Double-bromo and extraterminal (BET) domain proteins regulate dendrite morphology and mechanosensory function

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A complex array of genetic factors regulates neuronal dendrite morphology. Epigenetic regulation of gene expression represents a plausible mechanism to control pathways responsible for specific dendritic arbor shapes. By studying the Drosophila dendritic arborization (da) neurons, we discovered a role of the double-bromodomain and extraterminal (BET) family proteins in regulating dendrite arbor complexity. A loss-of-function mutation in the single Drosophila BET protein encoded by female sterile 1 homeotic [fs(1)h] causes loss of fine, terminal dendritic branches. Moreover, fs(1)h is necessary for the induction of branching caused by a previously identified transcription factor, Cut [Ct], which regulates subtype-specific dendrite morphology. Finally, disrupting fs(1)h function impairs the mechanosensory response of class III da sensory neurons without compromising the expression of the ion channel NompC, which mediates the mechanosensitive response. Thus, our results identify a novel role for BET family proteins in regulating dendrite morphology and a possible separation of developmental pathways specifying neural cell morphology and ion channel expression. Since the BET proteins are known to bind acetylated histone tails, these results also suggest a role of epigenetic histone modifications and the "histone code," in regulating dendrite morphology.

[Keywords: female sterile 1 homeotic; double bromodomain and extraterminal; histone acetylation; epigenetics; dendrite morphogenesis; mechanosensory function]

Supplemental material is available for this article.

Received February 13, 2014; revised version accepted August 4, 2014.

Dendrites are the primary site of information input to neural circuits, and the shape of dendritic arbors influences the electrophysiological responses of neurons (Häusser and Mel 2003). Due to the existence of highly diverse morphologies among different neuronal subtypes, a question of the relationship between form and function arises: By understanding how the shape of a neuron is specified, we can understand how morphology relates to neural function and how altered morphology relates to dysfunction.

Neurons can be defined by their physiology, morphology, and gene expression. Neuronal diversity is thought to arise from the combinatorial expression of genetic determinants (Hobert et al. 2010). The dendritic arborization (da) sensory neurons of the Drosophila peripheral nervous system (PNS) constitute a powerful system to study genetic determinants of dendritic arbor morphology (Corty et al. 2009; Jan and Jan 2010). In particular, the use of Drosophila genetic techniques to study the specification of stereotyped, subtype-specific dendritic arbor shapes (Grueber et al. 2002) resulted in the identification of multiple transcription factors, encoded by abrupt [ab], knot/collier [kn/coll], spineless [ss], and cut [ct], which regulate dendritic arbor morphology (Grueber et al. 2003; Li et al. 2004, Sugimura et al. 2004, Kim et al. 2006; Hattori et al. 2007; Crozatier and Vincent 2008). However, large-scale genomic analyses comparing the transcriptomes of various neural subtypes indicate a daunting amount of varied gene expression and implicate regulation by multiple transcription factors (Sugino et al. 2006; Hattori et al. 2013; Iyer et al. 2013). Thus, a particular...
neuronal morphology is likely the result of coordination between multiple genomic programs.

Epigenetic modifications are one mechanism that could allow coordinated, genome-wide expression profiles. Chromatin is packaged into nucleosomes, where DNA nucleotides wrap an octamer of histone proteins. The chromatin structure can be altered through three main types of modifications, consisting of direct methylation of DNA nucleotides, post-translational histone-tail modifications such as acetylation and methylation, and ATP-dependent chromatin remodeling (Ho and Crabtree 2010). ATP-dependent chromatin remodelers were first shown to regulate dendrite morphology when RNAi knockdown of brahma (brm)-associated protein 60kD (Bap60), Bap55, and the ATPase brm altered the dendritic arbors of class I da sensory neurons (Parrish et al. 2006). In mammalian neurons, the neural-specific Brg/Brm-associated factor (BAF) complex (nBAF), which contains BAF53b and the ATPase Brg, regulates activity-dependent dendrite growth [Wu et al. 2007]. In addition, the Drosophila BAF53a/b homolog Bap55 regulates dendritic targeting of olfactory projection neurons [PNs] [Tea and Luo 2011].

The post-translational modification of histone tails involves three types of molecules: The “writers” add methyl, acetyl, or phosphate groups and consist of histone methyl transferase (HMT), histone acetyltransferase (HAT), and kinase enzymes. The “erasers” remove these modifications and include demethylases (DMTs), histone deacetyltransferases (HDACs), and phosphatases. Finally, the “readers” are scaffolding proteins that recognize and bind acetyl, methyl, or phosphate modifications to position the “writer” and “eraser” enzymes along with transcriptional machinery to the correct genomic position and thereby modify gene expression (Borrelli et al. 2008). The discovery that the Polycomb repressor complex, which binds methylated histone tails, regulates da sensory neuron dendrite morphology indicates a role of histone methylation in dendrite development (Parrish et al. 2007) and a notion supported by the recent finding that the chromodomain Y-like (CDYL) protein negatively regulates dendritic complexity [Qi et al. 2014]. Regarding a role of histone acetylation in dendrite morphogenesis, both HDAC and HAT activities have been implicated in regulating dendrite morphology. Specifically, the BET protein regulates neural morphology/function.

Results

fs(1)h1112 reduces higher-order dendritic arbor complexity

The fs(1)h1112 allele was created by ethyl methanesulfonate (EMS) mutagenesis during a previous forward genetic screen of lethal X-chromosome mutations [Zheng et al. 2008]. To circumvent the early embryonic lethality associated with this allele and assess the cell-autonomous function of fs(1)h in dendritic development, we employed mosaic analysis with a repressible cell marker [MARCM] [Lee and Luo 1999]. This technique allows the generation of homozygous mutant neuron clones marked by GAL4-driven fluorescent proteins within an essentially heterozygous animal. Using the pan-da neuron GAL4109(2)80 driver [Gao et al. 1999] to express membrane-bound UAS-mCD8-GFP, we visualized the dendritic arbors of single-cell sensory neuron clones within live, intact third instar Drosophila larvae. We analyzed these dendritic arbors through confocal imaging followed by digital reconstruction and compared the morphologies of the fs(1)h mutant and wild-type control clones.

The da sensory neurons consist of four subtypes (classes) of neurons [class I, II, III, and IV], categorized by their dendritic arbor morphology [Grueber et al. 2002]. The fs(1)h1112 mutation alters the normal morphology of different classes of da neurons to a varying degree. In class I and class II da neurons, the dendritic arbor phenotype was mild but significant, causing a decrease (~13%) in total class I da neuron dendritic length [control: 1947 μm ± 57 μm, n = 5 cells vs. fs(1)h1112; 1699 μm ± 66 μm, n = 4 cells, P = 0.028] [Supplemental Fig. S1G] and an ~41% decrease in class II da neuron branch points [control: 40 ± 5 branch points, n = 6 cells vs. fs(1)h1112; 23 ± 3 branch points, n = 5 cells, P = 0.017] [Supplemental Fig. S1H]. There was no significant change in class I da neuron branch points [control: 30 ± 1 branch points, n = 5 cells vs. fs(1)h1112; 29 ± 3 branch points, n = 4 cells, P = 0.937] [Supplemental Fig. S1G] or class II da neuron dendritic length [control: 2009 μm ± 84 μm, n = 6 cells vs. fs(1)h1112; 1778 μm ± 86 μm, n = 7 cells, P = 0.034] [Supplemental Fig. S1F].
in total length but we also assessed whether spikes represent the higher-order, fine dendritic branches, thinner, fine dendritic terminal branches. The class III lower-order, thicker, primary dendrites and higher-order, also observed a significant decrease (39%) in primary sensitivity can be measured through in vitro recordings responsive to gentle touch stimuli and their touch appears to affect mainly the smaller, terminal dendritic points, n = 3 cells vs. control: 488 ± 17 branch points, n = 3 cells; P < 0.001 [Supplemental Fig. S2E] and had ~74% fewer branch points [fs(1)h^1112]; 129 ± 26 branch points, n = 3 cells vs. control: 488 ± 17 branch points, n = 3 cells; P < 0.001 [Supplemental Fig. S2F]. As indicated by a Sholl analysis [Supplemental Fig. S2G], this reduction in branching occurred uniformly throughout the dendritic arbor.

The most striking phenotype was observed in class III da neuron dendritic arbor, which are characterized by small, filopodia-like protrusions (“spikes”) sprouting from their primary dendrites [Grueber et al. 2002] and giving them a furry appearance. Whereas the overall arbor size of wild-type [Fig. 1A] and fs(1)h^1112 class III da neurons [Fig. 1B] appeared similar, the dendritic spikes were nearly completely abolished in fs(1)h^1112 neurons [Fig. 1A’,B’]. Indeed, fs(1)h^1112 caused a severe (>90%) reduction of the spikes compared with control [fs(1)h^1112, 24 ± 2 spikes, n = 8 cells vs. control: 261 ± 45 spikes, n = 6 cells; P < 0.001] [Fig. 1E]. Dendritic arbor consist of lower-order, thicker, primary dendrites and higher-order, thinner, fine dendritic terminal branches. The class III spikes represent the higher-order, fine dendritic branches, but we also assessed whether fs(1)h^1112 altered primary branch morphology. We observed a smaller (26%) but significant reduction in primary dendrite branch length [fs(1)h^1112, 2107 μm ± 137 μm, n = 8 cells vs. control: 2830 μm ± 184 μm, n = 6 cells; P = 0.011] [Fig. 1F]. We also observed a significant decrease [39%] in primary dendrite branch point number [fs(1)h^1112, 11 ± 1 branch points, n = 8 cells vs. control: 18 ± 2 branch points, n = 6 cells, P = 0.008] [Fig. 1G], but this reduction was much less severe than the nearly complete reduction in spike morphology. Therefore, considering the phenotypes observed in all classes of da neurons, the fs(1)h^1112 mutation appears to affect mainly the smaller, terminal dendritic protrusions, and this effect is stronger in dendritic arbor of higher complexity. Since the class III da neurons are responsive to gentle touch stimuli and their touch sensitivity can be measured through in vitro recordings [Yan et al. 2013], we decided to focus on the role of fs(1)h in regulating both the structure and the function of class III dendritic arbor.

There are two splice isoforms of fs(1)h: a short [Fsh-S] and a long [Fsh-L] isoform [Supplemental Fig. S3A]. Defects in embryonic segmental transformations caused by fs(1)h mutations can be rescued by expressing Fsh-S [Chang et al. 2007]. Therefore, we attempted to rescue the class III dendritic arbor phenotype of fs(1)h^1112 with a UAS transgene containing Fsh-S. Expressing UAS-Fsh-S resulted in a partial rescue (57% of control) of the reduction of spikes [fs(1)h^1112,UAS-Fsh-S: 149 ± 15 spikes, n = 8 cells vs. fs(1)h^1112; 24 ± 2 spikes, n = 8 cells, P = 0.004] [Fig. 1E] caused by fs(1)h^1112 with a negligible effect on primary branch length [fs(1)h^1112,UAS-Fsh-S: 2316 μm ± 81 μm, n = 8 cells vs. fs(1)h^1112; 2107 μm ± 137 μm, n = 8 cells; P = 0.712] [Fig. 1F], or primary dendrite branch point number [fs(1)h^1112,UAS-Fsh-S: 13 ± 1 branch points, n = 8 cells vs. fs(1)h^1112; 11 ± 1 branch points, n = 8 cells; P = 0.662] [Fig. 1G]. To look for possible effects of Fsh-S overexpression using the GAL4/UAS system, we examined the arbor of class III da neurons with UAS-Fsh-S expression in wild-type and fs(1)h backgrounds [Fig. 1D]. The expression of UAS-Fsh-S in a wild-type background caused a reduction of spike number [UAS-Fsh-S: 136 ± 27 spikes, n = 7 cells vs. control: 261 ± 45 spikes, n = 6 cells; P = 0.01] [Fig. 1E] but no change in primary branch length [UAS-Fsh-S: 2709 μm ± 183 μm, n = 7 cells vs. control: 2830 μm ± 184 μm, n = 6 cells; P = 0.945] [Fig. 1F] or branch point number [UAS-Fsh-S: 15 ± 2 branch points, n = 7 cells vs. control: 18 ± 2 branch points, n = 6 cells; P = 0.442] [Fig. 1G]. These findings indicate that the dendritic arbor defect in spike morphology caused by fs(1)h^1112 can be partially rescued by expression of UAS-Fsh-S.

The rescue of the fs(1)h^1112 dendrite phenotype by expression of UAS-Fsh-S is only a partial rescue. Moreover, UAS-Fsh-S expression in wild-type da neurons caused a reduction of dendritic spikes. One possible explanation is that Fsh-S is required at a specific expression level such that either too little or too much expression would alter dendritic morphogenesis, as is observed with the transcription factor Ct [Grueber et al. 2003]. Since the rescue employs GAL4/UAS-mediated expression of Fsh-S, the expression level is likely higher than endogenous wild-type expression levels. To address this possibility, we took advantage of the temperature dependence of GAL4/UAS activity [Duffy 2002]. In our initial experiments, all flies were raised at 25°C, but we posited that moderating the expression level may yield a more favorable rescue. Therefore, we repeated the same experiment while raising the flies at a lower temperature [20°C] to attenuate GAL4/UAS expression [Duffy 2002]. We observed a significant increase in the number of class III da neuron spikes in the dendritic arbor of neurons expressing UAS-Fsh-S from both fs(1)h^1112 [fs(1)h] and UAS-Fsh-S/+ at 20°C: 149 ± 15 spikes, n = 8 cells vs. 20°C: 202 ± 10 spikes, n = 9 cells; P = 0.004] [Supplemental Fig. S4] and wild-type [UAS-Fsh-S/+; 25°C: 136 ± 27 spikes, n = 7 cells vs. 20°C: 198 ± 7 spikes, n = 9 cells; P = 0.032] [Supplemental Fig. S4] neurons. However, we still observed only a partial rescue of the fs(1)h^1112 reduced spike phenotype with UAS-Fsh-S expression at 20°C [fs(1)h^1112,UAS-Fsh-S/+; 20°C: 202 ± 10 spikes, n = 9 cells vs. control, 20°C: 319 ± 20 spikes, n = 8 cells; P < 0.001] [Supplemental Fig. S4] and a reduced spike phenotype with UAS-Fsh-S expression in a wild-type background at 20°C [UAS-Fsh-S/+; 20°C: 198 ± 7 spikes, n = 9 cells vs. control, 20°C: 319 ± 20 spikes, n = 8 cells; P < 0.001] [Supplemental Fig. S4]. This suggests that Fsh-S is probably still overexpressed at 20°C. We were unable to use a lower temperature for these experiments because, at 20°C, we observed a variable reduction of GFP intensity between animals, which caused an inability to visualize the morphology of some neurons for quantification. Our observa-
tions of GAL4/UAS expression at two different temperatures indicate that dendrite morphology is sensitive to the level of Fsh-S expression and that both too little and too much expression causes abnormal dendrite morphology.

**fs(1)h1112 causes loss of Fsh-S expression in da sensory neurons**

Since there are two protein isoforms of *fs(1)h* and we observed a partial rescue of *fs(1)h1112* dendrite arbor complexity with expression of UAS-Fsh-S, we wanted to determine the localization of endogenous Fsh protein. We addressed this question through the use of antibody staining against *fs(1)h* protein products (anti-Fsh). Two antibodies (Supplemental Fig. S3A) were used that recognize either both the short and long isoforms (anti-Fsh-S+L) or only the long isoform (anti-Fsh-L) (Chang et al. 2007). Consistent with a previous study (Chang et al. 2007), nuclear staining intensity was observed with anti-Fsh-S+L (Fig. 2A–C). However, we failed to detect any specific signal with anti-Fsh-L (Supplemental Fig. S5A–C). Anti-Fsh-S+L staining was observed in all da sensory neurons as well as epithelial cell nuclei (Fig. 2A–C). Thus, Fsh protein is expressed in all da sensory neurons.

To identify the Fsh isoforms expressed by the da neurons, we repeated the immunohistochemical experiments using a mutant [*fs(1)h17*] that lacks Fsh-L but retains Fsh-S (Chang et al. 2007). In *fs(1)h17* hemizygous mutants, the diffuse labeling of anti-Fsh-L (Supplemental Fig. S5G–I) appeared identical to the pattern observed in *fs(1)h17/+* heterozygous (Supplemental Fig. S5D–F) and wild-type (Supplemental Fig. S5A–C) larvae. Therefore, we conclude that the anti-Fsh-L labeling is nonspecific in the larval PNS tissue. Moreover, anti-Fsh-S+L labeling exhibited nearly identical patterns of staining in *fs(1)h17* hemizygous mutants (Fig. 2G–I) and wild-type (Fig. 2A–C) or *fs(1)h17/+* heterozygous (Fig. 2D–F) larvae. Therefore, we conclude that the da neurons express Fsh-S but probably not Fsh-L.

While these data strongly suggest that the da neurons do not express Fsh-L, we wanted to test the functional contribution of Fsh-L to class III da neuron morphology. Using GAL419-12 to express UAS-CD4-tdGFP in class III da sensory neurons, we visualized their dendritic arbor morphology in *fs(1)h17* mutants. Overall, the dendritic arbors appeared normal between *fs(1)h17/+* heterozygous (Supplemental Fig. S5J,J9) and *fs(1)h17* hemizygous mutants (Fig. 2A–D). In addition, the number of spike protrusions was identical between hemizygous and heterozygous mutants (Supplemental Fig. S5K,K9) and *fs(1)h17/+* heterozygous (Fig. 2D–F) larvae. Therefore, we conclude that Fsh-L does not appear to regulate class III dendrite morphology. In contrast, our data suggest the Fsh-S isoform regulates dendrite morphology.

The *fs(1)h1112* mutation consists of a small deletion within the second bromodomain (BD2) of the *fs(1)h* gene,
causing a premature stop codon, which could lead to the generation of a truncated protein [Supplemental Fig. S3A]. Thus, it is possible that the \( fs(1)h^{1112} \) allele creates a protein with a dominant-negative or gain-of-function effect. To assess whether any Fsh protein product is expressed in da neuron MARCM clones, we used the anti-Fsh-S+L antibody to analyze \( fs(1)h^{1112} \) da neuron clones, which recognizes an epitope [Supplemental Fig. S3A] preceding the premature stop codon. Comparing \( fs(1)h^{1112} \) with wild-type neurons from adjacent segments, we noticed a loss of staining in \( fs(1)h^{1112} \) class III clones (Fig. 2J–K). Thus, \( fs(1)h^{1112} \) appears to be a complete loss-of-function mutation.

The human \( fs[1]h \) homolog (huBRD2) substitutes for Fsh protein function in regulating dendrite morphology

Since \( fs(1)h^{1112} \) causes a loss of anti-Fsh-S+L labeling (Fig. 2) and \( UAS-Fsh-S \) can partially rescue the reduction in dendrite arbor complexity [Fig. 1], we wanted to assess whether the human homolog can functionally substitute for Fsh-S. Comparing the protein sequence of \( Drosophila \) Fsh-S with its human homolog, BRD2 [huBRD2], indicates 69% similarity of FSH-S to huBRD2 and 54% similarity of huBRD2 to Fsh-S [Supplemental Fig. S3B]. Within domains of known functional importance [the two bromodomains
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[BD1 and BD2] and the extraterminal [ET] domain, there is >70% similarity, indicating a high degree of conservation between Drosophila and human sequences. To test whether huBRD2 can rescue the dendrite phenotype of \( fs(1)h^{1112} \), we created a UAS-huBRD2 transgene to express the huBRD2 coding sequence in da sensory neuron clones.

In \( fs(1)h^{1112} \) class III da neurons, the expression of UAS-huBRD2 significantly increased the number of dendritic spikes [Fig. 3C] compared with \( fs(1)h^{1112} \) alone [Fig. 3B] \((fs(1)h^{1112};UAS-huBRD2/+: 119 \pm 10 \) spikes, \( n = 7 \) cells vs. \( fs(1)h^{1112} \): 31 \pm 3 \) spikes, \( n = 9 \) cells, \( P < 0.001 \)] [Fig. 3E]. Expression of UAS-huBRD2 in \( fs(1)h^{1112} \) class III da neurons did not alter the primary dendrite length \((fs(1)h^{1112};UAS-huBRD2/+: 2610 \mu m \pm 270 \mu m, n = 7 \) cells vs. \( fs(1)h^{1112}: 1951 \mu m \pm 142 \mu m, n = 9 \) cells, \( P = 0.05 \)] [Fig. 3F] or branch point number \((fs(1)h^{1112};UAS-huBRD2/+: 14 \pm 2 \) branch points, \( n = 7 \) cells vs. \( fs(1)h^{1112}: 10 \pm 1 \) branch points, \( n = 9 \) cells, \( P = 0.146 \)] [Fig. 3G]. These findings reveal that huBRD2 partially rescues the \( fs(1)h^{1112} \) dendrite spike morphology phenotype.

\( fs(1)h^{1112} \) affects initial establishment of dendritic arbor complexity

The reduction in dendrite complexity caused by \( fs(1)h^{1112} \) could be the result of a failure to establish dendritic branch complexity or a compromised maintenance of dendritic complexity during development. Thus far, we only assessed the dendritic morphology at the late third instar stage, corresponding to \( \approx 100 \) h after egg laying (AEL). To determine whether \( fs(1)h^{1112} \) impairs establishment and/or maintenance of dendritic arbor complexity, we performed a time-course study comparing dendritic arbors of class III da neuron MARCM clones from wild-type control [Fig. 4A–C] and \( fs(1)h^{1112} \) [Fig. 4D–F] genotypes over three developmental stages (48, 72, and 96 h AEL). Class III wild-type da neuron arbors continually increased their number of spikes [Fig. 4G] from 48 to 96 h AEL (48 h AEL: 69 \pm 4 \) spikes, \( n = 4 \) cells; 72 h AEL: 118 \pm 16 \) spikes, \( n = 5 \) cells; 96 h AEL: 178 \pm 15 \) spikes, \( n = 4 \) cells) as their primary arbor lengthened [48 h AEL: 1100 \mu m \pm 77 \mu m, n = 4 \) cells; 72 h AEL: 2119 \mu m \pm 134 \mu m, \( n = 5 \) cells; 96 h AEL: 2201 \mu m \pm 65 \mu m, \( n = 4 \) cells] as their primary arbor lengthened [48 h AEL: 1100 \mu m \pm 77 \mu m, n = 4 \) cells; 72 h AEL: 2119 \mu m \pm 134 \mu m, \( n = 5 \) cells; 96 h AEL: 2201 \mu m \pm 65 \mu m, \( n = 4 \) cells] and their primary arbor lengthened [48 h AEL: 1100 \mu m \pm 77 \mu m, n = 4 \) cells; 72 h AEL: 2119 \mu m \pm 134 \mu m, \( n = 5 \) cells; 96 h AEL: 2201 \mu m \pm 65 \mu m, \( n = 4 \) cells] as their primary arbor lengthened [48 h AEL: 1100 \mu m \pm 77 \mu m, n = 4 \) cells; 72 h AEL: 2119 \mu m \pm 134 \mu m, \( n = 5 \) cells; 96 h AEL: 2201 \mu m \pm 65 \mu m, \( n = 4 \) cells] and their primary arbor lengthened [48 h AEL: 1100 \mu m \pm 77 \mu m, n = 4 \) cells; 72 h AEL: 2119 \mu m \pm 134 \mu m, \( n = 5 \) cells; 96 h AEL: 2201 \mu m \pm 65 \mu m, \( n = 4 \) cells] as their primary arbor lengthened [48 h AEL: 1100 \mu m \pm 77 \mu m, n = 4 \) cells; 72 h AEL: 2119 \mu m \pm 134 \mu m, \( n = 5 \) cells; 96 h AEL: 2201 \mu m \pm 65 \mu m, \( n = 4 \) cells].

Figure 3. The \( fs(1)h^{1112} \) dendritic arbor phenotype can be rescued by the human \( fs(1)h \) homolog huBRD2. (A–D) Dendritic arbors of class III da neuron MARCM clones in a control background (yw, FRT19A; sop-FLP, GAL4109(2)80,UAS-mCD8-GFP/+)) and crossed to various mutant and transgenic flies as indicated. Bar, 50 \mu m. The loss of spikes caused by \( fs(1)h^{1112} \) (B) is partially reversed by expression of UAS-huBRD2 (C) compared with the control (A). Bar, 50 \mu m. (E–G) Quantification of dendritic arbor morphology by number of spikes (E), primary branch length (F), and primary branch points (G). The loss of spikes in \( fs(1)h^{1112} \) clones is partially rescued by UAS-huBRD2. The \( fs(1)h^{1112} \)-induced decrease in primary dendritic branch length (F) and primary branch points (G) is not rescued by UAS-huBRD2 expression. The values are reported as mean \pm SEM, and P-value statistical significance is reported by results of one-way ANOVA followed by post-hoc Tukey test.
dendrite length (48 h AEL: 1496 μm ± 333 μm, n = 4 cells; 72 h AEL: 1448 μm ± 29 μm, n = 3 cells; 96 h AEL: 1959 μm ± 37 μm, n = 5 cells), although this increase was delayed compared with wild-type clones [Fig. 4H]. Since there is some increase in dendritic length without an increase in higher-order dendritic spikes, our data suggest that fs(1)h1112 impairs the initial establishment of dendritic arbor complexity.

A change in finer branch dynamics could explain the reduced dendritic complexity phenotype. To test this hypothesis, we performed acute time-lapse imaging of MARCM clones at 72 h AEL over a 30-min period. The terminal branch dynamics were classified as growing, retracting, or stable at each time point. We observed no change in the amount of class III da neuron spike growth between the control and fs(1)h1112 mutant neurons [wild type: 9.5% ± 1.0%, fs(1)h1112: 12.8% ± 1.4%, P = 0.06] [Fig. 4J]. However, the amount of retracting spikes increased in fs(1)h1112 neurons [wild type: 16.7% ± 1.9%, fs(1)h1112: 25.7% ± 2.3%, P = 0.005] [Fig. 4J], while the amount of stable spikes was reduced [wild type: 73.8% ± 2.5%, fs(1)h1112: 61.5% ± 2.6%, P = 0.002] [Fig. 4J]. Therefore, the spikes of class III da neurons lacking Fsh-S show a greater tendency to retract and reduced stability, which cause a failure to establish higher-order dendritic spikes.

fs(1)h interacts with genetic programs regulating dendritic development

We next wanted to explore the mechanism of how fs(1)h regulates dendritic morphology. One idea posits that neuron subtype-specific morphology arises from expression of specific combinations of molecules [Jan and Jan 2010]. Previous studies have identified a variety of proteins expressed in da neurons that regulate dendrite morphology [Grueber et al. 2003; Li et al. 2004; Sugimura et al. 2004; Kim et al. 2006; Hattori et al. 2007; Jinushi-Nakao et al. 2007]. Therefore, it is possible that fs(1)h could regulate
the combinatorial expression of molecular factors governing dendrite development. In particular, the transcription factor encoded by ct regulates the dendritic spikes of class III da neuron arbors. To test this hypothesis, we first examined the expression of Ct protein in fs(1)h1112 da neuron clones. We observed a loss of anti-Ct staining intensity in class III da neurons [Fig. 5A–F]. Next, we tested whether expression of UAS-Ct could rescue the fs(1)h1112 dendrite phenotype. Although expression of UAS-Ct in fs(1)h1112 clones seemed to mildly affect induction of spike formation [Fig. 5G, g,g''], this effect was variable throughout the dendritic arbor, and we did not observe a statistically significant increase in spike number [fs(1)h1112;UAS-Ct+/+: 48 ± 4 spikes, n = 7 cells vs. fs(1)h1112: 36 ± 4 spikes, n = 9 cells; P = 0.989] [Fig. 5I]. Since fs(1)h1 binds acetylated histones to alter transcription, it is possible that expression of the UAS-Ct transgene was altered in fs(1)h1112 mutants. To address this concern, we examined the expression of Ct protein with immunohistochemistry in fs(1)h1112 class III da neuron clones. In this experiment, we observed a high expression of Ct protein [Supplemental Fig. S6]. Thus, we confirmed that Ct protein was expressed by UAS-Ct, and therefore, Ct protein expression in fs(1)h1112 mutant neurons cannot rescue the loss of class III dendritic spike morphology.

The transcription factor Ct regulates class-specific dendrite morphology and is thought to act by regulating a cascade of molecular factors. Therefore, fs(1)h may regulate some of these downstream components, which could explain why expression of UAS-Ct did not rescue the class III dendrite phenotype caused by loss of Fsh. Ectopic expression of UAS-Ct in class I da neurons causes extensive overbranching of their normally simple arbors as well as the formation of ectopic dendritic spikes similar to those observed in class III da neurons [Grueber et al. 2003]. This paradigm can be used to probe whether fs(1)h1112 alters Ct-induced branching and spike formation. As previously shown [Grueber et al. 2003], expressing UAS-Ct in wild-type class I da neurons [Fig. 6B] caused an 86% increase in total dendrite length [UAS-Ct+/+: 3710 μm ± 265 μm, n = 4 cells vs. wild type: 1996 μm ± 71 μm, n = 5 cells; P < 0.001] [Fig. 6H], a 287% increase in branch point number [UAS-Ct+/+: 116 ± 13 branch points, n = 4 cells vs. wild type: 30 ± 1 branch points, n = 5 cells; P < 0.001] [Fig. 6G], and the formation of dendritic spike morphology [Fig. 6B]. In fs(1)h1112 class I da neuron clones, this induction of overbranching by UAS-Ct expression was abolished [Fig. 6C] with no increase in dendrite length [fs(1)h1112;UAS-Ct+/+: 1930 μm ± 23 μm, n = 4 cells vs. wild type: 1996 μm ± 71 μm, n = 5 cells; P = 0.985] [Fig. 6H], branch point number [fs(1)h1112;UAS-Ct+/+: 43 ± 6 branch points, n = 4 cells vs. wild type: 30 ± 1 branch points, n = 5 cells; P = 0.533] [Fig. 6F], or the emergence of spikes [Fig. 6C]. Finally, we tested whether UAS-Fsh-S expression can induce an overbranching phenotype in class I da neurons [Fig. 6D]. This did not occur, and dendritic arbors of fs(1)h1112 mutant class I da neurons expressing UAS-Fsh-S appeared identical to wild-type class I arbors with similar dendritic length [UAS-Fsh-S+/+: 2265 μm ± 95 μm, n = 4 cells vs. wild type: 1996 μm ± 71 μm, n = 5 cells; P = 0.517] [Fig. 6H], branch point number [UAS-Fsh-S+/+: 29 ± 2 branch points, n = 4 cells vs. wild type: 30 ± 1 branch points, n = 5 cells; P = 0.999] [Fig. 6G], and lack of spikes. Thus, fs(1)h is necessary for UAS-Ct-induced overbranching and spike formation in class I da neuron arbors, but UAS-Fsh-S overexpression does not cause any change in class I dendritic morphology.

The spike morphology of class III da neuron dendritic arbors is known to be F-actin-rich [Grueber et al. 2002] and can be altered by modulating the activity of the GTPase Rac1 [Andersen et al. 2005; Tsubouchi et al. 2012]. As mentioned earlier, ectopic expression of Ct causes spike formation in class I da neuron dendritic arbors [Grueber et al. 2003]. Ectopic Rac1 expression causes increased branching in class I da neuron arbors, which is similar but not identical to class III da neuron spikes. Interestingly, coexpression of Ct and Rac1 synergistically enhances the formation of spike morphology in class I da neurons [Jinushi-Nakao et al. 2007]. Therefore, Ct and Rac1 function in concert to specify the dendritic arbor shape of class III da neurons. For this reason, we wanted to test whether the loss of dendritic spikes in fs(1)h1112 mutant class III da neurons could be rescued by UAS-Rac1. Indeed, we observed a significant (57%) control) increase in spike number after UAS-Rac1 expression in fs(1)h1112 mutant class III da neuron clones [fs(1)h1112,UAS-Rac1+/+: 149 ± 16 spikes, n = 9 cells vs. fs(1)h1112, 36 ± 4 spikes, n = 9 cells; P < 0.001] [Fig. 6G, h, h', l]. This rescue was partial but similar to that achieved with UAS-Fsh-S expression in fs(1)h1112 class III da neurons [Fig. 1]. Moreover, in agreement with a previous report [Jinushi-Nakao et al. 2007], when we ectopically expressed UAS-Rac1 in class I da neurons [Fig. 6E], we observed a significant increase in branch points [UAS-Rac1+/+: 79 ± 10 branch points, n = 7 cells vs. wild type: 30 ± 1 branch points, n = 5 cells; P = 0.001] [Fig. 6G], although to a much less degree than observed with UAS-Ct [Fig. 6G, H]. In contrast to ectopic UAS-Ct, expression of UAS-Rac1 did not significantly alter total dendrite length [UAS-Rac1+/+: 2394 μm ± 94 μm, n = 7 cells vs. wild type: 1996 μm ± 71 μm, n = 5 cells; P = 0.118] [Fig. 6H]. Finally, we tested whether the UAS-Rac1-induced increase in class I da neuron dendritic branching was altered in fs(1)h1112 mutants [Fig. 6F]. Unlike the abolition of Ct-induced overbranching by fs(1)h1112, the Rac1-induced overbranching was unaltered [fs(1)h1112; UAS-Rac1+/+: 89 ± 5 branch points, n = 8 cells vs. UAS-Rac1+/+: 79 ± 10 branch points, n = 7 cells, P = 0.886] [Fig. 6G]. Thus, enhanced Rac1 expression can partially rescue the fs(1)h1112 class III da neuron dendritic phenotype, and fs(1)h is necessary for Ct-induced dendritic branching but not Rac1-induced branching.

Because Ct is also expressed in class II and IV da neurons, we also assessed whether the fs(1)h1112 mutation altered Ct expression in all da neuron subtypes. Similar to our observations in fs(1)h1112 mutant class III da neurons, Ct expression was lost in both class II [Supplemental Fig. S7B,E,H] and class IV [Supplemental
Fig. S7C,F,I) mutant da neurons. Since Ct is normally not expressed in class I da neurons, we asked whether the \(fs(1)h^{1112}\) mutation caused misexpression of Ct. This was not the case, and we did not detect any Ct expression in \(fs(1)h^{1112}\) mutant class I da neurons (Supplemental Fig. S7A,D,G). Therefore, \(fs(1)h\) regulates the expression of Ct in the da neurons that normally express Ct.

Because overexpression of Fsh-S also caused a dendrite phenotype similar to \(fs(1)h^{1112}\) mutant class III da neurons [Fig. 1], we tested whether overexpression of Fsh-S affected Ct expression and/or Ct-induced branching/spike formation. First, we examined the pattern of anti-Ct antibody labeling in larvae expressing \(UAS-Fsh-S\) under the control of a pan-da neuron GAL4 driver (GAL4\(^{109(2)80}\)). We did not observe a change in anti-Ct signal between control (Supplemental Fig. S8A,C,E) and \(UAS-Fsh-S\)-expressing (Supplemental Fig. S8B,D,F) da neurons. These data suggest that Fsh-S overexpression does not lead to decreased Ct expression as observed in \(fs(1)h^{1112}\) mutants and that Fsh-S overexpression does not induce misexpression of Ct in da neurons that normally do not express Ct.

Second, we assayed whether Fsh-S overexpression can affect Ct-induced branching/spike formation in class I da neurons. Using the class I da neuron GAL4\(^{2-23}\) driver [Gruene et al. 2003], we overexpressed \(UAS-Fsh-S\) along with \(UAS-Ct\) [Supplemental Fig. S9]. As observed previously [Gruene et al. 2003], \(UAS-Ct\) expression in class I da neurons caused an increase in total dendrite length [control: 1733 \(\mu m\) \(\pm\) 65 \(\mu m\), \(n = 10\) cells vs. \(UAS-Ct/+: 4149 \mu m \pm 143 \mu m, n = 9\) cells, \(P < 0.001\)] [Supplemental Fig. S9E] and branch points [control: 31 \(\pm\) 2 branch points, \(n = 10\) cells vs. \(UAS-Ct/+: 40 \pm 2\) branch points, \(n = 9\) cells, \(P < 0.001\)] [Supplemental Fig. S9F], with the production of class III da neuron-like morphology [Supplemental Fig. S9B,h’], which we also observed in our MARCM experiments [Fig. 6]. Similar to our MARCM
observations [Fig. 6], UAS-Fsh-S expression in class I da neurons caused no alteration in total dendrite length [control: 1733 μm ± 56 μm, n = 10 cells vs. UAS-Fsh-S/+: 1765 μm ± 37 μm, n = 9 cells; P = 0.995] [Supplemental Fig. S9E] or branch points [control: 31 ± 2 branch points, n = 10 cells vs. UAS-Fsh-S/+: 26 ± 1 branch points, n = 9 cells; P = 0.956] [Supplemental Fig. S9F]. When UAS-Fsh-S and UAS-Ct were overexpressed together in class I da neurons, we still observed the production of class III da neurons caused no alteration in total dendrite length [control: 1733 μm ± 56 μm, n = 10 cells vs. UAS-Ct/+;UAS-Fsh-S/+: 3588 μm ± 121 μm, n = 10 cells; P = 0.002] [Supplemental Fig. S9E] and branch points [UAS-Ct/+: 149 ± 8 branch points, n = 9 cells vs. UAS-Ct/+;UAS-Fsh-S/+: 109 ± 9 branch points, n = 10 cells; P < 0.001] [Supplemental Fig. S9F]. These data suggest that Fsh-S overexpression attenuates UAS-Ct-induced branching, which could explain why UAS-Fsh-S expression causes only a partial rescue of the fs(1)h1112 mutant phenotype and also why UAS-Fsh-S overexpression causes a dendrite phenotype similar to fs(1)h1112.

Having found dendritic phenotypes in all da neuron subtypes, we further examined whether fs(1)h1112 altered the expression of other transcription factors known to regulate the subtype-specific dendritic morphology of da neurons. Specifically, we used antibodies against the class I da neuron-specific transcription factor encoded by ab [Li et al. 2004] and the class IV-specific kn/col [Crozatier and Vincent 2008]. Interestingly, we observed a loss of both Ab and Kn/Col in fs(1)h1112 mutant class I [Supplemental Fig. S10] and class IV [Supplemental Fig. S11] da neurons, respectively. Collectively, these data suggest that fs(1)h regulates subtype-specific expression of transcription factors. It affects not only the expression of ct across multiple neural subtypes but also transcription factors in pathways acting in parallel to ct, such as ab and kn/col.

fs(1)h1112 impairs the mechanosensory response of da sensory neurons

A recent study discovered that NompC mediates gentle touch behavior and mechanosensitivity in class III da neurons [Yan et al. 2013]. Since the fs(1)h1112 mutation alters the morphology of class III da neurons, we tested whether fs(1)h modulates their touch sensitivity. Since fs(1)h1112 mutants are lethal, we could not use whole-
animal mutants. Instead, we used GAL4\textsuperscript{19-12} to express a UAS-RNAi construct (v51227) against fs(1)h in class III da neurons to knock down fs(1)h expression selectively in these neurons. As a control for the effectiveness of the RNAi construct, we tested whether UAS-RNAi expression altered class III da neuron spike morphology. Indeed, RNAi-mediated knockdown of fs(1)h caused a significant decrease in spike number (v51227 RNAi: 151 ± 9 spikes, n = 6 cells vs. wild type: 260 ± 9 spikes, n = 3 cells; P < 0.001) [Fig. 7A]. Using a separate RNAi line (v108662), we observed a similar decrease in spike number (v108662 RNAi: 151 ± 8 spikes, n = 6 cells vs. wild type: 260 ± 9 spikes, n = 3 cells; P < 0.001) [Fig. 7A]. This decrease was enhanced in a genetic background containing a heterozygous deletion mutant \((D/f(x)c128)\) of fs(1)h \(D/f(x)c128/v, v108662\) RNAi: 96 ± 9, n = 6 cells vs. wild type: 260 ± 9, n = 3 cells; \(P < 0.001\) [Fig. 7A]. Thus, RNAi-mediated knockdown of fs(1)h expression reduced spike morphology in class III da neuron dendritic arbors.

We next tested whether animals expressing RNAi constructs against fs(1)h exhibited any defects in gentle touch behavior. A previous genetic screen used a gentle touch assay to identify touch-insensitive mutants [Kernan et al. 1994], and this assay was used to determine that class III da neurons mediate this gentle touch behavior [Yan et al. 2013]. Using the same assay, we observed a significant \((P = 0.02)\) reduction in touch response score in animals expressing fs(1)h RNAi [Fig. 7B]. To determine whether the fs(1)h RNAi affects mechanosensation of class III da neurons as opposed to subsequent signaling in the CNS, we performed extracellular recordings from class III da neurons while delivering a touch stimulus of varying intensity. Control class III da neurons responded with a progressively higher frequency of action potentials [APs] as the touch stimulus intensity increased [Fig. 7C,D]. Although class III da neurons expressing fs(1)h RNAi also showed a dose-dependent response with increasing stimulus intensity [Fig. 7C], this response was significantly reduced compared with control neurons [Fig. 7C,D]. Therefore, knockdown of fs(1)h expression impairs the response of class III da neurons to touch.

Since NompC is the channel mediating the mechanosensitive response of class III da neurons, one possible explanation for their defective response is a loss or reduction of NompC expression. To address this possibility, we performed immunohistochemistry with an anti-NompC antibody in fs(1)h\textsuperscript{1112} class III clones. In both wild-type and fs(1)h\textsuperscript{1112} neurons, we observed anti-NompC signal [Supplemental Fig. S12] similar to a pre-mature signal (Supplemental Fig. S12G) similar to a previous study [Yan et al. 2013]. Therefore, the impaired sensory response of class III da neurons expressing fs(1)h RNAi is not due to a loss of NompC expression.

To test whether the defective response to gentle touch reflects an alteration of sensory neuron axon structure, we examined the axon terminals of fs(1)h\textsuperscript{1112} mutant class III da neurons. The axon terminals appeared similar between wild-type control [Fig. 7E] and fs(1)h\textsuperscript{1112} mutant [Fig. 7F] class III da neurons. In addition, we did not find a significant change in axon terminal length [fs(1)h\textsuperscript{1112}: 92 μm ± 13 μm, n = 13 cells vs. wild type: 108 μm ± 11 μm, n = 13 cells; \(P = 0.353\) ] (Fig. 7G). Therefore, the reduction of gentle touch response by fs(1)h\textsuperscript{1112} is not due to impaired axon terminal morphology. Rather, the fs(1)h\textsuperscript{1112} mutation altered dendrite morphology of class III da neurons without affecting NompC expression or axon morphology.

Recently, it was discovered that ectopic UAS-Ct expression in class I da neurons induces expression of NompC, which is normally expressed in class III, but not class I, da neurons. Since we observed a loss of Ct expression but normal expression of NompC in fs(1)h\textsuperscript{1112} mutant class III da neurons [Supplemental Fig. S12], we wanted to test whether fs(1)h is required for the Ct-induced expression of NompC in class I da neurons. When we performed anti-NompC immunostaining of class I da neuron fs(1)h\textsuperscript{1112} MARCM clones expressing UAS-Ct, we observed expression of NompC [Supplemental Fig. S13C,D], which was not observed in wild-type class I da neurons [Supplemental Fig. S13A,B].

We also observed UAS-Ct-induced NompC expression from class I da neurons in a fs(1)h\textsuperscript{1112} mutant background [Supplemental Fig. S13E,F]. Therefore, fs(1)h\textsuperscript{1112} does not alter Ct-induced NompC expression, which is interesting because fs(1)h\textsuperscript{1112} does block the Ct-induced overbranching [Fig. 6]. These data suggest that fs(1)h regulates genes involved in specifying dendritic arbor morphology but not ion channel expression.

Discussion

fs(1)h regulates dendritic arbor complexity

In the current study, we examined the role of fs(1)h in dendritic development. We analyzed the effect of a loss-of-function allele \([fs(1)h\textsuperscript{1112}]\) on the morphology of class III da sensory neurons in the Drosophila PNS. Overall, fs(1)h\textsuperscript{1112} causes a reduction in dendritic arbor complexity, most notably in the finer, higher-order branches. We were able to partially rescue this reduced morphological complexity by reintroducing Drosophila Fsh-S or the human homolog \((huBRD2)\) proteins.

Furthermore, we found one aspect of the genetic mechanism of action for fs(1)h to be regulating the expression of ct [and possibly other genes in the pathway] in multiple da neuron subtypes as well as subtype-specific transcription factors, such as Abrupt for class I and Knot/Collier for class IV da neurons, which in turn affect subtype-specific dendrite development. Our data show that fs(1)h regulates genetic pathways controlling dendritic arbor development but does not specify which ion channels are expressed. Finally, our results suggest that the subtype-specific spike morphology is important for an optimal response to relevant sensory stimuli in the mechanosensitive class III da neurons.

The development of a dendritic arbor involves multiple steps [Whitford et al. 2002; Corty et al. 2009; Puram and Bonni 2013] beginning with differentiation, where a neuronal precursor acquires a neural fate. Next, neurites...
begin to extend, and a neuron becomes polarized as neurites are designated as axon or dendrite. The immature axons and dendrites continue to grow as the neuron and the nervous system develop. Initially, the dendritic arbors are simple, with only a few primary dendrites, but as development progresses, the number of branches and overall arbor size increases. The terminal dendrite branches are dynamic throughout development, exhibiting growth, retraction, or stability. In addition, as the animal body size increases, the dendritic field area increases, and therefore a dendritic arbor must scale accordingly. Thus, dendritic development involves a complex plethora of processes, and dendritic morphology could be altered by affecting any of these processes. For instance, if the balance of dendrite dynamics is shifted such that retraction is greater than growth, then dendritic branching will become reduced over time. This appears to be the case in \(fs(1)h^{1112}\) mutants, since we observed an increase in retracting branches with no change in growth as well as a decrease in the proportion of stable branches [Fig. 4] in dendritic arbors of \(fs(1)h^{1112}\) mutant da neuron clones compared with wild-type clones. Alternatively, if scaling of the dendritic arbor is affected, the size of the dendritic arbor will become disproportionately small as the body size of the animal increases throughout development (Parrish et al. 2009). This does not seem to occur in \(fs(1)h^{1112}\) mutants because the primary dendrites of \(fs(1)h^{1112}\) arbors exhibited growth throughout development, although at a delayed rate [Fig. 4]. Instead, the number of spikes in class III da neuron arbors was reduced early in development and remained reduced throughout development [Fig. 4], probably due to the increased amount of dendritic branch retraction and reduced stability. Since the primary dendritic branches were not affected to a large degree by loss of \(fs(1)h\) function, we conclude that the major role of \(fs(1)h\) in dendritic development is to regulate dendritic complexity at the level of higher-order dendritic spikes. Moreover, our data suggest that \(fs(1)h\) affects dendritic arbor complexity by modulating the dynamics of terminal dendritic branches.

\(fs(1)h\) is necessary for Ct expression and Ct-induced spike formation

In the da neurons, many molecules are known to regulate dendrite morphology. In particular, Ct, Ss, Ab, and Kn have been shown to regulate subtype-specific morphology of the four classes of da sensory neurons, and these proteins act in parallel genetic pathways (Grueber et al. 2004). Figure 7. Altering the number of spikes correlates with a reduced mechanosensitive response. (A) Quantification of spike morphology in class III da neurons expressing \(fs(1)h\) RNAi in a control background (UAS-Dcr2/+;GAL4\(^{19-12}\),UAS-CD4-tdGFP/+). The expression of two different RNAi constructs (v51227 or v108662) causes a decrease in the number of spiked protrusions. This decrease is enhanced in a genetic background heterozygous for a \(fs(1)h\) deletion [Df(X)c128], while one-copy of Df(X)c128 alone did not cause a phenotype. (B) Expression of \(fs(1)h\) RNAi also caused a defect in the behavioral response to a gentle touch stimulus. (C,D) Extracellular electrophysiological recordings from class III neurons expressing \(fs(1)h\) RNAi indicated a reduction in the number of action potentials [APs] fired per second [no. of AP/s] for a mechanical stimulus of a particular intensity compared with baseline [Δ no. of AP/s] (shown in C). (D) A representative recording trace is shown from control and RNAi-expressing cells for a stimulus of 20-μm intensity. (E,F) Images of single axon terminals from class III (ddaF) da neuron MARCM clones. Bar, 10 μm. Axon terminals from wild-type control [E] or \(fs(1)h^{1112}\) mutant [F] neurons appear similar. (G) Quantification of axon terminal length shows no difference between control and \(fs(1)h^{1112}\) neurons. Values are reported as mean ± SEM, with statistical significance reported as results of Student’s t-test.
2003; Li et al. 2004; Sugimura et al. 2004; Kim et al. 2006; Hattori et al. 2007; Crozatier and Vincent 2008). Moreover, the expression of Ct and Ss regulates class III da neuron spike morphology (Grueber et al. 2003; Kim et al. 2006). We observed a loss of Ct expression in fs(1)h1112 mutant class III da neuron clones (Fig. 5), which suggests that fs(1)h regulates the induction or maintenance of Ct expression throughout class III da neuron development. However, reintroducing Ct expression to class III da neuron fs(1)h1112 clones did not rescue the nearly absent spike morphology (Fig. 5). Therefore, the class III da neuron dendritic phenotype caused by loss of fs(1)h cannot be solely attributed to the loss of Ct protein. Since it is thought that Ct is a component of a genetic pathway responsible for subtype-specific dendritic arbor development, it is possible that fs(1)h regulates Ct expression as well as expression of genes necessary for the Ct pathway to affect dendritic morphology. Therefore, the relationship between ct and fs(1)h does not appear to be a linear pathway, and fs(1)h might regulate both upstream and downstream components of ct. Our data indicating that fs(1)h is necessary for the Ct-induced overbranching and spike formation in class I da neuron dendrites (Fig. 6) support the idea that fs(1)h regulates the expression of downstream components of the Ct pathway, which are necessary for Ct-induced overbranching and spike formation. This hypothesis also explains why reintroducing Ct expression to fs(1)h1112 clones fails to rescue the dendrite phenotype. It is also known that Ct and Rac1 act synergistically to produce spike morphology (Jinushi-Nakao et al. 2007). We examined Rac1 overexpression in a fs(1)h1112 mutant background and found that Rac1 expression significantly rescued the loss of spikes in class III da neurons (Fig. 5). However, Rac1-induced overbranching in class I da neurons was not affected by fs(1)h1112 (Fig. 6). Therefore, fs(1)h does not appear to regulate genes downstream from Rac1 but does regulate genes downstream from ct. Since these pathways are known to converge in order to regulate dendritic spine formation, our data suggest that fs(1)h may be a crucial link between these two pathways. One possible scenario is that ct and Rac1 regulate parallel pathways, but ct may regulate the level of Rac1 expression such that increased Rac1 expression facilitates the formation of spikes. In this model, our results support the hypothesis that fs(1)h is necessary for the ct potentiation of Rac1 expression, which explains why increased expression of Rac1 with UAS-Rac1 causes a rescue of the class III da neuron dendritic phenotype in fs(1)h1112 mutants. Recent evidence indicates a role for reduced Rac1 expression in social defeat and depressive behavior in mice, possibly through regulating dendritic spine morphology (Golden et al. 2013). In these behavioral paradigms, reduced Rac1 expression occurred with altered epigenetic marks such that transcriptionally permissive histone H3 acetylation was reduced, while repressive histone H3 methylation was increased. Moreover, administering a class I HDAC inhibitor mitigated the reduced Rac1 expression. Thus, these data suggest that Rac1 expression can be regulated by histone acetylation. It is possible that epigenetic reader proteins, such as BET family proteins like fs(1)h, bind acetylated histone marks in the Rac1 promoter to recruit transcriptional machinery and in turn enhance Rac1 expression.

In addition, overexpression of UAS-Fsh-S in class I da neurons did not cause an overbranching phenotype similar to UAS-Ct (Fig. 6). In fact, there was no alteration of class I morphology, suggesting that Fsh-S is not sufficient to induce necessary components of the ct pathway to alter dendrite morphology. However, overexpression of Fsh-S in class III (Fig. 1) and class IV (Supplemental Fig. S2) da neurons did cause a decrease in dendritic spike numbers. These data indicate that dendrite morphology may be sensitive to the amount of Fsh-S expression, which we confirmed by modulating the amount of overexpression by reducing GAL4/UAS activity with lower temperature (Supplemental Fig. S4). This may explain why we can achieve only a partial rescue of the fs(1)h1112 dendritic phenotype with UAS-Fsh-S expression and why overexpression causes a dendritic phenotype similar to the phenotype caused by loss of Fsh-S. In support of this expression level hypothesis, we observed that Fsh-S overexpression can reduce Ct-induced branching in class I da neurons (Supplemental Fig. S9). Since BRD2 is known to be part of a protein complex (Denis et al. 2006), it is possible overexpression causes a gain-of-function or dominant-negative effect by altering the availability of complex components.

Another possible explanation for the partial rescue of Fsh-S expression concerns the developmental timing of expression. Since these experiments were completed using MARCM, GAL80 is expressed until mitotic recombination occurs to generate the mutant clones. It is likely that GAL80 protein may persist for some time after the clones are formed, and the presence of GAL80 would block GAL4/UAS activity. Therefore, our UAS-induced Fsh-S expression may occur at a delayed stage in embryonic development, which could produce a partial rescue. In actuality, a combination of both expression level and developmental timing probably explains the partial rescue of the fs(1)h1112 phenotype.

While we focused on the role of fs(1)h in regulating class III da neuron dendrite morphology, we did observe phenotypes in other classes of the da neurons as well as expression of Fsh-S in all da neuron classes. In fs(1)h1112 mutants, we observed a loss of Ct expression in all da neurons that normally express Ct (classes II, III, and IV) (Fig. 2, Supplemental Fig. S7), suggesting that fs(1)h regulates Ct expression broadly among different neural subtypes. We also observed a loss of the class I-specific transcription factor Ab (Supplemental Fig. S10) and the class IV-specific transcription factor Kn/Col (Supplemental Fig. S11). Thus, it appears that fs(1)h can regulate the expression of subtype-specific gene expression among various neuron subtypes. The loss of Ct or the loss of Kn/Col could explain the reduction in class IV da neuron dendritic arbor complexity, and this further illustrates the pleiotropic nature of the fs(1)h1112 phenotype. The loss of Ab from class I da neurons should produce an increase in dendritic complexity (Li et al. 2004), but interestingly, this did not occur in fs(1)h1112 mutants. Thus, these results consistently suggest that fs(1)h is necessary for
BET protein regulates neural morphology/function

Dendritic arbor morphology contributes to mechanosensory neuron function

Finally, our results suggest that the specific morphological shape of the class III da neuron dendrites is important for their ability to appropriately respond to sensory stimuli. Our results indicate that pathways regulating dendrite morphology, such as the ct pathway, are reduced in fs(1)h mutants, but other pathways involved in axon morphogenesis or cell type-specific physiology, such as NompC channel expression, remain active. Moreover, the number of spike protrusions correlates with the number of APs produced in response to a mechanosensitive stimulus [Fig. 7]. This was also observed in another study (Tsubouchi et al. 2012) involving manipulation of the number of spiked protrusions through modulating Rac1 activity. In that study, the gentle touch response increases as spike numbers increase, causing elevated calcium activity detectable with GCaMP fluorescence imaging. Conversely, decreasing the spike numbers results in a decrease of the gentle touch response and calcium activity. One potential caveat to this study is that Rac1 can modulate many aspects of dendritic cell biology through modulating actin cytoskeletal dynamics [Hotulainen and Hoogenraad 2010], and therefore it is unclear whether manipulating Rac1 activity alters the electrophysiological properties or localization of ion channels such as NompC. Our finding of a correlation between dendritic spike number and gentle touch/electrophysiological responses in fs(1)h mutant neurons with normal appearance of NompC expression implicates dendritic morphology in regulating touch sensitivity.

Interestingly, NompC is expressed in fs(1)h1112 mutants, and its distribution throughout the dendritic arbor resembles that of wild-type neurons [Supplemental Fig. S12]. While nompC mutants lack a mechanosensory response [Yan et al. 2013], neurons lacking fs(1)h still respond to mechanical stimuli, but the magnitude of the response (number of APs) is reduced for a given stimulus intensity. At the behavioral level, this manifests as a reduced response to gentle touch. Therefore, our data suggest that the unique dendritic spike morphology of class III dendrites contributes to their mechanical sensitivity.

While various proteins involved in epigenetic regulation of gene expression have been implicated in dendrite morphogenesis, our study provides evidence that “readers” of acetylated histone marks regulate dendrite morphology by demonstrating the involvement of BET family proteins in this process. Given the complexity of achieving a compre-
hensive view of molecularly defined neural subtypes, we need to identify genome-wide mechanisms for molecular diversity that regulate dendritic morphology in order to further understand how morphological diversity is specified. Epigenetic regulators are an intriguing possibility in this endeavor, and future studies comparing gene expression profiles in mutants for regulators of histone modifications among neurons with varied morphologies may be one step forward in answering this fundamental question.

Materials and methods

Fly stocks

All experimental fly crosses were maintained in circadian incubators (Darwin Chambers Company) at 25°C (or 20°C for GAL4 attenuation). The $\text{fs}(1)h^{1112}$ allele was generated through EMS mutagenesis and mapped as previously described (Zheng et al. 2008). We generated UAS-Fsh-S-eGFP and UAS-huBRD2-eGFP transgenes for rescue experiments with either the sequence of Fsh-S ($\text{Drosophila Genomics Resource Center}$ [DGRC] clone LD266482, primers $[5^\prime] \text{CGTAGCTATATCGTGCACAC} \text{TGGTGTCTGAC}$ and $[3^\prime] \text{ACCTCTGGATCGTGCACAC} \text{TGGTGTCTGAC}$) or huBRD2 ($\text{Open Biosystems}$ clone ID 6181728, primers $[5^\prime] \text{ATGCTGGCAAACTGACTCCCCAATAAGGTCC}$ or $[3^\prime] \text{GCCTGAGTCTGAGTCTGAC}$) using primers with 5' and 3' flanking sequences added for use in the Gateway cloning system (Invitrogen). The appropriate sequence was first subcloned into the pDONR-221 entry vector using BP-Clonase II (Invitrogen) and subsequently transferred to the pTWG vector (DGRC clone 1076) using LR-Clonase II (Invitrogen). The pTWG vector was modified to include an attB site for 9-attB1 and 3-attB2 flanking sequences added for use in the Gateway cloning system (Invitrogen). The appropriate sequence was first subcloned into the pDONR-221 entry vector using BP-Clonase II (Invitrogen) and subsequently transferred to the pTWG vector (DGRC clone 1076) using LR-Clonase II (Invitrogen). The pTWG vector was modified to include an attB site for 9-attB1 and 3-attB2 flanking sequences added for use in the Gateway cloning system (Invitrogen).

For live imaging dendrite morphology, larvae were whole-mounted on glass slides, sandwiched between the slide and coverglass in halocarbon oil to flatten and immobilize the larvae alive and intact (Grueber et al. 2002). A Leica SP5 confocal microscope equipped with a 20× oil immersion objective and an argon 488-nm emission laser was used to excite and image membrane-tagged fluorescent GFP expressed in da sensory neurons. Z-stacks containing the dendritic arbors of da sensory neurons were collected for analysis.

Single axon terminals were imaged as described previously (Kim et al. 2013) but with the following modifications: Larvae were filled with the CNS intact and fixed in 4% PFA for 30 min at room temperature. Larval filets were then mounted, and single da neuron axon terminals were imaged without immunostaining.

Time-course/time-lapse imaging

For time-sensitive experiments, staged embryo collections were performed on grape agar plates to synthesize groups of larvae (0 h AEL). For time-course experiments, larvae were grown on grape agar plates for 48, 72, or 96 h AEL at 25°C. Da sensory neuron MARCM clones were imaged with a 20× oil immersion objective and at these various developmental stages. For time-lapse imaging, synthesized larvae were collected at 72 h AEL and imaged using a 40× oil immersion objective. Z-stacks were collected every 5 min over a 30-min duration to visualize dendrite branch dynamics.

Morphological analysis/quantification

Morphological analysis of dendritic arbors was performed on maximum Z-projections of collected Z-stack image files. The arbors were reconstructed using the Simple Neurite Tracer plugin (Longair et al. 2011) of ImageJ (http://rsweb.nih.gov/ij/plugins/cell-counter.html). Total dendrite length refers to the summed length of all dendrite branches from a single neuron reconstruction. Branch points were determined using the Analyze Skeleton function. Sholl analysis was performed using the Sholl Analysis feature of the Simple Neurite Tracer plugin. The class III spiked protrusions were manually counted using the Cell Counter ImageJ plugin (http://rsweb.nih.gov/ij/plugins/cell-counter.html). For time-lapse imaging, all terminal branch points within the field of view of projected Z-stacks were compared between consecutive
time points and scored as growing (extending length), retracting (shortening length), or stable (no change in length). These events were counted across all time points over a 30-min period and summed to determine the number of “total events.” Growth, retraction, and stable branches were reported as a percentage of the “total events” sum.

Axon terminal length was quantified as described in Kim et al. (2013) using the Simple Neurite Tracer plugin of ImageJ.

Behavioral assay/electrophysiology

All behavioral and electrophysiological experiments were performed as reported previously (Yan et al. 2013). Briefly, for the gentle touch behavioral response assay, larvae were touched with an eyelash, and their subsequent behavior was categorically scored. The summed scores among larvae were averaged and reported as mean ± SEM. Extracellular electrophysiological recordings were performed on class III da neurons visualized by GAL4 19–12, UAS-CD4-tGFP expression while delivering a mechanical stimulus by depressing the body wall cuticle a defined distance increment (intensity) with a sealed glass tip pipet. During the 1-sec duration of stimulus application, the number of APs was recorded and reported as the change in AP frequency (Δ number of APs) compared with the spontaneous firing rate 1 sec prior to stimulus presentation.

Statistical analysis

Statistical tests for significance were conducted using the SPSS program. When comparing two groups, a Student’s t-test was used, whereas when comparing more than two groups, a one-way ANOVA followed by a post-hoc Tukey’s test was performed.

Acknowledgments

We thank D.H. Huang for providing the anti-Fsh antibodies, and J. Howard for providing the anti-NompC-NT antibody. We thank L. Cheng for generating the anti-NompC-EC antibody. We thank A. Vincent for providing the anti-kn/col antibody. We thank S. Younger, S. Barbel, and T. Cheng for technical support. We thank W.J. Kim, D. Goreczya, and all members of the Jan laboratory for discussion. We thank T. Uemura for fly lines. J.A.B. is the recipient of a National Science Foundation Graduate Research Fellowship Program under grant number 1144247. Z.Y. was the recipient of the Long-Term Fellowship from the Human Frontier Science Program. This work was supported by National Institutes of Health grant number R37NS040929 to Y.N.J., Y.N.J., and L.Y.J. are investigators of the Howard Hughes Medical Institute.

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