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Transposition Driven Genomic Heterogeneity in the *Drosophila* Brain: A Dissertation

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TRANSPOSITION DRIVEN GENOMIC HETEROGENEITY IN THE *DROSOPHILA* BRAIN

A Dissertation Presented
By

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NEUROSCIENCE PROGRAM

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A mis viejos,
quienes me inspiraron a realizar mis sueños.

To Heather,
my rock.

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ABSTRACT

In the *Drosophila* brain, memories are processed and stored in two mirror-symmetrical structures composed of approximately 5,000 neurons called Mushroom Bodies (MB). Depending on their axonal extensions, neurons in the MB can be further classified into three different subgroups: αβ, α'β' and γ. In addition to the morphological differences between these groups of neurons, there is evidence of functional differences too. For example, it has been previously shown that while neurotransmission from α'β' neurons is required for consolidation of olfactory memory, output from αβ neurons is required for its later retrieval. To gain insight into the functional properties of these discrete neurons we analyzed whether they were different at the level of gene expression. We generated an intersectional genetic approach to exclusively label each population of neurons and permit their purification. Comparing expression profiles, revealed a large number of potentially interesting molecular differences between the populations. We focused on the finding that the MB αβ neurons, which are the presumed storage site for transcription-dependent long-term memory, express high levels of mRNA for transposable elements and histones suggesting that these neurons likely possess unique genomic characteristics.

For decades, transposable elements (TE) were considered to be merely "selfish" DNA elements inserted at random in the genome and that they their sole function

was to self-replicate. However, new studies have started to arise that indicate TE contribute more than just “junk” DNA to the genome. Although it is widely believed that mobilization of TE destabilize the genome by insertional mutagenesis, deletions and rearrangements of genes, some rearrangements might be advantageous for the organism. TE mobilization has recently been documented to occur in some somatic cells, including in neuronal precursor cells (NPCs). Moreover, mobilization in NPCs seems to favor insertions within neuronal expressed genes and in one case the insertion elevated the expression. During the last decade, the discovery of the small RNA pathways that suppress the expression and mobilization of TE throughout the animal have helped to uncover new functions that TE play. In this work, we demonstrate that proteins of the PIWI-associated RNA pathway that control TE expression in the germline are also required to suppress TE expression in the adult fly brain. Moreover, we find that they are differentially expressed in subsets of MB neurons, being under represented in the αβ neurons. This finding suggests that the αβ neurons tolerate TE mobilization. Lastly, we demonstrate by sequencing αβ neuron DNA that TE are mobile and we identify >200 *de novo* insertions into neurally expressed genes. We conclude that this TE generated mosaicism, likely contributes a new level of neuronal diversity making, in theory, each αβ neuron genetically different. In principle the stochastic nature of this process could also render every fly an individual.

TABLE OF CONTENTS

List of Figures

LIST OF TABLES

List of Abbreviations

5-HT	serotonin
8-pCPT	8-(4-chloro-phentylthio)-2'-O-methyladenosine-3',5'-clyclic monophosphate
AGO2	Argonaute2
Ago3	Argonaute3
armi	Armitage
Aub	Aubergine
cAMP	3', 5' cyclic adenosine monophosphate
Dcr-2	Dicer 2
DPM	Dorsal Paired Medial
Epac	Exchange protein activated by cAMP
EPSC	excitatory postsynaptic current
FACS	Fluorescence activated cell sorting
FDR	False Discovery Rate
FLP	Flip recombinase enzyme
FRT	FLP recombinase target
GEF	guanine-nucleotide exchange factor
GEF	Guanosine Exchange Factor
GFP	Green fluorescent protein
LINE	Long interspersed nuclear elements

LTD	long-term depression
LTP	long-term potentiation
LTR	long terminal repeats
MB	Mushroom bodies
NPCs	Neuronal progenitor cells
pilRNA	piwi-like RNA
piRNA	piwi-associated interfering RNA
PKA	cAMP-dependent protein kinase A
RISC	RNA induced silencing complex
RNAi	RNA interference
shi^{ts1}	temperature-sensitive *shibire*
SINE	Short interspersed nuclear elements
siRNA	small interfering RNA
TE	Transposable elements
TIR	terminal inverted repeats

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Keene, A. C., Stratmann, M., Keller, A., **Perrat, P. N.**, Vosshall, L. B., & Waddell, S. (2004). Diverse odor-conditioned memories require uniquely timed dorsal paired medial neuron output. Neuron, 44(3), 521-533.

Pitman, J. L., DasGupta, S., Krashes, M. J., Leung, B., **Perrat, P. N.**, & Waddell, S. (2009). There are many ways to train a fly. Fly (Austin), 3(1), 3-9.

CHAPTER I

INTRODUCTION

The integrity of the genome is a foundation of cellular function. Consequently, cells have developed elaborate epigenetic (Muotri *et al.*, 2010) and post-transcriptional mechanisms (Vagin *et al.*, 2006; Yang and Kazazian, 2006; Brennecke *et al.*, 2008; Ghildiyal *et al.*, 2008; Kawamura *et al.*, 2008; Chung *et al.*, 2008; Czech *et al.*, 2008) to suppress the expression and disruptive activity of "parasitic" transposable elements that comprise a large percentage of most genomes; 42% in humans and 15-20% in flies. TE are believed to be central to genome evolution, in part because their mobilization has the capacity to dramatically influence genome organization (Kazazian, 2004; Cordaux and Batzer, 2009). Depending on their type, mobilized TE can act as insertional mutagens as well as create lesions where they once resided. Recombination between TE can also lead to deletion of intervening loci. As a result many individual cases of genetic disorders have been linked to de novo retrotransposition events. It is therefore of great interest to understand why transposon silencing is imperfect and what permits TE to wreak havoc in certain situations.

Several reports have indicated that specific regions of the mammalian brain, most evidently the hippocampus, might be particularly predisposed to LINE-1 (L1) element retrotransposition (Coufal *et al.*, 2009; Baillie *et al.*, 2011). L1 elements mobilized during neural differentiation appear to insert in the open chromatin of neurally expressed genes (Muotri *et al.*, 2005; Coufal *et al.*, 2009; Baillie *et al.*, 2011). Although some de novo insertions may contribute to neurological disease states, it is also conceivable that cells have harnessed TE to regulate gene expression (Muotri and Gage, 2006; Singer *et al.*, 2010). The potentially mosaic nature of neural TE mobilization could also provide advantageous neural diversity that could even manifest as behavioral variability at a population level (Muotri and Gage, 2006; Singer *et al.*, 2010). Having a model organism in which to investigate TE mobilization in the brain would accelerate our understanding of cause and consequence.

Functional requirement of MB subpopulations

It has been long known that in the *Drosophila* brain the structures called Mushroom Bodies (MB) are required for olfactory memory (Heisenberg *et al.*, 1985; de Belle and Heisenberg, 1994). The MB are two mirror-symmetrical structures formed by approximately 5000 neurons (Heisenberg, 2003). Depending on the morphology of their axonal projections, these 5000 neurons can be further subdivided into three subgroups: $\alpha\beta$, $\alpha'\beta'$ and γ (Crittenden *et al.*, 1998). This denomination corresponds to which of the five lobes of the MB a

neuron extends its axon. For example, the axon of a MB αβ neuron bifurcates and extends one branch into the vertical α lobe and the other into the horizontal β lobe (Figure I-1). In addition to having a different morphology, it has been shown that MB neurons from the different subgroups have different roles in memory processing. Current understanding suggests the γ neurons play a role in short-term memory (Blum *et al.*, 2009; Trannoy *et al.*, 2011) whereas the α'β' neurons are required to drive memory consolidation (Krashes *et al.*, 2007; Krashes and Waddell, 2008). The αβ neurons are critical for retrieval (McGuire *et al.*, 2001; Dubnau *et al.*, 2001), especially of long-term memory (Yu *et al.*, 2006; Krashes and Waddell, 2008; Trannoy *et al.*, 2011). In our lab, we used a number of enhancer-trap lines that express in these subgroups of neurons to induce the expression of the dominant temperature-sensitive *shibire*^{ts1} (*shi*^{ts1}) transgene (Kitamoto, 2001). At the restrictive temperature, *shi*^{ts1} blocks vesicle recycling therefore blocking synaptic transmission. When *shi*^{ts1} was expressed in α'β' neurons using the c305a{GAL4} enhancer-trap line, memory performance was only affected when flies where raised to the restrictive temperature either during training or during the first hour after training. In contrast, memory performance was affected during testing when *shi*^{ts1} was expressed in αβ neurons with the c739{GAL4} enhancer-trap line (Figure I-2). Therefore output from the α'β' neurons is required for acquisition and consolidation of olfactory memories whereas output from the αβ neurons is necessary to retrieve those memories. The difference in function of these two groups of neurons suggests that their

expression profiles should be different. We decided to further investigate this possibility. In this study we used cell-type specific gene expression profiling (Nagoshi *et al.*, 2010) to access molecular processes in these functionally distinct MB neurons.

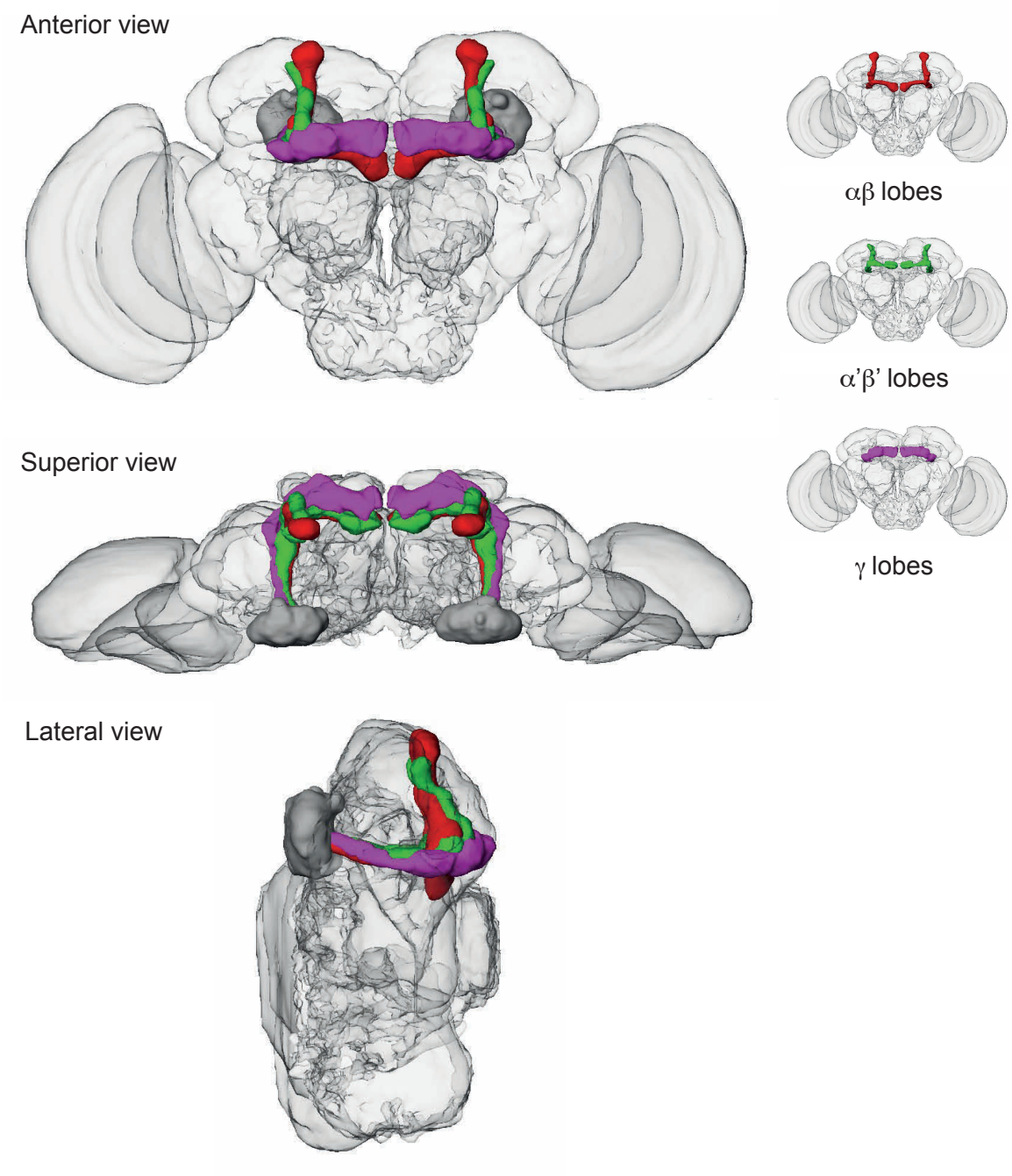


Figure I-1. *The Mushroom Bodies.* The panels illustrate the major anatomical subdivisions of the mushroom body. Kenyon cells extend their dendrites in the calyx (gray). Beyond the calyx, Kenyon cell axons bundle to form the peduncles, which project towards the anterior protocerebrum. The Kenyon cell axons then bifurcate to form the α and α' vertical lobes and the β, β' and γ medial lobes. Images were generated using Amira software (Wolf Huetteroth).

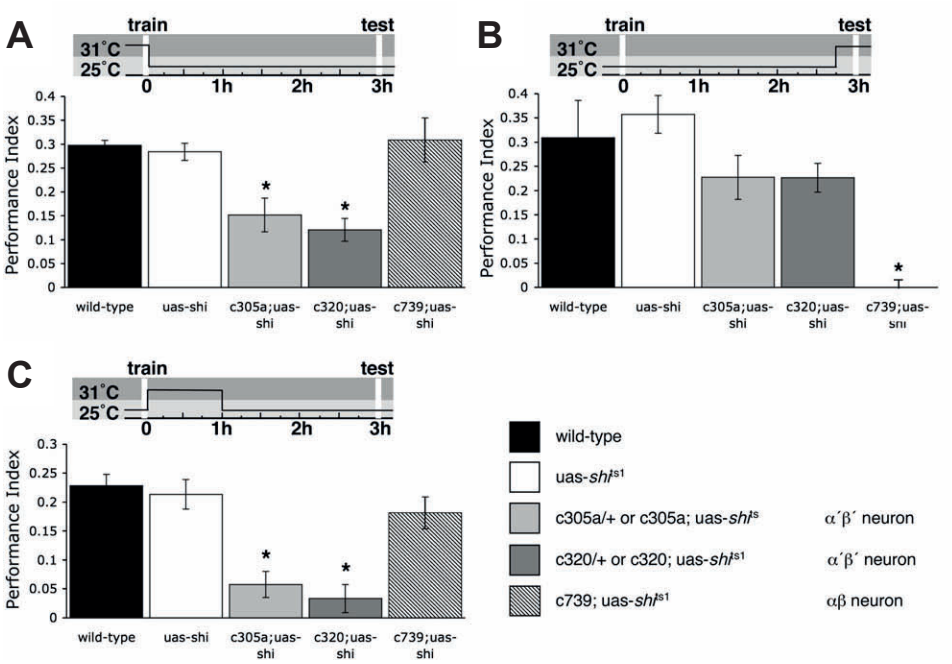


Figure I-2. *Mushroom Bodies subpopulations have different functions*. Blocking MB α'β' output during training (A) and one hour immediately after training (C) impairs 3-hour memory. In contrast, blocking MB αβ output during testing impairs 3-hour memory. The temperature shift protocols are shown pictographically above each graph. Error bars = SEM. Asterisks denote significant difference ($p < 0.05$) from all other unmarked groups. (Reproduced with permission from Krashes *et al.*, 2007).

PREFACE TO CHAPTER II

The work presented in this chapter is currently under review:

Paola N. Perrat, Shamik DasGupta, Jie Wang, Yuhua Shang, William Theurkauf, Zhiping Weng, Michael Rosbash and Scott Waddell (2012). Transposition driven genomic heterogeneity in the *Drosophila* brain. in revision.

Shamik DasGupta and Yuhua Shang contributed to the development of the intersectional strategy. Shamik DasGupta performed the brain immunostainings showed in Figure II-1B-D.

Jie Wang and Zhiping Weng performed the computational analysis and biostatistics of TE insertion sites.

Paola N. Perrat designed and performed all other experiments; fly genetics, cell type–specific expression profiling, immunostaining and molecular biology.

Scott Waddell, Michael Rosbash and Paola N. Perrat wrote the manuscript.

CHAPTER II

TRANSPOSITION DRIVEN GENOMIC HETEROGENEITY IN THE *DROSOPHILA* BRAIN

Exclusive labeling of MB neuron subpopulations

One of the greatest advantages of working with *Drosophila melanogaster* as a model is the easiness of manipulating gene expression. Since its development, the GAL4/UAS system has been a powerful tool to direct expression of a transgene of choice in a specific group of cells (Brand and Perrimon, 1993). One caveat to this system is that often the GAL4 enhancer-trap lines express in the desired group of cells but they also express in other cells. Such is the case of many of the widely used MB GAL4 enhancer-trap lines. These drivers express in other neurons in addition to a MB subgroup. In the behavioral analyses performed in our lab, we have been able to circumvent this problem by introducing the GAL80 repressor (Lee and Luo, 1999). We used a MB driven GAL80 transgene to block GAL4 expression in the MB and assess the contribution of the non-MB neurons to the behavior observed (Krashes *et al.*, 2007). This strategy was successful for the behavioral studies but it was necessary to develop a new approach to exclusively label MB neurons.

For this purpose, we wished to refine the drivers to exclude expression in non-MB neurons. We developed an intersectional genetic strategy using the

GAL4/UAS and LexA/lexAop transcriptional systems (Lai and Lee, 2006). We used a previously described MB-expressing LexA transgene, 247-LexA::VP16 (Pitman *et al.*, 2011) to express a lexA-operator driven FLP recombinase (lexAop-FLP) (Shang *et al.*, 2008) in combination with different MB-GAL4 drivers expressing the UAS>STOP>mCD8::GFP transgene (where ">" denotes a FLP Recombinase Target, FRT site). In order to observe mCD8::GFP expression the STOP cassette should be excised from the transgene by the FLP recombinase. This excision will occur in those neurons that express the lexA-FLP transgene. In other words, only a subgroup of MB neurons where both transcriptional systems overlap will show expression of mCD8::GFP (Figure II-1A). This FLP-out approach allowed us to refine the c739{GAL4}, c305a{GAL4} and NP1131{GAL4} drivers to exclusively label $\alpha\beta$, $\alpha'\beta'$ and γ neurons, respectively (Figure II-1).

Expression profiles of MB neuron subpopulations

After developing the means to label specific subpopulations of MB neurons, we used this approach to label and purify them to profile their gene expression. We dissected 60 brains from each genotype and the neurons were chemically and mechanically dissociated. GFP-positive neurons were subsequently isolated by Fluorescence Activated Cell Sorting (FACS). Polyadenylated RNA was isolated from these neurons and then amplified and hybridized to *Drosophila* GeneChips (Nagoshi *et al.*, 2010) (Figure II-2A).

Comparative analysis revealed striking differences in gene expression between MB α'β' neurons and MB αβ neurons. From the 75 top genes in the MB α'β' subpopulation we found thirty genes involved in transport of molecules across the membrane. From those thirty genes, fourteen are involved in synaptic transmission and nine in ion transport (Figure II-2B, Table II-1). The presence of high expression of a number of synaptic transmission and ion transport genes suggests that MB α'β' neurons may have a higher rate of activity and synaptic events than neighbouring MB neurons. This supports the model that MB α'β' neurons establish a recurrent activity loop with Dorsal Paired Medial (DPM) neurons that is necessary for consolidation of olfactory memories (Krashes *et al.*, 2007).

In contrast (and to our surprise), we found an abundance of transposable element and histone sequences in MB αβ neurons (Figure II-2B, Table II-2). From the top 80 transcripts, twenty-nine of these were derived from transposable elements. Alignment of the corresponding values from the γ and no MB profiles showed a similar bias in TE expression over these other samples (Figure II-3). The identified TE belong to both the Retrotransposons (class I) and the DNA transposons (class II) families. The Retrotransposons can be further subdivided into those with long-terminal repeats (LTR elements) and those without, the Long Interspersed Nuclear Elements (LINEs). We found evidence for expression of eleven LTR elements; roo, blood, gypsy, gypsy2, mdg1, Tabor, invader3, qbert,

412, microcopia and accord; and twelve LINE-like elements; Ivk, G6, baggins, HeT-A, Rt1b, Cr1a, R2, juan, Doc, Doc2, Doc3 and the *F* element. We also identified the DNA elements Bari1, pogo, Tc3 and transib3. Since the expression values showed no significant difference after multiple error corrections, we decided to verify some of the microarray data by quantitative RT-PCR. We assayed the MB subpopulations and non-MB neurons for expression of a group of six LTRs, six LINE1s and two DNA elements and in all case we found higher expression on TE in MB $\alpha\beta$ neurons than in the other populations (Figure II-4).

Expression of TE mRNA is ordinarily under tight control, being regulated by chromatin structure and by elaborate post-transcriptional mechanisms (Muotri *et al.*, 2007). Finding more abundant TE mRNA therefore suggests that normal TE expression control mechanisms are less functional in MB $\alpha\beta$ neurons. Several other mRNAs identified in $\alpha\beta$ neurons are consistent with a model of dysfunctional control of the expression of repeated loci. The mRNA for the translocated Stellate locus Stellate12D orphon (Ste12DOR) was greater than 10-fold more abundant in $\alpha\beta$ than $\alpha'\beta'$ neurons (Figure II-3). Stellate repeat transcripts are usually degraded by post-transcriptional mechanisms using small complementary RNAs produced from the Suppressor of Stellate, Su[Ste] locus (Aravin *et al.*, 2001; Aravin *et al.*, 2004). In addition, we identified transcripts from the two PlexinB pseudogenes present in the triplicated PlexinB locus as well as roadblock that has an inverted duplication known as roadblock similar 54B. Both

PlexinB and roadblock are neurally expressed genes (Ayoob *et al.*, 2006; Bowman *et al.*, 1999).

Our data also indicate that αβ neurons may have unusual chromatin organization. Six of the top twenty-four αβ enriched mRNAs encode four different histones, His1, His2B, His3 and His4 (Figure II-3). Histone loci are also highly repetitive with >20 copies of each gene. Histone mRNA expression is usually controlled by the cell cycle with 3´ end formation occurring via a stem-loop structure rather than polyadenylation (Akhmanova *et al.*, 1997). As the neuron expression profiling protocol uses poly-A priming, the result suggests that synthesis of histone transcripts occurs via a non-canonical mechanism in αβ neurons. It is also perhaps worth noting that the other recognizable transcripts that are enriched in αβ neurons come from the unextended and ubiquinol-cytochrome c reductase (Ucrh) genes, which reside in heterochromatin. Taken together with the expression of many TE and other repetitive sequences in these neurons, the data indicate that their genomes may be in a unique state relative to those of other neurons in the brain that required further investigation.

Transposable elements: from "good guys" to "bad guys" and everything in between

Transposable elements (TE) are DNA sequences of varied length (1-10 Kb) with the ability to insert new copies of themselves into new locations within the

genome. Depending on their form of transposition, TE can be divided in two classes. Class I or Retrotransposons utilize a “copy and paste” mechanism where proteins from an active element reverse transcribe an RNA intermediate and integrate it into a new location generating a net increase in copy number within an individual genome. Retrotransposons are further divided into those with long terminal repeats (LTRs) and those without them (LINEs and SINEs). Class II or DNA transposons use a “cut and paste” mechanism involving a DNA intermediate that is excised and inserted into a new genomic site by the transposon proteins thus generating no change in copy number within an individual. DNA transposons can be further classified according to the presence or absence of terminal inverted repeats (TIR) and DNA sequence conservation.

At first glance, such an ability of generating genomic rearrangements might be thought of as an entirely detrimental factor for the health of the genome and ultimately, the organism. But this is not how the story of TE started. In the 1950s, Barbara McClintock performed studies on the behavior of “mutable loci” capable of causing color variegation of maize kernels by “spontaneous translocation” (McClintock, 1950). She also observed that these translocations generated structural modifications in the chromosomes and suggested that the behavior of these “mutable loci” was the same in all organisms. This idea was further supported by the discovery of a large fraction of repetitive DNA in higher organisms (Britten and Kohne, 1968) and it was suggested that certain repetitive

DNAs might have a regulatory role in gene expression (Britten and Davidson, 1969).

This vision of a positive role for TE was seriously shifted during the 1970s. Studies in *Drosophila melanogaster* showed that the number and chromosomal locations of three different TE differed significantly between four phenotypically undistinguishable species and since they were not conserved, their function was considered to not be as important as it was previously thought (Strobel *et al.*, 1979). It was also demonstrated that the mobilization of specific TE (*P* elements and *I* elements) was responsible for increased mutation and recombination rates and sterility (Picard *et al.*, 1978; Kidwell, 1979; Rubin *et al.*, 1982; Engel, 1988). These discoveries contributed to the idea that TE were “selfish” DNA parasites spreading through the genome generating copies of themselves, introducing mutations and rearranging chromosomes (Doolittle and Sapienza, 1980; Orgel and Crick, 1980). For the most part, TE were believed to be deleterious to the organism. There were however a few exceptions where TE were demonstrated to be beneficial, as is the case of telomere protection by the TART and HeT-A elements (Biessmann *et al.*, 1992; Sheen and Levis, 1994; Pardue *et al.*, 2005).

Between 1990 and 2000 interest in TE appeared low, but this was changed by the introduction of fully sequenced genomes. It was found that TE are present in virtually all eukaryotic species studied and that they account for 3% to 80% of

total DNA (Hua-Van *et al.*, 2005; Biemont and Vieira, 2006; Piegu *et al.*, 2006). Currently, TE are believed to be central to genome evolution, in part because their mobilization has the capacity to dramatically influence genome organization (Kazazian, 2004; Cordaux and Batzer, 2009).

Mechanisms of TE regulation

Given the potentially harmful effect that TE possess by introducing mutations, causing deletions and chromosomal rearrangements, cells have developed several defense mechanisms to protect the integrity of the genome. At the DNA level, chromatin modifications such as DNA methylation, modifications of histone tails and alterations in chromatin packing and condensation repress transcription of TE (Muotri *et al.*, 2007). For example, methylation of histone H3 at lysine 9 is a signal for transcriptionally inactive chromatin and is found to be enriched in nucleosomes associated with TE (Gendrel *et al.*, 2002; Martens *et al.*, 2005). Another type of control of TE expression involves the production of small RNAs that guide a degrading machinery to complementary transcripts.

Post-transcriptional silencing of TE by RNAi

RNA interference (RNAi) is the mechanism by which proteins of the *dicer* family cleave dsRNA into small interfering RNAs (siRNA) of 21 nucleotides in length with a 3'-end modification. These siRNAs direct proteins from the *Argonaute* family, the catalytic component of the cleavage complex known as RNA induced

silencing complex (RISC) to their target transcripts. In *Drosophila*, the production of siRNAs is dependent on Dicer-2 (Dcr-2) and Argonaute-2 (AGO2) is responsible for silencing the target transcripts. In plants and animals, this mechanism is known to defend the organism against viral infections (Ding and Voinnet, 2007).

In recent years, an endogenous source of siRNA derived from TE, heterochromatin sequences, intergenic regions and mRNAs have been detected in *Drosophila* somatic and germ cells. High throughput sequencing analyzes of small RNAs in *Drosophila* tissues and cultured cells revealed an important fraction of sequences originated from TE of 21-nucleotide long with 3'-end modifications consistent with siRNAs structure. Moreover, expression of TE mRNA increases in *dcr-2* and *ago2* mutants suggesting that the siRNA pathway protects the organism by silencing TE (Ghildiyal *et al.*, 2008; Kawamura *et al.*, 2008; Chung *et al.*, 2008; Czech *et al.*, 2008).

Germline silencing of TE by piRNA

Piwi-interacting RNAs (piRNA) are another class of small RNAs. Differing from siRNAs, piRNAs are 24-32 nucleotides in length. They lack the 3'-end modification and they originate from fragmentation of longer transcripts in a Dicer-independent manner (Brennecke *et al.*, 2007; Vagin *et al.*, 2006; Thomson and Lin, 2009). In a similar way to the RNAi pathway, piRNA guide a cleavage

complex to the target transcript, but the proteins involved in the target degradation are different from those in the siRNA pathway. In this case, the role is played by the Piwi clade of the Argonaute proteins which is comprised of Piwi, Aubergine (Aub) and Argonaute3 (Ago3). Piwi proteins are expressed in *Drosophila* female and male gonads, and embryos (Williams and Rubin, 2002; Saito *et al.*, 2006; Brennecke *et al.*, 2007; Nishida *et al.*, 2007) and they are essential for germline development (Cox *et al.*, 1998; Harris and Macdonald, 2001) and female and male fertility (Cox *et al.*, 1998; Lin and Spradling, 1997; Schmidt *et al.*, 1999; Li *et al.*, 2009). Piwi proteins protect the germline from the potential harmful effects of TE mobilization thus maintaining DNA integrity. A number of studies have shown TE mobilization as consequence of mutations in the piwi proteins (Sarot *et al.*, 2004; Savitsky et al, 2006; Reiss *et al.*, 2004; Saito *et al.*, 2006; Vagin *et al.*, 2006).

The presence of piRNA in embryos and the germline is well characterized (Brennecke *et al.*, 2007; Gunawardane *et al.*, 2007; Brennecke *et al.*, 2008). Yet, its presence in somatic tissues is the subject of controversy. Only one specific type of somatic tissue: ovarian follicle cells have been shown to contain piRNA (Malone *et al.*, 2009; Li *et al.*, 2009). Two independent studies reported the presence of piRNA-like sequences (pilRNA) in the fly head and imaginal disc (Ghildiyal *et al.*, 2008; Yan *et al.*, 2011). These pilRNA have the same length and structure of piRNA but since their association with piwi proteins is yet to be

proven, they cannot be classified as such. To date, there is no report on the presence of piwi proteins in adult fly tissues outside the gonads. However, the presence of pilRNA suggests that it is conceivable that piwi proteins might act in other tissues.

Critical proteins of the piwi pathway are underrepresented in MB αβ neurons

The piRNA processing machinery prevents TE mobilization in the fly germline and loss of the key piRNA argonaute proteins, Aubergine (Aub) and Argonaute 3 (Ago3), leads to TE mobilization and derepression of repeated loci such as *Stellate* (Li *et al.*, 2009). We therefore stained Aub and Ago3 in the adult brain. We simultaneously co-localized CD8::GFP, which was expressed in specific subsets of MB neurons to assign argonaute protein signals to MB neuron type (Figure II-5). Strikingly, Aub protein was detectable throughout the processes of γ and α´β´ MB neurons but was much reduced in the αβ neurons. This differential abundance can be clearly seen at the level of the MB lobes and the peduncle, where the axons of the different MB neuron subtypes are anatomically discrete (Figure II-5F-H). Ago3 staining was most prominent in the proximal axonal segment of MB neurons in the peduncle, just below the dendritic region of the calyx. Ago3 staining differed to that of Aub and preferentially co-localized with γ and core αβ neurons (Figure II-6). Most importantly many αβ neurons labeled with the c739 FLP-out driven GFP were negative for Ago3. Therefore, the Aub

and Ago3 piRNA proteins are present in MB neurons but are of greatly lower abundance in the αβ subdivision. A similar pattern of Aub and Ago3 immunolabeling was observed in *D. erecta*, *D. sechellia* and the more distantly related *D. pseudoobscura* species (Figure II-7), indicating that this organization is conserved across the Drosophilids. We also immunostained *D. melanogaster* brains with antibodies directed towards PIWI (Cox *et al.*, 1998) but we were unable to detect expression in the brain (data not shown). The relative absence of the Aub and Ago3 proteins in αβ neurons is consistent with these neurons permitting the observed accumulation of TE and Ste12DOR mRNAs. Interestingly, mutations in the piRNA pathway genes such as *aubergine*, *ago3*, *armitage*, *spindle E*, *zucchini* and *squash* lead to the formation of Stellate protein crystals in spermatocytes (Li *et al.*, 2009; Schmidt *et al.*, 1999; Stapleton *et al.*, 2001; Tomari *et al.*, 2004; Pane *et al.*, 2007). Immunolabeling of the Stellate protein in the fly brain revealed punctate structures in the MB calyx that co-localizes with dendrites of αβ neurons. Consistent with previous observations, this suggests the formation of protein aggregates in these neurons as a result of *Stellate* (Ste12DOR) over expression (Figure II-8). We also explored expression of another piwi pathway component, the RNA helicase Armitage (armi) (Cook *et al.*, 2004). We found that armi co-localizes with γ neurons and parts of the αβ neurons in the MB lobes. In the peduncles, very low expression of armi is observed but most of the αβ neurons are negative for armi (Figure II-9). It is

interesting to note that armi is thought to be required for assembly of the silencing complex assembly (Tomari *et al.*, 2004).

To further test whether Aub, Ago3 and Armitage suppress TE expression in the brain, we assayed TE mRNA levels in transheterozygous *aub* (*aub*^{HN2}/*aub*^{QC42}), *ago3* (*ago3*^{t2}/*ago3*^{t3}) and *armi* (*armi*^{1}/*armi*$^{72.1}$) mutant flies (Figure II-11A). We tested 14 of the TE we had identified to be expressed in αβ neurons that represented the LTR, LINE-like and TIR groups. Head mRNA was prepared and assayed by quantitative RT-PCR with TE specific primers. Levels of the LTR elements *gypsy*, *Tabor* and *qbert*, the LINE-like *HeT-A*, *RT1B* and the *R2-element* and the TIR element *pogo* were significantly elevated in *ago3* mutants. In addition *blood*, *Tabor* and the *R2-element* were significantly elevated in *aub* mutants. Moreover, *blood*, *gypsy*, *invader3*, *qbert* and the *R2-element* were significantly elevated in *armi* mutants. We also tested mRNA levels of the same TE in *dcr-2* (*dcr-2*^{L811fsX}) and *ago2* (*ago2*^{414}) mutant flies (Figure II-11B). In *dcr-2* mutants we found significantly elevated level of *blood*, *gypsy*, *Tabor*, *qbert*, *HeT-A*, *R2-element* and *pogo*. In addition, *blood*, *gypsy*, *mdg1*, *Tabor*, *HeT-A*, *RT1B*, *Cr1a*, *R2-element*, *Doc3*, *Bari1* and *pogo* were significantly elevated in *ago2* mutants. Therefore, we conclude that the piRNA system contributes to repression of TE expression and that of other repetitive sequence mRNAs in MB α'β' and γ neurons. This conclusion is supported by both, the presence of piwi proteins in these neurons and over expression of TE observed in the piwi

pathway mutants. However, a contribution of the siRNA pathway in the control of TE in neurons cannot be discounted.

Transposable elements mobilization in MB αβ neurons

The relative absence of Aub and Ago3 in MB αβ neurons in addition to the high levels of TE expression are highly suggestive that TE are actively mobile in these neurons. To investigate TE mobility we tracked excision of *pogo* DNA elements by PCR. Several *pogo* insertions have been annotated in the fly genome. We established that *pogo* was present in the *kmn1*, *lilli*, *timeout* and *CG9413* loci in embryos from our strain but could not detect insertions in *cngl*, *snoo*, *nAChRα-30D* or *SKIP*. In mammals active L1 elements insert in neurally expressed genes (Muotri *et al.*, 2005; Coufal *et al.*, 2009; Muotri *et al.*, 2010). The *SKIP* locus encodes the Shal K^+ channel-interacting protein SKIP3 (Diao *et al.*, 2009). In addition the memory-relevant *GLD2* poly(A) polymerase gene (Kwak *et al.*, 2008) lies within the same intron of *SKIP* as the described *pogo* insertion. We therefore tested for brain-specific *pogo* insertions in *SKIP* by PCR and sequencing (Figure II-12). DNA from five individual embryos produced a single 460bp band corresponding to the intact *SKIP* locus. In contrast, DNA from ten individual fly brains produced the same band corresponding to intact *SKIP* plus additional larger bands resulting from insertion of *pogo* sequences into *SKIP*. The abundant 1.7kb band contains 1.2kb of *pogo* sequence that includes a 693bp ORF encoding a transposase and could therefore be autonomous. In

contrast the 1kb band appear to be either an insertion of a non-autonomous *pogo* or a remnant of a sequential insertion and partial excision. Either way, our experiments reveal heterogeneous brain-specific insertion of *pogo* sequences in the same position in the SKIP locus. Furthermore, the data suggest there is considerable heterogeneity of insertions within and between individual fly brains. Given the ability of SKIP3 to regulate Shal K^+ channel gating (Diao *et al.*, 2009), variable *SKIP* expression could provide additional range to the integrative and plastic properties of neurons.

In a more global approach, we decided to look for new TE insertions in MB $\alpha\beta$ neurons by genome-wide DNA sequencing. We identified new insertions by comparing reads from $\alpha\beta$ neurons to those in DNA from genetically identical embryos. We prepared DNA libraries from sorted GFP-positive cells (MB $\alpha\beta$ neurons) and GFP-negative cells as representative of the rest of the brain. DNA libraries from sibling embryos provide information regarding the position of inherited TE insertions. By subtracting existing TE insertions found in embryo DNA, we found 215 new insertions in MB $\alpha\beta$ neurons (Table II-4), and 219 new insertions in the rest of the brain. The position of each new TE insertion was analyzed relative to the closest gene. We found that 5% of insertions are located in promoter regions, 16% of insertions are located within exons and 32% of insertions mapped within introns. The remaining 47% are located in intergenic regions (Figure II-13A). A similar distribution was observed amongst the starting

embryonic insertions in embryo DNA and the new insertions in the rest of the brain (data not shown). We finally compared the chromosomal locations of new TE insertions in MB αβ neurons and in the rest of the brain (Figure II-13B). In general, no clustering to specific regions was observed for any of the groups, with TE seeming to randomly insert throughout all the chromosomes.

Interestingly, the genes receiving new TE insertions in MB αβ neurons are siginficantly enriched in the Gene Ontology (GO) terms neuronal differentiation, neuron projection development, neuron development, cell motion and plasma membrane (FDR < 0.05) (Table II-5). In comparison, the genes affected by new TE insertions in the rest of the brain were enriched in the GO terms synaptic vesicle transport, regionalization, exocytosis and pattern specification process. However these terms were not significant after the FDR correction. Perhaps more interestingly, MB αβ neurons revealed new TE insertions in the memory relevant genes *dunce*, *rutabaga*, *gilgamesh* and *derailed* (Dudai *et al.*, 1976; Tully and Quinn, 1985; Tan *et al.*, 2010; Moreau-Fauvarque *et al.*, 2002) as well as in the transmitter receptor genes nAcRalpha80B and GABA-B-R1 and the cAMP signaling relevant Gprk1 and cngl genes. Variability in the expression of this collection of genes alone would be expected to generate neural differences in input processing, reinforcement signaling and plasticity.

Prior work in mammals suggests that L1 expression occurs in neurons because the L1-promoter is released during neurogenesis (Muotri et al, 2005; Muotri *et al.*, 2010). Our data indicate that a broader explanation should be considered, at least for neural expression of TEs in the fly brain. Firstly, we find that TE expression is most pronounced in a functionally discrete class of MB αβ neurons. Importantly, finding both enhanced TE expression and a relative absence of piRNA machinery in these neurons strongly suggests that TE containing loci are released from transcriptional repression via relaxed post-transcriptional piRNA mechanisms. Our data also predict that the piRNA machinery, presumably functioning with previously characterized piRNAs, normally limits TE mRNA levels in much of the fly brain. It also seems unlikely that TE expression is only a product of neurogenesis in the fly brain because we find preferential TE expression in αβ neurons of adult flies. Moreover, the TE expressing αβ neurons are born before the αβ core neurons that have higher levels of Ago3 and after the γ and α´β´ neurons that have higher levels of Aub (Lee *et al.*, 1999). This birth order also argues against TE expression being a consequence of advancing neural age. We instead favor the possibility that TE expression reflects a specific property of αβ neurons related to their function. Establishing whether TE expression and mobilization confers a functional advantage on these cells and whether that translates to neural plasticity (Singer at al., 2010) and behavior at the organism level is of particular interest and will require further investigation.

Experimental procedures

Fly Strains

Fly stocks were raised on standard cornmeal food at 25°C and 40-50% relative humidity. The MB-LexA (aka 247-LexA) flies are described (Pitman *et al.*, 2011). MB-LexA labels most MB neurons but misses a significant population in the αβ core. We combined c305a-GAL4, c739-GAL4 and NP1131-GAL4 with MB-LexA to generate the driver lines c305a-GAL4; MB-LexA/TM3Sb, c739-GAL4; MB-LexA/TM3Sb and NP1131-GAL4; MB-LexA/TM3Sb, respectively. We combined lexAop-FLP^{21} with UAS>STOP>CD8::GFP (Stockinger *et al.*, 2005) to generate lexAop-FLP; UAS>STOP>CD8::GFP flies. We crossed lexAop-FLP; UAS>STOP>CD8::GFP females with males of the driver lines for the immunohistochemistry and gene expression profiling experiments. Transheterozygous aub^{HN2}/ aub^{QC42} flies were generated by crossing aub^{HN2}/CyO and aub^{QC42}/CyO flies (Schupbach and Wieschaus, 1991). Transheterozygous ago3^{t2}/ago3^{t3} flies were generated by crossing ago3^{t2}/TM6Tb and ago3^{t3}/TM6Tb flies (Li *et al.*, 2009). Transheterozygous armi^{1}/armi$^{72.1}$ flies were generated by crossing armi^{1}/TM3Sb and armi$^{72.1}$/TM3Hs-HID flies (Cook *et al.*, 2004). *dcr-2^{L811fsX}* and *ago2^{414}* flies were obtain from Phillip Zamore (UMASS). The wild-type *Drosophila* control strain used in the quantitative RT-PCR experiments is w1 (Li *et al.*, 2009). *D. sechellia* and *D. erecta* were obtained from Joel Levine

(University of Toronto) and *D. pseudoobscura* from Aki Ejima (Brandeis University).

Immunohistochemistry

Fly brains were dissected in chilled 1X Phosphate Buffered Saline (PBS) (1.86 mM NaH_2PO_4, 8.41 mM Na_2HPO_4, and 175 mM NaCl), 0.1% Triton X-100 (PBS-T) and fixed for 30 minutes in chilled 4% paraformaldehyde in 1XPBS. After fixation, brains were rinsed four times in PBS-T for 15 minutes at room temperature. The following primary antibodies were added to 1:200 final concentration in PBS-T and brains were incubated overnight at 4°C with gentle rocking: rabbit anti-Aub (Li *et al.*, 2009), rabbit anti-Ago3 (Li *et al.*, 2009), rabbit anti-Ste (Tomari *et al.*, 2004), and mAb anti-GFP (Invitrogen). Brains were rinsed in PBS-T and incubated overnight with the appropriate secondary antibodies (Jackson Laboratories). Confocal analysis was performed on a Zeiss LSM 5 Pascal confocal microscope. Confocal stacks were processed using ImageJ and Adobe Photoshop.

Cell sorting

RNA isolation and preparation protocol was performed as described previously (Nagoshi *et al.*, 2010). 50-60 brains were dissected from ~5 day old flies into ice-cold modified dissecting saline (9.9 mM HEPES-KOH buffer, 137 mM NaCl, 5.4 mM KCl, 0.17 mM NaH_2PO_4, 0.22 mM KH_2PO_4, 3.3 mM glucose, 43.8 mM

sucrose, pH 7.4) containing 50 μM d(–)-2-amino-5-phosphono-valeric acid (AP5), 20 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX), 0.1 μM tetrodotoxin (TTX), and immediately transferred them into modified SM^{active} medium (SM^{active} medium containing 5 mM Bis-Tris, 50 μM AP5, 20 μM DNQX, 0.1 μM TTX) on ice. Brains were digested with l-cysteine-activated papain (50 units/ml in dissecting saline; Worthington) for 30 minutes at 25°C. Digestion was stopped with five volumes of medium, and brains were washed twice with the chilled medium. Brains were triturated with a flame-rounded 1,000 μl pipetter tip with filter until most of the tissues were dissociated to single cells. The resulting cell suspension was filtered with a 40 μm Nylon Cell Strainer (BD Falcon). GFP-positive cells were sorted using a BD FACSAria Flow Cytometer with FACSDiVa 6.1.1 software.

Gene expression analysis

Total RNA was extracted from approximately 10,000 GFP-positive cells and poly(A) RNA was amplified by two-cycle linear amplification as previously described[25] and hybridized to a GeneChip *Drosophila* Genome 2.0 array (Affymetrix). Four biological replicates were profiled for each cell type. Scanned Affymetrix image data were processed with the Affymetrix GCOS software to convert to probe level signals. The probe signal files were then processed in CARMAweb (https://carmaweb.genome.tugraz.at/carma/index.jsp) (Rainer *et al*., 2006) using the GCRMA algorithm to normalize and calculate summary values for each probe set. CARMAweb tools were used to identify differentially

expressed genes. Genes from each population of cells with average value of replicates ≥7.0 were selected and compared between populations. Genes with a ≥2-fold change difference between populations are reported.

Real-Time PCR

Total RNA from adult fly heads was isolated with Trizol (Invitrogen) and cleaned with RNeasy Micro Kit (Qiagen) with DNAse I treatment. RNA (1 µg) was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and oligo(dT)$_{12\text{-}18}$. The cDNA was used for quantitative real-time PCR with ABI PRISM® 7000 Sequence Detection System (Applied Biosystems) with standard cycling parameters (2 min at 50 °C, 10 min at 95 °C, and 40 alternate cycles of 15 s at 95 °C and 60 s at 60 °C). The PCR mixture contained TaqMan® Gene Expression Master Mix and the appropriate Gene Expression Assay (Applied Biosystems). TaqMan custom-made qPCR assays were ordered for all transposons assayed. GAPDH (AB: Dm01841185_m1) was used as endogenous control for normalization of each gene (ΔC_T value). The increase in expression ($\Delta\Delta C_T$ value) was calculated and transformed to the exponential scale.

PCR

PCR reactions were carried out with 200pg genomic DNA from individual adult fly brains and 0.3µM of each primer in 10mM Tris pH8.3, 1.5mM $MgCl_2$, 50mM KCl, 0.2mM deoxyribonucleotides, and 1U Taq DNA polymerase (NEB) for 35 cycles

of 94°C for 30sec, 50°C for 30sec, and 72°C for 2min. Genomic DNA from sibling individual embryos was used as control. Primers were designed to span the *pogo* insertion in *SKIP*; 5´-ATCGTTACGGTTGGGCATTA-3´ and 5´-ACAGCACAAGCGTTTGAGAA-3´. PCR products were analyzed by agarose gel electrophoresis, eluted from the gel and sequenced.

DNA preparation for genome sequencing

DNA preparation for whole genome sequencing was done by shearing approximately 5000 sorted cells (GFP-positive and GFP-negative samples) and 10 µg DNA from embryos using the Sonifier 450 (Branson) with the following settings: Output 1, Duty Cycle 50%. Shearing was performed in 5 cycles of 20 pulses with 30 seconds rest. The sequencing library was prepared as previously described (Schmidt *et al.*, 2009) involving DNA end repair, addition of A bases to the 3' end and ligation to adapters (Illumina PE Adapter Oligo Mix). Adapter-ligated products were purified using DNA Clean&Concentrator-5 kit (Zymo Research) and amplified by PCR using Phusion DNA polymerase (New England Biolabs) for 30 cycles using the inPE PCR primers 1.0 and 2.0, and individual index primer for each sample (Illumina). PCR fragments of the desired range (200-400 base pairs) were purified using a 2% agarose gel. DNA quality was assessed and quantified using a High Sensitivity DNA Assay on a 2100 Bioanalyzer (Agilent Technologies). Computational identification of transposon insertion sites was performed as previously described (Khurana *et al.*, 2011).

Paired-end reads that were of sufficiently high sequencing quality were aligned against the unmasked *Drosophila* reference genome using the BWA algorithm (Li and Durbin, 2009), allowing insertions, deletions, and up to two mismatches per 100 nt read. For the detection of transposon insertions that were in the experimental genome but not in the reference genome, discordant read pairs were identified with one read mapping to a location in the reference genome and the other mapping to a transposon sequence.

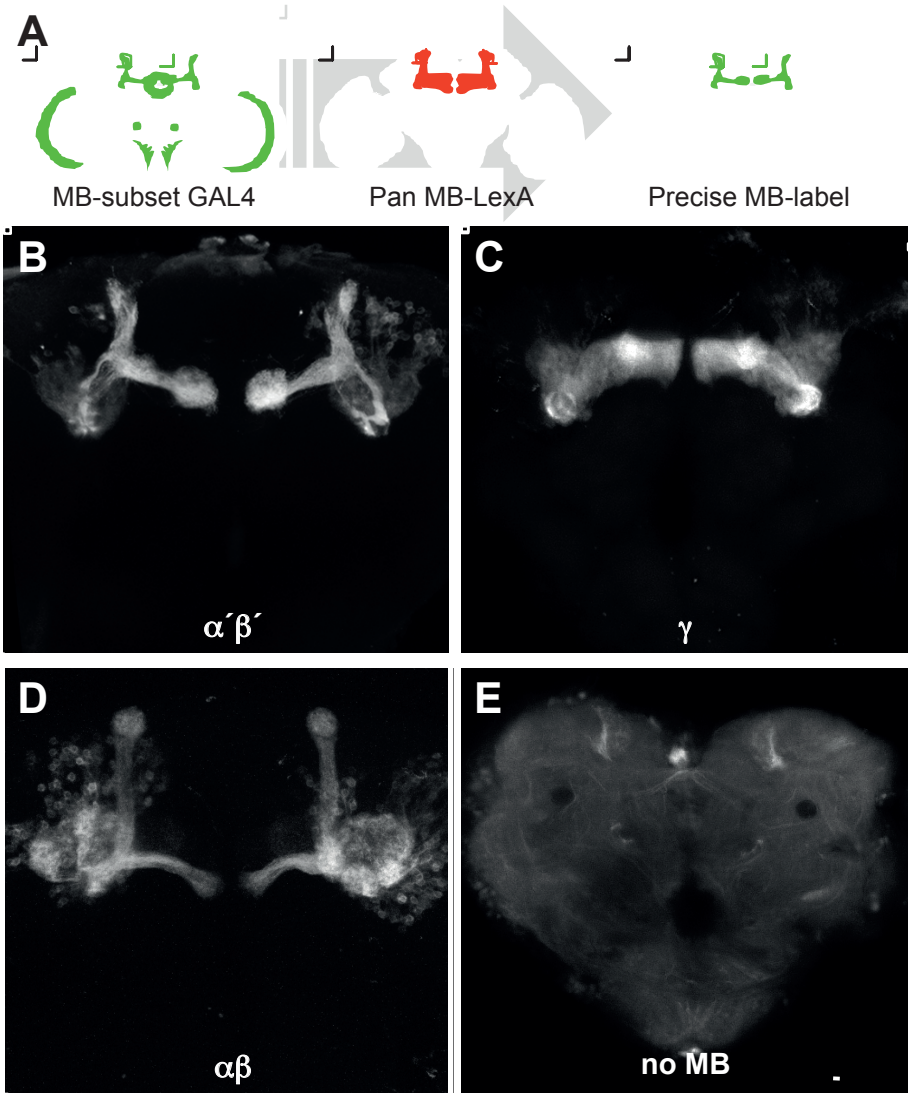


Figure II-1. *Exclusive labeling of mushroom body neurons in the fly brain with intersectional genetics.* **A.** MB-expressing GAL4s that also express elsewhere in the brain were intersected with an almost pan MB expressing LexA. A lexAop-FLP transgene was used to remove an FRT-flanked 'STOP' cassette in a uas>STOP>CD8::GFP transgene. This approach converts the **B.** c305a; **C.** NP1131 and **D.** c739 GAL4 drivers into clean labels for α´β´, γ and αβ neurons, respectively. We profiled these GFP labeled MB neurons and **E**, all neurons in the brain, except MB neurons from an elav-GAL4;MBGAL80;uas-CD8::GFP brain. Scale bar 40μm.

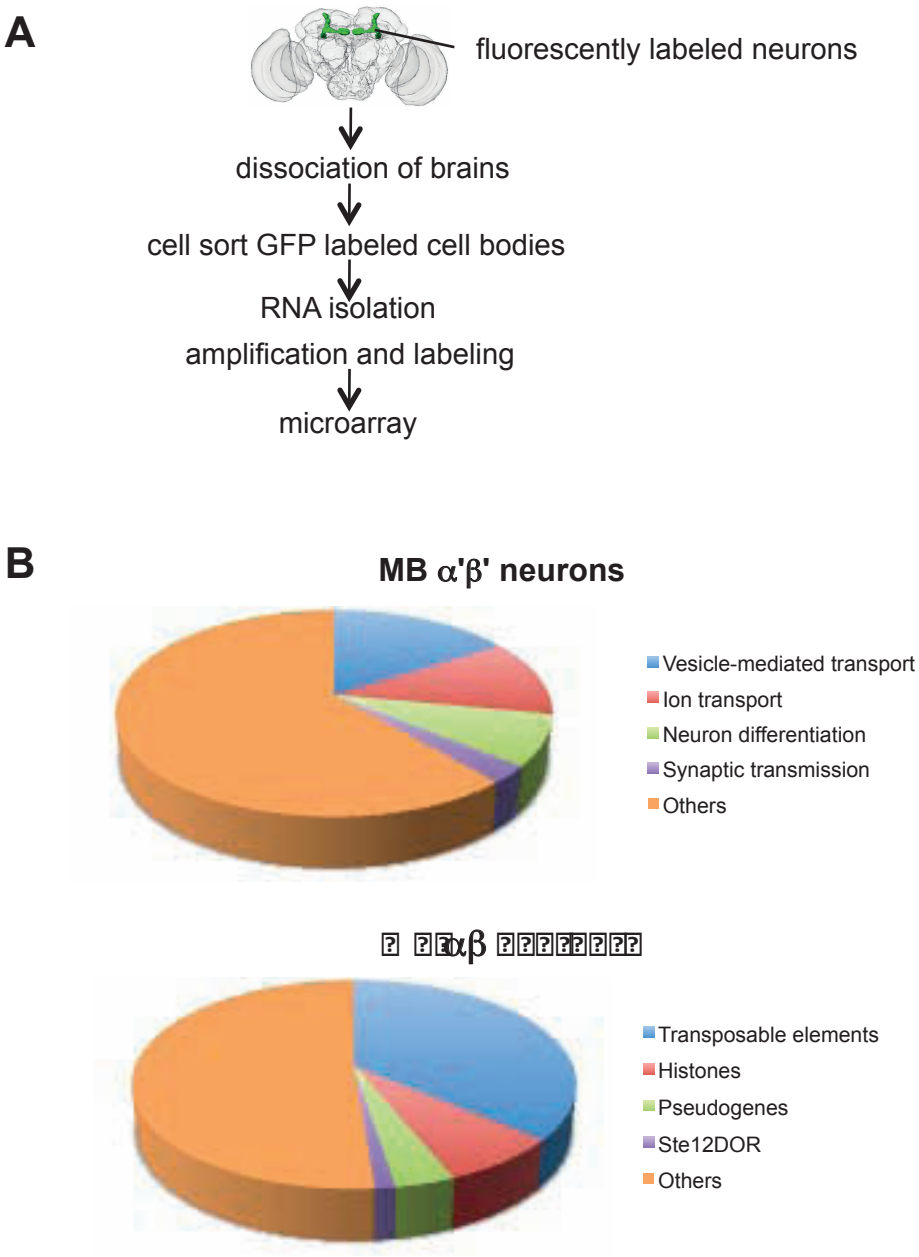


Figure II-2. *Gene expression profiling of MB neuron subsets in the Drosophila brain.* **A.** Approach for cell-type specific profiling. **B.** Approximately 40% of genes with higher expression in MB α'β' neurons are involved in neuronal communication processes. In contrast, almost half of the sequences with higher expression in MB αβ neurons belong to transposable elements or repetitive sequences, including histones.

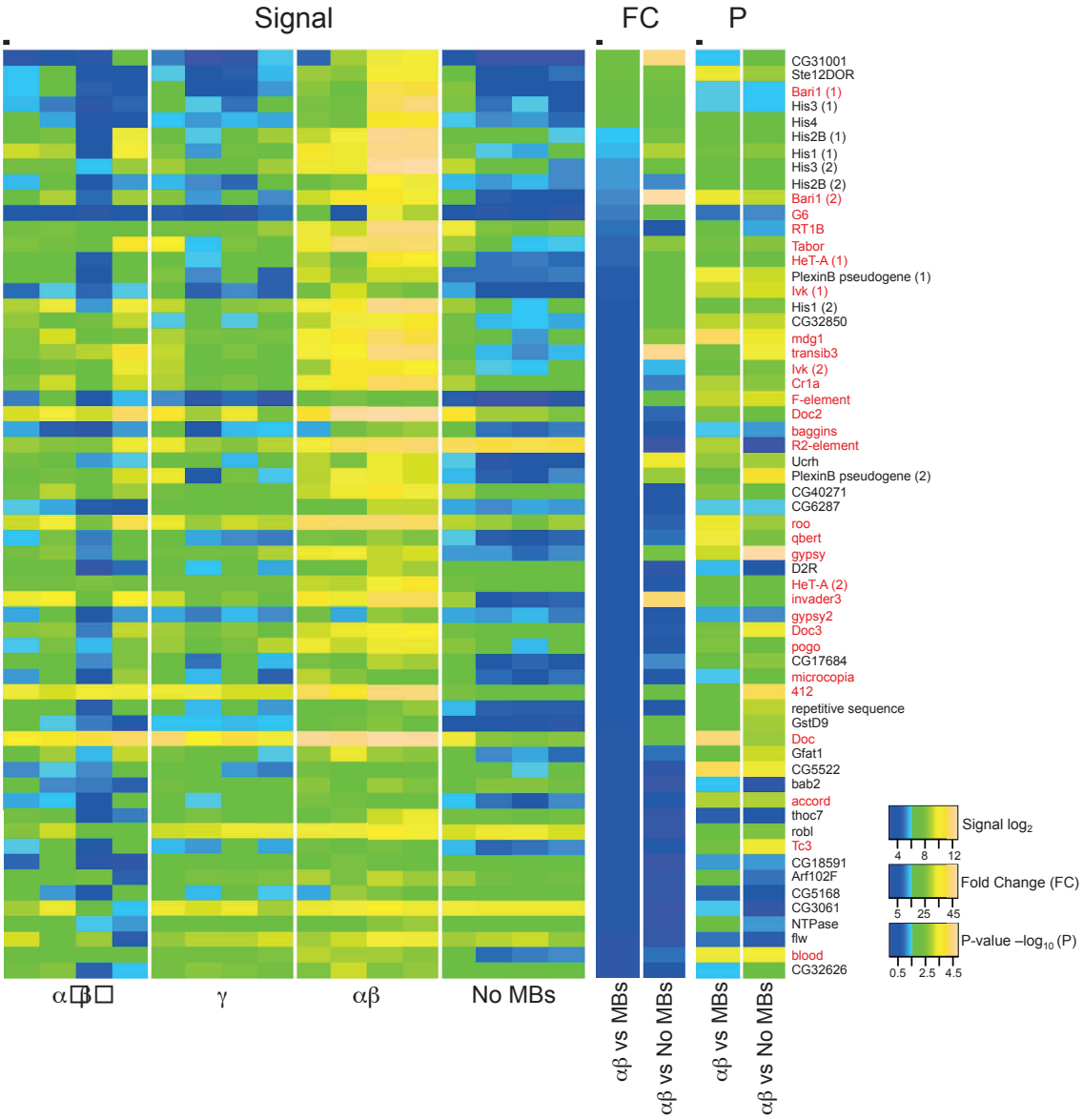


Figure II-3. *Transposable elements and histones are highly expressed in MB αβ neurons.* Microarray data emphasizing genes enriched in αβ neurons. The four 'Signal' columns per category represent independent replicates. Fold change (FC) and *t*-test *P* values are shown for αβ neurons versus other MB neurons (average value for α′β′ and γ neurons) and αβ neurons versus the rest of the brain (no MB group). The 60 genes shown represent those amongst the 150 most highly expressed αβ mRNAs that are greater than 2-fold more abundant in αβ than α′β′ neurons. The transposable elements are listed in red text. Scale bars are $\log_2$ of the signal level, linear for fold change and $-\log_{10}$ of P-value.

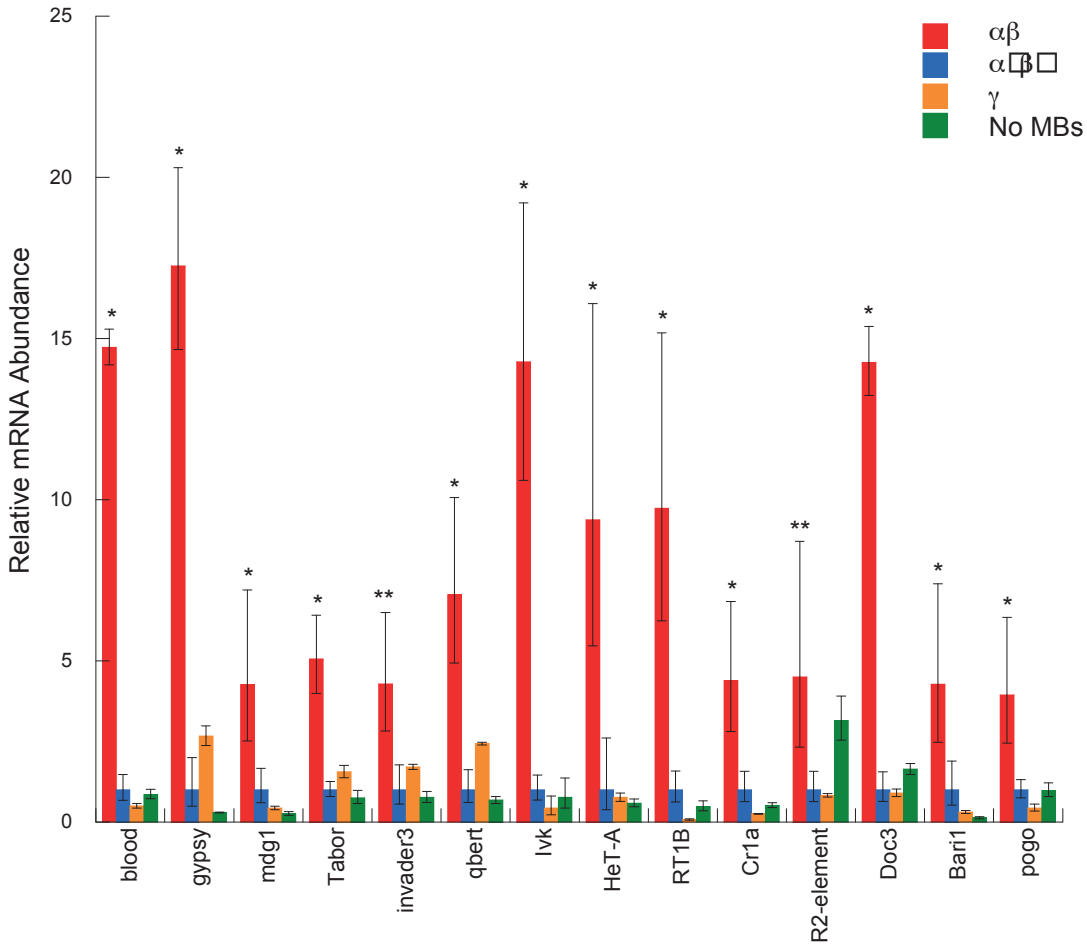
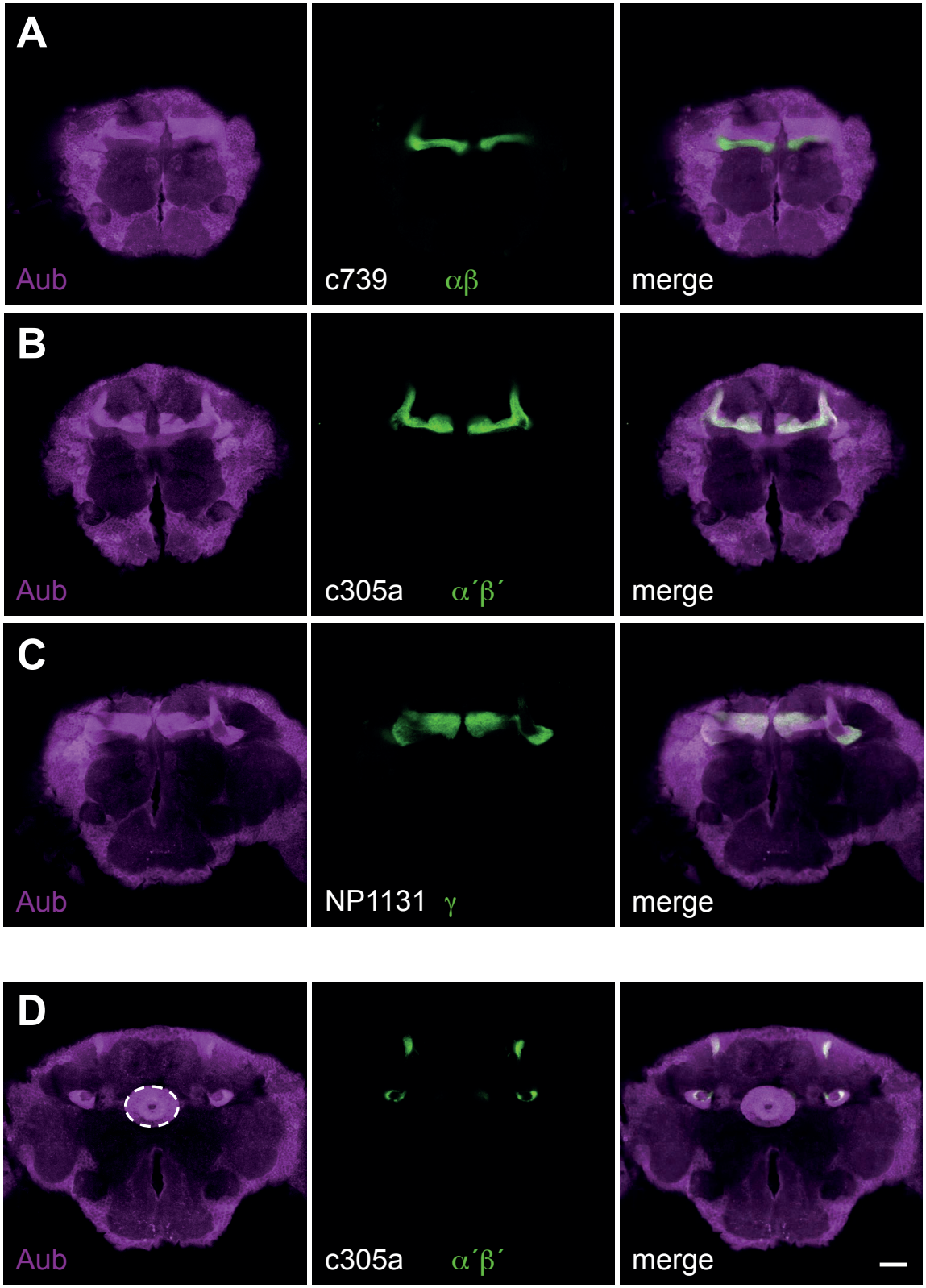


Figure II-4. *Validation of microarray results by RT-qPCR.* Expression of 14 TE mRNAs in the different MB subpopulations and non MB neurons were assayed by RT-qPCR. One asterisk denotes significant difference between αβ neurons and all other groups (*t*-test, p<0.05). Levels of invader3 were significantly higher in αβ neurons than in α'β' and non MB neurons whereas R2-element Levels were higher in αβ neurons than α'β' and γ neurons (***t*-test, p<0.05).


A
Aub
c739
αβ
merge
B
Aub
c305a
α′β′
merge
C
Aub
NP1131
γ
merge
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Aub
c305a
α′β′
merge

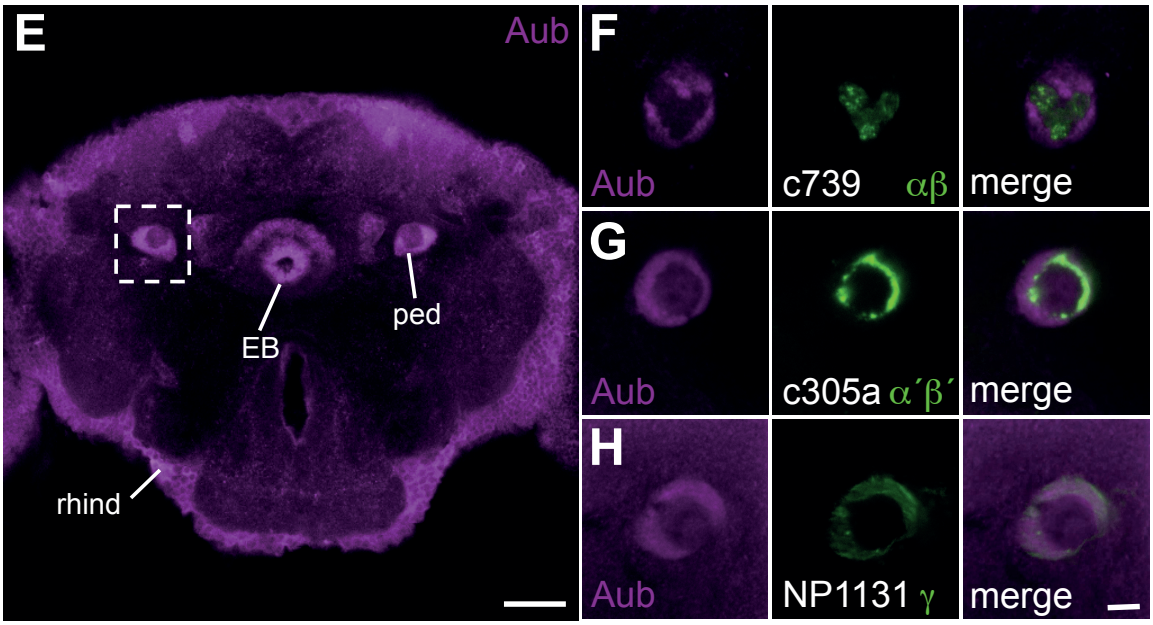
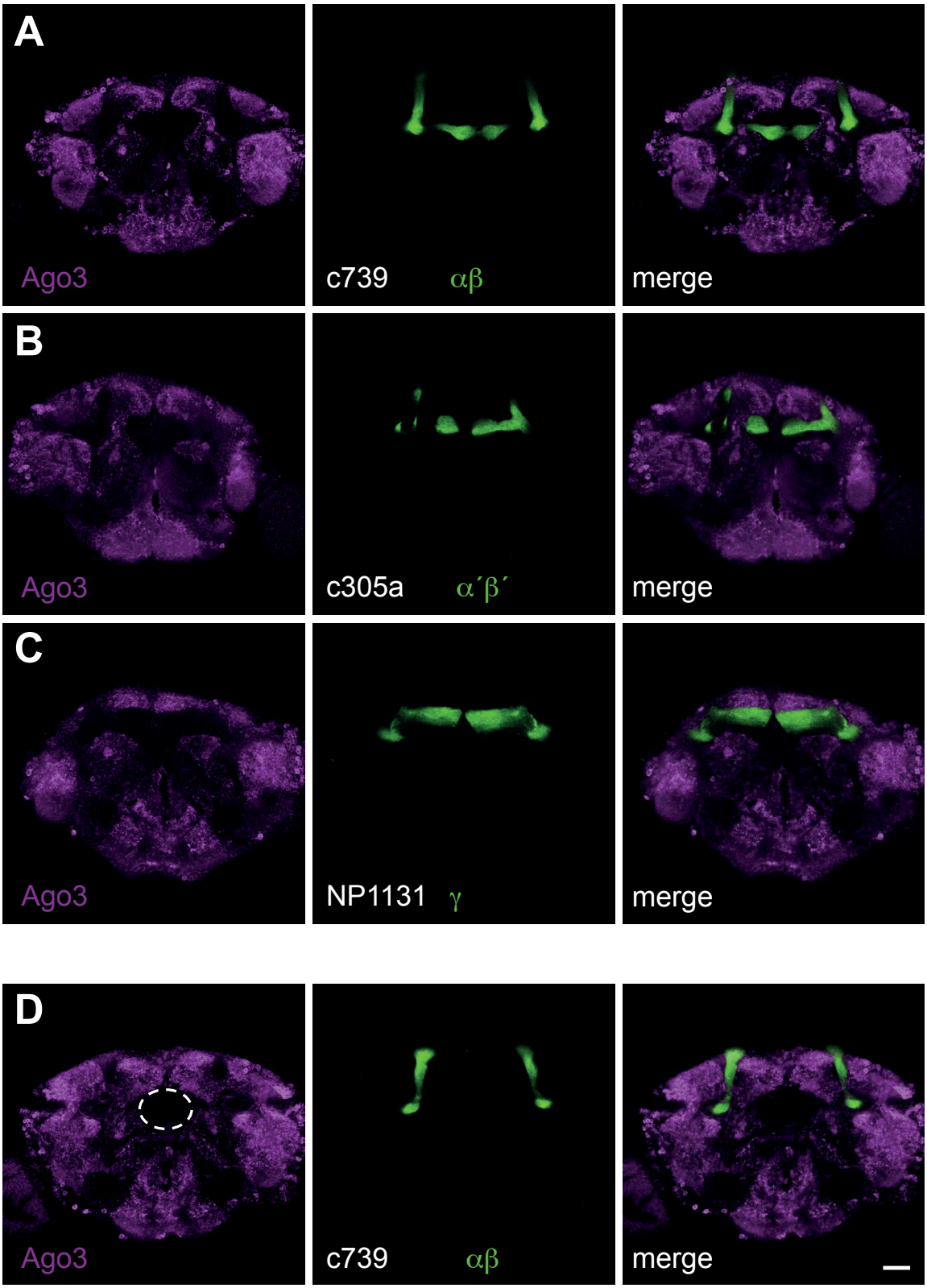


Figure II-5. *Aubergine expression in the MB.* Aub immunostaining labels the processes of α´β´ and γ neurons more strongly than those of the αβ neurons in the MB lobes. **A**. Aub immunostaining (magenta) does not co-localize with the αβ neurons labeled by c739 (green). **B**. Aub immunostaining (magenta) co-localizes with the α´β´ neurons labeled by c305a (green) and **C**. the γ neurons labeled by NP1131 (green). **D**. Aub also strongly labels the ellipsoid body of the central complex (dashed circle). **E**. Aub immunostaining strongly labels the ellipsoid body (EB) and prominent subdivision of neurons in the peduncle (ped) of the MB. **F-H**. Single confocal section through the brain at the level of the MB peduncle. Dotted box denotes approximate area of analysis. **F**. Aub labels α´β´ and g neurons in the peduncle more than the αβ neurons. Single confocal section at the level of the MB peduncle show that Aub staining is mutually exclusive to GFP expressing αβ neurons labeled with intersectional c739 but overlaps with **G**. α´β´ neurons labeled with intersectional c305a and **H**. γ neurons labeled with intersectional NP1131. A-D and F-H, left panels Aub staining; middle GFP; right panel merge. The MB neurons are labeled with flp-out GFP as in Figure II-1. Scale bars A-E, 20μm; F-H, 10μm.


A
Ago3
c739
αβ
merge
B
Ago3
c305a
α′β′
merge
C
Ago3
NP1131
γ
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D
Ago3
c739
αβ
merge

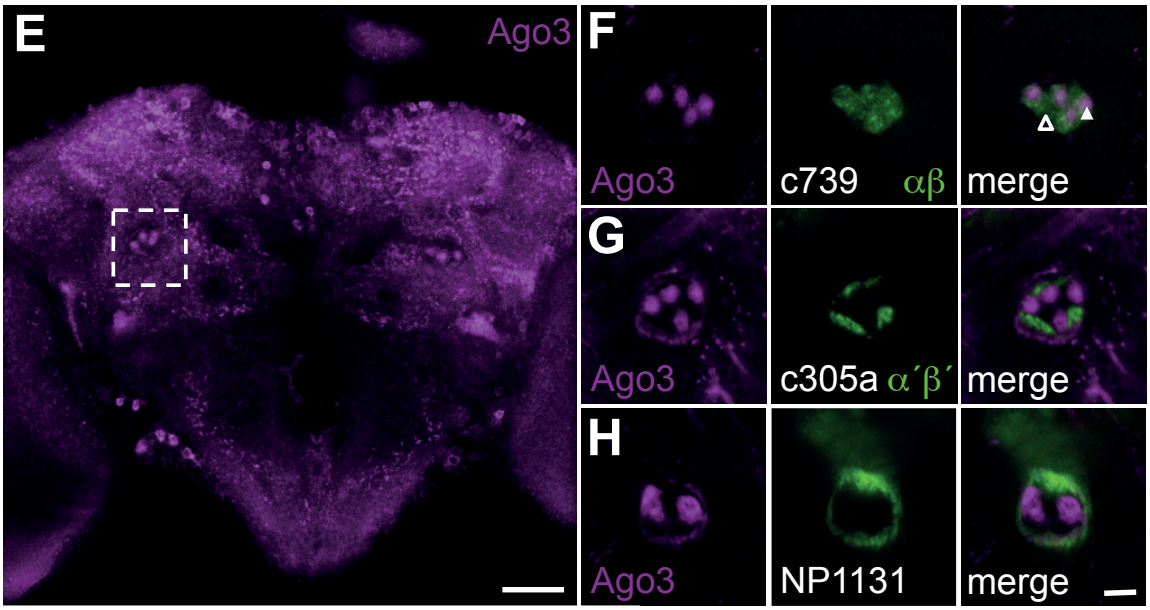

Figure II-6. *Argonaute3 expression in the MB.* Ago3 immunostaining (magenta) labels neurons throughout the brain and prominently labels subdivision of neurons in the peduncle of the MB. Ago3 immunostaining does not colocalize in the MB lobes with **A.** the αβ neurons labeled by c739, **B.** the the α´β´ neurons labeled by c305a or **C.** the γ neurons labeled by NP1131 (green). **D.** Ago3 immonolabeling is also notably absent from the ellipsoid body of the central complex (dashed circle). **F-H.** Single confocal section through the brain at the level of the MB peduncle. Dotted box denotes approximate area of analysis in panels. **F.** Ago3 staining is most prominent in the αβ core (closed triangle) and g neurons. Single confocal sections at the level of MB peduncle show that Ago3 staining **F.** does not overlap with most of the αβ neurons labeled with intersectional c739 (open triangle) nor with **G**. α´β´ labeled with intersectional c305a but overlaps with **H.** γ neurons labeled with intersectional NP1131. A-D and F-H, left panels Ago3 staining; middle GFP; right panel merge. The MB neurons are labeled with flp-out GFP as in Figure II-1. Scale bars A-E, 20μm; F-H, 10μm.

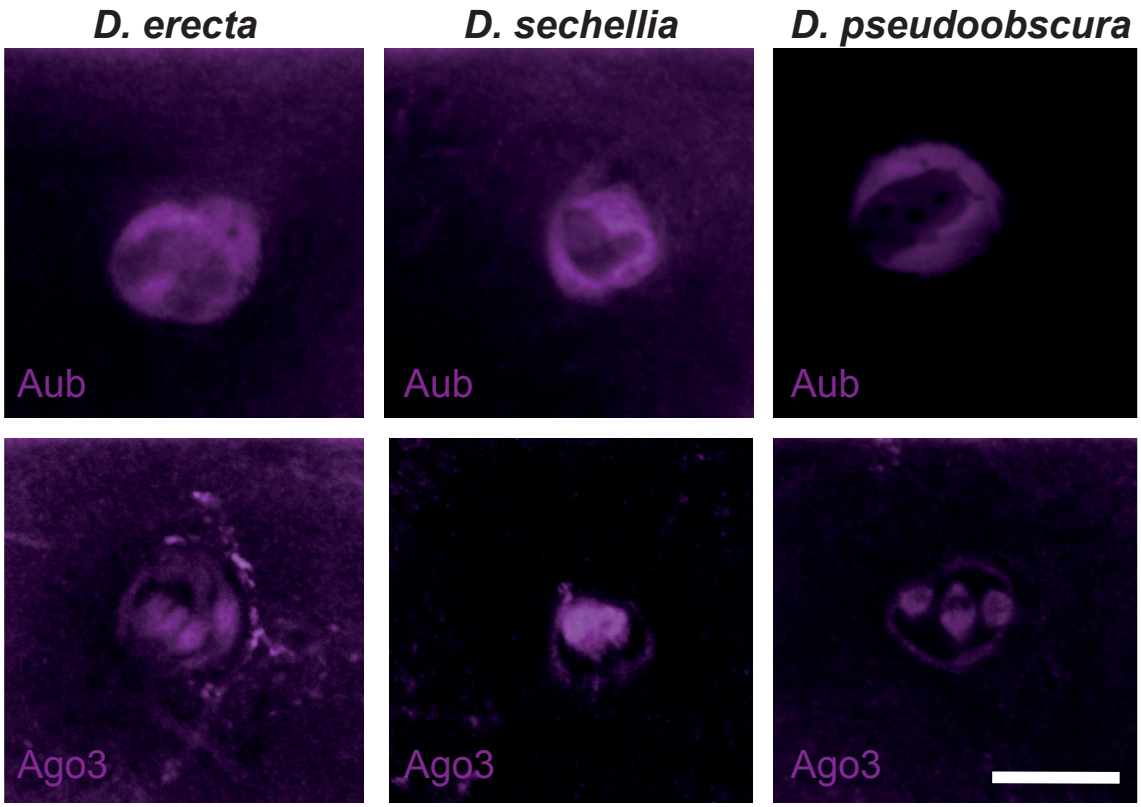

Figure II-7. *Aub and Ago3 label similarly organized neurons in the MB peduncle of other Drosophilids.* Immunostaining of adult brains from *D. erecta*, *D. sechellia* and *D. pseudoobscura*. Cross sections through the peduncle reveal weaker Aub labeling in the center of the peduncle than the periphery. Ago3 also labels the periphery of the peduncle and discrete bundles in the center. Both of these patterns are consistent with those seen in *D. melanogaster* (Figures II-5 and II-6) where Aub preferentially labels α'β' and γ neurons and Ago3 labels γ and αβ core neurons. Scale bar 10μm.

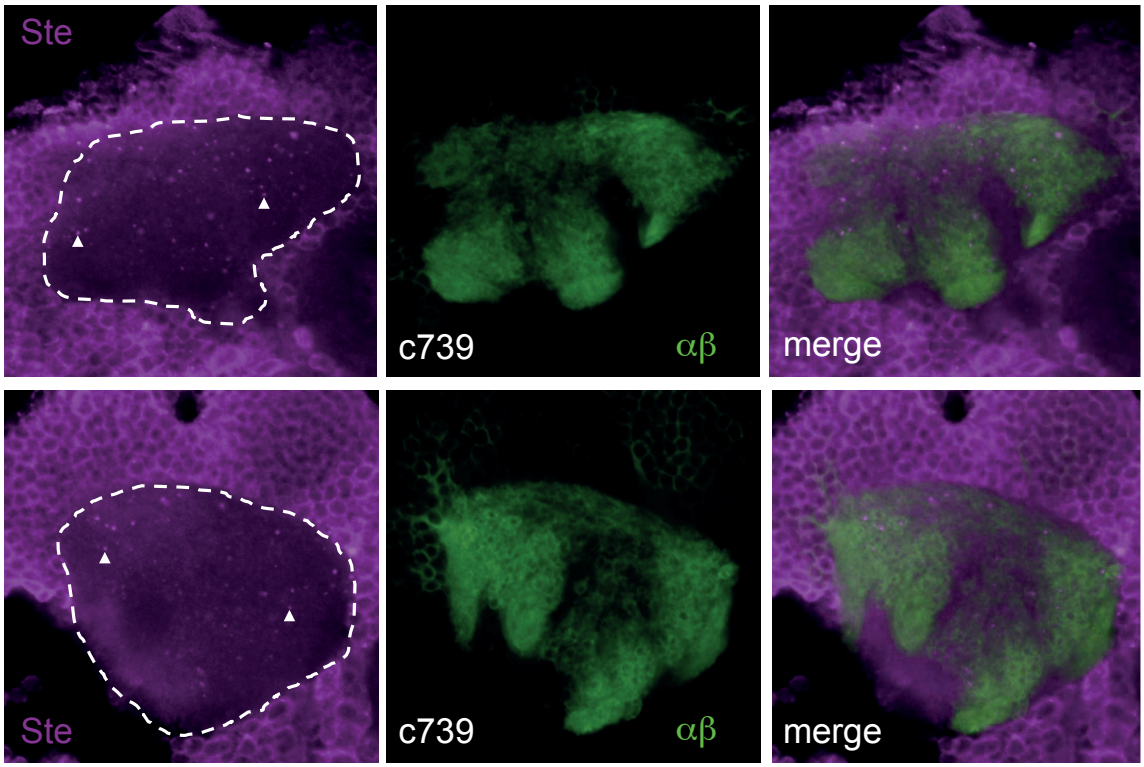

Figure II-8. *Stellate protein forms aggregates in the MB calyx.* Single confocal sections at the level of the MB calyx show Stellate protein aggregates (white arrowheads) that co-localize with αβ neurons. Scale bar 20μm. Left panels Ste staining; middle GFP; right panels merge.

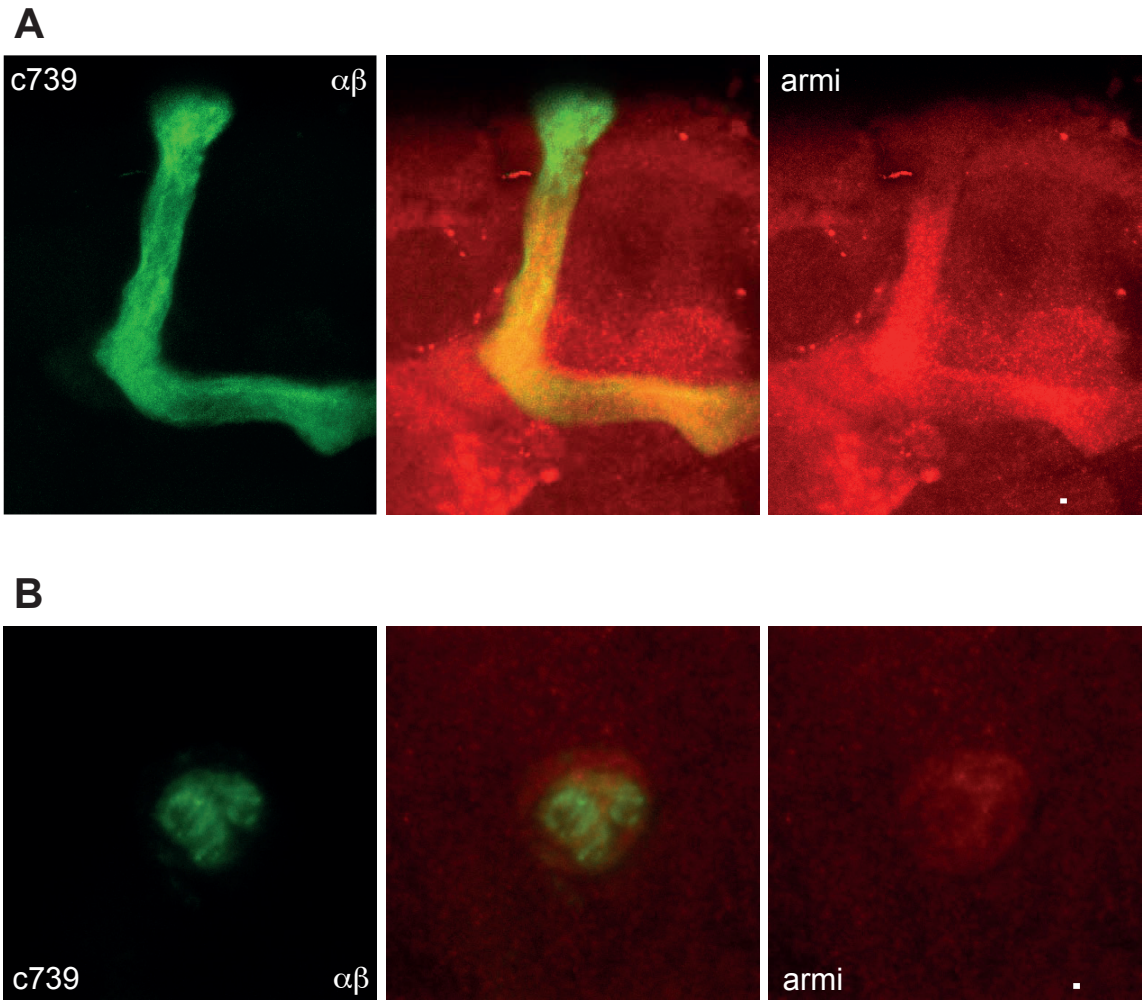

Figure II-9. *Armitage expression in the MB.* **A.** Armitage co-localizes with γ neurons and parts of the axonal projections of αβ neurons. Scale bar 20μm. **B.** Cross section of the peduncle show low levels of Armitage that are mostly excluded from αβ neurons. Scale bar 10μm. Left panels GFP; middle merge; right panels armi staining.

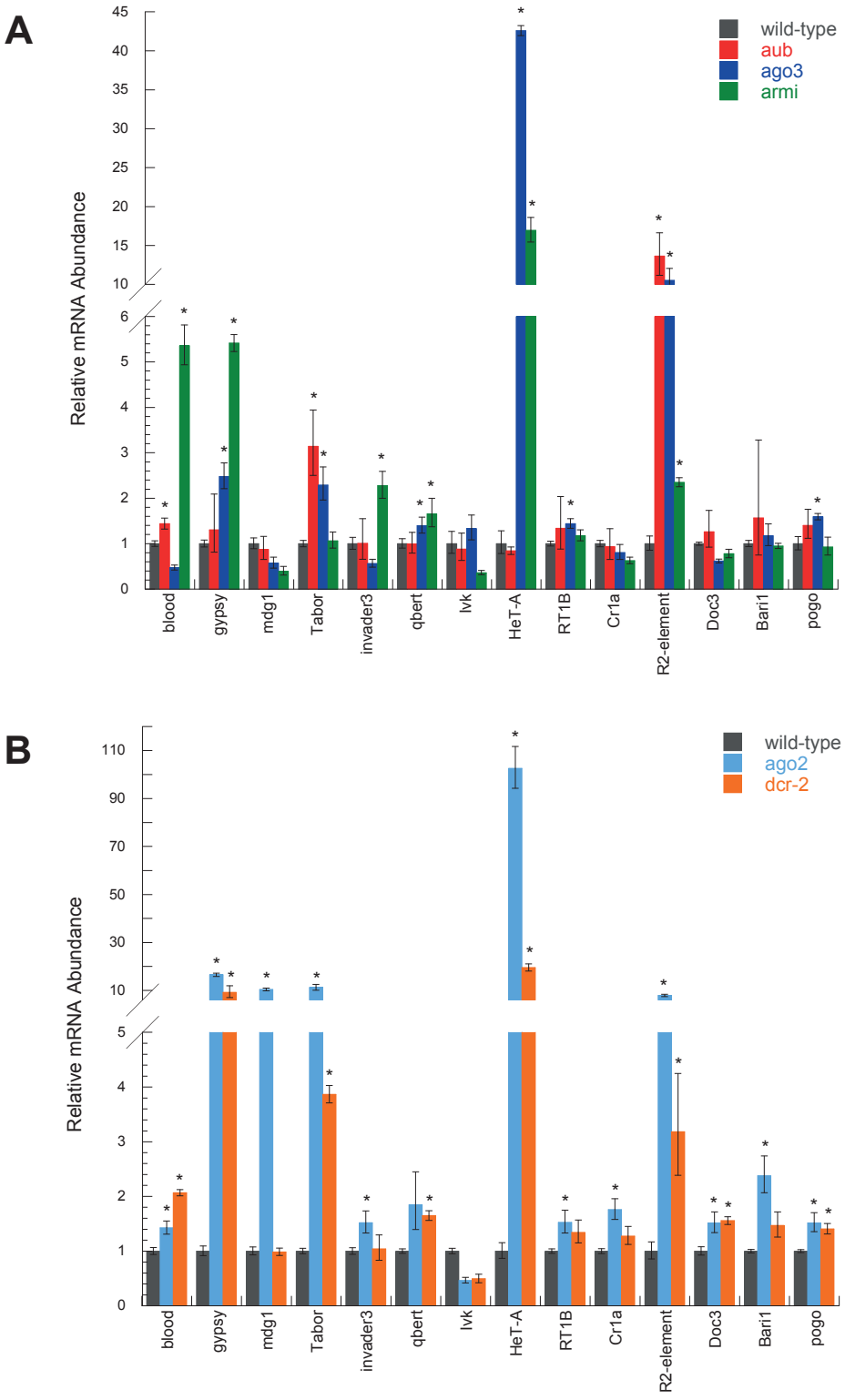
A
wild-type
aub
ago3
armi
Relative mRNA Abundance
45
40
35
30
25
20
15
10
6
5
4
3
2
1
0
blood
gypsy
mdg1
Tabor
invader3
qbert
Ivk
HeT-A
RT1B
Cr1a
R2-element
Doc3
Bari1
pogo
B
wild-type
ago2
dcr-2
Relative mRNA Abundance
110
90
70
50
30
10
5
4
3
2
1
0
blood
gypsy
mdg1
Tabor
invader3
qbert
Ivk
HeT-A
RT1B
Cr1a
R2-element
Doc3
Bari1
pogo

Figure II-10. *Transposable element expression in piRNA and siRNA pathway mutants.* **A.** Levels of several TE transcripts are elevated in *ago3* and *aub* mutant fly brains. Quantitative RT-PCR of TE transcripts from wild-type, aub^{HN2}/aub^{QC42} and $ago3^{t2}/ago3^{t3}$ mutant fly heads. Values normalized to those from wild-type flies. **B.** Some TE transcripts are also elevated in $ago2^{414}/ago2^{414}$ and $dcr\text{-}2^{L811fsX}/dcr\text{-}2^{L811fsX}$ mutants. Asterisks denote significant increase from wild-type samples, $P<0.05$ (*t*-test).

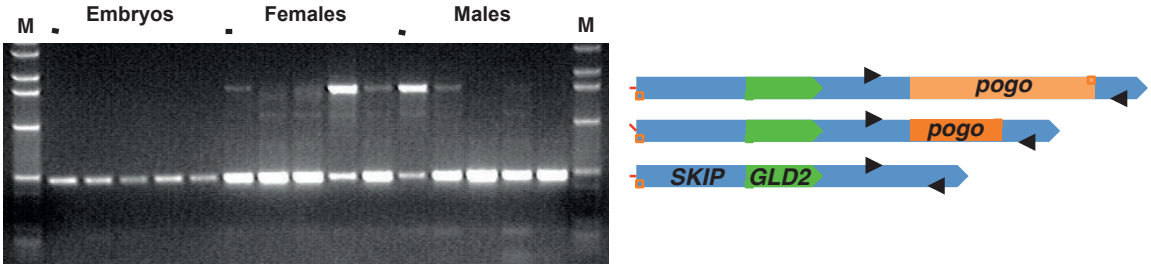

Figure II-11. *Heterogeneous insertion of pogo into the SKIP locus in adult brains.* 1.5% agarose gel showing PCR products from the *SKIP* locus from and five individual embryos 10 individual fly brains. M: DNA size markers. Schematics illustrate the nature of sequenced PCR products and the position of primers (black arrow heads).

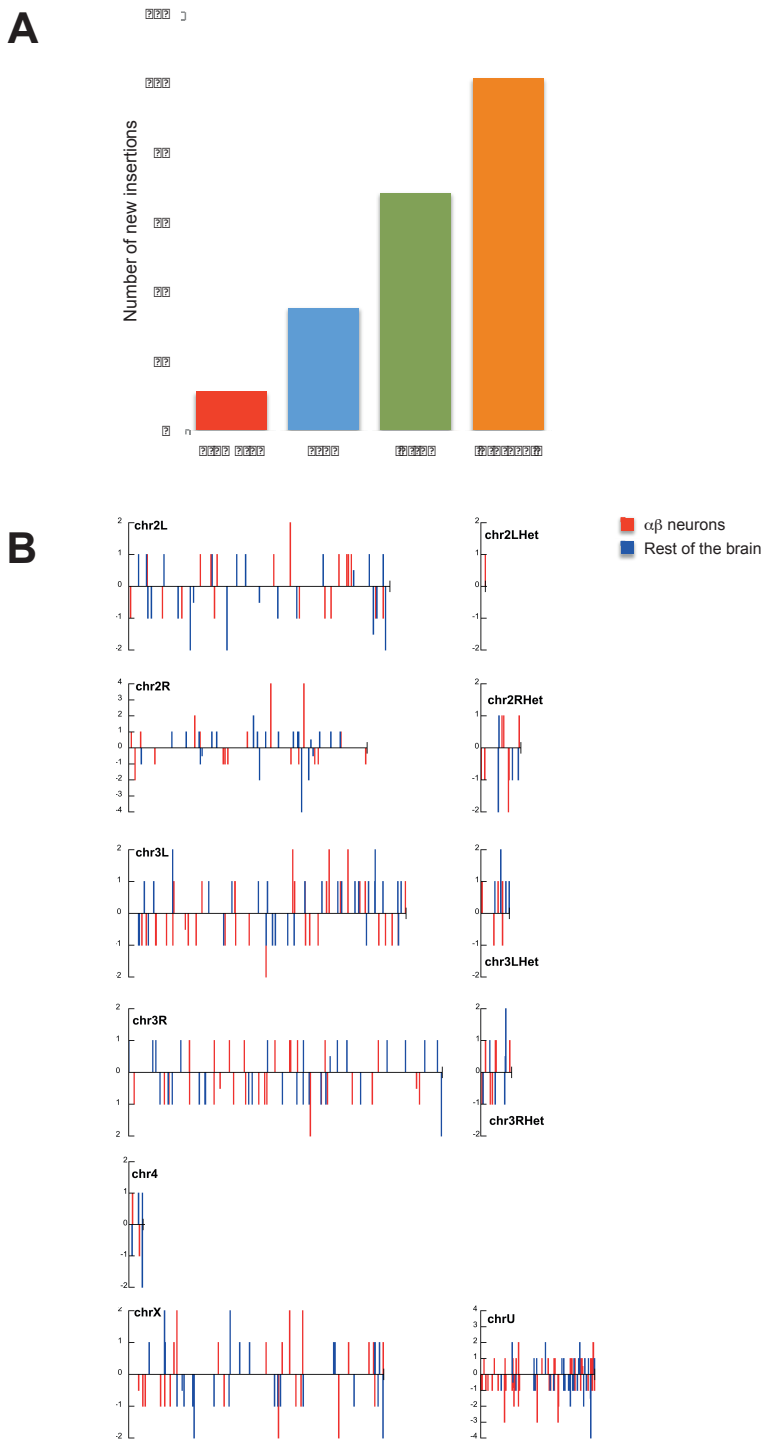

Figure II-12. *New transposons insertions in MB αβ neurons.* **A.** Distribution of new insertions relative to the closest gene. **B.** Mapping of new insertions in the adult brain according to their chromosomal locations.

Affy ID	Gene symbol	Chr	αβ Average	α'β' Average	γ Average	No MBs Average	Fold Change α'β' vs αβ
1637204_at	Ptp99A	3R	5.34	7.77	7.66	7.64	5.37
1628678_at	CG13920	3L	4.91	7.29	7.79	7.94	5.21
1628159_a_at	CG32206	3L	5.65	7.94	8.80	9.00	4.91
1624931_at	cib	X	6.42	8.50	8.58	8.22	4.24
1637525_s_at	Mpcp	3L	7.52	9.60	10.17	10.50	4.23
1635918_at	CG34401	X	5.52	7.60	7.93	7.90	4.22
1632279_at	Rac1	3L	5.72	7.79	7.70	8.53	4.21
1636967_a_at	CG5315	3R	6.25	8.29	9.46	8.89	4.11
1626902_a_at	Rab11	3R	5.36	7.38	7.94	9.09	4.06
1632429_at	Rab7	3R	5.21	7.13	7.57	7.94	3.78
1635183_at	Spn43Ab	2R	5.61	7.47	8.68	3.66	3.62
1630007_s_at	Plc21C	2L	6.63	8.46	8.23	8.48	3.55
1626304_at	Atox1	3L	6.20	7.98	8.19	8.36	3.42
1639292_at	Frq1	X	6.62	8.37	9.38	9.70	3.38
1637671_a_at	CG14767	2R	5.38	7.13	7.71	8.30	3.36
1636864_at	corto	3R	6.95	8.67	8.78	7.94	3.30
1625424_at	CG17360	3R	6.60	8.28	8.37	8.73	3.21
1632319_at	CG18598	3R	5.50	7.16	7.62	7.54	3.18
1624549_at	CG3523	2L	6.06	7.71	7.84	8.72	3.13
1623403_s_at	Cklα	X	8.43	10.07	10.19	10.18	3.12
1632731_at	CG8613	2R	6.45	8.08	8.39	8.95	3.09
1628859_at	shi	X	6.23	7.80	7.97	8.77	2.99
1640849_at	CG1458	3R	6.37	7.93	9.05	9.40	2.96
1640455_a_at	CG42446	3R	6.59	8.14	9.20	9.14	2.93
1624080_s_at	Trn	3L	7.73	9.28	9.57	9.45	2.93
1640774_a_at	Mob2	3L	7.49	9.02	9.85	10.17	2.90
1627955_a_at	Asator	4	6.77	8.29	8.25	8.65	2.86
1625131_s_at	Vha55	3R	7.44	8.95	9.39	9.63	2.85
1638445_a_at	SPoCk	3L	7.75	9.26	9.79	10.19	2.85
1635703_s_at	l(3)82Fd	3R	5.98	7.47	6.95	6.25	2.81
1639825_at	comt	X	6.30	7.79	8.49	8.88	2.81
1624726_s_at	CG7145	3L	7.14	8.62	9.33	9.84	2.80
1622984_at	Sod2	2R	5.82	7.27	8.30	8.28	2.72
1626619_at	CG9919	X	5.69	7.13	7.59	8.51	2.72
1635500_a_at	pros	3R	5.73	7.13	6.25	7.16	2.63
1624083_s_at	M(2)21AB	2L	7.47	8.84	9.38	10.32	2.58
1633443_s_at	CG2082	3R	6.25	7.57	7.48	7.46	2.49
1634670_at	CG33181	X	8.60	9.91	9.69	10.10	2.49
1629813_at	CG17691	Un	5.75	7.05	7.73	7.74	2.47
1632629_a_at	CG10804	X	7.19	8.49	9.20	9.88	2.46
1630285_at	RhoGAP100F	3R	6.13	7.42	7.16	7.63	2.45
1625646_at	CG11148	4	8.11	9.38	9.76	9.66	2.42
1637716_a_at	sqd	3R	7.38	8.66	9.26	8.98	2.41
1626970_at	Ugt35b	3R	5.84	7.11	7.43	3.51	2.41
1627228_at	CG15309	X	5.82	7.07	7.29	7.77	2.39
1632701_at	Pal	2R	6.89	8.15	8.84	8.10	2.39
1641294_a_at	CG3625	2L	6.90	8.16	8.95	9.13	2.38
1632117_s_at	Vha16	2R	7.21	8.46	8.61	9.57	2.36
1623683_at	mRpL33	X	7.24	8.48	9.81	9.41	2.36
1631142_a_at	Caps	4	7.61	8.85	8.68	8.94	2.36
1633582_at	Ih	2R	7.15	8.37	9.00	9.45	2.32
1633074_a_at	Moe	X	7.29	8.50	9.57	9.25	2.32
1629086_s_at	Scs-fp	2R	7.06	8.27	8.81	9.30	2.30
1631868_at	Vap-33-1	X	7.40	8.59	9.65	10.27	2.29
1623037_a_at	CG5594	2R	7.74	8.93	9.02	9.53	2.28
1630688_at	Hsp83	3L	6.75	7.94	9.23	8.46	2.27
1641116_at	Rsf1	2L	6.34	7.52	9.00	9.50	2.26
1625775_a_at	csw	X	6.00	7.16	7.84	6.79	2.24
1630008_at	CG18428	3R	7.14	8.30	8.56	8.56	2.24
1630470_s_at	CG9775	3R	6.26	7.42	8.09	8.37	2.23
1641355_a_at	RpL6	3R	7.37	8.48	10.10	9.46	2.16
1637767_at	CG3305	2L	9.51	10.61	10.73	10.90	2.14
1640435_at	eIF-1A	3R	8.88	9.97	10.12	10.58	2.13

Affy ID	Gene symbol	Chr	αβ Average	α'β' Average	γ Average	No MBs Average	Fold Change α'β' vs αβ
1627816_a_at	CG32264	3L	7.08	8.17	9.57	9.37	2.12
1640090_a_at	sIgA	X	7.51	8.60	9.28	9.56	2.12
1638004_at	Cbp53E	2R	8.18	9.26	10.47	11.05	2.11
1631408_at	SoxN	2L	6.32	7.40	7.48	7.09	2.10
1635360_at	CG10830	3R	6.57	7.63	7.76	7.44	2.09
1626449_s_at	exba	3R	6.01	7.06	7.82	8.11	2.07
1623373_at	CG14806	X	6.26	7.31	7.00	7.41	2.07
1634893_at	Gpdh	2L	8.18	9.22	9.73	9.94	2.06
1633143_s_at	Dyrk3	4	6.09	7.11	7.76	6.97	2.04
1636935_at	CG2219	4	6.80	7.83	8.52	8.27	2.04
1637059_s_at	DnaJ-1	3L	6.49	7.51	8.89	9.03	2.02

Table II-1. *MB α'β' neurons enriched genes.*

Affy ID	Gene symbol	Chr	αβ Average	α'β' Average	γ Average	No MBs Average	Fold Change αβ vs α'β'
1626867_at	His3	2L	9.44	4.34	6.03	5.30	34.23
1628230_x_at	CG31001	3R	8.16	3.69	3.86	2.86	22.05
1630855_s_at	Ste12DOR	X	9.14	4.79	4.78	4.69	20.38
1635715_at	His4	2L	9.04	4.79	5.27	5.00	18.96
1623831_x_at	Bari1	NA	9.09	4.98	4.60	4.71	17.35
1638308_s_at	His3	2L	10.94	7.07	7.68	6.62	14.61
1635666_at	His2B	2L	10.69	6.95	6.87	6.25	13.37
1623349_x_at	RT1B	NA	10.59	6.87	7.63	8.22	13.23
1623281_s_at	His2B	2L	8.69	5.15	5.16	5.19	11.63
1626205_s_at	G6	NA	7.04	3.59	3.66	3.11	10.89
1630585_s_at	HeT-A	NA	9.19	5.92	6.13	5.03	9.66
1629207_at	CG6287	2L	7.78	4.65	6.39	5.10	8.78
1629740_at	His1	2L	10.90	7.84	6.58	6.19	8.33
1623145_s_at	CG32009 (PlexinB pseudogene)	4	8.71	5.67	5.80	4.69	8.22
1630420_x_at	baggins	NA	7.26	4.30	5.19	5.08	7.79
1627112_s_at	Bari1	NA	9.96	7.00	6.00	4.55	7.78
1629641_s_at	Ivk	NA	7.99	5.08	5.02	3.79	7.53
1623250_at	CG18591	2L	7.09	4.23	6.86	6.55	7.23
1625195_s_at	mdg1	2R	10.68	7.86	7.86	6.12	7.05
1631349_s_at	Tabor	NA	10.92	8.11	7.38	6.35	7.00
1631321_s_at	His1	NA	10.98	8.24	8.03	6.83	6.68
1639054_s_at	pogo	NA	9.22	6.57	7.88	6.53	6.28
1630934_at	R2-element	NA	11.00	8.35	8.61	10.99	6.25
1627899_at	Ucrh	3LHet	8.74	6.15	6.41	3.89	6.04
1625632_at	robl	2R	9.95	7.40	9.34	9.27	5.88
1624819_s_at	gypsy	NA	9.35	6.81	7.62	4.89	5.83
1637749_at	D2R	X	7.53	5.04	5.77	6.41	5.61
1640606_x_at	Cr1a	NA	10.59	8.13	7.63	7.17	5.51
1624060_at	bab2	3L	7.61	5.19	6.79	7.36	5.36
1627745_s_at	Ivk	NA	9.92	7.50	6.83	6.24	5.35
1636708_at	CG3061	3R	9.71	7.39	9.06	9.64	4.97
1624390_a_at	thoc7	3L	7.95	5.73	6.97	7.20	4.67
1630948_s_at	Doc3	NA	9.65	7.45	7.74	6.70	4.61
1640702_at	CG32010 (PlexinB pseudogene)	4	9.25	7.07	6.61	4.59	4.54
1632295_s_at	Doc2	NA	11.99	9.83	8.98	8.81	4.47
1624755_a_at	flw	X	9.25	7.09	8.68	8.58	4.45
1633412_s_at	yu	X	7.00	4.85	7.10	7.18	4.44
1635829_s_at	gypsy2	NA	7.09	4.98	5.07	5.10	4.31
1624224_at	HeT-A	NA	9.52	7.45	7.36	6.79	4.19
1634633_s_at	accord	NA	7.18	5.15	5.98	4.77	4.07
1624693_at	Arf102F	4	8.36	6.36	7.37	7.55	4.00
1640932_s_at	CG32626	X	8.21	6.22	7.57	6.41	3.98
1630452_at	transib3	NA	10.89	8.91	7.30	5.55	3.93
1638615_s_at	RpS7	3R	9.83	7.87	10.42	9.79	3.89
1635135_at	roo	NA	11.31	9.38	8.86	8.25	3.81
1635589_at	CG7920	3R	8.00	6.08	8.17	7.95	3.79
1631379_a_at	ERp60	2R	7.54	5.64	7.62	7.94	3.74
1625877_s_at	uex	2RHet	9.70	7.81	6.86	6.82	3.69
1641464_s_at	CG32850	4	9.72	7.83	5.93	5.80	3.69
1624363_at	CG15201	X	9.27	7.39	9.47	3.46	3.68
1623575_at	F-element	NA	7.14	5.26	3.70	2.98	3.66
1628340_s_at	lark	3L	7.27	5.42	7.28	6.67	3.61
1636174_at	GstD9	3R	7.04	5.22	5.46	3.13	3.52
1641210_s_at	qbert	NA	7.77	5.97	5.28	4.46	3.48
1635388_s_at	RpS9	3L	10.34	8.55	10.67	10.46	3.45
1630857_s_at	NTPase	2L	7.61	5.84	6.51	6.61	3.43
1626528_at	CG40042	2LHet	8.72	6.94	7.99	8.66	3.43
1634095_at	Ef1beta	2R	7.62	5.85	8.55	8.73	3.41
1641450_s_at	micrococpia	NA	7.54	5.80	5.54	4.88	3.35

Affy ID	Gene symbol	Chr	αβ Average	α'β' Average	γ Average	No MBs Average	Fold Change αβ vs α'β'
1631775_at	CG11137	3L	7.88	6.15	7.90	7.63	3.32
1634249_s_at	Pdsw	2L	9.03	7.31	8.91	9.05	3.28
1639330_s_at	Tao-1	X	7.05	5.33	7.28	7.14	3.28
1632545_s_at	CG5522	2R	7.07	5.36	5.53	5.86	3.27
1624406_at	CG5168	2L	7.08	5.38	5.79	6.07	3.24
1628041_a_at	Dek	2R	8.01	6.32	8.74	6.75	3.23
1624440_at	repetitive sequence	NA	7.32	5.63	5.56	4.43	3.22
1635886_s_at	blood	NA	8.40	6.73	7.42	5.02	3.17
1625997_s_at	Doc	NA	12.31	10.68	10.58	8.39	3.09
1627513_at	CG17684	2RHet	7.93	6.31	5.73	4.45	3.07
1628705_at	PH4αEFB	3R	7.15	5.54	7.63	7.52	3.05
1638131_s_at	5-HT1A	2R	7.28	5.68	7.61	7.79	3.04
1640167_s_at	412	NA	11.22	9.61	9.36	7.04	3.03
1636564_at	CG14757	2R	7.09	5.50	7.50	7.24	3.01
1635177_at	CG7770	3L	7.09	5.52	7.52	7.32	2.97
1638469_s_at	CG3857	X	10.08	8.52	10.03	7.94	2.95
1637853_a_at	Hk	X	7.18	5.64	7.08	7.19	2.92
1629578_at	Tango7	2R	8.02	6.49	8.26	8.09	2.89
1627489_a_at	CG10433	2R	8.91	7.44	8.73	4.18	2.78
1629640_at	Tc3	NA	7.07	5.61	5.39	4.81	2.76
1627936_s_at	invader3	NA	10.51	9.05	7.80	5.24	2.75

Table II-2. *MB αβ neurons enriched genes.* Transposable elements are marked in red.

Transposon	Fold Change αβ vs α'β'	p-value	Fold Change αβ vs γ	p-value	Fold Change αβ vs No MBs	p-value
Bari1	17.35	0.02711	22.51	0.01999	20.85	0.03174
RT1B	13.23	0.00844	7.79	0.02244	5.18	0.04274
G6	10.89	0.08584	10.40	0.08755	15.18	0.06406
HeT-A	9.66	0.01712	8.38	0.02252	17.94	0.00667
baggins	7.79	0.01827	4.22	0.08882	4.54	0.04922
Bari1	7.78	0.04691	15.56	0.00563	42.56	0.00074
Ivk	7.53	0.00198	7.83	0.00725	18.39	0.00056
mdg1	7.05	0.00283	7.09	0.00024	23.59	0.00044
Tabor	7.00	0.04210	11.59	0.03824	23.73	0.00227
pogo	6.28	0.02773	2.53	0.04181	6.47	0.01546
R2-element	6.25	0.00275	5.24	0.00237	1.01	0.97928
gypsy	5.83	0.02758	3.32	0.00684	22.05	0.00001
Cr1a	5.51	0.01118	7.78	0.00159	10.67	0.00194
Ivk	5.35	0.03248	8.52	0.01020	12.84	0.00288
Doc3	4.61	0.08546	3.75	0.00434	7.71	0.00034
Doc2	4.47	0.02329	8.06	0.00968	9.08	0.00665
gypsy2	4.31	0.05941	4.06	0.05612	3.96	0.05961
HeT-A	4.19	0.02012	4.46	0.01972	6.62	0.00555
accord	4.07	0.01872	2.29	0.00510	5.31	0.00105
trans b3	3.93	0.05542	12.04	0.00590	40.47	0.00027
roo	3.81	0.07520	5.46	0.00133	8.35	0.00140
F-element	3.66	0.05103	10.83	0.00716	17.85	0.00055
qbert	3.48	0.05746	5.62	0.00194	9.88	0.00284
microcopia	3.35	0.16792	3.99	0.02983	6.31	0.01115
blood	3.17	0.00611	1.97	0.00215	10.42	0.00022
Doc	3.09	0.00188	3.30	0.01591	15.09	0.00144
412	3.03	0.00710	3.62	0.00507	18.13	0.00006
Tc3	2.76	0.13707	3.21	0.00187	4.80	0.00036
invader3	2.75	0.19324	6.54	0.00544	38.41	0.01018
rover	2.27	0.12854	2.67	0.03145	10.27	0.00287

Table II-3. *Comparison of transposable elements expression*. Expression of transposable elements is significantly higher in MB αβ neurons than in the others populations. TE expression in αβ neurons is also higher when compared with the rest of the brain (No MBs population) (*t*-test).

Transposon	Break point	Closest Gene	Distance to Gene
blood	chr2L:166531.167031	spen	0
Dm88	chr2L:1633907.1634407	chinmo	-16852
17.6	chr2L:2972495.2972995	gammaTub23C	0
3S18	chr2L:4682374.4682874	CG11929	0
Bari1	chr2L:6311178.6311678	Ddr	0
Quasimodo	chr2L:7234039.7234539	Ndae1	0
roo	chr2L:7551110.7551610	Rapgap1	0
297	chr2L:7789013.7789513	CG7149	0
HMS-Beagle2	chr2L:12777138.12777638	CG15483	-15298
1731	chr2L:14227307.14227807	smi35A	0
Doc	chr2L:15034199.15034699	GABA-B-R1	0
Doc	chr2L:17284369.17284869	CG15140	-1424
copia	chr2L:17819466.17819966	CadN2	0
Stalker4	chr2L:18521563.18522063	CG10211	0
Stalker	chr2L:18521563.18522063	CG10211	0
mdg3	chr2L:19208185.19208685	drl	0
F-element	chr2L:19357224.19357724	dnt	0
297	chr2L:19606291.19606791	Lar	0
mdg1	chr2L:21764351.21764851	CG31612	0
McClintock	chr2L:22415351.22415851	RpL5	11681
Stalker2	chr2LHet:368128.368628	CG40042	-199008
Ivk	chr2R:236965.237465	Gprk1	0
invader3	chr2R:568266.568766	CG17883	7121
micropia	chr2R:1056067.1056567	Nipped-A	8938
Doc	chr2R:2316078.2316578	jing	-73185
roo	chr2R:5850564.5851064	Pal1	0
rover	chr2R:6324027.6324527	RanBPM	0
roo	chr2R:8368193.8368693	Dh44-R2	0
17.6	chr2R:8529663.8530163	CG8785	0
roo	chr2R:8770581.8771081	Aats-asp	0
invader6	chr2R:10502719.10503219	Su(var)2-HP2	0
mdg1	chr2R:12571569.12572069	CG30463	0
F-element	chr2R:14360060.14360560	CG30116	0
roo	chr2R:15050897.15051397	CG10737	-254
FB	chr2R:15507018.15507518	sm	0
Burdock	chr2R:16464261.16464761	qsm	0
Idefix	chr2R:16780079.16780579	otp	0
copia	chr2R:18787172.18787672	nahoda	0
FB	chr2R:20977072.20977572	CG16778	0
Idefix	chr2RHet:26396.26896	CG40498	25914
Stalker4	chr2RHet:320886.321386	rl	68936
Stalker	chr2RHet:320886.321386	rl	68936
copia	chr2RHet:1738982.1739482	Scp1	0
Stalker4	chr2RHet:1883138.1883638	CG42596	0
Stalker	chr2RHet:1883138.1883638	CG42596	0
gypsy12	chr2RHet:2265991.2266491	CG41242	-108014
rover	chr2RHet:2314235.2314735	RYamide	-139463
297	chr2RHet:3152085.3152585	uex	0
BS	chr3L:1168829.1169329	bab2	0
copia	chr3L:1559391.1559891	CG7879	0
17.6	chr3L:2381853.2382353	CG13800	0
Doc	chr3L:2437752.2438252	CG42669	0
roo	chr3L:3342781.3343281	kst	0
HMS-Beagle	chr3L:3902321.3902821	Ubi-p63E	0
jockey	chr3L:3992210.3992710	scrt	3621
diver	chr3L:5003244.5003744	Con	0
297	chr3L:5268684.5269184	shep	0
jockey	chr3L:5959650.5960150	CG10479	490
gypsy	chr3L:6471091.6471591	CG42747	0
roo	chr3L:8521010.8521510	foi	0
Doc	chr3L:9376109.9376609	Hsp23	244
roo	chr3L:9399429.9399929	CG4022	0
Quasimodo	chr3L:9444197.9444697	CG3448	0
I-element	chr3L:10645582.10646082	CG32066	0
copia	chr3L:12132842.12133342	RhoGAP68F	0

Transposon	Break point	Closest Gene	Distance to Gene
mdg1	chr3L:12717131.12717631	SmD1	8827
mdg3	chr3L:14487633.14488133	bbg	0
Doc	chr3L:14660117.14660617	dlp	0
invader2	chr3L:15596698.15597198	RhoGAP71E	0
McClintock	chr3L:15617353.15617853	comm3	0
FB	chr3L:15991304.15991804	CG5830	0
gypsy	chr3L:16729590.16730090	CG9701	0
opus	chr3L:17474251.17474751	CG6333	0
Ivk	chr3L:17708342.17708842	Ccn	0
412	chr3L:18709823.18710323	CG12477	4669
297	chr3L:19369773.19370273	CG32206	0
roo	chr3L:20331329.20331829	Spn77Bc	-3904
roo	chr3L:20420698.20421198	CG5262	0
412	chr3L:20894520.20895020	CG11458	-3874
Circe	chr3L:22092327.22092827	msopa	16043
297	chr3L:22681494.22681994	CG14456	103
roo	chr3L:23275551.23276051	nAcRalpha-80B	0
roo	chr3L:23808385.23808885	CG40470	24073
G2	chr3L:24441956.24442456	nvd	47201
Doc	chr3LHet:112215.112715	ARY	69730
Doc	chr3LHet:1166228.1166728	CG40178	14411
Transpac	chr3LHet:1539209.1539709	CG41320	0
17.6	chr3LHet:1947749.1948249	CG40228	240666
Idefix	chr3LHet:1949300.1949800	CG40228	239115
1360	chr3LHet:2234356.2234856	vtd	-8714
BS	chr3R:484515.485015	CG43427	-289
1731	chr3R:3180466.3180966	CG43290	-5192
roo	chr3R:3575705.3576205	pyd3	0
3S18	chr3R:5408706.5409206	CG33654	0
FB	chr3R:5421083.5421583	CG8312	0
roo	chr3R:7598101.7598601	l(3)neo38	0
roo	chr3R:7617757.7618257	Sbf	0
Stalker4	chr3R:8134193.8134693	CG4066	-2381
Stalker	chr3R:8134193.8134693	CG4066	-2381
roo	chr3R:8965410.8965910	2mit	0
opus	chr3R:9330458.9330958	CG12538	17714
17.6	chr3R:10293437.10293937	cv-c	0
412	chr3R:10400992.10401492	CG7987	0
Quasimodo	chr3R:10985789.10986289	Mf	10057
Stalker4	chr3R:11569846.11570346	CG4520	-29
Stalker	chr3R:11569846.11570346	CG4520	-29
297	chr3R:12100543.12101043	gish	0
I-element	chr3R:12294043.12294543	CG42232	0
Circe	chr3R:13013275.13013775	Mur89F	-27605
invader1	chr3R:13020392.13020892	CG31262	21945
412	chr3R:14333427.14333927	fru	0
roo	chr3R:14421707.14422207	CG14304	0
Ivk	chr3R:15026575.15027075	CG31475	0
jockey	chr3R:16162851.16163351	Ir92a	-1637
Bari1	chr3R:17141897.17142397	CG16791	4084
Juan	chr3R:17476905.17477405	E2f	0
opus	chr3R:17689177.17689677	lsn	-1244
297	chr3R:19895033.19895533	4EHP	0
roo	chr3R:21677052.21677552	CG5886	0
jockey	chr3R:22213261.22213761	CG14239	13869
Burdock	chr3R:25611677.25612177	kay	0
roo	chr3R:25873641.25874141	CG7943	0
invader2	chr3RHet:111926.112426	CG40155	-19739
Transpac	chr3RHet:388979.389479	CG15831	0
Quasimodo	chr3RHet:775422.775922	CG42621	-103186
copia	chr3RHet:949353.949853	CG41281	-67069
HMS-Beagle2	chr3RHet:1200693.1201193	CG41056	0
Doc	chr3RHet:1261115.1261615	CG41056	0
297	chr3RHet:2372558.2373058	CG42402	-8923
roo	chr4:355358.355858	PMCA	0

Transposon	Break point	Closest Gene	Distance to Gene
rover	chr4:1006381.1006881	toy	-3472
297	chrU:8041.8541	CG42644	-796
I-element	chrU:124584.125084	CG40378	0
17.6	chrU:293972.294472	CG43676	10991
invader3	chrU:454927.455427	CG42623	0
F-element	chrU:640353.640853	CG40245	46995
G6	chrU:1010549.1011049	CG40249	-46685
roo	chrU:1197510.1198010	CG17691	12736
Tabor	chrU:1534914.1535414	CG41497	148391
Quasimodo	chrU:2096748.2097248	CG41087	-6669
Doc	chrU:2149169.2149669	CG41087	-59090
transib2	chrU:2224434.2224934	CG40188	-7316
412	chrU:2768860.2769360	CG41367	-8455
McClintock	chrU:2809375.2809875	CG40551	16683
Tabor	chrU:2809375.2809875	CG40551	16683
rover	chrU:2836710.2837210	CG40551	44018
Doc	chrU:2929447.2929947	CG40801	0
HMS-Beagle2	chrU:3053396.3053896	CG40813	-82365
Ivk	chrU:3127165.3127665	CG40813	-8596
Idefix	chrU:3328292.3328792	CG40813	191316
3S18	chrU:3336067.3336567	CG40813	199091
G2	chrU:3371005.3371505	CG40813	234029
F-element	chrU:3421939.3422439	CG40813	284963
X-element	chrU:4806365.4806865	CG40992	245301
TAHRE	chrU:4981366.4981866	CG40992	70300
Quasimodo	chrU:5390634.5391134	CG41562	-75961
HMS-Beagle	chrU:5940868.5941368	CG40625	428943
1360	chrU:6030465.6030965	CG40625	518540
Doc	chrU:6163395.6163895	SteXh:CG42398	-457625
mdg1	chrU:6462902.6463402	SteXh:CG42398	-158118
Juan	chrU:6476319.6476819	SteXh:CG42398	-144701
F-element	chrU:6494410.6494910	SteXh:CG42398	-126610
HMS-Beagle	chrU:6539777.6540277	SteXh:CG42398	-81243
invader2	chrU:6813353.6813853	SteXh:CG42398	191101
Tabor	chrU:6859328.6859828	SteXh:CG42398	237076
Ivk	chrU:7315703.7316203	CG40930	184983
Doc	chrU:7385878.7386378	CG40930	255158
copia	chrU:7894199.7894699	CG41020	94336
Dm88	chrU:7897892.7898392	CG41020	98029
412	chrU:7902466.7902966	CG41020	102603
Doc	chrU:8030195.8030695	CG40936	-10431
Doc	chrU:8251927.8252427	CG40936	210166
Quasimodo	chrU:8308353.8308853	CG40936	266592
Doc	chrU:8313288.8313788	CG40936	271527
17.6	chrU:8762860.8763360	CG40948	-97895
1731	chrU:8804303.8804803	CG40948	-139338
Doc	chrU:8831712.8832212	CG40948	-166747
Juan	chrU:8916289.8916789	CG40948	-251324
Stalker4	chrU:8981180.8981680	CG40948	-316215
Stalker	chrU:8981180.8981680	CG40948	-316215
micropia	chrU:9065620.9066120	CG40635	297659
roo	chrU:9463346.9463846	CG40635	-99249
roo	chrU:9686887.9687387	CG43176	-132262
412	chrU:9692164.9692664	CG43176	-126985
G2	chrU:9795744.9796244	CG43176	-23405
copia	chrU:9822645.9823145	CG43176	2759
roo	chrU:9910720.9911220	CG43176	90834
Stalker2	chrU:9934753.9935253	CG43176	114867
roo	chrU:10021095.10021595	CG43176	201209
Tabor	chrU:10033206.10033706	CG43176	213320
HMS-Beagle	chrX:878562.879062	CG18823	-5552
412	chrX:1244048.1244548	CG12773	0
roo	chrX:1461235.1461735	Mur2B	0
roo	chrX:1461238.1461738	Mur2B	0
roo	chrX:2802598.2803098	kirre	0

Transposon	Break point	Closest Gene	Distance to Gene
Ivk	chrX:3170314.3170814	dnc	0
Juan	chrX:3646994.3647494	tlk	0
Doc	chrX:3979221.3979721	CG43689	0
roo	chrX:4238901.4239401	norpA	0
hopper	chrX:7880261.7880761	sn	143
F-element	chrX:8387638.8388138	CG33181	0
blood	chrX:12082080.12082580	Ten-a	0
297	chrX:13168918.13169418	HDAC4	0
Transpac	chrX:13497708.13498208	up	-1172
blood	chrX:14147621.14148121	mud	0
Transpac	chrX:14152530.14153030	mud	0
Ivk	chrX:14699192.14699692	rut	0
jockey	chrX:15150541.15151041	PPYR1	-11595
17.6	chrX:15296536.15297036	cngl	0
gypsy	chrX:18466187.18466687	Bx	0
Bari1	chrX:19327336.19327836	kek5	0
Idefix	chrX:21125571.21126071	CG32521	0
412	chrX:21524582.21525082	DIP1	-23397
Tabor	chrX:21634083.21634583	DIP1	-132898
mdg3	chrX:22376780.22377280	stnA	5277

Table II-4. *New transposon insertions in MB $\alpha\beta$ neurons.*

A.

GO Term	Count	P-Value	Benjamini
Cell morphogenesis involved in neuron differentiation	10	1.90E-04	2.70E-02
Cell motion	10	2.90E-04	2.80E-02
Cell morphogenesis involved in differentiation	10	2.70E-04	3.10E-02
Cell projection morphogenesis	10	4.10E-04	3.40E-02
Neuron projection development	10	1.80E-04	3.50E-02
Neuron differentiation	11	5.80E-04	3.70E-02
Cell part morphogenesis	10	5.20E-04	3.80E-02
Neuron development	10	7.40E-04	4.20E-02
Axonogenesis	9	7.50E-05	4.30E-02
Plasma membrane	16	5.20E-04	4.70E-02
Cell projection organization	10	1.10E-03	5.00E-02
Neuron projection morphogenesis	10	1.80E-04	5.00E-02

B.

GO Term	Count	P-Value	Benjamini
Synaptic vesicle transport	5	2.20E-03	7.40E-01
Synaptic vesicle exocytosis	4	4.40E-03	7.40E-01
Regionalization	9	6.10E-03	7.10E-01
Exocytosis	4	8.00E-03	7.10E-01
Pattern specification process	9	8.50E-03	6.40E-01

Table II-5. *Gene Ontology clustering of new TE insertions.* **A.** Enrichment of GO terms in MB $\alpha\beta$ neurons. **B.** Enrichment of GO terms in the rest of the brain.

CHAPTER III

DISCUSSION

Basis of neuronal diversity and complexity

Our behavioral repertoire is testament of how complex our brains are. An intricate combination of genetic makeup and environmental influences make us unique individuals. Even identical twins, thought to have identical genetic blueprints are different. For the behavioral scientist, these differences are evident when a percentage of individuals behave in a different way to their isogenic counterparts, even when all the individuals experience the same controlled environmental influences of the laboratory. In a way that resembles a philosophical enigma, a neuroscientist asks the question: “what makes us individuals?” Of course, the short answer is the complexity of our brain with 100 billion neurons and 0.15 quadrillion (10^{15}) synapses. But that is an over simplified response. For instance, not all neurons have the same number of synaptic connections with the typical neuron having 5,000-200,000 synapses and it is estimated that there are 10,000 neuronal types, although the definition of an individual neuronal type is still debated. Moreover, neurons with similar morphology can possess different combinations of transmitter receptors, ion channels, etc that affect their firing patterns. There is no question that our brains are complex and diverse but how is this diversity is achieved?

Mechanisms of neuronal diversity

A number of well documented molecular mechanisms have being reported to contribute to neuronal diversity (Muotri and Gage, 2006). They act not only during development promoting neuronal differentiation, but also on the mature brain driven by environmental stimuli. These mechanisms orchestrate modifications in DNA, RNA and proteins making in theory each neuron unique. Aneuploidy, the gain or loss of chromosomes, can alter the DNA load of neurons. The presence of multiple promoters and regulatory sequences offers different possibilities on how a gene is expressed. In a similar way, alternative splicing and polyadenylation, and RNA editing work at the mRNA level. Finally, proteins undergo post-translational modifications that can affect their stability, activity and localization. Furthermore, gene expression can also be tuned by epigenetic mechanisms such as changes in DNA methylation and histone modifications (Figure III-1).

Transposable Elements as promoters of neuronal diversity

Transposable elements (TE) have the potential to promote a variety of modifications to the genome. Beyond insertional mutagenesis and deletion events, there are additional means in which TE can affect the genomic blueprint of each cell. For example, TE of high copy number have been reported to be the substrate for illegitimate homologous recombination, also known as ectopic recombination, causing rearrangements that can be deleterious, advantageous

or null (Boissinot *et al.*, 2006; Song and Boissinot, 2007). During mobilization, a retrotransposon can carry an adjacent gene and insert it into a new genomic location. Since retrotransposons mobilize through an RNA intermediate, the adjacent gene could be transposed as a pseudogene lacking intron sequences and containing a 3' poly(A) tail. It is estimated that the human genome contains approximately 8,000 pseudogenes and the majority of them appear to have been generated by LINE-1 (L1) retrotransposons (Zhang *et al.*, 2003; Ohshima *et al.*, 2003; Karro *et al.*, 2007). Moreover, it has been demonstrated that some of these pseudogenes are actually transcribed (Lin *et al.*, 2007). Finally, it has been reported that relics of TE can act as regulatory sequences in a process known as "exaption" (Nishihara *et al.*, 2006).

Recent research in transposition of L1 elements in the mammalian brain and brain stem cell cultures supports the idea of TE as promoters of somatic mosaicism in the brain. With the construction of a synthetic L1 transposon carrying a reporter gene that is only expressed after the transposition event has occurred, it was possible to show the mobilization of TE both *in vitro* and *in vivo* (Coufal *et al.*, 2009; An *et al.*, 2006; Muotri *et al.*, 2005). Interestingly, it was found that a high level of somatic transposition (An *et al.*, 2006) and a number of new transposition events occurred in neurally expressed genes (Muotri *et al.*, 2005). It is also important to note that the frequency of transposition was significantly different between different regions of the brain and individuals, and

the overall frequency in the brain was also higher when compared to the frequency of transposition in heart and liver (Muotri *et al.*, 2009).

Drosophila melanogaster as a model to study transposable elements and their effects in the brain

Flies have been used as a model to study the effects of transposable elements for years. But the vast majority of studies are focused in the germline and early embryogenesis. Alas, those cells were the place where TE were first discovered to act. In our search for specific genes expressed in MB subpopulations, that might be responsible for their differences in function, we uncovered a unique state for the long-term memory relevant $\alpha\beta$ subpopulation. MB $\alpha\beta$ neurons possess higher expression of TE relative to the rest of the brain suggesting that TE are actively moving throughout their genomes. In fact, we were able to show the presence of new TE insertions in the adult brain. Interestingly, a number of those insertions occurred in neurally expressed genes. In addition, we found that proteins of one of the mechanisms that control TE, the piwi proteins, are under represented in MB $\alpha\beta$ neurons, a characteristic that seems to be conserved across other *Drosophila* species. Our analysis showed that piwi proteins are required to silence the expression of TE in the brain. Therefore, we speculate that the down regulation of piwi proteins is responsible for the high expression of TE in MB $\alpha\beta$ neurons. These findings also suggest a possible role for the piRNA pathway in somatic tissues outside the gonad. Altogether, the work presented

here provides new prospects to further our knowledge of TE effects. The fact that mobilization of TE occurs in a subset of neurons with a well-defined behavioral function provides an excellent model to study how TE potentially contribute to neuronal and behavioral diversity.

Future work

Certainly, the work presented here opens many questions and avenues of future investigation. It would be interesting to determine if the unique state of MB αβ neurons where TE are derepressed is relevant to their functionality. If TE mobilization was adopted by these neurons as a means of generating diversity for their function in memory processing, it is conceivable that blocking TE actions might affect the capacity of these neurons to sparsely code odour stimuli, be plastic and to store long-term memories. Given that MB αβ neurons seem to be devoid of Aub and Ago3 expression, it might be possible to block TE mobilization by re-establishing expression of these proteins in the αβ neurons to test this hypothesis. However, expressing only Aub or Ago3 in MB αβ neurons might not be sufficient to repress TE mobilization because they are useless without the expression of the relevant piRNA clusters. Furthermore, other proteins of the piwi pathway may be required. However, other ways of blocking TE mobilization should certainly be investigated.

The fact that we observed TE expression and mobilization in MB αβ neurons where essential proteins of the piwi pathway are absent strongly suggests a functional link between the two. This view is also further supported by the finding that TE levels are comparably lower in the α'β' and γ MB subpopulations which express Aub and Ago3. Nevertheless, it will be worthwhile demonstrating a causative link between these observations. Analysis by sequencing of the small RNAs in the different MB subpopulations would allow one to determine whether the same, or different, classes of piRNA are expressed and utilized for TE silencing in the brain as those in the gonad.

Much of the data available on genes that are involved in memory processing results from behavioral screens for memory defective flies. Further analysis of the genes identified here could be transformative. Profiling MB gene expression provided a "snapshot" of the different MB subpopulations and generated new information that will be useful to uncover the connection between their unique functions in memory processing and their cellular components.

Final remarks

In recent years, thanks to the accumulation of full genome sequences from diverse organisms, the idea has begun to emerge that TE might provide some benefit to cells in addition to being detrimental for the health of the host in the long-term. It is possible that the bias towards viewing TE as promoters of harmful

effects arises from the over-representation of studies where an overt phenotypic change is observed. This would of course mean that instances involving silent transposition events where no overt change in the host is observed, have been missed. The new advances in DNA sequencing techniques allow scientists to obtain a global picture of TE contribution to the genome. Additionally, the discovery of the machinery that controls them allows a new angle of intervention to understand the range of TE actions. In terms of evolution, it is conceivable that down regulation of the expression of TE control machinery, and thus an increase in the propensity to mobilize TE in certain parts of the brain, has been selected as another means of generating useful neuronal diversity. It is possible that this down regulation occurs in a regulated spatial and temporal manner in order to promote diversity within a particular brain structure without affecting the overall health of the organism. A potential advantage of using such a system to generate neuronal diversity is that transposition events can occur in a somewhat random manner and thus allows evolution to ‘roll the dice’ in the brain while protecting potential progeny from inheriting a harmful mutation.

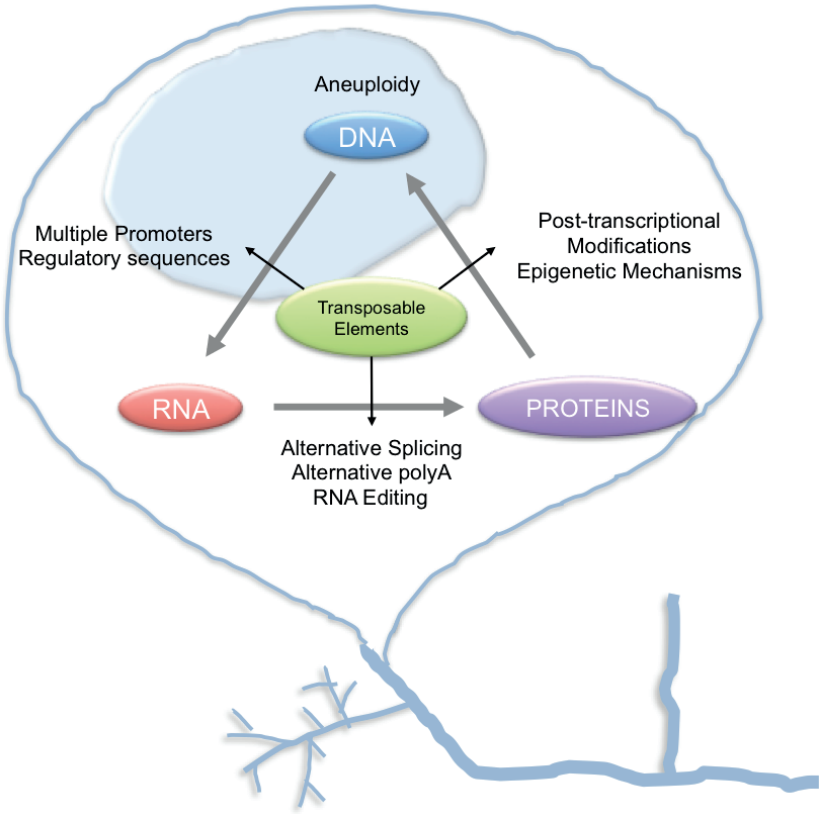

Figure III-1. *Mechanisms of neuronal diversity*. Neuronal diversity is orchestrated by a combination of mechanisms acting on DNA, RNA and proteins. Mobilization of transposable elements potentially contributes to neuronal diversity by altering the DNA blueprint of an individual neuron also affecting its RNA and proteins.

PREFACE TO APPENDIX I

The work presented in this chapter represents my initial project in the Waddell Lab. It intended to describe the role of the cAMP responsive Epac protein in learning and memory. Unfortunately, the inconsistencies between genotype and phenotype that we observed lead to us abandon the project.

This work has not been published elsewhere (although it deserves a spot in the Journal of Negative Confusing Data).

Perrat P.N. performed all the experiments and generated all the tools described in this chapter.

APPENDIX I

EPAC: A cAMP SENSOR INVOLVED IN *DROSOPHILA* MEMORY

Introduction

The second messenger, cAMP (3', 5' cyclic adenosine monophosphate), induces several processes in eukaryotic cells such as growth, differentiation, gene expression, secretion and neurotransmission. The activation of the cAMP signaling pathway plays a critical role in learning and memory processes in diverse organisms such as *Aplysia*, *Drosophila*, and rodents. Pharmacological, electrophysiological, and behavioral studies in these organisms confirm the involvement of cAMP signaling in learning and memory.

The most common target of action of cAMP is the cAMP-dependent protein kinase A, or PKA. However, cAMP is also found to be involved in the activation of cyclic-AMP-gated ion channels, which allow an influx of Na^+ in olfactory receptor neurons initiating a nerve impulse that travels along their axons. In addition, a guanine-nucleotide-exchange factor (GEF) named Epac (exchange protein directly activated by cAMP) was identified in 1998 (de Rooij *et al.*, 1998). This protein contains a cAMP binding domain and a homologous domain of GEFs for the proteins Ras and Rap1.

One of the Epac isoforms in mammals (Epac2 or cAMP-GEFII) is strongly expressed in both mature as well as developing brain (Kawasaki *et al.*, 1998). High levels of Epac2 mRNA were found in specific regions of the brain such as CA3 and the dentate gyrus of the hippocampus, both implicated in learning and memory. Moreover, Kaneko *et al.* (2004) observed an increase in excitatory postsynaptic currents (EPSC) when a cAMP analog (8-(4-chloro-phenylthio)-2'-O-methyl-cAMP) was used in cell recordings of brainstem preparations. This analog functions as a specific agonist of Epac (Enserink *et al.* 2002). Further experiments in the crayfish neuromuscular junction showed an enhancement in synaptic transmission when the same analog was applied along with inhibitors of cyclic nucleotide-gated ion channels and PKA. This enhancement was abolished when the Epac antagonist brefeldin A was used (Zhong and Zucker, 2005).

Drosophila melanogaster is an excellent model for studying the molecular and neural basis of both olfactory perception and olfactory learning (Keller and Vosshall, 2003; Heisenberg, 2003). The olfactory learning paradigm resembles a classical (Pavlovian) paradigm and is the most commonly used in memory behavioral test (Tully and Quinn, 1985). A number of *Drosophila* memory-defective mutants including *dunce*, *rutabaga*, and *amnesiac* have been identified using the olfactory conditioning paradigm (Quinn *et al.*, 1974), and their

molecular characterization showed that they all affect the cAMP cascade in different ways.

In flies there is only one gene encoding a protein homologous to the mammalian Epac proteins. The *Drosophila* Epac gene encodes a cAMP binding domain and a GEF domain. Moreover, Epac gene expression was shown in the *Drosophila* central nervous system (CNS) (Brody *et al.*, 2002). Therefore, we decided to investigate if the fly Epac had a role in memory processing. At the time we started this project, little was know about Epac and there were almost no tools available. However, there was a fly mutant available with which we started our preliminary studies. After confirmation that *Epac* mutant flies have a learning and memory phenotype, we developed a series of tools to further investigate the role of Epac in learning and memory. We chose to abandon the project due to several complications. In the last few years, studies have demonstrated a role for Epac in learning and memory. A summary will be provided in the discussion section of this chapter. But first, I will describe what we were able to demonstrate.

The importance of cAMP in memories

A number of experiments performed in *Aplysia*, *Drosophila* and other organisms showed a common molecule involved in the process of memory formation. This molecule is the second messenger 3' 5' cyclic adenosine monophosphate (cAMP). The importance of the cAMP-signaling pathway in olfactory associative

learning in *Drosophila* was demonstrated by studying mutations that affect olfactory learning. An olfactory conditioning paradigm (Quinn *et al.*, 1974) was used to isolate single-gene mutants defective for learning. Two of these mutants were *dunce* and *rutabaga* that performed poorly in several learning tasks (Dudai *et al.*, 1976; Tully and Quinn, 1985). The molecular and genetic analysis of *dunce* and *rutabaga* revealed that their genes encode a cAMP phosphodiesterase and a Ca^{2+}/Calmodulin-stimulated (type I) adenylate cyclase, respectively. These genes, like the majority of genes essential for memory, are preferentially expressed in the mushroom bodies and both mutations affect the cAMP-signaling pathway by changing the levels of intracellular cAMP (Dubnau and Tully, 1998; Tully, 1996). Furthermore, mutations in two other genes support the idea of the requirement of the cAMP-signaling pathway in olfactory learning. The genes *dco* and *PKA-RI* encode for the catalytic and one of the regulatory subunits of PKA, respectively. These genes are also preferentially expressed in the mushroom bodies and flies with mutations affecting these genes also have impaired olfactory learning (Connolly *et al.*, 1996; Goodwin *et al.*, 1997; Skoulakis *et al.*, 1993).

Epac: a cAMP sensor with lots of potential

Sometimes, it was observed that cAMP-induced activation works in the absence of PKA (de Rooij *et al.*, 1998). Sequence analysis for cAMP targets revealed several putative cAMP-binding domains. One of them was a genomic region

encoding a gene that includes a cAMP-binding domain and a guanine nucleotide exchange factor (GEF) domain named Epac (exchange protein directly activated by cAMP). Both *in vitro* and *in vivo* studies demonstrated that the Epac protein binds cAMP. Moreover, the binding of cAMP induces the GEF activity of Epac towards Rap1, a Ras-like GTPase (de Rooij *et al.*, 1998). In addition, a differential display protocol searching for brain-enriched genes in rats and human striatum, and screening of clones for second messenger motifs and GEF motifs revealed two close related genes named cAMP-GEFI and cAMP-GEFII for cAMP-regulated GEF proteins (Kawasaki *et al.*, 1998). These two proteins are also known as Epac1 and Epac2, respectively. Epac genes are expressed in several tissues in humans and rats. Epac1 is broadly expressed, but high levels of expression are found in the thyroid, kidney, ovary, skeletal muscle and certain regions of the brain. In contrast, Epac2 expression is more restricted. High levels of Epac2 are found in the adrenal gland, cerebral cortex, CA3 and dentate gyrus of the hippocampus, the habenula, and the cerebellum (Kawasaki *et al.*, 1998).

Even though the Epac cAMP-binding domain is highly conserved with respect to the PKA cAMP-binding domain, it was found that the Glu residue that forms the hydrogen bond with the 2'-OH group of the cAMP ribose, present in all known cAMP-binding domains, was absent in Epac proteins. This indicates that the Glu residue is not required for the binding of cAMP to Epac. Furthermore, mutation of the corresponding amino acid to Glu does not affect the binding of cAMP to Epac

(Rehmann *et al.*, 2003), but replacement of the Glu residue in PKA abolishes its high affinity for cAMP (Houge *et al.*, 1990). Since the 2'-OH group of the cAMP ribose is essential for its binding to PKA, a cAMP analog lacking the 2'-OH group should be able to specifically activate Epac. In fact, it was shown that the cAMP analog 8-(4-chloro-phenylthio)-2'-O-methyladenosine-3', 5'-cyclic monophosphate (8-pCPT) was a strong activator of Epac but not of PKA (Enserink *et al.*, 2002). Another difference between Epac and PKA is the concentration of cAMP required for their activation: Epac has lower affinity for cAMP than PKA.

Changes in synaptic potentiation play an important role in learning and memory formation. cAMP and PKA are central components that facilitate neurotransmitter release. However, is has also been reported that the release of hormones and neurotransmitters can occur in a cAMP-dependent PKA-independent manner. Intracellular loading of cAMP or Forskolin, an adenylyl cyclase agonist, in presynaptic nerve terminals at the calyx of Held of rats, is known to facilitate postsynaptic potentiation (Kaneko and Takahashi, 2004). But neither the PKA inhibitor H89 nor KT5720 abolishes postsynaptic potentiation suggesting the existence of another substrate for cAMP. Loading of 8-pCPT in presynaptic terminals promoted an increase of excitatory postsynaptic currents (EPSC) suggesting that Epac is involved in neurotransmitter release (Kaneko and Takahashi, 2004).

The role of Epac in synaptic potentiation was also supported by studies on crayfish neuromuscular junctions. The Epac agonist 8-pCPT enhances synaptic transmission in the presence KT5720 and ZD 7288, a PKA inhibitor and a cyclic nucleotide-activated ion channels inhibitor, respectively (Zhong and Zucker, 2005). In addition, the enhancement in synaptic transmission observed in the presence of 8-pCPT was abolished by brefeldin A (BFA). BFA acts on ADP-ribosylation factors, possible targets of Epac. Thus, it is possible that BFA blocks the downstream action of Epac (Zhong and Zucker, 2005).

How to teach a fly?

Even though it is possible to teach flies a number of tasks, olfactory learning is the tool most commonly used in these studies (Quinn *et al.*, 1974; Tully and Quinn, 1985). The olfactory learning paradigm consists in a simple procedure using a T-maze apparatus (Figure AI-1). First, flies are loaded in a tube with its inner surface covered with a copper grid. In this training tube, flies are exposed to an odor and to a series of foot electric shocks. After a short period of rest, flies are exposed to a new odor in the absence of the electric shock. Following training, flies are moved to the test section of the apparatus where they are placed at a choice point through an elevator. At the choice point, flies receive converging airflows of both odors. Flies that successfully associated the first odor

with the electric shock will try to avoid it by running towards the tube containing the second odor.

Drosophila Epac and memory

The *Drosophila* Epac gene was found in a differential cDNA screen for neural precursor genes followed by *in situ* hybridizations. *Drosophila* Epac is expressed in ventral cord midline glia cells in embryonic stage 14 (Brody *et al.*, 2002). A P-element insertion line was generated by the Gene Disruption Project (2001). This insertion is a P{SUPor-P} element at the 3' end of the open reading frame of the Epac gene (*Epac*^{KG00434}, referred from now on as *Epac*^{S}) disrupting the GEF domain of the Epac protein. I tested *Epac*^{S} heterozygous and homozygous flies using the olfactory conditioning paradigm and found that only *Epac*^{S} homozygous flies perform poorly in comparison to wild-type flies. *Epac*^{S} flies exhibited significant lower performance compared to wild-type flies when tested immediately after training and up to two hours after training (Figure AI-2). Importantly, control experiments revealed that *Epac*^{S} flies sense odors and shock as well as wild-type flies (Table AI-1). Altogether these data suggest that Epac is involved in learning and memory processes.

In addition, we analyzed Epac at the molecular level. Northern blot analysis of head and body extracts revealed the presence of one Epac mRNA of approximately 4 kb, consistent with the sequence prediction (Note: at the time we

performed the experiments, there was only one transcript predicted for Epac in the *Drosophila* genome) (Figure AI-3A). In contrast, we found two different mRNAs of approximately 2.6 kb and 4 kb in the *Epac*^{S} line (Figure AI-3B). Interestingly, the Epac transcripts were found only in the head extracts. We hypothesized, that the presence of two different mRNAs in *Epac*^{S} flies was due to aberrant splicing generated by the P-element insertion.

In a different approach, we analyzed Epac protein expression. We raised antibodies against the C-terminus of the protein including the GEF domain. We performed Western blot analysis on wild-type and *Epac*^{S} flies and observed a band of approximately 75 kDa in wild-type flies that was absent in *Epac*^{S} flies (Figure AI-3C). According to sequence prediction the expected band for the wild-type Epac protein was 94 kDa however a different mobility could easily arise from post-translational modifications. However, no remarks on this subject were found in the literature. We also determined that Epac is expressed in wild-type brains, but not in *Epac*^{S} brains (Figure AI-3D).

Epac^{S} Rescue

We used two different approaches to try to rescue the memory phenotype observed: I generated excision lines by mobilizing the P-element insertion in *Epac*^{S} flies and we used the UAS/GAL4 system (Brand and Perrimon, 1993) to restore Epac expression in the mutant flies. For the first approach, we crossed

the *Epac*^{S} flies with flies bearing the transposase encoded by the *Δ2-3* P-element. Our goal was to obtain new lines with precise excisions to test the reversion of the phenotype, and lines with imprecise excisions that would be useful as new mutant alleles. A number of excision lines were generated and analyzed for the presence of the P-element insertion by Southern blotting (Figure AI-4). We observed that a few lines seemed to revert to wild-type by precise excision, but a great number showed what appeared to be a mix of alleles with a precise excision and the intact insertion (Figure AI-4B). Nevertheless, we decided to test some of the "precise" excision lines for Epac expression and behavior (Figure AI-5). Western blot analysis showed that excision lines Ex8, Ex15, Ex20 and Ex24 had little to no expression of Epac even though they seemed to revert to the wild-type allele at the DNA level (Figure AI-5A). It is possible that the excisions of the P-element were slightly imperfect leaving a few nucleotides behind that would disrupt the open reading frame of Epac. If that was the case, we were unable to detect it by sequencing. Moreover, the "precise" excision lines showed a similar memory phenotype as the mutant flies (Figure AI-5B).

In the second approach, I attempted to rescue the memory phenotype by using the UAS/GAL4 system (Brand and Perrimon, 1993). I generated Epac-GAL4 driver lines by cloning different fragments from the promoter region of the Epac gene into the pG4PN2 vector and subsequent injection into fly embryos. In a

similar way, we created UAS-Epac transgene lines with the Epac open reading frame cloned into the pUAST vector. We tested the Epac-GAL4 driver lines by crossing them to UAS-mCD8::GFP flies and checked for GFP expression in the fly brain. Unfortunately, all the lines failed to drive GFP expression. It is possible that the upstream DNA region we selected for the GAL4 constructs contained an incomplete promoter and therefore they failed to drive GFP expression. On the other hand, the UAS-Epac line revealed some puzzling results. We crossed the UAS-Epac line with the pan-neural driver elav-GAL4 and the glial driver Repo-GAL4, and we performed Western blot analysis for Epac expression. Repo-GAL4 was the only driver capable of driving some expression, but the result was a duplex band instead of the single band previously observed in wild-type flies (Figure AI-6). These preliminary results suggested that the driver and transgene lines were inappropriate to use for the rescue experiments.

On a last ditch attempt to continue this project, we generated RNA interference (RNAi) transgenic flies (Kalidas and Smith, 2002) to silence Epac expression in wild-type flies and test whether they had a memory defect. We tested Epac down regulation by Western blotting but the results were inconclusive (data not shown). Taken together, the inconsistencies of the results, lack of additional information, and intense personal and PI frustration lead to us abandoning the project and focusing on more promising avenues.

Discussion

Since its discovery in 1998, a number of studies have shown that Epac is involved in several processes such as cell proliferation and differentiation, ion transport, gene transcription, secretion and more importantly, neuronal signaling (Roscioni *et al.*, 2008). But it was in recent years that its role in learning and memory have started to be uncovered. In the Hippocampus CA1 region, pharmacological treatment with the Epac specific agonist 8-pCPT induces long-term potentiation (LTP) and long-term depression (LTD) (Gelinas *et al.*, 2008; Ster *et al.*, 2009). Both LTP and LTD in synaptic strength are thought to be essential mechanisms required for memory formation. Behavioral studies in mice have also shown that 8-pCPT injection in the Hippocampus enhances memory in the context-dependent fear conditioning paradigm (Kelly *et al.*, 2009; Ostroveanu *et al.*, 2010). In another study, it was demonstrated that both PKA and Epac induction are necessary to rescue retrieval of contextual fear memory in noradrenaline-deficient dopamine β-*hydroxylase*-knockout mice (Ouyang *et al.*, 2008). In contrast, Ma and colleagues reported that Epac enhances long-term memory formation that appears to be PKA-independent and suggest that Epac is important in the memory consolidation process (Ma *et al.*, 2009). In a more recent study, it was shown that null mutations in mice for both EPAC1 and EPAC2 genes are responsible for defects in spatial learning and memory and social behaviors thus suggesting a redundant role for both Epacs (Yang et al, 2012). It is also interesting to note that a number of alterations of the Epac genes

including missense mutations, polymorphisms and changes in regulation, have been found in patients with neurological disorders such as Alzheimer's disease, autism, anxiety and depression (McPhee et al, 2005; Bacchelli et al, 2003; Woolfrey et al, 2009; Dwivedi et al, 2006; Middeldorp et al, 2010). It is therefore conceivable that Epac may represent an attractive therapeutic target for the treatment of these disorders.

Despite our unfortunate journey in trying to elucidate the role of Epac in the process of memory, we have been able to demonstrate that a mutant allele of Epac has memory defects in flies. The new data available and the design of new tools would allow us to further the knowledge of this fascinating gene.

Experimental procedures

Fly Strains. Fly stocks were raised on standard cornmeal food at 25°C and 40-50% relative humidity. y^1w^{67c23};P{SUPor-P}$Epac^{KG00434}$ (*Epac*^{S}) flies (Bloomington *Drosophila* Stock Center) were backcrossed to our Canton-S lab stock for 6 generations. Repo-GAL4 and elav-GAL4 (C155) were obtained from the Bloomington *Drosophila* Stock Center.

Behavioral Analysis. The olfactory avoidance paradigm was performed as previously described (Tully and Quinn, 1985). The Performance Index was calculated as described in Figure II-1. Odors were diluted in mineral oil 5:1000 for 3-Octanol (OCT) and 5:800 for 4-Methylcyclohexanol (MCH). The sensory acuity controls were performed as previously described in Keene *et al.*, 2004.

Northern Blotting. Total RNA from fly heads was isolated with Trizol (Invitrogen). 10 μg of RNA were separated by electrophoresis in a 0.8% agarose gel under denaturing conditions, transferred to a positively charged nylon membrane and fixed by UV cross-linking. Membranes were hybridized with the probe overnight at 68°C. The probe was synthesized using the DIG system for transcriptional labeling of RNA probes (Roche). After hybridization, the membranes were washed first with 2X SSC, 0.1% SDS twice for 5 minutes at room temperature; then with 0.2X SSC, 0.1% SDS twice for 5 minutes at room temperature; and two final washes of 15 minutes with 0.1X SSC, 0.1% SDS at 42°C. Detection was

performed according to Roche DIG system guidelines using a chemiluminescent alkaline phosphatase substrate (Pierce). For the mRNA Northern blot analysis, mRNA was isolated using the Oligotex® Qiagen kit (QIAGEN).

Southern Blotting. 5 μg of DNA were digested with XbaI overnight at 37°C and then separated by electrophoresis in a 0.8% agarose gel. Transfer to a positively charged nylon membrane, hybridization and detection were performed according to the Roche DIG system guidelines. The probe was synthesized by PCR using the PCR DIG Probe Synthesis Kit (Roche).

Polyclonal Antibody Production. The synthesis of polypeptides for antibody production was performed using the Glutathione-S Transferase (GST) Gene Fusion System. A fragment from the 5'-end corresponding to the Epac protein C terminus ($Epac_{Ct}$) was amplified by RT-PCR and cloned into the pGEX-5X-1 expression vector (Amersham Biosciences). The GST-$Epac_{Ct}$ fusion protein expression was induced in bacterial cultures and isolated with Glutathione Sepharose 4B beads. Two guinea pigs were immunized with the GST-$Epac_{Ct}$ fusion protein for antibody production (Covance).

Western Blotting. SDS-PAGE was performed as described in the Mini-PROTEAN® System protocol (Bio-Rad). Protein samples were prepared by grinding fly heads in 2X Lammeli Buffer (120 mM Tris-Cl (pH 6.8), 4% SDS, 20%

glycerol, 0.02% bromophenol blue) and boiling them for 5 minutes. The equivalent in volume of 4 heads was loaded into 10% Polyacrylamide gels. For immunoblotting, proteins were transferred to the Hybond^{TM}-C Extra nitrocellulose membrane (Amersham Biosciences). The latter was incubated with primary antibodies and followed by horseradish peroxidase (HRP)-linked secondary antibodies. The primary antibodies concentrations used were 1:10000 for guinea pig anti-Epac (#5544 2nd production bleed) and 1:1000 for mouse anti-β-tubulin (Invitrogen).

Excision lines. Epac^{S}/Epac^{S} females were crossed to transposase-bearing Sb(Δ2-3)/TM3Ser males. Dysgenic *Epac^{S}*;Sb(Δ2-3) males were crossed to W;CyO/Sp females, and excision chromosomes were selected by the absence of *w*$^{+}$ from the P{SUPor-P} element. In the next generation, we isolated putative Epac^{ex} males and prepared genomic DNA for Southern blot analysis.

Transgenic flies. The Epac-GAL4 constructs were generated by cloning a 1 Kb fragment and a 2 Kb fragment from the upstream region of the Epac gene into the pG4PN2 vector (a gift from Marc Freeman, University of Massachusetts, Worcester, MA). For the pUAST-Epac construct we amplified by PCR the Epac open reading frame from the cDNA clone GH01501 (*Drosophila* Genomics Resource Center) and cloned it into the pUAST vector (*Drosophila* Genomics Resource Center). To generate transgenic flies, the Epac-GAL4 and pUAST-

Epac constructs were then microinjected into embryos as previously described (Rubin and Spradling , 1982).

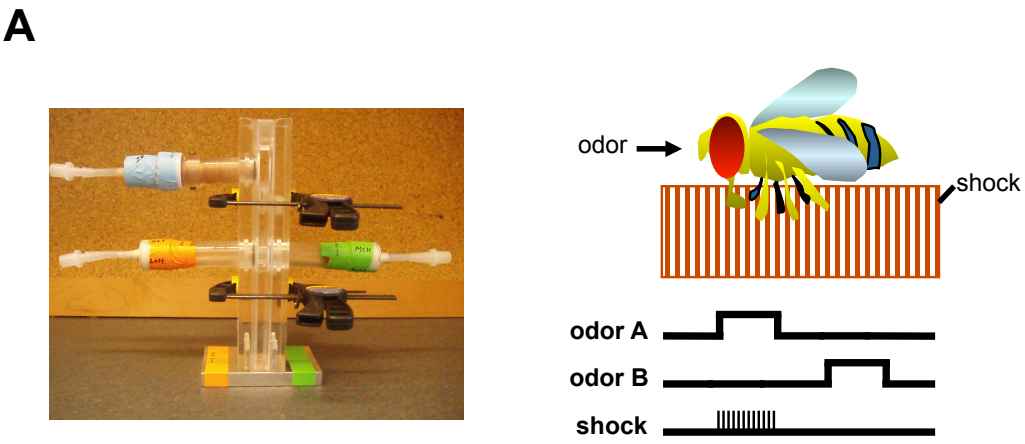

Figure AI-1. *Testing olfactory memory in flies*. **A.** The T-maze apparatus and training protocol. In the upper section, flies are trained pairing an odor with 12 pulses of electric shock. After a rest period, flies are exposed to the second odor. After training, flies are transported to the choice point in the lower section of the apparatus were they are exposed to both odors (Picture taken by Michael Krashes). **B.** The Performance Index is calculated as the number of flies avoiding the odor paired with the electric shock (odor A) minus the number of flies avoiding the unpaired odor (odor B), divided by the total number of flies. In order to avoid any odor bias, the reciprocal training is performed and the average score for the two odors is calculated.

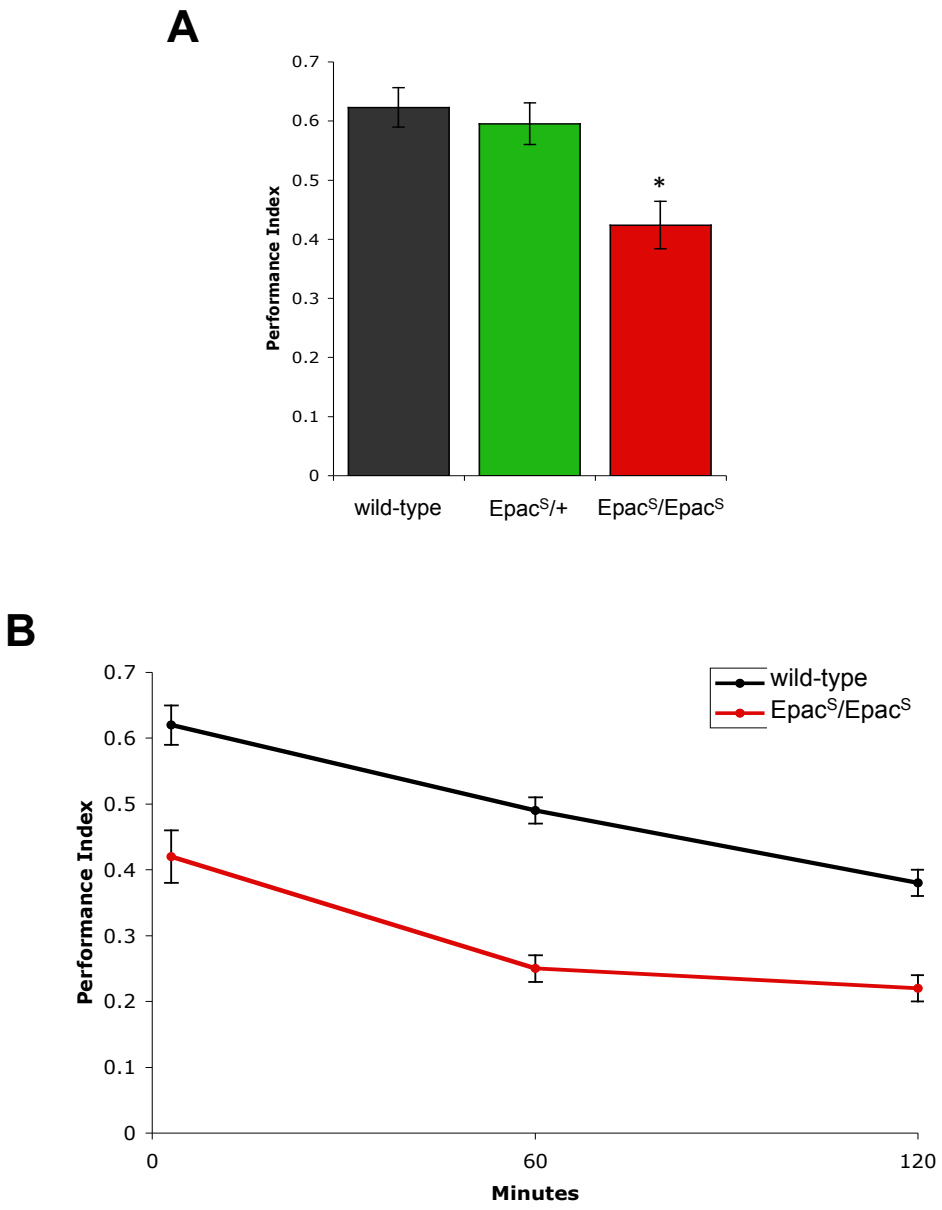

Figure AI-2. *Learning and memory defects in Epac^S flies*. **A.** Learning performance of wild-type and *Epac^S* heterozygous and homozygous flies (* $p<0.05$). **B.** Memory decay curve for wild-type and *Epac^S* homozygous flies. Flies were tested 3, 60 and 120 minutes after training ($p<0.05$).

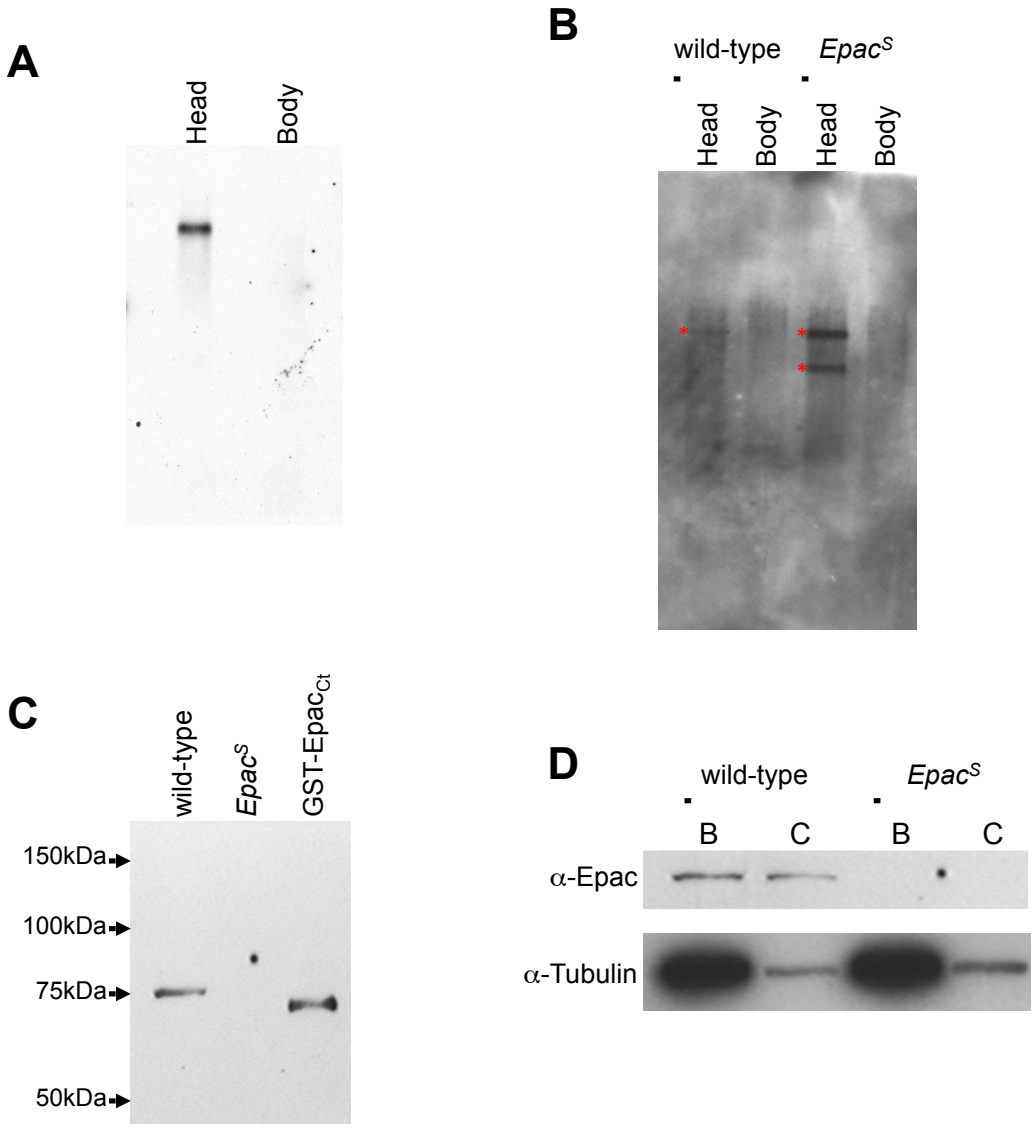

Figure AI-3. *Expression of Drosophila Epac*. **A.** Northern blot analysis of total RNA of head and body extracts from wild-type flies. **B.** Qualitative Northern blot analysis of mRNA of head and body extracts from wild-type and *Epac*^{S} flies. The asterisks indicate one 4kb transcript for wild-type and two transcripts of 4kb and 2.6kb for *Epac*^{S}. **C.** Western blot analysis of wild-type and *Epac*^{S} flies. GST-Epac$_{Ct}$ is the fusion protein used to raise the antibodies. **D.** Western blot analysis of dissected brains (B) and head capsules (C).

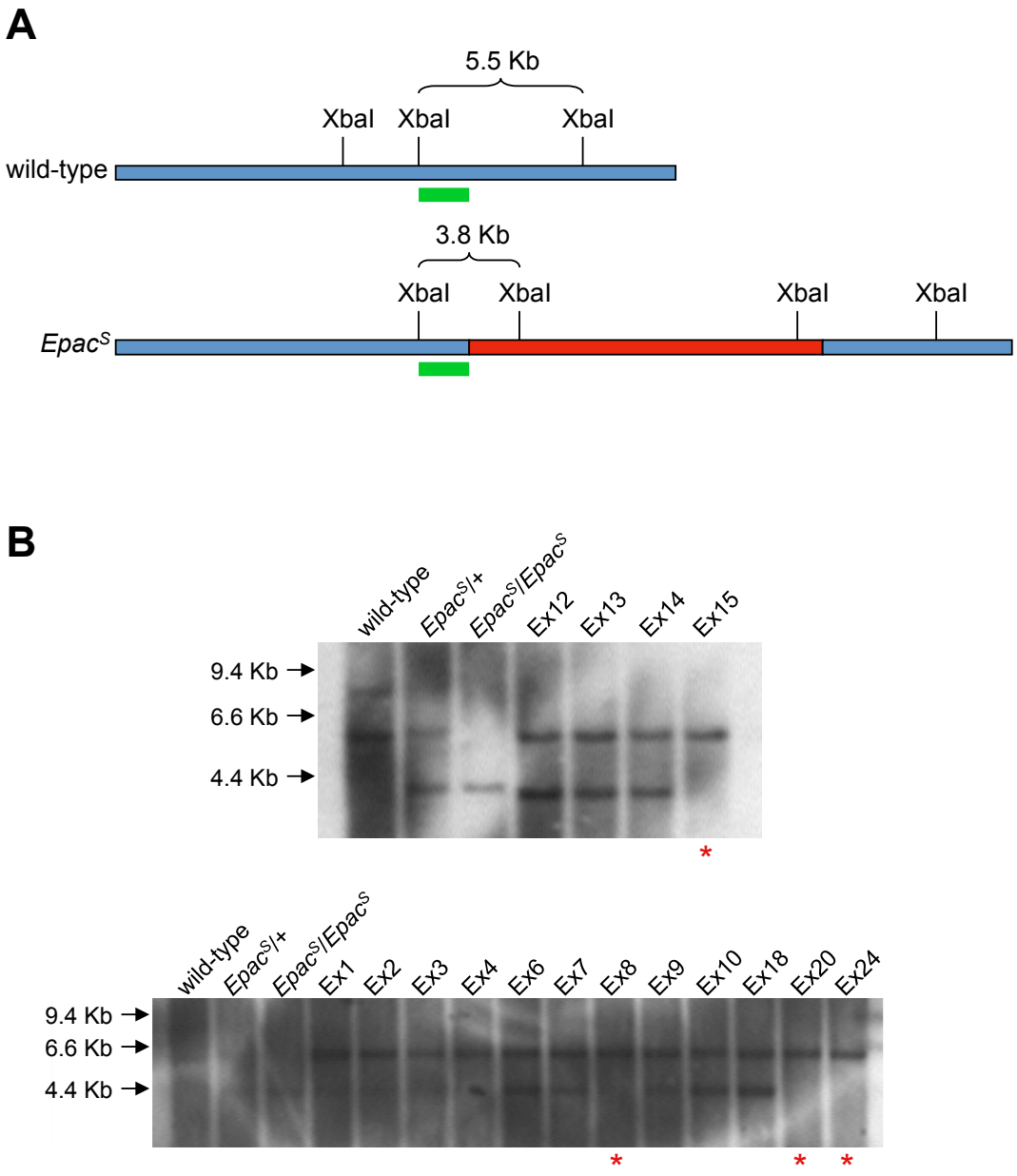

Figure AI-4. *Epac excision lines characterization*. **A.** Schematic of Southern blot analysis to test excision lines. The P{SUPor-P} insertion is marked in red. The sequence selected for the probe synthesis is marked by the green bar. **B.** Southern blot analysis of *Epac*^{S} excision lines. Lines Ex8, Ex15, Ex20 and Ex24 seem to revert to the wild-type allele (*).

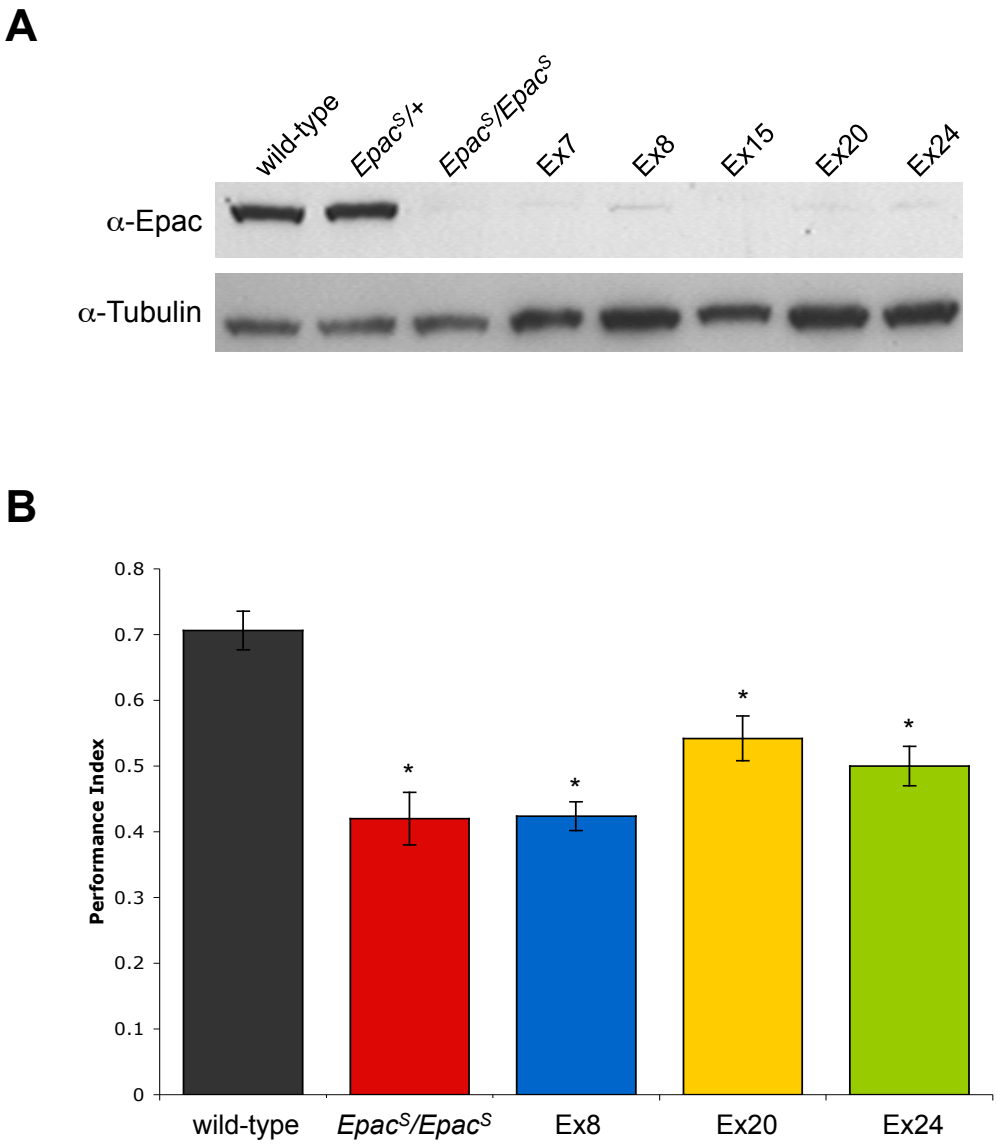

Figure AI-5. *Epac excision lines behavior*. **A.** Western blot anaysis of *Epac*^{S} excision lines. Excisions Ex8, Ex20 and Ex24 show a lower expression of Epac than wild-type. **B.** The low expression on Epac is not enough to rescue the memory phenotype of *Epac*^{S}. Flies were tested 3 minutes after training (* $p<0.05$).

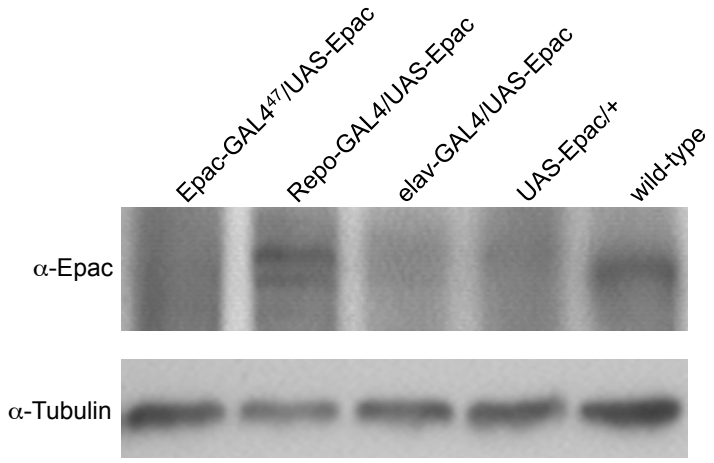

Figure AI-6. *Testing the UAS-Epac Transgene*. Western blot analysis of Epac expression. Different driver lines were used to drive expression of the UAS-Epac transgene. Repo-GAL4 (a glia driver) was the only one able to drive expression but the result is a duplex band instead of the single band of the wild-type.

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Genotype	OCT acuity	MCH acuity	Shock avoidance
wild-type	0.57±0.04	0.51±0.04	0.30±0.03
Epac^{S}/Epac^{S}	0.55±0.04	0.43±0.04	0.24±0.03

Table AI-1. *Sensory acuity controls*. There are not statistical differences between the two genotypes.

PREFACE TO APPENDIX II

This chapter has been published separately in:

Keene, A. C., Stratmann, M., Keller, A., **Perrat, P. N.**, Vosshall, L. B., & Waddell, S. (2004). Diverse odor-conditioned memories require uniquely timed dorsal paired medial neuron output. Neuron, 44(3), 521-533.

Perrat P.N. generated and analyzed the excision lines amn^{ex1} and amn^{ex39} described in Figure 1.

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Diverse Odor-Conditioned Memories Require Uniquely Timed Dorsal Paired Medial Neuron Output

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Summary

***amnesiac* mutant flies have an olfactory memory defect. The *amn* gene encodes a homolog of vertebrate pituitary adenylate cyclase-activating peptide (PACAP), and it is strongly expressed in dorsal paired medial (DPM) neurons. DPM neurons ramify throughout the mushroom bodies in the adult fly brain, and they are required for stable memory. Here, we show that DPM neuron output is only required during the consolidation phase for middle-term odor memory and is dispensable during acquisition and recall. However, we found that DPM neuron output is required during acquisition of a benzaldehyde odor memory. We show that flies sense benzaldehyde by the classical olfactory and a noncanonical route. These results suggest that DPM neurons are required to consolidate memory and are differently involved in memory of a volatile that requires multisensory integration.**

Introduction

Smelling influences the behavior of many animals. Olfactory cues are used for communication between animals, to find mates, and to avoid predation. Some odors, such as pheromones, have innate meaning. Others can be learned to become predictors of pleasant or unpleasant circumstance. Understanding how olfactory cues are perceived, integrated with other sensory cues, and stored as memories in the brain is a focus of considerable attention.

Drosophila melanogaster is an excellent model system to study the molecular and neural basis of olfactory perception and olfactory learning (Keller and Vosshall, 2003; Heisenberg, 2003). Olfactory memory-defective *Drosophila* mutants have been isolated using a paradigm in which flies associate an odor with electric shock punishment (Quinn et al., 1974; Tully and Quinn, 1985). The molecular characterization and anatomical localization of the affected gene products has highlighted the cyclic AMP cascade and the mushroom bodies (MBs) as key factors of olfactory memory (Nighorn et al., 1991; Han et al., 1992; Skoulakis et al., 1993). The MBs are third order neurons of the olfactory system. Odors are sensed by olfactory sensory neurons in the antennae and maxillary palps. Sensory neurons that express the same odorant receptor project axons to bilaterally symmetrical structures called glomeruli in the antennal lobe of the fly brain (Vosshall et al., 2000; Gao et al., 2000; Scott et al., 2001). From there, projection neurons in two tracts relay information to the mushroom bodies (MBs) and the lateral horn (Heimbeck et al., 2001; Wong et al., 2002; Komiyama et al., 2003). The MBs are required for olfactory learning (Heisenberg et al., 1985; de Belle and Heisenberg, 1994), and a functional cAMP cascade in the adult fly MBs is required and sufficient for olfactory memory (Zars et al., 2000; McGuire et al., 2003). Strikingly, MB output is required during retrieval of olfactory memory but is dispensable during acquisition and storage (Dubnau et al., 2001; McGuire et al., 2001; Schwaerzel et al., 2002). These data are consistent with the odor memories being represented in the presynaptic terminals of MB neurons.

Analysis of the *amnesiac* (*amn*) mutant uncovered another critical part of the memory circuit (Quinn et al., 1979; Waddell et al., 2000). The *amn* gene encodes a predicted preproneuropeptide with homology to mammalian pituitary adenylate cyclase-activating peptide (PACAP) (Feany and Quinn, 1995; Moore et al., 1998), and it is highly expressed in dorsal paired medial (DPM) neurons—large putative neuromodulatory neurons that ramify throughout the MB lobes (Waddell et al., 2000). *amn* mutant memory can be rescued with *amn* expression in DPM neurons (Waddell et al., 2000; Tamura et al., 2003), and blocking DPM output causes *amn*-like memory loss (Waddell et al., 2000). It is therefore plausible that DPM release of AMN peptide onto the MBs contributes to memory persistence. Here, we have determined the precise temporal requirement for DPM output in olfactory memory.

The vast majority of the memory mutants were isolated using a single odor pair—3-octanol (OCT) and 4-methylcyclohexanol (MCH) (Dudai et al., 1976; Quinn et al., 1979; Livingstone et al., 1984; Choi et al., 1991; Boynton and Tully, 1992; Dura et al., 1993; Folkers et al., 1993; DeZazzo et al., 2000; Dubnau et al., 2003). From a selection of 40 odors, Quinn et al. (1974) concluded that "not all odors work." OCT and MCH were chosen because they consistently produced good memory scores. It is not known why these odors are salient to the fruit fly and why they are potent conditioning stimuli. To our knowledge, no large-scale screen has asked whether odor-conditioned memories are relatively generic and can be formed with a variety of odors or whether pathways and genes that are required for memories are odor specific. Benzaldehyde (BA) is used by some groups in a BA-OCT combination (Skoulakis and Davis, 1996; Grotewiel et al., 1998; Cheng et al., 2001; Schwaerzel et al., 2002) or BA-MCH (Guo et al., 2000; Zars et al., 2000), and recently ethylacetate paired with isoamylacetate has been successfully employed to teach wild-type flies (Schwaerzel et al., 2003). However, it remains to be determined whether the existing mem-

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ory mutants have a general odor memory defect or differentially affect the coding of individual odors.

Published memory experiments with *amn* mutants have used OCT and MCH as odors (Quinn et al., 1979; Tully and Gergen, 1986; DeZazzo et al., 1999; Waddell et al., 2000; Tamura et al., 2003). We previously demonstrated that blocking DPM neurons did not affect immediate memory for these odors but abolished later memory (Waddell et al., 2000). Here, we show that prolonged DPM output is required for persistent OCT and MCH memory, consistent with a role for DPM neurons in the consolidation of odor memory. We found a different result with BA—an odor that we show is sensed by the classical olfactory pathway and a noncanonical route. *amn* flies have a short-term memory defect with BA. Strikingly, this BA memory defect can be mimicked in wild-type flies by blocking DPM output during acquisition, suggesting that DPM neurons have an additional function in BA memory.

Results

amn Is Not Required for DPM Neuron Specification and MB Targeting

Our demonstrated acute role for DPM neurons in memory (Waddell et al., 2000) led us to investigate whether the memory defect of *amn* mutant flies results from the absence of DPM neurons.

We used confocal microscopy to analyze DPM morphology in *amn* mutant fly brains by driving a uas-mCD8:GFP transgene with the DPM driver c316{GAL4} (Figure 1A). For these experiments, we used amn^{1}, a strong behavioral allele that has not been molecularly characterized in detail, as well as two new *amn* alleles, amn^{ex1} and amn^{ex39}, generated here by imprecise excision of the single P element in the amn^{c651} mutant (Waddell et al., 2000). The amn^{ex1} and amn^{ex39} are not predicted to produce any functional AMN peptide (Figure 1F).

We found that DPM neurons are present in *amn* mutants ($n > 10$ per genotype; Figure 1B shows a typical amn^{ex1} brain). In both wild-type and *amn* mutant fly brains, each DPM neuron sends a single large-diameter neurite toward the MB lobes. The neurite splits and projects to the vertical and horizontal MB lobes. These neurites further divide and extend toward the vertically arranged α and α' lobes and the horizontally arranged β, β', and γ lobes. The processes form a network of fibers and synaptic boutons throughout all of the lobes and into the spur and anterior region of the peduncle. These data indicate that *amn* is not essential for DPM targeting to the MBs during development. Furthermore, these data imply that *amn* mutant memory is not due to absence or gross maldevelopment of DPM neurons, and therefore the mnemonic phenotype may result from dysfunction of AMN peptide in adult flies.

Throughout this study, we have primarily analyzed DPM neurons in memory using the c316{GAL4} fly line (Figure 1A). c316 mostly expresses GAL4 in DPM neurons, and blocking transmission from these neurons produces an *amn*-like memory defect (Waddell et al., 2000). This is currently the most specific driver line available to investigate DPM neuron function. In some experiments, we have also used a less specific line, Mz717

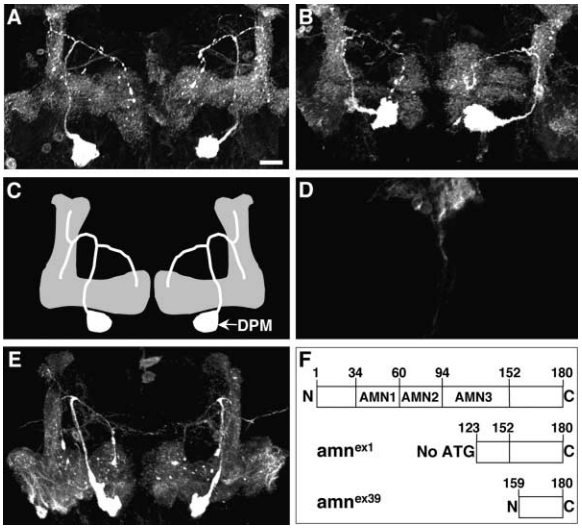

Figure 1. DPM Neurons Are Present in *amn* Mutants and Are Probably Cholinergic

(A) Morphology of DPM neurons in a wild-type fly brain revealed by driving mCD8:GFP with c316{GAL4}. The scale bar is 10 μm.

(B) Morphology of DPM neurons in an amn^{ex1} fly brain.

(C) Schematic representation of DPM neuron projections. Each neuron ramifies throughout the ipsilateral mushroom body lobe set, shown as gray.

(D) $Cha^{3.3kb}$-GAL80 represses c316{GAL4} activity in DPM neurons. Visible labeling is air sac material.

(E) Morphology of DPM neurons in a wild-type fly brain revealed by driving mCD8:GFP with Mz717{GAL4}.

(F) Schematic of putative peptides produced from the wild-type *amn* and amn^{ex1} and amn^{ex39} mutant loci. *amn* is predicted to encode a neuropeptide processed into three active peptides, AMN1, AMN2, and AMN3. The remaining *amn* gene in amn^{ex1} does not have an in-frame ATG. The remaining *amn* gene sequence in amn^{ex39} places an in-frame ATG before a potential 22 amino acid. However, this peptide falls after the putative amidation signal and is not expected to have function.

{GAL4} (Ito et al., 1998; Figure 1E) that labels DPM neurons and has additional expression in the antennal lobe. The c316 and Mz717 lines express GAL4 in the same DPM neurons because only two DPM neurons are visible when the driver lines are combined (data not shown). Other than DPM neurons, there is no obvious overlap between the neurons labeled in c316 and Mz717.

Although blocking DPM neurons produces an *amn*-like memory defect (Waddell et al., 2000), it is not known whether $shibire^{ts1}$ affects dense core vesicle (and, by extension, AMN peptide) release. We therefore asked whether DPM neurons corelease a fast-acting transmitter. Because acetylcholine (ACh) is the predominant transmitter of the *Drosophila* central nervous system (CNS), we performed a genetic experiment to test if DPM neurons are cholinergic. The choline acetyltransferase gene (*Cha*) is expressed in large subsets of cholinergic neurons, and a *Cha* promoter construct ($Cha^{3.3kb}$) drives expression of transgenes in these cholinergic neurons (Kitamoto et al., 1992, 1995; Kitamoto, 2002). We generated flies carrying a $Cha^{3.3kb}$ promoter driving expression of GAL80, a GAL4 repressor (Kitamoto, 2002), and our c316{GAL4} driver. We reasoned that if DPM neurons are cholinergic, $Cha^{3.3kb}$-GAL80 would inhibit c316{GAL4}-

driven reporter expression. We analyzed brains of mCD8:GFP;*Cha*$^{3.3kb}$-GAL80/c316{GAL4} flies for GFP expression in DPM neurons. Figure 1D shows that *Cha*$^{3.3kb}$-GAL80 completely suppresses GAL4 activity in DPM neurons (n = 14 brains). These data suggest that DPM neurons express *Cha* and are therefore likely cholinergic.

DPM Neuron Output Is Required during Consolidation for 3 Hr OCT-MCH Memory and Is Dispensable during Acquisition and Retrieval

In this study, we use the olfactory conditioning paradigm of Tully and Quinn (1985), because it produces a robust memory that allows a detailed analysis of specific memory phases. In this olfactory training protocol, a population of flies is exposed to one odor with an electric shock reinforcement followed by another odor without punishment. The flies are then tested for memory in a T maze, where they choose between the two odors used in training. Normal flies learn to avoid the shock-paired odor in a single training trial. Memory performance is calculated as the number of flies that avoid the shock-paired odor minus the number that avoid the non-shock-paired odor divided by the total number of flies. This memory score is a "half score" because normally a single performance index (PI) data point represents the average score of two experiments. In the second experiment, a new population of flies is taught to associate the other odor with shock. Score averaging eliminates odor bias; therefore, averaging half scores may obscure whether one odor is forgotten more quickly than the other. Later in this study (Figures 3D–6), we present half scores to highlight odor-specific effects. Until then, all data presented for OCT and MCH memory are average scores from reciprocal odors. We used OCT with MCH or OCT with BA, and we denote the odor pair used as either OCT-MCH or OCT-BA.

We used the GAL4-UAS system (Brand and Perrimon, 1993) to silence synaptic transmission in DPM neurons. We expressed the dominant temperature-sensitive *shibire*^{ts1} transgene, uas-*shi*^{ts1} (Kitamoto, 2001), in DPM neurons using the c316{GAL4} or Mz717{GAL4} DPM drivers (Waddell et al., 2000; Ito et al., 1998). The *shi* gene encodes a dynamin that is essential for endocytosis and synaptic vesicle recycling (van der Bliek and Meyerowitz, 1991; Chen et al., 1991). The *shi*^{ts1} allele has a vesicle recycling defect above 29°C that results in a rapid cessation of synaptic transmission (Koenig and Ikeda, 1989). High-temperature inactivation of *shi*^{ts1} is reversible and allows temporal control of neuron output by simply shifting flies between permissive and restrictive temperatures. Importantly, this allows us to test the role of DPM neurons in memory independent of *amn* mutation and therefore without confounding developmental defects that might arise from studying a nonconditional *amn* mutant.

We previously showed that blocking DPM output throughout an entire operant olfactory conditioning experiment (Quinn et al., 1974) did not affect learning (3 min memory) but abolished 1 hr OCT-MCH memory (Waddell et al., 2000). In this study, we first determined whether blocking DPM output caused a comparable memory defect in the classical conditioning paradigm of Tully and Quinn (1985). We conducted entire 3 hr memory experiments at 25°C (at which temperature we expected the neurons to function normally) and 31°C (under which conditions the *shi*^{ts1}-expressing neurons were expected to be synaptically silent). In each experiment, we compared the performance of c316;uas-*shi*^{ts1} double transgenic flies to wild-type and single transgenic c316 and uas-*shi*^{ts1} control flies. uas-*shi*^{ts1} flies at 31°C are a very appropriate control, because these flies often show a modest but significant reduction in performance at 31°C when compared to wild-type flies. We also included *amn*^{X8} flies to illustrate the effect of a null *amn* allele on 3 hr memory. At the permissive temperature of 25°C, both immediate (3 min) memory (wild-type = 0.64 ± 0.02; c316;uas-*shi*^{ts1} = 0.63 ± 0.04; uas-*shi*^{ts1} = 0.63 ± 0.03) and 3 hr memory of c316;uas-*shi*^{ts1} flies were statistically indistinguishable ($p > 0.7$) from wild-type, c316, and uas-*shi*^{ts1} control flies, while all groups showed greater memory than *amn*^{X8} mutant flies ($p < 0.02$) (Figure 2A). At the restrictive temperature of 31°C, immediate (3 min) memory of c316;uas-*shi*^{ts1} flies (0.67 ± 0.04) was statistically indistinguishable ($p > 0.7$) from wild-type (0.69 ± 0.02), and uas-*shi*^{ts1} flies (0.66 ± 0.04). However, 3 hr memory was statistically lower ($p < 0.01$ for all groups) than wild-type, c316, and uas-*shi*^{ts1} flies and statistically indistinguishable ($p > 0.7$) from that of *amn*^{X8} mutant flies (Figure 2B). These results are consistent with our previous findings (Waddell et al., 2000) and demonstrate that DPM output is required for 3 hr but not for short-term OCT-MCH memory.

We next used the reversibility of uas-*shi*^{ts1} to test whether DPM output during training (Figure 2C) or testing (Figure 2D) was required for memory. To block DPM neuron output during training, we incubated c316;uas-*shi*^{ts1} flies and all control flies at 31°C for 15 min prior to and during training. Flies were returned to 25°C immediately following training, and 3 hr memory was tested at 25°C. Blocking DPM output during training did not affect memory. The memory of c316;uas-*shi*^{ts1} flies was indistinguishable ($p > 0.9$) from uas-*shi*^{ts1} control flies that were trained at the restrictive temperature (Figure 2C). Therefore, memory acquisition does not require output from DPM neurons.

We similarly tested whether DPM output was required during memory recall (Figure 2D). We trained flies at 25°C, and 15 min before testing 3 hr memory we inactivated DPM neurons by shifting the flies to the restrictive temperature of 31°C. The 3 hr memory of c316;uas-*shi*^{ts1} flies was again indistinguishable ($p = 0.8$) from the uas-*shi*^{ts1} transgene control flies, suggesting that DPM output is not required for memory recall.

We also tested whether blocking DPM output during training and testing (Figure 2E) affected memory. We placed flies at 31°C 15 min prior to training and returned them to 25°C immediately after. Fifteen minutes before testing, we shifted them to 31°C again and tested olfactory memory. Strikingly, memory following this manipulation was no worse than that of flies receiving either manipulation alone and was indistinguishable ($p > 0.5$) from the memory of uas-*shi*^{ts1} control flies. Therefore, DPM output is not essential during training and testing for 3 hr OCT-MCH memory.

We finally tested whether DPM output was required in the period between training and testing (Figures 2F

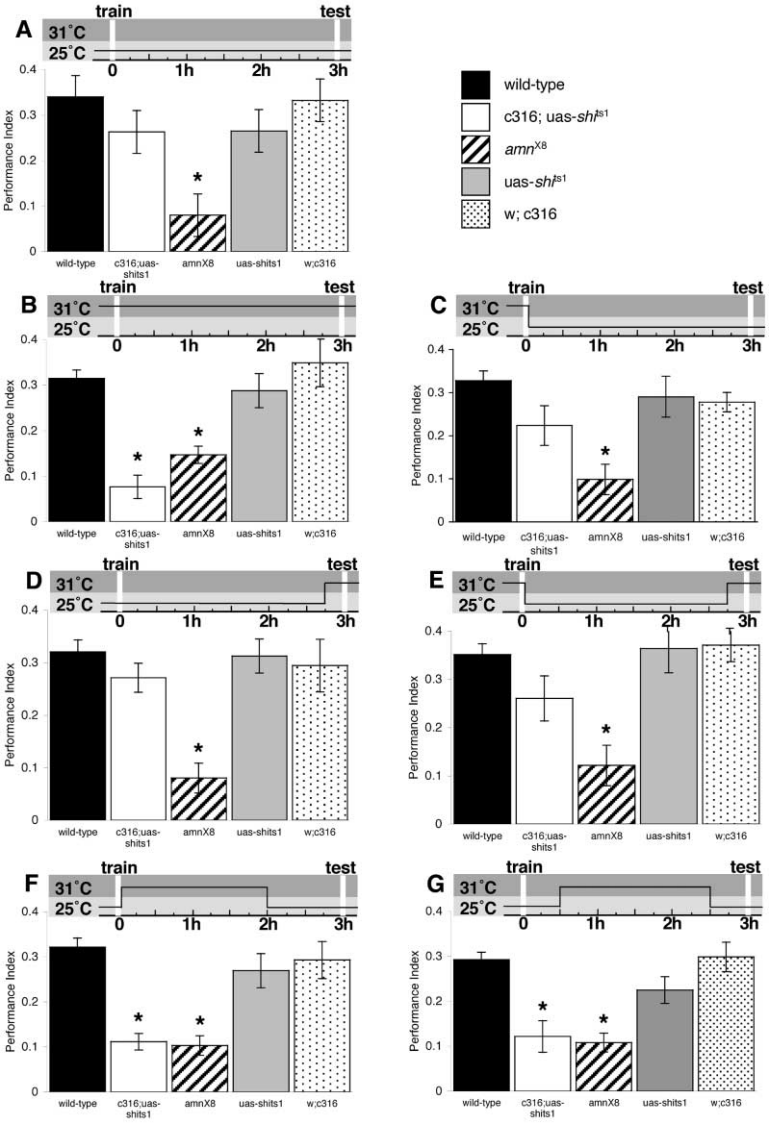

Figure 2. DPM Activity between Training and Testing Is Required for 3 Hr OCT-MCH Memory

All temperature shift protocols described below are shown pictographically above each graph. In each experiment, the genotypes shown were treated identically. (A) The permissive temperature of 25°C does not affect c316;uas-*shi*^{ts1} flies. All genotypes were trained and tested for 3 hr memory at 25°C. (B) Disrupting DPM output at the restrictive temperature of 31°C abolishes memory. All genotypes were trained and tested for 3 hr memory at 31°C. (C) Blocking DPM output during training does not affect 3 hr memory. Flies were incubated at 31°C for 15 min prior to and during training. Immediately after training, they were returned to 25°C and tested for 3 hr memory. (D) Blocking DPM output during testing does not affect 3 hr memory. Flies were trained at 25°C, and 165 min later they were shifted to 31°C. Fifteen minutes later, 3 hr memory was tested at 31°C. (E) Blocking DPM output during training and testing does not affect 3 hr memory. Flies were incubated at 31°C for 15 min prior to and during training. Immediately after training, they were returned to 25°C, and 165 min later they were shifted back to 31°C. Fifteen minutes later, 3 hr memory was tested at 31°C. (F and G) Blocking DPM output between training and testing impairs 3 hr memory. Flies were trained at 25°C, and immediately (F) or 30 min after training (G) they were shifted to 31°C for 2 hr. Flies were then returned to 25°C and tested for 3 hr memory at 25°C.

and 2G). This is the expected window of time in which memories become consolidated (Quinn and Dudai, 1976; Folkers et al., 1993; Tully et al., 1994). We trained flies at 25°C, and immediately following training we shifted them to 31°C for 2 hr. We then returned the flies to 25°C and tested them 1 hr later for 3 hr memory. Blocking DPM output between training and testing produced a dramatic loss of memory to levels statistically indistinguishable ($p = 1$) from that of *amn*^{X8} flies (Figure 2F). Therefore, DPM output is required between training and testing for 3 hr memory. We next tested whether blocking DPM output at later time points disrupted 3 hr memory. We delayed our 2 hr DPM blockade by 30 min into the middle of the experiment (Figure 2G). Blocking DPM output 30 min after training for 2 hr produced the same memory impairment as blocking output immediately after training.

To control for a nonspecific memory deficit produced by blocking DPM output for 2 hr at any point in the experiment, we incubated flies at 31°C for 2 hr, then returned them to 25°C and trained them 15 min later. This manipulation had no effect on 3 hr memory (wild-type flies = 0.33 ± 0.02; c316;uas-*shi*^{ts1} flies = 0.29 ± 0.06; $p = 0.4$). Therefore the intermediate 2 hr block likely causes a specific disruption of memory. None of the temperature manipulations that were used significantly impaired odor or shock acuity (Table 1). In conclusion, these data suggest that prolonged DPM output at least 30 min after training is required for wild-type 3 hr OCT-MCH memory, consistent with the idea that DPM neurons are involved in memory consolidation.

BA Is Sensed by the Olfactory Apparatus and a Noncanonical Pathway

Drosophila olfactory memory experiments typically involve a single odor pair. However, it is not known if results obtained with a single odor pair are representative of other odors. Some investigators use BA (the odor of bitter almond) instead of OCT or MCH. We discovered that BA is sensed by the classical olfactory route and a nonclassical route (Figure 3).

We tested whether BA avoidance behavior was de-

Table 1. Sensory Acuity Controls

Genotype	Temperature	OCT Acuity	MCH Acuity	BA Acuity	Shock Avoidance
wild-type	25°C	PD	PD	71 ± 6	73 ± 2
c316;uas-*shi*^{ts1}	25°C	PD	PD	81 ± 2	72 ± 2
Mz717;uas-*shi*^{ts1}	25°C	83 ± 3	83 ± 4	81 ± 7	72 ± 6
uas-*shi*^{ts1}	25°C	PD	PD	70 ± 6	65 ± 3
amn^{X8}	25°C	PD	PD	75 ± 6	70 ± 2
c316	25°C	PD	PD	69 ± 9	63 ± 2
wild-type	31°C	86 ± 3	89 ± 2	91 ± 3	83 ± 2
c316;uas-*shi*^{ts1}	31°C	79 ± 4	89 ± 3	81 ± 4	76 ± 4
Mz717;uas-*shi*^{ts1}	31°C	85 ± 5	83 ± 5	88 ± 3	62 ± 2
uas-*shi*^{ts1}	31°C	90 ± 5	92 ± 3	85 ± 2	71 ± 5
amn^{X8}	31°C	69 ± 2	77 ± 7	84 ± 5	76 ± 7
c316	31°C	76 ± 11	86 ± 6	85 ± 6	79 ± 3

PD, previously determined. Our earlier studies (Waddell et al., 2000) showed that the olfactory acuity of these strains is not significantly different at 25°C.

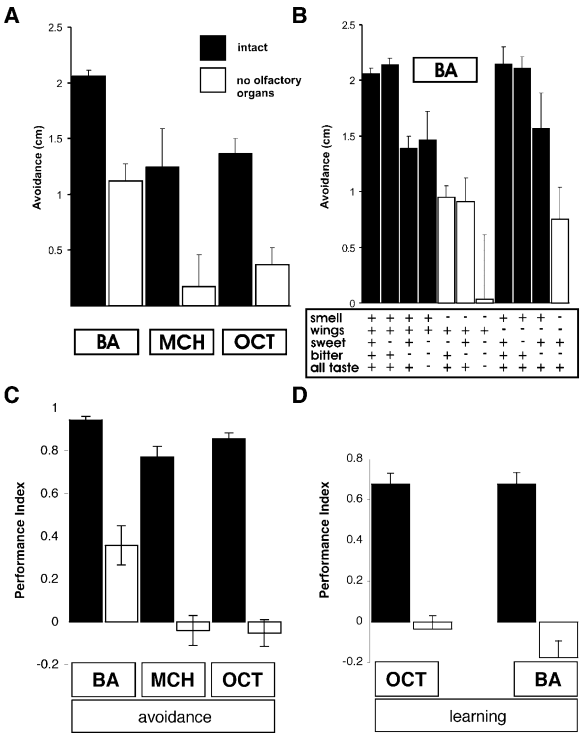

Figure 3. BA Is a Multimodal Stimulus

(A) BA, unlike MCH and OCT, elicits a substantial avoidance response in flies lacking olfactory organs ($n > 10$ flies). Intact naive wild-type and naive wild-type flies without olfactory organs were tested for avoidance of BA, MCH, and OCT in the arena paradigm.

(B) Genetic ablation and microsurgery identified three types of BA-sensitive neurons. Antennal and maxillary palp neurons were removed by surgery, bitter-sensitive neurons were ablated in *Gr66a-GAL4*, *uas-DTI* flies (no bitter taste) and all labelar chemosensory neurons were transformed in *poxn*$^{70\text{-}23}$*/Df(2R)WMG* mutant flies (no taste). Sweet-sensitive Gr5a-expressing neurons were ablated in *Gr5a-GAL4*, *UAS-DTI* flies (no sweet taste). Black bars represent flies without surgery. White bars are flies with organs removed.

(C) Flies lacking olfactory organs retain BA avoidance in the T maze. Intact wild-type flies and flies without olfactory organs were tested for BA, MCH, and OCT avoidance behavior in the T maze.

(D) Olfactory organs are required for olfactory conditioning with OCT and BA. Intact wild-type flies and flies without olfactory organs were tested for OCT and BA olfactory memory.

pendent on the classical olfactory apparatus—the antennae and maxillary palps. We surgically removed the antennae and palps from wild-type flies and tested avoidance of OCT, MCH, and BA in two different assays—the arena situation (Figures 3A and 3B) and the T maze used for olfactory learning (Figure 3C). Strikingly, significant BA responses were measured in both behavioral paradigms in the absence of olfactory organs, but OCT and MCH avoidance was abolished (Figures 3A, 3B, and 3C). This result suggests that OCT and MCH are classical odor stimuli sensed solely by the olfactory organs, but BA is also sensed by an entirely different mechanism that could be gustatory and/or somatosensory in nature.

To further define the nonolfactory BA-sensitive cells, we ablated other sites of chemosensation genetically or surgically. In homozygous *pox-neuro* (*poxn*) mutant flies, the chemosensory bristles on the wings, legs, and labelum are transformed into mechanosensory bristles (Awasaki and Kimura, 1997). *poxn* flies with an intact olfactory system show intermediate BA avoidance, whereas surgical removal of olfactory organs from *poxn* mutants abolishes BA avoidance (Figure 3B). Therefore *poxn*-affected neurons are responsible for the nonolfactory BA response.

We removed wings from flies to test whether *poxn*-expressing wing neurons mediate BA avoidance. Wing removal in flies lacking olfactory organs did not alter BA avoidance, suggesting that tarsal or labelar *poxn*-expressing neurons are more likely involved. We therefore independently ablated two subpopulations of labelar gustatory neurons by ectopically expressing a diphtheria toxin transgene (Wang et al., 2004). Ablating ~30 sweet-sensitive gustatory neurons (Gr5a-driven ablation) did not affect the response to BA, whereas ablating ~25 bitter-sensitive gustatory neurons (Gr66a-driven ablation) significantly reduced BA avoidance. However, ablating bitter gustatory neurons does not decrease the BA avoidance of flies lacking olfactory organs and is therefore not equivalent to *poxn* mutation. Taken together, these experiments demonstrate that BA is perceived by olfactory sensory neurons on the antennae and maxillary palps and by *poxn*-positive gustatory neurons located elsewhere. Some but not all of the *poxn* neurons are Gr66a-expressing labelar neurons. The additional neurons may reside in the pharynx, the mouth-

parts, or the legs. Since these BA-sensitive organs are unlikely to project to the antennal lobe (Thorne et al., 2004; Wang et al., 2004), BA information must be processed in parallel by multiple brain structures.

Following demonstration that BA avoidance was partially independent of the antennae and maxillary palps (Figures 3A, 3B, and C), we tested whether these organs were required for BA learning. We surgically removed antennae and maxillary palps from approximately 400 flies and tested their ability to associate OCT and BA with electric shock punishment (Figure 3C). Unlike naive avoidance behavior, the ability to associate BA with electric shock requires the antennae and maxillary palps. Flies lacking these structures do not learn with OCT or BA. However, it should be noted that the learning experiment without olfactory organs is not ideal, because flies lacking olfactory organs cannot sense OCT and therefore should only be able to partially sense one of the odors used in training and testing—BA.

amn Mutant Flies Learn Poorly with BA, and the Defect Is Partially DPM Dependent

The finding that BA is sensed differently to OCT and MCH raised the question of whether BA odor memory was acquired differently. We therefore tested wild-type and *amn* mutant fly learning with OCT-BA. We noticed a dramatic asymmetry in the learning scores (Figure 4A). The half score data revealed that, whereas wild-type flies learned well with OCT and BA, OCT learning of amn^{X8} flies was indistinguishable ($p = 1$) from wild-type flies but BA learning was greatly reduced ($p < 0.01$) (Figure 4A).

It has previously been reported that amn^{1} mutant flies have altered olfactory acuity following electric shock (Preat, 1998). It was therefore conceivable that our observed BA effect resulted from a selective loss of BA acuity or an increase in OCT acuity following electric shock. We tested relative odor acuity in amn^{X8} mutant flies both before and after electric shock.

Prior to conducting a learning experiment, the odors are balanced so that naive flies distribute evenly between the odors. Wild-type flies and amn^{X8} mutant flies distributed evenly between BA and OCT prior to shock (Figure 4B). We assayed the effect of shock on relative olfactory acuity by shocking flies in the absence of odor for 1 min (one shock every 5 s, total of 12 shocks as in the regular olfactory training protocol) and then allowing them to choose between OCT and BA. Shock did not change the distribution and hence did not change the relative odor acuity of wild-type or amn^{X8} mutant flies. Therefore, the BA learning defect of amn^{X8} flies cannot be explained by a change in relative odor acuity.

We also tested whether *amn* affected the alternate noncanonical pathway for sensing BA. We removed the antennae and palps from wild-type and amn^{X8} mutant flies and tested avoidance of BA, MCH, and OCT (Figure 4C). amn^{X8} flies without olfactory organs displayed BA avoidance that was indistinguishable from wild-type flies lacking olfactory organs ($p > 0.3$). These data suggest that *amn* does not affect BA sensation by the classical olfactory or the noncanonical route and instead is likely to affect neurons that are involved in processing BA information.

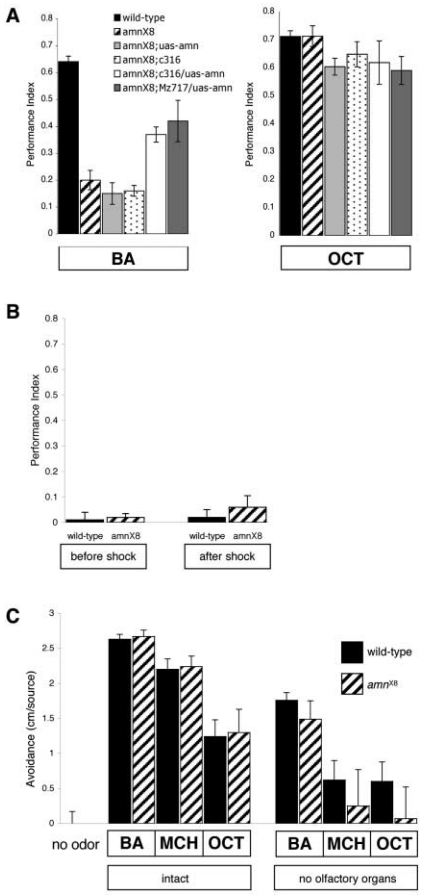

Figure 4. *amn* Mutant Flies Have a BA Learning Defect that Is Partially DPM Neuron Dependent

(A) Three minute OCT and BA memory. All genotypes shown were treated identically. Flies were trained to associate BA or OCT with shock and were tested for their preference between OCT and BA. Expressing the *amn* gene in DPM neurons (amn^{X8};c316/uas-*amn* and amn^{X8};Mz717/uas-*amn* flies) partially rescues the BA memory defect of *amn* mutant flies.

(B) Olfactory acuity of wild-type and amn^{X8} mutant flies before and after electric shock. Naive or previously electric-shocked flies were given the choice between OCT and BA in the T maze.

(C) amn^{X8} mutant flies without olfactory organs retain BA avoidance. Wild-type flies and amn^{X8} mutant flies with or without olfactory organs were tested for OCT, MCH, and BA avoidance in the arena apparatus.

Expressing *amn* in DPM neurons with c316{GAL4} rescues the OCT-MCH memory defect of *amn* mutant flies (Waddell et al., 2000). We therefore tested if DPM expression of *amn* restored BA immediate memory to *amn* mutant flies. In these experiments, we also used the Mz717 driver to increase the confidence that rescue could be ascribed to DPM neurons. We generated amn^{X8};c316/uas-*amn* and amn^{X8};Mz717/uas-*amn* flies and tested BA and OCT immediate memory (Figure 4A). The amn^{X8};c316/uas-*amn* and amn^{X8};Mz717/uas-*amn* flies learned to avoid BA significantly better than amn^{X8} flies ($p < 0.01$ for both), but their performance was still significantly worse than that of wild-type flies ($p < 0.01$ for both). Thus, expressing *amn* principally in DPM neurons partially restored BA immediate memory. In con-

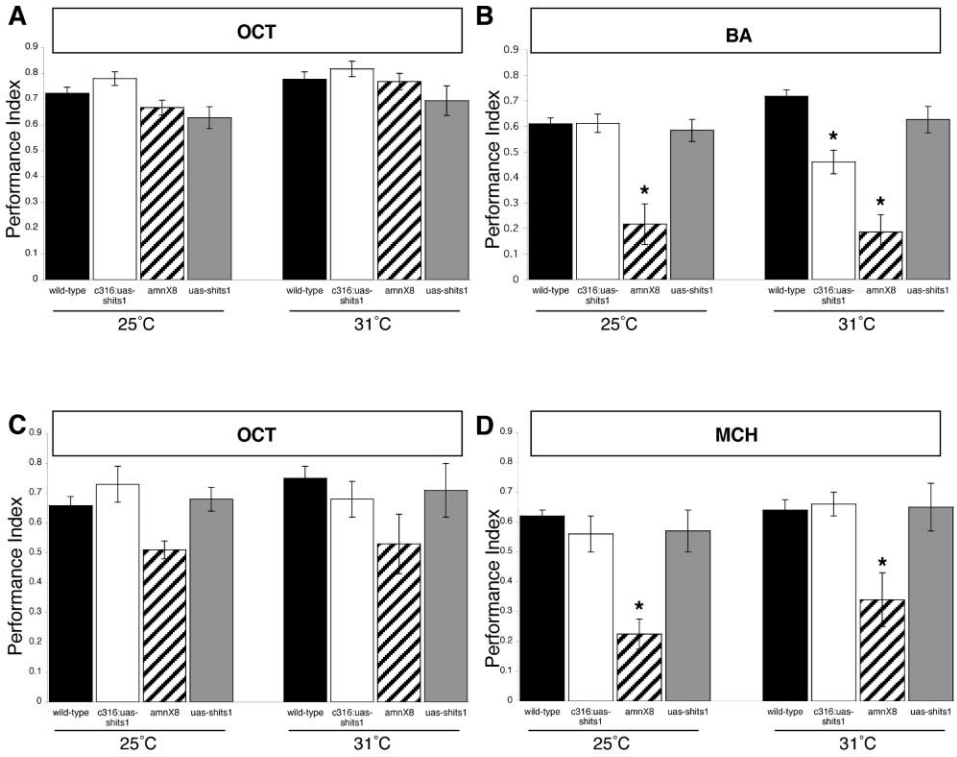

Figure 5. Blocking DPM Output Impairs BA Learning

(A) Blocking DPM output does not reduce OCT learning (3 min memory). All genotypes were trained to associate OCT with shock and tested for preference between OCT and BA.

(B) Blocking DPM output reduces BA learning. All genotypes were trained to associate BA with shock and tested for preference between BA and OCT. Asterisks denote significant difference ($p < 0.05$) from wild-type flies.

(C) Blocking DPM output does not reduce OCT learning. All genotypes were trained to associate OCT with shock and tested for preference between OCT and MCH.

(D) Blocking DPM output does not reduce MCH learning. All genotypes were trained to associate MCH with shock and tested for preference between MCH and OCT.

trast, OCT immediate memory of *amn*^{X8} flies was indistinguishable from wild-type flies and *amn*^{X8};c316/uas-*amn* or *amn*^{X8};Mz717/uas-*amn* flies. This result implies that DPM neurons are involved in BA learning.

Blocking DPM Output Impairs BA Learning

We tested if directly blocking DPM output impaired BA learning (Figure 5). We expressed uas-*shi*^{ts1} in DPM neurons with c316{GAL4}. We used the BA-OCT odor pair and tested immediate memory at both the permissive 25°C and the restrictive 31°C. At 25°C, the BA learning scores of all genotypes, except *amn*^{X8}, were not statistically different ($p > 0.1$) (Figures 5A and 5B). However, blocking DPM output with c316;uas-*shi*^{ts1} specifically reduced BA immediate memory ($p < 0.01$) (Figure 5B) and left OCT immediate memory intact ($p > 0.2$) (Figure 5A). Crucially, the uas-*shi*^{ts1} control flies do not have a defect with BA or OCT at 31°C (Figures 5A and 5B). For comparison, we also tested whether blocking DPM output impaired OCT and MCH immediate memory (Figures 5C and 5D). The OCT and MCH performance of c316;uas-*shi*^{ts1} flies is unaffected by temperature and is indistinguishable from the memory of wild-type flies ($p > 0.5$ for both odors). These data imply that DPM output is required to learn BA but not OCT or MCH. It is notable that *amn*^{X8} mutant flies also have a significant MCH immediate memory defect. However, this defect is not reproduced when DPM neurons are inactivated. Therefore, the MCH immediate memory defect is DPM independent and likely resides in other neurons that are affected by *amn* mutation.

Blocking DPM Output during Acquisition Impairs BA but Not OCT Memory

DPM output is required to stabilize OCT-MCH memory but is not required during acquisition of these odor memories (Figures 2F and 2G). Having observed a significant BA learning defect when we blocked DPM output (Figure 5B), we tested whether DPM output was required during acquisition of BA memory (Figure 6). We blocked DPM neuron output 15 min before training by incubating c316;uas-*shi*^{ts1} and Mz717;uas-*shi*^{ts1} flies at 31°C. We trained the flies with BA-OCT at 31°C and immediately returned the flies to 25°C to restore DPM function. We tested olfactory memory 1 hr later and again analyzed individual odor half scores separately. Blocking DPM output during acquisition did not affect 1 hr OCT memory: c316;uas-*shi*^{ts1} and Mz717;uas-*shi*^{ts1} fly memory was indistinguishable ($p > 0.8$ for both genotypes) from wild-type (Figure 6A). However, DPM blockade in c316;uas-

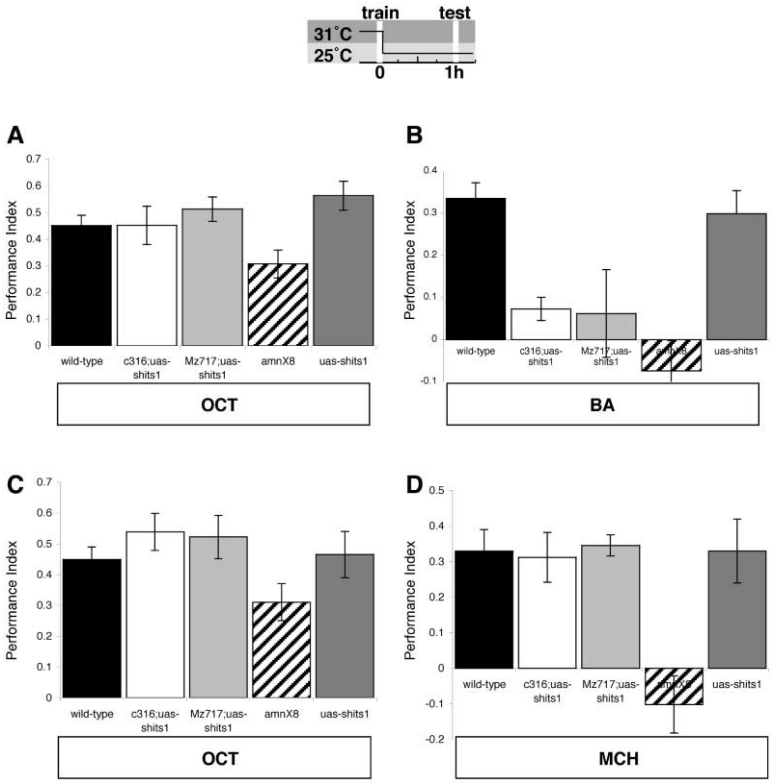

Figure 6. Blocking DPM Output during Acquisition Impairs BA Memory

All genotypes were incubated at 31°C for 15 min prior to and during training. Immediately after training, they were returned to 25°C, and they were tested for 1 hr memory at 25°C. (A) Blocking DPM output during acquisition does not affect OCT memory. Flies were trained to associate OCT with shock and tested for preference between OCT and BA. (B) Blocking DPM output during acquisition abolishes BA memory. Flies were trained to associate BA with shock and tested for preference between BA and OCT. (C) Blocking DPM output during acquisition does not affect OCT memory. Flies were trained to associate OCT with shock and tested for preference between OCT and MCH. (D) Blocking DPM output during acquisition does not affect MCH memory. Flies were trained to associate MCH with shock and tested for preference between MCH and OCT.

shi^{ts1} and Mz717;uas-*shi*^{ts1} flies severely impaired 1 hr BA memory ($p < 0.05$ for both) (Figure 6B). In contrast, DPM blockade during acquisition did not significantly affect memory with OCT-MCH (Figures 1C, 6C, and 6D). Furthermore, olfactory acuity and the response to electric shock of c316;uas-*shi*^{ts1} and Mz717;uas-*shi*^{ts1} flies were unaffected by temperature (Table 1). Therefore, neurons expressing GAL4 in c316 or Mz717 flies are not directly involved in sensing and avoiding BA. Instead, these data imply that DPM output is required during acquisition of BA memory but not for OCT and MCH memory.

Discussion

DPM Neurons and Consolidation of OCT-MCH Memory

Assaying MCH and OCT olfactory memory, we previously identified the DPM neurons—large putative modulatory neurons that innervate the mushroom bodies—as being the critical site of *amn* function in olfactory memory (Waddell et al., 2000). Using uas-*shibire*^{ts1} (Kitamoto, 2001) as a temperature-sensitive blocker of DPM neuron function, we showed that DPM output was not required for OCT-MCH learning but was required for extended (up to 1 hr) memory (Waddell et al., 2000). In this report, we demonstrate that DPM output is dispensable during training and recall for 3 hr OCT-MCH memory (Figures 2C, 2D, and 2E). Strikingly, DPM output is required at least 30 min into the period between training and testing (Figures 2F and 2G). This timing is consistent with the idea that DPM function and AMN neuropeptide is involved in memory consolidation.

It is noteworthy that the requirement for DPM output in memory differs from that of MB neuron output. MB output is not required for acquisition or during storage but is required for memory recall (Dubnau et al., 2001; McGuire et al., 2001; Schwaerzel et al., 2002). DPM neuron output is required during storage but is dispensable during acquisition and recall (Figure 2). Therefore, DPM neuron action on the MBs does not occur at a time when output from the MBs is required. This suggests that DPM-dependent memory processes in the MBs do not rely on transmitter release from MB synapses.

How Is AMN Involved?

Blocking DPM output with the uas-*shi*^{ts1} transgene produces a similar effect to mutation of the *amnesiac* gene (Waddell et al., 2000). Blocking DPM neurons does not affect immediate memory but abolishes later memory. With some variability between alleles, *amn* mutant flies have a near wild-type immediate memory but a pronounced later memory defect (Quinn et al., 1979; Tully and Gergen, 1986; DeZazzo et al., 1999). These data are consistent with the possibility that AMN peptides contribute to the memory process.

In support of an acute role for AMN peptides in odor memory, we found that DPM neurons are present in *amn*^{1}, *amn*^{ex1}, and *amn*^{ex39} mutant flies (Figure 1). The main branches of the major neurite are present, and the lobes of the MBs are ensheathed in the characteristic putative DPM-MB synapses. Therefore, *amn* is not required for DPM neuron survival or for the gross targeting of DPM neurons to the MB lobes. In addition, our behavioral analysis shows that DPM neurons are acutely required for memory. Restricting *amn* gene expression

spatially and temporally with TARGET (McGuire et al., 2003) or GeneSwitch (Osterwalder et al., 2001; Roman et al., 2001) should resolve whether *amn* has an acute function in adult flies and/or is involved in development.

Although blocking DPM neuron output with uas-*shi*^{ts1} produces an *amn* mutant-like memory defect (Waddell et al., 2000; and this study), it is not known if the *shi*^{ts1}-encoded dynamin blocks release of peptide-containing dense core vesicles (DCVs). DCVs, unlike typical synaptic vesicles, are derived from the trans-Golgi network. Dynamin is involved in endocytosis (Chen et al., 1991) and vesicle budding from the Golgi (for review, see Allan et al., 2002), but whether it is involved in DCV release is unclear. The amnesic effect of blocking DPM output suggests that uas-*shi*^{ts1} blocks AMN release and/or blocks release of an essential cotransmitter.

It is plausible that AMN peptides are coreleased from DPM synapses with a classical fast-acting transmitter. Glutamate is used in the CNS of *Drosophila*, but it is not the predominant transmitter (Strausfeld et al., 2003). Instead, this role appears to be taken by acetylcholine (Buchner et al., 1986; Gorczyca and Hall, 1987). We showed that a DPM neuron marker is coexpressed with a cholinergic neuron-specific marker (Figure 1D), suggesting that the DPM cotransmitter is Ach. Assuming that DPM neurons corelease Ach and AMN transmitters, DPM neuron release would trigger a postsynaptic response in receptive MB neurons that involves Ach receptors and AMN receptors. Genetic and pharmacological experiments suggest that the *amn* gene affects cAMP synthesis (Feany and Quinn, 1995; Moore et al., 1998), and we previously posited that memory stabilization may depend on prolonged cAMP cascade stimulation by AMN peptide (Waddell et al., 2000). Perhaps the role of Ach versus AMN peptides in DPM-dependent memory will rely on the evoked firing pattern of DPM neurons, with repetitive activity being required to release AMN (Zhong and Pena, 1995).

In mammals, the putative AMN homolog PACAP and the related vasoactive intestinal peptide (VIP) can be coreleased with ACh. In several neural systems, PACAP and VIP can potentiate both muscarinic and nicotinic ACh-evoked currents by a cAMP-dependent mechanism (Kawatani et al., 1985; Gurantz et al., 1994; Margiotta and Pardi, 1995; Liu et al., 2000). In a hippocampal slice preparation, PACAP38 enhances excitatory CA3-CA1 synaptic transmission, and the facilitation can be blocked by inhibiting muscarinic receptors (Roberto and Brunelli, 2000; Roberto et al., 2001). Perhaps AMN peptides fulfill a similar function in fly memory.

DPM Neuron Output and Acquisition of BA Memory

We discovered that DPM output is required during acquisition to associate BA with electric shock. Blocking DPM output during acquisition blocks BA memory but not OCT or MCH (Figure 6). Therefore, the temporal requirements for DPM output show some odor specificity. This finding implies that DPM neurons may be differentially involved in odor memory.

Why Is BA Different?

Our learning experiments suggested that BA might be unique for flies. Importantly, our data (Figures 3B and 3C) and a previous study (Charro and Alcorta, 1994) demonstrate that flies respond to BA independently of the antennae and maxillary palps. We found that the bitter almond smell of BA is sensed by the olfactory system, bitter-sensitive gustatory neurons, and *poxn*-affected neurons that are likely on the legs or mouthparts of the fly. This implies that *Drosophila* can use multiple neural pathways to sense some odors. In vertebrates, both the olfactory and a somatosensory system called the trigeminal system respond to most odorous chemicals. The free nerve endings of the trigeminal system are sensitive to thermal and mechanical stimuli as well as to very high and potentially harmful concentrations of chemicals. Trigeminal stimulation induces a reflex that stops inspiration to prevent inhalation of hazardous substances. Our finding that BA is a particularly potent somatosensory stimulus is consistent with the fact that BA is a drastically effective insecticide (Dettner et al., 1992) and also a potent trigeminal stimulus in humans (Doty et al., 1978). Therefore, our data suggest that *Drosophila* possess additional odor detecting systems that are perhaps analogous to the trigeminal system in vertebrates to detect potentially harmful chemicals.

What type of sensory neuron outside of the classical olfactory system is likely to detect BA? The fact that BA is a volatile stimulus would argue that the nonantennal/palp neurons are olfactory in nature, while our *poxn* results argue that they are gustatory. We feel that this apparent contradiction is purely semantic, because the strict division of sensory systems into olfactory and gustatory modalities is becoming increasingly blurred by new molecular and functional information. For instance, receptors from the same subclass function as odor receptors for amino acids in fish olfactory neurons (Speca et al., 1999), putative pheromone receptors in the vertebrate vomeronasal system (Dulac and Axel, 1995; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997), and taste receptors tuned to sweet and umami substances in the vertebrate tongue (Nelson et al., 2002). Similarly, several fly gustatory receptor genes are expressed selectively in olfactory neurons in the fly (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001; Thorne et al., 2004), and a prominent member of the insect odorant receptor gene family is expressed in the mosquito proboscis (Pitts et al., 2004), classically defined as a gustatory organ. Therefore, neither the class of molecular receptor expressed in a given sensory system nor the sensory organ itself is necessarily a clear indication of whether a given neuron is tasting or smelling a stimulus. In the nematode, chemosensory neurons have been divided into those responding to volatile stimuli and nonvolatile stimuli, corresponding to olfactory and gustatory senses, respectively (Bargmann et al., 1993; Bargmann and Horvitz, 1991). This division based on the stimulus type seems most relevant for the biology of terrestrial animals, and we favor the interpretation that chemosensory neurons of the olfactory class but lying outside of the classical olfactory system are tuned to BA. Future work will be aimed at characterizing these atypical sensory neurons and mapping their circuitry in the brain.

It is plausible that associative learning of BA involves signal integration of the electric shock pathway with BA information from all the systems that detect BA—an antennae/palp pathway, a bitter-sensitive pathway on

the labelum, and *poxn*-affected neurons located elsewhere. This multimodal BA information will be initially processed by distinct brain regions. Antennal and palp input projects to the antennal lobe (Gao et al., 2000; Vosshall et al., 2000), but labelar gustatory neurons project to the subesophageal ganglion (Thorne et al., 2004; Wang et al., 2004). We assume that tarsal chemosensory neurons will project to the ventral ganglion. We speculate that this unique and potentially integrative circuit specificity accounts for the different requirement of DPM neuron involvement in learning BA versus the memory of OCT and MCH. Alternatively, it is possible that DPM neurons differently process BA information that comes through the antennal and maxillary palp pathway. Future work will determine the importance of BA input through the noncanonical pathway in BA memory.

Previous studies have indicated that *Drosophila* process BA differently to other odors. Flies with a mutation in the *acj6* gene have a reduced olfactory jump response and a reduced electrophysiological response in the antennae and maxillary palps to all odors tested except BA (Ayer and Carlson, 1992). In contrast, mutation of the *ptg* gene produces a near reciprocal result to *acj6*. ptg^{7} mutant flies are defective in their response to BA but normal with other odors tested (Helfand and Carlson, 1989). In addition, disrupting olfactory receptor neuron expression of the Gqα heterotrimeric G protein subunit gene with region-restricted RNA interference abolished behavioral responses to isoamylacetate but not BA (Kalidas and Smith, 2002).

Is there any reason BA may have inherent meaning to an insect? BA is the odor of bitter almond. Many plants (including almond), when damaged, produce hydrogen cyanide and BA from a cyanogenic glycoside. This cyanogenesis is believed to protect against predation from herbivores (Gleadow and Woodrow, 2002). Perhaps it would be profitable for an organism that might otherwise lay its eggs on the fruits of a cyanogenic plant to be primed to associate the smell/taste of BA with the possibility of cyanide release. The detrimental effect of hydrogen cyanide is unquestioned—it causes a near universal respiratory arrest. BA, on the other hand, is considered more of a general irritant. In addition to plants, some insects use hydrogen cyanide and/or BA as a defensive/alert signal (Nahrstedt, 1988). For example, some millipede species release hydrogen cyanide and BA as defensive emissions (Conner et al., 1977). Perhaps more interesting, harvester ants release BA when agitated, and conditioned air suffused with this emission elicits an avoidance behavior in nonagitated naive ants (Blum et al., 1969). However, we have no evidence that BA is a constituent of a similar emission in *Drosophila*.

DPM Neurons and Odor Memories

In conclusion, the results presented here demonstrate that DPM neuron output is differentially involved in odor memory. DPM activity at least 30 min after training is required for normal OCT and MCH memory, supportive of a role for DPM neuron function (and presumably AMN peptide) in consolidation of OCT and MCH memory. In contrast, DPM output is required during acquisition of BA memory. Taken with our finding that BA is sensed by an olfactory and nonolfactory route, we speculate that DPM neurons are uniquely involved in the memory of odors that require multisensory integration.

Experimental Procedures

Fly Strains

Fly stocks were raised on standard cornmeal food at 25°C and 40%–50% relative humidity. The wild-type *Drosophila* strain used in this study is Canton-S and originated from W.G. Quinn's lab (Massachusetts Institute of Technology). The amn^{1}, amn^{c651}, and amn^{X8} alleles were described previously (Quinn et al., 1979; Waddell et al., 2000; Moore et al., 1998). amn^{X8} is a behavioral *amn* null allele generated by imprecise excision of the amn^{28A} P elements. DeZazzo et al. (1999) reported that amn^{X8} lacks the entire *amn* open reading frame (ORF). Imaging DPM neurons in amn^{X8} brains was not possible, because amn^{X8} flies have GAL4 activity in the mushroom bodies (data not shown). Therefore, amn^{X8} flies must retain P element sequence that was not described by DeZazzo et al. (1999). This residual GAL4 activity is not sufficient to rescue *amn* memory (Waddell et al., 2000; and this study; Figure 4A) but c316{GAL4};uas-CD8:GFP-labeled DPM projections into the MB lobes cannot be readily distinguished from intrinsic MB labeling. We therefore made new deletion alleles of the *amn* ORF by imprecise excision of the amn^{c651} P[w^{+}] element. Briefly, amn^{c651} females were crossed to transposase-bearing *Sb*(Δ2-3)/TM3*Ser* males. Dysgenic $amn^{c651;Sb}$(Δ2-3) males were crossed to FM7a females, and excision chromosomes were selected by the absence of the P[w^{+}] element. In the next generation, we isolated putative amn^{ex} males and prepared genomic DNA. We analyzed fifty of these putative excisions for the integrity of the *amn* locus by PCR and sequence analysis. Two of these lines—amn^{ex1} and amn^{ex39}—contained near complete deletion of the *amn* ORF. amn^{ex1} deletes a region of DNA extending from −661 nucleotides upstream of the ATG to position +369 within the *amn* ORF. amn^{ex39} deletes a region of DNA extending from −785 nucleotides upstream of the ATG to position +477 within the *amn* ORF. amn^{ex39} leaves only a small C-terminal fragment that is not expected to have function. The uas-mCD8:GFP flies are described in Lee and Luo (1999). The uas-shi^{ts1} flies were those previously used by us (Waddell et al., 2000) and described in Kitamoto (2001). We previously described the DPM neuron-restricted c316{GAL4} and the uas-*amn* flies (Waddell et al., 2000). The uas-*amn* flies are those previously denoted as "uas-*amn*#1." Mz717{GAL4} flies were described by Ito et al. (1998). Gr5a-Gal4;uas-DTI and Gr66a-Gal4;uas-DTI strains were tested as stable homozygous stocks generated as described in Wang et al. (2004). Transheterozygous *pox-neuro* mutant progeny from a cross between the hypomorphic allele and the deficiency were analyzed in behavioral assays.

Histochemistry

Adult brains expressing transgenic mCD8:GFP were removed from the head capsule and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (1.86 mM NaH_2PO_4, 8.41 mM Na_2HPO_4, 175 mM NaCl) for 15 min and rinsed in PBS-T (PBS containing 0.25% Triton X-100). Fixed brains were mounted in Vectashield. Confocal analysis was performed on a Leica TCS-SP laser scanning confocal microscope.

Preparation of Transgenic Flies for Behavioral Analysis

We generated flies expressing shi^{ts1} in DPM cells by crossing homozygous *w*,uas-shi^{ts1};uas-shi^{ts1} females to homozygous *w*;c316{GAL4} males. All progeny from this cross carry two uas-shi^{ts1} transgenes and one c316{GAL4}. Heterozygous *w*;c316{GAL4} and *w*,uas-shi^{ts1};uas-shi^{ts1} flies were generated by crossing homozygote females to *w* males. A mixed population of sexes was tested in the olfactory conditioning paradigm.

For rescue of the amn^{X8} memory defect, we crossed amn^{X8};c316{GAL4} and amn^{X8};uas-*amn* flies. All progeny from these crosses were homozygous for amn^{X8} and heterozygous for c316{GAL4} and uas-*amn*. Mixed sex populations were tested.

Behavioral Analysis

The olfactory avoidance paradigm was performed according to Tully and Quinn (1985) except that odors were delivered by bubbling air

through 15 ml scintillation vials containing odor dilutions in 10 ml of mineral oil. The PI was calculated as described in the text. A single PI value is usually the average score from flies of the identical genotype tested with each odor. In experiments highlighting odor-specific effects, individual odor scores were calculated separately. Experiments involving uas-*shi*^{ts1} were performed while the behavior room temperature was shifted from 25°C to 31°C.

For T maze experiments with olfactory organ-less flies, we removed the antennae and maxillary palps from several hundred wild-type flies. We mixed olfactory organ-less flies with a 5-fold excess of Cantonized *white* flies to obtain optimal numbers of flies for the experiments. We calculated the scores independently after sorting *white* from *white*$^{+}$ (olfactory organ-less) flies.

To test olfactory acuity, untrained flies were given 2 min to choose between a diluted odor (1:80 dilution in mineral oil of OCT, 1:107 of MCH, 1:210 of BA) as used in conditioning and air bubbled through mineral oil in the T maze. Percent avoidance was calculated according to Tully et al. (1994). Electroshock avoidance was performed and calculated similarly. Untrained flies chose between a tube containing an electrified grid and a tube containing a nonelectrified grid. To assess relative odor avoidance, we gave untrained (or previously electric-shocked) flies 2 min to choose between two diluted odors as used in conditioning in the T maze.

Odor avoidance was also tested in an arena by measuring the distance of single freely moving flies from an odor source. Odorants were placed on a piece of filter paper at the wall of a petri dish (8.5 cm diameter, 1.3 cm height). The fly's position was tracked at 6 Hz using a video camera and Ethovision tracking software (Noldus). The fly's average position relative to the stimulus was determined over 3 min. Avoidance was calculated by subtracting the average distance of a fly from an odorless filter paper from the value measured in the different experimental conditions. A zero avoidance value indicates that the flies behave like there is no odor stimulus.

Statistical analyses were performed using KaleidaGraph (Synergy Software). Overall analyses of variance (ANOVA) were followed by planned comparisons among the relevant groups with a Tukey HSD post hoc test. Unless stated otherwise, all experiments are $n \geq 8$, and all data points denoted as "statistically significant" are $p < 0.05$.

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