The Dorsal Raphe Regulates the Duration of Attack through the Medial Orbitofrontal Cortex and Medial Amygdala

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Abstract

The dorsal raphe (DR) is an evolutionarily conserved brain structure that is involved in aggressive behavior. It projects onto numerous cortical and limbic areas underlying attack behavior. The specific neurocircuit through which the DR regulates aggression, however, is largely unclear. In this study we show that DR neurons expressing CaMKIIα are activated by attack behavior in mice. These neurons project to the medial aspect of the orbitofrontal cortex (OFC; MeOC) and the medial amygdala (MeA), two key regions within the neural circuit known to control aggressive behavior. Using an in vivo optogenetic approach, we show that attack bouts are shortened by inhibiting CaMKIIα+ neurons in the DR and their axons at the MeOC and prolonged by stimulating the DR-MeOC axons during an attack. By contrast, stimulating the axons of CaMKIIα+ DR neurons at the MeA shortens attack. Notably, neither the DR-MeOC or DR-MeA pathway initiates attack when stimulated. These results indicate that the DR-MeOC and DR-MeA pathways regulate the duration of attack behavior in opposite directions, revealing a circuit mechanism for the control of attack by the DR.

Key words: aggression; dorsal raphe; medial amygdala; neurocircuit; optogenetics; orbitofrontal cortex

Significance Statement

The dorsal raphe (DR) is a major node in the brain circuit regulating multiple attack behaviors. The underlying neurocircuitry through which the DR acts on aggression, however, remains elusive. Here, we show that the DR regulates the duration of attack through the medial orbitofrontal cortex (OFC; MeOC) and the medial amygdala (MeA), areas known to play a key role in aggression. While neither pathway is sufficient to initiate an attack, silencing the DR-MeOC pathway or activating the DR-MeA pathway shortens an attack, and stimulation of the DR-MeOC circuit prolongs an already occurring attack. These findings identify two DR-mediated neural circuits that regulate attack behavior.

Introduction

The dorsal raphe (DR) nucleus is one of the raphe nuclei located on the midline of the brainstem. It is a phylogenetically conserved structure and plays a role in various types of aggressive behaviors, such as maternal and territorial aggression in rodents (Walletschek and Raab, 1982; Takahashi and Miczek, 2014; Holschbach et al., 2018; Muroi and Ishii, 2019). The role of the DR in aggression is complex and context dependent. For example, infusion of glutamate in the DR increases the frequency of attack bites against a conspecific, but with no effect on threatening behavior (Takahashi et al., 2015). Conversely, infusion of glutamate receptor agonists increases bite latency and decreases bite frequency in maternal aggression, with no effect on chasing behavior (Muroi and Ishii, 2019). Knock-
down of tyrosine receptor kinase receptors in DR neurons decreases latency to attack (Adachi et al., 2017). Prepartum lesion of the DR decreases the frequency of attack in maternal aggression, while postpartum lesion of the DR decreases the duration of an individual attack bout (Holschbach et al., 2018).

The DR contains a heterogenous population of neurons that release one or a combination of the neurotransmitters serotonin (5-hydroxytryptamine or 5-HT), dopamine, glutamate and GABA (Liu et al., 2014; Ren et al., 2018; Huang et al., 2019). Glutamatergic neurons of the DR innervate dopamine neurons of the ventral tegmental area (VTA) to reinforce instrumental responding and establish conditioned place preference (Qi et al., 2014). GABAergic interneurons of the DR mediate the acquisition of avoidance after social defeat, as demonstrated by optogenetic silencing of the GABAergic input to local 5-HT neurons (Challis et al., 2013). Dopaminergic neurons of the DR are required for rebound sociability after social isolation (Matthews et al., 2016). The serotonergic neurons of the DR are involved in aggressive behavior. In mice with reduced 5-HT release at the DR, defensive but not offensive aggression increases as determined by counter-attack bites (Chen et al., 1994). In maternal aggression, activation of 5-HT1A somatodendritic autoreceptors in the DR promotes lateral attacks, but with no effect on threatening behavior (da Veiga et al., 2011). Studies linking the DR to aggression are largely confined to the 5-HT neurons (Matthews et al., 2016). The serotonergic neurons of the DR are required for rebound sociability after social isolation (Matthews et al., 2016). The role of other DR cell types in aggression is unclear.

The prefrontal cortex and amygdala are densely innervated by the DR (Wilson and Molliver, 1991; Clarke et al., 2007; Ren et al., 2018). The orbitofrontal cortex (OFC) and medial amygdala (MeA) are subregions within these areas that receive DR inputs (Cádiz-Moretti et al., 2016; Murphy and Deutch, 2018; Ren et al., 2018) and regulate intermale aggression (Blair, 2004; Siever, 2008; Rosell et al., 2010; Hong et al., 2014; Rosell and Siever, 2015; Unger et al., 2015; Buades-Rotger et al., 2017; Haller, 2018). DR neurons projecting to the OFC and the MeA may control attack behavior in different ways. In support of this hypothesis, 5-HT signaling, the majority of which derives from neurons in the raphe, at the OFC and MeA appears to have different effects on attack behavior. In subsets of individuals with personality disorder that exhibit impulsive aggression, 5-HT2A expression is increased at the OFC, individual attack duration decreases latency to attack (Adachi et al., 2017). In OFC, the MeA mediate the acquisition of avoid-

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Table 1. Key resources table

| Resource type | Specific reagent or resource | Source or reference | Identifiers | Dilution or concentration |
|---------------|------------------------------|--------------------|-------------|--------------------------|
| Organism/strain | C57BL/6J | Charles River | Strain code: 556 |  |
| Antibody | CaMKIIa (Cba-2) mouse monoclonal antibody | Abcam | Catalog #137300 | 1:250 dilution |
| Antibody | c-Fos rabbit polyclonal antibody | Abcam | Catalog #ab190289 | 1:2000 dilution |
| Antibody | GFP polyclonal antibody | MBL | Catalog #598 | 1:1000 dilution |
| Antibody | Anti-TpH2 antibody | Abcam | Catalog #ab184505 | 1:2000 dilution |
| Antibody | Alexa Fluor 488 goat anti-mouse IgG | ThermoFisher | Catalog #A-10680 | 1:200 dilution |
| Antibody | Alexa Fluor 555 goat anti-mouse IgG | ThermoFisher | Catalog #A-21422 | 1:200 dilution |
| Antibody | Alexa Fluor 488 goat anti-rabbit IgG | ThermoFisher | Catalog #A-21428 | 1:200 dilution |
| Antibody | Alexa Fluor 555 goat anti-rabbit IgG | ThermoFisher | Catalog #A-11034 | 1:200 dilution |
| Antibody | Anti-TpH2 antibody | Abcam | Catalog #ab184505 | 1:2000 dilution |
| Antibody | GFP polyclonal antibody | MBL | Catalog #598 | 1:1000 dilution |
| Antibody | c-Fos rabbit polyclonal antibody | Abcam | Catalog #ab190289 | 1:2000 dilution |
| Antibody | CaMKIIa (Cba-2) mouse monoclonal antibody | Abcam | Catalog #137300 | 1:250 dilution |

Fibers were secured to the skull using Metabond (Parkell), stainless steel screws (PlasticsOne) and dental cement (DuraLay). After surgery, mice recovered on a heated pad until ambulatory and then were returned to their home cage for six weeks before optical stimulation.

**Resident intruder (RI) test**

Before the RI test, mice were individually housed for three weeks. All behavioral experiments took place during the dark cycle of the day, as this is the main activity phase of the mouse (Koolhaas et al., 2013). On the day of testing, mice were transferred in their home cage to a behavioral test room and allowed to acclimate for at least 1 h. Younger, group-housed target conspecific males were placed into the home cage of the resident mouse and the two were allowed to freely interact for 10 min. All animals were allowed to habituate to the patch cord for 20 min before introduction of the conspecific. Baseline aggression was tested at 1–4 d before the RI test. Animal behavior was captured with a video camera. If excessive tissue damage occurred, the test was prematurely terminated and not analyzed. Excessively aggressive mice, as determined by total attack time >40% during the RI test, were eliminated from further analysis (Hong et al., 2014; Nordman et al., 2020a).

**Optogenetic stimulation**

Optogenetic stimulation was performed via an optical fiber (ferrule fiber, ThorLabs) connected through a zirconia split sleeve and patch cord to a 473 nm laser (Coherent) or a 561 nm laser (CrystaLaser) under the control of an Optogenetics TTL Pulse Generator (Doric Lenses). Mice expressing ChR2 were stimulated for 5 ms using 1- to 3-mW 473 nm light pulsed at 10 Hz for 10 s. Mice expressing ArchT were delivered a 1- to 3-mW continuous 10-s 561 nm light pulse. Laser was manually turned on and the frequency and duration of light pulses were controlled by Doric Studio software (Doric).

**Immunohistochemistry**

Mice were transcardially perfused with 4% paraformaldehyde in PBS solution. Brains were removed and postfixed at 4°C overnight, then cryoprotected overnight in 15% sucrose (in PBS) followed by 30% sucrose in PBS. Brains were cut into 30-μm-thick sections using a cryostat (Leica CM3050-S), then either mounted onto silanized slides (KD Medical) or stored in PBS as floating sections (Leica CM3050-S). Sections were then stained for primary antibodies overnight at 4°C, followed by incubation with secondary antibodies for 1 h at room temperature.
and then a laser scanning confocal microscope (Zeiss objective to locate the areas with fluorescence signals, and then a laser scanning confocal microscope (Zeiss LSM510 and LSM780) with a 40× (NA 1.3 oil immersion) objective for high-magnification imaging in the region of interest. Z-stack confocal images were collapsed and analyzed with ImageJ by a researcher blind to the experimental conditions. c-Fos positive cells were identified using the “Analyze Particles” function of ImageJ and validated as cells by their overlap with DAPI. Cells co-labeled for DAPI and CaMKII were manually counted by a researcher blind to the experimental conditions.

### Table 2: Statistical table

| Data | Method | Factor | n   | T, U, or F stat | p value | Post hoc correction |
|------|--------|--------|-----|-----------------|---------|---------------------|
| Fig. 1C | Mann–Whitney | Resident vs control | 6, 7 | U = 2 | 0.008 |
| Fig. 1D | Mann–Whitney | Resident vs control | 6, 7 | U = 5 | 0.027 |
| Fig. 1E | Student’s t test | Resident vs control | 6, 7 | T_{11} = 0.164 | 0.872 |
| Fig. 1F | Student’s t test | Resident vs control | 6, 7 | T_{11} = 0.062 | 0.952 |
| Fig. 1H | One-way ANOVA | # of cells at bregma | 5, 5 | F_{2,14} = 13.339 | <0.001 | Tukey’s |
| Fig. 1I | One-way ANOVA | # of cells at bregma | 5, 5 | F_{2,14} = 6.357 | 0.013 | Tukey’s |
| Fig. 1J | One-way ANOVA | # of cells at bregma | 5, 5 | F_{2,14} = 168.490 | <0.001 | Tukey’s |
| Fig. 2C | Mann–Whitney | ChR2 vs GFP | 6, 6 | U = 0 | 0.005 |
| Fig. 2D | Student’s t test | ChR2 vs GFP | 6, 6 | T_{10} = 0.203 | 0.843 |
| Fig. 2G | One-way ANOVA | Tph2 vs ChR2 | 5, 5 | F_{2,17} = 13.714 | <0.001 | Tukey’s |
| Fig. 2K | Student’s t test | Opsin vs GFP | 5, 4 | T_{7} = 0.793 | 0.464 |
| Fig. 2O | Student’s t test | Before light onset (10 s) | 5, 4 | T_{7} = -0.607 | 0.563 |
| Fig. 2S | Student’s t test | Before light onset (10 s) | 5, 3 | T_{10} = 0.136 | 0.896 |
| Fig. 3L | Student’s t test | ChR2 vs GFP at bregma 2.68 | 6, 6 | T_{10} = 0.325 | 0.752 |
| Fig. 3M | Student’s t test | ChR2 vs GFP at bregma 2.34 | 6, 6 | T_{10} = 0.396 | 0.701 |
| Fig. 4L | Student’s t test | ChR2 vs GFP | 6, 6 | T_{10} = 0.474 | 0.646 |
| Fig. 4M | Student’s t test | ChR2 vs GFP | 6, 6 | T_{10} = 0.558 | 0.589 |
| Fig. 4N | Student’s t test | ChR2 vs GFP | 6, 6 | T_{10} = 0.258 | 0.801 |
| Fig. 4O | Student’s t test | ChR2 vs GFP | 6, 6 | T_{10} = 1.100 | 0.297 |
| Fig. 4P | Student’s t test | ChR2 vs GFP | 6, 6 | T_{10} = 1.203 | 0.257 |
| Fig. 4Q | Student’s t test | ChR2 vs GFP | 6, 6 | T_{10} = 4.693 | <0.001 |
| Fig. 4R | Student’s t test | ChR2 vs GFP | 6, 6 | T_{10} = 2.745 | 0.021 |
| Fig. 4S | Student’s t test | ChR2 vs GFP | 6, 6 | T_{10} = 3.104 | 0.011 |
| Fig. 4T | Student’s t test | ChR2 vs GFP | 6, 6 | T_{10} = 0.597 | 0.141 |
| Fig. 4U | Student’s t test | ChR2 vs GFP | 6, 6 | T_{10} = 2.525 | 0.030 |
| Fig. 5D | Student’s t test | Opsin vs GFP | 4, 3 | T_{9} = 1.101 | 0.321 |
| Fig. 5H | Student’s t test | Before light onset (10 s) | 4, 3 | T_{9} = 0.845 | 0.437 |
| Fig. 5L | Student’s t test | Before light onset (10 s) | 4, 3 | T_{9} = 0.632 | 0.555 |
| Fig. 6D | Student’s t test | Opsin vs GFP | 5, 4 | T_{7} = 0.137 | 0.895 |
| Fig. 6H | Student’s t test | Before light onset (10 s) | 4, 3 | T_{10} = 0.845 | 0.437 |
| Fig. 7D | Student’s t test | Opsin vs GFP | 4, 3 | T_{9} = 1.130 | 0.310 |
| Fig. 7H | Student’s t test | Before light onset (10 s) | 4, 3 | T_{9} = 1.425 | 0.214 |
| Fig. 7L | Student’s t test | Opsin vs GFP | 6, 4 | T_{8} = 0.010 | 0.342 |
| Fig. 7P | Student’s t test | Before light onset (10 s) | 6, 4 | T_{8} = 0.229 | 0.826 |

Temperature. Sections were mounted to slides with Vectashield HardSet Antifade Mounting Medium containing DAPI.

**Image acquisition and analysis**

Brain slices were imaged with a multi-slide fluorescent microscope (Zeiss Axio Scan) with a 10× (NA 0.45) objective to locate the areas with fluorescence signals, and then a laser scanning confocal microscope (Zeiss LSM510 and LSM780) with a 40× (NA 1.3 oil immersion) objective for high-magnification imaging in the region of interest. Z-stack confocal images were collapsed and analyzed with ImageJ by a researcher blind to the experimental conditions. c-Fos positive cells were identified using the “Analyze Particles” function of ImageJ and validated as cells by their overlap with DAPI. Cells co-labeled for DAPI and CaMKII and/or c-Fos were manually counted by a researcher blind to the experimental conditions.
Figure 1. CaMKIImα+ neurons of the DR are activated by attack. A–F, Mice (10 weeks of age) were examined for aggression using the RI assay. Mice were perfused 60 min after the assay for immunostaining. A, Representative images of brain sections stained for c-Fos and CaMKIImα in the DR from resident or control animals 1 h after the RI test. B, Representative high-magnification images of CaMKIImα cells in the DR that were positive for c-Fos. C, D, Percentage of c-Fos+ cells that colocalize with CaMKIImα− and DAPI− cells in resident and control mice for A, E, F, Quantification of CaMKIImα+ (C) and DAPI− (F) cells in the DR in resident and control mice. G, Representative images of brain sections stained for CaMKIImα and TpH2 throughout the DR (bregma –4.84 to –4.34 mm). High-magnification image of the DR is taken from a brain slice at bregma position –4.84, as this is where the majority of TpH2+ cells can be found. H, Quantification of total number of CaMKIImα+ and TpH2+ cells in the DR. Asterisks indicate statistical significance of CaMKIImα (green) or TpH2 (red) from merged set at the specified coordinate. One slice was quantified per area per animal. Animal number is indicated in parentheses. I, Percentage of DAPI− cells in the DR that co-label for CaMKIImα and TpH2. Asterisks indicate statistical significance of CaMKIImα (green) or TpH2 (red) from merged set at the specified coordinate. Only cells within the DR were counted. Scale bars: 200 μm (A, G) and 25 μm (B). Data are presented as mean ± SEM; *p < 0.05, **p < 0.01. Statistics can be found in Table 2.
Figure 2. Inhibition of CaMKIIα⁺ DR neurons shortens attack. **A**, Representative high-magnification and low-magnification images of ChR2-mCherry and ArchT-EYFP expression in the DR three weeks after viral injection. **B**, Representative images of brain sections stained for c-Fos in the DR of mice injected with ChR2-EYFP or GFP and photostimulated with 473 nm light (pulsed at 10 Hz for 10 s). **C, D**, Percentage of YFP⁺ or GFP⁺ cells that colocalize with c-Fos (C) and quantification of total DAPI⁺ cells (D) in the DR after photostimulation for B. Only cells within the DR were counted. **E, F**, Representative images of brain sections expressing ChR2 (E) or ArchT (F) under the CaMKIIα promoter co-stained for TpH2 in the DR. **G**, Quantification of ChR2 or ArchT (opsin) expressing cells that were stained positive for TpH2. Only cells within the DR were counted. One slice was quantified per area per animal. Animal number is indicated in parentheses. **H, L, P**, Schematic drawing of viral injection (AAV expressing ChR2 and ArchT or GFP control virus), placement of optic fiber, and stimulation procedure. Opsin expressing mice in H–K and opsin stimulated mice in L–S were injected with ChR2 virus and ArchT virus. The same mice were stimulated with 473 or 561 nm light on separate days. **I, Q, R**. Raster plots of attack events during each interaction episode (rows in the raster plot, defined as the period from 20 s before to 20 s after the onset of a spontaneous attack) for each mouse. All attack events during the testing period are shown. **J, R**, % of episodes (rows) in...
continued

which mice attacked at each time point in I; red and dark lines indicate the mean, pink and gray areas indicate SEM. K, Quantification of average attack time per mouse during the 10 s after the onset of a spontaneous attack, as represented in the boxed area in J, M. Raster plots of trials (rows) of mice photostimulated with 473 nm light at the DR when the mouse was not attacking. All trials are aligned to the onset of light. N, % of trials in which mice attacked at each time point in M; red and dark lines indicate the mean, pink and gray areas indicate SEM. O, Quantification of attack time per mouse before and during light stimulation for M, Q. Raster plots of trials (rows) of mice photostimulated with 561 nm light at the DR during an attack. All trials are aligned to onset of light. R, % of trials in which mice attacked at each time point in Q; red and dark lines indicate the mean, pink and gray areas indicate SEM. S, Quantification of attack time per mouse before and during light stimulation for Q. Scale bars: 200 μm (low-magnification image; top; A, E, F); 50 μm (high-magnification image; bottom; A, E, F); 10 μm (B). Data are presented as mean ± SEM; ***p < 0.001. Statistics can be found in Table 2.

Statistical analysis

All data were presented as individual data points and mean ± SEM SigmaPlot software was used for statistical analysis. Data were tested for normality and equal variance. Student’s t test (for data that satisfied normal distribution and equal variance) and Mann–Whitney U test (for data that did not satisfy normal distribution and equal variance) were used to compare two groups and one-way ANOVA was used to test for differences among three groups. Tukey’s test was used for post hoc multiple comparisons to identify groups that were significantly different; p < 0.05 was considered significant and all tests were two tailed. All statistical data can be found in Table 2.

Results

Excitatory neurons have been found in the DR (Commons, 2009; Qi et al., 2014; Zhou et al., 2015; Ren et al., 2018; Huang et al., 2019) and implicated in aggression (Chen et al., 1994). To better assess their function in aggressive behavior, we exposed C57BL/6 mice (male, 10 weeks of age) to the RI test and stained brain sections of the resident mice with antibodies against c-fos, which labels activated neurons, and CaMKIIα, a protein primarily expressed by excitatory neurons but not GABAergic neurons in many brain regions (Benson et al., 1992; Jones et al., 1994; Liu and Jones, 1996). The number of cells within the DR that were doubly positive for CaMKIIα and c-Fos significantly increased after the RI test, suggesting that CaMKIIα+ cells are activated by attack behavior (Fig. 1A–D). The total number of CaMKIIα+ and DAPI+ cells within the DR was comparable in resident and control mice (Fig. 1E,F). Because many DR cells co-release 5-HT and glutamate (Liu et al., 2014; Ren et al., 2018; Huang et al., 2019), we co-stained the DR sections for CaMKIIα and the 5-HT cell marker tryptophan hydroxylase type 2 (Tph2). No CaMKIIα+ cells co-localized with Tph2 throughout the DR, indicating that CaMKIIα+ neurons in the DR are not serotonergic (Fig. 1G–I).

To determine the function of CaMKIIα+ DR neurons in aggressive behavior, we opted for an optogenetic approach to alter their activities. Eight-week-old mice were injected with AAV expressing ChR2-mCherry (for neural activation) and ArchT-EYFP (for neural inhibition) under the CaMKIIα promoter into the DR for use in the RI test. Two-way crossed light pulses were delivered to the DR; 473 nm light pulses were delivered after attack had begun significantly increased attack behavior for the duration of the light pulse (Fig. 2L–O). However, 561 nm light stimulation (10-s constant light) delivered after attack had begun significantly reduced attack duration during the light pulse (Fig. 2P–S). Attack behavior during the 10-s prestimulation period did not differ between opsin and control mice (Fig. 2M–O,Q–S). These results indicate that inhibition of CaMKIIα+ DR neurons suppress ongoing attack.

CaMKIIα+ DR neurons project to the MeOC and MeA to regulate aggression

To investigate the circuit mechanism by which CaMKIIα+ DR neurons regulate attack behavior, we stimulated the DR axons in the OFC and MeA, two DR projection areas (Cádiz-Moretti et al., 2016; Murphy and Deutch, 2018; Ren et al., 2018) involved in aggressive behavior (Blair, 2004; Siever, 2008; Rosell et al., 2010; Hong et al., 2014; Rosell and Siever, 2015; Unger et al., 2015; Buades-Rotger et al., 2017; Haller, 2018). Mice were injected with AAV expressing ChR2-EYFP into the DR (Figs. 3A,B, 4A,B) and then examined for YFP expression in the OFC and MeA. YFP+ DR axons were found in the MeOC and throughout the MeA (Figs. 3C–E, 4C–E). Photostimulation of the DR using 473 nm light (5-msec pulse at 10 Hz for 10 s) activated cells within both regions as determined by c-Fos staining (Figs. 3F–M, 4F–U). The MeA can be divided into three main subdivisions: the anterior MeA (MeAa), the posterior-dorsal MeA (MeApd), and the posterior-ventral MeA (MeApv), all of which are involved in aggressive behavior.
Kollack-Walker and Newman, 1995; Lin et al., 2011; Hong et al., 2014; Miller et al., 2019; Nordman et al., 2020a). Analysis of c-Fos expression in the MeA revealed that all three regions are activated by photostimulation of the DR (Fig. 4L–U). DR neurons were injected with ChR2-mCherry and ArchT-EYFP virus and then implanted with optical fibers into the MeOC or MeA (Figs. 5A,E,I, 6A,E,I). The RI test was performed eight weeks later. Baseline attacks in opsin and GFP mice were comparable (Figs. 5B–D, 6B–D). During the RI test, mice were stimulated with 473 nm light when not attacking or 561 nm light after attack had begun. As with the DR, 473 nm light stimulation at the MeOC or MeA did not increase attack behavior (Figs. 5E–

Figure 3. DR neurons project to and activate the MeOC. A–E, Schematic drawing of ChR2-EYFP viral injection and representative images of ChR2 expression in DR neurons (B) and DR projections at the MeOC (C–E) three weeks later. F–K, c-Fos labeling of mice photostimulated at the DR-MeOC. L, M, Quantification of total number of cells (L) and percentage (M) of c-Fos+ cells in the MeOC for D–K. Only cells within the MeOC were counted. One slice was quantified per area per animal. Animal number is indicated in parentheses. Scale bars: 200 μm (A, B; low-magnification images in C, D) and 50 μm (high-magnification images in C, D). Data are presented as mean ± SEM; **p < 0.01, ***p < 0.001. Statistics can be found in Table 2.
Figure 4. DR neurons project to and activate the MeA. A–E, Schematic drawing of ChR2-EYPF injections into the DR and representative images of ChR2 expression in DR neurons (B) and axons at the MeA (C–E) three weeks later. F–K, c-Fos labeling of mice photostimulated at the DR-MeA. L–U, Quantification of total number of cells (L–M) and percentage (Q–U) of c-Fos+ cells in the MeA (MeAa, anterior MeA; MeApd, posteriordorsal MeA; MeApv, posteriormeatal MeA) for D–K. Only cells within the MeA were counted. One slice was quantified per area per animal. Animal number is indicated in bars of bar graphs. Scale
While 561 nm light had no effect on attack behavior when applied to the MeA, it shortened attack time when applied to the MeOC (Figs. 5I–L, 6I–L). The ChR2/ArchT and GFP control groups had comparable attack behavior before light stimulation (Figs. 5F–H, J–L, 6F–H, J–L). These results suggest that the input from DR CaMKIIα1 neurons to the MeOC is required for attack to continue.

Furthermore, stimulating the DR axons at the MeOC during an attack with 473 nm light pulses significantly increased attack duration, again with no differences in baseline attacks or in attack behavior during the pre-light stimulation period (Fig. 7A–H). These results indicate that the DR-MeOC pathway can prolong an already occurring attack.

Stimulation of the MeA has been shown to suppress aggression (Rodgers, 1977; Pucilowski et al., 1985; Hong et al., 2014). To assess whether stimulation of the input from DR CaMKIIα1 neurons to the MeA may inhibit ongoing attack, we stimulated the MeA of mice injected with AAV

**Figure 5.** Inhibition of CaMKIIα1 DR input to the MeOC shortens attack. A, C, I. Schematic drawing of viral injection (AAV expressing ChR2 and ArchT or GFP control virus), placement of optic fiber, and stimulation procedure. Opsin expressing mice in A–D and opsin stimulated mice in E–L were injected with ChR2 virus and ArchT virus. The same mice were stimulated with 473 or 561 nm light on separate days. B, Raster plots of attack events during each interaction episode (rows in the raster plot, defined as the period from 20 s before to 20 s after the onset of a spontaneous attack) for each mouse. All attack events during the testing period are shown.

C, % of episodes (rows) in which mice attacked at each time point in B; red and dark lines indicate the mean, pink and gray areas indicate SEM.

D, Quantification of average attack time per mouse during the 10 s after the onset of a spontaneous attack, as represented in the boxed area in C, F. Raster plots of trials (rows) of mice photostimulated with 473 nm light at the DR-MeOC when the mouse was not attacking. G, % of trials in which mice attacked at each time point in F; red and dark lines indicate the mean, pink and gray areas indicate SEM.

H, Quantification of attack time per mouse before and during light stimulation for F, J. Raster plots of trials (rows) of mice photostimulated with 561 nm light at the DR-MeOC during an attack. K, % of trials in which mice attacked at each time point in J; red and dark lines indicate the mean, pink and gray areas indicate SEM.

L, Quantification of attack time per mouse before and during light stimulation for J. Data are presented as mean ± SEM; **p < 0.01. Statistics can be found in Table 2.
ChR2 into the DR at the onset of an attack (Fig. 7M); 473 nm photostimulation of the MeA significantly shortened the duration of an attack during illumination. Baseline and prestimulation attack behaviors were comparable in ChR2 and GFP control mice (Fig. 7I–P). These results suggest that the DR-MeA pathway facilitates the termination of attack.

Taken together, these findings indicate that the projections from the CaMKIIα⁺ DR neurons to the MeOC and MeA have opposite effects on attack behavior, with the DR-MeOC inputs sustaining attack while the DR-MeA inputs shorten attack.

**Discussion**

The DR modulates attack behavior during aggressive encounters in rodents (Chen et al., 1994; Takahashi et al., 2010, 2015; da Veiga et al., 2011; Adachi et al., 2017; Balázsvi et al., 2018; Muroi and Ishii, 2019). The DR contains a heterogenous population of neurons that project to various brain regions to control social behavior and emotion (Challis et al., 2013; Qi et al., 2014; Matthews et al., 2016; Ren et al., 2018; Huang et al., 2019). However, the role of specific DR projections in aggressive behavior is incompletely understood. Here, we show that the DR-MeOC and DR-MeA pathways control attack duration in opposite directions.

The MeOC and MeA are subregions densely innervated areas by the DR (Cádiz-Moretti et al., 2016; Murphy and Deutch, 2018; Ren et al., 2018) and are key nodes in the processing of social interaction including aggression (Blair, 2004; Siever, 2008; Rosell et al., 2010; Hong et al., 2009).
Figure 7. Activation of the DR-MeOC prolongs attack and activation of the DR-MeA shortens attack. Mice were injected with ChR2 virus or GFP virus into the DR and optical fibers were placed into the MeOC (A, E) or the MeA (I, M). RI tests were performed eight weeks later. Separate groups of mice were used for A–H and I–P. B. Raster plots of attack events during each interaction episode (rows in the raster plot, defined as the period from 20 s before to 20 s after the onset of a spontaneous attack) for each mouse. All attack events during the testing period are shown. C. % of episodes (rows) in which mice attacked at each time point in B; red and dark lines indicate the mean, pink and gray areas indicate SEM. D. Quantification of average attack time per mouse for the 10 s after the onset of a spontaneous attack, as represented in the boxed area in C. F. Raster plots of trials (rows) of mice photostimulated with 473 nm light at the DR-MeOC during an attack. G. % of trials in which mice attacked at each time point in F; red and dark lines indicate the mean, pink and gray areas indicate SEM. H. Quantification of attack time per mouse before and during light stimulation for F, J. Raster plots of attack events during each interaction episode (rows in the raster plot, defined as the period from 20 s before to 20 s after the onset of a spontaneous attack) for each mouse. All attack events during the testing period are shown. K. % of episodes (rows) in which mice attacked at each time point in J; red and dark lines indicate the mean, pink and gray areas indicate SEM. L. Quantification of average attack time per mouse for the 10 s after the onset of a spontaneous attack, as represented in the boxed area in J. N. Raster plots of trials (rows) of mice photostimulated with 473 nm light at the DR-MeA during an attack. O. % of trials in which mice attacked at each time point in N; red and dark lines indicate the mean, pink and gray areas indicate SEM. P. Quantification of attack time per mouse before and during light stimulation for N. Data are presented as mean ± SEM; ***p < 0.001. Statistics can be found in Table 2.
2014; Rosell and Siever, 2015; Unger et al., 2015; Buades-Rotger et al., 2017; Haller, 2018). Activity within the OFC is negatively correlated with aggression and chronic inactivation or lesioning in this area heightens aggression in mice and humans (Anderson et al., 1999; Blair, 2004; Siever, 2008; Rosell et al., 2010; Beyer et al., 2015; Rosell and Siever, 2015; Kuniishi et al., 2016). The role of the MeA in aggression is better characterized. Within the MeA, activation of GABAergic neurons in the MeApd promotes attack, while activation of glutamatergic neurons suppress it (Hong et al., 2014; Padilla et al., 2016). Stimulation of dopamine D1 receptor (D1R)-expressing neurons within the MeApv projecting to the bed nucleus of the stria terminalis increases aggression, while stimulation of those projecting to the ventromedial hypothalamus decreases aggression (Miller et al., 2019). Potentiation of synapses between the MeApv and the ventromedial hypothalamus and bed nucleus of the stria terminalis underlies aggression priming and heightened aggression induced by traumatic stress (Nordman et al., 2020a,b). Notably, dysfunction within the OFC and MeA is associated with excessive and impulsive aggression in mice and humans (Grafman et al., 1996; Blair, 2004; New et al., 2004; Shalom et al., 2004; Coccaro et al., 2007; Mpakopoulou et al., 2008; Buades-Rotger et al., 2017; Herpertz et al., 2017). In this study, we chose to stimulate the axons of the CaMKIIαDR neurons at the MeOC and MeA to discriminate the effects of different pathways on attack behavior. We show that optogenetic silencing of CaMKIIαDR neurons in the DR and their projections to the MeOC reduces the duration of an attack while optogenetic activation of the DR-MeOC prolongs it. Conversely, activation of the DR-MeA projections reduces attack duration. These results raise the possibility that distinct groups of CaMKIIαDR neurons project to the MeOC and MeA. It is interesting that stimulating the DR-MeOC pathway mimics the effect of stimulating the DR on aggression, suggesting that the DR-MeOC pathway predominates over the DR-MeA pathway. It is noted that the MeA receives direct inputs from the OFC (Siever, 2008; Márquez et al., 2013; Cádiz-Moretti et al., 2016), leaving open the possibility that the DR-MeA pathway is suppressed by OFC input when the DR-MeOC pathway is activated.

The CaMKIIα promoter has been extensively used to drive gene expression in excitatory neurons, though it is also active in a small number of inhibitory neurons in the cortex (Scheyltjens et al., 2015; Watakabe et al., 2015). In addition, many glutamatergic cells of the DR co-release serotonin (Liu et al., 2014; Ren et al., 2018; Huang et al., 2019). Thus, one limitation of our study is that our AAV with the CaMKIIα promoter may transduce non-excitatory neurons in the DR. It is noted that in a previous study stimulation of DR neurons that were transduced with AAV expressing ChR2 under the pan neuronal promoter synapsin lead to a decrease in aggression and an increase in 5-HT and GABA release at the PFC (Balázsfi et al., 2018). These synapsin promoter targeted neurons likely overlap with the CaMKIIα promoter targeted neurons in this study. This raises an intriguing possibility that the CaMKIIαDR neurons may release neurotransmitters other than glutamate to regulate aggressive behavior. Our finding that the CaMKIIαDR neurons do not overlap with the serotonergic cell marker Tph2 would suggest that the CaMKIIαDR cells are non-serotonergic (Figs. 1G–I, 2E–G). The release of other neurotransmitters, however, cannot be ruled out.

The three main MeA subregions, MeAa, MeApd, and MeApv, are all involved in aggressive behavior (Kollack-Walker and Newman, 1995; Lin et al., 2011; Hong et al., 2014; Miller et al., 2019; Nordman et al., 2020b). While we observed that photostimulation of the DR can activate all three subregions, since the MeApd and MeApv have different effects on aggression (Kollack-Walker and Newman, 1995; Lin et al., 2011; Hong et al., 2014; Miller et al., 2019; Nordman et al., 2020a), it is worth considering that the DR may be shortening an attack through a specific subregion of the MeA. The photostimulation protocol used in this study does not allow for discrimination of these subregions because of their anatomic clustering.

Aggression is an adaptive behavior with the intention of preserving resources or protecting oneself from harm. Excessive aggression, however, is energetically unfavorable (Maynard Smith and Price, 1973; Haller, 1995; Miczek et al., 2013). In mice, prolonged attack is an indicator of excessive aggression (Miczek et al., 2013). The neural circuits that underlie prolonged aggression are poorly defined. Our study demonstrates that while neither the DR-MeOC or DR-MeA pathway can initiate an attack, both pathways regulate the duration of an already occurring attack. These findings suggest an intriguing possibility that dysfunction within these DR pathways may play a role in excessive aggression.

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