Abdominal-B Neurons Control *Drosophila* Virgin Female Receptivity

Jennifer J. Bussell[1], **Nilay Yapici**[1], **Stephen X. Zhang**[1,†], **Barry J. Dickson**[2], and **Leslie B. Vosshall**[1,3,*]

[1]Laboratory of Neurogenetics and Behavior, The Rockefeller University, 1230 York Avenue, Box 63, New York, NY 10065 USA

[2]Janelia Farm Research Campus, Howard Hughes Medical Institute, 19700 Helix Drive, Ashburn, VA 20147 USA

[3]Howard Hughes Medical Institute

SUMMARY

BACKGROUND—Female sexual receptivity offers an excellent model for complex behavioral decisions. The female must parse her own reproductive state, the external environment, and male sensory cues to decide whether to copulate. In the fly *Drosophila melanogaster*, virgin female receptivity has received relatively little attention, and its neural circuitry and individual behavioral components remain unmapped. Using a genome-wide neuronal RNAi screen, we identify a subpopulation of neurons responsible for pausing, a novel behavioral aspect of virgin female receptivity characterized in this study.

RESULTS—We show that Abdominal-B (Abd-B), a homeobox transcription factor, is required in developing neurons for high levels of virgin female receptivity. Silencing adult Abd-B neurons significantly decreased receptivity. We characterize two components of receptivity that are elicited in sexually mature females by male courtship: pausing and vaginal plate opening. Silencing Abd-B neurons decreased pausing, but did not affect vaginal plate opening, demonstrating that these two components of female sexual behavior are functionally separable. Synthetic activation of Abd-B neurons increased pausing, but male courtship song alone was not sufficient to elicit this behavior.

CONCLUSIONS—Our results provide an entry point to the neural circuit controlling virgin female receptivity. The female integrates multiple sensory cues from the male to execute discrete
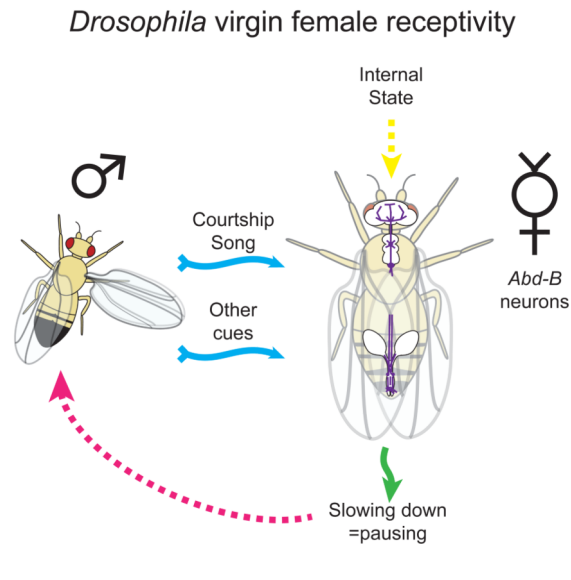
*Correspondence to: leslie.vosshall@rockefeller.edu.
†Current address: Department of Neurobiology, Harvard Medical School, 220 Longwood Avenue, Boston, MA 02115 USA

motor programs prior to copulation. Abd-B neurons control pausing, a key aspect of female sexual receptivity, in response to male courtship.

Graphical Abstract

Drosophila virgin female receptivity

INTRODUCTION

To choose their mates, male and female vinegar flies (*Drosophila melanogaster*) perform a duet of sexually dimorphic courtship behaviors that offers an excellent model for studying the neural circuitry of innate social behavior [1]. Male courtship is composed of a series of discrete and stereotyped motor programs such as following the female, producing courtship song using a single extended wing, tapping and licking her genitals, and finally, copulating [1]. The neural circuitry underlying several of these behavioral components has been identified and dissected [2]. In contrast, very little is known about how the female evaluates male courtship to decide whether to mate and how she executes that decision.

A female fly that engages in copulation is considered receptive to male courtship. Receptivity comprises the relative absence of obvious rejection behavior, slowing down to allow the male to initiate copulation, and opening cuticular vaginal plates to allow access to the genitalia [3]. However, prior work on receptivity measured only mating rate, a metric that provides little insight into the discrete motor programs females display in the context of courtship. Although classical genetic studies identified a region of the female brain [4] and genes required for receptivity [5-10], neurons with a clearly defined function specific to female behavior in courtship and mating have yet to be described.

Much of the effort to understand female receptivity has focused on its regulation. Female sexual maturity develops over the first few days after eclosion [11], and sexually immature adult females reject male courtship by running or jumping away and kicking and fluttering their wings [12]. After mating, female *Drosophila* temporarily switch into an unreceptive state in which they extrude the ovipositor to reject male courtship [12]. They also increase egg laying. This post-mating response is triggered by Sex Peptide, which activates the Sex

Peptide Receptor (*SPR*) [13] in a subset of female reproductive tract sensory neurons labeled by *pickpocket* (*ppk*), *fruitless* (*fru*), and *doublesex* (*dsx*) [14-16]. While recent work has continued to identify neurons that function to co-ordinately regulate receptivity and egg-laying [14, 17], neurons with specific functions restricted to virgin mating behavior are still unknown.

Males present an array of sensory cues during courtship, including contact and volatile pheromones and courtship song. It is not known which sensory signals trigger individual components of receptivity and whether these components are coordinately or independently controlled by female neural circuitry. Song seems critical to the courtship process since males muted by having their wings removed have dramatically reduced mating success [18]. However, it remains unclear to what degree females integrate sensory input from courtship song with other male courtship cues and how song affects their behavior [19-21].

To expand the mechanistic understanding of *Drosophila* virgin female receptivity, we set out to characterize its component motor programs and identify neurons directly involved in this behavior. Using a genome-wide neuronal RNA interference (RNAi) screen, we discovered a requirement for the homeobox (Hox) transcription factor *Abd-B* in developing neurons for female receptivity. Adult *Abd-BGal4* neurons located in the abdominal ganglion and reproductive tract project locally within these areas and to higher brain centers. These neurons are functionally required for female receptivity and act to induce pausing in response to multiple male courtship cues. In addition to demonstrating a role for *Abd-B* in sex-specific behavior, our results identify a neuronal subset responsible for a discrete component of virgin female receptivity.

RESULTS

An RNAi screen identifies genes required in neurons for female reproductive behaviors

We reasoned that genes and neural circuitry required for virgin female receptivity could be identified within hits of a genome-wide RNAi screen previously carried out to identify defects in female reproductive behaviors [13] (Figure S1). In this neuron-specific screen of the Vienna *Drosophila* RNAi library [22], males and females with pan-neuronal expression of each RNAi hairpin and Dicer2 to increase RNAi efficacy were allowed to mate, and egg-laying of female progeny was scored across 3 days (Figure S1A). Lines showing reduced egg-laying (Figure S1B) were re-screened twice to confirm the phenotype, and 28 candidates were pursued further.

Reduced egg-laying in these strains could have been caused by deficits in female receptivity, the female post-mating response, female fertility, or male mating success or fertility. To distinguish among these possible phenotypes, secondary assays for female receptivity, egg-laying, and remating were carried out (Figures S1C-1I). Of the 28 tested candidates, 10 showed decreased virgin female receptivity (Figure S1G), 10 showed decreased egg-laying without affecting receptivity (Figure S1H), and 3 were defective in post-mating responses and showed both decreased egg-laying and increased remating (Figures S1H and S1I). Among the latter group was *SPR* [13]. 5 candidates did not show a phenotype in secondary

assays (*CG13243*, *mad2*, *sec15*, *Rack1*, and *CG12338*), perhaps because they affected male mating success or fertility

Abd-B is required in neurons for female receptivity

Seven of 10 receptivity hits from Figure S1G showed a reduction in virgin female receptivity when rescreened in an assay pairing 1 virgin RNAi female with 2 wild-type males in a standard fly food vial for 1 h (Figures 1A and 1B). Of these, we chose the Hox transcription factor *Abd-B* for further analysis because it has well-studied functions in specifying cell identity [23].

A second RNAi hairpin targeting *Abd-B* and a second pan-neuronal driver, *neuronal synaptobrevin (nsyb)-Gal4,* both showed decreases in receptivity (Figures 1C and 1D). Anti-Abd-B antibody staining [24] revealed many *Abd-B*-expressing neurons within the adult female abdominal ganglion of the ventral nerve cord (Figures 1E and 1F) and a smaller number of neurons within the reproductive tract (data not shown). *Abd-B* RNAi strongly reduced *Abd-B* immunofluorescence signal in the abdominal ganglion, in both unreceptive virgin females and the small fraction that mated (Figure 1G).

To assess if *Abd-B* affects the development of the female receptivity circuit or is required for neuronal function in the adult, we temporally restricted *Abd-B* RNAi either to pre-adult stages or the adult using *Gal80^{ts}*, a temperature-sensitive repressor of *Gal4* [25] (Figure S2). *Abd-B* RNAi active only during development caused a reduction in receptivity (Figures 1H and 1I), while RNAi active only in the adult showed no effect on receptivity (Figure 1I). We conclude that *Abd-B* plays a developmental role in forming the female receptivity circuit.

Neuronal knockdown of *Abd-B* does not induce the post-mating response

We wished to learn if the decreased copulation success of *Abd-B* RNAi virgin females was because they were unattractive to males or because they were prematurely switched into the unreceptive post-mating state. Virgin *Abd-B* RNAi females tested in 1-cm plastic chambers with single wild-type males were as attractive to males as parental controls (Figures 2A and 2B). Mated, but not virgin, *Abd-B* RNAi females showed normal ovipositor extrusion (Figures 2C and 2D) and egg-laying (Figures 2E and 2F), and did not remate (data not shown). We conclude that the role of *Abd-B* in receptivity is independent from the post-mating response.

Abd-B receptivity neurons reside in the abdominal ganglion and reproductive tract

We accessed *Abd-B*-expressing neurons genetically with *Abd-B^{LDN}*, a *Gal4* insertion in the tethering element of the *Abd-B* promoter [26]. *Abd-B^{LDN}-Gal4* labeled 283 ± 10 (mean ± SEM, n = 3) cells in the adult female abdominal ganglion that were co-labeled with antibody to Abd-B protein as well as a small number of co-labeled cells in the reproductive tract (Figure 3A and data not shown).

To determine whether *Abd-B* RNAi restricted to *Abd-B^{LDN}-Gal4* positive neurons would yield receptivity defects, we limited *Abd-B^{LDN}>Abd-B* RNAi to neurons using the *nsyb* promoter. Briefly, we used the *lexA-lexAop* system [27] to express FLP recombinase in

neurons under the control of the *nsyb* promoter and "flipped-out" ubiquitous *Gal80* [28] to relieve repression of *Gal4* only in neurons (see Supplemental Experimental Procedures for complete genotypes). Virgin females with *Abd-B* RNAi in *Abd-B^{LDN}-Gal4* neurons showed reduced receptivity (Figure 3B). Thus, *Abd-B^{LDN}-Gal4* labels a subset of *Abd-B* neurons important for female receptivity. Due to the expression of *Abd-B^{LDN}-Gal4* in non-neuronal tissue [26], using *Abd-B^{LDN}-Gal4* to drive *Abd-B* RNAi without this neuronal restriction produced females with malformed genitalia that were unable to copulate (data not shown).

To describe the anatomy of the *Abd-B* receptivity neurons, we examined the expression of nuclear (Figures 3C-3H) and membrane-bound (Figures 3I-3N) green fluorescent protein (GFP) driven by *Abd-B^{LDN}-Gal4*. *Abd-B^{LDN}-Gal4* showed restricted labeling of 280 ± 5 (mean $\pm$ SEM, n = 4) neurons in the abdominal ganglion and a small number of neurons within the reproductive tract and along the vaginal plates, as well as non-neuronal cells in the reproductive tract (Figures 3F-3H and arrowheads in Figures 3G and 3H). We did not observe any *Abd-B^{LDN}* neuronal cell bodies in the brain (Figure 3C).

Abd-B^{LDN} neurons project to several higher brain areas including the subesophageal zone, the ventrolateral neuropils, and the superior neuropils (Figure 3I), with extensive processes both within the abdominal ganglion and throughout the ventral nerve cord (Figures 3J and 3K). Within the female reproductive tract and terminalia, *Abd-B^{LDN}-Gal4*-labeled tissue and neuronal processes innervating the oviducts, uterus, muscles near the vaginal plates, and the vaginal bristles (Figures 3L-3N).

We used *Abd-B^{LDN}*-driven expression of GFP fused to *nsyb* (Figures 3O-3T) and *Dscam* (Figures 3U-3Z), enriched in axons and dendrites, respectively [29, 30], to examine the polarity of *Abd-B^{LDN}* neurons. Extensive *nsyb-GFP* labeling was found throughout the abdominal ganglion and ventral nerve cord, as well as in the subesophageal zone, ventrolateral neuropils, and superior neuropils in the brain (Figures 3O-3Q) and in the reproductive tract, particularly along muscle fibers, including those near the vaginal plates (Figures 3R-3T). *Dscam-GFP* labeling was absent in the brain but abundant in the abdominal ganglion of the ventral nerve cord (Figures 3U-3W). In the reproductive tract and terminalia, *Dscam-GFP* labeling was sparse but present along uterine and vaginal tissues (Figures 3X-3Z). Our interpretation of these staining patterns is that abdominal ganglion *Abd-B^{LDN}* neurons ascend to terminate in the ventral nerve cord and brain, with dendritic labeling enriched within the abdominal ganglion. They may also project axons within the abdominal ganglion itself and descend to innervate targets in the reproductive tract. Although *Abd-B^{LDN}* neuronal cell bodies reside within the reproductive tract, it was not possible to establish the polarity of projections of these neurons with these methods. We did not observe any differences in the projections of *Abd-B^{LDN}* neurons in the brain, ventral nerve cord, abdominal ganglion, or reproductive tract between 1-day-old immature virgin females and 4-day-old mature virgin females (Figure S3).

A subset of *Abd-B* neurons is functionally required for virgin female receptivity

Having established that *Abd-B* has a role in the development of the receptivity neural circuit, we asked whether *Abd-B^{LDN}* neurons are functionally important for receptivity in adult females. We used *UAS-shi^{ts}*, a dominant-negative variant of dynamin that transiently blocks

chemical synaptic transmission at temperatures above 29°C [31], to silence $Abd-B^{LDN}$ neurons during courtship. At the restrictive temperature, there was a selective decrease in receptivity only in animals carrying both $Abd-B^{LDN}$ and shi^{ts} (Figure 4A). In females mated at the permissive temperature, acutely silencing $Abd-B^{LDN}$ neurons 48 hr later modestly increased remating (Figure 4B), but not to the ~75% level found in mated *SPR* RNAi females (Figure S1I), which are deficient in the post-mating response [13]. We used a second method of neuronal inactivation, *UAS-kir2.1* [32], to hyperpolarize $Abd-B^{LDN}$ neurons chronically and again found decreased virgin receptivity (Figure 4C). $Abd-B^{LDN}$ neurons are therefore functionally required for virgin female receptivity.

We carried out a series of experiments to restrict the expression of $Abd-B^{LDN}$ *Gal4* to a smaller subset of neurons that still decreased virgin receptivity using silencing with *kir2.1*. To confirm that $Abd-B^{LDN}>kir2.1$ is required in neurons for the receptivity phenotype, we used *elav-Gal80* [15] to suppress $Abd-B^{LDN}$-*Gal4* in neurons (Figure S4A) and rescued receptivity as expected (Figure 4C). *teashirt (tsh)-Gal80* is expressed in a large subset of ventral nerve cord cells [33] and suppressed *Gal4* expression in approximately half of the $Abd-B^{LDN}$ neurons, leaving 142 ± 2 (mean ± SEM, n = 4) neurons in the abdominal ganglion as well as those in the reproductive tract (Figure S4B and S4C). Silencing only this subset of $Abd-B^{LDN}$ neurons in the presence of *tsh-Gal80* was sufficient to reduce virgin female receptivity (Figure 4C).

Projections of $Abd-B^{LDN}$ neurons are found near the ovipositor, uterus, and vaginal plates. To ask if $Abd-B^{LDN}$ receptivity defects were due to function in descending motorneurons, we created a *Gal80* line using the *Drosophila* vesicular glutamate transporter (*VGlut*) promoter [34] to suppress $Abd-B^{LDN}$-*Gal4* expression in motorneurons. *VGlut-Gal80* removed the muscle-innervating $Abd-B^{LDN}$-*Gal4* projections in the female reproductive tract (Figures S4D and S4E) but receptivity remained strongly impaired in this strain, suggesting that motorneurons are not major contributors to the receptivity phenotype (Figure 4C).

We next asked if *ppk* sensory neurons in the reproductive tract involved in post-mating female behaviors [15, 16] contributed to our receptivity phenotype. Using *ppk-Gal80* [15, 16] (Figures S4F and S4G) we found no effect on the reduction in virgin female receptivity with $Abd-B^{LDN}>kir2.1$ (Figure 4C). Although these data suggest that neither motorneurons nor the *ppk*-expressing sensory neurons are major contributors to the receptivity phenotype, we cannot rule out a contribution from non-*ppk*-positive and non-*VGlut*-positive neurons in the reproductive tract and genitalia.

In addition to the *ppk* neurons, neurons labeled by *dsx-Gal4* have been shown to play a role in female mating behavior [35]. Specifically, silencing a subset of *dsx-Gal4* neurons in the abdominal ganglion by intersection with an enhancer-trap *FLP* recombinase line (Et^{FLP250}) blocks the post-mating response and increases mated female receptivity [14]. Since this neuronal subset is the intersection of Et^{FLP250} and *dsx-Gal4*, if it contributes to the $Abd-B^{LDN}$ receptivity phenotype, Et^{FLP250} should intersect a subset of $Abd-B^{LDN}$ neurons functionally important for virgin female receptivity. We therefore carried out intersectional neuronal silencing experiments using *UAS-FRT-STOP-FRT-kir2.1*. In control experiments, we showed that intersectional silencing of all $Abd-B^{LDN}$ neurons using *nsyb-lexA, lexAop-*

FLP (*neuronal FLP*) reproduced the receptivity phenotype (Figure 4D). While Et^{FLP250} does intersect a population of $Abd\text{-}B^{LDN}$ neurons (Figures 4E-4J), silencing this subset had no effect on virgin female receptivity (Figure 4D). Thus $Abd\text{-}B^{LDN}$ receptivity neurons comprise neither of the previously described *ppk* or $dsx \cap Et^{FLP250}$ neuronal subsets contributing to female-specific behaviors.

Given the central role of *fruitless*-labeled neurons in *Drosophila* courtship behavior [2] and the fact that silencing *fru-Gal4*-labeled neurons in females decreases receptivity [36], we tested whether $Abd\text{-}B^{LDN}\text{-}Gal4$ expression overlaps with *fru*. We intersected $Abd\text{-}B^{LDN}\text{-}Gal4$ with *fru* neurons using *fru-FLP* [2], and although there is a *fru* subset of $Abd\text{-}B^{LDN}$ neurons (Figures 4K-4P), this subset does not contribute to the $Abd\text{-}B^{LDN}$ receptivity phenotype (Figure 4D).

We also asked whether $Abd\text{-}B^{LDN}$ neurons play a role in male sexual behavior. $Abd\text{-}B^{LDN}\text{-}Gal4$ labels neurons in the male ventral nerve cord as well as neurons in the male reproductive tract (Figures S5A-S5J). However, these neurons are not functionally required for male courtship or copulation (Figure S5K and data not shown). Males have approximately the same number of $Abd\text{-}B^{LDN}$ ventral nerve cord neurons as females (280 ± 2, mean ± SEM, n = 3), but there is increased labeling in the flange within the subesophageal zone and the lateral protocerebrum in males and decreased labeling of projections in the superior medial protocerebrum (arrowheads in Figure S5A) relative to females.

Silencing *Abd-B* neurons decreases pausing during courtship

To determine the specific role of $Abd\text{-}B^{LDN}$ neurons in female receptivity, we examined the behavior of females with silenced $Abd\text{-}B^{LDN}$ neurons during courtship. We compared sexually mature virgin females to immature 1-day-old females, which are reported to run and jump away to avoid male courtship [12]. We first quantified two abdominal behaviors: vaginal plate opening and ovipositor extrusion (Figures S6C-S6F and Movie S1). While sexually immature virgin females did not open the vaginal plates, mature virgin females periodically opened their vaginal plates during courtship (Figure S6D). The transition to intermittent vaginal plate opening during courtship was intact in virgin females with silenced $Abd\text{-}B^{LDN}$ neurons, and we conclude that $Abd\text{-}B^{LDN}$ neurons are not functionally required for vaginal plate opening. Neither immature 1-day-old nor mature 4-day-old $Abd\text{-}B^{LDN}>kir2.1$ females showed significant ovipositor extrusion (Figure S6F).

To quantify slowing down during courtship, we tracked the movement of pairs of male and female flies using Ctrax software [37] (Figure 5A) in a large arena in which $Abd\text{-}B^{LDN}>kir2.1$ females also showed a strong receptivity defect (Figure 5B). Female walking speed during courtship did not differ with sexual maturity (Figure S6I), suggesting that receptive females do not generally slow their movement during courtship. Mature virgin $Abd\text{-}B^{LDN}>kir2.1$ females walked at the same speed as control females during courtship (n.s., not significant, one-way ANOVA with Tukey correction) (Figure S6I).

Instead, we identified periods in which the female "paused" during courtship (Figures 5C and 5D, Movie S2). The percent of time spent pausing was nearly doubled in control

receptive virgin females compared to unreceptive immature virgins (Figure 5D). Mature virgins paused more when being courted by a male than during periods when the male was present but not courting (Figure 5E), and they paused more during courtship than when in the arena alone (Figure 5F). Taken together, these data suggest that increased pausing is a specific hallmark of female receptivity and not a more generalized developmental motor change. Mature virgin Abd-B^{LDN}>$kir2.1$ females paused very little and were indistinguishable in this response from immature virgins (Figure 5D). Thus, Abd-B^{LDN} neurons are functionally required for the pausing component of receptivity.

Despite the decreased pausing of Abd-B^{LDN}>$kir2.1$ females, males attempted copulation as much with Abd-B^{LDN}>$kir2.1$ females as parental controls (Figures S6A and S6B) and displayed high levels of courtship as measured by courtship index (Figures S6G and S6H). Additionally, Abd-B^{LDN}>$kir2.1$ females did not run away from courting males: the distance between these females and courting males was the same as parental controls (Figures S6J).

Pausing is a response to multiple male courtship cues

To understand more about the regulation of female pausing by male sensory cues, we performed a cross-correlation analysis of female pausing during courtship and male touch and wing extension, which served as a proxy for courtship song (Figure 6A). Both of these behaviors were weakly correlated with female pausing. The normalized cross-correlation of wing extension was stronger than that of touch and was centered at zero time shift, suggesting that courtship song might provide sensory input to the neuronal circuit controlling female pausing.

To test this, we set up an assay to play recorded courtship song while tracking fly movement (Figure 6B). In control experiments, we determined that white noise sound playback did not affect female receptivity and that muting males by removing their wings decreased female receptivity (Figure 6C). As previously reported [38], playback of recorded courtship song rescued female receptivity with mute males (Figure 6C).

We next asked whether female pausing correlated with the effect of courtship song on receptivity and found that pausing was decreased when mute males were paired with white noise (Figure 6D). Pausing was rescued by playback of song during courtship with a mute male (Figure 6D). However, playback of recorded courtship song to females without a male present was not sufficient to increase pausing whether or not their Abd-B^{LDN} neurons were silenced (Figure 6E). From these data, we conclude that pausing requires the integration of song with other male sensory cues during courtship.

Activating *Abd-B* neurons induces pausing

To determine whether the activity of Abd-B^{LDN} neurons induces pausing, we synthetically activated them by expressing *Drosophila TrpA1*, a heat-activated non-selective cation channel [39]. Isolated females were assayed in our tracking arena (schematized in Figure 7A) at control temperatures or elevated temperatures that activated *TrpA1*. Although the higher temperature itself decreased pausing in control animals (p<0.001, Student's t-test, n=8-10), activation of Abd-B^{LDN} neurons counteracted this effect (Figure 7B). *TrpA1*

activation of $Abd\text{-}B^{LDN}$ neurons also increased pausing relative to the elevated-temperature control in the context of courtship with a wild-type male (Figures 7C and 7D). Thus, activation of $Abd\text{-}B^{LDN}$ neurons is sufficient to increase pausing in both the presence and absence of male courtship. However, females with $Abd\text{-}B^{LDN}>TrpA1$ activation did not copulate (Figures S7A and S7B), perhaps because this manipulation caused the vaginal plates to be locked in a spread open position and unable to open and close (Figures S7C and S7D and Movie S1). Although $Abd\text{-}B^{LDN}$ neurons are not required for vaginal plate opening during courtship, there is at least a subset of $Abd\text{-}B^{LDN}$ neurons that can affect the movement of the vaginal plates.

We sought to determine whether activation of $Abd\text{-}B^{LDN}$ neurons could compensate for the lack of song during courtship with a mute male (Figure 7E and 7G). $TrpA1$ activation had no effect on female walking speed (Figures 7H and 7I) and did not render the animals stationary, suggesting that $Abd\text{-}B^{LDN}$ neurons act within a receptivity pausing circuit rather than a more general locomotion control pathway. Activating $Abd\text{-}B^{LDN}$ neurons was sufficient to increase pausing during courtship with a mute male (Figures 7J and 7K). Thus, we conclude that $Abd\text{-}B^{LDN}$ neurons are both necessary and sufficient for the female pausing response to male courtship.

DISCUSSION

Female receptivity is a complex behavior comprising multiple motor programs and requiring the integration of sensory cues across several modalities. Nevertheless, *Drosophila* mating behavior is innate, and receptivity is likely controlled by hardwired neural circuits. We identified 7 candidate genetic markers of receptivity neurons using a neuronal RNAi screen. Our data suggest a central role for one of these, the transcription factor *Abd-B*, in forming a neural circuit that functions in receptivity.

We have refined the behavioral components of receptivity beyond mere copulation acceptance. We show that vaginal plate opening occurs throughout courtship and depends on sexual maturity. We also attribute the historically noted slowing down of receptive females to punctuated bouts of pausing during courtship rather than decreased walking speed. Pausing behavior is specific to female receptivity: it is decreased in both unreceptive females and in mature virgin females not being actively courted by a male. The increased level of pausing associated with receptivity requires the integration of multiple sensory inputs, including song, from a courting male. $Abd\text{-}B^{LDN}$ neuronal activity is both necessary for this pausing response and sufficient to induce it, thus establishing direct function of these neurons within the receptivity circuit.

How do *Abd-B* neurons control pausing? The $Abd\text{-}B^{LDN}$ neurons important for receptivity are not themselves motor neurons, and females with silenced $Abd\text{-}B^{LDN}$ neurons are not generally deficient in movement or posture. This suggests that $Abd\text{-}B^{LDN}$ neurons play a role downstream of the sensation of individual male courtship sensory inputs but upstream of motor output. The abdominal ganglion is emerging as a potential locus coordinating female-specific behavior [14, 40, 41], and $Abd\text{-}B^{LDN}$ neurons there are well-positioned to interact with other neurons involved in female behavior, including the post-mating response. These

neurons could therefore potentially function to integrate male courtship cues and internal inputs and promote pausing.

Silencing *Abd-B^{LDN}* neurons affects pausing but not vaginal plate opening, which demonstrates that it is possible to uncouple these two aspects of receptivity. However, activation of *Abd-B^{LDN}* neurons affects both pausing and the movement of the vaginal plates. It is therefore possible that *Abd-B^{LDN}* neurons, or subsets within them, function in both of these aspects of receptivity. There are likely to be additional circuit components involved in plate opening that may be able to act redundantly in the absence of *Abd-B^{LDN}* neurons, and the involvement of additional neurons in the control of the vaginal plates is consistent with the fact that *Abd-B^{LDN}* activation does not induce periodic vaginal plate opening but rather locks the plates in the open position. How the receptivity circuitry coordinates vaginal plate opening with pausing and male copulation attempts remains unknown. *Abd-B^{LDN}* neurons provide an important entry point to dissect the two female motor programs. We observed that vaginal plate opening occurs both while the female is moving and while she is stationary. Female movement has been shown to provide feedback to the male during courtship [42], and it could be that pausing provides an important connection between the sexes within the context of the courtship duet.

Abd-B is required in neurons during development for females to become highly receptive to male courtship. How does the Abd-B protein affect the receptivity circuitry? Our *Abd-B^{LDN}>Abd-B* RNAi experiments show that *Abd-B^{LDN}-Gal4* labels the neurons in which Abd-B functions during development to affect receptivity. However, we note that it is possible that these developmental *Abd-B^{LDN}* neurons are not identical to the adult *Abd-B^{LDN}* neurons we show to function in receptivity. In developing neuroblasts, *Abd-B* can have different, even opposing, functions, promoting cell death or promoting a particular cell fate or repressing it, depending on neuroblast identity and context [23]. In our *Abd-B^{LDN}>Abd-B* RNAi experiments, we did not observe any obvious changes in either the number or projections of *Abd-B^{LDN}* neurons in the adult, but this does not exclude the possibility of subtle anatomical changes or changes in cell identity.

Finally, we note that modularity in the control of complex innate behavior has been found across a variety of species and systems. From flies to mice, both aggression and mating are controlled by eliciting different modules in a sexually dimorphic way [2, 43, 44]. Thus, female fly receptivity fits into a larger pattern of sex-specific control of innate behavioral components.

EXPERIMENTAL PROCEDURES

Fly Stocks

Flies were maintained on conventional cornmeal-agar-molasses medium under a 12 h light: 12 h dark cycle (lights on 9am) at 25°C and 60% relative humidity, unless otherwise indicated. Canton-S was used as wild-type. Sources of all fly strains and detailed genotypes used in the paper can be found in the Supplemental Experimental Procedures.

Transgenic flies

VGlut-Gal80 was generated by PCR amplification of the 5.3 kb *dVGlut* promoter fragment from the *pC56-Kan dVGlut5* vector (kind gift of Richard Daniels, University of Wisconsin) [34] and cloned via the Gateway system (Life Technologies) into the *pBPGAL80Uw-6* vector [45]. The VGlut-Gal80 vector is available at AddGene (Plasmid #52555). Transgenic flies were generated using standard methods (Genetic Services Inc.).

Immunostaining and microscopy—Tissue was dissected in 4°C phosphate-buffered saline (PBS) (Ca^{+2}, Mg^{+2} free; Lonza BioWhittaker CAT#17-517Q), fixed in 4% paraformaldehyde in PBS for 20 min at 23°C, washed 4-6 times over 2 h in PBT (PBS and 0.1% Triton X-100), and blocked for 1 h in PBT + 5% goat serum at 23°C before incubation with primary antibodies diluted in PBT + 5% goat serum for 48 h at 4°C. Samples were washed 4-6 times over 2 h in PBT at 23°C before application of secondary antibodies for 48 h at 4°C. Samples were washed again 4-6 times over 2 h in PBT and mounted in VectaShield containing DAPI (Vector Labs) on glass slides with bridging cover slips. Confocal sections were acquired using a Zeiss LSM510 confocal microscope. For details on antibodies used, see the Supplemental Experimental Procedures.

RNAi screen

The genome-wide neuronal RNAi screen was carried out by N.Y. in the laboratory of B.J.D. at the Research Institute of Molecular Pathology (IMP) in 2005-2008 using the VDRC RNAi library [22]. Detailed information on the RNAi screen is in Supplemental Experimental Procedures.

Behavioral assays

These assays were carried out by J.J.B. in the laboratory of L.B.V. at The Rockefeller University in 2009-2014. All assays were performed at ZT3-9, and all genotypes and conditions were tested on at least 3 different occasions. Mature virgin females were collected within 6 h of eclosion, group-housed without males, and tested at 4-6 days old. Immature virgin females were tested at 24 h post-eclosion. Mated females were individually observed copulating with 1 of 2 males in a food vial at 4-5 days old and tested 48 h later. Wild-type Canton-S males were collected 0-2 days after eclosion and were group-housed away from females for 3-7 days. Temperature-shifted experiments were carried out in incubators (Insect model BC26-IN, BioCold Environmental).

Copulation assays

Single females were gently aspirated into standard fly food vials containing 2 wild-type males at 23°C. Individual pairs were visually scored for copulation at 5, 10, 15, 30, 45, and 60 min to determine the percent copulated during 1 h.

Temporally restricted RNAi

tub-Gal80^{ts} [25] crosses were reared at either 18°C or 30°C. Virgin females were collected at eclosion and then group-housed away from males for 4 days at either 18°C or 30°C before being tested for receptivity at 23°C or dissected.

Male courtship index

Courtship index was defined as the proportion of time the male followed and oriented towards the female within 5 min of courtship initiation, marked by the initial orientation towards and following of the female. Male courtship index in Figure 3B was scored from the same videos as ovipositor extrusion in Figure 3D.

Vaginal plate opening and ovipositor extrusion

Individual females were placed in one of eight 1-cm circular plastic chambers in a courtship wheel with a wild-type male and filmed for 15 min. To allow visualization of vaginal plate opening, uncompressed image sequences at 1600×1200 pixels and 30 frames per s and less than 10 ms exposure were recorded directly to disk with a Grasshopper-2 Firewire camera (Point Grey Research) with an Infinimite Alpha lens and 2X magnifier (Infinity Optics) using Streampix 5 (Norpix). Lighting was provided by angled low-flicker fluorescent lights (Coherent) and adjustable fiber optic lights from a dissecting microscope. Instances of vaginal plate opening and ovipositor extrusion were scored blind to genotype and mating status from frame-by-frame playback during the first 5 min of courtship or until copulation if it occurred within 5 min. Courtship initiation was defined as the male orienting towards and beginning to follow the female. Rare trials with fewer than 30 s of total courtship were discarded.

Egg-laying

Individual virgin females were observed to mate with a wild-type male in a fly food vial and then transferred singly into food vials at 25°C, 60% relative humidity, 12 h light: 12 h dark and allowed to lay eggs for 24 h. Adults were then transferred to a fresh vial and allowed to lay for another 24 h. The number of eggs was counted at the end of each 24 h period to determine total eggs laid per female in 48 h.

Acute neuronal silencing

Flies for *UAS-shi^{ts}* [31] silencing experiments were raised at 18°C and shifted to 18°C or 29°C 30 min prior to assays. Food vials were placed at 18°C or 29°C for 2 h prior to assays to reach the appropriate temperature.

Movement tracking

Fly movement in 2 dimensions was tracked during courtship in a custom 70 mm circular arena with sloping sides and a removable level center modified from published designs [46]. The arena was made of opaque white Delrin plastic (McMaster-Carr) custom-machined to uniform thickness to allow even backlighting from a light board (Smith-Victor Corporation CAT#A-5A) and topped with a piece of Plexiglas with a small hole for introducing flies. Plans are available upon request. Video was recorded with a consumer camcorder (Canon HFS20) mounted above the arena in an incubator at 60% relative humidity with the lights on. Movement was tracked using Ctrax open source software [37]. Technical details are in the Supplemental Experimental Procedures.

Sound playback

Recordings were played using Audacity software (http://audacity.sourceforge.net/) from a laptop computer on AX210 computer speakers (Dell). The 2 speakers were placed on opposite sides of an 8-chamber Plexiglass wheel (12 cm diameter) with mesh bottom. The song file was composed of 4 repeats of an approximately 20-min section of wild-type Canton-S male courtship song [47]. Intensity-matched white noise with amplitude 0.8 was generated using Audacity's noise generation function.

Mute males with wings removed

To generate mute males, 4-5 day old males were lightly anesthetized with carbon dioxide and their wings removed as close to the base as possible with dissecting scissors. Operated males were allowed to recover as a group for at least 24 h at 25°C, 60% relative humidity and used within 72 h of wing removal.

Neuronal activation

Flies for *UAS-TrpA1* activation experiments were raised at 22°C. Assays were conducted at 22°C or 30°C, with flies introduced to the appropriate temperature at the start of assays. The tracking arena was placed at 22°C or 30°C for 2 h prior to assays to reach the appropriate temperature.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism Software version 6.01 (GraphPad Software).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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HIGHLIGHTS

- Transcription factor *Abd-B* is required in neurons for *Drosophila* female receptivity

- *Abd-B* neurons in the VNC and genitalia are functionally required for receptivity

- Pausing and opening vaginal plates are separable components of female receptivity

- *Abd-B* neurons control pausing in response to multiple male courtship cues
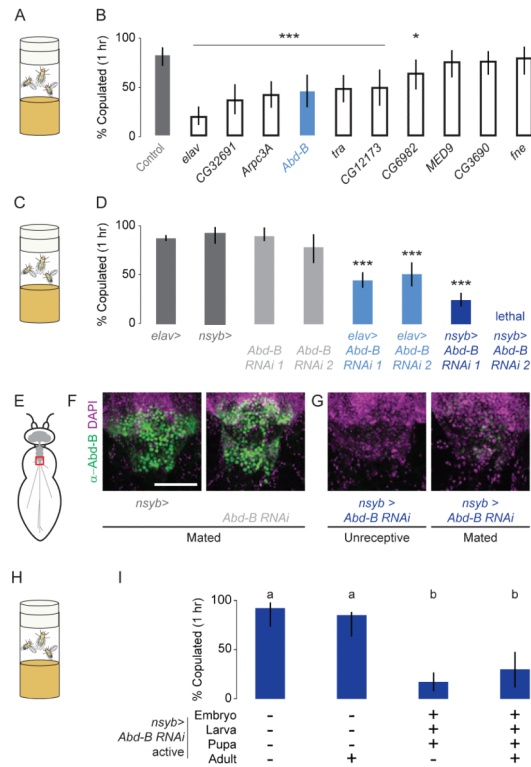
**Figure 1. *Abd-B* is required in neurons for virgin female receptivity**
(A,C,H) Schematic of food vial mating assay with 2 males. (B, D) Receptivity of virgin females with *elav-* (B, D) or *nsyb-* (D) *Gal4*-driven RNAi against the indicated gene [***p <0.0001 or *p <0.005 compared to control (B) or ***p <0.0001 compared to parental control (D): pairwise Fischer's exact test with Bonferroni correction; mean and 95% confidence interval are shown, n = 30-300 (B), n = 30-273 (D)]. *Abd-B* RNAi 1 is the original hairpin from the Vienna screen and was used in all other experiments. For unknown reasons, RNAi 2 when driven by *nsyb* was lethal, precluding further analysis. (E) Schematic of the fly nervous system (gray) indicating the abdominal ganglion (red). (F,G) Immunofluorescence of Abd-B (green) and nuclei (DAPI, magenta) in abdominal ganglia from females of the indicated genotype and mating status. Scale bar: 50 μm. (I) Receptivity of virgin females with *Abd-B* RNAi temporally restricted by shifts from 18°C to 30°C (***p <0.0001, pairwise Fischer's exact test with Bonferroni correction; mean and 95% confidence interval are shown, n = 23-32). See also Figures S1 and S2.
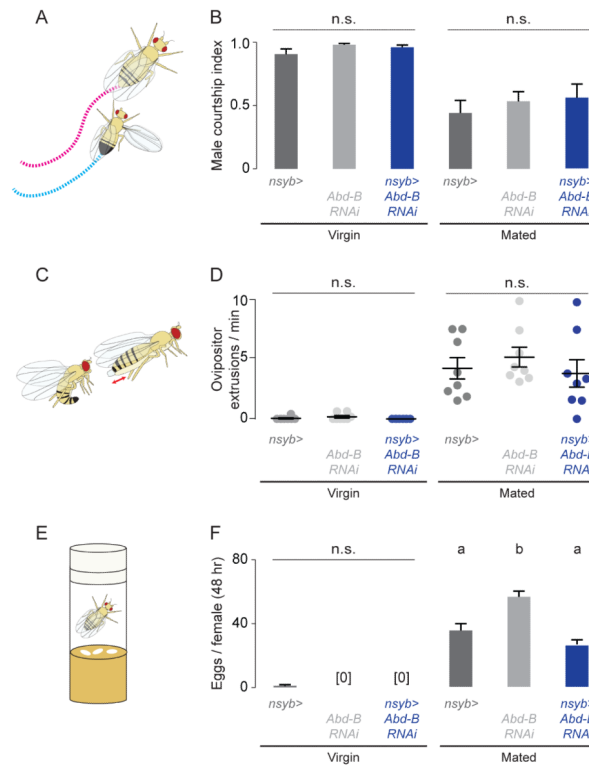
Figure 2. Neuronal *Abd-B* RNAi does not induce the post-mating response

(A, C, E) Schematics of assays in B, D, F. (B) Courtship index of wild-type males during the first 5 min of courtship of a female of the indicated genotype and mating status (n.s. = not significant, one-way ANOVA with Bonferroni correction, mean ± SEM, n = 8). (D) Female ovipositor extrusion during assays in A (n.s. = not significant, one-way ANOVA with Bonferroni correction, mean ± SEM, n = 8). (F) Egg-laying during the first 48 h after mating (n.s. = not significant; bars labeled with different letters are significantly different: p <0.01, one-way ANOVA with Bonferroni correction, mean ± SEM, n = 24-32).
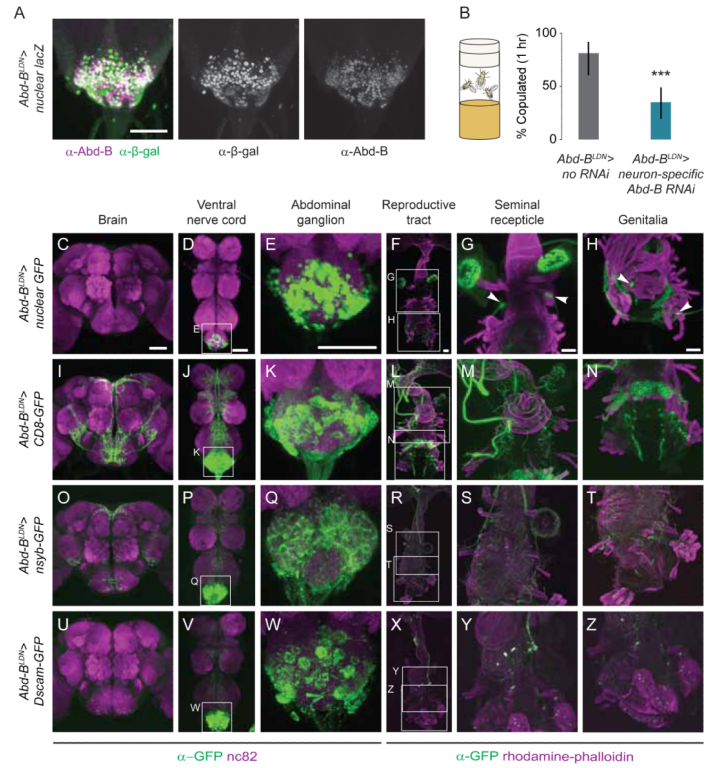
Figure 3. *Adb-B^{LDN}-Gal4* labels *Abd-B* receptivity neurons in the abdominal ganglion and reproductive tract

(A) Co-localization of Abd-B (magenta) and nuclear β-gal (green) driven by *Abd-B^{LDN}-Gal4* in the virgin female abdominal ganglion. Scale bar: 50 μm. (B) Receptivity of virgin females with RNAi against *Abd-B* driven by *Abd-B^{LDN}-Gal4* and *UAS-Dcr2*, limited to neurons by *nsyb-lexA*, *lexAop-FLP*, and *tub-FRT-Gal80-FRT-STOP* (***p <0.001, Fischer's exact test; mean and 95% confidence interval are shown, n = 16-40). See Supplemental Experimental Methods for complete genotypes. (C-Z) Immunofluorescence of GFP (green) and nc82 or rhodamine-phalloidin (magenta) in the indicated tissue in virgin females of the indicated genotype. Insets are separate z-stacks at higher magnification of approximate areas indicated. Arrowheads in G and H indicate neuronal cell bodies. Scale bars: 50 μm. See also Figure S3.
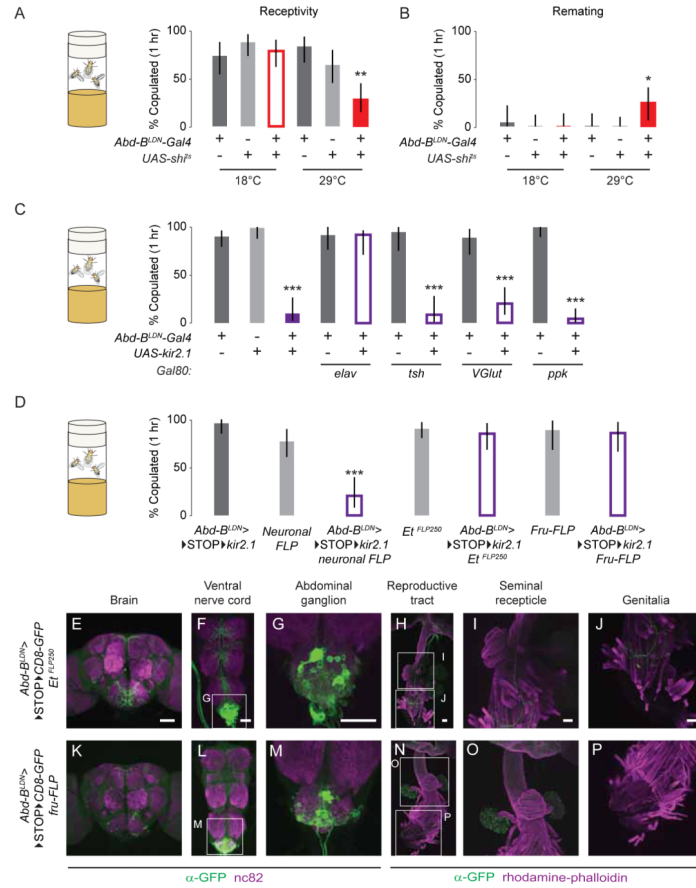
Figure 4. *Abd-B^LDN* neurons are functionally required for virgin female receptivity

(A-B) Temperature-shift receptivity of virgin females (A) or females 48 h after mating (B).
(C-D) Receptivity of virgin females. In A-D, *p <0.05, **p <0.01, ***p <0.001 compared to parental controls (at the same temperature in A-B), Fischer's exact test; mean and 95% confidence interval are shown. Sample sizes: n = 33-40 (A), n = 23-35 (B), n = 20-59 (C), n = 29-56 (D). (E-P) Immunofluorescence of GFP (green) and nc82 or rhodamine-phalloidin (magenta) in the indicated tissue in virgin females of the indicated genotype. Insets are separate z-stacks at higher magnification of approximate areas indicated. Scale bars: 50 μm. See also Figures S4 and S5.
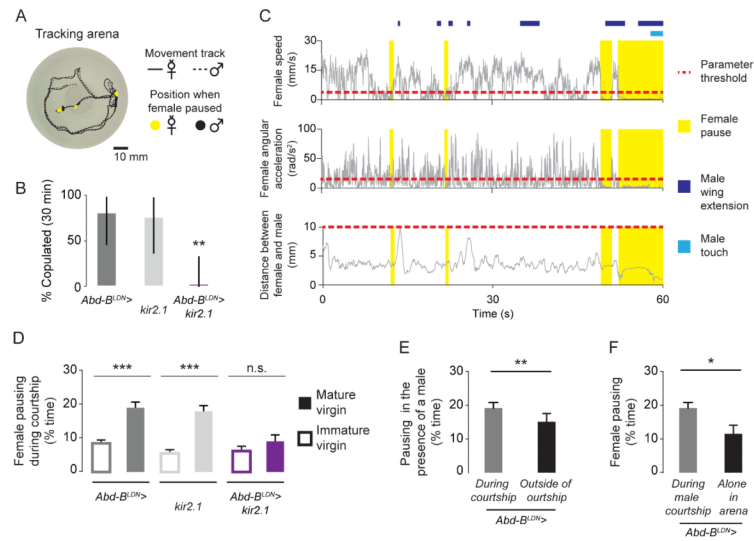
Figure 5. Silencing *Abd-B^LDN* neurons decreases pausing during courtship
(A) Tracking arena with fly positions during the last 60 s before copulation between *Abd-B^LDN-Gal4* mature virgin female and wild-type male. (B) Receptivity of virgin females in the tracking arena (**p <0.01 compared to parental controls, Fischer's exact test; mean and 95% confidence interval are shown, n = 8-10). (C) Per-frame parameters calculated from tracks in (A). (D) Female pausing during courtship (Student's t-test, mean ± SEM, n = 8-10, ***p <0.001). (E) Female pausing in the presence of a male (Student's t-test, mean ± SEM, n = 10, **p <0.01). (F) Female pausing (Student's t-test, mean ± SEM, n = 8-10, ***p <0.05). See also Figure S6 and Movies S1 and S2.
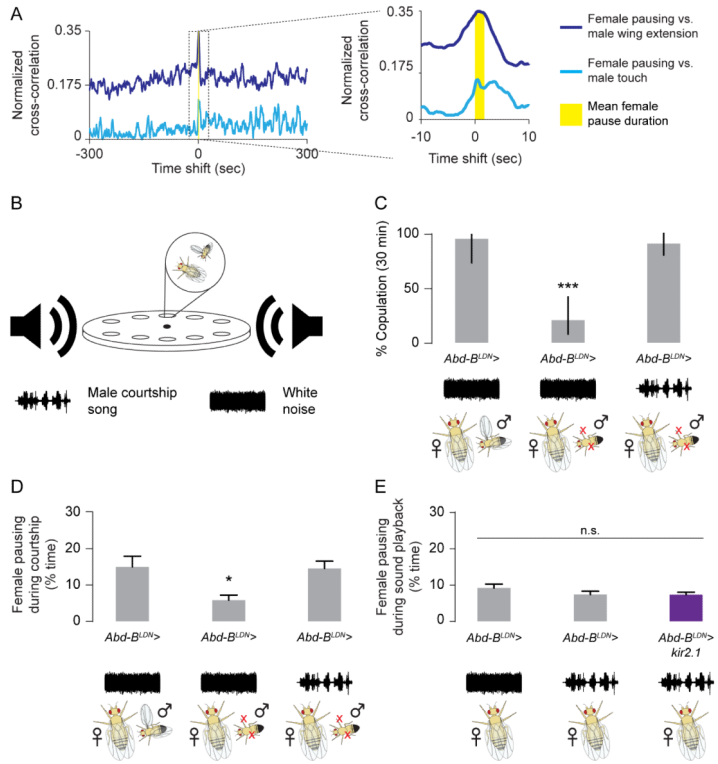
Figure 6. Pausing is a response to multiple male courtship cues

(A) Cross-correlation between female pausing and male wing extension or male touch during courtship tracking assays with *Abd-B^{LDN}-Gal4* mature virgin females and wild-type males (n = 5). (B) Schematic of sound playback assay. (C) Receptivity of *Abd-B^{LDN} Gal4* mature virgin females during sound playback with wild-type males with and without wings as indicated (***$p < 0.001$, Fischer's exact test; mean and 95% confidence interval are shown, n = 23-24 (D-E) Pausing of mature virgin females with wild-type males with and without wings as indicated (D) or in the absence of a male (E) during sound playback [n.s. = not significant, *$p < 0.05$, one-way ANOVA with Bonferroni correction, mean ± SEM; n = 6-7 (D), n = 22-23 (E)].
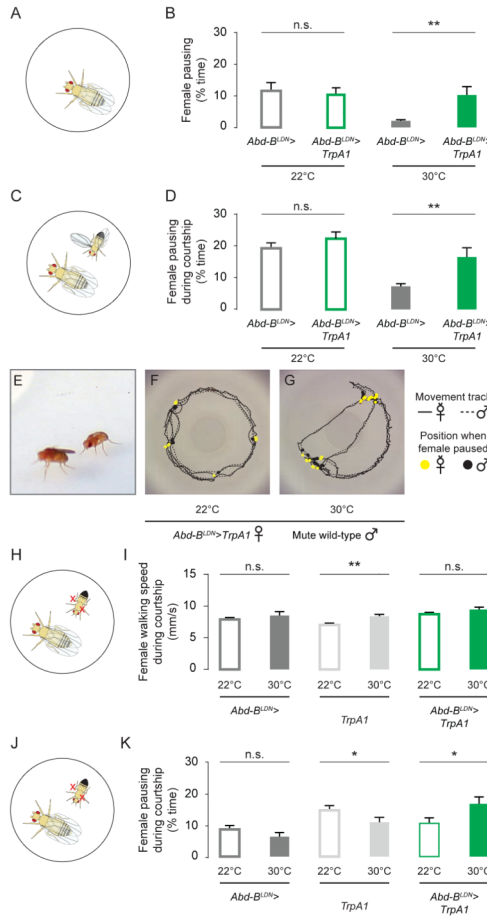
Figure 7. Activating *Abd-B* neurons induces pausing

(A) Schematic of assay in B. (B) Temperature-shifted pausing of mature virgin females in the absence of a male (n.s. = not significant, **p <0.01, Student's t-test, mean ± SEM, n = 7-10). (C) Schematic of assay in D. (D) Temperature-shifted pausing of mature virgin females during courtship from wild-type males (n.s. = not significant, **p <0.01, Student's t-test, mean ± SEM, n = 7-11). (E) Video frame of *Abd-B^{LDN}-Gal4* mature virgin female and wild-type male without wings in tracking arena. (F-G) Tracked fly positions during the fourth min of courtship between *Abd-B^{LDN}-Gal4>TrpA1* mature virgin female and wild-type male without wings at 22°C (F) or 30°C (G). (H) Schematic of assay in I. (I) Temperature-shifted female speed excluding frames classified as pausing during courtship from a male without wings (n.s. = not significant, **p <0.01, Student's t-test, mean ± SEM, n = 12-16). (J) Schematic of assay in (K). (K) Temperature-shifted pausing of virgin females during courtship from males without wings (n.s. = not significant, *p <0.05, Student's t-test, n = 12-16). See also Figure S7 and Movie S2.