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ORIGINAL ARTICLE

The absence of VGLUT3 predisposes to cocaine abuse by increasing dopamine and glutamate signaling in the nucleus accumbens

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Tonically active cholinergic interneurons (TANs) from the nucleus accumbens (NAc) are centrally involved in reward behavior. TANs express a vesicular glutamate transporter referred to as VGLUT3 and thus use both acetylcholine and glutamate as neurotransmitters. The respective roles of each transmitter in the regulation of reward and addiction are still unknown. In this study, we showed that disruption of the gene that encodes VGLUT3 (*Slc17a8*) markedly increased cocaine self-administration in mice. Concomitantly, the amount of dopamine (DA) release was strongly augmented in the NAc of VGLUT3$^{-/-}$ mice because of a lack of signaling by metabotropic glutamate receptors. Furthermore, dendritic spines and glutamatergic synaptic transmission on medium spiny neurons were increased in the NAc of VGLUT3$^{-/-}$ mice. Increased DA and glutamate signaling in the NAc are hallmarks of addiction. Our study shows that TANs use glutamate to reduce DA release and decrease reinforcing properties of cocaine in mice. Interestingly, we also observed an increased frequency of rare variations in *SLC17A8* in a cohort of severe drug abusers compared with controls. Our findings identify VGLUT3 as an unexpected regulator of drug abuse.

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INTRODUCTION

Drug addiction is a compulsive pattern of drug-taking/drug-seeking behavior with major adverse repercussions. Although the molecular mechanisms underlying addiction are not completely understood, all addictive substances regulate the reward circuit by increasing dopamine (DA) release in the nucleus accumbens (NAc).[1–3] GABAergic medium spiny neurons (MSNs) are the major input target as well as the output of the NAc. MSNs are dynamically regulated by dopaminergic fibers that originate from the ventral tegmental area (VTA) and by local interneurons, such as the tonically active cholinergic interneurons (TANs). Fine-tuning of the balance between DA and acetylcholine (ACh) is key for the correct processing of reward-directed behaviors. However, the precise role of TANs and ACh in the NAc is not well understood. VGLUT3 expression in TANs was recently found to increase ACh vesicular accumulation and release[4] and to confer on TANs the ability to signal with glutamate as well as with ACh.[5] Surprisingly, mice without the ability to secrete ACh experience minimal alterations in their behavioral responses to cocaine.[6] Interestingly, silencing VGLUT3 in mice results in marked cocaine-induced locomotor activity,[4] suggesting that glutamate released by TANs is instrumental in regulating reinforced responses to cocaine.

In this study, we analyzed the implications of VGLUT3 in reward and addiction. We found that the hedonic effects of cocaine were exacerbated in VGLUT3$^{-/-}$ mice. We also showed that TANs from the NAc used both glutamate and ACh to decrease or increase DA release, respectively. Finally, we reported a higher rate of non-synonymous mutations in the gene-encoding VGLUT3 in patients with severe addictions compared with controls. Our results suggest that TANs and VGLUT3 in the NAc act as protective filters against the reinforcing properties of substances of abuse such as cocaine.

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MATERIALS AND METHODS

Human populations

A total of 230 adult subjects with cocaine and/or opiate dependence from two different studies, METHADOSE ($n = 144$) and PSYCHOCOKE ($n = 86$), were included. Written informed consent was obtained from all participants. Institutional review board approval was obtained (Saint Louis Hospital Ethic Committee, Paris, France, 22 December 2008).

For inclusion in the METHADOSE study, patients had to be methadone-maintained opiate dependent with a stable methadone regimen for at least 3 months.

For inclusion in the PSYCHOCOKE study, patients had to be current cocaine users. Only cocaine-dependent patients were considered in the present genetic analysis.

Patients were primarily males (75%) with a mean age at interview of 40.5 ± 9.4 years. Most of them were Caucasian (88%), 9% were of sub-Saharan African origin and 3% were of Asian origin. All were 100% dependent with a mean duration of illness of 16 ± 10 years; 15% were cocaine-dependent only, 25% were opiate-dependent only and 60% were dependent on both cocaine and opiates. In addition, 55% of patients had a positive lifetime history of alcohol dependence, 43% had a lifetime history of benzodiazepine dependence and 64% had a lifetime history of cannabis dependence.

In addition, 213 unaffected controls of French origin were recruited from among blood donors attending the Henri Mondor Hospital (Créteil, France). The unaffected controls were assessed using the Diagnostic Interview for Genetic Studies (DIGS), and their family history of psychiatric disorders was determined with the Family Interview for Genetic Studies (FIGS). Only subjects with neither a personal nor a family history of affective disorders, severe addiction or suicide attempts were included. DNA from 390 controls of African origin (69 from Tunisia, 119 from Morocco, 75 from Algeria and 127 from sub-Saharan Africa) were generously provided by Dr Ryad Tamouza (Hôpital Saint Louis, Paris, France) and used to examine the allele frequency of rs45610843.

DNA from 265 patients with opiate dependence in methadone maintenance treatment have been used for the replication cohort. These subjects were included in a multi-center study in Switzerland as previously described in detail elsewhere[7] and a proportion of the patients was also cocaine abusers.

All subjects signed a written informed consent, and local institutional review boards approved this protocol.

SLC17A8 sequence analysis

For each individual, genomic DNA was isolated from blood leukocytes using a standard procedure. The *SLC17A8* coding regions were amplified before sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) and run on a 16-Capillary ABI PRISM 3130xl genetic analyzer (Life Technologies, Carlsbad, CA, USA). All primers used for the PCR amplifications and sequence analyses are available on request. Genotyping of 390 additional controls of African origin was performed to examine the allele frequency of rs45610843.

Animals

Animal studies were performed in accordance with the European Communities Council Directive (86/809/EEC) regarding the care and use of animals for experimental procedures in compliance with the *Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale* (permission # A 94-028-21).

The VGLUT3$^{-/-}$ mouse line has previously been described[4] and was backcrossed on a C57BL/6N background for 10 generations. DRD1a-EGFP heterozygous mice (C57BL/6 background, Gensat, New York, NY, USA) were crossed with VGLUT3$^{-/-}$ to allow the direct assessment of fluorescent MSN. All experiments were carried out with wild type (WT), WT:DRD1a-EGFP VGLUT3$^{-/-}$ and VGLUT3$^{-/-}$:DRD1a-EGFP 2- to 4-month-old male littermates, except for electrophysiological recordings that were performed with 30- to 40-day-old animals.

Animals were randomly allocated to experimental groups. Whenever possible, investigator was blinded during experimental procedures. Animals were excluded from the experimental data if and only when they were detected by outlier test (Grubb's test—GraphPad Prism Software, La Jolla, CA, USA).

Drugs

Cocaine hydrochloride (Sigma-Aldrich, Saint Louis, MO, USA) was dissolved in a saline solution (0.9% NaCl w/v), except for the *in vivo* electrophysiological studies in which it was dissolved in water. Quinpirole hydrochloride and eticlopride hydrochloride (Tocris Bioscience, Bristol, UK) were dissolved in water. The metabotropic glutamate receptor (mGLUR) broad-spectrum antagonist LY341495 (Sigma-Aldrich) was dissolved in artificial cerebrospinal fluid. Ketamine, xylazine, bicuculline and tetrodotoxin (Abcam, Cambridge, UK and Tocris, Bristol, UK) were dissolved in water, except for tetrodotoxin (1% citric acid).

Cocaine-induced locomotor activity and sensitization

Locomotor activity was measured in a cyclotron, which consisted of a circular corridor with four infrared beams placed at 90° angles (Imetronic, Pessac, France). Activity was counted as the consecutive interruption of two adjacent infrared beams (1/4 of a tour). To assess acute cocaine-induced locomotion, the animals (WT $n = 8$; VGLUT3$^{-/-}$ $n = 7$) were placed in the cyclotron for 30 min for habituation and then injected with cocaine (10 mg kg^{-1}, intraperitoneal (i.p.)). Locomotion was recorded for 60 min following the cocaine injection.

For the cocaine sensitization experiments, mice (WT $n = 6$; VGLUT3$^{-/-}$ $n = 6$) were injected with saline and placed in the cyclotron for 60 min over 3 consecutive days for habituation. Starting on day 4, the mice were split into two groups: one group was treated with saline and the other with cocaine (10 mg kg^{-1}, i.p., every day) for 5 consecutive days. This repeated exposure was followed by 5 days of withdrawal and by a cocaine challenge injection (10 mg kg^{-1}) on day 13. Locomotor activity was recorded 60 min after the saline or cocaine injection. Animals that underwent the cocaine sensitization protocol were used for spine labeling.

Cocaine conditioned place preference (CPP)

CPP was assessed using a Y-shaped apparatus (Imetronic, Pessac, France) as previously described.[8] Two chambers were distinguished by different patterns on the floors and walls and were separated by a central neutral area. In the preconditioning phase, animals were allowed to explore both chambers for 18 min. In the conditioning phase, mice were randomly treated three times (three pairings) with saline (WT $n = 8$; VGLUT3$^{-/-}$ $n = 7$), cocaine 2.5 mg kg^{-1} (WT $n = 10$; VGLUT3$^{-/-}$ $n = 11$) or cocaine 5 mg kg^{-1} (WT $n = 7$; VGLUT3$^{-/-}$ $n = 7$). Control animals received saline every day. After injections, they were confined to a given chamber for a period of 20 min. On the test day, the animals were again allowed to explore both chambers. Entries and time spent in each chamber were measured during both the pre- and post-conditioning phases. Data were expressed as the difference between the cocaine-paired minus the saline-paired chamber during pre- and post-conditioning.

Operant self-administration of cocaine

Cocaine self-administration experiments were performed as previously described.[9–11]

Mice were anesthetized with a ketamine/xylazine mixture (20 ml kg^{-1}, i.p.) and then implanted with indwelling intravenous silastic catheters. Each 2-h daily self-administration session started with a priming injection of the drug. Cocaine was infused in 23.5 µl over 2 s (0.5 mg kg^{-1} per injection, intravenously). Stimulation light (cue), located above the active hole, was paired with the delivery of the reinforcer. Mice (WT $n = 14$; VGLUT3$^{-/-}$ $n = 13$) were trained under a fixed ratio 1 schedule of reinforcement (FR1; one nose-poke lead to the delivery of one dose of cocaine) over 5 consecutive daily sessions and under a fixed ratio 3 (FR3) over 5 consecutive daily sessions. The timeout period after infusion delivery was 15 s. Responses on the inactive hole and all responses elicited during the 15-s timeout period were also recorded. The criteria for self-administration behavior were achieved when all of the following conditions were met: (1) mice maintained stable, responding with $< 20\%$ deviation from the mean of the total number of reinforcers earned in three consecutive sessions (80% of stability); (2) at least 75% of mice responding on the active hole; and (3) a minimum of 10 reinforcers per session. After the 10 FR sessions, animals were tested in a progressive ratio (PR) schedule of 3 h where the response requirement to earn the cocaine escalated according to the following series: 1–2–3–5–12–18–27–40–60–90–135–200–300–450–675–1000. On day 12, mice were moved from the cocaine self-administration/training phase to the extinction phase. The experimental conditions during the extinction phase were

similar to the cocaine self-administration sessions except that cocaine was not available, and light was not presented after nose poking in the active hole. Mice were given 2-h daily sessions until they achieved the extinction criterion with a maximum of 18 sessions. The criterion for extinction was achieved when, during 3 consecutive sessions, mice completed a mean number of nose pokes in the active hole consisting of $< 30\%$ of the mean responses obtained during the 3-day period to achieve the acquisition criteria for cocaine self-administration training. On day 28, all of the mice were tested under reinstatement induced by a cue during a 2-h session. The presentation of a conditioned environmental cue was performed to evaluate the reinstatement of cocaine-seeking behavior. The test for cue-induced reinstatement was conducted under the same conditions used in the training phase except that cocaine was not available. The reinstatement criterion was achieved when nose pokes in the active hole were double the number of nose pokes in the active hole during the 3 consecutive days when the mice acquired the extinction criteria.

In vivo voltammetry

Mice (WT $n = 14$; VGLUT3$^{-/-}$ $n = 16$) were anesthetized with chloral hydrate (400 mg kg^{-1}, i.p.), and voltammetric electrodes (Aldrich, Milwaukee, WI, USA) were implanted into the NAc (stereotactic coordinates AP: 1.3–1.5 mm, Lat: 0.4–0.6 mm, DV: 3.9-4.3). The voltammetric electrodes consisted of one 30-µm diameter carbon fiber coated with Nafion (Aldrich). Electrochemical measurements were performed using a high-speed chronoamperometric apparatus (Quanteon, Lexington, KY, USA) as previously described.[12] DA release was evoked by local injections of 200–300 nl of KCl (120 mM) at 10-min intervals in the presence or absence of the mGLUR broad-spectrum antagonist LY341495 (100µM). The results are presented as the mean ± s.e.m. of the difference in maximal DA variation after KCl ejection. The differences in DA release between the different groups of mice were assessed as a comparison with the differences in maximal variation for each group. The time to reach 80% of the maximal response (t80) was measured as an estimate of DA clearance.

Electrophysiological *in vivo* recording of VTA neurons

In vivo electrophysiological recordings of VTA neurons were performed as previously described.[13] For recordings of VTA neurons, extracellular recording electrodes were constructed from borosilicate glass tubing (1.5 mm OD/1.17 mm ID, Harvard Apparatus, Cambridge, UK) using a vertical electrode puller (Narishige, Tokyo, Japan). Tips were broken, and electrodes were filled with a 0.5% Na-acetate solution yielding impedances of 6–9 MΩ. Animals were anesthetized with chloral hydrate (400 mg kg^{-1}, i.p., supplemented as required to maintain optimal anesthesia throughout the experiment) and placed in a stereotaxic apparatus (Kopf Instrument, Tujunga, CA, USA). The left saphenous vein was catheterized for the intravenous administration of cocaine, and the right saphenous vein was catheterized for the intravenous administration of quinpirole and eticlopride. The electrophysiological activity was sampled in the central region of the VTA (coordinates: between 3.1 and 3.5 mm posterior to Bregma, 0.3 and 0.6 mm lateral to midline and 4 and 4.7 mm below brain surface). Spontaneously active DAergic neurons were identified on the basis of previously established electrophysiological criteria: (1) a typical triphasic action potential with a marked negative deflection; (2) a characteristic long duration (> 2.0 ms); (3) an action potential width from start to negative trough of > 1.1 ms; and (4) a slow firing rate (between 1 and 10 Hz) with an irregular single spiking pattern and occasional short, slow bursting activity.[14,15] At least 5 min of spontaneous baseline electrophysiological activity was recorded before the intravenous injection of cocaine (1 mg kg^{-1}). Fifteen minutes after the first cocaine injection, quinpirole (1 mg kg^{-1}) was administered intravenously. Five minutes later, eticlopride (1 mg kg^{-1}) was administered intravenously. For each cell, the firing frequency was rescaled as a percentage of its baseline value averaged over the 2 min before cocaine injection. The effect of cocaine was assessed as a comparison between the maximum variation of the firing rate and the spike within burst observed during the first 2 min after saline or cocaine injections. The analysis of the DAergic neurons' firing pattern was processed as previously described.[13] The results are presented as the mean ± s.e.m. of the difference in the maximal variation before and after cocaine. The differences in cocaine effects between the groups were assessed as a comparison between the differences in maximal variation for each group. We used a Student's *t*-test when both parameters followed a normal distribution (Shapiro test, $P > 0.05$) and a non-parametric Mann–Whitney test when they did not, as mentioned in the figure legends.

Electrophysiological *in vitro* recording of MSN

In vitro recordings of MSN were performed as previously described.[16,17] WT ($n = 6$), WT:DRD1a-EGFP ($n = 8$), VGLUT3$^{-/-}$ ($n = 8$) and VGLUT3$^{-/-}$:DRD1a-EGFP mice ($n = 10$) (3–4 weeks old) were anesthetized (150 mg kg^{-1} ketamine/10 mg kg^{-1} xylazine) for slice preparation. Coronal 300-µm slices were prepared in bubbled ice-cold 95% O_2/5% CO_2-equilibrated solution containing the following (in mM): 110 choline Cl; 25 glucose; 25 $NaHCO_3$; 7 $MgCl_2$; 11.6 ascorbic acid; 3.1 Na^+ pyruvate; 2.5 KCl; 1.25 NaH_2PO_4; and 0.5 $CaCl_2$. The slices were then stored at room temperature in 95% O_2/5% CO_2-equilibrated artificial cerebrospinal fluid containing the following (in mM): 124 NaCl; 26.2 $NaHCO_3$; 11 glucose; 2.5 KCl; 2.5 $CaCl_2$; 1.3 $MgCl_2$; and 1 NaH_2PO_4. The slices were kept at 32–34 °C in a recording chamber superfused with 2.5 ml min^{-1} artificial cerebrospinal fluid. Visualized whole-cell voltage-clamp recording techniques were used to measure the holding current and synaptic responses of MSNs of the nucleus accumbens shell with an upright microscope (Olympus, Hamburg, Germany). Currents were amplified, filtered at 5 kHz and digitized at 20 kHz and recorded at a holding potential of − 70 mV (IGOR PRO, Wavemetrics, Portland, OR, USA). Access resistance was monitored using a step of − 10 mV (0.1 Hz), and experiments were discarded if the access resistance increased by $> 20\%$. Miniature excitatory postsynaptic currents (EPSCs) were recorded at Vh = − 60 mV in the presence of tetrodotoxin (0.5 µM) using an internal solution containing the following (in mM): 140 potassium gluconate, 4 NaCl, 2 $MgCl_2$, 1.1 EGTA, 5 HEPES, 2 Na_2ATP, 5 sodium creatine phosphate and 0.6 Na_3GTP (pH 7.3 with KOH). The frequency, amplitude and kinetic properties of these currents were then analyzed using the Mini Analysis software package (Synaptosoft, Decatur, GA, USA). Synaptic currents were evoked by stimuli (60 µs) at 0.1 Hz through a glass pipette placed 200 µm from the patched neurons. The internal solution contained (in mM): 130 CsCl, 4 NaCl, 2 $MgCl_2$, 1.1 EGTA, 5 HEPES, 2 Na_2ATP, 5 sodium creatine phosphate, 0.6 Na_3GTP and 0.1 spermine. All experiments were performed in the presence of bicuculline (20 µM). α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA):*N*-methyl-D-aspartate (NMDA) ratios of evoked EPSCs were obtained by evoking pure AMPA-EPSC at − 60 mV and a mix of AMPA and NMDA at +40 mV. The NMDA component was extracted at 30–50 ms from EPSC onset when the AMPA component was back at baseline.[16]

Online/offline analyses were performed using IGOR-6 (Wavemetrics) and Prism (GraphPad Software, La Jolla, CA, USA). Compiled data are expressed as the mean ± s.e.m. Significance was set at $\alpha = 0.05$ using Student's *t*-test and the Kolmogorov–Smirnov test.

DRD1 and DRD2 autoradiography

Auto-radiographic detection of DRD1 and DRD2 was performed as previously described.[4] In short, fresh frozen sections of mice brains (WT $n = 5$; VGLUT3$^{-/-}$ $n = 5$) were pre-incubated in 50 mM Tris-HCl buffer, pH 7.4–7.5, with 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$. Then, the sections were incubated with either [^{3}H]SCH23390 (for DRD1 labeling) or [^{125}I]iodosulpride (DRD2 labeling), both of which were purchased from GE Healthcare (Milwaukee, WI, USA). [^{3}H]SCH23390-labeled sections were exposed to BAS-TR Fuji Imaging screens (Fuji Film Photo, Tokyo, Japan). Screens were then scanned with a Fuji Bioimaging Analyzer BAS-5000 (Fuji Film Photo). [^{125}I]iodosulpride-labeled sections were exposed to X-ray films (Biomax MR, Eastman Kodak, Sigma-Aldrich, Saint-Louis, MO, USA), scanned and converted to 16-bit images using a Umax PowerLook 1100 scanner (Umx System, Willich, Germany). Digitized images were analyzed with MCID software (Imaging Research, St Catharine's, ON, Canada). The results are expressed as the mean ± s.e.m. of optical density (in arbitrary units).

Immunofluorescence

Mice were anesthetized and intracardially perfused with phosphate-buffered saline containing 4% paraformaldehyde. Brains were dissected, post-fixed by immersion in the same fixative and cryoprotected in phosphate-buffered saline containing 10% sucrose. Coronal sections (20 µm) were cut using a vibratome and stored at − 20 °C in cryoprotective solution until use. Immunofluorescence was performed on free-floating sections as previously described.[18] Sections were incubated with VGLUT3 rabbit antiserum (1:2000, Synaptic Systems, Goettingen, Germany, catalog refs 135–203),[4] serotonin rat monoclonal antiserum (1:50, Millipore Bioscience Research Reagents, Billerica, MA, USA catalog ref. AB12) and home-made VAChT guinea pig antiserum (1:5000).[4] Primary antibodies were detected with anti-rabbit, anti-guinea pig or anti-mouse IgG coupled to Alexa Fluor 488, Alexa Fluor 555 or Cy5 (1:2000, Invitrogen, Waltham, Massachusetts, USA). Slices were mounted on glass slides

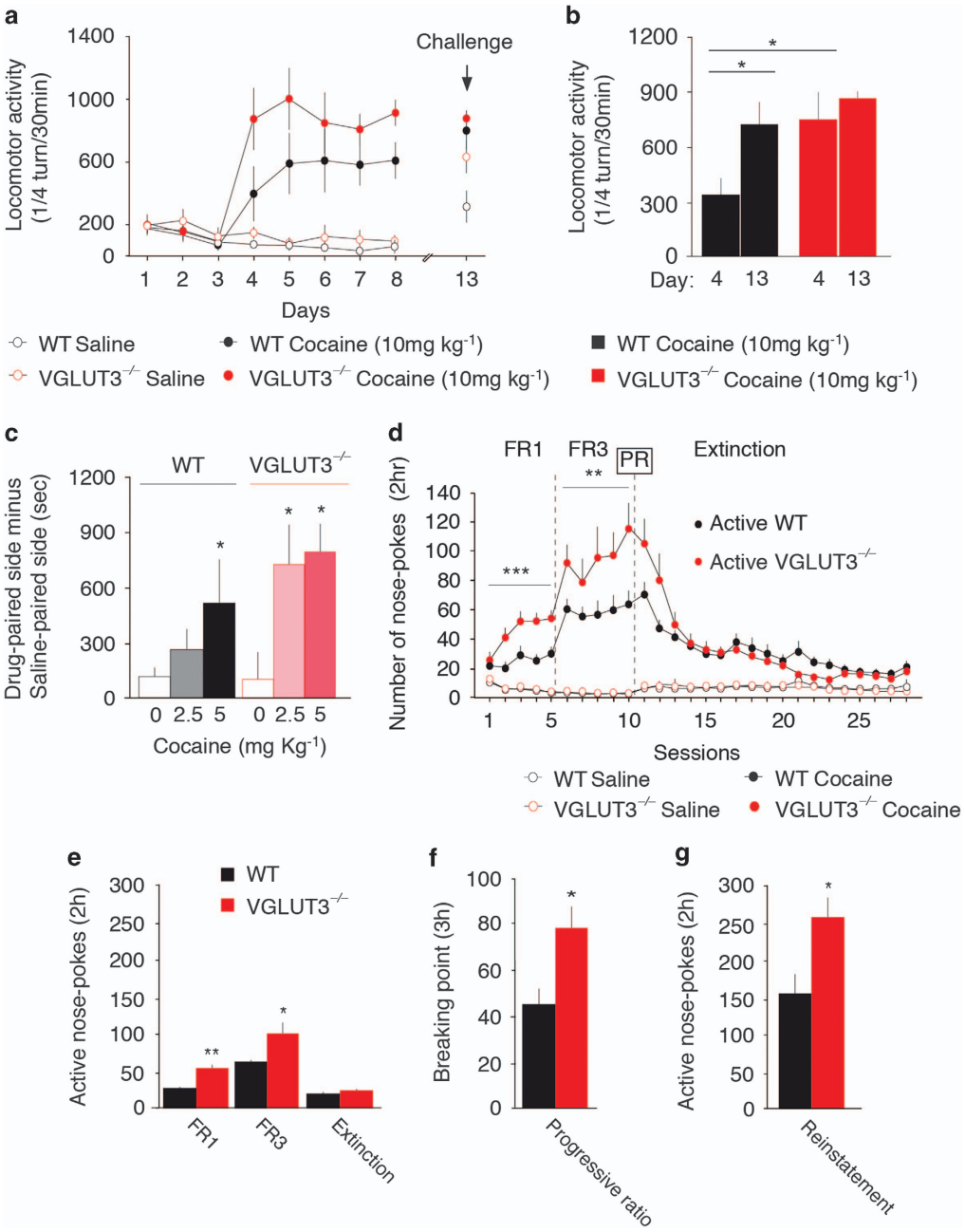

Figure 1. Enhanced rewarding properties of cocaine in VGLUT3$^{-/-}$ mice. (**a**) Repeated cocaine injections produced a progressive increase in locomotor sensitization (repeated-measures three-way analysis of variance (ANOVA), $F_{(8,156)} = 18.39$, $P < 0.001$). (**b**) Cumulative locomotion (30 min) following cocaine injection on the first day (day 4) versus challenge day (day 13). (Wild type (WT) ($n = 6$); VGLUT3$^{-/-}$ ($n = 6$); two-way ANOVA, $F_{(1,18)} = 9.52$, $P = 0.006$). (**c**) VGLUT3$^{-/-}$ mice established conditioned place preference when receiving a dose of 2.5 mg kg^{-1} cocaine ($n = 10$-11), whereas a dose of 5 mg kg^{-1} elicited preference in both genotypes (($n = 7$), two-way ANOVA, $F_{(2,47)} = 6.419$, $P = 0.003$, Bonferroni post-test). (**d**) Levels of active nose poking were significantly higher in VGLUT3$^{-/-}$ mice ($n = 13$) than in WT mice ($n = 14$) during the acquisition (fixed ratio 1 (FR1), FR3) of self-administration (three-way ANOVA, $F_{(1,25)} = 14.97$; $P < 0.01$). Both genotypes showed similar extinction (three-way ANOVA, $F_{(1,24)} = 0.08$, $P > 0.05$). (**e**) Number of active nose pokes during FR1, FR3 and extinction (**f**) Progressive ratio of cocaine self-administration. VGLUT3$^{-/-}$ mice were more motivated to seek cocaine than WT mice (one-way ANOVA, $F_{(1,25)} = 6.78$; $P < 0.05$). (**g**) Cue-induced reinstatement test. VGLUT3$^{-/-}$ mice displayed a higher rate of relapse than WT mice (three-way ANOVA, $F_{(1,25)} = 12.43$, $P < 0.01$). Newman–Keuls post-test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. All data are mean ± s.e.m. (Statistical analyses in Supplementary Tables S1 and S2.)

with Fluoromount-G (Southern-Biotech, Birmingham, AL, USA). Images were acquired using a Confocal Laser Scanning Microscope (Leica TCS SP5 AOBS, Leica Microsystems, Mannheim, Germany) equipped with a 63x, 1.4 NA oil immersion objective with the pixel size set to 60 nm and a z-step of 130 nm. Images were deconvoluted using a Maximum Likelihood Estimation algorithm with Huygens 3.6 software (Scientific Volume Imaging, Hilversum, The Netherlands). Settings were used such that background intensity was averaged from the voxels with lowest intensity and the signal to noise ratio was estimated to a value of 20. Labeled terminals were quantified using the 3D object counter plugin for ImageJ.[19] Colocalization analysis was performed based on the correlation of intensities between pairs of pixels in the two different channels as described by Jaskolski *et al.*[20] using the colocalization color map

plugin for ImageJ. Super-resolution imaging of sub-synaptic clusters of material enriched for VGLUT3 or VAChT or both was revealed with Structured Illumination Microscopy (Nikon N-SIM; Nikon Imaging Center at Institut Curie, Paris, France).

Diolistic labeling and dendritic spine analysis

Mice (WT $n=11$; VGLUT3$^{-/-}$ $n=14$) were anesthetized with pentobarbital, and a fast (2-min) transcardial perfusion with 30 ml of paraformaldehyde (1.5% in phosphate-buffered saline) was performed. The brains were removed and post-fixed for 1 h in the same fixative, and sections (150 µm) were cut with a vibratome.[21] Image stacks of MSN dendrites were acquired in the NAc shell using a 488 or 561-nm laser line for excitation of a Confocal Laser Scanning Microscope (SP5, Leica, Wetzlar, Germany). Image stacks were deconvoluted with Huygens 3.5 software (Scientific Volume Imaging). NeuronStudio software (CNIC, New York, NY, USA) was used to analyze dendritic spines.[22] After automated detection, manual checks were performed to avoid false positives.

Statistics

All statistical comparisons were performed with two-sided tests with Prism 5 (GraphPad Software, La Jolla, CA, USA). Mann–Whitney tests, one-way and repeated-measures two-way analysis of variance (ANOVA) were used when appropriate. Bonferroni's test for multiple comparisons *post-hoc* analysis was performed when required unless otherwise indicated.

In the case of sensitization, the statistical analysis was performed using StatView (SAS Institute, Cary, NC, USA). A three-way ANOVA with repeated measures was used followed by Tukey's/Kramer's test as a *post-hoc* test.

All results are expressed as the mean ± s.e.m., and differences were considered significant at $P<0.05$.

RESULTS

VGLUT3 deletion exacerbates cocaine-induced behaviors

In the NAc, TANs express VGLUT3 in addition to VAChT (Supplementary Figure S1). To assess whether VGLUT3 influences NAc-driven behaviors, we explored cocaine-related endophenotypes in VGLUT3$^{-/-}$ mice and WT littermates. Silencing VGLUT3 resulted in marked cocaine-induced locomotor activity (Supplementary Figure S2).[4] Sensitization to repeated cocaine administration[23] was evaluated in VGLUT3$^{-/-}$ and WT littermates (Figures 1a and b; Supplementary Table S1). WT mice displayed significantly higher levels of locomotor activity on the challenge day than on the first day of cocaine administration (Figure 1b). At the initial cocaine exposure, VGLUT3$^{-/-}$ mice exhibited higher drug-induced locomotor activity than WT mice (+103%, $P<0.05$). The cocaine-induced locomotor activity of the mutants remained similar on days 4 and 13. Thus, VGLUT3 deletion induced an immediate increase in cocaine-induced locomotor activity but occluded further sensitization to cocaine.

In addition to its locomotor effects, cocaine has reinforcing properties. The affective state in a drug-paired environment can be modeled in rodents using the CPP test.[24] VGLUT3$^{-/-}$mice, unlike WT littermates, showed a marked CPP at the lowest dose of cocaine (2.5 mg kg^{-1}, $P=0.003$; Figure 1c). No difference in CPP was observed for the two genotypes at a higher dose of cocaine (5 mg kg^{-1}). Thus, reinforcing properties of cocaine were increased by VGLUT3 deletion in CPP.

To further evaluate the reinforcing properties of psychostimulant drugs in VGLUT3$^{-/-}$ mice, we used the operant cocaine self-administration paradigm. During both FR1 and FR3, more VGLUT3$^{-/-}$ mice than WT mice met the acquisition criteria for the cocaine-maintained operant response (Supplementary Figure S3). Throughout the training, VGLUT3$^{-/-}$ animals showed more active nose-poking responses than WT littermates (Figures 1d and e; Supplementary Table S2). Animals were then tested in a PR schedule. The breaking point values were significantly higher in VGLUT3$^{-/-}$ mice (76.77 ± 9.69 nose pokes) than in WT mice (45.14 ± 7.49 nose pokes) (Figure 1f). Thus, VGLUT3$^{-/-}$ mice appeared to experience an enhanced motivation for cocaine. Following FR1, FR3 and the PR schedule, animals underwent extinction of operant responses to cocaine for 18 days. Responses of mutant mice at the active hole were comparable to those of WT mice and decreased across sessions in both genotypes (Figures 1d and e; Supplementary Table S2). After the 18 days of forced cocaine abstinence, mice were evaluated for cue-induced reinstatement of cocaine-seeking behavior. In this test, active nose-poking responses were significantly more pronounced in VGLUT3$^{-/-}$ mice than in WT littermates (Figure 1g) in a drug-associated environment.

Overall, VGLUT3$^{-/-}$ mice experienced a higher level of acquisition of self-administration, improved FR1 and FR3 performance, and had comparable levels of extinction. Furthermore, mice lacking VGLUT3 appeared to be more vulnerable to relapse. However, relapse vulnerability should be interpreted with caution given that VGLUT3$^{-/-}$ mice demonstrated a confounding higher degree of response with all schedules tested and with a higher PR breakpoint.

Dual regulation of DA release by TANs in the NAc

DA transmission is critical for enabling the reinforcing properties of cocaine. Using *in vivo* voltammetry, we measured the release of DA in response to KCl-induced depolarization in the NAc of VGLUT3$^{-/-}$ and WT mice. A single KCl pulse evoked a transient release of DA that was significantly higher in VGLUT3$^{-/-}$ mice than in WT animals (Figures 2a and b, +96%, $P=0.001$). No significant modification was observed in the clearance of extracellular DA at T80 (Figure 2c), indicating that DA reuptake was unaltered in VGLUT3$^{-/-}$ mice. *In vivo* electrophysiological recordings in VTA showed that this increased DA release was not related to a modified firing nor to an altered sensitivity to cocaine of DA neurons (Supplementary Figure S4).

TANs signal with both ACh and glutamate, and the synaptic storage of ACh is decreased in VGLUT3-null mice.[4,5] To further dissect the specific roles of ACh and glutamate in TANs, we tested DA release in mice without the ability to release ACh in the striatum because of selective elimination of VAChT.[6] In VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice, the amplitude of the KCl-induced DA release was markedly lower than in WT mice (Figures 2a and b; −69%, $P=0.00006$). No difference in T80 was observed for the two genotypes (Figure 2c). Thus, glutamate and ACh originating from TANs had opposing effects on DA efflux.

The electrochemical detection of DA by *in vivo* voltammetry reflects the dynamic balance between DA release, DA reuptake and DA receptor D2 (DRD2) presynaptic inhibition of release.[25] To investigate the functional consequences of the lack of VGLUT3 or VAChT on the DRD2-dependent accommodation of the response, the DA max release as well as the T80 were compared after four consecutive KCl ejections (K1–K4; Figures 2b and c) administered at 10-min intervals. As expected, repeated KCl ejections resulted in a progressive decline in responses for all three genotypes. After each successive depolarization, the DA release was always significantly higher in the NAc of VGLUT3$^{-/-}$ mice and lower in VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice than in WT mice. The DRD2 regulation appeared unaltered in these mutant mouse lines.

Consistent with their increased cocaine self-administration, VGLUT3$^{-/-}$ mice demonstrated a marked enhancement of DA efflux in the NAc. Thus, we explored whether this finding may be related to local effects in the NAc. Local application of a mGLUR antagonist has been shown to increase DA release in the NAc.[26] We thus investigated whether glutamate originating from TANs could exert a local inhibition of DA release by binding to mGLURs. As shown in Figures 2d and e, DA efflux was significantly increased by the local application of the broad-spectrum antagonist mGLUR LY341495[27] in WT mice (+96%, $P=0.00006$) but not in VGLUT3$^{-/-}$ mice. Therefore, blocking mGLUR had the same net effect on DA release as deleting VGLUT3. These findings suggest that VGLUT3-dependent glutamate release by TANs in the NAc of WT mice inhibits DA efflux by activating mGLUR.

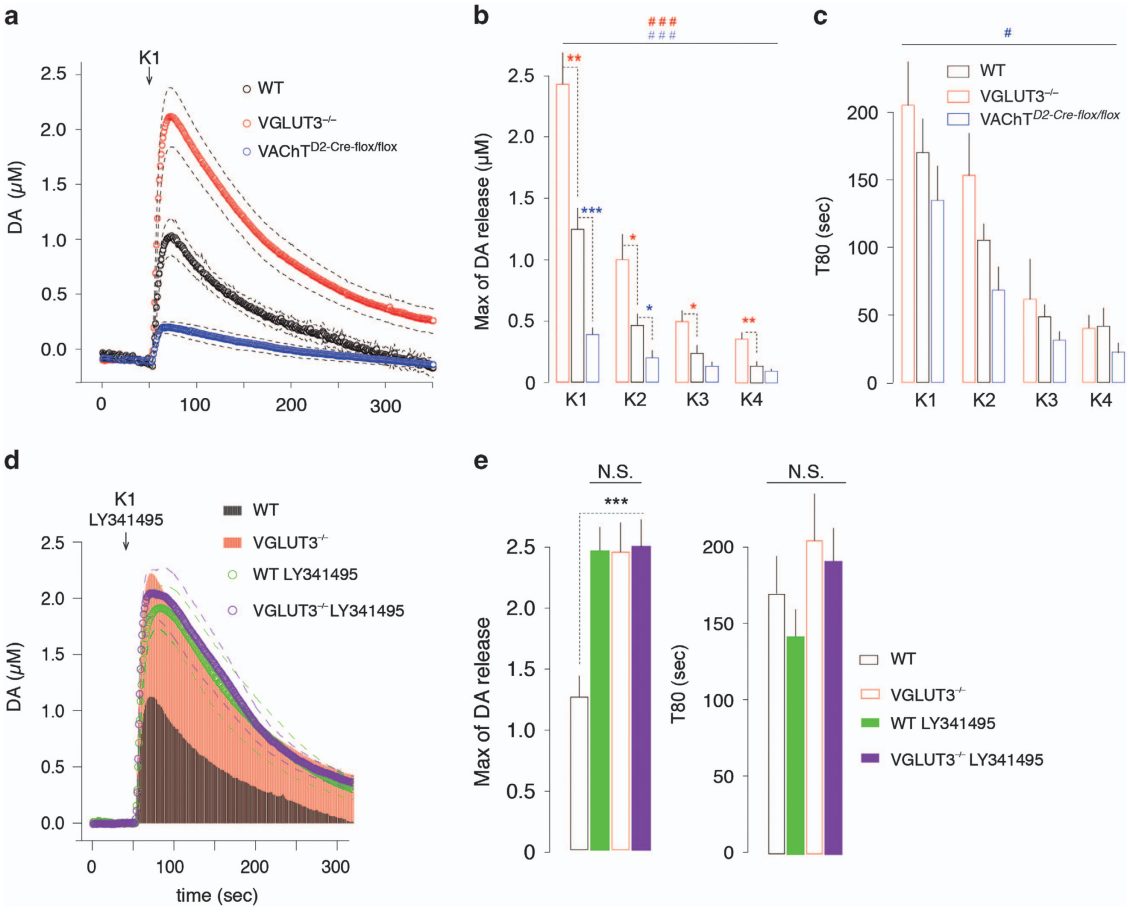

Figure 2. DA release is enhanced in the nucleus accumbens (NAc) of VGLUT3$^{-/-}$ mice. (**a**) Dopamine (DA) release induced by the first KCl injection (K1) in the NAc of wild type (WT) ($n=14$), VGLUT3$^{-/-}$ ($n=16$, two-way analysis of variance (ANOVA), $F_{(1,95)}=25.6$ $P<0.001$) and VACHT$^{D2\text{-}Cre\text{-}flox/flox}$ ($n=11$, two-way ANOVA, $F_{(1,73)}=23.8$, $P<0.001$) mice measured by *in vivo* chronoamperometry. (**b** and **c**) Accommodation of DA efflux in the NAc of WT, VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ and VGLUT3$^{-/-}$ mice after four consecutive KCl ejections (K1–K4). (**b**) Bar plots indicate the maximum of DA release and (**c**) the T80 induced by consecutive KCl stimulation. Two-way ANOVA genotype effect ($^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$), *post-hoc* Mann–Whitney analysis ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$: red asterisks WT vs VGLUT3$^{-/-}$, blue asterisks WT vs VACHT$^{D2\text{-}Cre\text{-}flox/flox}$). (**d** and **e**) Effects of the broad-spectrum metabotropic glutamate receptor (mGLUR) antagonist LY341495 (100 μM) on K^+-induced DA efflux in the NAc of WT mice and VGLUT3$^{-/-}$ mice. Black and red hatched areas indicate the release of K^+-induced DA efflux in WT mice and VGLUT3$^{-/-}$ mice, respectively, as shown in **a**. (**e**) Bar plots indicate the maximum of DA release and the T80 of the DA response induced by KCl in the presence or absence of LY341495 in WT mice and VGLUT3$^{-/-}$ mice. Mann–Whitney analysis ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$). (Statistics in Supplementary Table S3.)

The DA receptor D1 signaling cascade was activated in the NAc of VGLUT3$^{-/-}$ mice

The augmented behavioral response to cocaine and the increased DA release in the NAc of VGLUT3$^{-/-}$ mice prompted us to investigate the status of dopaminergic signaling cascades. The density of DRD2-binding sites did not differ between VGLUT3$^{-/-}$ and WT mice (Figure 3a). However, the density of DRD1 was significantly higher in the NAc of VGLUT3$^{-/-}$ mice (Figure 3b; +41%, $P=0.03$) but not in their dorsal striatum.

Cocaine-triggered DA release activates extracellular signal-regulated kinase (ERK) in DRD1-positive MSNs.[28] The activation of ERK following acute cocaine injection was monitored in the NAc and the dorsal striatum of VGLUT3$^{-/-}$ and WT littermates (Figure 3c). Interestingly, following a 2.5 mg kg^{-1} injection of cocaine, the number of phosphorylated ERK-positive cells was significantly higher in the NAc of VGLUT3$^{-/-}$ mice (+44%, $P<0.05$) than of WT mice (Figure 3c). At this dosage, no difference was observed between the dorsal striata of WT and mutants. A 10 mg kg^{-1} dose of cocaine caused a similar increase in phosphorylated ERK-positive neurons in the dorsal and ventral striata of both genotypes. Therefore, DRD1 receptor density and the ERK pathway were upregulated in the NAc of VGLUT3$^{-/-}$ mice.

VGLUT3 regulates morphological and functional properties of excitatory synapses onto MSNs

The cocaine-induced activation of the DRD1 cascade results in structural modifications of MSNs.[8,29,30] Therefore, we explored how chronic cocaine treatment may affect the remodeling of dendritic spines in the NAc of VGLUT3$^{-/-}$ and WT mice (Figure 3d). Spine density was measured in the NAc of saline-treated or cocaine-sensitized mice (as described in Figure 1a). As expected, spine density was significantly increased after cocaine sensitization in the MSNs of the NAc of WT animals (+50%, $P=0.006$). Surprisingly, the spine density of saline-injected VGLUT3$^{-/-}$ mice was comparable to that of cocaine-treated WT or VGLUT3$^{-/-}$ mice. Therefore, MSNs from the NAc of VGLUT3$^{-/-}$ mice presented constitutively elevated spine densities.

Glutamatergic afferents originating from the cortex, the thalamus, the hippocampus or the amygdala converge onto the spines of MSN from NAc to regulate reward behaviors.[31,32] Thus,

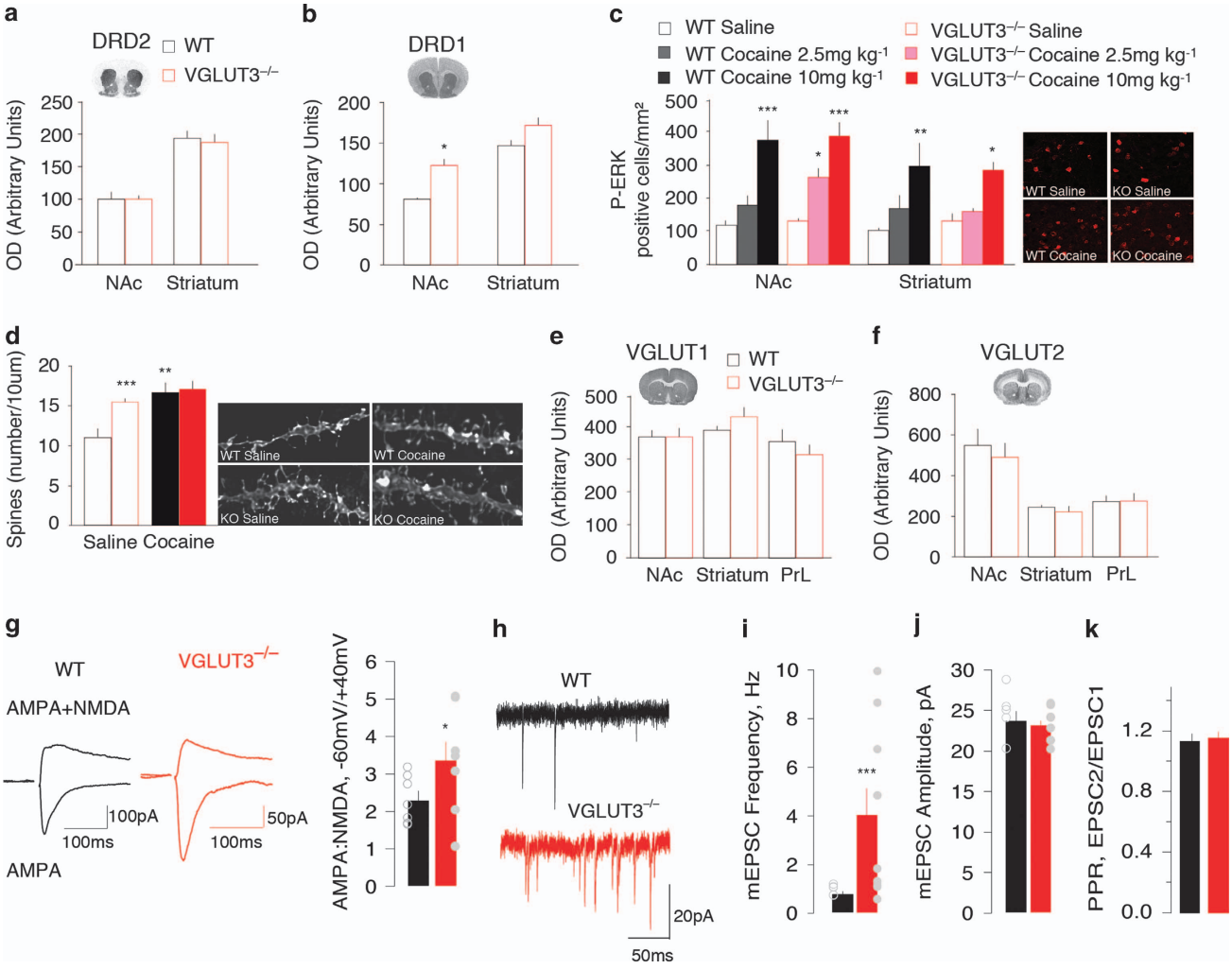

Figure 3. DRD1 signaling cascade, spine density and synaptic transmission in medium spiny neurons (MSNs) of VGLUT3$^{-/-}$ mice. Quantification of (**a**) [^{125}I]iodosulpride (DRD2) and (**b**) [^{3}H]SCH23390 (DRD1). The number of DRD1-binding sites increased in the nucleus accumbens (NAc) of VGLUT3$^{-/-}$ mice ($n=5$; Mann–Whitney, $P=0.0317$). (**c**) Cocaine administered at a dose of 2.5 mg kg^{-1} but not 10 mg kg^{-1} resulted in a higher number of P-ERK-positive cells in the NAc of VGLUT3$^{-/-}$ mice than in the NAc of wild-type (WT) mice ($n=5$; two-way analysis of variance (ANOVA), $F_{(2,\,22)}=30.22$, $P<0.0001$). (**d**) VGLUT3$^{-/-}$ mice had a higher density of MSN dendritic spines than WT mice ($n=11$-14; two-way ANOVA, $F_{(1,33)}=6.443$, $P<0.001$). Chronic cocaine administration increased spine density only in WT mice (two-way ANOVA, $F_{(1,33)}=14.21$, $P=0.006$). (**e** and **f**) VGLUT1 and VGLUT2 densities are unchanged in VGLUT3$^{-/-}$ mice. (**g**) Excitatory postsynaptic currents (EPSCs) recorded at −60 and +40 mV from WT and VGLUT3$^{-/-}$ mice. α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/*N*-methyl-D-aspartate (NMDA) ratios were higher in slices from mutant mice ($n=6$-8; WT, 2.3 ± 0.3; VGLUT3$^{-/-}$, 3.5 ± 0.4 $t_{12}=2.6$, $P<0.05$). (**h**) Miniature excitatory postsynaptic currents (mEPSCs) recorded in WT and VGLUT3$^{-/-}$ mice. (**i**) mEPSC frequency ($n=10$; WT, 0.82 ± 0.1 Hz; VGLUT3$^{-/-}$, 4.1 ± 1 Hz; $t_{18}=2.9$, $P<0.01$) and (**j**) amplitude ($n=10$; WT, 23.8 ± 1.1 pA; VGLUT3$^{-/-}$, 23.2 ± 0.6 pA; $t_{18}=0.4$, $P>0.05$). (**k**) Paired-pulse ratio ($n=7$-9; WT, 1.1 ± 0.1; VGLUT3$^{-/-}$, 1.2 ± 0.05; $t_{14}=0.3$, $P>0.05$). *$P<0.05$, **$P<0.01$, ***$P<0.001$. All data are mean ± s.e.m.

we investigated whether glutamatergic nerve endings were also modified in the NAc of VGLUT3$^{-/-}$ mice. Interestingly, postsynaptic changes were not accompanied by presynaptic modifications of VGLUT1 or VGLUT2 levels, either locally in the NAc or distally in the prefrontal cortex (Figures 3e and f).

Long-lasting changes in spine numbers are hypothesized to contribute to persistent synaptic adaptations.[30] The observed changes in spine number led us to perform whole-cell patch-clamp recordings from MSNs of the NAc (shell) of WT or VGLUT3$^{-/-}$ mice (Figures 3g and k). To probe for synaptic adaptations in the mutants, we evoked AMPA receptor-mediated responses at −60 mV and a mixture of AMPA and receptor-mediated responses at +40 mV. We compared the relative contribution of AMPA versus NMDA receptors with evoke EPSCs. The NMDA receptor-mediated component was computed by measuring the amplitude at +40 mV 30–50 ms after the onset of the EPSC. We detected a significantly higher AMPA/NMDA ratio in mutant mice than in WT mice (Figure 3g; $P=0.02$). To further examine the regulation of excitatory transmission in the NAc shell, we measured miniature EPSCs (Figures 3h and j). VGLUT3$^{-/-}$ mice demonstrated significantly increased miniature EPSCs frequency of AMPA receptor-mediated currents (Figure 3i; $P=0.007$). However, no alterations in the miniature EPSC amplitude (Figure 3j) nor in the paired-pulse ratio of evoked EPSCs (Figure 3k) were detected in VGLUT3$^{-/-}$ mice, which suggests the absence of presynaptic modifications and is consistent with an increased spine number. The strengthening of glutamatergic transmission in the mutants did not prevent cocaine-evoked plasticity of excitatory transmission. Indeed, as previously reported,[33] 24 h after the last cocaine injection, AMPA/NMDA ratios were reduced compared with saline-treated mice in both WT and VGLUT3$^{-/-}$ (Supplementary Figure S5). These morphological and electrophysiological changes

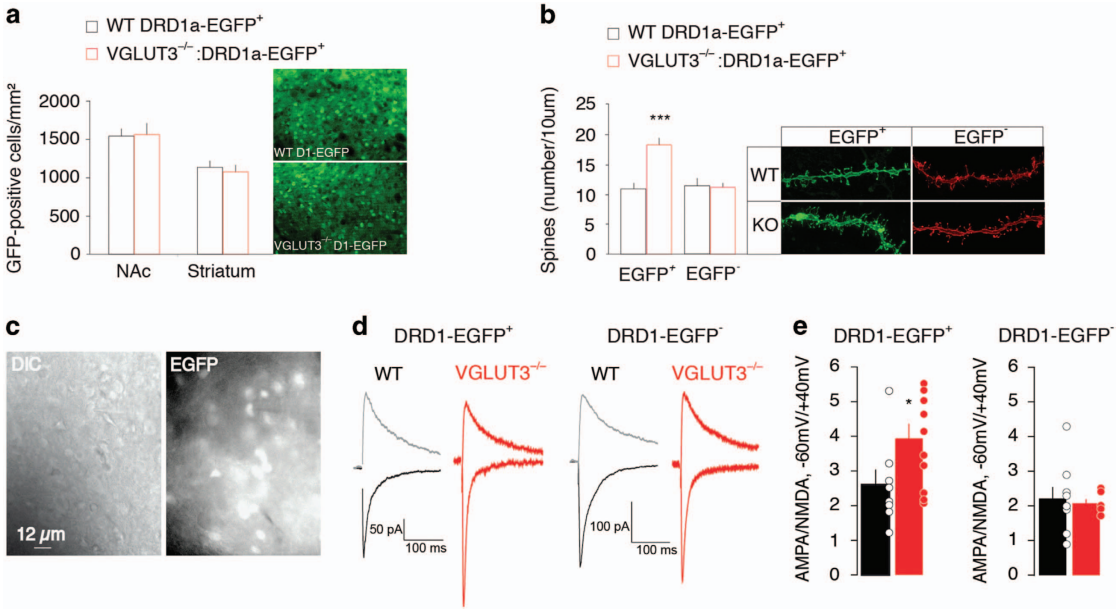

Figure 4. VGLUT3 deletion mediates structural and functional adaptations onto DRD1 medium spiny neurons (MSNs). (**a**) The number of DRD1a-positive neurons was not different in the nucleus accumbens (NAc) or in the dorsal striata of WT:DRD1a-EGFP$^+$ ($n=11$) and VGLUT3$^{-/-}$:DRD1a-EGFP$^+$ mice ($n=17$, two-way analysis of variance (ANOVA), $F_{(1,47)}=0.0823$, $P=0.775$). (**b**) VGLUT3$^{-/-}$ had a higher spine number in DRD1a-EGP-positive cells (EGFP$^+$) (WT $n=15$, VGLUT3$^{-/-}$ $n=19$, two-way ANOVA, $F_{(1,64)}=15.10$, $P<0.001$) but not in DRD1-negative MSN (DRD1a-EGFP$^-$, labeled with DiI; WT $n=13$, VGLUT3$^{-/-}$ $n=19$). (**c**) Differential interference contrast (DIC) and fluorescence channel image of a D1-EGFP$^+$ patched neuron. (**d**) Sample traces of excitatory postsynaptic currents (EPSCs) recorded at −60 and +40 mV in WT and VGLUT3$^{-/-}$ Drd1a-EGFP-positive and negative. (**e**) Bar graph and scatter plot for α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/*N*-methyl-D-aspartate (NMDA) ratios recorded in WT and VGLUT3$^{-/-}$ Drd1a-EGFP-positive and negative ($n=8$-10; WT—Drd1a-EGFP$^+$, 2.6 ± 0.4; VGLUT3$^{-/-}$—Drd1a-EGFP$^+$, 3.9 ± 0.4; $t_{16}=2.1$, $P<0.05$; WT—Drd1a-EGFP$^-$, 2.2 ± 0.3; VGLUT3$^{-/-}$—Drd1a-EGFP$^-$, 2.1 ± 0.1; $t_{15}=0.3$, $P>0.05$; *$P<0.05$, **$P<0.01$, ***$P<0.001$. All data are mean ± s.e.m.

Table 1. Summary of the variations identified in the human *SLC17A8* coding sequence

Populations	*Total number of subjects*	*Number of missense variations (% of alleles)*	*Number of subjects carrying missense variations (% of alleles)*	*Number of synonymous variations (% of alleles)*	*Number of subjects carrying synonymous variations (% of alleles)*
French addicts	230	7 (1.5)	12 (2.6)	2 (0.4)	4 (0.9)
Swiss addicts	265	4 (0.8)	4 (0.8)	2 (0.4)	5 (0.9)
French controls	213	1 (0.2)	1 (0.2)	1 (0.2)	1 (0.2)
1000 Genomes Project	1092	8 (0.4)	26 (1.2)	10 (0.5)	145 (6.6)

suggest that glutamatergic transmission in the NAc is strengthened in the absence of VGLUT3 but is still sensitive to cocaine exposure.

Cocaine experience modifies excitatory transmission specifically at glutamatergic synapses onto D1-positive MSNs.[34] To test whether VGLUT3 deletion-induced modifications are neuron specific, we crossed VGLUT3$^{-/-}$ mice with Drd1a-EGFP mice. This allowed us to visualize DRD1-positive neurons and discriminate them from other neuronal subtypes in the NAc. Using these double mutants, we found that the numbers of DRD1-positive and DRD1-negative MSNs were similar in the brains of WT and VGLUT3$^{-/-}$ mice (Figure 4a). Therefore, the augmented DRD1-binding site density (depicted on Figure 3b) was not related to an increased number of DRD1 and DRD1/DRD2-positive MSNs in the NAc. Furthermore, we found that the increased spine density reported in the NAc of VGLUT3$^{-/-}$ mice (see Figure 3d) was restricted to DRD1-positive MSNs (+63% in GFP-positive MSNs, $P=0.0003$; Figure 4b). In contrast, no significant changes in spine density were observed in DRD1-negative MSNs (putatively DRD2-positive MSNs; Figure 4b). Consistent with the morphological modifications of D1-MSNs in VGLUT3$^{-/-}$:Drd1a-EGFP mice, we found that AMPA/NMDA ratios were higher in the absence of VGLUT3 (Figures 4c and e). AMPA/NMDA ratios were comparable in EGFP-negative MSNs recorded from WT and VGLUT3$^{-/-}$ (Figures 4c and e). Taken together, these data suggest that the absence of VGLUT3 specifically modifies morphology as well as glutamatergic transmission onto DRD1-positive striato-nigral MSNs.

The frequency of rare variations in *SLC17A8* is increased in patients with severe addiction

Behavioral, biochemical, morphological and physiological analyses demonstrated that in the absence of VGLUT3, mice were more vulnerable to cocaine. These results suggest that allelic variations in VGLUT3 may be correlated with drug-seeking behavior in humans. Therefore, we sought rare mutations in the *SLC17A8* gene in a population of 230 French patients with severe addiction to cocaine or opiates. We identified seven mutations that predict an amino-acid change in the protein sequence and two synonymous variations (Figure 5, Table 1 and Supplementary Table S4). One of these variations, (p.T8I) was found in six non-related subjects. All mutations affected amino acids were highly conserved throughout evolution (average conservation of 0.973 in 16 vertebrate species). Five of these six amino acids (p.G71S, p.V104I, p.G130D, p.Y252S and p.P443L) were also conserved in VGLUT1 and VGLUT2 and predicted

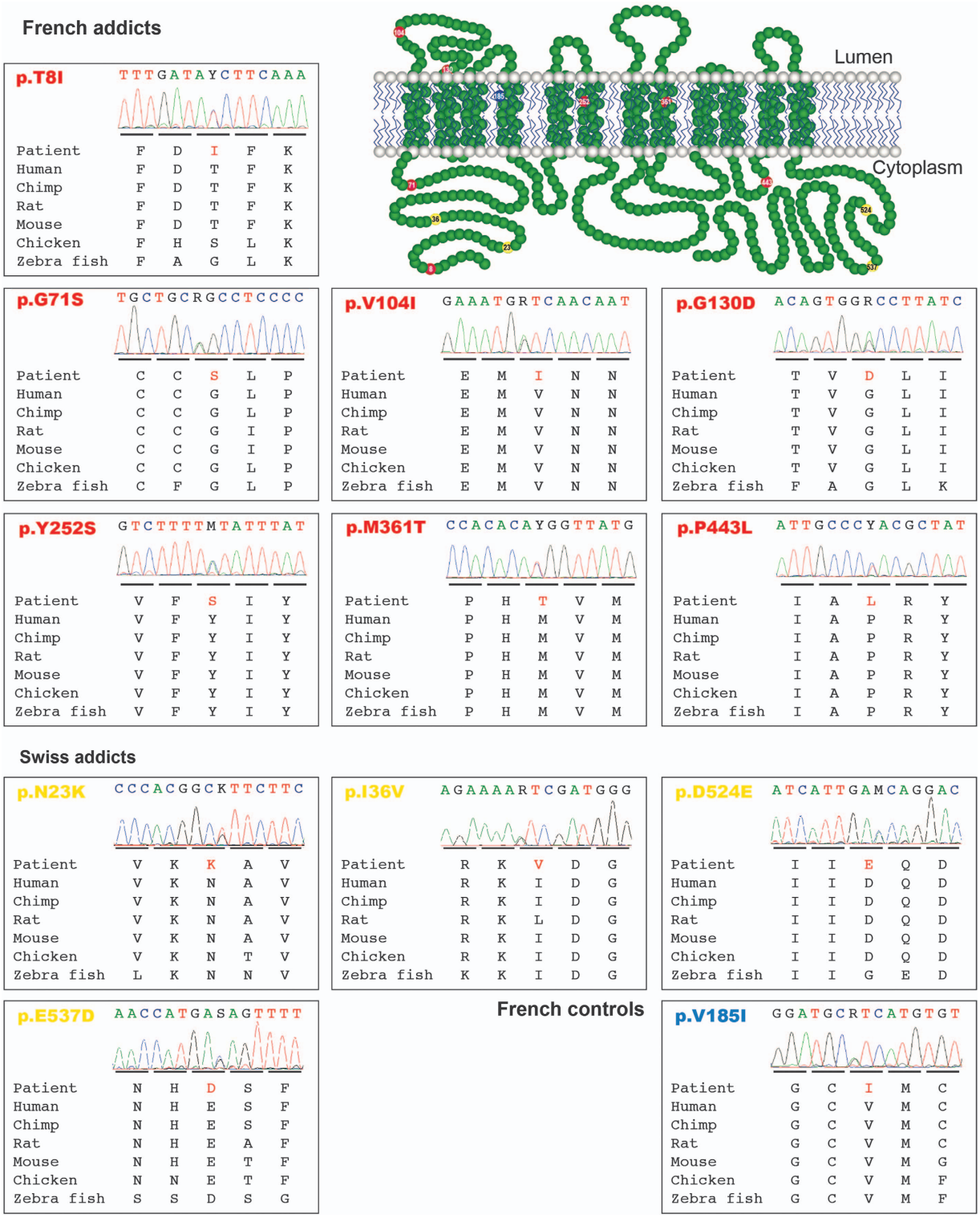

Figure 5. VGLUT3 mutations in humans with severe drug abuse. Seven mutations were identified in patients with severe addiction. The topological structure of VGLUT3 is depicted in the synaptic vesicle membrane based on protein structure described under accession Q8NDX2. Amino acids affected by the mutations are shown in red. The chromatogram corresponding to the DNA sequence observed in patients as well as the VGLUT3 protein alignment in six vertebrates is shown for each variation predicting an amino-acid change.

to be essential for the function of the VGLUT3 protein (Supplementary Table S4). None of the missense mutations were found in 213 control subjects of French origin without addiction, psychiatric disorder or suicidal behavior. In this control group, we identified only one missense mutation (p.V185I) and one synonymous variation (p.S410), suggesting that mutated alleles were significantly more frequent in subjects with severe addiction than in controls (2.6% and 0.2%, respectively, Fisher's exact test odds

ratio = 11.1, 95% confidence interval (CI) (1.4; 86.0), $P = 0.003$) (Table 1). We examined the mutation frequency observed in the 1000 Genomes Project.[35] Out of the 1092 subjects sequenced through phase 1, 8 missense variations were reported in 26 subjects and 10 synonymous variations were identified in 145 subjects (Table 1). Again, this demonstrated that the mutated allele frequency was significantly higher in subjects with severe addiction compared with the general population from the 1000 Genomes Project (2.6% and 1.2%, respectively, Fisher's exact test odds ratio = 2.2, 95% CI (1.1; 4.4), $P = 0.03$). We compared the non-synonymous and synonymous substitution rates between the patient and control populations using the ratio of the number of non-synonymous substitutions per non-synonymous site (d_N) to the number of synonymous substitutions per synonymous site (d_s) as an indicator of selective pressure acting on VGLUT3 (Supplementary Figure S6a). Interestingly, although the d_N/d_s ratio suggested a strong negative selection against *SLC17A8* in general population ($d_N/d_s = 0.23$), this ratio was fourfold higher ($d_N/d_s = 0.99$) in the population of subjects with severe addiction, confirming the higher number of amino-acid substitutions in this population compared with synonymous variations. To confirm our observations, we used a replication cohort of 265 opiate-dependent patients in methadone maintenance treatment from Switzerland, some with cocaine addiction. This mutation screening identified four new amino-acid changes and two synonymous variations (Figure 5, Table 1 and Supplementary Table S4), giving a mutation frequency of 0.8%. Although this frequency was higher than both of the frequencies observed in our French control population (0.2%) and in European subjects from the 1000 Genomes Project (0.5%), these differences were not significant (Fisher's exact test $P = 0.39$ and $P = 0.72$, respectively). However, we again noted a higher d_N/d_s ratio ($d_N/d_s = 0.57$) in the cohort from Switzerland than in the 1000 Genomes Project, confirming the increased frequency of non-synonymous variations in subjects with severe addiction.

Among the mutations identified in the cohort of subjects with severe addiction, one involved a change from a threonine at position 8 to an isoleucine [c.21C > T(p.T8I), rs45610843] and was found in six independent subjects. Five of these patients were of African origin, and one had two grandparents from Asia and two grandparents from Europe. To determine whether this variant was an ethnic-specific polymorphism or may be overrepresented in the population of patients with severe addiction, we screened the first exon of *SLC17A8* in 390 additional control subjects from Africa and compared the frequency of this allele in patients of identical origin. The I8 allele was found in 12 control subjects, revealing an allele frequency of 1.8% in the African population. This was consistent with the 1.2% frequency reported in subjects of African origin from the 1000 Genomes Project phase 1 (Supplementary Figure S6b; Fisher's exact test odds ratio = 1.2, 95% CI (0.4; 4.1), $P = 0.81$). Interestingly, the I8 allele was found at a frequency of 5.1% in patients of African origin with severe addiction, suggesting that this polymorphism was significantly overrepresented in patients with substance use disorders (Fisher's exact test odds ratio = 3.8, 95% CI (1.1; 10.9), $P = 0.02$) and increased the risk of addiction by a factor of three. Therefore, our results suggest that mutations that potentially alter VGLUT3 activity are associated with severe addiction in humans, a result that is consistent with the increased sensitivity to cocaine in VGLUT3 null mice.

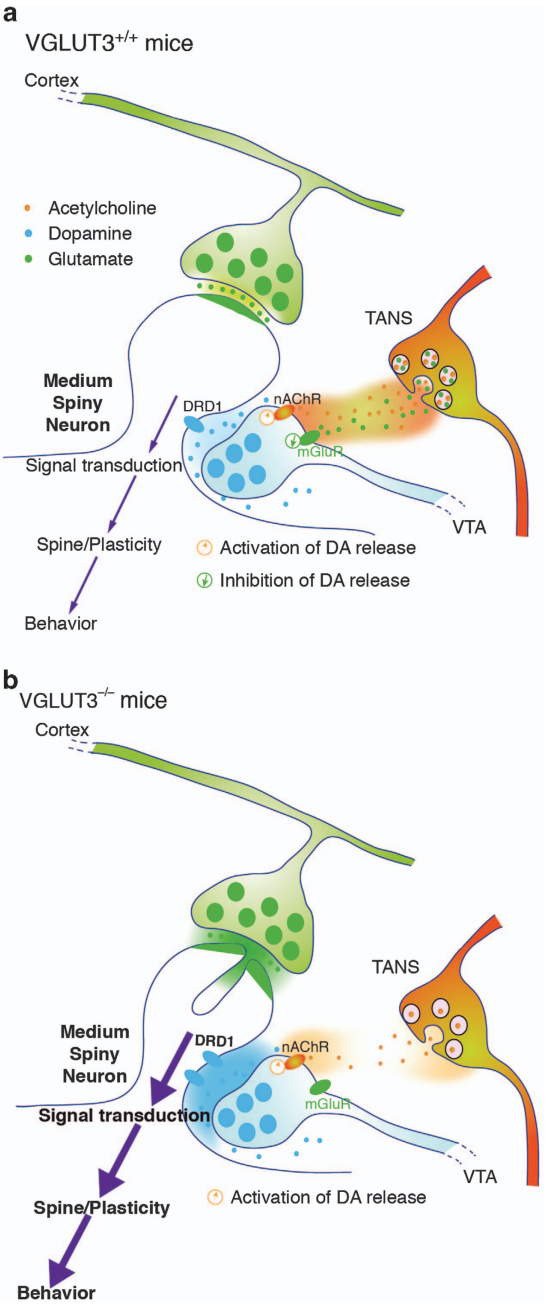

Figure 6. Dual regulation of dopamine (DA) efflux by acetylcholine (ACh) and glutamate released by tonically active cholinergic interneurons (TANs) in the nucleus accumbens (NAc). TANs express VAChT and VGLUT3 and therefore co-release ACh and glutamate. These two co-transmitters exert opposing effects on DA release. In wild-type (WT) mice, (**a**) DA efflux is stimulated by ACh (through nicotinic ACh receptors (nAChRs)) and inhibited by VGLUT3-dependent glutamate (through metabotropic glutamate receptors (mGLURs) most likely located on DA terminals). In VGLUT3$^{-/-}$ mice (**b**), the mGLUR-driven inhibition is lost and therefore DA efflux is markedly enhanced. Consequently the DRD1 signaling cascade is overactivated, and dendritic spine density (without modification of the number of excitatory terminals) and cortico-striatal glutamatergic activity are increased, leading to augmented sensitivity to the rewarding properties of cocaine.

DISCUSSION

It is well established that DA input from the VTA and local cholinergic TANs critically regulates reward-guided behaviors. DAergic and cholinergic neurons exhibit a characteristic alteration in pause-rebound activity in response to salient stimuli that is tightly controlled by GABAergic afferents from the VTA.[36,37] However, how TANs control the accumbal microcircuit is far from fully understood. In particular, little is known about the dual co-release of glutamate and Ach in the regulation of reward behavior.

This study provides evidence that VGLUT3 blunts cocaine behaviors by regulating DA and glutamate signaling in the NAc.

TANs from the NAc have long been recognized as key regulators of reward behavior and of the reinforcing properties of cocaine.[38,39] However, the mechanism by which TANs operate remains a matter of debate.[40] Our study suggests that one key to understanding this critical question resides in the fact that TANs use two different chemical codes to communicate with surrounding neural networks in the NAc: ACh and glutamate. We found that mice lacking ACh-release in the NAc exhibit a decreased DA release. This is consistent with previous observations showing that nicotinic ACh receptors exert stimulatory control over DA efflux (for review, see Exley and Cragg[41]). However, silencing ACh transmission in TANs minimally influenced reward behaviors,[6] and the marked decreased DA transmission in VAChT-null mice did not translate into a decrease in reward behavior. These observations will require further investigation.

In contrast, we herein report for the first time that TANs use VGLUT3-dependent glutamate and mGLURs to diminish DA release in the NAc and to modulate the reinforcing properties of cocaine. A negative regulation of DA release exerted by mGLURs in the NAc was previously reported;[26] however, the source of glutamate was not clearly identified. We herein establish that the stimulatory effects of a broad-spectrum mGLUR antagonist (LY341495) on DA efflux are observed exclusively in WT mice but not VGLUT3$^{-/-}$ mice. Therefore, this mGLUR-negative regulation of DA release is not influenced by glutamate coming from cortical or thalamic glutamatergic inputs but appeared to be strictly TANs- and VGLUT3 dependent.

It is now well established that TANs optogenetic stimulation results in monosynaptic glutamate- and ACh-mediated currents in striatal MSN and fast-spiking GABAergic interneurons.[5,42] Our results establish that glutamate/ACh co-transmission from TANs controls not only MSN and fast-spiking GABAergic interneuron but DA terminals as well.

The absence of VGLUT3 resulted in an increase in DA release with a concomitant activation of the pro-reward DRD1 signaling cascade. The augmented activity of DRD1-positive MSNs has been shown to promote reinforcement of behaviors in contrast to DRD2-positive MNs, which induce punishment.[43–46] Furthermore, VGLUT3 null mice demonstrated increased spine density and augmented glutamatergic signaling in the NAc. Interestingly, deletion of VGLUT3 appeared to exclusively affect the DRD1 pro-reward pathway without affecting DRD2-positive MNs. Therefore, the constitutive absence of VGLUT3 appears to produce the same effects as sustained cocaine intake. Excitatory cortical transmission is believed to encode for learning and the unmanageable motivation to seek drugs, whereas DA codes reward prediction and the 'wanting' of a drug.[47–49] Thus, with VGLUT3 and glutamate/ACh co-transmission, TANs use sophisticated machinery to shape accumbal dopaminergic and glutamatergic signals and thereby orchestrate reward seeking. The major findings of the present study are summarized in Figure 6.

Whether observations made in mouse models are relevant to humans remains to be fully established. Humans are not equivalent in their response to addictive drugs, and only a small percentage of susceptible individuals develop cocaine addiction.[50] Genetic variants may have an important role in the susceptibility to drug addiction. Given our observations, we hypothesize that allelic variations in VGLUT3 may contribute to the vulnerability of a subgroup of individuals who demonstrate heavy drug use. It is possible that VGLUT3 mutations may contribute to a presensitized reward circuit in humans as well. In support of this working hypothesis, we report that the frequency of rare variations within the gene-encoding VGLUT3 is higher in individuals with substance use disorders than in controls.

VGLUT3 is discretely distributed throughout the brain and may therefore be a relevant pharmacological target to treat addiction or other striatum-related diseases. A deeper understanding of the contributions of VGLUT3 to reward behaviors could pave the way for developing new therapeutic strategies for drug abuse or to allow the identification of individuals vulnerable to drug-seeking behavior.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

DYS, LJM, EM-G and JG-C performed the behavioral experiments with the help of SD. FM performed the *in vivo* electrophysiology and voltammetry with the help of DYS, LM and AG. DYS and LJM performed the anatomical experiments with the help of SB and EH. DYS, LJM and AB performed the biochemical measurements with the help of NH, PV and JC. MM and SL performed the *in vitro* electrophysiology experiments. The genetic studies were designed by SJ and performed by SJ with the help of FV, AH and FB. CBE, SC, FV and FB recruited subjects with severe addiction and unaffected controls. VFP and MAMP generated the VAChT mutant mice and helped with the manuscript. SEM designed the study and wrote the manuscript with the help of DYS, FM, RM, BG, MM and SJ.

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