A role for cerebral cortex in the suppression of innate defensive behaviour

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Abstract
The cerebral cortex is widely accepted to be involved in the control of cognition and the processing of learned information. However, data suggest that it may also have a role in the regulation of innate responses because rodents, cats or primates with surgical removal of cortical regions show excessive aggression and rage elicited by threatening stimuli. Nevertheless, the imprecision and chronic nature of these lesions leave open the possibility that compensatory processes may underlie some of these phenotypes. In the present study we applied a precise, rapid and reversible inhibition approach to examine the contribution of the cerebral cortex to defensive behaviours elicited by a variety of innately aversive stimuli in laboratory mice. Pharmacological treatment of mice carrying the pharmacogenetic inhibitory receptor hM4D selectively in neocortex, archicortex and related dorsal telencephalon-derived structures resulted in the rapid inhibition of cerebral cortex neural activity. Cortical inhibition was associated with a selective increase in defensive behaviours elicited by an aggressive conspecific, a novel prey and a physically stressful stimulus. These findings are consistent with a role for cortex in the acute inhibition of innate defensive behaviours.

KEYWORDS
avoidance, cortex, fear, predation, social approach

1 | INTRODUCTION
The cerebral cortex is an evolutionarily recent brain region that comprises the largest part of the mammalian forebrain (Merel et al., 2019; Molnár et al., 2014; Montiel & Aboitiz, 2015). Lesions of the cortex reduce cognitive and motor function in primates, but in lower mammals the motor effects of such lesions are minimal (Lopes et al., 2017; Walker & Fulton, 1938), suggesting that the executive motor control function of the cerebral cortex is a relatively recent adaptation. This distinction is supported by anatomical evidence that cortical projection neurons directly enervate motor neurons in spinal cord only in primates (Bernhard & Bohm, 1954; Lawrence & Kuypers, 1968; Yang & Lemon, 2003). As a result of the more severe effects of cortical lesions in higher mammals, it has been argued that the primary function of the cerebral cortex is better understood by examining its contributions to brain function in lower mammals (Lopes et al., 2017). Cortical lesions in cats, for example,
spare motor function and simple learning, although learning is slower and the animals show hesitation in executing motor actions that require three-dimensional visual-motor feedback (Bjursten et al., 1976). Decorticated animals eat and drink adequately, show normal sexual behaviour and maintain normal locomotor activity (Bjursten et al., 1976). They also perform visual discrimination (Winans, 1967) and are able to retain memories acquired before the lesion (Kawai et al., 2015). These data are consistent with the hypothesis that the primary role of cerebral cortex is to acquire experience-dependent models of the world and use these to guide behaviour in moments of uncertainty (Heindorf et al., 2018; Marple-Horvat et al., 1993; Schröder et al., 2019; van Bergen et al., 2015).

In contrast, subcortical brain regions are evolutionarily ancient, having their origins in the annelid and possibly pre-bilaterian ancestor (Tessmar-Raible et al., 2007). Notably, the hypothalamus contains a series of motor control networks that are necessary for the formation of internal states and the production of stimulus-appropriate adaptive behavioural and physiological responses, in particular those concerned with reproduction, defence and seeking (Swanson, 2000). Thus, the mammalian brain is able to elicit the majority of essential behavioural responses using subcortical circuits, whereas the cerebral cortex is likely to have developed to provide cognitive support for motor actions, culminating in the development of its ability to directly control motor behaviours in primates (Tomer et al., 2010).

Nevertheless, early lesion studies noted that removal of the cerebral cortex altered more than just cognitive function. In the first case in which such a lesion was carefully documented, damage to the human frontal cortex was associated with irritability, irreverence and disrespectfulness, with the attending physician noting that the ‘equilibrium between intellectual faculties and animal propensity had been destroyed’ (Harlow, 1869). Later, similar phenotypes were found in laboratory cats and dogs with targeted lesions of the cerebral cortex that consistently showed exaggerated defensive responses to otherwise innocuous stimuli (Dusser de Barenne, 1920; Goltz, 1892; Rothmann, 1923). Further studies showed that the phenotype extended to hyperexcitability caused by noise or by a physically stressful condition such as infection by worms (Schaltenbrand & Cobb, 1930). These so-called ‘sham rage’ phenomena were hypothesized to derive from the release from cortical inhibition of midbrain regions known to generate defensive behaviour (Bard, 1928, 1934; Bard & Riench, 1937; Cannon & Britton, 1925). Interestingly, when the decortication was performed during infancy, lesioned animals only started to show exaggerated defensive responses following sexual maturity (Bjursten et al., 1976), suggesting that this cortical inhibition function develops during adolescence.

However, cortical lesion studies to date have used physical removal of the cortical hemispheres, and it remains possible that the effects seen depend on long-term compensations that may have occurred in the remaining brain regions. At the same time, aspiration lesions of the cortex frequently suffer from imprecision that spares parts of cortex or removes adjacent subcortical regions (Goltz, 1881, 1888), leaving it unclear if the lesion phenotypes were strictly dependent on cortical function (Head, 1921). Finally, the impact of cortical lesions on innate behaviours has not been systematically examined. In particular, it remains unclear whether the increased threat responses seen in some studies generalize across stimuli. To overcome these limitations and directly assess whether cortex has a generalized role in the acute inhibition of threat responses, we developed a pharmacogenetic method to precisely, rapidly and reversibly suppress cortical function in mice and assess alterations in a variety of innate defensive behaviours. We found that acute inhibition of cortex caused increased defensive responses to social, non-social and physical threat cues.

2 | RESULTS

2.1 | Rapid pharmacogenetic inhibition of cerebral cortex

In order to precisely, rapidly and reversibly inhibit the entire cerebral cortex, we generated mice expressing the pharmacogenetic inhibitory receptor hM4D (Armbruster et al., 2007) in all cortical principal neurons. Experimental and control littermate mice were obtained by crossing mice expressing hM4D under a ubiquitous Cre-dependent promoter (RC::PDi; Ray et al., 2011) with mice expressing Cre recombinase under control of the Emx1 promoter (Rosa-CAG::LoxP-mCherry-STOP-LoxP:hM4D x Emx1::Cre; Figure 1a). Emx1::Cre drives expression in excitatory projection neurons of the cerebral cortex. Importantly, the driver line is active in both neocortex and archicortex, including hippocampus, piriform cortex, pallial amygdala and olfactory bulb, and thus allowed us to precisely direct hM4D expression across all dorsal telencephalon-derived structures in a manner not possible with earlier lesions (Iwasato et al., 2000, 2004). For brevity, we refer to these Emx1::Cre driver expressing brain regions as ‘cerebral cortex’ and their pharmacogenetic inhibition as ‘cortical inhibition’ throughout the paper. Control animals lacked the Emx1::Cre allele and thus did not express hM4D.
FIGURE 1  Transgenic mouse allowing rapid drug-induced cortical inhibition. (a) Schematic representation of (left) control and (right) hM4D experimental mice carrying, respectively, the Cre-dependent transgene expressing the pharmacogenetic inhibitory receptor hM4D alone or together with the tissue and cell-type specific Cre driver line Emx1::Cre. In the absence of Cre activity, mCherry is ubiquitously expressed, and hM4D is not expressed, whereas in the presence of Cre activity, mCherry is excised, and hM4D is expressed. (b) Histological analysis demonstrating (top) ubiquitous mCherry fluorescent reporter gene expression and background staining for the haemagglutinin-tagged hM4D receptor in (bottom left) cortex and (bottom right) thalamus. (c) Histological analysis demonstrating (top) selective deletion of mCherry fluorescent reporter gene expression in neocortical and archicortical structures and positive staining for the haemagglutinin-tagged hM4D receptor in (bottom left) cortex, but not (bottom right) thalamus (see also Figure S1). (d) Schematic representation of in vivo electrophysiology recording set-up showing stimulating and recording electrodes implanted contralaterally in the cortex; created with BioRender.com. (e) Quantification of field excitatory postsynaptic potentials (fEPSPs) recorded in control (N = 5) and hM4D (N = 4) mice at various times before and after CNO treatment (+P < 0.1, *P < 0.05)
(Figure 1a). As expected, fluorescence of the mCherry reporter was found throughout the brain in control mice (Figure 1b) but was reduced in the cortex of experimental double transgenic animals (Figures 1c and S1). Immunofluorescence staining against the haemagglutinin (HA) tag of hM4D showed expression in cortex of experimental, but not control, animals, although expression in thalamus was lacking in both, as expected (Figure 1b,c).

Treatment of mice expressing hM4D with clozapine-N-oxide (CNO) results in the activation of hM4D and the activation or inhibition, respectively, of somatodendritic G-coupled inward rectifying potassium (GIRK) and presynaptic voltage-gated calcium (VGCC) channels and inhibition of neural responsivity and neurotransmitter release (Armbuster et al., 2007; Mizutani et al., 2006). To confirm neural inhibition of cortex in our animals, we carried out in vivo electrophysiological measurements of evoked local field potential (fEPSP) responses in experimental and control littersmates treated with CNO. Recording and stimulation electrodes were implanted contralaterally in the motor cortex (Oh et al., 2014; Figure 1d) and fEPSP responses to repeated stimulation (0.1 Hz, 1–10 mA) were collected and analysed. Consistent with previous data in which fEPSPs were monitored to assess hM4D function (Madroñal et al., 2016), we observed a significant difference in fEPSP amplitude in experimental versus control animals starting 1 h after CNO delivery (3 mg/kg, i.p.; two-way repeated measures ANOVA—treatment: \( F[1,42] = 2.93, P = 0.131 \); time: \( F[6,42] = 14.29, P < 0.001 \); treatment \( \times \) time: \( F[6,42] = 2.78, P = 0.023 \); Figure 1e). Because evoked fEPSPs reflect fluctuations in the production of current sinks and sources at the recording electrode in response to the electrical stimulation of synaptic inputs, rather than action potential firing, the increase in fEPSP response observed in CNO-treated mice is consistent with the hyperpolarization of cortical target neurons by hM4D activation and a consequent larger change in current density at the target in response to afferent stimulation (Buzsáki et al., 2012; Fernández-Ruiz et al., 2013; Herreras, 2016).

### 2.2 Cerebral cortex suppresses social avoidance

To examine a possible role of cerebral cortex in the modulation of innate defensive responses, we treated experimental mice with CNO to suppress cortical neuron function and exposed them to three classes of innate threat stimuli: a social aggressor, a novel prey, and a physical stressor. For exposure to social threat, singly housed male hM4D and control mice were injected with saline or CNO (3 mg/kg, i.p.) and allowed to habituate to the testing room in their home cage for 1 h before behavioural testing. Testing comprised a 5-min habituation phase followed by a 5-min social phase when a CD1 aggressor was placed into the home cage of the mouse within a wire-mesh cage (Franklin et al., 2017; Figure 2a). Quantification of behaviour showed that CNO-treated hM4D mice showed a significant decrease in total locomotor activity during the social phase when compared with saline-treated hM4D mice (two-way ANOVA—group: \( F[1,26] = 0.451, P = 0.508 \); treatment: \( F[1,26] = 2.63, P = 0.117 \); group \( \times \) treatment: \( F[1,26] = 10.31, P = 0.003 \)) but that this effect was not present during the habituation phase (two-way ANOVA—group: \( F[1,26] = 1.55, P = 0.223 \); treatment: \( F[1,26] = 1.64; P = 0.211 \); group \( \times \) treatment: \( F[1,26] = 0.301; P = 0.588 \); Figure 2b,c). CNO-treated hM4D mice also showed a significant decrease in social approach (Mann–Whitney: \( U = 7.0, P = 0.026 \)) and increase in latency to investigate the threat (Kaplan–Meier: \( \chi^2(1) = 8.106, P = 0.004 \)) during the social phase when compared with saline-treated hM4D mice (Figure 2d,e). No significant modulation of social approach (Mann–Whitney: \( U = 24.0, P = 0.442 \)) or latency to investigate (Kaplan–Meier: \( \chi^2(1) = 0.275, P = 0.600 \)) or locomotor activity in the social phase was observed in control mice treated with CNO compared with saline, confirming that the effect of CNO in hM4D mice depends on the expression of the pharmacogenetic transgene (Figure 2b–e). Importantly, CNO-treated hM4D female mice exposed to the CD1 aggressor did not show any change in locomotor activity (two-way ANOVA—group: \( F[1,25] = 0.150, P = 0.702 \); treatment: \( F[1,25] = 1.089, P = 0.307 \); group \( \times \) treatment: \( F[1,25] = 0.0461, P = 0.832 \)), social approach (Mann–Whitney: \( U = 30.5, P = 0.878 \)) and avoidance (Mann–Whitney: \( U = 25.0, P = 0.505 \)) behaviour when compared with saline-treated hM4D littersmates, demonstrating that the effect of cortical inhibition is absent when the social interaction is affiliative, rather than antagonistic (Figure S2b–d). Nevertheless, a significant increase in latency to investigate in CNO-treated hM4D female mice when compared with saline-treated hM4D littersmates supports the idea that the cortex is involved in the inhibition of avoidance when the threat is unknown (Kaplan–Meier: \( \chi^2(1) = 6.884, P = 0.009 \); Figure S2e).

Next, we examined the effect of cortical inhibition on social approach in animals that had been habituated to the threat. Habituation reduces novelty-associated defensive behaviours, and if cortex were to have a selective effect on defence, then the effect of cortical inhibition should be attenuated under these conditions. The social approach test was repeated daily for 5 consecutive days with CNO or saline treatment on the first and last days,
under a pseudorandomized assignment (Figure S3a). To control for possible effects of stress associated with the treatment, all animals were given saline on the intervening days. On Day 5, CNO-treated hM4D mice showed a significant increase in social approach as compared with the first day, reaching the same level as controls (Mann–Whitney: $U = 0.00, P = 0.003$; Figure S3b). As expected, CNO treatment had no effect on total activity in the social phase on either day (two-way ANOVA—group: $F[1,27] = 0.496, P = 0.487$; treatment: $F[1,27] = 2.104, P = 0.158$; group $\times$ treatment: $F[1,27] = 0.0229, P = 0.881$; Figure S3c). This finding demonstrates that cortical inhibition selectively affects defensive behaviour under conditions of novelty and also supports earlier findings showing that habituation learning is intact in decorticated animals (Kawai et al., 2015).

Finally, we performed a within-subject analysis to examine whether cortical inhibition selectively
modulated defensive behaviours in a manner that was
time-locked to the introduction of the threat stimulus.
Although both CNO (Wilcoxon signed rank: Z = −2.366,
P = 0.018) and saline-treated hM4D mice significantly
increased freezing behaviour when exposed to the social
threat (Wilcoxon signed rank: Z = −2.070, P = 0.038),
only CNO-treated mice showed a significant increase in
avoidance behaviour in response to social threat (two-
way repeated measures ANOVA—treatment: F[1,12] =
7.25, P = 0.020; phase: F[1,12] = 19.061, P < 0.001;
treatment × phase: F[1,12] = 7.22, P = 0.020), as rev-
A | 2.3  Cerebral cortex suppresses prey avoidancen

Next, we examined the impact of cortical inhibition on
behavioural responses to a novel prey, a powerful non-
social threat stimulus. Mice are highly motivated to hunt,
kill and eat cockroaches but must learn to overcome
strong avoidance responses elicited by the prey during
the initial encounters (Rossier et al., 2021). To quantify
defensive responses to prey, hM4D and control mice were
An | treated with CNO and saline and allowed to habituate to
the testing room for 1 h before the introduction of a live
cockroach for 40 min (Figure 4a). CNO-treated hM4D
mice showed a trend for an increase in defensive freezing
(Mann–Whitney: U = 75.0, P = 0.069) and avoidance
(Mann–Whitney: U = 76.0, P = 0.079) behaviour during
the initial phase of exposure to the cockroach when com-
pared with saline-treated hM4D littermates (Figure 4b,c).
However, predatory aggression towards the cockroach
was not altered in CNO-treated hM4D mice when com-
pared with saline-treated hM4D mice as shown by an
absence of change in time spent attacking (Mann–Whit-
ney: U = 100.0, P = 0.847), mean attack duration
(Mann–Whitney: U = 98.0, P = 0.777), latency to attack
(Kaplan–Meier: χ²(1) = 0.748, P = 0.387) and latency to
kill (Kaplan–Meier: χ²(1) = 2.314, P = 0.128; Figures 4d,e
and S4). Neither change of the dynamic of predatory
aggression across the time was observed between
CNO-treated and saline-treated hM4D mice according to
the comparison of the time spent attacking (mixed linear
model—time: F = 1.326, P = 0.175; treatment: F = 3.798,
P = 0.052; time × treatment: F = 0.783, P = 0.783) and
the number of attacks (mixed linear model—time:
F = 0.996, P = 0.501; treatment: F = 0.358, P = 0.550;
time × treatment: F = 1.046.783, P = 0.438), measured
over the total duration of the test (Figure 5S).

Moreover, within-subject analysis revealed a signifi-
cant inverse correlation between defensive and hunting
behaviours in CNO-treated hM4D mice, suggesting a
potential mechanistic link between these measures
(Figure S6). As expected, CNO treatment did not cause
any change in freezing (Mann–Whitney: U = 111.0,
P = 0.953), avoidance (Mann–Whitney: U = 98.0,
P = 0.406), time spent attacking (Mann–Whitney:
U = 73.0, P = 0.106), mean attack duration (Mann–Whitney:
U = 77.0, P = 0.148), latency to kill (Kaplan–Meier:
χ²(1) = 1.706, P = 0.192) and latency to attack (Kaplan–Meier:
χ²(1) = 1.981, P = 0.159) in control mice (Figures 4b–e and S4).
These results support a selective role for cortex in the inhibition of defensive responses to both social and non-social threat, but not in
the modulation of predatory aggression.

2.4  Cerebral cortex promotes passive stress
coping behaviour

Finally, to detect whether cortex has a role in regulating
behavioural responses to a non-biological, physical
stressor, we subjected hM4D and control mice treated
with CNO and saline to the forced swim test (FST; Can
et al., 2012; Lucki et al., 2001; Porsolt et al., 1977). Mice
and rats typically struggle to escape during the initial
period of the FST and then settle into a period of alternat-
ing escape and immobility as the test continues (Can
et al., 2012; Lino-de-Oliveira et al., 2005). The amount of
immobility has been used as a measure of passive stress
coping (Molendijk & de Kloet, 2019). Mice were treated
with CNO or saline and left to habituate in their home
cage for 1 h before being placed into a beaker containing
3 L of tepid water where immobility was monitored for
4 min (Figure 5a). CNO-treated hM4D mice showed a
significant increase in time spent immobile when com-
pared with saline-treated hM4D mice, whereas the
drug treatment had no effect on control animals
(two-way ANOVA—group: F[1,54] = 0.307, P = 0.582;
treatment: F[1,54] = 0.819, P = 0.370; group × treatment: F[1,54] = 4.75, P = 0.034; Figure 5b).

Based on evidence that decorticated animals show
behavioural changes that emerge after sexual maturation
(Bjursten et al., 1976), we examined the impact of cortical
inhibition on FST responses in adolescent mice (postna-
tal Day 30). At this age, CNO-treated hM4D mice showed
no significant change in immobility when compared with
saline-treated hM4D littermates (two-way ANOVA—
group: F[1,55] = 0.573, P = 0.452; treatment: F[1,55] =
1.01, P = 0.320; genotype × treatment: F[1,55]
CNO treatment had no significant effect in control animals. These results argue for an involvement of cerebral cortex in the inhibition of innate defensive responses to threats regardless of whether they are social, non-social or physical and that this inhibition may mature during adolescence.
3 | DISCUSSION

In the present study, we examined the behavioural effects of acute pharmacogenetic inhibition of the cerebral cortex of mice. We tested the hypothesis that activity in the cerebral cortex suppresses subcortical structures that promote defensive behaviours and that lesions of cortex are associated with increased defensive responses to a variety of threats. Our findings confirm a selective role for cortex in suppressing innate defensive behaviours and failed to find any effect of cortical inhibition on other innate behaviours, including predatory aggression and affiliative social interactions. These findings suggest that earlier claims for a disinhibitory effect of cortical lesions on aggression, irritability and antisocial behaviour may be linked to a more specific effect on altering behavioural...
responses to threat. Our findings provide renewed support for the ‘release phenomenon’ concept described in the 18th century (Jackson, 1884) and revisited by scientists in the 20th century (Macmillan, 1992). This concept argued that the emotional excitement observed in decorticated animals in response to otherwise innocuous stimuli was due to the release of subcortical brain centres from cortical control.

The DREADD-dependent inhibition approach we chose to use allowed us to pharmacologically induce the precise inhibition of principal neurons across the entire neocortex and archicortex and thus overcome confounds associated with the imprecision of earlier aspiration, excitotoxic or pharmacological lesion studies. Moreover, our pharmacogenetic approach overcomes limitations of earlier irreversible lesions approaches that could have been confounded by long-term compensatory processes.

In light of our results in which cortical inhibition showed a selective impact on defensive behaviours, we speculate that either the wider phenotypes described with chronic lesions that frequently included aggression and irritability (Clemente & Lindsley, 1967) were the indirect result of a change in threat responses processes, or they were due to compensations arising from the chronic nature of the manipulations.

Our experiment examining the impact of cortical inhibition on prey approach, attack and capture suggests that cortex modulates prey avoidance, but not aggression. Pre-}

FIGURE 5  Acute cortical inhibition increases immobility during forced swim stress in adulthood, but not adolescence. (a) Experimental protocol for forced swim test. Mice were treated with saline or CNO and returned to their home cage for 1 h before introduction into a beaker of water and observation for 40 min. Time spent immobile for (b) adult mice (postnatal day 60, P60) and (c) adolescent mice (postnatal day 30, P30; N = 14–16; *P < 0.05; +P < 0.1)
aggression. Similar conclusions have been drawn from cortisol lesions performed in cats (Bard, 1928; Cannon & Britton, 1925; Dusser de Barenne, 1920; Rothmann, 1923). Interestingly, our observation that increased freezing and prey avoidance were associated with less successful hunting (Figure S4) supports the conclusion that these phenotypes are causally linked and that cerebral cortex is involved in the regulation of defence, rather than predatory aggression. We caution, however, that our social interaction test did not include measurements of social aggression (e.g. resident–intruder aggression) and thus did not allow us to draw conclusions about a role for cortex in defensive aggression.

Cortical inhibition significantly increased passive coping responses in the FST (Figure 5b), confirming previous reports of the cortical modulation of coping behaviour in this test (Duncan et al., 1993; Warden et al., 2012). The FST has been widely used as a pharmacological screening method for identifying antidepressants where it is proposed to measure the state of despair in mice (Lucki et al., 2001; Porstl et al., 1977). However, the tendency of mice to switch from initial struggling to immobile floating has also been interpreted as a passive coping strategy that animals adopt when exposed to an inescapable environmental stressor (Commons et al., 2017; Molendijk & de Kloet, 2019). This view suggests that the increased immobility associated with cortical inhibition that we observe reflects an increased tendency to choose a passive coping strategy to mitigate an aversive environment and, when combined with our other behavioural findings of increased avoidance responses, may indicate that cortical inhibition increases perceived threat intensity. Alternatively, the increased immobility in FST could reflect an adaptive response aimed at saving energy in the face of danger (de Kloet & Molendijk, 2016; West, 1990). In either case, increased immobility supports a role for cortex in suppressing either the perceived value or action motivation associated with threat responses.

Importantly, the effect of cortical inhibition was not observed in younger animals (Figure 5c). Previous studies have pointed to different mechanisms controlling FST behaviour in adolescent and mature animals (Doherty et al., 2017), and earlier decortication experiments noted the appearance of behavioural disturbances only after adolescence (Bjursten et al., 1976). We interpret this finding as consistent with the peri-adolescent development of cortical inhibition of behaviour reported in humans (Constantinidis & Luna, 2019) and supported by the delayed development of selected cortical-fugal projections in mammals (González-Maeso et al., 2007; Narboux-Nême et al., 2012; Piszczek et al., 2015).

Although our study did not allow us to identify the cortical projections involved in the behavioural effects we observed, our findings are consistent with studies implicating projections from prefrontal cortex to dorsal periaqueductal grey in the inhibition of social avoidance (Franklin et al., 2017) and to the midbrain dorsal raphe nucleus in the inhibition of FST escape (Warden et al., 2012). Meanwhile, similar inhibitory effects of cortex on brainstem regions involved in defensive behaviour have been inferred from human functional connectivity imaging studies. fMRI data in the context of virtual escape from predator threat demonstrate that the cingulate cortex, hippocampus and amygdala are recruited in the first phase of detection of potential threat and that these regions are subsequently inhibited in concert with the activation of periaqueductal grey when attack is imminent (Mobbs et al., 2007, 2009).

Our evoked electrophysiological recordings suggest that the transgenic pharmacogenetic inhibition approach we employed was likely to only partially inhibit cortical excitatory neurotransmission (Figure 1e). This finding was not entirely unexpected, as DREADD-based inhibition is frequently found to induce only partial inhibition of both baseline and evoked neural responses when examined in ex vivo brain slices. However, we are aware of only one other study in which hM4D-dependent suppression of neurotransmission was examined in vivo (Madroñal et al., 2016). The larger effect seen in this earlier study (60% vs. 25% here) may be related to the moderate expression levels achieved by the transgenic expression of hM4D that, for example, lacks the WPRE RNA stability element (Ray et al., 2011).

It is important to note that the Emx1::Cre driver line we used is active in both neocortex and archicortex and related dorsal telencephalon-derived structures. Thus, the full range of cortical regions were suppressed in our experimental animals, including hippocampus, basolateral amygdala, piriform cortex and olfactory bulb (Iwasato et al., 2000, 2004). This feature allowed us to systematically target all cortical regions (Garey, 1994; Gerfen & Wilson, 1996; Kapper, 1909; Swanson, 2000) in a manner not possible with earlier physical, chemical or pharmacological lesions (Bard, 1928, 1937; Bard & Mountcastle, 1948). However, it leaves open the relative roles of amygdala, hippocampus, olfactory bulb and neocortex in the behavioural effects we observe. Notably, lesions of amygdala and hippocampus have been reported to have anxiolytic effects that promote approach behaviour (Korn et al., 2017) and abolish innate defensiveness towards a threat (Martinez et al., 2011; Pentkowski et al., 2006), data that go in the opposite direction of the phenotypes we observed. Thus, we tentatively assign the inhibition of defensive behaviours to neocortex and speculate that it may be particularly dependent on midline neocortical structures such as...
prefrontal and cingulate areas that have prominent midbrain projections known to regulate defence (Franklin et al., 2017; Rozeses et al., 2018; Warden et al., 2012).

It remains possible that the increased defensive behaviour we observed was a non-specific consequence of a behavioural deficit in our mice. We do not favour such a non-specific effect for several reasons. First, spatial navigation appears not to have been significantly affected by our manipulation despite the known role of neocortical and archicortical structures in this behaviour (Chersi & Burgess, 2015; Lopes et al., 2017; Poucet et al., 2003). For example, we observed no detectable change in hunting behaviour (Figures 4d,e, S5 and S6) or social approach (Figure S2c), suggesting the ability of subcortical structures to support sophisticated spatial orientation and goal-directed behaviours. These findings are consistent with earlier cortical lesion studies that found intact navigation, spatial learning, social play and sexual behaviour (Bjursten et al., 1976; Kawai et al., 2015; Oakley, 1979; Terry et al., 1989; Whishaw & Kolb, 1983). Second, the persistence of sophisticated social behaviours and their proper habituation across experiences suggests unaffected olfaction. For example, female mice showed no differences in activity, social approach and avoidance towards male intruders (Figure S2), whereas males—whose interactions are known to include territorial threat responses—did (Figures 2 and 3), although we cannot entirely exclude the alternative possibility that a response in females was masked by a mating-associated affiliative component elicited by their exposure to the male intruder. Further support for a modification of threat rather than olfactory responses comes from our observation of an increase in escape responses in the FST where olfactory cues are not thought to play a role.

An important confound of our approach is that Emx1::Cre has been shown to be active in dorsal telencephalic glial cells as well as neurons, including astrocytes and oligodendrocytes that share a common origin with cortical excitatory neurons (Gorski et al., 2002; Iwasato et al., 2004). Although glial expression has not been verified in the driver line we used, we consider it likely that we have activated hM4D in cortical glia to some extent in our experiments. However, the impact of the acute activation of G-alpha-i signalling in non-excitable cells on neuronal circuit activity is not straightforward to predict, and the acute pharmacological activation of hM4D selectively in cortical astrocytes did not modulate innate behaviours, including baseline and shock-induced freezing and locomotion in a novel arena (Kol et al., 2020). One can also argue that if hM4D signalling did have an acute impact on local neuronal activity (see Kol et al., 2020 for evidence that it does not) such an effect would be superseded in our animals by the direct hM4D-mediated inhibition of local excitatory projection neurons. So, although we cannot rule out that the acute effects we see on innate behaviour are mediated in part by DREADD signalling in glia, we consider this unlikely to be the major mechanism underlying our findings.

In conclusion, our data point to the existence of a role for cortical structures in suppressing innate defensive responses to a variety of threats. Human functional imaging studies show that prefrontal cortical regions are hyperactivated during active escape from a virtual predator but quickly shut down when capture by the predator is imminent (Mobbs et al., 2007). This cortical shutdown is accompanied by a hyperactivation of midbrain defence control regions that are known to drive flight behaviour and be directly inhibited by prefrontal projections (Franklin et al., 2017). We speculate that this ability may be part of an adaptive mechanism aimed at inhibiting defensive responses under conditions in which cortical circuits can benefit from collecting more information about threat context to guide optimal response strategies but allow subcortical defensive regions to take over when survival is at stake.

4 | MATERIALS & METHODS

4.1 | Animals

All mice tested were obtained by internal colonies from the European Molecular Biology Laboratory. Mice were maintained in temperature and humidity-controlled condition with food and water provided ad libitum and on 12-h light–dark cycle (light on at 7:00). Experimental groups Emx1::Cre; RC::FPDi (called hM4D group) and RC::FPDi (called control group) consisted of littermate mice obtained by crossing homozygous RC::FPDi mice, kindly provided by Dr Susan Dymecki (Harvard Medical School, Boston, USA; Ray et al., 2011) and heterozygous Emx1::Cre mice (Piszczek et al., 2015). Adult CD1 aggressors used as intruders were selected based on a screening procedure previously described (Franklin et al., 2017). All experiments were performed in accordance with EU Directive 2010/63/EU and under approval of the EMBL Animal Use Committee and Italian Ministry of Health License 541/2015-PR to C.T.G.

4.2 | Behavioural testing

4.2.1 | Social avoidance test

Male and female animals, 2–4 months old, were singly housed 1 week before the test day and habituated to
skeletal saline i.p. injection for 2 days preceding the test. Experimental mice of the hM4D or control group were randomly treated with saline or CNO (3 mg/kg i.p. in 0.9% saline, Sigma-Aldrich) and placed for 1 h in their home cage where the test was subsequently carried out (Figure 2). After 5 min of habituation in which the activity of each subject was automatically recorded, an aggressive male CD1 intruder was placed into the resident home cage constrained within a wire-mesh cage. Mice interacted for 5 min while approach/avoidance behaviour was scored from videotape either automatically in case of activity, social approach, freezing and avoidance or manually for the latency to the first investigation using Solomon coder software. Male and female behavioural parameters were grouped separately, and the test was repeated for 5 consecutive days. Locomotor activity was calculated as the total time mice spent in activity (>0.1 cm/s calculated on a frame-by-frame basis) normalized for the test duration (300 s), and social approach was quantified as the time spent in the half of the available cage space closest to the intruder wire-mesh enclosure.

4.2.2 | FST

One hour after CNO or saline treatment (3 mg/kg, i.p.), mice (male and female) were placed in a glass cylindrical beaker (26 cm height × 16 cm diameter) filled with 3 L of tap water at 23–25 °C (Can et al., 2012). Rectangular white cardboard dividers were used in order to prevent mice from seeing each other while they were tested side by side. The duration of the test was 6 min, and the first 120 s was excluded from behavioural scoring because mice during this period show persistent escape activity (Can et al., 2012). Immobility was calculated by subtracting the time swimming from the total test duration. Swimming was scored whenever the mouse was actively moving its limbs but excluded periods when the animal made only limited circular movements of the legs aimed at maintaining its head above water. After the test, mice were dried with paper towels and placed for a short period under a heat lamp to recover. A subset of mice was tested at both time points, in which case the treatment at P60 was pseudorandomly scrambled.

4.2.3 | Hunting test

After treatment with saline or CNO (3 mg/kg, i.p.), 4- to 5-month-old male and female mice, previously group housed, were isolated for 1 h in a novel cage where the test occurred. Mice were exposed to a live cockroach (Blatta lateralis). The test ended when the mouse killed the cockroach or after 40 min, whichever was shorter. Aggression (time spent attacking, attack duration) was manually scored. Freezing and avoidance (time spent in the corner of the cage) were quantified during the first minute of exposure to the cockroach; percentage of time spent attacking was calculated as the time spent attacking divided by the total time of the test and attack duration as time spent attacking divided by the total number of attacks. Mice exposed to this test had previously performed FST, and in order to avoid possible confounds due to repeated pharmacological treatment, animals were randomly assigned to saline or CNO group, and an inter-subject analysis excluded that behavioural effects observed were somehow related to previous treatment (data not shown).

4.3 | In vivo electrophysiology

Synaptic field potentials (fEPSPs) were evoked by delivering 100 μs, square, biphasic pulses applied to the contralateral motor cortex (0.1 Hz, 40%–50% of maximum response amplitude, 4 min) at 40, 20 and 5 min before and 20, 40, 60 and 80 min after drug treatment using a pulse generator (CS-420, Cibertec) connected to an electrical stimulator (ISU-200bip, Cibertec). The neural signal was amplified (gain 1000×) and filtered (bandwidth 0.1–3 kHz) through a headstage 20× preamplifier and a 50× differential amplifier (OmniPlex, Plexon). Signals were digitized at 1600 Hz, and continuous recordings were collected for post hoc analysis. For initial studies, the peak-to-peak fEPSP amplitude values were extracted using commercial software (Spike2 and SIGAVG, Cambridge Electronic Design). Electrode placement was confirmed by eliciting an electrolytic lesion at the recording site (motor cortex), and the mice were anaesthetized 3 days later using 2.5% Avertin (400 mg/kg, i.p.; Sigma-Aldrich) and perfused transcardially (4.0% vol paraformaldehyde, 0.1 M phosphate buffer, pH 7.4). Serial coronal sections (50 μm) were collected using a vibratome (Leica VT1000S) and mounted with DAPI in the mounting medium (MOWIOL; 1:1000). Sections were visualized with a fluorescent microscope (Leica DFC 345 FX), and the green filter was used to visualize the autofluorescence generated by the damage created in the tissue by the electrolytic lesion.

4.4 | Histology

Mice were deeply anaesthetized with Avertin (400 mg/kg, i.p.; Sigma-Aldrich), perfused transcardially (4% paraformaldehyde in 0.1 M phosphate buffer,
pH 7.4), and brains were post-fixed in 4% paraformaldehyde. Brains were cryoprotected and incubated overnight in a solution containing 30% sucrose in 0.1 M PBS and then frozen in Tissue-Tek OCT compound. Coronal slices (50 µm) were cut with a sliding cryostat, mounted on SuperPlus slides, allowed to dry at 42°C on a flattening table (Leica H1220) and boiled in citrate buffer (10 mM). Sections were incubated with a blocking solution (1% BSA, 5% NGS in PBS, 0.4% Triton X-100) for 1 h. A primary antibody was used to detect HA (anti-rat, 1:200, 11867423001, Roche), and detection carried out with fluorescent-labelled secondary antibodies (goat anti-rat, 1:800, Alexa Fluor 488, A-101650, Invitrogen). For the detection of mCherry endogenous fluorescent protein, animals were anaesthetized with Avertin, transcardially perfused, and brains post-fixed overnight in 4% paraformaldehyde. Coronal slices (50 µm) were cut with a sliding cryostat (Leica), and mCherry was imaged with a fluorescent microscope (Leica DFC 345 FX, 10×/0.3, 40×/0.8).

4.5 Statistical analysis

All data analysis was performed using either SigmaPlot, SPSS or GraphPad Prism software, and all data are reported as mean ± SEM or as single data points. Statistical significance was determined by two-way ANOVA or t-test for measures that satisfied the Kolmogorov–Smirnov (KS) normality criterion ($P > 0.05$). Two-way ANOVA followed by Student–Newman–Keuls post hoc testing was used to determine the significance for locomotor activity in social avoidance test and immobility in FST; two-way repeated measures ANOVA followed by Student–Newman–Keuls post hoc testing was used for in vivo electrophysiological experiments and avoidance measure in social avoidance test. Two-tailed t-test was applied to compare freezing and avoidance among treatments in social avoidance test, measured as the difference between 1 min after and before the intruder exposure. In cases not satisfying the KS criterion, non-parametric Mann–Whitney U testing was used to examine treatment effects separately in hM4D and control groups, based on the a priori assumption that CNO would have a preferential effect in hM4D-expressing animals. Under the same assumptions above, Wilcoxon signed-rank non-parametric test was used to compare treatment effects in cases of repeated measures, and Kaplan–Meier testing was used to compare treatment effects for latency parameters. Mixed linear model statistics was applied to assess the evolution of hunting behaviour across the entire test duration. Correlations between behavioural measures were analysed by Spearman non-parametric testing.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

All behavioural experiments and data analysis were carried out by S.N.; M.E.M. and S.N. carried out electrophysiological experiments and together with C.T.G. designed the experiment; S.N. together with S.D. carried out the forced swim test; S.N., S.D. and C.T.G. designed the experiments; C.T.G. supervised the project and together with S.N. conceived the project and wrote the manuscript.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, CG, upon reasonable request.

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