Dorsal raphe nuclei to anterior cingulate cortex 5-HTergic neural circuit is implicated in consolation-like behaviors and sociability in mandarin voles

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Running title: DR→ACC 5-HTergic neural circuit modulate consolation

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ABSTRACT

Consolation is a common empathetic response in humans and some social animals, but the neural mechanisms underlying this behavior are not well characterized. Here, by using socially monogamous mandarin voles, we found that optogenetic or chemogenetic inhibition of 5-HTergic neurons in the dorsal raphe nuclei (DR) or optogenetic inhibition of 5-HT terminals in the anterior cingulate cortex (ACC) significantly decreased the allogrooming time in the consolation test and reduced sociability in the three-chamber test. Fiber photometry results showed that the release of 5-HT within the ACC and the activity of DR neurons were significantly increased when allogrooming and social approaching occurred. Finally, we found that the activation of 5-HT1A receptors in the ACC was sufficient to reverse consolation and sociability deficits induced by the chemogenetic inhibition of 5-HTergic neurons in the DR. Our study provided first direct evidence that DR→ACC 5-HTergic neural circuit is implicated in consolation-like behaviors and sociability in mandarin voles.

Keywords: Consolation; Empathy; Anterior cingulate cortex; Dorsal raphe nuclei; Serotonin; Fiber photometry

INTRODUCTION

Consolation behavior, which is referred to as increased in affiliative contact toward a distressed individual by an uninvolved bystander, is an important component of the social capabilities of humans (de Waal & Preston, 2017; Field, Diego, & Hernandez-Reif, 2009). Impaired consolation or empathy has been frequently observed in many psychiatric diseases, such as depression, autism, and schizophrenia (Young, Parsons, Stein, & Kringelbach, 2015). As a higher level of empathy-like behavior, consolation has long been assumed to exist in species possessing complex cognitive functions, such as humans, apes, dolphins and elephants (de Waal & Preston, 2017; Perez-Manrique & Gomila, 2018); but recent studies have indicated that it also exists in some socially-lived rodents, such as prairie voles (Burkett et al., 2016), mandarin voles (L. F. Li, Yuan, He, Wang, et al., 2019) and rats (Knapska, Mikosz, Werka, & Maren, 2010).

Currently, studies of the neural mechanisms underlying consolation and other empathy-like behaviors primarily focus on oxytocin systems (Burkett et al., 2016; L. F. Li, Yuan, He, Wang, et al., 2019). However, as a complex social behavior, consolation may require the coordinated actions of numerous neuromodulators and neurotransmitters. 5-HT is an evolutionarily ancient neurotransmitter that has long been implicated in a variety of emotional disorders (Faye et al., 2020; Garcia-Garcia et al., 2018; Meneses & Liy-Salmeron, 2012). According to recent studies, 5-HT transmission is also involved in a series of social behaviors such as social interaction (Walsh et al., 2018), social reward and aggression (Dolen, Darvishzadeh, Huang, & Malenka, 2013). Regarding empathy, a recent study revealed an association between the salivary 5-HT levels and the empathic abilities of people (Matsunaga et al., 2017); a polymorphism in the promoter region of the serotonin transporter gene has been linked to individual differences in empathy (Gyurak et al., 2013); MDMA (±3,4-methylenedioxymethamphetamine, better known as the recreational drug ''ecstasy''), which is well known to stimulate a feeling of closeness and empathy in its users (Carlyle et al., 2019), had been confirmed to robustly increase the

62 release of 5-HT in an activity-independent manner (Heifets & Malenka, 2016). In animal
63 studies, Kim, et al. found that microinjection of 5-HT into the anterior cingulate cortex
64 (ACC) impaired vicarious fear and altered the regularity of neural oscillations in mice
65 (Kim et al., 2014). Our recent study indicated that 5-HT1A receptors within the ACC are
66 involved in consolation deficits induced by chronic social defeat stress in mandarin voles
67 (L. F. Li, Yuan, He, Ma, et al., 2019). However, to our knowledge, direct evidence for an
68 association between 5-HT and consolation has yet to be obtained.
69 Dorsal raphe nuclei (DR) are a main source of 5-HT neurons and provide 70% of
70 5-HTergic projections in the forebrain (Fu et al., 2010; Luo, Zhou, & Liu, 2015). DR
71 5-HTergic neurons form dense, broad and bidirectional neural connections with a broad
72 range of forebrain and limbic structures, including the ACC (Celada, Puig, & Artigas,
73 2013; Charnay & Leger, 2010), which is a central hub for various types of empathy-like
74 behaviors. Therefore, direct modulation of the DR→ACC 5-HTegic circuit to investigate
75 its function role in consolation-like behaviors is interesting and meaningful.
76 The released 5-HT binds to pre- and postsynaptic receptors. To date, at least 14
77 different 5-HT receptor subtypes have been identified in the brain (Artigas, 2013).
78 Among which, 5HT1AR and 5HT2AR are the two main subtypes that are expressed at
79 high levels in the prefrontal cortex (Carhart-Harris & Nutt, 2017; Santana & Artigas,
80 2017). The distribution, signaling pathways and functions of these two receptors are
81 substantially different, and both receptors play critical roles in modulating cortical
82 activity and neural oscillations (Celada et al., 2013). Previous studies had indicated that
83 5HT2AR gene single nucleotide polymorphisms are associated with empathy-related
84 social communication abilities (Gong, Liu, Blue, Li, & Zhou, 2015), and a 5HT2AR
85 agonist increases emotional empathic ability (Dolder, Grunblatt, Muller, Borgwardt, &
86 Liechti, 2017). However, in animal studies by Kim, et al., blockade of serotonin receptors
87 in the ACC did not affect the observational fear response in mice (Kim et al., 2014).
88 Clearly, the specific functions of 5HT1AR and 5HT2AR in empathy-like behaviors still

require further examination.

The mandarin vole (*Microtus mandarinus*) is a socially monogamous rodent that is widely distributed across China (He et al., 2019). As shown in our previous studies, this species is capable of displaying consolation-like behaviors upon exposure to a distressed partner (L. F. Li, Yuan, He, Ma, et al., 2019; L. F. Li, Yuan, He, Wang, et al., 2019). In the present study, we first investigated the function of DR→ACC 5-HTegic circuits in consolation-like behaviors using optogenetic and chemogenetic approaches. To provide more direct evidence, we then monitored ACC 5-HT release and DR neuron activities during this behavior by using in vivo fiber photometry. Finally, we used chemogenetics plus pharmacological approaches investigated which types of 5-HT receptor in the ACC are involved in consolation-like behaviors in Mandarin Voles. In order to investigate any potential sex differences during these processes, both male and female subjects were involved in our study.

RESULTS

Optogenetic inhibition of DR 5-HT neurons in the DR→ACC neural circuit impaired consolation and reduced sociability

We first determined the 5-HTergic projection relationship between the DR and ACC in Mandarin Voles. For this experiment, the retrograde tracer CTB was injected into the ACC, followed by immunofluorescence staining of the DR sections with TPH2, a marker of 5-HTergic neurons. A substantial number of TPH2+ neurons colocalized with CTB, indicating the presence of DR→ACC 5-HTergic projections (Figure 1—figure supplement 1). We then used a novel dual-virus optogenetics approach to explore the function of DR→ACC 5-HTergic circuit in consolation-like behaviors and sociability, where double-floxed AAV-DIO-ChR2-mCherry (DIO-ChR2) or AAV-DIO-eNpHR3.0-mCherry (DIO-eNpHR3.0) were injected into the DR and retro-AAVs containing TPH2 promoter and Cre element (rAAV(Retro)-TPH2-Cre) were injected into the ACC (Figure 1A). This

virus strategy ensures that opsins (excitatory CHR2 or inhibitory eNpHR3.0) mainly expressed within the DR→ACC 5-HTergic circuit. Immunohistochemical staining showed that more than 75% of mCherry-labeled neurons expressed TPH2 in both male and female voles, and more than 80% of TPH2+ cells coexpressed mCherry (Figure 1—figure supplement 2). In the electrophysiological study, we found that the DR neurons reliably responded to pulses of 473 (activation)/593 (inhibition) nm light stimuli (Figure 1D-E). These results indicate the availability of this virus strategy.

To test whether modulation of DR 5-HT neuron activity alters consolation and sociability, five weeks after the virus injection, optic fibres were implanted above the DR (Figure 1A-B). In CHR2-expressing animals, activation of 5-HTergic neurons in the DR did not significantly affect allogrooming time in the consolation test (Figure 1G), sociability in the 3-chamber test (Figure 1I) and time spent in central area in the open-field test (Figure 1K) in both male and female voles. However, in the eNPHR3.0-expressing animals, optogenetic inhibition of 5-HTergic neurons in the DR significantly reduced the time spent in allogrooming towards a shocked partner (Figure 1H), which may indicate an impairment in consolation (Male: $t_{(14)} = 11.128$; Female: $t_{(14)} = 6.327$; all $P < 0.01$). The treatment also significantly reduced the chasing time ($t_{(14)} = 8.662$, 8.078, male ahead, all $P < 0.01$; Figure 1—figure supplement 3B), but did not significantly affect the self-grooming time ($t_{(14)} = 0.470$, 1.118, $P = 0.645$, 0.282; male ahead; Figure 1—figure supplement 3D). Furthermore, in the 3-chamber test, two-way ANOVA showed that 'light stimulation' exerted significantly effects on sociability in both male and female voles ($F_{(1,28)} = 12.286$, 10.838; all $P < 0.01$; male ahead). Optogenetic inhibition of DR 5-HT neurons reduced the social preference ratio in the eNPHR3.0-expressing voles (off *vs.* on; all $P < 0.01$; Figure 1J right bars), but had no effect on control voles (off *vs.* on; all $P > 0.05$ Figure 1J left bars). In the 5-min open-field test, the treatments ('virus' and 'light') did not significantly alter the time spent in central area (Figure 1L) and the total distance traveled (Figure 1—figure supplement 3F). In the following experiment, we

found the photoinhibitory effect disappeared within 24 h (Figure 1—figure supplement 4).

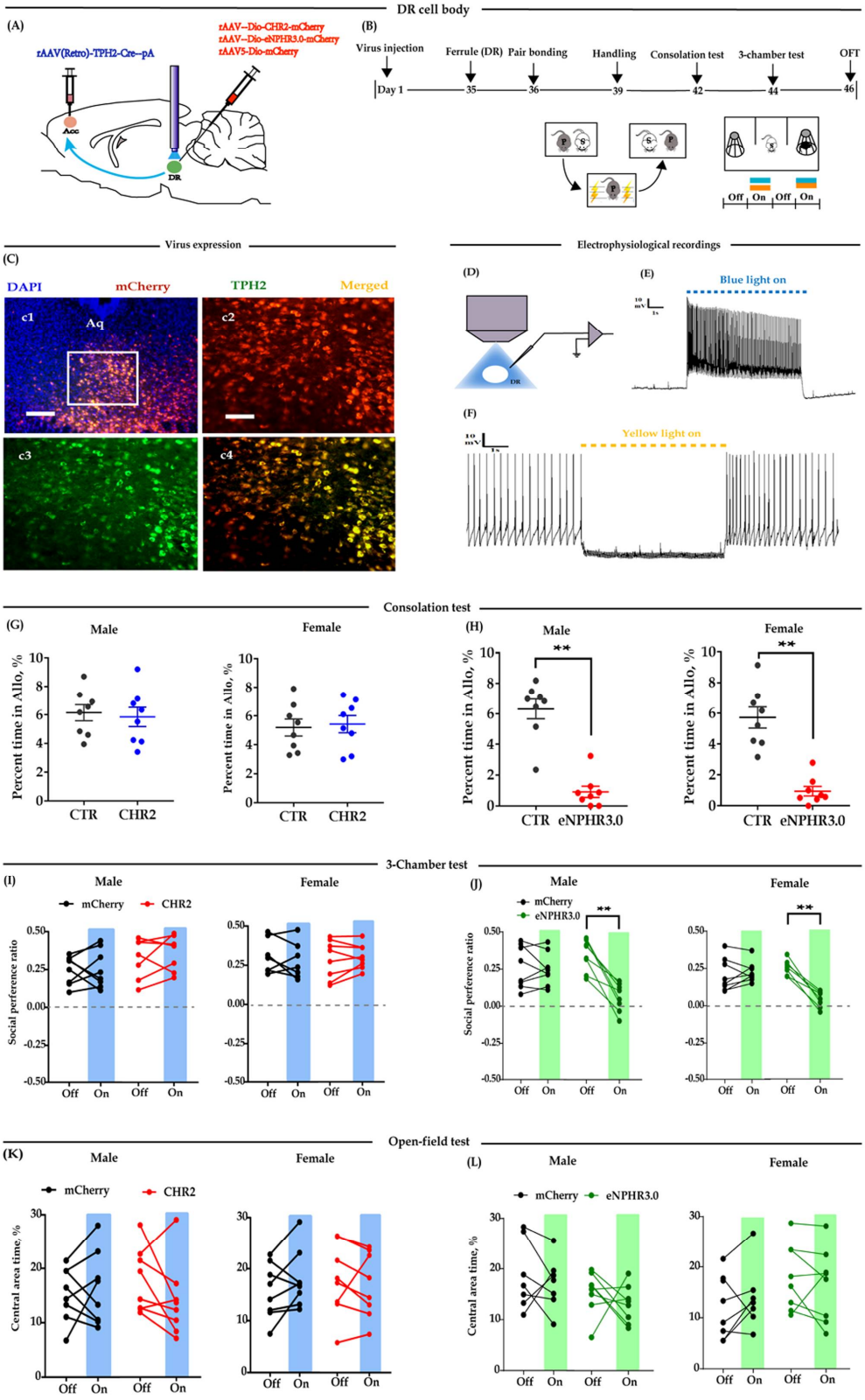
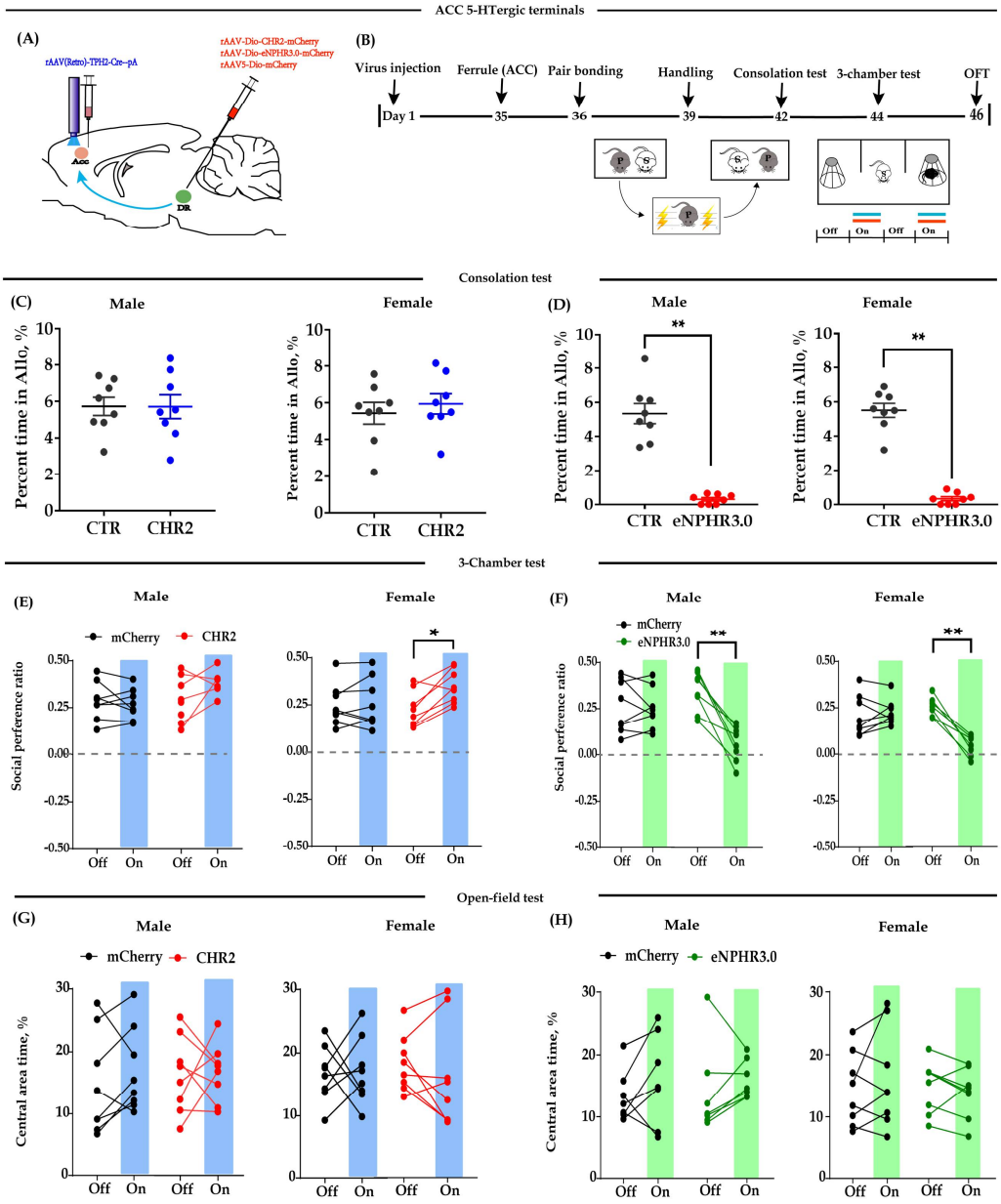


Figure 1. Optogenetic bidirectional modulation of 5-HT neuron in the DR in the DR→ACC neural circuit. (A): Schematic of optogenetic manipulation; (B): timeline of experiments; (C): immunohistological image showing virus expression in the DR (c1) and amplified images in the left box showing the mCherry, TPH2 and the colocaliztion of the two ('c2-c4, × 200); (D): electrophysiological recordings model; (E & F): representative traces from electrophysiological recordings showing photostimulation (E) and photoinhibition of a 5-HT neuron (F); (G-L): quantification of allogrooming time in the consolation test (G & H); social preference ratio in the three-chamber test (I & J) and time spent in the central area in the open-field test (K & L) during optogenetic modulations. Data are presented as mean ± SE, n = 7–8 in each group, $^{**}P < 0.01$. For G-H, two-tailed independent t tests; for I-L, two-way ANOVA with Bonferroni *pos-hoc* test. ACC: anterior cingulate cortex; CTR: control; DR: dorsal raphe nucleus; TPH2: tryptophan hydroxylase 2.

Optogenetic inhibition of ACC 5-HT terminals in the DR→ACC neural circuit similarly impaired consolation and reduced sociability

The modulation of 5-HT neurons may affect other neurons in the DR, and thus confound behavioral performance. In subsequent experiments, we placed optic fibers in the ACC and investigated whether the direct modulation of 5-HT terminals in this region would exert the same effects (Figure 2A). Similarly, optogenetic activation of DR-ACC 5-HT terminals did not significantly alter the behavioral performance in the consolation test, except that CHR2-expressing females spent more time chasing after their shocked partners (Figure 2—figure supplement 1A; $t_{(14)}$ = 2.458; P = 0.028). In the 3-chamber test, a significant interaction of 'light × virus' was observed in females ($F_{(1,28)}$ = 4.646, P = 0.04), and the *post hoc* comparison showed that optogenetic activation of 5-HT terminals in the ACC increased the social preference ratio in CHR2-expressing females but had no effect on control females (off *vs*. on, P = 0.012; Figure 2E, right bars), but had no effect on female control (off *vs*. on, P = 0.725, Figure 2E, left bars).

Optogenetic inhibition of DR-ACC 5-HT terminals significantly reduced the time spent in allogrooming (Figure 2D) and chasing the shocked partners in the consolation test (male: $t_{(14)}$= 8.441, 7.201; female: $t_{(14)}$ = 12.174, 8.695; all P < 0.01; allogrooming time ahead; Figure 2—figure supplement 1B), but had also no effect on selfgrooming time ($t_{(14)}$ = 0.438, 0.654; P = 0.799, 0.506; males ahead; Figure 2—figure supplement 1D). Similarly, the inhibitory effect disappeared within 24 h (Figure 2—figure supplement 2). In the 3-chamber test, two-way ANOVA showed a significant effect of 'light stimulation' on sociability in both male and female voles ($F_{(1,28)}$ = 9.8, 36.7; all P < 0.01; male ahead). Optogenetic inhibition of DR-ACC 5-HT terminals significantly reduced the social preference ratio in the eNPHR3.0-expressing animals (Figure 2F, all P < 0.01; off *vs*. on). Similarly, behavioral performance in the open-field test was unaffected by the manipulation of 5-HT terminals in both CHR2- and eNPHR3.0-expressing animals (Figure 2G, H; Figure 2—figure supplement 1E, F).



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186 **Figure 2. Optogenetic bidirectional modulation of 5-HT terminals within the ACC**

187 **in the DR→ACC neural circuit.** (A): Schematic of optogenetic manipulation; (B):

188 timeline of experiments; (C-H): quantification of time spent in allogrooming in the

189 consolation test (C & D), sociability in the three-chamber test (E & F) and time spent in

190 the central area in the open-field test (G & H) during optogenetic modulations. Data are

191 presented as mean ± SE, n = 7–8 in each group, *P < 0.05, **P < 0.01. For C-D, two-tailed

192 independent t tests; for E-H two-way ANOVA with Bonferroni *pos-hoc* test. ACC:

193 anterior cingulate cortex; DR: dorsal raphe nucleus; CTR: control.

Chemogenetic inhibition of DR 5-HT neurons in the DR→ACC neural circuit impaired consolation and reduced sociability

To confirm above optogenetic results over a longer time frame, we used a chemogenetic approach to selectively express 'Gq-DREADD' or 'Gi-DREADD' in the DR 5-HT neurons by injecting AAV-DIO-hM4Dq-mCherry (Gq-DREADD) or AAV-DIO-hM4Di-mCherry (Gi-DREADD) into the DR and rAAV(Retro)-TPH2-Cre into the ACC (Figure 3A). Immunohistochemical staining revealed that more than 65% TPH2 labeled neurons were infected by mCherry virus and more than 60% TPH2+ cells co-expressed mCherry (Figure 3—figure supplement 1). To determine whether the ligand CNO can activate or inhibit DR 5-HT neurons, whole-cell current-clamp recordings were performed. The results showed that addition of 10 μM CNO remarkably increased the number of action potentials in the Gq-DREADD-transfected neurons (Figure 3D). In contrast, CNO caused a significantly decrease in the number of spikes (Figure 3E) and increased the spike rheobase during current step injections in Gi-DREADD-transfected neurons (Figure 3F). These results indicate the specificity and applicability of this virus strategy.

In subsequent behavioral studies, we found chemogenetic inhibition of DR 5-HT neurons significantly reduced the allogrooming time of both sexes ($t_{(6)}$ = 5.0, $t_{(7)}$ = 4.2, all $P < 0.01$ *vs.* vehicle control, male ahead; Figure 3G), but did not affect the chasing and self-grooming times (Figure 3—figure supplement 2A&B) in the consolation test. Similarly, CNO-treated Gi-DREADD-expressing voles showed reduced sociability in the 3-chamber test ($t_{(6)}$ = 2.8, $P < 0.05$; $t_{(7)}$ = 6.1, $P < 0.01$; male ahead; Figure 3H). The CNO treatment did not exert significant effects on behavioral performance and distance traveled in the open-field test (Figure 3I; Figure 3—figure supplement 2C).

Based on the results of the chemogenetic and optogenetic experiments described above, inhibition of DR→ACC 5-HTergic circuit activity is sufficient to impair consolation and social abilities.

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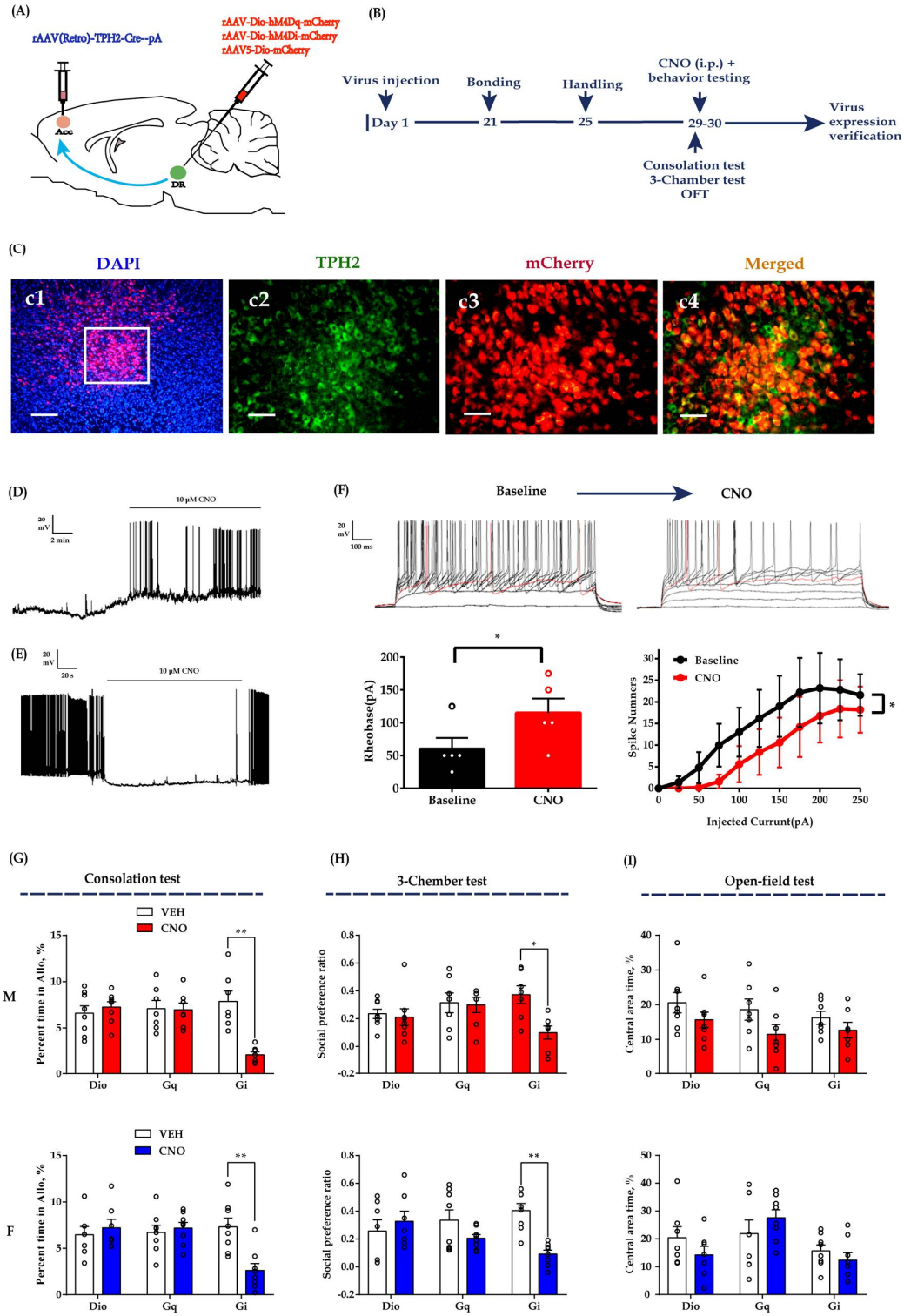

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Figure 3. Chemogenetic modulation of DR 5-HT neuron activities in the DR→ACC neural circuit. (A): Schematic of chemogenetical manipulations; (B): timeline of experiments; (C): immunohistological image showing virus expression in the DR (c1) and amplified images in the left white box showing the mCherry, TPH2 and the colocaliztion of the two (c2-c4, × 200); (D): representative trace from a Gq-DREADD neuron; (E): representative trace from a Gi-DREADD-transfected neuron; (F): quantification of spike rheobase and spike numbers under current step injections in Gi-DREADD-transfected neurons (n = 5 neurons, *P < 0.05); (G-I): quantification of allogrooming time in the consolation test (G); social preference ratio in the three-chamber test (H) and time spent in the central area in the open-field test (I) in male (upper panels) and female (down panels) voles. Data are presented as mean ± SE, n = 7–8 in each group, *P < 0.05, **P < 0.01 compared with vehicle control. For F, two-tailed independent t tests; for G-I, paired t test. ACC: anterior cingulate cortex; DR: dorsal raphe nucleus; TPH2: tryptophan hydroxylase 2; M: male; F: female.

Allogrooming and social approach were associated with increased DR neuron activity and ACC 5-HT release

To further establish the potential relevance of DR→ACC 5-HTergic circuit in consolation like behaviors, we then injected AAV-GCaMP6 into the DR and AAV-5HT2.1 into the ACC at the same time. Ten days latter, fibers were implanted above the injection sites. We then used multi-channel fiber photometry to simultaneously record DR fluorescent calcium transients and ACC 5-HT release in the consolation behavior test (Figure 4A). Immunohistochemical staining revealed that more than 60% GFP neurons co-expressed TPH2 in the DR (Figure 4—figure supplement 1) and the traces of 5-HT and calcium signals correlated well during the record (Figure 4D), suggesting that this strategy was feasible and effective.

During the behavioral test, we found the release of 5-HT and the activity of DR neurons were significantly increased when allogrooming (5-HT release: $t_{(4)} = –4.9, –4.7$, $P < 0.01$; DR activities: $t_{(4)} = –4.7$, $P < 0.01$, $t_{(4)} = –4.2$, $P < 0.05$; males ahead; Figure 4E) and social approaching occurred (5-HT release: $t_{(4)} = –3.1, –3.3$, $P < 0.05$; DR activities: $t_{(4)} = –3.0$, $P < 0.05$, $t_{(4)} = –4.6$, $P < 0.01$; Figure 4G). An increase in fluorescence during sniffing was only observed in females (5-HT release: $t_{(4)} = –3.8$, $P < 0.05$, Figure 4F2; DR calcium activities: $t_{(4)} = –3.7$, $P < 0.05$, Figure 4F4). We did not observe significant changes in fluorescence in both the ACC and DR during self-grooming (Figure 4H) and running (Figure 4I), suggesting that the increases in fluorescence were not due to movement artifacts.

In the following analysis, we observed significant larger fluorescence peaks for ACC 5-HT release and DR activities during allogrooming than during the other behaviors (5-HT release: $F_{(4,20)} = 15.3, 11.7$, all $P < 0.01$; DR activities: $F_{(4,20)} = 44.4, 32.0$, all $P < 0.01$; males ahead; *Pos hoc* comparisons: all $P < 0.01$ for allogrooming *vs.* other behaviors; Figure 4—figure supplement 2). These results provide direct evidence that DR 5-HT

267 systems are involved in consolation-like behaviors and some social behaviors in

268 mandarin voles.

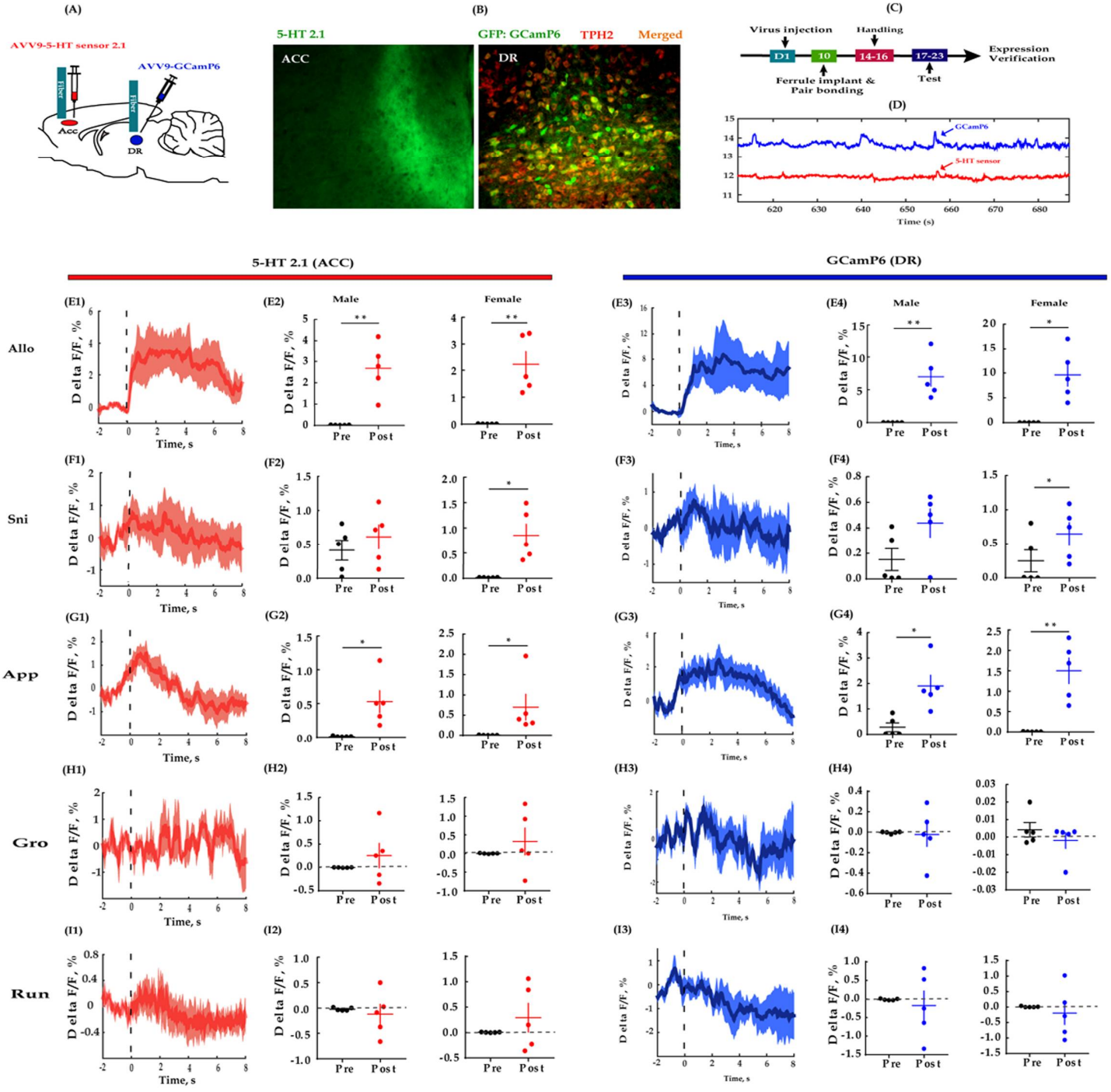

Figure 4. Multichannel fiber photometry simultaneously recording of 5-HT release in the ACC and DR neuron activities during the consolation test. (A): Schematic diagrams depicting the virus injection and recording sites; (B): histology showing the expression of 5-HT sensor in the ACC (left) and co-localization of GCaMP6$^+$ neurons (green) with TPH2$^+$ neurons (red) in the DR (right, scale bar = 200 μm); (C): experimental timeline for photometry experiments; (D): representative fluorescence changes of GCaMP6 (blue line) and 5-HT sensor (red line) during photometry recordings; (E1-I1): representative peri-event plot of 5-HT fluorescence signals aligned to onsets of various behavior (for all peri-event plots, the red line denotes the mean signals of 4-6 bouts of behaviors, whereas the red shaded region denotes the SE); (E2-I2): quantification of change in 5-HT fluorescence signals in the ACC before and after the events (n =5); (E3-I3): representative peri-event plot of calcium signals aligned to onsets of various behavioral events (for all peri-event plots, the blue line denotes the mean signals of 4-6 bouts of behaviors, whereas the blue shaded region denotes the SE); (E2-I2): quantification of change in calcium signals (GCaMP6 fluorescence) in the DR before and after the events. Data are presented as mean ± SE, n = 5 in each group, *P < 0.05, **P < 0.01; paired t test for E-I. ACC: anterior cingulate cortex; DR: dorsal raphe nucleus; TPH2: tryptophan hydroxylase 2; Allo: allogrooming; Sni: sniffing; App: approaching; Gro: selfgrooming; Run: running.

Serotonin in the ACC mediated consolation-like behaviors through 5-HT1A receptors

The most abundant 5-HT receptors expressed in the mPFC are 5-HT1AR and 5-HT2AR (Carhart-Harris & Nutt, 2017). 5-HT1A receptors are generally considered inhibitory, whereas 5-HT2A receptors are excitatory (Puig & Gulledge, 2011). To examine which kind of receptor mediated consolation-like behaviors within the ACC, we therefore infused a 5-HT1AR receptor agonist (8-OH-DPAT) or a 5-HT2AR antagonist (MDL 100907) into the ACC along with chemogenetic inhibition of DR 5-HT neurons before conducting behavioral assays (Figure 5A & B). The behavioral analysis indicated a significant 'drug treatment' effect on allogrooming and sociability (allogrooming: $F_{(3,19)}$ = 5.3, P = 0.01, $F_{(3,23)}$ = 4.2, P < 0.05; social preference ratio: $F_{(3,19)}$ = 9.0, $F_{(3,19)}$ = 6.4, all P < 0.01; males ahead). Not surprisingly, CNO elicited deficits in allogrooming and sociability (Figure 5C-G, left two bars). Pretreatment with 8-OH-DPAT reversed the dysfunctions to the normal level (all P > 0.05 for '8-OH-DPAT+CNO' *vs.* 'vehicle+saline' *pos hoc* comparisons; Figure 5C-D), whereas MDL 100907 did not exert a similar effect (all P < 0.01, for '8-OH-DPAT+CNO' *vs.* 'vehicle+saline' *pos hoc* comparisons except social preference ratio in female voles but also show such trend (0.25 ± 0.14 *vs.* 0.11 ± 0.09, P = 0.098; Figure 5F, G). The microinjected drugs and viruses did not alter the time spent in the central area and mobility in the open-field test (Figure 5E-H, Figure 5—figure supplement 2).

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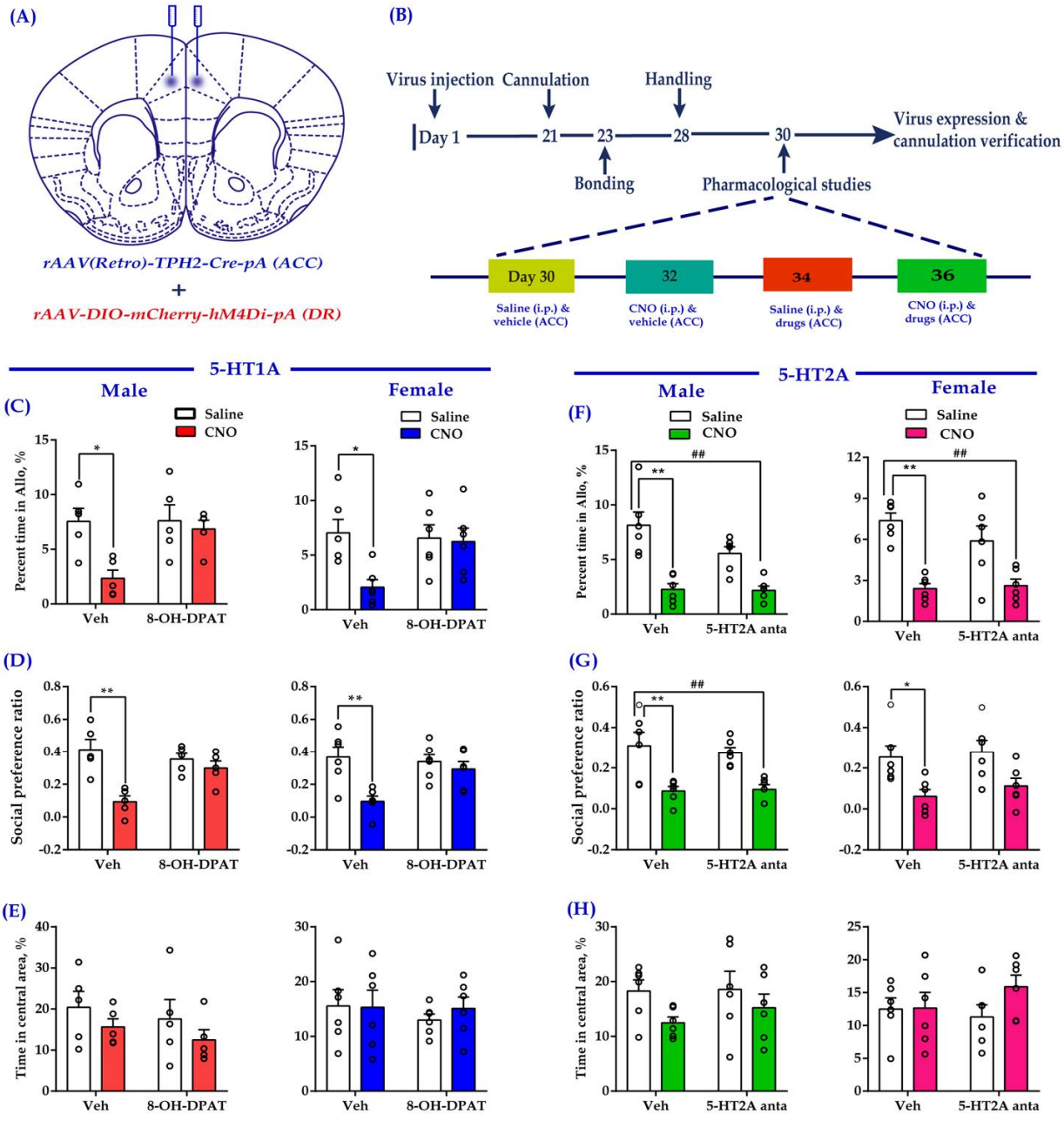

Figure 5. Intra-ACC injection of a 5-HT1AR agonist rescued sociability deficits induced by chemogenetic inhibition of DR 5-HT neurons in the DR→ACC neural circuit. (A): Schematic representation of ACC infusion sites and virus strategy; (B): timeline of experimental design; (C-E): effect of a 5-HT1AR agonist 8-OH-DPAT on allogrooming time in the consolation test (C), social preference ratio in the three-chamber test (D), and time spent in the central area in the open-field test (E) in male (left panels) and female (right panels) voles; (F-H): effect of a 5-HT2AR antagonist (MDL 100907) on allogrooming time in the consolation test (F), social preference ratio in the three-chamber test (G) and time spent in the central area in the open-field test (H) in male (left panels) and female (right panels) voles. Data are presented as mean ± SE, n = 5–6 in each group; for C-H one-way ANOVA with Bonferroni *pos-hoc* test. *$P < 0.05$, **$P < 0.01$ compared with 'vehicle + saline' *vs.* 'vehicle + CNO'; ##$P < 0.01$, '5-HT2AR antagonist + CNO' *vs.* 'vehicle + saline'. Anta: antangonist; ACC: anterior cingulate cortex; DR: dorsal raphe nucleus.

DISCUSSION

In the present study, we demonstrated a crucial role for the DR→ACC 5-HTergic neural circuit in the regulation of consolation-like behaviors and sociability in mandarin voles for the first time. Our major findings are listed below. First, inhibition of DR 5-HT neurons or their terminals in the ACC decreased allogrooming behavior and reduced sociability. Second, DR neuron activity and ACC 5-HT release increased during allogrooming and social approaching. Third, direct activation of ACC 5-HT1A receptors was sufficient to ameliorate deficits in consolation and sociability induced by the chemogenetic inhibition of DR 5-HT neurons.

We found that optogenetic activation DR 5-HT neurons (Figure 1) or ACC 5-HT terminals (Figure 2) did not elicit corresponding increases in allogrooming and sociability. Chemogenetic activation of DR 5-HT neurons produced similar results (Figure 3). One possible explanation is that various 5-HT receptors expressed in the ACC and some have opposite functional effects (Puig & Gulledge, 2011; Tian, Yamanaka, Bernabucci, Zhao, & Zhuo, 2017). For example, 5-HT1AR coupled to the Gi family of G proteins induces the hyperpolarization of pyramidal neurons, whereas 5-HT2AR coupled to Gq proteins induces depolarization in the same neurons (Carhart-Harris & Nutt, 2017). In addition, these two types of receptors are expressed in both pyramidal and GABAergic neurons of the mPFC (Santana, Bortolozzi, Serrats, Mengod, & Artigas, 2004). Therefore, the net behavioral effects of 5-HT release must result from the comprehensive effects of all these receptors. However, in a previous study by Kim, et al., the directly administration of 5-HT into the ACC impaired observational fear learning, which is an empathy-like behavior (Kim et al., 2014). This inconsistency will require further investigations, and species differences (mice *vs.* voles), methodological differences (pharmacology *vs.* optogenetics and chemogenetics) or the use of different behavioral indicators (consolation *vs.* emotional contagion) may be the main explanations for the

discrepancy. Furthermore, optogenetic or chemogenetic activation of DR 5-HT neurons is also distinct from pharmacological intervention with MDMA, which robustly induces 5-HT releases in the whole brain and enhances closeness and empathy in its users in human studies (Carlyle et al., 2019; Heifets & Malenka, 2016). Nevertheless, at least some effects of activation were visible. For example, optogenetic activation of ACC 5-HT terminals increased sociability in CHR2-expressing females. Clearly, effects of activation of the DR→ACC 5-HTergic neural circuit on sociability and empathy-like behaviors still require further in-depth study.

In contrast to activation manipulations, we found inhibition of the DR→ACC 5-HTergic neural circuit significantly decreased intimate behaviors (allogrooming and chasing) toward their distressed partners and reduced sociability (Figures 1-3). Consistent with our results, Walsh et al found that optogenetic inhibition of DR 5-HT neurons reduced social interactions in the three-chamber test (Walsh et al., 2018). However, we still do not know whether the reduced consolation is due to the overall decrease in sociability induced by the current paradigm. Another open question is how this process occurs, namely, what are the neural mechanisms underlying this process? The neocortical excitatory/inhibitory (E/I) balance hypothesis may help address this question. The hypothesis indicated that an increase in the cortical cellular E/I balance, for example through increased activity in excitatory neurons or reduction in inhibitory neuron function, is the common etiology and final pathway for some psychiatric diseases, such as autism and schizophrenia (Bozzi, Provenzano, & Casarosa, 2018; Vattikuti & Chow, 2010). This hypothesis has recently been verified in mice, as optogenetic excitation of glutamatergic neurons in the mPFC elicited a profound impairment in sociability, while compensatory excitation of inhibitory neurons in this region partially rescued social deficits caused by an increase in the E/I balance (Yizhar et al., 2011). 5-HT tends to inhibit prefrontal pyramidal activity (Puig & Gulledge, 2011; Tian et al., 2017). For example, according to Puig et al., electrical stimulation of the DR inhibits approximately

two-thirds of pyramidal neurons in the mPFC (Puig, Artigas, & Celada, 2005). Therefore, a reasonable hypothesis is that inhibition of the DR→ACC 5-HTergic neural circuit may increase the E/I balance, which ultimately leads to abnormal social behaviors in mandarin voles. Clearly, this hypothesis should be verified in electrophysiological studies in the future.

Our results show that neither activation nor inhibition DR→ACC 5-HTergic neural circuit exerted any significant effect on some control behaviors (Figure 1—figure supplement 3; Figure 2—figure supplement 1; Figure 3—figure supplement 2), i.e., self-grooming in the consolation test or behavioral performance in the open-field test, consistent with pervious studies (for self-grooming please refer to (Correia et al., 2017); for open-field test please refer to (Walsh et al., 2018)). However, inconsistent results have also been reported. For example, Ohmura, et al found that optogenetic activation of 5-HT neurons in the median raphe nucleus enhanced anxiety-like behaviors in mice (Ohmura, Tanaka, Tsunematsu, Yamanaka, & Yoshioka, 2014); in the study by Correia, et al, the activation of 5-HTergic neurons in the DR affected behavior in the open-field test, but not anxiety (Correia et al., 2017). Different stimulation protocols or manipulatings of different groups of 5-HT neurons may account for the discrepancies.

Our results obtained using *in vivo* multichannel fiber photometry indicated that an increase in DR neuron activity during allogrooming and social approach (Figure 4–E4 and G4). This result is consistent with previous studies showing that increases in the activity of 5-HTergic neurons in the DR during nonaggressive social interactions (Y. Li et al., 2016; Walsh et al., 2018). Furthermore, using the highly sensitive 5-HT fluorescent sensors developed by Wan et al (Chaki, 2017), we provided the first evidence that allogrooming and social approach elicited robust 5-HT release in the ACC (Figure 4–E1 and G1). Notably, 5-HT and calcium signals correlated well with each other during the recording period (Figure 4D), providing additional evidence that the DR→ACC 5-HTergic neural circuit is implicated in consolation-like behaviors and sociability.

However, self-grooming and chasing did not induce 5-HT release within the ACC (Figure 4–H2, I2). This finding may indicate the behavioral relevance of the 5-HT sensors used in our study to some extent. Clearly, additional evidence is needed to confirm the effectiveness of 5-HT sensors in future studies.

Although both 5-HT1AR and 5-HT2AR are expressed at high levels in the mPFC and work together to modulate cortical network activity (Celada et al., 2013), we found only the 5-HT1AR receptor agonist reversed the consolation and sociability deficits induced by chemogenetic inhibition of 5-HTergic neurons in the DR (Figure 5). This result is consistent with our previous finding that the administration of WAY-100635 (a 5-HT1AR antagonist) into the ACC attenuated consolation and sociability in mandarin voles (L. F. Li, Yuan, He, Ma, et al., 2019). The 5-HT1AR within the mPFC has frequently been reported to exert antidepressant and anxiolytic effects (Artigas, 2013; Fukumoto et al., 2020; Fukumoto, Iijima, & Chaki, 2014; Wang et al., 2019), but our findings clearly indicate that this type of 5-HT receptor within the ACC is also involved in regulating some social behaviors. Clearly, this effect should be verified in many other species in future studies. The administration of the 5-HT2AR antagonist into the ACC did not exert an obvious effect on the social deficits induced by the chemogenetic inhibition of DR 5-HT neurons (Figure 5F-H). Thus, 5-HT2AR within the ACC may not play a major role in consolation and sociability. However, this conclusion should be interpreted very cautiously because 5-HT2AR expression within the mPFC shows clearly rostral-to-caudal gradients in mice (Weber & Andrade, 2010) and we were unable to determine its functional role in this dimension in the present study. Furthermore, the effects of 5-HT in the ACC on consolation and sociability probably involve other subtypes of 5-HT receptors, such as 5-HT1BR and 5-HT3R, which require further examination.

In conclusion, our findings establish the importance of the DR→ACC 5-HTergic neural circuit in consolation-like behaviors and sociability in mandarin voles. Considering the widespread innervation of 5-HT terminals and abundant distribution of

5-HT receptors in the whole brain, detailed knowledge of cellular and circuit mechanisms of the 5-HTergic system, will not only improve our understanding of its complicated features and functions but also have implications for the development of novel therapies for the treatment of prevalent neuropsychiatric disorders, such as depression, autism, and schizophrenia. Additionally, although both male and female subjects were included in this study, sexually dimorphic effects were rarely observed. This finding may provide additional evidence of cooperative evolution to adapt to environmental challenges, particularly in species that adapt monogamous relationships and require cooperative breeding.

METHODS AND MATERIALS

Key Resources Table

Reagent type	Source	Identifier	Additional information
Antibodies			
Anti-TPH2 goat polyclonal antibody	Abcam	ab121013	1/500
Donkey anti-goat secondary antibody conjugated with Dylight 488	Jackson	705-545-147	1/300
Donkey anti-goat secondary antibody conjugated with TRITC	Jackson	705-025-003	1/300
DAPI	Boster	AR1177	Original fluid
Drugs and compounds			
8-OH-DPAT	Sigma	H8520	Injection site : ACC; volume: 200 nL/side; concentration: 1.5 mg/mL
MDL 100907	Tocris Bioscience	Cat#4173	Injection site : ACC; volume: 200 nL/side; concentration: 1 mg/mL

CNO	BrainVTA	Cat# CNO-02	1 mg/kg, i.p.
Cholera Toxin Subunit B (CTB)594	Thermo Fisher	Cat#C34777	Injection site : ACC ; volume : 400 nL/side ; expression time : 10 days
Optogenetic virus			
rAAV(Retro)-TPH2-Cre-WPRE-pA	BrainVTA	R-396-K181127	Injection site : ACC ; volume : 300 nL/side ; expression time : 5 wks
rAAV-Ef1α-DIO-ChR2-mCherry-WPRE-pA	BrainVTA	9-2-K190827	Injection site : DR; volume : 500 nL ; expression time : 5 wks
rAAV-Ef1α-DIO-eNpHR3.0-mCherry-WPRE-pA	BrainVTA	rAAV9-7-5-1	Injection site : DR; volume : 500 nL ; expression time : 5 wks
rAAV-Ef1α-DIO-mCherry-WPRE-pA	BrainVTA	9-13-K190430	Injection site : DR; volume : 500 nL ; expression time : 5 wks
Chemogenetic virus			
rAAV(Retro)-TPH2-Cre-WPRE-pA	BrainVTA	R-396-K181127	Injection site : ACC ; volume : 300 nL/side ; expression time : 5 wks
rAAV-Ef1α-DIO-hM4Di(Gi)-mCherry-WPRE-pA	BrainVTA	9-43-K190521	Injection site : DR; volume : 500 nL ; expression time : 5 wks
rAAV-Ef1α-DIO-hM4Dq(Gq)-mCherry-WPRE-pA	BrainVTA	9-42-K190530	Injection site : DR; volume : 500 nL ; expression time : 5 wks
Fiber photometry virus			
rAAV-hSyn-5HT2.1-WPRE-hGHpA (5-HT sensor)	BrainVTA	9-1826-K190620	Injection site : ACC ; volume : 400 Nl ; expression time : 3 wks
rAAV-hSyn-GCamp6m-WPRE-PA	BrainVTA	rAAV9-148-1-1	Injection site : DR; volume : 500 nL ; expression time : 5 wks

439 **Animals**

440 The mandarin voles used in this study were laboratory bred strains (F2-F3) whose
441 ancestors derived from a wild population from Lingbao city (Henan, China). The voles
442 were weaned on postnatal day 21, socially housed in same-sex in each polycarbonate
443 cage (44×22×16 cm) and housed on a 12-h light/dark cycle with food and water ad
444 libitum. Voles used for experiments were about 70-90 days old at the time. All breeding,
445 housing, and experimental procedures were in accordance with Chinese guidelines for
446 the care and use of laboratory animals and were approved by the Animal Care and Use
447 Committee of Shaanxi Normal University.

448 **Stereotaxic surgery and virus infusions**

449 The kind of virus, total injection volumes and the expression time were listed in Key
450 Resources Table. For surgery, voles (about 50 days) were anesthetized with 1.5%-3.0%
451 isoflurane and placed in a stereotaxic instrument. Next, thirty-three gauge syringe
452 needles (Hamilton) were used for virus delivery. The injection rates were set at 50
453 nL/min. After each injection, the needle was left in the brain for 5 min before being
454 slowly withdrawn in order to prevent the virus from leaking out. The bregma
455 coordinates for the virus injection were as follows: ACC: A/P: 1.6, M/L: 0.5, D/V: 1.6;
456 DR: AP: -4.5; DV: -3.3, ML: +1.2, with a 20 angle toward the midline in the coronal plane.

457 **Microinjection and drugs**

458 The 5-HT1AR agonist 8-OH-DPAT was prepared in saline with a final concentration of
459 1.5 mg/mL. The 5-HT2AR antagonist MDL 100907 was prepared in 0.01 M PBS (adjust
460 PH value with 0.1 M HCl to 6.4) with a final concentration of 1 mg/mL. All the
461 microinjections were administered 30 min before the behavioral test. The speed of
462 injection was 0.1 μL/min, and the total injection volume were 0.2 μL per side for all the
463 drugs. The injector tips remained in situ for another 2 min for drug diffusions. The dose

and timing of drug administration were chosen based on previous studies with 8-OH-DPAT (Cooper, McIntyre, & Huhman, 2008; L. F. Li, Yuan, He, Ma, et al., 2019) and MDL 100907 (Ishii, Ohara, Tobler, Tsutsui, & Iijima, 2015; Pockros, Pentkowski, Swinford, & Neisewander, 2011), which adjusted according to preliminary studies. In chemogenetic studies, CNO (1 mg/kg) was dissolved in saline and delivered intraperitoneally 30 min before the behavioral test.

Optogenetic studies

For optogenetic activation, ferrules were connected (by patch cords) to a 473 nm laser diode through a FC/PC adaptor and a fibre optic rotary joint. The output parameters were: 10 ms, 20 Hz, 8 s on and 2 s off cycle, ~10 mW for terminal stimulation, ~5 mW for somatic stimulation. For optogenetic inhibition, ferrules were connected to a 593 nm laser diode. The output parameters were: 10 ms, 20 Hz, constant, ~10 mW for both terminal and somatic inhibition. The optogenetic stimulation parameters were chosen and slightly modified based on previous studies (Garcia-Garcia et al., 2018; Walsh et al., 2018; Zhao et al., 2011).

Behavioral assays

Generally, all the behavioral experiments were performed under dim light during the dark phase of the light-dark cycle. For optogenetic and fibre photometry studies, all subjects were allowed to habituate to patch cords for at least three days and allowed 30 min acclimation to the connection before the experiment started. The lasting effects of the laser were measured 24 h after the stimulation. In all tests, all groups of experimental mice were randomly selected and the observers were blinded to the treatments.

Consolation test

The consolation test was performed as previously described (Burkett et al., 2016). Briefly, five days before the experiment, age-matched adult male and female voles were

cohoused together to promote the formation of a pair bond (Yu et al., 2012). On the testing day, the subjects' partners were gently transferred in a cup to a sound-proof electric shock chamber and then subjected to 10 rounds of light foot shocks (3 s, 0.8 mA, 2 min intertrial intervals). At the same time, the test voles were left undisturbed in their home cages. After the separation, the pairs were reunited, and the behavior of the subjects was recorded using a video camera for 10 minutes in the test room. The digital videos were viewed and quantified using J Watcher software (http://www.jwatcher.ucla.edu/). According to previous studies (Burkett et al., 2016; Li et al., 2019), allogrooming is designated as behavioral indicators of consolation, which defined as head contact with the body or head of their partner, accompanied by a rhythmic head movement.

For optogenetic tests, the consolation test was designed as a between-subjects test in which both groups (mCherry or opsin) received laser stimulation.

Three-chamber test

The three-chamber test was used to assess the sociability of a subject (Horie et al., 2019; Walsh et al., 2018). The apparatus consisted of a rectangular box with three separate chambers (20 × 40 × 20 cm each). One side of each chamber contained a circular metal wire cage (stimulus animal cage, 11 cm high and 9 cm in diameter). One day before the test, all subjects were habituated to the arena for 5 min and age- and sex-matched voles (stimulus voles) also habituated to the wire cages for 5 min. On the testing day, the test vole was placed in the center chamber and a stimulus vole was randomly placed into one of the wire cages. After 2 min, the partitioning walls between the chambers were

511 removed and the test voles were allowed to explore freely for a 10 min session. The time
512 spent exploring each chamber was automatically recorded using a video tracking system
513 (Shanghai Xinruan, China). Sociability was calculated as follows: (time spent in the
514 stimulus vole side − time spent in the empty side)/(time spent in the stimulus vole side +
515 time spent in the empty side).

516 For optogenetic tests, voles received four epochs of light beginning with a light OFF
517 baseline epoch (OFF-ON-OFF-ON). Each epoch lasted for 5 min and the total duration
518 was 20 min.

519 Open-filed test

520 The anxiety level and locomotor function of the subjects were assessed by using the
521 open-field test (Flanigan et al., 2020; Walsh et al., 2018). Briefly, a square open field (50
522 cm × 50 cm × 25 cm) was virtually subdivided into 16 even square. The four central
523 squares were designated as central area. At the beginning of the test, the test vole was
524 placed into the center area facing away from the experimenter. Behavior was recorded
525 for 5 min. Outcome measures were distance traveled, frequency entries and time spent in
526 the central area.

527 For optogenetic tests, voles were tested twice in the same day in both light-off and
528 light-on conditions with at least 2 h between sessions (within-subjects design).

529 **Multi-channel fibre photometry**

530 The multi-channel fiber photometry recording set-up (ThinkerTech, Nanjing, China) was
531 generated and used as previously described (Feng et al., 2019; Yuan et al., 2019). Briefly,
532 the emission light was generated by a 480 LED, reflected with a dichroic mirror and

delivered to the brain in order to excite the GCaMP6m and 5-HT sensor. The emission light passed through another band pass filter, into a CMOS detector (Thorlabs, Inc. DCC3240M) and finally recorded by a Labview program (TDMSViewer, ThinkerTech, Nanjing, China).

On the test day, voles were mildly anesthetized with isoflurane and connected to a muti-mode optic fiber patch cord (ThinkerTech Nanjing Bioscience®, NA: 0.37, OD: 200 μm) which the other end connected to fiber photometry apparatus. After 30 min habituation, the voles were subjected to consolation test and the behavioral recording consisted of allogrooming, sniffing, approaching, self-grooming and running.

Fiber photometry signals were processed with custom-written MATLAB software. Briefly, all the data were segmented based on the behavioral events and baseline phase. The change in fluorescence (ΔF/F) was calculated as (F–F0)/F0, where F0 represents the baseline fluorescence signal averaged over a 10 s-long control time window. We first segmented the data based on the behavioral events. Then, we calculated the average 5-HT and calcium signals in both the pre- and postphases. The response elicited during a behavior was calculated as the average ΔF/F during all trials of a particular behavior. The peak response during a behavior was calculated as the maximum ΔF/F during the behavior minus F0.

In vitro electrophysiological recordings

To verify the optogenetic and chemogenetic manipulations, we performed in vitro whole-cell patch-clamp recordings from DR neurons. Neurons expressing ChR2, eNpHR3.0, hM3Dq and hM4Di were visually identified by fluorescence of mCherry. The voles were anesthetized with isoflurane. Brains were quickly dissected and 300 μm coronal slices containing the DRN were prepared in a chamber filled with ACSF (32-34 ℃) using vibratome (Campden 7000 smz). The recordings were obtained using a Multiclamp 700B amplifier, filtered at 5 kHz and sampled at 10 kHz with Digidata 1440A. Clampex

559 10.5 was used for analysis.

560 Current-clamp recordings were performed to measure evoked action potentials. For
561 photoactivation and photoinhibition, the light protocols used during behavioral tests
562 were delivered through a 200 µm optical fiber close to recorded neurons. For CNO
563 activation, spontaneous firing of action potentials in the cell was recorded in current
564 clamp mode at -60 mV. After 5 min of recording, the slices were perfused with 10 µM
565 CNO. The total recording time for each cell was 10 min. For CNO inhibition, we applied
566 currents in steps of 25 pA, ranging from 0 pA to 250 pA. Neurons were allowed to
567 recover for 10 min then perfused with 10 µM CNO. The same current procedure was
568 performed. Afterwards, the CNO was removed by washes with ASCF and cells were
569 recorded for another 10 min.

570 **Data analysis**

571 All data are represented as mean ± SEM. All data were assessed for normality using a
572 one-sample Kolmogorov-Smirnov test, and Levene's test was used to confirm
573 homogeneity of variance. Unless other indicated, two-tailed independent *t* tests or paired
574 *t* tests were used for single value comparisons. ANOVA was used to perform group
575 comparisons with multiple measurements. Bonferroni corrections were conducted for
576 multiple comparisons when appropriate. Data are considered to be statistically
577 significant if $P < 0.05$.

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AUTHOR CONTRIBUTIONS

Prof. F.D.T. designed the study; L.F.L. conducted the majorities of experiments and wrote the original draft; L.Z.Z participated in the electrophysiological experiment; Z.X.H. and R.J. discussed the results and provided constructive comments; H.M., Y.F.X, W.Y., J.Z., W.J.H., Z.J.L. and L.Y.T. participated in the behavior study and helped to collect and analyze the data. All authors contributed to and have approved the final manuscript.

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