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Dopaminergic Circuitry Underlying Mating Drive

Highlights

- Male flies calibrate mating drive to reflect reproductive potency
- Localized dopaminergic activity reflects recent mating history
- Dopamine tone gates courtship initiation at the P1 locus
- Sensory and internal state inputs converge to drive behavior

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In Brief

Zhang et al. show that male flies, like mammals, calibrate mating drive to reproductive capacity. Localized dopaminergic activity is a functional correlate of mating drive, gating the ability of sensory stimuli to trigger courtship behavior.

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Dopaminergic Circuitry Underlying Mating Drive

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SUMMARY

We develop a new system for studying how innate drives are tuned to reflect current physiological needs and capacities, and how they affect sensory-motor processing. We demonstrate the existence of male mating drive in *Drosophila*, which is transiently and cumulatively reduced as reproductive capacity is depleted by copulations. Dopaminergic activity in the anterior of the superior medial protocerebrum (SMPa) is also transiently and cumulatively reduced in response to matings and serves as a functional neuronal correlate of mating drive. The dopamine signal is transmitted through the D1-like DopR2 receptor to P1 neurons, which also integrate sensory information relevant to the perception of females, and which project to courtship motor centers that initiate and maintain courtship behavior. Mating drive therefore converges with sensory information from the female at the point of transition to motor output, controlling the propensity of a sensory percept to trigger goal-directed behavior.

INTRODUCTION

Most of our behaviors are flexible. Rather than being stereotyped responses to a given stimulus, our actions are contingent on internal states and external conditions. We eat primarily when we are hungry and can acquire quality food—not whenever we see food, and not regardless of quality. Even when hungry, we will not eat quality food if doing so exposes us to danger—unless we are hungry enough to accept the risk. Given the variety of potential inputs into these types of decisions, hardwired solutions for all possible situations are impractical, if not impossible. Instead, the nervous system creates drive states such as hunger to bias choices so that they reflect internal needs and capacities. These states determine how readily sensory stimuli trigger goal-directed motor output. In this work, we investigate where and how this regulation occurs in a novel, reductionist system.

Male mating behavior in *Drosophila* has been studied for over a hundred years (Sturtevant, 1915), but the last decade has seen a dramatic increase in our knowledge of the underlying neural circuitry. Much of this progress has followed from gaining genetic access to neurons expressing the sexually dimorphic transcription factor Fruitless (Ito et al., 1996; Ryner et al., 1996; Stockinger et al., 2005). Fruitless expression is thought to delineate circuitry extending from the sensory neurons that detect females to the motor output neurons that execute the various aspects of the courtship ritual (Cachero et al., 2010; Clowney et al., 2015; Kallman et al., 2015; Manoli et al., 2005; Ruta et al., 2010; Stockinger et al., 2005; Yu et al., 2010). The ability to target and manipulate small groups of neurons within the Fruitless circuit has begun providing insight into how sensory information is translated into motor output in the context of a goal-oriented behavior (Clowney et al., 2015; Coen et al., 2016; Fan et al., 2013; Kallman et al., 2015; Kimura et al., 2008; Kohatsu et al., 2011; Pan et al., 2012; von Philipsborn et al., 2011; Ruta et al., 2010; Zhou et al., 2015). Here we adapt this system to ask where and how sensory-motor transformations are gated by internal state. We show that male sexual behaviors are transiently and cumulatively reduced as reproductive fluid stores are depleted by repeated matings. The activity of dopaminergic neurons that project to the anterior superior medial protocerebrum (SMPa) reflects the male's recent mating history and is used to instruct mating drive. The dopamine signal is sensed and interpreted by a group of ~40 Fruitless neurons called P1 (Kimura et al., 2008) (also known as pMP4; Yu et al., 2010; or as a subset of pC1; Pan et al., 2012; Zhou et al., 2015). P1 neurons receive stimulatory input from female-derived sensory information (Clowney et al., 2015; Kallman et al., 2015; Kohatsu et al., 2011) and project to motor command neurons (Kohatsu et al., 2011; von Philipsborn et al., 2011). Dopaminergic internal state input therefore gates courtship initiation through a numerically compact neuronal node that lies at the transition between sensory input and motor output. The coincidence of sensory and internal state information activates P1, driving levels of mating behavior that reflect the quality of the female percept and the reproductive capacity of the male. At P1, then, analog information about two fundamental and necessary conditions for behavioral selection (appropriate external object and internal state) converges to determine the propensity for a binary output: whether or not to court. This system may provide deep insight into the generation of motivational states and their impact on sensory-motor circuitry.

RESULTS

Reproductive Satiety in Male *Drosophila*

To look for evidence of reproductive satiety in *Drosophila*, single males that had been isolated from females for 3–6 days were placed in food vials containing ~25 virgin females and examined

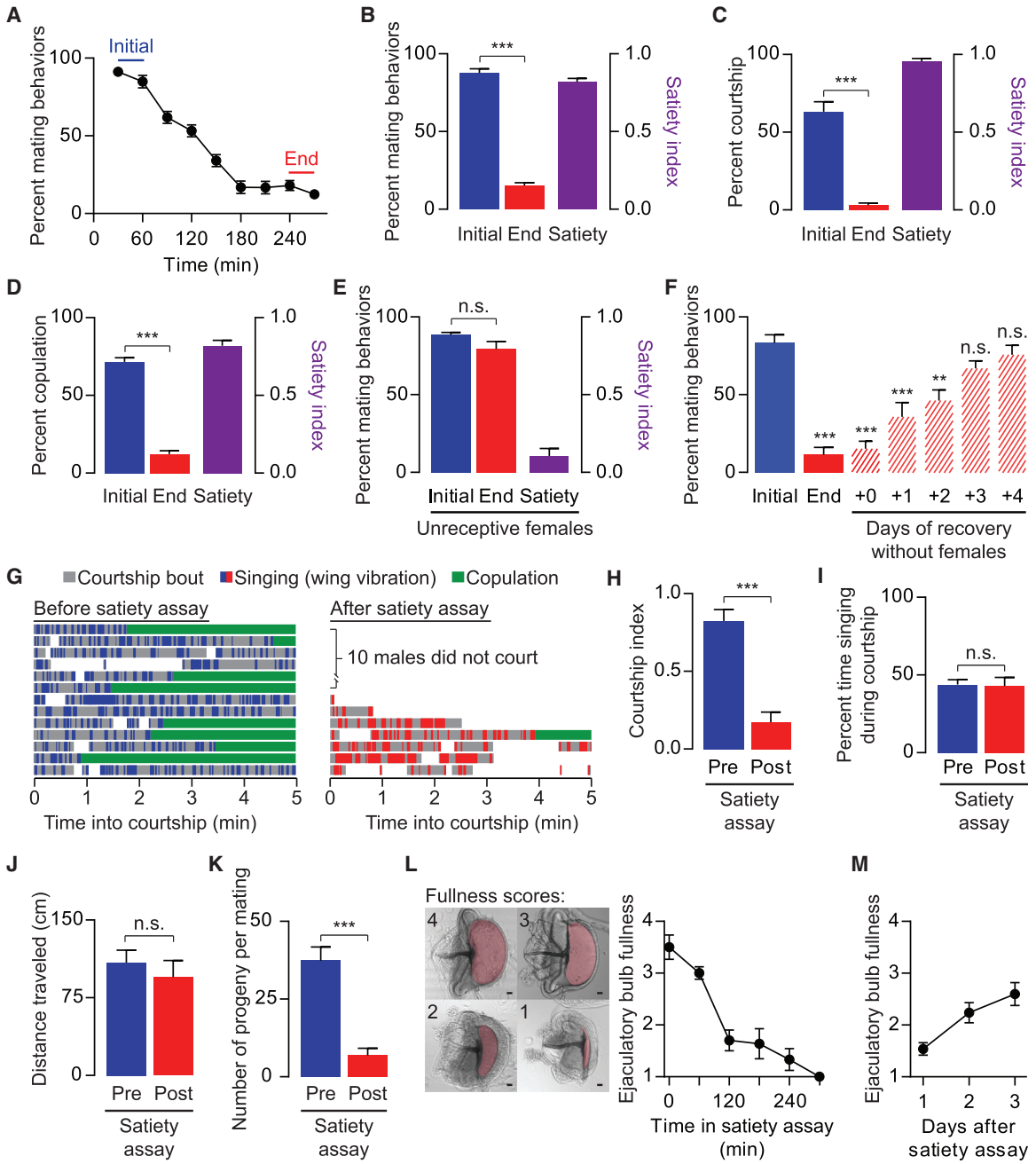

Figure 1. Reproductive Satiety in Male Flies

(A) The percent of time that wild-type males spend in mating behaviors (courtship or copulation) decreases over the course of the satiety assay (n = 9–17 groups of 6–8 assays. Each assay consists of 1 male with ~25 females).

(B–D) Left axis (red and blue bars): percent of time that wild-type males spend in mating behaviors (B), courtship (C) and copulation (D), decreases from the first (initial) to the last (end) 60 min of the satiety assay. Right axis (purple bar): fraction of the corresponding behavior reduced from the first to the last 60 min of the satiety assay (satiety index) (t test, n = 16 groups of 6–8 assays).

(E) Sexually unreceptive females (elav > sex peptide) (Nakayama et al., 1997) do not induce satiety (t test, n = 5 groups of 5–7 assays).

(F) After the satiety assay (blue and red bars), male flies were removed and housed without females for 0 to 4 days. When reintroduced to ~25 fresh females for 60 min, they showed increasing mating drive over the course of the recovery period (striped bars) (one-way ANOVA, n = 5–7 groups of 6–8 assays).

(G) Ethograms of male courtship, wing vibration, and copulation before (n = 13, all courted) and after the satiety assay (n = 17, 7 courted).

(H and I) Satiety decreases courtship (t test, n = 13–17) (H), but does not change the percent of time singing within a courtship bout (t test, n = 7–13) (I).

(J) Distance traveled by wild-type males within a 10 min time period does not change after the satiety assay (t test, n = 14–16). No females are present during the locomotion assay.

(legend continued on next page)

for mating behaviors (courtship or copulation) every 30 min. Initially, males spent ~80% of their time engaged in mating behaviors (courtship and copulation), but the percentage gradually declined to ~10% over 4 hr (Figures 1A and S1A, available online). During the assay, males mated an average of 3.3 ± 0.3 times with an average copulation duration of 24.6 ± 0.4 min that did not change with repeated matings. We use the term satiety to reflect the gradually diminishing propensity to engage in mating behaviors as the assay progresses. To quantify this change, we calculate a satiety index: the fraction by which mating behavior is reduced in the last 60 min relative to the first 60 min of the assay, which is 0.82 ± 0.02 for wild-type flies (Figures 1A and 1B; see Experimental Procedures). Robust reproductive satiety is seen at the level of both courtship (satiety index 0.96 ± 0.02; Figure 1C) and copulation (satiety index 0.82 ± 0.03; Figure 1D). Unlike other aspects of male mating behavior, satiety is not affected by the male's prior social experience with other males (Dankert et al., 2009; Inagaki et al., 2014; Kim et al., 2012) (Figure S1B).

Satiety is not the result of habituation to the presence of females, but requires copulation. Preventing copulation through the use of low-receptivity females (Nakayama et al., 1997), or males that have physical defects in external genitalia (Castrillon et al., 1993), resulted in high levels of courtship that were maintained throughout the satiety assay (Figures 1E and S1D). The reduction in mating behavior seen at the end of the assay was not altered by placing the satiated male with fresh virgin females (Figure 1F), but gradually recovered over the course of 3–4 days if the male was removed from females (Figure 1F). The reduction in mating drive in satiated males was also seen in standard courtship assays (one male and one female in a 15 min assay, used throughout this study when assaying for hyposexuality; see Experimental Procedures) (Figures 1G and 1H). When satiated males did court, they spent a comparable proportion of courtship time performing unilateral wing vibration ("singing"), but their courtship was often transient (Figures 1G and 1I). Satiety is unlikely to be a consequence of physical exhaustion, because locomotor activity was not changed by completion of the satiety assay (Figure 1J). We conclude that, similar to mammals (Beach and Jordan, 1956), male *Drosophila* exhibit a transient reproductive satiety induced by mating, a hallmark of sexual motivation.

Mating Drive Reflects, but Is Not Set by, Reproductive Capacity

Reproductive satiety parallels the male's diminishing reproductive capacity after repeated matings. This reduced potency is evident in males that have recently completed the satiety assay, as their subsequent copulations result in few, if any, progeny (Figure 1K) (also see Crickmore and Vosshall, 2013; Lefevre and Jonsson, 1962; Linklater et al., 2007). A physical basis of this diminishing fertility is found in the male's ejaculatory bulb, which houses mature sperm and seminal fluid (Demerec, 1950). The volume of the bulb gradually decreases over the course of repeated matings, with kinetics redolent of the decline in mating behaviors (Figure 1L). By the end of the satiety assay, reproductive fluids are largely depleted and the lumen of the bulb has shrunken substantially, a presumptive cause of the impaired fertility of males in a state of reproductive satiety. The fullness of the bulb recovers after the satiety assay, though only to ~75% after 3 days away from females (Figure 1M).

Since mating drive roughly tracks ejaculatory bulb volume, we asked if information about the volume of the bulb is used to gate the execution of sexual behaviors. To allow copulation but prevent depletion of reproductive fluids from the bulb, we performed a modified satiety assay wherein copulations were artificially terminated 5 min after initiation—before reproductive fluids are transferred (Crickmore and Vosshall, 2013; Gilchrist and Partridge, 2000; Tayler et al., 2012). As expected, the ejaculatory bulb remains full throughout this modified assay (Figure 2A). Unexpectedly, though, we find that truncated copulations trigger satiety to the same degree as normal copulations: the number of matings per male in the assay remained unchanged when copulations were disrupted at 5 min (Figure 2B), despite the cumulative time spent in copulation being dramatically reduced (Figure 2C). Normal satiety in males with truncated copulations is also seen in a courtship assay (Figure 2D). We then examined the consequences of artificially emptying the ejaculatory bulb without allowing the male to copulate. Thermogenetic activation of corazonin (crz) neurons causes repeated ejaculation, even in the absence of copulation (Tayler et al., 2012). Ejaculatory bulbs of males in which the warmth-sensitive cation channel TrpA1 (Hamada et al., 2008) was targeted to crz neurons (crz > TrpA1) were depleted after incubation at 30°C for 3 days, but this treatment had no effect on courtship behavior (Figure 2E). Moreover, recovery of mating drive after the satiety assay was unimpeded by artificially maintaining the empty status of the ejaculatory bulb (Figure 2E). The finding that ejaculatory bulb fullness is entirely dissociable from courtship vigor is consistent with the delayed recovery kinetics of bulb fullness compared to mating drive (Figure 1M). Together, these results argue against a direct role of sperm and seminal fluid accumulation or depletion in instructing mating drive. To ask whether reproductive organs have any role in the acute determination of mating drive, we surgically removed the posterior abdomen, which contains the internal and external reproductive organs. This surgery had no major detrimental effect on courtship in naive males, and did not stimulate courtship in satiated males (Figure 2F; Movie S1). These results show that though mating drive is calibrated to reflect reproductive capacity, the reproductive organs themselves have no acute influence on the male's response to the presentation of a virgin female. Though sensory information

(K) Number of progeny sired by wild-type males per mating decreases significantly after the satiety assay (t test, n = 8–9).

(L and M) Fullness scores of ejaculatory bulbs from wild-type males decrease over the course of the satiety assay (n = 6–11) (L) and recover gradually afterward (n = 20–28) (M). Representative images of ejaculatory bulb scores are shown on the left, with lumina highlighted in red.

In all figures, scale bars represent 20 μm; in all figures, error bars represent SEM; in all figures, *p < 0.05, **p < 0.01, ***p < 0.001, n.s., not significant for all statistical tests. Detailed genotypes can be found in the Supplemental Experimental Procedures online. In all figures, one-way ANOVA was used with Tukey's post-test.

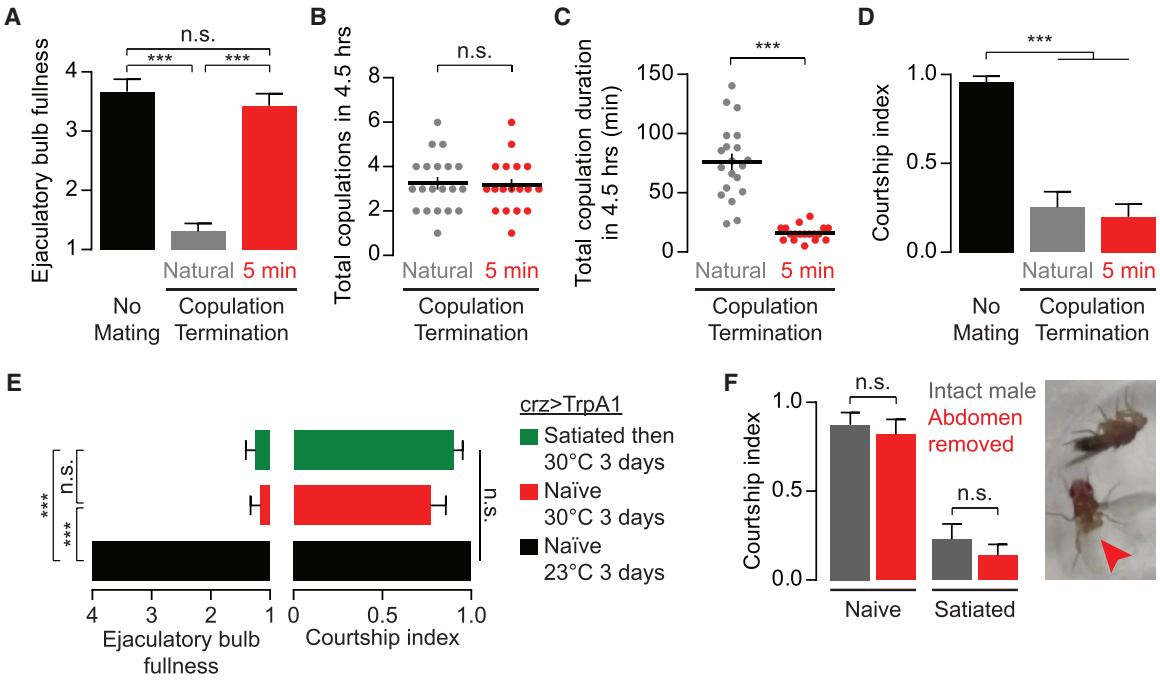

Figure 2. Mating Drive Reflects, but Is Not Acutely Instructed by, Reproductive Status

(A) Artificially terminating copulations at 5 min prevents depletion of ejaculatory bulbs. Black bar shows data for handling control flies that did not go through the satiety assay but went through the same procedure used to terminate copulations (same in D; see Experimental Procedures for details) (one-way ANOVA, n = 12–20).

(B and C) Artificially terminating copulations at 5 min does not change the number of copulations (B) but decreases total copulation duration (C) in the satiety assay (t tests, n = 18–20).

(D) Males whose copulations were terminated at 5 min still show decreased courtship index after the satiety assay (one-way ANOVA, n = 12–20).

(E) Ejaculatory bulb fullness is separable from mating drive. Thermogenetic stimulation of crz neurons (crz > TrpA1) at 30°C in naive males depletes the ejaculatory bulb but does not diminish sex drive (red). The same manipulation in satiated males prevents refilling of the bulb during the recovery from the satiety assay but does not prevent the recovery of sex drive (green) (one-way ANOVA, n = 6–14).

(F) Abdomen removal (red arrowhead in inset) has no effect on the courtship indices of wild-type males in either the naive or satiated state (one-way ANOVA, n = 13–18).

from the genitalia may signal the onset of copulation, the resulting adjustments to and maintenance of mating drive are carried out within the CNS.

Dopaminergic Neurons Control Mating Drive

To identify the genes and neurons that control mating drive in male *Drosophila*, we examined over 1,500 genetic manipulations (see Experimental Procedures) in the satiety assay in search of animals that maintained high levels of mating behaviors after repeated copulations. The only hit obtained in this screen resulted from thermogenetic activation of dopaminergic neurons. When dopaminergic neurons were stimulated at the end of the satiety assay using tyrosine hydroxylase-Gal4 (TH > TrpA1; TH is an enzyme required for dopamine synthesis), satiated males showed a dramatic rebound in mating behaviors (Figure 3A). The rebound is seen in both copulation (58% decrease in satiety) and courtship (84% decrease in satiety) (Figures S2A and S2B). This finding parallels previous work showing that dopaminergic neurons of the ventral nerve cord (VNC) promote the male's motivation to sustain individual copulation bouts (Crickmore and Vosshall, 2013). In that study, dopaminergic function was localized to the VNC due to the ability of TshGal80 (which inhibits the activity of Gal4 in most VNC neurons) to block Gal4-mediated phenotypes. In contrast, TshGal80 has no effect on the reversal of satiety seen in TH > TrpA1 males (Figures 3A, S2A, and S2B). Together, these findings reveal two functionally and anatomically distinct roles for dopamine in promoting mating drive in flies. The initial drive to mate is set by dopamine neurons of the brain, and once copulation is initiated, dopaminergic neurons of the VNC determine the persistence and duration of the mating.

To identify which brain dopaminergic neurons underlie mating drive, we thermogenetically activated four subsets of dopaminergic neurons using Gal4 lines derived from enhancer elements of the *pale* locus, which encodes TH (Liu et al., 2012). Stimulation of either of two subsets of TH neurons (TH-C' and TH-D'; Figures 3B and 3C) resulted in partial recovery from satiety. This reversal of satiety is not a secondary effect of increased locomotion, as stimulating either population does not significantly impact basal activity (Figure S3A). The activity of neurons within these populations is necessary for mating drive, as adult-specific silencing of TH-C' or TH-D' neurons with the potassium channel Kir2.1 decreased courtship (Figure 3D). Dopamine itself is likely the mating drive signal, as lowering TH production in TH-C' or TH-D' neurons with pale-RNAi dramatically reduced courtship (Figure S4).

To ask whether the mating drive dopaminergic neurons lie within the Fruitless circuitry, we introduced a Gal80 transgene whose expression pattern is indirectly controlled by the *cis*-regulatory elements of *fruitless* (see Figure 3B legend). Thermogenetic stimulation in these flies (TH-C' > TrpA1;FruGal80 and TH-D' > TrpA1;FruGal80) fails to reverse satiety (Figure 3B), arguing that mating drive dopamine neurons are Fruitless positive. In both TH-C' and TH-D', FruGal80 blocked Gal4 activity in a single neuron per brain hemisphere, and in both cases this neuron was located in the PAL dopaminergic cluster (Mao and Davis, 2009) (Figures 3E and S5). In adults, no TH-C' or TH-D' neurons stain with Fruitless antibodies (data not shown), but in both populations, an FLP recombinase transgene inserted into the *fruitless* locus (FruFLP) identifies single dopaminergic neurons belonging to the PAL dopaminergic cluster (Figure 3F) (Keleman et al., 2012; Mao and Davis, 2009). This neuron is called aSP4 (Keleman et al., 2012; Yu et al., 2010) and has been shown to impair courtship behavior when feminized in males (von Philipsborn et al., 2014). TH-F and TH-G do not label any neurons in the PAL cluster, including aSP4 (Liu et al., 2012). aSP4 projects to several dorsal brain regions (von Philipsborn et al., 2014), including the anterior of the superior medial protocerebrum (SMPa) (Ito et al., 2014) (Figure 3F).

While activation of aSP4 is necessary to reverse satiety in this system, it does not appear to be sufficient, as thermogenetic activation of VT02857-Gal4, which targets aSP4 (Keleman et al., 2012), did not increase mating behavior in satiated males (Figure 3G), even when two copies of the Gal4 were used to increase expression (data not shown). This suggests the existence of other dopaminergic neurons that work with aSP4 to promote mating drive. We therefore screened ~30 Gal4 lines labeling dopaminergic neurons, looking for lines that could overcome satiety when activated together with aSP4. We identified NP5945-Gal4 (Kuo et al., 2015), which does not label aSP4 and did not reverse satiety when stimulated alone, but did increase mating behavior from satiated males when stimulated together with VT02857 (Figure 3G). Silencing either VT02857 or NP5945 neurons in adults using Kir2.1 decreased courtship behavior (Figure 3H), suggesting that the combined activity of these two neuronal populations is necessary to promote the normal accrual of mating drive. NP5945 labels non-Fruitless dopaminergic neurons in four clusters (PPL2ab, PPL2c, PPM2, and PPM3) and has projections to the SMPa (Figure 3I). Although we cannot currently isolate the neurons within NP5945 that promote mating drive together with aSP4, our results suggest the SMPa as a locus for the dopaminergic control of sexual motivation in males.

Dopamine Signals Mating Drive to P1 Neurons

As a first step toward identifying the neuronal targets of dopamine signaling relevant to mating drive, we examined courtship in animals carrying mutations in the four *Drosophila* dopamine receptors. When tested at 3 days old, a combination of mutations in three of the receptors showed normal levels of courtship, whereas a deletion of the fourth, DopR2 (a D1-like receptor) (Han et al., 1996; Keleman et al., 2012), resulted in dramatically reduced courtship without affecting locomotor activity (Figures 4A and S3B). These data initially seemed to be in conflict with a previous study reporting normal levels of courtship in these same DopR2 mutants in the same genetic background (Keleman et al., 2012). Further examination of these mutants led us to a likely explanation for this discrepancy: we found that the mating drive of DopR2 animals gradually increased over 10–15 days (Figure 4B), as did, to a lesser extent, the mating drive of wild-type males (Figure S1C). This suggests that as mating drive increases with age, additional dopamine receptors gradually become capable of receiving the mating drive signal. DopR2 functions in neurons (as opposed to non-neuronal cells) to promote the timely accumulation of mating drive, as knockdown of DopR2 with the pan-neuronal elav-Gal4 driver phenocopied the deletion mutant (Figure 4C). Matings involving 3-day-old DopR2 mutant males were fully fertile (data not shown), so the behavioral phenotype is not a secondary consequence of sterility.

The identification of a receptor that receives dopamine input relevant to reproductive state presents an opportunity to investigate how motivation signals impinge on sensory-to-motor circuitry to gate the execution of behavior. In this system, the dopamine-receiving neurons are Fruitless positive, as RNAi knockdown of DopR2 in Fruitless neurons reduced courtship behavior in mature males (Figure 4D).

Within the Fruitless circuitry, much attention has focused on a locus composed of ~40 neurons, called P1, that is required for mating behavior (von Philipsborn et al., 2011). Stimulation of a subset of P1 neurons (labeled by the Gal4 line R71G01, here referred to as P1-B in reference to the Baker lab where it was first characterized) has been shown to drive courtship behaviors toward moving, fly-sized inanimate objects (Kohatsu and Yamamoto, 2015; Pan et al., 2012). In our hands, activation of P1-B neurons drives robust courtship behavior toward even stationary rubber band pieces—if they are painted black (Figure 5A; Movie S2). This phenotype is distinct from activation of the entire fruitless circuit, which drives courtship in the absence of a target (Figure S6A), and from activation of dopamine neurons, which does not cause courtship of inanimate objects (Figure 5A).

Based on anatomy and the Fruitless wiring diagram, the Dickson lab suggested that the aSP4 dopamine neuron may connect to P1 neurons (von Philipsborn et al., 2014; Yu et al., 2010). We found that activation of P1-B neurons drives robust courtship in satiated males (Figure 5B), as well as in 3-day-old males that lack DopR2 (Figure 5C). Activating P1 neurons therefore bypasses the requirement for state-dependent dopaminergic input, indicating that they are functionally downstream of the dopamine mating drive signal.

Are P1 neurons the direct targets of dopaminergic signaling? As noted by the Dickson lab (von Philipsborn et al., 2014; Yu et al., 2010), co-labeling P1-B neurons and dopaminergic neurons (Figure 5D) shows intermingling of neuronal processes between these two populations. We detected a strong GRASP (GFP reconstitution across synaptic partners) (Feinberg et al., 2008) signal between the two populations, indicating potential sites of synaptic connectivity (Figures 5E and S6B). Much of the GRASP signal was located in the SMPa, a region that is targeted by the aSP4 dopaminergic neuron (Figure 3F) as well as the NP5945 neurons (Figure 3I). When we knocked DopR2 down in P1 neurons, we saw a profound reduction of courtship behavior that recovered with age (Figure 5G), phenocopying

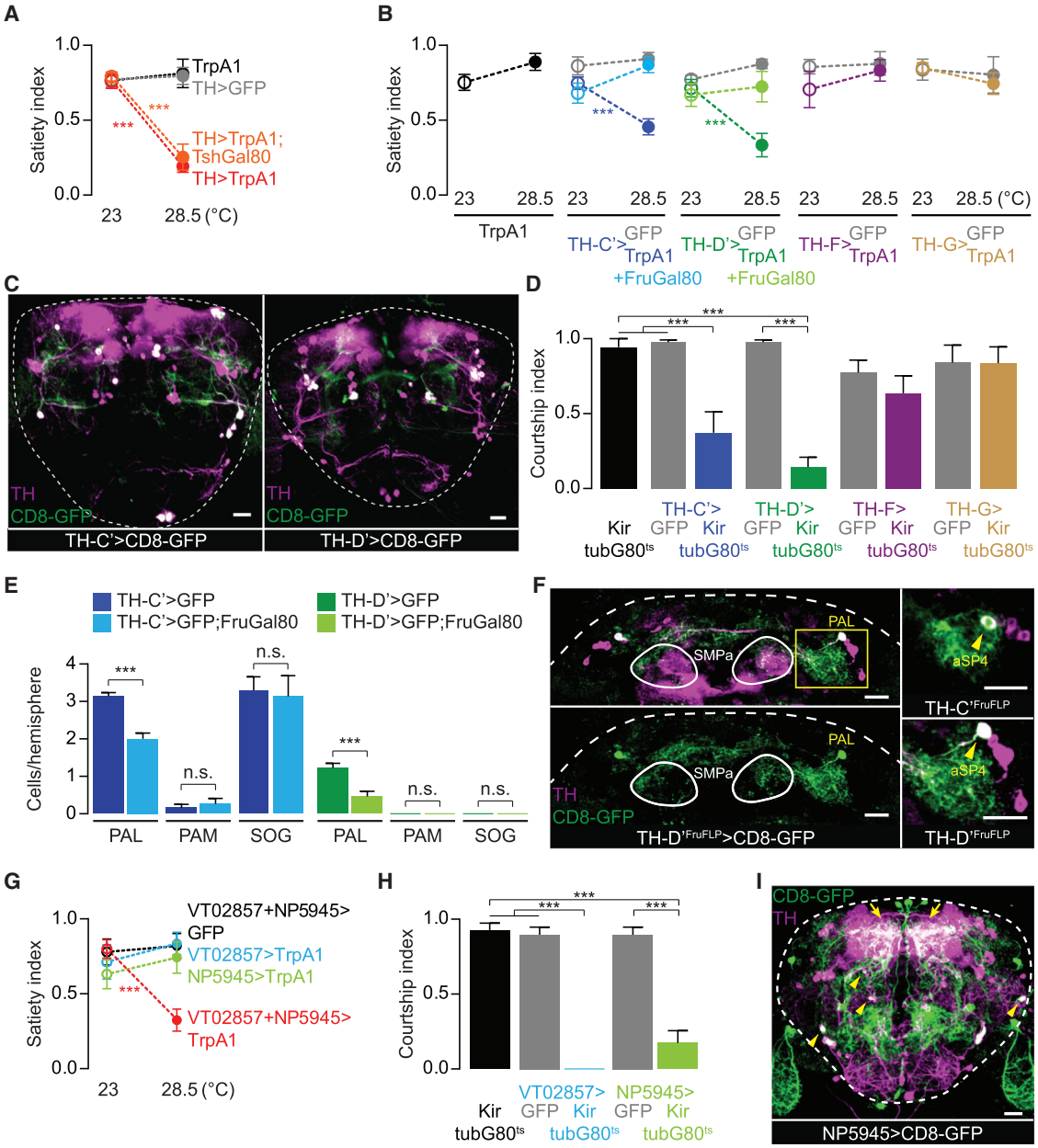

Figure 3. Dopamine Controls Mating Drive

(A) Thermogenetic stimulation of dopaminergic neurons (red) at 28.5°C decreases the satiety index of male flies. This satiety reversal effect is not blocked by TshGal80 (orange), which suppresses Gal4 activity in the VNC. No satiety reversal is seen in parental controls (gray and black) (two-way ANOVA, n = 6–13 groups of 6–8 males). See Figures S2A and S2B for the satiety reversals of copulation (Figure S2A) and courtship (Figure S2B).

(B) Thermogenetic stimulation of neurons labeled by TH-C' and TH-D', but not TH-F or TH-G, reverses satiety. The satiety reversal effects are blocked by FruGal80, which was constructed by combining Fru-LexA (Mellert et al., 2010) and LexAop-Gal80 transgenes (two-way ANOVA, n = 5–13 groups of 6–8 males).

(C) Immunostaining of GFP (green) and TH (magenta) in TH-C' > CD8-GFP and TH-D' > CD8-GFP male brains.

(D) Silencing TH-C' or TH-D', but not TH-F or TH-G neurons, with the potassium channel Kir2.1 in adult males decreases courtship. Males were raised at 23°C until eclosion, when they were moved to 30°C for 3 days to inactivate GAL80^{ts} and allow Kir2.1 expression before testing (one-way ANOVA, n = 8–24).

(E) FruGal80 blocks GFP expression in one TH-C' and one TH-D' cell per hemisphere in the PAL cluster, but not in any other anterior clusters (t test, n = 13–18 hemispheres per genotype) or posterior clusters (see Figure S5).

(F) Left: immunostaining of GFP (green) and TH (magenta) in the anterior brain of a TH-D'^{FruFLP} > CD8-GFP male. Yellow rectangle outlines a dopaminergic PAL cluster, which is magnified on the right. Solid white lines circumscribe the SMPa. Right: same as left, but images are magnified for TH-C'^{FruFLP} > CD8-GFP (top) and TH-D'^{FruFLP} > CD8-GFP (bottom). Arrowheads point to neurons that are positive for both CD8-GFP and TH. CD8-GFP labeling is seen in 7 out of 23 TH-C'^{FruFLP} > CD8-GFP (all unilateral) and 4 out of 14 TH-D'^{FruFLP} > CD8-GFP brains (two unilateral and two bilateral).

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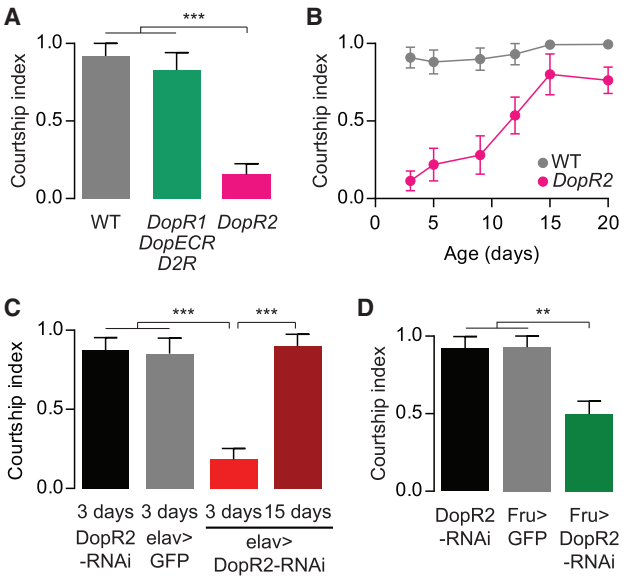

Figure 4. Dopamine Signals Mating Drive through DopR2

(A) Males mutant for *DopR2* (*damb*) (magenta; $DopR2^{attp}$), but not males mutant for all other dopaminergic receptors in combination (green; $DopR1^{attp}$, $DopECR^{c02142}$, $D2R^{f06521}$) show decreased courtship when compared to the wild-type background strain for the $DopR2^{attp}$ deletion (Keleman et al., 2012) (one-way ANOVA, n = 10–26). See Figure S3B for locomotor activity data.

(B) Courtship gradually increases in $DopR2^{attp}$ mutants, reaching near-wild-type levels by 15 days (n = 6–25).

(C) Pan-neuronal RNAi knockdown of DopR2 decreases courtship in 3-day-old males, but normal courtship is seen in these animals by 15 days (one-way ANOVA, n = 14–29).

(D) RNAi knockdown of DopR2 in Fruitless cells decreases courtship (one-way ANOVA, n = 13–23).

the DopR2 mutant. This is strong evidence that P1 neurons receive the dopaminergic mating drive signal.

The P1-B Gal4 line labels some cells outside of the sexually dimorphic P1 cluster (Pan et al., 2012). When FruGal80 is introduced, P1-B labeling within the P1 cluster is greatly reduced, but expression outside the cluster is not obviously affected (Figure 5F). Knocking down DopR2 in these remaining neurons had no effect on courtship behavior (Figure 5H), confirming that the Fruitless-positive P1 neurons receive and interpret the dopamine signal. To ask whether additional neurons receive the mating drive signal through DopR2, we blocked Gal4 activity in P1-B neurons (see Figure 5I legend) while knocking down DopR2 in all other neurons. Blocking pan-neuronal DopR2 knockdown in P1-B neurons restored courtship to normal levels (Figure 5I), arguing that the dopaminergic mating drive signal is received exclusively by P1.

Dopaminergic Activity in the SMPa Is a Neuronal Correlate of Mating Drive

The presumptive synaptic contacts between dopaminergic and P1 neurons in the SMPa region led us to monitor the activity of dopamine neurons in this area in males of varying reproductive states. We drove the calcium sensor GCaMP6s (Chen et al., 2013) in dopaminergic neurons and found that fluorescence levels in the SMPa tracked the male's level of satiety. Similar to mating drive, fluorescence levels declined gradually over the course of the assay, to the point that they were reduced by ~80% after 4.5 hr (Figures 6A and 6B). Also similar to mating drive, dopaminergic activity in the SMPa gradually recovered from the satiety assay over the course of 3 days of isolation from females (Figure 6B). The aSP4 neuron accounts for much of the dopaminergic activity in the SMPa, as FruGal80 blocks ~65% of the fluorescence in naive animals (Figure 6C). Fluorescence did not noticeably change with mating history in an unrelated region, the optic lobe (Figure 6A), or in a neighboring neuropil, the medial lobe of the mushroom body (Figure 6B). Nor were SMPa fluorescence changes observed in the calcium-insensitive fluorescent protein tdTomato ($p > 0.95$) or with immunostaining of fixed TH > GCaMP6s brains ($p > 0.92$) (Figures S7A and S7B). The mating-history-dependent GCaMP6s fluorescence change in SMPa was also detected using VT02857-Gal4, which labels aSP4 and a few other dopaminergic neurons (Figure 6D) (Keleman et al., 2012; Yu et al., 2010), as well as NP5945-Gal4, which labels neurons in four dopaminergic clusters (Figures 6E and 3I) (Kuo et al., 2015). These results show that dopaminergic activity in the vicinity of the connection to P1 neurons serves as a neuronal correlate of male mating drive.

The decreased basal activity of dopaminergic projections in the SMPa suggests that these neurons may become less excitable following repeated matings. To test this idea directly, we drove the red-light-sensitive ion channel CsChrimson (Hoopfer et al., 2015; Klapoetke et al., 2014) together with GCaMP6s in dopaminergic neurons and used two-photon microscopy to image optogenetically induced calcium transients in the SMPa. At all stimulation lengths, dopaminergic SMPa fluorescence peaks were lower in satiated brains than in naive brains, or in brains tested 3 days after completion of the satiety assay (Figures 6F and 6G). Fluorescence decay kinetics did not change with mating history (Figure 6H). Mating therefore decreases the excitability and/or calcium influx of SMPa-projecting dopaminergic neurons while leaving calcium buffering properties unchanged. These long-lasting changes suggest a molecular mechanism acting either on the inputs to dopamine neurons, or within the dopamine neurons themselves. The resulting dopamine tone in the SMPa serves as a neuronal correlate of mating drive, gating

(G) Thermogenetic co-stimulation of VT02857 (labeling aSP4 and a few other dopaminergic neurons) and NP5945 neurons (red), but not of either population alone (blue or green), decreases the satiety index of male flies (two-way ANOVA, n = 5–7 groups of 5–8 males).

(H) Silencing VT02857 or NP5945 neurons with the potassium channel Kir2.1 decreases courtship. Males were raised at 23°C until 1 day after puparium formation, when they were moved to 30°C and kept isolated from females until 3 days after eclosion, to inactivate $GAL80^{ts}$ and allow Kir2.1 expression before testing (one-way ANOVA, n = 10–20).

(I) Immunostaining of GFP (green) and TH (magenta) in an NP5945 > GFP male brain showing co-labeling in ~8 neurons per hemisphere, in four clusters (arrowheads). Arrows point to SMPa.

In all figures, a dashed line circumscribes the central brain unless otherwise stated. In all figures, interaction terms between genotype and temperature are tested by two-way ANOVA with a Bonferroni post-test.

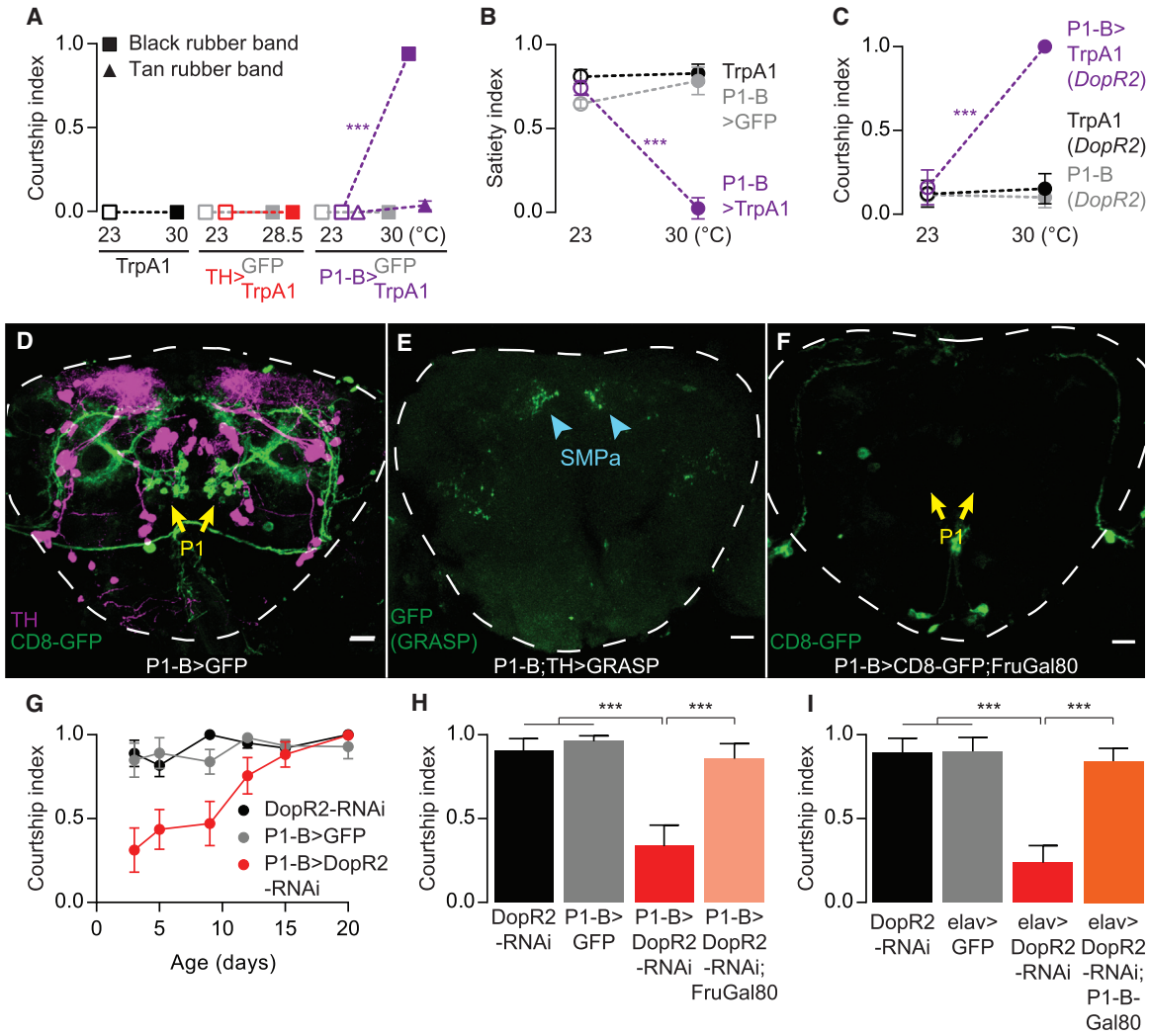

Figure 5. Dopamine Signals Mating Drive to P1 Neurons

(A) Thermogenetic stimulation of P1 neurons (purple), but not dopaminergic neurons (red), elicits courtship toward a fly-sized piece of black rubber band (squares). No courtship is seen toward a tan rubber band (triangles) (two-way ANOVA, n = 8).

(B) Thermogenetic stimulation of P1-B neurons reverses satiety (two-way ANOVA, 5–12 groups of 6–8 males).

(C) Thermogenetic stimulation of P1 neurons in 3-day-old males carrying the *DopR2*^{attp} deletion increases courtship (two-way ANOVA, n = 13–19).

(D) Immunostaining of GFP (green) and TH (magenta) in P1-B > CD8-GFP male brains. Arrows point to the P1 neuron cell bodies.

(E) Immunostaining of GRASP (GFP reconstitution across synaptic partners) between TH-Gal4 and P1-B-LexA neurons. Arrowheads point to the SMPa. See Figure S6B for parental controls.

(F) Immunostaining of GFP (green) and TH (magenta) in P1-B > CD8-GFP;FruGal80 males. Arrows point to the approximate area of the P1 neuron cell bodies.

(G) RNAi knockdown of DopR2 in P1-B neurons decreases courtship in 3-day-old males, but normal levels of courtship are seen by 15 days (n = 8–25).

(H) FruGal80 blocks the courtship deficit caused by knocking down DopR2 in P1 neurons (one-way ANOVA, n = 13–15).

(I) The courtship deficit caused by pan-neuronal RNAi knockdown of DopR2 is restored if Gal4 activity is blocked in P1-B neurons. P1-B-Gal80 was constructed by combining P1-B-LexA and LexAop-Gal80 transgenes (one-way ANOVA, n = 12–17).

the activation of P1 neurons in response to sensory stimulation from females.

DISCUSSION

To ensure the appropriateness of goal-directed behavior, information about needs and capacities must control the propagation of activity from sensory to motor systems. We have developed male mating behavior in *Drosophila* as a model to study this regulation at the molecular and circuit level. This system is attractive because the expression of sexually dimorphic transcription factors extends from sensory input neurons to motor output neurons, delineating circuitry that controls many aspects of sexually dimorphic behavior and facilitating the identification of underlying circuit elements (Cachero et al., 2010; Clowney et al., 2015; Crickmore and Vosshall, 2013; Kallman et al.,

2015; Manoli et al., 2005; Pan et al., 2012; Shirangi et al., 2006; Stockinger et al., 2005; Vrontou et al., 2006; Yu et al., 2010; Yuan et al., 2014; Zhou et al., 2014, 2015). Our finding that regulation by internal state occurs through the influence of dopamine on P1 has many implications for understanding motivational regulation in this and other systems.

In his *Ethics* (Spinoza, 1677), Spinoza drew a distinction between perception and conception: "the word perception seems to imply that the mind is passive in respect to the object; whereas conception seems to express an activity of the mind." While Spinoza was referring to the human mind, if we substitute "internal state" for "mind," the logical framework seems to hold in the system described here. The perception of a female conspecific likely occurs independently of internal state, but conception as a potential mating partner is dependent on recent mating history. The mechanistic basis of this distinction is apparent in the circuitry: regulation by internal state occurs at the point where processed, multi-modal female sensory inputs converge onto a locus that is capable of driving courtship behavior (Figure 7). Whether or not the percept of a female is translated into the concept of a courtship target is determined by the ability of sensory input to stimulate P1, which is determined by local dopamine tone in the SMPa, which is set by mating history. In this circuit logic, changes in mating drive require neither sensory nor motor processing streams to be altered by internal state; the gating occurs at the point where sensory input can stimulate motor output. Once courtship behavior has been selected, the interplay between sensory and motor systems continues as the male calibrates his singing and other actions to the changing position of the female (Coen et al., 2014, 2016).

Two general questions about dopaminergic control of motivation are made addressable in this system: (1) How is dopamine tone set and maintained by transient experience? (2) How does dopamine tone gate the execution of goal-directed behavior? There likely exist neurons in the male that detect the onset of copulation and induce decrements in SMPa dopaminergic activity with each mating. This transient signal is then maintained as diminished dopaminergic activity that steadily recovers over tens of hours. We have identified the relevant dopaminergic locus and have genetic access to the neurons. The upstream inputs that convey mating information to the dopaminergic neurons are likely to be sexually dimorphic—and therefore identifiable and targetable. A thorough interrogation of the molecular and circuit changes that occur in response to mating history may lead to broadly useful principles about how transient events cause long-lasting changes in motivation and behavior.

Equally promising is the study of how P1 interprets the local dopamine tone. When dopamine neurons are active, males court females; when P1 neurons are synthetically activated, males will court even inanimate objects. Clearly, dopamine does not simply activate P1 neurons, but allows their activation by appropriate sensory input. In mammals, dopamine has a well-established role in motivating behaviors (Volkow and Morales, 2015; Wise, 2004), but the targets of this regulation are distributed across many neurons and brain regions (Volkow and Morales, 2015; Wise, 2002). The compact P1 locus will allow a deep understanding of the cellular mechanisms that interpret local dopamine tone to determine behavioral responses to external stimuli.

The unaltered mating drive seen in males lacking reproductive organs demonstrates that dopaminergic projections in the SMPa are set to reflect reproductive potency without receiving a direct measurement of reproductive fluid stores. There are other instances in which representations of the internal and external world are found to be based on inference instead of direct measurement. For example, the retinal blind spot in humans is filled in using surrounding visual information (Ramachandran and Gregory, 1991). While experimenters can easily measure the amount of reproductive fluid stored in the ejaculatory bulb of a dissected male, for the fly itself it may be more reliable or efficient to infer the predictable changes in reproductive potency that occur with mating history, similar to the way the circadian system models the predictable rotation of the earth (Sollberger, 1965). Here the male fly's nervous system uses the predictable rates of loss and recovery of reproductive fluid that occur during copulation and abstinence to generate a neuronal representation of reproductive potency in the SMPa.

There are many additional similarities in studies of humans and other mammals to the motivational system we describe here. In *Awakenings*, Oliver Sacks described hypersexuality as an invariant consequence of administering L-DOPA to his patients (Sacks, 1999), demonstrating the conservation of dopamine's role in mating drive across phyla. Classic work in cats showed that stimulating a specific hypothalamic area elicits aggression, even toward inanimate "dummy" cats (Brown et al., 1969), similar to the courtship not only toward females, but also toward fly-sized inanimate objects, when dopamine-receiving neurons are activated. More recently, optogenetic techniques were used to stimulate an aggression locus within the murine hypothalamus, causing aggression toward other mice, or even toward an inflated rubber glove (Lin et al., 2011). In humans, anxiety levels exert a strong influence on the categorization of various objects and situations (Bar-Haim et al., 2007). We suggest that these effects on object classification result from a general principle of motivational circuitry: when the intensity of a behavioral state is high, the demand for accurate sensory input is lowered (Lorenz, 1950). In the concise circuitry we describe here, we find that this trade-off results from the direct influence of internal state information on the propensity for perceptual input to drive goal-directed behavior.

EXPERIMENTAL PROCEDURES

Fly Stocks

Flies were maintained on conventional cornmeal-agar-molasses medium under a 12 hr light/12 hr dark cycle at 25°C and ambient humidity. Unless otherwise stated, males were collected 0–3 days after eclosion and group housed away from females for 3 days before testing. Virgin females were generated by heat-shocking a w^{1118} stock with a hs-hid transgene integrated on the Y chromosome (Bloomington stock #24638) in a 37°C water bath for 90 min. Virgins were group housed for 3–13 days before use. All behavioral experiments were carried out between ZT 1 and ZT 10 (ZT, Zeitgeber time; lights are turned ON at ZT 0 and turned OFF at ZT 12). Detailed genotypes of all strains used in the paper are listed in the Supplemental Experimental Procedures, available online.

Satiety Assays

In general, we used satiety assays when looking for hypersexuality and standard courtship assays (below) for hyposexuality. In the satiety assay, individual

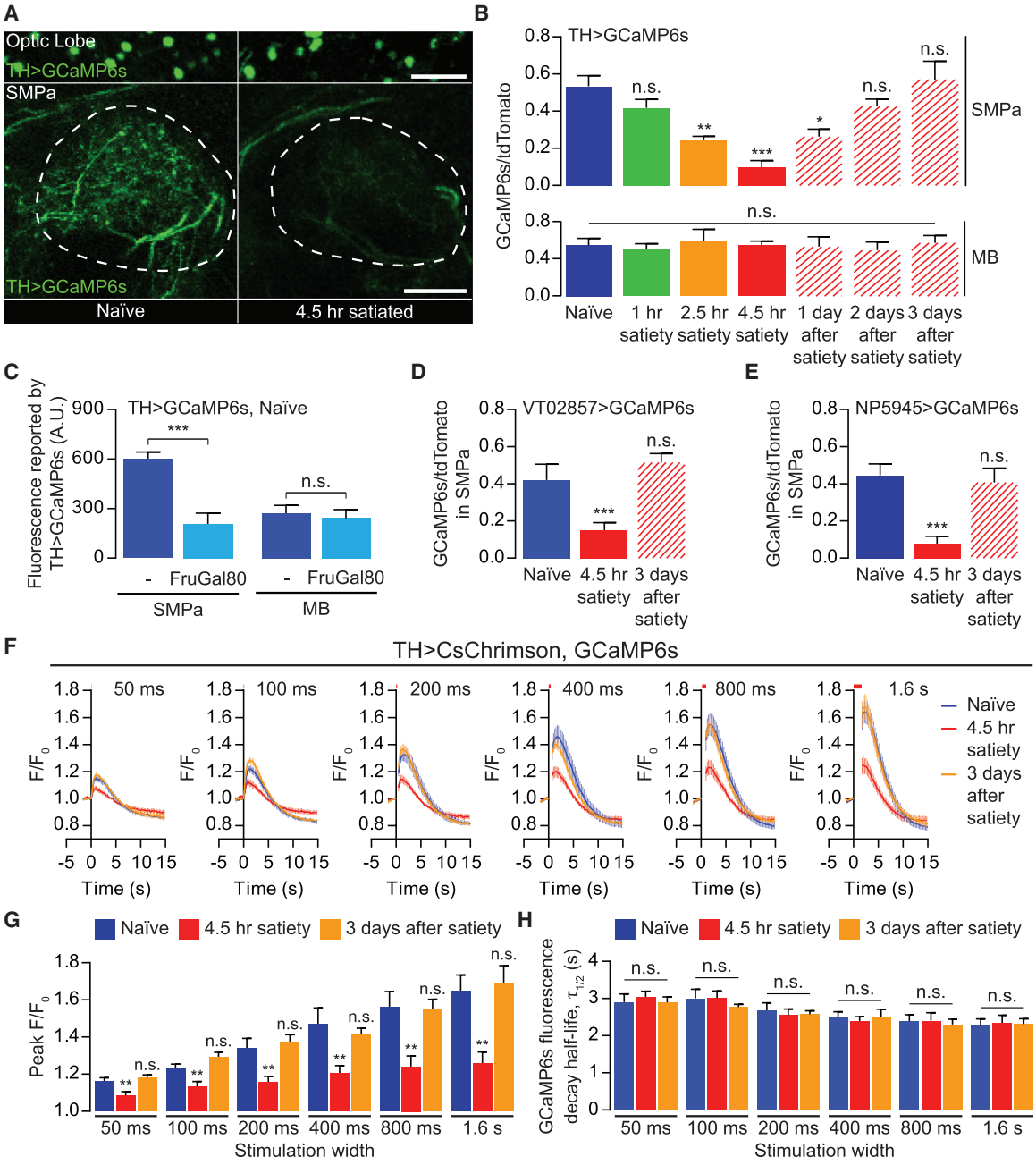

Figure 6. Dopaminergic Activity at the Sensory-Motor Interface Reflects Reproductive Capacity

(A) Representative images of fluorescence reported by GCaMP6s in the optic lobe (top) and the anterior superior medial protocerebrum (SMPa) (bottom) in 5- to 10-day-old males that were naive (left) or had just completed the satiety assay (right). SMPa (dashed outline) fluorescence decreases with satiety, while optic lobe fluorescence (from the same stack) is not noticeably changed.

(B) Top: average pixel fluorescence reported by TH > GCaMP6s (normalized to TH > tdTomato fluorescence) in SMPa in 3- to 8-day-old males decreases with time in the satiety assay and gradually recovers after isolation from females for 3 days. See Figure S7A for quantification of tdTomato fluorescence. Bottom: no changes were seen in the medial lobe of the mushroom body, a neighboring region innervated by dopaminergic neurons (one-way ANOVA, n = 13–16).

(C) FruGal80 blocks much of the TH > GCaMP6s fluorescence in the SMPa, but not in the mushroom body (t test, n = 8–10).

(D and E) Average tdTomato-normalized SMPa pixel fluorescence reported by VT02857 > GCaMP6s (5–10 days old) (D) and NP5945 > GCaMP6s (3–8 days old) (E) males decreases after the satiety assay and recovers after isolation from females for 3 days (one-way ANOVA, n = 8–10 for D and n = 11–20 for E).

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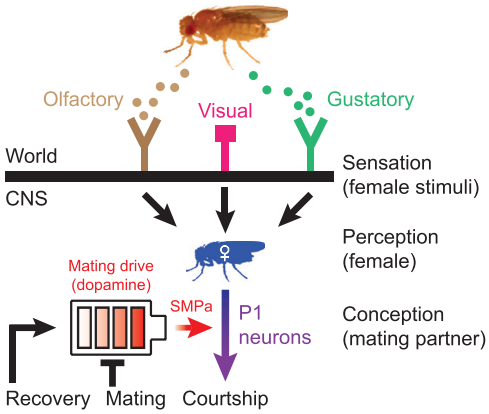

Figure 7. Dopaminergic Circuitry Underlying Mating Drive

male flies (3–6 days old unless otherwise stated) were placed with ~25 virgin females in a food vial at 23°C and ambient humidity for 4.5 hr. Mating behaviors (courtship and copulation) were scored manually every 30 min, in groups of 6–8 vials per genotype. A within-group percentage was generated for each time point. The first two and last two time points were averaged to generate Initial and End percentages, respectively. A satiety index was calculated for each group as (%Initial − %End)/%Initial. To generate separate satiety indices for copulation or courtship (Figures 1C, 1D, S2A, and S2B), percentages of copulating or courting but non-copulating flies were used, respectively. In single-versus-group rearing experiments (Figure S1B), males were aged either in isolation or groups of ~10 before the assay. In thermogenetic experiments, the temperature was raised after the last time point, and mating behaviors were scored 20 min after the incubator reached the appropriate temperature. In satiety assays involving artificial copulation termination, each mating pair was allowed to copulate for 5 min before being manually separated by repeatedly aspirating into and out of a pipette tip. As a handling control, naive males were aspirated similarly before scoring in Figures 2A and 2D.

Satiety Screen

The screen for genes and neurons that control satiety was initially a candidate screen, looking at mutations affecting reproductive function, memory, and sensory perception. In a second phase, we screened an RNAi collection targeted to all receptors in the *Drosophila* genome. In a third phase, we initiated an unbiased RNAi screen. The bulk of this screening was carried out in Leslie Vosshall's lab at Rockefeller University. After these screens failed to produce a hit that that prevented satiety, we screened biogenic amine pathways, discovering the TH > TrpA1 combination that gave a dramatic satiety reversal phenotype.

Courtship Assays

Courtship assays were carried out and videotaped in cylindrical courtship chambers (10 mm diameter and 3 mm height) at 23°C and ambient humidity. Unless otherwise stated, a male fly (3–6 days old) was paired with a w^{1118} virgin female fly in each chamber. The courtship index was manually scored as the fraction of time during which the male fly is engaged in mating behaviors (courtship and copulation) within 5 min once the male fly started courting. In this paper, we manually scored courtship assays to keep consistent with the courtship scoring in the satiety assays, whose 3D environment and large number of animals complicate automated scoring. A bout of courtship was scored as initiated when the male oriented toward the female, began tracking her, and unilaterally extended his wing to sing to her. A bout was scored as terminated if the male did not sing in the subsequent 30 s or turned away from the female. If the male fly did not court within the first 15 min of each assay, the courtship index was scored as 0. Using these criteria, two experimenters (M.A.C. and S.X.Z.) routinely gave near-identical courtship indices in pilot assays. The same criteria were applied when producing the ethograms in Figure 1G, where percent time singing during courtship was calculated as time_spent_singing/total_time_spent_courting × 100%. In thermogenetic experiments, the courtship assays were performed first at 23°C and then again at the higher temperature. For experiments involving tubGal80^{ts} (Figures 3D and 3H), male flies were moved to 30°C after eclosion (Figure 3D) or 1 day after puparium formation (Figure 3H) and kept there (isolated from females) until just before the assay, which took place at 23°C. For experiments involving DopR2 mutants or RNAi knockdown, only 3-day-old males were used to avoid the confounding effects of age. The *DopR2^{attp}* animals had previously been introgressed into a wild-type Canton-S background (Keleman et al., 2012), which served as the wild-type background control in our behavioral experiments. To further ensure background consistency, we re-crossed the mutant into the Canton-S background three more times, with no effect on courtship. We therefore combined the courtship data from before and after the second introgression.

Courtship Assays with Abdomen Removal

Canton-S male flies were used for this experiment. For each group, experimental (abdomen removed) and control (intact) flies were collected and aged together. Mature naive males were collected 0–3 days after eclosion and group housed without females for 3 days before the surgery. Mature satiated males were collected and aged in the same way as the mature naive males, but were run through the satiety assay before surgery. For the surgery, flies were anesthetized by incubating on ice, and the posterior 60%–80% of the abdomens was surgically removed with forceps. After the flies were allowed to recover in food vials for ~20 min, the vials were quickly inverted, and only flies that could climb up the vial within 5 s were used for courtship assays. Courtship assays were conducted as above, with the exception that two virgin females were used per male. Experimental males were dissected post hoc to ensure that all reproductive organs were removed during the surgery.

Rubber Band Courtship Assays

Rubber band courtship assays were carried out and scored as above, except that they took place in larger cylindrical courtship chambers (30 mm diameter and 3 mm height). The rubber bands (OfficeMax #OM97346) were cut into chunks of ~1 mm^3. The "tan" rubber bands were not painted; the "black" rubber bands were colored black by a black felt-tip Sharpie and left to dry for at least 1 day before use.

Fertility Assays

Canton-S males (3–6 days old), half of which had just completed the satiety assay, were individually paired with virgin females at 23°C and ambient humidity. Immediately after copulation, the females were individually isolated in food vials at 25°C. After 10 days, the females were removed from the vials and the adult progeny were scored 10 days later.

Locomotion Assays

Locomotion assays were carried out in the cylindrical chambers (30 mm diameter and 3 mm height) at 23°C and ambient humidity. Canton-S males (3–6 days old), half of which had just completed the satiety assay, were individually gently aspirated into the chambers and allowed to explore the chambers for 1 min before their locomotion was recorded with a standard video camera at 30 fps for 10 min. Video segmentation and tracking were carried out using

(F) Calcium transients (measured using TH > GCaMP6s) in dopaminergic projections in the SMPa after optogenetic stimulation (TH > CsChrimson) using light pulses between 50 and 1,600 ms long (see Experimental Procedures for details). Different colors correspond to the satiety states of the flies prior to dissection (n = 6–9 flies per satiety state for all pulse widths).

(G) CsChrimson-induced GCaMP6s transients peak lower in satiated brains (red) than in naive ones (blue), or in brains tested 3 days after the satiety assay (orange) (one-way ANOVA, n = 6–9).

(H) The decay kinetics of CsChrimson-induced GCaMP6s transients are unchanged by the satiety state of the animal (one-way ANOVA, n = 6–9).

FIJI (Schindelin et al., 2012) with the MTrack2 plugin (http://valelab.ucsf.edu/~nstuurman/ijplugins/MTrack2.html).

Ejaculatory Bulb Volume Scoring

Male flies were fixed in PBS solution with 0.3% Triton X-100 (PBST) and 4% paraformaldehyde at room temperature for 1 hr. Ejaculatory bulbs were dissected out, washed with PBST, and mounted on slides using standard procedures. Bulbs were imaged and scored blind to treatment on a scale from 1 (empty) to 4 (full).

Thermogenetic Bulb Depletion

Male crz > TrpA1 flies were collected 0–3 days after eclosion and group housed without females for 3 days at 23°C and ambient humidity. A subset of these males was run through the satiety assay. Ejaculatory bulbs were then depleted by raising the temperature to 30°C for 3 days. Control crz > TrpA1 males were housed at 23°C during the same time period.

Immunostaining and Microscopy

Fly brains were removed in PBS or Schneider's medium (ThermoFisher, 21720-024) and immediately fixed in PBST and 4% paraformaldehyde at room temperature for 20 min. After washing with PBST three times (20 min each), the brains were incubated with primary antibodies (diluted in PBST) at 4°C for 48 hr. Following a PBST wash (three times for 20 min), the brains were incubated with secondary antibodies (diluted in PBST) at 4°C for 48 hr. After three more PBST washes (20 min each), the brains were mounted with VectaShield (Vector Labs) on glass slides using standard procedures. Confocal sections were acquired using an Olympus Fluoview 1000 microscope or an Olympus Fluoview 1200 microscope at 3–5 μm intervals, and maximum projections of image stacks were obtained in FIJI (Schindelin et al., 2012).

Calcium Imaging of Dissected Brains

Fly brains were dissected from 3- to 8-day-old males in external saline (Wilson and Laurent, 2005) and mounted, anterior side down, onto the base of a glass-bottom petri dish (MatTek, P35G-1.5-20-C) housing 4 mL static external saline. To stabilize the samples, optic lobes were adhered to the coverslip with UV glue (Kemxert, KOA 300) solidified with a 5 mw purple laser pointer (Oxlasers, OX-B005). To image the SMPa, four to seven confocal sections spanning this region were acquired using an Olympus Fluoview 1000 microscope at 3 μm intervals, and maximum projections of image stacks were obtained in FIJI (Schindelin et al., 2012). After one of us (S.X.Z.) took the images, another of us (D.R., blind to the treatment) selected regions of interest (ROIs) of ipsilateral SMPa, mushroom body medial lobe, and background (near the antennal lobe, where dopamine projections are sparse) using the tdTomato channel (Figures 6B, 6D, and 6E) or the GCaMP6s channel (Figure 6C). ROI sizes were kept roughly the same between samples. Average pixel fluorescence in each ROI was calculated with a custom-written program MATLAB (Mathworks). Normalized fluorescence was calculated as (GCaMP6s_SMPa − GcaMP6s_background)/(tdTomato_SMPa − tdTomato_background). The same formula was used for the mushroom body medial lobe as well. The program is available upon request.

Two-Photon Calcium Imaging with CsChrimson Stimulation

Two-photon laser-scanning microscopy was performed using a custom microscope as previously described (Carter and Sabatini, 2004). Shortly after eclosion, flies were transferred to vials containing rehydrated potato flakes (Carolina Biological Supply, 173200) and 100 μL of all-trans-retinal stock solution (Sigma, R2500; 35 mM in ethanol). All experiments were conducted with adult male flies within 3–6 days after eclosion. Prior to imaging, fly brains were carefully dissected under low-light conditions and mounted, posterior side down, onto a coated petri dish (Thermo Scientific, 150318) containing 3 mL HL3.1 saline solution (Feng et al., 2004). GCaMP6s was excited at 920 nm and 11.8 mW at the sample, and light was collected in 128 × 128 frames at 256 ms per frame. A basal level of retinal-dependent CsChrimson activation was observed at 920 nm (and at all wavelengths tried between 860 and 940 nm) that typically plateaued within the first 20 frames (~5 s). At frame #24, a pulse of red light (655 nm) was delivered using an LED (Luxeon Star LEDs, LXM3-PD01-0350), which was driven by a 720 mW BuckPuck (Luxdrive, 3021-D-E-700), and collimated with a lens (Carclo, 13193) ~3 cm away from the sample. No fluorescence data were collected during stimulation, in order to protect the photomultiplier tube. For each experimental series, light pulses of different widths were delivered in increasing, decreasing, or random order. Fluorescence data were analyzed in MATLAB using a semi-automated program modified from a published independent component analysis algorithm (Mukamel et al., 2009). Decay half-life was estimated by fitting the fluorescence decay data with a single-exponential function. All time constants measured in Figure 6H are longer than that of GCaMP6s itself (<1.2 s in *Drosophila*) (Chen et al., 2013). The program is available upon request.

Antibodies

Primary antibodies were chicken anti-GFP (CAT# GFP-1010, Aves Labs, 1:1,000 dilution), rabbit anti-GFP (CAT# A-11122, Invitrogen, 1:1,000 dilution), mouse anti-tyrosine hydroxylase (TH) (CAT# 22941, ImmunoStar, 1:1,000 dilution), and rabbit anti-fru^M "male2" (from Daisuke Yamamoto, 1:500 dilution). For GRASP experiments, mouse anti-GFP clone 3E6 (CAT# A11120, Invitrogen, 1:2,000 dilution) primary antibody was used instead. Secondary fluorescent antibodies were Alexa Fluor 488 donkey anti-chicken (CAT# 703-545-155, Jackson ImmunoResearch, 1:400), Alexa Fluor 488 goat anti-rabbit (CAT# A11008, Invitrogen, 1:400 dilution), Alexa Fluor 488 donkey anti-mouse (CAT# A21202, Invitrogen, 1:400 dilution), and Cy3 donkey anti-mouse (CAT# 715-166-150, Jackson ImmunoResearch, 1:400 dilution).

Statistical Tests

All statistical tests were performed using Graphpad Prism 6. In all one-way ANOVA tests, statistical significance was corrected for multiple comparisons using post hoc Tukey's range tests. All two-way ANOVA tests use post hoc Bonferroni corrections. In all figures, error bars represent SEM. In all figures, statistical significance is denoted as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; n.s., not significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2016.05.020.

AUTHOR CONTRIBUTIONS

S.X.Z. and M.A.C. performed the experiments. All authors designed the experiments, analyzed the data, and wrote the paper.

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