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A neurotrophin functioning with a Toll regulates structural plasticity in a dopaminergic circuit

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eLife Assessment

This **important** study identifies neurotrophin signaling as a molecular mechanism underlying previous findings of structural plasticity in central dopaminergic neurons of the adult fly brain. The authors present **solid** evidence for neurotrophin signaling in shaping the structure and synapses of certain dopaminergic circuits. The work suggests an intriguing potential link between neurotrophin signaling and experience-induced structural plasticity but further research will be necessary to establish this connection definitively.

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Abstract

Experience shapes the brain, as neural circuits can be modified by neural stimulation or the lack of it. The molecular mechanisms underlying structural circuit plasticity and how plasticity modifies behaviour, are poorly understood. Subjective experience requires dopamine, a neuromodulator that assigns a value to stimuli, and it also controls behaviour, including locomotion, learning and memory. In *Drosophila*, Toll receptors are ideally placed to translate experience into structural brain change. *Toll-6* is expressed in dopaminergic neurons (DANs), raising the intriguing possibility that Toll-6 could regulate structural plasticity in dopaminergic circuits. *Drosophila* neurotrophin-2 (DNT-2) is the ligand for Toll-6 and Kek-6, but whether it is required for circuit structural plasticity was unknown. Here, we show that *DNT-2* expressing neurons connect with DANs, and they modulate each other. Loss of function for *DNT-2* or its receptors *Toll-6* and kinase-less Trk-like *kek-6* caused DAN and synapse loss, impaired dendrite growth and connectivity, decreased synaptic sites and caused locomotion deficits. By contrast, over-expressed *DNT-2* increased DAN cell number, dendrite complexity and promoted synaptogenesis. Neuronal activity modified DNT-2, it increased synaptogenesis in DNT-2-positive neurons and DANs, and over-expression of DNT-2 did too.

Altering the levels of DNT-2 or Toll-6 also modified dopamine-dependent behaviours, including locomotion and long-term memory. To conclude, a feedback loop involving dopamine and DNT-2 sculpted the circuits engaged, and DNT-2 with Toll-6 and Kek-6 induced structural plasticity in this circuit modifying brain function and behaviour.

Introduction

The brain can change throughout life, as new cells are formed or eliminated, axonal and dendritic arbours can grow or shrink, synapses can form or be eliminated (Wiesel 1982, Feldman and Brecht 2005, Holtmaat and Svoboda 2009, Gage 2019). Such changes can be driven by experience, that is, neuronal activity or the lack of it (Wiesel 1982, Maguire et al. 2000, Cotman and Berchtold 2002, Feldman and Brecht 2005, Sur and Rubenstein 2005, Holtmaat and Svoboda 2009, Woollett and Maguire 2011, Chen and Brumberg 2021, Bharmauria et al. 2022). Structural changes result in remodelling of connectivity patterns, and these bring about modifications of behaviour. These can be adaptive, dysfunctional or simply the consequence of opportunistic connections between neurons (Kuner and Flor 2016, Leemhuis et al. 2019, Yang et al. 2020). It is critical to understand how structural modifications to cells influence brain function. This requires linking with cellular resolution molecular mechanisms, neural circuits and resulting behaviours.

In the mammalian brain, the neurotrophins (NTs: BDNF, NGF, NT3, NT4) are growth factors underlying structural brain plasticity (Poo 2001, Lu et al. 2005, Park and Poo 2013). They promote neuronal survival, connectivity, neurite growth, synaptogenesis, synaptic plasticity and Long-Term Potentiation (LTP), via their Trk and $p75^{NTR}$ receptors (Poo 2001, Lu et al. 2005, Park and Poo 2013). In fact, all anti-depressants function by stimulating production of BDNF and signalling via its receptor TrkB, leading to increased brain plasticity (Casarotto et al. 2021, Castren and Monteggia 2021). Importantly, NTs have dual functions and can also induce neuronal apoptosis, neurite loss, synapse retraction and Long-Term Depression (LTD), via $p75^{NTR}$ and Sortilin (Lu et al. 2005). Remarkably, these latter functions are shared with neuroinflammation, which in mammals involves Toll-Like Receptors (TLRs)(Squillace and Salvemini 2022). TLRs and Tolls have universal functions in innate immunity across the animals (Gay and Gangloff 2007), and consistently with this, TLRs in the CNS are mostly studied in microglia. However, mammalian *TLRs* are expressed in all CNS cell types, where they can promote not only neuroinflammation, but also neurogenesis, neurite growth and synaptogenesis and regulate memory – independently of pathogens, cellular damage or disease (Ma et al. 2006, Rolls et al. 2007, Okun et al. 2010, Okun et al. 2011, Patel et al. 2016, Chen, CY et al. 2019). Whether TLRs have functions in structural brain plasticity and behaviour remains little explored, and whether they can function together with neurotrophins in the mammalian brain is unknown.

Progress linking cellular and molecular events to circuit and behavioural modification has been rather daunting and limited using mammals (Wang et al. 2022). The *Drosophila* adult brain is plastic and can be modified by experience and neuronal activity (Technau 1984, Barth and Heisenberg 1997, Barth et al. 1997, Sachse et al. 2007, Kremer et al. 2010, Sugie et al. 2015, Linneweber et al. 2020, Baltruschat et al. 2021, Coban et al. 2024). Different living conditions, stimulation with odorants or light, circadian rhythms, nutrition, long-term memory and experimentally activating or silencing neurons, modify brain volume, alter circuit and neuronal shape, and remodel synapses, revealing experience-dependent structural plasticity (Heisenberg et al. 1995, Barth and Heisenberg 1997, Barth et al. 1997, Devaud et al. 2001, Gorska-Andrzejak et al. 2005, Sachse et al. 2007, Fernandez et al. 2008, Kremer et al. 2010, Bushey et al. 2011, Sugie et al. 2015, Duhart et al. 2020, Baltruschat et al. 2021, Vaughen et al. 2022, Coban et al. 2024). Furthermore, the *Drosophila* brain is also susceptible to neurodegeneration (Bolus et al. 2020). However, the molecular and circuit mechanisms underlying structural brain plasticity are mostly unknown in *Drosophila*.

Toll receptors are expressed across the *Drosophila* brain, in distinct but overlapping patterns that mark the anatomical brain domains (Li et al. 2020). Tolls share a common signalling pathway downstream, that can drive at least four distinct cellular outcomes – cell death, survival, quiescence, proliferation – depending on context (McIlroy et al. 2013, Foldi et al. 2017, Anthoney et al. 2018, Li et al. 2020). They are also required for connectivity and structural synaptic plasticity and they can also induce cellular events independently of signalling (McIlroy et al. 2013, Ward et al. 2015, McLaughlin et al. 2016, Foldi et al. 2017, Ulian-Benitez et al. 2017, Li et al. 2020). These nervous system functions occur in the absence of tissue damage or infection. This is consistent with the fact that - as well as universal functions in innate immunity - Tolls also have multiple non-immune functions also outside the CNS, including the original discovery of Toll in dorso-ventral patterning, cell intercalation, cell competition and others (Meyer et al. 2014, Pare et al. 2014, Anthoney et al. 2018, Tamada et al. 2021). The Toll distribution patterns in the adult brain and their ability to switch between distinct cellular outcomes means they are ideally placed to translate experience into structural brain change (Li et al. 2020).

We had previously observed that in the adult brain, *Toll-6* is expressed in dopaminergic neurons (McIlroy et al. 2013). Dopamine is a key neuromodulator that regulates wakefulness and motivation, experience valence, such as reward, and it is essential for locomotion, learning and memory (Riemensperger et al. 2011, Waddell 2013, Adel and Griffith 2021). In *Drosophila*, Dopaminergic neurons (DANs) form an associative neural circuit together with mushroom body Kenyon cells (KCs), Dorsal Anterior Lateral neurons (DAL) and Mushroom Body Output neurons (MBONs) (Chen et al. 2012, Aso et al. 2014a, Boto et al. 2020, Adel and Griffith 2021). Kenyon cells receive input from projection neurons of the sensory systems, they then project through the mushroom body lobes where they are intersected by DANs to regulate MBONs to drive behaviour (Heisenberg 2003, Aso et al. 2014b, Boto et al. 2020). This associative circuit is required for learning, long-term memory and goal-oriented behaviour (Chen et al. 2012, Guven-Ozkan and Davis 2014, Adel and Griffith 2021). During experience, involving sensory stimulation from the external world and from own actions, dopamine assigns a value to otherwise neutral stimuli, labelling the neural circuits engaged (Boto et al. 2020). Thus, this raises the possibility that a link of Toll-6 to dopamine could enable translating experience into circuit modification to modulate behaviour.

In *Drosophila*, Toll receptors can function both independently of ligand-binding and by binding Spätzle (Spz) protein family ligands, also known as *Drosophila* neurotrophins (DNTs), that are sequence, structural and functional homologues of the mammalian NTs (DeLotto and DeLotto 1998, Weber et al. 2003, Hoffmann et al. 2008a, Hoffmann et al. 2008b, Zhu et al. 2008, Lewis et al. 2013, McIlroy et al. 2013, Foldi et al. 2017). Like mammalian NTs, DNTs also promote cell survival, connectivity, synaptogenesis and structural synaptic plasticity, and they can also promote cell death, depending on context (Zhu et al. 2008, McIlroy et al. 2013, Sutcliffe et al. 2013, Foldi et al. 2017, Ulian-Benitez et al. 2017). As well as Tolls, DNTs are also ligands for Kekkon (Kek) receptors, kinase-less homologues of the mammalian NT Trk receptors and are required for structural synaptic plasticity (Ulian-Benitez et al. 2017). Importantly, the targets regulated by Tolls and Keks - ERK, NFκB, PI3K, JNK, CaMKII – are shared with those of mammalian NT receptors Trk and $p75^{NTR}$, and have key roles in structural and functional plasticity across the animals (Park and Poo 2013, Foldi et al. 2017, Ulian-Benitez et al. 2017, Yang et al. 2020, Tamada et al. 2021).

Here, we focus on Drosophila neurotrophin −2 (DNT-2), proved to be the ligand of Toll-6 and Kek-6, with in vitro, cell culture and in vivo evidence (McIlroy et al. 2013, Foldi et al. 2017, Ulian-Benitez et al. 2017). Here we asked how DNT-2, Toll-6 and Kek-6 are functionally related to dopamine, whether they and neuronal activity – as a proxy for experience - can modify neural circuits, and how structural circuit plasticity modifies dopamine-dependent behaviours.

Results

DNT-2A, Toll-6 and Kek-6 neurons are integrated in a dopaminergic circuit

To allow morphological and functional analyses of *DNT-2* expressing neurons, we generated a *DNT-2Gal4* line using CRISPR/Cas9 and drove expression of the membrane-tethered-GFP *FlyBow1.1* reporter. We identified at least 12 DNT-2+ neurons and we focused on four anterior DNT-2A neurons per hemi-brain (**Figure 1A, B**). Using the post-synaptic marker Denmark, DNT-2A dendrites were found at the prow (PRW) and flange (FLA) region, whereas axonal terminals visualised with the pre-synaptic marker synapse defective 1 (Dsyd1-GFP) resided at the superior medial protocerebrum (SMP) (**Figure 1C, C''**). We additionally found post-synaptic signal at the SMP and pre-synaptic signal at the FLA/PRW (**Figure 1C, C'**), suggesting bidirectional communication at both sites. Using Multi-Colour Flip-Out (MCFO) to label individual cells stochastically (Nern et al. 2015, Costa et al. 2016), single neuron clones revealed variability in the DNT-2A projections across individual flies (**Figure 1D**), consistently with developmental and activity-dependent structural plasticity in *Drosophila* (Heisenberg et al. 1995, Kremer et al. 2010, Sugie et al. 2015, Mayseless et al. 2018, Li et al. 2020, Linneweber et al. 2020, Baltruschat et al. 2021). We found that DNT-2A neurons are glutamatergic as they express the vesicular glutamate transporter vGlut (**Figure 1E**, Figure S1A) and lack markers for other neurotransmitter types (Figure S1). DNT-2A terminals overlapped with those of dopaminergic neurons (**Figure 1G**), suggesting they could receive inputs from neuromodulatory neurons. In fact, single-cell RNA-seq revealed transcripts encoding the dopamine receptors *Dop1R1, Dop1R2, Dop2R* and/or *DopEcR* in DNT-2+ neurons (Croset et al. 2018). Using reporters, we found that Dop2R is present in DNT-2A neurons (**Figure 1F**, Figure S1B), but not Dop1R2 (Figure S1E). Altogether, these data showed that DNT-2A neurons are glutamatergic neurons that could receive dopaminergic input both at PRW and SMP. DNT-2 functions via Toll-6 and Kek-6 receptors, and *Toll-6* is expressed in DANs (McIlroy et al. 2013).

To identify the cells expressing *Toll-6* and *kek-6* and explore further their link to the dopaminergic system, we used *Toll-6Gal4 (*Li et al. 2020*)* and *kek-6Gal4* (Ulian-Benitez et al. 2017) to drive expression of membrane-tethered *FlyBbow1.1* and assessed their expression throughout the brain. Using anti-Tyrosine Hydroxilase (TH) - the enzyme that catalyses the penultimate step in dopamine synthesis - to visualise DANs, we found that Toll-6+ neurons included dopaminergic neurons from the PAMs, PPL1 and PPL2 clusters (**Figure 1I**, Figure S2D,F Table S1), whilst Kek-6+ neurons included PAM, PAL, PPL1, PPM2 and PPM3 dopaminergic clusters (**Figure 1J**, Figure S2B,E,F Table S1). DNT-2 can also bind various Tolls and Keks promiscuously (McIlroy et al. 2013, Foldi et al. 2017) and other *Tolls* are also expressed in the dopaminergic system: PAMs express multiple *Toll* receptors (Figure S2F) and all PPL1s express at least one *Toll* (Figure S2F). Using MCFO clones revealed that both *Toll-6* and *kek-6* are also expressed in Kenyon cells (Li et al. 2020) (Figure S2I,J, Table S1), DAL neurons (Figure S2G,H, Table S1) and MBONs (Figure S2A-C). In summary, *Toll-6* and *kek-6* are expressed in DANs, DAL, Kenyon cells and MBONs (**Figure 1H**). These cells belong to a circuit required for associative learning, long-term memory and behavioural output, and DANs are also required for locomotion (Riemensperger et al. 2011, Chen et al. 2012, Aso et al. 2014b, Boto et al. 2014, Adel and Griffith 2021, Huang et al. 2024). Altogether, our data showed that DNT-2A neurons are glutamatergic neurons that could receive dopamine as they contacted DANs and expressed the *Dop2R* receptor, and that in turn DANs expressed the DNT-2 receptors *Toll-6* and *kek-6*, and therefore could respond to DNT-2. These data suggested that there could be bidirectional connectivity between DNT-2A neurons and DANs, which we explored below.

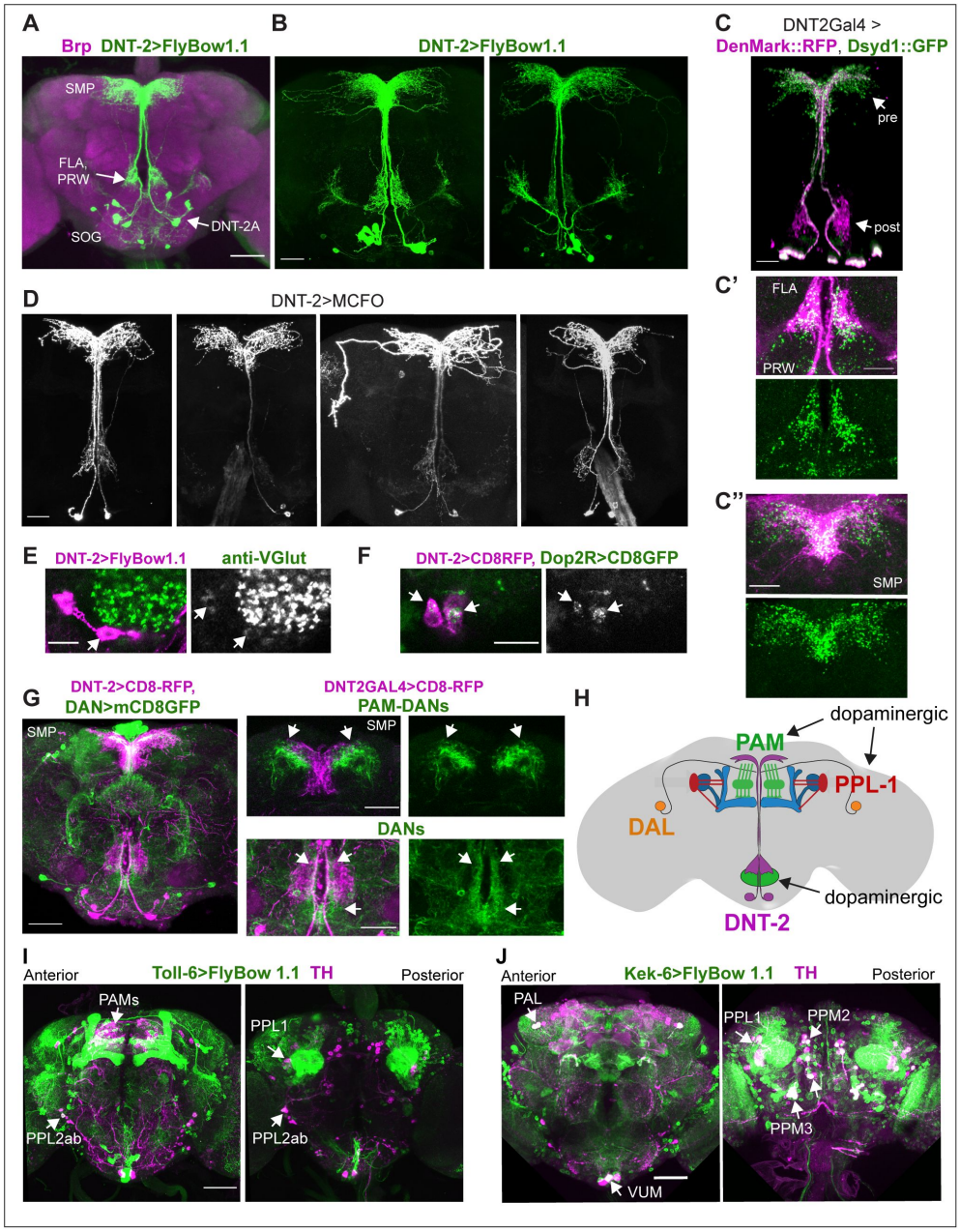

Figure 1

Neurons expressing *DNT-2* and its receptors *Toll-6* and *kek-6* in the adult brain

(A, B) *DNT-2A* expressing neurons (*DNT-2>FlyBow1.1* in green; anti-Brp in magenta) have cell bodies in SOG and project to FLA/PRW and SMP. **(C, C', C'')** Pre-synaptic (green) and post-synaptic (magenta) terminals of DNT-2A neurons seen with *DNT-2>DenMark::RFP, Dsyd1::GFP*, higher magnification in **(C',C'')**, different specimens from (C). DNT-2A projections at SMP and PRW have both pre- and post-synaptic sites. **(D)** Single neuron DNT-2A>MCFO clones. **(E)** DNT-2A neurons have the vesicular glutamate transporter vGlut (arrows). **(F)** Colocalization between *Dop2RLex>AopCD8-GFP* and *DNT2Gal4>UASCD8-RFP* in cell bodies of DNT-2A neurons (arrows). **(G)** Terminals of dopaminergic neurons (*TH>mCD8GFP*) abut and overlap those of DNT-2A neurons (*DNT2>CD8-RFP*, magenta), arrows; magnified projections on the right. **(H)** Illustration of neurons expressing DNT-2 (magenta) and KCs, DAN PAM and PPL1, and DAL neurons **(I)** *Toll-6>FlyBow1.1* is expressed in Kenyon cells, PPL1, PPL2 and PAM DANs, as revealed by co-localization with anti-TH. **(J)** *kek-6>FlyBow1.1* co-localises with TH in MB vertical lobes, dopaminergic PALs, VUMs, PPL1, PPM2 and PPM3. SMP: superior medial protocerebrum, PRW: Prow, FLA: Flange, SOG: sub-aesophageal ganglion. Scale bars: (A, H, I) 50µm; (B,C,C'', D,G) 30µm (C', F,) 25µm.

Bidirectional connectivity between DNT-2A neurons and DANs

To verify the connectivity of DNT-2A neurons with DANs, we used various genetic tools. To identify DNT-2A output neurons, we used TransTango (Talay et al. 2017) (**Figure 2A** and Figure S3). DNT-2A RFP+ outputs included a subset of MB α'β' lobes, αβ Kenyon cells, tip of MB β'2, DAL neurons, dorsal fan-shaped body layer and possibly PAM or other dopaminergic neurons (**Figure 2A**, Figure S3). Consistently, these DNT-2A output neurons express *Toll-6* and *kek-6* (Supplementary Table S1). To identify DNT-2A input neurons, we used BAcTrace (Cachero et al. 2020). This identified PAM-DAN inputs at SMP (**Figure 2B**). Altogether, these data showed that DNT-2A neurons receive dopaminergic neuromodulatory inputs, their outputs include MB Kenyon cells, DAL neurons and possibly DANs, and DNT-2 arborisations at SMP are bidirectional.

To further test the relationship between DNT-2A neurons and DANs, we reasoned that stimulating DANs would provoke either release or production of dopamine. So, we asked whether increasing DNT-2 levels in DNT-2 neurons could influence dopamine levels. For this, we over-express *DNT-2* in full-length form (i.e. *DNT-2FL*), as it enables to investigate non-autonomous functions of DNT-2 (Ulian-Benitez et al. 2017). Importantly, DNT-2FL is spontaneously cleaved into the mature form (McIlroy et al. 2013, Foldi et al. 2017) (see discussion). Thus, we over-expressed *DNT-2FL* in DNT-2 neurons and asked whether this affected dopamine production, using mRNA levels for *TH* as readout. Using quantitative real-time PCR (qRT-PCR) we found that over-expressing *DNT2-FL* in DNT-2 neurons in adult flies increased *TH* mRNA levels in fly heads (**Figure 2C**). This showed that DNT-2 could stimulate dopamine production.

Next, we wondered whether in turn DNT-2A neurons, that express *Dop2R*, could be modulated by dopamine. Binding of dopamine to D2-like Dop2R (also known as DD2R) inhibits adenylyl-cyclase, decreasing cAMP levels (Hearn et al. 2002, Neve et al. 2004). Thus, we asked whether DNT-2A neurons received dopamine and signal via Dop2R. Genetic restrictions did not allow us to activate PAMs and test DNT-2 neurons, so we activated DNT-2 neurons and tested whether *Dop2R* knock-down would increase cAMP levels. We used the FRET-based cAMP sensor, Epac1-camps-50A (Shafer et al. 2008). When Epac1-camps-50A binds cAMP, FRET is lost, resulting in decreased YFP/CFP ratio over time. Indeed, *Dop2R* RNAi knock-down in DNT-2A neurons significantly increased cAMP levels (**Figure 2D**), demonstrating that normally Dop2R inhibits cAMP signalling in DNT-2A cells. Importantly, this result meant that in controls, activating DNT-2A neurons caused dopamine release from DANs that then bound Dop2R to inhibit adenylyl-cyclase in DNT-2A neurons; this inhibition was prevented with *Dop2R* RNAi knock-down. Altogether, this shows that DNT-2 up-regulated TH levels (**Figure 2E**), and presumably via dopamine release, this inhibited cAMP in DNT-2A neurons (**Figure 2F**).

In summary, DNT-2A neurons are connected to DANs, DAL and MB Kenyon cells, all of which express DNT-2 receptors *Toll-6* and *kek-6* and belong to a dopaminergic as well as associative learning and memory circuit. Furthermore, DNT-2A and PAM neurons form bidirectional connectivity. Finally, DNT-2 and dopamine regulate each other: DNT-2 increased dopamine levels (**Figure 2E**), and in turn dopamine via Dop2R inhibited cAMP signalling in DNT-2A neurons (**Figure 2F**). That is, an amplification was followed by negative feedback. This suggested that a dysregulation in this feedback loop could have consequences for dopamine-dependent behaviours and for circuit remodelling by the DNT-2 growth factor.

DNT-2 and Toll-6 maintain survival of PAM dopaminergic neurons in the adult brain

Above we showed that DNT-2 and PAM dopaminergic neurons are connected, so we next asked whether loss of function for *DNT-2* or *Toll-6* would affect PAMs. In wild-type flies, PAM-DAN number can vary between 220-250 cells per *Drosophila* brain, making them ideal to investigate changes in cell number (Liu et al. 2012). Maintenance of neuronal survival is a manifestation of

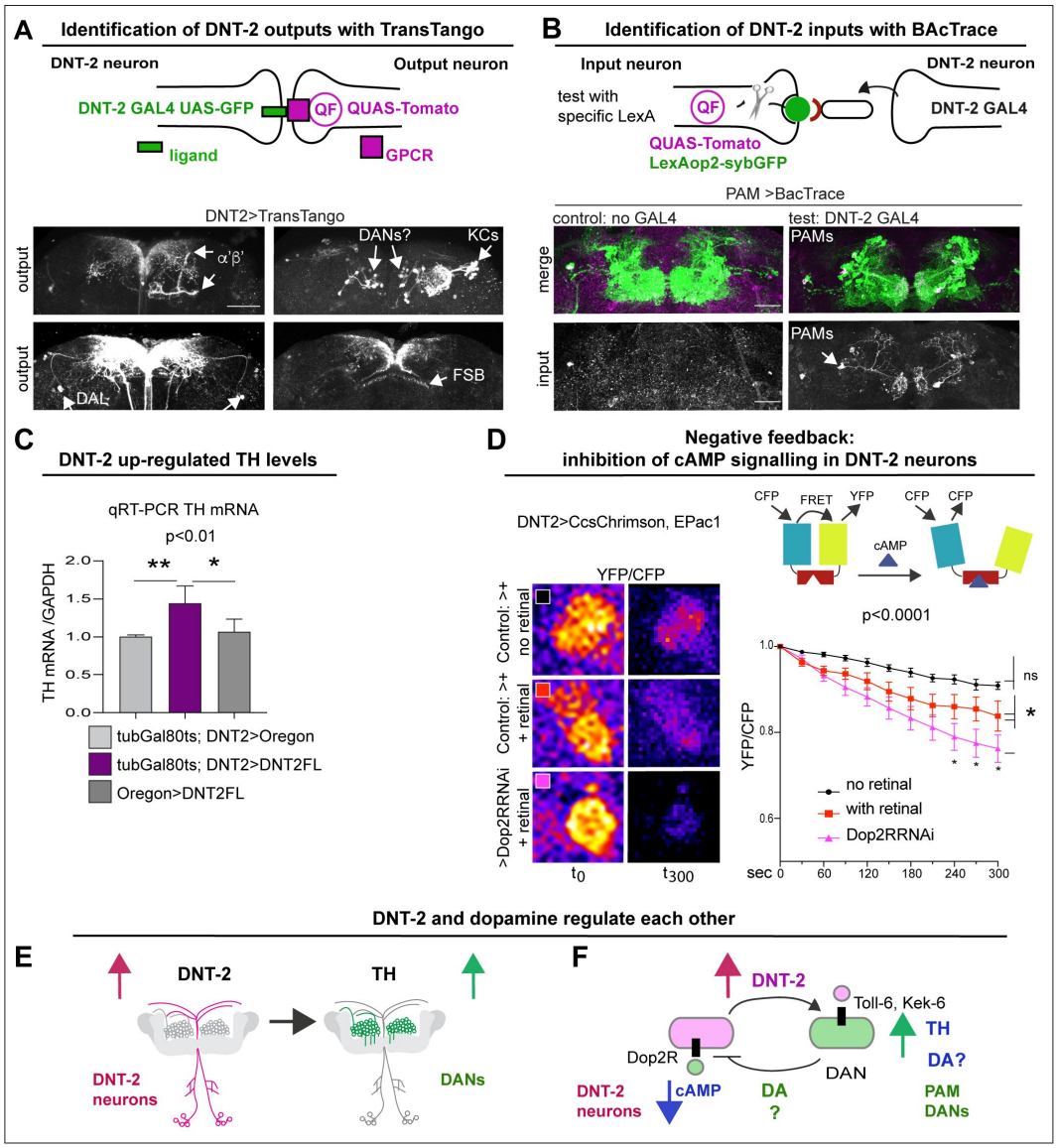

Figure 2

DNT-2 neurons are functionally connected to dopaminergic neurons

(A) TransTango revealed out-puts of DNT-2 neurons. All neurons express *TransTango* and expression of the TransTango ligand in DNT-2 neurons identified DNT-2 out-puts with Tomato (anti-DsRed). TransTango identified as DNT-2 outputs KC α'β' MB lobes (anterior brain, arrow, **top left**); Kenyon and possibly DAN cell bodies (posterior, arrows **top right**); DAL neurons **(bottom left,** arrows) and the dorsal layer of the fan shaped body **(bottom right,** arrow). See also Supplementary Figure S3 for further controls. **(B)** BAcTrace tests connectivity to a candidate neuron input visualised with *LexAop>sybGFP* by driving the expression of a ligand from *DNT-2GAL4* that will activate *QUASTomato* in the candidate input neuron (Cachero et al. 2020). Candidate PAM neurons visualised at SMP with GFP (green): Control: *R58E02LexA>BAcTrace 806*, no *GAL4*. Test: *R58E02LexA, DNT-2GAL4>BAcTrace 806* revealed PAMs are inputs of DNT-2A neurons at SMP (Tomato, bottom). Magenta shows QUAS-Tomato. **(C)** qRT-PCR showing that TH mRNA levels increased with DNT-2 over-expression at 30°C *(tubGAL80^{ts}, DNT-2>DNT-2FL).* One way ANOVA, P=0.0085; post-doc Dunnett's multiple comparison test. **(D)** FRET cAMP probe Epac1 revealed that *DNT-2>Dop2R-RNAi* knockdown decreased YFP/CFP ratio over time in DNT-2A neurons, meaning that cAMP levels increased. Two-way ANOVA, genotype factor p<0.0001, time factor p<0.0001; post-doc Dunnett's. **(E,F)** Summary: DNT-2 neurons and DANs are functionally connected and modulate each other. **(E)** DNT-2 can induce *TH* expression in DANs; **(F)** this is followed by negative feedback from DANs to DNT-2 neurons (question marks indicate inferences). TH: tyrosine hydroxylase. Scale bars: (A) 50µm; (B) 30µm; (D) 20µm. P values over graphs in (C) refer to group analyses; stars indicate multiple comparisons tests. *p<0.05,**p<0.01, ***p<0.001. For sample sizes and further statistical details, see Supplementary Table S2.

structural brain plasticity in mammals, where it depends on the activity-dependent release of the neurotrophin BDNF (Lu et al. 2005, Wang et al. 2022). Importantly, cell number can also change in the adult fly, as neuronal activity can induce neurogenesis via Toll-2, whereas DANs are lost in neurodegeneration models (Feany and Bender 2000, Li et al. 2020). Thus, we asked whether DNT-2 influences PAM-DAN number in the adult brain. We used *THGal4; R58E02Gal4* to visualise nuclear Histone-YFP in DANs (**Figure 3A**) and counted automatically YFP+ PAMs using a purposely modified DeadEasy plug-in developed for the adult fly brain (Li et al 2020). DeadEasy plug-ins were developed and used before to count cells labelled with sparsely distributed nuclear markers in embryos (Zhu et al. 2008, Forero et al. 2009, Forero et al. 2010a, Forero et al. 2010b, McIlroy et al. 2013), larvae (Kato et al. 2011, Forero et al. 2012, Losada-Perez et al. 2016) and adult (Li et al. 2020) *Drosophila* brains. Here we show that $DNT2^{37}/DNT2^{18}$ mutant adult brains had fewer PAMs than controls (**Figure 3B**). Similarly, *Toll-6* RNAi knock-down in DANs also decreased PAM neuron number (**Figure 3C**). DAN loss was confirmed with anti-TH antibodies and counted manually, as there were fewer TH+ PAMs in $DNT2^{37}/DNT2^{18}$mutants (**Figure 3D**). Importantly, PAM cell loss was rescued by over-expressing activated $Toll\text{-}6^{CY}$in DANs in *DNT-2* mutants (**Figure 3D**). Altogether, these data showed that DNT-2 functions via Toll-6 to maintain PAM neuron survival.

To ask whether DNT-2 could regulate dopaminergic neuron number specifically in the adult brain, we used $tubGal80^{ts}$ to conditionally knock-down gene expression in the adult. PAMs were visualised with either *R58E02LexA>LexAop-nls-tdTomato* or *THGal4; R58E02Gal4 >histone-YFP* and counted automatically. Adult specific *DNT-2* RNAi knock-down decreased Tomato+ PAM cell number (**Figure 3E**). Similarly, RNAi *Toll-6* knock-down in DANs also decreased PAM neuron number (**Figure 3F**). Furthermore, knock-down of either *Toll-6* or *DNT-2* in the adult brain caused loss of PAM neurons visualised with anti-TH antibodies and counted manually (**Figure 3G**). Cell loss was due to cell death, as adult specific *DNT-2 RNAi* knock-down increased the number of apoptotic cells labelled with anti-*Drosophila* Cleave caspase-1 (DCP-1) antibodies compared to controls, including Dcp-1+ PAMs and other TH+ cells (**Figure 3H**). Dcp-1+ cells also included TH-negative cells, consistent with the expression of *Toll-6* and *kek-6* also in other cell types. By contrast, *DNT-2FL* over-expression in DNT2 neurons did not alter the incidence of apoptosis (**Figure 3G**), consistently with the fact that DNT-2FL spontaneously cleaves into the mature form (McIlroy et al. 2013, Foldi et al. 2017). Instead, and importantly, over-expression of DNT-2FL increased PAM cell number (**Figure 3G**). Thus, *DNT-2* and *Toll-6* knock-down specifically in the adult brain induced apoptosis and PAM-neuron loss, whereas DNT-2 gain of function increased PAM cell number.

Altogether, these data showed that PAM cell number is plastic, sustained PAM neuron survival in development and in the adult brain depends on DNT-2 and Toll-6, and a reduction in their levels causes DAN cell loss, characteristic of neurodegeneration.

DNT-2 and its receptors are required for arborisations and synapse formation

We next asked whether DNT-2, Toll-6 and Kek-6 could influence dendritic and axonal arbours and synapses of dopaminergic neurons (**Figure 4A**). Visualising the pre-synaptic reporter Synaptotagmin-GFP (Syt-GFP) in all DANs, we found that $DNT\text{-}2^{18}/DNT\text{-}2^{37}$mutants completely lacked DAN synapses in the MB β,β' and γ lobes (**Figure 4B**). Interestingly, DAN connections at α,α' lobes were not affected (**Figure 4B**). This means that DNT-2 is required for synaptogenesis and connectivity of PAMs to MB β,β' and γ lobes.

PAM-β2β'2 neuron dendrites overlap axonal DNT2 projections. *Toll-6* RNAi knock-down in PAM-β2β'2 (with split-GAL4 *MB301BGal4* (Aso et al. 2014a)), reduced dendrite complexity (**Figure 4C**). To test whether DNT-2 could alter these dendrites, we over-expressed mature *DNT-2CK*. DNT-2CK is not secreted (from transfected S2 cells), but it is functional in vivo (Zhu et al. 2008,

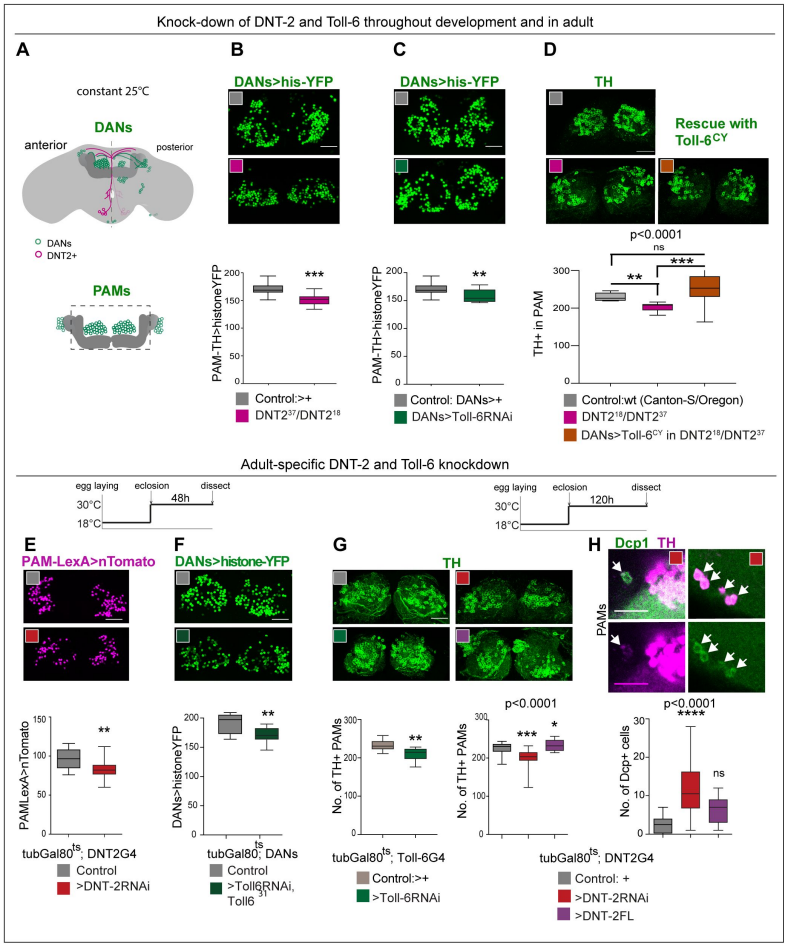

Figure 3

DNT-2 and Toll-6 maintain PAM neuron survival in the developing and adult brain.

(A) Illustration of PAM neuronal cell bodies and experimental temporal profile. DANs are shown in green, DNT-2A neurons in magenta and MB in dark grey. The left hemisphere shows the anterior brain with PAL and PAM DAN neurons (green) and DNT-2 neurons (magenta); the right shows the posterior brain, with the calyx and other DAN neurons (PPM1, PPM2, PPM3, PPL1, PPL2, green). **(B-D)** Fruit-flies were kept constantly at 25°C, from development to adult. Analyses done in adult brains. **(B)** *DNT-2^{37}/DNT-2^{18}* mutants had fewer histone-YFP+ labelled PAM neurons (*THGAL4, R58E02-GAL4>hisYFP).* Un-paired Student t-test. **(C)** *Toll-6* RNAi knock-down in all DANs (*THGAL4, R58E02-GAL4>hisYFP, Toll-6 RNAi)* reduced Histone-YFP+ labelled PAM cell number. Un-paired Student t-test. **(D)** *DNT-2^{37}/DNT-2^{18}* mutants had fewer PAMs stained with anti-TH antibodies. Over-expressing *Toll-6^{CY}* in DANs (*THGAL4, R58E02 GAL4>hisYFP, Toll-6^{CY}*) rescued TH+ PAM neurons in *DNT-2^{37}/DNT-2^{18}* mutants, demonstrating that DNT-2 functions via Toll-6 to maintain PAM cell survival. Welch ANOVA p<0.0001, post-hoc Dunnett test. **(E-H)** Adult specific restricted over-expression or knock-down at 30°C, using the temperature sensitive GAL4 repressor *tubGAL80^{ts}*. **(E)** Adult specific *DNT-2* RNAi knockdown in DNT-2 neurons decreased Tomato+ PAM cell number (*tubGAL80^{ts}, R58E02-LexA, DNT-2 GAL4>LexAOP-Tomato, UAS DNT-2-RNAi*). Un-paired Student t-test, p= 0.005. **(F)** Adult specific *Toll-6* RNAi knock-down in *Toll-6^{31}* heterozygous mutant flies in DANs, reduced Histone-YFP+ PAM cell number (*tubGAL80^{ts}; THGAL4, R58E02-GAL4>hisYFP, Toll-6 RNAi/Toll6^{31}*). Un-paired Student t-test. **(G)** PAMs were visualised with anti-TH. Left: *tubGAL80^{ts}, Toll-6>Toll-6-RNAi* knock-down decreased TH+ PAM cell number. Un-paired Student t-test. Right: *tubGAL80^{ts}, DNT-2>DNT-2-RNAi* knock-down decreased TH+ PAM cell number, whereas *DNT-2FL* over-expression increased PAM cell number. Kruskal-Wallis ANOVA, p=0.0001, post-hoc Dunn's test. **(H)** Adult-specific *tubGAL80^{ts}, DNT-2>DNT-2RNAi* knock-down increased the number of apoptotic cells in the brain labelled with anti-DCP-1. Dcp-1+ cells co-localise with anti-TH at least in PAM clusters. One Way ANOVA, p<0.0001, post-doc Bonferroni's multiple comparisons test. DANs>histone-YFP: all dopaminergic neurons expressing histone-YFP, genotype: *THGal4 R58E02Gal4>UAS-histoneYFP*. *PAMsLexA>tomato*: restricted to PAM DANs: *R58E02LexA>LexAop-nlstdTomato.* Controls: *GAL4* drivers crossed to wild-type Canton-S. Scale bars: (B-G) 30µm; (H) 20µm. Graphs show box-plots around the median. P values over graphs in (D, G right, H) refer to group analyses; stars indicate multiple comparisons tests. *p<0.05, **p<0.01, ***p<0.001. For further genotypes, sample sizes and statistical details, see Supplementary Table S2.

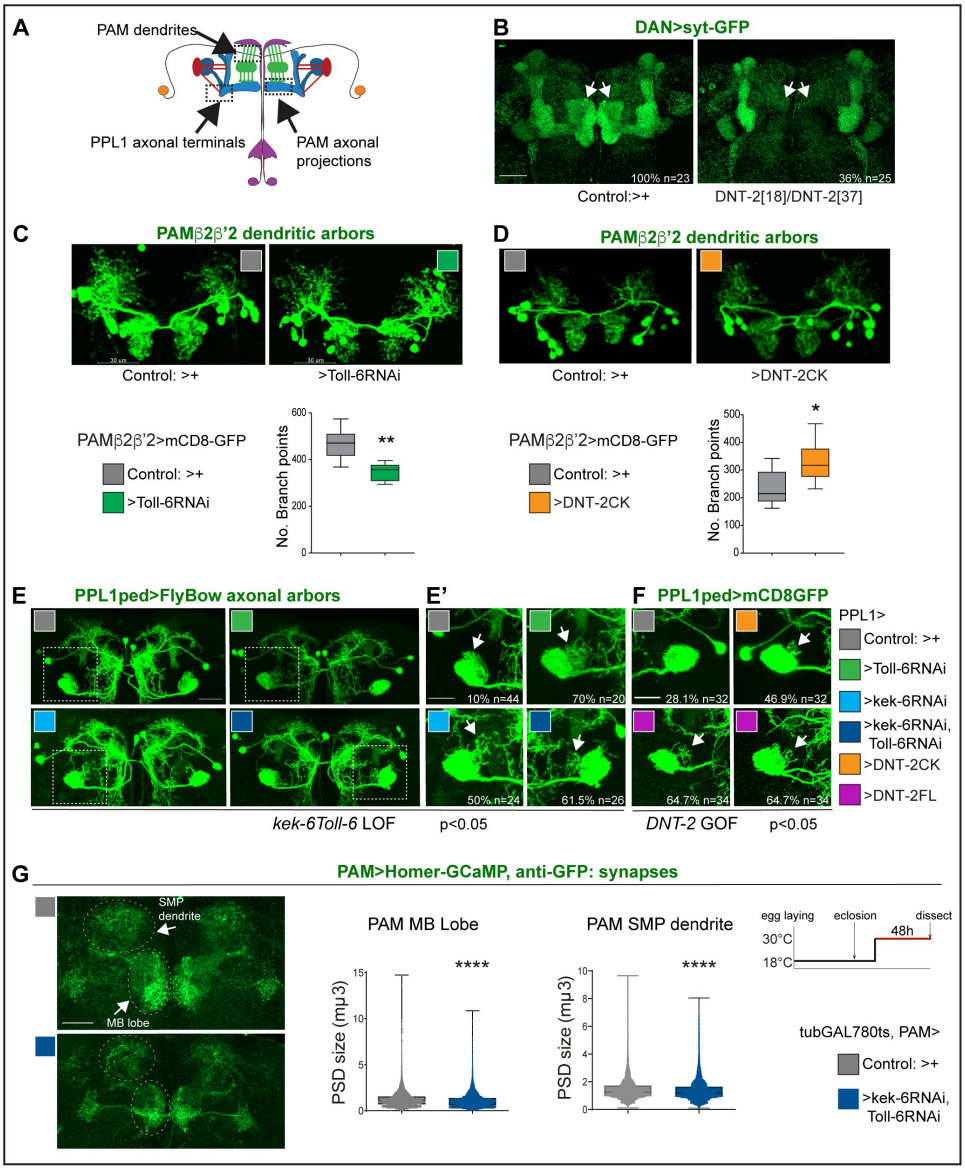

Figure 4

DNT-2, Toll-6 and Kek-6 are required for arborisations and synapse formation.

(A) Illustration showing the ROIs (dashed lines) used for the analyses, corresponding to dendrites and axonal endings of PAMs and axonal terminals of PPL1ped neurons. **(B-F)** Fruit-flies were kept constantly at 25°C. **(B)** Complete loss of DAN synapses *(TH, R58E02>sytGFP)* onto α,α MB lobe in *DNT-2* null mutants. **(C)** *Toll-6* RNAi knockdown in PAM β2β'2 neurons (*MB301B>FB1.1, Toll-6-RNAi*) decreased dendrite complexity. Un-paired Student t-test. **(D)** Over-expression of cleaved *DNT-2CK* in PAM β2β'2 neurons *(MB301B>CD8-GFP, DNT-2CK)* increased dendrite complexity. Un-paired Student t-test. **(E,E',F)** PPL1ped axonal misrouting was visualised with *split-GAL4 MB320CGal4>FlyBow1.1.* Images show PPL1-yped neurons and some PPL1-α2α'2. **(D,D')** RNAi knock-down of *Toll-6, kek-6* or both (e.g. *MB320CGal4>FlyBow1.1, Toll-6RNAi)* in PPL1-yped neurons caused axonal terminal misrouting (arrows). **(E')** Higher magnification of **(E,** dotted squares**)**. Chi-square for group analysis: p = 0.0224, and multiple comparisons Bonferroni correction control vs *Toll-6RNAi* p<0.01; vs *kek-6RNAi* p<0.05; vs *Toll-6RNAi kek-6RNAi* p<0.01, see Table S2. **(F)** PPL1 misrouting was also induced by over-expressed *DNT-2CK* or *DNT-2FL* (e.g. *MB320CGal4>FlyBow1.1, DNT-2FL)*. Chi-square for group analysis: p<0.05, Bonferroni correction control vs *DNT-2CK* ns, vs *DNT-2FL* *p<0.05, see Table S2. **(G)** Adult-specific *Toll-6 kek-6 RNAi* knockdown in PAM neurons decreased size of post-synaptic density sites (*PAM>Homer-GCaMP* and anti-GFP antibodies). Temperature regime shown on the right. **(C,D)** Graphs show box-plots around the median; **(G)** are box-plots with dot plots. (C,D,G) *p<0.05, **p<0.01, ****p<0.0001. Scale bars: (B,C,D,E) 30μm; (E',F,G) 20μm. For genotypes, sample sizes and statistical details, see Supplementary Table S2.

Foldi et al. 2017, Ulian-Benitez et al. 2017). Importantly, over-expressed *DNT-2CK* functions cell-autonomously whereas *DNT-2FL* functions also non-autonomously, but they have similar effects (Zhu et al. 2008, Foldi et al. 2017, Ulian-Benitez et al. 2017). Over-expression of *DNT-2CK* in PAM-β2β'2 increased dendrite arbour complexity (**Figure 4D**). Thus, DNT-2 and its receptor Toll-6 are required for dendrite growth and complexity in PAM neurons.

To ask whether DNT-2 could affect axonal terminals, we tested PPL1 axons. PPL1-γ1-pedc neurons have a key function in long-term memory gating (Aso et al. 2012, Placais et al. 2012, Aso et al. 2014a, Boto et al. 2020, Huang et al. 2024) and express both *Toll-6* and *kek-6* (Table S1). Using split-GAL4 line *MB320C-Gal4* to visualise PPL1 axonal arbours, RNAi knock-down of either *Toll-6*, *kek-6* or both together caused axonal misrouting away from the mushroom body peduncle (**Figure 4E,E'**, Chi Square p<0.05, see Table S2). Similarly, *DNT-2FL* over-expression also caused PPL1 misrouting (**Figure 4F**, Chi-Square p<0.05, see Table S2). Thus, DNT-2, Toll-6 and Kek-6 are required for appropriate targeting and connectivity of PPL1 DAN axons.

To test whether this signalling system was required specifically in the adult brain, we used *tubGAL80^{ts}*to knock-down *Toll-6* and *kek-6* with RNAi conditionally in the adult and visualised the effect on synaptogenesis using the post-synaptic reporter Homer-GCaMP and anti-GFP antibodies. Adult-specific *Toll-6 kek-6* RNAi knock-down in PAM neurons did not affect synapse number (not shown), but it decreased post-synaptic density size, both at the MB lobe and at the SMP dendrite (**Figure 4G**). These data meant that the DNT-2 receptors *Toll-6* and *kek-6* continue to be required in the adult brain for appropriate synaptogenesis.

Altogether, these data showed that DNT-2, Toll-6 and Kek-6 are required for dendrite branching, axonal targeting and synapse formation. The shared phenotypes from altering the levels of *DNT-2* and *Toll-6 kek-6* in arborisations and synapse formation, support their joint function in these contexts. Importantly, these findings showed that connectivity of PAM and PPL1 dopaminergic neurons depend on DNT-2, Toll-6 and Kek-6.

DNT-2 neuron activation and *DNT-2* over-expression induced synapse formation in target PAM dopaminergic neurons

The above data showed that DNT-2, Toll-6 and Kek-6 are required for DAN cell survival, arborisations and synaptogenesis in development and adults. This meant that the dopaminergic circuit remains plastic in adult flies, consistently with their functional plasticity (Boto et al. 2014). Thus, we wondered whether neuronal activity could also induce remodelling in PAM neurons. In mammals, neuronal activity induces translation, release and cleavage of BDNF, and BDNF drives synaptogenesis (Poo 2001, Lu et al. 2005, Lu et al. 2013, Wang et al. 2022). Thus, we first asked whether neuronal activity could influence DNT-2 levels or function. We visualised tagged DNT-2FL-GFP in adult brains, activated DNT-2 neurons with TrpA1 at 30°C, and found that DNT-2 neuron activation increased the number of DNT-2-GFP vesicles produced (Supplementary Figure S4A). Furthermore, neuronal activity also facilitated cleavage of DNT-2 into its mature form. In western blots from brains over-expressing *DNT-2FL-GFP*, the levels of full-length DNT-2FL-GFP were reduced following neuronal activation and the cleaved DNT-2CK-GFP form was most abundant (Supplementary Figure S4B). These findings meant that, like mammalian BDNF, also DNT-2 can be influenced by activity.

Thus, we asked whether neuronal activity and DNT-2 could influence synapse formation. We first tested DNT-2 neurons. Activating DNT-2 neurons altered DNT-2 axonal arbours (**Figure 5A**) and it increased Homer-GFP+ synapse number in the DNT-2 SMP arbour (**Figure 5B** and Figure S5). Next, as DNT-2 and PAMs form bidirectional connexions at SMP (**Figure 1**, **2**), we asked whether activating DNT-2 neurons could affect target PAM neurons. To manipulate DNT-2 neurons and visualise PAM neurons concomitantly, we combined *DNT-2GAL4* with the *PAM-LexAOP* driver. However, there were no available *LexA/OP* post-synaptic reporters, so we used the pre-synaptic

LexAOP-Syt-GCaMP reporter instead, which labels Synaptotagmin (Syt), and GFP antibodies. Activating DNT-2 neurons with TrpA1 increased the number of Syt+ synapses at the PAM SMP arbour (**Figure 5C**) and reduced their size (**Figure 5C**). This was consistent with the increase in Homer-GFP+ PSD number in stimulated DNT-2 neurons (**Figure 5B**). Neuronal activity can induce ghost boutons, immature synapses that are later eliminated (Fuentes-Medel et al. 2009). Here, the coincidence of increased pre-synaptic Syt-GFP from PAMs and post-synaptic Homer-GFP from DNT-2 neurons at SMP suggests that newly formed synapses could be stable. PAM neurons also send an arborisation at the MB β, β', γ lobes, but DNT-2 neuron activation did not affect synapse number nor size there (**Figure 5C**). These data showed that activating DNT-2 neurons induced synapse formation at the SMP connection with PAMs.

Finally, we asked whether, like activity, DNT-2FL could also drive synaptogenesis. We over-expressed *DNT-2FL* in DNT-2 neurons and visualised the effect in PAM neurons. Over-expression of *DNT-2FL* in DNT-2 neurons did not alter Syt+ synapse number at the PAM SMP dendrite, but it increased bouton size (**Figure 5D**). By contrast, at the MB β, β' lobe arborisation, over-expressed *DNT-2* did not affect Syt+ bouton size, but it increased the number of output synapses (**Figure 5D**). This data showed that DNT-2 released from DNT-2 neurons could induce synapse formation in PAM target neurons.

Altogether, these data showed that neuronal activity induced synapse formation, it stimulated production and cleavage of DNT-2, and DNT-2 could induce synapse formation in target neurons.

Structural plasticity by DNT2 modified dopamine-dependent behaviour

Circuit structural plasticity raises the important question of what effect it could have on brain function, i.e. behaviour. Data above showed that DANs and DNT-2 neurons are functionally connected, that loss of function for *DNT-2* or its receptors caused dopaminergic neuron loss, altered DAN arborisations and caused synapse loss or reduction in size, and that DNT-2 could induce dendrite branching and synaptogenesis, altogether modifying circuit connectivity. To measure the effect of such circuit modifications on brain function, we used dopamine-dependent behaviours as readout.

Startle-induced negative geotaxis (also known as the climbing assay) is commonly used as a measure of locomotor ability and requires dopamine and specifically PAM neuron function (Riemensperger et al. 2013, Sun et al. 2018). We tested the effect of *DNT-2* or *Toll-6* and *kek-6* loss of function in climbing, and both $DNT\text{-}2^{37}/DNT\text{-}2^{18}$mutants and flies in which *DNT-2* was knocked-down in DNT-2 neurons in the adult stage had lower climbing ability than controls (**Figure 6A**). Similarly, when *Toll-6* and *kek-6* were knocked-down with RNAi in the adult using a *Toll-6*- or a *PAM-GAL4* neuron driver, climbing was also reduced (**Figure 6B**). Importantly, over-expressing activated $Toll\text{-}6^{CY}$in DANs rescued the locomotion deficits of *DNT-2* mutants, showing that DNT-2 functions via Toll-6 in this context (**Figure 6C**).

We also tracked freely moving flies in an open arena (Eyjolfsdottir et al. 2014). Interestingly, in that setting, locomotion of homozygous $DNT\text{-}2^{37}/DNT\text{-}2^{18}$mutants was similar to that of controls, but over-expression of $Toll\text{-}6^{CY}$in their DANs increased locomotion as flies walked longer distances and spent less time immobile (**Figure 6D**). Adult flies over-expressing *DNT2-FL* walked faster (**Figure 6E** and Figure S5) and so did those where DNT-2 neurons were activated with TrpA1 (**Figure 6F** and Figure S6), consistently with the fact that neuronal activity increased DNT-2 production (Figure S4A) and that DNT-2FL increased TH levels (**Figure 2C**). Therefore, increased $Toll\text{-}6^{CY}$ levels in DANs increase locomotion and increased DNT-2 levels are sufficient to boost walking speed. Interestingly, both loss and gain of function for *DNT-2* also caused seizures (Figure S7). Thus, dopamine-dependent locomotion is regulated by the function of DNT-2, Toll-6 and Kek-6.

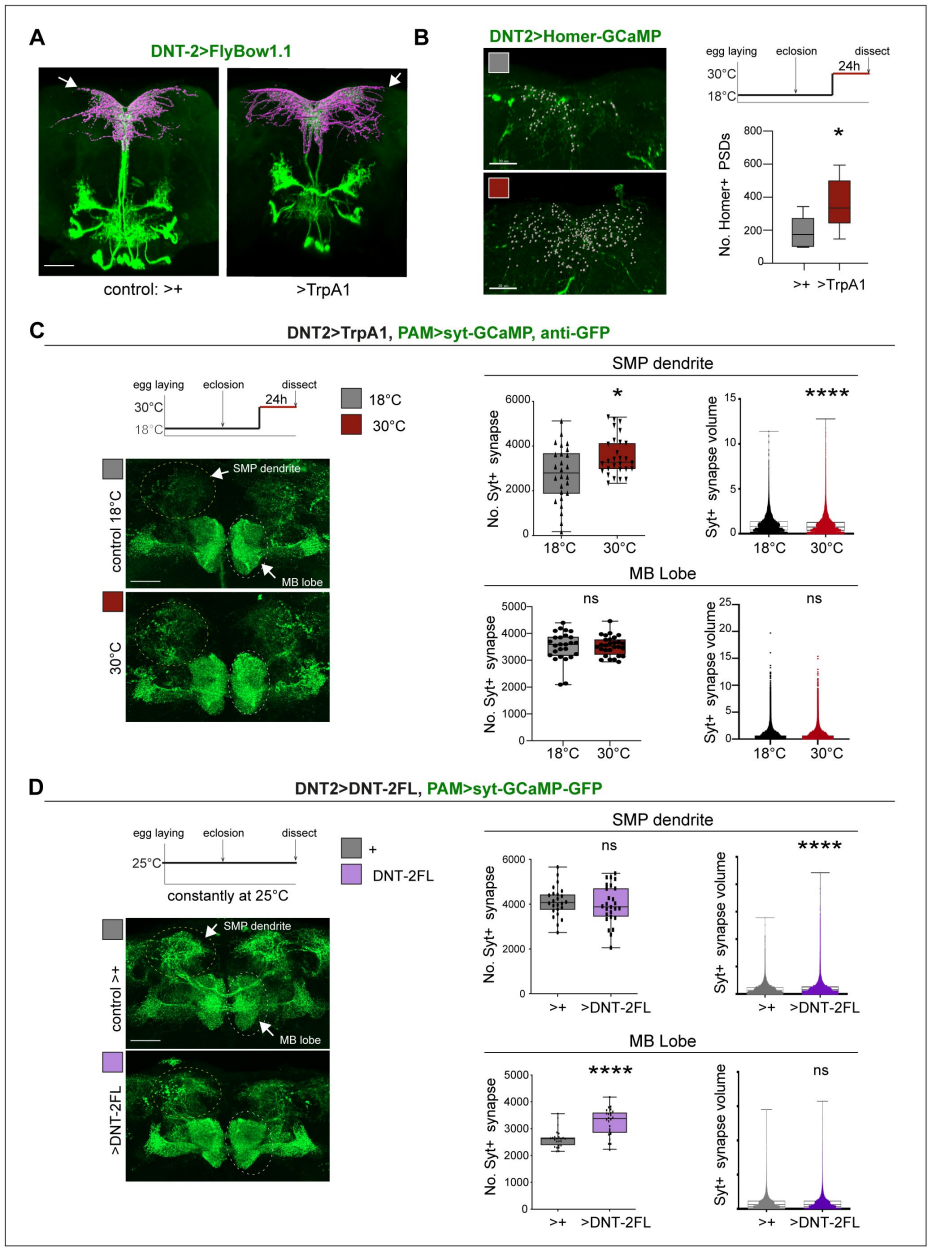

Figure 5

DNT-2 neuron activation and DNT-2 over-expression induced synaptogenesis.

(A) Thermo-activation of DNT-2 neurons at 30°C altered DNT-2 arborisations, here showing an example with smaller dendrites and enlarged axonal arbours (magenta shows 3D-rendering of axonal arborisation done with Imaris, merged with raw image in green). (Genotype: *DNT-2>FlyBow1.1, TrpA1*). **(B)** Thermogenetic activation of DNT-2 neurons increased the number of Homer+ PSDs in DNT-2 neurons, at SMP (*DNT-2>Homer-GCAMP3, TrpA1*, anti-GFP). Test at 30°C 24h: Unpaired Student t test. See Table S2. **(C)** Thermogenetic activation of DNT-2 neurons induced synaptogenesis in PAM target neurons at SMP, but not at MB lobe. (Genotype: *PAM(R58E02)LexA/LexAOP-sytGCaMP; DNT-2GAL4/UASTrpA1*). At SMP: No. Syt+ synapses: Mann Whitney-U; Syt+ synapse volume: Mann Whitney-U. At MB lobe: No. Syt+ synapses: Unpaired Student t ns; Syt+ synapse volume: Mann Whitney-U ns. **(D)** Over-expression of *DNT-2FL* in DNT-2 neurons increased synapse volume at SMP dendrite and induced synaptogenesis at MB lobe. (Genotype: *PAM(R58E02)LexA/LexAopSytGcaMP6; DNT-2Gal4/UAS-DNT-2FL*). At SMP: No. Syt+ synapses: Unpaired Student t ns. Syt+ synapse volume: Mann Whitney-U. At MB lobe: No. Syt+ synapses: Unpaired Student t; Syt+ synapse volume: Mann Whitney-U ns. Graphs show box-plots around the median, except for PSD volume data that are dot plots. *p<0.05, ****p<0.0001; ns: not significantly different from control. Scale bars: 30µm. For Genotypes, sample sizes, p values and other statistical details, see Supplementary Table S2.

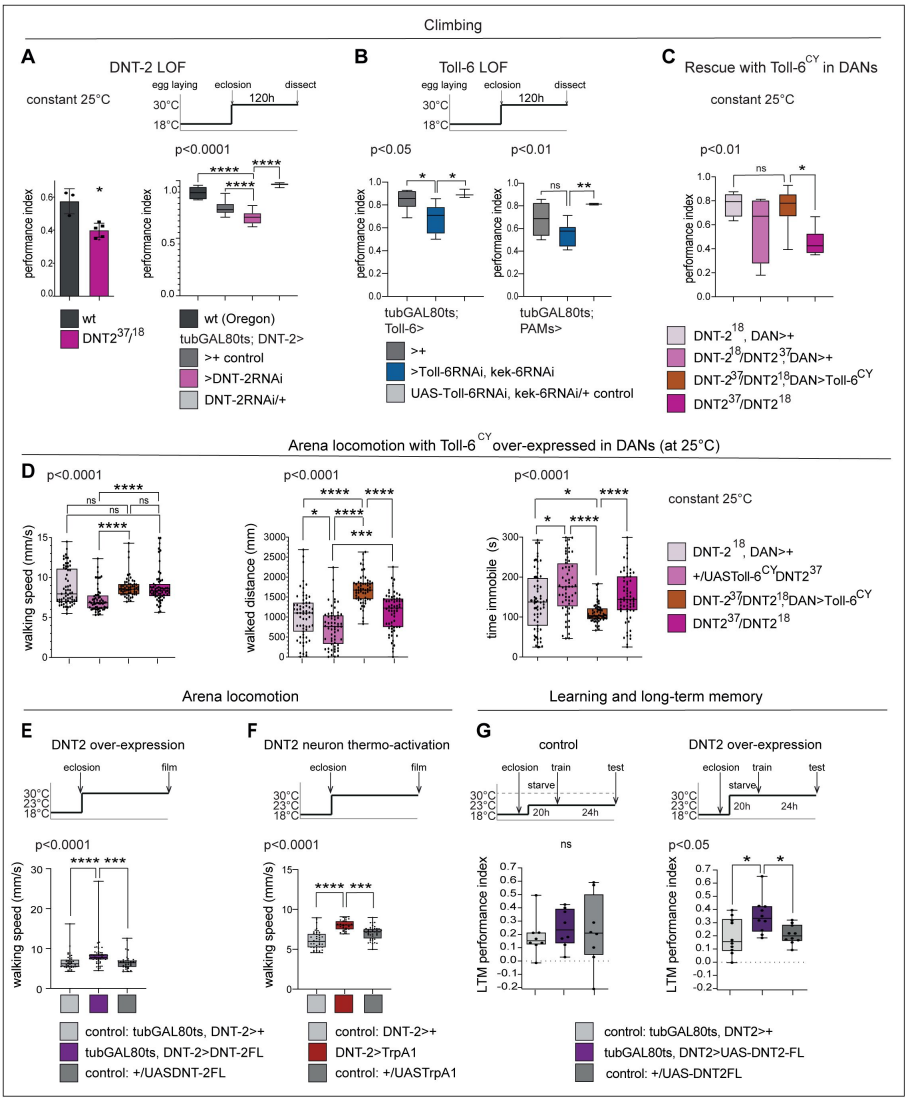

Figure 6

DNT-2-induced circuit plasticity modified dopamine-dependent behaviour.

(A) *DNT-2* mutants (left, *DNT-2^{37}/DNT-2^{18}*) and flies with adult-specific RNAi knock-down of *DNT-2* in DNT-2 neurons (right, *tubGAL80^{ts}; DNT-2>DNT-2RNAi*), had impaired climbing. Left Mann Whitney U; Right: One Way ANOV, post-hoc Dunnett. **(B)** Adult-specific *Toll-6* and *kek-6* RNAi knock-down in Toll-6 *(tubGAL80^{ts}; Toll-6>Toll-6RNAi, kek-6RNAi,* left) or PAM (*tubGAL80^{ts}; R58E02>Toll-6RNAi, kek-6RNAi,* right) neurons impaired climbing. Left: Welch ANOVA, post-hoc Dunnett. Right: Welch ANOVA, post-hoc Dunnett. **(C)** The climbing impairment of *DNT-2* mutants could be rescued with the over-expression of *Toll-6^{CY}* in dopaminergic neurons (Rescue genotype: UASToll-6^{CY}/+; DNT-2^{18}*THGAL4 R58E02GAL4/DNT-2^{37}).* Welch ANOVA, post-hoc Dunnett. **(D)** Adult specific over-expression of *Toll-6^{CY}* in DANs increased locomotion in *DNT-2^{37}/DNT-2^{18}*mutants. (Test genotype: *UASToll-6^{CY}/+; DNT-2^{18}THGAL4 R58E02GAL4/DNT-2^{37}*). Walking speed: Kruskal Wallis ANOVA, post-hoc Dunn's. Distance walked: Kruskal Wallis ANOVA, post-hoc Dunn's. Time spent immobile: Kruskal Wallis ANOVA, post-hoc Dunn's. **(E)** Adult specific *DNT-2FL* overexpression in DNT-2 neurons increased fruit-fly locomotion speed in an open arena at 30°C (see also Supplementary Figure S6A for further controls). (Test genotype: *tubGAL80^{ts}, DNT-2>DNT-2FL*) Kruskal-Wallis, post-hoc Dunn's test. **(F)** Thermogenetic activation of DNT-2 neurons at 30°C *(DNT-2>TrpA1)* increased fruit-fly locomotion speed. (See also Supplementary Figure S6B for further controls). One Way ANOVA p<0.0001, post-hoc Dunnett's test. **(G)** Over-expression of *DNT-2-FL* in DNT-2 neurons increased long-term memory. (Test genotype: *tubGAL80^{ts}, DNT-2>DNT-2FL*). Left: 23°C controls: One Way ANOVA p=0.8006. Right 30°C: One Way ANOVA, post-hoc Dunnett's test. Graphs show box-plots around the median, under also dot plots. P values over graphs refer to group analyses; asterisks indicate multiple comparisons tests. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; ns: not significantly different from control. For further genotypes, sample sizes, p values and other statistical details, see Supplementary Table S2.

Next, as dopamine is an essential neurotransmitter for learning and memory (Adel and Griffith 2021), we asked whether DNT-2 might influence appetitive olfactory conditioning. Starved flies were trained to associate a sugar reward with an odour (CS+) while another odour was presented without sugar (CS-), and their preference for CS+ versus CS-was measured, 24h after training (Tempel et al. 1983, Krashes and Waddell 2008, 2011). Remarkably, over-expression of *DNT-2FL* in DNT-2 neurons in adults enhanced appetitive long-term memory (**Figure 6G**), consistent with the positive role of DNT-2 in synaptogenesis that we demonstrated above.

In summary, we have shown that alterations in DNT-2, Toll-6 and Kek-6 levels that caused structural phenotypes in DANs also modified dopamine-dependent behaviours, locomotion and long-term memory.

Discussion

Our findings indicate that structural plasticity and degeneration in the brain are two manifestations of a shared molecular mechanism that could be modulated by experience. Loss of function for *DNT-2, Toll-6* and *kek-6* caused cell loss, affected arborisations and synaptogenesis in DANs and impaired locomotion; neuronal activity increased DNT-2 production and cleavage and remodelled connecting DNT-2 and PAM synapses; and over-expression of *DNT-2* increased TH levels, PAM cell number, dendrite complexity, and synaptogenesis, and it enhanced locomotion and long-term memory.

It was remarkable to find that the number of dopaminergic neurons in the *Drosophila* adult brain is plastic, and this is functionally relevant as it can influence behaviour. We showed that PAM cell number is variables across individuals, that adult-specific gain of *DNT-2* function increases, whereas loss of *DNT-2* or *Toll-6* function decreases, PAM cell number. Loss of *DNT-2* function in mutants, constant loss of *Toll-6* function in DANS and adult-restricted knock-down of either *DNT-2* (in DNT-2 neurons) or *Toll-6* (in Toll-6 neurons and in DANs) all resulted in DAN cell loss, verified with three distinct reporters, and consistently with the increase in DAN apoptosis. Furthermore, DAN cell loss in *DNT-2* mutants could be rescued by the over-expression of *Toll-6* in DANs. Cell loss was also verified using two reporter types (ie GAL4 based nuclear reporters and cytoplasmic anti-TH antibodies), multiple GAL4 drivers and mutants, and multiple cell counting methods, including automatic cell counting with DeadEasy plug-ins for His-YFP and nls-Tomato (where the signal was of high contrast and sphericity) and software assisted manual cell counting for anti-TH (where the signal is more diffuse and less regular in shape). DeadEasy plug-ins have been used before for reliably counting His-YFP labelled cells in both larval CNS and adult brains, including Kenyon cells (Kato et al. 2011, Forero et al. 2012, Losada-Perez et al. 2016, Li et al. 2020, Harrison et al. 2021). Thus, the finding that loss of *DNT-2* and *Toll-6* function in the adult brain cause dopaminergic neuron loss is robust. Our findings are reminiscent of the increased apoptosis and cell loss in adult brains with *Toll-2* loss of function (Li et al. 2020), and the support of DAN survival by Toll-1 and Toll-7 driven autophagy (Zhang et al. 2024). They are also consistent with a report that loss of function for *DNT-2* or *Toll-6* induced apoptosis in the third instar larval optic lobes (McLaughlin et al. 2019). This did not result in neuronal loss - which was interpreted as due to Toll-6 functions exclusive to glia (McLaughlin et al. 2019) - but instead of testing the optic lobes, neurons of the larval abdominal ventral nerve cord (VNC) were monitored (McLaughlin et al. 2019). In the VNC, Toll-6 and −7 function redundantly and knock-down of both is required to cause neuronal loss in embryos (McIlroy et al. 2013), whereas in L3 larvae and pupae the phenotype is compounded by their pro-apoptotic functions (Foldi et al. 2017). It is crucial to consider that the DNT-Toll signalling system can have distinct cellular outcomes depending on context, cell type and time, i.e. stage (Foldi et al. 2017, Li et al. 2020, Li and Hidalgo 2021). Our work shows that in the adult *Drosophila* brain DAN neurons receive secreted growth factors that maintain cellular integrity, and this impacts behaviour. Consistently with our findings, *Drosophila* models of Parkinson's Disease reproduce the loss of DANs and locomotion impairment

of human patients (Feany and Bender 2000, Riemensperger et al. 2011, Riemensperger et al. 2013, Sun et al. 2018). Dopamine is required for locomotion, associative reward learning and long-term memory (Riemensperger et al. 2011, Riemensperger et al. 2013, Waddell 2013, Sun et al. 2018, Boto et al. 2020, Adel and Griffith 2021). In *Drosophila*, this requires PAM, PPL1 and DAL neurons and their connections to Kenyon Cells and MBONs (Heisenberg 2003, Chen et al. 2012, Placais et al. 2012, Aso et al. 2014b, Placais et al. 2017, Boto et al. 2020, Adel and Griffith 2021, Huang et al. 2024). DNT-2 neurons are connected to all these neuron types, which express *Toll-6* and *kek-6,* and modifying their levels affects locomotion and long-term memory. Altogether, our data demonstrate that structural changes caused by altering DNT-2, Toll-6 and Kek-6 modified dopamine-dependent behaviours, providing a direct link between molecules, structural circuit plasticity and behaviour.

We used neuronal activation as a proxy for experience, but the implication is that experience would similarly drive the structural modification of circuits labelled by neuromodulators. Similar manipulations of activity have previously revealed structural circuit modifications. For example, hyperpolarising olfactory projection neurons increased microglomeruli number, active zone density and post-synaptic site size in the calyx, whereas inhibition of synaptic vesicle release decreased the number of microglomeruli and active zones (Kremer et al. 2010). There is also evidence that experience can modify circuits and behaviour in *Drosophila.* For example, natural exposure to light and dark cycles maintains the structural homeostasis of presynaptic sites in photoreceptor neurons, which breaks down in sustained exposure to light (Sugie et al. 2015). Prolonged odour exposure causes structural reduction at the antennal lobe and at the output pre-synaptic sites in the calyx, and habituation (Devaud et al. 2001, Pech et al. 2015). Similarly, prolonged exposure to CO2 caused a reduction in output responses at the lateral horn and habituation (Sachse et al. 2007). Our findings are also consistent with previous reports of structural plasticity during learning in *Drosophila.* Hypocaloric food promotes structural plasticity in DANs, causing a reduction specifically in connections between DANs and Kenyon cells involved in aversive learning, thus decreasing the memory of the aversive experience (Coban et al. 2024). By contrast, after olfactory conditioning, appetitive long-term memory increased axonal collaterals in projection neurons, and synapse number at Kenyon cell inputs in the calyx (Baltruschat et al. 2021). Our data provide a direct link between a molecular mechanism, synapse formation in a dopaminergic circuit and behavioural performance. Since behaviour is a source of experience, the discovery that a neurotrophin can function with a Toll and a neuromodulator to sculpt circuits provides a mechanistic basis for how experience can shape the brain throughout life.

Importantly, in humans structural brain plasticity (e.g. adult neurogenesis, neuronal survival, neurite growth and synaptogenesis) correlates with anti-depressant treatment, learning, physical exercise and well-being (Cotman and Berchtold 2002, Woollett and Maguire 2011, Castren and Monteggia 2021, Cheng et al. 2022). Conversely, neurite, synapse and cell loss, correlate with ageing, neuroinflammation, psychiatric and neurodegenerative conditions (Holtmaat and Svoboda 2009, Lu et al. 2013, Wohleb et al. 2016, Forrest et al. 2018, Vahid-Ansari and Albert 2021, Wang et al. 2022). Understanding how experience drives the switch between generative and destructive cellular processes shaping the brain is critical to understand brain function, in health and disease. In this context, the mechanism we have discovered could also operate in the human brain. In fact, there is deep evolutionary conservation in DNT-2 vs mammalian NT function (e.g. BDNF), but some details differ. Like mammalian NTs, full-length DNTs/Spz proteins contain a signal peptide, an unstructured pro-domain and an evolutionarily conserved cystine-knot of the NT family (Zhu et al. 2008, Arnot et al. 2010, Foldi et al. 2017). Cleavage of the pro-domain releases the mature cystine-knot (CK). In mammals, full-length NTs have opposite functions to their cleaved forms (e.g. apoptosis vs cell survival, respectively). However, DNT-2FL is cleaved by intracellular furins, and although DNT-2 can be found both in full-length or cleaved forms in vivo, it is most abundantly found cleaved (Zhu et al. 2008, McIlroy et al. 2013, Foldi et al. 2017). As a result, over-expressed *DNT-2FL* does not induce apoptosis and instead it promotes cell

survival (Foldi et al. 2017). The same functions are played by over-expressed mature *DNT-2CK* as by *DNT-2FL* (Zhu et al. 2008, Foldi et al. 2017, Ulian-Benitez et al. 2017). In S2 cells, transfected DNT-2CK is not secreted, but when over-expressed in vivo it is functional (Zhu et al. 2008, Foldi et al. 2017, Ulian-Benitez et al. 2017) (and this work). In fact, over-expressed *DNT-2CK* also maintains neuronal survival, connectivity and synaptogenesis (Zhu et al. 2008, Foldi et al. 2017, Ulian-Benitez et al. 2017) (and this work). Similarly, over-expressed mature *spz-1-C106* can rescue the *spz-1*-null mutant phenotype (Hu et al. 2004) and over-expressed *DNT-1CK* can promote neuronal survival, connectivity and rescue the *DNT-1* mutant phenotype (Zhu et al. 2008). Consistently, both DNT-2FL and DNT-2CK have neuro-protective functions promoting cell survival, neurite growth and synaptogenesis (Zhu et al. 2008, Sutcliffe et al. 2013, Foldi et al. 2017, Ulian-Benitez et al. 2017) (and this work). Importantly, over-expressing *DNT-2FL* enables to investigate non-autonomous functions of DNT-2 (Ulian-Benitez et al. 2017). We have shown that *DNT-2FL* can induce synaptogenesis non-autonomously in target neurons. Similarly, DNT-2 is a retrograde factor at the larval NMJ, where transcripts are located post-synaptically in the muscle, DNT-2FL-GFP is taken up from muscle by motoneurons, where it induces synaptogenesis (Sutcliffe et al. 2013, Ulian-Benitez et al. 2017). Importantly, we have shown that neuronal activity increased production of tagged DNT-2-GFP, and its cleavage. In mammals, neuronal activity induces synthesis, release and cleavage of BDNF, leading to neuronal survival, dendrite growth and branching, synaptogenesis and synaptic plasticity (ie LTP)(Poo 2001, Horch and Katz 2002, Lu et al. 2005, Arikkath 2012, Wang et al. 2022). Like BDNF, DNT-2 also induced synaptogenesis and increased bouton size.

It may not always be possible to disentangle primary from compensatory phenotypes, as Hebbian, homeostatic and heterosynaptic plasticity can concur (Forrest et al. 2018, Jenks et al. 2021). Mammalian BDNF increases synapse number, spine size and long-term potentiation (LTP), but it can also regulate homeostatic plasticity and long-term depression (LTD), depending on timing, levels and site of action (Poo 2001, Lu et al. 2005, Wang et al. 2022). In this context, neuronal stimulation of DNT-2 neurons induced synapse formation in PAM neuron SMP dendrites, whereas DNT-2 over-expression from DNT-2 neurons increased synapse size at SMP and synapse number in PAM outputs at the mushroom body lobes. These distinct effects could be due to the combination of plasticity mechanisms and range of action. Neuronal activity can induce localised protein synthesis that facilitates local synaptogenesis and stabilises emerging synapses (Forrest et al. 2018). By contrast, DNT-2 induced signalling via the nucleus can facilitate synaptogenesis at longer distances, in output sites. In any case, synaptic remodelling is the result of concurring forms of activity-dependent plasticity altogether leading to modification in connectivity patterns (Forrest et al. 2018, Jenks et al. 2021). Long-term memory requires synaptogenesis, and in mammals this depends on BDNF, and its role in the protein-synthesis dependent phase of LTP (Poo 2001, Minichiello 2009, Wang et al. 2022). BDNF localised translation, expression and release are induced to enable long-term memory (Poo 2001, Lee et al. 2004, Minichiello 2009, Wang et al. 2022). We have shown that similarly, over-expressed *DNT-2FL* increased both synaptogenesis in sites involved in reward learning, and long-term memory after appetitive conditioning.

The relationship of NTs with dopamine is also conserved. DNT-2 and DAN neurons form bidirectional connectivity that modulates both DNT-2 and dopamine levels. Similarly, mammalian NTs also promote dopamine release and the expression of DA receptors (Blochl and Sirrenberg 1996, Guillin et al. 2001). Furthermore, DAN cell survival is maintained by DNT-2 in *Drosophila,* and similarly DAN cell survival is also maintained by NT signalling in mammals and fish (Hyman et al. 1991, Sahu et al. 2019). Importantly, we showed that activating DNT-2 neurons increased the levels and cleavage of DNT-2, up-regulated DNT-2 increased *TH* expression, and this initial amplification resulted in the inhibition of cAMP signalling via the dopamine receptor Dop2R in DNT-2 neurons. This negative feedback could drive a homeostatic reset of both DNT-2 and dopamine levels, important for normal brain function. In fact, we showed that alterations in DNT-2 levels could cause seizures. Importantly, alterations in both NTs and

dopamine underlie many psychiatric disorders and neurodegenerative diseases in humans (Hyman et al. 1991, Guillin et al. 2001, Berton et al. 2006, Forrest et al. 2018, Wang et al. 2022).

We have uncovered a novel mechanism of structural brain plasticity, involving a NT ligand functioning via a Toll and a kinase-less Trk-family receptor in the adult *Drosophila* brain. Toll receptors in the CNS can function via ligand dependent and ligand-independent mechanisms (Anthoney et al. 2018). However, in the context analysed, Toll-6 and Kek-6 function in structural circuit plasticity depends on their ligand DNT-2. This is also consistent with their functions promoting axonal arbour growth, branching and synaptogenesis at the NMJ (Ulian-Benitez et al. 2017). Furthermore, Toll-2 is also neuro-protective in the adult fly brain, and loss of *Toll-2* function caused neurodegeneration and impaired behaviour (Li et al. 2020). There are six *spz/DNT*, nine *Toll* and six *kek* paralogous genes in *Drosophila* (Tauszig et al. 2000, MacLaren et al. 2004, Mandai et al. 2009, Ulian-Benitez et al. 2017), and at least seven *Tolls* and three adaptors are expressed in distinct but overlapping patterns in the brain (Li et al. 2020). Such combinatorial complexity opens the possibility for a fine-tuned regulation of structural circuit plasticity and homeostasis in the brain.

Drosophila and mammalian NTs may have evolved to use different receptor types to elicit equivalent cellular outcomes. In fact, in mammals, NTs function via Trk, $p75^{NTR}$ and Sortilin receptors, to activate ERK, PI3K, NFκB, JNK and CaMKII (Lu et al. 2005, Wang et al. 2022). Similarly, DNTs with Tolls and Keks also activate ERK, NFκB, JNK and CaMKII in the *Drosophila* CNS (McIlroy et al. 2013, Foldi et al. 2017, Ulian-Benitez et al. 2017, Anthoney et al. 2018). However, alternatively, NTs may also use further receptors in mammals. Trks have many kinase-less isoforms, and understanding of their function is limited (Fryer et al. 1996, Stoilov et al. 2002). They can function as ligand sinks and dominant negative forms, but they can also function independently of full-length Trks to influence calcium levels, growth cone extension and dendritic growth and are linked to psychiatric disorders, e.g. depression (Ferrer et al. 1999, Yacoubian and Lo 2000, Ohira et al. 2006, Carim-Todd et al. 2009, Ernst et al. 2009, Ohira and Hayashi 2009, Fenner 2012, Tessarollo and Yanpallewar 2022). Like Keks, perhaps kinase-less Trks could regulate brain plasticity vs. degeneration.

A functional relationship between NTs and TLRs could exist also in humans, as in cell culture, human BDNF and NGF can induce signalling from a TLR (Foldi et al. 2017) and NGF also functions in immunity and neuroinflammation (Levi-Montalcini et al. 1996, Hepburn et al. 2014). Importantly, TLRs can regulate cell survival, death and proliferation, neurogenesis, neurite growth and collapse, learning and memory (Okun et al. 2011). They are linked to neuroinflammation, psychiatric disorders, neurodegenerative diseases and stroke (Okun et al. 2011, Figueroa-Hall et al. 2020, Adhikarla et al. 2021). Intriguingly, genome-wide association studies (GWAS) have revealed the involvement of TLRs in various brain conditions and potential links between NTs and TLRs in, for example, major depression (Sharma 2012, Mehta et al. 2018, Chan et al. 2020, Garrett et al. 2021). Importantly, alterations in NT function underlie psychiatric, neurologic and neurodegenerative brain diseases (Lu et al. 2005, Martinowich et al. 2007, Krishnan and Nestler 2008, Lu et al. 2013, Park and Poo 2013, Wohleb et al. 2016, Yang et al. 2020, Casarotto et al. 2021, Wang et al. 2022) and BDNF underlies the plasticity inducing function of anti-depressants (Lu et al. 2013, Casarotto et al. 2021, Wang et al. 2022). It is compelling to find out whether and how these important protein families – NTs, TLRs and kinase-less Trks - interact in the human brain.

Conclusion

To conclude, we provide a direct link between structural circuit plasticity and behavioural performance, by a novel molecular mechanism. The neurotrophin DNT-2 and its receptors Toll-6 and the kinase-less Trk family Kek-6 are linked to a dopaminergic circuit. Neuronal activity boosts DNT-2, and DNT-2 and dopamine regulate each other homeostatically. Dopamine labels the circuits

engaged and DNT-2, a growth factor, with its receptors Toll-6 and Kek-6, drives structural plasticity in these circuits, enhancing dopamine-dependent behavioural performance. These findings mean that DNT-2 is a plasticity factor in the *Drosophila* brain, that could enable experience-dependent behavioural enhancement. Whether NTs can similarly functions with TLRs and Kinase-less Trks remains to be explored. As behaviour is a source of experience, this has profound implications for understanding brain function and health.

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Data availability statement

All data are contained within the manuscript; metadata and statistical analysis details including full genotypes, sample sizes, statistical tests and p values have been provided in Table S2. This work generated fly stocks and molecular constructs, which we will distribute on request. Raw data will be distributed on request.

Additional information

Author contributions

J.S., V.C and A.H. designed and/or executed experiments and/or analysed data; S.U-B, G.L, D.S, X.W., F.R.C, M.M. executed experiments and analysed data; M.G.F, S.C., A.H. developed tools; D.K., G.J, V.C., A.H. supervised; J.S and A.H. wrote the manuscript. A.H. conceived and directed the project. All authors provided feedback on, and contributed to improvements to, the manuscript.

Declaration of interests

The authors declare that they have no conflict of interest.

Materials and methods

Genetics

Mutants: *DNT2*^{37} and *DNT2*^{18}are protein null(Foldi et al. 2017, Ulian-Benitez et al. 2017). *Toll6*^{31}is a null mutant allele(McIlroy et al. 2013). **Driver lines:** *DNT2-Gal4* is a CRISPR/Cas9-knock-in allele, with GAL4 at the start of the gene (this work, see below). *Toll-6-Gal4* was generated by RMCE from *MIMIC Toll-6*^{MIO2127}*; kek6-Gal4* from *MIMIC Kek6*^{MI12953}(Ulian-Benitez et al. 2017, Li et al. 2020). *MB320C-Gal4 (BSC68253), MB301B-Gal4 (BSC68311), R58E02-Gal4 (BSC41347), MB247-Gal80 (BSC64306), R58E02-LexA (BSC52740), R14C08-LexA (BSC52473)* were

from the *Drosophila* Bloomington Stock Centre (BSC). *Dop1R2-LexA, Dop2R-LexA, Gad-LexA* were kindly provided by Yi Rao; *TH-LexA* (gift of Ron Davis), *TH-Gal4,R58E02-GAL4* (gift of Serge Birman); *G0431-Gal4 (DAL-GAL4), VT49239 nls LexA (DAL LexA)* (gifts from Ann-Shyn Chiang); tubGal80ts, Tdc-LexA. **Reporter lines:***UAS-CD8::GFP,* for membrane tethered GFP; *UAS-histone-YFP*, for YFP-tagged nuclear histone; *UASFlybow1.1*, constant membrane tethered expression (gift of Iris Salecker); *13xLexAop-nls-tdTomato,* nuclear Tomato (gift of B. Pfeiffer); *UASCD8::RFP, LexAopCD8::GFP* (BSC32229), for dual binary expression; *UAS-homer-GCaMP* for post-synaptic densities (gift of André Fialá), *UAS-syt.eGFP* (BSC6925) and *LexAop-Syt-GCaMP* (BSC64413) for presynaptic sites. **For connectivity:***UAS-DenMarkRFP, UAS-Dsyd1GFP* (gift of Carolina Rezaval); TransTango: *yw UAS-myrGFP,QUAS-mtdTomato-3xHA attP8; Trans-Tango@attP40* (BSC77124); BAcTrace 806 *(w;LexAop2-Syb::GFP-P10(VK37) LexAop-QF2::SNAP25::HIVNES::Syntaxin(VK18)/CyO; UAS-B3Recombinase (attP2) UAS<B3Stop<BoNT/A (VK5) UAS<B3Stop<BoNT/A(VK27) QUAS-mtdTomato::HA/TM2*): **MCFO clones**: *hs-FLPG5.PEST;; 10xUAS (FRT.stop) myr::smGdP-OLLAS 10xUAS (FRT.stop) myr::smGdP-HA 10xUAS (FRT.stop) myr::smGdP-V5-THS-10xUAS (FRT.stop) myr::smGdP-FLAG* (BSC64086). **Optogenetic activation:***20x UAS-V5-Syn-CsChrimson td tomato* (gift of B. Pfeiffer). **For thermogenetic activation:***UASTrpA1@attP2 (BSC26264)* and *UAS-TrpA1@attP216 (BSC26263).***UAS gene over-expression**: *UAS-DNT2CK, UAS-DNT2FL-GFP, UAS-DNT2-FL-47C, UAS-Toll-6^{CY}*(McIlroy et al. 2013, Foldi et al. 2017). **UAS-RNAi knockdown:***UAS-DNT2RNAi* (VDRC49195), *UAS-Toll6RNAi* (VDRC928), *y^1v^1; UAS-Toll6RNAi[Trip.HMS04251]* (BSC 56048*), UAS-kek6RNAi* (VDCR 109681), *y^1v^1; UAS-Dop2R-RNAi[TRiP.HMC02988](*BSC50621).

Molecular biology

DNT-2GAL4 was generated by CRISPR/Cas9 enhanced homologous recombination. 1 kb long 5' and 3' homology arms (HA) were amplified by PCR from genomic DNA of wild type flies, using primers for 5'HA: atcgcaccggtttttacaggcaccccatgtctga containing AgeI cutting site and cttgacgcggccgcTGTCAATTCATTCGCCGTCGAT containing NotI cutting site. 3'HA primers were: tattaggcgcgccATGACAAAAAGTATTAAACGTCCGCCC containing AscI cutting site and tactcgactagtgaagcacacccaaaatacccagg containing SpeI cutting site. HAs were sequentially cloned by conventional cloning into pGEM-T2AGal4 vector (Addgene #62894). For gRNA cloning, two 20 nucleotide gRNA oligos (gtcGACAAGTTCTTCTTACCTATG and aaacCATAGGTAAGAAGAACTTGTC) were designed using Optimal Target Finder. BbsI enzyme sites were added: gtc(g) at the 5' end of sense oligo, and aaac at the 5' end of the antisense oligo. gRNA is located at 41 bp downstream of the start codon of DNT-2 within the first coding exon. The gRNA was cloned into pU6.3 using conventional ligation. The two constructs were injected in Cas9 bearing flies and red fluorescent (3xP3-RFP) transformants were selected and balanced, after which 3xP3-RFP was removed with CRE-recombinase.

qRT-PCR

qRT-PCR was carried out from 20 whole adult fruit-fly heads, frozen in liquid nitrogen before homogenising in 50 ml Trizol (Ambion #AM9738), followed by a stand RNA extraction protocol. RNA was treated with DNase treatment (ThermoFisher # AM1906) to remove genomic DNA. 200 ng of RNA was used for cDNA synthesis following Goscript Reverse Transcriptase (Promega #237815) protocol. Sample was diluted 1:4 with Nuclease free H_2O. Standard qPCR was performed with SensiFast syb Green Mix (Bioline #B2092020) in ABI qPCR plate (GeneFlow #P3-0292) and machine. To amplify TH mRNA the following primers were used: TH-F: CGAGGACGAGATTTTGTTGGC and TH-R: TTGAGGCGGACCACCAAAG. GAPDH was used as a housekeeping control. Reactions were performed in triplicate. Specificity and size of amplification products were assessed by melting curve analyses. Target gene expression relative to reference gene is expressed as value of $2^{-\Delta\Delta Ct}$ (where Ct is the crossing threshold).

Conditional expression

Multiple Colour Flip Out clones: *DNT2-Gal4, Toll6-Gal4* and *kek6-Gal4* were crossed with *hsFLP::PEST;;MCFO* flies, female offspring were collected and heat-shocked at 37°C in a water bath for 15 mins, then kept for 48h at 25°C before dissecting their brains. ***TransTango:*** DNT2-Gal4 or Oregon female virgins were crossed with *TransTango* males, progeny flies were raised at 18°C constantly and 15 days after eclosion, female flies were selected for immunostaining. **Thermogenetic activation with *TrpA1:*** Fruit-flies were bred at 18°C from egg laying to 4 days post-adult eclosion, then shifted to 29°C in a water bath for 24h followed by 24h recovery at room temperature for over-expressed *DNT-2FL-GFP*; for the other experiments, after breeding as above, adult flies were transferred to an incubator at 30°C, kept there for 24h and then brains were dissected. **Conditional gene over-expression and RNAi knockdown:** Flies bearing the temperature sensitive GAL4 repressor tubGal80^{ts} were kept at 18°C from egg laying to adult eclosion, then transferred to 30 °C incubator for 48h for Dcp-1+ and cell counting experiments and for 120h for TH+ cell counting.

Immunostainings

Adult fruit-fly female brains were dissected (in PBS), fixed (in 4% para-formaldehyde, room temperature, 20-30min) and stained following standard protocols. Primary antibodies and their dilutions were as follows: Mouse anti-Brp (nc82) 1:10 (DSHB); Rabbit anti-GFP 1:250 (Thermofisher); Mouse anti-GFP 1:250 (Thermofisher); Chicken anti-GFP 1:500 (Aves); Rabbit anti-FLAG 1:50 (Sigma); Mouse anti-V5 1:50 (Invitrogen); Chicken anti-HA 1:50 (Aves); Rabbit anti-VGlut 1:500 (gift of Hermann); Mouse anti-TH 1:250 (Immunostar); Rabbit anti-TH 1:250 (Novus Biologicals); Rabbit anti-DsRed 1:250 (Clontek); Mouse anti-ChAT4B1 1:250 (DSHB); Rabbit anti-5-HT 1:500 (Immunostar); Rabbit Anti-DCP-1 1:250 (Cell Signalling). Seconday antibodies were all used at 1:500 and all were from Thermofisher: Alexa Flour 488 Goat anti-mouse, Alexa Flour 488 donkey anti-rabbit, Alexa Flour 488 Goat anti-rabbit (Fab')2, Alexa Flour 488 Goat anti-chicken, Alexa Four 546 Goat anti-rabbit, Alexa Four 546 Goat anti-mouse, Alex Four 647 Goat anti-rabbit, Alex Four 647 Goat anti-mouse.

Microscopy and Imaging

Laser scanning confocal microscopy

Stacks of microscopy images were acquired using laser scanning confocal microscopy with either Zeiss LSM710, 900 or Leica SP8. Brains were scanned with a resolution of 1024×1024, with Leica SP8 20x oil objective and 1 mm step for whole brain and DCP-1 stainings, 40x oil objective and 1 mm step for central brain. Resolution of 1024×512 was used for analysing PAM clusters with 0.96 mm step for cell counting; 0.5 mm step for neuronal morphology; and 63x oil objective with 0.5mm step for neuronal connections. Acquisition speed in Leica SP8 was 400Hz, with no line averaging. Resolution of 3072×3072 was used for single image analysis of synapses, using either Leica SP8 or Zeiss LSM900 and Airyscan acquisition with 40x water objective speed 6, and average 4, or with 1024×512, with 40x oil lens 2x zoom and 0.35mm step. TH counting in PAM were scanned with Zeiss 710 with a resolution 1024×1024, 40x oil objective, step 1 mm, speed 8. Zeiss LSM900 Airyscan with a resolution of 1024×1024, 40x water objective, speed 7, 0.7 zoom and 0.31µm step size was used for acquisition of optical sections of synapses in PAM neurons.

Optogenetics and Epac1 FRET 2-photon imaging

To test whether DNT-2 neurons can respond to dopamine via the Dop2R inhibitory receptor, we used the cAMP sensor Epac1 and 2-photon confocal microscopy. Epac1 is FRET probe, whereby data are acquired from CFP and YFP emission and lower YFP/CFP ratio reveals higher cAMP levels. DNT-2Gal4 flies were crossed to *UAS-CsChrimson UAS Epac1* flies, to stimulate DNT-2 neurons and

detect cAMP levels in DNT2 neurons. 1-3 day-old *DNT2Gal4>UASCsChrimson, UAS Epac1* flies were collected and separated in two groups. Flies bearing *DNT2Gal4 UASCsChrimson UAS Epac1 UASDop2RRNAi* were fed on 50μM all-trans retinal food for at least 3 days prior to imaging and kept in constant darkness prior to the experiment.

Optogenetic stimulation of fly brains expressing CsChrimson in DNT-2 neurons was carried out using a sapphire 588nm laser, in a 2-photon confocal microscope. For acquisition of YFP and CFP data from Epac1 samples, a FV30-FYC filter was applied, using a 925nm laser for both YFP and CFP imaging. The stimulation laser was targeted onto DNT-2 neuron projections in SMP region for 20s. Acquisition ROI was at DNT-2A cell bodies with a frame rate of around 10Hz. The first acquisition started 10s before the 20s stimulation and consequential acquisition was done every 30s for 10 cycles.

Image analysis of Epac data was carried out using ImageJ. The two channels (YFP and CFP) were separated, and the ratio of YFP/CFP for each pixel was calculated using the ImageJ>Image Calculator by diving YFP channel by CFP channel. The obtained result of YFP/CFP ratio was saved and the mean ratio of YFP/CFP in the ROI was calculated for each time point and 11 time points were used. The 11 values represent the ratio of YFP/CFP change in the cell body upon stimulation, with 30s interval and repeated 10 times.

Cell counting

To count cells labelled with nuclear reporters (e.g. Histone-YFP, nls-tdTomato) and Dcp-1+ cells, where signal is of high intensity, contrast and sphericity, we adapted the DeadEasy Central Brain ImageJ plug-in (Li et al. 2020) for automatic cell counting in adult brains. DeadEasy plug-ins automatically identify and count cells labelled with nuclear reporters in 3D stacks of confocal image in the nervous system of embryos (Forero et al. 2010a, Forero et al. 2010b), larvae (Kato et al. 2011, Forero et al. 2012, Losada-Perez et al. 2016, Harrison et al. 2021) and adult (Li et al. 2020) *Drosophila*. DeadEasy plug-ins are accurate at counting cells sparsely labelled with nuclear markers, and importantly, treat all genotypes objectively and equally yielding reliable data. Here, adult brains expressing Histone-YFP or nls-td-tomato reporters were dissected, fixed and scanned without staining them. DeadEasy Central Brain was used with threshold set to 75.

To count the TH labelled PAMs, where both the signal and the labelled-cell shape are more irregular, we used assisted manual cell counting using two methods. First, we developed a plug-in called DeadEasy DAN as follows. A median filter was used to reduce Poison noise, without having large losses at the edges. Then, a 3D morphological closing was performed. Next, all very dark pixels were assigned a value of zero. To mark each cell, each chasm in the image was found using a 3D extended h-minimal transform. As more than one local minimum can be found within each cell, which would result in counting a cell multiple times, a 3D inverse dome detection was performed, and then labelled. Thus, each inverse dome was used as a seed to identify each cell. Once the seeds were obtained, a 3D watershed transformation was performed to recover the shape of the cells. Then, we ran DeadEasy DAN on our raw data, to obtain a results stack of images and formed a merged stack between the raw and result stacks, to manually add any missing cells. This assisted cell counting method was effective at producing accurate cell counts with less labour and time than conventional manual counting and worked well for some genotypes (**Figure 3D**). However, it was less effective with RNAi knock-down genotypes, where the signal can be less intense, for which TH+ cells were counted manually, assisted by the ImageJ cell counter instead.

Dendrite analysis with Imaris

To analyze dendritic complexity, image data were processed with Imaris using the “Filaments” module with the default algorithm and “Autopatch function”. A simple region of interest (ROI) with parallelepiped shape was delimited. Thresholds were set for the largest and the smallest diameter of the dendrite, and this was consistent across samples within the same experiment. The starting

point threshold was adjusted to only represent the soma of the neurons, and the "Seed Points threshold" to match the branches of the neurons. "Remove Seed Points Around Starting Points" and "Remove Disconnected Segments" were chosen, keeping the default values. The threshold for background substation and local contrast was consistent across all samples within an experiment. The "Edit function" within the Filaments module was used to correct any inaccuracy detected in the resulting tracing. Number of dendritic branches, dendritic segments, dendritic branch points and dendritic terminal points were collected to compare differences between the groups.

Vesicles, synapses and PSD analysis with Imaris

To analyse the number and volume of Homer-GCaMP GFP+ post-synaptic densities (PSD), Syt-GCaMP GFP+ presynaptic sites and DNT-2FLGFP+ vesicles, optical section images of confocal stacks through the brain were processed with the Imaris "Spot function". To analyze the number and volume of Homer-GCaMP GFP+ post-synaptic densities (PSD), the "Surface module" from Imaris was used to restrict a region of interest (ROI). Then, "Absolute Intensity Thresholding" method was applied to each sample choosing the same cutoff each time. The resulting surface was applied to mask the original scan. The masked image was processed using "Image Processing module" from Imaris. Background subtraction followed by threshold cutoff filters were applied. Afterwards, the "Spots module" was used as explained below.

An ROI was determined for Syt-GCaMP GFP+ presynaptic sites and DNT-2FLGFP+ vesicles using the "Surface module" with the "Edit Manually" option "Algorithm". The ROI for the SMP region started in the slide immediately after the last slide where the soma of PAM neurons was detectable, and finished in the last one where the dendrite was visible. The SMP region laterally was delimited by the black space given by the α lobe position. For the MB lobe region, the ROI started in the first slide where γ5,β'2 and β2 was visible (Aso et al., 2014) and finished in the last slide where this structure was appreciable. The surface was used to create a "Masked Channel", which a posteriori was used to determine the spots using the Spots module.

The "Spots module" algorithm was set to "Different Spot Sizes". An Estimated XY Diameter was set according to each experiment group, using the same within an experiment. "Background Subtraction" option was selected. "Intensity Center Filter" was used. "Spot Region" type was determined from "Local Contrast", and the "Region Threshold" according to the "Region Border". Setting of the threshold was consistent across genotypes.

Behaviour

1. Startle induced negative geotaxis Assay

was carried out as described in (Sun et al. 2018). Groups of approximately 10 male flies of the same genotype were placed in a fresh tube one night before the test, after which flies were transferred to a column formed with two empty tubes 15cm long and 2cm and then habituated for 30mins. Columns were tapped 3-4 times, flies fell to the bottom and then climbed upwards. Multiple rounds of testing were performed 3-7 times in a row per column. The process was filmed and films were analysed. Flies were scored during the first 15s after the tapping, and those that climbed above 13cm and those that climbed below 2cm were counted separately. Results given are mean ± SEM of the scores obtained with ten groups of flies per genotype. The performance index (PI) is defined as 1⁄2[(ntot + ntop − nbot)/ntot], where ntot, ntop, and nbot are the total number of flies, the number of flies at the top, and the number of flies at the bottom, respectively. The assay was carried out at 25°C, 55% humidity. Flies with tubGal80^{ts} to conditionally overexpress or knock-down were shifted from 18 to 30°at eclosion and kept for 5 days at 30°C to induce Gal4. Experiments were carried in an environmental chamber at 31°C, 60% humidity or in a humidity and temperature-controlled behaviour lab always kept at 25°C.

2. Spontaneous Locomotion in an open arena

Male flies of each genotype were collected and kept in groups of 10-20 flies, in vials containing fresh fly food for 5-9 days. Before filming, three male flies from one genotype were transferred into a 24 mm well of a multi-well plate using an aspirator and habituated for 15 – 20 mins. The multi-well plate with transparent lid and bottom was placed on a white LED light pad (XIAOSTAR Light Box) and either inside a light-shielding black box (PULUZ, 40*40*40cm) in a room with constant temperature (25°C) and humidity (55%) to maintain stable environmental conditions (Figure 7D), or inside a temperature controlled environmental chamber at 18°C or 30°C (Figure 7E,F,). The locomotion behaviour of freely-moving flies was filmed with a camera (Panasonic, HC-V260) in the morning from ZT1-ZT4 and for 10 min at a frame-rate of 25 fps. The 10-min videos were trimmed (from 00:02:00 to 00:07:00) to 5-min videos for analysis using Flytracker software(Eyjolfsdottir et al. 2014). For the *DNT-2* mutants and over-expression of Toll-6^{CY} experiments, flies were bred and tested at 25 °C. To test over-expression of *DNT-2* with *tubGAL80^{ts}; DNT2-Gal4>UAS-DNT-2FL*, flies were raised at 18°C until eclosion, and controls were kept and tested at 18°C; test groups were transferred directly after eclosion to 30°C for 5 days and were tested in an environmental chamber kept at 30°C, 60%. For thermo-genetic activation of DNT2 neurons using TrpA1 (DNT-2GAL4>UASTrpA1), flies were bred at 18°C and kept at 18°C for 7-9 days post-eclosion. Following habituation at 18°c for 20 mins in the multi-well plates, they were transferred to the 30°C chamber 10 mins before filming to activate TrpA1 and then filmed for the following 10 minutes. Fly locomotion activity was tracked using FlyTracker and calculated (distance and speed) in Matlab(Eyjolfsdottir et al. 2014) using the raw data generated from the tracking procedure. The “walking distance” was calculated as the sum of the distance flies moved and the “walking speed” was the speed of flies only when they were walking, and it was calculated using only frames where flies moved above 4 mm/s (which corresponds to two body lengths).

3. Appetitive long term memory test

Appetitive long-term memory was tested as described in Krashes and Waddell(Krashes and Waddell 2011). The two conditioning odours used were isoamyl acetate, Sigma-Aldrich #24900822, 6mL in 8mL mineral oil (Sigma-Aldrich #330760) and 4-methylcyclohexanol (Sigma-Aldrich # 153095, 10mL in 8mL mineral oil). Groups of 80-120 mixed sex flies were starved in a 1% agar tube filled with a damp 20 x 60 mm piece of filter paper for 18-20 hour before conditioning. During conditioning training, one odorant was presented with a dry filter paper (unconditioned odour, CS-) for 2 minutes, before a 30 second break, and presentation of a second odorant with filter paper coated with dry sucrose (conditioned odour, CS+). The test was repeated pairing the other odorant with sucrose, with a different group of flies to form one replicate. After training, flies were transferred back to agar tubes for testing 24 hours later. Performance index (PI) was calculated in the same way as in Krashes and Waddell(Krashes and Waddell 2011), as the number of flies approaching the conditioned odour minus the number of flies going in the opposite direction, divided by the total number of flies. A single PI values is the average score from the test with the reverse conditioning odour combination. Groups for which the total number of flies among both odorants was below 15 were discarded. For the *DNT-2* over-expression experiments with *tubGAL80^{ts}; DNT-2>DNT-2FL*, flies were raised at 18°C until 7-9 days post-eclosion. They were then either transferred to and maintained at 23°C (controls) or 30°C for 18-20h starvation, training, and up to testing 24h later.

Statistical analysis

Statistical analyses were carried out using Graph-Pad Prism. Confidence interval was 95%, setting significance at $p<0.05$. Chi-square tests were carried out when comparing categorical data. Numerical data were tested first for their type of distributions. If data were distributed normally, unpaired Student t-tests were used to compare means between two groups and One Way ANOVA or Welch ANOVA for larger groups, followed by post-doc Dunnett test for multiple comparisons to

a fixed control. Two Way ANOVA was used when comparisons to two variables were made. If data were not normally distributed, non-parametric Mann-Whitney U-test for 2 two group comparisons and Kruskal Wallis ANOVA for larger groups, followed by post-hoc Dunn's multiple comparisons test to a fixed control. Statistical details including full genotypes, sample sizes, tests and p values are provided in Supplementary Table S2.

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Reviewer #1 (Public review):

Summary:

Sun et al. are interested in how experience can shape the brain and specifically investigate the plasticity of the Toll-6 receptor-expressing dopaminergic neurons (DANs). To learn more about the role of Toll-6 in the DANs, the authors examine the expression of the Toll-6 receptor ligand, DNT-2. They show that DNT-2 expressing cells connect with DANs and that loss of function of DNT-2 in these cells reduces the number of PAM DANs, while overexpression causes alterations in dendrite complexity. Finally, the authors show that alterations in the levels of DNT-2 and Toll-6 can impact DAN-driven behaviors such as climbing, arena locomotion, and learning and long-term memory.

Strengths:

The authors methodically test which neurotransmitters are expressed by the 4 prominent DNT-2 expressing neurons and show that they are glutamatergic. They also use Trans-Tango and Bac-TRACE to examine the connectivity of the DNT-2 neurons to the dopaminergic circuit and show that DNT-2 neurons receive dopaminergic inputs and output to a variety of neurons including MB Kenyon cells, DAL neurons, and possibly DANS.

Weaknesses:

(1) To identify the DNT-2 neurons, the authors use CRISPR to generate a new DN2-GAL4. They note that they identified at least 12 DNT-2 plus neurons. In Supplementary Figure 1A, the DNT-2-GAL4 driver was used to express a UAS-histoneYFP nuclear marker. From these figures, it looks like DNT-2-GAL4 is labeling more than 12 neurons. Is there glial expression? This question is relevant as it is not clear how many other cell types are being manipulated with the DNT-2-GAL4 driver is used in subsequent experiments. For example, is DNT-2-GAL4--> DNT-2-RNAi is reducing DNT2 in many neurons or glia effects could be indirect.

(2) In Figure 2C the authors show that DNT-2 upregulation leads to an increase in TH levels using q-RT-PCR from whole heads. However, in Figure 3G they also show that DNT-2 overexpression also causes an increase in the number of TH neurons. It is unclear whether TH RNA increases due to expression/cell or number of TH neurons in the head.

(3)DNT-2 is also known as Spz5 and has been shown to activate Toll-6 receptors in glia (McLaughlin et al., 2019), resulting in the phagocytosis of apoptotic neurons. In addition, the knockdown of DNT-2/Spz5 throughout development causes an increase in apoptotic debris in the brain, which can lead to neurodegeneration. Indeed Figure 3H shows that an adult-specific knockdown of DNT-2 using DNT2-GAL4 causes an increase in Dcp1 signal in many neurons and not just TH neurons.

Comments on revisions:

The authors have made some changes in the text to tone down their claims. They have also provided additional images to support their work. However, requested controls are not provided, and new experiments are not added to address reviewer concerns.

https://doi.org/10.7554/eLife.102222.2.sa3

Reviewer #2 (Public review):

This paper examines how structural plasticity in neural circuits, particularly in dopaminergic systems, is regulated by Drosophila neurotrophin-2 (DNT-2) and its receptors, Toll-6 and Kek-6. The authors show that these molecules are critical for modulating circuit structure, dopaminergic neuron survival, synaptogenesis, and connectivity. They demonstrate that the loss of DNT-2 or Toll-6 function leads to the loss of dopaminergic neurons, reduced dendritic arborization, and synaptic impairment, whereas overexpression of DNT-2 increases dendritic complexity and synaptogenesis. Additionally, DNT-2 and Toll-6 influence dopamine-dependent behaviors, including locomotion and long-term memory, suggesting a link between DNT-2 signaling, structural plasticity, and behavior.

A major strength of this study is the impressive cellular resolution achieved. By focusing on specific dopaminergic neurons, such as the PAM and PPL1 clusters, and using a range of molecular markers, the authors were able to clearly visualize intricate details of synapse formation, dendritic complexity, and axonal targeting within defined circuits. Given the critical role of dopaminergic pathways in learning and memory, this approach provides a valuable foundation for exploring the role of DNT-2, Toll-6, and Kek-6 in experience-dependent structural plasticity. While the manuscript hints at a connection to experience-induced plasticity, the study does not establish a direct causal link between neurotrophin signaling and experience-driven changes. To support this idea, it would be necessary to observe experience-induced structural changes and demonstrate that downregulation of DNT-2 signaling prevents these changes. The closest attempt in this study was the artificial activation of DNT-2 neurons using TrpA1, which resulted in overgrowth of axonal arbors and an increase in synaptic sites in both DNT-2 and PAM neurons. However, whether the observed structural changes were dependent on DNT-2 signaling remains unclear.

In conclusion, this study demonstrates that DNT-2 and its receptors play a role in regulating the structure of dopaminergic circuits in the adult fly brain. Whether DNT-2 signaling contributes to experience-dependent structural plasticity within these circuits remains an exciting open question and warrants further investigation.

Comments on revisions:

I appreciate the authors' responses to my previous comments and have no further suggestions.

https://doi.org/10.7554/eLife.102222.2.sa2

Reviewer #3 (Public review):

Summary:

The authors used the model organism Drosophila melanogaster to show that the neurotrophin Toll-6 and its ligands, DNT-2 and kek-6, play a role in maintaining the number of dopaminergic neurons and modulating their synaptic connectivity. This supports previous findings on the structural plasticity of dopaminergic neurons and suggests a molecular mechanism underlying this plasticity.

Strengths:

The experiments are overall very well designed and conclusive. Methods are in general state-of-the-art, the sample sizes are sufficient, the statistical analyses are sound, and all necessary controls are at place. The data interpretation is straight forwards, and the relevant literature is taken into consideration. Overall, the manuscript is solid and presents novel, interesting and important findings.

Weaknesses:

There are three technical weaknesses that could perhaps be improved.

First, the model of reciprocal, inhibitory feedback loops (figure 2F) is speculative. On the one hand, glutamate can act in flies as excitatory or inhibitory transmitter (line 157!), and either situation can be the case here. On the other hand, it is not clear how an increase or decrease in cAMP level translates into transmitter release. One can only conclude that two type of neurons potentially influence each other.

Second, the quantification of bouton volumes (no y-axis label in Figure 5 C and D!) and dendrite complexity are not convincingly laid out. Here, the reader expects fine-grained anatomical characterizations of the structures under investigation, and a method to precisely quantify the lengths and branching patterns of individual dendritic arborizations as well as the volume of individual axonal boutons.

Third, figure 1C shows two neurons with the goal of demonstrating between-neuron variability. It is not convincingly demonstrated that the two neurons are actually of the very same type of neuron in different flies, or two completely different neurons.

Review of the revised manuscript:

The authors have addressed some points of concern raised by the reviewers. I would like to emphasize that I find the overall research study highly interesting and important.

https://doi.org/10.7554/eLife.102222.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *Sun et al. are interested in how experience can shape the brain and specifically investigate the plasticity of the Toll-6 receptor-expressing dopaminergic neurons (DANs). To learn more about the role of Toll-6 in the DANs, the authors examine the expression of the Toll-6 receptor ligand, DNT-2. They show that DNT-2 expressing cells connect with DANs and that loss of function of DNT-2 in these cells reduces the number of PAM DANs, while overexpression causes alterations in dendrite complexity. Finally, the authors show that alterations in the levels of DNT-2 and Toll-6 can impact DAN-driven behaviors such as climbing, arena locomotion, and learning and long-term memory.*
>
> *Strengths:*
>
> *The authors methodically test which neurotransmitters are expressed by the 4 prominent DNT-2 expressing neurons and show that they are glutamatergic. They also use Trans-Tango and Bac-TRACE to examine the connectivity of the DNT-2 neurons to the dopaminergic circuit and show that DNT-2 neurons receive dopaminergic inputs and output to a variety of neurons including MB Kenyon cells, DAL neurons, and possibly DANS.*

We are very pleased that Reviewer 1 found our connectivity analysis a strength.

> *Weaknesses:*
>
> *(1) To identify the DNT-2 neurons, the authors use CRISPR to generate a new DN2-GAL4.*
>
> *They note that they identified at least 12 DNT-2 plus neurons. In Supplementary Figure 1A, the DNT-2-GAL4 driver was used to express a UAS-histoneYFP nuclear marker. From these figures, it looks like DNT-2-GAL4 is labeling more than 12 neurons. Is there glial expression?*

Indeed, we claimed that DNT-2 is expressed in at least 12 neurons (see line 141, page 6 of original manuscript), which means more than 12 could be found. The membrane tethered reporters we used – UAS-FlyBow1.1, UASmcD8-RFP, UAS-MCFO, as well as UAS-DenMark:UASsyd-1GFP – gave a consistent and reproducible pattern. However, with DNT-2GAL4>UAS-Histone-YFP more nuclei were detected that were not revealed by the other reporters. We have found also with other GAL4 lines that the patterns produced by different reporters can vary. This could be due to the signal strength (eg His-YFP is very strong) and perdurance of the reporter (e.g. the turnover of His-YFP may be slower than that of the other fusion proteins).

We did not test for glial expression, as it was not directly related to the question addressed in this work.

> *(2) In Figure 2C the authors show that DNT-2 upregulation leads to an increase in TH levels using q-RT-PCR from whole heads. However, in Figure 3H they also show that DNT-2 overexpression also causes an increase in the number of TH neurons. It is unclear whether TH RNA increases due to expression/cell or the number of TH neurons in the head.*

Figure 3H shows that over-expression of DNT-2 FL increased the number of Dcp1+ apoptotic cells in the brain, but not significantly (p=0.0939). The ability of full-length neurotrophins to induce apoptosis and cleaved neurotrophins promote cell survival is well documented in mammals. We had previously shown that DNT-2 is naturally cleaved, and that over-expression of DNT-2 does not induce apoptosis in the various contexts tested before (McIlroy et al 2013 Nature Neuroscience; Foldi et al 2017 J Cell Biol; Ulian-Benitez et al 2017 PLoS Genetics). Similarly, throughout this work we did not find DNT-2FL to induce apoptosis.

Instead, in Figure 3G we show that over-expression of DNT-2FL causes a statistically significant increase in the number of TH+ cells. This is an important finding that supports the plastic regulation of PAM cell number. We thank the Reviewer for highlighting this point, as we had forgotten to add the significance star in the graph. In this context, we cannot rule out the possibility that the increase in TH mRNA observed when we over-express DNT-2FL could not be due to an increase in cell number instead. Unfortunately, it is not possible for us to separate these two processes at this time. Either way, the result would still be the same: an increase in dopamine production when DNT-2 levels rise.

We have now edited the abstract lines 38-39 adding that “By contrast, over-expressed DNT-2 increased DAN cell number,…”, within the main text in Results page 10 lines 259-265 and in the Discussion section page 15 lines 391, 393-396.

> *(3) DNT-2 is also known as Spz5 and has been shown to activate Toll-6 receptors in glia (McLaughlin et al., 2019), resulting in the phagocytosis of apoptotic neurons. In addition, the knockdown of DNT-2/Spz5 throughout development causes an increase in apoptotic debris in the brain, which can lead to neurodegeneration. Indeed Figure 3H shows that an adult specific knockdown of DNT-2 using DNT2-GAL4 causes an increase in Dcp1 signal in many neurons and not just TH neurons.*

Indeed, we did find Dcp1+ TH-negative cells too (although not widely throughout the brain), although this is not shown in the images of Figure 3H where we showed only TH+ Dcp+ cells.

That is not surprising, as DNT-2 neurons have large arborisations that can reach a wide range of targets; DNT-2 is secreted, and could reach beyond its immediate targets; Toll-6 is expressed in a vast number of cells in the brain; DNT-2 can bind promiscuously at least also Toll-7 and other Keks, which are also expressed in the adult brain (Foldi et al 2017 J Cell Biology; Ulian-Benitez et al 2017 PLoS Genetics; Li et al 2020 eLife). Together with the findings by McLaughlin et al 2019, our findings further support the notion that DNT-2 is a neuroprotective factor in the adult brain. It will be interesting to find out what other neuron types DNT-2 maintains.

We have made some edits on these points in page 10 lines 259-265.

We would like to thank Reviewer 1 for their positive comments on our work and their interesting and valuable feedback.

> ***Reviewer #2 (Public review):***
>
> *This paper examines how structural plasticity in neural circuits, particularly in dopaminergic systems, is regulated by Drosophila neurotrophin-2 (DNT-2) and its receptors, Toll-6 and Kek-6. The authors show that these molecules are critical for modulating circuit structure and dopaminergic neuron survival, synaptogenesis, and connectivity. They show that loss of DNT-2 or Toll-6 function leads to loss of dopaminergic neurons, dendritic arborization, and synaptic impairment, whereas overexpression of DNT-2 increases dendritic complexity and synaptogenesis. In addition, DNT-2 and Toll-6 modulate dopamine-dependent behaviors, including locomotion and*

long-term memory, suggesting a link between DNT-2 signaling, structural plasticity, and behavior.

A major strength of this study is the impressive cellular resolution achieved. By focusing on specific dopaminergic neurons, such as the PAM and PPL1 clusters, and using a range of molecular markers, the authors were able to clearly visualize intricate details of synapse formation, dendritic complexity, and axonal targeting within defined circuits. Given the critical role of dopaminergic pathways in learning and memory, this approach provides a good opportunity to explore the role of DNT-2, Toll-6, and Kek-6 in experience-dependent structural plasticity. However, despite the promise in the abstract and introduction of the paper, the study falls short of establishing a direct causal link between neurotrophin signaling and experience-induced plasticity.

Simply put, this study does not provide strong evidence that experience-induced structural plasticity requires DNT-2 signaling. To support this idea, it would be necessary to observe experience-induced structural changes and demonstrate that downregulation of DNT-2 signaling prevents these changes. The closest attempt to address this in this study was the artificial activation of DNT-2 neurons using TrpA1, which resulted in overgrowth of axonal arbors and an increase in synaptic sites in both DNT-2 and PAM neurons. However, this activation method is quite artificial, and the authors did not test whether the observed structural changes were dependent on DNT-2 signaling. Although they also showed that overexpression of DNT-2FL in DNT-2 neurons promotes synaptogenesis, this phenotype was not fully consistent with the TrpA1 activation results (Figures 5C and D).

In conclusion, this study demonstrates that DNT-2 and its receptors play a role in regulating the structure of dopaminergic circuits in the adult fly brain. However, it does not provide convincing evidence for a causal link between DNT-2 signaling and experience-dependent structural plasticity within these circuits.

We would like to thank Reviewer 2 for their very positive assessment of our approach to investigate structural circuit plasticity. We are delighted that this Reviewer found our cellular resolution impressive. We are also very pleased that Reviewer 2 found that our work demonstrates that DNT-2 and its receptors regulate the structure of dopaminergic circuits in the adult fly brain. This is already a very important finding that contributes to demonstrating that, rather than being hardwired, the adult fly brain is plastic, like the mammalian brain. Furthermore, it is remarkable that this involves a neurotrophin functioning via Toll and kinase-less Trks, opening an opportunity to explore whether such a mechanism could also operate in the human brain.

We are very pleased that this Reviewer acknowledges that this work provides a good opportunity to explore the role of DNT-2, Toll-6, and Kek-6 in experience-dependent structural plasticity. We provide a molecular mechanism and proof of principle, and we demonstrate a direct link between the function of DNT-2 and its receptors in circuit plasticity. We also showed a link of DNT-2 to neuronal activity, as neuronal activity increased the production of DNT-2GFP, induced the cleavage of DNT-2 and a feedback loop between DNT-2 and dopamine, and both neuronal activity and increased DNT-2 levels promoted synaptogenesis.

As the Reviewer acknowledges this approach provides a good opportunity to explore the role of DNT-2, Toll-6, and Kek-6 in experience-dependent structural plasticity. Finding out the direct link in response to lived experience is a big task, beyond the scope of this manuscript, and we will be testing this with future projects. Nevertheless, it is important to place our findings within this context together with the link to mammalian neurotrophins (as explained in the discussion), as it is here where the findings have deep and impactful implications.

To accommodate the criticism of this Reviewer, we have now toned down our narrative. This does not diminish the importance of the findings, it makes the argument more stringent. Please see edits in: Abstract page 2 lines 42-44; and Discussion page 22 line 586 – which were the only points were a direct claim had been made.

We would like to thank Reviewer 2 for the positive and thoughtful evaluation of our work, and for their feedback.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *The authors used the model organism Drosophila melanogaster to show that the neurotrophin Toll-6 and its ligands, DNT-2 and kek-6, play a role in maintaining the number of dopaminergic neurons and modulating their synaptic connectivity. This supports previous findings on the structural plasticity of dopaminergic neurons and suggests a molecular mechanism underlying this plasticity.*
>
> *Strengths:*
>
> *The experiments are overall very well designed and conclusive. Methods are in general state-of-the-art, the sample sizes are sufficient, the statistical analyses are sound, and all necessary controls are in place. The data interpretation is straightforward, and the relevant literature is taken into consideration. Overall, the manuscript is solid and presents novel, interesting, and important findings.*

We are delighted that Reviewer 3 found our work solid, novel, interesting and with important findings. We are also very pleased that this Reviewer found that all necessary controls have been carried out.

> *Weaknesses:*
>
> *There are three technical weaknesses that could perhaps be improved.*
>
> *First, the model of reciprocal, inhibitory feedback loops (Figure 2F) is speculative. On the one hand, glutamate can act in flies as an excitatory or inhibitory transmitter (line 157), and either situation can be the case here. On the other hand, it is not clear how an increase or decrease in cAMP level translates into transmitter release. One can only conclude that two types of neurons potentially influence each other.*

Thank you for pointing out that glutamate can be inhibitory. In response, we have removed the word ‘excitatory’ from the only point it had been used in the text: page 7 line 167.

In mammals, the neurotrophin BDNF has an important function in glutamatergic synapses, thus we were intrigued by a potential evolutionary conservation. Our evidence that DNT-2A neurons could be excitatory is indirect, yet supportive: exciting DNT-2 neurons with optogenetics resulted in an increase in GCaMP in PAMs (data not shown); over-expression of DNT-2 in DNT-2 neurons increased TH mRNA levels; optogenetic activation of DNT-2 neurons results in the Dop2R-dependent downregulation of cAMP levels in DNT-2 neurons. Dop2R signals in response to dopamine, which would be released only if dopaminergic neurons had been excited. Accordingly, glutamate released from DNT-2 neurons would have been rather unlikely to inhibit DANs.

cAMP is a second messenger that enables the activation of PKA. PKA phosphorylates many target proteins, amongst which are various channels. This includes the voltage gated calcium channels located at the synapse, whose phosphorylation increases their opening probability.

Other targets regulate synaptic vesicle release. Thus, a rise in cAMP could facilitate neurotransmitter release, and a downregulation would have the opposite effect. Other targets of PKA include CREB, leading to changes in gene expression. Conceivably, a decrease in PKA activity could result in the downregulation of DNT-2 expression in DNT-2 neurons. This negative feedback loop would restore the homeostatic relationship between DNT-2 and dopamine levels.

We agree with this Reviewer that whereas our qRT-PCR data show that over-expression of DNT-2 increases TH mRNA levels, this does not demonstrate that originates from PAM neurons. Similarly, although our EPAC data imply that dopamine must be released from DANs and received by DNT-2 neurons to explain those data, the evidence did not include direct visualisation of dopamine release in response to DNT-2 neuron activation. To accommodate these criticisms, we have edited the summary Figure 2E adding question marks to indicate inference points and page 9 line 221.

Our data indeed demonstrate that DNT-2 and PAM neurons influence each other, not potentially, but really. We have provided data that: DNT-2 and PAMs are connected through circuitry; that the DNT-2 receptors Toll-6 and kek-6 are expressed in DANs, including in PAMs; that alterations in the levels of DNT-2 (both loss and gain of function) and loss of function for the DNT-2 receptors Toll-6 and Kek-6 alter PAM cell number, alter PAM dendritic complexity and alter synaptogenesis in PAMs; alterations in the levels of DNT-2, Toll-6 and kek-6 in adult flies alters dopamine dependent behaviours of climbing, locomotion in an arena and learning and long-term memory. These data firmly demonstrate that the two neuron types DNT-2 and PAMs influence each other.

We have also shown that over-expression of DNT-2 in DNT-2 neurons increases TH mRNA levels, whereas activation of DNT-2 neurons decreases cAMP levels in DNT-2 neurons in a dopamine/Dop2R-dependent manner. These data show a functional interaction between DNT-2 and PAM neurons.

> *Second, the quantification of bouton volumes (no y-axis label in Figure 5 C and D!) and dendrite complexity are not convincingly laid out. Here, the reader expects fine-grained anatomical characterizations of the structures under investigation, and a method to precisely quantify the lengths and branching patterns of individual dendritic arborizations as well as the volume of individual axonal boutons.*

Figure 5C, D do contain Y-axis labels, all our graphs in main manuscript and in supplementary files contain Y-axis labels.

In fact, we did use a method to precisely quantify the lengths and branching patterns of individual dendritic arborisations, volume of individual boutons and bouton counting. These analyses were carried out using Imaris software. For dendritic branching patterns, the “Filament Autodetect” function was used. Here, dendrites were analysed by tracing semi-automatically each dendrite branch (ie manual correction of segmentation errors) to reconstruct the segmented dendrite in volume. From this segmented dendrite, Imaris provides measurements of total dendrite volume, number and length of dendrite branches, terminal points, etc. For bouton size and number, we used the Imaris “Spot” function. Here, a threshold is set to exclude small dots (eg of background) that do not correspond to synapses/boutons. All samples and genotypes are treated with the same threshold, thus the analysis is objective and large sample sizes can be analysed effectively. We had already provided a description of the use of Imaris in the methods section.

We have now exapanded the protocol on how we use Imaris to analyse dendrites and synapses, in: Materials and Methods section, page 28 lines 756-768 and page 29 lines 778-799.

> *Third, Figure 1C shows two neurons with the goal of demonstrating between-neuron variability. It is not convincingly demonstrated that the two neurons are actually of the very same type of neuron in different flies or two completely different neurons.*

We thank Reviewer 3 for raising this interesting point. It is not possible to prove which of the four DNT-2A neurons per hemibrain, which we visualised with DNT-2>MCFO, were the same neurons in every individual brain we looked at. This is because in every brain we have looked at, the soma of the neurons were not located in exactly the same location. Furthermore, the arborisation patterns are also different and unique, for each individual brain. Thus, there is natural variability in the position of the soma and in the arborisation patterns. Such variability presumably results from the combination of developmental and activity-dependent plasticity. Importantly, for every staining we carried out using DNT-2GAL4 and various membrane reporters and MCFO clones, we never found two identical DNT-2 neuron profiles.

To increase the evidence in support of this point, we have now expanded Figure 1, adding one more image of DNT-2>FlyBow (Figure 1A) and two more images of DNT-2>MCFO (Figure 1D). In total, seven images in Figure 1 and two further images in Figure 5A demonstrate the variability of DNT-2 neurons.

We would like to thank Reviewer 3 for the very positive evaluation of our work and the interesting and valuable feedback.

> ***Recommendations for the authors:***
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> ***Reviewer #1 (Recommendations for the authors):***
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> *In the fly list, several fly lines are missing references and sources.*

Apologies for this over-sight, this has now been corrected.

We thank Reviewer 1 for their effort and time to scrutinise our work, and for their very positive and helpful feedback.

> ***Reviewer #2 (Recommendations for the authors):***
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> *(1) Here I provide some more specific comments that I hope will help the authors further improve the study.*
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> *(2) L148: "single neuron clones revealed variability in the DNT-2A". How do the authors know that they are labeling the same subtype of DNT-2A neurons?*

There are four anterior DNT-2A cells per hemibrain, that project from the SOG area to the SMP. It is not possible to verify that every time we look at exactly the same neuron, because the exact position of the somas and the arborisation patterns vary from brain to brain. We know this from two sources of data: (1) when using DNT-2GAL4 to visualise the expression of membrane reporters (e.g. UAS-FlyBow, UAS-mCD8-GFP, UAS-CD8-RFP) no brain ever showed a pattern identical to that of another brain, neither in the exact position of the somas nor in the exact arborisation patterns. (2) When we generated DNT-2>MCFO clones to visualise 1-2 cells at a time, no single neuron or 2-neuron clones ever showed an identical pattern. The most parsimonious interpretation is that the exact location of the somas and the exact arborisation patterns vary across individual flies. Developmental variability in neuronal patterns has also been reporter by Linneweber et al (2020) Science.

To make our evidence more compelling, and in response to this Reviewer's query, we have now added further images. Please find in revised Figure 1 A,B three examples of three

different brains expressing DNT-2>FlyBow1.1. In Figure 1D, two more examples (altogether 4) of DNT-2>MCFO clones. Here it is clear to see that no neuron shape is identical to that of others, demonstrating variability in individual fly brains. We now show four images in Figure 1 and two more in Figure 5A that demonstrate the variability of DNT-2A neurons.

> *(3) Figure 1E: Are all DNT-2A neurons positive for vGlut and Dop2R? This figure shows only two DNT-2A neurons.*

Yes, all four DNT-2A neurons per hemibrain are vGlut positive and we have now added more images to Supplementary Figure S1A (right), also showing that presynaptic DNT-2A endings at SMP also coincide with a vGlut+ domain (Figure S1A left).

Yes, all all four DNT-2A neurons per hemibrain are Dop2R positive and we have now added more images to Supplementary Figure S1B.

> *(4) L156: Glutamate is generally considered to be inhibitory in the adult fly brain. More evidence is needed before the authors can claim that "DNT-2A neurons are excitatory glutamatergic neurons".*

Thank you for pointing this out. Although our data do not conclusively demonstrate it, they are consistent with DNT-2A neurons being excitatory. BDNF is most commonly released from glutamatergic neurons in mammals, its release is activity-dependent and leads to formation and stabilisation of synapses. The phenotypes we have observed are consistent with this and reveal functional evolutionarily conservation: (1) exciting DNT-2 neurons with TrpA1 results in increased production and cleavage of DNT-2GFP and de novo synaptogenesis; (2) over-expression of DNT-2 in the adult induces de novo synaptogenesis; (3) down-regulation or loss of DNT-2 and its receptors Toll-6 and Kek-6 impair synaptogenesis. Furthermore, we show that DNT-2 dependent synaptogenesis is between DNT-2 and dopaminergic neurons, which are involved in the control of locomotion, reward learning and long-term memory, and dopamine itself is required for such behaviour. Consistently with this we found that: (1) over-expression of DNT-2 increases TH mRNA levels, which would lead to the up-regulation of dopamine production; (2) exciting DNT-2 neurons increases locomotion speed in an arena; (3) knock-down of DNT-2 and its receptors decreases locomotion, whereas over-expression of DNT-2 increases locomotion; (4) over-expression of DNT-2 increases learning and long-term memory. Finally, in a previous version in bioRxiv, we also showed using optogenetics and calcium imaging that exciting DNT-2 neurons induced GCaMP signalling in their output PAM neurons, and in this version we show that exciting DNT-2 neurons regulates cAMP in DNT-2 neurons via dopamine-release dependent feedback. Altogether, the most parsimonious interpretation of these data is that vGlut+ DNT-2 neurons are excitatory.

In any case, to address this reviewer's point, we have now removed the word 'excitatory' from page 7 line 167.

> *(5) Figure 1H, I: A more detailed description of the Toll-6 and Kek-6 expressing neurons will be helpful. Are they expressed in specific types of PAM and PPL1 DANs? The legend in Figure S2 mentions labeling in γ2α'1 zones, but it seems to be more than that.*

This information had been already provided, presumable this Reviewer overlooked this. This was already described in great detail by comparing our microscopy data with the single cell RNA-seq data available through Fly Cell Atlas (https://flycellatlas.org) and Scope (https://scope.aertslab.org/#/b77838f4-af3c-4c37-8dd9-cf7a41e4b034/*/welcome).

Please see our previously submitted Table S1 "Expression of Tolls, keks and Toll downstream adaptors in cells related to DNT-2A neurons".

> *(6) Figure S3 should be controls for Figure 2A. It is incorrectly labeled as controls for Figure 3A.*

Thank you for pointing out this typo, this has now been corrected.

> *(7) L197: The authors state, "This showed that DNT-2 could stimulate dopamine production in neighboring DANs". However, the results do not fully support this conclusion because the experiments measure overall TH levels in the brain, not specifically in neighboring DANs. The observed effect could be indirect via other neurons.*

Indeed, we have now edited the text to: “This showed that DNT-2 could stimulate dopamine production”: page 8 line 208.

> *(8) Figure 3: If Toll-6 is expressed in specific subtypes of PAM DANs, are they the dying cells when Toll-6 was knocked down? I think the paper will be significantly improved if the authors provide a more in-depth analysis of the phenotype. Also, permissive temperature controls are missing for the experiments in (E)-(H). Permissive controls are essential to confirm that the observed effects are due to adult-specific RNAi knockdown.*

Current tools do not enable us to visualise Toll-6+ neurons at the same time as manipulating DNT-2 neurons and at the same time as monitoring Dcp1. Stainings with Dcp1 in the adult brain are not trivial. Thus, we cannot guarantee this. However, Toll-6 is the preferential receptor for DNT-2, and given that apoptosis increases when we knock-down DNT-2, the most parsimonious interpretation is that the dying cells bear the DNT-2 receptor Toll-6. Even if DNT-2 can promiscuously bind other Toll receptors, the simplest way to interpret these data remains that DNT-2 promotes cell survival by signalling via its receptors, as no other possible route is known to date. This would be consistent with all other data in this figure.

We thank this Reviewer for the feedback on the controls. Unfortunately, these are not trivial experiments, they require considerable time, effort, dedication and skill. This manuscript has already taken 5 years of daily hard work. We no longer have the staff (ie the first author left the lab) nor resources to dedicate to address this point.

> *(9) Figure 4B: This phenotype in DNT-2 mutants is very striking. Did the neurons still survive and did their axonal innervation in the lobes remain intact?*

Homozygous DNT-2 mutants are viable and have impair climbing, as we had already shown in Figure 7C.

> *(10) L261: The authors mention that "PAM-β2β'2 neurons express Toll-6 (Table S1)". However, I cannot find this information in Table S1.*

Unfortunately, I cannot identify the source of that statement at present and the first authors has left the lab. In any case, although the fact that knocking down Toll-6 in these neurons causes a phenotype means they must, it does not directly prove it. We have now corrected this to: “PAM-b2b'2 neuron dendrites overlap axonal DNT2 projections”, page 11 line 280.

> *(11) Figure 4C, D: What about their synaptogenesis? Do they agree with the result in Figure 4B?*

This was not tested at the time. Unfortunately, these are not trivial experiments and require considerable time, effort, dedication and skill. Addressing this point experimentally is not possible for us at this point. In any case, given the evidence we already provide, it is highly

unlikely they would alter the interpretation of our findings and the value of the discoveries already provided.

> *(12) L270: The authors state: "To ask whether DNT-2 might affect axonal terminals, we tested PPL1 axons." However, it is unclear why the focus was shifted to PPL1 neurons when similar analyses could have been performed on PAM DANs for consistency. In addition, it would be beneficial to assess dendritic arbor complexity and synaptogenesis in PPL1-γ1-pedc neurons to provide a more comprehensive comparison between PPL1 and PAM DANs. Performing parallel analyses on both neuron types would strengthen the study by providing insight into the generality and specificity of DNT-2 in different dopaminergic circuits.*

The question we addressed with Figure 4 was whether the DNT-2 and its receptors could modify axons, dendrites and synapses, ie all features of neuronal plasticity. The reason we used PPL1-g1-pedc to analyse axonal terminals was because of their morphology, which offered a clearer opportunity to visualise axonal endings than PAMs did. An exhaustive analysis of PPL1-g1-pedc is beyond the scope of this work and not the central focus.

> *(13) Figure 4G lacks a permissive temperature control, which is essential to confirm that the observed effects are due to adult-specific RNAi knockdown.*

We thank this Reviewer for this feedback, which we will bear in mind for future projects.

> *(14) Figure 5A requires quantification and statistical comparison.*

We thank this Reviewer for this feedback. We did consider this, but the data are too variable to quantify and we decided it was best to present it simply as an observation, interesting nonetheless. This is consistent as well with the data in Figure 1, which we have now expanded with this revision, which show the natural variability in DNT-2 neurons.

> *(15) Figure 5B: Many green signals in the control image are not labeled as PSDs, raising concerns about the accuracy of the image analysis methods used for synapse identification. While I trust that the authors have validated their analysis approach, it would strengthen the study if they provided a clearer description or evidence of the validation process.*

This was done using the Imaris “Spot function”, in volume. A threshold is set to exclude spots due to GFP background and select only synaptic spots. The selection of spots and quantification are done automatically by Imaris. All spots below the threshold are excluded, regardless of genotype and experimental conditions, rendering the analysis objective. We have now provided a detailed description of the protocol in the Materials and Methods section: page 29 lines 778-799.

> *(16) Figure 5C lacks genotype controls (i.e., DNT2-GAL4-only and UAS-TrpA1-only). These controls are essential because elevated temperatures alone, without activation of DNT2 neurons, could potentially increase Syt-GCaMP production, leading to an increase in the number of Syt+ synapses. Including these controls would help ensure that the observed effects are truly due to the activation of DNT2 neurons and not temperature-related artifacts.*

We thank this Reviewer for this feedback, which we will bear in mind for future projects.

> *(17) L314-316: The authors state, "Here, the coincidence of... revealed that newly formed synapses were stable." I think this statement needs to be toned down because there is no*

> *evidence that these pre- and post-synaptic sites are functionally connected.*

The Reviewer is correct that our data did not visualise together, in the same preparation and specimen, both pre- and post-synaptic sites. Still, given that PAMs have already been proved by others to be required for locomotion, learning and long-term memory, our data strongly suggest that synapses between them at the SMP are functionally connected.

Nevertheless, as we do not provide direct cellular evidence, we have now edited the text to tone down this claim: “Here, the coincidence of increased pre-synaptic Syt-GFP from PAMs and post-synaptic Homer-GFP from DNT-2 neurons at SMP suggests that newly formed synapses could be stable”, page 13 line 351.

> *(18) Figure 5D lacks permissive temperature controls. Also, the DNT-2FL overexpression phenotypes are different from the TpA1 activation phenotypes. The authors may want to discuss this discrepancy.*

Regarding the controls, these are not appropriate for this data set. These data were all taken at a constant temperature of 25°C, there were no shifts, and therefore do not require a permissive temperature control. We thank this Reviewer for drawing our attention to the fact that we made a mistake drawing the diagram, which we have now corrected in Figure 5D.

Regarding the discrepancy, this had already been discussed in the Discussion section of the previously submitted version, page 19 Line 509-526. Presumably this Reviewer missed this before.

> *(19) Figure 6A, B lack permissive temperature controls. These controls are important if the authors want to claim that the behavioral defects are due to adult-specific manipulations. In addition, there is no statistical difference between the PAM-GAL4 control and the RNAi knockdown group. The authors should be careful when stating that climbing was reduced in the RNAi knockdown flies (L341-342).*

We thank this Reviewer for this feedback, which we will bear in mind for future projects.

Point taken, but climbing of the tubGAL80ts, PAM>Toll-6RNAi flies was significantly different from that of the UAS-Toll-6RNAi/+ control.

> *(20) Figure 6C: It seems that the DAN-GAL4 only control (the second group) also rescued the climbing defect. The authors may want to clarify this point.*

The phenotype for this genotype was very variable, but certainly very distinct from that of flies over-expressing Toll-6[CY].

We thank Reviewer 2 for their very thorough analysis of our paper that has helped improve the work.

> ***Reviewer #3 (Recommendations for the authors):***
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> *Overall, the manuscript reports highly interesting and mostly very convincing experiments.*

We are very grateful to this Reviewer for their very positive evaluation of our work.

> *Based on my comments under the heading "public review", I would like to suggest three possible improvements.*

> *First, the quantification of structural plasticity at the sub-cellular level should be explained in more detail and potentially improved. For example, 3D reconstructions of individual neurons and quantification of the structure of boutons and dendrites could be undertaken. At present, it is not clear how bouton volumes are actually recorded accurately.*

Thank you for the feedback. The analyses of dendrites and synapses were carried out in 3D-volumes using Imaris "Filament" module and "Spot function", respectively. Dendrites are analysed semi-automatically, ie correcting potential branching errors of Imaris, and synapses are counted automatically, after setting appropriate thresholds. Details have now been expanded in the Materials and Sections section: page 28 lines 756-768 and page 29 lines 780-799.

We would also like to thank Imaris for enabling and facilitating our remote working using their software during the Covid-19 pandemic, post-pandemic lockdowns and lab restrictions that spanned for over a year.

> *Second, the variability between DNT-2A-positive neurons with increasing sample size compared to a control (DNT-2A-negative neurons) should be demonstrated. Figure 2C does currently not present convincing evidence of increased structural variability.*

It is unclear what data the Reviewer refers to. Figure 2C shows qRT-PCR data, and it does not show structural variability, which instead is shown with microscopy. If it is the BacTrace data in Figure 2B, the controls had been provided and the data were unambiguous. If Reviewer means Figure 1C, it is unclear why DNT-2GAL4-negative flies are needed when the aim was to visualise normal (not genetically manipulated) DNT-2 neurons. Thus, unfortunately we do not understand what the point is here.

The observation that DNT-2 neurons are very variable, naturally, is highly interesting, and presumably this is what drew the attention of Reviewer 3. We agree that showing further data in support of this is interesting and valuable. Thus, in response to this Reviewer's comment we have now increased the number of images that demonstrate variability of DNT-2 neurons:

(1) We have added an extra image, altogether providing three images in new Figure 1A showing three different individual brains stained with DNT-2GAL4>UAS-FlyBow1.1. These show common morphology and features, but different location of the somas and distinct detailed arborisation patterns. Two more images using DNT-2GAL4 are provided in Figure 5A.

(2) We have now added two further MCFO images, altogether showing four examples where the somas are not always in the same location and the axons arborise consistently at the SMP, but the detailed projections are not identical: new Figure 1D.

These data compellingly show natural variability in DNT-2 neuron morphology.

> *Third, I propose to simplify the feedback model (Figure 2F) to be less speculative.*

Indeed, some details in Figure 2F are speculative as we did not measure real dopamine levels. Accordingly, we have now edited this diagram, adding question marks to indicate speculative inference, to distinguish from the arrows that are grounded on the data we provide.

Accordingly, we have also edited the text in:

- page 9, lines 221: "Altogether, this shows that DNT-2 up-regulated TH levels (Figure 2E), and presumably via dopamine release, this inhibited cAMP in DNT-2A neurons (Figure 2F)".

> *- page 20, lines 515: "Importantly, we showed that activating DNT-2 neurons increased the levels and cleavage of DNT-2, up-regulated DNT-2 increased TH expression, and this initial amplification resulted in the inhibition of cAMP signalling via the dopamine receptor Dop2R in DNT-2 neurons."*

As minor points:

(1) Appetitive olfactory learning is based on Tempel et al., (1983); Proc Natl Acad Sci U S A. 1983 Mar;80(5):1482-6. doi: 10.1073/pnas.80.5.1482. This paper should perhaps be cited.

Thank you for bringing this to our attention, we have now added this reference to page 14 line 394.

> *(2) Line 34: I would add ..."ligand for Toll-6 AND KEK-6,".*

Indeed, thank you, now corrected.

> *(3) Line 39: DNT-2-POSITIVE NEURONS.*

Now corrected, thank you.

> *(4) The levels of TH mRNA were quantified. Why not TH or dopamine directly using antibodies, ELISA, or HPLC? After all, later it is explicitly written that DNT modulates dopamine levels (line 481)!*

We thank this Reviewer for this suggestion. We did try with HPLC once, but the results were inconclusive and optimising this would have required unaffordable effort by us and our collaborators. Part of this work spanned over the pandemic and subsequent lockdowns and lab restrictions to 30% then 50% lab capacity that continued for one year, making experimental work extremely challenging. Although we were unable to carry out all the ideal experiments, the DNT-2-dependent increase in TH mRNA coupled with the EPAC-Dop2R data provided solid evidence of a DNT-2-dopamine link.

> *(5) Line 271: The PPL1-g1-pedc neuron has mainly (but not excusively) a function in short-term memory!*

They do, but others have also shown that PPL1-g1-pedc neurons have a gating function in long-term memory (Placais et al 2012; Placais et al 2017; Huang et al 2024) and are required for long-term memory (Adel and Griffith 2020; Boto et al 2020).

> *(6) Line 401: Reward learning requires PAM neurons. PPL1 neurons are required for aversive learning.*

Indeed, PPL1 neurons are required for aversive learning, but they also have a gating function in long-term memory common for both reward and aversive learning (Adel and Griffith, 2020 Neurosci Bull; Placais et al, 2012 Nature Neuroscience; Placais et al 2017 Nature Communications; Huang et al 2024 Nature).

> *Overall, the manuscript presents extremely interesting, novel results, and I congratulate the authors on their findings.*

We would like to thank this Reviewer for taking the time to scrutinise our work, their helpful feedback that has helped us improve the work and for their interest and positive and kind works.

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