cAMP-Fyn signaling in the dorsomedial striatum direct pathway drives excessive alcohol use

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ABSTRACT

Fyn kinase in the dorsomedial striatum (DMS) of rodents plays a central role in mechanisms underlying excessive alcohol intake. The DMS is comprised mostly of medium spiny neurons (MSNs) that are divided into dopamine D1 receptor expressing neurons (dMSNs), and dopamine D2 receptor expressing neurons (iMSNs). Here, we examined the cell-type specificity of Fyn's actions on the development of alcohol use. First, we knocked down Fyn selectively in DMS dMSNs or iMSNs of mice and measured the level of alcohol consumption. We found that downregulation of Fyn in dMSNs, but not in iMSNs, reduces excessive alcohol but not saccharin intake and preference. D1Rs are coupled to Gαs/olf, which activate cAMP signaling. To examine whether Fyn's actions are mediated through cAMP signaling, DMS dMSNs were infected with GαsDREADD, and the activation of Fyn signaling was measured following CNO treatment. We found that remote stimulation of cAMP signaling in DMS dMSNs activates Fyn and promotes the phosphorylation of the Fyn substrate, GluN2B. Next, we tested whether remote activation of GαsDREADD in DMS dMSNs or iMSNs alters alcohol intake, and observed that CNO-dependent activation of GαsDREADD in DMS dMSNs but not iMSNs increases alcohol but not saccharin intake and preference. Finally, we examined the contribution of Fyn to GαsDREADD-dependent increase in alcohol intake, and found that systemic administration of the Fyn inhibitor, AZD0503 blocks GαsDREADD-dependent increase in alcohol consumption. Our results suggest that the cAMP-Fyn axis in the DMS dMSNs is a molecular transducer of mechanisms underlying the development of excessive alcohol consumption.

Introduction

The dorsomedial striatum (DMS) is critically involved in processes such as locomotion (Grillner *et al*, 2005), and goal-directed behaviors (Luft and Buitrago, 2005; Redgrave *et al*, 2010). The DMS is comprised primarily of GABAergic medium spiny projection neurons (MSNs) that receive dopaminergic input from the midbrain (Bolam *et al*, 2000). MSNs can be divided into two populations of neurons that take part in opposing activities (Gerfen and Surmeier, 2011); MSNs that project directly to the substantia nigra pars reticula (SNr) facilitate actions and are defined as direct MSNs (dMSNs) (Gerfen *et al*, 2011), and MSNs that project indirectly to the SNr gate actions and are defined as indirect MSNs (iMSNs) (Gerfen *et al*, 2011). dMSNs selectively express the dopamine D1 receptors (D1Rs) whereas iMSNs express the dopamine D2 receptors (D2Rs) (Gerfen *et al*, 2011). In the striatum, D1Rs are coupled to Gαolf, a homolog of Gαs (Herve, 2011). Stimulation of Gαs/olf-coupled receptors results in the production of the second messenger cyclic adenosine monophosphate (cAMP) (Herve, 2011; Neve *et al*, 2004), which binds to, and activates, protein kinase A (PKA) (Taylor *et al*, 2013), a kinase that plays an important role in the adult brain (Brandon *et al*, 1997; Kandel, 2012; Waltereit and Weller, 2003). In contrast, D2Rs are coupled to Gαi, which inhibits cAMP signaling (Neve *et al*, 2004). dMSNs and iMSNs exert balanced influence on locomotion and goal-directed behaviors (Gerfen *et al*, 2011), and an imbalance of dMSNs and iMSNs function has been implicated in neurodegenerative disorders such as Parkinson's disease (Gerfen *et al*, 2011; Ryan *et al*, 2018), as well as psychiatric disorders such as obsessive compulsive disorder, anxiety and addiction (Bocarsly *et al*, 2019; Gunaydin and Kreitzer, 2016; Lobo and Nestler, 2011).

We previously observed that Fyn kinase is activated in DMS dMSNs upon stimulation of D1Rs (Phamluong *et al*, 2017). Fyn belongs to the Src family of non-receptor protein tyrosine kinases (PTKs) (Ingley, 2008; Resh, 1998), and is highly expressed in the developing and adult brain in regions such as cortex, hippocampus and cerebellum as well as in the striatum (Umemori *et al*, 1992; Yagi *et al*, 1993). Fyn plays an important role in the CNS (Ohnishi *et al*, 2011), as it modulates excitatory and inhibitory synaptic transmission and participates in learning and memory processes (Chattopadhyaya *et al*, 2013; Grant *et al*, 1992; Hildebrand *et al*, 2016; Kojima *et al*, 1997; Ohnishi *et al*, 2011; Salter and Kalia, 2004; Trepanier *et al*, 2012; Yaka *et al*, 2002). Dysfunction of Fyn signaling has been associated with Alzheimer's disease (Kaufman *et al*, 2015) and pain (Hildebrand *et al*, 2016).

Accumulating data in humans and rodents also suggest that Fyn plays a central role in cellular neuroadaptations that underlie alcohol use disorder (AUD) (Morisot and Ron, 2017; Ron and Barak, 2016). Specifically, genetic mutations within the Fyn gene have been associated with increased susceptibility for the development and severity of AUD in humans (Ishiguro *et al*, 2000; Pastor *et al*, 2009; Schumann *et al*, 2003), and gene network association studies identified a link between Fyn and alcohol dependence (Han *et al*, 2013). Animal data suggest that Fyn plays a role in the acute tolerance to the hypnotic sedative effect of alcohol (Miyakawa *et al*, 1997; Yaka *et al*, 2003b), as well as in alcohol drinking behavior (Morisot *et al*, 2019; Wang *et al*, 2007; Wang *et al*, 2010). Molecularly, excessive consumption of alcohol activates Fyn specifically in the DMS of mice and rats (Darcq *et al*, 2014; Gibb *et al*, 2011; Wang *et al*, 2010). Once activated by alcohol, Fyn phosphorylates its substrate, GluN2B (Darcq *et al*, 2014; Gibb *et al*, 2011; Wang *et al*, 2010). Alcohol-dependent Fyn-mediated phosphorylation of GluN2B results in forward trafficking of the channel and in long-lasting enhancement of GluN2B activity

in the DMS (Wang *et al*, 2010). Inhibition of Fyn in the DMS of rats attenuates operant self-administration of alcohol (Wang *et al*, 2010), and systemic administration of the Fyn inhibitor, AZD0530, attenuates goal-directed alcohol seeking and facilitates extinction in mice (Morisot *et al*, 2019). Together, these data suggest that Fyn in the DMS plays a central role in neuroadaptations that underlie alcohol use.

This study was aimed to explore the cellular specificity of Fyn-dependent molecular and behavioral neuroadaptations that drive AUD.

Methods

The description of purchased reagents and animals, mouse breeding, collection of brain samples, westernblot analysis, preparation of solutions and the preparation of FLEX-shRNA-Fyn and FLEX-SCR is detailed in the Supplementary Information section.

*Infection of the DMS with FLEX-shFyn and FLEX-SC*R

D1-Cre/Ai1 and A2A-Cre/Ai14 mice were anesthetized using a mixture of ketamine (120 mg/kg) and xylazine (8 mg/kg). Bilateral microinfusions were made using stainless steel injectors (33 gauge, Small Parts) into the DMS (the stereotaxic coordinates were anterioposterior +1.1 mm and +1.3 mm from bregma; mediolateral ±1.2 mm from bregma and dorsoventral -2.8 and -3 mm from the skull surface). Animals were infused with lenti-virus expressing FLEX-shFyn or its scramble control (FLEX-SCR) (1.2 µl/site with 4 sites of injection per hemisphere) at a concentration of 10^7 pg/ml at an injection rate of 0.1 µl/minute (Phamluong *et al*, 2017). After each infusion, the injectors were left in place for an additional 10 minutes to allow the virus to diffuse.

Infection of the DMS with AAV-DIO-rM3D(Gs)-mCherry

Drd1a-Cre or A2A-Cre mice were anesthetized using a mixture of ketamine (120 mg/kg) and xylazine (8 mg/kg). Bilateral microinfusions were made using stainless steel injectors (33 gauge, Small Parts) into the DMS (the stereotaxic coordinates were anterioposterior +1.1 mm and +1.3

mm from bregma; mediolateral ±1.25 mm from bregma and dorsoventral -2.8 and -3 mm from the skull surface). Mice were infused with AAV-DIO-rM3D(Gs)-mCherry (AAV-DIO-Gs-DREADD) (1 µl per hemisphere) at a concentration of 10^{13} vg/ml and at an injection rate of 0.1 µl/minute. After each infusion, the injectors were left in place for an additional 10 minutes to allow the virus to diffuse.

Drinking Paradigm

Two bottle choice - 20% alcohol

Mice underwent four weeks of 2-bottle choice 20% (v/v) alcohol drinking paradigm (IA20%2BC) as described in (Laguesse *et al*, 2017; Warnault *et al*, 2013). Specifically, one month after stereotaxic surgery and infection of the DMS with FLEX-shFyn or FLEX-SCR, mice had 24 hours access to one bottle of 20% alcohol and one bottle of water on Monday, Wednesday and Friday with alcohol drinking sessions starting 2 hours into the dark cycle. During the 24 or 48 hours (weekend) of alcohol deprivation periods, mice had access to two bottles of water (**Figure 1b**).

Two bottle choice - 10% alcohol

One month after stereotaxic surgery and the infection of the DMS of Drd1a-Cre or A2A-Cre with AAV-DIO-Gs-DREADD, mice were habituated by intraperitoneal (IP) injection of saline for three days. On test day, mice received a systemic administration of vehicle (0.5% DMSO) or CNO (3 mg/kg). Fifteen minutes later, mice had access to one bottle of 10% (v/v) alcohol and one bottle of water. Alcohol and water intake were measured 4 hours later (**Timeline, Figure 3a**). Mice were then given water only for one week and were tested again using a counterbalanced, within-subject design.

A separate cohort of Drd1a-Cre mice were infected with AAV-DIO-Gs-DREADD in the DMS. One month later, mice were systemically administered with vehicle (20% HPBCD) or AZD0530 (10 mg/kg) 3 hours prior to the beginning of the drinking session. Subsequently, animals received a systemic administration of vehicle (0.5% DMSO) or Clozapine N-Oxide (CNO, 3 mg/kg) 15 minutes before the beginning of the drinking session, and alcohol and water intake were measured 4 hours later (**Timeline, Figure 4a**). Mice were tested in a counterbalanced, within-subjects design.

Two bottle choice - 0.03% Saccharin

Mice underwent a saccharin intake procedure as described in (Ben Hamida *et al*, 2012). Specifically, 2 weeks after the end of the alcohol drinking paradigm during which mice consumed only water, mice had access to one bottle of water and one bottle of saccharin (0.03%) every other day for 1 week (2 sessions) (**Timeline, Figure 1b**).

The DMS of a separate cohort of Drd1a-Cre mice were infected with AAV-DIO-Gs-DREADD. On test day, mice received a systemic administration of vehicle (0.5% DMSO) or CNO (3 mg/kg). Fifteen minutes later, mice had access to a bottle of saccharin (0.03%) and one bottle of water. Saccharin and water intake were measured 4 hours later (**Timeline, Figure 3a**). Mice were then given water only for one week and were tested again using a counterbalanced, within-subject design.

Alcohol (g/kg), saccharin (ml/kg) and water (ml/kg) intake were recorded at the end of each 4-hour drinking session, and preference ratio (alcohol/alcohol+water or saccharin/saccharin+water) was calculated. The placement (right or left) of the bottles was alternated in each session to control for side preference. Spillage was monitored using an additional water bottle in a nearby unused cage.

Statistical analysis

GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to plot and analyze the data. D'Agostino–Pearson normality test and *F*-test/Levene tests were used to verify the normal distribution of variables and the homogeneity of variance, respectively. Data were analyzed using the appropriate statistical test, including two-tailed unpaired or paired t-test, two-way analysis of variance (ANOVA) followed by post hoc tests as detailed in the Figure Legends. Experiments designed to evaluate the consequence of Fyn knockdown using FLEX-shFyn were analyzed with unpaired t-test. Experiments designed to test the contribution of cAMP signaling on alcohol intake using AAV-DIO-Gs-DREADD were analyzed with paired t-test since within subject design was used. All data are expressed as mean ± SEM, and statistical significance was set at $p<0.05$.

Results

To determine whether Fyn's actions are localized to DMS dMSNs and/or iMSNs, we used transgenic mice which express Cre recombinase and tdTomato specifically in dMSNs (Drd1-Cre-Ai14), or that express Cre recombinase and tdTomato specifically in iMSNs (A2A-Cre-Ai14), in combination with a Cre-dependent Flip Excision (FLEX) approach to downregulate *Fyn* mRNA in dMSNs or iMSNs, respectively, as described in (Phamluong *et al*, 2017). First, we examined the consequence of Fyn knockdown in DMS dMSNs on alcohol drinking. To do so, the DMS of Drd1-Cre-Ai14 was infected bilaterally with a lentivirus expressing a short hairpin mRNA sequence targeting Fyn inserted in a FLEX cassette (FLEX-shFyn) (10^7 pg/ml, 1.2 µl per site) or with a FLEX virus expressing a scramble sequence which was used as a control (FLEX-SCR, 10^7 pg/ml, 1.2 µl per site) (**Figure 1a**). After 4 weeks, which enabled maximal viral infection and knockdown of the gene (Phamluong *et al*, 2017), mice were subjected to 4 weeks of IA20%2BC and alcohol intake was measured at the final 4-hours drinking session (**Timeline, Figure 1b**). Alcohol intake (**Figure 1c, Supplementary Table 1**) and preference (**Figure 1d**) were significantly reduced in Drd1-Cre-Ai14 mice infected with FLEX-shFyn in dMSNs as compared to the FLEX-SCR infected mice.

Next, to determine if downregulation of Fyn in iMSNs also affects alcohol intake, the DMS of A2A-Cre-Ai14 mice was infected with FLEX-shFyn or FLEX-SCR. Four weeks later, mice underwent an IA20%2BC for 4 weeks and alcohol intake and preference were measured

(**Timeline, Figure 1b**). Knockdown of Fyn in iMSNs did not alter alcohol intake (**Figure 1e, Supplementary Table 1**) or preference (**Figure 1f**). Together, these data suggest that Fyn participates in mechanisms underlying alcohol consumption through its actions in dMSNs but not in iMSNs.

We then assessed whether Fyn's action in DMS dMSNs is specific for alcohol or is shared with other rewarding substances. To examine this question, we tested the consequences of Fyn knockdown in DMS dMSNs on the consumption of 0.03% saccharin (**Timeline Figure 1b**). As shown in Figure **1g-h**, knockdown of Fyn in DMS dMSNs did not alter saccharin intake (**Figure 1g, Supplementary Table 1**) or preference (**Figure 1e**) suggesting that Fyn's regulation of consummatory behavior in DMS dMSNs is specific for alcohol, and is not generalized to other reinforcing agents.

As stated above, D1Rs are selectively expressed in dMSNs (Gerfen *et al*, 2011). D1Rs are coupled to Gαs/olf, and stimulation of Gαs/olf-coupled receptors activates cAMP/PKA signaling (Herve, 2011; Neve *et al*, 2004). *Ex vivo* data suggest that Fyn is activated in the hippocampal neurons through the cAMP/PKA pathway (Goebel-Goody *et al*, 2012; Thornton *et al*, 2004; Trepanier *et al*, 2012; Yaka *et al*, 2003a). We therefore postulated that stimulation of cAMP/PKA signaling in DMS dMSNs activates Fyn. To test this possibility, we utilized the Designer Receptor Exclusively Activated by Designer Drug (DREADD) system to remotely activate Gαs in DMS dMSNs or iMSNs (Farrell *et al*, 2013). First, the DMS of Drd1-Cre mice was infected bilaterally with AAV8-hSyn-DIO-rM3D(Gs)-mCherry (10^{13} vg/ml, 1 μl per hemisphere) (**Figure 2a**). Four weeks after surgery, vehicle or CNO (3 mg/kg) was administered systemically, and the DMS was harvested 30 minutes later (**Timeline, Figure 2b**). As shown in **Figure 2c-d**, CNO administration produced a robust activation of Fyn in the DMS. We also

measured the phosphorylation level of the Fyn substrate, GluN2B (Trepanier *et al*, 2012), and found that Fyn activation in dMSNs was accompanied by the phosphorylation of GluN2B (**Figure 2e-f**). In contrast, CNO administration did not alter Fyn's activity or GluN2B phosphorylation in the DLS, a neighboring striatal region which was not infected with AAV8-hSyn-DIO-rM3D(Gs)-mCherry (**Figure 3c-f**). Together, these data suggest that Fyn signaling is enhanced in response to remote activation of GαsDREADD in DMS dMSNs.

We then determined whether the cAMP-dependent activation of Fyn in dMSNs alters alcohol drinking. As Drd1-Cre mice consume large quantities of 20% alcohol ((Wang *et al*, 2015), **Figure 1c**), we used a lower concentration (10% v/v) in order to avoid a confounding ceiling effect of alcohol intake due to GαsDREADD activation. The DMS of Drd1-Cre mice was infected bilaterally with AAV-hSyn-DIO-rM3D(Gs)-mCherry, four weeks later, vehicle or CNO (3 mg/kg) was administered systemically 15 minutes before the beginning of a 10% alcohol drinking session, and alcohol intake was measured after 4 hours (**Timeline, Figure 3a**). As shown in **Figure 3b-c**, remote activation of GαsDREADD in DMS dMSNs significantly increased alcohol intake (**Figure 3b**, **Supplementary Table 1**) and preference (**Figure 3c**). We then examined whether remote activation of GαsDREADD in DMS iMSNs also alters alcohol intake. Interestingly, CNO administration did not alter alcohol intake (**Figure 3d, Supplementary Table 1**) or preference (**Figure 3e**) in DMS iMSNs suggesting that cAMP signaling in dMSNs but not iMSNs contributes to the development of alcohol consumption.

Gomez et al. reported that CNO is converted to clozapine prior to binding and activating DREADDs (Gomez *et al*, 2017), and furthermore, CNO was reported to produce some behavioral effects on its own (Goutaudier *et al*, 2019; MacLaren *et al*, 2016). Therefore, to ensure that the increase in alcohol intake was not due to off-target effects of the drug, we

measured the level of alcohol consumption upon vehicle or CNO (3 mg/kg) treatment in Drd1-Cre mice that were not infected with AAV-hSyn-DIO-rM3D(Gs)-mCherry (**Timeline**, **Supplementary Figure 1a**). CNO administration did not alter alcohol intake or preference in uninfected Drd1-Cre mice (**Supplementary Figure 1b-c, Supplementary Table 1)** suggesting that the increase in alcohol intake by CNO-dependent activation of Gα*s*DREADD in DMS dMSNs is not due to off target effects of the drug itself or its metabolite, clozapine.

Next, we examined whether remote activation of GαsDREADD in DMS dMSNs alters the consumption of saccharin. Two weeks after the end of the alcohol drinking experiment, vehicle or CNO (3 mg/kg) was administered systemically 15 minutes prior to the initiation of the saccharin (0.03%) drinking session, and saccharin intake was measured after 4 hours (**Timeline, Figure 3a**). Activation of GαsDREADD in dMSNs did not alter saccharin intake (**Figure 3f, Supplementary Table 1**) or preference (**Figure 3f**) suggesting that the increase in consumption upon activation of GαsDREADD in DMS dMSNs is specific for alcohol.

Finally, we set out to test whether Fyn is required for GαsDREADD-dependent increase in alcohol consumption. To test this possibility, the DMS of Drd1-Cre mice was infected bilaterally with AAV-hSyn-DIO-rM3D(Gs)-mCherry, four weeks later, vehicle or the Src/Fyn inhibitor AZD0530 (10 mg/kg) (Hennequin *et al*, 2006; Kaufman *et al*, 2015; Morisot *et al*, 2019) was administered 3 hours before the beginning of the 10% alcohol drinking session followed by the administration of vehicle or CNO (3 mg/kg) 15 minutes prior to the start of the session, and alcohol intake was measured after 4 hours (**Timeline**, **Figure 4a**). In accordance with **Figure 3b**, the amount of alcohol consumed by mice was elevated after the remote activation of GαsDREADD in DMS dMSNs, but the increase in alcohol intake was blocked

when AZD0530 was administered prior to CNO (**Figure 4b, Supplementary Table 1**). These results suggest alcohol intake is driven through the cAMP/PKA/Fyn signaling in DMS dMSNs.

Discussion

Here, we present data to suggest that cAMP/PKA-dependent activation of Fyn kinase in the DMS dMSNs participates in mechanisms underlying the development of excessive alcohol intake. Specifically, we show that downregulation of Fyn levels in DMS dMSNs prior to the initiation of the IA20%2BC regimen attenuates alcohol intake. We further report that the stimulation of Gαs signaling in DMS dMSNs activates Fyn/GluN2B signaling, and enhances alcohol intake in a Fyn-dependent manner. In contrast, knockdown of Fyn or activation of cAMP signaling in iMSNs does not alter alcohol intake and preference. Finally, we show that the cAMP/Fyn signaling in DMS dMSNs is specific for alcohol and does not contribute to mechanisms underlying consummatory behavior *per se*. Based on previous data showing that Fyn is activated in the DMS through the stimulation of D1R in DMS dMSNs (Phamluong *et al*, 2017), and since dopamine levels in the dorsal striatum are elevated by drugs of abuse including alcohol (Di Chiara and Imperato, 1988; Lof *et al*, 2007), we propose a model in which dopamine released in the DMS in response to alcohol exposure stimulates D1R/cAMP/PKA signaling in dMSNs which in turn activates Fyn to initiate neuroadaptations such as GluN2B phosphorylation that promote the development of alcohol use (**Supplementary Figure 2**).

Our data suggest that Fyn in DMS dMSNs contributes to the development of alcohol consumption. The DMS is essential for goal-directed behaviors (Luft *et al*, 2005; Redgrave *et al*,

2010), and dMSNs contribute to reward learning and reinforcement (Hikida *et al*, 2010; Kravitz *et al*, 2012). Thus, it is plausible that Fyn in dMSNs promotes reward learning which in turn initiates and maintains goal-directed alcohol seeking. This possibility is in line with recent findings demonstrating that systemic administration of the Src/Fyn inhibitor, AZD0530, attenuates goal-directed alcohol self-administration in mice (Morisot *et al*, 2019). Xie et al. previously showed that administration of the Src/Fyn inhibitor PP2 into the dorsal hippocampus attenuates context-dependent cocaine seeking (Xie *et al*, 2013), and more recently, Belin-Rauscent reported that oral administration of the Src PTKs inhibitor, Masitinib, attenuates self-administration of cocaine (Belin-Rauscent *et al*, 2018). Thus, it would be of interest to determine whether Fyn in DMS dMSNs contributes to goal-directed seeking of other drugs of abuse. Furthermore, Goto et al. previously reported that mating behavior increases PKA activity in dorsal striatal dMSNs (Goto *et al*, 2015). As PKA in DMS dMSNs is upstream of Fyn, it is plausible that the Fyn in these neurons plays a role in other goal-directed behaviors such as mating. Finally, recently, Bocarsly et al. recently showed that enhanced D1R signaling in the dorsal striatum of mice is required for the consumption of alcohol despite negative consequences, and for the enhancement of alcohol-dependent hyperlocomotion (Bocarsly *et al*, 2019). Therefore, it plausible that Fyn in DMS dMSNs also contributes to other alcohol-dependent behaviors.

We report that stimulation of the cAMP/PKA signaling in DMS dMSNs activates Fyn signaling. To do so, we utilized anti-phosphoTyrosine417/420 antibodies which recognize the autophosphorylated active form of the kinase (Ingley, 2008; Resh, 1998). The anti-phosphoTyrosine417/420 antibodies do not distinguish between active Fyn and Src. However, we are confident that we detected the specific activation of Fyn and not Src as neither alcohol

exposure (Wang *et al*, 2007) nor D1R stimulation (Phamluong *et al*, 2017) activates Src in the dorsal striatum.

The data herein and previous findings (Morisot *et al*, 2017) suggest that Fyn exerts its action on alcohol drinking in dMSNs through the phosphorylation and activation of GluN2B. However, we cannot exclude the possibility that other molecular transducers of Fyn contribute to the development of alcohol use. For example, protein translation plays a critical role in mechanisms underlying AUD (Laguesse and Ron, 2019), and Fyn was shown to enhance protein translation in oligodendrocytes (White *et al*, 2008) and in neurons (Li and Gotz, 2017). Fyn was also shown to promote ERK1/2 phosphorylation in oligodendrocytes (Peckham *et al*, 2016), and NfkB signaling in microglia (Panicker *et al*, 2015); both signaling cascades have been linked to alcohol use (Crews *et al*, 2011; Ron *et al*, 2016). Finally, Fyn was reported to phosphorylate the metabotropic glutamate receptor 1 (mGluR1) (Jin *et al*, 2017) and the Collapsin Response Mediator Protein 2 (CRMP2) (Uchida *et al*, 2009), which have also been implicated in alcohol's actions in the brain (Liu *et al*, 2017; Olive, 2010). Exploring the contribution of these substrates and others in DMS dMSNs to the neuroadaptations underlying AUD merit further investigation.

Finally, it is highly likely that the activation of PKA in DMS dMSNs produces additional cellular consequences. For example, PKA phosphorylates the Striatal-Enriched Protein Tyrosine Phosphatase (STEP) (Goebel-Goody *et al*, 2012) resulting in the inhibition of the activity of the phosphatase (Goebel-Goody *et al*, 2012). STEP is an endogenous Fyn inhibitor and is responsible for the termination of Fyn activation (Goebel-Goody *et al*, 2012). We previously reported that alcohol increases PKA phosphorylation of STEP in the DMS, and that knockdown of STEP in the DMS (Darcq *et al*, 2014), or global knockout of the phosphatase (Legastelois *et al*, 2015) increases alcohol intake. It is therefore plausible that one of the consequence of PKA

activation in DMS dMSNs is the phosphorylation of STEP, enabling Fyn in these neurons to stay active for a prolonged period of time.

Using the DREAD/CNO methodology, we report that the activation of GαsDREADD in dMSNs but not in iMSNs initiates the consumption of alcohol. We further show that GαsDREADD-dependent increase in alcohol intake dependent, at least in part, on Fyn. To our knowledge this is the first study that provides a link between GαsDREADD activation and alcohol consummatory behavior. Cheng et al. previously showed that GαiDREADD-mediated inhibition of iMSNs enhances alcohol intake (Cheng *et al*, 2017). Stimulation of Gαs-couled receptors activates adenylate cyclase that increases cAMP production, whereas the stimulation of Gαi-coupled receptors inhibits adenylate cyclase activity and cAMP production (Neve *et al*, 2004). Thus, it is plausible that the development of alcohol consumption depends on the inhibition of cAMP signaling in iMSNs and on the activation of cAMP signaling in dMSNs.

Interestingly, neither Fyn knockdown in dMSNs nor CNO-dependent activation of GαsDREADD in DMS dMSNs alters the intake of saccharin suggesting that the contribution of this signaling cascade cannot be generalized to all consummatory behaviors. Our model (**Supplementary Figure 2**) suggests that alcohol activates Fyn in DMS dMSNs through dopamine-dependent D1R stimulation. Dopamine is not required for sucrose and saccharin consumption (Cannon and Palmiter, 2003). Thus, it is plausible that the requirement of dopamine release in the dorsal striatum is the differentiating factor between the molecular mechanisms underlying the consumption of alcohol vs. sweetened solutions.

Our data suggest that alcohol-dependent molecular adaptations are highly specific and are segregated to a subpopulation of neurons. Future studies are necessary to determine whether this

cell-type specificity is unique for the cAMP/PKA/Fyn signaling or that this is a common feature shared by some or all of the molecular targets of alcohol (Ron *et al*, 2016).

Although we provide a strong evidence linking PKA signaling in dMSNs to alcohol drinking behaviors, we cannot exclude the possibility that other cAMP effectors such as the guanine nucleotide exchange factor EPAC (exchange protein directly activated by cAMP) (Schmidt *et al*, 2013), and/or cyclic nucleotide-gated ion channels (CNGC) (Kaupp and Seifert, 2002) also contribute to the development of excessive alcohol use. Finally, further research is required to monitor cAMP production and PKA activation in behaving animals. Recent advances in the development of reporters for cAMP (Muntean *et al*, 2018), and PKA (Ma *et al*, 2018) will enable a spatial and temporal analysis of cAMP/PKA signaling in animals consuming alcohol.

Funding and Disclosure

This research was supported by the National Institute of Alcohol Abuse and Alcoholism,

UO1 AA023489 (D.R. and V.A.A).

The authors have no conflict of interest.

Acknowledgements

The authors thank Ellanor Whiteley for her contribution.

Author Contributions

Y.E designed experiments, contributed to the design of the experiments, the acquisition of data, data analysis, and to the preparation of the manuscript. N.M, S.A.S and K.P contributed to the design of the experiments, the acquisition of the data and data analysis. M.F.A. contributed to the acquisition of data. V.A.A contributed to the conception of the study. D.R. contributed to the conception of the study, the design of the experiments and wrote the manuscript.

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Figure Legends

Figure 1 Knockdown of Fyn in DMS dMSNs but not in iMSNs attenuates the development of alcohol but not saccharin intake

(a) The DMS of Drd1-Cre-Ai14 mice was infected bilaterally with a lenti-virus expressing FLEX-shFyn or FLEX-SCR (10^7pg/ml, 1.2 µl per side), and viral infection was evaluated 4 weeks later. Infection was visualized on a EVOS FL microscope (Life Technologies, Carlsbad, CA), scale: 2x. (**b**) Timeline of experiments. Four weeks after surgery, Drd1-Cre-Ai14 (**c-d**) or A2A-Cre-Ai14 mice (**e-f**) underwent 4 weeks of home cage IA20%2BC drinking paradigm, and alcohol and water intake were recorded 4 hours after the initiation of the alcohol drinking session. At the completion of the alcohol drinking regimen, Drd1-Cre-Ai14 mice had access to water only for two weeks, followed by one week of home cage intermittent access to 0.03% saccharin. Saccharin and water intake were measured at the 4 hours time point. (**c-d**) Knockdown of Fyn in the DMS of Drd1-Cre-Ai14 mice attenuates alcohol intake (**c)** (two-tailed unpaired t-test, t=2.491, p=0.0234), and preference (**d**) (two-tailed unpaired t-test, t=3.325, p=0.0209). (**e-f**) Knockdown of Fyn in the DMS of A2A-Cre-Ai14 does not affect alcohol intake (**e**) (two-tailed

unpaired t-test, t=0.583, p=0.5692,) and preference (**f**) (two-tailed unpaired t-test, t=1.024, p=0.3232). (**g-h**) Knockdown of Fyn in the DMS of Drd1-Cre-Ai14 mice does not alter saccharin intake (**g**) (two-tailed unpaired t-test, t=0.2163, p=0.8327), and preference (**h**) (two-tailed unpaired t-test, t=0.4592, p=0.6550). Data are presented as individual data points and mean ± SEM. * $p<0.05$. (**c-d**) FLEX-shFyn n=8, FLEX-SCR n=11. (**e-f**) FLEX-shFyn n=8, FLEX-SCR n=8. **(g-h**) FLEX-shFyn n=7, FLEX-SCR n=6.

Figure 2 Stimulation of GαsDREADD in DMS dMSNs activates Fyn/GluN2B signaling

(a) The DMS of Drd1-Cre mice was infected bilaterally with AAV-DIO-rM3D(Gs)-mCherry (10^{13} vg/ml, 1 μl per side), and infection was evaluated 4 weeks later. Infection was visualized on a EVOS FL microscope (Life Technologies, Carlsbad, CA), scale: 2x. (**b**) Timeline of experiment. Four weeks after surgery, vehicle (0.5% DMSO) or CNO (3 mg/kg) was systemically administered, and the DMS and the DLS were dissected 30 minutes later. The phosphorylation of Fyn (**c-d**) and GluN2B (**e-f**) were measured by Western blot analysis using anti-Tyr417/420[Src/Fyn] and anti-Tyr1472[GluN2B] antibodies, respectively. Total protein levels of Fyn (**c-d**), GluN2B (**e-f**) and actin, which was used as a loading control, were measured in parallel. Representative image of Fyn (**c**) and GluN2B (**e**) phosphorylation in the DMS (top) and DLS (bottom). (**d,f**) Data are presented as the individual data points and mean densitometry values of the phosphorylated protein divided by the densitometry values of the total protein ± SEM and expressed as % of vehicle. Activation of GαsDREADD in DMS dMSNs increases Fyn

activation (**d**) (two-tailed unpaired t-test, t=7.148, p<0.001, and GluN2B phosphorylation (**f**) (two-tailed unpaired t-test, t=6.948, p<0.001). n=10 per treatment. ***p<0.001.

Figure 3 Stimulation of GαsDREADD in DMS dMSNs but not in iMSNs increases alcohol but not saccharine intake

(**a**) Timeline of experiment. The DMS of Drd1-Cre or A2A-Cre mice was infected bilaterally with AAV-DIO-rM3D(Gs)-mCherry (10^{13} vg/ml, 1 μl per side). (**b**-e) Four weeks after surgery, Drd1-Cre (**b-c**) or A2A-Cre (**d-e**) received a systemic administration of vehicle (0.5% DMSO) or CNO (3 mg/kg) 15 minutes before the beginning of the first 10% alcohol drinking session, and alcohol intake was measured 4 hours later. (**f-g**) At the completion of the alcohol drinking regimen, Drd1-Cre-Ai14 mice had access to water only for two weeks. Afterwards, mice received a systemic administration of vehicle (0.5% DMSO) or CNO (3 mg/kg) 15 minutes before the beginning of a 0.03% saccharine drinking session, and saccharine and water intake were measured 4 hours later. (**b-c**) Activation of GαsDREADD in DMS dMSNs increases alcohol intake (**b**) (two-tailed paired t-test, t=3.501, p=0.003), and preference (**c**) (two-tailed paired t-test, t=2.241, p=0.0395). (**d-e**) Activation of GαsDREADD in the DMS of A2A-Cre does not affect alcohol intake (**d**) (two-tailed paired t-test, t=1.482, p=0.1724,) and preference (**e**) (two-tailed paired t-test, t=0.1579, p=0.878). (**f-g**) Activation of GαsDREADD in DMS dMSNs does not alter saccharine intake (**f**) (two-tailed paired t-test, t=0.2501, p=0.8097), and preference (**g**) (two-tailed paired t-test, t=0.5072, p=0.6276). Data are presented as individual data points and mean ± SEM. (**b-c**) n=17 per treatment, (**d-e**) n=10 per treatment, (**f-g**) n=8 per treatment. * p<0.05, ** p<0.01.

Figure 4 Enhancement of alcohol intake by GαsDREADD stimulation in DMS dMSNs requires Fyn

(**a**) Timeline of experiment. The DMS of Drd1-Cre mice was infected bilaterally with AAV-DIO-rM3D(Gs)-mCherry (10^{13} vg/ml, 1μl per side). Four weeks after surgery, animals received a systemic administration of vehicle (20% HPBCD) or AZD0530 (10 mg/kg) 3 hours before the beginning of a 10% alcohol drinking session. Subsequently, animals received a systemic administration of vehicle (0.5% DMSO) or CNO (3 mg/kg) 15 minutes before the beginning of the drinking session, and alcohol and water intake were measured 4 hours later. (**b**) AZD0530 blocks GαsDREADD-dependent enhancement of alcohol intake (Two-way RM ANOVA, effect of AZD0530: $F_{1,10}$=9.177, p=0.0127; effect of CNO: $F_{1,10}$=19.43, p=0.0013). Data are presented as individual values and mean ± SEM. n=11. ** p<0.01. ns: non-significant.

Figure 1

Knockdown of Fyn DMS dMSNs but not in iMSNs attenuates the development of alcohol but not Saccharin intake

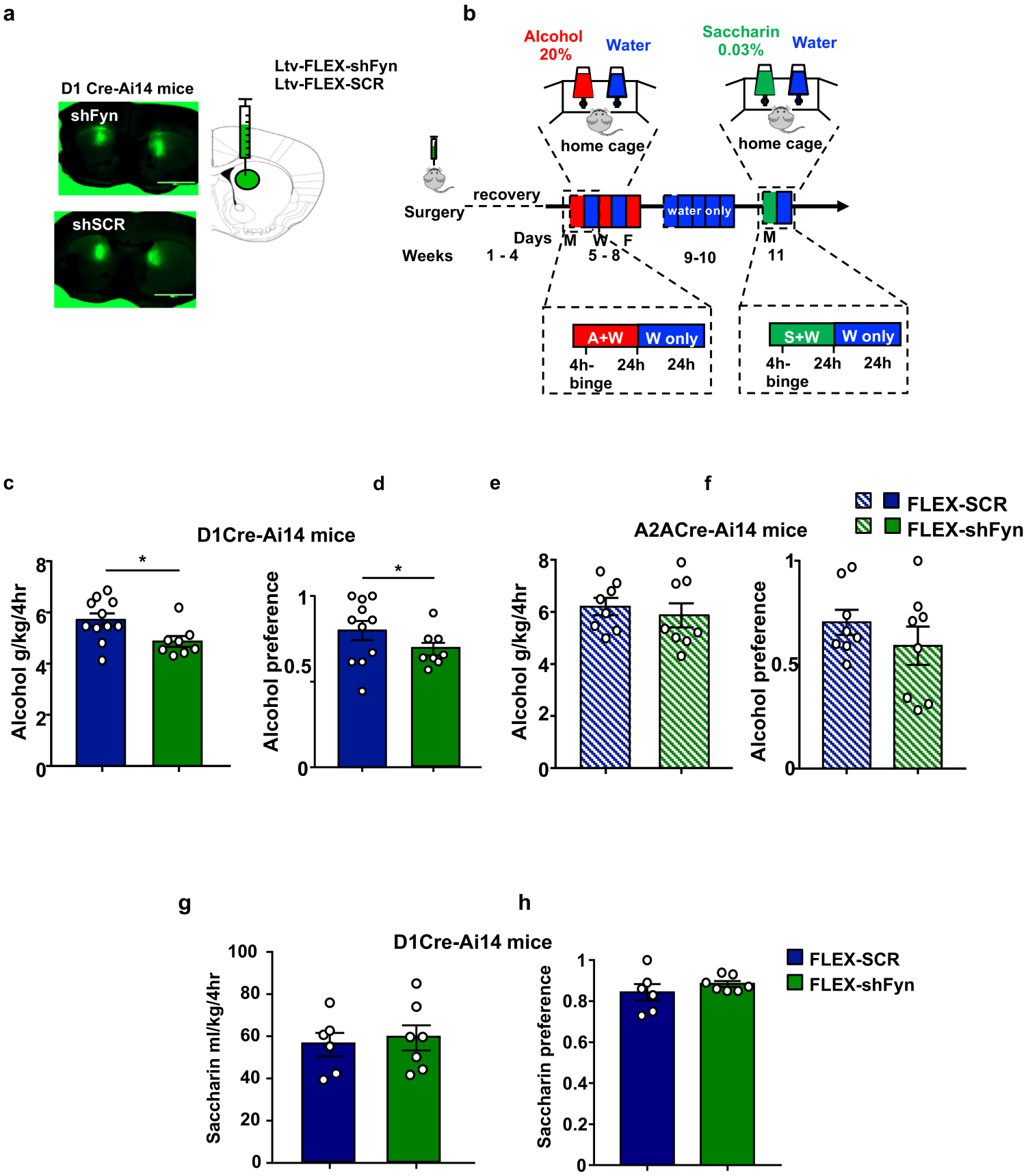


Figure 2

Stimulation of GαsDREADD in the DMS dMSNs activates Fyn/GluN2B/CamKII signaling

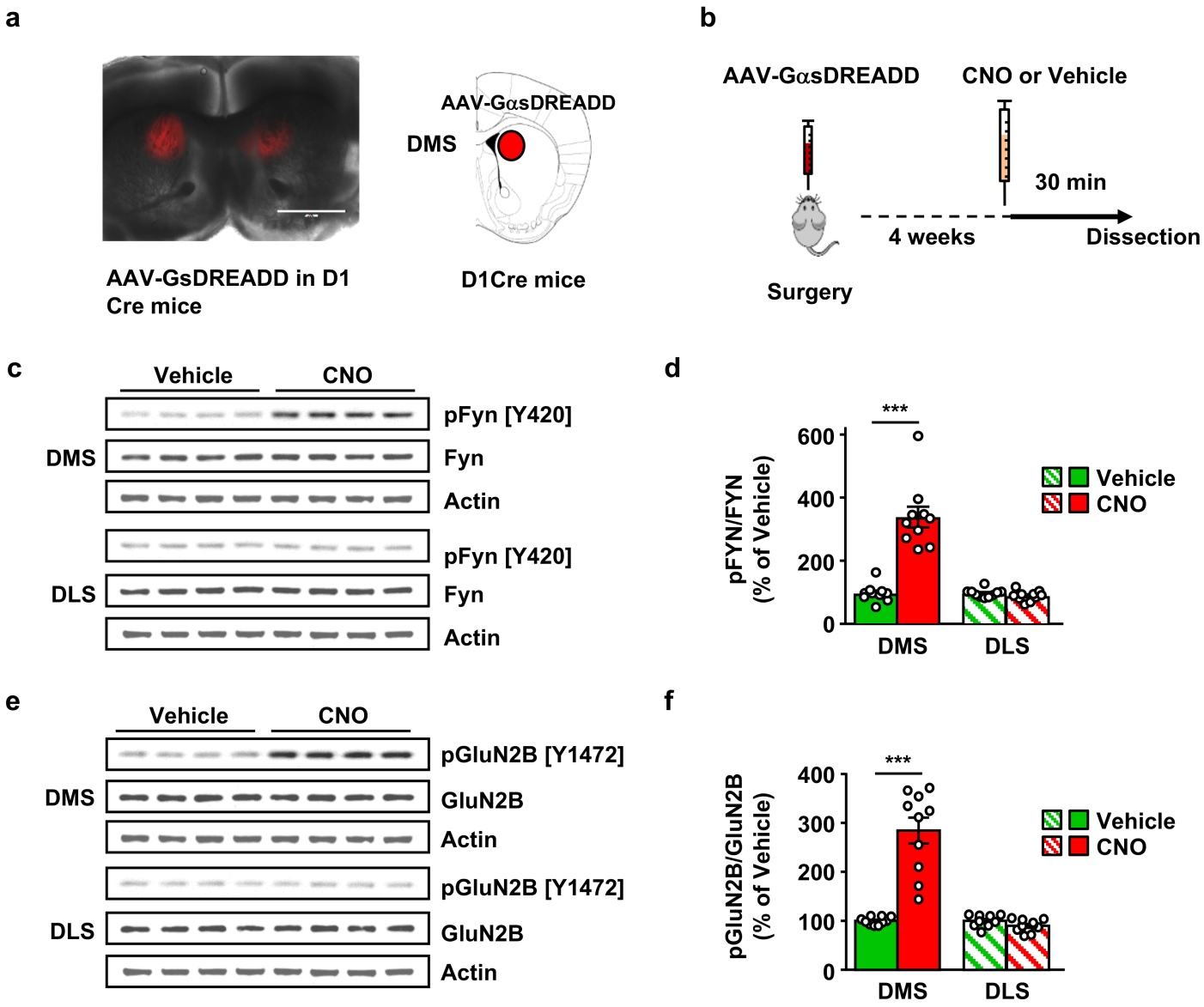


Figure 3

Stimulation of GαsDREADD in DMS dMSNs but not in iMSNs increases alcohol but not Saccharin intake

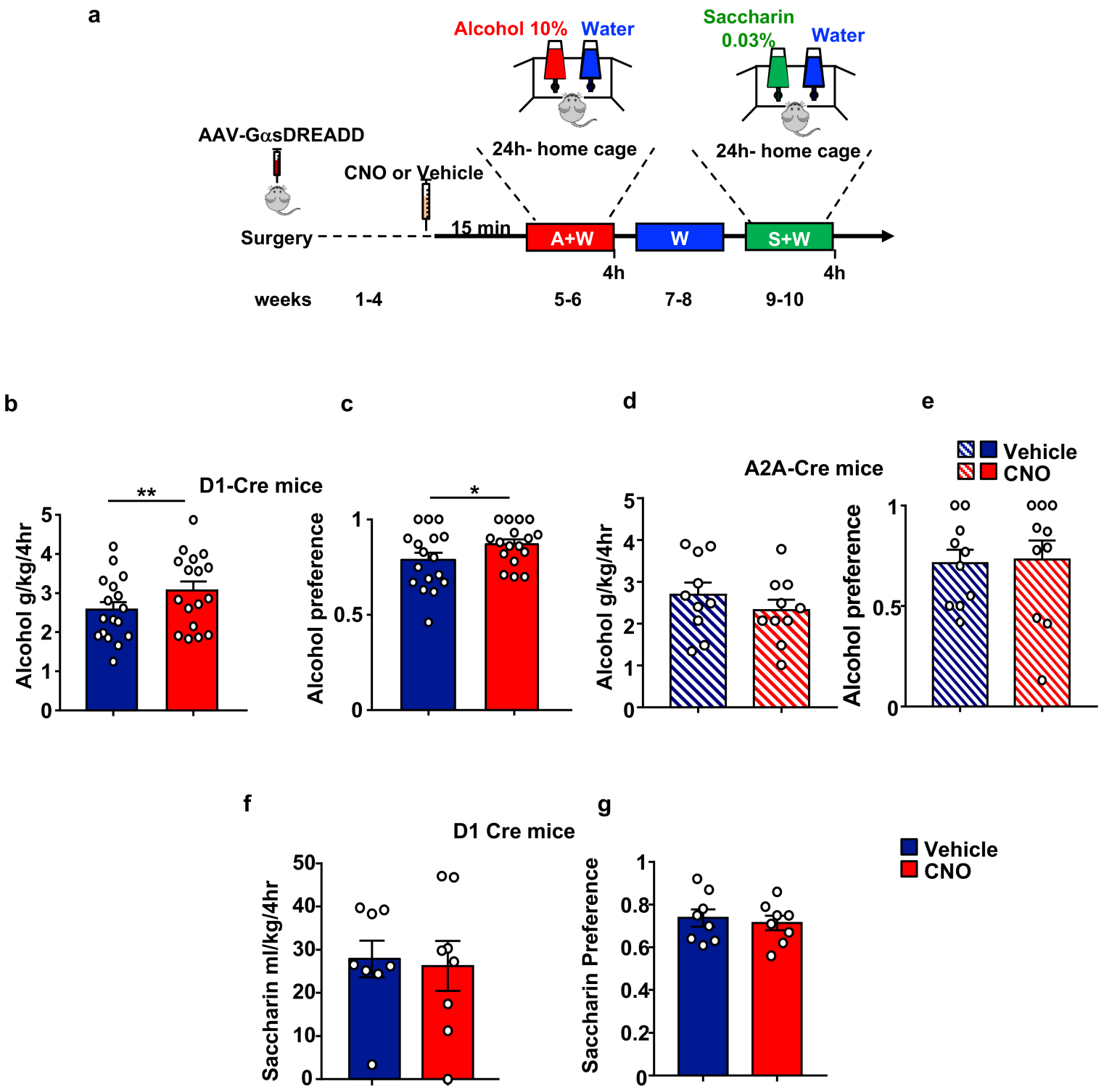
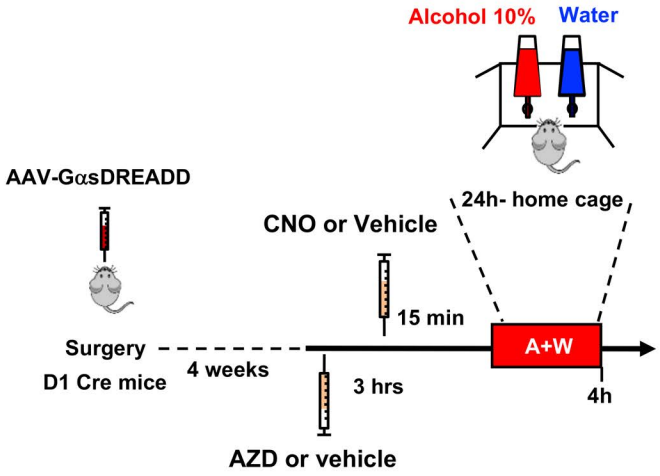
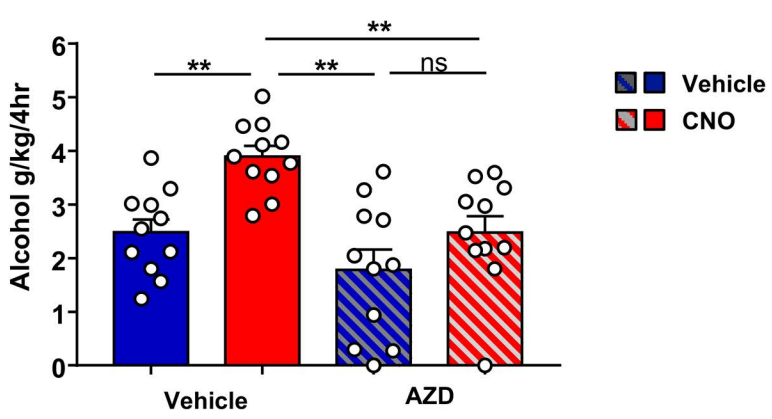


Figure 4

a


Alcohol 10%
Water
AAV-GαsDREADD
24h- home cage
CNO or Vehicle
15 min
Surgery
D1 Cre mice
4 weeks
3 hrs
A+W
4h
AZD or vehicle

b


Alcohol g/kg/4hr
0
1
2
3
4
5
6
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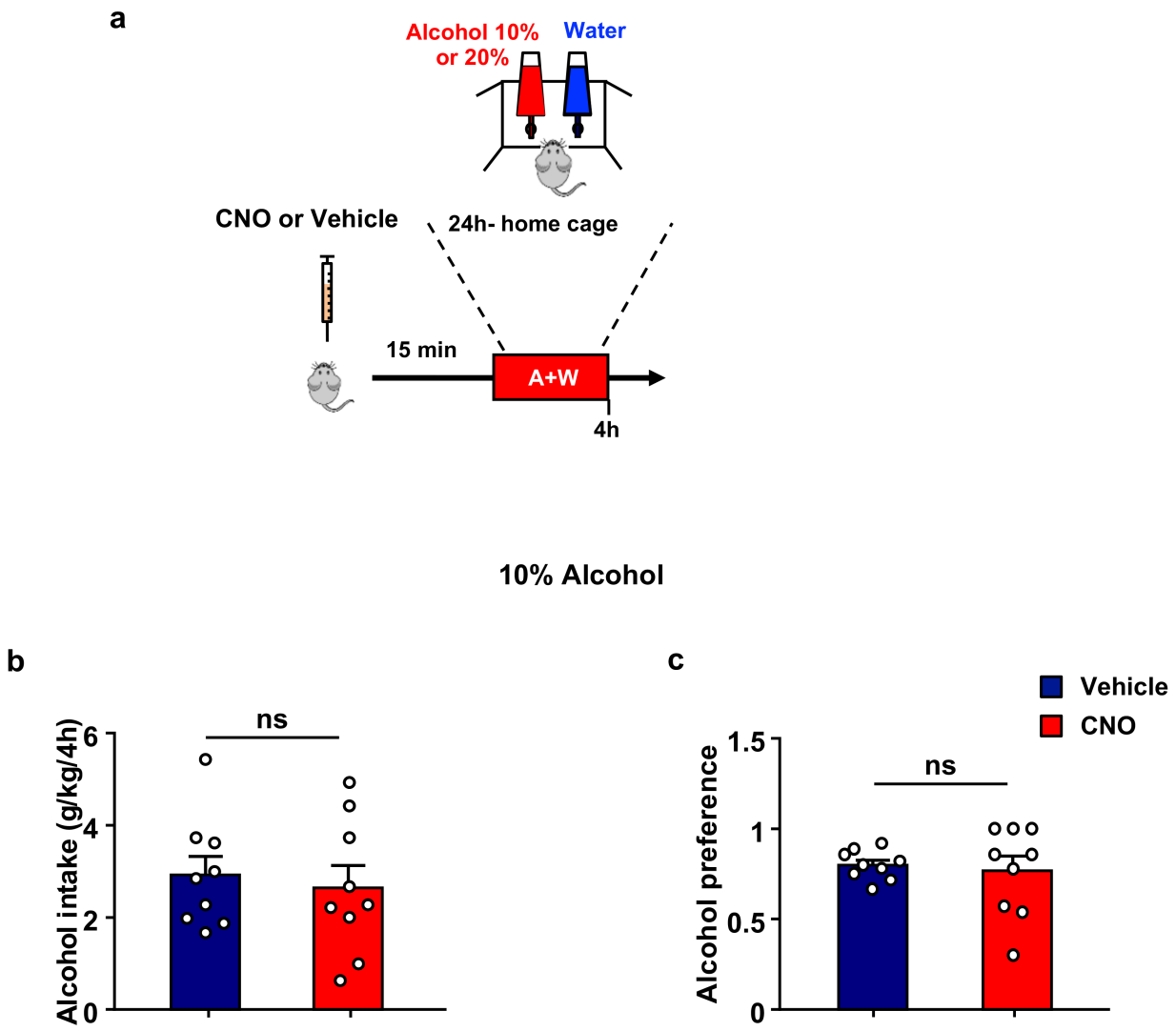
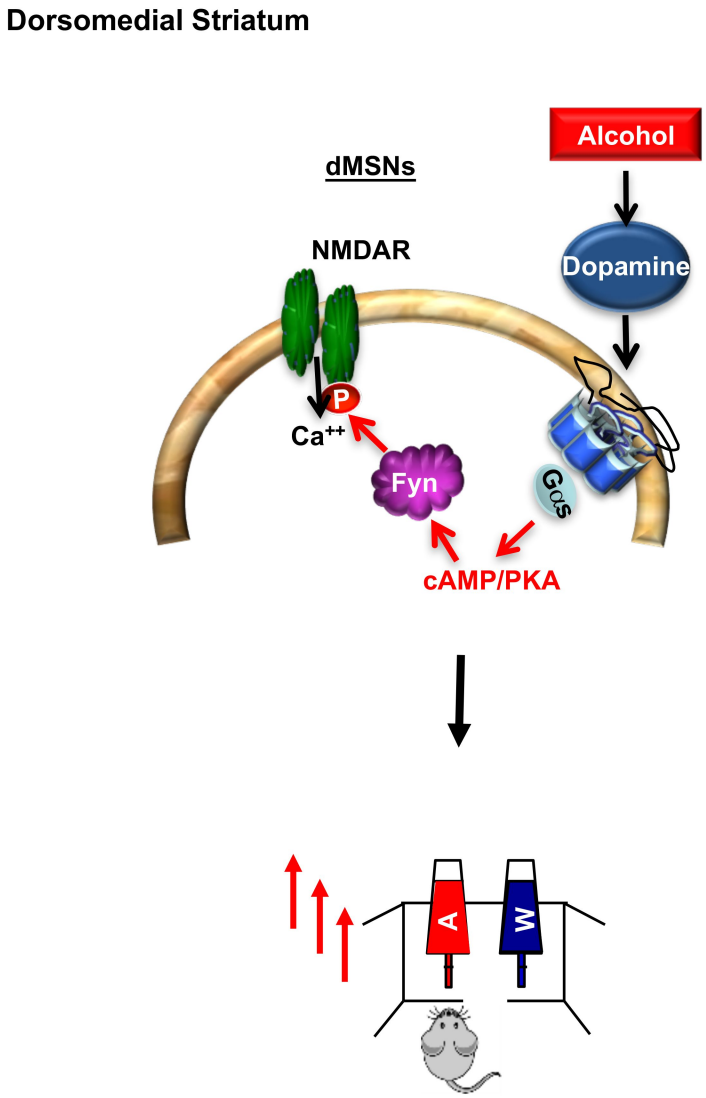
ns
Vehicle
AZD
Vehicle
CNO

Supplementary Figure 1



Supplementary Figure 2

Activation of Fyn signaling in dMSNs promotes alcohol consumption