Divergent medial amygdala projections regulate approach–avoidance conflict behavior

Samara M. Miller¹, Daniele Marcotulli¹,², Angela Shen³ and Larry S. Zweifel¹,⁴*

Avoidance of innate threats is often in conflict with motivations to engage in exploratory approach behavior. The neural pathways that mediate this approach–avoidance conflict are not well resolved. Here we isolated a population of dopamine D1 receptor (D1R)-expressing neurons within the posteroverentral region of the medial amygdala (MeApv) in mice that are activated either during approach or during avoidance of an innate threat stimulus. Distinct subpopulations of MeApv-D1R neurons differentially innervate the ventromedial hypothalamus and bed nucleus of the stria terminalis, and these projections have opposing effects on investigation or avoidance of threatening stimuli. These projections are potently modulated through opposite actions of D1R signaling that bias approach behavior. These data demonstrate divergent pathways in the MeApv that can be differentially weighted toward exploration or evasion of threats.

Animals across all levels of complexity possess approach mechanisms that evoke social and exploratory behavior and avoidance behaviors that evoke withdrawal and flight. In order to maximize gains, animals will engage in risky exploratory behavior, ignoring signs of potential threat, to exploit resources in their environment. Animals exposed to competing environmental cues must navigate between mutually incompatible behaviors, such as feeding, reproduction, flight or defense. In some contexts, threatening stimuli, such as predators or aggressive conspecifics, will suppress appetitive behavior, such as reproduction. Conversely, hunger and food-seeking will override defensive behaviors.

Defensive responses can be adaptive depending on the fed state of an animal, and evidence suggests that these adaptations specifically involve neurons in subregions of the medial amygdala (MeA). Thus, animals require integrated circuits to generate gradients of defensive responses appropriate to the contextual threat and approach responses proportional to potential gains. A major conflicting drive of innate avoidance is an inherent motivation to approach and explore the unknown. In the classical sense of approach–avoidance conflict, exploratory drive is an essential feature of maximizing an animal’s ability to thrive, whereas avoidance is essential for survival.

Early lesion and electrical stimulation studies have provided intriguing insight into potential loci of approach–avoidance interactions. In rats, electrolytic lesion of the corticomedial amygdala, which encompassed the MeA, elicited a reduction in freezing behavior and an increase in exploratory interactions with a live cat, a natural predator of the rat. Similarly, more restricted excitotoxic lesion of the MeA greatly reduces defensive behavior in rats during exposure to a live cat and increases exploratory locomotion. These findings support the idea that innate approach of the unfamiliar cat is actively suppressed by the innate avoidance impulse. In contrast to lesions, electrical stimulation of subcortical brain regions can elicit either approach, avoidance, or in some cases ambivalent responses. Intriguingly, the regions that produced these ambivalent responses are innervated by the MeA, including the bed nucleus of the stria terminalis (BNST) and the ventromedial hypothalamus (VMH).

In addition to lesion studies, activity mapping using the immediate–early gene Fos has strongly implicated the MeApv in the processing of innate threat cues. Retrograde tracing and Fos expression analysis revealed that MeApv neurons that were activated by threat stimuli projected to hypothalamic nuclei and brainstem ascending pathways to hypothalamic nuclei are implicated in both appetitive (reproduction) and aversive behaviors. In contrast, MeApv neurons activated by reproductive stimuli did not project to these regions. Based on these observations, it was hypothesized that the MeA–hypothalamic pathway may be an important ‘gate’ between conflicting appetitive behavior and threatening stimuli, either through interneurons within the VMH or through indirect modulation by the BNST.

We recently identified the MeA as a brain region activated in response to stimulation of midbrain dopamine neurons. The midbrain dopamine system plays an essential role in reward learning, motivation, fear learning and avoidance of conditioned stimuli. Recent data support a role for dopamine in modulating approach–avoidance conflict. Increasing dopamine activity by lesioning an inhibitory input onto dopamine neurons in the VTA causes animals to display increased approach to a predator odor threat. Additionally, increasing dopamine with the use of monoamine oxidase inhibitors is an effective treatment for specific phobias, disorders likely caused by dysfunction in innate fear circuitry.

The MeA is considered part of the caudal striatum, or more generally a component of the striatopallidal complex. This anatomical association suggests that the MeA may be functionally involved in dopamine-dependent behaviors. Given the role of the MeA in regulating innate defensive responses, along with the observation that dopamine neurons of the midbrain are connected to this striatopallidal-like structure, we hypothesized that dopamine-receptive neurons in this region would probably contribute to innate approach and avoidance behavior. We identified dopamine-receptive neurons in the MeA that express D1R and are strongly biased in their localization to the MeApv. Using viral tracing methods, calcium imaging, and optogenetic–pharmacological manipulations, we find that MeApv-D1R neurons segregate into distinct populations that regulate either approach or avoidance of innate threat stimuli. These populations regulate...
approach and avoidance behaviors through two separate projections, one largely inhibitory projection to the BNST and one largely excitatory projection to the dorsomedial VMH (VMHdm).

Results
Identification of dopamine receptive neurons in the MeApv.
Using cell-type-specific expression of the stimulatory designer receptor exclusively activated by designer drugs (DREADD) HM3Dq in dopamine neurons of the ventral midbrain, we recently demonstrated that activation of dopamine neurons induces Fos in the MeA\(^\text{(10)}\). To confirm the presence of dopamine-receptive neurons in this region, we searched the Allen Institute for Brain Science Mouse Brain in situ hybridization atlas\(^\text{(14)}\) for dopamine receptor expression in the amygdala. Within the MeA, Drd1 was expressed prominently in the MeApv; high levels of Drd1 were also observed in the intercalated cell clusters and, to a lesser extent, in the basolateral amygdala (BLA) and central amygdala (CeA). In contrast, we did not observe appreciable levels of expression of Drd2, Drd3, Drd4 or Drd5 in the MeA, though varying degrees of expression of these genes were seen in other amygdala nuclei. To confirm the presence of Drd1-expressing neurons in the MeA, we crossed Drd1\(^\text{Cre/\text{floxed}}\) (D1R-Cre\(^\text{19}\)) mice with the fluorescent reporter line Ai14 (ref. \(^\text{(20)}\))
MeApv-D1R neurons are activated during approach and avoidance. It has been shown that exposure to innate threat stimuli, such as predator odors and intruder conspecifics, induces Fos expression in the MeApv5,24,25. To determine whether MeApv-D1R neurons are specifically activated by innate threat stimuli, we virally labeled MeApv-D1R neurons are activated during approach and avoidance to predator odor and robobug. MeApv-D1R neurons were detected in the MeApv, and we observed a significantly biased distribution of tdTomato-positive neurons in the MeApv compared with that in the posterodorsal MeA (MeApd) (Fig. 1a,b).

Consistent with innervation of the MeA by dopamine-producing neurons, injection of a Cre-dependent adeno-associated virus (AAV1) containing an expression cassette for the fluorescent reporter synaptophysin (syn)-GFP (AAV1-FLEX-Syn-GFP) into the ventral tegmental area–substantia nigra pars compacta (VTA/SNC) of mice expressing Cre recombine from the endogenous dopamine transporter locus, Slc6a3Cre+ (DAT-Cre21), revealed a significant bias of syn-GFP puncta localized to the MeApv compared with the MeApd (Fig. 1c). To test for functional D1R signaling in the MeApv, we injected the D1R agonist SKF-81297 intraperitoneally (i.p., 7.5 mg/kg) and immunostained for Fos protein. We observed robust Fos levels in the MeA relative to saline controls and higher Fos in the MeApv relative to the MeApd (Fig. 1d).

To further establish the localization of D1R within the MeA and its relationship to other previously identified genetic markers within this region5,24,25, we performed fluorescence in situ hybridization analysis for mRNA localization using RNAscope26. Consistent with the localization of MeA-D1R neurons observed in the D1R-Cre::Ai14 line and Fos localization after SKF-81297 administration, we observed a strongly biased expression of Drd1 in the MeApv compared with the MeApd (Fig. 1e–g). In contrast to Drd1, Lhx6 expression was biased toward the MeApd (Fig. 1e), and Cyp19a1 (aromatase) did not show a differential expression pattern (Fig. 1f).

Similarly to Drd1, Npy1r was differentially localized with biased expression in the MeApv (Fig. 1g). Npy1r showed more overlap with Drd1 in the MeApv than did either Lhx6 or Cyp19a1 (Figs. 1e–g; 40.1 ± 3.5%, 61.1 ± 1.2% and 23.6 ± 3.2%, respectively, one-way ANOVA, P < 0.0001).

MeApv-D1R neurons are activated during approach and avoidance. It has been shown that exposure to innate threat stimuli, such as predator odors and intruder conspecifics, induces Fos expression in the MeApv5,24,25. To determine whether MeApv-D1R neurons are specifically activated by innate threat stimuli, we virally labeled these neurons with GFP and exposed mice to a variety of multi- 

Fig. 2 | MeApv-D1R neurons are activated during approach and avoidance to predator odor and robobug. a, Schematic of miniscope, lens and viral injection for calcium imaging and behavior. b, Field of view (FOV) of MeApv-D1R:GCaMP6m neurons from each of the four designated behavioral epochs. Open circles represent designated regions of interest (ROIs) from a subset of cells categorized as active during the behavioral epoch. c, d, Top: individual traces of a subset of cells activated during approach (c) or hide box (d) (n = 1 mouse). Bottom: average of the cells presented above (n = 10 cells/behavioral epoch from 1 mouse). e, Activity profiles of cells from all mice during robobug investigation (n = 4 mice). Top: probability of distribution of peak fluorescence for all cells active during approach to robobug (dashed line represents initiation of investigation; n = 58 cells from 4 mice). Middle: heat map of calcium responses during an approach and investigation epoch for all active cells (n = 58 cells from 4 mice). Bottom: average traces of all cells activated prior to an approach and investigation epoch; shaded areas represent s.e.m. (n = 58 cells from 4 mice). f, Activity profiles of cells from all mice during hide box period from robobug assay. Top: probability of distribution of peak fluorescence for all cells active following entry into hide box (dashed line represents entry into hide box; n = 40 cells from 4 mice). Middle: heat map of calcium responses following entry into hide box for all active cells aligned to a hide box entry (n = 40 cells from 4 mice). Bottom: average traces of all cells activated following an entry to the hide box; shaded areas represent s.e.m. (n = 40 cells from 4 mice). g, Selectivity of cells active during robobug assay (n = 4 mice). h, i, Same as e–g for predator odor assay. For approach and investigation behavior, n = 57 cells from 4 mice. For hidebox behavior, n = 35 cells from 4 mice. Shaded areas represent s.e.m. j, Selectivity of cells active during predator odor assay (n = 4 mice).
neurons, we bilaterally injected AAV-FLEX-Syn-GFP into the MeApv of D1R-Cre mice. Consistent with previous reports of MeApv projections\textsuperscript{31}, we observed dense GFP-positive puncta in the VMH and BNST (Fig. 3a), with a significant bias toward a higher-density projection to the BNST (integrated pixel density, BNST = 655.5 ± 105.4 versus VMH = 320.5 ± 105.9, \( P = 0.039, n=3 \) mice). Analysis of the anatomical distribution of MeApv-D1R fibers revealed projections localized most prominently to the transverse (tr) nucleus of the BNST (Fig. 3b) and the dorsomedial (dm) subdivision of the VMH (Fig. 3c), consistent with previous anatomical analysis of MeApv projections\textsuperscript{15,31,32}. To determine whether projections from the MeApv to the VMH and BNST represent collaterals or independent projections, we co-injected retrogradely transported red fluorescent beads (RetroBeads) into the VMH and...
green RetroBeads into the BNST of wild-type mice (Fig. 3d). Both red and green RetroBeads were observed in the MeApv, with a stronger labeling of BNST projection neurons. We observed very few neurons with overlapping red and green fluorescent beads (Fig. 3d).

To characterize the synaptic connectivity of MeApv-D1R neuron projections to the VMHdm and BNST, we transduced MEApv-D1R neurons with a conditional channelrhodopsin33 (ChR2)-expressing virus (AAV1-FLEX-ChrR2-mCherry). After viral expression, we...
Activation of distinct MeApv-D1R pathways differentially drives approach and avoidance. 

**a, b.** Schematic of viral injection of AAV1-FLEX-ChR2-mCherry, fiber-optic implant, and behavioral experiments for threat exposure. 

**c.** Optogenetic activation of VMH-projecting MeApv-D1R neurons increases avoidance of predator odor, whereas optogenetic activation of BNST-projecting MeApv-D1R neurons decreases approach to predator odor (n = 17, 9, 10 mice/group; grooming: one-way ANOVA F(2, 33) = 19.18, P < 0.0001; latency to approach: one-way ANOVA F(2, 33) = 5.501, P = 0.0087; investigation (inv) time: one-way ANOVA F(2, 33) = 111, P = 0.0002; frequency of investigations: one-way ANOVA F(2, 33) = 7.949, P = 0.0015). 

**d.** Optogenetic activation of VMH-projecting MeApv-D1R neurons increases avoidance of robobug. Optogenetic activation of BNST-projecting MeApv-D1R neurons increases approach to robobug (n = 17, 9, 10 mice; time spent in hide box: one-way ANOVA, Kruskal–Wallis test with Dunn’s multiple-comparison test, P = 0.0007; latency to approach: one-way ANOVA, Kruskal–Wallis test with Dunn’s multiple-comparison test, P = 0.0039; investigation time: one-way ANOVA F(2, 26) = 2.607, P = 0.0929).

**e.** Optogenetic activation of VMH-projecting MeApv-D1R neurons increases grooming of conspecific during resident-intruder assay, and optogenetic activation of BNST-projecting MeApv-D1R neurons increases fighting of conspecific during resident-intruder assay (n = 16, 10, 11 mice/group; grooming: one-way ANOVA F(2, 30) = 16.88, P < 0.0001; fighting: one-way ANOVA F(2, 30) = 5.048, P = 0.012; investigation: one-way ANOVA F(2, 30) = 0.6207, P = 0.5435). Unless otherwise indicated, for all panels, Tukey’s multiple-comparisons test was used. For **a-e**, center values represent mean and error bars represent s.e.m.

**Fig. 4.**

photostimulated MeApv-D1R terminals and performed whole-cell recordings in the BNST and the VMH (Fig. 4e,f and Supplementary Fig. 4a,b). In the BNST, 15 of 31 cells displayed a light-evoked inhibitory postsynaptic current (IPSC) that was blocked by the GABA$_A$ receptor antagonist picrotoxin (Fig. 3c). A smaller proportion of cells showed short latency excitation (3 of 31). In a subset of cells (4 of 31), we observed delayed inhibitory input that was blocked by the AMPA glutamate receptor antagonist CNQX, suggesting feedforward inhibition (Supplementary Fig. 4c). In the VMH, 9 of 15 cells displayed a light-evoked excitatory postsynaptic current (EPSC) that was blocked by CNQX (Fig. 3f). We also observed one cell with delayed inhibition and one cell with delayed excitation, consistent with feedforward synaptic transmission. These data demonstrate an excitatory MeApv projection to the VMH and predominantly inhibitory projection to the BNST that is consistent with distinct projection populations of MeApv-D1R neurons.

**VMH- and BNST-projecting MeApv-D1R neurons differentially regulate innate fear.** To determine whether BNST- and VMH-projecting MeApv-D1R neurons differentially regulate innate fear, we injected RetroBeads into either the BNST or VMH and exposed mice to predator odorant, robobug or the conspecific intruder assay (Supplementary Fig. 5a). Consistent with our projection mapping, a larger number of cells projecting to the BNST than to the VMH was observed (Supplementary Fig. 5b). BNST- and VMH-projecting MeApv-D1R neurons were both activated by innate threat stimuli in all assays (Supplementary Fig. 5c); however, a significantly larger proportion of VMH-projecting MeApv-D1R neurons compared with BNST-projecting MeApv-D1R neurons was observed (Supplementary Fig. 5b). BNST- and VMH-projecting MeApv-D1R neurons were both activated by innate threat stimuli in all assays (Supplementary Fig. 5c); however, a significantly larger proportion of VMH-projecting MeApv-D1R neurons compared with BNST-projecting MeApv-D1R neurons was observed in D1R-Cre mice, and an optic fiber was implanted directly over the VMH or the BNST (Fig. 4a and Supplementary Fig. 6a–c). Direct stimulation of MeApv-D1R→VMH projections enhanced defensive avoidance behaviors (that is, increased time spent in hide box and decreased exploration of the threat) in response to a predator odor (Fig. 4c) and robobug (Fig. 4d). In contrast, MeApv-D1R→BNST stimulation decreased avoidance of these threats and increased exploratory investigations (Fig. 4c,d).

In response to a conspecific threat in the resident–intruder assay, male mice will display territorial aggression toward an intruder. To assess whether MeApv-D1R projections to the VMH or BNST influence this behavior, we stimulated these projections during a resident–intruder
assay. MeApv-D1R→VMH-stimulated mice showed significantly increased grooming of the male conspecific intruder and less aggression (Fig. 4c), whereas MeApv-D1R→BNST-stimulated mice showed a significantly increased aggression phenotype (Fig. 5c). Across the groups, we did not observe significant differences in total time spent investigating the intruder (Fig. 4c).

To establish whether MeApv-D1R→VMH or MeApv-D1R→BNST connections are inherently rewarding or aversive, we performed a real-time place preference (RTPP) assay during terminal stimulation. We observed an increased avoidance of the light-paired chamber in MeApv-D1R→VMH-stimulated mice relative to the unpaired side (Supplementary Fig. 7a,b). MeApv-D1R→VMH-stimulated mice exhibited a small but significant reduction in distance traveled compared with MeApv-D1R→BNST-stimulated mice, but not compared with controls (Supplementary Fig. 7c). In contrast, MeApv-D1R→BNST stimulation had no significant effect on RTPP behavior (Supplementary Fig. 7a,b).

Our connectivity analysis indicates that MeApv-D1R neurons are heterogeneous and dominantly release glutamate at VMH terminals and GABA at BNST terminals. We also find that these pathways together bidirectionally regulate innate defensive behaviors. To determine whether these projections are truly functionally opposed or whether one can dominate the other, we optogenetically stimulated MeApv-D1R cell bodies (Supplementary Fig. 8a) in the presence of innate threat stimuli. Similarly to MeApv-D1R→BNST terminal stimulation, collective stimulation of MeApv-D1R cell bodies reduced fear in the predator odor and robobug assays and enhanced aggression in the resident–intruder assay (Supplementary Fig. 8b–d). MeApv-D1R cell body stimulation also did not induce a change in behavior in the RTPP assay (Supplementary Fig. 8e). These data suggest that when co-activated, the BNST pathway overrides the VMH projection to bias behavior toward approach.

To establish whether inhibiting these pathways alters approach and avoidance behaviors related to threats, we performed projection-specific inhibition of MeA-D1R→BNST and MeA-D1R→VMH pathways using the inhibitory opsin Jaws35. The retrograde transducing AAV AAV2-retro36, containing a Cre-dependent expression cassette for Jaws, was injected into either the VMH or BNST of D1R-Cre mice (Fig. 5a,b and Supplementary Fig. 9a–d). Cells bodies projecting to the VMH or BNST were inhibited during each assay with red light (1 s on with a 1-s ramp-down, 2s off) delivered to the MeApv. Consistent with activation of the VMH pathway eliciting increased avoidance behavior, inhibition of this projection reduced avoidance behavior in the predator odorant assay (Fig. 5c) and the robobug assay (Fig. 5d). Inhibition of the VMH projection neurons did not alter grooming behavior, but significantly enhanced
aggression (Fig. 5c). In contrast to inhibiting the VMH projection population, inhibition of the BNST projections did not significantly alter these behaviors (Fig. 5c–e). Thus, under these conditions, the MeApv-D1R→VMH pathway appears to be predominantly regulating avoidance behavior.

**DIR signaling biases activation of the MeA→BNST approach pathway.** It is well established that transient elevated levels of dopamine release in the striatum promote a conditioned approach to rewarding stimuli that is mediated in part by dopamine signaling through DIR to enhance incentive motivational drive. To establish whether DIR signaling modulates the excitability of MeApv projections to the VMH or BNST, we recorded MeApv-D1R neurons in an acute brain slice preparation. MeApv-D1R neurons were identified by expression of tdTomato using the D1R-Cre::Ai14 line. Projection specificity was determined by injection of green RetroBeads into either the BNST or VMH (Fig. 6a,b). Excitability was measured as the number of action potentials elicited by increasing current injection (10 pA steps, 10 to 60 pA) in the absence (in artificial cerebrospinal fluid (ACSF) only) or presence of the DIR agonist SKF-81297 (10 μM). As expected, SKF-81297 bath application increased the excitability of BNST-projecting neurons (9 of 16 cells; Fig. 7a,c–e). Paradoxically, SKF-81297 application reduced the excitability of VMH-projecting neurons (8 of 14 cells; Fig. 6b–e) that was associated with a hyperpolarization of the resting membrane potential (Fig. 6f).

To determine whether increasing dopamine DIR signaling on MeApv-D1R alters defensive behavior, we bilaterally infused the DIR agonist SKF-81297 (0.1 μg or 1 μg/0.5 μl per side) directly into the MeApv of wild-type mice through stereotaxically implanted cannula (Fig. 6g and Supplementary Fig. 10). Infusion of SKF-81297 suppressed fear responses after exposure to innate threat stimuli and enhanced approach and aggression in a dose-dependent manner (Fig. 6h–j). SKF-81297 infusion did not alter locomotor behavior in these assays (Supplementary Fig. 10). Taken together, these data support a role for MePV-DIR neurons in the modulation of innate fear behavior and demonstrate that the MeA-D1R→VMH and MeA-D1R→BNST pathways are not equivalent in their opposition, but rather can be biased toward approach. We propose that during periods of high-incentive motivation, such as during the drive to acquire rewards, behavior is biased toward approach in the face of potential danger that is predicated on state-dependent levels of dopamine in the amygdala (Fig. 6k,l).

**Discussion**

We find that MeApv-D1R neurons can be parsed into inhibitory and excitatory projections to the BNST and VMH, respectively, which differentially regulate defensive behavior. Our data are consistent with the striatopallidal-like designation of the MeA and its potential as a caudal extension of the striatum, though there are some clear distinctions. Like the striatum, the MeA contains dopamine-receptive neurons and appears to possess a type of direct and indirect pathway. First, MeApv-D1R neurons send a direct excitatory projection to the VMHdm to regulate innate defensive behaviors. Second, MeApv-D1R neurons send an inhibitory projection to the BNST, which may serve as an indirect pathway. Consistent with previous anatomical analysis of MeApv projections, MeApv neurons preferentially innervate the transverse BNST (BNSTtr) and to a lesser extent the interfascicular BNST (BNSTif), both of which project to the defensive nuclei in the hypothalamus; notably, the BNSTif projects to the VMHdm. The manner in which GABAergic projections from the MeApv-D1R neurons to the BNST suppress defensive behavior remains unclear. One possibility is that these projections would inhibit intra-BNST GABAergic interneurons to disinhibit GABAergic outputs to the hypothalamus. Alternatively, inhibitory MeApv-D1R→BNST projections could inhibit GABAergic projections to appetitive behavioral centers, thus providing a disinhibitory gate as proposed previously. Interestingly, the BNSTif has also been shown to send a significant projection to the VTA, and both the BNSTif and BNSTst project back to the MeA, providing multiple potential feedback loops.

In contrast to the striatum, in which D2Rs inhibit the indirect pathway and D1Rs stimulate the direct pathway, we find DIR signaling enhances the activity of the putative BNST indirect pathway and inhibits the putative VMH direct pathway. Though paradoxical, the inhibition of VMH-projecting neurons by the DIR is not unprecedented. Notably, DIR signaling has been shown to inhibit neurons within the amygdala in a cell-autonomous manner. Future investigations to determine the cell-autonomous effects of DIR activation in MeApv-D1R neurons will provide additional insight into the organization of this system and the role of dopamine in modulating circuit function.
Dopamine has been highly implicated in incentive motivational processes underlying reward seeking and conditioned fear\(^{41,43}\), but whether dopamine is a key modulator of circuits underlying approach and avoidance conflict has not been established. Here we demonstrate a role for dopamine in innate fear behavior through the modulation of dopamine D1R signaling in the MeApv. Specifically, we show that increasing D1R signaling onto MeApv-D1R neurons increases approach behavior, likely through the simultaneous suppression of the VMH pathway and activation of the BNST pathway. This finding is consistent with previous studies demonstrating that a significant fraction of dopamine neurons are inhibited by aversive stimuli and activated by appetitive stimuli\(^{41}\). Under these
conditions, dopamine levels would be low during threat encounters and high during increased-incentive motivation. Thus, when an animal is threatened, reduced dopamine would promote the activation of the VMH avoidance pathway, and under increased appetitive motivational states, elevated dopamine levels would promote activation of approach pathways and suppression of avoidance pathways. Interestingly, it has recently been shown that activation of distinct inhibitory subpopulations within the laterodorsal tegmentum oppositely regulate olfactory cue-induced freezing to an innate threat56. Given that the laterodorsal tegmentum is a potent regulator of the midbrain dopamine system, this represents a putative mechanism for differential regulation of MeApv dopamine levels through inhibitory and disinhibitory projections of the laterodorsal tegmentum.

MeApv-D1R neurons that evoke GABA-mediated inhibition of the BNST elicit approach behavior to threat stimuli, including aggression toward conspecífics. Conversely, MeApv-D1R neurons that evoke glutamatergic excitation of the VMHdm enhance avoidance of threatening stimuli and grooming of conspecífics. Consistent with our observation, stimulation of the VMHdm enhances innate fear behaviors such as hiding and freezing58. We found that inhibition of the MeApv-D1R→VMH pathway suppressed defensive behavior, consistent with these neurons directly influencing defensive behavior. We did not find an effect of inhibiting the BNST pathway during exposure to innate threats. One potential explanation for this observation is that if the BNST serves as a gate to permit exploratory behavior, and the internal drive is sufficiently low, closing this gate further may have a minimal effect.

The amygdala is composed of multiple distinct and interconnected nuclei involved in threat detection, as well as hedonic and consummatory processes49,50. The MeApd and MeApv are thought to be differentially involved in defensive responses to either conspecífics or predators15,16. In rodents, the MeApd is robustly activated by aggressive conspecífics, while the MeApv is strongly activated by a live cat or its odor51,52. Anterograde tracing studies have demonstrated that the MeApd projects to regions implicated in reproduction and conspecific responsive behavior, including the ventrolateral VMH (VMHvl)53,54. In contrast, the MeApv projects to distinct hypothalamic nuclei involved in predator-responsive and general avoidance behavior, including the VMHdm17,18—though projections to the VMHvl have also been reported55. Activation of independent pathways from the VMHdm to either the periaqueductal gray or the anterior hypothalamic nucleus can evoke either freezing or avoidance in response to threats, respectively19. Based on previous hypotheses that conspecific and predatory threats are differentially regulated by the MeApd and MeApv20, we were surprised to find that projection-specific pathways from the MeApv differentially regulated conspecific male aggression. However, our data are consistent with previous reports showing Fos induction in the MeApv in response to a male conspecific21.

Further supporting the diverse functions of MeApv-D1R neurons, our in vivo imaging data demonstrate that these cells respond to a variety of threatening and appetitive stimuli. Subsets of neurons were activated during the hide box period, and largely non-overlapping neurons were activated proximal to the investigation periods. These data support the hypothesis that distinct populations regulate approach and avoidance behavior. Cells activated proximal to investigation were largely activated leading up to the investigation, but were largely silent once the active investigation was initiated. Based on this observation we suggest that these neurons play an important role in approach. Cells activated during the hide box period showed largely constrained activity that peaked shortly after entry into the hide box. Although this activity is consistent with the avoidance of the stimulus, it remains possible that these cells represent a type of safety signal engaged once an escape has been successfully completed. In addition to responding to threat stimuli, MeApv-D1R neurons were also activated by conspecific odors, and projection-specific activation of MeApv-D1R neurons significantly impacted male–male interactions. Additional calcium imaging studies with larger sample sizes are required to assess the selectivity of MeApv-D1R neurons in male and female mice to both same- and opposite-sex conspecifics. Sex-specific social processing within the MEA likely gates state-dependent behaviors to differentially drive aggression versus investigation in particular contexts17,27. Future investigations are needed to understand how MeApv-D1R neurons regulate male–female and female–female social behaviors and this type of information processing.

Aggressive behavior toward intruder conspecífics was once viewed as a purely defensive response; however, recent studies have revealed that aggression in rodents generates a conditioned place preference and increases nucleus accumbens dopamine levels66,67. Specifically, microdialysis experiments in the nucleus accumbens in aggressive males reveal that DA levels increase prior to an anticipated fight and can remain elevated during and after a single fight68. Additionally, local dopamine receptor blockade in the nucleus accumbens decreases aggression-motivated operant responding69. Our findings that activation of the MeApv-D1R→BNST pathway increases aggression may reflect a parallel or integrated mechanism by which increased amygdalar dopamine generates a motivational state to drive aggression. However, we cannot exclude the possibility that the small number of D1R neurons localized to the MeApd is regulating in the observed aggressive behavior.

In conclusion, our data demonstrate that distinct approach and avoidance pathways exist within the MeApv. These two pathways segregate based on distinct projection patterns and neurotransmitter phenotype, but represent interspersed ensembles at the level of the MEA. The identification of a brain locus underlying approach–avoidance conflict permits further resolution of upstream and downstream pathways critical for resolving these behavioral conflicts.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41593-019-0337-z.

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Author contributions

S.M.M. and L.S.Z designed the experiments. S.M.M. and L.S.Z wrote the manuscript. S.M.M. performed all viral injection surgeries, behavior experiments, and slice electrophysiology. D.M. analyzed all calcium imaging data. Behavioral analysis and histology was performed by S.M.M. and A.S. L.S.Z purified all viral vectors.

Competing interests

The authors declare no competing interests.

Additional information

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Methods

Animals. All experiments were approved in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Washington. DrlalCre mice and Slc6a3Cre−/− (DAT-Cre) mice have been characterized previously55. The Slc6a3Cre-dependent reporter strain, Rosa26CreERt2 was purchased from Jackson Laboratories (stock #007914). Approximately equal numbers of male and female mice were used for all experiments, with the exception of the resident–intruder assay, in which only male mice were used. Mice were housed on a 12-h light cycle and given ad libitum access to food and water. Behavioral mice were 8 weeks old or older, and mice used for slice electrophysiology were 5–8 weeks old. Mice were group housed with the exception of resident male mice, which were singly housed 2–3 weeks prior to the resident–intruder assay. Mice were bred onto a C57BL/6j background, and mice used for social interaction assays were wild-type C57BL/6j. Mice were assigned randomly to control or experimental groups, and experimenters were blinded during data analysis.

Viruses and neuronal tracers. All viruses were produced in house, with titers of 1–3 × 1010 particles per milliliter as described13. RetroBeads were obtained from Lumafurol and injected with a Hamilton syringe (0.5 µl/hemisphere). In accordance with manufacturers protocol, red RetroBeads were injected at a dilution of 1:4 and green RetroBeads were injected undiluted.

Surgery. Mice were anesthetized with isoflurane and secured on a stereotaxic device (David Kopf instruments) before injection. Body temperature was maintained with a heating pad during surgery, and 1.5–2% isoflurane was delivered continuously through a nose port. Injection coordinates were as follows from bregma in mm: A: ±2, M: ±2.5, V: −5.6; BNST, A: ±0.2, M: ±1.25, D: −4.25; VMHdm, A: −1.15, M: ±0.5, D: −5.5; VTA, A: −3.25, M: ±0.5, D: −4.5. For in vivo ChR2 and Jaws experiments, fiber optic cannulae were manufactured in house as described14. For ChR2 experiments, optic fibers were implanted unilaterally 0.5 mm above the coordinates listed for viral injection. For Jaws experiments, optic fibers were implanted bilaterally 0.5 mm above the coordinates for the MeApv. For microinfusion experiments, cannulae were implanted lateral to MeApv at M: ±2.6 mm from bregma. Behavioral testing and slice electrophysiology experiments began after a minimum of 2-weeks surgical recovery period, with the exception of RetroBead and microinfusion surgeries, in which recovery was only 1 week. After behavioral testing, injection sites and fiber implant placements were confirmed using immunohistochemistry on collected brain tissue sections. Mice with mistargeted injections or implants were excluded from this study.

Calcium imaging surgeries were performed as described previously56. DrlalCre mice were injected with AAV1-FLEX-ECFP64 and AAV1-FLEX-HM3-mCherry using the stereotaxic protocol described above. Three weeks later, mice were implanted with a microendoscope lens (length: 7.3 mm, diameter: 0.6 mm; Inscopix). Animals were injected with 1 mg/kg CNO at the start of the surgery to visualize neuronal activity during implantation. The lens was implanted ~200–300 µm above the viral injection site. One week after the lens implant, mice were anesthetized and a fluorescent dissecting microscope (Inscopix) could be implanted on top of the lens to serve as an interface between the miniature microscope and the lens during experiments. A dummy miniature microscope (Inscopix) was used to habituate animals for ~5 d prior to any behavioral experiments. Only animals with successful lens implants were used for this study.

Immunohistochemistry. Primary antibodies were against GFP (mouse, 1:1,000, Invitrogen A11200 (ref. 12); or rabbit, 1:1,000, Invitrogen A11220), c-Fos (rabbit 1:2,000, Milipore ABE457)30, or TH (mouse, 1:1,000, Milipore MAB318)58. Secondary antibodies were against GFP (AAV1-FLEX-synaptoGFP)60 was used to characterize downstream projections of MeApv-D1R neurons. Pixel density in downstream target areas was quantified using ImageJ software. The “Measure” feature of ImageJ was used to quantify the integrated pixel density within a standardized ROI drawn based on previous classifications from the Allen Brain Atlas and other literature59.

Behavior. For all ChR2 behavioral experiments, blue light stimulation parameters were 10 Hz, 5 µs, 3× on, 3× off. For all behavioral experiments, red light stimulation parameters were a 2 s pulse with a 1 s ramp down (to prevent rebound excitation), and 1 s off. Experiments were conducted during the light cycle. Animals from each cohort were exposed to all of the following behavioral paradigms in the same order, with at least one week of separation in between each. Only male mice from each cohort were tested in the resident-intruder assay. Unless otherwise indicated, all behavioral videos were scored using Ethovision (XT10).

Pretender odor. For the pretender odor assay, mice were habituated for 3–4 d to a behavioral chamber with an odorant dish filled with clean cat litter and a hidebox on the opposite side of the chamber. On the test day, mice were placed in the chamber for a 5-min habituation period with an empty odorant dish, followed by a 5-min test period with blue light stimulation in which the mice were exposed to an odorant dish filled with cat litter saturated in cat urine. Behaviors scored were time spent in hide box, total time investigating the odorant dish, frequency of approach toward the odorant dish and latency to first approach of the odorant dish. Investigation and approach behavior was scored based on an animal’s proximity (≤28 cm) and orientation to the odorant, which were automatically detected by the Ethovision software.

Robogus. For the robogus assay, mice were placed in a behavioral chamber to which they had been previously habituated. On the test day, mice were placed in the chamber for a 2-min habituation period followed by a test period with blue light stimulation including a 30 s period in which a robogus apparatus (H: 11 cm; W: 11 cm; D: 13 cm; HEXBUG) was remotely activated. Mouse remained in the chamber with the stationary robogus for an additional 2 min. Scored behavior included latency to investigate the robogus after the remote activation period, time spent in hide box, and total time investigating the robogus. As in the predator odor assay, investigation and approach behavior was scored based on animal’s proximity (≤28 cm) and orientation to the robogus.

Resident–intruder. Resident mice were singly housed for at least 2 weeks, with a total of 7 of no cage change, were sexually experienced, and were 3–4-weeks older than intruder mice, which were group housed17. Encounters took place during the light cycle. Mice were allowed a 10 min non-scored habituation period after connection to the fiber optic cable. Intruders were placed into the resident’s home cage, and the blue light was turned on. Encounters lasted for 20 min, and videos were scored using an experienced observer and to the same standards. Behaviors scored were resident-initiated investigation, grooming, and fighting (which included attacking/mounting, high-speed chasing and nipping).

RTTP! Mice were placed in a two-chambered arena with partial walls dividing the two sides that allowed passage of the fiber optic cable53. One side of the arena had horizontal black and white stripes, and the other side had vertical stripes. One side was randomly assigned to be paired with blue light stimulation, and the other was unpaired. The assay lasted for 20 min.
Calcium imaging studies. A total of 24 mice were injected with AAV1-FLEX-GCaMP6m and AAV1-FLEX-HM3-mCherry. Of those mice, CNO-evoked calcium signals in the MeA was observed in nine animals that were implanted with GRIN lenses. Of the implanted animals, seven mice showed detectable GCaMP signals, with four mice displaying detectable signals during multiple behavioral epochs, where individual cells could be reliably isolated. All calcium imaging data were collected using 30–50% LED power using a miniature microscope from Inscopix (nVista), and calcium recordings were collected at 5 frames per second and a 200-ms exposure time. Behavioral videos and calcium recordings were synchronized using Ethovision (Noldus, XT10). Calcium videos were collected 2 min at a time to reduce file size. Behavioral assays were similar to those described above, with the exception of conspecific stimuli. Animals were exposed to an odorant dish containing soiled bedding from cages containing groups of either male or female mice.

Animals were habituated to a dummy miniature microscope in the behavior chamber for 5 d prior to testing. On test day, animals were hooked up to the miniature microscope and placed in the behavior chamber for approximately 1 h. To minimize the chance of altering the field of view of the lens, an animal was exposed to all stimuli on the same day, with a period of 30–60 min in between each assay. All animals were exposed to stimuli in the same order: robobug, predator odor, opposite-sex bedding, same-sex bedding. All behavioral assays for the calcium imaging studies lasted 10 min and included a 2 min pre- and post-test to collect baseline activity of the cells in which there was no stimulus in the behavior chamber. All behavior was hand scored to generate time bins (epochs), designating when an animal approached/investigated or avoided (spent time in the hide box) the stimulus. Investigation and approach behavior was scored based on an animal’s proximity (≤5–10 cm) and orientation to the stimulus. The 10-cm designation is one mouse body length in distance. When a mouse displayed a clear orientation toward the stimulus and was within one body length (10 cm) from the stimulus, it was designated as actively approaching. An approach was designated only if the animal continued to within 5 cm (one half a body length) of the stimulus and maintained an orientation to the stimulus (active investigation range). An additional approach required moving at least 5 cm away from the stimulus and oriented away from the stimulus followed by a reorientation and subsequent approach.

Calcium imaging analysis. For each behavioral assay, calcium imaging videos were concatenated and spatially downsampled by a factor of 2 in Mosaic (Inscopix). To correct for lateral displacements of the brain and prevent motion-induced artifacts, we applied frame-by-frame rigid-body registration using the TurboReg plugin in ImageJ. To prevent the artificial detection of ROIs along high-contrast borders, we cropped surrounding black borders after registration. After motion correction and cropping of the concatenated videos, neurons were automatically detected using a constrained non-negative matrix factorization method for microendoscopic data (CNMF-e)\(^{63,64}\). Merging threshold was set to automatically detected using a constrained non-negative matrix factorization after motion correction and cropping of the concatenated videos, neurons were synchronized using Ethovision (Noldus, XT10). Calcium videos were collected 2 min at a time to reduce file size. Behavioral assays were similar to those described above, with the exception of conspecific stimuli. Animals were exposed to an odorant dish containing soiled bedding from cages containing groups of either male or female mice.

Animals were habituated to a dummy miniature microscope in the behavior chamber for 5 d prior to testing. On test day, animals were hooked up to the miniature microscope and placed in the behavior chamber for approximately 1 h. To minimize the chance of altering the field of view of the lens, an animal was exposed to all stimuli on the same day, with a period of 30–60 min in between each assay. All animals were exposed to stimuli in the same order: robobug, predator odor, opposite-sex bedding, same-sex bedding. All behavioral assays for the calcium imaging studies lasted 10 min and included a 2 min pre- and post-test to collect baseline activity of the cells in which there was no stimulus in the behavior chamber. All behavior was hand scored to generate time bins (epochs), designating when an animal approached/investigated or avoided (spent time in the hide box) the stimulus. Investigation and approach behavior was scored based on an animal’s proximity (≤5–10 cm) and orientation to the stimulus. The 10-cm designation is one mouse body length in distance. When a mouse displayed a clear orientation toward the stimulus and was within one body length (10 cm) from the stimulus, it was designated as actively approaching. An approach was designated only if the animal continued to within 5 cm (one half a body length) of the stimulus and maintained an orientation to the stimulus (active investigation range). An additional approach required moving at least 5 cm away from the stimulus and oriented away from the stimulus followed by a reorientation and subsequent approach.

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For all assays, cell activity was analyzed from the first 10 s of the longest hide box period. For each animal, only a single hide box period was analyzed. For robobug and predator odors assays, cell activity was analyzed from ≥5 s of investigation periods or series of investigation periods lasting longer than 20 s. For each animal, 3–5 investigation periods were analyzed. For conspecific behavioral assays, cell activity was analyzed across all investigation periods. To categorize cells as having calcium signals selective for these epochs, the activity of each cell was shuffled in time to generate a null distribution of Ca\(^{2+}\) activity. This null distribution was registered to the behavioral epoch, and only cells that showed calcium signals during behavioral epochs with unshuffled data and complete inactivity during these epochs with shuffled data were categorized as selective. The threshold for inactivity was established by generating iterative multiples of the mean activity of each cell during the entire recording session, until a threshold in which no cell was categorized as selective during an epoch using shuffled data across all animals and all sessions was obtained. The least common multiple was determined to be 5-times the mean. Thus, cells were only considered selective for a given epoch if the average ΔF exceeded this conservative threshold\(^{65}\). Heat maps represented z-scores of denoised fluorescent activity±1.5 s of the corresponding hide box event. Events for heat plot selection across mice were determined by matching investigation or hide box epochs based on the duration of the event. Probability distribution curves were generated by plotting the kernel density estimate of a cell’s peak activity occurring ±1.5 s of the corresponding hide box of investigation event used for the heat maps.

Statistics. All statistical analyses were performed using Prism software (GraphPad). For comparison of two groups, an unpaired Student’s t test was used, except where noted. For comparison of multiple groups, one-way ANOVA was used, followed by Tukey’s or Bonferroni’s post hoc analysis. For comparison of two or more groups across treatment condition or time, a two-way repeated measure ANOVA was used, followed by Bonferroni post hoc analysis. Data distributions were tested for normality using the Kolmogorov–Smirnov normality test. If a dataset was not normally distributed, nonparametric analyses were applied. This is indicated where necessary in the manuscript. The sample sizes were determined based on those previously reported in studies related to our experimental assays\(^{63,64}\).

Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Datasets supporting the findings in this study and custom codes used for imaging analysis are available from the corresponding author upon reasonable request.

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- Clearly defined error bars
  - State explicitly what error bars represent (e.g. SD, SE, CI)

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Software and code

Policy information about availability of computer code

Data collection

Data was collected using Ethovision XT 8.5 and nVista recording software. Electrophysiological recordings were collected using Clampex v10.5

Data analysis

Data was analyzed using GraphPad Prism v6.07, Ethovision XT 8.5, ImageJ64, Mosaic, Python and Matlab. Open source CNMF-e code from Zhou et al was used in conjunction with custom code written by Daniele Marcotulli (available upon request).

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| Sample size | Power analysis was not used, instead sample sizes were based upon previously published variability of assays and sufficient numbers for duplication. |
|-------------|-----------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | Animals were excluded from behavioral assays based on incorrect targeting with virus or incorrect placement of cannula. |
| Replication | Behavioral assays and Fos experiments were repeated a minimum of two times with successful replication of results. |
| Randomization | Randomization was not relevant to our study because all animals were from the same genetic background and arbitrarily assigned to control or experimental groups. |
| Blinding | Behavior was analyzed by an investigator blind to the testing condition of the animal and conditions were only revealed after behavior had been scored. The experimenter was not blinded during data collection. |

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
|     | Unique biological materials |
|     | Antibodies |
|     | Eukaryotic cell lines |
|     | Palaeontology |
|     | Animals and other organisms |
|     | Human research participants |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
|     | ChIP-seq |
|     | Flow cytometry |
|     | MRI-based neuroimaging |

Antibodies

| Antibodies used | Primary antibodies were against GFP (mouse, 1:1000, Invitrogen A11120; or rabbit, 1:1000, Invitrogen A11122) c-Fos (rabbit 1:2000, Milipore ABE457) or TH (mouse, 1:1000, Milipore MAB3818). Secondary antibodies were conjugated to DyLight488 or CY3 (1:250, Jackson Immunolabs). RNAscope probes used were Probe-Dr-drd1a-01 #463851, MM-Npy1r-C3 #427021, Mm-Cyp19a1-02 #430821. |
| Validation | These are widely used antibodies and evidence for their validation, including numerous references (which are cited in the manuscript) can be found on manufacturer’s website (Invitrogen and Milipore). |

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | Male and female Cre-driver line animals were bred onto C57BL/6J background (JAX Laboratories) in University of Washington animal facilities. Cre-driver lines used were: Drd1a-Cre/+ and Slc6a3-Cre/+. The Ai14 cre dependent reporter strain, Rosa26Sor-fs.Tdt, was also provided by JAX laboratories. All behavioral mice were 8 weeks or older. Animals used for slice electrophysiology were 5-8 weeks old. |
| Wild animals | This study did not involve the use of wild animals. |
| Field-collected samples | This study did not involve samples that were collected from the field. |