Dendritic refinement of an identified neuron in the *Drosophila* CNS is regulated by neuronal activity and Wnt signaling

Ajeet Pratap Singh¹, K. VijayRaghavan²,,* and Veronica Rodrigues¹,²

SUMMARY
The dendrites of neurons undergo dramatic reorganization in response to developmental and other cues, such as stress and hormones. Although their morphogenesis is an active area of research, there are few neuron preparations that allow the mechanistic study of how dendritic fields are established in central neurons. Dendritic refinement is a key final step of neuronal circuit formation and is closely linked to emergence of function. Here, we study a central serotonergic neuron in the *Drosophila* brain, the dendrites of which undergo a dramatic morphological change during metamorphosis. Using tools to manipulate gene expression in this neuron, we examine the refinement of dendrites during pupal life. We show that the final pattern emerges after an initial growth phase, in which the dendrites function as ‘detectors’, sensing inputs received by the cell. Consistent with this, reducing excitability of the cell through hyperpolarization by expression of Kᵥ2.1 results in increased dendritic length. We show that sensory input, possibly acting through NMDA receptors, is necessary for dendritic refinement. Our results indicate that activity triggers Wnt signaling, which plays a ‘pro-retraction’ role in sculpting the dendritic field: in the absence of sensory input, dendritic arbors do not retract, a phenotype that can be rescued by activating Wnt signaling. Our findings integrate sensory activity, NMDA receptors and Wingless/Wnt5 signaling pathways to advance our understanding of how dendritic refinement is established. We show how the maturation of sensory function interacts with broadly distributed signaling molecules, resulting in their localized action in the refinement of dendritic arbors.

KEY WORDS: Dendritic refinement, *Drosophila*, Neuronal activity, NMDA receptors, Wnt signals

INTRODUCTION
In the central nervous system (CNS), dendrites are major sites of synaptic input from partner neurons, and their development is intimately linked to the emergence of a functional network. The molecular and cellular mechanisms that regulate dendritic growth and refinement are an area of intense research (Corty et al., 2009; Wong and Ghosh, 2002). The development of precise dendritic patterns is believed to be regulated by interplay between an intrinsic genetic program, extrinsic factors and neuronal activity.

The mechanisms that underlie the activity-dependent regulation of dendritic growth and refinement could be the same as those that regulate neuronal plasticity in the mature nervous system. Activity levels are maintained at circuit-specific set points by homeostatic mechanisms: the system tries to counterbalance any deviations by adjusting overall synaptic strength in multiple ways. Recently, Tripodi et al. suggested that during development, dendrites in embryonic *Drosophila* motoneurons act as homeostatic devices that regulate their size and shape in response to inputs (Tripodi et al., 2008). Refinement, by contrast, involves the strengthening of optimum connections between pre- and postsynaptic neurons and the elimination of suboptimal connections. The mechanisms of synaptic maturation and refinement are suggested to be similar to Hebbian plasticity, which underlies learning and memory (Espinosa et al., 2009; Constantine-Paton and Cline, 1998). Thus, overall dendritic patterns are established through a combination of homeostatic plasticity, which maintains the functional range of excitability, and Hebbian plasticity, which could act to regulate activity-dependent refinement within this range (Turrigiano and Nelson, 2004). The value of these distinctions and their extent of overlap will only become clear as the molecular and cellular mechanisms of dendritic patterning are revealed.

The molecular mechanisms by which some neurites are stabilized while others are removed are not yet fully understood. One view is that this could depend on the ability of certain connections to ‘capture’ trophic molecules released in the vicinity of their targets (Poo, 2001). The relative contribution of ‘activity-dependent’ versus ‘activity-independent’ processes (Hebb’s rule versus Sperry’s chemoaffinity hypothesis) during circuit formation has been a matter of some debate (Cline, 2003), although there is a growing body of evidence to suggest the recruitment of developmental signaling pathways in response to activity. Recent observations that Wnts are secreted at the synapse in response to neural activity (Ataman et al., 2008; Chen et al., 2006) suggest a novel role for the Wnt signaling pathway downstream of activity during nervous system development and plasticity (Tang, 2007). Activity-dependent Wnt release and concomitant β-catenin stabilization have been shown to influence dendritic branching (Yu and Malenka, 2003) and, recently, Hayashi et al. have implicated the release of Wnt as a trophic agent regulating synaptic stability (Hayashi et al., 2009). The fact that Wnts have been shown to act as pro- as well as anti-synaptogenic factors in different contexts (Klassen and Shen, 2007; Packard et al., 2002) makes them interesting candidates for examination in the context of dendritic refinement.

¹Department of Biological Sciences, Tata Institute of Fundamental Research, Mumbai–5, India. ²National Centre for Biological Sciences, TIFR, GKVK Campus, Bangalore–65, India.

*Author for correspondence (vijay@ncbs.res.in)

Accepted 8 February 2010
In the current study, we identify a wide-field serotonergic neuron (CSDn) in the CNS of Drosophila as a model with which to study dendritic growth and refinement. Dendrites of the CSDn in Drosophila undergo remodeling during metamorphosis and, as the adult neuron has very few dendrites projecting to the antennal lobe, deviations from the wild-type pattern can be quantified easily. We demonstrate a key role for neuronal activity in the refinement of dendritic branches of the CSDn during metamorphosis. Cell-autonomous reduction in excitability results in a lack of refinement of dendritic arbors. Dendritic refinement and maturation of the aristal-derived presynaptic inputs are temporally coincident, suggesting that these neurons could trigger activation of the CSDn.

Dendritic branches of the CSDn in the CNS of Drosophila undergo remodeling during metamorphosis. Cell-autonomous reduction in excitability results in a lack of refinement of dendritic arbors. Dendritic refinement and maturation of the aristal-derived presynaptic inputs are temporally coincident, suggesting that these neurons could trigger activation of the CSDn. Consistent with this observation, phenotypes observed in mutants lacking aristal neurons can be rescued by manipulations that have been shown to lead to increased levels of activity. Cell-autonomous knockdown of N-methyl-D-aspartate-type glutamate receptors (NMDARs) in the CSDn also affects the refinement process. We show that target interactions are likely to lay the field for the action of Wnt signaling. We find that Wingless (Wg; Drosophila Wnt1) levels are elevated in response to increased neural activity in the developing antennal lobe, and compromising Wg and Wnt5 signaling phenocopies reduced excitability, suggesting that the Wnt signaling cascade is recruited by neural activity to drive dendritic refinement. In summary, we present a new and valuable neuron preparation for the genetic dissection of dendritic patterning mechanisms. Using this identified central neuron, we demonstrate that neuronal activity, mediated through identified receptors and sensory inputs, along with Wnt signaling, acts to refine dendritic pattern to its ultimate functional state in the adult animal.

MATERIALS AND METHODS

Drosophila strains

RN2flp, tub>CD2-Gal4, UASSmCD8::GFP and RN2flp, tub>CD2-Gal4, UAS-mRFP (Roy et al., 2007) flies were used to uniquely label the CSDn. UAS-Apc2-GFP, UAS-nod-GFP, UAS-Syr-GFP, UAS-para.dtTCPΔN, UAS-Axen GFP, UAS-ugg-S914 MB14, UAS-Tryp-A1, dsb8, dsb4, wg; T; T(2;3) C202, al1SM1 and al1 dp2 b1 pr1 c1 px1 were obtained from the Bloomington Drosophila Stock Center, Indiana University, IN, USA. UAS-Kc2.1 was kindly provided by Richard Baines (Baines et al., 2001). UAS-fas-GFP by Roel Nusse (Cadijan et al., 1998), UAS-dsNR2 by Tim Tully (Wu et al., 2007; Xia et al., 2005), UAS-Calpain A RNAi (v35262) from the Vienna Drosophila RNAi Center (Dietzl et al., 2007), UAS-Calpain B RNAi from the National Institute of Genetics, Japan [courtesy of Lucas Waltzer (Osman et al., 2009)] and UAS-lgslf7E by Konrad Basler (Kramps et al., 2002; Mosimann et al., 2006). The UAS-eag-DN, UAS-Sl-hD recombinant was provided by Subhabrata Sanyal (Hartwig et al., 2008) and SG18.1-Gal4 by B. Shyamala (Shyamala and Chopra, 1999). The mutant stocks Wnt5550, dp601 and fz5f6/TM3Sb were obtained from Bassem Hassan (Srahna et al., 2005). To generate al1 homoyzgous animals, either al1 dp2 b1 pr1 c1 px1 homoyzgotes or the progeny of T(2;3) C202, al1 SM1 and al1 dp2 b1 pr1 c1 px1 were used. Both combinations gave similar results and are referred to throughout as al1 homoyzgotes.

All flies were maintained under standard conditions at 25°C unless otherwise indicated. For pupal timing, white pupa [0 hours after puparium formation (APF)] were collected and placed on a moist filter paper in humid conditions. This stage lasts ~1 hour, thus setting the accuracy of staging; the pupal stage lasts 100 hours under the conditions in our laboratory.

Immunochemistry

Brains from 2- to 4-day-old adults were dissected and stained as described (Roy et al., 2007; Wu and Luo, 2006). Primary antibodies used were mouse anti-Bruchpilot [mAbnc82; 1:20; Developmental Studies Hybridoma Bank (DSHB)], rabbit anti-GFP (1:10,000; Molecular Probes Invitrogen, Delhi, India), rabbit anti-RFP (1:500; Abcam, Cambridge, UK), mouse anti-Futsch (mAb22e10; 1:20; DSHB), mouse anti-Wingless (1:200; DSHB) and mouse anti-Fz2 (1:50; DSHB). Secondary antibodies used were Alexa Fluor 488-, Alexa Fluor 568- and Alexa Fluor 647-coupled antibodies generated in goat (Molecular Probes; 1:400). Samples were mounted between coverslips with a spacer in Vectashield (Vector Labs, Peterborough, UK) anti-fading agent.

RESULTS

Dendrites of the CSDn undergo a defined pattern of activity-dependent refinement during metamorphosis

A pair of serotoninergic neurons associated with the antennal lobes are labeled in the RN2flp, tub>CD2-Gal4, UASSmCD8GFP strain (Roy et al., 2007) (see Fig. S1 in the supplementary material). FLP recombinase activity labeled either both or a single neuron in each animal. Analysis of preparations bearing a unilateral ‘flip-out’ allowed us to analyze dendrites, which show a characteristic change in pattern from larval to adult stages. Processes close to the soma were identified as dendrites based on selective localization of the well-established markers Apc2-GFP and nod-GFP (see Fig. S1B-D in the supplementary material) (Rolls et al., 2007; Sanchez-Soriano et al., 2005). Synaptotagmin-GFP is excluded from the dendrites and is enriched specifically in the presynaptic terminals (see Fig. S1C in the supplementary material).

The sparse dendrites innervating the adult antennal lobe, as compared with that seen in the larva, makes the CSDn a promising system for analysis of the regulation of dendritic patterning during metamorphosis (Fig. 1). We quantified the cumulative length of dendritic branches by measuring the number of bins occupied by the neurites (see Fig. S2A,A’ in the supplementary material). There was some variability in the dendritic pattern of the adult CSDn across animals (see Fig. S2B-D in the supplementary material), although this was not so large that it precluded quantitative analysis.

At the larval stage, the dendritic field is composed of several branches (Fig. 1A,M), which are pruned such that at 10 hours APF only a few fine arbors remain (Fig. 1B,M). At 30 hours APF, there is an increase in fine, long arbors (Fig. 1C,M; see Fig. S3A1-6 in the supplementary material). At this time, the cumulative dendritic length is at its maximum and begins to show a reduction by 50 hours APF (Fig. 1D,M; see Fig. S3B1-6 in the supplementary material).
and resembles that of the adult by ~70 hours APF (Fig. 1E,F,M). We refer to this process as ‘refinement’ to distinguish it from pruning, which occurs earlier during pupal life to remove larval arbors from the main trunk of the axon.

The refinement period coincides with the ingrowth of sensory neurons, which are known to invade the antennal lobe by ~30 hours APF; glomerular formation continues until ~50 hours APF (Rodrigues and Hummel, 2008). It is therefore tempting to speculate that, as in vertebrates, refinement in *Drosophila* is regulated by neuronal innervation (Wong and Ghosh, 2002; Zhang and Poo, 2001). In order to test the role of activity, we silenced the CSDn by targeted expression of the inward rectifying human K⁺ channel Kᵣ₂.₁ (also known as KCNJ2 and IRK1 – Human Genome Nomenclature Database), which hyperpolarizes membrane potential (Baines et al., 2001). In these animals, development of the larval dendritic arbors occurred normally (compare Fig. 1G with 1A,M; *P* >0.05) and these were pruned as in the wild-type controls (compare Fig. 1H with 1B,M; *P* >0.1). Morphology appeared normal even at 30 hours APF (compare Fig. 1I with 1C,M; *P* >0.2) and the subsequent growth phase is normal (I, *n*=7, *P* =0.24). At (J) 50 hours APF (*n*=5, *P* =0.004), (K) 72 hours APF (*n*=3, *P* =0.011) and (L) adult (*n*=10, *P* =0.0001) the dendrites remain exuberant. Images are oriented as indicated in A, D, dorsal; M, medial. Arrowheads indicate the dendritic arbors. Scale bar: 20 μm. (M) Bar chart showing quantitation of dendritic arbors. Bars show the mean ± s.e.m. (for sample numbers, see Table S1 in the supplementary material), *, *P* <0.05; **, *P* <0.01; ***, *P* <0.001; n.s., not significant (*P* >0.05).

### Fig. 1. Metamorphosis of the dendrites of the CSDn during pupal life.

(A-F) Development of the CSDn in ‘wild-type’ (WT) *Drosophila* of genotype RN2/+, tub>CD2>Gal4, UASmCD8::GFP/+ Larval dendrites (∗, *n*=6) are pruned by 10 hours after puparium formation (APF) (B, *n*=6). (C) At 30 hours APF, fine dendritic arbors appear in the growth phase (∗, *n*=4). (D) Fifty hours APF (*n*=4). (E) Seventy-two hours APF (∗, *n*=5). (F) A 2- to 4-day-old adult (∗, *n*=19). (G-L) Development of the CSDn in *Drosophila* of genotype RN2/+, tub>CD2>Gal4, UASmCD8::GFP/UAS-Kᵣ₂.₁. Larval dendrites (∗, *n*=4) are pruned normally at 10 hours APF (H, *n*=4, *P* =0.12) and the subsequent growth phase is normal (I, *n*=7, *P* =0.24). At (J) 50 hours APF (*n*=5, *P* =0.004), (K) 72 hours APF (*n*=3, *P* =0.011) and (L) adult (*n*=10, *P* =0.0001) the dendrites remain exuberant. Images are oriented as indicated in A, D, dorsal; M, medial. Arrowheads indicate the dendritic arbors. Scale bar: 20 μm. (M) Bar chart showing quantitation of dendritic arbors. Bars show the mean ± s.e.m. (for sample numbers, see Table S1 in the supplementary material), *, *P* <0.05; **, *P* <0.01; ***, *P* <0.001; n.s., not significant (*P* >0.05).
necessary, is not sufficient for dendritic refinement, raising the possibility that additional inputs might be required from presynaptic partners, which arrive at the lobe ~30 hours APF.

Taken together, these results suggest that the dendritic pruning that occurs during the early phase of metamorphosis is independent of neuronal activity. Neuronal activity is essential for dendritic refinement at the time when synapse formation and maturation occur in the developing antennal lobe.

**Sculpting of the dendrites of the CSDn requires NMDA receptors and presynaptic input**

Dendritic refinement is thought to follow Hebb’s postulate, whereby coincident pre- and postsynaptic firing results in strengthening of synaptic connections, whereas non-coincident pre- and postsynaptic activity results in weakening of synapses (Constantine-Paton and Cline, 1998). N-methyl-D-aspartate-type glutamate receptors (NMDARs) have been suggested to act as ideal coincident detectors, as opening of these channels requires simultaneous release of glutamate from the presynaptic neuron and depolarization of the postsynaptic neuron (Espinosa et al., 2009; Zhang and Poo, 2001). The *Drosophila* NMDAR is composed of two subunits, dNR1 and dNR2 (Nmdar1 and Nmdar2 – FlyBase), which are widely expressed in the brain, and RNAi-mediated knockdown of these subunits results in memory defects (Wu et al., 2007). Simultaneous knockdown of both subunits in the CSDn using RNAi resulted in an increase in the dendritic field in adult flies (Fig. 3B,C; compare with 3A; see Fig. S4B in the supplementary material; \( P < 0.0001 \)). This suggests a requirement for a presynaptic glutamatergic input that is sensed by NMDARs on the CSDn. What could be the source of the input to the NMDARs?

Dendrites of the CSDn invade the coarse neuropile in the posterior region of the antennal lobe, where glomerular identity is not very clear with normal synaptic markers. Axonal projections from aristral sensory neurons are known to project to this region to glomeruli VP2 and VP3 (Stocker et al., 1983). We backfilled the arista with rhodamine-dextran (Fig. 3E, magenta) and noted that the terminals in VP lie in close proximity to the CSDn dendrites (Fig. 3E,F). However, the sensory neurons from the arista are likely to be cholinergic in nature and therefore their activity is unlikely to directly activate the NMDARs. The resolution of our methods does not allow us to assess whether connections with the aristral neurons are direct or are through local interneurons, most of which are known to be GABAergic (Wilson and Laurent, 2005) and several of which co-express glutamate (R. Priya and A. Chiang, personal communication) (Daniels et al., 2008). We examined the morphology of the CSDn dendrites in *aristaless (al1)* mutants, which have poorly developed aristae and reduced sensory input (Fig. 3G, n=3). The phenotype is comparable to that shown in the wild type in Fig. 1 (\( P < 0.3 \)). Images are oriented as indicated in G. D, dorsal; M, medial. Dotted lines outline the borders of the antennal lobe. Arrowheads indicate the dendritic arbors. Scale bar: 20 μm. (E, H). Bar charts showing quantitation of dendritic arbors for the different genotypes. Bars show the mean ± s.e.m. In H, the extent of the dendritic arbors is compared between the wild type and situations in which eag-DN and Sh-DN transgenes are ectopically expressed. *, \( P < 0.05 \); ***, \( P < 0.0001 \); n.s., not significant (\( P > 0.05 \)).
neurons either make direct contact with CSDn dendrites or act through borders of the antennal lobe. Arrowheads indicate the dendritic arbors. 

Inhibiting Wnt signaling results in increased dendritic arbor

Work in Drosophila and in mouse has suggested a role for the activity-dependent recruitment of Wnt at the synapse (Chiang et al., 2009; Ataman et al., 2008; Chen et al., 2006; Wayman et al., 2006). Wingless (Wg) and the receptor Frizzled 2 (Fz2) can be detected by immunostaining in the developing glomeruli (see Fig. S6A-B in the supplementary material) and levels of Wg were found to increase in the neuropile upon KCl-induced global depolarization (see Fig. S6C-E in the supplementary material). The observation that Wg levels at the terminals of neurons are influenced by activity even during development, led us to test the role of Wnt signaling during dendritic morphogenesis.

We expressed Fz2-GPI, a GPI-anchored form of the Wg receptor that acts in a dominant-negative manner by titrating away the ligand (Cadigan et al., 1998). Compared with wild type (Fig. 4A), the Fz2-GPI-expressing CSDn showed an increase in the dendritic field (Fig. 4B-I; \( P<0.0001 \)). Signaling downstream of Wnt receptors occurs through the cytoplasmic protein Dishevelled (Dsh), which is an essential component of canonical as well as non-canonical Wnt signaling pathways (Wallengord and Habas, 2005). Heterozygotes of \( \text{wg}^{1114/+} \); Fig. 4C) as well as of \( \text{dsh}^{h^{99}}/+ \); Fig. 4D) showed mild, but significant, dendritic phenotypes (Fig. 4I; \( P<0.006 \)) and activated Shaggy (Sgg; Fig. 4HI; \( P<0.0001 \)); see Fig. S4D in the supplementary material). Downregulation of Wnt signaling by ectopic expression of activated Sgg in the CSDn did not affect the branching pattern of larval dendrites (see Fig. S7A in the supplementary material) or the pruning of larval arbors during early metamorphosis (see Fig. S7B in the supplementary material). However, dendritic refinement during later pupal stages was compromised and ectopic arbors were seen to persist at 50 hours APF (see Fig. S7C in the supplementary material).

These results suggest that Wnt signaling impinges upon the mechanisms that culminate in the refinement of dendritic processes. Recent studies (Abe and Takeichi, 2007) have demonstrated that neuronal activity can also trigger Wnt signaling by activation of Calpain proteases, which result in \( \beta \)-catenin/TCF-dependent nuclear activity. In order to test whether this mechanism operates in our system, we examined the dendritic field of the CSDn after RNAi-mediated knockdown of Calpain A (Dietzl et al., 2007) or Calpain B (Osman et al., 2009). The dendritic arbors were normal in adults, demonstrating that downregulation of the Calpain pathway does not affect the refinement process (see Fig. S8A-C in the supplementary material; \( P>0.5 \)). Furthermore, abrogation of the transcriptional route of the Wnt pathway by ectopic expression of dTCFAN (van de Wetering et al., 1997) or a mutant form of legless (\( lgs^{17E} \)) that has weak Armadillo-binding capacity (Mosimann et al., 2006; Kramps et al., 2002), did not compromise dendritic refinement (see Fig. S8D,E,G in the supplementary material). The dendritic field was also normal in \( dsh^{h^{1}} \) mutants (see Fig. S8F,G in the supplementary material), in which the planar cell polarity pathway is compromised but Dsh function in the canonical pathway is unaffected (Boutros et al., 1998). This observation implicates a role for the canonical Wnt pathway in normal refinement. However, as TCF-dependent nuclear activity is also dispensable in this process, we suggest that refinement of dendritic arbors requires the action of the divergent canonical pathway downstream of Wnt action (Salinas and Zou, 2008).

Fig. 3. Dendritic refinement requires NMDAR activation and the presence of aristal neuron terminals. (A) CSDn dendritic field of a wild-type 2- to 4-day-old adult. (B) In UAS-\( \text{dsNR2}^{+/+} \); RN2\( \text{flp}^{+} \), tub\( >\)CD2\( >\)Gal4, UASmCD8::GFP;UASdnNR1, the dendritic field is enlarged (\( n=5, P<0.0001 \)). (D) Pupal antenna, 27 hours APF, labelled with anti-Futsch (mAb22c10). Arrow indicates aristal sensory neurons.

with controls (Fig. 3H,I; see Fig. S4C in the supplementary material; \( P<0.001 \)). As discussed above, aristal neurons may or may not make direct contact with the CSDn and these might be part of the local circuit involving the CSDn and lobe interneurons. Our data can be interpreted to mean that appropriate activation of the CSDn by direct/indirect input from aristal neurons, together with activation of NMDARs, probably by populations of glutamatergic neurons, mediate refinement. One possibility is that refinement occurs by removal of the dendrites that are not ‘stabilized’ by contact with presynaptic inputs.

Inhibiting Wnt signaling results in increased dendritic arbor

Work in Drosophila and in mouse has suggested a role for the activity-dependent recruitment of Wnt at the synapse (Chiang et al., 2009; Ataman et al., 2008; Chen et al., 2006; Wayman et al., 2006). Wingless (Wg) and the receptor Frizzled 2 (Fz2) can be detected by immunostaining in the developing glomeruli (see Fig. S6A-B in the supplementary material) and levels of Wg were found to increase in the neuropile upon KCl-induced global depolarization (see Fig. S6C-E in the supplementary material). The observation that Wg levels at the terminals of neurons are influenced by activity even during development, led us to test the role of Wnt signaling during dendritic morphogenesis.

We expressed Fz2-GPI, a GPI-anchored form of the Wg receptor that acts in a dominant-negative manner by titrating away the ligand (Cadigan et al., 1998). Compared with wild type (Fig. 4A), the Fz2-GPI-expressing CSDn showed an increase in the dendritic field (Fig. 4B-I; \( P<0.0001 \)). Signaling downstream of Wnt receptors occurs through the cytoplasmic protein Dishevelled (Dsh), which is an essential component of canonical as well as non-canonical Wnt signaling pathways (Wallengord and Habas, 2005). Heterozygotes of \( \text{wg}^{1114/+} \); Fig. 4C) as well as of \( \text{dsh}^{h^{99}}/+ \); Fig. 4D) showed mild, but significant, dendritic phenotypes (Fig. 4I; \( P<0.006 \)) and activated Shaggy (Sgg; Fig. 4HI; \( P<0.0001 \)); see Fig. S4D in the supplementary material). Downregulation of Wnt signaling by ectopic expression of activated Sgg in the CSDn did not affect the branching pattern of larval dendrites (see Fig. S7A in the supplementary material) or the pruning of larval arbors during early metamorphosis (see Fig. S7B in the supplementary material). However, dendritic refinement during later pupal stages was compromised and ectopic arbors were seen to persist at 50 hours APF (see Fig. S7C in the supplementary material).

These results suggest that Wnt signaling impinges upon the mechanisms that culminate in the refinement of dendritic processes. Recent studies (Abe and Takeichi, 2007) have demonstrated that neuronal activity can also trigger Wnt signaling by activation of Calpain proteases, which result in \( \beta \)-catenin/TCF-dependent nuclear activity. In order to test whether this mechanism operates in our system, we examined the dendritic field of the CSDn after RNAi-mediated knockdown of Calpain A (Dietzl et al., 2007) or Calpain B (Osman et al., 2009). The dendritic arbors were normal in adults, demonstrating that downregulation of the Calpain pathway does not affect the refinement process (see Fig. S8A-C in the supplementary material; \( P>0.5 \)). Furthermore, abrogation of the transcriptional route of the Wnt pathway by ectopic expression of dTCFAN (van de Wetering et al., 1997) or a mutant form of legless (\( lgs^{17E} \)) that has weak Armadillo-binding capacity (Mosimann et al., 2006; Kramps et al., 2002), did not compromise dendritic refinement (see Fig. S8D,E,G in the supplementary material). The dendritic field was also normal in \( dsh^{h^{1}} \) mutants (see Fig. S8F,G in the supplementary material), in which the planar cell polarity pathway is compromised but Dsh function in the canonical pathway is unaffected (Boutros et al., 1998). This observation implicates a role for the canonical Wnt pathway in normal refinement. However, as TCF-dependent nuclear activity is also dispensable in this process, we suggest that refinement of dendritic arbors requires the action of the divergent canonical pathway downstream of Wnt action (Salinas and Zou, 2008).
Fig. 4. Wnt signaling is necessary for dendritic refinement. (A) Dendritic field of a wild-type CSDn from a 2- to 4-day-old adult. (B) UAS-fz2-GPI; RN2-flp, tub>CD2>Gal4, UASmCD8::GFP+. Dendritic arbors are increased in the adult (n=4, P<0.0001). (C) wgl114/+; RN2-flp, tub>CD2>Gal4, UASmCD8::GFP+. wgl114 heterozygotes show a weak dendritic phenotype (n=4, P=0.002). (D) dsh1/+; RN2-flp, tub>CD2>Gal4, UASmCD8::GFP+. dsh1 heterozygotes show a mild phenotype (n=8, P=0.018). (E) RN2-flp, tub>CD2>Gal4, UASmCD8::GFP+; wgl114/+; dsh1/+; transheterozygotes show greatly increased dendritic field (Fig. 5E; see Fig. S4E in the supplementary material; n=10, P=0.006). (F) UAS-sgg-act+/+; RN2-flp, tub>CD2>Gal4, UASmCD8::GFP+; sgg-act/+; transheterozygotes have an enlarged dendritic field (n=8, P<0.0001). Images are oriented as indicated in A. D, dorsal; M, medial. Dotted lines outline the borders of the antennal lobe. Arrowheads indicate the dendritic arbors. Scale bar: 20 μm. (I) Bar chart showing quantitation of dendritic arbors. Bars show mean ± s.e.m. *, P<0.05; **, P<0.01; ***; P<0.0001; n.s., not significant (P>0.05).

Wg and Wnt5 signaling genetically interact in refining dendrites of CSDn
Wnt5, an additional ligand, is expressed together with Wg in the developing antennal lobe (Yao et al., 2007). We find that Wnt5 and its receptor Derailed (Drl) (Bonkowsky et al., 1999) participate in dendritic refinement of the CSDn. An additional transgenic line, drlRed2 (Fig. 5B) and Wnt5400 (Fig. 5C; see Fig. S4E in the supplementary material) mutants showed mild, but significant, increases in dendritic fields (Fig. 5H; P<0.0001). Wnt5400/+ heterozygotes were comparable to wild-type animals (Fig. 5D; P=0.09). Wnt5400/+; wgl114/+ transheterozygotes (Fig. 5E) demonstrated haploinsufficient interactions (Fig. 5H; P<0.0001), suggesting that signaling by Wnt5 and Wg ligands collaborate during dendritic refinement. Further genetic evidence for this conclusion arose from experiments in which either the dosage of wg (Wnt5400; wgl114/+; Fig. 5F; H; see Fig. S4F in the supplementary material; P<0.0001) or that of fz2 (Wnt5400; fz2[E4]/+; Fig. 5G; H; P<0.0001) was reduced in a Wnt5-null background. Together, these genetic data lead us to conclude that both Wnt5 and Wg/Fz2 act synergistically to regulate dendritic refinement.

The aristal sensory neurons trigger activation of the CSDn and Wnt signaling is recruited to regulate dendritic refinement
What is the link between presynaptic input, neuronal activity and the Wnt signaling pathway in regulating dendritic refinement? The experiments described above do not allow us to decipher whether these are contributing factors that act independently on the refinement process resulting in incremental changes in phenotype. In order to test for a link between presynaptic input and Wnt signaling, we targeted overexpression of Dsh, which is known to trigger Wnt signaling (Wehrli et al., 2000), in the CSDn of animals mutant for al1. As expected, al1 control progeny from a cross between al1/CyO; UAS-dsh and al1/CyO; RN2-flp, tub>CD2>Gal4, UASmCD8/GFP showed a greatly enlarged dendritic field (Fig. 6B; P=0.005). Experimental siblings (al1/al1; UAS-dsh/RN2-flp, tub>CD2>Gal4, UASmCD8::CD8GFP) showed an entirely normal morphology, indicating complete rescue of the al phenotype (Fig. 6C; P=0.15). Ectopic expression of Dsh in a wild-type neuron did not affect the dendritic morphology (Fig. 6D; P=0.5). This means that the arrival of the aristal sensory neurons within the antennal lobe recruits Wnt signaling in the CSDn, either by inducing postsynaptic activity or by the physical presence of these terminals within the lobe.

In order to distinguish between these possibilities, we exploited the temperature-sensitive dTrp-A1 (Trp-A1 – Flybase) channel, which, upon ectopic expression, has been shown to trigger activity in animals exposed to temperatures above 25°C (Pulver et al., 2009; Hamada et al., 2008). al1 homozygous animals expressing dTrp-A1 in the CSDn were reared at 22°C (at which temperature dTrp-A1 is not active) until the third larval instar, and then shifted to 25°C until eclosion. The dendritic field in this genotype (al1/al1; UAS-Trp-A1/RN2-flp, tub>CD2>Gal4, UASmCD8::GFP) showed strong rescue of the al phenotype (Fig. 6E compared with 6B) and was similar to that of the wild type (P=0.03). Ectopic expression of dTrp-A1 in a wild-type background had no effect (Fig. 6F; J). The ability of autonomous activation of CSDn to compensate for a lack of sensory innervation provides evidence for the triggering of neuronal activity by the aristal neurons, either directly or through the action of local interneurons.

How does activation of the CSDn relate to Wnt signaling? We activated the CSDn by targeted expression of the eag-DN and Sh-DN transgenes in Wnt5400 homozygous mutants. The dendritic phenotype seen in Wnt5 mutants (Fig. 6G) was not rescued by activation of the CSDn (Fig. 6H; P=0.18 compared with Wnt5). Expression of eag-DN and Sh-DN has been used in several recent studies (Chiang et al., 2009; Duch et al., 2008; Hartwig et al., 2008) and has been found to cause hyperactivation. Two possible explanations for this observation are that Wnt5 signaling is...
Regulation of dendritic refinement

Fig. 5. Genetic interaction between Wg and Wnt5 in the regulation of dendritic form. (A) Wild-type adult CSDn. (B) drRed2, RN2flp, tub>CD2>Gal4, UASmCD8::GFP+ showing dendritic phenotype (n=10, P<0.0001). (C) Wnt5400, RN2flp, tub>CD2>Gal4, UASmCD8::GFP+ animals exhibit a mild increase in dendritic branches (n=13, P<0.0001). (D) Wnt5400; RN2flp, tub>CD2>Gal4, UASmCD8::GFP+ animals have a dendritic field comparable to wild-type. (E) Wnt5400; wgI114++; RN2flp, tub>CD2>Gal4, UASmCD8::GFP+. Wnt5400; wgI114++; RN2flp, tub>CD2>Gal4, UASmCD8::GFP+. The reduced dosage of Wg in Wnt5 hemizygous animals causes strong dendritic phenotypes (n=8, P<0.0001). (F) Wnt5400, RN2flp, tub>CD2>Gal4, UASmCD8::GFP+/z2[2]; Reduced z2[2] dosage in Wnt5400 hemizygotes causes an enhanced phenotype (n=5, P<0.0001). Images are oriented as indicated in A. D, dorsal; M, medial. Dotted lines outline the borders of the antennal lobe. Arrowheads indicate the dendritic arbors. Scale bar: 20 μm. (H) Bar chart showing quantitation of dendritic arbors. Bars show mean ± s.e.m. *, P<0.05; **, P<0.01; ***, P<0.0001; n.s., not significant (P>0.05).

downstream of CSDn activation or that both Wnt5 and activity act in parallel to trigger dendritic refinement. This needs to be investigated further.

The results from experiments involving epistatic interactions allow us to propose the model shown in Fig. 7. The aristal sensory neurons, acting either directly or through local circuits, trigger neuronal activity in the CSDn. The activity of the aristal neurons also in some way stimulates Wnt signaling within the CSDn. Activation of the CSDn is not sufficient to lead to refinement in the absence of Wnt5.

Fig. 6. Sensory input, autonomous neuronal activity and signaling act together in regulating the refinement of CSDn dendrites. (A) Wild-type adult CSDn. (B) alI; RN2flp, tub>CD2>Gal4, UASmCD8::GFP+ from a cross between alI; UAS-dsh and alI; RN2flp, tub>CD2>Gal4, UASmCD8::GFP show an enlarged dendritic field (n=8, P<0.0051). (C) alI; RN2flp, tub>CD2>Gal4, UASmCD8::GFPUAS-dsh shows rescue of the alI phenotype and the neuron resembles that of the controls (n=5, P=0.15). (D) RN2flp, tub>CD2>Gal4, UASmCD8::GFPUAS-dsh. The dendritic arbors are unaffected (n=5, P=0.51). (E) alI; RN2flp, tub>CD2>Gal4, UASmCD8::GFPUAS-Trp-A1 reared at 25°C from third instar larval stage until eclosion. The excess dendritic branching in alI mutants is partially rescued and is similar to that of controls (n=2, P=0.03). (F) RN2flp, tub>CD2>Gal4, UASmCD8::GFPUAS-Trp-A1 shows a normal branching pattern (n=4, P=0.4). (G) Wnt5400; RN2flp, tub>CD2>Gal4, UASmCD8::GFP+ animals show an increased dendritic field (n=13, P<0.0001). (H) Wnt5400, UAS-eag-DN, UAS-Sh-DN+/+; RN2flp, tub>CD2>Gal4, UASmCD8::GFP+ Expression of UAS-eag-DN, UAS-Sh-DN in the mutant background does not rescue this phenotype (n=6, P<0.0001). (I) UAS-eag-DN, UAS-Sh-DN+/+; RN2flp, tub>CD2>Gal4, UASmCD8::GFP+ control animals do not show any mutant phenotype (n=4, P=0.3). Images are oriented as indicated in A. D, dorsal; M, medial. Dotted lines outline the borders of the antennal lobe. Arrowheads indicate the dendritic arbors. Scale bar: 20 μm. (J) Bar chart showing quantitation of dendritic arbors. Bars show mean ± s.e.m. *, P<0.05; **, P<0.01; ***, P<0.0001; n.s., not significant (P>0.05).
**DISCUSSION**

In this study, we focus on a specific phase during the metamorphosis of the dendrites of a central serotonergic neuron, in which excess growth is removed by a process that we term refinement. Genetic analyses using loss-of-function mutants and RNAi-mediated knockdown of specific genes allow us to postulate a link between neuronal activity, synaptic input and Wnt signaling in this process (Fig. 7). The dendrites present on the CSDn during the larval stage are removed early in pupation by pruning, followed by a period of exuberant growth. The arrival of sensory neurons at the antennal lobe correlates well with when growth of the CSDn dendrites ceases and removal of the excess branches occurs. The CSDn must be active for the refinement process to occur, as refinement fails when neuronal activity is inhibited or when the sensory neurons are absent. Phenotypes observed in the latter case can be rescued by ectopic activation of the neuron using the temperature-sensitive dTrp-A1 channel. We suggest that activity within the CSDn, possibly together with activity in presynaptic neurons, acts to provide the correlated activity required to trigger activation of NMDARs. We know that knockdown of NMDARs affects the refinement process, although identifying its specific action requires further study. A possible consequence of the activity-dependent process is activation of the Wg pathway, as the phenotype observed in α/ mutants can also be rescued by ectopic expression of Dsh in the CSDn. It seems unlikely that activity within the CSDn leads to the release of Wnt ligands, but rather that dendrites respond locally to Wnt ligands in the region of a dendrite that is receiving input. Although other interpretations of the data are possible, we favor a hypothesis whereby specific synapses are stabilized as a result of correlated neuronal activity, and that excess dendritic branches are removed by Wnt signaling (Fig. 7C).

**Neuronal activity shapes dendritic form**

Perturbations in neuronal activity can be compensated by changes at multiple levels, including alterations in the expression of ion channels and in synaptic strength (for reviews see Davis, 2006; Turrigiano and Nelson, 2004). Tripodi et al. provide evidence for structural homeostasis whereby alterations in afferent input during development can be compensated by changes in dendritic geometry (Tripodi et al., 2008). This suggests that dendritic arbors serve as sensors for input levels, thus allowing the self-organization of circuits that is necessary for robust behavioral outputs (Tripodi et al., 2008). Our studies in the CSDn support these observations: reduced activation of the cell by targeted expression of Kir2.1 results in a greatly enlarged dendritic field in the adult. This phenotype can be explained by a mechanism in which the absence of electrical activity results in a failure of the signaling mechanisms that stop growth of the arbors and that remove additional branches. Reduced excitability could also drive the homeostatic mechanisms towards making more arbors and to suppress the refinement program.

**Dendritic remodeling requires NMDAR function**

Dendritic growth and refinement are closely associated with input activity and synapse formation during development. Activity-dependent development of circuits is thought to utilize mechanisms similar to those involved in Hebbian learning and plasticity. NMDARs are ideal candidates for detecting correlated pre- and postsynaptic activity, which is crucial in the Hebbian model of learning and plasticity. Here, strengthening of synapses leads to the stabilization and extension of dendrites, whereas weakening of synapses leads to the destabilization and elimination of dendritic branches (Espinosa et al., 2009; Cline and Haas, 2008; Constantine-Paton and Cline, 1998). During vertebrate hippocampal development, NMDAR activation has been shown to limit synapse
number and reduce dendritic complexity (Luthi et al., 2001). The stabilization of a particular synapse or arbor possibly attenuates the formation of new branches or synapses, thus limiting further dendritic growth. In such a scenario, knocking down NMDAR levels would be expected to result in increased dendritic complexity, as indeed we have observed. The mechanism by which ‘appropriately connected’ synapses are strengthened, whereas suboptimal contacts are eliminated, needs to be studied in our system. In other systems, Ca\(^{2+}\), which is released upon NMDAR activation, impinges on various intracellular effectors that regulate dendritic morphogenesis (Konur and Ghosh, 2005). In addition, selective stabilization/distabilization of dendritic arbors could be affected by the local release of growth factors in response to activity.

Wnt signaling in dendritic refinement

We demonstrate that activity-dependent activation of the Wnt pathway facilitates retraction of dendritic arbors. Arbors that receive appropriate input are somehow protected and stabilized. Our experiments suggest that Wnt-dependent refinement mechanisms through a non-nuclear pathway and could act by impinging directly on cytoskeletal dynamics (Schlessinger et al., 2009; Salinas and Zou, 2008). Disruption of the microtubule cytoskeleton is a key feature of dendritic pruning in Drosophila during metamorphosis (Williams and Truman, 2005). GSK3β (Shaggy in Drosophila) is an intracellular inhibitor of the Wnt pathway, has been shown to act as a sensor of inputs for neuronal activity (Chiang et al., 2009) and a potent regulator of microtubule dynamics in axons (Ciani et al., 2004). In the Drosophila embryonic CNS, the Src family of tyrosine kinases (SFKs) is required for Wnt5/Drl-mediated signaling (Wouda et al., 2008). Interestingly, SFKs seem to act as a crucial point of convergence for multiple signaling pathways that enhance NMDAR activity and hence are thought to act as molecular hubs for the control of NMDARs (Salter and Kalia, 2004). It is tempting to envisage a scenario in which there is cross-talk between Wnt5/Drl signaling-mediated activation of SFKs and NMDAR signaling during refinement.

In summary, we show that the dendritic refinement of a central modulatory serotonergic neuron is regulated by electrical activity, NMDAR and Wnt signaling. Similar mechanisms have been implicated in dendritic growth and refinement of excitatory neurons in vertebrates (Wong and Ghosh, 2002; Luthi et al., 2001; Constantine-Paton and Cline, 1998). We provide a model neuron preparation in which the dendritic growth and refinement of a modulatory neuron can be analyzed genetically. We demonstrate that the dendrites of CSDN receive input from sensory neurons from the arista, supporting previous suggestions (Hill et al., 2002) that mechanosensory input could alter sensitivity to odorant stimuli. In both Drosophila (Dacks et al., 2009) and the mammalian olfactory bulb (Petzold et al., 2009), serotonin gates the odor-evoked sensory response. CSDN sends projections to higher brain centers and multimodal projections to the contralateral antennal lobe and hence it is likely to influence the overall properties of the olfactory circuit. Our study suggests that the structural and resulting functional properties of this neuron emerge from an interaction between partner neurons, together with input from intrinsic and extrinsic cues.

Acknowledgements

We thank Abhijit Das, Prabhat Tiwari and Keshava Subramanya for technical help with dye backfilling experiments and microscopy; Mani Ramaswami, Subhabrata Sanjal and Mathreyi Narasimha for discussions and comments on the manuscript; reviewers for comments on a previous version that have helped to provide better insight into the mechanisms of dendritic refinement; Rashi Priya and Albert Chiang for sharing unpublished data; the Bloomington Drosophila Stock Center and several laboratories for generous supply of fly strains; and the Centre for Nanotechnology (Department of Science and Technology grant No. SR/55/NM-36/2005) for the Olympus FV1000 microscopes in the Central Imaging and Flow Facilities (NCBS). This work was supported by core funding from TIFR.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.044131/-/DC1

References


Table S1. The P-values are calculated using the unpaired t-test versus the wild-type control (Glφ; Tw) unless otherwise stated.

<table>
<thead>
<tr>
<th>Sample shown in figure</th>
<th>Genotype (number of samples)</th>
<th>Mean±s.e.m</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFP+ (6)</td>
<td>224.75±42.65</td>
<td>N.A.</td>
</tr>
<tr>
<td>1B</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFP+ (6)</td>
<td>94.92±17.23</td>
<td>N.A.</td>
</tr>
<tr>
<td>1C</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFP+ (4)</td>
<td>512.15±70.26</td>
<td>N.A.</td>
</tr>
<tr>
<td>1D</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFP+ (4)</td>
<td>9.25±26.22</td>
<td>N.A.</td>
</tr>
<tr>
<td>1E</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFP+ (5)</td>
<td>6.7±4.84</td>
<td>N.A.</td>
</tr>
<tr>
<td>1F</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFP+ (19)</td>
<td>39.63±8.29</td>
<td>N.A.</td>
</tr>
<tr>
<td>1G</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (4)</td>
<td>186.13±48.98</td>
<td>0.57 with respect to 1A</td>
</tr>
<tr>
<td>1H</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (4)</td>
<td>4.4±2.48</td>
<td>0.12 with respect to 1B</td>
</tr>
<tr>
<td>1I</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (7)</td>
<td>74.5±54.07</td>
<td>0.24 with respect to 1C</td>
</tr>
<tr>
<td>1J</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (5)</td>
<td>945.1±176.9</td>
<td>0.004 with respect to 1D</td>
</tr>
<tr>
<td>1K</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (3)</td>
<td>116±438.02</td>
<td>0.011 with respect to 1E</td>
</tr>
<tr>
<td>1L</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (10)</td>
<td>1062±257.45</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>2A</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFP+ (9) grown at 29°C</td>
<td>113±32.82</td>
<td>0.0073</td>
</tr>
<tr>
<td>2B</td>
<td>para¹,², RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFP+ (8) grown at 25°C</td>
<td>43.3±10.54</td>
<td>0.82</td>
</tr>
<tr>
<td>2C</td>
<td>para¹,², RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (3) grown at 29°C</td>
<td>435.6±110.37</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>2D, 6I</td>
<td>UAS-egfp DN, US-Am-DN+, RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFP+ (4)</td>
<td>69.4±8.88</td>
<td>0.3</td>
</tr>
<tr>
<td>2F</td>
<td>UAS-egfp DN, US-Am-DN+, RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (5)</td>
<td>57.8±84.51</td>
<td>0.48</td>
</tr>
<tr>
<td>2G</td>
<td>UAS-egfp DN, US-Am-DN+, RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (3)</td>
<td>140.3±29.58</td>
<td>0.35</td>
</tr>
<tr>
<td>2H</td>
<td>SSB1-3, RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (5)</td>
<td>449±250.13</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3A</td>
<td>a², RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFP+ (5)</td>
<td>776±342.18</td>
<td>0.0002</td>
</tr>
<tr>
<td>3B</td>
<td>a², RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (5)</td>
<td>465.7±75.56</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3C</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFP+ (4)</td>
<td>115.5±26.94</td>
<td>0.002</td>
</tr>
<tr>
<td>3D</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFP+ (8)</td>
<td>110.5±39.29</td>
<td>0.018</td>
</tr>
<tr>
<td>3E</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (5)</td>
<td>24.4±5.15</td>
<td>0.38</td>
</tr>
<tr>
<td>3F</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (13)</td>
<td>235.9±37.21</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3G</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (10)</td>
<td>359.4±149.93</td>
<td>0.006</td>
</tr>
<tr>
<td>3H</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (8)</td>
<td>241.5±36.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3I</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (10)</td>
<td>264±87.23</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3J</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (13)</td>
<td>218.2±30.86</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3K</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (15)</td>
<td>89.6±70.73</td>
<td>0.09</td>
</tr>
<tr>
<td>3L</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (8)</td>
<td>319.6±338.69</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3M</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (8)</td>
<td>828.5±201.25</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3N</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (5)</td>
<td>610±140.43</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3O</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (5)</td>
<td>806.7±395.61</td>
<td>0.0051</td>
</tr>
<tr>
<td>3P</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (4)</td>
<td>66.2±14.4</td>
<td>0.15</td>
</tr>
<tr>
<td>3Q</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (5)</td>
<td>28.2±10.93</td>
<td>0.51</td>
</tr>
<tr>
<td>3R</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (2)</td>
<td>116±81</td>
<td>0.03</td>
</tr>
<tr>
<td>3S</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (4)</td>
<td>23.5±10.35</td>
<td>0.4</td>
</tr>
<tr>
<td>3T</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (4)</td>
<td>218.2±30.86</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3U</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (6)</td>
<td>298.6±54.12</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3V</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (4)</td>
<td>69.4±8.88</td>
<td>0.3</td>
</tr>
<tr>
<td>3W</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (5)</td>
<td>46.6±15.07</td>
<td>0.7</td>
</tr>
<tr>
<td>3X</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (5)</td>
<td>36.8±14.03</td>
<td>0.87</td>
</tr>
<tr>
<td>3Y</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (4)</td>
<td>27.9±19.68</td>
<td>0.51</td>
</tr>
<tr>
<td>3Z</td>
<td>RN2flp; tub;CD2&gt;Ga4; USAmCD8:EFPUS-A2.7 (6)</td>
<td>67.8±25.75</td>
<td>0.18</td>
</tr>
</tbody>
</table>

*P-values are calculated using the unpaired t-test versus the wild-type control (Glφ; Tw) unless otherwise stated.