Supplemental Information

Supplemental Methods and Materials

Human brains

A total of 73 postmortem brain specimens were obtained from two separate resources of known drug abusers and respective controls and were separately evaluated in Study I (n = 29) and Study II (n = 44). Study I, defined here as a multidrug population, represents three groups of drug abuse subjects — heroin, cocaine, and heroin-cocaine. Study II represents a group of heroin abusers. Cause of death, toxicological analysis at the time of death and documented drug history were used to categorize subjects into the specific drug groups studied. It, however, cannot be excluded that these individuals could have used other drugs during their lives, but had negative blood and urine toxicology for such substances at the time of death. Aside from documented abuse history, no posthumous DSM-IV dependence diagnosis was given as it was not possible to fully characterize the behavioral pattern of the subjects' drug use during life.

Multiple drug abuse population (Study I)

Post-mortem brain specimens from heroin, cocaine, and heroin-cocaine polysubstance users as well as normal control subjects were collected approximately 24 h after death as part of the routine autopsy process under a protocol approved by Wayne State University's Human Investigation Committee. Cause and manner of death were determined after medicolegal examination by a forensic pathologist after evaluating the circumstances of death, toxicological report and autopsy results (included physical evidence like needle marks, blackened lungs, heart size, etc.). Information was also evaluated from police reports as well as family and friends and medical records when available. All cases were assessed for common drugs of abuse (including

alcohol) and also screened for many therapeutic drugs depending upon initial general urine assessment. Blood concentrations were assessed by gas chromatography–mass spectrometry (GC-MS). Heroin, cocaine, and cocaine-heroin users had positive blood toxicological evidence for heroin, cocaine, and combination of cocaine and heroin and/or their metabolites (for heroin: 6-monoacetyl morphine, morphine, morphine glucuronide; for cocaine: benzoylecgonine, ethyl cocaine, ecgonine methylester, and methylecgonidine), respectively. Heroin abuse subjects were negative for cocaine and cocaine abusers tested negative for opiates. Control subjects tested negative for opiates and cocaine. All subjects tested negative for other common drugs of abuse including barbiturates, benzodiazepines, and phencyclidine. Of the subjects studied, four control, three heroin and two heroin-cocaine subjects tested positive for sub-intoxicating levels of alcohol (control, 0.08 ± 0.09 g/dl; heroin, 0.05 ± 0.08 g/dl; heroin-cocaine, 0.03 ± 0.07 g/dl: mean ± SD) but did not exhibit common signs of chronic alcohol abuse such as liver pathology. The cause of death for all drug groups was drug-related (intoxication and/or abuse) except for 2 subjects in the heroin group for which the cause was gun shot wound to the chest and atherosclerotic cardiovascular disease; these subjects had evidence of recent drug use based on blood toxicological analysis and a documented history of heroin abuse. The general characteristics for control, heroin, cocaine and heroin-cocaine subjects (Study I, n = 7-8/group) are described in Table 1 of main text.

Heroin abuse population (Study II)

Brain specimens from a larger population of drug abusers were also studied of subjects who died from heroin intoxication. Brain samples from heroin abusers and control subjects were collected at the Department of Forensic Medicine at Semmelweis University, Hungary, as well as from the

National Institute of Forensic Medicine, Karolinska Institutet, Stockholm, Sweden. The specimens were collected at autopsy within 24 h after death under the guidelines approved by the Semmelweis University Human Ethical Committee and the Ethics Committee at Karolinska Institutet and the Swedish Board of Social Welfare, respectively. The cause of death was determined by a forensic pathologist after evaluating the circumstances of death, toxicological report and autopsy results. Information was also evaluated from police reports, interviews of family and friends and medical records when available. All cases were assessed for common drugs of abuse (including alcohol) and also for therapeutic agents. Heroin subjects died from heroin intoxication (as verified by toxicological analysis), had physical signs of heroin use such as needle track marks, and most had a history of heroin abuse. Blood toxicological analysis was conducted by GC-MS. The heroin group represented a unique drug abuse population as they were predominantly heroin users not receiving methadone treatment. The blood morphine/codeine concentration ratio was 16.2 ± 7.6 and 3.62 ± 1.2 μg/ml in the urine and blood, respectively; > 1 morphine/codeine concentration ratio normally indicates heroin usage rather than medication with codeine (1). Only a few subjects had positive blood levels of 6-monoacetylmorphine, the rapid metabolite of heroin. The control group had negative blood levels of opiates or other drugs of abuse except alcohol in very few cases in which ethanol concentrations (1.3 g/l blood) were similar to the limited alcohol-positive subjects identified in the heroin group (1.2 ± 0.6 g/l blood) and none of those subjects exhibited common signs of chronic alcohol abuse such as liver pathology. The demographics and general characteristics of inclusion criteria for control (n = 15) and heroin (n = 29) subjects (Study II) are described in Table 2 of the main text.

Brain tissue processing

For the molecular studies, immediately after autopsy the brains were cut coronally in 1.5 cm slabs, frozen and kept at -70°C. Twenty mm sections were taken from the amygdala blocks using a Microm HM560 cryostat (Microm International GmbH, Walldorf, Germany), quickly mounted onto Superfrost plus-glass (Brain Research Laboratories, Newton, MA) and then kept at –30°C until analysis. After sectioning, tissue punches were taken from the remaining caudal lateral amygdala for protein measurements. All molecular and biochemical procedures were carried out blinded to the subject group.

In situ hybridization histochemistry

Homer 1 and PSD-95 riboprobes were synthesized from a human cDNA library using nested polymerase chain reaction (PCR). All inner primer pairs were flanked with SP6 or T7 promoter sequences. To allow *in vitro* transcription the Homer 1 probe spanned from base 781 to base 997 in NM_004272. The outer primer pair corresponded to bases 713-734 + 2023-2046, and the inner pair to bases 781-804 + 974-997. The outer primer pair for the PSD-95 probe (U83192 bases 2854-3102) corresponded to bases 2793-2815 + 3106-3126, and the inner pair to bases 2854-2879 + 3078-3102. Riboprobes were generated by *in vitro* transcription using SP6 or T7 polymerase and [^{35}S]-aUTP (Amersham Biosciences, Europe). *In situ* hybridization was performed as previously described (2, 3). Briefly, prior to hybridization duplicate brain sections/subject were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), incubated in 0.25% acetic anhydride in 0.1 M triethanolamine/saline, dehydrated through a series of graded ethanol, and delipidated with chloroform. Cover-slipped sections were incubated with 270 ml ($20x10^3$ CPM/ml) overnight at 55ºC. Hybridization was terminated by washes in a graded

series of SSC solutions followed by dehydration in a graded series of ethanol. Subsequently, the slides were dried and exposed to Kodak Biomax MR film for 5 to 15 days depending on the probe of interest. The specificity of the antisense probes was verified by their distinct anatomical distribution patterns and by the use of sense riboprobes that showed no positive hybridization signals. This specificity highlights the good quality of the postmortem human specimens which is also demonstrated by the lack of significant impact of storage time on the mRNA expression levels of the current markers studied.

Image analysis

Optical density values were measured using Scion Image (PC based version of NIH ImageJ; National Institutes of Health, Bethesda, MD) from digitalized images with a resolution of 300 dpi (dots per inch; films scanned by ScanMaker III; Microtek Electronics, Düsseldorf, Germany) The optical density values were converted to DPM (disintegrations per minute)/mg by reference to co-exposed C^{14} standards (American Radiolabeled Chemicals, Inc., St. Louis, MO). Measurements were taken within discrete amygdala subnuclei (accessory basal, basal, and lateral, Figure 1) with the help of published sources of the human amygdala (4, 5). DPM/mg values from duplicate slides were averaged.

Western blot analysis

Tissue samples were sonicated in sodium dodecyl sulfate (SDS) extraction buffer (67.5 mM Tris-HCl, pH 6.8, 1% SDS, phosphatase and 5 x protease inhibitors). Protein extracts were aliquoted and kept at -80°C until usage. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Rockford, IL; Pierce). Aliquots of tissue extracts

were mixed with SDS sample buffer to a final concentration of 1X [2X; 120 mM Tris HCl, 20% glycerol, 4% SDS, 10%, 0.01% Bromophenol Blue, 50 mM DTT (pH 6.8)] and heated for 15 min at 65°C or at 15 min 85°C (mGluR5 and GluA1). Solubilized protein (10 - 60 μg per lane) was subjected to 10% Criterion Precast Tris-HCl gel SDS-PAGE electrophoresis (Bio-Rad Laboratories, Hercules, CA). Reference samples consisting of protein extracts from one control subject were loaded onto two wells and their density values were used to ascertain reproducibility on each blot and interblot comparison. Proteins were transferred to nitrocellulose membranes (BioRad Laboratories and Whatman International Ltd, part of GE Healthcare, Piscataway, NJ) and stained with Memcode Reversible Protein Stain Kit (Thermo Fisher Scientific). The membranes were then blocked in 2.5-5% non-fat milk/PBS (1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4 or 50% Odyssey Blocking Buffer/50% PBS) (LI-COR, Lincoln, NE) for 2 h and probed with primary antibodies at 4°C overnight. Rabbit polyclonal antibodies were used against Homer 1, PSD-95 [1:5000;1:2000; Synaptic Systems GmbH (SYSY) Goettingen, Germany], or mGluR5 and GluA1 (1:200;1:1000 Millipore (Upstate), Billerica, MA). In addition we used mouse monoclonal antibodies against GluN1 (114 011, 1:1000, SYSY) and GAPDH (MAB374, 1:60,000 Millipore (Upstate)). Membranes were washed three times for 15 min in PBST buffer and incubated with goat anti- rabbit or goat anti-mouse IRDye 680 or IRDye 800 secondary antibodies (LI-COR) for 1 h at room temperature. Each protein analyzed was identified as a single or a double band with the predicted molecular size ~ 45 (Homer 1), 95 (PSD95), 130 (mGluR5), 106 (GluA1), 110 (GluN1-analyzed as a double band), ~ 100 (dynamin-3) and 35 (GAPDH) kDa. No protein degradation (evident by smearing) was detected in the blots for any of the postmortem samples. For inter-gel comparison, the optical density of proteins in each sample was expressed as a percentage of reference samples

on a given gel, and these values were used for statistical analysis. GAPDH and/or Memcode optical density were used to control for total protein content. Membranes were developed with the LI-COR infrared imaging system (LI-COR) and images quantified using average integrated intensity values with the Odyssey application software (Odyssey application software (version 2.0) and the ImageJ software (NIH).

Immunoprecipitation

Tissue punches from the lateral amygdala of human subjects were homogenized in lysis buffer (1% IGEPAL CA-360, 50 mM Tris, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, pH 7.4) with 1 μg/ml pepstatin, complete protease inhibitor cocktail (Roche, Indianapolis, IN) and Halt™ phosphatase inhibitor cocktail (Thermo Scientific), solubilized for 15 min and cleared by low-speed centrifugation (1000 x g) for 5 min at 4°C. Both Homer 1b/c (Santa Cruz, Santa Cruz, CA) and control mouse IgG (Santa Cruz) antibodies were crosslinked to Dynabeads (3 μg antibody per 30 μl beads; Invitrogen, Carlsbad, CA) using the crosslinking reagent Bis(Sulfosuccinimidyl) suberate (Thermo Scientific). The protein concentration of the brain samples was measured using the BCA protein assay (Thermo Scientific) and 500 μg of protein was added to 30 μl of the Dynabead-antibody complex for 4 hours at 4°C with constant rotation. The Dynabeads were washed with lysis buffer and PBS prior to elution of complex proteins by boiling in 1x SDS-PAGE sample buffer. The protein samples were separated by SDS-PAGE (10.5-14% Tris-HCl gel; Bio-Rad, Hercules, CA), transferred and the nitrocellulose membranes were stained for protein using Memcode (Thermo Scientific), scanned and then blocked as described above. The membranes were split after blocking and probed with either 1 μg/ml Homer 1b/c or 1 μg/ml

Dynamin 3 (Abcam, Cambridge, MA) antibodies overnight at 4°C. The blots were developed and analyzed as described above.

Statistical analysis

Shapiro-Wilk's W test was performed to assess the normal distribution of the data. In those data sets that were not normally distributed, natural log transformation rendered the data into normal distribution. General linear stepwise regression analysis was used to evaluate statistical groups in relation to the potential influence of various variables: age, brain pH, sex, blood ethanol and brain freezer storage time. GAPDH and/or Memcode optical density examined for western blot were also assessed in regard to potential influence of the markers studied. Variables with a significant association with group were included in the final statistical model as covariates. Student's *t*-test was used to assess differences between groups when no covariates were found. Correlation analyses were performed to determine the association between blood drug levels and the glutamatergic markers in the drug abusers as well as to evaluate the relationship between individual glutamatergic marker pairs between drug abusers and control subjects. To compare the correlation structure between heroin and control brains a structural equation multi-group analysis was performed (structural equation modeling is an extension of general linear regression which takes into account the modeling of interactions and as such integrates path analysis and factor analysis). Two models were estimated, one in which the correlations were constrained to be the same across the two groups and one where the correlation parameters were unconstrained. Akaike's information criteria (AIC) and chi-square test (likelihood ratio test) were used to identify the best model fit. If the correlations were found to be invariant across groups, a Z-statistic (critical ratio test) was used for pairwise comparisons between single correlation

estimates. Significance was set at $P < 0.05$ and trends considered for $P < 0.10$. Statistical evaluations were carried out using the JMP [SAS Institute, Cary, NC), Statistica (StatSoft Scandinavia, Uppsala, Sweden) and AMOS version 16.0 (SPSS Inc., Chicago, IL] software packages. Data was expressed as mean (%) ± SEM relative to respective controls, which were arbitrarily set at 100%.

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Supplemental Figure

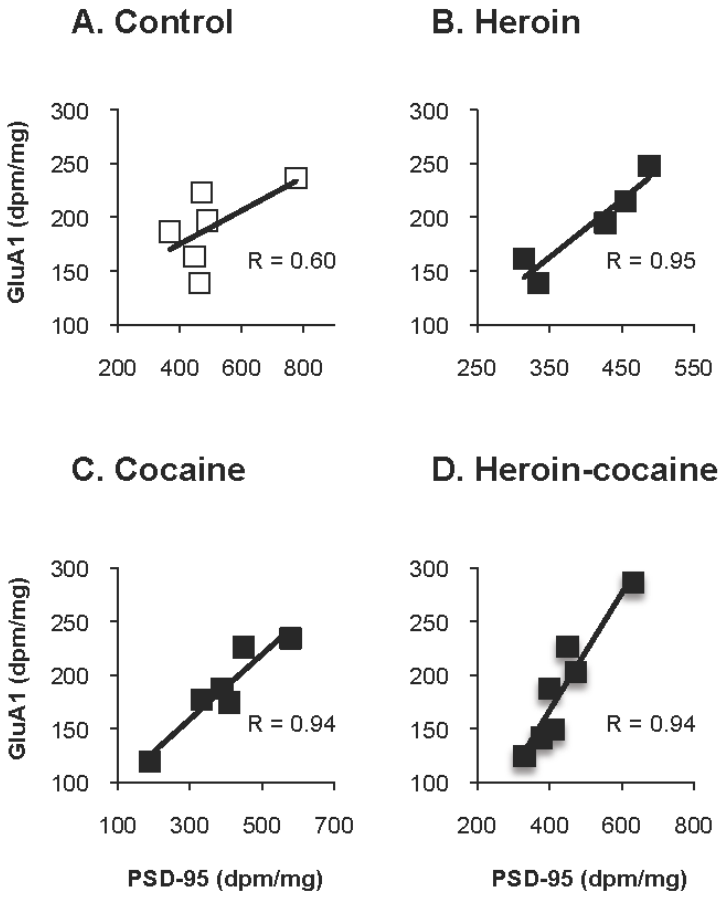

Figure S1. Correlation between GluA1 and PSD-95 mRNA levels in the lateral amygdala nucleus in control (n = 6; **A**), heroin (n = 5; **B**), cocaine (n = 6; **C**) and heroin-cocaine (n = 7; **D**) subjects from the multi drug abuse population (Study I).