Chemogenetic Inactivation of Dorsal Anterior Cingulate Cortex Neurons Disrupts Attentional Behavior in Mouse

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Attention is disrupted commonly in psychiatric disorders, yet mechanistic insight remains limited. Deficits in this function are associated with dorsal anterior cingulate cortex (dACC) excitotoxic lesions and pharmacological disinhibition; however, a causal relationship has not been established at the cellular level. Moreover, this association has not yet been examined in a genetically tractable species such as mice. Here, we reveal that dACC neurons causally contribute to attention processing by combining a chemogenetic approach that reversibly suppresses neural activity with a translational, touchscreen-based attention task in mice. We virally expressed inhibitory hM4Di DREADD (designer receptor exclusively activated by a designer drug) in dACC neurons, and examined the effects of this inhibitory action with the attention-based five-choice serial reaction time task. DREADD inactivation of the dACC neurons during the task significantly increased omission and correct response latencies, indicating that the neuronal activities of dACC contribute to attention and processing speed. Selective inactivation of excitatory neurons in the dACC not only increased omission, but also decreased accuracy. The effect of inactivating dACC neurons was selective to attention as response control, motivation, and locomotion remain normal. This finding suggests that dACC excitatory neurons play a principal role in modulating attention to task-relevant stimuli. This study establishes a foundation to chemogenetically dissect specific cell-type and circuit mechanisms underlying attentional behaviors in a genetically tractable species.

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INTRODUCTION

Attention deficits are commonly observed across several psychiatric conditions, including schizophrenia (Chen and Faraone, 2000; Cornblatt and Malhotra, 2001), attention deficit hyperactivity disorder (Huang-Pollock *et al*, 2012), and several mood disorders (Brown *et al*, 1994). Although the neurobiological basis of attention has been heavily investigated, the regions implicated in these studies have not yet been tested for their mechanistic casual contributions. A genetically tractable animal model, particularly in a thoroughly examined species such as mouse, could test the causal contribution of neuronal activity to attentional behavior. In turn, an attention task in mice that has been highly characterized on a cellular basis could facilitate diagnosis, prevention, treatment, and cure of deficits in psychiatric disorders.

Mechanistic neural contributions to behavior can be addressed using engineered G protein-coupled receptors known as DREADDs (designer receptors exclusively activated

by designer drugs) by providing chemogenetic means to achieve a high level of temporal, reversible, and noninvasive manipulation of neuronal activity (Zhu and Roth, 2014). The utility of this approach is substantiated in accumulating studies that have applied technology to specific cell types and neural circuits in the context of behavioral assays of brain function following its inception (Urban and Roth, 2015). Human M4 muscarinic DREADD receptor coupled to Gi (hM4Di) has been used to silence neuronal firing *in vivo* (Ferguson *et al*, 2011; Krashes *et al*, 2011), and has successfully elucidated neural mechanisms mediating complex cognitive processes, such as goal-directed behavior (Parnaudeau *et al*, 2013; Parnaudeau *et al*, 2015). This powerful chemogenetic tool has not been employed to examine the neural basis of attention processes. Establishing a chemogenetic manipulation in conjunction with an attention task could advance understandings of the neural basis of attention processing.

Accumulating evidence across species points to a conserved role of the dorsal anterior cingulate cortex (dACC), a dorsal part of medial prefrontal cortex, in normal attention processing. Cytoarchitectural similarities across human, primate, and rodent dACC are accompanied by shared functional findings (Uylings *et al*, 2003; Vogt and Paxinos, 2014). Observational studies indicate that the dACC selectively responds to task-relevant stimuli. Human studies

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show that dACC activity correlates with performance when study participants attend to task-relevant stimuli (Alexander *et al*, 2005; Corbetta *et al*, 1991; Weissman *et al*, 2005). Furthermore, the dACC is hypoactivated in patients with attention-deficit/hyperactivity disorder during an attention task (Bush *et al*, 1999). Shrinkage of the dACC that is commonly observed in multiple psychiatric disorders—including schizophrenia, bipolar disorder, depression, addiction, obsessive-compulsive disorder, and anxiety—correlates with poor executive functioning including sustained attention (Goodkind *et al*, 2015). Electrophysiologic single-unit recording in both primates and rodents also demonstrate dACC activity during attention tasks, specifically in anticipation of oncoming task-relevant visual cues (Johnston *et al*, 2007; Totah *et al*, 2009). These observations are corroborated by lesion observations. In humans, anterior cingulotomy produces attentional deficits (Alexander *et al*, 2005; Cohen *et al*, 1999; Ochsner *et al*, 2001). In rodents, both excitotoxic lesions and pharmacologic GABAergic disinhibition of rat dACC activity result in attentional deficits (Chudasama *et al*, 2003; Passetti *et al*, 2002; Pehrson *et al*, 2013).

A chemogenetic approach can further these findings by providing mechanistic insights about how neuronal features mediate behavioral observations. Permanent lesions disrupt normal psychophysiology and may induce compensatory changes that confound observed cognitive alterations. Although pharmacologic approaches are temporary, they can produce off-target effects because of non-cell type-specific binding and lack of spatial control. Studies in mice facilitate chemogenetic approaches that can introduce alterations in activity with high temporal control based on specific cellular features. When coupled to behavioral paradigms, this methodology affords the opportunity to examine behavioral outcomes in conjunction with molecular-, cellular-, and circuit-level disruptions. Addressing these concerns, we expressed hM4Di in dACC neurons and subjected mice to the five-choice serial reaction time task (5CSRTT) that measures sustained visuospatial attention alongside processing speed and response control (Bari *et al*, 2008; Robbins, 2002). Our study was conducted in Bussey–Saksida touch-screen operant chambers with a high degree of automation and standardization, applying similar stimulus and response characteristics to those used by the analogous Cambridge Neuropsychological Test Automated Battery in humans and non-human primates (Bussey *et al*, 2012; Mar *et al*, 2013; Romberg *et al*, 2011; Silverman *et al*, 2013). By coupling DREADD technology to attentional behavior for the first time, we demonstrated that the dACC neurons play an important causal role in attention to relevant stimuli. Establishing a chemogenetic approach in a genetically tractable species opens avenues to identify specific cell-type and circuit mechanisms underlying attentional behaviors in future studies.

MATERIALS AND METHODS

Animals

Male C57Bl/6 mice (Charles River Laboratories, Massachusetts) were housed under a standard 12 h light/dark cycle in a temperature- and humidity-controlled vivarium. Training

was initiated when mice were 10–12 weeks old. Mice were allowed access to water for 2 h each day during behavioral testing. All animals were originally group-housed, but two mice (one mouse each from two cohorts subjected to behavior tests) that strongly attacked cagemates were singly housed through the duration of the experiment. Food was available *ad libitum* throughout the experiment. All animal protocols were approved by IACUC at Icahn School of Medicine at Mount Sinai.

Drugs

Clozapine-*N*-oxide (CNO; Tocris Bioscience, Bristol, UK) and pentylentetrazole (PTZ; Sigma Aldrich, St Louis, MO) were fully dissolved in normal saline. For behavior experiments, CNO was injected intraperitoneally (i.p.) at 10 mg/kg 30 min before testing. For DREADD validation, mice were first separated into individual cages 1 h before CNO or vehicle injection (2–3 mice per group) to reduce variability in neuronal activation. PTZ was then administered subcutaneously 30 min after CNO injection at 65 mg/kg. Mice were perfused 2 h later when tonic-clonic seizure was observed.

Viral Injection

Mice were anesthetized with isoflurane and head-fixed in a mouse stereotaxic apparatus (Narishige, East Meadow, NY). Bilateral injections were made at the following coordinates: AP +1.7 mm, ML $\pm$ 0.2 mm and DV $-$ 0.7 mm; AP +1.1 mm, ML $\pm$ 0.2 mm and DV $-$ 0.7 mm; AP +0.4 mm, ML $\pm$ 0.2 mm and DV $-$ 0.7 mm. Then, 500 nl of a mixture of AAV8-hSyn-DIO-hM4D(Gi)-mCherry and AAV8-hSyn-GFP-Cre or AAV8-CaMKII α -GFP-Cre (University of North Carolina Vector Core) in a 1 : 1 ratio was infused in each coordinate site at 150 nl/min using a microinjector set and 2.5 μ l Hamilton syringe. The syringe was left in place for 1 min following the injection to reduce backflow of virus. We waited at least 3 weeks to allow for maximal viral expression.

Five-Choice Serial Reaction Time Task

Apparatus. Testing was conducted in the Bussey–Saksida chamber (Lafayette Instrument, Lafayette, IL), a black plastic trapezoid (walls 20 cm high $\times$ 18 cm wide (at screen-magazine) $\times$ 24 cm wide (at screen) $\times$ 6 cm wide (at magazine)). Stimuli were displayed on a touch-sensitive screen (12.1 inch, screen resolution 600 $\times$ 800) divided into five response windows by a black plastic mask (4.0 $\times$ 4.0 cm², positioned centrally with windows spaced 1.0 cm apart, 1.5 cm above the floor) fitted in front of the touchscreen. Schedules were designed and data were collected and analyzed using ABET II Touch software (Lafayette Instrument). The inputs and outputs of the multiple chambers were controlled by WhiskerServer software (Lafayette Instrument).

Habituation. Before training on 5CSRTT, mice were initially trained to touch the screen. Mice were acclimated to the chambers for 3 days in 30-min sessions. During this habituation phase, the food magazine was illuminated and diluted sweetened condensed milk (Eagle Brand) was dispensed every 40 s. Mice were then trained to touch the

screen. A stimulus was randomly displayed in one of the five response windows. If the mouse touched the stimulus, the milk reward was delivered in conjunction with a tone and magazine light. Touches to nonstimuli had no consequence. After reaching criterion on this phase (20 touches in 30 min for 2 consecutive days), mice moved onto 5CSRTT training phase.

Training. Mice were tested 5 days a week, 100 trials a day (or up to 30 min). Each trial began with the illumination of the magazine light. When the mouse made a nose poke in the food magazine, the stimulus was delivered after an intertrial interval (ITI) period of 5 s. If a mouse touched the screen during this ITI period, the response was recorded as premature and the mouse was punished with a 5-s time-out (house light on). After the time-out period, the magazine light illumination and house light switch off signaled onset of the next trial. After the ITI period, a stimulus appeared randomly in one of the five response windows for a set stimulus duration (this varied from 32 to 2 s). A limited-hold period followed by the stimulus duration was 5 s, during which the stimulus was absent but the mouse was still able to respond to the location. Responses during stimulus presence and limited holding period could be recorded either as correct (touching the stimulus window) or incorrect (touching any other windows). A correct response was rewarded with a tone, and milk delivery, indicated by the illumination of the magazine light. A failure to respond to any window over the stimulus and limited-hold period was counted as an omission. Incorrect responses and omissions were punished with a 5-s time-out. In addition, animals could make perseverative responses that are screen touches after a correct or incorrect response. Animals started at a stimulus duration of 32 s. With a goal to baseline mice at a stimulus duration of 2 s, the stimulus duration was sequentially reduced from 32, 16, 8, 4, to 2 s. Animals had to reach a criterion (>50 trials, $>80\%$ accuracy, $<20\%$ omissions) over 2 consecutive days to pass from one stage to the next. After reaching baseline criterion with the 2 s stimulus duration (5 consecutive days), mice were injected with virus.

Reduced stimulus test. Following viral injections, mice were reestablished to baseline and then challenged with an increased attentional demand by reducing the stimulus duration to 2, 1.5, 1, and 0.8 s (reduced stimulus test). They then underwent 8 days of testing, with half of the mice receiving vehicle injections on odd days and the other half on even days. After 4 days of testing, mice were subjected to 2 s stimulus duration training to confirm that the mice maintain stable baseline performance. In case of under baseline criteria, the mice were reestablished to baseline, and then were tested. Attention and response control were assessed by measuring the following performance: percentage accuracy ($100 \times \text{correct responses} / (\text{correct responses} + \text{incorrect responses})$), percentage omission ($100 \times \text{omissions} / (\text{omissions} + \text{correct responses} + \text{incorrect responses})$), number of premature responses, number of perseverative responses, latency to collect response, and latency to reward collection after correct choices.

Open Field Test

Locomotor activity was measured for 30 min in a square apparatus (43 cm $\times$ 43 cm $\times$ 33 cm) equipped with a panel of infrared beams (16 beams) located in the horizontal direction along the sides of each square apparatus. Data were collected with Fusion v4 software (Omnitech Electronics, Columbus, OH). Mice were subjected to the open field test more than 1 week after the completion of 5CSRTT. Activity was assessed twice for each mouse (with vehicle and CNO injection respectively). The order of vehicle *versus* CNO administration was counterbalanced across mice. Activity sessions were separated by at least 1 week drug-free days.

Immunohistochemistry

Anesthetized mice were transcardially perfused with cold 4% paraformaldehyde (PFA) dissolved in 0.1 M phosphate buffer. The brains were post-fixed in 4% PFA at 4 °C, and cryoprotected in 30% sucrose solution. The frozen brains were sectioned into 40- μ m-thick coronal sections using a cryostat (CM3050, Leica). Free-floating sections were washed in tris-buffered saline (TBS), pH 7.5, and then blocked in 1% bovine serum albumin in TBST (0.25% Triton X-100 in TBS) for 1 h. The sections were incubated with anti-c-Fos antibody (1 : 500; sc-52, Santa Cruz Biotechnology) or anti-CamKII antibody (1 : 200; 05-532, EMD Millipore) overnight at room temperature. They were washed in TBST, followed by incubation with Alexa 647-conjugated goat anti-rabbit antibody or Alexa 647-conjugated donkey anti-mouse antibody (Thermo Fisher Scientific). To visualize all neurons, we used NeuroTrace 435/455 blue fluorescent Nissl stain (Thermo Fisher Scientific). This fluorescent probe has been reported to only label neurons as stained cells do not colocalize with markers of glial cells, particularly astrocytes, microglia, or oligodendrocytes (Lazo *et al*, 2010).

Double Fluorescence *In Situ* Hybridization

In situ hybridization was performed as described previously (Demars and Morishita, 2014). cDNA fragments of mouse vGlut1 and Cre were amplified by PCR. Probes were synthesized using T3/T7 RNA polymerase (Roche) labeled with digoxigenin or fluorescein and hybridized to frozen sections. After overnight hybridization of RNA probes at 72 °C, sections were incubated with anti-fluorescein POD antibodies (1 : 2000) followed by TSA Plus DNP signal amplification (Perkin Elmer), and then incubated with anti-DIG-alkaline phosphatase and anti-DNP-KLH-488 (both 1 : 1000, Roche). Following incubation, slides were incubated in fast red solution (Roche). Finally, slides were washed in water and coverslipped using CC mount solution (Sigma Aldrich). For colocalization counting, four images/mouse from two mice were taken around injection sites.

Imaging and Quantification

Imaging was performed using LSM780 confocal microscopes (Zeiss). All images were processed and analyzed on ImageJ software (NIH). Images were thresholded using the Max Entropy algorithm. Total cell numbers were estimated using the 'Analyze Particle' function. For analysis of colocalization

Figure 1 Viral transduction of hM4Di in the dorsal anterior cingulate cortex neurons. (a) Experimental approach for Cre-dependent inhibitory DREADD showing stereotactic injection coordinates. (b, top panel) Representative confocal images showing native fluorescent signals from dACC cells coexpressing hM4Di-mCherry (magenta) and GFP-Cre (green). GFP-Cre was transduced with the hSyn promoter (left panel) or CaMKII α promoter (right panel). Arrowheads indicate cells coexpressing hM4Di and Cre (white). Scale bar = 20 μ m. (b, bottom panel) Quantification of the percentage of hM4Di-positive cells that coexpress Cre. Data are represented as mean $\pm$ SEM ($n = 6$). (c) Representative confocal images showing native hM4Di-mCherry fluorescence (magenta) in the prefrontal cortex of hSyn-hM4Di and CaMKII-hM4Di mice. MOs, secondary motor; dACC, dorsal anterior cingulate; vACC, ventral anterior cingulate; PL, prelimbic; IL, infralimbic. Scale bar = 500 μ m. (d) Relative distribution of hM4Di-positive cells transduced by AAV-hSyn-GFP-Cre (left panel) or AAV-CaMKII α -GFP-Cre virus (right panel) among prefrontal cortices. Data are represented as mean $\pm$ SEM ($n = 6$).

with Nissl or GFP-Cre, the analysis was performed with three sections/mouse from six mice for each group. For CaMKII immunohistochemistry, 8–10 images/each mouse were taken from 2 hSyn mice and 2 CaMKII mice. For hM4Di distribution, to ensure the coverage of the anterior–posterior axis for our counting, three sections per hemisphere were collected from three different areas around each injection site (one section each from each injection site) spanning anterior–posterior axis (AP+1.7, +1.1, +0.4 mm). Six images were collected from each animal, and were averaged from six mice for the quantification. Although our quantification does not include the most anterior (AP+2.2 mm) and posterior (AP–0.1 mm) portion of ACC, we expect the reported widespread transduction of AAV8 (Aschauer *et al*, 2013) allows the viral transduction to extend up to 500 μ m from the injection sites close to the anterior and posterior boarder of ACC. Nonetheless, we cannot rule out the possibility that our hM4Di distribution analysis overestimated the transduction rate. For c-Fos/hM4Di colocalization analysis, 4–6 images/mouse were taken around injection sites (vehicle- and PTZ-treated hSyn-hM4Di mice, $n = 3$; CNO- and PTZ-treated hSyn-hM4Di mice, $n = 3$; vehicle- and

PTZ-treated CaMKII-hM4Di mice, $n = 2$; CNO- and PTZ-treated CaMKII-hM4Di mice, $n = 3$).

Statistical Analyses

Statistical analyses were performed using Prism (Graphpad). The 5CSRTT data (accuracy and omission) were analyzed using a two-way repeated measures analysis of variance (ANOVA) with Drug (vehicle, CNO) and Stimulus duration (2, 1.5, 1, 0.8 s) as within-subjects factors, followed by *post hoc* Bonferroni's multiple comparisons test when F-ratios of the interaction were significant ($p < 0.05$). In the analysis of other 5CSRTT data and locomotion, a paired *t*-test was used. Immunohistochemistry data were analyzed using Student's *t*-test. All data are expressed as means $\pm$ SEM.

RESULTS

Targeting Inhibitory DREADD to dACC Neurons

To achieve cell type-specific suppression of dACC neuronal activation in mice, we bilaterally injected

AAV8-hSyn-DIO-hM4Di-mCherry with AAV8-hSyn-GFP-Cre (hSyn-hM4Di mice) for pan-neuronal transduction or AAV8-CaMKII α -GFP-Cre (CaMKII-hM4Di mice) for excitatory neuron-specific transduction (Figure 1a). hM4Di was expressed in a Cre-dependent manner: $91 \pm 2\%$ and $96 \pm 1\%$ of hM4Di-positive cells coexpressed Cre in hSyn-hM4Di and CaMKII-hM4Di mice, respectively (Figure 1b). hM4Di transduction was neuron specific: $95 \pm 2\%$ and $97 \pm 1\%$ hM4Di-positive cells were costained with Nissl in hSyn-hM4Di and CaMKII-hM4Di mice respectively (Supplementary Figure S1a and b). We also confirmed that AAV-CaMKII α -GFP-Cre was selectively expressed in excitatory neurons: $94 \pm 2\%$ of hM4Di-positive cells were immunolabeled with an excitatory neuron marker CaMKII protein in CaMKII mice, whereas $86 \pm 4\%$ of hM4Di-positive cells coexpressed CaMKII in hSyn mice consistent with the promotor selectivity (Supplementary Figure S1c and d). Double *in situ* hybridization of Cre with *vGlut1*, a marker of glutamatergic neurons (Supplementary Figure S1e and f), also showed $89 \pm 4\%$ of the total Cre-positive cells in the dACC coexpressed *vGlut1* mRNA in CaMKII mice, demonstrating that AAV-CaMKII α -GFP-Cre transfection was highly selective toward excitatory neurons. In addition, $17 \pm 3\%$ and $26 \pm 4\%$ of Nissl-positive dACC neurons expressed hM4Di in hSyn-hM4Di and CaMKII-hM4Di mice, respectively (data not shown). We thus found that hM4Di expressed in a pan-neuronal manner in hSyn-hM4Di mice and in excitatory neurons in CaMKII-hM4Di mice.

To assess specificity of transduction to the dACC, we mapped the pattern of hM4Di expression in the prefrontal cortex (Figure 1c and d). The largest percentage of hM4Di-expressing cells was in the dACC in hSyn-hM4Di ($58 \pm 3\%$) and CaMKII-hM4Di mice ($57 \pm 5\%$). Expression ratios of hM4Di in secondary motor (MOs), ventral ACC (vACC), prelimbic (PL), and infralimbic (IL) cortices in hSyn-hM4Di mice were $12 \pm 2\%$, $12 \pm 2\%$, $4 \pm 1\%$, and $14 \pm 2\%$, respectively. Expression ratios of CaMK2-hM4Di in MOs, vACC, PL, and IL were $8 \pm 2\%$, $14 \pm 2\%$, $9 \pm 2\%$, and $12 \pm 2\%$, respectively. Our quantification shows that hM4Di was predominantly expressed in the dACC in both hSyn- and CaMKII-hM4Di mice.

Although the inhibitory DREADD virus that we used has been reported to suppress firing of infected neurons (Perova *et al*, 2015), we additionally verified its functional effect *in vivo* by assaying expression of c-fos, a recognized marker of neuronal activation, upon CNO administration. As previous reports suggest that high baseline c-fos activation is required to detect the reduced c-fos activation by CNO (Koch *et al*, 2015; Sasaki *et al*, 2011), we elevated the baseline c-fos activation with PTZ (65 mg/kg), a GABA_A receptor antagonist (Andre *et al*, 1998; Li *et al*, 2014), to allow for *in vivo* quantification. We found that CNO (10 mg/kg) significantly decreased the percentage of total hM4Di-expressed cells that coexpress c-fos in both hSyn- and CaMKII-hM4Di mice injected with PTZ (Supplementary Figure S2). Furthermore, c-Fos immunohistochemistry in mice perfused 60 min after the reduced stimulus 5CSRTT showed that CNO slightly, but not significantly, suppressed the reduced stimulus test-induced c-Fos expression (% of c-Fos+ cells in hM4Di+ cells: $13 \pm 3\%$ with CNO vs $18 \pm 9\%$ with saline in hSyn-hM4Di mice. CNO or vehicle was given

30 min before the test; 3 mice each). Based on prior reports (Koch *et al*, 2015; Sasaki *et al*, 2011), we concluded that this finding is likely because of the far lower baseline c-fos activation of ACC neurons by attention task compared with PTZ-induced c-Fos activation ($18 \pm 9\%$ +5CSRTT vs $29 \pm 3\%$ +PTZ; Supplementary Figure S2). Although hM4Di suppression of PTZ-induced c-Fos expression does not directly prove that CNO suppresses attention-dependent neural activity, it does however provide a proof of principle that CNO-dependent inactivation of neural activity in cells expressing inhibitory DREADD applies to viral introduction to the dACC *in vivo*.

Reversible Chemogenetic Inactivation of the dACC Neurons Disrupts Attentional Behavior

Once we established that inhibitory DREADD was functioning to suppress dACC neuronal activity, we subjected mice to the 5CSRTT. After training mice to baseline (see the detailed method in Materials and Methods section), AAVs were injected as shown in Figure 1. Mice were maintained at a performance baseline for 3 weeks in order to achieve maximal hM4Di expression before testing. During the test phase, CNO or saline was administered at 10 mg/kg 30 min before each of test sessions. The injection schedule was counterbalanced across mice (Figure 2a). Because pharmacokinetic studies report that CNO given i. p. at 1 mg/kg is eliminated from plasma within 2 h and reaches peak efficacy for 10 min to 10 h, we waited 30 min until testing and allowed 24 h for the drug effect to dissipate fully (Farrell and Roth, 2013; Ray *et al*, 2011). In the absence of DREADD expression, CNO administration at dose of ≥ 10 mg/kg has been reported to have no measurable effect across many monitored behavioral and physiological assays (Alexander *et al*, 2009; Mahler *et al*, 2014; Ray *et al*, 2011; Richards *et al*, 2014).

In each test session, mouse attention demand was taxed by reducing the stimulus duration to 2, 1.5, 1, and 0.8 s in a pseudorandomized order. Prior studies have shown that attention deficits can be measured quantitatively as a decrease in accurate responses and an increase in omitted responses to light stimulus, and that performance decreases with shortened stimulus duration. Consistent with previous findings, as the stimulus duration decreased, all mice showed an expected increase in the percentage of omission and decrease in the percentage of accuracy (omission: hSyn-hM4Di mice: $F(3, 15) = 22.06$, $p < 0.001$; CaMKII-hM4Di mice: $F(3, 15) = 11.52$, $p < 0.001$; accuracy: hSyn-hM4Di mice: $F(3, 15) = 24.40$, $p < 0.001$, CaMKII-hM4Di mice: $F(3, 15) = 29.86$, $p < 0.001$) (Figure 2b–e). We observed a difference between hSyn-hM4Di mice and CaMKII-hM4Di mice in the omission rate at 2.0 s when treated with vehicle, but we saw no difference in the omission rate between mice injected with DREADD and controls injected with GFP (H Koike and H Morishita, unpublished observation: GFP vs hSyn DREADD ($p = 0.40$, *t*-test), GFP vs CaMKII DREADD ($p = 0.17$, *t*-test)). We therefore consider this difference to represent normal performance variability across the population of injected mice subjected to the 5CSRTT.

We next assessed accurate and omitted trials as stimulus duration decreased in both groups upon CNO administration (Bari *et al*, 2008; Mar *et al*, 2013). Compared with

Figure 2 DREADD inactivation of dACC neurons produces visual attention deficits. (a) Schematic overview of the experimental design. All mice were injected with AAVs after reaching baseline criteria. Baseline performance was maintained whereas hM4Di expression reached full expression. Animals were divided into counterbalanced groups that received either vehicle or CNO on the first day of testing; both groups alternated between CNO and vehicle for each testing session. Injections were administered 30 min before testing and one testing session was executed each day. (b, c) CNO administration increases the percentage of trials omitted. (d, e) CNO administration does not affect accuracy when hM4Di was expressed pan-neuronally, but reduces the percentage of accurate responses when expressed in excitatory neurons. Veh, vehicle; CNO, clozapine-*N*-oxide. *** $P < 0.01$, * $p < 0.05$ vs vehicle. Data are represented as mean $\pm$ SEM ($n = 6$).

vehicle delivery, CNO administration significantly increased the number of trials omitted in both hSyn-hM4Di and CaMKII-hM4Di mice. In hSyn-hM4Di mice we observed a significant drug effect across all stimulus durations (main effect of drug: $F(1, 5) = 8.743$, $p < 0.05$; interaction between drug and stimulus duration: $F(3, 15) = 0.40$, $p = 0.76$) and in CaMKII-hM4Di mice the drug effect gained significance as stimulus duration shortened below baseline (main effect of drug: $F(1, 5) = 3.915$, $p = 0.10$; interaction: $F(3, 15) = 11.21$,

$p < 0.001$; *post hoc* comparison for 1.5, 1.0, 0.8 s $p < 0.01$) (Figure 2b and c). CNO administration similarly significantly decreased accuracy in CaMK2-hM4Di mice but not in hSyn-hM4Di mice (CaMK2-hM4Di mice: main effect of drug: $F(1, 5) = 10.52$, $p < 0.05$; interaction: $F(3, 15) = 0.87$, $p = 0.48$; hSyn-hM4Di mice: main effect of drug: $F(3, 15) = 0.99$, $p = 0.37$; interaction: $F(3, 15) = 0.11$, $p = 0.95$) (Figure 2d and e). Of note, 5CSRTT performances at 0.8 s were not changed throughout testing (hSyn-hM4Di: effect of day for omission: $F(3, 36) = 2.14$, $p = 0.11$; effect of day for accuracy: $F(3, 36) = 0.21$, $p = 0.88$; CaMKII-hM4Di mice: effect of day for omission: $F(3, 30) = 2.54$, $p = 0.07$; effect of day for accuracy: $F(3, 30) = 1.32$, $p = 0.28$) (Supplementary Figure S3), indicating repeating tests and CNO treatment did not influence each other's tests. Collectively, these results suggest that chemogenetic inactivation of the dACC neurons disrupts attention behavior.

Reversible Chemogenetic Inactivation of dACC Neurons Did Not Cause Deficits in Motivation and Locomotion

Because an increase in omission can also arise because of altered motor function and decreased motivation to obtain a reward, we next sought to evaluate motivation and motor deficits through latency to collect rewards as well as the open field test (Bari *et al*, 2008; Mar *et al*, 2013). Although we observed a significant lengthening of latency to produce a correct response in hSyn-hM4Di mice ($p < 0.05$), but not in CaMK2-hM4Di mice ($p = 0.13$), CNO administration did not affect latency to collect reward (hSyn-hM4Di, $p = 0.40$; CaMKII-hM4Di, $p = 0.13$) (Figure 3a–d). CNO administration did not affect the distance moved in the open field test (hSyn-hM4Di, $p = 0.88$; CaMKII-hM4Di, $p = 0.29$ Figure 3e and f), ruling out motor deficits. Collectively, these results demonstrate that increased omission and processing speed deficit were likely not induced secondarily by lack of motivation or gross motor deficits.

Reversible Chemogenetic Inactivation of dACC Neurons Did Not Induce Impulsive and Compulsive Behavior

To examine whether the effect of chemogenetic inactivation of dACC neurons is specific to attention processing, we also assessed for alterations in impulsivity and compulsivity upon CNO administration by measuring the number of premature responses and perseverative responses, respectively (Bari *et al*, 2008; Mar *et al*, 2013). No effect of CNO administration on the number of premature responses (Figure 4a and b: hSyn-hM4Di, $p = 0.71$; CaMKII-hM4Di, $p = 0.50$) and perseverative responses (Figure 4c and d: hSyn-hM4Di, $p = 0.15$; CaMKII-hM4Di, $p = 0.30$) was observed in either hSyn-hM4Di or CaMKII-hM4Di mice, indicating that response control was not affected by the dACC pan-neuronal or excitatory inactivation.

DISCUSSION

By combining a chemogenetic neural silencing approach with a translational, touchscreen-based attention task in mice, we demonstrated that DREADD inactivation of the dACC neurons disrupts attentional behavior. Our chemogenetic approach circumvents the difficulties in interpretations

Figure 3 DREADD inactivation of dACC neurons does not affect processing speed, motivation, or locomotion. (a, b) In hSyn-hM4Di but not CaMKII-hM4Di mice, CNO administration increased collect response latency. (c, d) CNO administration does not affect the latency to collect reward for a correct response collection latency. (e, f) In a separate open field test, the average distance traveled is not altered by DREADD inhibition. Veh, vehicle; CNO, clozapine-N-oxide. * $P < 0.05$ vs vehicle; NS, nonsignificant. Data are represented as mean $\pm$ SEM ($n = 6$).

associated with the lesion or pharmacological studies by directly establishing a causal role of dACC neural activities on attentional behavior. Our study supports the possibility that the changes in dACC observed in many psychiatric disorders (Bush *et al*, 1999; Goodkind *et al*, 2015) can causally contribute to cognitive dysfunction. As far as we are aware, this is the first study that directly demonstrates that dACC neurons play a causal role in attention processing in mice. Compared with optogenetic techniques, chemogenetic approaches are less invasive and permit neural activity manipulations over extended periods of time; this approach is thus ideally suited to examine the long-term impacts of sustained altered neural activity over the time course of developmental time windows. Such manipulations will enable study of how normal developmental contributions

Figure 4 DREADD inactivation of dACC neurons does not alter impulsivity and compulsivity. (a, b) The number of premature responses before the display of the light stimulus does not change under the influence of CNO. (c, d) Repetitive nose-poking in response to the light stimulus is not affected by drug administration. Veh, vehicle; CNO, clozapine-N-oxide; NS, nonsignificant. Data are represented as mean $\pm$ SEM ($n = 6$).

produce long-lasting impacts on attention, and additionally, how aberrant developmental signaling could be targeted to prevent attentional deficits in preclinical models. DREADD can be expressed in cell-type and circuit-specific manners via viral approaches (Urban and Roth, 2015). Because of this capability, our study provides a template for future studies to chemogenetically dissect the neural circuit basis of attentional behavior in mice.

We observed that dACC neuronal inactivation by DREADD decreases accuracy and increases omissions in mice during attention behavior assayed with the 5CSRTT (Figure 2). Although omissions can indicate an attention deficit as mice fail to detect the presented stimulus, it is also altered when mice have motor deficits that prevent them from responding in a timely manner, or lack motivation to obtain a reward received following a correct response (Robbins, 2002). Given that latency to reward and locomotor activity were not affected by the DREADD inactivation (Figure 3), it is highly likely that the observed increase in omissions by dACC inhibitory DREADD primarily reflects a deficit specific to attention. Minor inactivation of ~20% of the dACC neuronal population could induce attention deficits, suggesting that the dACC provides prominent contributions to normal attention-based cognition. The specificity of our DREADD inactivation also points to a specific role for the activity of dACC neurons in attention-based behavior, and not in impulsivity and compulsivity (Figure 4). Our results are consistent with a previously

reported lesion study in rat dACC (Chudasama *et al*, 2003), but more conclusively demonstrated that dACC neuronal activity is a key contributor to attention-based cognition without the confounding nonspecific lesions that accompany lesions.

Our pan-neuronal and excitatory inactivation produced overlapping yet separate effects on attention processing (Figure 2), suggesting that more elaborate cell type-specific inactivation will be important in future studies to draw conclusions about the neural mechanisms underlying this type of cognition. Pan-neuronal dACC inactivation induced mild attentional deficits and slowed processing speed. In contrast, excitatory neuron-specific inactivation produced more severe effects on attention with no impact on processing speed. Because transduction efficacy was comparable between hSyn-hM4Di and CaMKII-hM4Di mice (Figure 1: $p=0.11$, paired *t*-test), the different attention deficits between the two groups appear to reflect differences in behavioral contributions from infected cell types. The accuracy deficit we observed in the inhibition of excitatory neurons may be masked by pan-neuronal inactivation: inhibitory neuron inactivation often produces excitatory cell disinhibition, and this effect may in fact compensate for the primary action of DREADD inhibition on excitatory neurons. To our surprise, pan-neuronal inactivation, compared with excitatory neuron-specific inactivation, produced a greater processing speed deficit with a milder attention deficit. Interestingly, a similar phenotype (increased correct response latency without accuracy deficits in the 5CSRTT) was previously reported following microinjection in rat PFC of a NMDA receptor antagonist that preferentially binds and inhibits GABAergic neural activity and enhances glutamatergic neural activity (Carli *et al*, 2006; Homayoun and Moghaddam, 2007). As hM4Di likely inactivates nonexcitatory GABAergic neurons in hSyn mice, but not in CaMKII mice (Supplementary Figure 1c and d), we speculate that GABAergic neuron inactivation could contribute to the increased correct response latency in hSyn mice. Furthermore, an activity balance between glutamatergic and GABAergic neurons could differentially contribute to the dissociation between the processing speed and attention phenotypes in hSyn and CaMKII mice. Taken together, our findings suggest that activity balances of excitatory and inhibitory neurons in the dACC play a critical role in attentional processes and processing speed (Pehrson *et al*, 2013; Totah *et al*, 2009). More elaborated cell type-specific inactivation by specific gene promoters in excitatory and inhibitory neurons could shed light on the neural mechanisms of attention and processing speed in the dACC in future studies.

The dACC is connected with many different brain regions and attention processing likely requires signaling in additional areas. Interestingly, the dACC is reciprocally connected with visual area, and ‘top-down’ dACC projections modulate sensory processing in the visual cortex (Zhang *et al*, 2014; Zingg *et al*, 2014). Thus, one possibility is that attention deficits observed in our study could be attributed to dACC inactivation or alteration of top-down visual processing. Another potential explanation is that dACC inactivation disrupts the passage of visual information based on the stimulus to connected somatomotor areas that mediate responsive motor activity (Zingg *et al*, 2014). It is

also possible that a functional interaction between ACC and PL neurons, associated with preparatory attention in rats, could be disrupted in the present study (Totah *et al*, 2013). Our Cre-dependent viral approach in mouse provides a template for future circuit-specific chemogenetic studies for alternative combinatorial approaches to address such possibilities; replacing AAV8-Cre with retrograde traveling Canine adenovirus type 2 (CAV)-Cre in axonal projecting areas will permit Cre expression only within unique projection circuits (Boender *et al*, 2014; Carter *et al*, 2013; Penzo *et al*, 2015). This chemogenetic dissection approach can serve as a powerful tool to deduce which circuits inhibited by global dACC inactivation primarily contribute to attention deficits. Finally, noninvasive nature of chemogenetic approach is particularly well-suited to examine how established dynamic changes in network activity across adolescence contribute to brain development (Booth *et al*, 2003; Fair *et al*, 2007).

Although our study proposes a new neurobiological basis underlying 5CSRTT performance in mice, it would be fruitful to extend a chemogenetic approach to other attention tasks in future studies. Although 5CSRTT is the most widely used task to study the neural basis of attention in rodents (Lustig *et al*, 2013; Moore *et al*, 2013), there are some limitations (Young *et al*, 2012; Young and Geyer, 2015). First, 5CSRTT does not contain nontarget stimuli requiring subjects to inhibit their responses that are included in the continuous performance task (CPT), a gold standard of attention task in human patients. A variant of the 5CSRTT, the rodent five-choice continuous performance test (5C-CPT), requires response inhibition to irrelevant stimuli and provides greater consistency with the human CPT (Young *et al*, 2009). Second, the primary measure of attention deficits in 5CSRTT performance is inconsistent between humans and rodents (Young *et al*, 2013b). In the human 5CSRTT, schizophrenia patients show increased variability in reaction times with no accuracy and omission deficits (Barnett *et al*, 2010); however, attention performance in rodent 5CSRTT is assessed mainly by accuracy and omission. As the 5C-CPT, similar to the human CPT, permits performance analysis using signal detection theory, it may have a better translational validity for the human CPT (Young *et al*, 2013a). Applying the DREADD technology we used here to 5C-CPT would greatly facilitate the translation of findings in animal studies for potential treatments for humans. Finally, the National Institute of Mental Health Research Domain Criteria (RDoC) project recommends a divided attention task as a primary measurement of attention in humans (Cuthbert, 2015; Insel *et al*, 2010). Future efforts to establish a well-designed divided attention task (target selection in the presence of competition) (Arnold *et al*, 2003) together with chemogenetic manipulation would be another important direction.

In the present study, we demonstrated a causal relationship between the dACC neuronal activity and visual attention, and established a foundation for applying a chemogenetic approach in genetically tractable species. Combining chemogenetic technology with translational behavior tasks in mouse will be a useful strategy to understand the neural basis of attention in more detail at cell-type and circuit levels to reveal how attentional deficits are caused in psychiatric disorders, and also enable the more

precise delineation of drug effects on attention deficits by identifying the mechanism of treatment-induced improvement of attentional control in preclinical animal models.

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