of semaphorin genes, produced through alternative splicing, may be used as regulators of that gene's activity. Furthermore, neuronal activity alters splice patterns (Daoud et al., 1999) and cocaine administration stimulates this activity. Together this may further complicate the pattern and to some extent explain the large diversity of expression pattern of the axon guidance cues identified in this study.

Eph receptors and related Ephrin ligands display significant changes after drug treatment, but of lower intensity than the semaphorins: The Eph family receptor tyrosine kinases include at least 14 receptors and 8 Ephrin ligands (Zhou, 1998) that guide migrating cells and neuronal growth cones to specific destinations by repulsive mechanisms during development of the nervous system (Cheng et al., 1995, Drescher et al., 1995, Feldheim et al., 1998, Gao et al., 1996, Gao et al., 1998). The ligands and receptors are often expressed in opposing gradients, and the ligand–receptor interaction restricts receptor-rich axons to ligand poor areas. Because Eph receptors and ephrins have complementary expression, bidirectional activation of Eph receptors and ephrin-B occur at interfaces of their expression domains (Mellitzer et al., 1999).

In our study we found complementary up-regulation of Eph and their partner ligands, the Ephrin in the Ventral Tegmental Area and in the Nucleus Accumbens after all drug treatments. The up-regulation is very significant, although of lower magnitude than changes observed with semaphorins. Similar complementary expression of EphB1 and Ephrin-B2 has been described in the

nigrostriatal and mesolimbic pathways in the adult after cocaine treatment (Yue et al., 1999). Dopamine activity regulates developmental expression of EphB1 and Ephrin-B2, whereas cocaine administration produced a significant elevation in Ephrin-B2 expression in the Nucleus Accumbens.

Highest changes after drug treatment are found in the Nucleus Accumbens with EphB4, EphB3 and EphB1 in chronically treated animals. By contrast down regulated in the striatum (CPu) is observed with specific EphA and EphrinA. The nature of the specific neural pathways implicated remains to be clarified in more details.

EphA4 and Ephrin-A3 are both up-regulated in the hippocampus. Murai has shown that expression of and signalling through Ephrin-A3/EphA4 are critical for spine morphology of CA1 pyramidal neurons in the hippocampus (Murai et al, 2003) and for maintaining a stable and dynamic organization of spines during synaptic remodelling in the adult brain, whereas EphB receptor activation induces modest changes in dendritic spines in CA1. Changes in hippocampal spine density and dimensions occur in several mental disorders, including depression, schizophrenia and addiction.

Another study has shown that EphB1, EphB2, and EphB3 are involved all three, although to varying degrees, in dendritic spine morphogenesis and synapse formation in the hippocampus (Henkemeyer et al, 2003). All these candidates are up regulated in that region in all protocols of drug administration

Drug-induced Expression Changes of axon guidance cues may be related to plasticity and learning in addiction: In drug addiction the value of the drug and drug-related stimuli is enhanced, at the expense of other reinforcers, as a consequence of conditioned learning. The memory of the expected reward results in over activation of the reward and motivation circuits while decreasing the activity in the cognitive control circuit, which contributes to an inability to inhibit the drive to consume the drug (Volkow et al., 2003). Thus conditioned-learned associations and cognitive control is a key process in drug addiction. On the other hand several studies have implied axon guidance cues in synaptic plasticity and learning (Gerlai et al., 2001). EphB2 regulates NMDA-dependent synaptic function and is up regulated in hippocampal pyramidal neurons by stimuli known to induce changes

in synaptic structure (Henderson et al., 2001). Mice lacking EphB2 have normal hippocampal synapse morphology, but display defects in synaptic plasticity (Grunwald et al, 2001), long-term potentiation (LTP) is impaired, and two forms of synaptic depression are completely extinguished (Gosh, 2002, Grunwald et al., 2001, Henderson et al., 2001). Conditional ablation of Ephrin-B2 and Ephrin-B3, or of EphA4, a high-affinity receptor for these ligands, causes comparable defects in LTP (Grunwald et al., 2004). By contrast, in our study we found up to four-fold induction of EphB2 in the hippocampus, which theoretically should result in improved LTP and thus may be linked to improved cue-mediated conditioned learning as observed in addiction.

On the other hand regulation of neurogenesis is a form of plasticity in the adult rat hippocampus. It has been proposed that drug regulation of neurogenesis in the adult rat hippocampus may be one mechanism by which drug exposure influences hippocampal function (Eisch et al., 2000). Thus drug-induced expression of axon guidance cues, particularly the Ephs and Ephrins, may be part of this process, which may recapitulate expression patterns of cues induced in earlier stages during development. In the adult brain, for example, EphB signalling regulates neural stem cell migration and possibly proliferation and EphB2 and Ephrin-B2, which both are induced by drug treatment in our study, has been shown to substantially increase the number of neural stem cells (Hinamen et al., 2003).

Effect of the drug administration paradigm on gene regulation: In this study, different drug administration paradigms have been used to assess expression changes of plasticity-related cell surface guidance cues. In most cases, chronic treatment or S4 sensitization groups display highest intensities in expression changes compared to other treatments within a given area. Expression changes of a given candidate within a specific brain region do not qualitatively differ from one paradigm to another, whereas only the intensity of the observed change is affected and strongly depends upon the type of treatment, with some exceptions. Therefore, under most circumstances, changes observed with a candidate in one paradigm will be observed also upon other treatments, but at a different intensity: wherever a candidate is up- or down-regulated, the

regimen does not influence this effect, only its magnitude. Some exceptions are observed, however, but mainly where expression changes of the candidate are of small intensity (<2-fold change).

In several conditions, acute treatment induces strongest changes, while the other treatments have small effects, i.e. for Sema3A and Sema3F in the Nucleus Accumbens, Sema4A and Sema4G in the Ventral Tegmental Area, Sema7A (several regions), and Ephrin-A3 in the Caudate Putamen, etc. It is therefore somewhat surprising that a single dose of drug may induces strong changes, where repeated doses produce lower expression change of the same candidate. This may imply a complex program of compensatory regulation and temporal adaptations of these guidance cues.

In conclusion, we describe differential regulation of a large array of guidance cues in several brain regions in response to cocaine. The involvement of multiple Semaphorins, Neuropilins, Ephs and Ephrins in cocaine action suggests the activation of a complex program of synaptic rearrangements induced by the drug, which may recapitulate the plastic changes occurring during development in terms of mechanisms and of the molecular players involved. Local expression changes of these cues may mediate cytoskeleton rearrangement via signalling and induce synaptic plasticity, through mechanisms similar to synaptic targeting during development (Yue et al, 2002). These cues display a high range of functional characteristics, illustrating the complexity and the variability of neuroadaptations induced in the brain by psychostimulant treatment. Furthermore alternative splicing forms of these molecules have been described in many cases, which affect their binding and signalling properties, further complicating the pattern. The changes observed in this study are consistent with a role in addiction and may contribute to drug-related reinforcing of the mesolimbic circuits. Clearly, important neuroplastic changes occur in the reward- and memory-related brain centers following drug action. Together, these finding help clarifying the molecular and cellular events involved in early stages of drug addiction. The fact that the candidates investigated in this study regulate both axon guidance, during

development, and postnatal synaptic function emphasizes the varied roles of these cues in regulating cell-cell interaction within the CNS.

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Figure Legends

Figure 1. Protocols of Drug administration.

A) Acute Treatment. Animals (n=6) are injected a single dose of cocaine (15 mg/kg) and sacrificed 24 h after injection.

B) Binge Treatment. Animals (n=6) receive four injections of high dose cocaine (30 mg/kg) and are sacrificed 24 h after the last injection.

C) Chronic Treatment. Animals (n=6) receive a single dose of cocaine (15 mg/kg) every day over 15 days and are sacrificed 24 h after the last injection. In each treatment (acute, binge or chronic), control animals were injected saline. In each cases aRNA samples of brain regions from controls (reverse transcribed as Cy3-dCTP) and from treated animals (reverse-transcribed as Cy5-dCTP) were prepared, hybridized on microarrays and the ratios measured.

D) Sensitization Treatment. Four groups of animals (n=6) are used. Each group receives initially five daily injections of either saline (groups S1 and S2) or cocaine (15 mg/kg, groups S3 and S4).

Short withdrawal groups: three days after the last injection, groups S1 and S3 are challenged with a single dose of either saline (group **S1**) or cocaine (15 mg/kg, group **S3**) and sacrificed 24 h after the injection.

Long withdrawal groups: fourteen days after the last injection, groups S2 and S4 are challenged a single dose of either saline (group **S2**) or cocaine (15 mg/kg, group **S4**) and sacrificed 24 h after the last injection.

Groups S1 and S2 are animals sensitized with saline and challenged after a short withdrawal period (3 days, group S1) or a long withdrawal period (14 days, group S2). Groups S3 and S4 are animals sensitized with cocaine and challenged after a short withdrawal period (3 days, group S3) or a long withdrawal period (14 days, group S4). aRNA samples of brain regions from controls (challenged with saline, reverse transcribed as Cy3-dCTP) and from treated samples (challenged with cocaine, reverse-transcribed as Cy5-dCTP) were prepared, hybridized on microarrays and the ratios measured.

Figure 2. Expression Patterns of Controls and Semaphorins.

Upper panel: Expression changes of the 10 positive control genes are represented as ratios of cocaine-treated versus saline-treated samples from tissues of the four regions under investigation, each at the seven different conditions of drug administration.

Lower panel: Expression changes of the Semaphorins, in the same regions and conditions as in the upper panel.

Microarrays were scanned with a scanning laser microscope (Methods section). Each bar represents the means of ratios from 8 hybridization spots per gene from three independent tissue samples for a single treatment paradigm, with standard deviation (see Material and Methods section for details). Standard deviation was <8.5% in all cases. Color codes: as indicated in the figure, corresponding each to different drug administration protocols.

For all control genes no significant change of expression is observed under any condition and the ratio between samples from cocaine-treated and saline-treated tissues is almost 1.0.

E2a: Transcription factor E2   LDH-A   : Lactate dehydrogenase A, *HSP70:* Heat Shock Protein 70, *GP62:* Nuclear Pore Glycoprotein 62,

H: Hippocampus, **A:** Nucleus Accumbens, **C:** Caudate Putamen, **V:** Ventral Tegmentum Area.

Figure 3. Expression Patterns of Neuropilins, Eph's and Ephrins.

Upper panel: Expression changes of the Neuropilins, NP-1 and NP-2.

Middle panel: Expression changes of the Eph-A and their corresponding ligands, the Ephrin-A.

Lower panel: Expression changes of the Eph-B and their corresponding ligands, the Ephrin-B.

Same representations as in figure 2, in the same regions and conditions of drug administration, with standard deviations. The same color codes in figure 2 (as indicated) are used for representing the different treatment conditions. Each bar represents the means of ratios (sample from cocaine-treated tissue versus sample from saline treated tissue) from 8 hybridization spots per gene from three independent tissue samples for a single treatment paradigm, with standard deviation. Standard deviation was <8.5% in all cases.

_NP1:_ Neuropilin1, _NP2:_ Neuropilin2.

H: Hippocampus, **A:** Nucleus Accumbens, **C:** Caudate Putamen, **V:** Ventral Tegmentum Area.

Figure 4. mRNA Quantification of Candidate Transcripts: Comparison of Quantitative Real-Time PCR and Microarray.

QRT-PCR: quantification of gene transcripts by means of quantitative real-time PCR. _Microarray:_ quantification of gene transcripts on microarray (see figures 2 and 3 and Material and Methods).

Colour codes correspond to the different protocols (as indicated within the figure), and are as in Figure 2 & 3. **H:** Hippocampus; **A:** Nucleus Accumbens; **C:** Caudate Putamen; **V:** Ventral Tegmental Area. Standard deviation for RT-PCR was <4%, and for microarrays <8.5%, in all cases.

For qRT-PCR, the relative abundance of candidate transcripts in total RNA from different tissue samples and from different protocols was determined by real-time RT-PCR and expressed relative to GAPDH for replicate determinations (as described in the "Materials and Methods"). After normalization, the ratios between samples from cocaine-treated and saline-treated tissues were calculated.

Fig. 1

Group	Sensitization	Withrawal	Challenge	Sub-Group					
S1	*Saline*	3 Days	Saline (dCTP-Cy3)	*Control*	S2	*Saline*	14 Days	Saline (dCTP-Cy3)	*Control*
			Cocaine (dCTP-Cy5)	Treated				Cocaine (dCTP-Cy5)	Treated
S3	*Cocaine*	3 Days	Saline (dCTP-Cy3)	*Control*	S4	*Cocaine*	14 Days	Saline (dCTP-Cy3)	*Control*
			Cocaine (dCTP-Cy5)	Treated				Cocaine (dCTP-Cy5)	Treated

Saline Injection **Cocaine Injection** **Saline Challenge** **Cocaine Challenge**

Sacrifice - Dissection

Fig. 2

Fig. 3

Fig. 4

H	qRT-PCR	Microarray
Sema3B	4.1	4.7
Sema3C	-2.5	-2.7
Sema3C	-1.4	-1.2
Sema3E	2	2.3
Sema3E	4.2	3.5
Sema3E	6.2	5
Sema3E	2.1	2.4
Sema3E	2.1	2.7
Sema3E	4.2	4.8
Sema3E	7.1	5.8
Sema3F	1.7	1.1
Sema4C	8.5	7.4
Sema4F	4.0	3.0
Sema4G	-8.0	-7.5
Sema5A	5.6	5.7
Sema5A	10.1	8.9
Sema6C	-6.0	-5.1
Sema7A	0.9	1.1
NP-1	2.2	2.2
NP-1	3.1	2.7
NP-1	4.6	3.8
NP-1	2.1	2.3
NP-1	4.5	4.0
NP-1	4.9	4.9

A	qRT-PCR	Microarray
Sema3A	-4	-4.5
Sema3B	7.6	8
Sema3E	-4.1	-3.5
Sema3E	-2.7	-2.2
Sema3E	-2	-1.4
Sema3E	-4	-3.5
Sema3E	-3.3	-2.5
Sema3E	-3.0	-2.7
Sema3E	-3.2	-2.6
Sema3F	-8.0	-6.6
Sema3F	-2.1	-1.7
Sema4A	4.5	4.0
Sema4B	9.0	8.0
Sema4C	9.1	7.8
Sema4G	-5.3	-5.7
Sema4G	-3.3	-2.8
Sema5B	-3.6	-3.5
Sema5B	-3.6	-2.9
Sema6C	-10.5	-10.5
Sema7A	-6.6	-5.5
NP-1	4.2	3.6
NP-1	3.9	3.2
NP-1	6.1	4.9
NP-1	2.9	3.0
NP-1	4.6	4.1
NP-1	4.5	4.2

C	qRT-PCR	Microarray
Sema3A	-3.2	-3.6
Sema3E	4.1	3.0
Sema3E	4.0	3.5
Sema3E	4.1	4.3
Sema3E	3.1	2.2
Sema3E	4.2	3.9
Sema3E	4.3	4.0
Sema3E	7.1	5.6
Sema3F	4.9	4.6
Sema4C	8.2	7.4
Sema4D	-7.9	-6.8
Sema4G	3.6	2.6
Sema4G	4.6	3.8
Sema5A	-2.8	-2.1
Sema5A	-1.8	-1.6
Sema5B	2.2	1.9
Sema6C	-6.1	-6.0
NP-1	4.1	3.6
NP-1	3.9	3.2
NP-1	4.2	3.8
NP-1	4.2	3.5
NP-1	3.9	3.6
NP-1	5.2	5.0

V	qRT-PCR	Microarray
Sema3C	8.2	7.3
Sema3C	5.5	3.9
Sema3E	-8.3	-6.5
Sema3E	-3.1	-2.4
Sema3E	-3.2	-2.5
Sema3E	-4.9	-4.0
Sema3E	-4.6	-3.8
Sema3E	-3.9	-3.1
Sema3E	-6.5	-5.6
Sema3F	6.4	6.2
Sema4A	-3.5	-4.0
Sema4B	3.5	3.3
Sema4D	3.5	2.8
Sema4F	10.2	9.5
Sema4G	-9.2	-7.8
Sema4G	-6.1	-5.3
Sema5A	6.3	5.4
Sema5A	6.0	5.1
Sema5A	9.2	9.6
Sema5B	-8.1	-6.8
Sema5B	-5.9	-5.5
Sema6C	-7.9	-7.1
Sema7A	-9.4	-7.8
NP-1	2.0	1.5
NP-1	4.1	3.6
NP-1	4.1	3.3
NP-1	2.2	2.2
NP-1	4.1	4.4
NP-1	7.2	6.3

Legend: Acute (black), Binge (red), Chronic (pink), S1 (pink), S2 (dark green), S3 (dark blue), S4 (yellow), Sensitization (gray)

H	qRT-PCR	Microarray
EphA3	1.8	1.4
EphA5	1.1	1.2
EphA5	1.5	1.4
EphA5	2.7	2.4
EphA5	3.0	2.9
EphB2	4.0	3.5
EphB2	4.2	3.5
EphA8	3.2	2.8
EphA8	1.9	1.5
EphA8	2.6	2.2
EphA8	1.8	1.4
EphA8	2.5	2.4
EphA8	3.2	3.0
EphB1	5.2	5.0
EphB3	3.3	3.4
EphB4	6.1	5.4
EphB6	1.9	2.3
EphB1	4.3	3.8
EphrinB2	7.2	6.7
EphrinB3	6.2	5.6

A	qRT-PCR	Microarray
EphA1	1.5	1.4
EphA3	2.0	1.8
EphA8	3.5	3.2
EphA8	2.2	3.3
EphA8	3.7	3.0
EphA8	2.1	1.9
EphA8	2.7	2.5
EphA8	2.6	2.6
EphB1	5.6	5.3
EphB1	4.5	4.5
EphB2	4.1	5.1
EphB3	5.2	5.8
EphB4	8.8	8.5
EphB6	4.1	3.9
EphrinA5	2.2	2.9
EphrinA5	4.3	4.0
EphrinB2	4.7	4.2
EphrinB3	2.6	3.5

C	qRT-PCR	Microarray
EphA1	-8.3	-6.6
EphA3	-6.1	-5.7
EphA3	-4.2	-4.4
EphA5	-3.9	-3.5
EphA5	-3.2	-3.1
EphA5	-3.2	-3.0
EphA5	-2.5	-2.2
EphA8	2.9	2.6
EphA8	1.7	1.7
EphA8	1.8	1.9
EphA8	2.1	1.7
EphA8	1.8	1.8
EphA8	2.5	2.5
EphB1	3.6	3.2
EphB1	3.8	4.3
EphB2	3.9	3.2
EphrinA1	-8.0	-6.6
EphrinA3	-8.1	-6.6
EphrinB2	4.3	4.5
EphrinB3	3.9	4.5

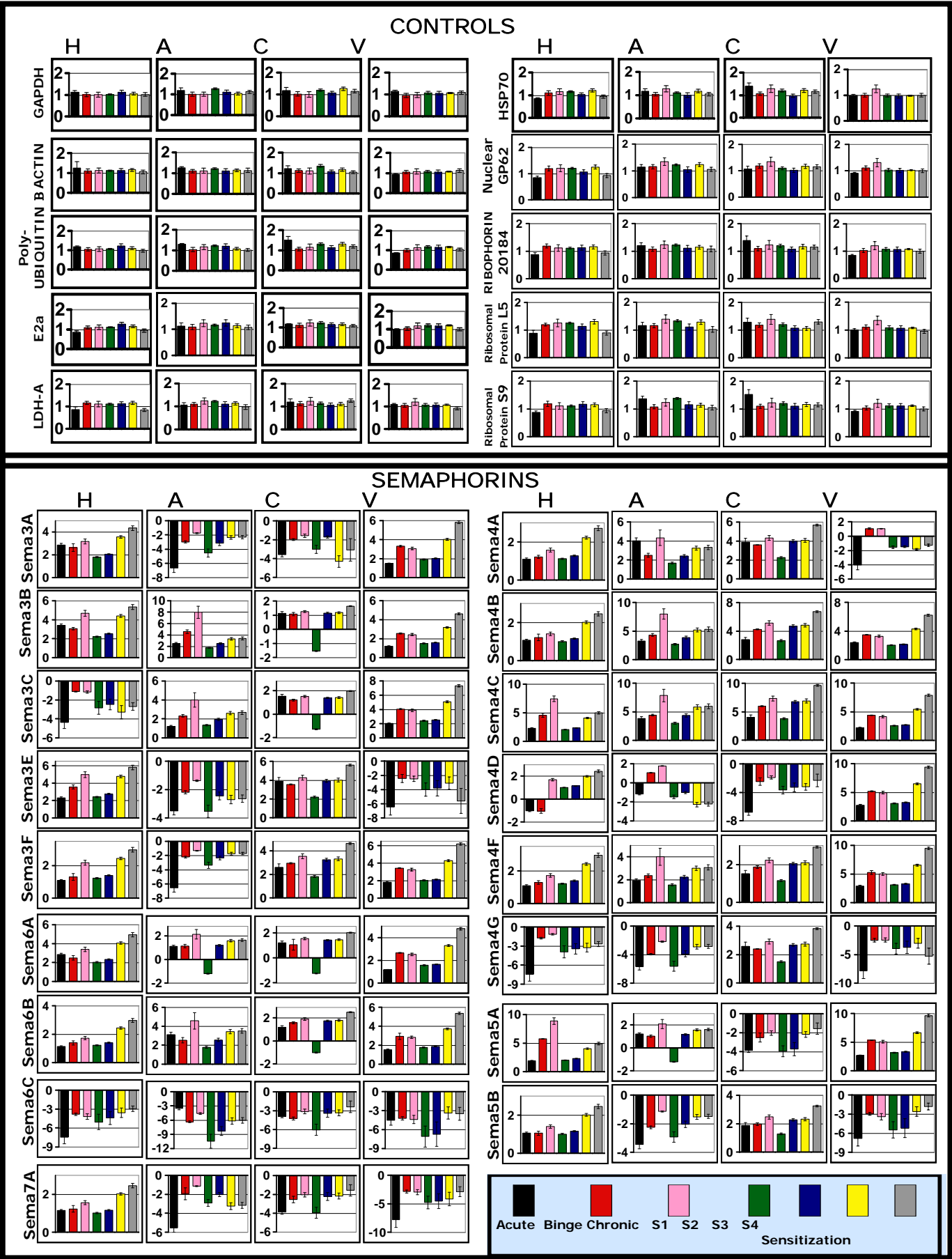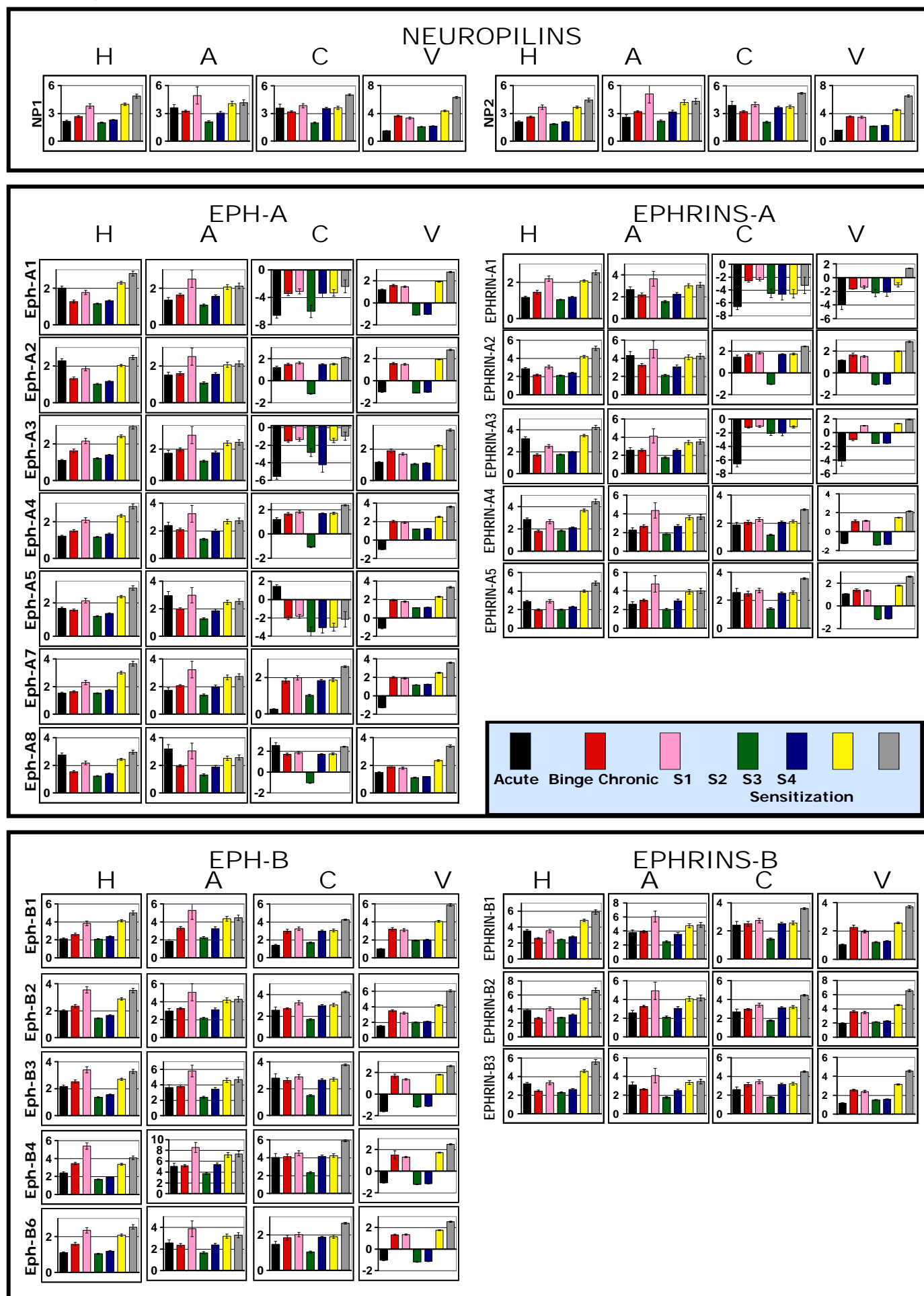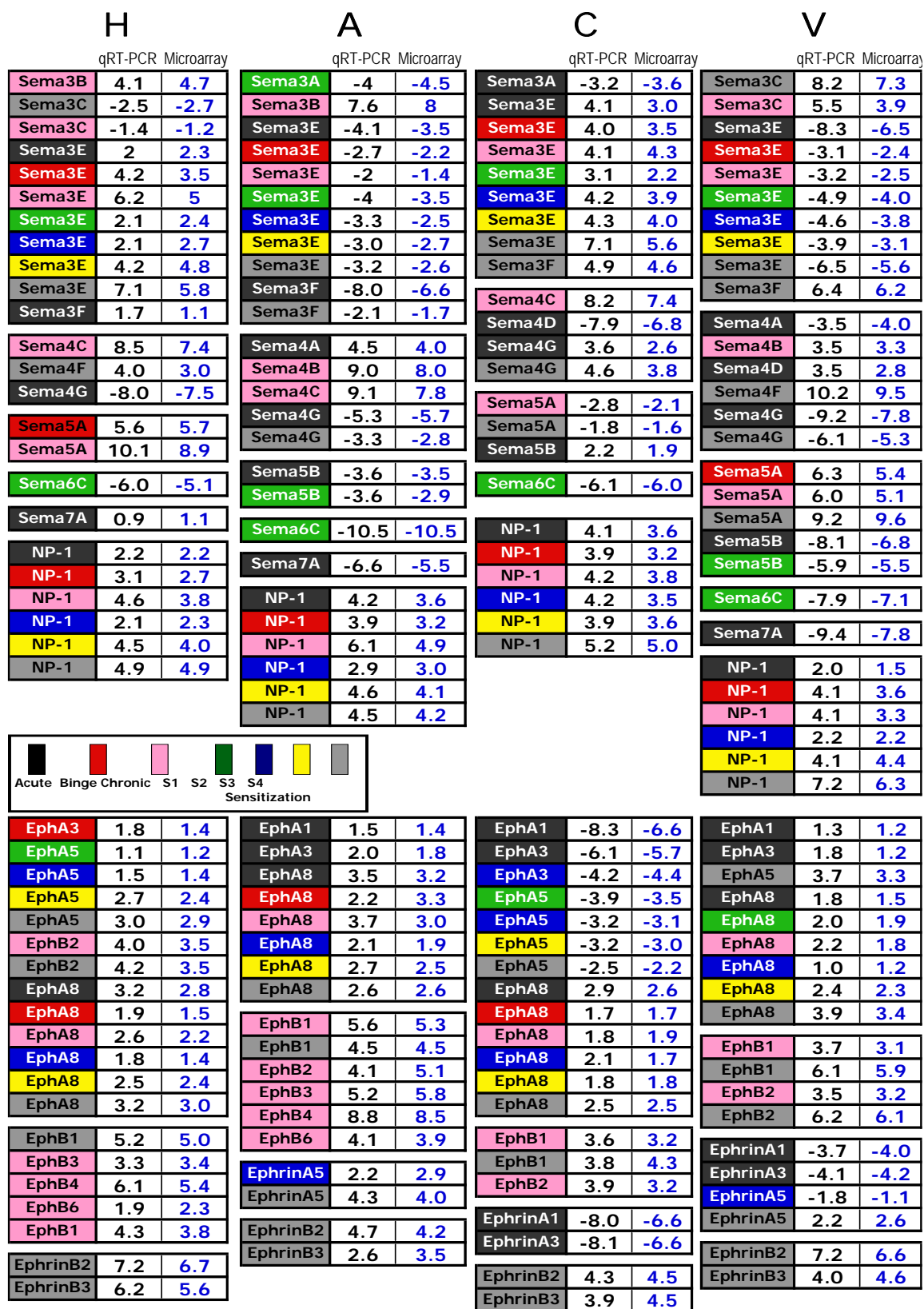| V	qRT-PCR	Microarray
EphA1	1.3	1.2
EphA3	1.8	1.2
EphA5	3.7	3.3
EphA8	1.8	1.5
EphA8	2.0	1.9
EphA8	2.2	1.8
EphA8	1.0	1.2
EphA8	2.4	2.3
EphA8	3.9	3.4
EphB1	3.7	3.1
EphB1	6.1	5.9
EphB2	3.5	3.2
EphB2	6.2	6.1
EphrinA1	-3.7	-4.0
EphrinA3	-4.1	-4.2
EphrinA5	-1.8	-1.1
EphrinA5	2.2	2.6
EphrinB2	7.2	6.6
EphrinB3	4.0	4.6

Table 1. Gene candidates and Oligonucleotides.

Gene candidates, species and Gene ID (NCBI) are listed. Oligonucleotides for the different candidates were designed and checked ("Materials and Methods"). Oligonucleotides synthesized with 5'-amino modification, were arrayed onto QMT Aldehyde Slides with an OmniGrid Accent™ Microarrayer. aRNA samples (from samples of cocaine-treated and of saline-treated tissues) were reverse-transcribed in the presence of Cy3-dCTP (samples from saline-treated tissues) or Cy5-dCTP (samples from cocaine-treated tissues) and hybridized. See Material and Methods for details.

Name	Species	Gene_Id	Oligo Sequence
Sema3A	Rat	X95286	GAAGAAGGATTCAGGCAATTACCTGTGTCACGCCGTGGAGCACGGTTTCATGCAAACTCTTCTTAAGGTG
Sema3B	Mouse	X85990	CCAGCACCCTATGCTCTGGAGACTCTTCTCACTCTGTGCTGCTGGAGAAGAAGGTGTTGGGTGTGGAGAG
Sema3C	Mouse	X85994	CAATGGAAATCTCATCCAAAAAGCAACAGTTGTACGTGAGCTCCAATGAGGGGGTTTCCCAAGTCTCTCT
Sema3E	Mouse	Z80941	CTTAAGCGGTATGTCTCAGGGGACAAAACCGTGGTACAAGGAATTCTTGCAGCTGATTGGCTACAGCAAC
Sema3F	Mouse	AF080090	TCAATTCCAATGCAAACAAGAATGCCGTAGAGTCTGTGCAGTACGGAGTGGCCGGCAGCGCAGCTTTCCT
Sema4A	Mouse	X85991	GTTTGGACAGATTGTTATTATTGTCTCTGCCCTGGCTAGAATGGGGGCATAATCTGAGCCTTGTTCCCTT
Sema4B	Mouse	X85992	CAAGAATTCCTCATACAAGGCCCGGTTTCTTGTGCCAGGTAAGCCATGTAAACAAGTCCAGATCCAACCA
Sema4C	Mouse	S79463	ACTTTCCTGTCTCTATCACGTCTTCCCTTGGCCTGGGGTCCTCCTGGTTGAGTCTTTGGAGCTATGAAGG
Sema4D	Mouse	U69535	CAAATGTTTCCAAAAGTCCAACCTAGCCCGGGTGGTATGGAAGTTCCAGAATGGCGAGTTGAAGGCCGCA
Sema4F	Rat	AB002563	CTAACCTAGCAGCATTGATCGTTCATTGTAAAGTGGGGATATACCTACCTCAGGGTTGCTGCAAGGATCA
Sema4G	Mouse	AF134918	AGATTTCCCTTCCCATTTATCGGTCCTCACTGAACTAGGACAAGTGGCCAACACTGCACTCAATGAGCCA
Sema5A	Mouse	X97817	GCTCTTATACTTTGGGCTCGTTGTAAACTCGCTGCTCCTCAAACCGTGCTCCATGGCTGCCCCATATTTC
Sema5B	Mouse	X978181	GTAGAACCACTTTGGTTTACCCTCTGCACTGGGCCAAGAACTTGCTGCCTAGCCAGTAAGGGTCCCATCT
Sema6A	Mouse	AF030430	GGCTGCCACCCTGGAGTATAAGACCATCAAAGAGCACCTGAGTAGCGAGAGTTCACCCTATGTTCTGAAG
Sema6B	Rat	AB000776	GAACCTACTTCTAAAGGGCTTGTGGGGGGGTTTTGGGAGGGTGGAAGTGGGCAGACACCCCTGTAAATACA
Sema7A	Mouse	AB017532	TATTTTTTCGAAATCTCACACTTGACTTCATGCAGTGATGCACCTGGCCTGAGAGCCCATCAGCCTGGCA
Sema6C	Rat	AB000817	GCCTTCCCGGACTCCAAGAGTCTCCCGGGGTCCCCTCTCGCCTCGGTTTATTTATTGACTGTCTTTCCCC
EphA1	Mouse	U18084	GGAATAGCTCTGCTGATCGGGATTTATGTCTTCCGTTCAAGGAGAGGCCAGAGACAGAGACAGCAGAGGC
EphA2	Mouse	X78339	AGGTCAACACAGTGGGGATTCCTATCTGAGTCCATTGGGGCCGTGCCCAACAATACTTGAAGAGCCACAG
EphA3	RAT	U69278	TGGACCAAAGCAATGTCGATATTGCTACGTTCCACACAACAGGTGATTGGCTTAACGGTATGAGGACAGC
EphA4	Mouse	X65138	GGCCCATTCATGCTGCCTCTTCAATGGCCAGGACACCTGGTGGATTTTTATGACATAGGGGGGGGTTAGAA
EphA5	Rat	X78689	GTTTGAAGACACTGGTGAACGCGTCAAGCAGGGTATCTACTTTGTTGGCAGAACATGGATCTTTGGGGTC
EphA7	Rat	U21954	GACCAGAGCACTCCCGACTTCACTGCCTTCTGTTCAGTGGGAGAGTGGTTGCAGGCTATTAAAATGGAGA
EphA8	Rat	X59290	GGTACTCCTCCCTTGGCATGGTGCTTCACATGAACGCCCAGGATGTTCGTGCCCTCGGCATCACCCTCAT
EphB1	Rat	X13411	TTTCTCGGCCATGGAAGTGTGTAGAAGCCGTGGGAAACCAAACGCGTAATGATTGACGATAAAGACAGAG
EphB2	Mouse	L25890	TTTGCCAACGCCGGCTTCACCTCTTTCGACGTTGTATCTCAGATGATGATGGAGGACATTCTCCGCGTTG
EphB3	Mouse	Z49086	CCCAGGAAGTGTGCCCCAAACCTCTTCATATTGAAGATGGATTAGAAGAGGGGGTGATGTCCCCTCCCCA
EphB4	Mouse	Z49085	GACAGTTCCCCGGAATTCCGGCAAGGATGCGCCCGCTATATTAAGAATCCTCGAATTGTGCTATTGGATTC
EphB6	Rat	L77867	TCCCCAGGCCTGGCTTTCAGCCATTGGACTAGAGTGCTACCAGGACAACTTCTCCAAGTTTGGTCTTTCC
Ephrin A1	Rat	D38056	TGCTGCAAACTCAGTGAAGGTATTTGCTTGCCCTGGCTTACGGATTGGCACGGGACTAAGGGGCAGGGTT
EphrinA2	Mouse	U14941	ACTCGCTTTCACCAGGCCACCAGGGCCATCCAGTGTTGTTTAATTACAGTCGGAAAGACTTAAGGTTTTT
EphrinA3	Mouse	U90666	TGGACATTATTTGTCCGCACTACAACAGCTCAGGGCCTGGCGGCGGGGCGGACAGGTACGTGCTGTACAT
EphrinA4	Mouse	U90663	TCCAGCGCTGGAATTGCTCGATGCCTTTTGCCCCCTTTCAGCCCTGTTCGATTCTCAGAAAAGATTCAGCG
EphrinA5	RAT	U69279	CAAAGGGTTCAAGAGATGGGAATGTAACCGGCCTCACTCTCCAAACGGACCGCTGAAGTTCTCGGAGAAA
EphrinB1	Rat	U07560	GCTCTCTTAACCCTAAGTTCCTAAGTGGGAAGGGCTTGGTGATCTACCCGAAGATTGGAGATAAGCTGGA
EphrinB2	Mouse	U16819	AGAGGTACTGGAAATAAAAAGCGCAGCGCAGAGCTGTGGGAGAGTCCGTCTGCTTTGGGAGATGTTTTAA
EphrinB3	Mouse	AF025288	TCCTCTCCTAGTTATGAGTTCTACAAACTGTACCTGGTAGAGGGTGCCCAGGGTCGGCGTTGTGAGGCAC
Neuropilin1	Mouse	D50086	CTGTGGTGGATTCTTCTCGGAAACTGCAAAATCCAAGATGCTGGCACTAGGCGTTATTCAGTGGGCCCTT
Neuropilin2	Rat	AF016297	ACAAGGTCAAGATCAATCACCAGAAGTGCTGCTCGGAGGCATGACCGATTGTGTCTGAATCGCTTCTGGC

Table 2. Primer sets designed for quantitative RT-PCR.

See Material and Methods.

	Forward Primer	Reverse Primer
GAPDH	ATGACTCTACCCACGGCAAG	CATACTCAGCACCAGCATCAC
β-Actin	AGCCATGTACGTAGCCATCC	CTCTCAGCTGTGGTGGTGAA
HSP-70	CCAGCTACGTGGCCTTCAC	GCTTCATGTCCGACTGCAC
GP62	TCTCAGCTCCAGCGACAAC	GCAGTAGTAGTGGTGGTGGTAG
LDHA	ACTCTAACACGCTGGCTGAG	CACCCGCACCCTGATACAG
Sema3A	GGCTCCTGCTTCGTAGTCTG	GCCATCTCCATCGTCATCTT
Sema4F	TTATGCTTGCGAGTGTCAGG	GAGGAGAGTGAGGGATGCTG
Sema7A	GCTCCATTGCAGAAGGTTTC	GTTGAGCCTCACGGAGGTAG
Neuropilin1	GGCACCAAGGAGACCACTG	CATCCACAGCAATTCCACCAAG
EphA3	GCTGGCAGAAAGACAGGAAC	ACCGTTAAGCCAATCACCTG
Sema6B	GCTTATGCAGAATGGCTGGAC	GCGTGTGGGTGTGTTGTG
Sema6C	TCAGCCTCCCGATCCATCC	ACAAGCACAGGACACCAAGAG
Sema5A	GGACCCCTGTCAACATCTCTG	CCTCTGTCTTCCTACTTCCAGC
EphA5	GCAGACCTGGGTTCTTCAAAG	CATTGTGGGCGGATCAGATTC
EphB1	CCAGTTTGACCATCCCAACATC	GTCTAAAGCGCCATTCTCCATG
EphrinA1	CCTCTCTTGGGTCTGTGCTG	TCCTCGTAATGTGGGCAGATG
EphrinB1	CAAGCTCCGCAAACGACATC	TGTTCTCTGTAGTCCGTAAGGG
EphB2	AGAATGGTGCCATCTTCCAG	CCTTGAAGGTTCCTGATGGA
EphrinB2	AGGAATCACGGTCCAACAAG	GTCTCCTGCGGTACTTGAGC
EphrinA5	GCTGCTCTTTCTGGTGCTCT	GGGCAGAAAACATCCAGGTA

Table 3. Statistical Analysis of Genes modified by Cocaine Treatments and confirmed by qRT-PCR.
Statistical significance of the genes candidates modified by cocaine from microarray and confirmed by qRT-PCR (see Fig 4) was evaluated by the two-tailed Student's test (see Methods). -P≥0.05; *P<0.05; **P<0.01; ***P<0.005.

	Hippocampus							Nucleus Accumbens							Caudate Putamen							Ventral Tegmentum Area						
	A	B	C	S1	S2	S3	S4	A	B	C	S1	S2	S3	S4	A	B	C	S1	S2	S3	S4	A	B	C	S1	S2	S3	S4
Sema3A	-	-	-	-	-	-	-	-	-	-	*	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-
Sema3B	-	-	**	-	-	-	-	-	-	***	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sema3C	-	-	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**	-	-	-	***
Sema3E	*	**	***	*	*	**	***	**	*	*	*	*	*	*	**	*	**	*	**	**	***	***	*	*	**	**	*	***
Sema3F	-	-	-	-	-	-	-	***	-	-	-	-	-	*	-	-	-	-	-	**	-	-	-	-	-	-	-	***
Sema4A	-	-	-	-	-	-	-	**	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-	-
Sema4B	-	-	-	-	-	-	-	-	-	***	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-
Sema4C	***	-	-	-	-	-	-	-	-	***	-	-	-	-	-	-	***	-	-	-	-	-	-	-	-	-	-	-
Sema4D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	***	-	-	-	-	-	-	*	-	-	-	-	-	-
Sema4F	-	-	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	***
Sema4G	***	-	-	-	-	-	-	-	-	-	-	-	-	*	*	-	-	-	-	**	-	***	-	-	-	-	-	***
Sema5A	-	**	***	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-	***	**	-	-	-	***
Sema5B	-	-	-	-	-	-	-	*	-	-	*	-	-	-	*	-	-	-	-	-	-	***	-	-	**	-	-	-
Sema6C	-	-	-	**	-	-	-	-	-	-	***	-	-	-	-	-	-	***	-	-	-	-	-	-	***	-	-	-
Sema7A	-	-	-	-	-	-	-	***	-	-	-	-	-	-	-	-	-	-	-	-	-	***	-	-	-	-	-	-
NP-1	*	*	**	-	*	**	**	**	*	**	-	*	**	**	**	*	**	-	**	**	**	*	**	**	-	*	**	***

	Hippocampus							Nucleus Accumbens							Caudate Putamen							Ventral Tegmentum Area						
	A	B	C	S1	S2	S3	S4	A	B	C	S1	S2	S3	S4	A	B	C	S1	S2	S3	S4	A	B	C	S1	S2	S3	S4
EphA1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	***	-	-	-	-	-	-	-	-	-	-	-	-	-
EphA3	-	-	-	-	-	-	-	*	-	-	-	-	-	-	***	-	-	-	**	-	-	-	-	-	-	-	-	-
EphA5	-	-	-	-	-	*	*	-	-	-	-	-	-	-	-	-	-	*	*	*	*	-	-	-	-	-	-	*
EphA8	*	**	*	-	-	*	*	*	*	-	-	*	*	*	*	-	-	-	*	-	*	-	-	-	*	-	*	*
EphB1	-	-	**	-	-	-	**	-	-	**	-	-	-	**	-	-	*	-	-	-	*	-	-	*	-	-	-	***
EphB2	-	-	**	-	-	-	-	-	-	**	-	-	-	-	-	-	*	-	-	-	-	-	-	*	-	-	-	***
EphB3	-	-	*	-	-	-	-	-	-	**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EphB4	-	-	***	-	-	-	-	-	-	***	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EphB6	-	-	-	-	-	-	-	-	-	**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EphrinA1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	***	-	-	-	-	-	-	*	-	-	-	-	-	-
EphrinA3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	***	-	-	-	-	-	-	**	-	-	-	-	-	-
EphrinA5	-	-	-	-	-	-	-	-	-	-	-	*	-	**	-	-	-	-	-	-	-	-	-	-	-	-	-	*
EphrinB2	-	-	-	-	-	-	***	-	-	-	-	-	-	**	-	-	-	-	-	-	**	-	-	-	-	-	-	***
EphrinB3	-	-	-	-	-	-	***	-	-	-	-	-	-	*	-	-	-	-	-	-	*	-	-	-	-	-	-	*