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Social Behavioral Deficits with Loss of *Neurofibromin* Emerge from Peripheral Chemosensory Neuron Dysfunction

Emilia H. Moscato[1], **Christine Dubowy**[1], **James A. Walker**[2], **Matthew S. Kayser**[1,3,4,5,*]

[1]Department of Psychiatry, Perelman School of Medicine at University of Pennsylvania, Philadelphia, PA 19104, USA

[2]Center for Genomic Medicine, Massachusetts General Hospital, Boston, MA 02114, USA

[3]Department of Neuroscience, Perelman School of Medicine at University of Pennsylvania, Philadelphia, PA 19104, USA

[4]Chronobiology and Sleep Institute, Perelman School of Medicine at University of Pennsylvania, Philadelphia, PA 19104, USA

[5]Lead Contact

SUMMARY

Neurofibromatosis type 1 (NF1) is a neurodevelopmental disorder associated with social and communicative disabilities. The cellular and circuit mechanisms by which loss of neurofibromin 1 (Nf1) results in social deficits are unknown. Here, we identify social behavioral dysregulation with Nf1 loss in *Drosophila*. These deficits map to primary dysfunction of a group of peripheral sensory neurons. Nf1 regulation of Ras signaling in adult ppk23^{+} chemosensory cells is required for normal social behaviors in flies. Loss of Nf1 attenuates ppk23^{+} neuronal activity in response to pheromones, and circuit-specific manipulation of Nf1 expression or neuronal activity in ppk23^{+} neurons rescues social deficits. This disrupted sensory processing gives rise to persistent changes in behavior beyond the social interaction, indicating a sustained effect of an acute sensory misperception. Together our data identify a specific circuit mechanism through which Nf1 regulates social behaviors and suggest social deficits in NF1 arise from propagation of sensory misinformation.

Graphical Abstract

*Correspondence: kayser@pennmedicine.upenn.edu.

AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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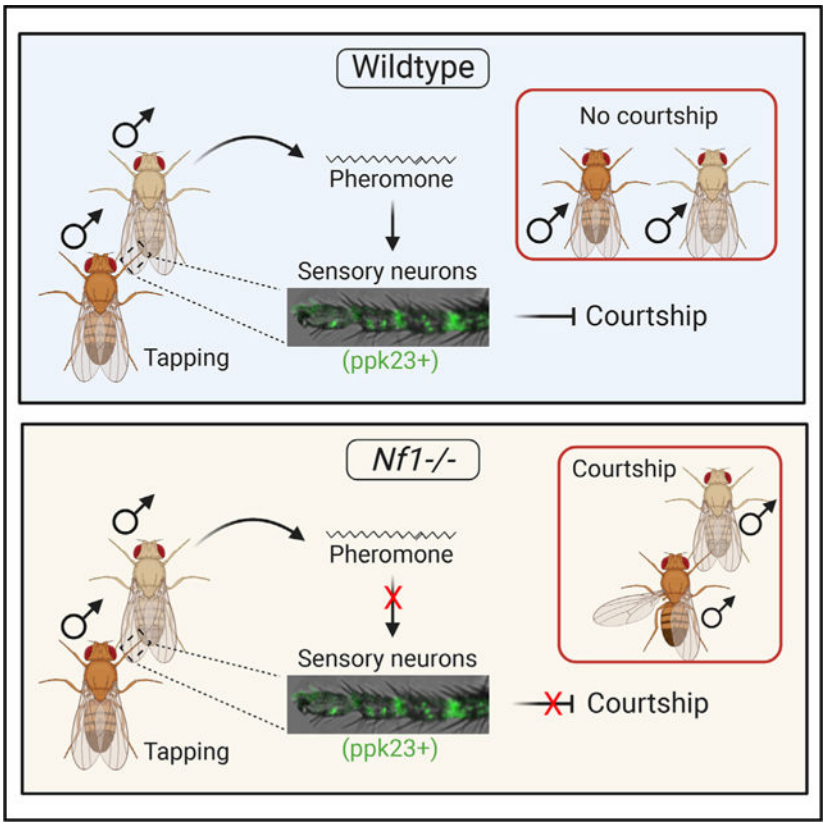

In Brief

Cellular and circuit mechanisms of social behavioral deficits in neurofibromatosis type 1 (NF1) are unknown. Moscato et al. identify a specific circuit mechanism through which *Nf1* acts to regulate social behaviors in *Drosophila melanogaster* and suggest social deficits in NF1 may arise in part from dysfunction in peripheral sensory neurons.

INTRODUCTION

Neurofibromatosis type 1 (NF1) is a common and debilitating neurodevelopmental disorder (NDD) caused by loss-of-function mutations in the *neurofibromin 1* (*Nf1*) gene (Xu et al., 1990). *Nf1* is highly expressed in cells of the nervous system, where it functions as a tumor suppressor by negatively regulating the Rasmitogen-activated protein kinase (Ras-MAPK) signaling pathway (Xu et al., 1990; DeClue et al., 1992). The disease is predominantly characterized by neurofibromas and other tumors of the nervous system, but broad deficits in neurocognitive functioning significantly degrade quality of life. Compared with the general population, children with NF1 have greatly increased rates of autism spectrum disorder (ASD) (Adviento et al., 2014; Plasschaert et al., 2015; Morris et al., 2016). Studies suggest rates of ASD are 25%–50% in NF1 (1%–2% in general population), with NF1 patients being 13 times more likely to exhibit highly elevated ASD symptom burden (Morris et al., 2016). Social and communicative disabilities stemming from ASDs in NF1 patients are among the greatest contributors to disease morbidity (Plasschaert et al., 2015). Children with NF1

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experience increased isolation and bullying (Noll et al., 2007), difficulties on social tasks, and poorer social outcomes (Barton and North, 2004; Huijbregts et al., 2010; Plasschaert et al., 2015). Yet, the mechanisms by which loss of *Nf1* results in ASD and social deficits remain largely unknown.

The *Drosophila melanogaster* homolog of *Nf1* is highly conserved, and *Nf1* mutant flies exhibit a range of cellular and behavioral phenotypes with relevance to the human disease. For example, loss of *Nf1* in *Drosophila* is associated with impaired growth, circadian and sleep abnormalities, learning and memory deficits, hyperactivity, and repetitive grooming behavior (The et al., 1997; Guo et al., 2000; Williams et al., 2001; Walker et al., 2006; Buchanan and Davis, 2010; Bai and Sehgal, 2015; King et al., 2016; van der Voet et al., 2016; Bai et al., 2018). Disruption of the conserved signaling pathways downstream of *Nf1*, Ras-MAPK and/or cyclic AMP-protein kinase A (cAMP-PKA), has been implicated in these phenotypes. The genetic tractability of *Drosophila* also provides unique opportunities to study how social behaviors are affected by the function of genes, such as *Nf1*, that are associated with NDDs and ASDs. Previous work has described social deficits with mutation of fly homologs of the fragile X mental retardation gene (*dFMR1*) and ASD candidate genes, such as *neurobeachin* (*rugose*) and *Neuroligin* (*Dnlg-2* and *Dnlg-4*) (Bolduc et al., 2010; Hahn et al., 2013; Wise et al., 2015; Corthals et al., 2017). The neuronal circuitry that underlies social interactions in flies is well studied (Clowney et al., 2015; Kallman et al., 2015), facilitating the examination of how *Nf1* modulates such behaviors with circuit-level resolution.

Normally, a male fly responds to a female fly with courtship and to another male fly with rejection. Peripheral sensory neurons in antenna, legs, and mouth detect sex-specific cuticular hydrocarbons (CHCs) on the cuticle of other animals. This flow of sensory information modulates the activity of neurons in the brain to promote or suppress social interactions. In wild-type (WT) *Drosophila melanogaster* males, chemosensory detection of female-specific pheromones activates P1 “command” neurons to promote social interaction and courtship, while detection of male-derived pheromones suppresses P1 activity and inhibits courtship (Billeter et al., 2009; Clowney et al., 2015; Kallman et al., 2015). Disrupted function of specific groups of sensory neurons leads to aberrant social interactions between flies (Moon et al., 2009; Wang et al., 2011; Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012; Fan et al., 2013; Dweck et al., 2015; Hu et al., 2015). Interestingly, sensory processing errors and social communication deficits are both prominent symptoms in NDDs, including ASDs (American Psychiatric Association, 2013), and there is growing recognition that the sensory deficits can actually drive social dysfunction (Hilton et al., 2010; Baranek et al., 2013; Orefice et al., 2016; Ronconi et al., 2016). The cellular and circuit mechanisms that couple sensory and social deficits in NDDs are not well understood.

Here, we use *Drosophila* to examine how *Nf1* functions in a circuit to regulate a social interaction. We find that *Nf1* mutant males exhibit aberrant courtship behavior, and that Nf1 acts in a Ras-dependent manner in neurons controlling this behavior. Restoring Nf1 expression only during adulthood rescues courtship behavior of *Nf1* mutants, suggesting Nf1 has an ongoing role in coordinating social functions in the adult. Behavioral and physiological data reveal that Nf1 acts in peripheral sensory neurons, and its loss results in

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sensory errors underlying social deficits. *In vivo* monitoring of neural activity demonstrates that *Nf1* mutants show decreased chemosensory neuronal activation in response to specific pheromonal cues, giving rise to disinhibition of brain neurons that direct social decisions. Circuit-specific manipulations to restore activity in ppk23^{+} sensory neurons or suppress activity in P1 courtship "command" neurons rescue errors in social interaction. Surprisingly, disrupted sensory processing in *Nf1* mutants is associated with a persistent behavioral change that outlasts the social interaction. These findings indicate that social deficits in a fly model of NF1 arise from peripheral sensory neuron dysfunction.

RESULTS

Nf1 Mutant Males Have Defects in Social Interaction Behaviors

As an entry point into the function of Nf1 in social functions, we monitored courtship, which consists of stereotyped and selective behavioral routines dependent on social cues. We focused on intermale social interaction by pairing two males of the same genotype to determine whether mutants are impaired in social behaviors. We first assayed male flies carrying different mutant alleles of *Nf1*. $Nf1^{P1}$ and $Nf1^{P2}$ are null alleles generated by mobilization of a P element (The et al., 1997) (Figure 1A). The parental P element strain, *K33*, served as a control genotype. Mutant male flies homozygous for either the $Nf1^{P1}$ or the $Nf1^{P2}$ allele displayed a dramatic increase in single-wing extensions toward another mutant male, compared with control male pairs, which performed few single-wing extensions (Figure 1B; Figure S1A; Videos S1, S2, and S3). Wing extensions are an early step in the courtship ritual in which a male vibrates one wing to produce a mating song, usually directed toward females. Trans-heterozygous mutants, carrying one $Nf1^{P1}$ allele and one $Nf1^{P2}$ allele, also exhibited enhanced single-wing extensions. In contrast, heterozygous flies with only one mutated allele of *Nf1* were not significantly different from control flies. We also tested $Nf1^{E1}$, an allele generated by ethyl methanesulfonate (EMS)-induced mutations in which the protein is truncated upstream of the catalytic domain (Walker et al., 2006) (Figure 1A). We assayed transheterozygous flies that carry $Nf1^{E1}$ and $Nf1^{P1}$ alleles and found these mutants also show increased single-wing extensions (Figure 1B; Figure S1A). Together these results demonstrate that loss of Nf1 is associated with aberrant courtship behavior between male flies.

Do *Nf1* mutant males show enhanced courtship activity toward partners of either sex? To measure copulation frequency, individual *Nf1* mutant males were paired with single WT females in a courtship arena for 60 min. The time to copulate was slower and the overall frequency of copulation decreased in *Nf1* mutant males compared with controls (Figure 1C). Next, we paired individual males with single females for 10 min or until copulation occurred and analyzed videos for specific courtship parameters. The number of single-wing extensions toward a female and overall courtship index (the proportion of time spent courting a female during the assay) were decreased in *Nf1* mutant male flies compared with control flies (Figure 1D; Figure S1A). The latency to initiate courtship toward a female was significantly increased in mutant males, as was the latency to copulate (Figure 1D). Thus, *Nf1* mutant males do not simply show increased levels of courtship overall.

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We next tested *Nf1* mutant males in competitive copulation assays to determine whether they are less effective than control males at courting a female target. In every assay, the control male "won" over the mutant and copulated with the female (data not shown). We hypothesized that the *Nf1* mutant male flies might "lose" because they are spending time courting the control males, rather than directing their efforts solely toward the female. However, both control and mutant males directed the vast majority of their courtship behavior toward the female target (Figure S1B). This result suggests that *Nf1* mutant male flies are not "genderblind," as has been described in unrelated mutants that also engage in male-male courtship (Grosjean et al., 2008). Rather, mutant males can differentiate female from male targets and, in the presence of a female, appropriately direct their courtship efforts toward it.

In addition to differentiating between male and female, successful reproduction in the wild requires *Drosophila melanogaster* males to distinguish females of their own species from other drosophilids. Related but distinct species have evolved unique pheromone codes to aid in this recognition: females of the closely related species *Drosophila simulans* produce 7-T, the CHC also produced by *Drosophila melanogaster* males (Jallon and David, 1987; Shirangi et al., 2009). 7-T is aversive to *Drosophila melanogaster* males and helps inhibit both intraspecific male-male courtship through ppk23 receptors and interspecific male-female courtship through Gr32a receptors (Lacaille et al., 2007; Thistle et al., 2012; Toda et al., 2012; Fan et al., 2013). As expected, in a social preference assay, control males preferred to court *D. melanogaster* females over *D. simulans* females (Figure S1C). This preference remained intact in *Nf1* mutant males. In contrast, males with a mutation in the chemoreceptor *Gr32a*, previously shown to be necessary for interspecies recognition (Fan et al., 2013), showed reduced preference for *D. melanogaster* over *D. simulans* compared with controls. These results suggest that mechanisms for interspecies discrimination are not disrupted in *Nf1* mutant males. Moreover, 7-T guides social behaviors through multiple circuit pathways, which may be differentially impacted by loss of Nf1.

Loss of Nf1 Is Associated with Impaired Detection of Sensory Cues

Altered male-male interactions in pairs of *Nf1* mutants could arise from impaired detection of sensory cues. Alternatively, *Nf1* mutants might produce abnormal cues such that these flies no longer appear male to their partner, leading to nonproductive courtship from other males. For example, male flies without oenocytes fail to produce CHCs, and thus elicit courtship from WT males (Billeter et al., 2009). To distinguish between these possibilities, we examined whether *Nf1* mutant males elicit single-wing extensions from control male tester flies (Figure 1E). If mutants no longer produced the correct blend of CHCs to mark them as male, then we would predict they would elicit courtship from control males. However, we found that control males performed rare single-wing extensions when paired with either *Nf1* mutants or other control target flies (Figure 1F, left). Mutant tester flies directed single-wing extensions toward *Nf1* mutants, but, unexpectedly, few wing extensions toward control targets ($p < 0.01$). We suspected that the apparent preference of *Nf1* mutant males for mutant targets might be due to rejection behavior from control targets. To address this variable, we assessed wing extensions of tester males paired with decapitated male targets (Figure 1F, right). *Nf1* mutant tester flies indeed displayed the same elevated level of

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courtship toward both control and *Nf1* mutant targets under these conditions. Surprisingly, control male testers performed significantly more wing extensions toward an *Nf1* mutant target than a control target (Figure 1F, right; $p < 0.05$), demonstrating that the genotype of the target fly does influence the behavior of the tester, although not in a consistent manner. These results do not demonstrate Nf1 function in courtship to be exclusively fly autonomous (controlling the behavior) or non-fly autonomous (eliciting the behavior). Rather, these data suggest that Nf1 has a potential role in both the behavioral response to male cues and the elicitation of courtship from other flies. Below, we use matched-genotype male-male pairs to focus on Nf1's role in detecting sex-specific cues that should normally serve to direct appropriate courtship behaviors.

Nf1 Acts in Fru$^+$ Neurons to Promote Normal Male-Male Social Interactions

The previous experiments suggest that *Nf1* mutant males cannot detect and/or process inhibitory chemosensory cues from male flies. We next sought to determine where Nf1 is acting. First, we determined whether Nf1 is functioning in neurons. We used a neuron-specific Gal4, *n-synaptobrevin (n-syb)-Gal4*, to re-express Nf1 and observed a suppression of *Nf1* mutant male-male single-wing extensions (Figure 2A). Nf1 re-expression in neurons also rescued courtship index and copulation success in male-female pairs compared with genetic controls (although latency to copulation was not significantly different; Figures S2A and S2C). These experiments indicate that Nf1 expression within neurons is sufficient to correct mutant courtship behaviors in both male-male and male-female interactions.

Many of the neural structures and circuits that orchestrate social interaction behavior are sexually dimorphic. A principle transcription factor in the sex-determination hierarchy is *fruitless* (*fru*) (Demir and Dickson, 2005). The male-specific form of *fru* is expressed throughout the central nervous system (CNS) in ~1,700 neurons, including circuits that have been implicated in courtship and other sexually dimorphic social behaviors. We used fru^{NP21}-*Gal4* to drive expression of Nf1 in sexually dimorphic neurons in mutant flies, and observed a complete rescue of male-male single-wing extensions compared with genetic controls (Figure 2B). To ensure that overexpressing Nf1 in Fru$^+$ circuitry did not broadly suppress all courtship behavior, we tested male-female courtship. Courtship index, latency to copulation, and copulation percentage were indistinguishable between genotypes (Figures S3A and S3C). Consistent with a role for Nf1 in Fru$^+$ neurons, RNAi-mediated knockdown of Nf1 using *fru-Gal4* phenocopied the *Nf1* mutant male social interaction phenotype (Figure 2C). Together, these data show that Nf1 is both necessary and sufficient in sexually dimorphic Fru$^+$ neural circuits to suppress male-male courtship interactions.

We next sought to more precisely localize Nf1 function within the male CNS. The circuitry that transforms socially relevant sensory information into courtship motor programs is well mapped (Clowney et al., 2015; Kallman et al., 2015). Gustatory sensory neurons that detect male pheromones activate mAL (medially located, just above antennal lobe) interneurons, which in turn inhibit P1 neurons (Figure 2D), a male-specific population of cells known for their ability to trigger courtship behavior. We rescued Nf1 expression in P1 neurons using *R71G01-Gal4* and unexpectedly found no rescue of mutant male-male social interaction behavior (Figure 2E). Expressing Nf1 in mAL interneurons (*R43D01-Gal4* and *R25E04-*

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Gal4) failed to rescue mutant behavior as well (Figure 2F). Thus, restoring Nf1 function to two central nodes of courtship circuitry in the brain is not sufficient to normalize *Nf1* male social interaction behavior. Although we cannot rule out the possibility that Nf1 is required in multiple subsets of central courtship neurons concurrently for normal male-male social behavior, these results raise the possibility of a role for Nf1 outside of the brain.

NF1-Related Social Interaction Deficits Are Caused by Disrupted Ras-Dependent Signaling in Adulthood

In humans with NF1, many disease features are thought to be of developmental origin. To distinguish between a developmental versus an adult physiological function of Nf1 in regulating social behaviors, we examined the morphology of courtship neural circuitry in the central brain. We visualized sexually dimorphic neuronal circuitry with membrane-bound CD8:GFP (Figure 3A, top panels) and detected no gross changes between controls and mutants. Next, we used more restricted expression of GFP to examine specific courtship-related circuits, P1 and mAL, but observed no changes to overall gross morphology (Figure 3A, middle and bottom panels, respectively).

Considering that *Nf1* mutant male brains had no obvious morphological abnormalities, we tested whether re-expressing Nf1 only in adulthood was sufficient to rescue mutant male social behavior. We used the RU-486 hormone-inducible driver *daughterless-GeneSwitch* (*da-GS*) to gain temporal control over Nf1 expression; in this system, Nf1 is expressed only when flies are fed RU-486. For adult-only rescue of Nf1 function, RU-486 was fed to mutant male flies after eclosion (Figure 3B), which we found was sufficient to restore normal male-male interactions (Figure 3C). Together, our morphological and behavioral data argue against a prominent developmental effect of Nf1 on social behavioral regulation, and instead indicate an ongoing role for Nf1 in adult neuronal physiology.

Nf1 operates in a variety of signaling cascades in the CNS, most notably in its role as a Ras-GTPase activating protein (Ras-GAP); loss-of-function mutations in Nf1 lead to hyperactive Ras signaling (Martin et al., 1990; Xu et al., 1990; Bollag et al., 1996). In *Drosophila*, mutating the receptor tyrosine kinase Anaplastic lymphoma kinase(dAlk)decreases Ras signaling and rescues sizeand some behavioral phenotypes in *Nf1* mutant flies (Gouzi et al., 2011; Walker et al., 2013; Bai and Sehgal, 2015). To test whether elevated Ras signaling contributes to mutant male-male interaction, we assayed *Nf1* mutant male flies that were heterozygous for loss-of-function *dAlk* mutations. Two different mutant alleles of *dAlk* rescued the enhanced single-wing extensions of the *Nf1* mutants, demonstrating that normalizing levels of active Ras can reverse social behavioral dysfunction (Figure 3D). To further confirm this finding, we expressed the catalytic domain of Nf1, the GAP-related domain (GRD), using *fru-Gal4* in *Nf1* mutant males, and observed a decrease in single-wing extensions (Figure 3E), comparable with the magnitude of rescue with full-length Nf1. These data establish that the Ras-GAP activity of Nf1 is sufficient to rescue *Nf1* mutant male courtship behavior, and that this function can be specifically localized to Fru^+ neurons.

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Nf1 Is Required in Ppk23^{+} Neurons for Chemosensory Function

Our findings suggest that Nf1 acts during adulthood to coordinate social behaviors, but not within examined courtship circuits of the central brain. Disruption of specific sensory neurons in flies results in similar phenotypes to those observed in *Nf1* mutants, so we reasoned that Nf1 might be acting in peripheral sensory neuron function. Our top candidate was Ppk23^{+} gustatory sensory neurons (GSNs). The *ppk23* gene is a member of the degenerin/epithelial sodium channel (Deg/ENaC) ion channel family and is expressed in sexually dimorphic Fru^{+} GSNs (Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012). During social interactions, including courtship, a male fly taps his target using foreleg tarsi to detect CHCs. A subset of tarsal Ppk23^{+} neurons respond specifically to the male pheromone 7-T, ultimately inhibiting P1 courtship neurons to suppress male-male courtship (schematized in Figure 4A) (Thistle et al., 2012; Kallman et al., 2015). Like loss of Nf1, mutations to the *ppk23* gene result in enhanced male-male courtship (Thistle et al., 2012; Toda et al., 2012). When we rescued expression of Nf1 in Ppk23^{+} neurons in *Nf1* mutants, we observed a reduction in male-male single-wing extensions compared with genetic controls (Figure 4B). This rescue did not reflect a broad reduction in all courtship behavior, because there was no effect on the male-female courtship index, latency to copulation, or copulation frequency (Figures S4A–S4C). RNAi-mediated knock down of Nf1 using *ppk23-Gal4* phenocopied the *Nf1* mutant male social interaction phenotype (Figure 4C). Together, these data show that Nf1 is both necessary and sufficient in ppk23^{+} neural circuits to suppress male-male courtship interactions.

As further evidence of a role for Nf1 in Ppk23^{+} neurons, we tested whether *Nf1* mutant male-male courtship relies on olfactory information. Specifically, aversive CHCs detected by Ppk23^{+} neurons normally mask attractive olfactory signals and prevent male-male courtship; *ppk23* mutant males lacking antennae show complete suppression of intermale courtship (Thistle et al., 2012). Similarly, *Nf1* mutant male-male courtship was nearly abolished in flies lacking antennae (Figure S4D). In our surgeries, we removed the arista along with the antenna; therefore, audition and olfaction should be compromised. This finding supports the idea that ectopic courtship in *Nf1* mutant males, as in *ppk23* mutants, results from detection of an attractive olfactory or auditory cue that should normally be masked by gustatory detection of aversive CHCs secreted by male flies.

We hypothesized that in the absence of Nf1, Ppk23^{+} neurons might not be properly activated in response to male pheromones. In support of this hypothesis, constitutive activation of Ppk23^{+} neurons with NaChBac, a bacterial sodium channel, resulted in a decrease in *Nf1* mutant male single-wing extensions (Figure 4D). The partial rescue could be due to the fact that Ppk23^{+} neurons comprise two functionally and genetically distinct groups that exert opposing effects on P1 neurons. M cells respond to male pheromones and inhibit activity of P1 neurons, while F cells respond to female pheromones and increase P1 activity (Kallman et al., 2015). M cells can be genetically accessed by combining *ppk23-Gal4* with *vGlut-Gal80*, which suppresses Gal4 expression in F cells (Kallman et al., 2015). When we selectively activated M cells in an *Nf1* mutant background, single-wing extensions were significantly decreased (Figure 4E). In contrast, activation of Or67d^{+} neurons, a class of olfactory receptor neurons that also convey an inhibitory signal to P1 upon sensing the

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volatile male pheromone cVA (Clowney et al., 2015), did not rescue *Nf1* mutant male courtship (Figure S4E). These data suggest that in *Nf1* mutant males, Ppk23$^+$ neurons are hypoactive in the presence of male pheromones, and argue that Nf1 functions specifically in peripheral gustatory sensation to modulate male-male courtship interactions.

To test whether elevated Ras signaling specifically in ppk23$^+$ neurons contributes to male-male courtship in *Nf1* mutants, we expressed the catalytic GRD of Nf1 with *ppk23-Gal4* in an *Nf1* mutant background. This caused a significant decrease in male-male courtship compared with genetic controls (Figure 4F). Additionally, we selectively decreased Ras activation by expressing a dominant-negative Alk transgene (UAS-Alk-EC) with *ppk23-Gal4* in an *Nf1* mutant background and again observed a significant decrease in male-male courtship (Figure 4F). These data show that normalizing levels of active Ras in ppk23$^+$ neurons can rescue *Nf1* mutant male-male courtship behavior.

We next expressed UAS-mCD8::GFP in Ppk23$^+$ neurons and imaged their axonal projections through the ventral nerve cord into the brain. In *Nf1* mutant males, Ppk23$^+$ axons retained their male-specific midline crossing and terminated in the sub-esophageal ganglion similar to control males (Figure 4G). The number and localization of Ppk23$^+$ cell bodies in the distal four foreleg tarsi segments were also identical in control and mutant males (Figure 4H). Loss of Nf1 therefore does not disrupt Ppk23$^+$ neuronal morphology.

Ppk23$^+$ neurons are known to provide inhibitory tone to P1 neurons (Clowney et al., 2015; Kallman et al., 2015). We wondered whether the *Nf1* mutant behavioral phenotype ultimately emerges from aberrant P1 output because of loss of Nf1-dependent upstream inhibition from Ppk23$^+$ neurons. To test this idea, we examined how experimental inhibition of P1 neurons affects *Nf1* mutant behavior. We found that blockade of synaptic output from P1 neurons using tetanus toxin (UAS-TNT) and *R71G01-Gal4* decreased male-male courtship in *Nf1* mutant flies (Figure S4F). These findings suggest that P1 hyperactivity contributes to NF1-related male-male social dysfunction, although not because of a role for Nf1 in P1 neurons themselves.

To directly test the hypothesis that Nf1 is required for detection of male pheromones by Ppk23$^+$ neurons, we monitored calcium signals with GCaMP6 slow (GCaMP6s). In males, two Ppk23$^+$ neurons, one M cell and one F cell, are housed beneath each chemosensory bristle on the foreleg tarsi (Thistle et al., 2012; Kallman et al., 2015). Single chemosensory bristles were stimulated with 7-T or hexane vehicle, and fluorescent changes in the Ppk23$^+$ neurons underneath the bristle were analyzed (Figure 5A). As shown previously (Thistle et al., 2012), in control males, the calcium increase upon stimulation with 7-T was significantly greater in the male-responsive cell (cell A) than the female-responsive cell (cell B) (Figure 5B). The responses to hexane alone were not significantly different between cells A and B. Similarly, in *Nf1* mutant males, we observed a greater response to 7-T in cell A versus cell B, and no change in the response to hexane. However, the magnitude of the calcium response to 7-T in the male-responsive Ppk23$^+$ neuron was significantly reduced in *Nf1* mutant males, confirming that Ppk23$^+$ neurons are not properly activated in response to 7-T upon loss of Nf1 (Figures 5B and 5C; Figure S5A). Thus, Nf1 is required for detection of

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aversive male CHCs by a class of chemosensory neurons, demonstrating a role for Nf1 in gating the flow of sensory input to produce correct courtship behavior output.

Persistent Behavioral Change in *Nf1* Mutants

After initiation of male-male courtship behaviors, we noticed that *Nf1* mutant males frequently continued single-wing extensions even when not oriented to the target. Importantly, *Nf1* mutants never exhibited wing extensions in isolation without preceding exposure to a target male. We hypothesized that aberrant sensory function might lead to a persistent behavioral change that outlasts the stimuli that elicited it. To test this, we paired male flies and allowed them to interact for 5 min. We then removed one of the males and monitored the remaining male for 5 more minutes. *Nf1* mutants frequently displayed single-wing extensions even after being isolated (Figure 5D; Videos S4, S5, and S6). In contrast, control males never performed single-wing extensions following removal of the target, even when analysis was restricted to flies that performed at least one single-wing extension while paired. The absence of sustained wing extensions in control males did not simply reflect low courtship at baseline, because pairing control males with females for 5 min (which induced robust courtship) still did not elicit persistent wing extensions (Figure 5D). Surprisingly, although *Nf1* mutant males court a female target, they exhibited no persistent behaviors once the female was removed. These data suggest that during social interactions, improper coding of chemosensory information at the periphery leads to a persistent behavioral change in *Nf1* mutant males that outlasts the initial error in sensory detection.

DISCUSSION

Social behavioral deficits represent a common comorbidity in children with NF1, although the etiology remains unknown. Here, we find that *Drosophila Nf1* mutants exhibit social behavioral dysfunction that can be traced to a cell-autonomous role for Nf1 in chemosensory neurons. Nf1 acts in a Ras-dependent manner in sexually dimorphic, Fru^+ neural circuits to inhibit male-male courtship. Our data localize Nf1 function to $Ppk23^+$ GSNs in foreleg tarsi, known to be critical for appropriate social decisions (Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012; Fan et al., 2013). We observe no gross structural abnormalities in these neurons, and restoring Nf1 expression only in adulthood is sufficient to rescue mutant male courtship behavior, indicating that Nf1 acts in the adult nervous system to promote normal male-male social interactions. Loss of Nf1 results in attenuated response of $Ppk23^+$ neurons to social cues and subsequent non-productive intermale courtship, likely because of failed propagation of a crucial inhibitory signal to the brain. Surprisingly, this behavior persists beyond the social interaction itself, suggesting that an acute sensory misperception gives rise to a sustained behavioral error.

Sensory Processing and Social Communication in ASDs

In humans, difficulty processing sensory information across modalities greatly impedes social functioning. For example, face-and gaze-processing impairments make social gestures like eye contact and attention difficult. Atypical auditory processing can disrupt speech recognition and discrimination of emotionally charged speech. Odor preferences can affect the initiation and maintenance of conversation (Thye et al., 2018). Although our studies

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were focused on disease mechanisms in NF1, a near-universal feature of ASDs is heightened or impaired sensitivity to sensory stimuli (Baranek et al., 2014). Not all patients with NF1 harbor ASD symptomatology, but the incidence and severity of ASD in NF1 is strikingly high (Morris et al., 2016). Our work therefore provides important insights into the mechanisms of social interaction behavior and sensory-processing deficits that could be relevant to the broader population with ASD.

Numerous lines of evidence suggest that dysfunction in peripheral sensory neurons contributes to core behavioral features across a range of ASD models (Han et al., 2016; Orefice et al., 2016, 2019; Bhattacherjee et al., 2017; Oginsky et al., 2017; Dawes et al., 2018; Perche et al., 2018). One influential model is that sensory impairments give rise to errors in brain development, leading to behavioral deficits in adulthood (Orefice et al., 2016, 2019). Social and cognitive deficits can be mapped to primary dysfunction of somatosensory neurons rather than neurons within the brain (Orefice et al., 2016). Deletion of *Mecp2*, which causes a syndromic form of autism, solely in peripheral mechanosensory neurons leads to behavioral deficits that recapitulate core symptom domains of ASDs; these social behavioral phenotypes are not seen when *Mecp2* is deleted from mechanosensory neurons in adulthood. More recent work suggests the early post-natal period in mice is the critical window during which mechanosensation influences later behavior (Orefice et al., 2019); pharmacological approaches to normalize mechanoreceptor function during this developmental time can improve behavioral deficits, including social impairments in *Mecp2* mutant mice.

Our work likewise identifies errors in sensory coding at the periphery as a mechanism of social deficits, but, in contrast, primarily implicates an ongoing role for Nf1 in mature sensory neurons. These possibilities are not mutually exclusive. In one model, normal sensory experiences promote typical brain development, and developmentally timed sensory disturbances result in aberrant brain wiring and behavioral output (e.g., social and cognitive deficits). In another model, neurodevelopmental disorder-associated genes such as *Nf1* have an ongoing role in sensory function; loss of function causes ongoing sensory processing errors, aberrant encoding of social cues to the brain, and behavioral output deficits, all in the absence of structural brain abnormalities. These distinctions point toward different therapeutic strategies; our data suggest adult correction of sensory function in NF1 patients might abrogate certain social deficits.

Transformation of Sensory Errors into Behavioral Output Errors

How is a graded change in sensory neuron responsiveness transformed into a dramatic behavioral change? P1 functions as a courtship command center by virtue of receiving input from diverse sensory modalities (contact, vision, olfaction); the summed balance of excitation and inhibition onto P1 dictates behavioral output, not simply the signal strength of each labeled line (Clowney et al., 2015; Kallman et al., 2015). In this way, the quantitatively small attenuation of inhibitory Ppk23^{+} neuron output in the presence of a male leads to fundamentally altered signal structure reaching P1. Genetic silencing of P1 suppresses *Nf1* mutant male courtship, suggesting these neurons are hyperactive in mutants. Future work will directly examine P1 activity in *Nf1* mutants during social interactions and test whether

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rescue of Nf1 in Ppk23$^+$ neurons alone restores net P1 suppression by a male target. Supporting this idea, specific activation of Ppk23$^+$ M cells suppresses mutant intermale courtship. The fly NF1 model will, moreover, facilitate examination of the neural computations taking place at each successive level of the courtship circuit with loss of Nf1.

It is possible that Nf1 acts in other courtship-relevant circuits aside from Ppk23$^+$ neurons, and convergence of these aberrant signals amplifies P1 and courtship dysfunction in response to a male cue. Several lines of evidence support this possibility. First, we do not observe complete suppression of male-male courtship with re-expression of Nf1 in Ppk23$^+$ neurons. This is in contrast with rescue using the broader drivers *nsyb-Gal4* or *fru-Gal4*. This difference could reasonably be attributed to variable driver strength and expression levels of Nf1. However, there might be additional neurons in which Nf1 is necessary to fully suppress male-male courtship. There are several other chemosensory neurons that normally detect aversive pheromones and inhibit nonproductive courtship, and Nf1 may affect signaling from these neurons similar to its effects in Ppk23$^+$ neurons. Although rescue of Nf1 expression in either mAL or P1 does not suppress intermale courtship, Nf1 may also play additional roles in other Fru$^+$ neurons downstream of gustatory sensation, or may be required simultaneously in multiple central brain circuits. Ongoing work will more broadly examine Nf1 function within chemosensory systems.

Further studies will also address the non-fly autonomous role of Nf1 in eliciting courtship behaviors from other flies. Although live *Nf1* mutant targets do not elicit courtship from control flies, decapitated mutant targets do stimulate courtship from controls. In contrast, decapitated *Nf1* mutant targets do not elicit more courtship than decapitated control targets from mutant testers. These contradictory results necessitate additional studies investigating a possible role for Nf1 in the development or function of oenocytes, secretory cells on the abdominal cuticle that produce sex-specific hydrocarbons.

Dissociable Mechanisms of Response to Male or Female Cues in *Nf1* Mutants

Surprisingly, restoring Nf1 expression to Fru$^+$ or Ppk23$^+$ neurons did not rescue male-female courtship behaviors, in contrast with pan-neuronal restoration of Nf1. This finding suggests that Nf1 does not act only in Fru$^+$ neurons to promote normal male-female social behaviors, but perhaps has a role in Fru- gustatory circuits or modulatory central brain neurons as well (Fan et al., 2013; Zhang et al., 2016). In *ppk23* mutant males, a decrease in male-female courtship is observed along with an increase in male-male courtship (Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012). Therefore, Ppk23$^+$ neurons seemed well positioned to account for both types of courtship deficits observed in *Nf1* mutants. Yet, consistent with *fru-Gal4* rescue data, Nf1 re-expression in Ppk23$^+$ neurons did not rescue male-female courtship. *ppk25* and *ppk29* are two additional members of the Deg/Enac family of ion channels, along with *ppk23*, and Nf1 could function downstream of these receptors. However, Ppk25 and Ppk29 are co-expressed in Ppk23$^+$ neurons and act in a complex with Ppk23 to detect the female aphrodisiac 7,11-HD (Liu et al., 2020); re-expression of Nf1 using *ppk23-Gal4* should have restored Nf1 function in Ppk25$^+$/Ppk29$^+$ cells. Importantly, *Nf1* mutant males are able to discriminate the sex of their target to preferentially court females, arguing that there is still a robust detection of attractive stimuli

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from females. *Nf1* mutant males are also able to perform all steps in the courtship sequence, including copulation, demonstrating that the motor output from the central courtship circuitry is intact. A more likely explanation is the decrease in male-female courtship behavior in *Nf1* mutants is secondary to deficits in global processes, such as arousal, motivation, or attention, all of which can be affected in NF1 patients.

Nf1 Function in Sensory Neurons

What intracellular function does Nf1 serve within ppk23^{+} sensory neurons? There are many steps between ligand binding and intracellular calcium increases, any of which could potentially be impacted by loss of Nf1 signaling. Given that 7-T induces a response in ppk23^{+} neurons of *Nf1* mutants, albeit diminished, we conclude that ppk23^{+} channels must still be expressed in the cell membrane. Our data show Nf1-mediated Ras signaling is key to its role in sensory neuron function. In the absence of Nf1, hyperactive Ras might modulate ppk23 receptor expression levels or expression of other receptor complex components, alter excitability through phosphorylation-dependent post-translational modification of channels, or interfere with downstream signaling cascades, ultimately affecting intracellular calcium. Indeed, previous work suggests a role for Nf1 in regulating intracellular calcium levels in *Drosophila* neurons and mammalian astrocytes (Bai et al., 2018). Furthermore, studies from isolated mouse dorsal root ganglion cells demonstrate changes to N-type calcium currents in *Nf1*$^{+/-}$ sensory neurons compared with controls, likely involving post-translational modification to these channels (Duan et al., 2014). Related work reported increased expression levels of specific voltage-dependent sodium channels (Hodgdon et al., 2012). These results suggest increased excitability and transmitter release in cells from *Nf1*$^{+/-}$ mice, perhaps relevant for enhanced pain sensitivity in NF1 (Créange et al., 1999; Wolkenstein et al., 2001; Wang et al., 2005; Hingtgen et al., 2006). Together, such findings support the idea that Nf1 modulates chemosensory neuron responsiveness in a Ras-dependent manner, and underscore the need for more detailed examination of sensory processing in humans with NF1.

Persistent Behavioral Effects of Transient Sensory Misinformation

In *Drosophila*, transient sensory information can lead to persistent behavioral states that outlast the stimulus (Anderson and Adolphs, 2014). For example, brief optogenetic stimulation of P1 neurons can produce a persistent aggressive state in the absence of continued photoactivation (Hoopfer et al., 2015). Likewise, single-wing extensions continue after termination of P1 neuron photostimulation (Inagaki et al., 2014). More recent work has begun to define the molecules and circuits responsible for these persistent behaviors (Zhang et al., 2019; Jung et al., 2020). We find that *Nf1* mutants exhibit persistent courtship behaviors even after termination of a social encounter with another male; this behavior was never observed after interaction with a female target or in control tester flies following encounters with male or female targets. It will be interesting to determine whether this persistence arises from sustained alterations in activity of specific neurons following the improperly encoded social stimulus. *Nf1* mutants vigorously court males and females; the dichotomous persistence behavior of *Nf1* mutants following these social interactions reinforces that nuanced disruptions to sensory processes can manifest as pronounced changes in behavioral output. Social deficits in humans with NF1 and other ASDs could

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similarly reflect sustained alterations to neural encoding of social cues emanating from primary sensory disturbances.

Data are expressed as boxplots or mean ± SEM in all figures.

STAR★METHODS

RESOURCE AVAILABILITY

Lead Contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Matthew Kayser (kayser@pennmedicine.upenn.edu).

Materials Availability—This study did not generate unique reagents.

Data and Code Availability—This study did not generate datasets or code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Flies were maintained in bottles on cornmeal molasses food obtained from Lab Express (Fly Food R, recipe available at http://lab-express.com/DIS58.pdf) at 25°C on a 12h:12h light:dark cycle. *nsyb-Gal4*, *fru^{NP21}-Gal4*, *R71G01-Gal4*, *R25E04-Gal4*, *R43D01-Gal4*, *or67d-Gal4*, *UAS-TNT*, *UAS-mCD8::GFP*, *UAS-NaChBac*, *UAS-GCaMP6S*, and NF1 RNAi (BDSC #53322) were obtained from Bloomington stock center and outcrossed into an *iso31* background. *daughterless-GeneSwitch, Nf1^{E1}, Nf1^{E2}, Alk$^{\beta}$* and *Alk^{9}* mutant alleles, *UAS-dNf1* and *UAS-GRD* (Lorén et al., 2003; Walker et al., 2006; Tricoire et al., 2009) were previously described. Wild-type Canton-S flies were generously provided by Dr. Edward Kravitz. *K33, Nf1^{P1}* and *Nf1^{P2}* mutant alleles (The et al., 1997) were generously provided by Dr. Amita Sehgal and outcrossed into an *iso31* background. *Gr32a$^{-/-}$* was generously provided by Dr. Hubert Amrein. *Drosophila simulans* stock was generously provided by Dr. Jean-Christophe Billeter. *ppk23-Gal4/CyO* was generously provided by Dr. Barry Dickson. *ppk23-Gal4/TM6,Sb* was generously provided by Dr. Kristin Scott.

METHOD DETAILS

Courtship assays—Male flies were collected as virgins within 4 hours of eclosion and raised in isolation for 4–7 days. Female CS flies were collected as virgins and maintained in vials in groups of 10–15 for 3–5 days. Paired flies were gently aspirated into a well-lit porcelain mating chamber (25 mm diameter and 10mm depth) covered with a glass slide. Assays were performed at 25°C and 50% humidity, recorded for 10 minutes on a Sony camcorder and scored blind to experimental condition. For male-male assays, the total number of single wing extensions in pairs of flies was quantified except for Figure 1F, where only the number of single wing extensions performed by the tester fly was quantified. For male-female assays, courtship index was calculated as the percentage of time a male was engaged in courtship behavior during a period of 10 minutes or until copulation. Courtship index and single wing extension scoring methodologies have been described previously (Billeter et al., 2006; Pan et al., 2011). Copulation percentage was calculated as percentage of flies in each condition that successfully copulated during the 10 min assay. Preference

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index was calculated as the difference in time spent courting each target divided by the total time spent courting. For copulation frequency (Figure 1), assays were monitored for 1 hour or until copulation occurred. Copulation events were binned into 5-minute intervals. For experiments with mated females, CS females were paired for 48 hours with CS males, then separated under CO2 anesthesia and used 24 hours later in experiment. Assays were recorded for 1 hour and the first and last 5 minutes were scored. For experiments to monitor persistent courtship behavior, flies were paired for 5 minutes, then one fly was removed by aspiration and remaining fly was monitored for an additional 5 minutes. In the M+F condition females were decapitated to prevent copulation. For antenna-less experiments, the antenna and arista were surgically removed bilaterally with forceps 24 hours prior to experiment. In the mock-surgical condition flies were anesthetized for the same period no surgery performed. For interspecies discrimination experiments, *Drosophila melanogaster* and *Drosophila simulans* females were anesthetized and labeled with a dot of acrylic paint on the thorax 24 hours prior to assay. For competitive copulation and courtship elicitation experiments, males of different genotypes were labeled with a dot of acrylic paint on the thorax 24 hours prior to assay. For courtship assays using *daughterless-GeneSwitch*, crosses were set up on standard food. Upon eclosion, males were isolated as virgins and transferred to food containing 500 μM mifepristone (RU-486) or ethanol control for 4–7 days until use in experiment.

Immunohistochemistry and imaging—Brains were dissected in PBS, fixed in 4% paraformaldehyde for 30 min at room temperature, washed in 0.1% PBS-Tx and incubated with primary antibody at 4°C overnight. Following washes in 0.1% PBS-Tx, brains were incubated with secondary antibody for 2 hours at room temperature, washed and incubated with 70% glycerol for 2 hours at room temperature, and mounted in Vectashield. Primary antibodies included mouse anti-nc82 (1:100, Developmental Studies Hybridoma Bank) and rabbit anti-GFP (1:1000, Molecular Probes). Secondary antibodies included donkey anti-rabbit Alexa488 (1:500, Invitrogen) and donkey anti-mouse Alexa647 (1:500, Invitrogen). Brains were visualized with a TCS SP8 confocal microscope and images processed in NIH FIJI/ImageJ. For visualizing GFP fluorescence in forelegs, tarsi were removed with forceps and mounted in vectashield. Imaging was performed within 20 minutes of removal and mounting.

GCaMP6S imaging—Tarsi calcium imaging was performed as previously described with slight modifications (Thistle et al., 2012). Flies expressing *UAS-GCaMP6S* and *ppk23-Gal4* were mounted on their side to a glass slide with nail polish, and forelegs extended and fixed with thin ribbons of tape over the 1st and 5th tarsal segments, exposing the 2nd-4th segments for imaging. Slides were mounted on a TCS SP8 confocal microscope. 7-tricosene (10 ng/mL, Cayman chemicals) or hexane vehicle (Fisher) was applied to single chemosensory bristles for 25 s with a pipet pulled from a borosilicate glass capillary tube. The maximum change in fluorescence (ΔF/F) was calculated by dividing the peak intensity change by the average intensity 6 s immediately prior to stimulation. Cell A and Cell B were defined according to Thistle et al. (2012). All image analysis was performed on manually selected ROIs using FIJI/ImageJ.

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QUANTIFICATION AND STATISTICAL ANALYSIS

Data were plotted and analyzed using GraphPad Prism software. Details of statistical tests, including p values and *n*, are included in figure legends. Significance was defined as *p < 0.05 and **p < 0.01.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *Drosophila melanogaster* males with mutations in *Nf1* show social behavior impairments
- *Nf1* regulation of Ras signaling is required for normal male-male social behavior
- Ppk23^{+} neuron activity in response to pheromones is attenuated in *Nf1* mutant males
- Social deficits in a fly model of NF1 arise from sensory neuron dysfunction

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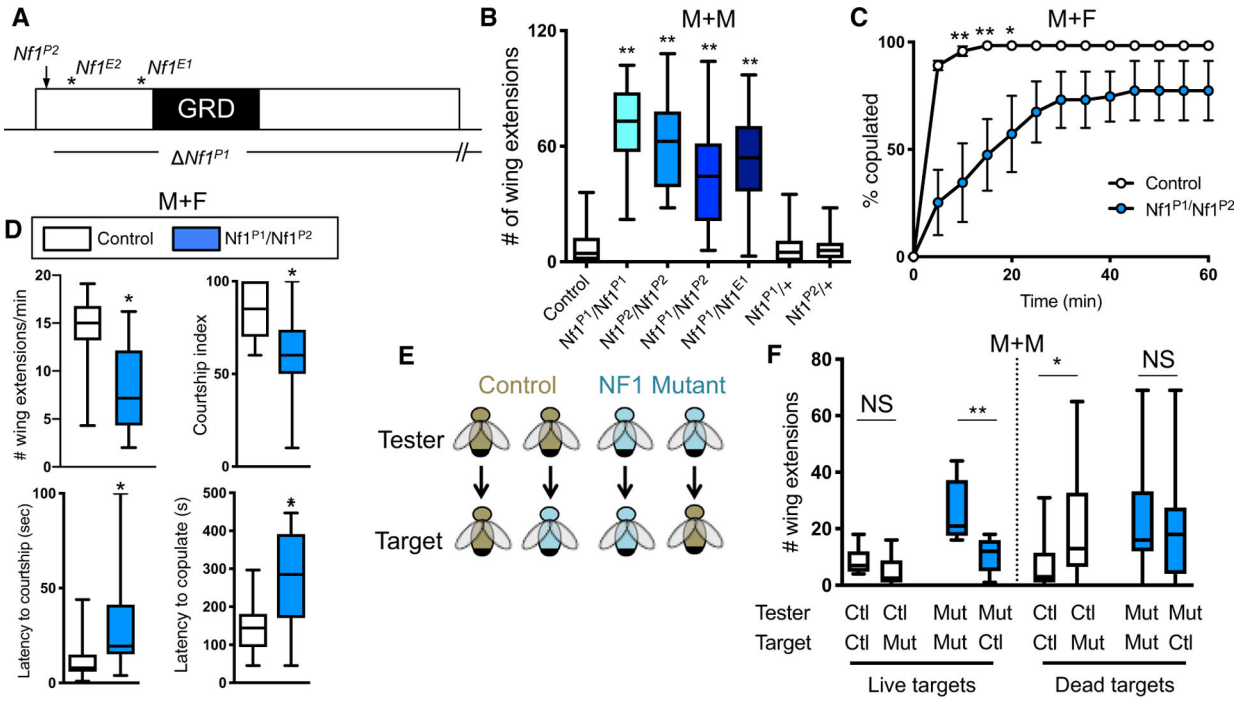

Figure 1. ***Nf1*** **Mutant Male Flies Display Courtship Deficits**

(A) Schematic of *Nf1* mutant alleles. $Nf1^{P1}$ has a deletion that removes the entire *Nf1* gene except for the first exon. $Nf1^{P2}$ contains a P element in the first intron (arrow). Neither allele expresses Nf1 protein. $Nf1^{E1}$ and $Nf1^{E2}$ mutant alleles (asterisks) introduce non-sense mutations upstream of the catalytic domain (GAP-related domain [GRD]) and result in a loss of protein product.

(B) Quantification of single-wing extensions in male-male (M+M) pairs. The genotypes $Nf1^{P1}/Nf1^{P1}$, $Nf1^{P2}/Nf1^{P2}$, $Nf1^{P1}/Nf1^{P2}$, and $Nf1^{E1}/Nf1^{P1}$ show a significant increase in the number of wing extensions compared with control flies. $Nf1^{P1/+}$ and $Nf1^{P2/+}$ heterozygotes are not significantly different from controls. n = 16–25 pairs per genotype.

(C) Quantification of copulation frequency of control and $Nf1^{P1}/Nf1^{P2}$ mutant male flies paired with females (M+F). Mutants are slower to copulate, and fewer pairs successfully mate by the end of the assay. n = 45–60 pairs per genotype.

(D) Quantification of courtship parameters in male-female pairs. $Nf1^{P1}/Nf1^{P2}$ males show a decrease in the number of single-wing extensions per minute and overall courtship index, an increased latency to begin courting, and an increased latency to copulate compared with control flies. n = 17–20 pairs per genotype.

(E) Schematic of experiment. Tester control (brown) or *Nf1* mutant males (blue) were paired with target controls or mutants. Flies were distinguished with a dot of acrylic paint on the thorax.

(F) The number of wing extensions performed by control tester males toward control or mutant target males is not significantly different. Wing extensions performed by mutant testers toward control target males is significantly decreased compared with mutant pairs. If target flies are decapitated, control male testers display significantly increased courtship toward a mutant target, while mutant male courtship index toward a mutant or control target is not significantly different. n = 6–21 pairs per genotype.

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*p < 0.05, **p < 0.01 by Kruskal-Wallis test followed by Dunn's multiple comparisons test (B), Mann-Whitney test (D and F), or repeated-measures ANOVA followed by Bonferroni's multiple comparisons test (C). See also Figure S1.

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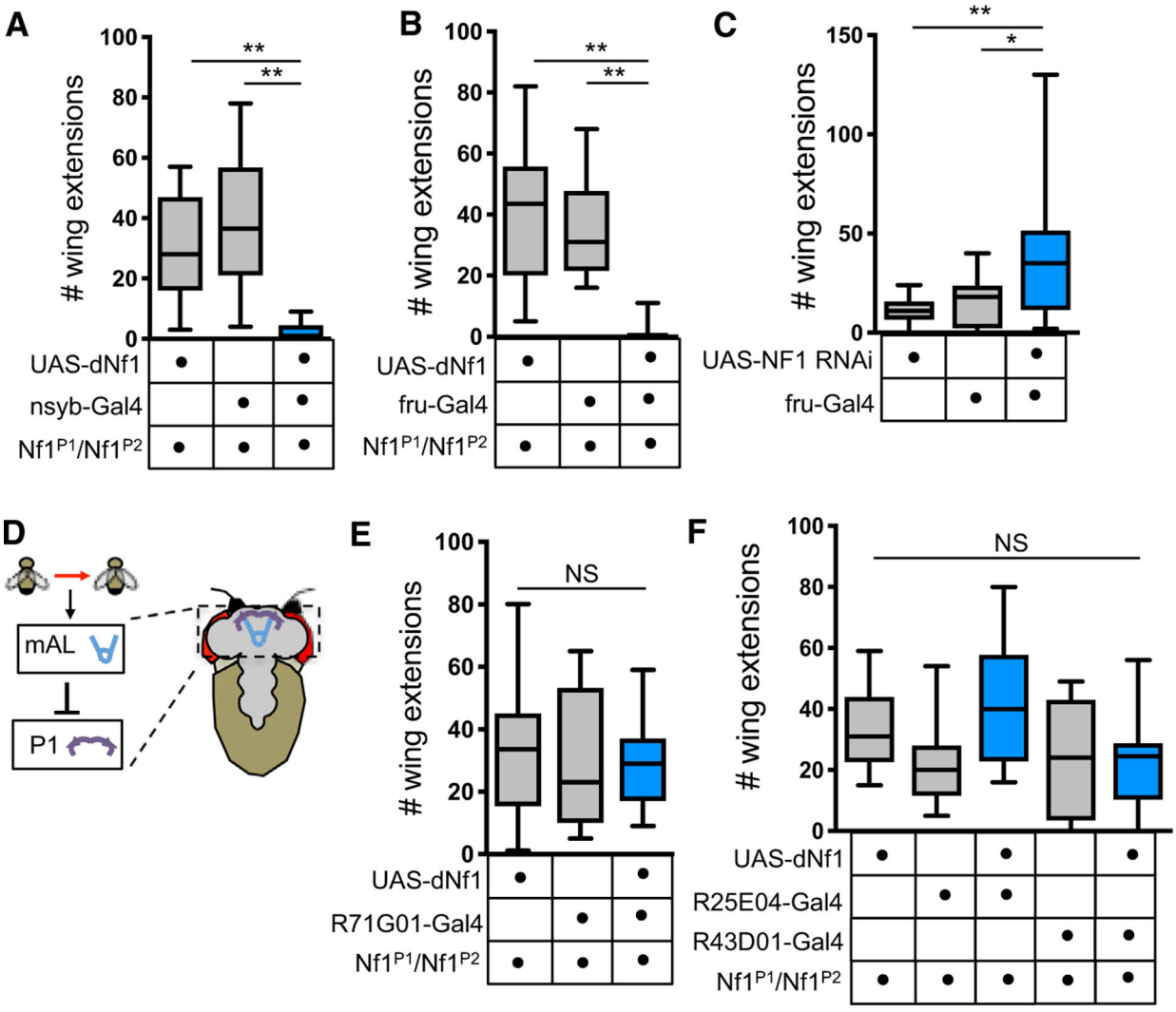

Figure 2. Nf1 Acts in Fru^{+} Neurons to Promote Normal Social Function

(A) Quantification of single-wing extensions in matched-genotype male-male pairs. When *nsyb-Gal4* was used to drive *UAS-dNf1* pan-neuronally in an *Nf1* mutant background, the number of wing extensions performed by males was significantly decreased compared with *nsyb-Gal4* or *UAS-dNf1* expression alone in a mutant background. n = 13–44 pairs per genotype.

(B) Quantification of single-wing extensions in matched-genotype male-male pairs. When *fru-Gal4* was used to drive *UAS-dNf1* in sexually dimorphic neurons in an *Nf1* mutant background, the number of wing extensions performed by males was significantly decreased compared with *fru-Gal4* or *UAS-dNf1* expression alone in a mutant background. n = 14–21 pairs per genotype.

(C) Quantification of single-wing extensions in matched-genotype male-male pairs. When *fru-Gal4* was used to knock down Nf1 expression in sexually dimorphic neurons, the number of wing extensions performed by males was significantly increased compared with *fru-Gal4* or *UAS-Nf1-RNAi* alone. n = 21–22 pairs per genotype.

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(D) When two males are paired (red arrow indicates social interaction), detection of volatile and non-volatile pheromones results in activation of mAL neurons, which leads to net inhibition of P1 neurons within the brain of the tester fly. Boxed region shows a schematic of the relevant neural circuitry.

(E) Quantification of single-wing extensions in matched-genotype male-male pairs. When *R71G01-Gal4* was used to drive *UAS-dNf1* in P1 neurons in an *Nf1* mutant background, the number of wing extensions performed by males was not significantly different compared with *R71G01-Gal4* or *UAS-dNf1* alone in a mutant background. n = 20–28 pairs per genotype.

(F) Quantification of single-wing extensions in matched-genotype male-male pairs. When *R25E04-* or *R43D01-Gal4* was used to drive *UAS-dNf1* in mAL neurons in an *Nf1* mutant background, the number of wing extensions performed by males was not significantly different compared with the *Gal4* or *UAS-dNf1* alone in a mutant background. n = 9–14 pairs per genotype.

*p < 0.05, **p < 0.01 by Kruskal-Wallis test followed by Dunn's multiple comparisons test (A–C, E, and F). See also Figures S2 and S3.

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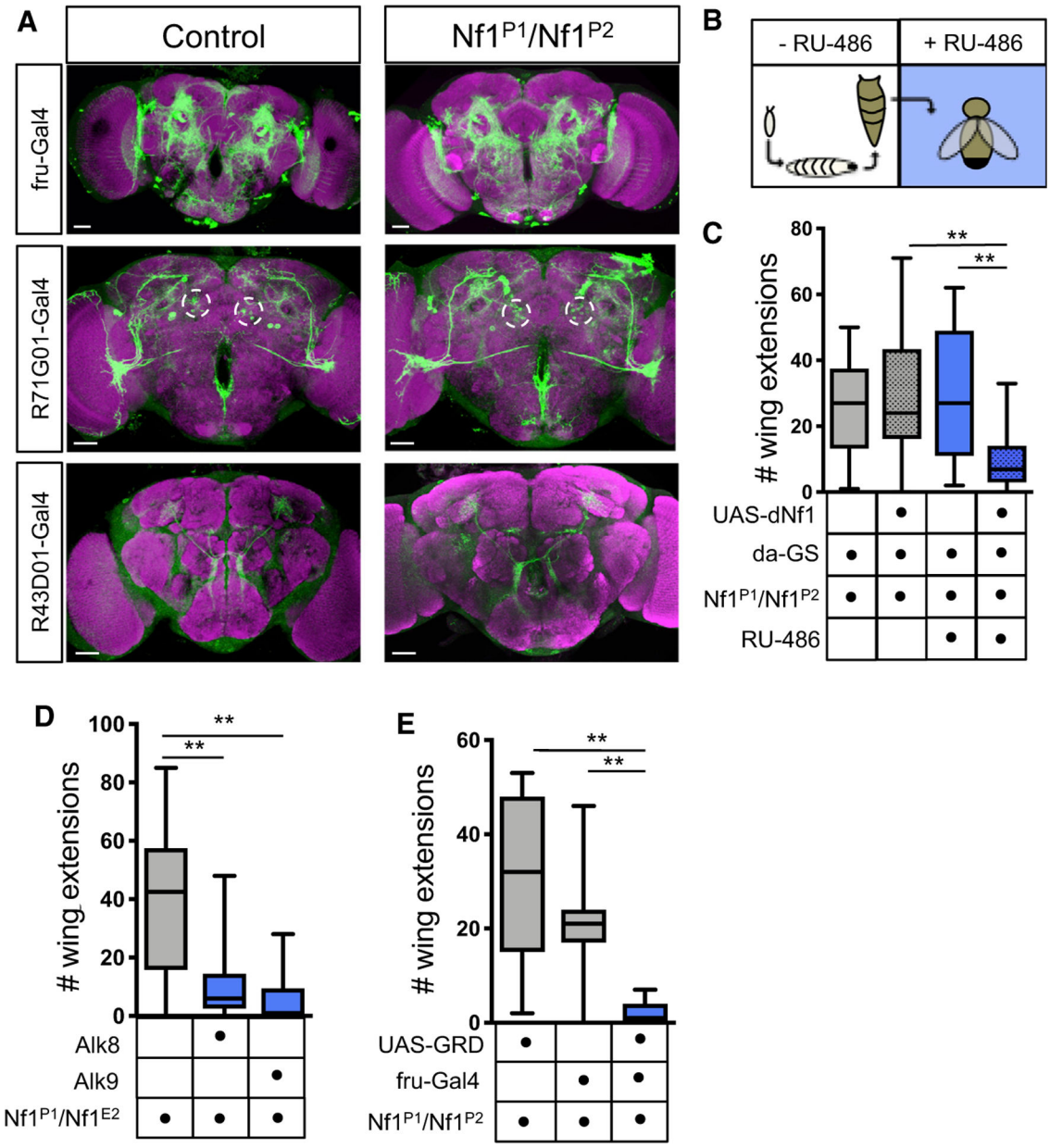

Figure 3. NF1-Related Social Deficits Are Caused by Disrupted Ras-Dependent Signaling in Adulthood

(A) Courtship-relevant sexually dimorphic circuitry is grossly unchanged in *Nf1* mutant males. Top panels show Fru$^+$ circuitry; left, *fru-Gal4>UAS-mCD8::GFP*; right, *fru-Gal4>UAS-mCD8::GFP; Nf1^{P1}/Nf1^{P2}*. Middle panels show P1 neurons, and relevant cell bodies are circled; left, *R71G01-Gal4>UAS-mCD8::GFP*; right, *R71G01-Gal4>UAS-mCD8::GFP; Nf1^{P1}/Nf1^{P2}*. Bottom panels show mAL neurons; left, *R43D01-Gal4>UAS-mCD8::GFP*; right, *R43D01-Gal4>UAS-mCD8::GFP; Nf1^{P1}/Nf1^{P2}*. Scale bars, 30 μm.

(B) Schematic of RU-486 time course. Males are not exposed to RU-486 during embryonic, larval, and pupal stages (pre-eclosion), and there is no expression of *UAS-dNf1*. Upon eclosion, adult males are moved to food containing RU-486 to activate gene expression of *UAS-dNf1*.

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(C) Quantification of single-wing extensions in matched-genotype male-male pairs. Gray bars, controls never exposed to RU-486; blue bars, flies exposed to RU-486 post-eclosion. When Gal4-mediated expression of *UAS-dNf1* is turned on by *daughterless-GeneSwitch* (*da-GS*) in a *Nf1* mutant background, the number of wing extensions performed by males is significantly decreased compared with *da-GS* genetic controls (with and without RU-486) and controls of the same genotype not fed RU-486. n = 15–25 pairs per condition.
(D) Quantification of single-wing extensions in matched-genotype male-male pairs. Loss of one copy of the receptor tyrosine kinase Alk using two different mutant alleles, $Alk8^{+/-}$ or $Alk9^{+/-}$, in an *Nf1* mutant background results in a significant decrease in the number of wing extensions. n = 21–26 pairs per genotype.
(E) Quantification of single-wing extensions in matched-genotype male-male pairs. When *fru-Gal4* was used to drive just the catalytic domain of Nf1 (*UAS-GRD*) in an *Nf1* mutant background, the number of wing extensions performed by males was significantly decreased compared with *fru-Gal4* or *UAS-GRD* alone in a mutant background. n = 11–30 pairs per genotype.
**p < 0.01 by Kruskal-Wallis test followed by Dunn's multiple comparison test (C–E).

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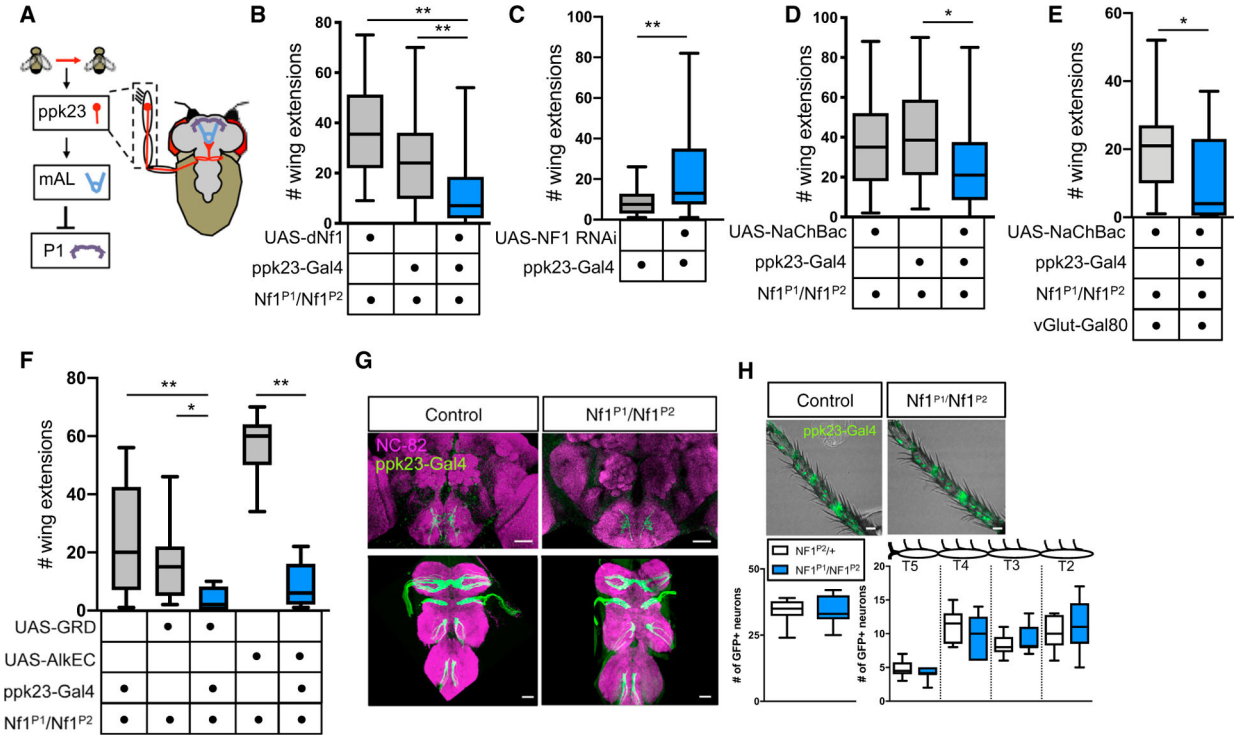

Figure 4. Nf1 Functions in Ppk23^{+} Neurons to Regulate Social Behaviors

(A) When two males are paired (red arrow indicates social interaction), detection of non-volatile pheromones results in activation of Ppk23^{+} gustatory sensory neurons, which leads to activation of mAL neurons and net inhibition of P1 neurons within the brain. Boxed region shows a schematic of the relevant neural circuitry in foreleg tarsi.

(B) Quantification of single-wing extensions in matched-genotype male-male pairs. When *ppk23*-Gal4 was used to drive *UAS-dNf1* in an *Nf1* mutant background, the number of wing extensions performed by males was significantly decreased compared with *ppk23-Gal4* or *UAS-dNf1* expression alone in a mutant background. n = 20–26 pairs per genotype.

(C) Quantification of single-wing extensions in matched-genotype male-male pairs. When *ppk23-Gal4* was used to knock down Nf1 expression, the number of wing extensions performed by males was significantly increased compared with *ppk23-Gal4* alone. n = 28–37 pairs per genotype.

(D) Quantification of single-wing extensions in matched-genotype male-male pairs. When Ppk23^{+} gustatory sensory neurons were constitutively activated with *UAS-NaChBac* in an *Nf1* mutant background, the number of wing extensions performed by males was significantly decreased. n = 25–40 pairs per genotype.

(E) Quantification of single-wing extensions in matched-genotype male-male pairs. When *vGlut-Gal80* was used to restrict expression of *UAS-NaChBac* to Ppk23^{+} M cells in an *Nf1* mutant background, the number of wing extensions performed by males was significantly decreased compared with genetic controls lacking *ppk23-Gal4*. n = 17–19 pairs per genotype.

(F) Quantification of single-wing extensions in matched-genotype male-male pairs. When *ppk23-Gal4* was used to express the catalytic domain of Nf1 (*UAS-GRD*) or a dominant-negative Alk (*UAS-AlkEC*) in an *Nf1* mutant background, the number of wing extensions

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performed by males was significantly decreased compared with genetic controls. n = 10–18 pairs per genotype.

(G) Ppk23^{+} sensory neuron projections into the ventral nerve cord and brain are grossly unchanged in *Nf1* mutant male flies. Top panels show projections terminating in the sub-esophageal ganglia in the brain; bottom panels show projections through the ventral nerve cord; left, *ppk23-Gal4>UAS-mCD8::GFP*; right, *ppk23-Gal4>UAS-mCD8::GFP; Nf1^{P1}/Nf1^{P2}*. Scale bars, 30 μm.

(H) Ppk23^{+} neuronal cell bodies in foreleg tarsi (segments 2–5) in the male fly. Left, *ppk23-Gal4>UAS-mCD8::GFP*; right, *ppk23-Gal4>UAS-mCD8::GFP; Nf1^{P1}/Nf1^{P2}*. The total number of cell bodies in tarsal segments 2–5 in the foreleg tarsi is unchanged between control and mutant males. The number in individual tarsal segments is also unchanged. n = 8–9 flies per genotype, 1 foreleg per fly analyzed. Scale bars, 20 μm.

*p < 0.05, **p < 0.01 by Kruskal-Wallis test followed by Dunn's multiple comparison test (B, D, and F); p > 0.05, *p < 0.05 by Mann-Whitney test (C, E, and H). See also Figure S4.

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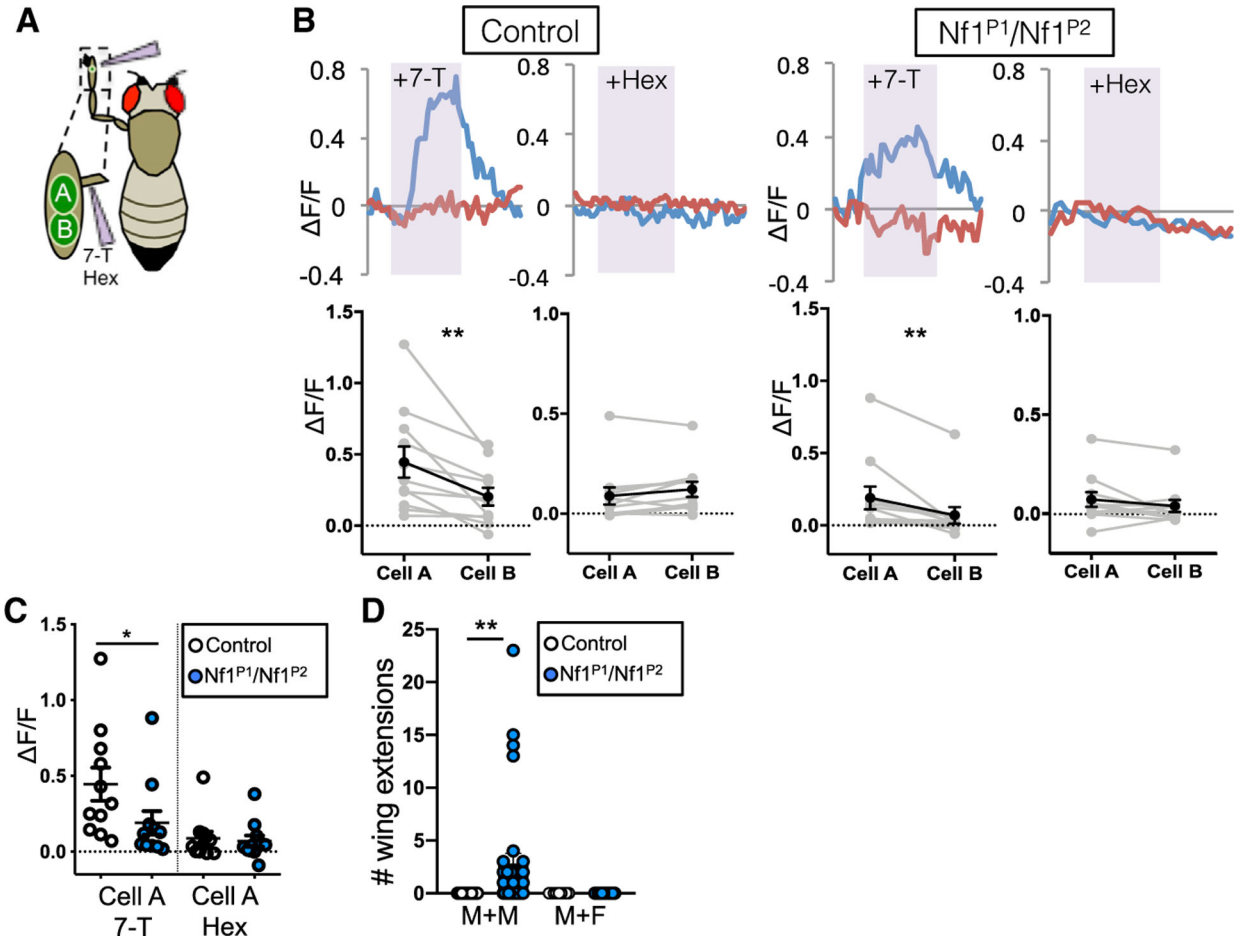

Figure 5. Diminished Ppk23^{+} Activity in Nf1 Mutants in Response to Social Cues

(A) Schematic of the experimental preparation for stimulation and imaging of Ppk23^{+} neurons in foreleg tarsi. A pipet was used to apply 7-T or hexane vehicle to single chemosensory bristles, and *UAS-GCaMP6S* signal in the pair of Ppk23^{+} neurons underneath was imaged.

(B) Representative traces (top) and quantification (bottom) of Ppk23^{+} neuronal responses to 7-T or hexane vehicle in control (left) or mutant (right) male flies. In control flies, upon stimulation with 7-T, there is a large change in the fluorescent GCaMP6S signal in cell A (blue, the putative M cell) and a significantly smaller response in cell B (red, the putative F cell). In response to hexane, there is no significant difference in ΔF/F between the two neurons. In *Nf1* mutant flies, there is also a significant difference in GCaMP6s signal between cell A and cell B in response to 7-T, and no change in response to hexane. n = 11 flies per genotype, 1 bristle per fly.

(C) Quantification of ΔF/F in cell A in control and *Nf1* mutant flies. Control males show a significantly greater response to 7-T than mutant males. There is no change in response to hexane.

(D) Quantification of single-wing extensions after termination of courtship assay by removing the target fly. After a matched-genotype male target is removed from the courtship arena, control flies never perform single-wing extensions, while *Nf1* mutant males display a

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significantly greater number (left, M+M; n = 15–17 per genotype). After a decapitated female target is removed from the courtship arena, flies do not perform single-wing extensions (right, M+F; n = 12 per genotype).

*p < 0.05, **p < 0.01 by Mann-Whitney test (C and D) and Wilcoxon matched-pairs signed rank test (B). See also Figure S5.

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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-GFP	EMSCO/Fisher	A11122; RRID: AB_221569
Mouse anti-NC82	Developmental Studies Hybridoma Bank	DSHB Cat# nc82; RRID: AB_2314866
Donkey anti-Rabbit IgG Secondary Antibody, Alexa Fluor 488	ThermoFisher Scientific	A21206; RRID: AB_2535792
Donkey anti-Mouse IgG Secondary Antibody, Alexa Fluor 647	ThermoFisher Scientific	A31571; RRID: AB_162542
Chemicals, Peptides, and Recombinant Proteins		
7(Z)-tricosene	Cayman Chemicals	9000313
Mifepristone	Thermofisher scientific	H11001
16% Paraformaldehyde Aqueous Solution, EM Grade (PFA)	Electron Microscopy Science (EMS)	15710
Hexane, HPLC grade	Sigma-Aldrich	H303SK-4
Experimental Models: Organisms/Strains		
Drosophila melanogaster. fru^{NP21}-GAL4: w [1118]; P{w[+mW.hs] = GawB}fru[NP0021]	Bloomington Drosophila Stock Center	30027
Drosophila melanogaster. R71G01-GAL4: w[1118]; P{y[+t7.7] w[+mC] = GMR71G01- GAL4}attP2	Bloomington Drosophila Stock Center	39599
Drosophila melanogaster: R25E04-GAL4: w [1118]; P{y[+t7.7] w[+mC] = GMR25E04- GAL4}attP2	Bloomington Drosophila Stock Center	49125
Drosophila melanogaster: R43D01-GAL4: w [1118]; P{y[+t7.7] w[+mC] = GMR43D01- GAL4}attP2	Bloomington Drosophila Stock Center	64345
Drosophila melanogaster. Or67d-GAL4: P {w[+mC] = Or67d-GAL4.F}57.1, y[1] w[*]	Bloomington Drosophila Stock Center	9997
Drosophila melanogaster. UAS-Tetanus Toxin: w[*]; P{w[+mC] = UAS-TeTxLC.tnt} G2	Bloomington Drosophila Stock Center	28838
Drosophila melanogaster. UAS-CD8::GFP: y[1] w[*]; P{w[+mC] = UAS-mCD8::GFP.L} LL5, P{UAS-mCD8::GFP.L}2	Bloomington Drosophila Stock Center	5137
Drosophila melanogaster. UAS-NachBac: y [1] w[*]; P{w[+mC] = UAS-NaChBac}2	Bloomington Drosophila Stock Center	9469
Drosophila melanogaster. UAS-GCaMP6S: w[1118]; P{y[+t7.7] w[+mC] = 20XUAS-IVS- GCaMP6s}attP40	Bloomington Drosophila Stock Center	42746
Drosophila melanogaster. UAS-Calexa: w[*]; P{w[+mC] = LexAop-CD8-GFP-2A- CD8-GFPJ2; P{w[+mC] = UAS-mLexA- VP16-NFATJH2, P{w[+mC] = lexAop-rCD2- GFP}3/TM6B, Tb[1]	Bloomington Drosophila Stock Center	66542
Drosophila melanogaster. UAS-NF1 RNAi y [1]v[1];P{y[+t7.7]v[+t1.8] = TRiP.HMC03551}attP40	Bloomington Drosophila Stock Center	53322
Drosophila melanogaster. daughterless- GeneSwitch	Tricoire et al., 2009	FBtp0057039
Drosophila melanogaster: $NF1^{E1}$	Walker et al., 2006	FBal0197114
Drosophila melanogaster. $NF1^{E2}$	Walker et al., 2006	FBal0197115
Drosophila melanogaster. Alk^{8}	Loren et al., 2003	FBal0157357
Drosophila melanogaster. Alk^{9}	Loren et al., 2003	FBal0157356
Drosophila melanogaster. UAS-dNF1	Walker et al., 2006	FBtp0023091
Drosophila melanogaster. UAS-dNF1-GRD	Walker et al., 2006	FBtp0023101
Drosophila melanogaster. K33	Theetal., 1997	

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Drosophila melanogaster. $NF1^{P1}$	Theetal., 1997	FBal0061708
Drosophila melanogaster. $NF1^{P2}$	Theetal., 1997	FBal0061707
Drosophila melanogaster. *Gr32a−/−*	Miyamoto and Amrein, 2008	FBal0241775
Drosophila simulans	Dr. Jean-Christophe Billeter	
Drosophila melanogaster. Iso31	Dr. Amita Sehgal	
Drosophila melanogaster. Canton-S	Dr. Edward Kravitz	
Drosophila melanogaster. Ppk23-GAL4/CyO	Dr. Kristin Scott	FBtp0079723
Drosophila melanogaster. Ppk23-GAL4/TM6C,Sb	Dr. Barry Dickson	FBtp0085295
Drosophila melanogaster. Nsyb-GAL4	Dr. Julie Simpson	FBtp0041245
Software and Algorithms		
Prism	GraphPad Software	RRID: SCR_002798
FIJI	US National Institute of Health	RRID: SCR_002285