Title: Hypodopaminergic state of the nigrostriatal pathway drives compulsive alcohol use

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Abstract: The neurobiological mechanisms underlying compulsive alcohol use, a cardinal feature of alcohol use disorder, remain elusive even though they have often been suggested to involve dopamine (DA). Here, we found that rats expressing compulsive alcohol-related behavior, operationalized as punishment-resistant self-administration, showed a decrease in DA levels restricted to the dorsolateral territories of the striatum, the main output structure of the nigrostriatal DA pathway. We then causally demonstrated that a chemogenetic-induced selective hypodopaminergia of this pathway results in compulsive alcohol self-administration in rats otherwise resilient, accompanied by the emergence of alcohol withdrawal-like motivational impairments. These results demonstrate a major implication of tonic nigrostriatal hypodopaminergic state in alcohol addiction and provide new insights into our understanding of the neurobiological mechanisms underlying compulsive alcohol use.

One-Sentence Summary:
Tonic dopamine deficiencies within nigrostriatal pathway drives compulsive alcohol use

More than 300 million people suffer from alcohol use disorder (AUD) worldwide (*1*) a pathology that encompasses a broad spectrum of health, social and economic problems with various degrees of severity (*2*). The most severe form is frequently referred as alcohol addiction, a chronic relapsing disease occurring only in a subset of vulnerable users (*3*). It is notably characterized by compulsive alcohol seeking and drinking, that is the continuation of using alcohol despite having significant negative consequences, and the presence of a negative motivational and affective state in absence of alcohol (*2*, *4*). While neuroscientists have identified a plethora of actors in AUD, treatments are limited mainly due to the poor understanding of the psychobiological mechanisms underlying the shift from controlled to compulsive alcohol seeking and taking (*3*). At the neural systems level, dopamine (DA) is considered as a prominent actor in the pathophysiology of addiction mainly through its role in incentive motivation and reinforcement (*4*). However, forty years of research have hitherto failed to provide a clear idea of its exact contribution (*5*). While alcohol consumption transiently increases DA levels in the ventral striatum (*6*), the main output of the mesolimbic system, extended consumption instead leads to an overall and prolonged decrease of DA levels upon abstinence in this structure (*4*). Over the past decade, tonic hypodopaminergic states of the mesolimbic pathway have been causally linked to the emergence of both acute drug withdrawal symptoms and excessive alcohol seeking and taking (*7*, *8*). However, implication of these DA hypofunctions in protracted abstinence and in the compulsive dimension of AUD remains controversial (*9*, *10*)

Beyond the mesolimbic DA pathway, a growing body of research had recently demonstrated the strong implication in motivated and affective behaviors of the neighboring nigrostriatal DA pathway, originally restricted to motor functions (*11*, *12*). Interestingly, clinical studies in abstinent individuals with AUD not only have shown an altered DA function in the ventral striatum, but also within the dorsal striatum (*13*, *14*), the major output of the

nigrostriatal DA pathway assumed to play a prominent role in compulsive drug use (*3*, *15*, *16*). More specifically in rodents, compulsive alcohol seeking has been found to depend on the anterior part of the dorsolateral striatum (aDLS) and DA signaling in this structure (*17*). In striking contrast with the mesolimbic system however, study of potential tonic hypodopaminergic states of the nigrostriatal system in AUD has been neglected so far (*16*). We therefore hypothesized here that nigrostriatal hypodopaminergia would contribute to the development of compulsive alcohol use and of a negative affective state.

Compulsive alcohol use is specifically associated with a decrease in DA levels in the aDLS

We first tested whether compulsive alcohol use is associated with a hypodopaminergic state of the nigrostriatal pathway, by assessing striatal DA levels in rats expressing, or not, compulsive alcohol seeking behavior. Briefly, animals were first trained to voluntary consume high levels of alcohol under an intermittent access 20%-ethanol two-bottle choice (IA 20%-EtOH 2BC), before being challenging instrumentally to respond for alcohol in operant chambers (*18*, *19*). When operant performances reached stable levels, lever presses for alcohol were coupled to mild footshocks to identify rats expressing compulsive alcohol use, operationalized as resistance to punishment of alcohol self-administration (*20*, *21*) (Fig. 1A). During these punished sessions, a bimodal distribution progressively appeared, which clearly indicates the existence of two sub-populations: one with a strong decrease in lever presses, the footshock-sensitive (FS) rats (58%), while the other, the footshock-resistant (FR) rats (42%), maintained their responses despite footshocks (Fig. 1, C and D, fig. S1A; see also (*21*)). No differences were observed on the second, inactive lever, indicating that the tendency to resist to the punishment was specifically related to the search for alcohol (fig. S1B). Importantly, and as already observed for alcohol (*17*) and cocaine (*22*), the history of intake cannot account for these two distinct phenotypes. Indeed, we found a similar escalation of alcohol consumption during IA 20%-EtOH 2BC (Fig. 1B and fig. S1C), as well as similar lever presses levels during

unpunished self-administration sessions between FR and FS rats (Fig. 1, C and D). While DA levels measured by ELISA assays on striatal extracts did not differ between FS and FR rats in the nucleus accumbens (NAc) or dorsomedial striatum (DMS) and appeared similar to those obtained from a non-exposed alcohol condition (water control rats), FR rats showed a significant decrease in DA levels in the aDLS (Fig. 1E). In addition, a robust negative correlation was found between the degree of resistance to footshocks and the amount of aDLS DA (Fig. 1F). Thus, in line with our hypothesis, compulsive alcohol seeking is associated with a decrease in aDLS DA level, suggesting an important link between a tonic hypodopaminergic state of the nigrostriatal pathway and the emergence of this maladaptive behavior.

Chemogenetically-induced nigrostriatal hypodopaminergia induces compulsive alcohol use

Consequently, we next investigated whether this DA hypofunction is causally implicated in the emergence of compulsive alcohol use. To that end, an experimental and reversible nigrostriatal hypodopaminergia was induced using the inhibitory DREADD (designer receptor exclusively activated by designer drug) hM4Di (*23*). TH-Cre rats were transduced in the substantia nigra pars compacta (SNc) with AAVs that Cre-dependently express hM4Di-mCherry, or mCherry alone (control condition), allowing its specific expression in SNc DA neurons (Fig. 2, A-C). hM4Di was activated by the synthetic ligand Compound 21 (C21) (*24*), at a dose that we previously reported as potent and selective to inhibit SNc neurons in TH-Cre rats (*25*). We first confirmed with *in vivo* microdialysis that this approach efficiently decreases aDLS DA levels (Fig. 2D and fig. S2). In hM4Di rats, a 30%-decrease in aDLS DA level was observed between 90 and 135 minutes after C21 injection (Fig. 2E), which is consistent with the temporal inhibition of SNc neurons previously reported (*25*). Importantly, this strategy did not influence NAc DA levels (Fig. 2E), confirming that chemogenetic inhibition of SNc DA neurons induces a reversible and selective hypodopaminergic state of the nigrostriatal pathway.

Then, we tested whether induction of this experimental nigrostriatal hypodopaminergia state is sufficient to induce compulsive alcohol seeking in FS rats. TH-Cre rats were therefore trained in IA 20%-EtOH 2BC and operant ethanol-self-administration procedures as in the first experiment (Fig. 3A), and then transduced with DREADDs ensuring a same history of alcohol exposure between hM4Di and mCherry control rats (Fig. 3, B and C). After selection of FS-hM4Di and FS-mCherry rats under punishment sessions preceded by saline injections, we assessed their tendency to persist in seeking alcohol despite punishment over the course of a second series of sessions, this time following administration of C21 (Fig. 3D). In these sessions, we found that chemogenetic inhibition of SNc DA neurons progressively increased the resistance of FS rats to punishment (Fig. 3, D and E), while it did not affect their intrinsic footshocks sensitivity (fig. S3A). Interestingly, this increase was observed for each hM4Di FS rat (Fig. 3F) and correlated only with the level of hM4Di expression within the distal SNc (Fig. 3, G and H), the main DA input of the DLS (*26*). In marked contrast, effect of the chemogenetic manipulation was found neither on the inactive lever during punishment sessions (fig. S3B), nor on operant alcohol self-administration under baseline conditions (fig. S3C), confirming that this effect was related to punishment and not to a non-selective or general change in operant behavior. Taken together, these results demonstrated that an hypodopaminergic state of the nigrostriatal pathway is sufficient to induce compulsive alcohol seeking behavior in animals that were otherwise resilient.

Chemogenetically-induced nigrostriatal hypodopaminergia leads to a prolonged negative motivational, but not affective, state

Because we previously showed that a partial DA denervation of the nigrostriatal pathway leads to motivational impairments as well as depression- and anxiety-related behaviors (*11*), three core features of the negative psychological state experienced during withdrawal (*27*), we finally test whether chemogenetically-induced nigrostriatal hypodopaminergia recapitulates

such phenotype (Fig. 4, A and B). Motivated behavior was evaluated in an operant sucrose self-administration task as previously performed (*11*, *12*). In comparison to the control groups, hM4Di-C21 treated rats exhibited a prolonged decrease in their performance to obtain sucrose, that persisted beyond the last operant session under C21 (Fig. 4, C and D). This result was not due to a motor deficit associated with nigrostriatal hypodopaminergia, as revealed by the absence of motor alterations in an open arena and fine use of the forepaws in a stepping test (Fig. 4, E and F). It cannot be attributed either to an inability to discriminate between the active and inactive lever that was preserved throughout the task (Fig. S4A), or to a decrease in sensitivity to the reinforcing properties of sucrose, as preference (Fig. 4G) and consumption (fig. S4B) for the sucrose solution was unchanged in a two-bottle choice paradigm. Taken together, these data confirm that the chemogenetically-induced decrease in operant sucrose self-administration reflects a motivational deficit (*11*). In sharp contrast, no increase in anxiety- or depression-related behaviors were observed in light/dark avoidance and elevated-plus maze tests (Fig 4. H and fig. S4C) and in forced swim test (Fig. 4I) respectively. Finally, in addition to its implication in compulsive alcohol use, hypodopaminergic state of the nigrostriatal pathway leads to a prolonged negative motivational, but not affective, state.

Conclusion

At a time of great controversies about the exact role of DA in addiction (*10*), we shed to light here hitherto overlooked implication of the DA nigrostriatal pathway in AUD. Our findings indicate that chronic exposure to alcohol leads to the development of a tonic hypodopaminergic state in the nigrostriatal pathway in vulnerable individuals that directly contributes to the development of compulsive alcohol use and probably participates to the maintenance of the negative motivational state experienced during abstinence.

Mesolimbic tonic hypodopaminergic states have been proposed to be part of the allostasis phenomenon observed during abstinence that underlines the emergence of a negative

motivational and affective state and participates to the development of excessive alcohol seeking and intake (*7*, *28*). In regard to the present data, it suggests that, in vulnerable chronic users, these DA hypofunctions might eventually propagate or migrate to the nigrostriatal pathway through different possible interconnexions (*29*, *30*). This transition from the ventromedial to the dorsolateral territories of the striatum has already been observed for DA phasic signals, that emerge progressively in the aDLS over the course of drug taking, while they concomitantly fade in the ventral portion (*31*).

Interestingly, DA phasic signals are directly modulated by the DA tonic state (*32*) and the relation between these two complementary modes of signaling has been suggested to be important to consider for alcohol and other substance use disorder (*33*). Emergence of the hypodopaminergic state of the mesolimbic pathway in the early phase of the disorder might therefore lead to an aberrant enhanced DA phasic signaling in the NAc, that would contribute to aberrant learning (*34*) and sensitization of the mesolimbic DA pathway to drugs or associated cue (*35*, *36*). Within this framework, optogenetic studies conducted within the mesolimbic pathway have shown that increasing DA phasic firing promotes excessive alcohol consumption, whereas increasing DA tonic firing decrease consumption and annihilates the effect of DA phasic signal when induced simultaneously (*37*). In the ultimate stages of AUD, the hypodopaminergic state of the nigrostriatal pathway revealed in our study, would strengthen similar abnormal phasic DA signals in the aDLS engaged by drug-paired cues, facilitating the formation of maladaptive incentive habits and the ensuing rigidity in drug seeking (*17*, *30*, *38*) exacerbated by negative urgency during abstinence (*39*) This finally suggests that DA-based therapy normalizing DA tonic firing may eliminate aberrant DA phasic signals and suppress excessive as well as compulsive alcohol seeking and drinking. In this respect, normalization of DA tone in the mesolimbic pathway, using GDNF or OSU6162, has already been shown effective at suppress excessive alcohol consumption in rodents and diminish alcohol craving in

humans (*7*, *40*, *41*). Further studies, build on the present findings, would be of high interest to determine whether normalization of tonic DA state in the nigrostriatal pathway with new DA-based therapy is efficient to limit compulsive alcohol use.

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Visualization: RG, SC

Funding acquisition: SC

Project administration: SC

Supervision: BC, SC

Writing – original draft: RG, SC

Writing – review & editing: RG, DM, SB, CD, BC, SC

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Supplementary Materials

Materials and Methods

Supplementary Text

Figs. S1 to S4

Tables S1

References (11, 17 – 20, 25, 32, 43 – 49)

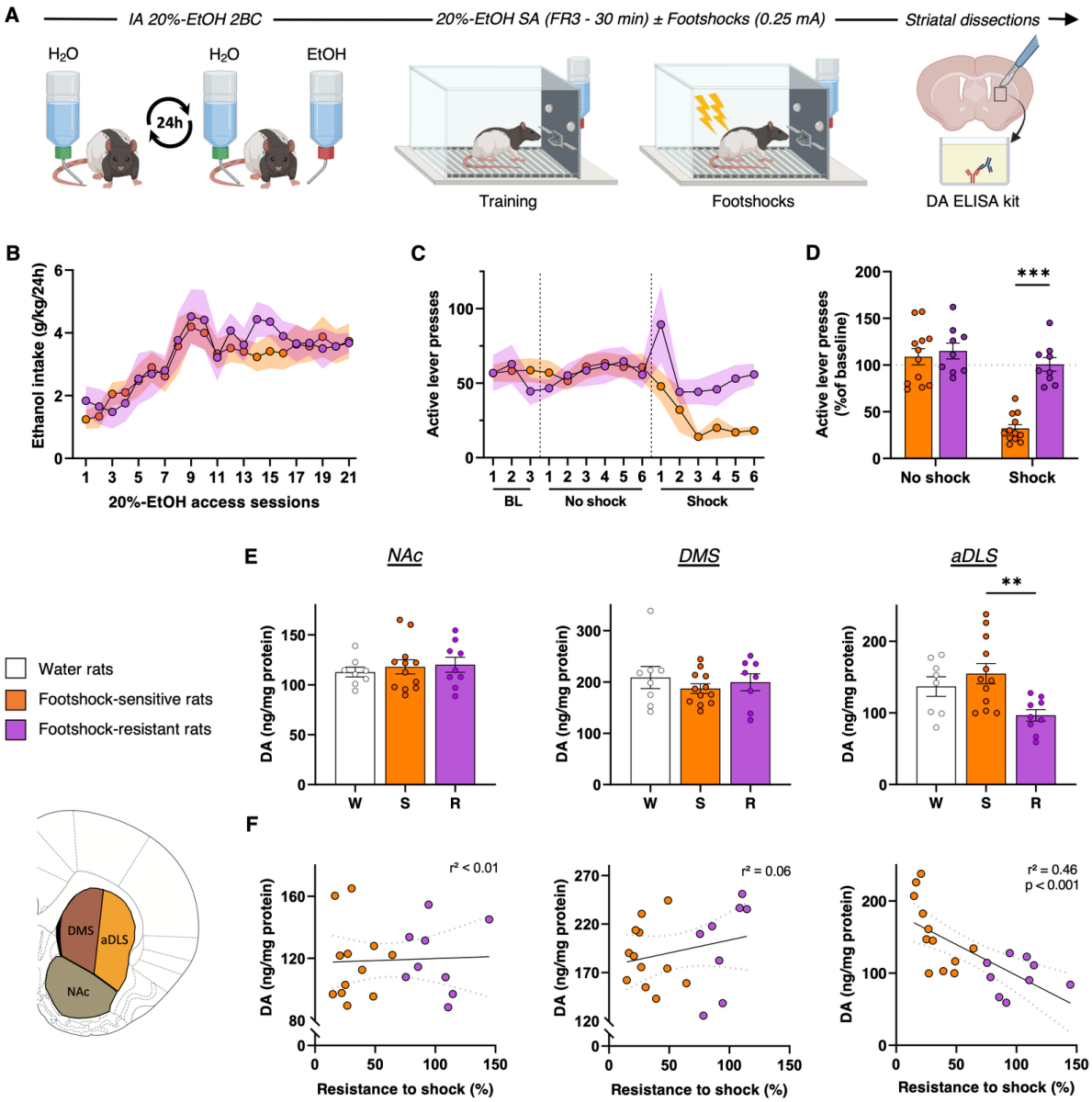

Fig. 1: Compulsive alcohol use is specifically associated with a decrease in DA levels in the aDLS. (A) Experiment timeline. **(B)** Ethanol intake during intermittent-access 20%-ethanol two-bottle-choice (*IA 20%-EtOH 2BC*). RM two-way ANOVA showed a significant effect of session [$F_{(6, 109)}$=7.82, P<0.001, partial η^2=0.29] but neither effect of group, nor session x group interaction [F_s<0.47, P>0.5, partial η^2<0.02]. **(C)** Number of active lever presses in 30-min self-administration sessions (SA) of 20% EtOH (FR3), during baseline, "no-shock" and "shock" sessions (see supplementary material for details) in *footshock-sensitive* (FS, orange, *n*=12) and *footshock-resistant* (FR, purple, *n*=9) rats. RM two-way ANOVA showed a

significant group x session interaction [$F_{(14, 266)}$=2.86, P<0.001, partial η^2=0.13]. **(D)** Mean active lever presses during the three last "no-shock" sessions and the three last "shock" sessions normalized to baseline. RM two-way ANOVA showed a significant shock condition x group interaction [$F_{(1, 19)}$=20.89, P<0.001, partial η^2=0.52] **(E)** NAc, DMS and aDLS DA levels for *FR*, *FS* and *Water* rats (white, *n*=8). One-way ANOVA showed a main effect of group in the aDLS [$F_{(2,26)}$=5.56, P<0.01, partial η^2=0.3], but not in the NAc and DMS [F_s<0.55, P>0.5, partial η^2<0.04]. **(F)** Linear regression between resistance to footshocks and DA level in NAc, DMS or aDLS. A significant correlation was found in the aDLS [$F_{(1,19)}$=16.18, P<0.001], but not in the NAc or DMS [F_s<1.14, P>0.3]. Data are expressed in mean ± SEM. Bonferroni correction post-hoc analysis: **, P<0.01; ***, P<0.001. BL: baseline, DMS: dorsomedial striatum, aDLS: anterior dorsolateral striatum, FR: fixed ratio, NAc: nucleus accumbens.

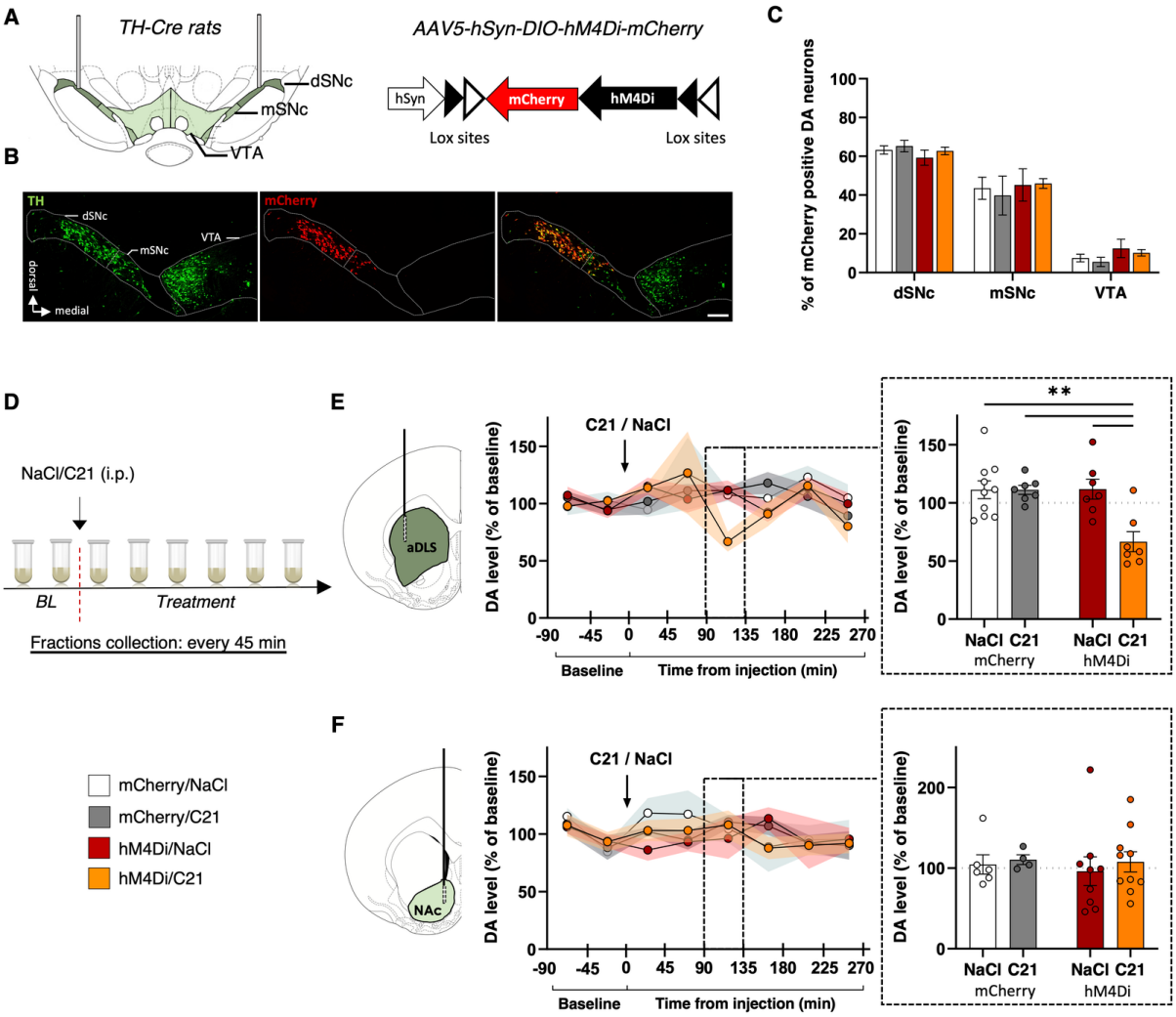

Fig. 2: Chemogenetic inhibition of the SNc DA neurons induces selective nigrostriatal hypodopaminergia. (A-C) hM4Di-mCherry and mCherry expression in SNc DA neurons. **(A)** TH-Cre rats received bilateral injection of Cre-dependent hM4Di-mCherry or Cre-dependent mCherry virus in the SNc. **(B)** Representative images of TH immunostaining and hM4Di-mCherry expression. Scale bar: 250 µm. **(C)** Quantification of transgene expression in distal (dSNc), medial SNc (mSNc) and VTA. Three-way ANOVA showed a significant effect of the area [$F_{(2,129)}$=158, P<0.001, η^2=0.71], but no effect of transgene, treatment or any interaction between these factors [F_s<1.04, P>0.36, partial η^2<0.02]. **(D-G)** Extracellular DA concentrations in aDLS and NAc throughout eight 45min-fractions collected by microdialysis. Data are normalized to baseline. **(D)** Design of the microdialysis experiment. **(E)** Time course of extracellular DA concentrations in the aDLS of *hM4Di* rats treated with C21 (orange, *n*=7)

or NaCl (red, *n*=7) and *mCherry* rats treated with C21 (grey, *n*=7) or NaCl (white, *n*=10). **(F)** Time course of extracellular DA concentrations in the NAc of *hM4Di* rats treated with C21 (orange, *n*=10) or NaCl (red, *n*=9) and *mCherry* rats treated with C21 (grey, *n*=4) or NaCl (white, *n*=6). In the fraction collected between 90 and 135 minutes after injection (dotted squares), two-way ANOVA found a treatment x transgene interaction in the aDLS [$F_{(1,27)}$=8.63, P<0.01, partial η^2=0.24], but not in the NAc [$F_{(1,25)}$=0.03, P=0.87, partial η^2=0.001]. Data are expressed in mean ± SEM. Bonferroni correction post-hoc analysis: **, P<0.01. C21: compound 21, SNc: substantia nigra pars compacta, TH: tyrosine hydroxylase, VTA: ventral tegmental area.

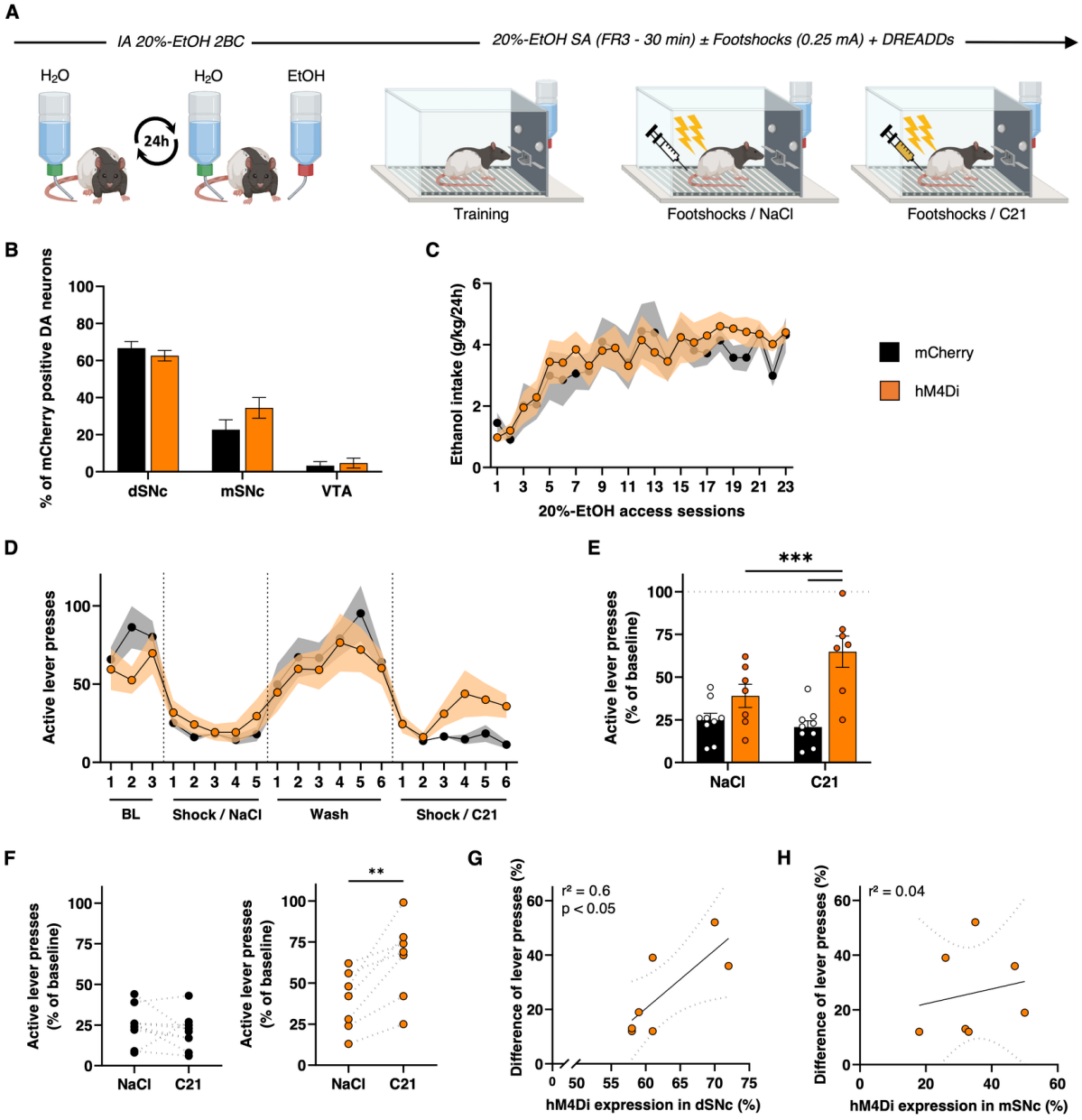

Fig. 3: Chemogenetically-induced nigrostriatal hypodopaminergia induces compulsive alcohol use. (A) Experiment timeline. **(B)** Quantification of hM4Di-mCherry and mCherry expression in distal (dSNc), medial SNc (mSNc) and VTA. Two-way ANOVA showed significant effect of the area [$F_{(2,90)}$=116.4, P<0.001, η^2=0.72], but no effect of transgene, treatment or any interaction between these factors [F_s<1.99, P>0.14, partial η^2<0.04]. **(C)** Ethanol intake during intermittent-access 20%-ethanol two-bottle-choice (*IA2BC 20%-EtOH*) in *hM4Di* rats (orange, *n*=7) and *mCherry* rats (black, *n*=10). RM two-way ANOVA showed a significant effect of session [$F_{(4, 59)}$=10.56, P<0.001, partial η^2=0.43), but neither effect of

transgene, nor session x transgene interaction [F_s<0.57, P>0.5, partial η^2<0.04]. **(D)** Number of active lever presses in 30-min self-administration sessions of 20%-EtOH (FR3), during baseline, "Shock/NaCl", "Wash" and "Shock/C21" sessions. RM two-way ANOVA showed a significant session x transgene interaction [$F_{(19,266)}$=2.18, P<0.01, partial η^2=0.13]. **(E - F)** Mean active lever presses during the three last "Shock/NaCl" and "Shock/C21" sessions normalized to baseline **(E)** and individual trajectories during these two periods **(F)**. RM two-way ANOVA reported a significant session x transgene interaction [$F_{(1,14)}$=18.41, P<0.001, partial η^2=0.57], while paired t-test reported a significant effect of C21 in *hM4Di* [t=4.3, P<0.01] but not in *mCherry* rats [t=1.02, P=0.34]. **(G - H)** Correlation between the difference of active lever presses during "Shock/C21" and "Shock/NaCl" sessions and the percent of hM4Di expression within dSNc **(G)** or mSNc **(H).** A significant correlation was found for dSNc [$F_{(1,5)}$=7.64, P<0.05], but not for mSNc [$F_{(1,5)}$=0.18, P=0.69]. Data are expressed in mean ± SEM. Bonferroni correction post-hoc analysis: **, P<0.01; ***, P<0.001.

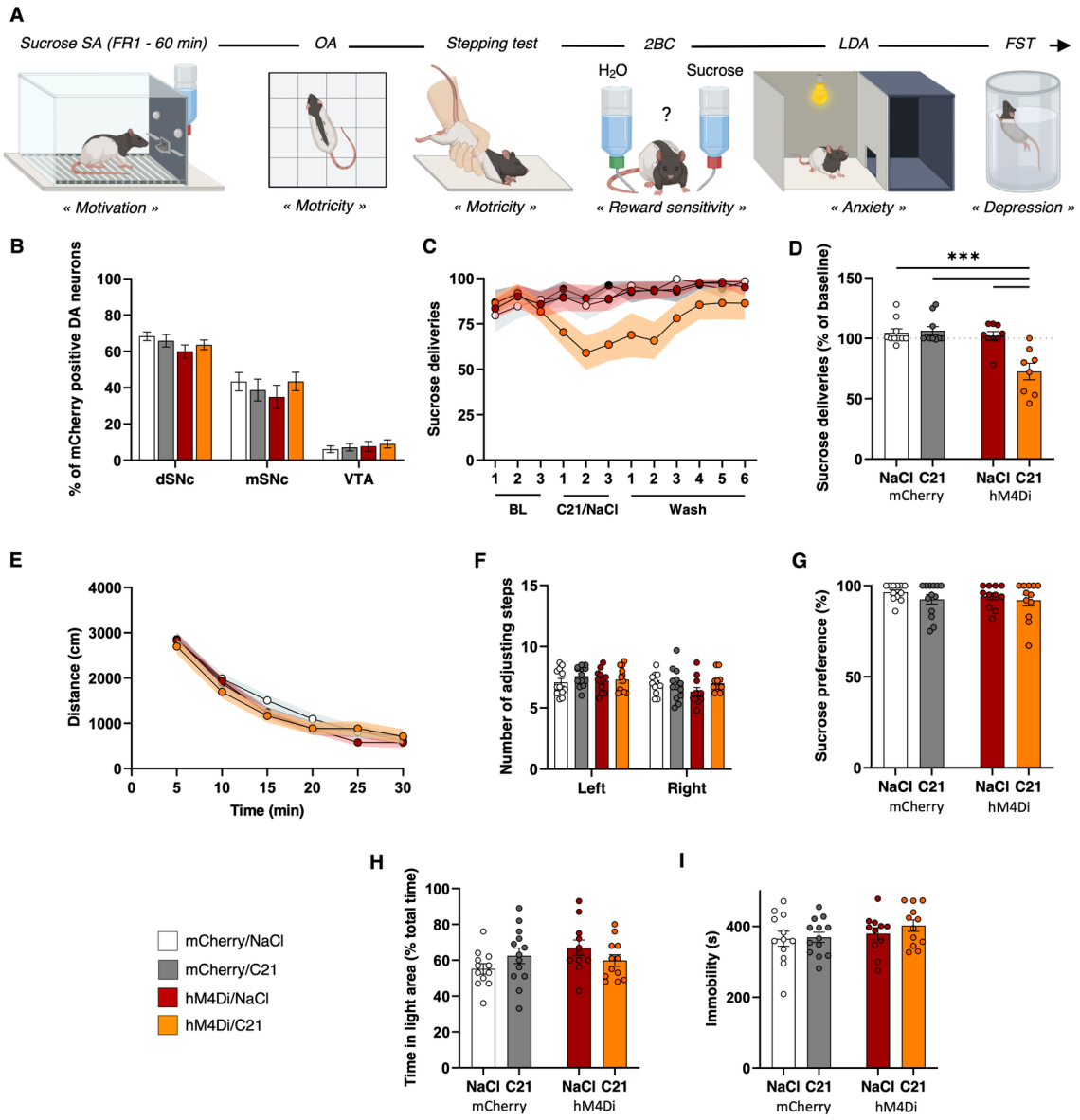

Fig. 4: Chemogenetically-induced nigrostriatal hypodopaminergia leads to a prolonged negative motivational, but not affective, state. (A) Behavioral screening timeline. **(B)** Quantification of hM4Di-mCherry and mCherry expression in distal (dSNc), medial SNc (mSNc) and VTA. Three-way ANOVA showed significant effect of the area [$F_{(2, 140)}$=346.7, P<0.01, η^2=0.83], but no effect of transgene, treatment or any interaction between these factors [F_s<1.31, P>0.27, partial η^2<0.02]. **(C)** Number of 2.5%-sucrose deliveries obtained in 60-min self-administration (SA) sessions under continuous reinforcement (FR1), during baseline, "C21/NaCl" and "Wash" sessions, in *hM4Di* rats treated with C21 (orange, *n*=8) or NaCl (red, *n*=9) and *mCherry* rats treated with C21 (grey, *n*=10) or NaCl (white, *n*=10). RM three-way

ANOVA showed a significant session x transgene x treatment interaction [$F_{(11, 363)}$=2.67, P<0.01, partial η^2=0.07]. **(D)** Mean sucrose deliveries obtained during "C21/NaCl" sessions normalized to baseline. Two-way ANOVA showed a significant transgene x treatment interaction [$F_{(1, 33)}$=13.38, P<0.001, partial η^2=0.29]. **(E - I)** Distance traveled over the course of a 30-min session in an open area (OA) **(E)**, number of adjusting left and right forepaws in a stepping test **(F)**, sucrose preference measured over a 60-min 2.5%-sucrose two-bottle-choice (2BC) drinking session **(G),** percentage of time spent in the light area in a light/dark avoidance test (LDA) **(H)** and time spent immobile in a forced swim test (FST) **(I)** in *hM4Di* rats treated with C21 (orange, *n*=12) or NaCl (red, *n*=11) and *mCherry* rats treated with C21 (grey, *n*=13) or NaCl (white, *n*=12). Two- or three-way ANOVA found no interaction implicating the transgene and the treatment [F_s<1.3 , P>0.26, partial η^2<0.03] in these different tests, except a marginal side x transgene x treatment interaction in stepping test [$F_{(1, 44)}$=3.55, P=0.07, partial η^2=0.07] and a transgene x treatment interaction in LDA [$F_{(1, 44)}$=3.68, P=0.06, partial η^2=0.08], mainly driven by small size effects related to the transgene condition and not to a C21 effect on *hM4Di* rats. Data are expressed in mean ± SEM. Bonferroni correction post-hoc analysis: ***, P<0.001.