SUBSETS OF DEVELOPMENTAL TRANSCRIPTION NETWORKS MAINTAIN CELLULAR SUBTYPE IDENTITY IN THE MATURE NERVOUS SYSTEM

by

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ABSTRACT

The diversification of cellular subtype during development is directed by combinatorially acting transcription factors and signaling pathways that act to regulate subtype specific gene expression profiles in post-mitotic cells. These key transcription factors and signaling pathways operate in a transcriptional network, which act to establish cellular subtype identity over the course of a developing cellular lineage. Lineage progression towards ever increasing cellular diversity is often viewed as a ratchet mechanism of irreversible steps resulting in the specification and then terminal differentiation of cell subtype identities. From this viewpoint, terminally differentiated cells have long been considered as irreversibly locked into their identity. In a landmark article, Blau and Baltimore (Blau and Baltimore, 1991) postulated that a cell's identity, or differentiated status, requires persistent active regulation, rather than lapsing into a passive 'locked-in' state. While little genetic evidence was available at the time, sufficient evidence has since accumulated to propose that the terminally differentiated state, or identity, of a cell subtype indeed requires active maintenance. Currently, however, we have only the most rudimentary understanding of the regulatory mechanisms that maintain neuronal identity. This thesis presents a systematic effort to characterize the role of the transcriptional networks that differentiate neuronal identity in the mature neurons of the adult nervous system. Using the *Drosophila* Tv cluster neurons I show the persistent requirement of 1) target derived signals and 2) networks of transcription factors for the maintenance of the cellular subtype specific expression profiles of terminal differentiation genes, genes that define these neuron's function and identity. This work establishes one of the most comprehensive transcriptional models for maintenance of cell identity to date. It also provides novel mechanistic insights showing that cellular differentiation is a persistent process that requires active maintenance, rather than being passively 'locked-in' or unalterable. As such, the work of this thesis provides critical insight that provides a strong foundation for further efforts to determine how neuronal identity is maintained.

PREFACE

Chapter 2. A version of this has been published as: Eade, K. Allan, D. (2009) Neuronal phenotype in the mature nervous system is maintained by persistent retrograde signaling. J Neurosci. 29*:*3852–3864.
For this publication I performed all experiments and analyses. Douglas Allan and I conceived experiments and methods of analysis, as well as wrote the paper.

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Hailey Fancher acquired confocal images in figure 3.1. Marc Ridyard performed the experiment and the analysis of figure 3.3J. I performed all other experiments and analysis with assistance in dissection from Hailey Fancher in figures 3.2 through 3.5. Douglas Allan and I conceived experiments and methods of analysis, as well as wrote the paper.

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LIST OF ABBREVIATIONS

Aβ	amyloid beta
ALS	amyotrophic lateral sclerosis
AP	anterior-posterior
Ap	apterous
APP	amyloid precursor protein
BAC	bacterial artificial chromosome
bHLH	bascic helix-loop-helix
BMP	bone morphogenic protein
Cas	castor
CBF	cholinergic basal forebrain
Col	collier
Dac	dachshund
Dimm	dimmed
DN	dominant negative
DNH	dorsal neurohaemal organ
dsRNAi	double stranded RNAi
DV	dorsal-ventral
Eya	eyes absent
FISH	fluorescent in situ hybridization
FSH β	follicle-stimulating hormone subunit β
Gbb	glass bottom boat
GMC	ganglion mother cell
Grh	grainy head
HAT	histone acetyltransferase
HDAC	histone deacytlyase
Hb	hunchback
Hth	homothorax
Kr	kruppel
NGF	nerve growth factor
PcG	polycomb group

pMad	phosphorlyated Mad
PMN	postmitotic neuron
Sax	saxophone
SCG	superior cervical ganglion
Sqz	squeeze
TDG	terminal differentiation gene
TGFβ	transforming growth factor-β
TF	transcription factor
Th	thoracic segment
Tkv	thickveins
TrxG	trithorax group
VNC	ventral nerve cord
Wit	wishful thinking

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And to all the thousands of flies that died in the name of science.

DEDICATION

For the Eades

1. INTRODUCTION

Neurons exhibit an enormous diversity in their form and function. This diversity is underpinned by differences in the gene expression profiles of the many distinct neuronal subtypes. Of particular importance to a neuronal subtype's unique function is the battery of terminal differentiation genes (TDGs) that it expresses. These genes include the neuropeptides, neurotransmitter biosynthetic enzymes, ion channels and specific sets of receptors that perform the unique functions of that neuronal subtype. Work over many years has shown that the gene expression profile of a neuron is directed by the combinatorial action of multiple sequence-specific transcription factors. From *Drosophila* to vertebrates, very few transcription factors have been described that are uniquely expressed in any single neuronal subtype and are singly responsible for critical aspects of the neuron's identity and function. Instead, the *cis*-regulatory regions of most TDGs serve essentially as a coincidence detector for the co-expression of multiple different transcription factors (Biggin, 2011). In this way, a specific 'code' of multiple transcription factors is required to initiate the subtype-specific expression of any terminal differentiation gene. In addition, target derived signaling has also been shown to be required for the expression of certain TDGs.

How diverse postmitotic neuronal subtypes arise from progenitor neuroepithelial/ neuroectodermal cells has been characterized in many model systems, and a number of core, conserved mechanisms have been uncovered (Bertrand et al., 2002; Pearson and Doe, 2004). In all cases, the process by which specific codes of transcription factors are established in postmitotic neuronal subtypes is initiated during early embryogenesis, and involves spatially and temporally-patterned transcription factors and signaling pathways that become progressively refined throughout the progression of each lineage. In stark contrast to these early events, we know very little regarding how subtype identity is maintained for the life of the organism, after differentiation. Not only do we have little information regarding how the TDGs required for function are maintained, but we have even less information on the transcriptional basis for maintained neuronal identity and function. This problem is especially intriguing in the nervous system where many of the neurons are born in the embryo and must survive and maintain their function throughout the life of the organism. Moreover, this question is clinically relevant because the progressive loss of neuronal function that occurs with age has enormous societal impact.

Following cellular differentiation, two main mechanisms may be at play to maintain the

expression of subtype-specific gene expression; i) persistent activity of the transcription factors and signaling pathways that were required for their initiation, or ii) specific maintenance mechanisms that act independently of the initiating factors. In this thesis, I provide evidence that target-derived signaling and the transcription factors that are directly involved in the initiation of subtype TDG expression, are persistently required for the maintenance of subtype TDG expression throughout the life of the organism. However, we also find that the network of transcription factors required for the specification and differentiation of a specific neuronal subtype is altered after differentiation to a unique maintenance configuration. First, a number of transcription factors required for initiation of certain other transcription factors are no longer expressed. Second, amongst the transcription factors that are retained, cross-regulatory interactions that were essential during differentiation are no longer found to be essential in the adult. Thus, a maintenance-specific network of transcription factors is established that is distinct from the differentiating network required to establish it. This indicates the existence of a final step of development in which a progressive transcriptional developmental cascade is resolved into a stable transcriptional maintenance state.

Using well-characterized neuronal subtypes in *Drosophila*, I directly tested the mechanisms that maintain mature neuronal identity by conditionally knocking down transcription factors that had been required for the specification and differentiation of those neurons. This is a targeted approach to delineating transcriptional maintenance mechanisms that, until the recent development of appropriate genetic technologies, we were unable to address. As the goal of this thesis was to examine the role of developmental mechanisms in maintaining mature neuronal identity, this approach required foreknowledge of the mechanisms that established those neurons' subtype identity. I will briefly introduce our current state of knowledge of the mechanisms that diversify neuronal lineages, focusing mainly on evidence from *Drosophila* while drawing on certain subtypes of vertebrate neurons to emphasize the conservation of core mechanisms. In this introduction, I will focus specifically on five major themes: 1) The establishment of spatial and temporal patterning events and their integration to generate subtype-specific codes of transcription factors in postmitotic neurons; 2) The direct activation of TDGs at their *cis*-regulatory region by combinatorial codes of transcription factors; 3) The requirement of target-derived signaling for establishing terminal identity in neurons; 4) Evidence from a number of models supporting the hypothesis that maintenance of TDGs is governed by developmental transcription factors; 5) The potential role of mechanisms that may maintain gene expression in the absence of the initiating transcription factors.

1.1 SPATIAL AND TEMPORAL PATTERNING

1.1.1 Spatial patterning

In the early *Drosophila* embryo, dorsal-ventral (DV) and anterior-posterior (AP) patterning through transcription factor expression patterns and signaling gradients forms a 'Cartesian grid' of positional information within the neuroectoderm. DV patterning is established by gradients of Dorsal, BMP and EGFR signaling (von Ohlen and Doe, 2000). These establish three distinct DV domains of homeodomain transcription factor expression in the developing neuroectoderm; from dorsal to ventral these are *msh (muscle specific homeobox), ind (intermediate nervous system defective) and vnd* (ventral nervous system defective). In the AP axis, patterning is established by the hierarchical actions of maternal genes (eg, *bicoid* and *hunchback*), gap genes (eg. *kruppel*, *giant* and *knirps*), and pair rule genes (eg, *even-skipped* and *fushi tarazu*) that establish domains of Hox gene expression and ultimately parasegment identity. Each segment is further subdivided by the local AP-patterned expression domains of segment polarity genes (eg *wingless*, *engrailed*, *gooseberry*, *patched*, *hedgehog*, and *naked cuticle*) that become expressed by each neuroblast and regulate the subsequent potential of a given lineage. Together, the combination of DV- and AP-patterned transcription factors and morphogens within the neuroectoderm and delaminating neuroblasts determines the subsequent lineage potential of each neuroblast (Akam, 1987; Nusslein-Volhard and Wieschaus, 1980).

The developing vertebrate nervous system utilizes the same principles of spatial axial patterning to determine the competence of neuronal lineages. In the developing neural tube, opposing gradients of Sonic hedgehog (ventral) and BMP (dorsal) establish discrete domains of partially overlapping homeodomain and bHLH transcription factor expression within the neuroepithelial neuronal progenitors. These domains correspond to specific neural progenitor pools with unique competencies to become specific neuronal subtypes, along the DV axis (Lee and Pfaff, 2001). Hox gene expression is established along the AP axis by intersecting gradients of retinoic acid, fibroblast growth factors and Wnts secreted from regional organizers, and plays an important role in patterning neuronal progenitor pools along the AP axis (Liu et al., 2001; Nordstrom et al., 2006).

1.1.2 Temporal patterning

In the *Drosophila* ventral nerve cord (VNC), neuroblasts delaminate from the neuroectoderm in 5 waves, generating 30 neuroblasts per hemisegment, each one with a specific spatial identity. These neuroblasts then undergo a successive series of asymmetric divisions to produce a ganglion mother cell (GMC) and a neuroblast. The GMC only divides once to produce two postmitotic neurons or glia (Fig 1.1). The birth order of GMCs provides an additional temporal 'axis of information' that further diversifies the neurons that are generated from a single neuroblast lineage (Bossing et al., 1996; Doe, 1992; Doe and Goodman, 1985; Schmid et al., 1999). Underlying this temporal code is sequential expression of the transcription factors *hunchback* (*hb*), *kruppel (kr)*, *pdm*, *castor (cas)* and *grainy head (grh)* at each neuroblast division. These temporal transcription factors can be expressed over single or multiple neuroblast divisions, and may be partially overlapping (Fig 1.1). The specific expression profile of temporal transcription factors in neuroblasts is referred to as an expression window. Although, the duration of these transcription factor expression windows may differ between lineages, the sequential order of expression of each temporal transcription factor does not appear to differ. Overall, these temporal changes result in the generation of GMCs with discrete spatial and temporal transcription factor expression profiles depending on birth order (Isshiki et al., 2001).

Within a given neuroblast lineage, the expression of a specific temporal transcription factor corresponds directly to the generation of a specific neuronal/glial cell type . Neuroblast NB7-3, identified by expression of the transcription factor *eagle*, produces four characteristic neurons from three neuroblasts (Fig 1.1A). GMC-1 produces a serotonergic interneuron EW1 and a motor neuron GW from a Hb/Kr expressing neuroblast. At the next neuroblast/GMC division, the Kr-expressing GMC-2 produces a serotonergic interneuron EW2, and a sister cell that undergoes apoptosis. At the end of the lineage GMC-3 differentiates directly into a corazonin-expressing interneuron EW3 in a Pdm expression window (Isshiki et al., 2001; Lundell and Hirsh, 1998). It is important to emphasize that the outcome of temporal transcription factor activity is not correlated with a specific neuronal subtype. For example, while the Kr-expressing GMC in lineage 7-3 produces a serotonergic interneuron, the Kr-expressing GMC in the neuroblast 7-1 lineages produces the U3 motoneuron (Fig 1.1B). To further illustrate this point, depending on the identity of the neuroblast, Hb-expressing GMCs can produce interneurons, motoneurons or glia (Isshiki et al., 2001).

The proper regulation of temporal transcription factor expression windows in neuroblast lineages is critical to the proper differentiation of postmitotic neurons derived from each window. Transcription factor expression windows in the temporal cascade are controlled in part through cross-regulation, where the activation and repression of temporal transcription factors is regulated by succeeding and preceding temporal transcription factors (Fig 1.1C) (Grosskortenhaus et al., 2005; Isshiki et al., 2001). Interestingly, different neuronal subtypes can be generated within the expression window of temporal transcription factors. This is in part mediated by differences in the relative expression levels of the temporal transcription factor in a GMC, which appears to be informative with regards to the neuronal subtypes generated. For example, Tv cluster neurons (the focus of this thesis) are generated by the neuroblast 5-6T (NB 5-6T) lineage in an expression window of *cas* and *grh*. In *cas* mutants, the entire Tv neuronal cluster is not generated, however in *grh* mutants, only the last neuron, Tv4, is not generated. Detailed analysis has shown that *grh* expression levels progressively increase as the neuroblast lineage proceeds from Tv1-Tv4 generation. It appears that by Tv4, Grh expression reaches a specific threshold that is necessary for generating the Tv4 neuron subtype fate. (Baumgardt et al., 2009; Brody and Odenwald, 2000; Kambadur et al., 1998; Karlsson et al., 2010). This cluster of neurons is the focus of my thesis and will be described in greater detail throughout this thesis.

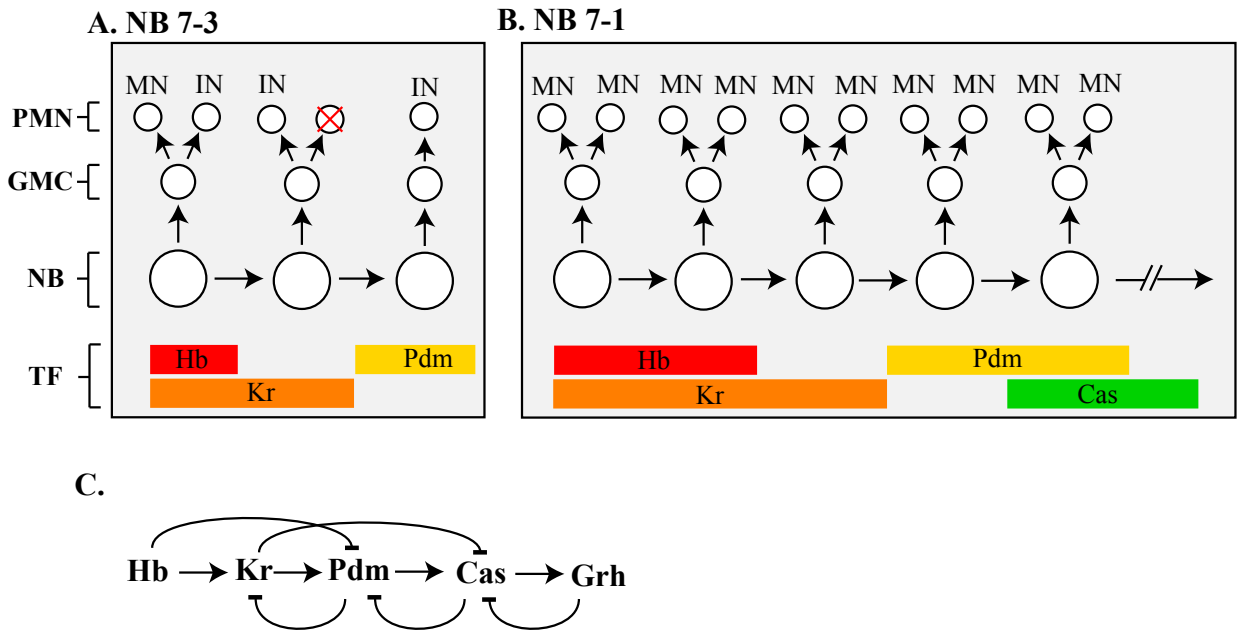

Figure 1.1 Temporal patterning of Drosophila neuroblast lineages 7-3 and 7-1.

Neuroblast (NB) lineages propagate by NBs dividing asymmetrically to produce another NB and a GMC. The GMC typically divides to produce two postmitotic neurons (PMNs) or glia, which differentiate into mature neurons/glia or undergo cell death (red cross). The type of neuron/glia generated, within a given lineage, corresponds to the transcription factors expressed at the time of NB division (coloured bars). Transcription Factors expression windows are always expressed in the same sequential order but the length of their expression window varies between NB lineages. Between NB lineages similar transcription factor expression windows generate different neuronal/glial types. (A) NB 7-3 produces motoneurons (MN) and interneurons (IN). (B) The first 5 NB divisions of the NB 7-1 lineage only produce MNs.
(C) Temporal transcription factors mutually activate and repress each other to restrict expression windows throughout a neuroblast lineage.

1.2 TRANSCRIPTION FACTOR NETWORKS ESTABLISH CELL SUBTYPE IDENTITY

1.2.1 Integration of spatial and temporal patterning events establish transcription factor networks that determine neuronal subtype identity

Spatial and temporal patterning events establish progenitor neuron lineages with specific transcription factor expression patterns, as described above. These transcription factors, referred to as lineage specifying transcription factors, initiate the expression of a cellular subtype specific combination of transcription factors in postmitotic neurons. These postmitotic transcription factors, referred to as differentiating transcription factors, directly regulate cell specific expression profiles of terminal differentiation genes (TDGs), thus determining the specific form and function of a cell. Throughout this thesis, I refer to the combination of lineage specifying transcription factors and postmitotic differentiating transcription factors as a 'subtype transcription network'. Understanding how these subtype transcriptional networks establish cellular subtype identity in postmitotic neurons is critical to understanding the work of this thesis, as it is the role of these types of transcription factors that I directly test in the maintenance of TDG expression throughout life.

One of the more fully characterized models of neuronal subtype specification and differentiation in vertebrates are the spinal motoneurons (Dasen and Jessell, 2009). By virtue of the unique and experimentally accessible axonal trajectories that are characteristic of motoneuron subtypes, early studies could assign specific axon pathfinding phenotypes to transcription factor mutants. These studies identified the roles of multiple transcription factors in the assignment of motoneuron subtype identities. The pMN progenitor lineage, that will give rise to motoneurons, is generated by a Shh gradient from the floor plate that establishes a Pax6, Nkx6.1, Nkx6.2, and Olig2 expression domain. These factors establish a transcription factor cascade within the pMN progenitors that directs the activation of the bHLH transcription factor Ngn2 and the homeodomain transcription factors Lhx3, Isl1, Isl2 and HB9 within nascent and postmitotic motoneurons (Dasen and Jessell, 2009; Lee and Pfaff, 2001). These transcription factors combinatorially regulate features that are common to all spinal motor neurons including sending an axon out of the spinal cord as well as expression of choline acetyltransferase. Distinct motoneuron subtype identities are further generated by the subset-specific emergence of discrete combinatorial expression patterns of Hox gene and LIM-homeodomain transcription factors in postmitotic neurons (Dasen et al., 2003; Kania and Jessell, 2003). These subtype-specific

transcriptional codes then regulate underlying molecular mechanisms of axon guidance (Dasen et al., 2005). For example, in lateral motor columns, the cooperative action of LIM-homeodomain transcription factors with Hox genes then regulate the expression of EphA receptors, which in turn regulate the neuron's response to ephrin-A, a DV pathfinding cue, thus directing DV limb projections.

1.2.2 Specification of Tv cluster neurons in Drosophila

In the Drosophila nervous system a discrete cluster of neurons known as the Tv neurons, the focus of this thesis, provide another excellent example of cellular subtype differentiation through the convergence of lineage specifying and differentiating transcription factors. Despite differences in axonal trajectories and possibly neuronal physiology between Tv cluster neurons, subtype identity can easily be discriminated by differential expression of the neuropeptides Nplp1 and FMRFa in Tv1 and Tv4 neurons, respectively (Fig 1.2) (Baumgardt et al., 2007; Schneider et al., 1991). This has allowed for a detailed analysis of how different complex combinatorial codes of transcription factors converge onto single subtype-defining TDGs, providing a high resolution in understanding of the genetic circuitry that defines subtype identity (Fig 1.3) (Allan et al., 2003; Baumgardt et al., 2009; Baumgardt et al., 2007; Benveniste et al., 1998; Hewes et al., 2003; Karlsson et al., 2010; Marques et al., 2003; Miguel-Aliaga et al., 2004). To date, the specification and differentiation of postmitotic Tv cluster neurons from the NB5-6T lineage is one of the most comprehensively understood models of neuronal subtype development by subtype transcription factor networks. Here, I will introduce the transcriptional networks that specify the Tv cluster neuronal subtypes.

The NB 5-6T lineage expresses the spatial transcription factors *gooseberry-neuro* and *ladybird early*. As stated previously, the Tv cluster neurons are born sequentially, Tv1 to Tv4, within a *cas* and *grh* temporal transcription factor expression window, at the end of the NB 5-6T lineage (Fig 1.3). This specification however, only occurs in the thoracic segments of the VNC (Fig 1.2). In abdominal segments, the NB5-6 lineage is truncated prior to generation of Tv cluster neurons due to the presence of the abdominally restricted Hox genes, *ultrabithorax* and *abdominal A*. In thoracic segments, the Hox gene *antennaepedia* functions coordinately with Pbx/Meis Hox cofactors *homothorax* (*hth*), *extradenticle* and the temporal transcription factor *cas* to extend the neuroblast lineage and induce the transcription factors *collier* (*col), squeeze (sqz), grh,* and the cofactor *nab* (Karlsson et al., 2010). The sequential expression of *col, sqz, nab* and *grh* in nascent Tv neuron neuroblasts then specifies the generation of three distinct Tv

neuronal subtypes Tv1, Tv2/3 and Tv4 (Baumgardt et al., 2009; Baumgardt et al., 2007; Karlsson et al., 2010). Details of these processes are provided in the next paragraph. Following each Tv neurons' final mitotic division, each neuron's subtype initiates expression of the transcription factors *apterous* (*ap*) and *eyes absent* (*eya*) (Baumgardt et al., 2009). Additionally, Tv1 and Tv4 neurons initiate the expression of the neurosecretory determinant transcription factor *dimmed* (*dimm*) within a few hours of becoming postmitotic (Baumgardt et al., 2007). The cascade of transcription factors initiated in the nascent Tv neuroblasts is refined within postmitotic Tv neurons, so that by stage 17 embryos (when subtype-specific terminal differentiation genes are activated), each Tv neuron subtype has a specific transcription factor code. Tv1 neurons express *col, eya, ap and dimm*, Tv4 neurons express *grh, eya, ap, dimm, dac* and *sqz*, whereas Tv2/3 both express *eya, ap, dac* and *sqz* (Allan et al., 2003; Baumgardt et al., 2009; Baumgardt et al., 2007; Hewes et al., 2003; Miguel-Aliaga et al., 2004). Establishing these unique profiles of transcription factor expression is essential for the proper specification of these neurons.

The specification of the Tv1 neuron primarily revolves around *col,* which is required for initiation of *ap* and *eya* expression. Initiation of *dimm* then requires the collective action of *col, ap* and *eya*. Then all four transcription factors are required for initiation of Nplp1expression (Fig 1.3) (Baumgardt et al., 2007). Although *col* is essential for the specification of the Tv1 neuron and retained for the differentiation of Nplp1 expression, it is only transiently expressed in Tv2/3 and Tv4 where it initiates *ap* and *eya* expression, then disappears (Fig 1.3) (Baumgardt et al., 2009; Baumgardt et al., 2007). The specification of Tv2/3 and Tv4 requires the repression of *col* by *sqz* and *nab*. In the absence of *sqz* or *nab*, an extra Tv1 neuron expressing *col* is generated at the expense of a Tv2/3 neuron. Here *sqz* and *nab* act together to oppose the transcriptional cascade that leads to Tv1 specification and differentiation (Fig 1.3). Further specification of the Tv4 neuron requires elevated levels of *grh* expression. In the absence of *grh*, the Tv4 neuron is not generated and supernumerary Tv3 neurons are formed (Baumgardt et al., 2009; Baumgardt et al., 2007). Functionally, the transcription factors *cas, col, grh, sqz*, and *nab* can be viewed as specifying transcription factors, because in their absence the neuron is not specified normally, or is transfated to a different neuronal subtype. In the Tv1 neuron *col* performs a dual function; it is first required for the specification of all Tv cluster neurons through activation of *ap* and *eya* but is then is then subsequently required in Tv1 together with *eya, ap* and *dimm* for the initiation of Nplp1. In the Tv4 neuron, *sqz* also plays a dual role. It is required for transitioning the neuroblast lineage to generate a Tv2 neuron after Tv1 neuron generation (in the absence of *sqz*, two Tv1

neurons are generated) *sqz* is then required in the Tv4 neuron for expression of FMRFa, in combination with *ap, eya, dimm, dac* and BMP signaling. In both the Tv1 and Tv4 neurons *dimm*, a bHLH transcription factor, has a transcriptional sub-routine to initiate key genes required for the peptidergic phenotype (Hewes et al., 2003).

In this thesis, I directly test for the presence and necessity of BMP signaling in the Tv4 neuron, as well as all transcription factors that are known to be required to specify and differentiation of Tv1 and Tv4 neurons in mature adult neurons for the continued expression of Nplp1 and FMRFa.

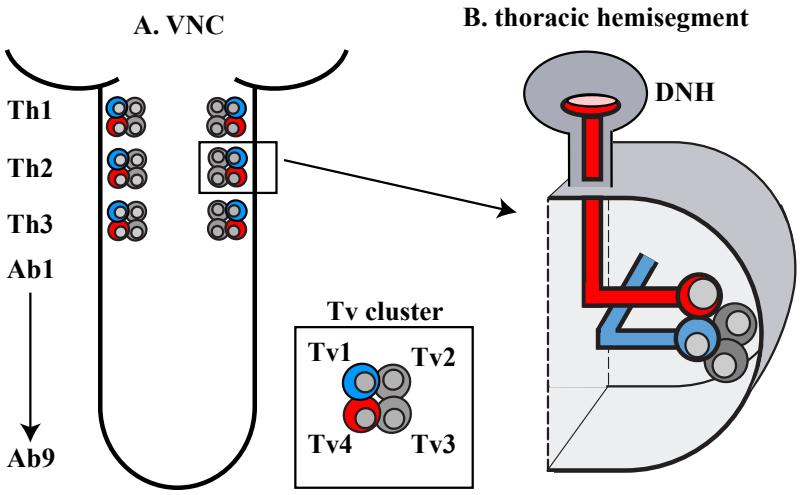

Figure 1.2 The Tv cluster neurons of the *Drosophila* ventral nerve cord

(A) Cartoon representing a dorsal view of the *Drosophila* ventral nerve cord (VNC) in late embryonic and larval stages. Each Tv cluster contains four neurons: a Tv1 neuron expressing Nplp1 (blue); a Tv4 neuron expressing FMRFa (red); and a Tv2 and a Tv3 neuron. The VNC contains six Tv clusters in thoracic segments (Th) 1-3. Tv cluster neurons are not present in abdominal segments (Ab) 1-9.
(B) Cross section of a thoracic VNC hemisegment. Tv clusters in the late embryonic and larval stages are identical. The Tv4 axon projects to the midline then dorsally to innervate the dorsal neurohaemal organ (DNH). The Tv4 axon projects to the midline then anteriorly to an unknown target.

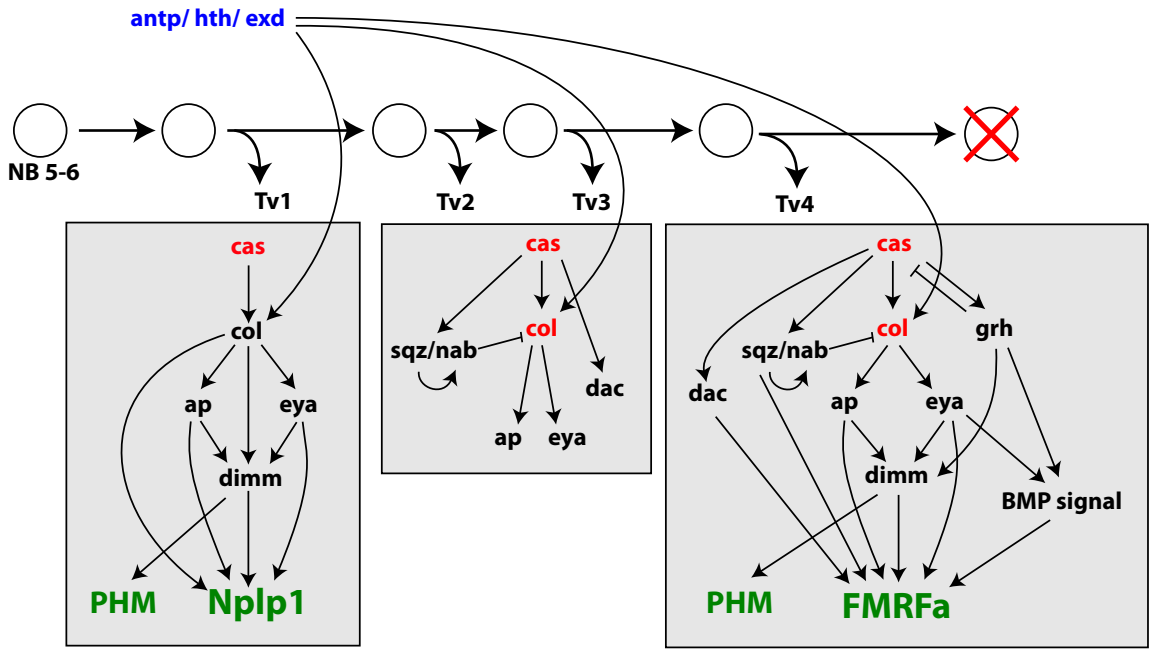

Figure 1.3 Schematic representing transcription factor induction cascades in neuroblast 5-6 (NB 5-6) lineage specifying Tv1, Tv2, Tv3, Tv4 neurons.

Spatial transcription factors *antp*, *hth*, *exd* (blue) interact with temporal transcription factors *cas* and *col*, which are transiently expressed in neurons Tv2-4 (red). The cooperative action of these transcription factors establishes a post-mitotic network of transcription factors (black) that differentially initiate the expression of TDGs (green).

1.2.3 Combinatorial codes of transcription factors act in postmitotic neurons to direct subtype-specific gene expression profiles through specific cis-regulatory motifs.

Transcription factors required for the selective expression of a given TDG are present in various cell types, but not all the cell types in which they are present express that particular TDG. The transcriptional initiation of TDG expression typically requires the combinatorial action of multiple sequence-specific transcription factors. This is governed by the *cis*-regulatory DNA motifs in the enhancer regions of TDGs and the manner in which transcription factors that bind these motifs lead to gene transcription. The *cis*-regulatory DNA motifs act as binding sites for a unique combination of specific transcription factors. These transcription factors then recruit the mediator complex and/or chromatin-modifying enzymes to either increase or suppress the transcriptional activity of targeted genes (Berger, 2007; Biggin, 2011; Casamassimi and Napoli, 2007). Thus, through additive and synergistic mechanisms, the binding of multiple specific transcription factors at *cis*-regulatory sequences of TDGs only occurs in neuronal subtypes where specific sets of transcription factors are co-expressed. In this way, the expression of specific combinatorial transcription factor codes directly regulates the function and identity of specific neuronal subtypes.

In the simple model of the AIY sensory neurons of *C. elegans*, the transcription factors TTX-3 (homeodomain) and CEH-10 (paired-like homeodomain) bind cooperatively to a 16bp regulatory module, the AIY motif, that is present in the majority of AIY subtype-specific TDGs. TTX-3 has low affinity for the AIY motif alone but has high affinity when bound with CEH-10, allowing for activation of AIY specific genes (Wenick and Hobert, 2004). TTX-3 and CEH-10 are together necessary and sufficient for AIY-specific gene expression, but do not regulate pan-neuronal properties of the AIY neuron. In TTX-3 and CEH-10 mutants, the AIY neurons are still fated as neurons but do not differentiate as AIY-specific neurons (Bertrand et al., 2002; Hobert et al., 2010).

In *Drosophila* and vertebrates, the combinatorial action of these transcription factors appears to be considerably more complex and requires increased numbers of transcription factors and signaling pathways for TDG expression. In vertebrates noradrenergic sympathetic neurons derived from neural crest cells express the subtype markers Tyrosine Hydroxylase (TH) and Dopamine β-Hydroxylase (DBH). During their differentiation BMP signals secreted from the dorsal aorta initiate expression of the bHLH transcription factor Mash1 and the homeodomain transcription factor Phox2b (Goridis and Rohrer, 2002). Mash1 and Phox2b act combinatorially

to initiate a cross-regulatory induction cascade of transcription factors Phox2a, dHand, and Gata3. All of these transcription factors then directly regulate the expression of TH and DBH by binding at their *cis*-regulatory enhancer regions (Goridis and Rohrer, 2002). In different cell types, TDG expression can be dependent on different transcription factors that act at different *cis*-regulatory regions. In midbrain dopaminergic neurons that are characterized by their expression of TH but not DBH, TH expression is directly activated by the cooperative binding of transcription factors Nurr1, Pitx3, FOXA1, and FOXA2, to an alternate c*i*s-regulatory region of the TH enhancer (Lee et al., 2010; Lin et al., 2009).

It is well established that the appropriate expression of TDGs requires the complete combinatorial code of transcription factors to activate each other and ultimately directly activate TDG expression. Following initiation however, it is not established to what extent the developmental regulatory cascade or the cis-regulatory acting transcription factors are required to maintain TDG expression, if any.

1.3 TARGET-DEPENDENT INDUCTION OF POSTMITOTIC NEURONAL IDENTITY.

In many cases following lineage specification, the terminal differentiation of a neuron is dependent on target-derived retrograde signals from the cells that they innervate. These signals trigger the expression of various cell-specific TDGs (Hippenmeyer et al., 2004; Nishi, 2003; Xu and Hall, 2006). Notably, the majority of those TDGs are mediators of neuronal communication (neuropeptides, neurotransmitter biosynthetic enzymes and ion channels). The integration of target-derived signals with combinatorial codes of transcription factors for the initiation of TDGs in post-mitotic neurons is a critical step for the differentiation and function of many neurons. It was not known, however, whether these target-derived signals are persistently required for the continued expression of the TDGs they initiate or if they are merely transient cues that are only developmentally required to trigger terminal differentiation.

In *Drosophila*, retrogradely trafficked target-derived bone morphogenetic protein (BMP) signaling has been increasingly found to be a common mechanism for the terminal differentiation of neurons. Retrograde BMP signaling is essential for the transcriptional induction of neuropeptides FMRFa in the Tv4 neuron, Dilp7 in dMP2 neurons, as well as CCAP, MIP, and Bursicon β in CCAP neurons. (Allan et al., 2003; Miguel-Aliaga et al., 2008; Veverytsa and Allan, 2011). Retrograde BMP signaling has also been shown to alter the

structure and function of neuromuscular junctions, in part through transcriptional regulation of a guanine exchange factor Trio in motoneurons (Ball et al., 2010). In the case of FMRFa and Dilp7 initiation, retrograde BMP signaling has been shown to act in concert with subtype-defining transcription factors that differentiate the neuronal subtype (Allan et al., 2003; Miguel-Aliaga et al., 2004; Miguel-Aliaga et al., 2008). In vertebrates, target-dependent initiation of TDGs is known to occur through a number of different target-derived signals including cytokines (Habecker et al., 1997), neurotrophins (Patel et al., 2000), and transforming growth factor-β (TGFβ) ligands, such as activins and BMPs (Ai et al., 1999). In the absence of the target-derived signal, neurons are still specified as their specific neuronal subtype, but are unable to fully differentiate and function appropriately.

Indirect evidence had suggested that retrograde signaling may be persistently required for the maintenance of the adult neuronal phenotype following differentiation. In the vertebrate adult superior cervical ganglionic neurons, terminal differentiation is dependent on retrograde BMP signaling. Upon axotomy of adult neurons, these neurons had an altered neuropeptide expression profile, an effect that was partially blocked by direct BMP administration (Pavelock et al., 2007). Neurotrophins have also been implicated in maintaining continued TDG expression through target-derived signaling. Blocking target-derived signaling through axotomy of adult sympathetic or through target ablation of cholinergic neurons in the basal forebrain alters their neurotransmitter expression profile (Sofroniew et al., 1993; Zigmond et al., 1998). These results indicate that target-derived retrograde signaling may be persistently required to maintain neuronal identity following their role in differentiation. However, many of these experiments are correlative or fail to account for changes in gene expression that are downstream of the injury response. Following the initiation of TDG expression by target-derived retrograde signaling it is not known whether persistent TDG expression is maintained by the same signal or if expression is maintained by an intrinsic mechanism.

In Chapter 2 of my thesis I directly test whether the target derived retrograde BMP signal that initiates the expression of the TDG FMRFa is persistently required to maintain the expression of FMRFa throughout the life of the neuron.

1.4 MAINTENANCE OF NEURONAL IDENTITY BY COMBINATORIAL CODES OF TRANSCRIPTION FACTORS

The selective expression of TDGs requires the combinatorial activity of sequence specific transcription factors that regulate TDG expression through the binding of TDG *cis*-regulatory sequences. These transcription factors, and the cascades in which they operate, have been identified for many lineages, but once cell identity is attained, how expression of TDGs is faithfully maintained throughout the cell's lifetime is unresolved.

Recent technical advances have allowed for the conditional knockout of transcription factors exclusively in the post-developmental period. Use of these techniques has led to a slow accumulation of evidence that the transcription factors directly required for the initiation of TDG expression are persistently required for the maintenance of TDG expression, and therefore cell identity in the mature cell. In all of these models, individual transcription factors are tested for their necessity in maintenance of TDG expression. *However, TDG expression is initiated by a network of transcription factors that act not only to directly regulate TDG expression but also the expression of other each other. There is little evidence to show how a network of differentiation transcription factors maintain cellular subtype identity.*

Work in *C. elegans* provides an elegant model that addresses how networks and feedback loops can potentially control the maintenance of cell identity. Subtype-specific batteries of TDGs in the ASE and dopaminergic neurons are initiated by sequence-specific transcription factors Che-1 and Ast-1, respectively (Etchberger et al., 2007; Uchida et al., 2003). Conditional knockouts of these transcription factors in the larva following terminal differentiation shows that these factors are then subsequently required to maintain the expression of their respective TDGs (Etchberger et al., 2009; Flames and Hobert, 2009). In the case of the ASE neuron, once Che-1 expression is initiated it maintains it's own expression by binding it's own gene's *cis*-regulatory region in an autoregulatory positive feedback loop (Etchberger et al., 2007). This provides an elegantly simple mechanism for maintaining subtype-specific transcription factor expression, and subsequently cellular identity through a self-perpetuation loop. It is worth noting however, that these experiments were all performed in the developing larva, soon after terminal differentiation and not in the fully mature worm.

In the vertebrate nervous system, midbrain dopaminergic neurons require the nuclear hormone factor, Nurr1, for developmental induction of dopaminergic specific genes including TH, discussed in section 1.2.3. Conditional ablation Nurr1 in mature midbrain dopaminergic

neurons showed that loss of Nurr1 in adult midbrain dopaminergic neurons resulted in progressive loss of TH expression and other dopaminergic specific markers (Kadkhodaei et al., 2009). The loss of TH expression appears to be the result of a loss of direct transcriptional regulation as there weren't significant levels of cell death at the time of TH downregulation, although there was a progressive loss of pan-neuronal markers. Similar results have also been found for the maintenance of TH expression, by Hand2, in terminally differentiated neonatal sympathetic neurons (Schmidt et al., 2009). In serotonergic neurons of the raphe nucleus, the ETS domain transcription factor Pet1 has also been shown to maintain expression of critical TDGs. During differentiation, Pet1 is required to activate a serotonergic gene battery of TDGs, including tryptophan hydroxylase (Tph1/2), and the serotonin transporter (Sert) (Hendricks et al., 2003). A conditional knockout of *Pet1* in adult mice resulted in a downregulation of Tph1/2, Sert and subsequently serotonin (Liu et al., 2010). Similarly Lmx1b knockouts in adult neurons led to a down regulation in Tph2, Sert, and Vmat2 that ultimately lead to a decrease in serotonin (Song et al., 2011). In all three models, midbrain dopaminergic, sympathetic, and serotonergic neurons, differentiating transcription factors have been shown to be able to bind *cis*-regulatory sequences in the enhancer regions of the TDGs they regulate for initiation (Goridis and Rohrer, 2002; Hendricks et al., 1999; Lee et al., 2010) but it is not known if such binding is required for maintenance.

In non-neuronal models, accumulating evidence in lymphatic endothelial cells (Johnson et al., 2008) as well as B-cells and long-lived plasma cells (Schebesta et al., 2007; Shapiro-Shelef et al., 2005) shows that the active maintenance of cellular identity by differentiating transcription factors is a universal mechanism. Notably, the requirement of the homeodomain transcription factor Pdx1 is persistently required for adult β-cells to regulate insulin and glut2 expression (Holland et al., 2005). Adult loss of Pdx1 leads to β-islet cell dysfunction and diabetic complications in mice. Additionally, Pdx1 and several other key pancreatic transcription factors also employ autoregulatory feedback loops to maintain their own expression, similar to the models shown in *C. elegans* (Marshak et al., 2000). Thus, the available evidence is beginning to show that mature cells retain certain transcription factors required during differentiation in order to maintain their subtype-specific gene expression profile, and hence their function, through out the life of the cell. In spite of this growing evidence, current models have mostly only addressed single transcription factors in cells. This leaves uncertain the function of the developmental transcriptional networks within which each transcription factor operates.

In Chapter 3 of my thesis I directly test whether transcription factors in the Tv cluster

neurons maintain the same developmental cross-regulatory relationships, required for differentiation, in the adult.

1.5 MAINTENANCE OF GENE EXPRESSION THROUGH MECHANISMS INDEPENDENT OF THE INITIATING TRANSCRIPTION FACTORS

During development, transcriptional profiles in mitotically dividing cells can be maintained independently of the transcription factors that initiate their expression. Such mechanisms of maintenance, known as inheritance, provide a possible alternative to our working hypothesis that genes are maintained actively by the transcription factors that initiate their expression. These mechanisms are commonly referred to as epigenetic modifications. The best understood mechanisms for this include polycomb group (PcG) and trithorax group (TrxG) proteins, chromatin acetlylation states, and CpG methylation (Jaenisch and Bird, 2003; Kouzarides, 2007; Ringrose and Paro, 2004). With regards to CpG DNA methylation, it is largely associated with the permanent silencing of transcriptional activity, such as the generation of heterochromatin (Jaenisch and Bird, 2003). It is also important to note that CpG methylation does not occur in *Drosophila*, and as such will not be addressed further. Although I do not directly address the possible role of these mechanisms for the maintenance of subtype identity in my thesis, it is important to consider this issue as it provides a possible alternative mechanism, albeit not necessarily a mutually exclusive mechanism, to an active transcription factor regulated state of maintenance.

1.5.1 HDACs and HATs

Mediation of histone acetylation states through histone acetyltransferases (HATs) and histone deactylases (HDACs) are associated with active and repressive transcriptional states, respectively (Kouzarides, 2007; Li et al., 2007). Increased histone acetylation is thought to regulate transcriptional activity by increasing histone displacement or dissociation, referred to as "opening the chromatin", on stretches of DNA in the promoter regions of genes. This opening of the chromatin structure at promoter regions allows for the increased binding and recruitment of transcriptional activators, thus promoting transcription.

Increasing evidence has shown that regulation of histone acetylation is essential for the continued regulation of TDG expression following differentiation. In *C. elegans*, ASE gustatory neurons are laterally differentiated to express groups of *gcy* chemoreceptor genes that are left and right specific. This left/right asymmetry is established through transcription factors, *che*-1 and *die*-1 that establish and maintain two separate negative feedback loops which regulate either a left or right specific expression pattern of *gcy* genes (Johnston and Hobert, 2003; Johnston et al., 2005; O'Meara et al., 2010). This results in the initiation and maintenance of left and right specific *gcy* expression profiles. It has been proposed that these sequence specific transcription factors *che*-1 and *die*-1 then recruit the MYST-type HAT, Lys-12 to the enhancer region of *gcy* genes to maintain left/right specific *gcy* expression (O'Meara et al., 2010). When *lys-12* function was knocked out in the adult worm, the ASE right specific *gcy* genes were expressed in the ASE left neurons. This suggests that maintenance of cell subtype identity is dependent on continuous maintenance of histone acetylation states, although the mechanism by which this is done is unclear. In the vertebrate dopaminergic neurons of the midbrain, the binding of the transcription factor Pitx3 to the TH enhancer decreases Nurr1 association with SMRT, a HDAC associated protein that promotes the repressive deacetylated state, at the TH *cis*-regulatory region (Jacobs et al., 2009). This presumably increases the transcriptional activity of the TH gene.

Although these examples show histone acetylation states are associated with maintenance of gene expression, in both cases there is also a persistent requirement of the sequence specific transcription factors for continued TDG expression (Kadkhodaei et al., 2009; O'Meara et al., 2010). It has been noted (Ptashne, 2007), that HDACs and HATs are not sequence specific and therefore not self-targeting to specific regions of the genome. Furthermore they have a relatively high turnover rate and are not known to be self-renewing. This suggests that histone acetylation states are merely facilitative, instead of instructive, for the maintenance of appropriate subtype gene expression profiles during development, or in mature fully differentiated cells.

1.5.2 PcG and TrxG regulation

During development Hox gene expression patterns are maintained throughout dividing cellular lineages independently of the transcription factors that initiated their expression (Akam, 1987). A Hox gene's expression domain is established in early embryonic development by a cascade of transient maternal and zygotic transcription factors including the gap and pair rule

genes (Akam, 1987). Although the expression of these transcription factors is lost soon after Hox gene expression domains are established, Hox genes expression persists in the cellular lineages of those domains throughout subsequent cell divisions. The maintenance of appropriate Hox gene expression patterns are dependent upon a group of transcriptional regulators called PcG and TrxG proteins (Schuettengruber and Cavalli, 2009).

PcG and TrxG proteins are antagonistic heterogeneous complexes of proteins that regulate Hox gene expression through regulating chromatin modifications and recruiting transcriptional regulators (Ringrose and Paro, 2007). These complexes are sequence specific and bind the closely integrated but discrete sequences in the *cis*-regulatory regions of Hox genes, called polycomb and trithorax response elements (PREs) and (TREs) (Schuettengruber et al., 2007; Tillib et al., 1999). PcG mediated silencing at *cis-regulatory* regions of Hox genes has been shown to occur through histone methylation and ubiquitylation of specific lysine residues of histones H3 and H2 respectively (Schuettengruber and Cavalli, 2009). However this repression is a dynamic and reversible process (Klymenko and Muller, 2004; Schmitt et al., 2005). TrxG proteins have been shown to promote transcriptional activation through methylation of alternate lysine groups on histone H3, leading to recruitment of chromatin remodeling complexes (Schuettengruber et al., 2011). A key feature of TrxG proteins is that they are not required for the initiation of gene expression, rather only to maintain it. Although they have been shown to mediate maintenance of transcriptional expression through development it is not known whether this regulation is required for cells to maintain their identity when fully differentiated.

1.6 THESIS OBJECTIVES

Following neurogenesis, subtype-specific gene expression profiles that persist throughout the life of the cell can be maintained by either 1) the same transcription factors and signaling pathways that initiated their expression or 2) mechanisms independent of those initiating factors. There is increasing evidence that the subtype transcription factor networks that direct differentiation during development are subsequently required for long-term maintenance of identity. Using the well-defined *Drosophila* neuronal model of the Tv cluster neurons I will directly test whether: 1) the persistent expression of the Tv4 specific terminal differentiation gene FMRFa is maintained retrograde BMP signaling; 2) Terminal differentiation genes Nplp1 in the Tv1 neuron and FMRFa in the Tv4 neuron are maintained by the same subtype specific transcription factor

network required for their initiation; 3) the cross regulatory interaction of the subtype specific transcription factor network in Tv1 and Tv4 remains the same in the mature neurons following differentiation. This study will provide novel insight into the maintenance of subtype identity as an active process underpinned by combinatorially-acting developmental transcriptional factors and signaling pathways. These findings will have broad implications for understanding the maintenance of all long-lived cell types.

2. Neuronal phenotype in the mature nervous system is maintained by persistent retrograde bone morphogenetic protein signaling.

2.1 SYNOPSIS

The terminal differentiation of many developing neurons occurs after they innervate their target cells, and is triggered by secreted target-derived signals that are transduced by presynaptic receptors. Such retrograde signaling induces the expression of genes that are often distinctive markers of neuronal phenotype and function. However, whether long-term maintenance of neuronal phenotype requires persistent retrograde signaling remains poorly understood. Previously, we found that retrograde BMP signaling induces expression of a phenotypic marker of *Drosophila* Tv neurons, the neuropeptide FMRFa. Here, we utilized a genetic technique that enables precise spatiotemporal targeting of transgene expression in *Drosophila* to test the role of persistent BMP signaling in the maintenance of Tv phenotype. We show that expression of dominant blockers of BMP signaling selectively in adult Tv neurons dramatically downregulated FMRFa expression. Moreover, adult-onset expression of mutant Glued, which blocks dynein/dynactin-mediated retrograde axonal transport, eliminated retrograde BMP signaling and dramatically downregulated FMRFa expression. Finally, we found that BMP deprivation did not affect Tv neuron survival and that FMRFa expression fully recovered to control levels after BMP blockade or Glued expression was terminated. Our results show that persistent retrograde BMP signaling is required to induce and to subsequently maintain the expression of a stably-expressed phenotypic marker in a subset of mature *Drosophila* neurons. We postulate that retrograde maintenance of neuronal phenotype is conserved in vertebrates, and as a consequence, neuronal phenotype is likely vulnerable to neurodegenerative disease pathologies that disrupt neuronal connectivity or axonal transport.

2.2 INTRODUCTION

Maintenance of neuronal phenotype is critical to nervous system function. However, our understanding of the mechanisms that maintain the differentiated state of neurons is rudimentary. Terminal differentiation of many neurons requires retrograde signals from the target cells that they innervate, including bone morphogenetic proteins (BMPs), activins, cytokines, and neurotrophins (Ernsberger and Rohrer, 1999; Hippenmeyer et al., 2004; Nishi, 2003). Target-induced genes are often stably-expressed, distinguishing phenotypic markers with critical roles in synaptic transmission, including neuropeptides (Allan et al., 2003; Coulombe and Kos, 1997; Duong et al., 2002; Patel et al., 2000), neurotransmitter biosynthetic enzymes (Ernsberger and Rohrer, 1999; Stanke et al., 2006), and ion channels (Martin-Caraballo and Dryer, 2002). Does the maintenance of such target-induced genes require persistent retrograde signaling in the adult nervous system?

Members of the TGFβ superfamily, including BMPs and activins, are the only known conserved mediators of retrograde neuronal differentiation from *Drosophila* to vertebrates (Hippenmeyer et al., 2004; Nishi, 2003; Xu and Hall, 2006). In vertebrates, target-derived activin induces the neuropeptide calcitonin gene related peptide (CGRP) in cutaneous afferents (Ai et al., 1999). Activin retrogradely induces the neuropeptide somatostatin in choroid neurons (Darland and Nishi, 1998). BMP4 acts retrogradely to determine transcription factor expression in trigeminal neurons (Hodge et al., 2007). In *Drosophila*, target-derived BMP induces expression of neuropeptides, FMRFa in Tv neurons (Allan et al., 2003), and dILP7 in subsets of MP2 neurons (Miguel-Aliaga et al., 2008).

In the adult vertebrate nervous system, activin and BMP signaling modulate dendritic spine morphology and synaptic efficacy (Shoji-Kasai et al., 2007; Sun et al., 2007). Moreover, activin is upregulated in the skin after injury, and acts retrogradely to increase the number of cutaneous afferents that express CGRP (Xu et al., 2005). However, only indirect evidence supports a role for retrograde TGFβ superfamily signaling in maintenance of adult neuronal phenotype. Axotomy of adult superior cervical ganglion neurons (SCG) alters their neuropeptide expression profile, an effect that is partially blocked by BMP administration (Pavelock et al., 2007). Intriguingly, BMP ligands are expressed at those neurons' target tissues (Victor May, personal communication). Similarly, systemic statins reduce CGRP expression in adult cutaneous afferents *in vivo*, likely by inhibition of BMP signaling (Bucelli et al., 2008). These

results led the authors of both studies to propose that retrograde BMP signaling is required to maintain normal neuropeptide levels.

Here, we directly tested the hypothesis that persistent retrograde BMP signaling maintains adult neuronal phenotype. Previously, we demonstrated that induction of the neuropeptide FMRFa in *Drosophila* Tv neurons requires target-derived BMP signaling (Allan et al., 2003). Here, by targeting transgenic dominant blockers of BMP signaling selectively in adult Tv neurons, after their normal development, we show that maintenance of FMRFa is absolutely dependent upon persistent BMP signaling. Moreover, adult-onset blockade of retrograde axonal transport inhibited nuclear BMP signaling and eliminated FMRFa expression. These data demonstrate that persistent retrograde BMP signaling is required to induce and subsequently maintain the mature phenotype of subsets of *Drosophila* neurons.

2.3 MATERIALS AND METHODS

2.3.1 Fly genetics

Flies were maintained on standard cornmeal food and maintained at stable temperatures in environment rooms set at 70% humidity at 18^0C, 25^0C or 29^0C. The following fly strains were used: *FMRFa-lacZ* (WF3-T2) (Allan et al., 2003); *apterous*^{md544} (referred to as *ap*^{GAL4} here (Allan et al., 2003)); *tubP>GAL80*^{TS} (temperature sensitive GAL80 under the control of the *Drosophila* tubulin 84B promoter (McGuire et al., 2003)); *UAS-nEGFP* (nuclear localized EGFP); *UAS-wit*$^{\Delta I}$ (intracellular domain deletion (McCabe et al., 2003), referred to as *UAS-wit*^{DN}*; UAS-tkv*$^{\Delta GSK}$ (GS-box and kinase domain deletion (Haerry et al., 1998), referred to here as *UAS-tkv*^{DN}); *UAS-Mad*^{1} (*Mad*^{1} mutant that cannot bind DNA (Takaesu et al., 2005), referred to here as *UAS-Mad*^{DN}); *UAS-Glued*$^{\Delta 84}$ (referred to here as *UAS-Glued*^{DN}; (Allen et al., 1999)); *UAS-ANF-GFP* (prepro-atrial natruietic factor fused to emerald GFP (Rao et al., 2001)). The X chromosome insertion (Rao et al., 2001) was hopped (DWA) to chromosome III using standard methods of P-element mobilization. Flies were generously provided by Paul Taghert, Thomas Schwarz, Justin Kumar, Stuart Newfeld, Graeme Davis and the Bloomington *Drosophila* Stock Centre (Indiana).

2.3.2 Spatial and temporal regulation of transgene expression using the TARGET system.

Flies for TARGET-mediated transgene induction (**Fig. 3-7)** were generated by crossing *FMRFa-lacZ,ap^{GAL4}/CyO, actin-GFP ; tub-GAL80^{TS},UAS-nEGFP* (**Fig. 3**) or *ap^{GAL4}/CyO, actin-GFP ; tub-GAL80^{TS},UAS-nEGFP* (**Fig. 4-7**) to *w^{1118}* for the control group or *UAS-tkv^{DN};UAS-wit^{DN}* (**Fig. 3-6**), or *UAS-Mad^{DN} ;UAS-Mad^{DN}* (**Fig. 4-6**), or *UAS-Glued^{DN}* (**Fig. 7**). Experiments were performed on resulting progeny bearing the appropriate genotypes (screened by loss of the *Cyo, actin-GFP* chromosome), as detailed in Results section. All experimental and control flies were raised at 18^{0}C to suppress GAL4/UAS-mediated transgene expression until after eclosion (hatching from the pupal case). Adult flies were collected every 24hrs to ensure accuracy of age for experimental conditions. Non-induced flies were subsequently maintained at 18^{0}C for continued suppression of transgene expression for the duration of the experiment. Induced flies were switched to 29^{0}C for the entire duration of the induction period indicated, for continuous transgene expression.

2.3.3 Antibodies

Primary antibodies: sheep anti-digoxygenin (1:1500; Roche); mouse anti-Eya (1:100; clone 10H6; Developmental Studies Hybridoma Bank); rabbit anti-GFP (1:100; A6455 Invitrogen); rabbit α-FMRFa (1:1000; T-4757 Peninsula Labs); rabbit anti-pMad (1:1000; a generous gift from C-H Heldin); mouse anti-β-Galactosidase (1:100; 40-1a). Secondary antibodies: donkey anti-sheep Alexa 555 (1:10; Invitrogen); donkey anti-mouse Cy5 and donkey anti-rabbit Cy2 (1:200; Jackson Immunoresearch).

2.3.4 Antisense DIG-RNA Probe

DIG-Uracil tagged RNA probes were generated using T3 RNA polymerase from clone RH03963 (DGRC: *Drosophila* Genomic Resource Centre, Indiana) containing a 1584bp FMRFa cDNA (using the Roche DIG-U-RNA Labelling Kit). Probe synthesis was confirmed using gel electrophoresis.

2.3.5 Multiplex Fluorescent in situ hybridization (FISH) and immunohistochemistry

All tissues that were compared for fluorescence intensity were processed at the same time using the same aliquots of all solutions under the same conditions. They were then mounted on the same slide. Adult ventral nerve cords were dissected in ice cold PBS (phosphate buffered saline), then incubated for 50 mins in ice cold 4% paraformaldehyde (PFA) in PBTw (PBS; 0.1% Tween-20; 0.1% Diethyl polycarbonate (DEPC) treated ddH_2O). Samples were then PBTw washed and stored overnight in 100% methanol at -20^{0}C. Samples were rehydrated and placed in 4% PFA-PBTw for 20 mins. Samples were washed in PBTw and incubated in HYBE solution at 55^{o}C for 1 hour. Hybridization with Digoxigenin (DIG)-tagged antisense RNA probes to FMRFa was performed at 55^{0}C overnight on a rotating platform in a Bambino Hybridization Oven. Samples were PBTw washed and incubated for 1hr in PBTw with 5% donkey serum (PBTw-DS). Tissues were incubated overnight at 4^{0}C in PBTw-DS containing primary antibodies. Tissues were washed (PBTw) and blocked (PBTw-DS), then incubated in secondary antibody (in PBTw-DS) at room temperature for 3hrs. Tissues were washed in PBTw, then PBS, and slide-mounted in Vectashield (Vector).

2.3.6 Immunohistochemistry

Ventral nerve cords were dissected in ice cold PBS and fixed in ice cold 4% PFA in 0.1% PBT (PBS - 0.1% Triton X-100) for 40 minutes, washed in PBT and incubated in PBT with 5% donkey serum (PBT-DS). Primary antibodies were incubated overnight at 4^{o}C and secondary antibodies were incubated at 3hrs at room temperature. Antibodies were incubated in PBT-DS. Tissues were washed in PBT then PBS and mounted in Vectashield (Vector).

2.3.7 Image analysis

All images were acquired on an Olympus FV1000 confocal microscope as multiple TIFF files representing individual Z-stacks. For each data set, we quantified every identifiable Th1 and Th3 Tv neuron from each control and experimental animal. Raw files were imported into Image J (US National Institutes of Health) for analysis. For each Tv neuron, we compressed all Z-slices

spanning whole Tv neurons using the Z-projector function, set to sum the pixel intensities from each Z-slice. Each Tv neuron was outlined and the mean of the summed pixel intensity for each neuron was measured. Background fluorescence intensity was corrected for by subtracting the average of three regions of background (of equal size to the Tv neuron) from the same summed Z-stack for each Tv neuron. The resulting value for each Tv neuron was then incorporated as a single datum point towards the mean FMRFa intensity for each experiment (shown in 2S.1-3). To normalize data across multiple timepoints and genotypes, we further expressed each datum point as a percentage of the mean of the w^{1118} control for that experiment (Percentage intensity measurements are provided in Table 2S.1-3). For every experiment, we also collected intensity measurements for an additional pair of FMRFa-expressing neurons in Th3 in every nerve cord, using the same settings as for FMRFa in the Tv neurons. These neurons do not show expression for ap^{GAL4};*UAS-nEGFP,* and were therefore not subjected to transgene expression. We tabulated the expression of FMRFa in these neurons as Internal Controls in Tables 2S.1-3. These data show that there was no significant difference in the expression of FMRFa in these neurons between different genotypes, irrespective of GAL4 induction. For images shown in Figs 1-7, we chose representative images from Tv neurons that were stacked to show the entire *ap*-cluster. Images that were directly compared were further processed in an identical way, simultaneously, using Adobe Photoshop CS2.

2.3.8 Statistical analysis

Normally distributed unpaired experimental groups data were compared using a two-tailed T-test assuming equal variance, to identify significant differences between groups. For data groups that are not normally distributed, we used the non-parametric Mann-Whitney test. Normal distribution was determined using the D'Agostino and Pearson omnibus test. All statistical analysis and graphs data were performed using Prism 5 software (Graphpad)

2.4 RESULTS

2.4.1 Initiation of FMRFa expression in the Tv4 neuron

The *Drosophila* nervous system contains six Tv neurons, one in each of the six thoracic hemisegments (Th1-3) of the ventral nerve cord (VNC) (**Fig. 2.1A**). Prior to metamorphosis, Tv4 axons project to the ipsi-segmental dorsal neurohaemal organ (DNH), which protrudes dorsally from each thoracic VNC segment (Allan et al., 2003; Benveniste et al., 1998). In adults, after DNH retraction into the VNC, Tv4 axons form neurohaemal contacts along the dorsal VNC neural sheath (Brown et al., 2006; Lundquist and Nassel, 1990). Developing and adult Tv4 neurons are phenotypically characterized by expression of the neuropeptide FMRFa (O'Brien et al., 1991; Schneider et al., 1991), which they secrete into the haemolymph at neurohaemal endings (Predel et al., 2004; Wegener et al., 2006). In each thoracic hemisegment, the Tv4 neuron is one of a cluster of four neurons that co-express the transcription factors, *apterous* and *eyes absent*, termed the Tv cluster (Benveniste et al., 1998; Miguel-Aliaga et al., 2004). FMRFa expression in Tv4 neurons is determined by a specific set of transcriptional regulators and target (DNH)-derived BMP signaling (Allan et al., 2005; Allan et al., 2003; Miguel-Aliaga et al., 2004). Tv4 axons access the BMP ligand, Glass bottom boat (Gbb), at the DNH. Gbb engages the Type II BMP receptor Wishful thinking (Wit) and the Type I BMP receptors, Thickveins (Tkv) and Saxophone (Sax), resulting in Type I receptor-mediated Mad phosphorylation (pMad) in Tv4 neurons (Allan et al., 2003; Marques et al., 2003). Retrograde trafficking of the BMP signal, by an unknown mechanism, translocates pMad to the Tv4 neuron nucleus, to induce FMRFa expression (Allan et al., 2003).

2.4.2 BMP signaling persists in mature Tv4 neurons.

In order to test whether BMP signaling is maintained in Tv4 neurons, we examined pMad immunoreactivity in the nuclei of larval and adult Tv4 neurons (Allan et al., 2003). Tv4 neurons were identified by co-expression of FMRFa (*FMRFa-lacZ* reporter) and *apterous* (*ap^{GAL4}; UAS-nEGFP*) (Allan et al., 2003). We observed strong immunoreactivity to pMad in Tv4 nuclei at all ages tested up to adult day 20 (Fig. 2.1B-F). These data indicate that a BMP signal is transduced and trafficked into Tv4 neuronal nuclei through the life of the fly.

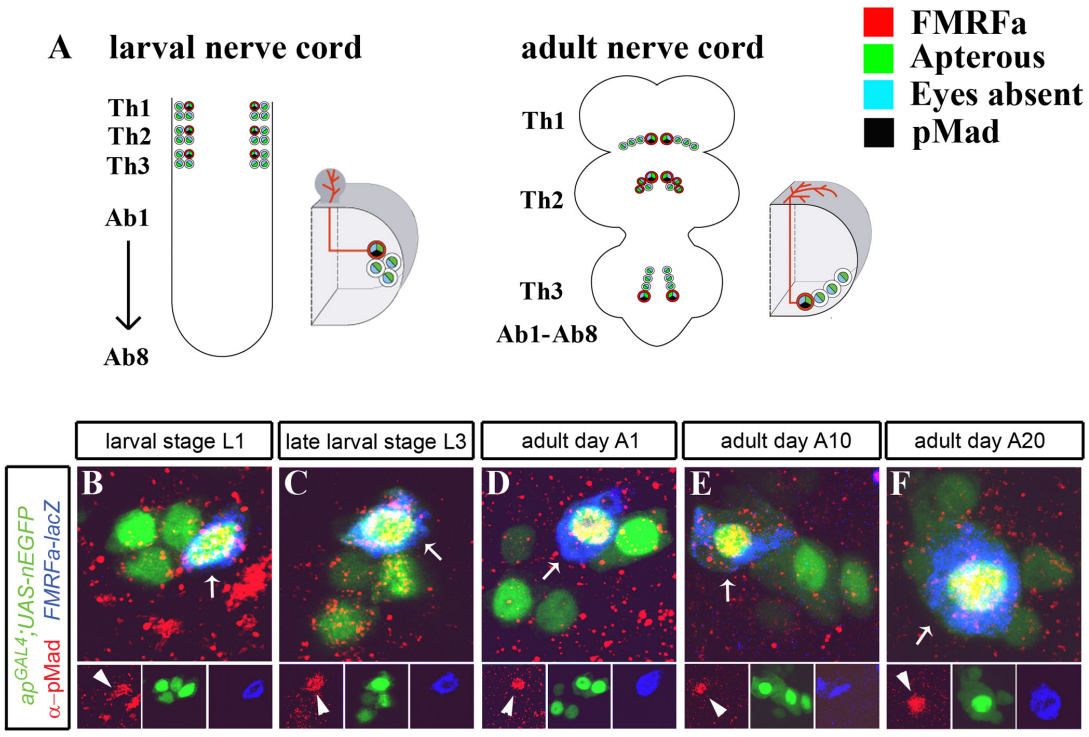

Figure 2.1 BMP signaling and FMRFa expression persists in Tv4 neurons throughout larval and adult stages

(a) Cartoons of the larval and adult ventral nerve cord, shown in their full antero-posterior extent from thoracic segment Th1 to abdominal segment Ab8, and also in a transverse section through a thoracic hemisegment to show the relative position of each Tv-cluster and the innervation of the dorsal neurohaemal organ by Tv4 axons. These cartoons show the six thoracic Tv-neuron clusters that co-express the transcription factors *apterous* (green) and *eyes absent* (blue) within Th1 to Th3. Each *Tv-cluster* comprises Tv4, Tv1, and Tv2/3 neurons. In larvae, only the Tv4 neuron expresses FMRFa (red) and nuclear pMad (black), and innervates the dorsal neurohaemal organ (DNH). In adult Th1 and Th3 segments, only the Tv4 neuron expresses FMRFa and nuclear pMad. In adult Th2, FMRFa is expressed in Tv4, and Tv2/3, but only the Tv4 neuron has nuclear pMad. There are no Tv-clusters in abdominal segments, Ab1 to Ab8.
(b-f) The Tv4 neuron (arrow) is identified by co-expression of *apterous* (green; ap^{GAL4}*;UAS-nEGFP*) and FMRFa (blue; *FMRFa-lacZ*; anti-β-Gal) in Tv-clusters. Nuclear accumulation of pMad (red; arrowhead; anti-pMad) in the Tv4 neuron is evident in **(b)** larval stage L1, **(c)** late larval stage L3, **(d)** adult day A1, **(e)** A10, and **(f)** A20 (the oldest age examined). Flies were maintained at 25°C.

2.4.3 Transgenic blockade of BMP signaling in Tv4 neurons

We tested the efficacy of numerous transgenes to ablate BMP signaling in Tv4 neurons. In control L1 larvae (*ap^{GAL4}/+; UAS-nEGFP/+*), FMRFa was expressed by 5.7±0.1 Tv4 neurons (n=29 Tv4 neurons), and nuclear pMad was observed in 6.0±0.0 Tv4 neurons (n=44) (**Fig. 2.2A,B**). Using *ap^{GAL4}* to express transgenic blockers of BMP signaling in the Tv cluster, we found that co-expression of dominant negative versions *thickveins* (*UAS-tkv^{DN}*) (Haerry et al., 1998) and *wishful thinking* (*UAS-wit^{DN}*) (McCabe et al., 2003) resulted in 100% loss of both pMad immunoreactivity (n=34) and FMRFa expression (n=10; $p=3.2x10^{-28}$) in L1 Tv4 neurons (**Fig. 2.2A,C**). Expression of two copies of dominant-negative *Mad* (*UAS-Mad^{DN}*) (Takaesu et al., 2005) resulted in 100% loss of FMRFa expression in Tv4 neurons (n=10; $p=3.2x10^{-28}$), whereas pMad immunoreactivity was observed in 100% of Tv4 neurons (n=30 Tv4 neurons) (**Fig. 2.2A,D**). Mad^{DN} encodes a protein in which DNA-binding is disrupted. Thus, our data show that BMP signaling upstream of pMad DNA-binding had been maintained, but that DNA binding of pMad is required for FMRFa expression. FMRFa was only partially downregulated by expression of either *UAS-tkv^{DN}* or *UAS-wit^{DN}* alone (**Fig. 2.2A**). In summary, transgenic overexpression of *Mad^{DN}* or *tkv^{DN}/wit^{DN}* in the Tv cluster prevented the induction of FMRFa expression in developing Tv4 neurons.

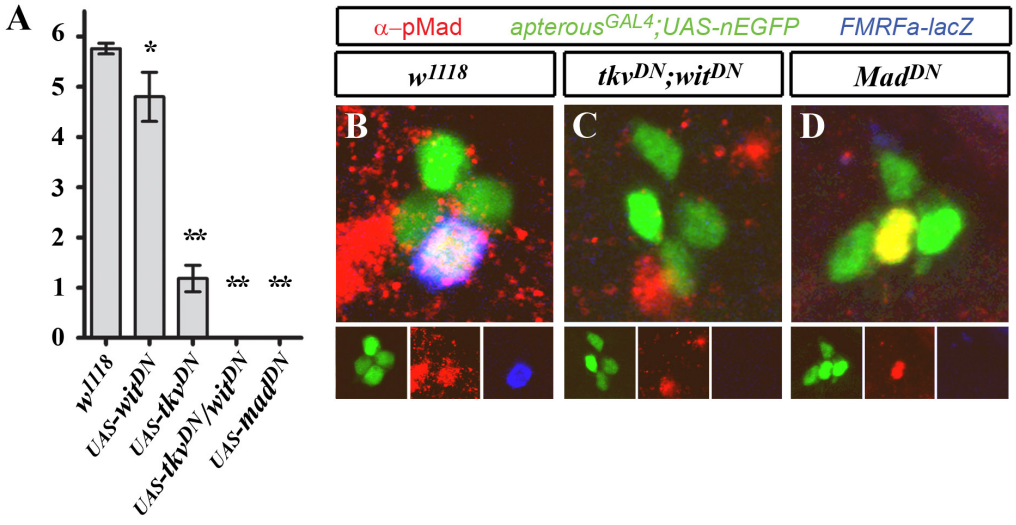

Figure 2.2 Larval expression of dominant negative-Mad and -BMP receptors blocked FMRFa expression in Tv4 neurons.

(a) Quantification of Tv4 neurons that express *FMRFa-lacZ* (anti-βGal) in L1 larvae. There are six Tv4 neurons per nerve cord. In w^{1118} control larvae (*FMRFa-lacZ,apterous*^{GAL4}/+), FMRFa expression was seen in all Tv4 neurons. We used *apterous*^{GAL4} to express dominant-negative BMP pathway transgenes in *ap*-neurons, and counted the number of Tv4 neurons in which FMRFa-lacZ was expressed. Expression of dominant negative BMP Type II receptor, *wishful thinking* (*UAS-wit*^{DN}) caused a small, significant reduction in FMRFa expression (* P<.01). Dominant negative BMP Type I receptor *thickveins* (*UAS-tkv*^{DN}) caused a great reduction in FMRFa expression. FMRFa expression was 100% eliminated by co-expression of *UAS-tkv*^{DN} and *UAS-wit*^{DN}, or by expression of two copies of a dominant-negative version of the BMP-dependent transcription factor, Mad (*UAS-Mad*^{DN}*;UAS-Mad*^{DN}). ** P<.0001.
(b-d) *ap*-neurons were identified by *apterous*^{GAL4}-mediated expression of *UAS-nEGFP* (green). *FMRFa-lacZ* expression was determined using anti-βGal (blue). Immunoreactivity to pMad (red) showed its accumulation in Tv4 neuron nuclei (arrowhead); indicative of active BMP signaling. **(b)** In w^{1118} controls, nuclear pMad accumulation was always observed in *FMRFa*-expressing Tv4 neurons. **(c)** Co-expression of dominant-negative BMP receptors *UAS-tkv*^{DN}*;UAS-wit*^{DN} resulted in 100% loss of *FMRFa-lacZ* and nuclear pMad in the Tv-cluster. There was no loss of *Tv-cluster* neurons. **(d)** Expression of *UAS-Mad*^{DN} resulted in 100% loss of *FMRFa-lacZ* expression, but nuclear pMad was unaffected, indicating that BMP pathway activity was unaffected upstream of nuclear pMad accumulation. See text for details.

2.4.4 Temporal regulation of BMP signaling in adult Tv4 neurons.

The TARGET system (McGuire et al., 2003) enables temperature-dependent switching of GAL4/UAS-mediated transgene expression, by use of a temperature-sensitive allele of the yeast GAL4-repressor, GAL80 ($GAL80^{TS}$) (see Fig. 2.3A,D for details). We generated the following genotype to co-regulate tkv^{DN} and wit^{DN} expression in adult Tv4 neurons *in vivo* using the TARGET system; *FMRFa-lacZ,* ap^{GAL4}*/UAS-*tkv^{DN}*; UAS-nEGFP, tub>*$GAL80^{TS}$*/UAS-*wit^{DN}. The w^{1118} control genotype was ap^{GAL4}*/+ ;UAS-nEGFP, tub>*$GAL80^{TS}$*/+*.

The efficacy of adult-onset BMP signaling blockade in Tv4 neurons was examined by pMad nuclear immunoreactivity and FMRFa-lacZ reporter expression in w^{1118} controls (Fig. 2.3B,E,G) and tkv^{DN};wit^{DN} flies (Fig. 2.3C,F,H). Flies were raised at 18°C until adult day A1 (first day post-eclosion). At that time, ap^{GAL4}-mediated GAL4/UAS activity was repressed by $GAL80^{TS}$, as shown by lack of nEGFP expression (Fig. 2.3B,C); FMRFa-lacZ and nuclear pMad expression was observed in 100% of Tv4 neurons in w^{1118} control (n=15 Tv4 neurons) and tkv^{DN};wit^{DN} flies (n=18 Tv4 neurons) (Fig. 2.3B,C).

At A1, we switched flies to 29°C to induce GAL4 activity. After both 12 and 48 hours at 29°C, we observed robust nEGFP expression in both genotypes (Fig. 2.3E-G). In w^{1118} controls, FMRFa-lacZ and nuclear pMad expression was observed in 100% of Tv4 neurons after 12 and 48hrs at 29°C (12hrs; n=16 Tv4 neurons. 48hrs; n=24 Tv4 neurons Fig. 2.3E,G). In contrast, in tkv^{DN};wit^{DN} flies, we observed 100% loss of pMad nuclear immunoreactivity at 12hrs (n=16 Tv4 neurons; Fig. 2.3F), and 48hrs (n=17 Tv4 neurons; Fig. 2.3H). In tkv^{DN};wit^{DN} flies, FMRFa-lacZ was maintained at 12 hours (Fig. 2.3F), but was profoundly reduced in 100% of Tv4 neurons by 48 hours (Fig. 2.3H).

In summary, the TARGET system provides robust temporal regulation of transgene expression in Tv4 neurons. We found that adult-onset blockade of BMP signaling ablated nuclear pMad immunoreactivity within 12 hours in Tv4 neurons, and greatly reduced an FMRFa reporter in Tv4 neurons within 48hrs.

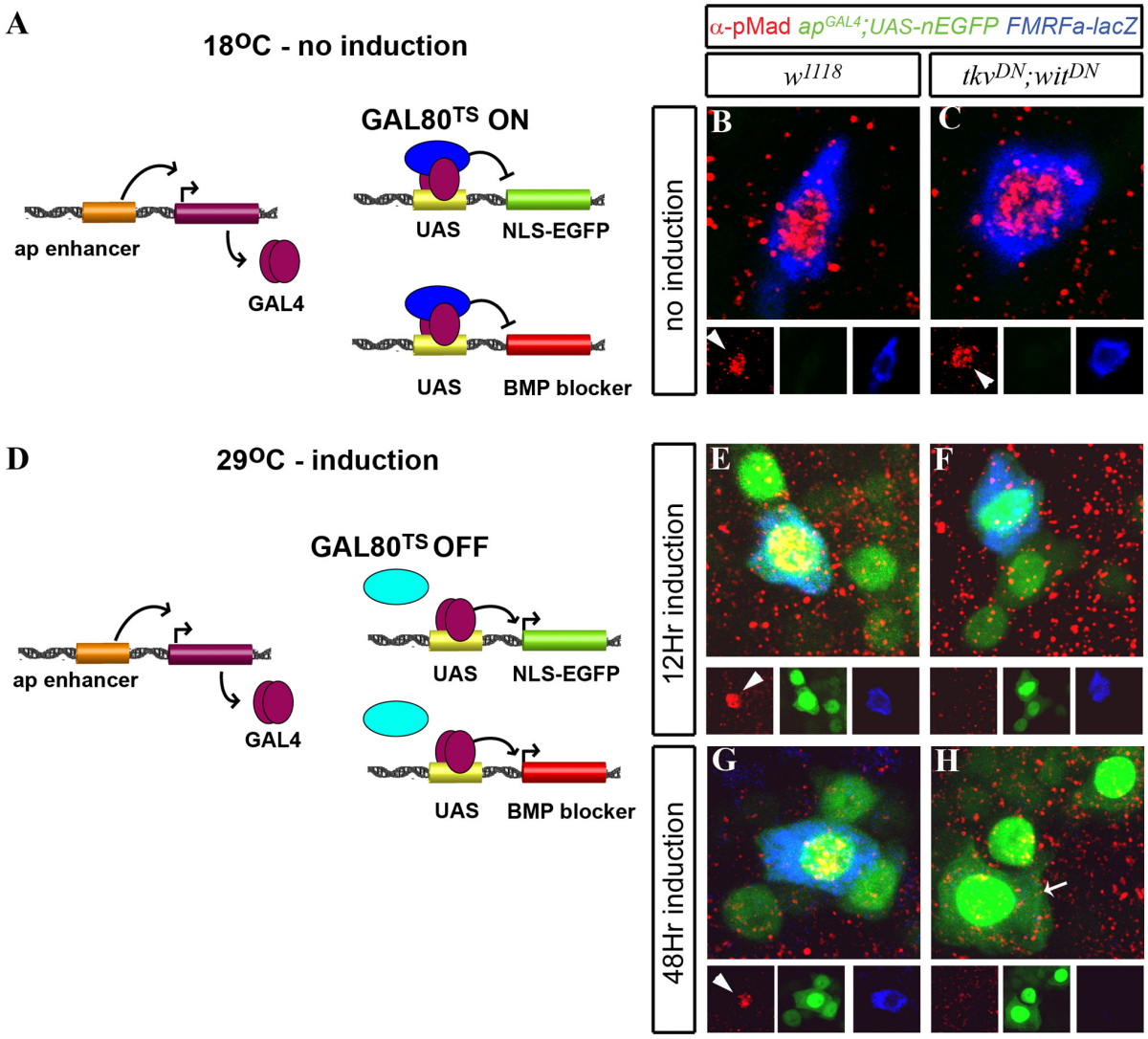

Figure 2.3 Adult induction of BMP blockade in Tv4 neurons eliminated nuclear pMad accumulation and FMRFa reporter expression.

We generated flies of genotype *FMRFa-lacZ, ap^{GAL4}/CyO; tub-GAL80^{TS},UAS-nEGFP*. These were crossed to either control *w^{1118}* flies **(b,e,g)** or *UAS-tkv^{DN},UAS-wit^{DN}* flies **(c,f,h)** to generate progeny that were raised at 18°C. **(a,d)** Schematic of the TARGET system for regulated spatiotemporal expression of transgenes in *ap*-neurons. GAL4 was expressed in *ap*-neurons only under control of the genomic *apterous* enhancer. GAL80^{TS} was expressed ubiquitously by the *tubulin* promoter. **(a)** At 18°C, GAL4 activity is not induced; GAL80^{TS} binds GAL4 and blocks GAL4-mediated gene transactivation from UAS sequences. **(d)** At 29°C, GAL4 activity is

induced; loss of GAL80^{TS} binding to GAL4 allows GAL4 to transactivate gene expression from UAS sequences, in this case in *ap*-neurons.

(b,c) No induction: In adult day A1 animals raised at 18^{o}C, GAL80^{TS} blocked *ap^{GAL4}*-mediated induction of *UAS-nEGFP* (green) and *UAS-tkv^{DN}/UAS-wit^{DN}* (in c). Note lack of nEGFP expression in both genotypes. In *w^{1118}* control flies **(b)** and *UAS-tkv^{DN};UAS-wit^{DN}* flies **(c)**, there was no difference in the expression of *FMRFa-lacZ* (blue; anti-βGal) or nuclear pMad (red; anti-pMad; arrowhead) in Tv4 neurons. See text for details.

(e,f) After 12 hours induction at 29^{o}C, nEGFP expression was activated (green) in both genotypes. **(e)** *FMRFa-lacZ* and nuclear pMad (arrowhead) expression was maintained in Tv4 neurons in *w^{1118}* control flies. **(f)** Nuclear accumulation of pMad was completely lost in *UAS-tkv^{DN};UAS-wit^{DN}* flies. FMRFa-lacZ was unaffected by 12 hours induction. These data show that the BMP pathway had been blocked within 12 hours of GAL4 induction.

(g,h) After 48 hours induction at 29^{o}C, BMP pathway blockade in *UAS-tkv^{DN};UAS-wit^{DN}* flies **(h)** resulted in a loss of pMad nuclear accumulation and a profound downregulation of *FMRFa-lacZ* in Tv4 neurons (arrow). In *w^{1118}* control flies **(g)**, nuclear pMad (arrowhead) and *FMRFa-lacZ* expression were unaffected.

2.4.5 BMP blockade in adults dramatically reduced FMRFa transcript and peptide.

Using the TARGET system, we tested the effect of adult-onset BMP blockade in Tv4 neurons on levels of FMRFa transcript (fluorescent *in situ* hybridization, FISH; Fig. 2.4) and FMRFa peptide (anti-FMRFa immunofluorescence; Fig. 2.5). To quantify FMRFa expression, we measured the relative pixel intensity of FISH or immunofluorescence in Tv4 soma in all genotypes for each experiment (tabulated in Tables 2S.1,2). Data acquisition and quantification described in Methods. In order to standardize our results across multiple timepoints, here we have expressed FMRFa fluorescence per Tv4 neuron as a percentage of the mean of the w^{1118} control for each experiment.

We examined FMRFa expression in non-induced flies at 18°C at 10, 20 and 30 days after A1. In non-induced flies, GAL4 activity was blocked at all timepoints, as shown by lack of nEGFP expression (Fig. 2.4A-C, 2.5A-C). Strong FMRFa FISH (Figs. 2.4A,B,C) and peptide immunoreactivity (Figs. 2.5A,B,C) was observed in non-induced flies of all genotypes, at all timepoints. Quantification of FISH (Fig 2.4D) and peptide immunoreactivity (Fig 2.5D) showed that there was no significant difference in FMRFa levels between genotypes at each timepoint. Thus genotype alone, in the absence of GAL4 induction, had no effect on FMRFa expression.

We next examined FMRFa FISH (Fig. 2.4E-H) and peptide immunoreactivity (Fig. 2.5E-H) in w^{1118} controls, tkv^{DN};wit^{DN} flies and Mad^{DN} flies that had been switched to 29°C at A1, and then maintained at 29°C for 5, 10 and 15 days. These ages are comparable to non-induced flies at 10, 20 and 30 days; flies maintained at 18°C age at about half the rate of flies at 29°C. Flies switched to 29°C expressed nEGFP in the Tv cluster (Fig. 2.4E-G, 2.5E-G). After 15 days at 29°C, w^{1118} controls had high level FMRFa FISH (100±6.4%; n=30; Fig.4E) and peptide immunofluorescence (100±8.8%; n=45; Fig. 2.5E). In contrast, expression of tkv^{DN};wit^{DN} for 15 days at 29°C ablated FMRFa transcript to 3.2±0.5% of w^{1118} controls (n=18 Tv4 neurons; P= 2.5×10^{-15}; Fig. 2.4F), and ablated FMRFa immunofluorescence to 8.2±2.7% of w^{1118} controls (n=36 Tv4 neurons; P= 6.4×10^{-14}; Fig. 2.5F). Similarly, expression of Mad^{DN} for 15 days at 29°C greatly reduced FMRFa transcript to 20.1±1.6% of w^{1118} controls (n=32 Tv4 neurons; P = 2.5×10^{-18}; Fig. 2.4G) and reduced FMRFa immunofluorescence to 37.9±4.6% of w^{1118} controls (n=32 Tv4 neurons; P=3.6×10^{-7}; Fig. 2.5G).

We further quantified FMRFa transcript and peptide levels in w^{1118} control, tkv^{DN};wit^{DN} and Mad^{DN} flies after 5 and 10 days at 29°C (Fig. 2.4H, 2.5H; Tables 2S.1,2). We observed a dramatic decline of transcript levels after induction of BMP blockade at A1. In tkv^{DN};wit^{DN} flies,

FMRFa transcript levels fell 3.5-fold by Day 5, 7.4 fold by Day 10, and 30-fold by Day 15, when compared to w^{1118} controls (Fig. 2.4H). Further, FMRFa peptide levels fell 3-fold by Day 5, 5.4-fold by Day 10, and 12-fold by Day 15, when compared to w^{1118} controls (Fig. 2.5H). In Mad^{DN} flies, FMRFa transcript levels fell 2.2-fold by Day 5, 4.1-fold by Day 10, and 5-fold by Day 15, when compared to w^{1118} controls (Fig. 2.4H). Further, FMRFa peptide levels fell 2.1-fold by Day 5, 2.2-fold by Day 10, and 2.6-fold by Day 15, when compared to w^{1118} controls (Fig. 2.5H).

We confirmed that loss of BMP signaling in adult Tv4 neurons did not cause gross retraction of Tv4 axons from their terminal field. We expressed a transgenic marker of dense-core vesicles, atrial natruietic factor (*UAS-ANF-GFP*) (Rao et al., 2001) in the Tv cluster (using ap^{GAL4}) in w^{1118} control, tkv^{DN};wit^{DN} and Mad^{DN} flies. After 15 days of induction at 29°C, we observed normal distribution of Tv4 axons in all genotypes (Fig 2S.1), showing that Tv4 axons had not retracted.

In summary, we show that maintenance of FMRFa expression in adult Tv4 neurons requires persistent BMP signaling. Interestingly, the decline of FMRFa peptide closely parallels that of FMRFa transcript. The simplest explanation is that FMRFa peptide is rapidly translated and trafficked to Tv4 axon termini for secretion into the haemolymph, and that a high level of FMRFa transcription is required to maintain that.

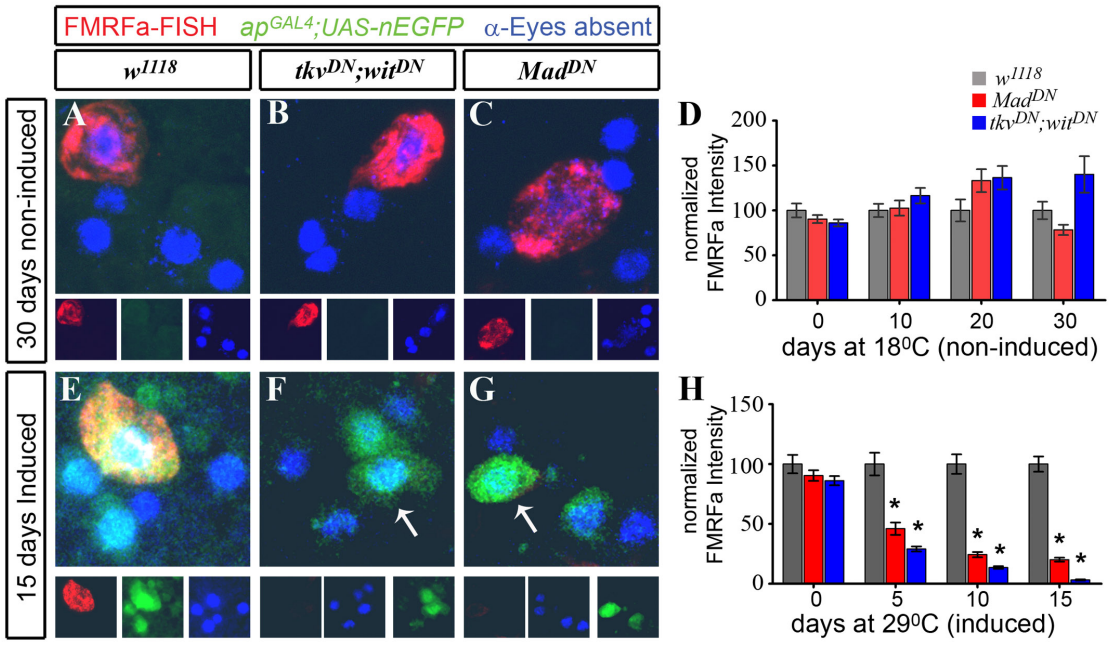

Figure 2.4 Acute blockade of the BMP pathway in adults dramatically reduced FMRFa transcript.

Genotypes: w^{1118} control flies (**a,e**; ap^{GAL4}/+; $tub\text{-}GAL80^{TS}$,$UAS\text{-}nEGFP$/+); tkv^{DN};wit^{DN} flies (**b,f**; ap^{GAL4}/$UAS\text{-}tkv^{DN}$; $tub\text{-}GAL80^{TS}$, $UAS\text{-}nEGFP$/$UAS\text{-}wit^{DN}$); Mad^{DN} flies (**c,g**; ap^{GAL4}/$UAS\text{-}Mad^{DN}$; $tub\text{-}GAL80^{TS}$, $UAS\text{-}nEGFP$/$UAS\text{-}Mad^{DN}$). Flies were raised at 18°C until A1. They were then **(a-d)** kept at 18°C for up to 30 days, or **(e-h)** switched to 29°C for up to 15 days. **(d,h)** Experimental results comparing relative pixel intensity of FMRFa FISH at each timepoint, for each genotype. Each datum point was normalized to the percentage of the mean of the w^{1118} control for each timepoint. Data for each genotype, at each timepoint, is presented as mean ± SEM. See Table 1 for details.

(a-c) In non-induced flies maintained at 18°C for 30 days, GAL4 activity was repressed, as shown by lack of nEGFP (green) expression in Tv-cluster neurons (anti-Eyes absent, blue). High level FMRFa FISH (red; anti-DIG) was observed in control w^{1118} flies **(a)**, tkv^{DN};wit^{DN} flies **(b)** and Mad^{DN} flies **(c)**. **(d)** Quantification of FMRFa FISH intensity for flies kept at 18°C, at A1 (time 0), and at 10, 20 and 30 days later. No significant difference was observed between genotypes at any timepoint.

(e-g) In induced flies maintained at 29°C for 15 days, GAL4 activity was induced, as shown by nEGFP expression (green) in Tv-cluster neurons (anti-Eyes absent, blue). FMRFa FISH (red) was absent in Tv4 neurons (arrows) in tkv^{DN};wit^{DN} **(f)** and Mad^{DN} flies **(g)**, in contrast to w^{1118}

control flies **(e)**. **(h)** Quantification of FMRFa FISH intensity for flies induced at 29^{o}C, at A1 (time 0), and at 5, 10 and 15 days later. Over the induction period, there was a progressive, dramatic reduction of FMRFa transcript levels after BMP pathway blockade until it was almost entirely absent. *P<0.0001 compared to control at each timepoint.

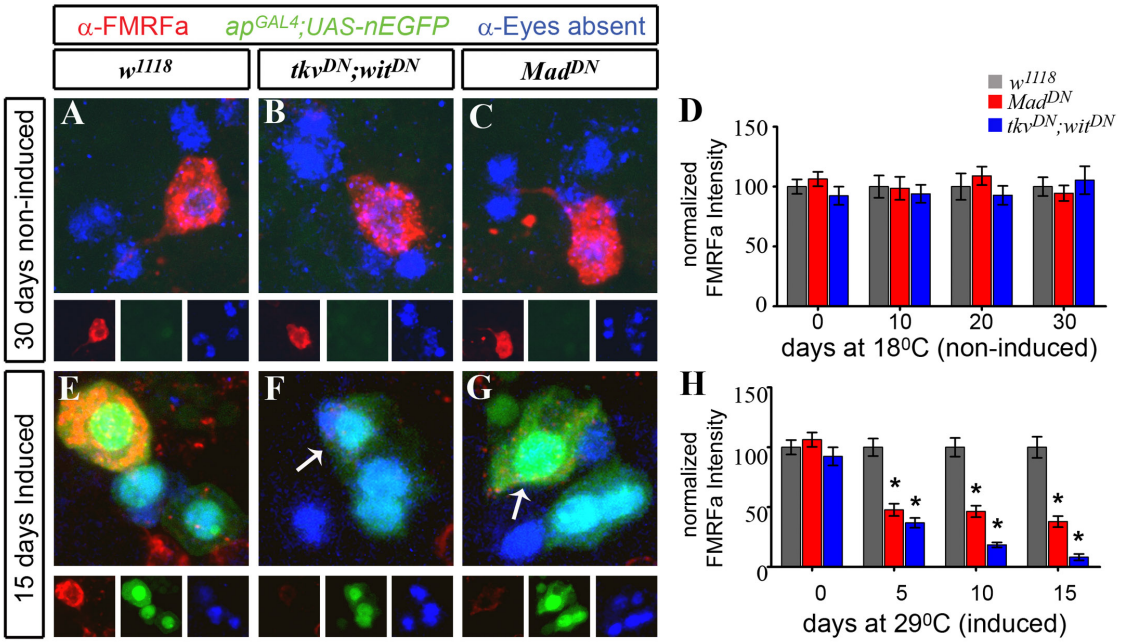

Figure 2.5 Acute blockade of the BMP pathway in adults dramatically reduced FMRFa peptide. Genotypes: w1118 control flies (a,e; apGAL4/+; tub-GAL80TS,UAS-nEGFP/+); tkvDN;witDN flies (b,f; apGAL4/UAS-tkvDN; tub-GAL80TS, UAS-nEGFP/UAS-witDN); MadDN flies (c,g; apGAL4/UAS-MadDN; tub-GAL80TS, UAS-nEGFP/UAS-MadDN). Flies were raised at 18°C until A1. They were then (a-d) kept at 18°C for up to 30 days, or (e-h) switched to 29°C for up to 15 days. (d,h) Experimental results comparing relative pixel intensity of FMRFa immunoreactivity at each timepoint, for each genotype. Each datum point was normalized to the percentage of the mean of the w1118 control at each timepoint. Data for each genotype at each timepoint is presented as mean ± SEM. See Table 2 for details.

(a-c) In non-induced flies maintained at 18°C for 30 days, GAL4 activity was repressed, as shown by lack of nEGFP (green) expression in Tv-cluster neurons (anti-Eyes absent, blue). High level FMRFa immunoreactivity (red; anti-FMRFa) was observed in control w^{1118} flies **(a)**, $tkv^{DN};wit^{DN}$ flies **(b)** and Mad^{DN} flies **(c)**. **(d)** Quantification of FMRFa immunoreactivity intensity for flies kept at 18°C, at A1 (time 0), and at 10, 20 and 30 days later. No significant difference was observed between genotypes at any timepoint.

(e-g) In induced flies maintained at 29°C for 15 days, GAL4 activity was induced, as shown by nEGFP expression (green) in Tv-cluster neurons (anti-Eyes absent, blue). FMRFa immunoreactivity (red) was absent in Tv4 neurons (arrows) in $tkv^{DN};wit^{DN}$ **(f)** and Mad^{DN} flies **(g)**, in contrast to w^{1118} control flies **(e)**. **(h)** Quantification of FMRFa immunoreactivity intensity for flies induced at 29°C, at A1 (time 0), and at 5, 10 and 15 days later. Over the induction period,

there was a progressive, dramatic reduction of FMRFa peptide levels after BMP pathway blockade until it was almost entirely absent. *P<0.0001 compared to control at each timepoint.

2.4.6 Restoration of FMRFa transcript after recovery from acute BMP blockade in adults.

We tested the capacity of Tv4 neurons to restore FMRFa transcript after a period of BMP blockade. We raised w^{1118} control, $tkv^{DN};wit^{DN}$ and Mad^{DN} flies at 18°C until A1. They were switched to 29°C for 10 days to induce GAL4 activity, and reduce FMRFa transcript (~13% and ~24% of w^{1118} control levels in $tkv^{DN};wit^{DN}$ and Mad^{DN} flies, respectively; **Fig. 2.4H, 2.6A-D, Table 2S.1**). Flies were then returned to 18°C for a further 10 days of "recovery", in which GAL4 no longer drove transgene expression and transgene levels presumably decline. Remarkably, after recovery, FMRFa FISH levels in $tkv^{DN};wit^{DN}$ and Mad^{DN} flies was fully restored to control levels (**Fig. 2.6E-H; Table 2S.1**). In w^{1118} controls, FMRFa FISH intensity was 100±7.3% of the mean of w^{1118} controls (n=53 Tv4 neurons). In $tkv^{DN};wit^{DN}$ flies, FMRFa FISH was 109.8±10.3 (n=53 Tv4 neurons; no significant difference to control (NSD); **Fig. 6F**). In Mad^{DN} flies, FMRFa intensity was 111.2±7.4% (n=53 Tv4 neurons; NSD **Fig. 2.6G**). We noted a weak persistent EGFP expression the Tv cluster in "recovered" flies (**Fig. 2.6E-G**), confirming that transgene expression had been induced previously.

In summary, these data show that adult Tv4 neurons retain the capacity to re-establish normal FMRFa expression after an acute 10 day block of BMP signaling that had dramatically reduced FMRFa transcript levels.

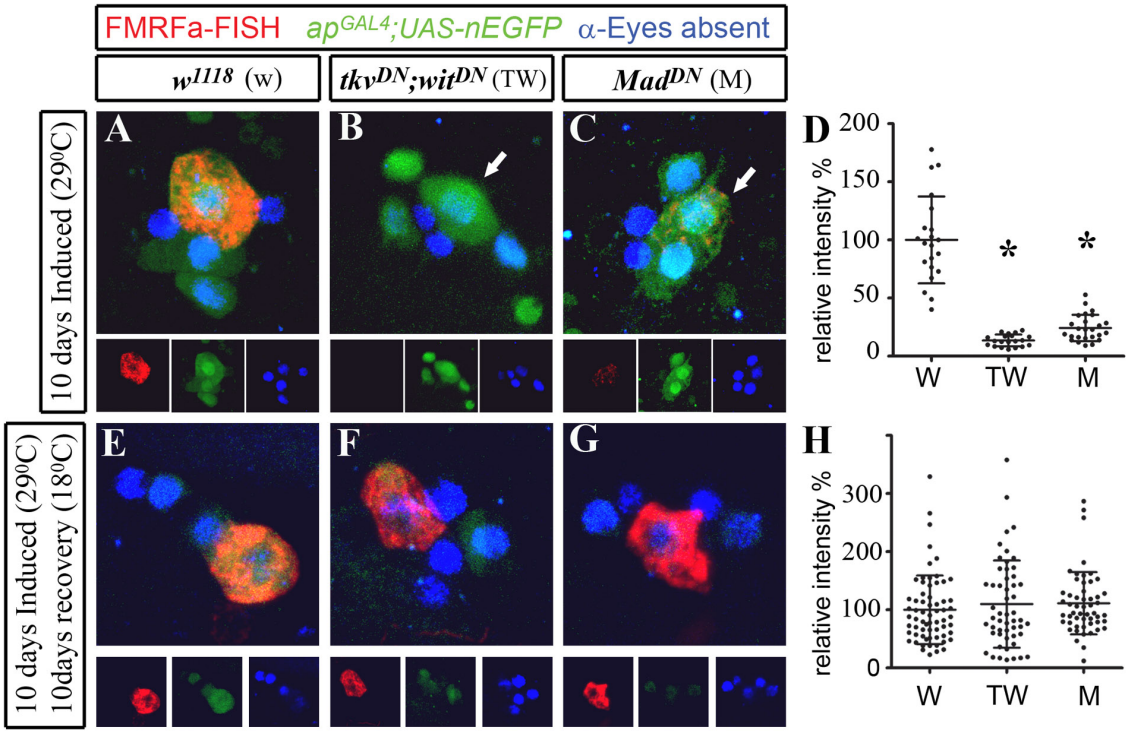

Figure 2.6 FMRFa transcript was fully restored after recovery from acute BMP blockade in adults.

Genotypes: w^{1118} control flies (**a,e**; ap^{GAL4}/+; tub-$GAL80^{TS}$,UAS-nEGFP/+); tkv^{DN};wit^{DN} flies (**b,f**; ap^{GAL4}/UAS-tkv^{DN}; tub-$GAL80^{TS}$, UAS-nEGFP/UAS-wit^{DN}); Mad^{DN} flies (**c,g**; ap^{GAL4}/UAS-Mad^{DN}; tub-$GAL80^{TS}$, UAS-nEGFP/UAS-Mad^{DN}). Flies were raised at 18°C until A1. They were then **(a-d)** switched to 29°C to induce GAL4 activity for 10 days, or **(e-h)** switched to 29°C for 10 days to induce GAL4 activity, and then returned to 18°C for 10 days to allow neurons to recover from BMP blockade. **(d,h)** Scatter plots show relative pixel intensity of FMRFa FISH in individual Tv4 neurons of w^{1118} control (w), tkv^{DN};wit^{DN} flies (TW), or Mad^{DN} flies (M). FMRFa FISH intensity was expressed as a percentage of the mean of the control for each experiment. Mean ± SEM is shown. See Table 1 for details.

(a-c) Induction of BMP blockade in adult Tv-cluster neurons (blue, anti-Eyes absent) for 10 days reduced FMRFa transcript (red) in Tv4 neurons (arrow). High nEGFP (green) expression showed GAL4 activity. **(d)** Scatter plot shows that BMP blockade in tkv^{DN};wit^{DN} flies (TW) and Mad^{DN} flies (M) significantly reduced FMRFa expression in all Tv4 neurons, when compared to w^{1118} control flies (w). Note that these data are included in Fig 4h for the 10 day timepoint. *P<0.0001 compared to w^{1118} control.

(e-g) After 10 days of recovery from BMP blockade, FMRFa FISH (red) was fully restored to control levels. Note low level nEGFP expression (green), indicative of nEGFP expression during the induction period. **(h)** Scatter plot shows that recovery from BMP blockade resulted in a complete restoration of FMRFa FISH levels, with no significant difference seen between w^{1118} control flies (w), tkv^{DN};wit^{DN} flies (TW), or Mad^{DN} flies (M).

2.4.7 Late-onset expression of mutant Glued in adult Tv4 neurons blocked retrograde BMP signaling and downregulated FMRFa, in a reversible manner.

The above data led us to the hypothesis that neurodegenerative disease pathologies can disrupt target-dependent gene expression in neurons by disruption of retrograde transport of essential target-derived signals. We tested this hypothesis using mutant Glued ($Glued^{DN}$) (Allen et al., 1999). Glued has been linked to familial and sporadic amyotrophic lateral sclerosis (ALS) (Munch et al., 2004; Puls et al., 2003). Mutant Glued disrupts dynein motor function in

Drosophila (Martin et al., 1999) and vertebrates (Levy et al., 2006) and recapitulates key features of ALS in mouse models (Chevalier-Larsen et al., 2008; Laird et al., 2008).

We generated flies to spatiotemporally control Glued^{DN} expression (*UAS-Glued^{DN}*) in adult Tv4 neurons using the TARGET system (genotype: (*FMRFa-lacZ),ap^{GAL4}/UAS-Glued^{DN}; UAS-nEGFP, tub>GAL80^{TS}/+*). We tested the effect of late-onset Glued^{DN} expression on maintenance of FMRFa expression. We raised *w^{1118}* control and *Glued^{DN}* flies at 18^{o}C until A1. First, we maintained flies at 18^{o}C for 10 days to control for genotype in the absence of GAL4 activity, which was verified by lack of nEGFP expression (Fig. 2.7A,B). After 10 days at 18^{o}C, the intensity of FISH for FMRFa transcript, normalized to the mean of *w^{1118}* control, was not significantly different between *w^{1118}* control (100±12.4; n=15 Tv4 neurons) and *Glued^{DN}* flies (106.3±14.2%; n=20; NSD; Fig. 2.7C, Table 2S.3). Second, we induced *Glued^{DN}* for 5 days at 29^{o}C; FMRFa expression in *Glued^{DN}* flies fell to 28.8±4.9% (n=20; P=1.7x10^{-13}), compared to that of *w^{1118}* control flies (100±4.7%; n=34; Fig. 2.7D-F, Table 2S.3). Third, we tested whether Tv4 neurons were able to recover from an acute period of *Glued^{DN}* induction that downregulated FMRFa expression. We raised *w^{1118}* control and *Glued^{DN}* flies at 18^{o}C until A1. They were then switched to 29^{o}C for 5 days to induce GAL4 activity. Flies were then returned to 18^{o}C for a further 10 days of "recovery". Remarkably, after the 10 day recovery period, FMRFa levels were restored to 194.6 ± 24.9% (n=15; P=0.00011) of *w^{1118}* control flies (100±4.8%; n=21; Fig. 2.7G-I, Table 2S.3). In summary, overexpression of GluedDN in adult Tv4 neurons led to a profound downregulation of FMRFa expression.

We next examined whether BMP retrograde signaling is blocked by Glued^{DN}, by testing nuclear pMad accumulation in Tv4 neurons in *w^{1118}* control and *Glued^{DN}* flies (Fig 2.7J-M). We raised *w^{1118}* control and *Glued^{DN}* flies at 18^{o}C until A1. First, we examined pMad immunoreactivity in Tv4 nuclei at A1, prior to induction at 29^{o}C. In both *w^{1118}* control and *Glued^{DN}* flies, 100% of Tv4 neurons expressed FMRFa-lacZ and pMad immunoreactivity (n=6, n=12 Tv4 neurons, respectively). Next, we induced *ap^{GAL4}* activity by switching animals to 29^{o}C. After just 12 hours of induction, we found that pMad nuclear accumulation was observed in 0% of Tv4 neurons in *Glued^{DN}* flies (n=29 Tv4 neurons), as opposed to 100% of Tv4 neurons in *w^{1118}* control (n=15). This shows that acute Glued^{DN} expression in adult Tv4 neurons rapidly blocked persistent retrograde transport of the BMP signal in adult Tv4 neurons.

In summary, these data support the hypothesis that maintenance of FMRFa expression in adult Tv4 neurons requires persistent retrograde BMP signaling. Moreover, we provide the first direct evidence that neurodegenerative disease-related disruption of axonal transport leads to the

downregulation of phenotypic markers, such as the neuropeptide FMRFa, that depend upon persistent target-dependent signaling. These findings highlight a novel mechanism by which neurodegenerative diseases may disrupt normal neuronal function. Importantly, our data further demonstrate that the effect of *Glued*^{DN} is fully reversible, in that neurons can restore normal target-dependent FMRFa expression after recovery from mutant Glued expression.

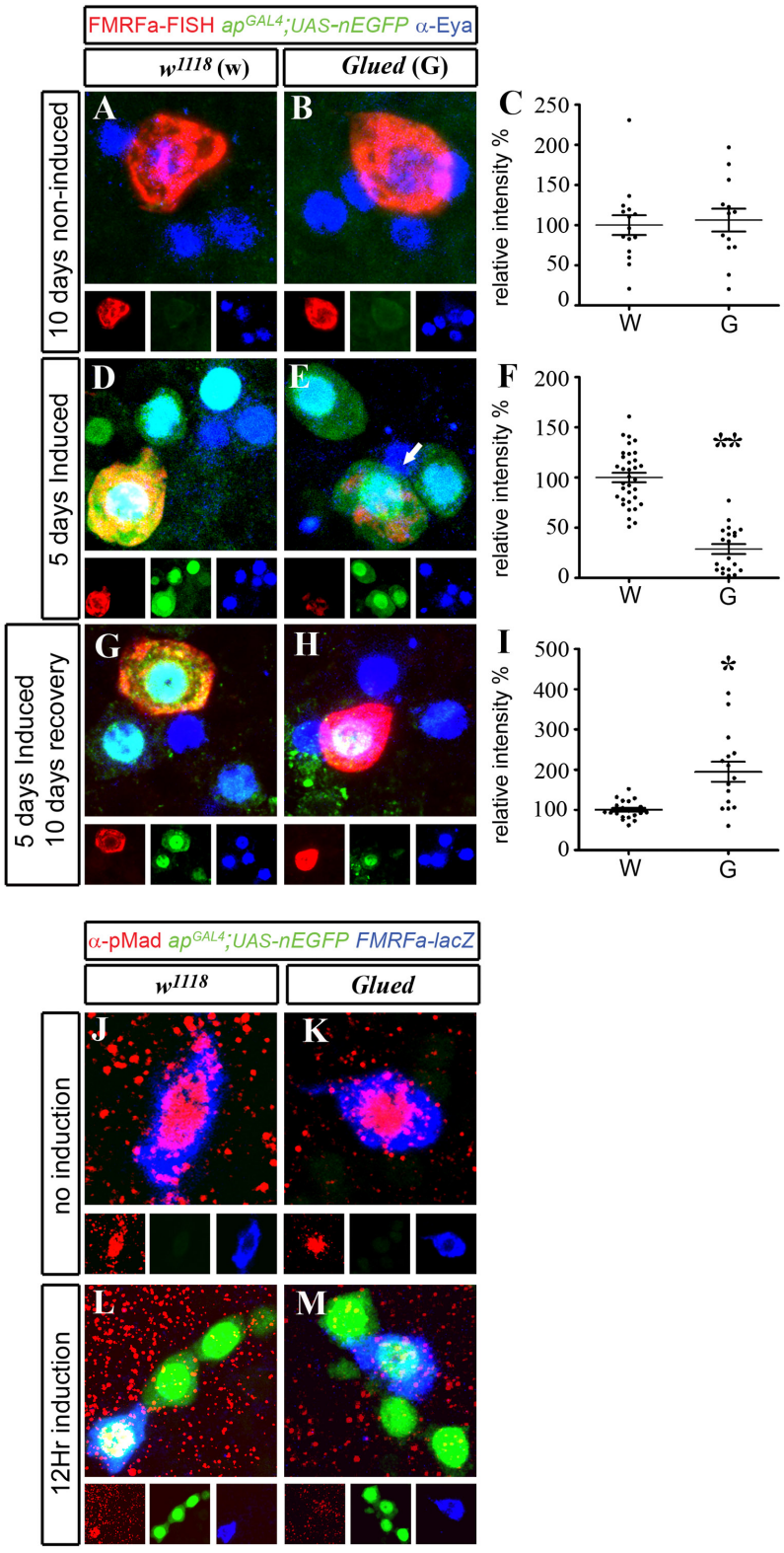

Figure 2.7 FMRFa expression was reduced by expression of mutant p150/Glued, and was fully restored after recovery from mutant p150/Glued expression.

Genotypes: w^{1118} control flies (**a,d,g**; ap^{GAL4}/+; *tub-GAL80*^{TS}*,UAS-nEGFP*/+); $Glued^{DN}$ flies (**b,e,h**; ap^{GAL4}/*UAS-Glued*$^{\Delta 84}$; *tub-GAL80*^{TS}*, UAS-nEGFP*/+). Flies were raised at 18^{o}C until A1. They were then **(a-c)** kept at 18^{o}C for 10 days to block GAL4 induction, **(d-f)** switched to 29^{o}C for 5 days to induce GAL4 activity, or **(g-i)** switched to 29^{o}C for 5 days, and then returned to 18^{o}C for 10 days to allow neurons to recover from Glued^{DN} expression. **(c,f,i)** Scatter plots show relative pixel intensity of FMRFa FISH in individual Tv4 neurons of w^{1118} control (w), and $Glued^{DN}$ flies (G). FMRFa FISH intensity was expressed as a percentage of the mean of the control for each experiment. Mean ± SEM is shown. See Table 1 for details.

(a,b) Flies were kept at 18^{o}C for 10 days. Note the lack of nEGFP (green) in Tv-cluster neurons (blue; anti-Eyes absent), showing lack of GAL4 activity. FMRFa FISH (red) was high in non-induced w^{1118} control flies **(a)** and $Glued^{DN}$ flies **(b)**. **(c)** Scatter plot shows that there was no significant difference in FMRFa transcript in individual Tv4 neurons between non-induced w^{1118} control (w), and $Glued^{DN}$ flies (G).

(d,e) 5 days of induced $Glued^{DN}$ expression in adult Tv-cluster neurons (blue; anti-Eyes absent) significantly reduced FMRFa FISH (red) in Tv4 neurons (arrow). High nEGFP (green) expression showed induction of GAL4 activity. **(f)** Scatter plot shows that FMRFa transcript was downregulated in all Tv4 neurons in $Glued^{DN}$ flies (G), compared with w^{1118} control flies (w). ** P<.0001 compared to control.

(g,h) After 10 days of recovery from Glued^{DN} expression, FMRFa FISH (red) was restored to above control levels (194.6 ± 24.9%). Note low nEGFP expression (green), indicative of nEGFP expression during the induction period. **(i)** Scatter plot shows that after recovery from Glued^{DN} expression, FMRFa transcript was fully restored to above control levels (G), when compared to w^{1118} control flies (w). *P<0.001compared to control.

(j-m) Flies of genotype *FMRFa-lacZ,* ap^{GAL4}*/CyO; tub-GAL80*^{TS}*,UAS-nEGFP* were crossed to either control w^{1118} flies **(j,l)** or $UAS\text{-}Glued^{DN}$ flies **(k,m)** to generate progeny that were raised at 18^{o}C. **(j,k)** No induction, A1 flies maintained at 18 ^{o}C. Note lack of nEGFP expression in both genotypes. In w^{1118} control flies **(j)** and $UAS\text{-}Glued^{DN}$ flies **(k)**, there was no difference in the expression of *FMRFa-lacZ* (blue; anti-βGal) or nuclear pMad (red; anti-pMad) in Tv4 neurons.

(l,m) After 12 hours induction at 29^{o}C, nEGFP expression was activated (green) in both genotypes. **(l)** In w^{1118} control flies, *FMRFa-lacZ* and nuclear pMad (arrowhead) expression was maintained in Tv4 neurons. **(m)** In $UAS\text{-}Glued^{DN}$ flies, *FMRFa-lacZ* expression was maintained, but nuclear accumulation of pMad was entirely absent in Tv4 neurons.

2.5 DISCUSSION

Previously, we demonstrated that target-dependent neuronal differentiation is conserved from invertebrates to vertebrates by showing that expression of FMRFa in developing *Drosophila* Tv4 neurons is induced by retrograde BMP signaling (Allan et al., 2003). FMRFa is a stably-expressed phenotypic marker of Tv4 neurons expressed throughout *Drosophila* life (Schneider et al., 1993). In spite of the growing number of identified target-induced genes in vertebrates and *Drosophila*, it is unclear whether maintenance of these genes' expression requires persistent retrograde signaling, or switches to cell-autonomous maintenance. Here, we address this question for FMRFa in Tv4 neurons. By cell-autonomous blockade of BMP signaling or retrograde axon transport in Tv4 neurons, selectively in adult flies, we show that persistent retrograde BMP signaling is absolutely required to maintain FMRFa expression. To our knowledge, this is the first explicit demonstration that a specific retrograde signaling pathway is required to induce and then to maintain the expression of a stably-expressed phenotypic marker in neurons.

2.5.1 TGFβ-superfamily ligands are conserved regulators of circulating peptide hormone levels.

Tv4 neurons secrete FMRFa into the fly circulatory system, the haemolymph, akin to the secretion of peptide hormones from the mammalian pituitary and pancreas into the circulation. Intriguingly, persistent BMP signaling is required to maintain expression of insulin in pancreatic β-islet cells (Goulley et al., 2007), and persistent activin signaling is required to maintain follicle-stimulating hormone subunit β (FSH β) expression in pituitary gonadotrophs (DePaolo et al., 1992a; Depaolo et al., 1992b; Guo et al., 1998; Kumar et al., 2003). These data, together with our results showing that BMP signaling maintains neuropeptide expression in *Drosophila* neuroendocrine neurons, now demonstrates that TGFβ-superfamily signaling is a conserved mechanism for maintenance of circulatory peptide hormone levels in adults.

Does BMP signaling participate in homeostatic regulation of FMRFa? A precedent for homeostatic peptide hormone regulation by TGFβ-superfamily signaling comes from the well-characterized transcriptional regulation of FSHβ during the oestrous cycle by activin. FSHβ expression depends upon activin signaling via activin receptors, which in turn is modulated by the balance of locally-produced activin and circulating levels of inhibin, an activin inhibitor secreted from the gonads (Bilezikjian et al., 2006; Gregory and Kaiser, 2004). In *Drosophila*, the

level of BMP ligand or antagonist accessible to Tv4 neurons may be responsive to an unknown cue that reads through to physiologically-instructive levels of FMRFa. We have not detected any periodicity in FMRFa expression suggestive of homeostatic modulation, but neither have we ruled out such a possibility. BMP signaling has a well-documented role in neuromuscular efficacy in *Drosophila* larvae (Keshishian and Kim, 2004; McCabe et al., 2003), and pharmacological administration of FMRFa enhances neuron-evoked contractility of larval *Drosophila* body wall muscles (Clark et al., 2008; Hewes et al., 1998). These findings have led to the untested proposal that activity-dependent BMP signaling feeds back to FMRFa transcription in order to influence neuromuscular efficacy (Keshishian and Kim, 2004). However, there is currently no evidence to suggest that retrograde BMP signaling may not simply function to maintain high level FMRFa transcription independent of any homeostatic regulatory role. Future studies will need to discriminate between these models and define the role(s) of FMRFa in adult flies.

2.5.2 Retrograde maintenance of neuronal phenotype in adults

After neurons differentiate, they must maintain their differentiated state for the lifetime of the animal. Blau and Baltimore (1991) postulated that a cell's differentiated state requires persistent active regulation, rather than lapsing into a passive 'locked-in' state. Evidence has emerged from work in *C.elegans* to show that core 'terminal selector' transcription factors, which differentiate neuron subset-specific identity, are subsequently required to maintain that identity. Notably, a cell-autonomous autoregulatory mechanism operates to actively maintain cell-specific 'terminal selector' transcription factor expression (Etchberger et al., 2009; Hobert, 2008). Maintenance of extrinsically-induced gene expression poses a distinct problem for maintenance. In neuronal progenitors, genes induced by inductive extrinsic signals are subsequently maintained by intrinsic mechanisms (Edlund and Jessell, 1999). In contrast, our data now shows that the stable phenotype of a subset of postmitotic *Drosophila* neurons requires persistent retrograde BMP signaling. This is the first demonstration that persistent TGFβ-type signaling is required to maintain neuronal phenotype, and highlights the possibility that many of target-induced genes in vertebrate and invertebrate nervous systems may require persistent retrograde signaling for their maintenance.

Work in vertebrates has provided compelling evidence that neurotrophins act retrogradely to maintain adult neuronal phenotype. Axotomy of adult sympathetic and sensory

neurons alters their expression of neurotransmitters and neuropeptides (Zigmond et al., 1998). As nerve growth factor (NGF) is expressed at those neurons' targets, it is notable that NGF administration partially blocks axotomy-induced phenotypic changes (Verge et al., 1995) and that NGF function-blocking antibodies partially recapitulate axotomy-induced changes in intact neurons (Shadiack et al., 2001). Similarly, cholinergic markers are downregulated in murine cholinergic basal forebrain (CBF) neurons in transgenic mice that express an anti-NGF antibody or after target-tissue ablation (Capsoni et al., 2000; Sofroniew et al., 1993).

These vertebrate studies strongly suggest that retrograde signaling maintains neuronal phenotype, but in most cases it has been difficult to discriminate between i) loss of gene expression as a direct consequence of loss of a specific retrograde signal, and ii) loss of gene expression as an indirect consequence of neuronal injury, degeneration or regeneration. The use of conditional mouse knockouts and transgenics will circumvent many of these issues and promises a wealth of insight. For example, conditional BDNF knockouts have been used to uncover a role for anterograde BDNF signaling in the maintenance of serotonin receptor expression in the adult prefrontal cortex (Rios et al., 2006). In our study, we have circumvented these issues using the TARGET spatiotemporal transgene targeting system (McGuire et al., 2004) which provides several advantages: i) Using reversible temperature-dependent transgene expression, we were able to block retrograde BMP signaling cell-autonomously at any timepoint in intact Tv4 neurons in freely-moving flies. ii) We were able to examine FMRFa expression in the same individually-identifiable neurons between experimental groups. iii) The use of cell-specific markers that are not BMP-dependent, *apterous* and *eyes absent*, allowed us to identify Tv4 neurons in the absence of FMRFa expression, confirming that Tv4 neurons do not require retrograde BMP signaling for survival. Our results provide very strong support for work in vertebrates that has indicated a role for retrograde signaling in the maintenance of neuronal phenotype.

2.5.3 Inhibition of persistent retrograde signaling: A mechanism for disrupting neuronal phenotype in neurodegenerative disease.

This study has demonstrated a requirement for persistent retrograde signaling in the maintenance of a gene critical to subset-specific neuronal phenotype and function. These data are of particular significance in light of the many neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS), Alzheimer's disease and Huntington's disease, that have been

increasingly attributed to defects in axon transport (Chevalier-Larsen and Holzbaur, 2006; De Vos et al., 2008; Gunawardena and Goldstein, 2004). In order to block retrograde transport, we overexpressed a dominantly-acting Glued mutant in Tv4 axons. Importantly, dominantly-acting Glued mutations have been linked to ALS (Munch et al., 2004; Puls et al., 2003) and mouse models recapitulate many cellular and functional deficits of ALS (Chevalier-Larsen et al., 2008; Laird et al., 2008; Levy et al., 2006). Glued is a critical component of the dynactin complex necessary for dynein motor function in retrograde axon transport (Chevalier-Larsen and Holzbaur, 2006; De Vos et al., 2008), and given that similar phenotypes have been described in mouse dynein mutants or upon dynamitin overexpression (Hafezparast et al., 2003; Laird et al., 2008; LaMonte et al., 2002), numerous lines of evidence converge to indicate that axonal transport defects may be causative for ALS.

It is still unclear how reduced axon transport leads to neuronal dysfunction and degeneration. A widely-held hypothesis proposes that disrupted axon transport diminishes target-derived neurotrophin signaling (Blesch, 2006; Salehi et al., 2003), depriving neurons of necessary trophic support and leading to degeneration. Evidence for this link has come from elegant work utilizing mice trisomic for amyloid precursor protein (APP) to model early-onset Alzheimer's disease in Down's syndrome. This showed that APP overexpression inhibited retrograde NGF transport, leading to presynaptic neuron atrophy and reduced expression of the p75 low affinity NGF receptor (Cooper et al., 2001; Salehi et al., 2006).

Our data show that blockade of dynein-mediated axonal transport eliminates retrograde BMP signaling in adult neurons. We further show that this directly impacts neuronal phenotype by eliminating the expression of a stably-expressed, target-dependent gene that defines the neuron's identity and function. Importantly, this occurs in the absence of overt neuronal degeneration. Thus, we postulate that the maintenance of neuronal phenotype may be particularly vulnerable to pathologies that diminish retrograde signaling, even in the absence of overt neuronal degeneration or death. However, both our work and that of others suggest that the loss of neuronal phenotype resulting from disrupted axonal transport may be reversible. Cooper et al (2001) found that reduced retrograde NGF transport in cholinergic basal forebrain neurons led to neuronal atrophy and reduced p75 expression, but that both could subsequently be fully reversed by NGF infusion. Similarly, our 'recovery' data shows that FMRFa expression was fully restored after retrograde BMP signaling was re-established. Both studies strongly suggest that loss of neuronal phenotype in neurodegenerative disease may be treatable.

3. Developmental transcriptional networks are required to maintain neuronal subtype identity in the mature nervous system.

3.1 SYNOPSIS

During neurogenesis, transcription factors combinatorially specify neuronal fates and then differentiate subtype identities by inducing subtype-specific gene expression profiles. But how is neuronal identity maintained in mature neurons? Modeling this question in two *Drosophila* neuronal subtypes (Tv1 and Tv4), we test whether the subtype transcription factor networks that direct differentiation during development are required persistently for long-term maintenance of subtype identity. By conditional transcription factor knockdown in adult Tv neurons after normal development, we find that most transcription factors within the Tv1/Tv4 subtype transcription networks are indeed required to maintain Tv1/Tv4 subtype-specific gene expression in adults. Thus, gene expression profiles are not simply 'locked-in', but must be actively maintained by persistent developmental transcription factor networks. We also examined the cross-regulatory relationships between all transcription factors that persisted in adult Tv1/Tv4 neurons. We show that certain critical cross-regulatory relationships that had existed between these transcription factors during development were no longer present in the mature adult neuron. This points to key differences between developmental and maintenance transcriptional regulatory networks in individual neurons. Together, our results provide novel insight showing that the maintenance of subtype identity is an active process underpinned by persistently active, combinatorially-acting, developmental transcription factors. These findings have implications for understanding the maintenance of all long-lived cell types and the functional degeneration of neurons in the aging brain.

3.2 INTRODUCTION

Tremendous progress has been made in delineating the transcriptional mechanisms that diversify neuronal subtype identities during development. Spatiotemporally patterned transcription factor cascades act within increasingly diversified progenitor populations to specify postmitotic neuron subtype fate. Within postmitotic neurons, subtype-specific sets of transcription factors act combinatorially to differentiate subtype identity by initiating expression of the genes that define subtype form and function (di Sanguinetto et al., 2008; Hobert, 2010; Landgraf and Thor, 2006). These so-called terminal differentiation genes include subtype-specific neuropeptides, neurotransmitter enzymes and ion channels (Hobert et al., 2010).

Developmental transcriptional cascades are progressive and typically nonlinear; many transcription factors act at multiple levels, they exhibit extensive cross-regulation, and their expression undergoes considerable refinement in maturing postmitotic neurons (Alavian et al., 2008; Baumgardt et al., 2009; di Sanguinetto et al., 2008; Habener et al., 2005). Here, we apply the term 'subtype transcription network' to refer to the transcription factors that direct subtype specification and differentiation, their cross-regulatory relationships (or configuration), and the manner in which they direct the expression of subtype-specific sets of terminal differentiation genes.

After subtype-specific gene expression profiles are established by differentiation, continued neuronal function throughout life depends upon their maintenance of subtype gene expression profiles. However, we currently have only a rudimentary understanding of the mechanisms of long-term subtype-specific gene maintenance. Two extreme models would posit that subtype identity is either actively maintained by persistent subtype transcription network activity or passively maintained by for example stabilized chromatin structure, independent of a subtype transcription network. Here, we test the active model in two *Drosophila* neuronal subtypes to address the following largely unanswered questions: Do developmental subtype transcription networks persist in adult neurons or are they dispensed with? Are they required to maintain the expression of subtype-specific sets of terminal differentiation genes? If they are required, does maintenance of terminal differentiation genes require the same complex combinatorial codes of transcription factors as for their initiation, or a simplified code involving fewer transcription factors? Finally, do persisting developmental transcription factors retain the same cross-regulatory relationships that regulated their expression during development?
We model these questions in *Drosophila* Tv1 and Tv4 neurons. There are six clusters of Tv neurons in the *Drosophila* ventral nerve cord, each comprising four distinct subtypes (Tv1-Tv4). Tv1 and Tv4 express subtype-specific terminal differentiation genes, the neuropeptides Nplp1 (Tv1) and FMRFa (Tv4) and the neuropeptide amidase PHM (peptidylglycine alpha-hydroxylating monooxygenase; in Tv1/Tv4). Using these genes as markers for subtype-specific differentiation, previous work had revealed the elaborate subtype transcription networks that direct Tv1/4 subtype specification and differentiation (Allan et al., 2005; Allan et al., 2003; Baumgardt et al., 2009; Baumgardt et al., 2007; Karlsson et al., 2010; Miguel-Aliaga et al., 2004). The expression of FMRFa (Eade and Allan, 2009), Nplp1 and PHM (herein) are stably maintained in Tv1/4 neurons throughout *Drosophila* life. Thus, our detailed understanding of their subtype-specific initiation in the embryo provides an ideal background to investigate how

such terminal differentiation genes, and hence subtype identity, are maintained in the adult. We previously established that persistent retrograde BMP signaling is required to initiate and maintain FMRFa in Tv4 neurons (Eade and Allan, 2009). Here, we examined the adult function of Tv1 and Tv4 subtype transcription networks in the maintenance of Nplp1, FMRFa and PHM. We further examined whether the cross-regulatory interactions observed between the Tv1/Tv4 network transcription factors during development were maintained in adults. We found that each subtype transcription network is largely retained in adult Tv neurons and is required to actively maintain subtype-specific gene expression. Thus, the combinatorial transcription codes for subtype-specific gene expression are not 'simplified' or dispensed with for maintenance. Further, we find that certain critical developmental cross-regulatory interactions between transcription factors are no longer utilized in adults for transcription actor maintenance. Thus, we observe a post-developmental switch to a distinct maintenance configuration between individual transcription factors. Collectively, these data provide novel insight relevant to understanding how long-lived cell types maintain their subtype identity.

3.3 MATERIALS AND METHODS

3.3.1 Fly Stocks

Flies were maintained on standard cornmeal food and maintained at stable temperatures in environment rooms set at 70% humidity at 18°C, 25°C or 29°C.

3.3.2 Fly strains

apterous^{md544} (referred to as *ap*^{GAL4}); *ap*^{P44}*; sqz*^{IE}*; sqz*^{GAL4}*; UAS-thickveins activated* (*UAS-tkv*^{A}*); UAS-saxophone activated* (*UAS-sax*^{A}) (Allan et al., 2003); *dac*^{GAL4} (Heanue et al., 1999); *rev4; UAS-dimm* (Hewes et al., 2003)*; dac4* (Mardon et al., 1994); *; eya*^{CliIID} (Pignoni et al., 1997); *grh*^{GAL4} (Baumgardt et al., 2009). *tubP>GAL80*^{TS} (temperature sensitive GAL80 under the control of the *Drosophila* tubulin 84B promoter (McGuire et al., 2003); *UAS-nEGFP* (nuclear localized EGFP);

3.3.3 dsRNAi lines

Strains used for primary data: *UAS-col#24E* (Baumgardt et al., 2007); *UAS-ap*^{dsRNAi} *8376R-2; UAS-dac*^{dsRNAi} *4952R-2*; *UAS-sqz*^{dsRNAi} *5557R-2 (*NIG-FLY); *UAS-dimm*^{dsRNAi} *GD44470; UAS-eya*^{dsRNAi} *GD43911* (VDRC)

Strains used secondarily to verify data: *UAS-dimm^{dsRNAi} KK103356; UAS-eya^{dsRNAi} 108071KK* (VDRC)*; UAS-eya^{dsRNAi} JF03160; UAS-dac^{dsRNAi} JF02322* (TRiP).

3.3.4 Spatial and temporal regulation of transgene expression using the TARGET system

Flies for TARGET-mediated transgene induction were generated by crossing utility flies (*UAS-dicer/UAS-dicer; ap^{Gal4}; tubP>GAL80^{TS}, UAS-nEGFP/SM6-TM6,Tb*) or (*UAS-dicer/UAS-dicer; dac^{Gal4}; tubP>GAL80^{TS}, UAS-nEGFP/SM6-TM6,Tb*) to *UAS-dsRNAi* (experimental group) or *w^{1118}* flies (control). Experiments were performed on resulting progeny bearing appropriate genotypes (screened by loss of *SM6-TM6, Tb* balancer chromosome). All experimental and control flies were raised at 18°C until eclosion (hatching from the pupal case). On adult day 1 (A1), flies were switched to 29°C for the duration of the induction period indicated.

3.3.5 Antibodies

Primary antibodies: sheep anti-digoxygenin (1:1500; Roche); rabbit anti-GFP (1:100; A6455 Invitrogen); rabbit anti-FMRFa (1:1000; T-4757 Peninsula Labs); mouse anti-β-Galactosidase (1:100; 40-1a); chicken anti-Nplp1 (1:1000), guinea pig anti-Dimm (1:1000); rat anti-Cas (1:1000 (all gifts from S. Thor, Linkoping U, Sweden); guinea pig anti-Collier (1:1000, a gift from Adrian Moore, RIKEN, Japan); rabbit anti-nab (Fernando Jimenez Diaz-Benjumea); mouse anti-Eya (1:100; clone 10H6); mouse anti-Dac (1:2; Mab Dac 2-3 (both from Developmental Studies Hybridoma Bank; Iowa U. Iowa)
Secondary antibodies: donkey anti-sheep Alexa 555 (1:10; Invitrogen, Carlsbad, USA); donkey anti-mouse Cy5 and donkey anti-rabbit Cy2 (1:200; Jackson Immunoresearch, West Grove, USA). Antisense DIG-RNA Probe: DIG-Uracil tagged RNA probes were generated using T3 RNA polymerase from clone RH03963 (DGRC: *Drosophila* Genomic Resource Centre, Indiana, USA) containing a 1584bp FMRFa cDNA (using the Roche DIG-U-RNA Labelling Kit). Probe synthesis was confirmed using gel electrophoresis.

3.3.6 Multiplex Fluorescent in situ hybridization (FISH) and immunohistochemistry

Standard in situ and immunohistochemistry protocols were carried out as described (Eade and Allan, 2009). All tissues compared for fluorescence intensity were processed at the same time using the same aliquots of all solutions under the same conditions. They were then mounted on the same slide and confocal settings were calibrated to control staining levels.

3.3.7 Image and statistical analysis

All images acquired on an Olympus FV1000 confocal microscope. Fluorescent intensity of individual neurons was measured using Image J (US National Institutes of Health). The mean pixel intensity for each neuron was measured from compressed Z-slices, and corrected for background fluorescence. Analysis was performed on every identifiable Tv1 and Tv4 neuron excluding Tv4 neurons in thoracic segment 2, due to lack of markers distinguishing Tv4 from Tv2/3. The resulting value for each Tv neuron was then incorporated as a single datum point towards the mean intensity for each experiment. Each datum point is represented as a percentage of the mean of the w^{1118} control for that experiment. Data are presented as Mean ± SEM. Representative images of Tv neurons that were directly compared in figures were processed in an identical way, simultaneously, using Adobe Photoshop CS4. Normally distributed unpaired data were compared using a two-tailed T-test assuming equal variance, to identify significant differences between means. All statistical analysis and graphs data were performed using Prism 5 software. (Graphpad).

3.4 RESULTS

3.4.1 Terminal differentiation of Tv cluster neurons is regulated by a sub-type transcription factor network.

Genetic analysis has defined cascades of transcription factors that specify Tv1 and Tv4 neuron fates and then differentiates their subtype-specific terminal differentiation gene expression profiles (Figure 3.1A,B) (Allan et al., 2005; Allan et al., 2003; Baumgardt et al., 2009; Baumgardt et al., 2007; Karlsson et al., 2010; Miguel-Aliaga et al., 2004). Tv1-4 are born sequentially from the NB5-6T neuroblast lineage within an expression window of the 'temporal' transcription factors *castor* (*cas*) and *grainy head* (*grh*) (Baumgardt et al., 2009; Brody and Odenwald, 2005). These specify Tv subtype generation together with *collier* (*col*), *squeeze* (*sqz*) and *nab* (Allan et al., 2003; Baumgardt et al., 2009; Baumgardt et al., 2007; Terriente Felix et al., 2007). Within postmitotic Tv1 neurons, *ap, eya, dimmed (dimm)* and *col* then differentiate Tv1 identity in part by initiating Nplp1 expression (Baumgardt et al., 2007). In Tv4 neurons *ap, eya, dimm, dachshund (dac), sqz* and *grh* act combinatorially with target-derived BMP signaling to differentiate Tv4 identity, in part by initiating FMRFa expression (Allan et al., 2005; Allan et al., 2003; Baumgardt et al., 2009; Benveniste et al., 1998; Hewes et al., 2003; Marques et al., 2003; Miguel-Aliaga et al., 2004). Also, in both Tv1 and Tv4, *dimm* acts independently of other

regulators to induce expression of the neuropeptide amidase PHM (Allan et al., 2005; Park et al., 2008). We refer to these two transcription factor cascades as the Tv1 and Tv4 'subtype transcription networks' (outlined in Figure 3.1A,B). Genetic analysis has placed these transcription factors into two partially overlapping categories; those that are necessary for directing the specification, or generation, of Tv subtypes around the time of postmitotic neuron birth, and those that thereafter direct differentiation of Tv subtypes by initiating subtype-specific terminal differentiation gene expression. Tv1 neurons are not specified in *cas* or *col* mutants, whereas *eya*, ap, *col* and *dimm* all act combinatorially thereafter to initiate Nplp1 expression (Baumgardt et al., 2009; Baumgardt et al., 2007). Tv4 neurons are not specified in *cas*, *col* or *grh* mutants. In *sqz* and *nab* mutants, Tv4 neurons are not specified in a segment-specific manner (Allan et al., 2003; Baumgardt et al., 2009). Thereafter, *eya*, *ap*, *sqz*, *dimm*, *dac*, *grh* and BMP signalling all appear to combinatorially initiate FMRFa expression.

3.4.2 Subtype transcription network retention in adult Tv1 and Tv4 neurons.

We previously reported that adult Tv4 neurons maintain FMRFa, *ap* and *eya* expression, as well as retrograde BMP-signaling. We also demonstrated that FMRFa maintenance in adult Tv4 neurons requires persistent retrograde BMP-signaling (Eade and Allan, 2009). Here, we determined the adult expression of the other transcriptional regulators implicated in Tv1 and Tv4 development (Figure 3.1C-J). We found that adult Tv4 neurons retained *ap, eya, dimm, dac* and *sqz* (Figure 3.1C-E,J), but no longer expressed *grh*, *cas* or *nab* (Figure 3.1H-I). Additionally, adult Tv1 neurons retained expression of Nplp1, as well as *ap, eya, dimm* and *col* (Figure 3.1C,D,F). Previously, *cas* expression was shown to be lost in all Tv neurons prior to neuropeptide initiation (Baumgardt et al., 2007), and here we find that it does not become not re-expressed in adult Tv1 or Tv4 neurons (Figure 3.1I). These data are summarized (Figure 3.1K,L). Further analysis found that the adult complement of transcription factors was established by the start of the L1 larva stage (Figure 3S.1), shortly after Tv1/4 terminal differentiation.

Which transcription factors persist in adult neurons is interesting. All those previously implicated in the postmitotic differentiation of Tv1 and/or Tv4 neurons persist. In contrast, transcription factors that act within the neuroblast and newborn postmitotic neuron to specify the fate of Tv1 (*cas*) or Tv4 (*cas, col, grh, nab*) neurons are not retained in the adult. The exceptions to this are *col* and *sqz*. Both are implicated in Tv subtype specification, but it is notable that both transcription factors have also been implicated, by loss and gain of function genetics, as part of the combinatorial transcription factor codes that initiate Nplp1 or FMRFa expression (Allan et al.,

2003; Baumgardt et al., 2009; Baumgardt et al., 2007). Thus, we find that only regulators implicated in postmitotic subtype differentiation are maintained in adult neurons.

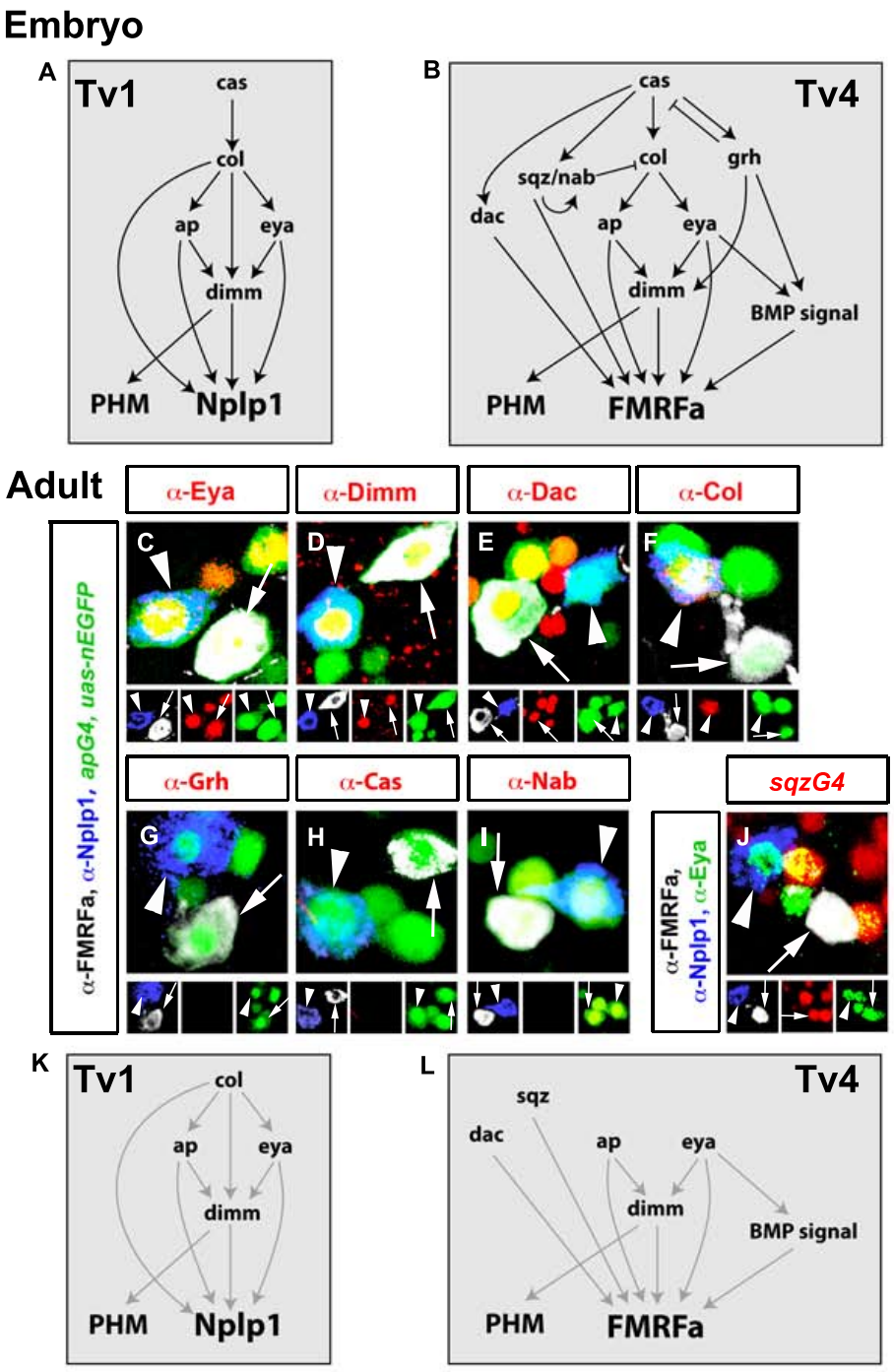

Figure 3.1 Adult Tv1 and Tv4 neurons maintain Nplp1 and FMRFa and a subset of embryonic transcription factors.

(**A-B**) Outline of transcription factor network configuration during specification and differentiation of Tv1 (A) and Tv4 (B) neurons in embryonic stages. Tv1 expresses Nplp1 (blue). Tv4 expresses FMRFa (red). Tv1 and Tv4 neurons both express PHM.
(**C-J**) Representative images from Th1/Th3 Tv clusters in adults (green). Tv4 neurons (arrows) express FMRFa (white). Tv1 neurons (arrowheads) express Nplp1 (blue). Tv1 and Tv4 retain expression of transcription factors *ap* (**C-I**, green), *eya* (**C** red**, J** green) and *dimm* (**D**, red). Tv1 neurons retain *col* (**F**, red). Tv4 neurons retain *dac* (**E**, red) and *sqz* (**J**, red). Tv1 and Tv4 do not express transcription factors *grh* (**G**, red), *cas* (**H** red) or nab (**I**, red) in adult stages .
Genotype: (**C-I**) *FMRFa-LacZ,ap*^{Gal4}*/+; UAS-nEGFP/+*. (**J**) *UAS-nEGFP/+;* sqz^{Gal4}*/+*.
(**K,L**) Outline of transcription factors present in fully differentiated adult Tv1 (**K)** and Tv4 (**L**) neurons. Grey arrows indicate known developmental interactions between transcription factors in embryonic Tv cluster neurons that are not known whether to persist into adult stages.

3.4.3 Conditional dsRNAi knockdown in adult Tv neurons

To test the function of each transcription factor in maintaining terminal differentiation gene expression in Tv1 and Tv4 neuronal subtypes, we used ap^{GAL4} (except where noted) to express *UAS-dsRNAi* transgenes (abbreviated to *dsRNAi*) targeted to each transcription factor. To selectively induce *dsRNAi* in adults, we utilized the TARGET system wherein a temperature-sensitive GAL4-repressor GAL80 ($GAL80^{TS}$) controls the activity of GAL4 (McGuire et al., 2004). Flies were raised at 18ºC to allow functional $GAL80^{TS}$ to repress GAL4 activity throughout development. Then at adult day 1 (A1), flies were switched to 29ºC and kept at that temperature for the remainder of each experiment. At this temperature, $GAL80^{TS}$ becomes dysfunctional and thus GAL4 is allowed to induce *dsRNAi* expression (Figure 3.2A) (Eade and Allan, 2009).

3.4.4 eya, ap, and sqz are required for FMRFa maintenance in adults

We induced eya^{dsRNAi} at adult day 1 (A1) and quantified FMRFa transcript levels by fluorescent *in situ* hybridization, relative to the mean of controls. In adults, eya^{dsRNAi} dramatically reduced FMRFa transcript to 19.7 ± 4.4% of control ($p<0.0001$) by adult day A15 (Figure 3.2B-D). No further FMRFa downregulation was observed from adult day A15 to A20 (not shown). We observed a similar downregulation of immunoreactivity to the mature amidated FMRFa peptide (Table 3S.1). We tested for phenotypic enhancement of eya^{dsRNAi} in an *eya* heterozygous background to confirm *dsRNAi* specificity, and found that FMRFa was indeed further reduced to 4.6 ± 0.5% of control ($p<0.0001$ to eya^{dsRNAi} alone), even though *eya* heterozygosity alone had no effect on FMRFa expression (Figure 3.2D). Eya immunoreactivity was eliminated in all cases (Table 3S.4). Previous studies demonstrated that FMRFa is severely downregulated in *eya* mutants by late embryogenesis (Miguel-Aliaga et al., 2004). Our data now show that FMRFa maintains this critical dependence on *eya* in adulthood.

As ap^{GAL4} is a strong hypomorphic *ap* allele (O'Keefe et al., 1998), we expressed ap^{dsRNAi} using dac^{GAL4} in wildtype and heterozygous *ap* backgrounds. We observed a significant enhancement of FMRFa downregulation by ap^{dsRNAi} in *ap* heterozygotes, falling to 66.8 ± 4.2% of control ($p<0.0001$ from control or *ap* heterozygote alone, and $p<0.005$ from ap^{dsRNAi} alone). No loss of FMRFa was observed in either *ap* heterozygotes or ap^{dsRNAi} alone (Figure 3.2H-J). Similar results were obtained for downregulation of the mature FMRFa amidated peptide (Table 3S.1). When we used ap^{GAL4} induced ap^{dsRNAi} using at A1 we observed a significant reduction of FMRFa

peptide to 40.0 ± 2.2% of control by A20 (w^{1118} control n=40, ap^{dsRNAi} n=38; p<0.0001). Additionally, FMRFa downregulation was also noted using an ap^{dsRNAi} targeting different *ap* sequences (Table 3S.2). The FMRFa downregulation (but not elimination) observed here is comparable to that reported in embryonic *ap* null mutants (Allan et al., 2003; Benveniste et al., 1998), thus, we conclude that *ap* maintains a persistent role in FMRFa regulation. We were unable to determine the extent of Ap knockdown, by either ap^{dsRNAi} transgene, due to a lack of suitable Ap-specific antibodies. Therefore, we tested ap^{dsRNAi} efficacy by examining another *ap* phenotype. As we found that ap^{dsRNAi} expression in the developing wing, using ap^{GAL4}, precisely phenocopied *ap* mutants (Figure 3S.3) (Butterworth and King, 1965), we conclude that ap^{dsRNAi} is highly effective. However, we could not quantitate Ap downregulation in Tv neurons, thus we cannot discount the possibility that FMRFa would be further downregulated if Ap were entirely eliminated.

To test the role of *sqz* in adult Tv4 neurons, we expressed sqz^{dsRNAi} at A1 and observed a partial downregulation of FMRFa expression, but only in a *sqz* heterozygotic background, to 53.0 ± 4.8% of control (p<0.0001 from control, *sqz* heterozygote or sqz^{dsRNAi} alone) (Figure 3.2E-G). Similar results were obtained for immunoreactivity to the mature amidated FMRFa peptide (Table 3S.1). Previous reports established that FMRFa is partially downregulated in embryonic *sqz* mutants (Allan et al., 2003). Thus, our data indicate that *sqz* maintains its partial requirement for FMRFa expression. Due to ubiquitous but weak Sqz expression in the thoracic nerve cord, we were not able to adequately quantitate Sqz downregulation in Tv neurons. Thus, we do not discount the possibility that Sqz may not have been entirely eliminated, and therefore may be underestimating its effect on FMRFa expression. Taken together, our data demonstrate that *eya*, *ap* and *sqz* are required to maintain wildtype FMRFa levels in the adult.

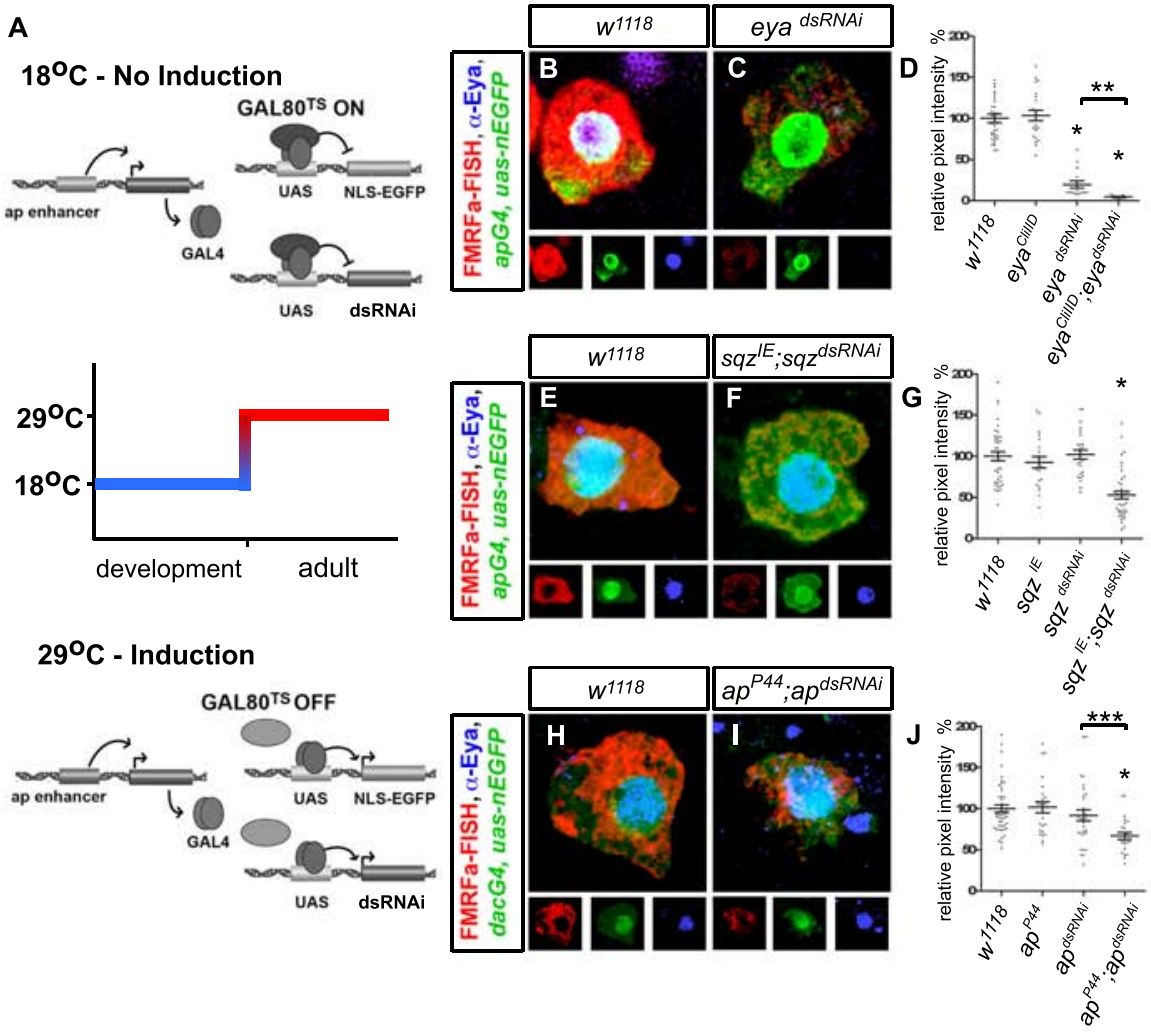

Figure 3.2 Transcription factors *ap*, *eya*, *sqz* are required for persistent FMRFa expression in the adult Tv4 neuron.

(**A**) Cartoon illustrating adult induction of dsRNAi constructs in adult Tv neurons using the TARGET system.

(**B,C,E,F,H,I**) Images of adult Tv4 neurons expressing FMRFa transcript (red), ap^{Gal4},*UAS-nlsEGFP* (green) and Eya (blue) at A15 (**B-G**) or A20 (**H-J**) of *dsRNAi* expression at 29°C.

(**B,C**) Eya immunoreactivity is eliminated and FMRFa is downregulated in eya^{dsRNAi} (**C**) compared to w^{1118} control (**B**).

(**E,F**) FMRFa is downregulated in sqz^{IE},sqz^{dsRNAi} (**F**) compared to w^{1118} control (**I**).

(**H,I**) FMRFa is downregulated in ap^{P44};ap^{dsRNAi} (**I**) compared to w^{1118} control (**H**).

(**D,G,J**) Quantification of FMRFa transcript in individual adult Tv4 neurons at A15 (**D,G**) or A20 (**J**) at 29°C.

**(D) *** p<0.0001 eya^{dsRNA} (n=15) and *eya^{dsRNAi}; eya^{CliIID}* (n=13) compared to *w^{1118}* (n=35) and *eya^{CliIID}* control. ****** p<0.0001 compared to eya^{dsRNAi} alone.
**(G) *** p<0.0001 *sqz^{IE}; sqz^{dsRNAi}* (n=39) compared to *w^{1118}* (n=40), *sqz^{IE}* (n=21), and, sqz^{dsRNAi} (n=25) control.
**(J) *** p<0.0001 *ap^{P44}; ap^{dsRNAi}* (n=25) compared to *w^{1118}* (n=47) and *ap^{P44}* (n=25) control. ******* p<0.005 compared to ap^{dsRNAi} (n=32) alone.

***Genotypes*:** *w^{1118}* (*UAS-dicer2/+; ap^{Gal4}/+; tub-Gal80^{TS}, UAS-nEGFP*) (**A-C**) *eya^{CliIID}* (*UAS-dicer2/+; ap^{Gal4}/eya^{CliIID}; tub-Gal80^{TS}, UAS-nEGFP/+*); *eya^{dsRNAi}* (*UAS-dicer2/UAS-eya^{dsRNAi}; ap^{Gal4}/+; tub-Gal80^{TS}, UAS-nEGFP/+*); *eya^{dsRNAi}; eya^{CliIID}* (*UAS-dicer2/UAS-eya^{dsRNAi}; ap^{Gal4}/ eya^{CliIID}; tub-Gal80^{TS}, UAS-nEGFP/+*).
(**E-G**) *sqz^{IE}* (*UAS-dicer2/+; ap^{Gal4}/sqz^{IE}; tub-Gal80^{TS}, UAS-nEGFP/+*); *sqz^{dsRNAi}* (*UAS-dicer2/+; ap^{Gal4}/+; tub-Gal80^{TS}, UAS-nEGFP/UAS-sqz^{dsRNAi}*); *sqz^{IE}; sqz^{dsRNAi}* (*UAS-dicer2/+; ap^{Gal4}/ sqz^{IE}; tub-Gal80^{TS}, UAS-nEGFP/UAS-sqz^{dsRNAi}*).
(**H-I**) *ap^{P44}* (*UAS-dicer2/+; ap^{Gal4}/ap^{P44}; tub-Gal80^{TS}, UAS-nEGFP/+*); *ap^{dsRNAi}* (*UAS-dicer2/+; ap^{Gal4}/+; tub-Gal80^{TS}, UAS-nEGFP/UAS-ap^{dsRNAi}*) ; *ap^{P44}; ap^{dsRNAi}* (*UAS-dicer2/+; ap^{Gal4}/ap^{P44}; tub-Gal80^{TS}, UAS-nEGFP/UAS-ap^{dsRNAi}*).

3.4.5 dimm maintains FMRFa peptide processing.

We induced *dimm*^{dsRNAi} at A1 and found that immunoreactivity to the mature amidated FMRFa peptide was profoundly reduced by *dimm*^{dsRNAi} to 24.0±3.2 % of control by A10 ($p<0.0001$), and this was enhanced to 9.8 ± 1.6% of control in *dimm* heterozygotes ($p<0.0001$ to control and *dimm* heterozygotes, $p<0.001$ to *dimm*^{dsRNAi} alone) (Figure 3.3A-C, Figure S3). Immunoreactivity to Dimm demonstrated that it had been eliminated (Table 3S.4). In contrast, FMRFa transcript in adults was downregulated, only in *dimm* heterozygotes, to 67.1 ± 2.9% of control by A20 ($p<0.0001$) (Figure 3.3D). Similar effects were observed using a *dimm*^{dsRNAi} that targets different *dimm* sequences (Table 3S.2). In late Stage 17 embryonic *dimm* mutants, immunoreactivity to the mature amidated FMRFa peptide was profoundly reduced, but the extent to which FMRFa transcript was affected had not been quantified (Allan et al., 2005; Hewes et al., 2003). Here, we find that FMRFa transcript was only modestly downregulated in late Stage 17 embryonic *dimm* mutants to 71.6±3.9% of controls (wild type control n=54, *dimm* mutant n=34 ($p<0.0001$)). Thus, we conclude that *dimm* retains its role in the initiation and maintenance of both FMRFa transcript and mature peptide. Why is the mature peptide more responsive to *dimm*^{dsRNAi} than is the transcript? We postulated that this was due to *dimm*'s regulation of proprotein convertases and peptide amidases in secretory neurons, both of which are required to process the FMRFa prepropeptide into amidated neuropeptides (Allan et al., 2005; Hewes et al., 2006; Park et al., 2008). We tested this in adults by examining expression of peptidylglycine α-hydroxylating monooxygenase after *dimm*^{dsRNAi} induction (PHM). Confirming our hypothesis, *dimm*^{dsRNAi} entirely eliminated PHM immunoreactivity in Tv4 neurons (Figure 3.3E,F). Thus, the maintenance of neuropeptide-processing enzyme expression and biosynthesis of the amidated FMRFa peptide is dependent upon persistent *dimm* function in adult Tv neurons.

3.4.6 dac is necessary for FMRFa maintenance

Previous studies found that FMRFa was only modestly downregulated in *dac* mutants during development (Miguel-Aliaga et al., 2004). In confirmation, we found here that in L1 larvae, FMRFa immunofluorescence per Tv4 neuron was 68.3 ± 8.5% of control (Figure 3.3J; $P<0.02$). We tested *dac* function in adults and found that *dac*^{dsRNAi} dramatically downregulated FMRFa immunoreactivity in adults to 14.8 ± 2.5% of controls as early as A10 ($p<0.0001$) (Figure 3.3G-I). Correspondingly, FMRFa transcript was reduced to 24.9 ± 4.2% of controls ($p<0.0001$ to controls), and this was enhanced in *dac* heterozygotes to 6.5 ± 0.8% ($p<0.001$ to *dac*^{dsRNAi} alone)

(Figure 3S.3). Notably, by A15, FMRFa peptide and transcript were entirely eliminated (not shown). In all cases, we found that Dac immunoreactivity was eliminated (Table 3S.4). Moreover, similar effects were observed using a dac^{dsRNAi} that targets different *dac* sequences (Figure 3S.2). Thus, *dac* appears to be unique amongst the Tv4 subtype transcription network factors in that it assumes an increasingly essential role in maintenance compared to developmental initiation.

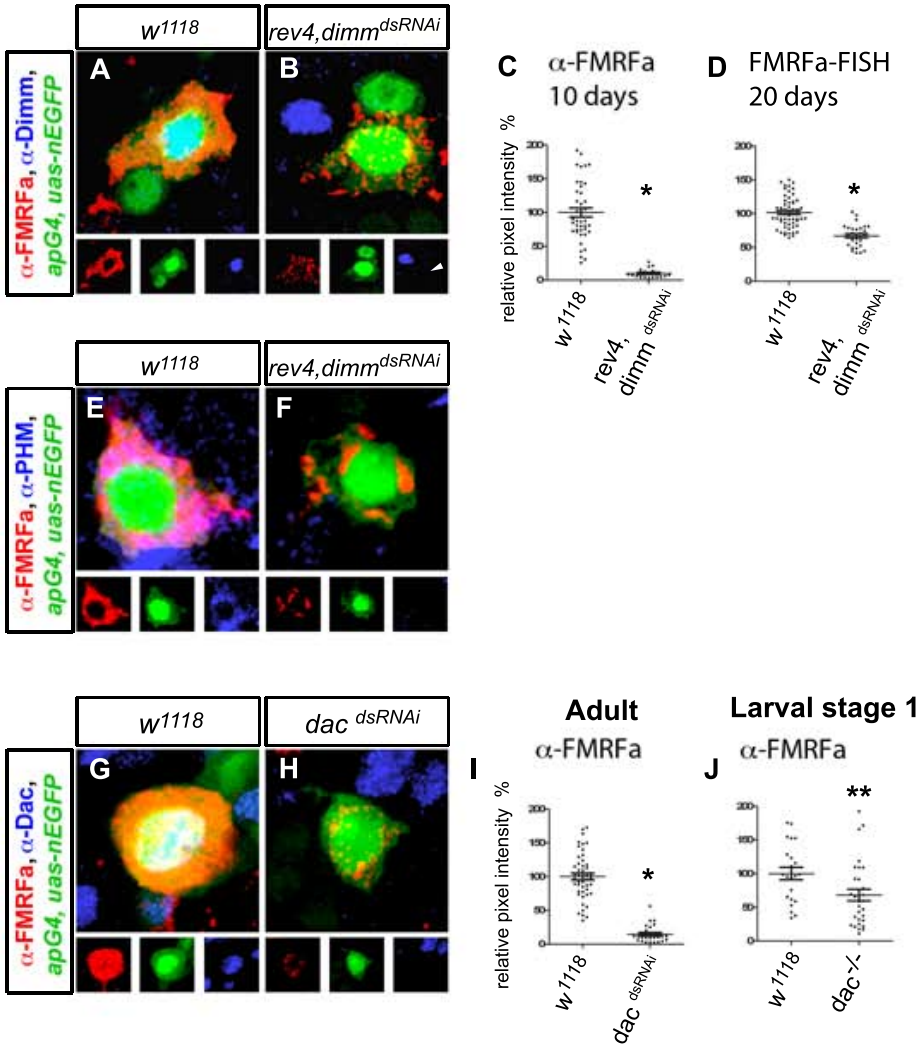

Figure 3.3 *dimm* maintains peptidergic phenotype and *dac* has an enhanced maintenance function.

(**A,B**) Images of adult Tv4 neurons expressing FMRFa peptide (red), ap^{Gal4},*UAS-nlsEGFP* (green) and Dimm (blue) at A10 at 29°C. FMRFa is downregulated and Dimm is lost in $rev4,dimm^{dsRNAi}$ (**B**) compared to w^{1118} control (**A**).

(**C,D**) Quantification of FMRFa peptide (at A10) **(C)** and FMRFa transcript (at A20) **(D)** in individual adult Tv4 neurons at 29°C. (**C**)* p<0.0001 $rev4,dimm^{dsRNAi}$ (n=19) compared to w^{1118} (n=42) control. (**D**)* p<0.0001 $rev4,dimm^{dsRNAi}$ (n=30) compared to w^{1118} (n=58) control.

(**E,F**) Image of Tv4 neurons expressing mature FMRFa peptide (red), ap^{Gal4},*UAS-nlsEGFP* (green) and PHM (blue) in adult Tv4 neurons at A10 at 29°C. PHM is lost in $rev4,dimm^{dsRNAi}$ (n=26) (**E**) compared to w^{1118} control (n=30) (**F**).

(**G,H**) Images of adult Tv4 neurons expressing FMRFa peptide (red), *ap^{Gal4},UAS-nlsEGFP* (green) and Dac (blue) at A10 at 29°C. FMRFa is downregulated and Dac immunostaining is lost in *dac^{dsRNAi}* (**H**) compared to *w^{1118}* control (**G**).

(**I,J**) Quantification of FMRFa peptide in individual adult Tv4 neurons at A10 at 29°C, and in individual L1 larval Tv4 neurons in *dac* null mutants. (**I**) * p<0.0001 *dac^{dsRNAi}* (n=26) compared to *w^{1118}* (n=47) control. (**J**) ** P=0.02 *dac$^{-/-}$* (n=31) compared to *w^{1118}* (n=22)control.

Genotypes**:** *w^{1118}* (*UAS-dicer2/+; ap^{Gal4}/+; tub-Gal80^{TS}, UAS-nEGFP/+*); *rev4,dimm^{dsRNAi}* (*UAS-dicer2/+; ap^{Gal4}/rev4, UAS-dimm^{dsRNAi}; tub-Gal80^{TS}, UAS-nEGFP/+*); *dac^{dsRNAi}* (*UAS-dicer2/+; ap^{Gal4}/UAS-dac^{dsRNAi}; tub-Gal80^{TS}, UAS-nEGFP/+*); *dac$^{-/-}$* (*dac^{3}/dac$^{Df(3L)EXEL\ 7066}$*).

3.4.7 Post-developmental changes to Tv4 subtype transcription network configuration.

The Tv4 subtype transcription network acts through hierarchical and feedforward transcription factor activity, which we refer to here as the network's configuration (summarized in Figure 3.1A,B) (Allan et al., 2005; Baumgardt et al., 2009). Initiation of *grh, dac, sqz* and *col* requires transient *cas* activity (Baumgardt et al., 2009). Expression of *ap* and *eya* requires transient *col* expression (Baumgardt et al., 2007). The induction of *dimm* then requires *eya*, *ap* and *grh* (Allan et al., 2005; Baumgardt et al., 2009; Baumgardt et al., 2007). BMP signaling is dependent upon *eya* (Miguel-Aliaga et al., 2004). Finally, *ap, eya, dimm, dac, sqz, grh* and BMP signaling are all required for FMRFa initiation (Allan et al., 2003; Baumgardt et al., 2009; Benveniste et al., 1998; Hewes et al., 2003; Miguel-Aliaga et al., 2004). This cascade represents a progressive and dynamic set of interactions during Tv4 neuron specification and differentiation. However, for long-term maintenance of subtype gene expression the subtype transcription network presumably resolves into a stable configuration. As *grh*, *cas* and *col* are lost by early L1 (Figure 3S.1), network configuration must change as the remaining transcription factors become independent of those that initiated their expression. However, we wished to ask whether the developmental cross-regulatory interactions between the persisting transcription factors are retained in the adult to help stabilize the network post-developmentally. Thus, we examined the configuration (cross-regulatory interactions) of all Tv4 subtype transcription network factors.

BMP signaling in embryonic Tv4 neurons is dramatically reduced in *eya* mutants (Miguel-Aliaga et al., 2004). We expressed eya^{dsRNAi} in adults until A15 and found that nuclear pMad, an indicator of BMP activity (Allan et al., 2003), was significantly downregulated to 47.9% ± 2.9 of control (Figure 3.4A,B,D). As BMP signaling is required for FMRFa expression in embryos and adults, we asked whether *eya*-dependence of FMRFa in adults is due to reduced BMP signaling. To do this, we simultaneously expressed eya^{dsRNAi} and restored BMP signaling, using constitutively-activated type I BMP-receptors, *thickveins* and *saxophone*. Even though nuclear pMad was robustly activated in all Tv neurons, eya^{dsRNAi}-induced FMRFa downregulation was not rescued (Figure 3.4C,E). Thus, in adults, *eya* independently maintains both BMP signaling and FMRFa expression.

In the embryo, initiation of *dimm* expression in Tv4 is absolutely dependent upon *eya* and *grh* (Baumgardt et al., 2009; Baumgardt et al., 2007) and partially dependent upon *ap* (Allan et al., 2005). As *grh* is not expressed in adult Tv4 neurons, *dimm* maintenance must become independent of *grh*. However, as *eya* and *ap* are retained, we tested their role in *dimm*

maintenance. We expressed ap^{dsRNAi} in adults until A20 using ap^{GAL4} (a strong hypomorphic allele) and found that Dimm immunoreactivity was significantly downregulated to 58.5% ± 6.6 of control (Figure 3.4I-K). In contrast, we found that Dimm expression in adult Tv4 neurons was entirely unaffected by Eya knockdown (Figure 3.4F-H). These date indicate that Dimm becomes independent of Eya in adult Tv4 neurons, even though *eya* expression persists in adults and *eya* is absolutely required for the initiation of Dimm expression (Baumgardt et al., 2007). We conclude that maintenance of *dimm* remains dependent on *ap* but becomes independent of *eya* and *grh* post-developmentally.

We also examined all other potential cross-regulatory relationships within the Tv4 subtype transcription network (Table 3S.3), but found no instances of a transcription factor requiring the presence of another for its maintenance.

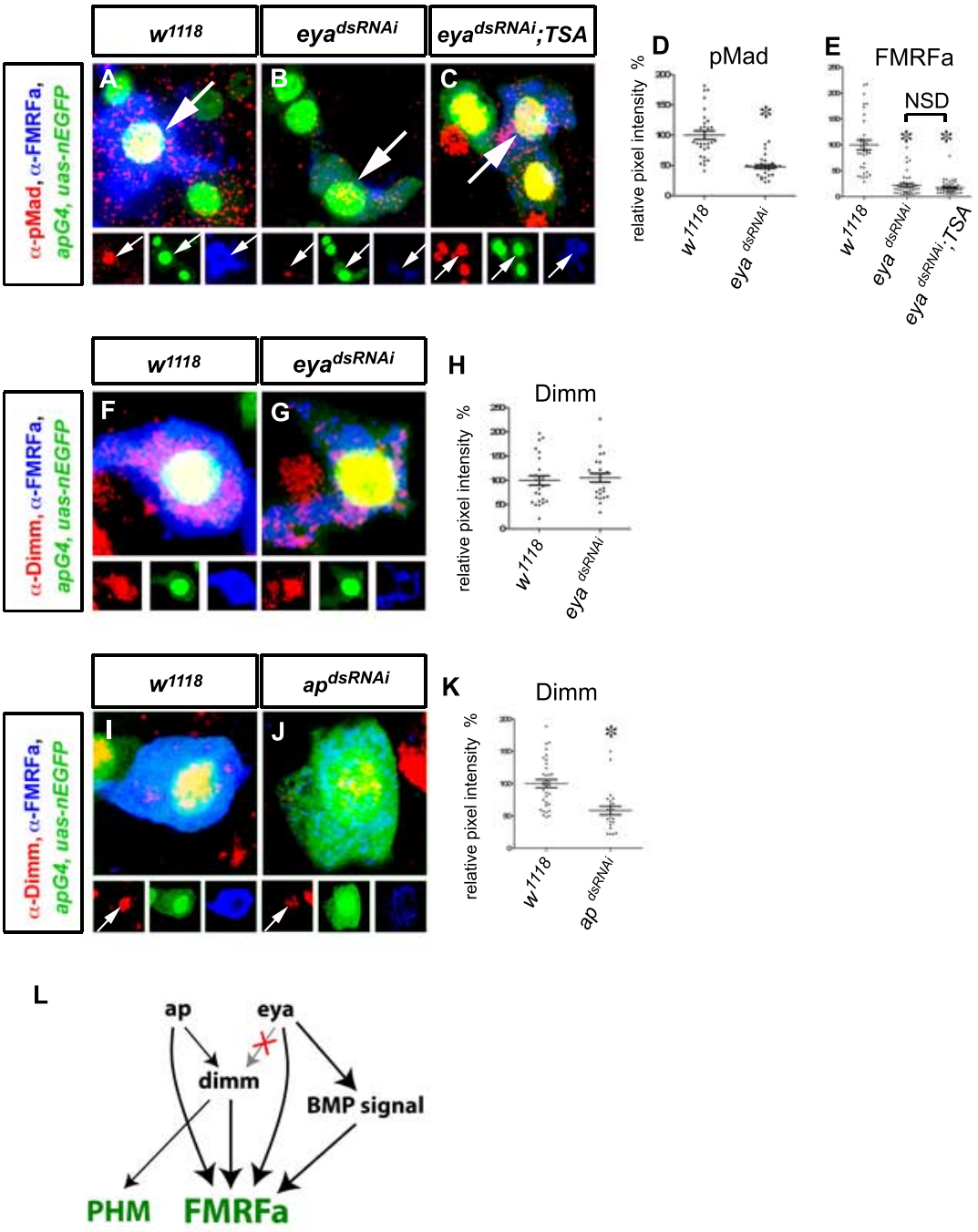

Figure 3.4 Changes in Tv4 network configuration for maintenance.

(**A-E**) *eya* regulates FMRFa independently of BMP signaling. (**A,B**) Nuclear pMad accumulation (red) in Tv4 (arrowhead) was downregulated in eya^{dsRNAi} flies (**B**) compared to w^{1118} control (**A**). (**C**) Expression of eya^{dsRNAi} in the presence of constitutively-activated Thickveins and Saxophone BMP type I receptors (*TSA)* activated pMad accumulation (red) in all Tv neurons (including Tv4; arrowhead) but failed to rescue FMRFa (blue) compared to w^{1118} control. nsd – no significant difference.

(**D**) Quantification of pMad immunoreactivity in Tv4 nucleus in w^{1118} and eya^{dsRNAi} flies.
***** p<0.0001 eya^{dsRNAi} (n=30) compared to w^{1118} (n=30) control.
(**E**) Quantification of FMRFa immunoreactivity in Tv4 in w^{1118}, eya^{dsRNAi}, and eya^{dsRNAi}; *TSA*.
***** p<0.0001 eya^{dsRNAi} (n=38) and eya^{dsRNAi}; *TSA* (n=42) compared to w^{1118} (n=34) control.
NSD no significant difference eya^{dsRNAi} compared to eya^{dsRNAi}; *TSA*.
(**F-H**) *eya* does not regulate *dimm* in adult Tv4. *dimm* (red) in Tv neurons (blue) is maintained in eya^{dsRNAi} (**G**) compared to w^{1118} control (**F**). (**H**) Quantification of Dimm immunoreactivity in Tv4. There is no significant difference between eya^{dsRNAi} (n=24) and w^{1118} (n=25) control p=0.67.
(**I-K**) *ap* regulates *dimm* in adult Tv4. *dimm* (red) in Tv neurons (blue) is downregulated in ap^{dsRNAi} (**J**) compared to w^{1118} control (**I**). (**K**) Quantification of Dimm immunoreactivity in Tv4.
***** p<0.0001 ap^{dsRNAi} (n=24) compared to w^{1118} (n=35).
(**L**) Model depicting configuration changes of *ap, eya, dimm* and BMP signaling from embryonic to adult Tv4. Notably, the dependence of *dimm* on *eya* expression is not maintained (X).

Genotypes: w^{1118} (**A-K**) (*UAS-dicer2/+;* ap^{Gal4}*/+;* $tub\text{-}Gal80^{TS}$*, UAS-nEGFP/+*); eya^{dsRNAi} flies (**B,G**) (*UAS-dicer2/UAS-*eya^{dsRNAi}*;* ap^{Gal4}*/+;* $tub\text{-}Gal80^{TS}$*, UAS-nEGFP/+*); eya^{dsRNAi}*; TSA* flies (**C**) (*UAS-dicer2/UAS-*eya^{dsRNAi}*;* ap^{Gal4}*/UAS-*tkv^{A}*, UAS-*sax^{A}*;* $tub\text{-}Gal80^{TS}$*, UAS-nEGFP/+*). ap^{dsRNAi} flies (**J**) (*UAS-dicer2/+;* ap^{Gal4}*;* $tub\text{-}Gal80^{TS}$*, UAS-nEGFP/UAS-*ap^{dsRNAi}).

3.4.8 Maintenance of subtype transcription network output but not configuration in mature Tv1 neurons.

Is the maintained role of the Tv4 subtype transcription network common to other subtype transcription networks? To determine this, we examined the output and configuration of the adult Tv1 subtype transcription network. In the neuroblast lineage that gives rise to the Tv1 neuron, *cas* induces *col*. Upon birth of the postmitotic Tv1 neuron, *col* initiates *eya* and *ap* expression. Initiation of *dimm* is then absolutely dependent on each of *col, ap* and *eya*. Then all four regulators are required for Nplp1 initiation (Baumgardt et al., 2009; Baumgardt et al., 2007).

In adult Tv1 neurons, *col, eya, ap* and *dimm* were maintained (Figure 3.1). Induction of col^{dsRNAi} (Baumgardt et al., 2007) at A1 reduced Nplp1 to 10.7±1.7% of control by A15 (Figure 3.5A-C). We also found that induction of ap^{dsRNAi}, eya^{dsRNAi} or $dimm^{dsRNAi}$ significantly reduced Nplp1 expression levels to 22.7 ± 2.7%, 45.3 ± 4.2%, and 19.0 ±2.4% of control, respectively (all $p<0.0001$ to control) (Figure 3.5G,J,O). We verified that Col, Eya and Dimm immunoreactivity in Tv1 were eliminated by their respective *dsRNAi* (Table 3S.4). In addition we found that $dimm^{dsRNAi}$ also eliminated PHM expression in Tv1 neurons (Figure 3.5N). Thus, the Tv1 subtype transcription network is required to maintain the expression of Tv1-specific terminal differentiation gene expression.

Next, we examined the configuration of the adult Tv1 subtype transcription network. Intriguingly, even through col is essential for *eya*, *ap* and *dimm* expression in the embryo, col^{dsRNAi} had no effect on *dimm, ap,* or *eya* expression in Tv1 (Figure 3.5D-F). In contrast, expression of either ap^{dsRNAi} or eya^{dsRNAi} led to a significant downregulation of Dimm levels in Tv1 to 52.4 ± 5.1% and 42.9 ± 3.7% (Figure 3.5H,K). These data are intriguing with regards to *eya* because it regulation of *dimm* was lost in adult Tv4 neurons. Genetic studies in the embryo had established that *col, ap, eya* and *dimm* act non-redundantly to initiate Nplp1 expression during development (Baumgardt et al., 2009). We tested whether these transcription factors also act non-redundantly in the adult. As col^{dsRNAi} dramatically downregulated Nplp1 but did not affect *ap*, *eya* or di*m*m, we conclude that *col* acts non-redundantly in this case. However, *dimm* is dependent on both *ap* and *eya* in Tv1. Therefore, to test for redundancy between these transcription factors, we restored *dimm* (*UAS-dimm*) in either ap^{dsRNAi} or eya^{dsRNAi} backgrounds. *UAS-dimm* expression was found to only partially rescue Nplp1 expression in an ap^{dsRNAi} background, from 22.7 ± 2.7%, to 57.0 ± 5.4% ($p<0.0001$ compared to ap^{dsRNAi} and also w^{1118} control) (Figure 3.5I). However, *UAS-dimm* expression failed to rescue Nplp1 expression in an

eya^{dsRNAi} background (Figure 3.5L). Thus, as during development, all regulators are required combinatorially for normal Nplp1 expression in adult Tv1 neurons.

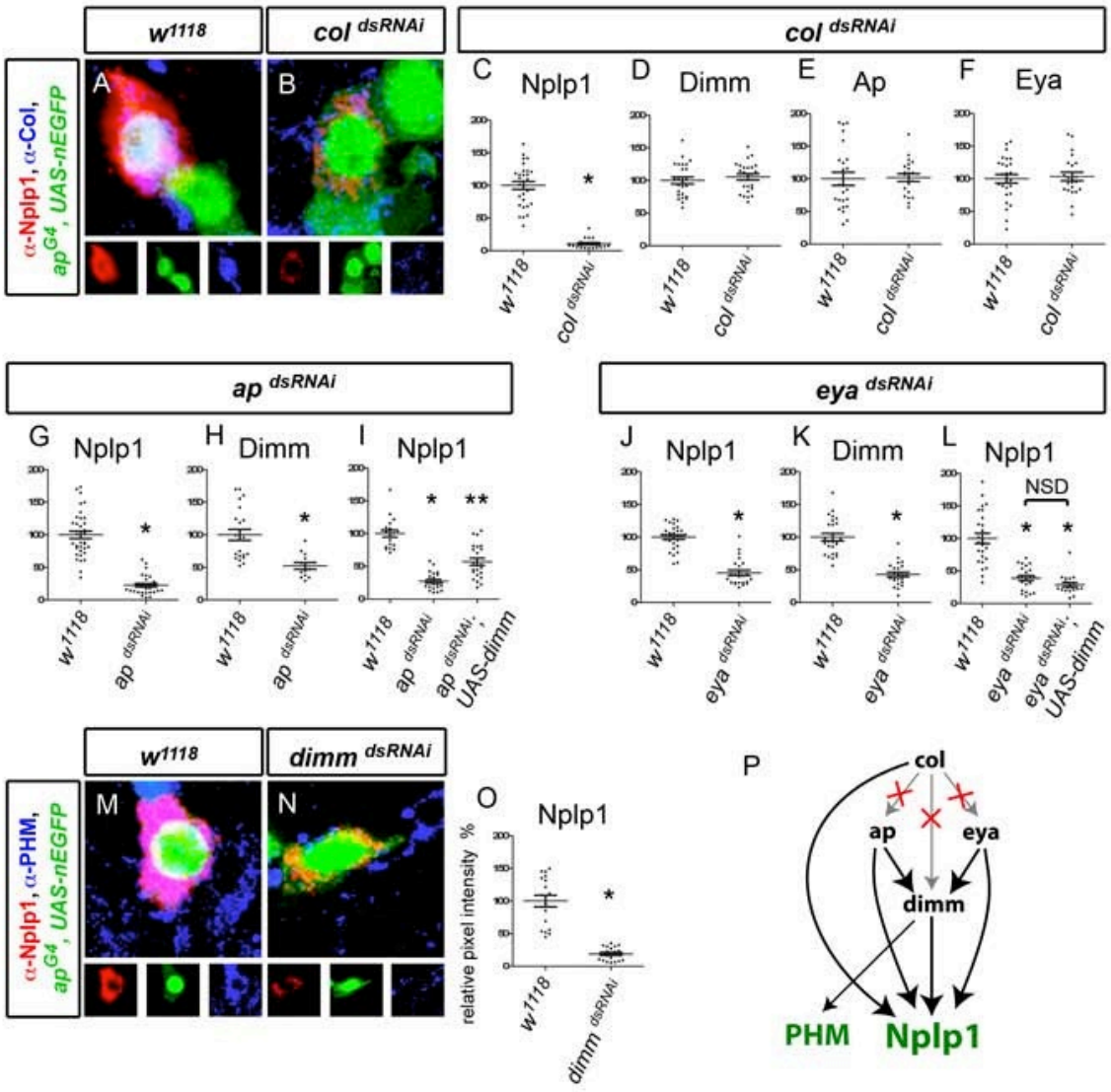

Figure 3.5 Transcriptional regulation of Nplp1 in adult Tv1 neurons.

(**A-F**) col^{dsRNAi} downregulated Nplp1 but not *ap*, *eya*, or *dimm* in adult Tv1.

(**A,B**) Expression of Nplp1 (red), ap^{Gal4},*UAS-nlsEGFP* (green) and Col (blue) in adult Tv4 neurons at A10 at 29°C. Col expression is lost and Nplp1 is downregulated in col^{dsRNAi} (**B**) compared to w^{1118} control (**A**).

(**C**) Quantification of Nplp1 immunoreactivity in w^{1118} (n=29) and col^{dsRNAi} (n=20) at A10 at 29°C. (**D-F**) col^{dsRNAi} (**D** n=24; **E** n=22; **F** n=21) did not affect Dimm immunoreactivity (**D**), ap^{Gal4},*UAS-nEGFP* fluorescence (**E**), or Eya immunoreactivity (**F**) compared to w^{1118} (**D** n=25; **E** n=27; **F** n=24)at A10 at 29°C.

(G-I) ap^{dsRNAi} significantly reduced Nplp1 (**G**) and Dimm (**H**) immunoreactivity compared to w^{1118} at A20 at 29°C. (**G**) * p< 0.0001 ap^{dsRNAi} (n=30)compared to w^{1118} control (n=36). (**H**) * p<

0.001 ap^{dsRNAi} (n=13)compared to w^{1118} control (n=22)

(I) Dimm restoration *(UAS-dimm)* in ap^{dsRNAi} background only partially rescued Nplp1 downregulation at A15 at 29°C. * p< 0.0001 ap^{dsRNAi} (n=22)compared to w^{1118} control (n=19). ** p< 0.0001 ap^{dsRNAi}*; UAS-dimm* (n=23) compared to ap^{dsRNAi} and w^{1118} control.

(J-L) eya^{dsRNAi} significantly reduced Nplp1 (**J**) and Dimm (**K**) immunoreactivity compared to w^{1118} at A10 at 29°C. **(L)** Dimm restoration *(UAS-dimm)* in eya^{dsRNAi} background failed to rescue Nplp1 immunoreactivity at A15 at 29°C. * p< 0.0001 eya^{dsRNAi} (**J** n=23; **K** n=23; **L** n=22) and eya^{dsRNAi}; *UAS-dimm* (n=19) compared to w^{1118} control (**J** n=30; **K** n=25; **L** n=26). NSD no significant difference eya^{dsRNAi} compared to eya^{dsRNAi}; *UAS-dimm.*

(**M-O**) $dimm^{dsRNAi}$ downregulated Nplp1 and PHM in adult Tv1.

(**M,N**) Tv1 neurons expressing Nplp1 (red), ap^{Gal4}*,UAS-nlsEGFP* (green) and PHM (blue) in adult Tv4 neurons at A10 at 29°C. Nplp1 is downregulated and PHM is lost in $dimm^{dsRNAi}$ (n=18) (**M**) compared to w^{1118} control (n=15) (**N**).

(O) $dimm^{dsRNAi}$ significantly reduced Nplp1 immunoreactivity compared to w^{1118} at A10 at 29°C. * p< 0.0001 $dimm^{dsRNAi}$ (n=19)compared to w^{1118} control (n=18)

(P) Model depicting regulation of Nplp1 and PHM and the configuration changes between *ap, eya, dimm*, and *col* from embryonic to adult Tv1. Notably, *col* no longer regulates *dimm*, *eya* or *ap* expression (denoted by X).

Genotypes: w^{1118} (*UAS-dicer2/+;* ap^{Gal4}*/+;* $tub\text{-}Gal80^{TS}$*, UAS-nEGFP/+*); col^{dsRNAi} (*UAS-dicer2/+;* ap^{Gal4}*/+;* $tub\text{-}Gal80^{TS}$*, UAS-nEGFP/UAS-*col^{dsRNAi}*);* ap^{dsRNAi} *(UAS-dicer2/+;* ap^{Gal4}*/+;* $tub\text{-}Gal80^{TS}$*, UAS-nEGFP/UAS-*ap^{dsRNAi}); ap^{dsRNAi}*; UAS-dimm (UAS-dicer2/+;* ap^{Gal4}*/UAS-dimm;* $tub\text{-}Gal80^{TS}$*, UAS-nEGFP/UAS-*ap^{dsRNAi}); eya^{dsRNAi} (*UAS-dicer2/UAS-*eya^{dsRNAi}*;* ap^{Gal4}*/+;* $tub\text{-}Gal80^{TS}$*, UAS-nEGFP/+*); eya^{dsRNAi}; *UAS-dimm* (*UAS-dicer2/UAS-*eya^{dsRNAi}*;* ap^{Gal4}*/UAS-dimm;* $tub\text{-}Gal80^{TS}$*, UAS-nEGFP/+*); $dimm^{dsRNAi}$ (*UAS-dicer2/+;* ap^{Gal4}*/UAS-*eya^{dsRNAi}*;* $tub\text{-}Gal80^{TS}$*, UAS-nEGFP/+*); *rev4,*$dimm^{dsRNAi}$ (*UAS-dicer2/+;* ap^{Gal4}*/rev4, UAS-*$dimm^{dsRNAi}$*;* $tub\text{-}Gal80^{TS}$*, UAS-nEGFP/+*).

3.4.9 Adult neurons respond predictably to ectopic reconstitution of subtype transcription network activity.

During development, late-acting subtype transcription networks can override earlier-acting transcriptional codes to dominantly activate ectopic expression of their target genes and/or subtype identity (Baumgardt et al., 2009). For example, in embryos over-expression of *col* in all Tv neurons ectopically activated Nplp1 in Tv4, presumably reconstituting the *col/ap/eya/dimm* Tv1 subtype transcription network (Baumgardt et al., 2007). Interestingly, this did not disrupt native FMRFa expression in Tv4 neurons, nor its known subtype transcription network profile. Here, we verify these data in embryos (Figure 3.6A,C). Additionally, we demonstrate here that the reciprocal subtype transcription network reconstitution, *dac* and BMP activation in all Tv neurons, is sufficient to initiate FMRFa expression in embryonic Tv1 neurons. Interestingly, we found that this also occurred without a concomitant disruption of Nplp1 expression in Tv1 (Figure 3.6A,B).

We tested whether Tv1 and Tv4 subtype transcription networks retained this capacity in adult neurons. This would prove that these subtype transcription networks are capable of inducing expression of their target gene in a cell that never had expressed that neuropeptide. We found that activation of BMP signaling and *dac* in adult Tv1 neurons for 5 days robustly activated ectopic FMRFa expression in 100% of Tv1 neurons (Figure 3.6D,E), without affecting Nplp1 in Tv1. We next ectopically expressed *col* in adult Tv neurons, however this failed to induce ectopic Nplp1 expression (n=21 Tv4 neurons) (data not shown). However, co-expression of *col* and *dimm* in adult Tv neurons was sufficient to trigger ectopic Nplp1 expression in 100% of Tv4 neurons (n=42) (Figure 3.6D,F). These data suggest that subtype transcription networks are sufficient to initiate pertinent target gene expression, even in adult neurons that had never expressed the gene.

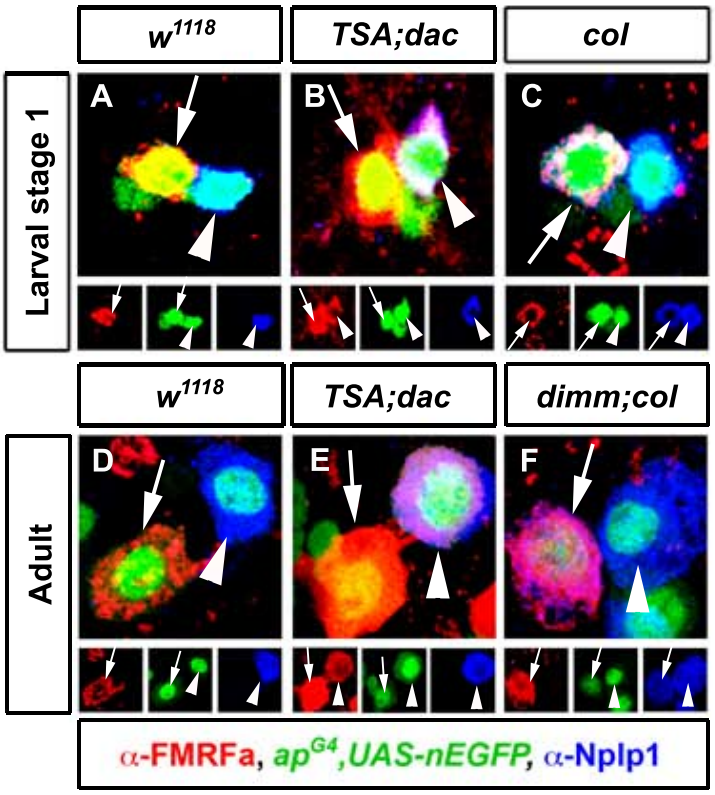

Figure 3.6 Differentiation networks have different abilities to activate ectopic gene expression in adult neurons.

(**A-F**) Representative images of Tv4 neurons (arrows) and Tv1 neurons (arrowheads) at larval stage 1 (**A-C)** and adults (**D-F**).

(**A,B,D,F**) Misexpression of *dac* and constitutively-activated Thickveins and Saxophone BMP receptors (*TSA)* In embryos (**B** n=33) and for 5 days in adults (**E** n=31),initiated ectopic FMRFa expression (arrowhead, red) in Tv1 (identified with Nplp1, blue).

(**A,C**) Misexpression of *col* in embryonic Tv neurons using ap^{Gal4} initiates ectopic Nplp1 expression (arrow, blue) in Tv4 neurons (identified with FMRFa, red) (**C** n=24).

(**D,F**) Misexpression of *col* and upregulation of *dimm* for 5 days in adults using ap^{Gal4} initiates ectopic Nplp1 expression (arrow, blue) in Tv4 neurons (identified with FMRFa, red) (**F** n=42).

Genotypes: (**A-C**) Larval stage 1 w^{1118} (ap^{Gal4}/+; *UAS-nEGFP*/+); *TSA;dac* (ap^{Gal4}/ $UAS\text{-}tkv^{A}$, $UAS\text{-}sax^{A}$; *UAS-nEGFP/UAS-dac*); *col* (ap^{Gal4}/*UAS-col; UAS-nEGFP*/+).

(**D-F**) Adult w^{1118} (ap^{Gal4}/+; $tub\text{-}Gal80^{TS}$, *UAS-nEGFP*/+); *TSA;dac* (ap^{Gal4}/ $UAS\text{-}tkv^{A}$, $UAS\text{-}sax^{A}$; $tub\text{-}Gal80^{TS}$, *UAS-nEGFP/UAS-dac*); *dimm;col* (ap^{Gal4}/*UAS-col;* $tub\text{-}Gal80^{TS}$, *UAS-nEGFP/ UAS-dimm*).

3.5 DISCUSSION

Our data provide novel insight supporting the view of Blau and Baltimore (1991) that cellular differentiation is a persistent process that requires active maintenance, rather than being passively 'locked-in' or unalterable. We make two primary findings regarding the long-term maintenance of neuronal identity. First, we find that all known developmental transcription factors acting in postmitotic Tv1 and Tv4 neurons to initiate the expression of subtype terminal differentiation genes are then persistently required to maintain their expression. Second, we found that key cross-regulatory developmental relationships that initiated the expression of certain transcription factors were no longer required for their maintained expression in adults.

In this study all transcription factors implicated in the initiation of subtype-specific neuropeptide expression in Tv1 and Tv4 neurons were found to maintain subtype terminal differentiation gene expression in adults (summarized in Figure 3.7). In Tv1, *col, eya, ap* and *dimm* are required for Nplp1initiation during development (Figure 3.1A). In this study, knockdown of each transcription factor in adult Tv1 neurons was shown to dramatically downregulate Nplp1. In Tv4 neurons, FMRFa initiation during development requires *eya, ap, sqz, dac, dimm* and retrograde BMP signaling (Figure 3.1B). Together with our previous work showing that BMP signaling maintains FMRFa expression in adults (Eade and Allan, 2009), we now demonstrate that all six regulatory inputs are required for FMRFa maintenance. Most regulators, except for *dac*, also retained their relative regulatory input for FMRFa and Nplp1 expression. In addition, individual transcription factors also retained their developmental subroutines. For example, as previously found for its developmental role (Allan et al., 2005; Miguel-Aliaga et al., 2004; Park et al., 2008), *dimm* was required in adults to maintain PHM (independently of other regulators) and FMRFa/Nplp1 expression (combinatorially with other regulators).

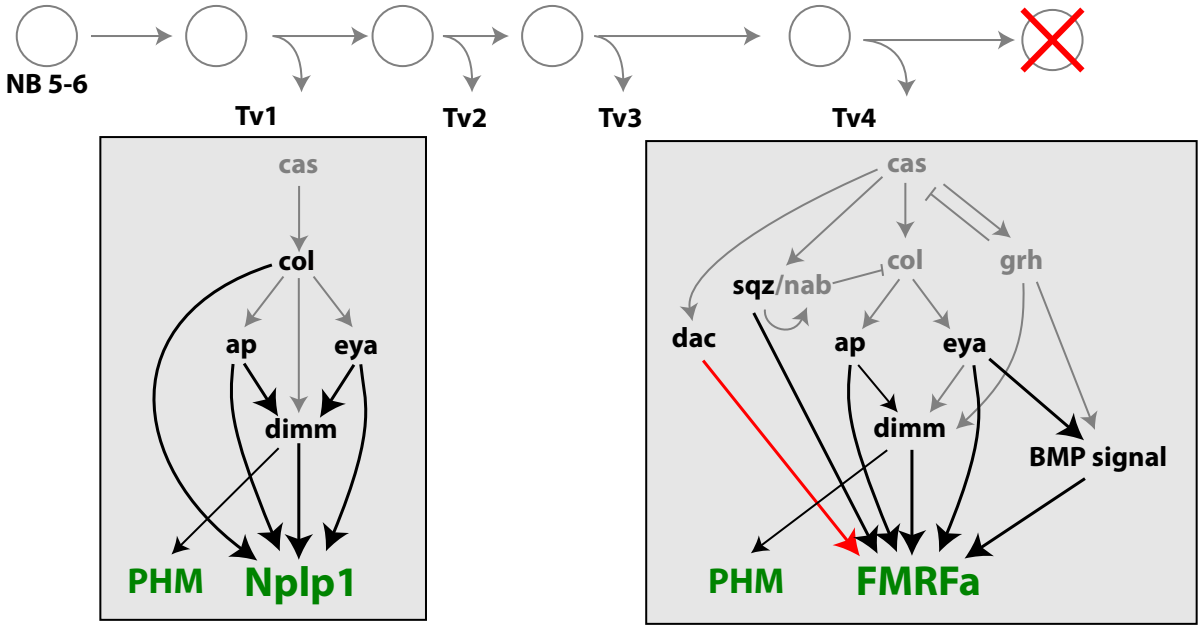

Figure 3.7 Summary of changes in subtype transcription network configuration between initiation and maintenance of subtype identity.

Following terminal differentiation, Tv1 and Tv4 maintain expression of the terminal differentiation genes, PHM as well as Nplp1 and FMRFa, respectively (green). The expression of transcription factors in grey text is lost by larval stages, all transcription factors in black text are retained in adult Tv1/4 neurons. Notably, all black transcription factors have been implicated in differentiation of subtype-specific neuropeptide expression in postmitotic Tv1/4 neurons. In contrast, all grey transcription factors have been implicated in the specification of Tv neuron subtype fates, and not in direct differentiation of subtype neuropeptide gene expression. All remaining transcription factors are required for maintained Nplp1 and FMRFa expression (black arrows), but their cross-regulatory relationships are mostly lost in adults (grey arrows). The only transcription factors that maintain their embryonic configuration are *ap* and *eya's* requirement for *dimm* in Tv1, and *eya's* continued regulation of active BMP signaling in Tv4. Dac plays an enhanced role in FMRFa expression in adult Tv4 neurons (red arrow).

The few genetic studies that test a persistent role for developmental transcription factors support their role in initiating and maintaining terminal differentiation gene expression. In *C.elegans*, where just one or two transcription factors initiate most neuronal subtype-specific terminal differentiation genes, they then also appear to maintain their target terminal differentiation genes.

In ASE and dopaminergic neurons respectively, CHE-1 and AST-1 initiate and maintain expression of pertinent subtype-specific terminal differentiation genes through larval stages (Etchberger et al., 2007; Flames and Hobert, 2009). In vertebrate neurons, where increased numbers of combinatorially-acting transcription factors initiate gene expression in a subtype-specific manner, certain transcription factors have been demonstrated to be required for maintenance of subtype identity. These are Hand2 that initiates and maintains tyrosine hydroxylase and dopa β-hydroxylase expression in mouse sympathetic neurons (Schmidt et al., 2009), Pet-1, Gata3 and Lmx1b for serotonergic marker expression in mouse serotonergic neurons (Liu et al., 2010; Song et al., 2011), and Nurr1 for dopaminergic marker expression in murine dopaminergic neurons (Kadkhodaei et al., 2009).

However, while these studies confirm a role for certain developmental transcription factors in subtype maintenance, it had remained unclear whether the elaborate developmental subtype transcription networks observed in *Drosophila* and vertebrates are retained in their entirety for maintenance, or whether they become greatly simplified. Our analysis of all known subtype transcription network factors in Tv1 and Tv4 neurons now indicates that the majority of a developmental subtype transcription network is indeed retained and required for maintenance. Why would an entire network of transcription factors be required to maintain subtype-specific gene expression? The combinatorial nature of subtype-specific gene expression entails cooperative transcription factor binding at clustered cognate DNA sequences and/or synergism in their activation of transcription. In such cases, our data would indicate that this cannot be dispensed with for maintaining terminal differentiation gene expression in mature neurons.

How the transcription factors of the subtype transcription networks are maintained is less well understood. An elegant model has emerged from studies in *C.elegans*, wherein transcription factors stably auto-maintain their own expression and also maintains the expression of subtype terminal differentiation genes (Hobert, 2011). The transcription factor CHE-1 is a key transcription factor that initiates and maintains subtype identity in ASE neurons. CHE-1 binds selectively to a cognate DNA sequence motif (the ASE motif) in subtype terminal differentiation genes as well as in its own c*i*s-regulatory region. Notably, a promoter fusion of the *che-1* transcription factor failed to express in *che-1* mutants, indicative of CHE-1 autoregulation. (Etchberger et al., 2009; Etchberger et al., 2007) Similar observations were made for *ast-1* (Flames and Hobert, 2009), and for the cooperatively-acting TTX-3 and CEH-10 transcription factors in AIY neurons (Bertrand and Hobert, 2009). Thus, subtype maintenance in *C.elegans* is anchored by auto-maintenance of the transcription factors that initiate and maintain terminal

differentiation gene expression.

In contrast, all available evidence in Tv1 and Tv4 neurons fails to support such a role for autoregulation. An *ap* reporter (*apC-τ-lacZ*) is expressed normally in *ap* mutants (Allan et al., 2003; Lundgren et al., 1995), and in this study ap^{dsRNAi} did not alter ap^{GAL4} reporter activity. Moreover, *col* transcription was unaffected in *col* mutants that express a non-functional Col protein (Baumgardt et al., 2007). This leaves unresolved the question of how the majority of the transcription factors are stably maintained. For transcription factors that are initiated by transiently expressed inputs, a shift to distinct maintenance mechanisms have been invoked and in certain cases shown (Bertrand and Hobert, 2009). In this study, this was found for the loss of *cas* expression in Tv1 (required for *col* initiation) and the loss of *cas, col* and *grh* in Tv4 (required for *eya, ap, dimm, sqz, dac* initiation). However, we were surprised to find that the cross-regulatory relationships between persistently-expressed transcription factors was also significantly altered in adults. Notably, *eya* initiated but did not maintain *dimm* in Tv4. In Tv1, *col* initiated but did not maintain *eya*, *ap* or *dimm*. This was particularly unexpected as *eya* remained critical for FMRFa maintenance and *col* remained critical for Nplp1 maintenance. Indeed, although we tested for cross-regulatory interactions between all transcription factors in both the Tv1 and Tv4 subtype transcription networks, only Dimm was found to remain dependent upon its developmental input; Eya and Ap in Tv1 as well as Ap in Tv4. However, even in this case, the regulation of Dimm was changed; it no longer required *eya* in Tv4, and in Tv1 it no longer required *col*, in spite of the fact that both inputs are retained in these neurons. We anticipate that these findings will be pertinent for many *Drosophila* and vertebrate neurons, which exhibit high complexity in their subtype transcription networks (Alavian et al., 2008; di Sanguinetto et al., 2008). Indeed, recent evidence has found that in murine serotonergic neurons, the initiation of Pet-1 requires Lmx-1b, but ablation of Lmx-1b in adults does not perturb the maintenance of Pet-1 expression (Song et al., 2011).

Ongoing work is aimed at determining a potential role for autoregulation for the other factors in the Tv1/Tv4 subtype transcription networks. However, we are also pursuing three potentially overlapping models for subtype transcription network maintenance. First, regulators may act increasingly redundantly upon one another. Second, unknown regulators may become increasingly sufficient for transcription factor maintenance. Third, transcription factors may be maintained by dedicated maintenance mechanisms, as been shown for the role of trithorax group genes in the maintenance of Hox genes and Engrailed (Breen et al., 1995; Mihaly et al., 2006). Moreover, chromatin modification is undoubtedly involved and likely required to maintain high-

level transcription of Tv1 transcription factors as well as FMRFa, Nplp1 and PHM. However, the extent to which these are instructive as opposed to permissive has yet to be established (Ptashne, 2007). In this light, it is intriguing that MYST-HAT complexes, in addition to the subtype transcription factors Che-1 and Die-1, are required for maintenance of ASE-Left subtype identity in *C.elegans* (Song et al., 2011).

Taken together, our studies have identified two apparent types of maintenance mechanism that are operational in adult neurons. On one hand, there are sets of genes that are maintained by their initiating set of transcription factors. These include the terminal differentiation genes and the transcription factor *dimm*. On the other, most transcription factors appear to no longer require regulatory input from their initiating transcription factor(s). Further work will be required to better understand whether these differences represent truly distinct modes of gene maintenance or reflect a lack of understanding of the regulatory input onto these transcription factors at initiation and maintenance. One issue worth considering here is that the expression of certain terminal differentiation genes in neurons can be plastic throughout life, with changes commonly occurring in response to a developmental switch or physiological stimulus (Borodinsky et al., 2004; Sprecher and Desplan, 2008; Xu et al., 2005). However, FMRFa, Nplp1 and PHM are stably expressed at high levels in Tv1/4 neurons, and we have not found any conditions that alter their expression throughout life. Thus, we consider these to be stable terminal differentiation genes akin to serotonergic or dopaminergic markers in their respective neurons that define those cells' functional identity and, where tested, are actively maintained by their developmental inputs (Kadkhodaei et al., 2009; Liu et al., 2010). Tv1/4 neurons undoubtedly express a battery of terminal differentiation genes, and sets of unknown transcription factors are likely required for their subtype-specific expression. We consider subtype transcription networks to encompass all regulators required for differentiating the expression of all subtype-specific terminal differentiation genes. Further, we view differentiation of subtype identity as the completion of a multitude of distinct gene regulatory events in which each gene is regulated by a subset of the overall subtype transcription network. As highly restricted terminal differentiation genes expressed in Tv1 and Tv4 neurons, we believe that Nplp1, FMRFa and PHM provide a suitable model for the maintenance of overall identity, with the understanding that other unknown terminal differentiation genes expressed in Tv1 and Tv4 may not be perturbed by knockdown of the transcription factors tested herein. In the future, it will be important to incorporate a more comprehensive list of regulators and terminal differentiation genes for each neuronal subtype. However, we believe that the principles uncovered here for FMRFa, Nplp1 and PHM

maintenance will hold for other terminal differentiation genes.

Finally, we propose that the active mechanisms utilized for maintenance of subtype differentiation represent an Achilles heel that renders long-lived neurons susceptible to degenerative disorders. Nurr1 ablation in adult mDA neurons reduced dopaminergic markers and promoted cell death (Kadkhodaei et al., 2009). Notably, Nurr1 mutation is associated with Parkinson's disease (Grimes et al., 2006), and its downregulation is observed in Parkinson's disease mDA neurons (Le et al., 2008). Adult mDA are also susceptible to degeneration in *foxa2* heterozygotes, another regulator of mDA neuron differentiation that is maintained in adult mDA neurons (Kittappa et al., 2007). Studies in other long-lived cell types draw similar conclusions. Adult conditional knockout of Pdx1 reduced insulin and β-cell mass (Holland et al., 2005; Lottmann et al., 2001) and, importantly, heterozygosity for *Pdx1* leads to a rare monogenic form of non-immune diabetes, MODY4 (Ahlgren et al., 1998). Similarly, *NeuroD1* haploinsufficiency is linked to MODY6 (Malecki et al., 1999) and adult ablation of NeuroD in β-islet cells results in β-cell dysfunction and diabetes (Gu et al., 2010). These data, together with our results here, underscore the need to further explore the transcriptional networks that actively maintain subtype identity, and hence the function, of adult and aging cells.

4. DISCUSSION

The diversification of cellular subtype identity during development is directed by combinatorial codes of transcription factors and intersecting signaling pathways that regulate subtype-specific TDG expression profiles. This thesis directly tests the requirement of developmental transcription factors and target-derived retrograde signaling in the persistent maintenance of subtype specific TDG expression profiles in the mature nervous system. Using a highly characterized model for neuronal developmental, the Tv cluster neurons in *Drosophila,* I have established one of the most comprehensive transcriptional models to date for the maintenance of cellular subtype identity.

During development, lineage progression towards ever increasing cellular diversity is often viewed as a ratcheted mechanism of irreversible steps resulting in the differentiation of cell subtype identities. From this viewpoint, terminally differentiated cells are considered irreversibly 'locked' into their identity. In a perspective review article Blau and Baltimore postulated that a cell's identity, or differentiated status, requires persistent active regulation, rather than lapsing into a passive 'locked-in' state (Blau and Baltimore, 1991). Since then, genetic studies have made it increasingly clear that cells require constant input from developmental cues to retain their identity and that terminal differentiation is a plastic cellular state. These findings carry with them profound implications. While it suggests that degradation of the active maintenance mechanisms may contribute to progressive degeneration of cell identity and function, it also suggests that gene regulation in mature cells may be sufficiently plastic for therapeutic intervention.

4.1 TV CLUSTER NEURONS AS A MODEL SYSTEM

A number of characteristics of the Tv cluster neurons make them a particularly well-suited model for the characterization of transcriptional maintenance networks in mature neurons. Primarily, the Tv neuronal cluster is one of the most comprehensively studied models for developmental transcriptional networks that specify and differentiate neuronal subtype identity. Moreover, the transcriptional complexity evident in Tv neuron development is more reflective of the known complexity of vertebrate systems than is the well-characterized neuronal models of *C. elegans*. Although the vertebrate nervous system has multiple well-characterized developmental transcriptional networks for neuronal lineages, it is considerably more difficult to analyze an entire transcriptional network. The use of the TARGET system in *Drosophila* for conditional manipulation of multiple transcription factors and a retrograde signaling cascade has allowed me

to rapidly address fundamental concepts of transcriptional maintenance by complex networks. Recent work in well-defined vertebrate models has utilized inducible Cre recombinase to excise targeted genes flanked by LoxP sites to similarly test the persistent requirement of transcription factors. This is a powerful genetic tool but has only been used to address individual transcription factors in large neuronal populations. Unlike Tv neurons, where individual neurons can be reproducibly identified and manipulated in every animal tested, these large populations could contain as yet undefined subpopulations with subtly different transcriptional circuitries. Indeed, work in serotonergic raphe neurons and midbrain dopaminergic neurons makes it clear that such subtype diversity is observed (Kadkhodaei et al., 2009; Liu et al., 2010).

Another distinct advantage of *Drosophila* in addressing neuronal maintenance is that following insult to transcription factors expression or disruption of signaling pathways *Drosophila* neurons do not appear to undergo cell death. Thus, we have no difficulty in separating cell death processes from specific loss of gene expression and identity. This issue has proven to be a considerable hurdle in testing the persistent requirement of target-derived signals and certain transcription factors for continued TDG expression, where target-derived signals may be required for survival and removal of transcription factors can result in apoptosis.

In vertebrate and invertebrate models, an increasing body of work is emerging that aims to address how transcriptional networks maintain cell subtype identity. These studies will undoubtedly unveil the underlying transcriptional mechanisms that maintain cell subtype identity for cells from all germ layers. My work provides a strong foundation for increasingly focused ongoing work.

4.2 PERSISTENT REQUIREMENT OF TARGET DERIVED SIGNALING

In Chapter 2, I directly address the persistent requirement of retrogradely trafficked BMP signaling for the maintenance of FMRFa expression. In section 2.5.3, I discuss our findings in the context of other models in the field and how disruption of persistent retrograde targeting as a potential mechanism for neuronal dysfunction in neurodegenerative diseases. In the following two sections, I shall discuss certain caveats in interpretations of experiments performed in chapter 2, and the implications of this work that will direct ongoing and future work.

4.2.1 Caveats to experimental approaches or interpretations

A major caveat to this model is our lack of understanding of the axon targeting of the Tv4 neuron in the adult and the unknown source of BMP ligand, Glass bottom boat (Gbb) that

acts at the Tv4 axonal terminal. In data that is not presented, I had found that the axon of the Tv4 neuron remodels during metamorphosis (Truman, 1990). Initially, in late embryonic and larval stages, the axon of Tv4 neuron exclusively innervates the dorsal neurohaemal organ (DNH), which is situated dorsally and just outside of the VNC. Following metamorphosis, the DNH is reabsorbed into the VNC along with the Tv4 axons. The Tv axons then spread extensive projections across the dorsal most region of the VNC, but with maintained contact with the DNH. I had demonstrated this by generating a transgenic reporter for the DNH, using a GAL4 reporter driven from an enhancer region of the *buttonless* transcription factor, that is restricted to the DNH in development (Chiang et al., 1994). I found that this reporter expression persists in adults and the location of this structure at the dorsal nerve cord midline was verified. One issue that has not been resolved from studies in Tv4 neuron development is the exact source of Gbb ligand. Previous studies have shown that Tv4 neurons must innervate the DNH to access the Gbb ligand. However, there is no compelling evidence that Gbb derives from the DNH rather than from a source that possibly secretes Gbb into the haemolymph. Interestingly, during development, misrouting Tv4 axons out of the CNS via lateral motor nerves is sufficient for Tv4 neurons to access Gbb ligand and express FMRFa (Allan et al., 2003). Thus, in the adult it is unclear whether the DNH continues to provide Gbb ligand or, alternatively the source of Gbb may be the haemolymph or other cells within the CNS. The origin of Gbb ligand could be determined in larval and adult stages using readily available tissue specific *GAL4* drivers to express *UAS-dsRNAi* to *gbb*. In this way the entire animal could be screened for sources of Gbb that is required for persistent FMRFa expression. In spite of this, the evidence presented in this thesis strongly suggests that BMP signaling retrogradely trafficked from the terminals of the Tv4 axon are persistently required for continued expression of FMRFa.

4.2.2 Future directions - Disease screen

In section 2.5.3, I discuss disruption of persistent retrograde targeting as a potential mechanism for neuronal dysfunction in neurodegenerative diseases. The sensitivity of FMRFa expression to a disruption in retrograde signaling in the Tv4 neuron provides a potential reporter system to screen for disruption of retrograde signaling by gain of function genetic mutations linked to neurodegenerative diseases. Using ap^{GAL4} to express *UAS*-constructs of known neurodegenerative disease linked alleles, FMRFa expression could be used as a rapid readout of retrograde trafficking disruption. Many human neurodegenerative disease genes under the control of *UAS* already exist, and others can be made rapidly. Any positive hits from this screen

would further allow for genetic enhancer/suppressor screens and pharmacological screens using libraries of small molecules. A caveat to this approach is whether or not a human gene would be properly processed in *Drosophila* neurons to produce a functional equivalent to the gene in humans. In some cases the appropriate factors could be artificially supplemented or mimetic alleles could be made of orthologous *Drosophila* genes.

4.3 PERSISTENT REQUIREMENT OF SUBTYPE SPECIFIC TRANSCRIPTION FACTORS FOR TDG EXPRESSION.

Chapter 3 of this thesis addresses the persistent requirement of developmental transcription factors for the maintenance of the expression of FMRFa, Nplp1, and PHM in the Tv cluster neurons. As the technical caveats are addressed throughout section 3.4, I will focus in the next section on caveats in the interpretation of the data based on my experimental approach. I will also suggest potential experimental approaches to clarify our current understanding of the model.

4.3.1 Caveats to experimental approaches or interpretations

A primary focus of this thesis is the persistent requirement of developmental transcription factors networks for the maintenance of TDG expression profiles in the mature neuron. However, I only addressed the role of transcriptional networks in the maintenance of a very select group of TDGs related to neuropeptide identity. Neuronal identity is determined by a myriad of other factors including gross cell morphology, connectivity, electrophysiology, and neurotransmitter expression. Undoubtedly, the transcription factors assayed in this study are required for regulation of various other aspects of neuronal identity. For example *eya* is known to be required for axon pathfinding during development of the Tv4 neuron. As discussed previously, the axon morphology of the adult Tv cluster neurons are not well characterized, nor are the numerous other aspects that are required for its normal function. To test how these neuronal characteristics are affected after the downregulation of various transcription factors, further characterization of the Tv cluster neurons would be required. Additionally, new assays to measure these parameters would need to be developed. Although the Tv cluster has been remarkable for it's contribution to delineate the role of transcription factor networks in gene expression it would be a relatively poor model for analyzing various other neural physiological properties. Alternatively, other neuronal models could be adapted as specialized models to test the persistent requirement of transcription factors for a given cellular features may be preferred.

Following transcription factor knockdown, it is not known if the resultant down regulation of TDG expression is a transient effect or causes a permanent alteration to the transcriptional profile of the cell. To control for this, restoring transcription factor expression after a period of downregulation and subsequent testing for the resurgence of TDG expression would address whether or not the cell was permanently altered/damaged. Additionally, This would have profound therapeutic implications for degenerative diseases. Following an insult to normal gene expression through loss of a transcription factor or factors, can the cell recover? These experiments were attempted, however when *dsRNAi* expression was switched off following downregulation of the targeted transcription factor, I was unable to recover expression of the targeted transcription factor. A major caveat of this approach is that the RNAi machinery is self-propagating and may not be able to be turned off following a cease in *dsRNAi* production (Vastenhouw et al., 2006). In addition to this, considering the long periods of *dsRNAi* induction in my experiments and the short life span of the fly, the cells will have accumulated a considerable level of *dsRNAi* transcript that may not have time to be cleared. The use of *dsRNAi*-mediated knockdown of gene expression does not seem a viable option for this experiment. The use *dsRNAi* for transcription factor knockdown also poses a number of other caveats regarding target specificity and possible incomplete knock down of the target. Although I controlled for these caveats it would be useful in future experiments when characterizing other systems to consider other techniques that would allow for greater control over gene expression.

An alternate approach to would be to directly mutate the transcription factor of interest then rescue it back utilizing the inducible TARGET system. Libraries of *Drosophila* genomic sequences contained within bacterial artificial chromosomes (BACs) are available through the BACPAC Resource Center. An insertion of a BACs transgene containing an entire gene locus, including the enhancer and gene coding regions, in a null background of that gene would presumably rescue gene expression to endogenous levels. This transgenic BAC would act as a 'substitute gene'. Current recombineering techniques used on large BAC fragments allow for the ability to directly mutate specific sequences with in the context of an entire gene locus, in our case a transcription factor. This would allow for the insertion of a recombinase recognition site, FRT sites, to flank the transcription factor coding region. This complete gene locus including an FRT flanked coding region could then be used as faithful transgenic substitute for the expression of transcription factors in a transcription factor null background. Using a conditionally expressed GAL4 under the control of the TARGET system, the expression of an inducible recombinase, *UAS-flippase,* could be pulsed in order to excise the coding region of the transgenic transcription

factor in select cells in the adults. This would conditionally knock out the transgenic transcription factor only in specific cells. This method would allow for the complete eradication of transcription factors in a very selective manner without the issues associated with *dsRNAi*.

Following a period of clearance of the transcription factor from the cell, the transcription factor could be rescued by the use of the same TARGET system by conditionally expressing a separate *UAS* construct of the same transcription factor. Although the initial activation of the TARGET system to excise the coding region would also activate the *UAS* transcription factor construct, the activation would only be for a brief enough period to allow for the excision, after which it would be deactivated. There would not be appreciable accumulation of the UAS controlled transcription factor to levels that could not be cleared along with the transgenic "endogenous" transcription factor following a period of clearance. A major assumption of this technique is that transcription factors do not perdure for longer than the life of the fly. This has not yet been tested and would render this technique ineffective for *Drosophila*.

4.4 FUTURE DIRECTIONS - MAINTENANCE OF THE TRANSCRIPTION FACTOR NETWORK

A major conclusion of this thesis is that the subtype transcription factor network changes its configuration to a distinct maintenance cascade. Notably, key transcription factors lose their dependence on the transcription factors that were required for their initiation. This conclusion poses a critical issue that can be addressed in future studies, what regulates the transcription factors following the switch to a stable maintenance specific cascade? This could include two distinct but not mutually exclusive models 1) autoregulation, where transcription factors maintain their own expression either directly or indirectly through a feedback loop (2) independent maintenance elements, a separate transcriptional maintenance mechanism distinct from the action of the initiating transcription factors themselves. In the following sections I will discuss possible experimental approaches to test for the presence of either of these two models in the Tv cluster neurons.

4.4.1 Autoregulation

C. elegans has provided a very simple model whereby cell specific transcription factors bind *cis*-regulatory motifs that are present in terminal differentiation genes and their own regulatory region. Here, key regulatory transcription factors have an autoregulatory feedback mechanism that maintains cell specific patterns of transcription factors and terminal identity genes (Hobert, 2011). In *Drosophila* and mouse models, there is some evidence to support

transcription factor autoregulation through either direct or indirect mechanisms (Crews and Pearson, 2009). However, it is not known whether this is how entire gene regulatory networks are maintained in fully differentiated cells. Conceptually, only one 'master' regulator transcription factor could be required to maintain it's own expression and then the expression of all subsequent transcription factors. As the work of this thesis was being completed, additional transcription factors were discovered that are essential for early development of Tv cluster neurons; *homothorax*, *extradenticle*, and *antennaepedia* (discussed in section 1.2.2) (Karlsson et al., 2010). These transcription factors are required to specify the Tv cluster neurons and initiate the expression of the transcription factor *col*. Testing for the continued presence and necessity of these transcription factors in the mature Tv cluster neurons is an essential next step. These may be key to maintaining Tv1 and/or Tv4 transcription factors and thereby provide the stabilizing input to the adult network configuration. However, even if these transcription factors are found to be present and persistently required for maintenance of *col* and perhaps other transcription factors, the question still remains: What regulates the expression of those transcription factors?

Delineating the stable regulation of a transcription factor network in a cellular model where not every transcription factor is known is a major caveat. Unless we can cleanly close the loop on the maintenance of all known transcription factors, there could always be another unknown factor 'X' that is unaccounted and fulfills such a role. Future efforts to address the regulation of transcriptional networks will require increasingly complete identification of transcriptional cascades required for differentiation of cellular subtypes. Using a transcriptomic approach to characterize cellular expression profiles, such as by RNA-seq, would provide a thorough method to determining all the transcription factors present in a fully differentiated cell. The presence of only six Tv neurons in the *Drosophila* nervous system provides a considerable technical hurdle with regards to this approach. Currently, techniques are not yet available to isolate six neurons from the *Drosophila* nervous system with enough fidelity for identification of the Tv1 or Tv4 transcriptome. With the increasing pace of sequencing and cell isolation technologies, it is foreseeable that this technology is close to being viable. Alternatively, this work could be done in another neuronal subpopulation with a large and homogeneous population and well characterized mechanisms of specification and differentiation.

One other approach that could be performed with relative ease would be a functional screen for essential transcription factors using the *UAS-dsRNAi* libraries for *Drosophila*. This could directly identify the majority of transcription factors required cell-autonomously for maintenance of Tv1 or Tv4 neuronal identity. Logistically, the approximately 750 transcription

factors in the *Drosophila* genome could be prescreened for CNS-specific expression. The Berkeley Drosophila Genome Project has performed in situ hybridization for all transcription factors and made these data available. Moreover, the Dow lab at the University of Glasgow has performed tissue-specific microarray (including the adult and larval central nervous system) of all genes in the fly and posted these data online as 'FlyAtlas'. Based upon data from these resources, a select subgroup of transcription factors could then be screened for their effect on TDG expression, using a strong postmitotic cell specific *GAL4* driver to express the targeting *dsRNAi*. As Nplp1 and FMRFa are downstream of all known transcription factors in Tv1 and Tv4 neurons, respectively, their expression would be an excellent readout for subtype identity. Subsequent work could probe further into the exact mechanisms through which novel transcription factors may operate to maintain cellular identity. As with all screens, there are certain caveats to this approach, for example *UAS-dsRNAi* strains are not always effective, and it may not be feasible to notice slight variations in the expression of TDGs assayed, missing out on transcription factors with minor or redundant roles. This screen would be more amenable to high throughput by using a neuronal population in which a loss of identity resulted in a behavioral phenotype.

4.4.2 Maintenance mechanisms independent of initiating transcription factors

Although transcription factor autoregulation provides a conceptually tidy model for the maintenance of an autonomous transcriptional network, a transcription factor network could potentially be regulated through transcription factor independent mechanisms. Regulation through Polycomb Group (PcG) and Trithorax Group (TrxG) genes offers an intriguing solution to the maintenance question, as they are known to maintain the expression of certain axial patterning genes through lineage progression and in the absence of the initiating transcriptional regulators. It is important to note, though, that PcG and TrxG factors are well established for their role in gene maintenance through cell division (Brock and Fisher, 2005), but to my knowledge their role in long-term maintenance in postmitotic cells has not been addressed. In *Drosophila*, the mechanisms and consequences of gene regulation through PcG and TrxG has largely been delineated in the regulation of Hox genes, although PcG and TrxG proteins are known to bind to the regulatory sequences of genes other than the Hox genes (Boyer et al., 2006). PcG and TrxG proteins bind to *cis-regulatory* sequences of Hox genes, termed polycomb and trithorax response elements (PREs) and (TREs) (Tillib et al., 1999). These sequences are tightly

integrated but physically separable. Although the consensus sequences that PcG and TrxG proteins bind are still somewhat poorly defined, certain studies have taken a bioinformatic approach to identify potential binding sites that may act to control a further breadth of non-homeotic genes. These have identified many late stage non-homeotic genes as potential pcG/TrxG-regulated genes. Importantly, these include *eya*, *dac* and *homothorax* (Janody et al., 2004; Ringrose et al., 2003). Further functional analysis verified that *eya*, *dac* and *hth* are *bona fide* direct targets of PcG and TrxG in the eye (Janody et al., 2004). Mutational analysis of these candidate sequences in transgenic reporters for these and other transcription factors (in the Tv1 and Tv4 neurons) could test if these sites were required for the continued maintenance of these transcription factors in terminally differentiated Tv neurons. If reporter expression were found to be initiated, but not maintained, compared to a wild type reporter, it would suggest that these sequences are required for the maintenance of transcription factor expression. In combination with testing the effect of targeted knockdown of PcG and TrxG genes, this approach could test the putative role of these genes in Tv1/Tv4 subtype transcription network maintenance. Although this approach could provide a step forward, developing faithful transgenic reporters for potentially large genomic regions is not trivial, although this has become technically feasible using integrase-based BAC transgenesis. A potential caveat to this approach is if PcG and TrxG proteins have broad effects on multiple gene regulatory regions. A knockdown of these protein complexes could lead to wide array of cellular dysfunction that could be difficult to control for. However, to my knowledge this would be the first example where these complexes would be shown to regulate gene expression in a postmitotic cell for the maintenance of cellular identity.

4.5 MAINTENANCE SPECIFIC CIS-REGULATORY MOTIFS

Ultimately, subtype-specific gene expression is determined by the cumulative action of combinatorial codes of transcriptional regulators at *cis*-regulatory elements of subtype-specific genes. One way to further the work of this thesis would be to address whether there are initiation and maintenance-specific *cis*-regulatory regions. For genes that are initiated and persistently-regulated by the same transcription factors, such as FMRFa, Nplp1, PHM or *dimm,* it seems likely that those transcription factors will act at the same *cis*-regulatory regions throughout development and maintenance. Ongoing work in the Allan laboratory has identified many of these DNA sequences for the FMRFa gene and similar approaches could be taken for Nplp1 and other genes. Conversely, for genes that lose their dependence on the transcription factors that are required for their induction, it would be informative to see whether the factors that maintain the

expression of these genes act at the same *cis*-regulatory domains as were involved in initiation. This could be addressed by testing for the sufficiency and necessity of cis-regulatory elements in transgenic enhancer reporters for faithful reporter expression throughout the life of the cell. Initially, using selected enhancer regions as reporters to test for sufficiency of *cis*-regulatory modules would isolate functional units required for transcription factor expression. Following this characterization, these select regions could be tested for their necessity in the context of the entire enhancer region. Delineating the components of a maintenance-specific cis-regulatory region would give powerful insights into the factors that retain their expression, and could distinguish between the numerous alternative maintenance models posited above.

Utilizing recombineering techniques on large fragments of *Drosophila* genomic sequences contained within BACs, as discussed in *section 4.3.1*, would allow for the ability to directly mutate select sequences within an entire enhancer region. These large enhancer fragments with selectively mutated regions could then be used as faithful transgenic reporters of transcription factor expression. The use of recombineering to precisely alter specific regions in large fragments also allows for conditional sequence mutation by introducing FRT recombination sites to flank targeted regions. Conditional induction of flippase would allow for temporal and spatial cell-specific excision of targeted regions. If excision of these regions resulted in a loss of reporter expression, this would suggest that these regions are critical for the maintenance of expression. This approach circumvents issues in constitutive mutations where lack of reporter expression at later time points could be the result of a lack of initiation of expression. As discussed in the caveats of section 4.5.2, this approach only addresses the requirement of specific sequences and does not address the factors that act upon them. While this is an ambitious approach, I believe that conditional sequence mutations in concert with conditional transcription factor knockdown and signaling disruption will provide powerful insights into fundamental mechanisms of transcriptional networks in the future.

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APPENDIX

A.1 Supplemental data for chapter 2

Tv neurons	w^{1118} Control	$tkv^{DN};wit^{DN}$	Mad^{DN}
A1 NON-Ind: %	100 ± 5.8% (n=28)	86.1± 4.2% (n=20) P=0.05	90.4 ± 4.5% (n=23) *P=0.55
MRD	8978.6 ± 523.3	7554.0±372.9	8116.8 ±402.5
A1-A5 Ind %	100 ± 8.1% (n=34)	29.1± 2.1% (n=18) *P=8.8 $x10^{-8}$	46.0 ± 5.2% (n=23) *P=6.2 $x10^{-6}$
MRD	9439.5 ± 768.3	2743.3 ± 202.4	4345.8 ± 489.7
A1-A10 Ind %	100 ± 8.1% (n=21)	13.5 ± 1.1% (n=20) P=1.2$x10^{-12}$	24.3 ± 2.3% (n=25) P=2.1$x10^{-12}$
MRD	15603.1 ± 1271.3	2115.0 ± 176.5	3797.0 ± 345.2
A1-A15 Ind %	100 ± 6.4% (n=30)	3.2 ± 0.5% (n=18) P=2.5$x10^{-15}$	20.1 ± 1.6% (n=32) P=2.5$x10^{-18}$
MRD	18233.3 ± 1166.6	583.4 ± 86.0	3670.8 ± 289.9
A10 NON-Ind %	100 ± 7.4% (n=55)	116.5 ± 8.6% (n=26) P=0.16	102.7 ± 8.4% (n=49) P=0.85
MRD: Exp 1	6744.1 ± 718.2 (n=31)		7740.9 ± 727.1 (n=35)
Exp 2	27680.39±2782.7 (n=24)	31857.2 ± 2382.0 (n=26)	19906.8 ± 1767.8 (n=14)
A20 NON-Ind %	100 ± 12.3% (n=25)	136.5 ± 13.1% (n=17) *P=0.03	133.2 ± 12.8% (n=26) *P=0.05
MRD	8043.9 ± 989.6	10976.8 ± 1052.9	10710.5 ± 1029.2
A30 NON-Ind %	100 ± 9.7% (n=28)	140.1 ± 20.3% (n=26) *P=0.24	78.36 ± 5.6% (n=23) *P=0.19
MRD	7140.9 ± 692.9	10005.4 ± 1449.7	5596.0 ± 398.0
A1-10 Ind %	100 ± 7.3% (n=65)	109.8 ± 10.3% (n=53) P=0.43	111.2 ± 7.3% (n=53) P=0.29
A10-20 MRD: Exp 1	8216.9 ± 811.4 (n=31)	10628.4 ± 1179.0 (n=22)	10203.5 ± 1183.3 (n=24)
Recovery Exp 2	13521.3 ± 1470.5 (n=34)	12970.0 ± 1895.0 (n=31)	13582.2 ± 779.7 (n=29)
Internal controls (all MRD)			
A1 NON-Ind	3558.4 ± 427.8(n=10)	2669.8 ± 384.7 (n=12) P=0.13	3583.8 ± 397.4 (n=8) P=0.97
A1-A5 Ind	3947.2 ± 245.7(n=16)	4008.9 ± 185.9 (n=9) P=0.86	4138.9 ± 375.8 (n=7) P=0.67
A1-A10 Ind	6414.4 ± 637.4 (n=7)	5680.8 ± 378.4 (n=11) P=0.31	5073.8 ± 345.6 (n=11) P=0.06
A1-A15 Ind	4233.8 ± 618.7(n=10)	3157.0 ± 266.5 (n=12) P=0.11	2868.9 ± 334.5 (n=12) P=0.06
A10 NON-Ind: Exp 1	2181.9 ± 158.9 (n=13)		3293.6 ± 382.4 (n=15) P=0.70
Exp 2	6618.7 ± 660.2 (n=10)	6741.8 ± 778.2 (n=11) P=0.91	6430.5 ± 701.7 (n=7) P=0.85
A20 NON-Ind	3536.6 ± 485.6 (n=11)	2788.7 ± 567.4 (n=7) P=0.41	3923.0 ± 611.5 (n=9) P=0.62
A30 NON-Ind	4162.8 ± 348.0 (n=12)	4662.7 ± 392.3 (n=13) P=0.35	4014.0 ± 342.0 (n=15) P=0.77
A1-10 Ind Exp 2	5640.3 ± 560.7 (n=11)	4462.8 ± 332.6 (n=13)	5784.7 ± 813.7 (n=8)
A10-20 Exp 1	4079.3 ± 612.4 (n=13)	3893.7 ± 335.5 (n=14)	3942.7 ± 460.4 (n=12)
Recovery:			

Table 2S.1 Relative Pixel Intensity of FMRF FISH in Tv neurons following the expression of BMP pathway blockers at adult day 1 for denoted periods of time.

All flies were raised at 18°C up to adult day A1. For non-induced conditions, flies were then maintained at 18°C for the denoted period. For induced conditions, flies were then maintained at 29°C for the denoted period. For Recovery conditions, flies were initially maintained at 29°C for the denoted period, and then maintained at 18°C for the denoted recovery period. Data is expressed as MRD or % ± standard error of the mean. MRD (Mean of the Raw Data); the mean of FMRFa FISH relative pixel intensities for all Tv neurons analyzed for that experiment. %: the MRD for each experiment expressed as the mean of the control for that specific experiment. All data was statistically analyzed using a two-tailed T-test of normally distributed data. * These data points were not normally distributed and were compared to the appropriate control using the Mann-Whitney test.

Tv neurons	w^{1118} Control	$tkv^{DN};wit^{DN}$	Mad^{DN}
A1 NON-Ind			
%	100 ± 6.1% (n=44)	92.4 ± 7.6% (n=31) P=0.43	106.4 ± 6.0% (n=26) P=0.54
MRD: Exp 1	11874.1 ± 1056.6 (n=22)	11711.5 ± 1591.5 (n=15)	12632.9 ± 710.3 (n=26)
Exp 2	16033.5 ± 1354.4 (n=22)	13873.24 ± 1265.8 (n=16)	
A1-A5 Ind %	100 ± 7.3% (n=27)	36.9 ± 4.1% (n=17) P=$8.9x10^{-8}$	47.7 ± 5.0% (n=19) P= $8.4x10^{-6}$
MRD	21915.7 ± 1600.6	8082.7 ± 888.7	10464.1 ± 1147.5
A1-A10 Ind			
%	100 ± 7.8% (n=28)	18.5 ± 2.1% (n=25) P=$5.3x10^{-13}$	46.4 ± 4.8% (n=30) P=$2.0x10^{-7}$
MRD: Exp 1	7417.0 ± 1179.4 (n=10)	1410.2 ± 223.5 (n=14)	3497.9 ± 411.2 (n=14)
Exp 2	16983.7 ± 1483.5 (n=18)	3012.3 ± 463.5 (n=11)	7770.7 ± 1321.6 (n=16)
A1-A15 Ind			
%	100 ± 8.8% (n=45)	8.2 ± 2.7% (n=36) *P=$6.4x10^{-14}$	37.9 ± 4.6% (n=32) *P=$3.6x10^{-7}$
MRD: Exp 1	3951.2 ± 367.2 (n=22)	421.6 ± 149.2 (n=17)	1842.7 ± 259.2 (n=18)
Exp 2	8106.5 ± 1206.8 (n=23)	485.4 ± 106.1 (n=19)	2167.2 ± 421.2 (n=14)
A10 NON-Ind		94.0 ± 7.5% (n=24) P=0.62	98.6 ± 9.6% (n=19) P=0.92
%	100 ± 9.3% (n=16)	485.4 ± 106.1	2167.2 ± 421.2
MRD	16190.3 ± 1501.9		
A20 NON-Ind	100 ± 11.0% (n=22)	92.8 ± 7.9% (n=28) *P=0.59	109.0 ± 7.7% (n=21) P=0.51
%	13795.9 ± 1519.6	12795.7 ± 1087.7	15036.9 ± 1060.5
MRD			
A30 NON-Ind	100 ± 8.5% (n=30)	105.4 ± 11.7% (n=21) P=0.91	94.5 ± 6.5% (n=19) P=0.44
%	13857.1 ± 1176.1	14599.5 ± 1619.4	13094.0 ± 897.8
MRD			
Internal controls (all MRD)			
A1 NON-Ind:			
Exp 1	4928.5 ± 824.5(n=12)	5573.8 ± 659.1 (n=7) P=0.60	6161.9 ± 579.7(n=11) P=0.25
Exp 2	6724.2 ± 701.7(n=11)	5195.4 ± 523.2 (n=9) P=0.11	
A1-A5 Ind	14913.3 ± 1275.2(n=11)	14055.0 ± 1429.9(n=10) P=0.66	14979.4 ± 1596.5 (n=8) P=0.97
A1-A10 Ind:	31260.8 ± 3439.0 (n=7)	37150.3 ± 2839.5 (n=8) P=0.21	45947.2 ± 11666.9 (n=5) P=0.19
Exp 1	26215.3 ± 3471.4(n=8)	18512.9 ± 1672.7 (n=6) P=0.10	36675.8 ± 5409.4 (n=7) P=0.11
Exp 2			
A1-A15 Ind:	21150.3 ± 3307.2 (n=13)	27787.1 ± 3550.2 (n=9) P=0.20	16360.7 ± 2945.9 (n=7) P=0.34
Exp 1	15165.2 ± 1783.0(n=13)	13766.9 ± 2069.7 (n=8) P=0.62	11618.2 ± 1322.1(n=11) P=0.14
Exp 2			
A10 NON-Ind	20030.5 ± 2235.4 (n=7)	15756.0 ± 1462.6 (n=11) P=0.11	19354.2 ± 2039.0 (n=11) P=0.83
A20 NON-Ind	32995.2 ±2254.4 (n=12)	22130.5 ± 2695.5 (n=9) P=0.005	14835 ± 1488.6 (n=16) P= $2.0x10^{-7}$
A30 NON-Ind	24529.2 ±2834.3 (n=14)	17017.1 ± 2626.2 (n=11) P=0.07	24446.2 ± 2380.0 (n=11) P=0.98

Table 2S.2 Relative Pixel Intensity of FMRF immunofluorescence in Tv neurons following the expression of BMP pathway blockers at adult day A1 for denoted periods of time.

All flies were raised at 18°C up to adult day A1. For non-induced conditions, flies were then maintained at 18°C for the denoted period. For induced conditions, flies were then maintained at 29°C for the denoted period. For Recovery conditions, flies were initially maintained at 29°C for the denoted period, and then maintained at 18°C for the denoted recovery period. Data is expressed as MRD or % ± standard error of the mean. MRD (Mean of the Raw Data); the mean of FMRFa immunofluorescence relative pixel intensities for all Tv neurons analyzed for that experiment. %: the MRD for each experiment expressed as the mean of the control for that specific experiment. All data was statistically analyzed using a two-tailed T-test of normally distributed data. * These data points were not normally distributed and were compared to the appropriate control using the Mann-Whitney test.

Tv neurons		**w^{1118} control**	***Glued*^{DN}**
A1-A5 INDUCED	%	100 ± 4.7% (n=34)	28.7 ± 4.9% (n=20) P=1.7x10^{-13}
	MRD	11704.2 ± 551.7	3366.7 ± 579.1
A10 NON-INDUCED	%	100 ± 12.4% (n=15)	106.3 ± 14.2% (n=13) P=0.74
	MRD	30256.7 ± 3737.9	32164.3 ± 4309.2
A1-A5 INDUCED	%	100 ± 4.8% (n=21)	194.6 ± 24.9% (n=15) P=0.00011
A10-20 Recovery:	MRD	11549.0± 553.6	22477.6 ± 2873.2
Internal controls			
A1-A5 INDUCED	MRD	5186.6 ± 235.0 (n=12)	4603.7 ± 224.4 (n=11) P=0.08
A10 NON-INDUCED	MRD	7653.4 ± 786.2 (n=10)	7038.2 ± 1021.6 (n=8) P=0.63
A1-A5 INDUCED	MRD	6574.4 ± 543.2 (n=10)	6023.8 ± 549.8 (n=9) P=0.48
A10-20 Recovery:			

Table 2S.3 Relative Pixel Intensity of FMRF FISH in Tv neurons following the expression of Glued^{DN} at Adult day 1 for demoted periods of time.

All flies were raised at 18^{o}C up to adult day A1. For non-induced conditions, flies were then maintained at 18^{o}C for the denoted period. For induced conditions, flies were then maintained at 29^{o}C for the denoted period. For Recovery conditions, flies were initially maintained at 29^{o}C for the denoted period, and then maintained at 18^{o}C for the denoted recovery period. Data is expressed as MRD or % ± standard error of the mean. MRD (Mean of the Raw Data); the mean of FMRFa FISH relative pixel intensities for all Tv neurons analyzed for that experiment. %: the MRD for each experiment expressed as the mean of the control for that specific experiment. All data was statistically analyzed using a two-tailed T-test of normally distributed data.

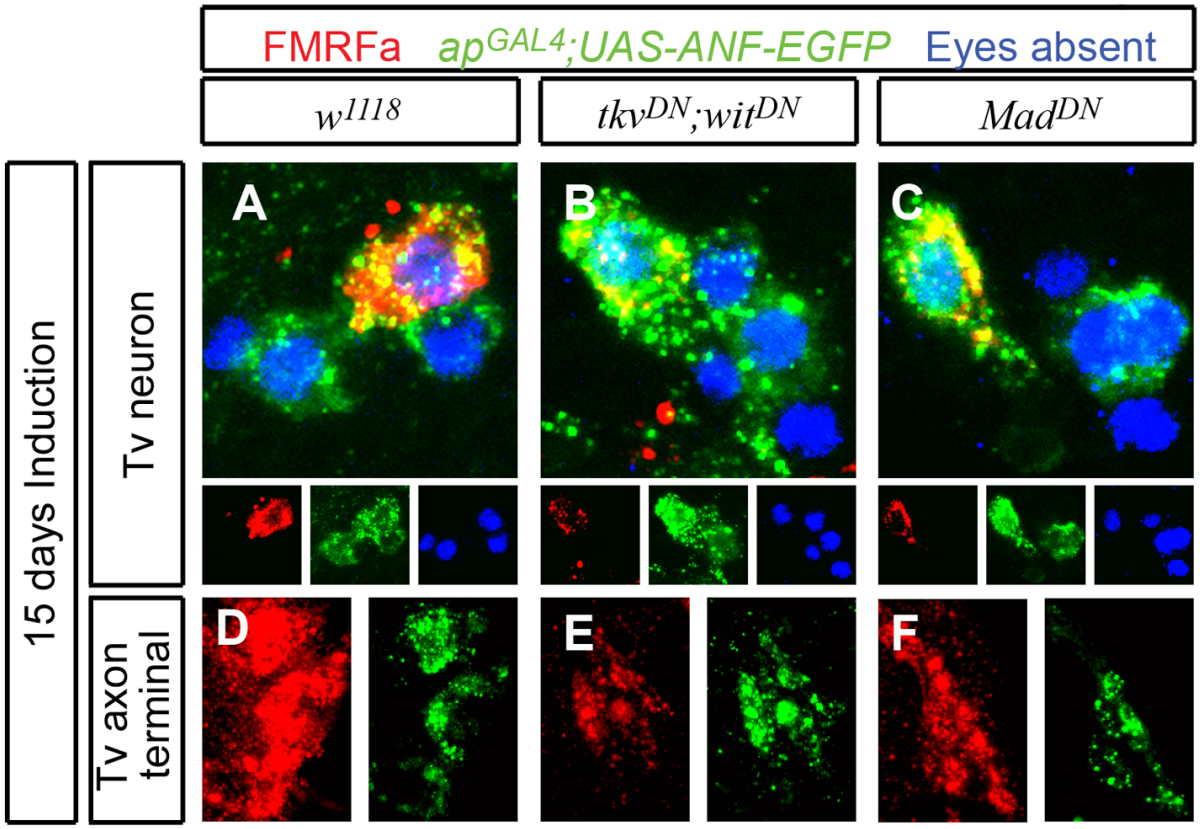

Figure 2S.1 The Tv axon projections remain unchanged following acute blockade of the BMP pathway in adults.

Genotypes: w^{1118} control flies (**a,d**; ap^{GAL4}, $tub\text{-}GAL80^{TS}$/+;,$UAS\text{-}ANF\text{-}GFP$/+); $tkv^{DN};wit^{DN}$ flies (**b,e**; /$UAS\text{-}tkv^{DN}$; $UAS\text{-}ANF\text{-}GFP/UAS\text{-}wit^{DN}$); Mad^{DN} flies (**c,f**; ap^{GAL4}, $tub\text{-}GAL80^{TS}$ /$UAS\text{-}Mad^{DN}$; $UAS\text{-}RPN\text{-}GFP/UAS\text{-}Mad^{DN}$). **(a-f)** Flies were raised at 18°C until A1. They were then incubated at 29°C for 15 days to induce GAL4, as shown by ANF-GFP expression (green) in Tv-cluster neurons (anti-Eyes absent, blue). **(a-c)** FMRFa immunoreactivity (red) was reduced in the soma of Tv neurons in $tkv^{DN};wit^{DN}$ **(b)** and Mad^{DN} flies **(c)**, in contrast to w^{1118} control flies **(a)**. **(d-f)** FMRFa immunoreactivity (red) in the Tv axon termini (visualized by expression of ANF-GFP; green) was also reduced in $tkv^{DN};wit^{DN}$ **(e)** and Mad^{DN} flies **(f)**, in contrast to w^{1118} control flies **(d)**.

A.2 Supplemental Data for chapter 3

FMRFa peptide				
control	n	experimental	n	P value
apP44; ap-dsRNAi				
100.0±10.0%	20	65.2±5.4%	16	0.007
eya-dsRNAi				
100.0±9.3%	34	22.2±3.6%	38	<0.0001
sqz-dsRNAi; sqzIE				
100.0±11.4%	15	51.1±11.9%	15	0.008

Table 3S.1 Downregulation of eya, ap, and sqz has the same effect on FMRFa peptide as FMRFa transcript.

To verify that downregulation of *eya, ap*, and *sqz* equally affects FMRFa transcript and peptide levels, we measured the fluorescence intensity of FMRFa peptide following experimental conditions outlined in **Fig 2**.
Table columns: *dsRNAi* line for each TF; Fluorescent intensity for FMRFa in **Control** and **Experimental** groups normalized as a percentage of the mean of the control, and presented as mean ± SEM; Sample size where **n**= number of neurons.

dsRNAi line	Wild type	n	experimental	n	P value	Induction time
ap-dsRNAi						
JF02311	100.0±8.2%	34	61.25±6.7%	29	<0.0001	20days
eya-dsRNAi						
JF03160	100.0±8.0%	32	50.6±5.9%	27	<0.0001	20 days
108071KK	100.0±8.0%	32	27.0±4.7%	27	<0.0001	20 days
dac-dsRNAi						
JF02322	100.0±6.0%	19	52.9±8.5%	13	<0.0001	15 days
dimm-dsRNAi						
103356KK	100.0±5.6%	27	40.8±3.1%	36	<0.0001	15 days

Table 3S.2 Multiple UAS-dsRNAi lines targeting different regions of transcription factors downregulate FMRFa immunoreactivity in Tv4.

Utility flies (*UAS-dicer2/UAS-dicer2; ap^{Gal4}; tub-Gal80^{TS}, UAS-nEGFP/SM6-TM6,Tb*) were crossed to various *UAS-dsRNAi* fly lines (experimental group) and *w^{1118}* flies (control). F1 generation were raised at 18°C until they eclosed as adults, then kept at 29°C for specified time (Induction time).
Table columns: *UAS-dsRNAi* line for each transcription factor; Fluorescence intensity for FMRFa in **Control** and **Experimental** groups normalized as a percentage of the mean of the control, and presented as mean ± SEM; Sample size where **n**= number of neurons; **Induction time:** duration of time adult flies were maintained at 29°C prior to sampling**.**

Tv4					
TF	control	n	experimental	n	P value
ap-dsRNAi					
Eyes absent	100.0±6.4%	11	119.4±9.3%	14	0.12
Dachshund	100.0±8.0%	22	117.0±10.8%	15	0.20
eya-dsRNAi					
Apterous	100.0±8.5%	16	95.1±8.0%	16	0.68
Dachshund	100.0±7.3%	20	95.3±5.7%	22	0.61
dac-dsRNAi					
Apterous	100.0±6.2%	47	109.0±14.6%	24	0.51
Eyes absent	100.0±6.1%	44	82.1±8.5%	26	0.08
Dimmed	100.0±5.6%	35	94.9±8.0%	19	0.60
dimm-dsRNAi					
Apterous	100.0±10.1%	18	102±8.1%	18	0.88
Dachshund	100.0±7.8%	14	95.0±8.0%	11	0.66
Eyes absent	100.0±6.4%	18	166.5±13.4%	18	<0.0001
sqz-dsRNAi; sqzIE					
Apterous	100.0±11.4%	13	111.0±9.1%	14	0.46
Dachshund	100.0±7.7%	14	91.2±2.6%	18	0.25
Eyes absent	100.0±15.8%	16	95.2±7.3%	18	0.77
Dimmed	100.0±7.5%	13	114.2±7.5%	14	0.25

Tv1					
TF	control	n	experimental	n	P value
ap-dsRNAi					
Eyes absent	100.0±6.5%	16	88.8±7.0%	15	0.24
Collier	100.0±7.2%	17	142.3±9.5%	20	0.25
eya-dsRNAi					
Apterous	100.0±11.3%	17	148.1±16.0%	19	0.02
Collier	100.0±6.4%	17	123.9±8.6%	19	0.03
dimm-dsRNAi					
Apterous	100.0±11.4%	29	92.3±10.3%	23	0.62
Collier	100.0±10.1%	15	107.2±5.9%	15	0.54
Eyes absent	100.0±4.8%	29	122.4±6.3%	23	0.006

Table 3S.3 Configuration of transcription factor cross-regulation within Tv1 and Tv4 terminal selector networks

To test the potential effect of the downregulation of each transcription factor on every other transcription factor, we measured transcription factor immunoreactivity or reporter activity following induction of *UAS-dsRNAi* of other transcription factors.
Table columns: headings for *UAS-dsRNAi* line for each TF; Fluorescent intensity for each **TF** in **Control** and **Experimental** groups normalized as a percentage of the mean of the control, and presented as mean ± SEM; Sample size where **n**= number of neurons.
Genotypes: w^{1118} (*uas-dicer/+; ap*^{Gal4}*/+; tub-Gal80ts, UAS-nEGFP/+*). *eya*^{dsRNAi} (*uas-dicer/uas-eya dsRNAi 43911; ap*^{Gal4}*/+; tub-Gal80ts, UAS-nEGFP/+*). *sqz*^{dsRNAi} (*uas-dicer/+; ap*^{Gal4}*/+; tub-*

Gal80ts, UAS-nEGFP/uas-sqz dsRNAi 5557R-2). *apP44; ap^{dsRNAi}* (*uas-dicer/+; ap^{Gal4}/apP44; tub-Gal80ts, UAS-nEGFP/uas-ap dsRNAi 8376R-2*). *dimm^{dsRNAi}* (*uas-dicer/+; ap^{Gal4}/uas-dimm dsRNAi 44470; tub-Gal80ts, UAS-nEGFP/+*). *dac^{dsRNAi}* (*uas-dicer/+; ap^{Gal4}/uas-dac dsRNAi 4952R-2; tub-Gal80ts, UAS-nEGFP/+*). *col^{dsRNAi}* (*uas-dicer/+; ap^{Gal4}/+; tub-Gal80ts, UAS-nEGFP/uas-col dsRNAi#24E)*

***dsRNAi* lines**	**Tv4**		
	w^{1118}	expt	days ind
eya-dsRNAi			
43911	36/36	0/26	15d
eyaCliIID; 43911	36/36	0/38	15d
JF03160	40/40	0/27	20 d
108071KK	40/40	025	20 d
dac-dsRNAi			
4952R-2	52/52	0/27	10d
dac3, 4952R-2	52/52	0/41	10d
JF02322	35/35	0/29	15 d
dimm-dsRNAi			
44470	44/44	0/14	10d
rev4,44470	44/44	0/20	10d
103356KK	27/27	0/39	15 d

***dsRNAi* lines**	**Tv1**		
	w^{1118}	expt	days ind
col-dsRNAi			
#24E	29/29	0/20	10d
eya-dsRNAi			
43911	25/25	0/20	20d
dimm-dsRNAi			
44470	18/18	0/19	10d
rev4,44470	11/11	0/20	10d

Table 3S.4 Configuration of transcription factor cross-regulation within Tv1 and Tv4 terminal selector networks

Utility flies (*UAS-dicer2/UAS-dicer2; ap^{Gal4}; $tub\text{-}Gal80^{TS}$, UAS-nEGFP/SM6-TM6,Tb*) were crossed to various *UAS-dsRNAi* fly lines (experimental group(**expt**)) and $\boldsymbol{w^{1118}}$ flies (control). F1 generation were raised at 18°C until they eclosed as adults, then kept at 29°C for specified time (Induction time).
Table columns: *UAS-dsRNAi* line for each transcription factor; Cell counts for the presence of immuno staining of the transcription factor being targeted by dsRNAi lines in $\boldsymbol{w^{1118}}$ flies and Experimental groups. In Tv1 and Tv4 neurons Presented as a fraction (cells with immuno staining/total number of cells counted)
Induction time: duration of time adult flies were maintained at 29°C prior to sampling**.**

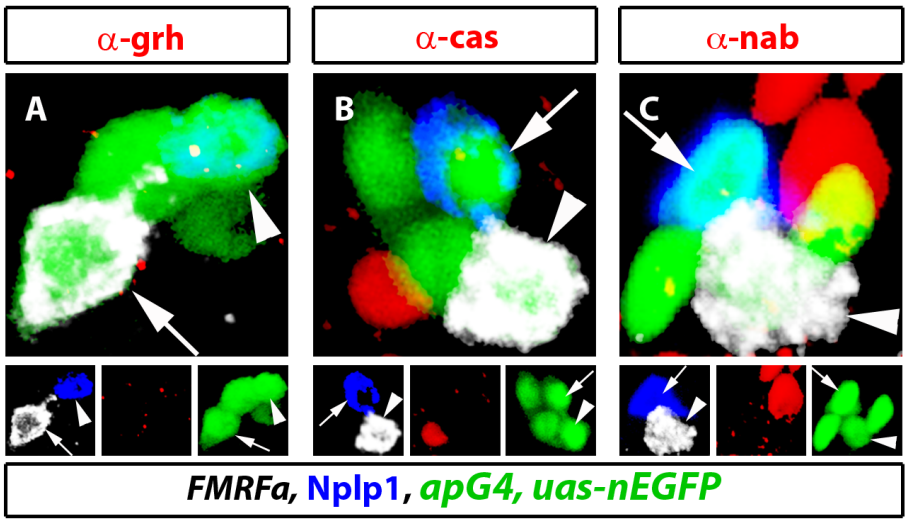

Figure 3S.1 Expression of grh, cas and nab are lost in Tv4 neurons by early L1 stages.

(**A-C**) Representative Confocal images of adult Th1 and Th3 Tv clusters (green) in larval stage. Tv4 neurons (arrows) express FMRFa (white), Tv1 neurons (arrow heads) express Nplp1 (blue). Tv1 and Tv4 do not express transcription factors *grh* (**A**, red), *cas* (**B**, red) or nab (**C**, red) in larval stages . Flies were maintained at 25°C.
Genotype: (**A-F**) *+/+;FMRFa-LacZ* ap^{Gal4}*; UAS-nEGFP*

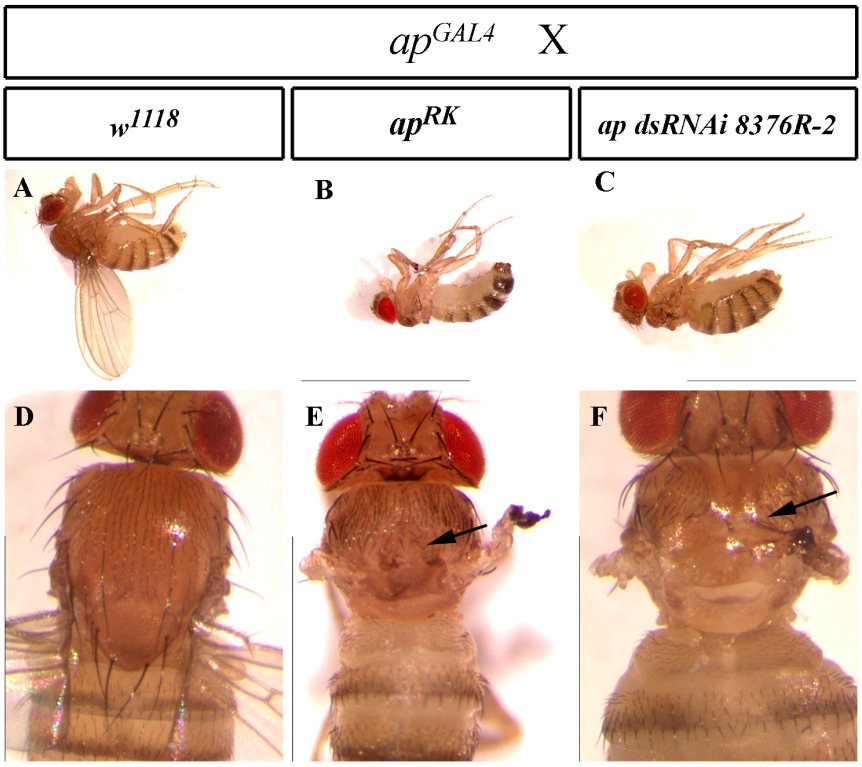

Figure 3S.2 UAS-ap^{dsRNAi} phenocopies strong ap hypomorphs**.**

(**A-F**) Strong *ap* hypomorphs *ap^{Gal4}/ap^{RK568}* (**B**) and *ap^{Gal4,}/UAS-ap^{dsRNAi}* (**C**) flies did not develop wings (**A-C**), and develop the same thoracic dorsal mid line defects (arrows) (**D-F**)

Genotypes: (**A,D**) *ap^{Gal4}/+*; (**B,E**) *ap^{Gal4}/ap^{RK568}*; (**C,F**) *ap^{Gal4}/+; uas-ap-dsRNAi 8376R-2/+*. Flies were incubated at 25°C.

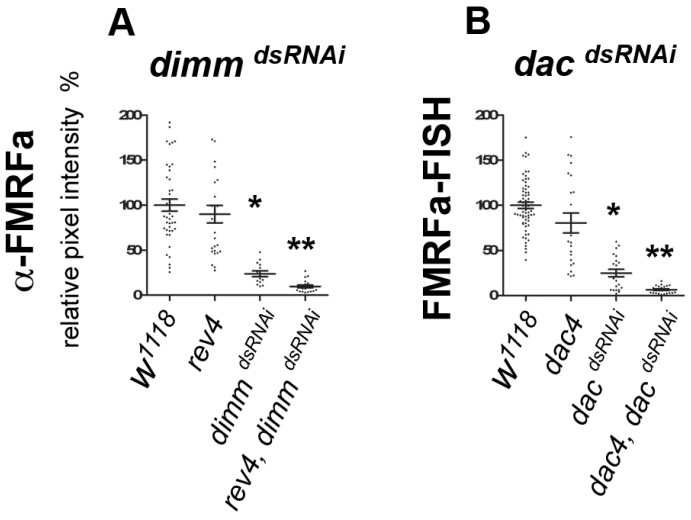

Figure 3S.3 Down regulation of FMRFa by dac^{dsRNAi} and dimm^{dsRNAi} lines is enhanced in a heterozygous background for the pertinent transcription factor.

Experimental results compare relative pixel intensity of FMRFa peptide (**A**) and FMRFa transcript (**B**) of individual Tv4 neurons. Each datum point was normalized to the percentage of the mean of the *w^{1118}* control. Data for each genotype is presented as mean ± SEM.
* FMRFa levels are significantly different from *w^{1118}* control p<0.0001
** FMRFa levels are significantly different from *dsRNAi* only p<0.001

Genotypes: (**A,B**) *w^{1118}* (*uas-dicer/+; ap^{Gal4}/+; tub-Gal80ts, UAS-nEGFP/+*).
(**A**) *rev4* (*uas-dicer/+; ap^{Gal4}/rev4; tub-Gal80ts, UAS-nEGFP/+*); *dimm^{dsRNAi}* (*uas-dicer/+; ap^{Gal4}/uas-dimm dsRNAi 44470; tub-Gal80ts, UAS-nEGFP/+*); *rev4,dimm^{dsRNAi}* (*uas-dicer/+; ap^{Gal4}/rev4, uas-dimm dsRNAi 44470; tub-Gal80ts, UAS-nEGFP/+*).
(**B**) *dac4* (*uas-dicer/+; ap^{Gal4}/dac4; tub-Gal80ts, UAS-nEGFP/+*); *dac^{dsRNAi}* (*uas-dicer/+; ap^{Gal4}/uas-dac dsRNAi 4952R-2; tub-Gal80ts, UAS-nEGFP/+*); dac4, *dac^{dsRNAi}* (*uas-dicer/+; ap^{Gal4}/dac4, uas-dac dsRNAi 4952R-2; tub-Gal80ts, UAS-nEGFP/+*).