Convergent evidence from alcohol-dependent humans and rats for a hyperdopaminergic state in protracted abstinence

Natalie Hirth[a,1], Marcus W. Meinhardt[a,1], Hamid R. Noori[a,1], Humberto Salgado[b], Oswaldo Torres-Ramirez[b], Stefanie Uhrig[a], Laura Broccoli[a], Valentina Vengeliene[a], Martin Roßmanith[a], Stéphanie Perreau-Lenz[a,2], Georg Köhr[a], Wolfgang H. Sommer[a,c], Rainer Spanagel[a,1], and Anita C. Hansson[a,1,3]

[a]Institute for Psychopharmacology at Central Institute for Mental Health, Medical Faculty Mannheim, University of Heidelberg, 68159 Mannheim, Germany; [b]Neurosciences Department, Autonomous University of Yucatán, 97000 Merida, Yucatan, Mexico; and [c]Department of Addictive Behavior and Addiction Medicine at Central Institute for Mental Health, Medical Faculty Mannheim, University of Heidelberg, 68159 Mannheim, Germany

Edited by Tomas G. M. Hokfelt, Karolinska Institutet, Stockholm, Sweden, and approved January 25, 2016 (received for review March 30, 2015)

A major hypothesis in addiction research is that alcohol induces neuroadaptations in the mesolimbic dopamine (DA) system and that these neuroadaptations represent a key neurochemical event in compulsive drug use and relapse. Whether these neuroadaptations lead to a hypo- or hyperdopaminergic state during abstinence is a long-standing, unresolved debate among addiction researchers. The answer is of critical importance for understanding the neurobiological mechanism of addictive behavior. Here we set out to study systematically the neuroadaptive changes in the DA system during the addiction cycle in alcohol-dependent patients and rats. In postmortem brain samples from human alcoholics we found a strong down-regulation of the D1 receptor- and DA transporter (DAT)-binding sites, but D2-like receptor binding was unaffected. To gain insight into the time course of these neuroadaptations, we compared the human data with that from alcohol-dependent rats at several time points during abstinence. We found a dynamic regulation of D1 and DAT during 3 wk of abstinence. After the third week the rat data mirrored our human data. This time point was characterized by elevated extracellular DA levels, lack of synaptic response to D1 stimulation, and augmented motor activity. Further functional evidence is given by a genetic rat model for hyperdopaminergia that resembles a phenocopy of alcohol-dependent rats during protracted abstinence. In summary, we provide a new dynamic model of abstinence-related changes in the striatal DA system; in this model a hyperdopaminergic state during protracted abstinence is associated with vulnerability for relapse.

alcoholism | translational studies | dopamine release | in silico analysis | postmortem brain tissue

About 10% of the total burden of disease in developed countries is caused by alcohol use alone (1). A large proportion of alcohol-related disability results from alcohol addiction. The condition affects more than 12% of the United States population at some point in their lives and is one of the most prevalent psychiatric disorders in Europe (2, 3). The relapsing course of alcoholism is associated with compulsive drinking, loss of control over intake, and emergence of a negative emotional state during abstinence (4). Afflicted individuals go through repeated cycles of alcohol intoxication and withdrawal leading to persistent alterations in brain activity that are hypothesized to drive relapse and compulsive alcohol use even long after detoxification (5).

Seminal studies in experimental animals established that alcohol's rewarding properties are associated with increased dopamine (DA) in regions such as the nucleus accumbens (Acb) (6), whereas withdrawal after chronic alcohol use decreases DA neurotransmission (7). In humans, the binding of a DA receptor ligand, typically one for the D2-like receptor subgroup, i.e., [^{11}C] raclopride, can be monitored by PET. Displacement of the radioligand provides an indirect measure of DA release and has been used to demonstrate alcohol-evoked DA release in the accumbens of healthy social drinkers (8, 9). On the other hand, a blunted response of the DA system and reduced availability of the D2-like receptor was found in alcoholics (10–17). The collective interpretation of these studies postulates a hypodopaminergic state, characterized by low extracellular DA levels and reduced D2-like receptor availability in areas of the mesolimbic system, which may drive relapse behavior in alcoholism (18, 19). However, this interpretation of in vivo receptor availability seen in PET studies is inherently ambiguous, because decreased signal can be caused either by a reduced number of receptors or by increased ligand concentration. Furthermore, unchanged or even increased striatal D2-like binding in alcohol-abstinent patients has been found also (20–22). Naltrexone, one of the few medications approved by US Food and Drug Administration for treatment of relapse, reduces alcohol-induced accumbal DA release (23), and this effect seems argue against the importance of a hypodopaminergic state for increased propensity for relapse. Thus, clarification of the role of DA during abstinence is highly important for the development of novel therapeutic strategies.

Here, we used brain samples from deceased alcoholics and controls to study both transcriptional and binding levels for DA

Significance

A major hypothesis in the addiction field suggests there are deficits in dopamine signaling during abstinence. This hypodopaminergic state is considered a driving mechanism for the relapsing course of the disorder. Experimental support for this view comes mostly from human PET studies that found reduced striatal D2-like receptors in alcoholics. Here we report on surprising findings from postmortem brains of deceased alcoholics and alcohol-dependent rats that show no differences in D2-like receptor binding during withdrawal and prolonged abstinence. Instead we observe a dynamic regulation of D1 receptors, dopamine transporter, dopamine release properties, and phenotypic characteristics that all are in line with a hyperdopaminergic state during protracted abstinence. We propose that both hypo- and hyperdopaminergia are states of vulnerability to relapse.

Author contributions: W.H.S., R.S., and A.C.H. designed research; N.H., M.W.M., H.R.N., H.S., O.T.-R., S.U., L.B., V.V., M.R., S.P.-L., and A.C.H. performed research; N.H., M.W.M., H.R.N., H.S., O.T.-R., S.U., V.V., G.K., W.H.S., and A.C.H. analyzed data; and N.H., M.W.M., H.R.N., S.U., W.H.S., R.S., and A.C.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]N.H., M.W.M., H.R.N., R.S., and A.C.H. contributed equally to this work.

[2]Present address: SRI Biosciences, Division of SRI International, Center for Neuroscience, Menlo Park CA 94025.

[3]To whom correspondence should be addressed. Email: anita.hansson@zi-mannheim.de.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1506012113/-/DCSupplemental.

D1 and D2-like receptors and transporter (DAT). To investigate the time course of neuroadaptations within the DA system during abstinence, we used an established animal model in which dependence is induced by chronic intermittent intoxication with alcohol vapor leading to long-lasting neuronal and behavioral adaptations that persist even in the absence of the drug (24–27). Although this model is based on experimenter-controlled intoxication, as opposed to the largely voluntary drinking seen in humans, it has been valuable in establishing mechanisms underlying the high propensity for relapse in addicted individuals, i.e., a chronic hyperactivity of central stress systems when access to the drug is prevented (25, 26, 28), a phenomenon that Koob and Le Moal (29) term the "dark side" of addiction. In this animal model we performed microdialysis, electrophysiology, and behavioral studies to demonstrate a hyperdopaminergic state—a condition in which extracellular DA is elevated—during protracted abstinence. The hyperdopaminergic state here refers to the basal tonic state of the system and may underlie a diminished response capability for DA release (30).

Results

Postmortem Brain Analysis Suggests a Hyperdopaminergic State in Human Alcoholics. Ten alcoholic and 10 control subjects were included in the study. All alcoholics had a history of daily alcohol intake of more than 80 g/d, and the control cases had an average daily consumption of less than 20 g. Subjects were free of detectable alcohol levels at their time of death (see highlighted core sample set in Table S1). There was no significant difference in age, postmortem interval, or brain pH between the groups (Welch's t test for all variables: $P > 0.1$).

Sections from the ventral striatum (VS, including the Acb) and nucleus caudatus (NC) were used to measure ligand binding for D1 ([^{3}H]-SCH23390) and D2-like ([^{3}H]-raclopride) receptors and for DAT ([^{3}H]-mazindol) by autoradiography. The number of D1-binding sites in both the VS and NC was strongly decreased in alcoholics as compared with controls (VS: 59%, $F_{1,15} = 31.7$, $P < 0.001$; NC: 61%, $F_{1,16} = 104.2$, $P < 0.001$) (Fig. 1*A*). In contrast, no differences were observed for D2-binding sites (VS: $F_{1,16} = 0.005$, $P > 0.5$; NC: $F_{1,15} = 1.3$, $P > 0.5$) (Fig. 1*B*). Furthermore, samples from alcoholics showed a highly significant reduction in DAT-binding sites (VS: 62%, $F_{1,14} = 139.8$, $P < 0.001$; NC: 56%, $F_{1,14} = 65.4$, $P < 0.001$) (Fig. 1*C*).

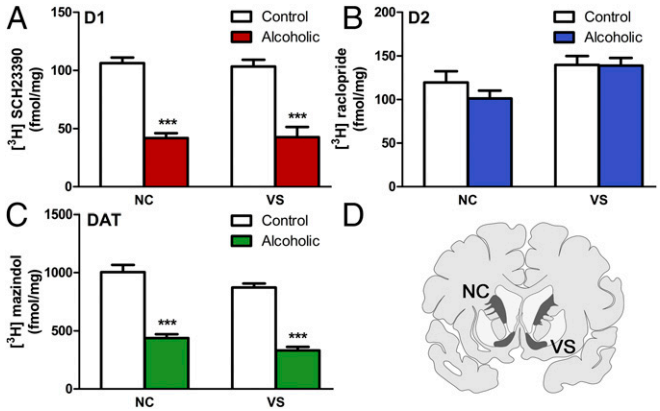

Fig. 1. Expression analysis of the dopaminergic system in postmortem brains of heavy alcoholics suggests a hyperdopaminergic state. (*A*–*C*) Bar graphs showing quantitative analysis of D1 receptors (*A*), D2-like receptors (*B*), and DAT-binding sites (*C*) in postmortem striatal brain sections of heavy alcoholics (red bar in *A*, blue bar in *B*, green bar in *C*) and controls (white bars). D1 and DAT are strongly decreased in striatal brain regions, but D2 is not altered. Data are expressed as means (expressed in femtomoles per milligram) ± SEM, $n = 8$ or 9 per group. (*D*) Schematic illustration showing a coronal section of the human striatal forebrain region including the NC and VS (including the accumbens).

To support the finding of reduced striatal D1 binding further, we studied a separate, larger cohort of subjects including alcoholics either positive or negative for blood alcohol concentration (BAC) (0.183 ± 0.145 g/100 mL or zero in intoxicated and nonintoxicated alcoholics, respectively, $n = 10$ per group) and controls ($n = 30$) (Table S1). As in the first cohort, both striatal regions showed reduced D1 binding in alcoholics with no differences between intoxicated and nonintoxicated subjects (NC: $F_{2,43} = 7.6$, $P = 0.001$; VS: $F_{2,43} = 10.4$, $P = 0.0002$) (Fig. S1). Potentially confounding factors such as tissue pH, postmortem interval, age, and smoking status were included as covariates in the analysis but did not cause any significant effects. In contrast to the protein findings, quantitative real-time PCR (qRT-PCR) to assess mRNA levels for *DRD1* and *DRD2* did not show any differences between the groups (Table S2). *SLC6A3* mRNA encoding the DAT was not determined, because transcripts are located mostly in cell bodies of the nigrostriatal and ventral tegmental area (VTA) neurons.

To provide convergent evidence for this surprising finding, we next performed a systematic search and a meta-analysis of existing literature on DA concentrations and its metabolites during abstinence in alcohol-dependent rats and then examined the dopaminergic system at different time points in an established animal model of alcoholism.

Alcohol-Dependent Rats Mirror the Hyperdopaminergic State Observed in Human Alcoholics. The meta-analysis was based on 16 published studies in rats (a total of 192 rats chronically exposed to ethanol). This analysis revealed an increase in DA release on day 0, followed by a decrease on days 1–3, and an increase again on days 7 and 21 of abstinence (Fig. 2*A*; for detailed information, see *SI Materials and Methods*). Accumbal release of DA metabolites [3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA)] followed a pattern similar to that observed for DA (Fig. S2). In summary, the meta-analysis suggests that dynamic changes in accumbal DA levels occur during abstinence with a hypodopaminergic state during acute withdrawal followed by a hyperdopaminergic state later on.

We then performed a time-course experiment for D1-, D2-like–, and DAT-binding sites during abstinence in alcohol-dependent rats. To be in line with our human studies we focused on the Acb shell (AcbS) and core (AcbC) and the caudate putamen (CPu) (31). Rats exposed to intermittent cycles of alcohol vapor for 7 wk (26, 27) were killed at day 0, 1, 3, 7, or 21 of abstinence as in refs. 24 and 32. DA receptor/transporter-binding sites were quantitatively analyzed (33) and are presented as normalized data respective to control group and time in Fig. 2 *B*–*D*. Raw data for controls are summarized in Table S3, and representative images of autoradiographies are shown in Fig. S3.

AcbS. D1 and DAT varied as a function of time from exposure (two-way ANOVA, treatment × time; D1 main effect: $F_{4,54} = 4.6$, $P < 0.01$; DAT main effect: $F_{4,54} = 4.8$, $P < 0.01$). D1- and DAT-binding sites were strongly regulated at several time points between acute intoxication (day 0) and day 21 of abstinence. On day 0, animals were killed immediately after the last cycle of exposure to ethanol vapor, having positive BACs of 273 ± 52 mg/dL. In comparison to controls, D1 was significantly reduced, by 11%, at this time point but reached control levels 1 d later (day 1). After 3 d of abstinence (day 3), D1 increased (10%, $P = 0.07$), and this effect reached significance after 7 d. After a further 2 wk of abstinence (day 21), D1 was decreased by 14% (Fig. 2*B*). The binding sites of DAT at these time points were regulated differentially. On day 0, DAT was increased by 22%; however, this effect failed to reach significance ($P = 0.07$). On day 1, DAT was significantly decreased by 33% and returned to control levels on day 7. After 21 d of alcohol abstinence, DAT again was significantly reduced by 35% (Fig. 2*B*). For the AcbC, the D1- and DAT-binding sites followed a pattern similar to that in the AcbS (D1 main effect: $F_{4,58} = 7.9$, $P < 0.001$; DAT main effect $F_{4,61} = 6.2$, $P < 0.001$). Post hoc analysis revealed that D1 was decreased

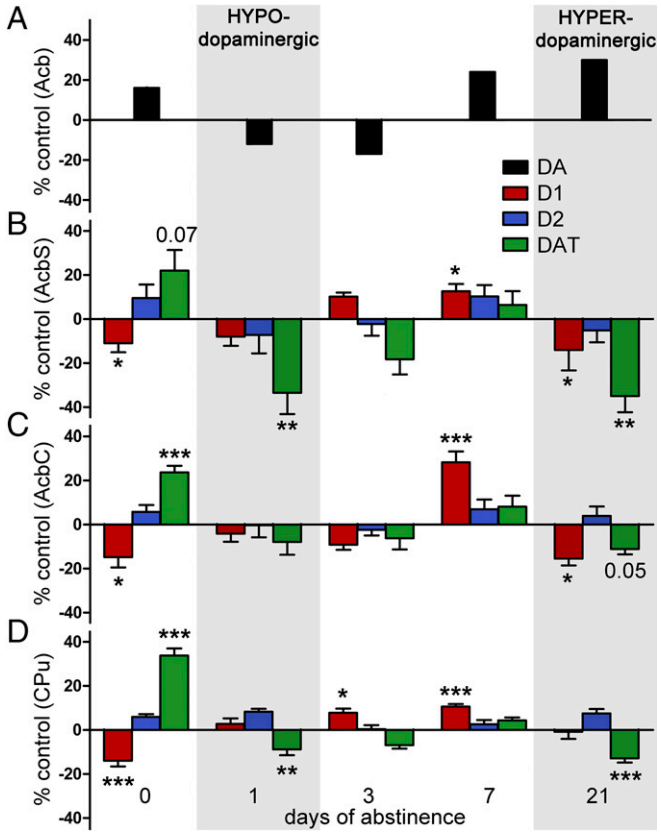

Fig. 2. Analyses of the dopaminergic system in alcohol-dependent rats reveals a hyperdopaminergic state in long-term abstinence. (*A*) The time course of DA release in the Acb was modeled by a meta-analysis of 192 rats derived from 16 animal studies by continuous interpolation of the averages of experimental values with respect to the time of measurement during abstinence. This quantitative analysis suggests a robust pattern of dynamic changes in DA concentrations. During the first 6 d of withdrawal, the DA concentrations decline to 30% of the baseline concentrations (hypodopaminergic state) but then increase again to a hyperdopaminergic state. (*B–D*) Regulation of D1 receptors (red bars), D2-like receptors (blue bars), and DAT-binding sites (green bars) at different days of abstinence in the AcbS (*B*), AcbC (*C*), and CPu (*D*) of alcohol-dependent rats vs. control rats (set as 0% baseline at each time point). Rats were intermittently exposed to ethanol vapor for 7 wk and were killed immediately after the last exposure cycle (on day 0) or after 1, 3, 7, or 21 d of abstinence. D1 and DAT are dynamically increased and decreased at different times during abstinence, but D2-like binding levels remain unaffected. Statistical analysis was performed by two-way ANOVA followed by Fisher's post hoc test. Data are expressed as percent of controls ± SEM, $n = 4–8$ per group. For expression levels in controls at each time point, see Table S3. The shaded areas in the figure indicate a hypo- or a hyperdopaminergic state during abstinence.

on day 0 by 15% and increased on day 7 by 30% relative to baseline. On day 21 D1 again was decreased by 15%. DAT binding was significantly increased by 24% on day 0, returned to control levels on days 1–7, and then tended to decrease (11%, $P = 0.05$) on day 21 of abstinence (Fig. 2*C*). In the CPu and in the dorsal striatum D1 and DAT binding paralleled the temporal pattern in the AcbS with the exception that D1 binding was not down-regulated at day 21 (D1 main effect: $F_{4,58} = 10.8$, $P < 0.001$; DAT main effect: $F_{4,55} = 25.2$, $P < 0.001$). With chronic alcohol use, D1 binding decreased by 14% on day 0 and increased significantly on day 3 (8%) and on day 7 (11%) of abstinence. DAT binding was strongly increased on day 0 (34%) and decreased on day 1 (9%) and day 21 (13%) (Fig. 2*D*). D2-like binding sites were not changed at any time point in any region (Fig. 2 *B–D*).

Together these data support our notion derived from the meta-analysis that abstinence is characterized by changes in the dynamics of the components of the striatal DA system, with the profile of the intoxicated state being clearly separable from that of acute withdrawal and that of protracted abstinence. The human postmortem findings appear most closely related to those seen with 21-day protracted abstinence in rodents.

Characterization of the Hyperdopaminergic State in Protracted Abstinence. Extracellular DA levels in the AcbS after 21 d of abstinence were analyzed via in vivo microdialysis. The basal dialysate DA concentrations were significantly elevated in alcohol-dependent rats ($F_{1,26} = 2.7$, $P < 0.05$) (Fig. 3*A*). When different doses of ethanol (0, 1, or 2 g/kg, i.p.) were applied, control rats showed an ethanol-induced increase in extracellular DA (on average a 49 ± 33% increase from baseline) after the injection of 2 g/kg ethanol (i.p.), whereas alcohol-dependent rats displayed a blunted response to ethanol treatment (on average a 9 ± 49% increase from baseline) (Fig. 3*B*). Repeated-measurement ANOVA revealed a significant effect of alcohol injections ($F_{1,14} = 7.1$, $P < 0.05$), a trend for treatment (alcohol-dependent vs. control, $F_{1,14} = 3.8$, $P = 0.07$), but no interaction effect ($F_{1,14} = 0.8$, $P > 0.5$). Further support for increased striatal DA release came from in situ hybridization for *TH* (tyrosine hydroxylase) mRNA showing an increase in the substantia nigra pars compacta (SNc) by 31% in 3-wk-abstinent rats ($F_{1,10} = 18.6$, $P < 0.01$) but no changes in the VTA (Fig. S4).

We also assessed basal motor activity following 21 d of abstinence. In the first 20 min in an open-field exposure, i.e., under conditions of novelty, alcohol-dependent and control rats showed no difference in total distance traveled (Fig. S4). However, after habituation, alcohol-dependent rats had significantly enhanced locomotor activity compared with controls ($F_{1,14} = 5.6$, $P < 0.05$) (Fig. 3*C* and Fig. S4). Also the alcohol-dependent rats displayed higher activity in the home cage ($F_{1,28} = 8.3$, $P < 0.01$) (Fig. 3*D*). This difference resulted exclusively from higher activity during the active phase of the circadian cycle (Fig. S4). Cue-induced reinstatement of alcohol seeking, an established model of relapse (34), was significantly higher in 3-wk-abstinent alcohol-dependent rats than in controls ($t_{54} = 3.8$; $P < 0.001$) (Fig. 3*E*).

Finally, we validated the down-regulation of D1 in protracted abstinence at the synaptic level by examining glutamatergic inputs to medium spiny neurons (MSNs) of the AcbS in brain slices during electrical stimulation of the AcbS (Fig. 3 *F–H*). Ethanol perfusion (25 mM; 25 min) increased excitatory postsynaptic currents (EPSCs) similarly in both groups of rats. Subsequent perfusion of the D1 agonist SKF81297 (5 μM; 20 min) in the presence of ethanol further enhanced the EPSCs in control rats but, importantly, not in alcohol-dependent rats (control: $F_{2,20} = 2115$, $P < 0.001$ vs. baseline and vs. ethanol; alcohol-dependent: $F_{2,20} = 270$, $P < 0.001$ vs. baseline and $P > 0.05$ vs. ethanol), as also was apparent from the differences in EPSCs in alcohol-dependent vs. control rats perfused with ethanol plus SKF81297 ($P < 0.05$).

In summary, these data suggest that 21-d-abstinent alcohol-dependent rats have increased accumbal DA levels but blunted DA responses to alcohol at both presynaptic (DA release) and postsynaptic (D1 agonist) sites. On the behavioral level these plasticity changes are associated with hyperactivity and an increased propensity for relapse in protracted abstinence.

A Transgenic Rat Model for Hyperdopaminergia Is Similar to Rats in Protracted Abstinence. For further validation of the behavioral consequences of a hyperdopaminergic state, we used a genetic rat model in which a single point mutation in the *Slc6a3* gene was introduced. This functional mutation led to an amino acid exchange (N157K) and subsequently to a loss of function of DAT. This loss of function was confirmed by strongly reduced [^{3}H]-mazindol binding to DAT in the Acb in DAT N157K mutants as compared with that in WT rats ($t_{17} = 10.8$, $P < 0.001$) (Fig. 4*A*). [^{3}H]-SCH23390 binding demonstrated that the mutation also caused a significant reduction in D1 binding ($t_{16} = 16.0$, $P < 0.001$)

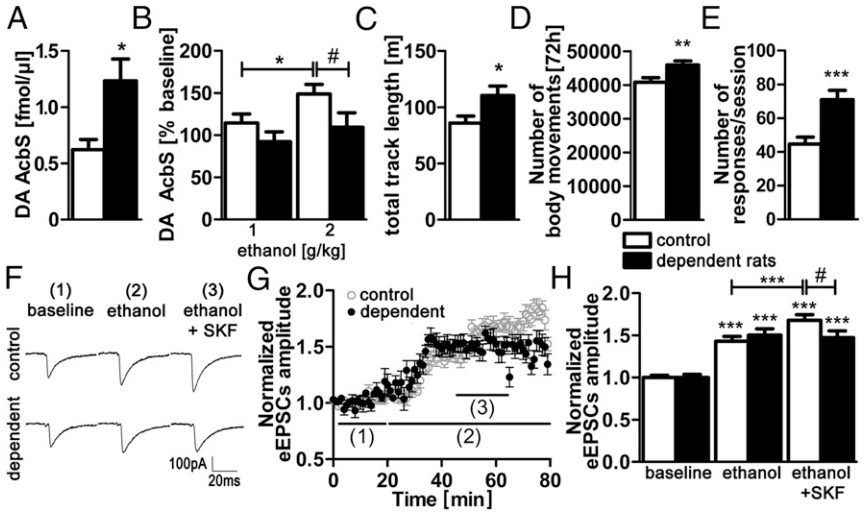

Fig. 3. The hyperdopaminergic state in 3-wk alcohol-abstinent rats. DA microdialysis displays increased DA levels and a blunted response to ethanol treatment in alcohol-dependent rats. (*A*) Basal extracellular DA levels within the AcbS are markedly increased in alcohol-dependent rats ($n = 15$ per group). (*B*) AcbS DA levels after the application of consecutive doses of ethanol (1 or 2 g/kg, i.p.). Control animals show an increase in extracellular DA levels after ethanol (2 g/kg, i.p.), whereas alcohol-dependent rats show a blunted response to the treatment ($n = 8$ per group). (*C* and *D*) Hyperlocomotion in 3-wk-abstinent rats was detected by records of locomotor activity in the open field (*C*) and home cage (*D*). The bar graph in *C* represents the total track length measured over a 60-min interval in the open-field setting. The graph in *D* represents the number of body movements per hour during a 72-h period detected using a home cage e-motion system. For the respective time courses, see Fig. S4. (*E*) Enhanced ethanol cue-induced reinstatement in abstinent rats compared with controls. The graph shows the mean number of responses after the presentation of stimuli previously paired with ethanol. (*F*) Representative EPSCs recorded at −80 mV in MSNs were evoked by electrical stimulation in the AcbS before (baseline) and during perfusion with 25 mM ethanol or 25 mM ethanol plus 5 µM SKF81297. Current traces represent the average of 10 sweeps. (*G*) Time courses of the effects shown in *F* for normalized EPSCs. (*H*) Summary of the effects on EPSCs (control, $n = 12$; alcohol-dependent, $n = 7$). Data are expressed as means ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. baseline or vs. ethanol; $^{\#}P < 0.05$ alcohol-dependent vs. control.

(Fig. 4*B*), which was likely caused by compensatory decrease of this receptor resulting from elevated extracellular DA concentration ($t_{10} = 24.1$, $P < 0.001$) (Fig. 4*C*). The behavioral consequences of this hyperdopaminergic state in DAT N157K mutants are manifested by enhanced locomotor activity in the open-field test ($t_{32} = 8.3$, $P < 0.001$) (Fig. 4*D*) and enhanced alcohol drinking as compared with WT animals ($t_{32} = 2.5$, $P < 0.05$) (Fig. 4*D*). This genetic model thus shares several features with alcohol-dependent rats during protracted abstinence.

Discussion

In this translational study we provide evidence for the development of a hyperdopaminergic state during protracted alcohol abstinence. We found striatal D1 receptor and DAT binding to be strongly decreased in postmortem brain tissue from alcoholics. These results were supported by a dynamic regulation of D1 and DAT at different times during abstinence in an animal model of alcohol dependence, with pronounced reductions of D1 and DAT during protracted abstinence. Functionally, the reduction in D1 receptors resulted in a lack of modulation of glutamatergic transmission upon D1 stimulation. Further, we found elevated basal extracellular DA levels in the AcbS associated with a blunted DA response to alcohol challenges. On the behavioral level we saw hyperactivity and enhanced alcohol-seeking during protracted abstinence. A transgenic rat model for hyperdopaminergia demonstrated a decrease in accumbal DAT and D1, increased extracellular DA levels and locomotor activity, and increased alcohol intake similar to that seen in 3-wk-abstinent rats. Together with a meta-analysis of rodent studies, our data provide convergent evidence that a hyperdopaminergic state occurs during protracted abstinence. According to the literature and the time-course studies we present here, we propose that dynamical changes take place in the mesolimbic DA system during withdrawal and protracted abstinence, resulting in a hypodopaminergic state that characterizes acute withdrawal (19, 35) and a hyperdopaminergic state that characterizes protracted abstinence (Fig. 2). These dynamic alterations of the mesolimbic DA system in the course of the addiction cycle have implications for our understanding of the mechanisms underlying alcoholism, the interpretation of PET results in alcoholic patients, and the development of effective therapeutic strategies.

A major hypothesis suggests deficits in DA signaling, leading to a hypodopaminergic state, as a driving mechanism for the relapsing course of the disorder (18, 19). Experimental support for this view comes from animal work (35) and human PET studies that found reduced availability of striatal D2-like receptors compared with controls (10–16). Because these PET studies do not provide a coherent picture (20–22), we used saturated receptor autoradiography techniques to measure the number of DA receptor and DAT-binding sites in postmortem brain tissues of alcoholics and controls. Surprisingly, we found a highly significant reduction in both D1- and DAT-binding sites in striatal tissue but no change in D2-like binding; these results imply a hyper- rather than a hypodopaminergic state, especially in protracted abstinence. These findings show that PET studies should be interpreted with caution, because the commonly used low-affinity radiotracer can be displaced by competing levels of endogenous DA even though the number of binding sites remains unchanged. In fact, such competition has been observed for [^{11}C]raclopride upon pharmacological manipulation of DA levels (36). Thus, psychostimulant-increased DA levels lead to a reduction in striatal D2-binding potential. In line with our own data from human postmortem brain tissue, a recent study with the high-affinity D2 ligand [^{18}F]fallypride, which is less sensitive to endogenous DA levels (37), found unaltered striatal binding potential in alcoholics during protracted abstinence as compared

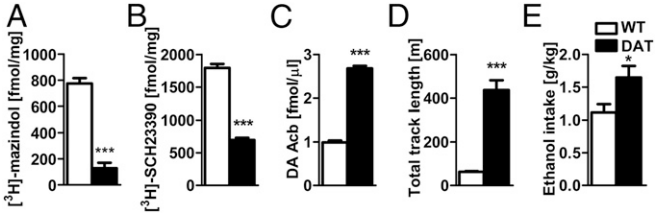

Fig. 4. A hyperdopaminergic state in DAT N157K mutant rats is associated with hyperlocomotion and increased alcohol consumption. (*A* and *B*) Quantitative analysis of DAT ([^{3}H]-mazindol)-binding sites (*A*) and D1 ([^{3}H]-SCH23390)-binding sites (*B*) (expressed in femtomoles per milligram) in the Acb of WT and DAT N157K mutant rats. (*C*) Basal extracellular DA levels (expressed in femtomoles per microliter) in the Acb of WT and DAT N157K mutant rats. (*D*) Total track length (in meters) during 60-min testing in the open field in WT and DAT N157K mutant rats. (*E*) Intake of total ethanol (expressed in grams per kilogram body weight per day) in WT and DAT N157K mutant rats during 6 wk of continuous concurrent access to water and 5%, 10%, and 20% ethanol solutions. Data are expressed as means ± SEM; $^{*}P < 0.05$, $^{***}P < 0.001$ vs. WT.

with healthy controls (22). In vivo data for D1 are not available for alcoholics. Similar to our data, Tupala et al. (38) found reduced striatal D1 binding in postmortem brain samples from alcoholics. In apparent contrast to our data, however, they found decreased D2 in striatal regions. This difference could be caused by various factors. We noticed that most of the subjects in their sample had high alcohol or medication levels at the time of death. Further, human in vivo studies found increased DA synthesis rates in alcoholics as assessed by the uptake of [^{18}F]DOPA, an immediate precursor of DA synthesis, as well as reduced DAT availability (39). Reduction of DAT is in line with our observations and those of others (40) in postmortem samples.

Animal studies performed during withdrawal consistently found reward deficits associated with the suppression of accumbal DA release (4, 41, 42). Remarkably, although protracted abstinence is the most relevant clinical condition in alcohol and other substance use disorders (4, 5, 43), it has not been the focus of preclinical DA research. To investigate the neuroadaptations of the DA system in more detail, we first performed a meta-analysis of the existing rodent literature on DA concentrations and its metabolites in the AcbS at different time points during abstinence. We found evidence for alcohol-induced DA elevation on day 0 followed by a decline during acute withdrawal, but around the sixth day of abstinence an increase in DA is found, which is most augmented during protracted abstinence. Although in this quantitative evaluation of the literature the methods used to induce dependence in the rats vary among studies, the general pattern of dynamic changes appears to be robust and seems to follow an oscillatory-like mode over time.

To confirm such oscillatory-like regulation of DA at the membrane level, we analyzed binding sites of D1, D2-like receptors, and DAT in three striatal regions of alcohol-dependent rats at various time points during abstinence according to our previous studies (24, 32). A similar temporal pattern of regulation was found for D1 and DAT (Fig. 2). During protracted abstinence (day 21), both D1- and DAT-binding sites are decreased. D2-like receptors were not changed at any time during abstinence, a finding that is in agreement with our human postmortem brain data. Functional evidence is given by electrophysiological data showing a blunted modulation of glutamatergic transmission upon D1 activation in the presence of ethanol in the MSNs of the AcbS and thus confirming a strong reduction of D1 in these neurons in response to elevated DA during protracted abstinence.

Our microdialysis studies in the AcbS of 21-d-abstinent rats found elevated extracellular DA levels. In addition, we found a blunted accumbal DA response to acute alcohol in abstinent rats, a finding that is in line with human PET data obtained after psychostimulant challenge in alcoholics (15, 16). This lack of responsiveness could be interpreted in two ways, reflecting state-specific response dynamics dependent on low or high extracellular DA levels or a relative DA deficit caused by high chronic demands that have exhausted compensatory mechanisms. The latter interpretation is supported by a recent metabolomics study showing deficits in central energy metabolism in the AcbS of alcohol-abstinent rats (44). In fact, it has long been proposed that blunted DA responses represent an endophenotype for substance use disorders (17, 18), and thus the blunted DA response to alcohol may represent a potential factor for enhanced alcohol-seeking behavior. It is worth noting that these reciprocal effects of increased steady-state activity and blunted challenge-induced responses occur in other biological systems as well. For example, some alcoholic populations show enhanced basal production of cortisol but have a blunted response to acute intervening stress, leading to impaired or inappropriate stress responses (45).

Mechanistically, the reduction we observed in D1 and DAT densities in alcoholics and alcohol-dependent rats after 21 d of abstinence can be explained in several ways. Chronic stimulation of D1 by repeated intoxication may lead to internalization and degradation of D1. Such a mechanism has been demonstrated after repeated administration of DA agonists and produces a lack of sensitivity to subsequent administration of DA agonists on behavioral, biochemical, and electrophysiological levels (46, 47). Also, DAT and D1 expression seem intrinsically related: Postsynaptic D1 is reduced in both DAT N157K mutant rats and DAT-KO mice (48).

On the behavioral level, alcohol-dependent rats in protracted abstinence show enhanced motor activity, enhanced alcohol consumption (24, 25, 27, 49, 50), and augmented reinstatement of cue-induced alcohol seeking, a finding that has been replicated consistently in several studies (51–53). A similar phenotype is observed in DAT N157K mutant rats. These mutant rats exhibit hyperdopaminergia, resembling on a molecular level alcohol-dependent rats in protracted abstinence (i.e., strongly reduced DAT and D1), and also display enhanced motor activity and augmented alcohol consumption. Thus, on both the molecular and the behavioral level, DAT-mutant rats represent a phenocopy of alcohol-dependent rats during protracted abstinence.

Taken together, our studies provide convergent evidence for a hyperdopaminergic state of the reward system during protracted abstinence. This hyperdopaminergic state is associated with increased motor activity and augmented alcohol seeking and use. We suggest that an enhanced risk for relapse exists both during acute withdrawal and long into protracted abstinence, but, according to our data, this vulnerability can be associated with either hypo- or hyperdopaminergia. Although the link between the early withdrawal phenomena and subsequent dysregulations remains unclear, many biological functions depend on homeostatic regulation, so that either a deficit or an excess in regulation results in worsening performance. Such a model was proposed for the role of DA in cognitive functioning in ref. 54. In this sense, an increased risk for relapse in a hypodopaminergic state could be caused by reward deficiency, whereas hyperdopaminergia might cause hyperactivity, which often is associated with poor impulse control.

In conclusion, our study extends the current neurobiological understanding of alcohol dependence, proposing that a hyperdopaminergic state may exist during protracted abstinence, at least in some alcoholics. We show dynamic changes within the mesolimbic DA system during alcohol exposure, withdrawal, and prolonged abstinence. Enhanced dopaminergic activity during alcohol exposure is followed by a hypodopaminergic system that characterizes the first few days of acute withdrawal; subsequently, counter adaptive changes that involve D1-, DAT-, and DA-releasing properties ensue, leading to a hyperdopaminergic state during protracted abstinence. Clinical studies are now warranted to define whether this hyperdopaminergic state is a marker for vulnerability to craving and relapse and whether it provides a window for specific intervention.

Materials and Methods

Human postmortem brain samples of males of European ancestry were used for expression analysis. Dependence was induced in male Wistar rats by cyclic intermittent ethanol vapor exposure, and the animals were used for expression analysis, microdialysis, electrophysiology, and behavioral tests. A meta-analysis was performed for accumbal DA concentration during abstinence. DAT N157K mutant rats and all experimental procedures are described in detail in *SI Materials and Methods*. Human postmortem brain experiments were approved by the institutional review board (IRB study no. 2009-238-MA licensed to the Institute for Psychopharmacology, Central Institute for Mental Health, Mannheim, Germany), and all animal experiments were approved by the local animal care committee (Regierungspräsidium Karlsruhe, Germany, license numbers: 35-9185.81/G-183/09 and 35-9185.81/G-126/13).

ACKNOWLEDGMENTS. We thank the Sydney Brain Bank, and especially C. Harper, D. Sheedy, and J. J. Kril, for providing brain samples; O. Stählin for support in statistical analysis; and E. Röbel, F. Matthäus, and G. Plotzky for technical support. This work was supported by the Bundesministerium für Bildung und Forschung [BMBF; Grants 01EB0110; NGFN Plus, FKZ: 01GS08152; e:Med program, FKZ: 01ZX1311A (55), ERA-Net TRANSALC, FKZ 01EW1112], the Ministerium für Wissenschaft, Forschung und Kunst in Baden-Württemberg, Deutsche Forschungsgemeinschaft (DFG) Grant HA6102/1-1, DFG Center Grant SFB1134, and the European Union's Horizon 2020 Program 668863-SyBil-AA.

1. Rehm J, et al. (2009) Global burden of disease and injury and economic cost attributable to alcohol use and alcohol-use disorders. *Lancet* 373(9682):2223–2233.
2. Wittchen HU, et al. (2011) The size and burden of mental disorders and other disorders of the brain in Europe 2010. *Eur Neuropsychopharmacol* 21(9):655–679.
3. Hasin DS, Stinson FS, Ogburn E, Grant BF (2007) Prevalence, correlates, disability, and comorbidity of DSM-IV alcohol abuse and dependence in the United States: Results from the National Epidemiologic Survey on Alcohol and Related Conditions. *Arch Gen Psychiatry* 64(7):830–842.
4. Koob GF (2013) Theoretical frameworks and mechanistic aspects of alcohol addiction: Alcohol addiction as a reward deficit disorder. *Curr Top Behav Neurosci* 13:3–30.
5. Spanagel R (2009) Alcoholism: A systems approach from molecular physiology to addictive behavior. *Physiol Rev* 89(2):649–705.
6. Di Chiara G, Imperato A (1988) Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc Natl Acad Sci USA* 85(14):5274–5278.
7. Diana M, Pistis M, Carboni S, Gessa GL, Rossetti ZL (1993) Profound decrement of mesolimbic dopaminergic neuronal activity during ethanol withdrawal syndrome in rats: Electrophysiological and biochemical evidence. *Proc Natl Acad Sci USA* 90(17): 7966–7969.
8. Ramchandani VA, et al. (2011) A genetic determinant of the striatal dopamine response to alcohol in men. *Mol Psychiatry* 16(8):809–817.
9. Boileau I, et al. (2003) Alcohol promotes dopamine release in the human nucleus accumbens. *Synapse* 49(4):226–231.
10. Hietala J, et al. (1994) Striatal D2 dopamine receptor binding characteristics in vivo in patients with alcohol dependence. *Psychopharmacology (Berl)* 116(3):285–290.
11. Volkow ND, et al. (1996) Decreases in dopamine receptors but not in dopamine transporters in alcoholics. *Alcohol Clin Exp Res* 20(9):1594–1598.
12. Heinz A, et al. (2004) Correlation between dopamine D(2) receptors in the ventral striatum and central processing of alcohol cues and craving. *Am J Psychiatry* 161(10): 1783–1789.
13. Heinz A, et al. (2005) Correlation of alcohol craving with striatal dopamine synthesis capacity and D2/3 receptor availability: A combined [18F]DOPA and [18F]DMFP PET study in detoxified alcoholic patients. *Am J Psychiatry* 162(8):1515–1520.
14. Volkow ND, et al. (2002) Effects of alcohol detoxification on dopamine D2 receptors in alcoholics: A preliminary study. *Psychiatry Res* 116(3):163–172.
15. Volkow ND, et al. (2007) Profound decreases in dopamine release in striatum in detoxified alcoholics: Possible orbitofrontal involvement. *J Neurosci* 27(46): 12700–12706.
16. Martinez D, et al. (2005) Alcohol dependence is associated with blunted dopamine transmission in the ventral striatum. *Biol Psychiatry* 58(10):779–786.
17. Volkow ND, Wang GJ, Fowler JS, Tomasi D, Telang F (2011) Addiction: Beyond dopamine reward circuitry. *Proc Natl Acad Sci USA* 108(37):15037–15042.
18. Koob GF, Volkow ND (2010) Neurocircuitry of addiction. *Neuropsychopharmacology* 35(1):217–238.
19. Diana M (2011) The dopamine hypothesis of drug addiction and its potential therapeutic value. *Front Psychiatry* 2:64.
20. Repo E, et al. (1999) Dopamine transporter and D2-receptor density in late-onset alcoholism. *Psychopharmacology (Berl)* 147(3):314–318.
21. Guardia J, et al. (2000) Striatal dopaminergic D(2) receptor density measured by [(123)I]iodobenzamide SPECT in the prediction of treatment outcome of alcohol-dependent patients. *Am J Psychiatry* 157(1):127–129.
22. Rominger A, et al. (2012) [18F]Fallypride PET measurement of striatal and extrastriatal dopamine D 2/3 receptor availability in recently abstinent alcoholics. *Addict Biol* 17(2): 490–503.
23. Gonzales RA, Weiss F (1998) Suppression of ethanol-reinforced behavior by naltrexone is associated with attenuation of the ethanol-induced increase in dialysate dopamine levels in the nucleus accumbens. *J Neurosci* 18(24):10663–10671.
24. Hansson AC, et al. (2010) Long-term suppression of forebrain neurogenesis and loss of neuronal progenitor cells following prolonged alcohol dependence in rats. *Int J Neuropsychopharmacol* 13(5):583–593.
25. Sommer WH, et al. (2008) Upregulation of voluntary alcohol intake, behavioral sensitivity to stress, and amygdala crhr1 expression following a history of dependence. *Biol Psychiatry* 63(2):139–145.
26. Meinhardt MW, Sommer WH (2015) Postdependent state in rats as a model for medication development in alcoholism. *Addict Biol* 20(1):1–21.
27. Rimondini R, Arlinde C, Sommer W, Heilig M (2002) Long-lasting increase in voluntary ethanol consumption and transcriptional regulation in the rat brain after intermittent exposure to alcohol. *FASEB J* 16(1):27–35.
28. Vendruscolo LF, et al. (2012) Corticosteroid-dependent plasticity mediates compulsive alcohol drinking in rats. *J Neurosci* 32(22):7563–7571.
29. Koob GF, Le Moal M (2005) Plasticity of reward neurocircuitry and the 'dark side' of drug addiction. *Nat Neurosci* 8(11):1442–1444.
30. Goto Y, Otani S, Grace AA (2007) The Yin and Yang of dopamine release: A new perspective. *Neuropharmacology* 53(5):583–587.
31. Noori HR, Spanagel R, Hansson AC (2012) Neurocircuitry for modeling drug effects. *Addict Biol* 17(5):827–864.
32. Hermann D, et al. (2012) Translational magnetic resonance spectroscopy reveals excessive central glutamate levels during alcohol withdrawal in humans and rats. *Biol Psychiatry* 71(11):1015–1021.
33. Sommer WH, Costa RM, Hansson AC (2014) Dopamine systems adaptation during acquisition and consolidation of a skill. *Front Integr Nuerosci* 8:87.
34. Epstein DH, Preston KL, Stewart J, Shaham Y (2006) Toward a model of drug relapse: An assessment of the validity of the reinstatement procedure. *Psychopharmacology (Berl)* 189(1):1–16.
35. Spiga S, et al. (2014) Hampered long-term depression and thin spine loss in the nucleus accumbens of ethanol-dependent rats. *Proc Natl Acad Sci USA* 111(35): E3745–E3754.
36. Laruelle M, et al. (1997) Microdialysis and SPECT measurements of amphetamine-induced dopamine release in nonhuman primates. *Synapse* 25(1):1–14.
37. Cropley VL, et al. (2008) Small effect of dopamine release and no effect of dopamine depletion on [18F]fallypride binding in healthy humans. *Synapse* 62(6):399–408.
38. Tupala E, Tiihonen J (2005) Striatal dopamine D1 receptors in type 1 and 2 alcoholics measured with human whole hemisphere autoradiography. *Brain Res* 1031(1):20–29.
39. Tiihonen J, et al. (1998) Striatal presynaptic dopamine function in type 1 alcoholics measured with positron emission tomography. *Mol Psychiatry* 3(2):156–161.
40. Bergström KA, Tupala E, Tiihonen J (2001) Dopamine transporter in vitro binding and in vivo imaging in the brain. *Pharmacol Toxicol* 88(6):287–293.
41. Weiss F, et al. (1996) Ethanol self-administration restores withdrawal-associated deficiencies in accumbal dopamine and 5-hydroxytryptamine release in dependent rats. *J Neurosci* 16(10):3474–3485.
42. Schulteis G, Markou A, Cole M, Koob GF (1995) Decreased brain reward produced by ethanol withdrawal. *Proc Natl Acad Sci USA* 92(13):5880–5884.
43. Heilig M, Egli M, Crabbe JC, Becker HC (2010) Acute withdrawal, protracted abstinence and negative affect in alcoholism: Are they linked? *Addict Biol* 15(2):169–184.
44. Meinhardt MW, et al. (2015) The neurometabolic fingerprint of excessive alcohol drinking. *Neuropsychopharmacology* 40(5):1259–1268.
45. Wand GS, Dobs AS (1991) Alterations in the hypothalamic-pituitary-adrenal axis in actively drinking alcoholics. *J Clin Endocrinol Metab* 72(6):1290–1295.
46. Dumartin B, Caillé I, Gonon F, Bloch B (1998) Internalization of D1 dopamine receptor in striatal neurons in vivo as evidence of activation by dopamine agonists. *J Neurosci* 18(5):1650–1661.
47. Cooper JR, Bloom FE, Roth RH (2003) *The Biochemical Basis of Neuropharmacology* (Oxford Univ Press, Oxford, UK), 8th Ed.
48. Dumartin B, et al. (2000) Dopamine tone regulates D1 receptor trafficking and delivery in striatal neurons in dopamine transporter-deficient mice. *Proc Natl Acad Sci USA* 97(4):1879–1884.
49. Hansson AC, Rimondini R, Neznanova O, Sommer WH, Heilig M (2008) Neuroplasticity in brain reward circuitry following a history of ethanol dependence. *Eur J Neurosci* 27(8):1912–1922.
50. Björk K, Hansson AC, Sommer WH (2010) Genetic variation and brain gene expression in rodent models of alcoholism implications for medication development. *Int Rev Neurobiol* 91:129–171.
51. Meinhardt MW, et al. (2013) Rescue of infralimbic mGluR2 deficit restores control over drug-seeking behavior in alcohol dependence. *J Neurosci* 33(7):2794–2806.
52. Vengeliene V, et al. (2015) The Calpain Inhibitor A-705253 Attenuates Alcohol-Seeking and Relapse with Low Side-Effect Profile. *Neuropsychopharmacology*, 10.1038/npp.2015.225.
53. Ciccocioppo R, Lin D, Martin-Fardon R, Weiss F (2003) Reinstatement of ethanol-seeking behavior by drug cues following single versus multiple ethanol intoxication in the rat: Effects of naltrexone. *Psychopharmacology (Berl)* 168(1-2):208–215.
54. Mattay VS, et al. (2003) Catechol O-methyltransferase val158-met genotype and individual variation in the brain response to amphetamine. *Proc Natl Acad Sci USA* 100(10):6186–6191.
55. Spanagel R, et al.; Addiction GWAS Resource Group (2013) A systems medicine research approach for studying alcohol addiction. *Addict Biol* 18(6):883–896.
56. Salgado H, Köhr G, Treviño M (2012) Noradrenergic 'tone' determines dichotomous control of cortical spike-timing-dependent plasticity. *Sci Rep* 2(417):1–7.
57. Brand I, Fliegel S, Spanagel R, Noori HR (2013) Global ethanol-induced enhancements of monoaminergic neurotransmission: a meta-analysis study. *Alcohol Clin Exp Res* 37: 2048–2057.
58. Noori HR, Helinski S, Spanagel R (2014) Cluster and meta-analyses on factors influencing stress-induced alcohol drinking and relapse in rodents. *Addict Biol* 19:225–232.
59. Spanagel R, Noori HR, Heilig M (2014) Stress and alcohol interactions: animal studies and clinical significance. *Trends Neurosci* 37:219–227.
60. Noori HR, Fliegel S, Brand I, Spanagel R (2012) The impact of acetylcholinesterase inhibitors on the extracellular acetylcholine concentrations in the adult rat brain: a meta-analysis. *Synapse* 66:839–901.
61. Hall H, Wedel I, Halldin C, Kopp J, Farde L (1990) Comparison of the in vitro receptor binding properties of N-[3H]methylspiperone and [3H]raclopride to rat and human brain membranes. *J Neurochem* 55(6):2048–2057.
62. Hall H, et al. (1994) Distribution of D1- and D2-dopamine receptors, and dopamine and its metabolites in the human brain. *Neuropsychopharmacology* 11(4):245–256.
63. Schulz DW, Stanford EJ, Wyrick SW, Mailman RB (1985) Binding of [3H]SCH23390 in rat brain: Regional distribution and effects of assay conditions and GTP suggest interactions at a D1-like dopamine receptor. *J Neurochem* 45(5):1601–1611.
64. Javitch JA, Blaustein RO, Snyder SH (1984) [3H]mazindol binding associated with neuronal dopamine and norepinephrine uptake sites. *Mol Pharmacol* 26(1):35–44.
65. Donnan GA, et al. (1991) Distribution of catecholamine uptake sites in human brain as determined by quantitative [3H] mazindol autoradiography. *J Comp Neurol* 304(3): 419–434.