MASTERARBEIT

Titel der Masterarbeit

„Identification of sexually shared components of the

Drosophila courtship circuit"

Verfasser

David Mahringer, BSc

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Synopsis

Neuronal circuits are the basis for animal behavior. Male and female animals both share and distinguish themselves in behavioral patterns. This raises the question if neuronal circuits are completely separated between sexes or to what extent they share certain components and how they are differentially activated to produce adequate behavioral output. Little is known about shared components and their signal transduction within a neuronal network that influence behavioral patterns observed in both sexes. The neuronal circuitry underlying the courtship ritual of Drosophila melanogaster has been partially explored. A male-specific neuron, P1, is believed to be a decision making component for male courtship behavior. Similar, pMP8, a central neuron in the female brain of Drosophila, is seen as an important neuron for female courtship behavior. Artificial activation of both neurons causes either male or female flies to follow other flies. This following behavior is usually seen during male courtship behavior. We hypothesized that a neuron responsible for signal transduction during following behavior should be common to both sexes, although naturally, differentially activated. With the work described in this thesis the objective was to gain more insight into the circuit that harbors shared components responsible for following behaviors in both sexes of Drosophila. In order to identify such a component we targeted a variety of genetic tools to specific neurons of interest using a previously-annotated Gal4 enhancer lines known as VT lines. By performing an epistasis screen in which we activated pMP8 and simultaneously silenced a neuronal cluster overlapping with pMP8, we could identify 12 VT lines that showed no following behavior upon pMP8 activation. We were also interested to see if pMP8 can be directly integrated into a potential neuronal circuit for female receptivity and tested this by performing anatomical studies and labeling experiments.

Synopsis

Neuronale Schaltkreise sind die Grundlage für das Verhalten von Tieren. Männliche und weibliche Tiere teilen sowohl, aber unterscheiden sich auch in Verhaltensmustern. Dies wirft die Frage auf, ob neuronale Schaltkreise komplett zwischen den Geschlechtern getrennt sind oder in welchem Umfang sie bestimmte Komponenten teilen und wie diese differentiell aktiviert werden, um angemessene Verhaltensweisen zu erzeugen. Wenig ist über gemeinsam genutzte Komponenten und deren Signaltransduktion in einem neuronalen Netzwerk bekannt, welche Verhaltensmuster beeinflussen und die in beiden Geschlechtern beobachtet werden können. Die neuronale Grundlage des Balzverhaltens in Drosophila wurde teilweise erforscht. Von einem spezifisch männlichen Neuron, P1, wird angenommen, dass es eine entscheidungsrelevante Komponente für männliches Balzverhalten ist. Ähnlich dazu ist pMP8, eine zentrale Neuron im weiblichen Gehirn von Drosophila, welches als ein wichtiges Neuron für weibliches Balzverhalten angesehen wird. Künstliche Aktivierung beider Neuronen in entweder männlichen oder weiblichen Fliegen resultiert in einem gegen andere Fliegen gerichteten Folge-Verhalten. Dieses Folge-Verhalten kann in der Regel während männlichem Balzverhalten beobachtet werden. Wir stellten die Hypothese auf, dass ein Neuron das für die Signaltransduktion während dem Folge-Verhalten verantwortlich ist, beiden Geschlechter gemeinsam sein sollte, obwohl es unter natürlichen Umständen differentiell aktiviert wird. Ziel dieser Arbeit war es mehr Einsicht in den Schaltkreis, welcher gemeinsam genutzte Komponenten, die für das Folge-Verhalten in beiden Geschlechtern von Drosophila verantwortlich gemacht werden können, beherbergt, zu bekommen. Um eine solche Komponente zu identifizieren, verwendeten wir eine Vielzahl von genetischen Werkzeugen in spezifischen Neuronen von Interesse, unter der Verwendung von im Vorfeld bekannt gemachten „Gal4 enhancer lines", die als „VT-lines" bekannt sind. Mit der Durchführung eines „epistasis-screens", in dem wir pMP8 aktivierten und gleichzeitig mit pMP8 überschneidende, neuronale Cluster still-schalteten, konnten wir 12 „VT-lines" identifizieren, die kein Folge-Verhalten während der pMP8 Aktivierung zeigten. Auch waren wir daran interessiert, ob pMP8 direkt in einen möglichen, neuronalen Schaltkreis für weibliche Empfänglichkeit integriert werden kann und testeten dies mithilfe anatomischer Studien und Markierungsexperimenten.

Chapter 1: Introduction

Behavioral neuroscience

A general goal of neuroscience is to elucidate the unknown mechanisms in our brain that contribute to cognition, behavior and other higher brain functions. First descriptions of our brain were found in a papyrus from ancient Egypt, the so called "Edwin Smith Papyrus" (Kandel, 2012). The document describes cases of human brain injuries and their medical treatments, but no descriptions of specific cognitive functions of our brain can be found in this document. The discussion about brain functions started with Hippocrates, thinking of the brain as the basis of mental processes. Already at this time, opinions about the function of our brain diverged (Finger, 1994). Aristotle argued against Democritus and Plato, stating that the heart was the center for our thoughts and the brain was an organ controlling the cooling of blood (Bear et al., 2007). Neuroscience developed further by anatomical descriptions from Galen. He divided the brain into cerebrum and cerebellum and believed that the cerebrum was the center for memories whereas the cerebellum controlled muscles (Bear et al., 2007; Finger, 1994). Although not experimentally proven at that time, his view was not so far away from the truth. Many more scientists worked over the centuries on the description of our brain, still it was challenging to characterize mechanisms of perception or behavior in detail.

The complexity of the human brain and the lack of experimental methods made it difficult over the past few hundred years to overcome pure anatomical studies and gain insight into neuronal networks. Although research on the architecture of neuronal cells, especially by Ramón y Cajal and others led to the basis of modern theory of how neurons connect and signal to each other (Strausfeld, 2012), studies on lesions and brain injuries made it first possible to ask which parts of our brain harbor certain functions (Finger, 1994; Kandel, 2012). Pierre Paul Broca and Karl Wernicke were among the first to correlate behavior and areas for brain function. Broca observed that patients with a common lesion in a brain area now known as Broca's area (posterior inferior frontal gyrus of the left hemisphere) were unable to speak consecutively. He concluded form this observation that this area is important for speech production (Purves, 2012). Soon after, Wernicke discovered, also by lesion studies, another area that is responsible for the ability to understand language, the so called Wernicke area in the posterior section of the superior temporal gyrus (Purves, 2012). These findings formed a model with interconnected

centers on the surface of the brain for language perception: the Wernicke area responsible for understanding and the Broca area for forming language. This example illustrates how first attempts to understand and localize different functions of our brain were made and how scientists tried to draw circuits for neuronal networks of behavior.

In modern neuroscience new methods enable scientists to ask detailed questions about brain mechanisms and their underlying principles (Luo et al., 2008). They allow targeting individual neuronal cells (Pfeiffer et al., 2008) and analyzing their function in a given context. Though accurate functional analysis of neurons responsible for a certain behavior is difficult, focusing on robust innate behavior and parts of more complex behavior is a feasible way to get insight into the neuronal basis of behavior. This is possible as it is believed that these behaviors are governed by specific neuronal circuits that guide the decision making process of an animal. Innate behavior of invertebrates offers the opportunity to analyze robust decision making processes and their underlying principles, as their nervous systems are less complex than those of vertebrates (Sattelle and Buckingham, 2006). *Drosophila melanogaster* has been widely used as a model organism for a broad range of research topics including neuroscience, as the fly is able to perform a variety of robust innate behaviors (Amrein and Thorne, 2005; Hall, 1994; Sokolowski, 2001; Zwarts et al., 2012). Drosophila exhibits an elaborate courtship ritual, consisting of several steps until successful mating is achieved, wherein both flies have to integrate internal and external information in order to make a decision about mating (Dickson, 2008). This enables scientist, combined with the entire well developed genetic tools for this model organism, to ask specific questions about how each step is accomplished on a neuronal basis. In the end analysis of neurons involved in decision making processes will allow to explain how neuronal networks or brains process signals to guide decisions and thus behavior.

Drosophila courtship ritual

Courtship behavior ensures that only the fittest individuals reproduce and therefore enhance the evolutionary success of the species (Bastock, 2007). The Drosophila courtship ritual is an innate behavior that consists of six steps performed by the male fly (Hall, 1994; Sokolowski, 2001), in which female flies judge the quality of the male courtship and decide whether to allow the male to copulate or not.
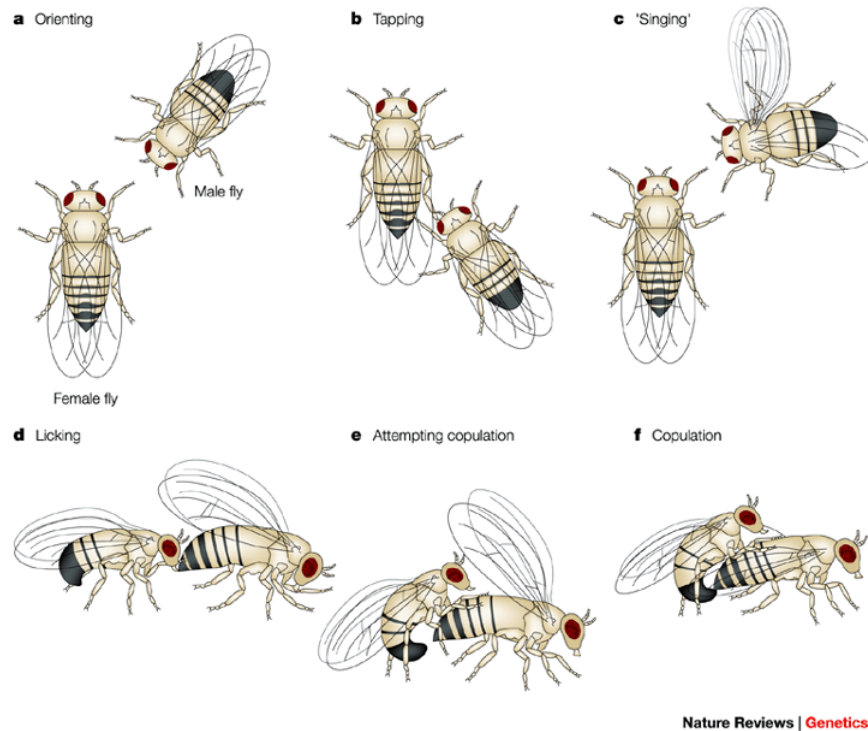
Figure 1.1 Courtship ritual of Drosophila melanogaster consisting out of six steps. Adapted from (Sokolowski, 2001)

The ritual starts as soon as the male fly recognizes the female and orients towards her. Next the male taps the female abdomen with one of his forelegs. Males produce a specific courtship song by unilaterally vibrating one wing. Also they lick the female genitalia and then try to copulate with the female before the actual copulation takes place. The sequence presented in Figure 1.1 is not entirely fixed, illustrated steps can alternate and vary in their contributing levels. Courtship steps will be repeated until the male fly successfully mounts onto the female or he is finally rejected by her (Bastock and Manning, 1955)

A characteristic part of the courtship behavior in Drosophila is the courtship song. Male flies unilaterally extend their wing during courtship and vibrate in order to create a specific song pattern that is recognized by the female (Bennetcl.Hc and Ewing, 1967). The quality of the song is a critical parameter for successful mating and will determine the female's decision to copulate or not (Bennetcl.Hc and Ewing, 1969). The song itself is composed of two units, the sine song and the pulse song (Vonschilcher, 1976). Von Schilcher suggested that the function of the sine song is priming the female fly for the pulse song and thus increasing receptivity. It has a typical sinusoidal frequency of 140-170 Hz and can be heard as a humming sound after amplification. In contrast to this the pulse song has a higher frequency of 150-300 Hz and can be seen in a train of 2-50 pulses with one to three cycles per pulse. The pulses are highly structured and spaced by specific interpulse-intervals (IPIs). These IPIs last around 34ms and provide a recognition pattern for the song that ensures species specificity (Kyriacou and Hall, 1982).

Sex pheromones play another important part in the courtship ritual. Among the best studied ones are cVA (cis-vaccenyl acetate) and 7,11-HD (7,11-heptacosadiene). cVA is transferred during copulation in the seminal fluid of the male to the female (Butterworth, 1969; Everaerts et al., 2010) and acts as an repellent for male flies, indicating that the female recently mated (Zawistowski and Richmond, 1986). It is important as male flies learn to discriminate between unmated and mated females to increase their mating success (Keleman et al., 2012). cVA is also involved in other social behaviors of Drosophila. It promotes male aggression behavior (Wang and Anderson, 2010) and may act as an aggregation signal for female flies (Bartelt et al., 1985; Benton, 2007; Schlief and Wilson, 2007). 7,11-HD is present in the female cuticle and acts as an aphrodisiac for males (Antony et al., 1985). It is detected by neurons in the male forelegs and promotes courtship (Thistle et al., 2012; Toda et al., 2012).

As female flies are not stationary during the courtship ritual males chase their target and try to present their qualities by keeping track of the female. Female flies slow down their movement to allow copulation in the end (Tompkins et al., 1982). This following behavior during the courtship ritual is male specific and not seen by female flies. Still females follow other flies in a similar manner as male flies do (Cook, 1975, 1981). Female following is seen in 3-5 day old virgins and directed to both males and females. Following bouts are shorter than observed in males and directionality is focused on the posterior part of the fly. In some cases wing extension and flickering and a more head to head orientation as seen in males during courtship can be observed (Cook, 1981). It is clear from these findings that female flies show following behavior. Whether or not this can be attributed to a female courtship behavior remains unclear.

Neuronal courtship circuit

The central nervous system of the adult Drosophila fly consists of a brain and a ventral nerve cord (VNC). The brain can be divided into 3 parts, namely a central brain surrounded by two optic lobes. The VNC is made up by 5 units, beginning with the prothoracic neuromere anteriorly, followed by the wing-, mesothoracic-, metathoracic neuromere and ending with the abdominal ganglion in the most posterior region (Ito reference). The total mass of the central nervous system is estimated to consist of about 100.000 cells (North and Greenspan, 2007). This network of specialized cells coordinates actions and decisions during behavior and produces motor output. Compared to other model organisms the overall cell number in the Drosophila nervous system is low, making it an ideal model to investigate and manipulate neuronal circuits for courtship behavior. Two genes provide an entry point to identify components of the underlying neuronal circuit that guides the courtship ritual in Drosophila. The two transcription factors, fruitless (fru) and doublesex (dsx), broadly influence circuit wiring in Drosophila for sexual behavior (Burtis and Baker, 1989; Cachero et al., 2010; Ito et al., 1996; Ryner et al., 1996; Yu et al., 2010). Moreover fruitless has been shown to regulate sexual identity and courtship behavior (Demir and Dickson, 2005). By expressing the male variant of fruitless Fru^M in females, female flies started showing courtship behavior towards other females. Interestingly this putative transcription factor is expressed in approximately 1500 neurons in the flies central nervous system and has been linked not only to courtship behavior in male flies, but also to aggression behavior, another sex specific behavioral pattern (Certel et al., 2007; Chan and Kravitz, 2007; Wang and Anderson, 2010). These implications of fruitless in the nervous system provide an entry point to study the neuronal circuits involved in courtship behavior.

Intersectional genetic methods allowed labeling and classifying approximately 100 types of fru$^+$ neurons. In combination with data from neuronal polarity staining it is possible to draw a circuit diagram for courtship behavior in Drosophila (Yu et al., 2010). This diagram is a basis for functional analysis of the circuit components within the fly's nervous system. A central component of this diagram is pMP4 (Figure 2), which has been analyzed for its role in courtship behavior. pMP4 neurons (or P1) respond to female contact and their artificial activation of in male flies leads to courtship song production by unilateral wing vibration (Kohatsu et al., 2011; von Philipsborn et al., 2011). This example shows that genetic approaches allow the identification and functional analysis of different components within a neuronal network for certain behaviors. These studies have been carried out in male flies and do not necessarily
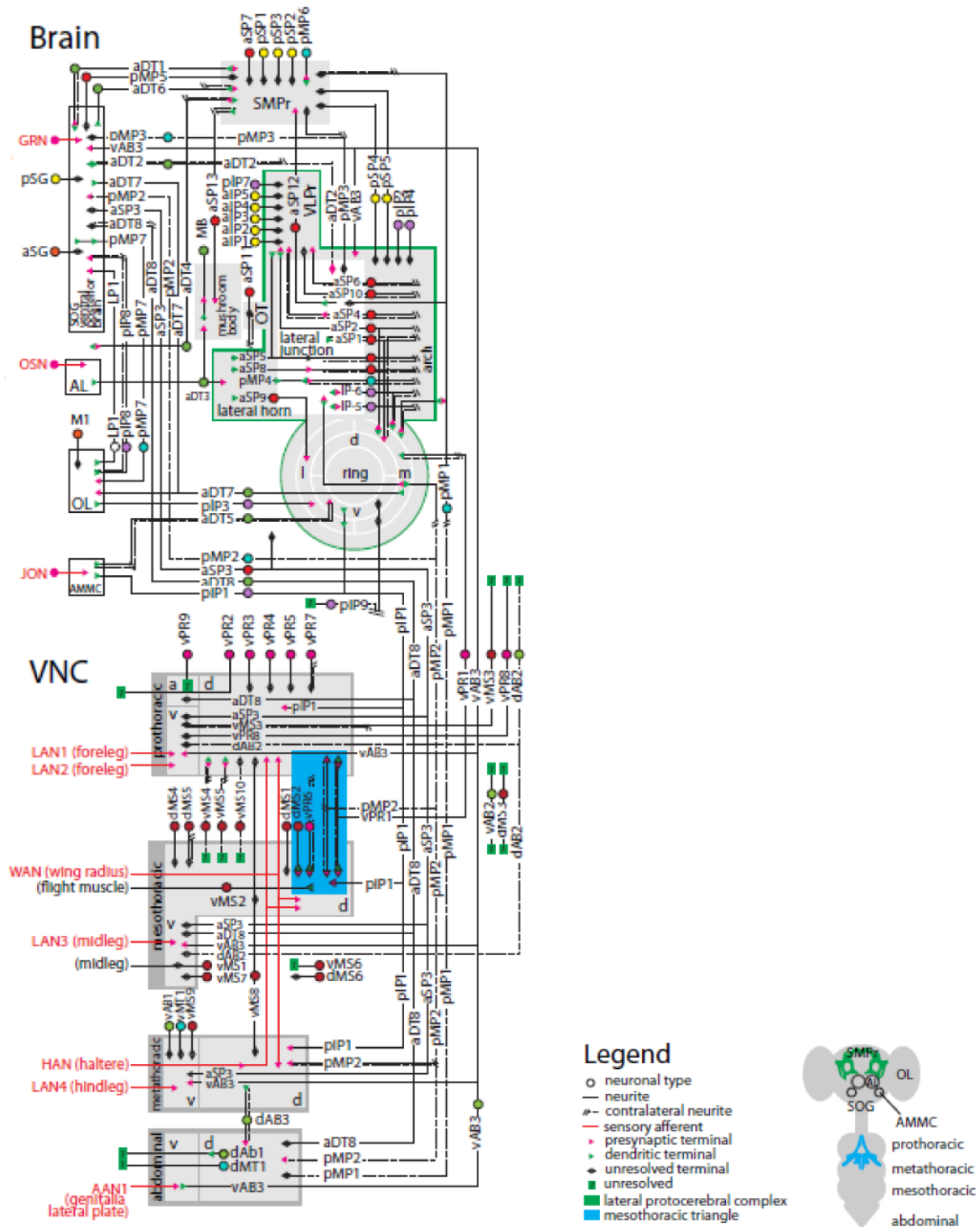
Figure 1.2. Neuronal circuit diagram for fru+ neurons in the Drosophila brain. Adapted from (Yu et al., 2010).

reflect the situation in females. Some identified components do not exist in female flies (von Philipsborn et al., 2011), moreover sex specific wiring of individual neurons within the courtship circuit can be observed (Cachero et al., 2010). This difference is partly explained by the sex specific splicing of *fruitless* in Drosophila (Kimura et al., 2005).

Sex specific wiring contributes to different processing of internal and external signals and explains why certain behaviors normally don't occur in both sexes. Complete separated sets of neurons for sex specific behavior is unlikely as studies showed that sex specific behavioral patterns can be elicited also in the opposite sex (Clyne and Miesenbock, 2008; Kimura et al., 2008). As introduced before, P1 neurons resemble a sex specific neuronal cluster for males that produce courtship behavior (Kohatsu et al., 2011; von Philipsborn et al., 2011). Genetic rescue of this cluster in female flies leads to courtship behavior of females towards other females (Kimura et al., 2008). Only the P1 cluster has been masculinized in the nervous system of these flies. This result suggests that circuit components downstream of P1 are shared between both sexes. Other evidence comes from a study in which fru$^+$ neurons of decapitated female flies were artificially activated and produced courtship song (Clyne and Miesenbock, 2008). This male specific behavior is not seen in females under normal conditions. The finding that females are in principle capable of performing this behavioral pattern, points also towards the hypothesis that components within the circuit for courtship behavior in Drosophila are shared to a certain degree.

Tools

Functional and physiological analysis of neurons within a neuronal network is important for the characterization of individual elements in order to understand their behavioral relevance. Specific targeting of components is required to apply manipulating tools in experiments. This approach is necessary to gain further insight into basic processing of circuits for behavior.

Vienna Tiles

The binary expression system GAL4/UAS can be used to drive expression of a reporter gene in the tissue of interest (Brand and Perrimon, 1993). The expression of GAL4 itself is regulated by cis-regulatory elements that define the expression pattern of the reporter gene within the tissue. This system has been used in combination with a random P-element insertion strategy (Rorth, 1996; Rorth et al., 1998), which allowed using the local genomic enhancer environment to drive GAL4 expression in a subset of cells. As the ability to characterize and manipulate a defined subset of cells or neurons is dependent on the specificity of the genetic access to those cells, this method has two disadvantages. Random insertion into the genome creates dependency of GAL4 expression strength on the local gene enhancer environment. This strategy usually labels a wide population of cells, meaning a broad expression pattern of the reporter gene can be observed, decreasing specificity of any targeting approach. To overcome these drawbacks we used an enhancer bashing method (Pfeiffer et al., 2008) (Masser, 2011; Bidaye, 2012). Overlapping enhancer tiles of the Drosophila genome were each cloned upstream of the GAL4 transcription factor and integrated into the Drosophila genome by site specific recombination (Groth et al., 2004) (Masser, 2011; Bidaye, 2012). The strategy ensures the reproducible expression of GAL4 by its upstream enhancer tile and the local genomic environment. It also refines the expression pattern of the reporter gene and thereby increases specificity of the transgenic GAL4 lines (Masser, 2011; Bidaye, 2012). This enhancer bashing approach also allows exchange of the binary expression system with e.g. the yeast Hap1/HBS (Martin Haesemeyer and Barry Dickson, unpublished) or the bacterial LexA/LexAop system (Yagi et al., 2010). Independent expression of reporter genes by different binary systems is advantageous if more complicated genetic experiments are required to characterize the role of neurons in behavioral assays.

Split Gal4 system

To further increase refinement of the reporter expression in a tissue it is useful to use the split GAL4 system (Luan et al., 2006). This method makes us of the advantage that Gal4 can be divided into an activation (AD) and a DNA binding domain (DBD). In combination with two different drivers, dimerization of the two domains via a linker region into a functional, reconstituted Gal4 will only occur in overlapping parts of the driver expression and result in transcription of the reporter. The obtained expression pattern will be sparser compared to the individual one before and will increase specificity of any targeting approach.

Manipulating tools

Analysis and characterization of neurons for behavior requires physiological manipulation to alter their activity. Genetically encoded manipulators offer a non-invasive opportunity to shift a neuron's activity to a higher or lower level. Activation of neuronal cells can be facilitate with optogenetic tools as channelrodophins (Boyden et al., 2005) or heat sensitive ion channels as dTrpA1 (Pulver et al., 2009). Activation of both channels leads to an increased inward current generating action potential in the targeted neuron. Using dTrpA1 in experiments has the advantage that multiple animals can be tested at the same time in a temperature gradient by heating up the flies. In order to lower a neuron's activity hyperpolarization of the neuron's membrane potential with an inwardly rectifying potassium channel, namely Kir2.1 (Baines et al., 2001; Johns et al., 1999) can be applied. Hyperpolarization leads to a profound decrease in chances for action potential propagation, thus creating a silent neuron with less signal transduction. Another way is the transgenic expression of tetanus toxin (TNTe) in a neuron to block synaptic transmission. This toxin, originally found in Clostridium tetani bacteria (Wright, 1955), cleaves SNARE proteins required for synaptic vesicle fusion (Schiavo et al., 1992) thereby preventing synaptic vesicle release into the synaptic cleft and current induction in the postsynaptic cell (Sweeney et al., 1995).

pMP8

pMP8 is a central neuron in the female brain of Drosophila (Figure 1.3a). This neuronal cluster harbors 4 cells with their cell bodies located on the posterior surface of the brain. The cell bodies send their projections to the dorsal part of the brain, where aborizations proceed to extend across large parts of both hemispheres. Ring structures can be found in the dorsomedial protocerebrum with arbors reaching down to the subesophageal ganglion. pMP8 neurons are only found in the female nervous tissue. They were identified by using our VT line library to target and activate a subset of neurons in the Drosophila brain with dTrpA1 (Pulver et al., 2009). Artificial activation of pMP8 triggers persistent following behavior and wing flickering of female flies towards other flies (Yang Wu and Barry Dickson, unpublished). This behavior is usually only seen in male flies during courtship (Hall, 1994).
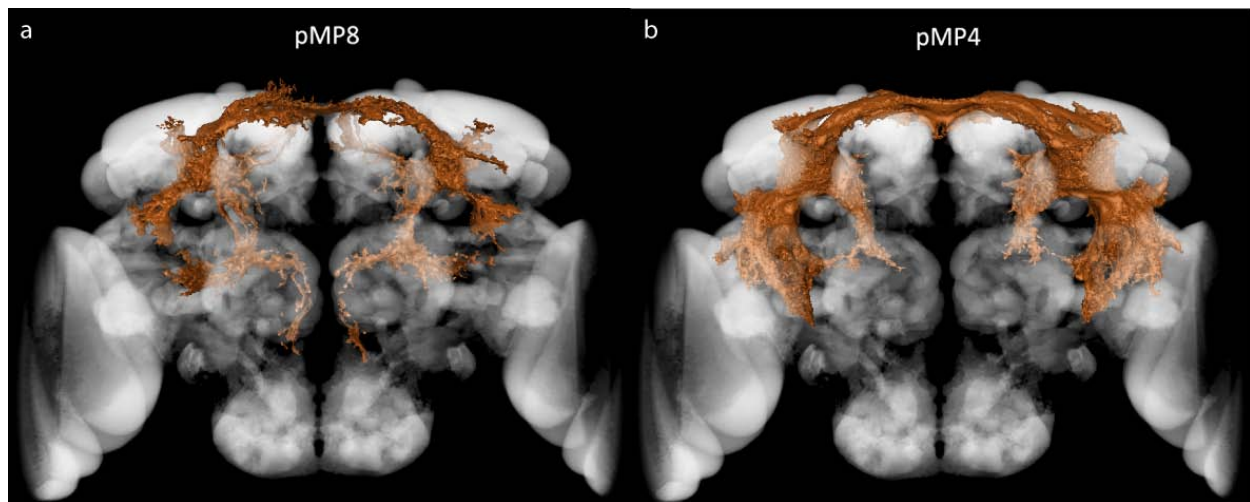
Figure 1.3. Segmented arborizations of pMP8 and pMP4 (P1).

Both neurons arborize extensively in both hemispheres of the fly's brain. Lateral ring structures are formed by both neurons. pMP8 (a) projects down to the suboesphageal ganglion, which cannot be observed in pMP4 (b).

Similar to pMP8, pMP4 (or P1) is a sex specific neuron and exclusively present in male brains (Figure 1.3b). This neuronal cluster consists out of 25-30 cells featuring very similar anatomical structures compared to pMP8. Both neurons aborize extensively in both hemisphere and form central ring

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structures. Artificial activation of P1 neurons leads to full courtship song production of male flies in isolation (von Philipsborn et al., 2011). Moreover courtship behavior of males towards other male flies, showing also following behavior, can be observed (Liu, 2012). These observations led to the hypothesis that P1 is a decision making component within neuronal circuit for courtship behavior in male flies. As similar behavioral patterns are observed upon pMP8 activation in female flies, we hypothesized that pMP8 is a central component within the courtship circuit of females.

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Chapter 2: Identification of sexually shared components of the Drosophila courtship circuit

Introduction

It is likely that Drosophila shares circuit components common to both sexes for courtship behavior. Evidence for this comes from a study that induced courtship song production in females (Clyne and Miesenbock, 2008). This behavioral pattern is not observed in females under normal conditions, thus their principle capability points towards the hypothesis that flies share circuitry in part downstream of central decision making components. Identification of pMP8 in female flies and its activation phenotype allowed us to use it as a model to identify sexually shared components within the courtship circuit. In our experiments we performed an epistasis screen by activation pMP8 with dTrpA1 (Pulver et al., 2009) and simultaneously silencing an overlapping neuronal cluster with either Kir2.1 or TNT (Baines et al., 2001; Johns et al., 1999; Sweeney et al., 1995). We aimed to identify an output neuron of pMP8 that is responsible for the signal transduction during following behavior and common to both sexes.

Results

Selection of VT lines

We selected 450 candidate VT lines for our genetic epistasis screen. Lines included were either found by annotations of following defects observed in previous screens carried out by Christopher Masser and Ines Ribeiro Martins Albes or by overlapping expression patterns visualized with Braingaizer software (Yu et al., 2010). This program was written to create the possibility to have a 3-dimensional look at the expression pattern of a VT line within the brain and VNC. Moreover it allows to search specifically for VT lines that have overlapping expression patterns with neurons of interest, in our case pMP8. We performed an overlap search with pMP8 in Braingaizer not only for neurons in the female nervous system, but also in the male nervous system. This search automatically scores lines according to their overlapping area and ranks them from highest to lowest. The potential disadvantage of this setting is

that lines with only a small overlap have a low score and will show up at the end of the search list. In contrast a small overlapping area might be still potent to form synaptic connections between two neurons. In order not to miss them and give those lines a higher score, we subdivided the anatomical structure of pMP8 into 11 subdivisions (Supplementary Figure 1.1 and 1.2) and performed the overlap search in the Braingaizer program once more. We compared the obtained lists for duplicates and selected in total 450 VT lines for our screen.

The VT line driver line for pMP8 activation resides in the same landing site as the VT-Gal4 driver line for neuronal silencing, thereby making the phenomenon of transvection possible. Transvection is caused by somatic paring of homologous chromosomes, which influences transcription of regulatory gene sequences in *trans* (Duncan, 2002; Mellert and Truman). In our case we would thereby either silence pMP8, leading to a false positive result in our screen, or activate the candidate overlapping VT, which we indented to silence. To prevent this possible contrarily acting effects on our targeted neuronal cells, we chose to screen all our 450 lines additionally with a LexA expression system (Yagi et al., 2010) instead of GAL4 (meaning LexA is replaced by GAL4). VT driver lines with the downstream LexA expression system were cloned into a landing site on the second chromosome (the VT driver line for pMP8 sits on the third chromosome), making them ideal for our epistasis screen.

Qualitative analysis

The primary screen was carried out with two tester female flies with the same genotype per arena. Flies were heated up from around 25°C to around 32°C for 10 minutes (Supplementary Figure 2). We chose this temperature gradient experiment for our primary screen, as it allows screening a VT line at different temperatures while looking at its behavioral output. This allows preliminary conclusions about the strength of the phenotype observed. Upon thermal activation of pMP8 flies started to show the following phenotype, resulting not only in a pure following behavior, moreover circling events could be observed. This phenotype can still be seen and scored as following, as it is only caused by the fact that both flies try to follow each other. Nevertheless this circling behavior is not detected by Matebook, a program written in our lab to automatically quantify fly behavior data (Christian Machacek, Christian Schusterreiter and Barry Dickson, unpublished). For this reason we decided to score each VT line manually for the following phenotype.

Positive hits in our primary screen, which showed no following behavior, were selected upon manual, qualitative scoring. This scoring was based on the number of flies per genotype, which showed no following phenotype at less than 30°C, around 30°C and around 32°C. To make this scoring method more objective, two criteria, based on the following phenotype, had to be fulfilled. Upon thermal activation of pMP8 flies orient towards the other fly and follow the target fly even if it changes the movement direction. Only VT-lines that did not show this orientation and persistent following were scored as positive. Moreover these lines were retested two more times to confirm the result.

We scored 12 lines as positives by this qualitative analysis approach (Figure 2.1 and 2.2). The majority of these lines showed no following behavior upon pMP8 activation and simultaneous silencing of overlapping neurons labeled by the VT driver line. With some lines orientation and following at higher temperatures could be observed, which might indicate that neuronal silencing can be overcome by strong thermal activation of the putative upstream neuron pMP8. Despite this fact that it is possible to overcome the silencing, all lines show suppression of the following phenotype when compared to the positive control. The positive control is clearly showing following behavior throughout the temperature gradient (Figure 2.1 and 2.2). We hypothesized that an output neuron responsible for following behavior is common in both sexes and that we might be able to find such a neuron with our screen. Behavioral data from other lab members shows that silencing neurons labeled by VT2041, VT50242, VT21421 in male flies, causes following and orientation defects. This suggests that we are indeed identifying neurons common to both sexes that have a role in following behavior. To have a more precise measurement of the differences between positive control and our hits, we modified our assay to be able to quantify this suppression with Matebook.

driver	UAS-Kir2.1 ; VT8469Hap1p65,HBS-TrpA1		
	<30°C	~30°C	~32°C
	n following/n total	n following/n total	n following/n total
CS	70/70	70/70	70/70
VT2041-Gal4	0/132	2/132	8/132
VT21421-Gal4	0/104	2/104	6/104
VT36876-Gal4	0/42	0/42	0/42
VT8528-Gal4	0/50	0/50	0/50
VT50242-Gal4	2/74	10/74	32/74
VT48352-Gal4	24/134	68/134	114/134
VT63546-Gal4	14/58	36/58	56/58
VT42866-Gal4	8/76	42/76	60/76
VT26732-Gal4	6/12	8/12	12/12

driver	LexAop-TNTE ; VT8469Hap1p65,HBS-TrpA1		
	<30°C	~30°C	~32°C
	n following/n total	n following/n total	n following/n total
CS	70/70	70/70	70/70
VT8172-LexA	0/78	0/78	0/78
VT22104-LexA	0/44	0/44	0/44
VT12412-LexA	0/50	0/50	0/50

Figure 2.1. Positive VT-Gal4 lines.

Qualitative scoring of Gal4 lines positive for the absence of following behavior. Number of flies was annotated that showed following phenotype.

Figure 2.2. Positive VT-LexA lines.

Qualitative scoring of LexA lines positive for the absence of following behavior. Number of flies was annotated that showed following phenotype.

Quantification

For quantification of our primary positives we choose to test them at a constant temperature of 30°C for 10 minutes. Instead of testing two female flies with the same genotype per arena, we modified the assay to testing only one female fly per arena with a white-eyed Canton S male (w⁻ CS). This gave us the advantage to track the following phenotype with Matebook, as the male fly will run away from the female fly as it tries to follow it. This pure following is well detected by the program and can be used to compare following indexes of control versus testes lines. To further ensure that the program is able to quantify this behavior we analyzed a fraction of the positive control and one hit manually (Figure 2.3). Both quantifications show a robust detection of the following suppression phenotype.

Compared to the positive controls in Figure 2.4 all VT lines show strong and robust suppression of the following phenotype when silenced. Although some variability can be observed, as presented in the box-whisker plot, differences are highly significant. A potential argument against this result might be that following is not detected because the flies are not moving during the assay. This argument can be disproved by looking at the average fly locomotion speed during the behavioral assay (Figure 2.5). Except 4 VT-lines that have a reduced average speed, all other tested lines were moving normally when compared to control levels, validating the previously detected results.

To further show that the suppression is due to silencing, we performed another control experiment. Here we activated pMP8 by dTrpA1, drove again expression of Gal4 in the candidate overlapping VT lines, but did not silence them as we didn't include a neuronal silencer – Kir2.1 or TNTe (Figure 2.6). Leaving out specifically the silencing in these VT lines caused them to come back to control levels of following behavior. This shows that the suppression of the following phenotype (Figure 2.4) is specific to silencing of overlapping neuronal cells. Also as expected flies have normal average speed levels compared to our positive control (Supplementary Figure 3).

following manual quantification

p < 0.0001
unpaired student's t-test
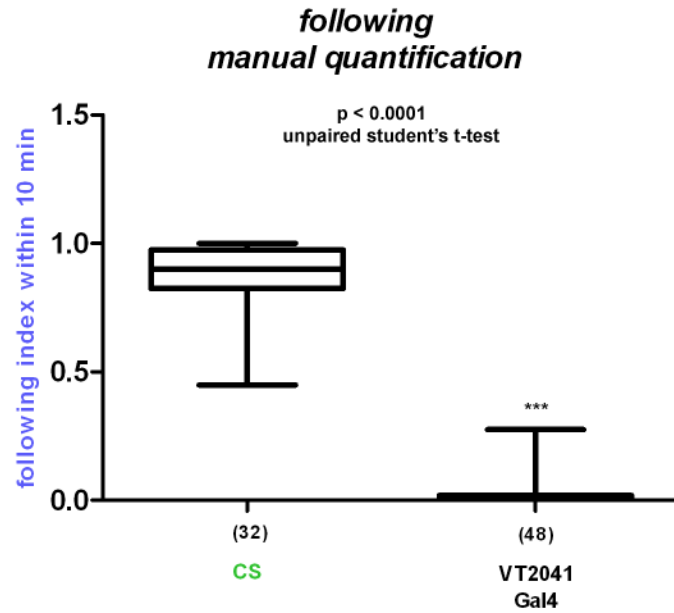
| VT8469Hap1p65,HBS-dTrpA1 | + | + |
| UAS-Kir2.1 | + | + |

Figure 2.3. Manual quantification of the positive control and one VT line showing no following behavior.

Fly behavior was scored for following phenotype every 10 seconds and averaged for every fly over 10 minutes. Statistical analysis was performed using Graphpad Prism 5 (unpaired student's t-test, p < 0.0001, n [positive control] = 32, n [VT2041-Gal4] = 48).
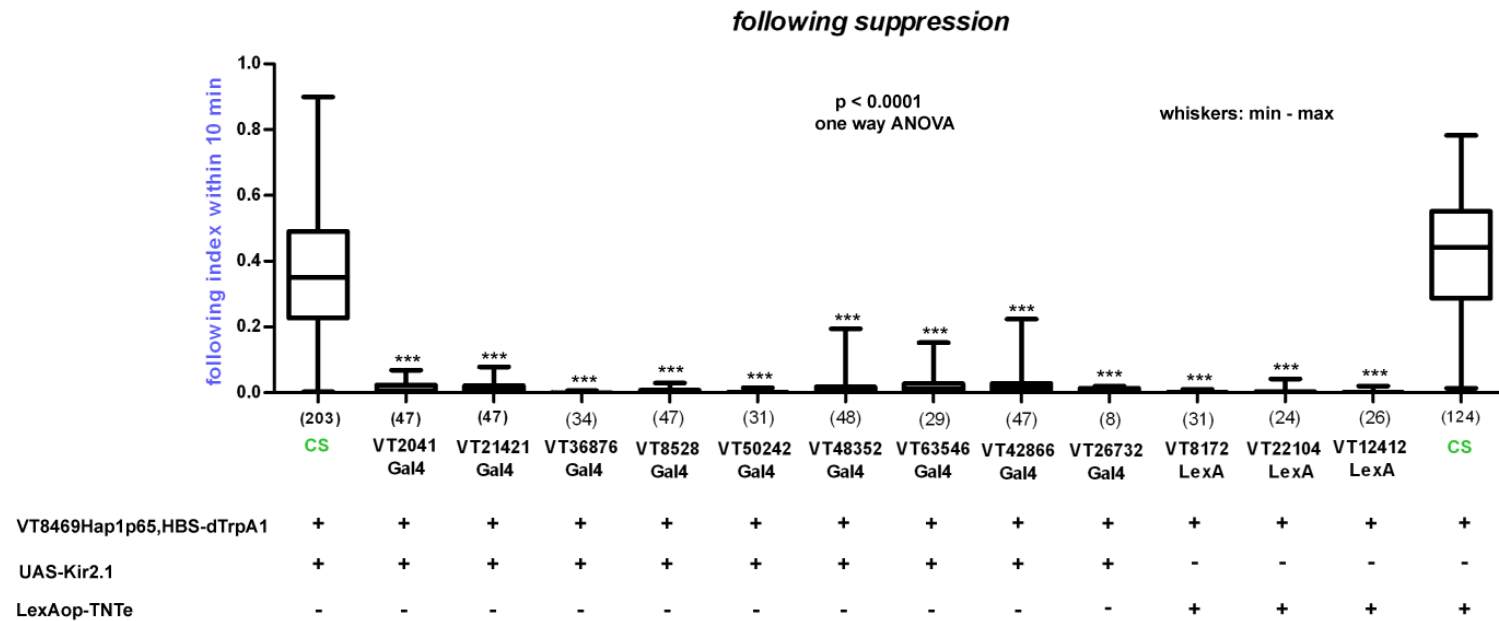
Figure 2.4. Silencing suppresses following behavior.

Following behavior was quantified using automated tracking of fly behavior by Matebook v2011. Numbers in parentheses represent number of flies tested. Statistical analysis was performed using Graphpad Prism 5 (one way ANOVA, p < 0.0001, whiskers represent minimum and maximum of following behavior detected).
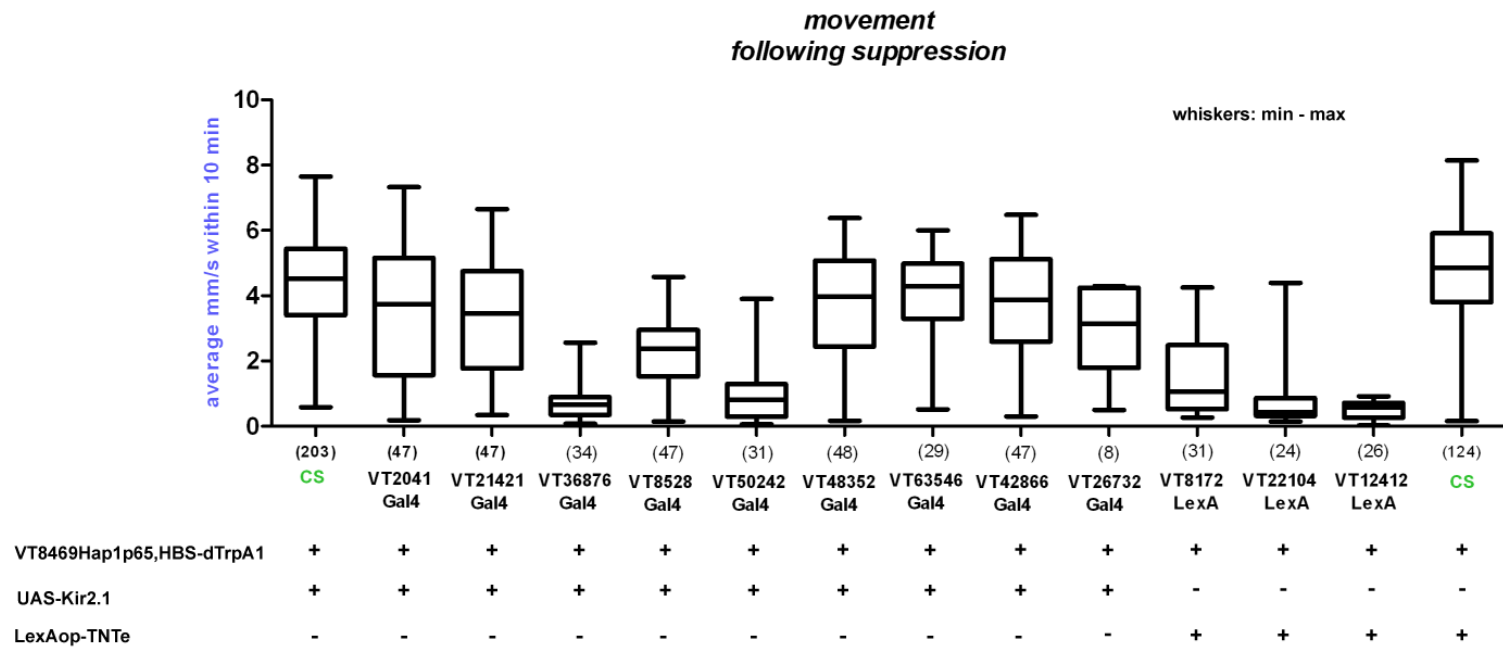
Figure 2.5. Silencing does not affect locomotion.

Fly locomotion was quantified using automated tracking of fly behavior by Matebook v2011.Numbers in parentheses represent number of flies tested. Statistical analysis was performed using Graphpad Prism 5 (one way ANOVA, p < 0.0001, whiskers represent minimum and maximum of following behavior detected).

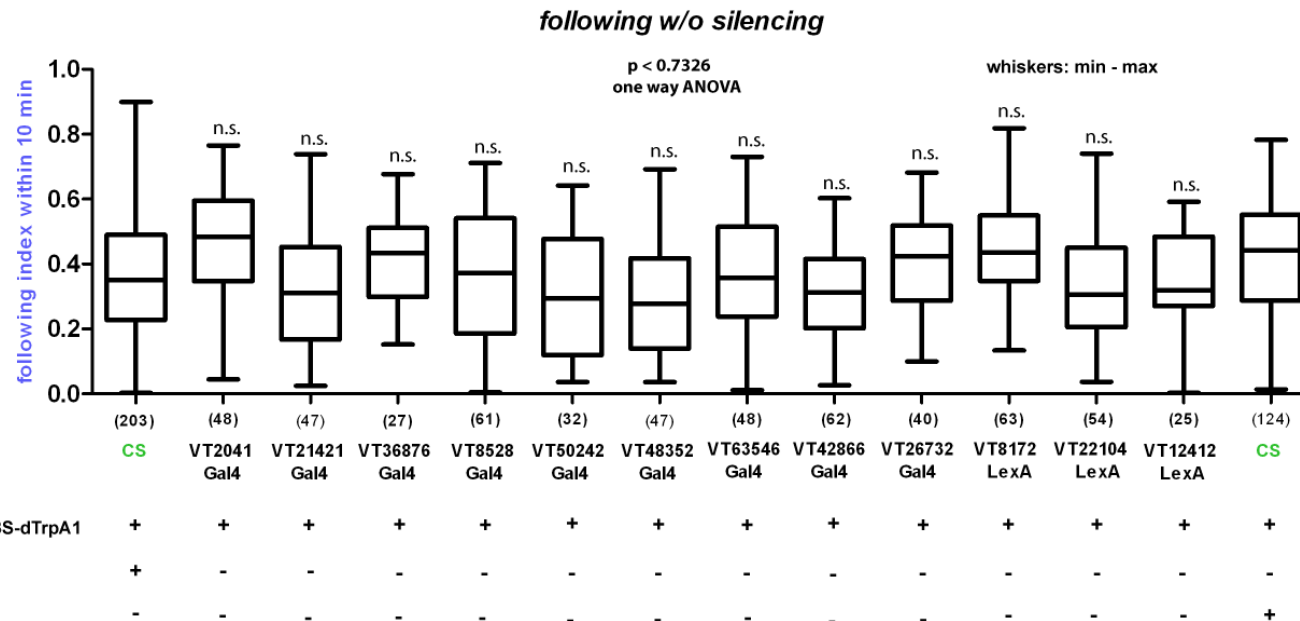**Figure 2.6.** Silencing specifically affects following phenotype.

Following behavior was quantified using automated tracking of fly behavior by Matebook v2011. Numbers in parentheses represent number of flies tested. Statistical analysis was performed using Graphpad Prism 5 (one way ANOVA, $p < 0.0001$, whiskers represent minimum and maximum of following behavior detected).
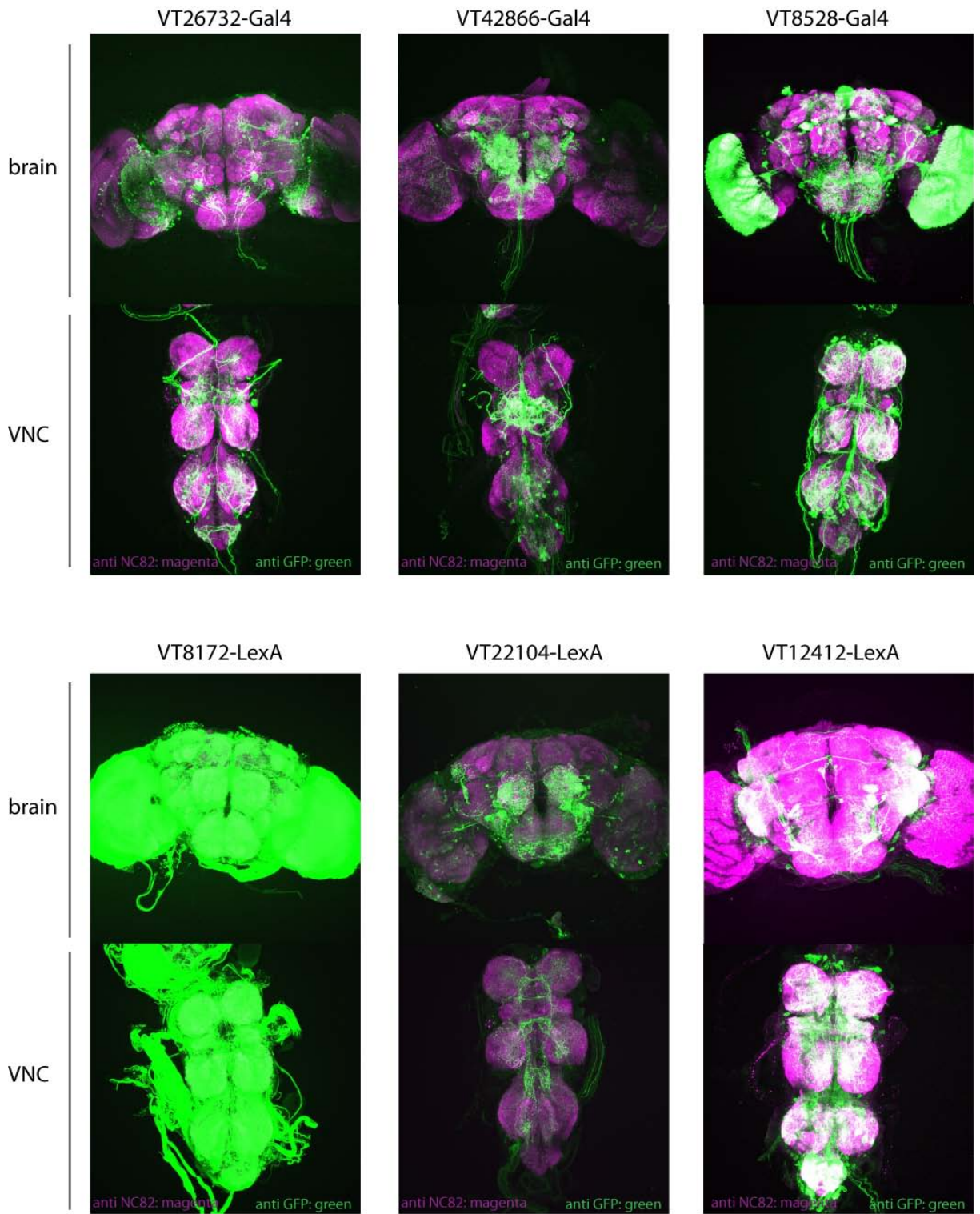
Expression pattern of positive VT lines

We further looked at the expression of our positive VT-lines to gain insight into involved neurons. Expression in the brain and ventral nerve cord of the fly could be observed in all positives lines (Figure 2.7 and 2.8). Expression varies from sparse .g. VT50242-GAL4 (Figure 2.7) to very broad e.g. VT8172-LexA (Figure 2.8). Looking at labeled neurons reveals that our epistasis screen does not exclusively identify downstream neurons. Expression can also be observed in many parts of the fly's brain. Positive results in our screen for those lines indicate that in general any neuronal subpopulation upstream, in parallel or downstream (in respect to signal processing) that interferes with the behavioral phenotype in the assay contributes to the result. This conclusion leads to one important aspect when commenting on the relevance of our results and identifying a responsible neuron. Our hits label more than one neuronal population in the fly's brain and ventral nerve cord that cause the observed phenotype and therefore it is not possible to say which neuron is our putative output neuron for pMP8. Further genetic refinement of the neuronal labeling has to be done in order to test which neuron exactly is involved in following behavior.
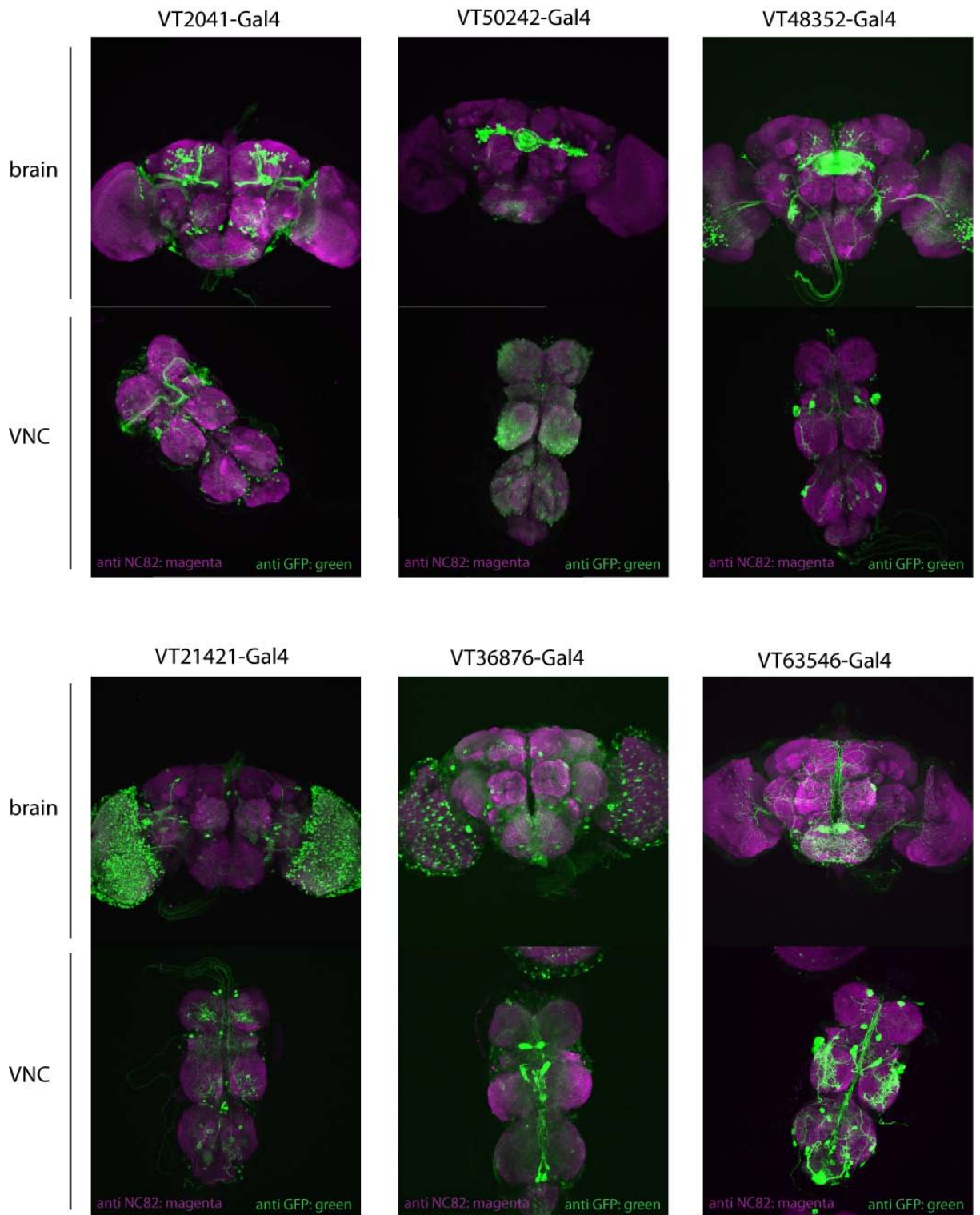
Figure 2.7. Expression pattern of positive VT lines.

Expression pattern of VT lines that do not show the following phenotype upon neuronal silencing.

Figure 2.8. Expression pattern of positive VT lines.

Expression pattern of VT lines that do not show the following phenotype upon neuronal silencing.

Discussion

In our screen we found 12 VT lines that showed no following behavior upon pMP8 activation and simultaneous silencing of overlapping neuronal cluster. Quantification was done at constant temperature (30°C) and females were paired with a w⁻ Canton S male fly in the behavior arena. All lines show strong suppression of the following behavior (Figure 2.4). In contrast qualitative scoring at 30°C our primary screen shows that some following behavior can be observed with VT48352, VT63546, VT42866, and VT26732 (Figure 2.1 and 2.2). Two arguments can explain this obvious discrepancy. One reason for the observation of following at 30°C in the primary screen can be inaccurate temperature control and measurement. This is possible because temperature was controlled manually and controlled by a thermometer electrode measuring the temperature inside the behavior arena. Inaccurate placing (too close to arena opening on the bottom where cool air circulated) of this electrode might have caused lower measurements of temperature as actually present. As neuronal activation increases with temperature (Pulver et al., 2009) this might explain why following behavior could be observed. Another reason that explains this finding is the difference between the assays for our primary screen and the quantification. In our primary screen we aspirated two female tester flies into one behavior arena whereas for quantification we used one tester female together with a w⁻ Canton S male as a target for following. This was necessary to be able to track the following behavior automatically. We speculate that when two female tester flies are placed together into a behavior arena, following behavior is initiated more easily as both files will try to follow each other.

The decision to use the female nervous system as a model for our experiments instead of the male has several advantages. First we can specifically target pMP8, as the VT line labeling pMP8 is spare. In contrast all lines for P1 show a broad expression pattern, which make it difficult to look for specific output. Male flies initiate courtship behavior upon input of several signals from their environment and the female fly (Ewing, 1983). Their courtship behavior consists out of several actions like orientation, tapping and singing, during which they are following the female fly (Hall, 1994; Sokolowski, 2001). As we intended to perform neuronal silencing experiments to look for a neuron responsible for following behavior, silencing candidate neurons in males potentially interferes with any other courtship action and thus lead to reduced following. By activation pMP8 in female flies we exclusively observe following, which increases the chances of finding a neuron specifically transmitting signals for following behavior.

This specific design of our experiments should help us to identify a neuronal population, which we can then integrate in a circuit diagram for courtship behavior in Drosophila.

Another interesting finding in our results can be made, when positive lines are compared to behavioral data from other lab members. VT2041, VT50242 and VT21421 show almost no following phenotype upon pMP8 activation and neuronal silencing in female flies. Silencing experiments of neurons labeled by these VT-lines in male flies also leads to following deficiencies in courtship assays. Both results indicate that our hypothesis and experimental design to identify a neuron that is common to both sexes and responsible for following go into the right direction.

Sexual dimorphisms in the fly brain have been detected in a set of neurons that express *fruitless* (Cachero et al., 2010). As fruitless is an important regulator of sexual behavior (Demir and Dickson, 2005) these dimorphisms are thought to contribute to distinct sexual behavioral patterns. This raises the question if neuronal circuits underlying such patterns are separated or shared and differentially activated between the sexes. Similar behavioral patterns can be observed in both males and females. For example following behavior is seen in females (Cook, 1981) and males (Hall, 1994). Also females flies can be forced to sing (Clyne and Miesenbock, 2008) as males do during courtship. Little is known about to what extend neuronal circuits are shared between both sexes, but the capability of e.g. female flies to show male specific behavior suggests that some circuit components are indeed shared. In order to control sex specific behavior these circuit components have to be differentially activated. This differential activation could be facilitated by sex specific key integration sites for sensory processing within the fly's brain. Potential neuronal candidates for these important integration sites could be seen in P1 (Kohatsu et al., 2011; von Philipsborn et al., 2011) and pMP8 neurons (Yang Wu and Barry Dickson, unpublished), as activation of both neurons induces behavioral patterns that can be observed during courtship.

Experimental procedures

Fly stocks

Flies were reared at 22°C and 60% humidity in a 12/12 hour light circle on yeast enriched standard cornmeal agar medium. VT8469Hap1p65, HBS-dTrpA1, UAS-Kir2.1, LexAop-TNTe were created in our lab. LexAop-myrGFP was obtained from B.Pfeiffer and G.Rubin (Janelia Farm). UAS-mCD8GFP flies were used for visualizing expression patterns of positive lines (Lee and Luo, 1999). A Canton S laboratory strain in w⁻ background was used for target males during quantification.

Epistasis experiments

Flies were collected shortly after eclosion and aged for 9-12 days at 22°C on yeast enriched, modified standard agar medium. For the temperature gradient behavior assay around 30 flies were tested per video by placing two tester females together into a 10 mm behavior arena. Starting temperature, time point of 30°C and time when 32°C were reached was controlled by a thermometer measuring the temperature inside the behavior arena and annotated manually for each video. Constant temperature behavioral assays were done similar, except one female tester fly was placed together with a w⁻ Canton S male into a 10 mm behavior arena. Flies were heated up by running current through a glass plate covering the behavior chambers. Current flow was controlled manually (temperature gradient) or automatically by a feedback loop connected to the power supply (constant temperature).

Video analysis and statistics

Recorded videos for temperature gradient experiments were scored manually by detecting the number of flies showing following behavior below 30°C, around 30°C and around 32°C. Constant temperature experiments were scored by Matebook v2011, an automated tracking software created in the lab (Schusterreiter, Machacek and Dickson, unpublished). Standard fly tracking settings were used except for:

Arena Detection	Events	
border size: 1,00 mm	copulation persistence:	600,00 seconds
interior: bright	following:	min distance: 0.1 mm
		max angle: 90°
		min speed: 1 mm/s

Statistical analysis of our data was performed using Graphpad Prims 5. One way ANOVA test and unpaired student's t-test were used to calculate significance values.

Immunohistochemistry and image analysis

Flies were aged for 5-7 days after eclosion at 25°C, 60% humidity on powerfood before dissection of their brain and VNC. Staining procedure was done as described in chapter 2. Antibodies used were:

rabbit anti GFP (1:6000, Torri Pines)

mouse monoclonal NC82 (1:20, Hybridoma Bank)

secondary Alexa 488, 647 (all 1:1000, Invitrogen).

Confocal stacks were obtained with Zeiss LSM700 with a Multi Immersion NeoFluor 25x/0.8 objective on a multislide holder. ImageJ (NIH) was used to analyze stacks and z-projections.

A detailed, stepwise description of the staining protocol and mounting procedure can be found in the supplement.

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Chapter 3: Testing connectivity between SPAbG and pMP8 neurons

Introduction

pMP8 was first discovered in an activation screen utilizing VT-Gal4 lines. Multiple VT lines showing the following phenotype labeled pMP8. Silencing experiments of these VT lines in a receptivity assay resulted in decreased receptivity of female flies. This result lead to the hypothesis that pMP8 is involved in controlling female receptivity. In our experiments we hypothesized that pMP8 and SPAbG, a neuron responsible for the mating switch in Drosophila (Kai Feng, Mark Palfreman, Martin Haesemeyer and Barry Dickson, unpublished) are connected and form a circuit for female receptivity. Here we used a VT-Hap1 line that labels pMP8 and fewer additional neurons in the female brain.

Results

Double labeling of SPAbG and pMP8 neurons in the fly brain

SPAbG neuronal cell bodies sit in the abdominal ganglion of the ventral nerve cord in Drosophila nervous system (Figure 3.1a). These neurons send their projections to the dorsal part of the brain and arbor in both hemispheres of the fly brain. pMP8 neurons have their cell bodies in the ventrolmedial protocerebrum and they send their projections to dorsal and central part of the brain, with prominent ring structures in the dorsomedial protocerebrum (Figure 3.1b). Expression seen in the VNC of the VT8469Hap1p65 line is ectopic and not related to pMP8 neurons in the brain. Segmented images of both SPAbG and pMP8 give a more detailed view of their anatomical structure, making it possible to have a look at overlapping regions. Both neurons have arborizations in the dorsal part of the brain, which create the possibility for synaptic connections (Figure 3.2). Comparing segmentations of both neurons is not enough to argue about connectivity, visualizing both neurons in the same brain and the look for overlap is a better way for a detailed analysis of the anatomical structure.

In order to get better insight how the anatomical structure of both neurons look like in the same brain, we performed a double labeling experiment. Co-labeling in the same brain and scanning a z-stack, allowed us to track single projections and arbors of both neurons that can be overlaid and compared for overlapping regions. Analysis of the z-stack showed that SPAbG and pMP8 do not overlap for most of their arborizations. Surprisingly even if they innervate the same anatomical region, they run along close next to each other. Nevertheless overlapping regions in the dorsal part of the brain could be found as expected. The overlap found was punctuated, suggesting only small overlapping regions (Figure 3.3). This result is very informative as from the neuronal segmentation images we expected a bigger area of overlap. As a next step we used GRASP to see if the overlapping regions of SPAbG and pMP8 are close enough together to allow a reconstituted GFP signal, potentially indicate a synaptic connection between these two neurons.

UAS-mCD8GFP/VT50405AD ; VT45154DBD
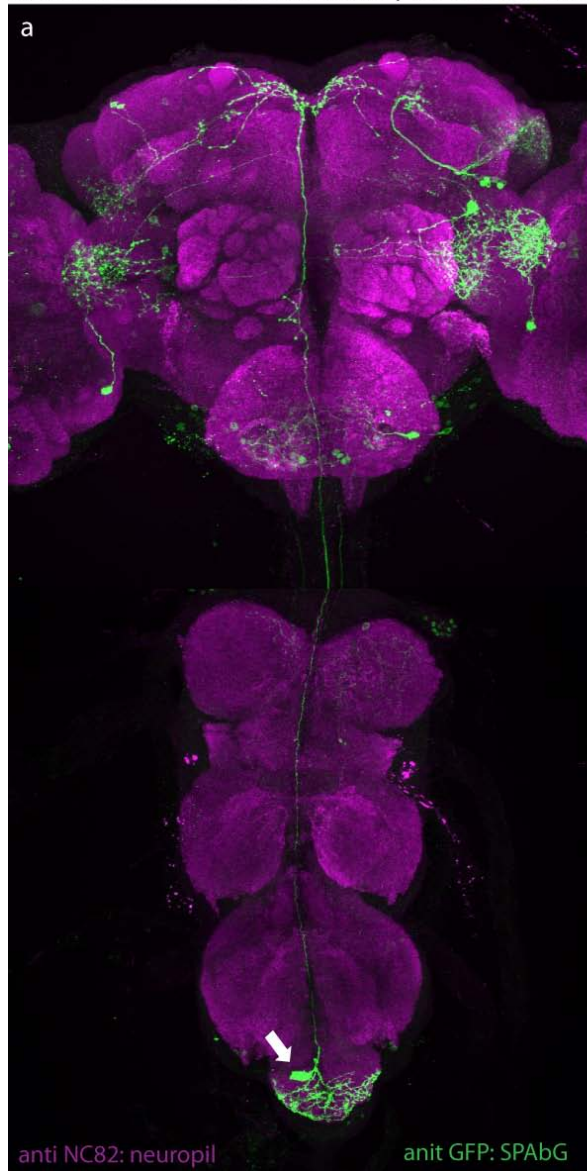
HBS-Gal4/UAS-GFP ; VT8469Hap1p65

a

b

anti NC82: neuropil   anit GFP: SPAbG

anti NC82: neuropil   anit GFP: pMP8

Figure 3.1 Expression of VT lines labeling SPAbG and pMP8.

(a) GFP expression of VT50405p65ADZp together with VT45154ZpGAL4DBD as a split Gal4 driver that labels SPAbG neurons. Cell body position in the abdominal ganglion is indicated by white arrow (image kindly obtained from Kai Feng)

(b) GFP expression of a VT8469Hap1p65 amplified with HBS-GAL4 to label pMP8 neurons. Cell body position is indicated by white arrows (image kindly obtained from Yang Wu).

SPAbG segmentation
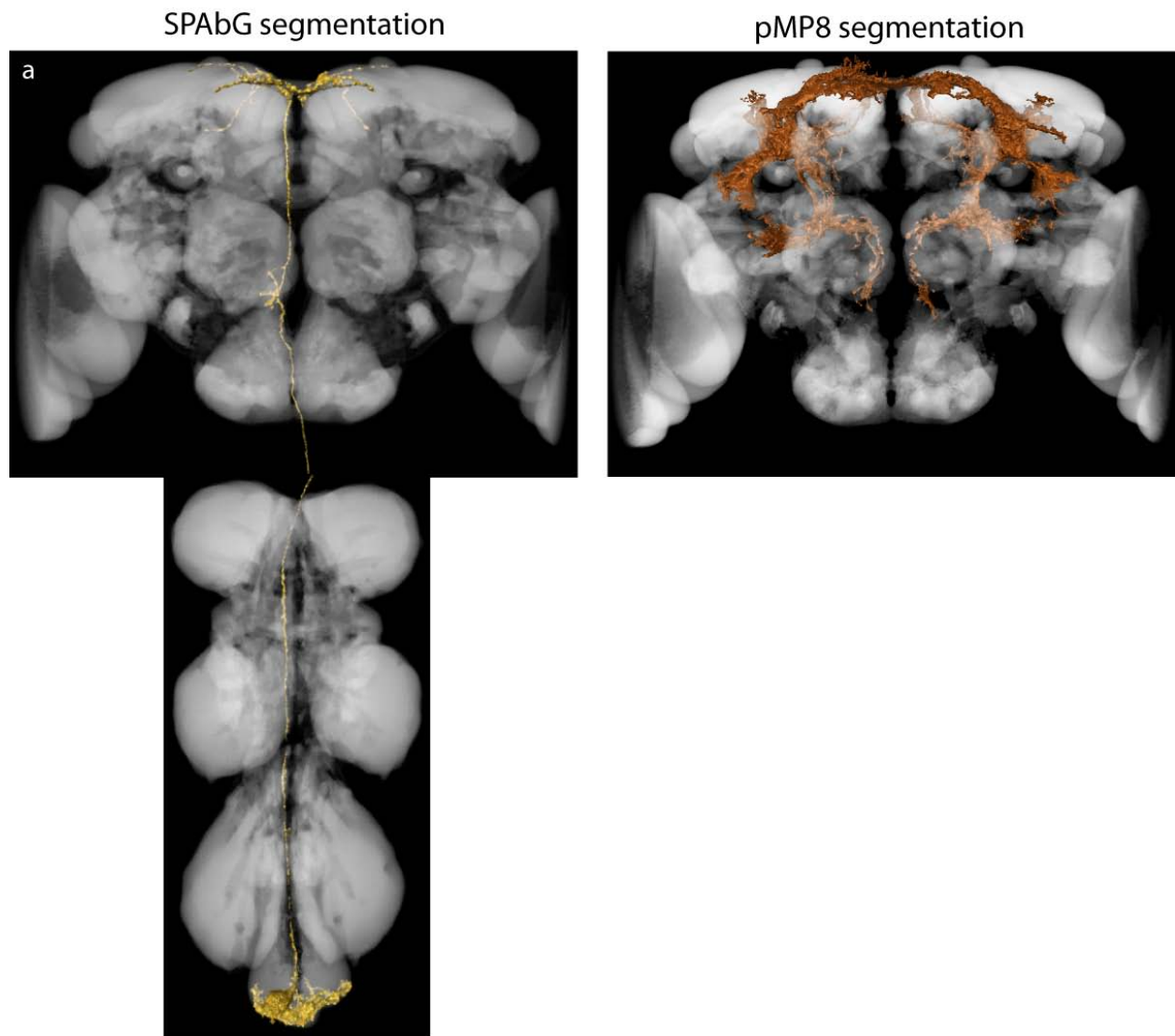
pMP8 segmentation

Figure 3.2 Segmentations of SPAbG and pMP8.

Segmented images of SPAbG (a) and pMP8 (b) show possible overlapping regions in the dorsal part of the brain. No VNC segmentation for pMP8 as the neuronal cluster is exclusively located in the central brain (images kindly obtained from Kai Feng and Yang Wu).

UAS-mCD8GFP/VT50405AD ; VT8469-Hap1,HBS-mCherry/VT45154DBD
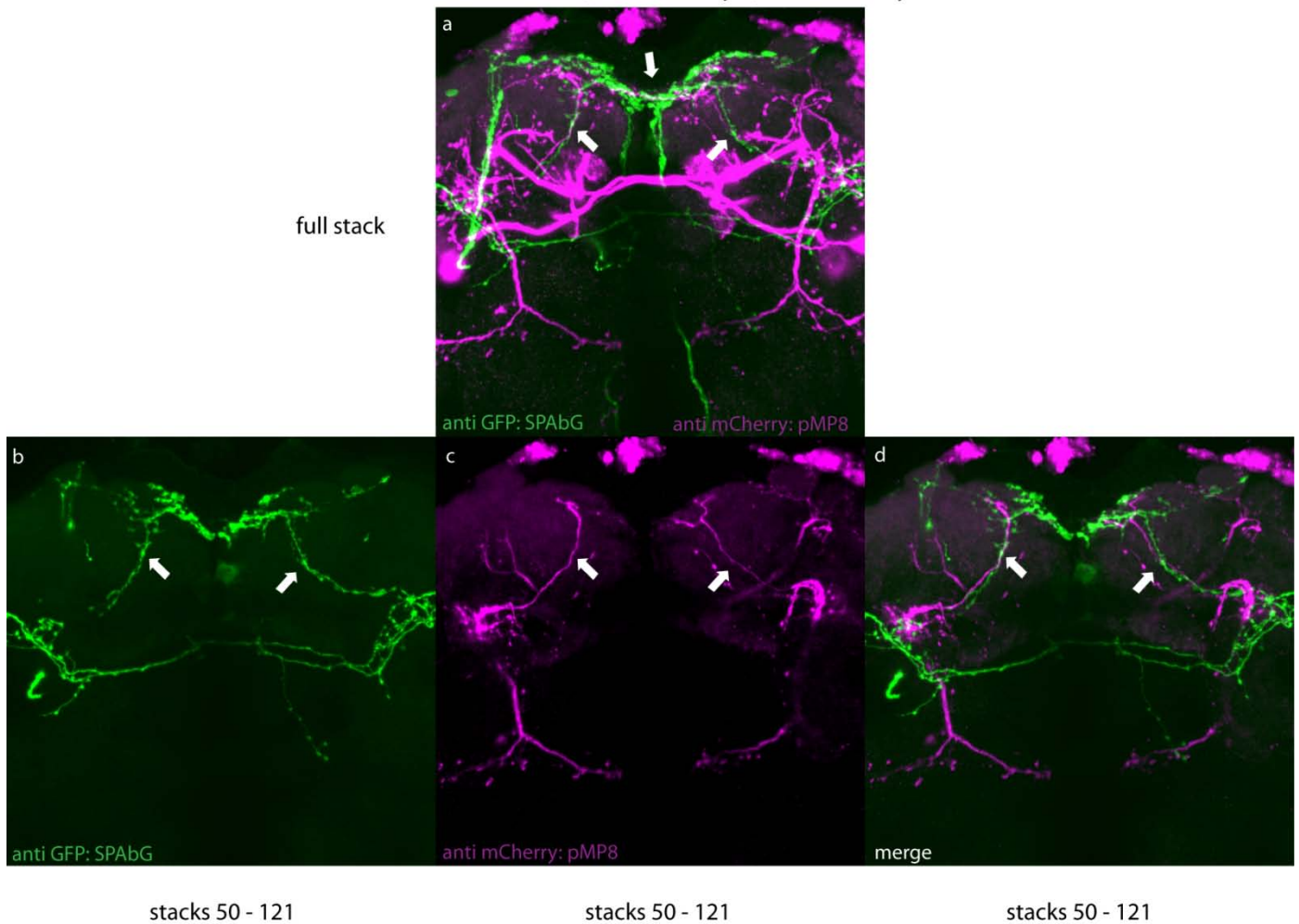
Figure 3.3 Double labeling of SPAbG and pMP8 neurons.

(a) Full z-stack (180 slices) of the dorsal part of the brain showing overlapping regions indicated by white arrows.

(b) Partial stack (slices 50-121) in which arbors of SPAbG can be seen that innervate a more ventral part of the dorsal brain.

(c) Partial stack (slices 50-121) in which arbors from the cell bodies of pMP8 project to the dorsal part of the brain.

(d) Merged image from (b) and (c) showing overlapping areas of both neurons.

Using GRASP to visualize potential connectivity

Another technique to see if two neurons form connections is GRASP (GFP reconstitution across synaptic partner) (Feinberg et al., 2008). This method uses the advantage that the GFP construct can be split into two non active halves and attached to an outer-membrane anchor. Each of this half's is expressed in a different neuronal cell, causing a reconstitute GFP signal only if those two cells are forming connections, in case of neurons synapses, or are very close to each other. In order to ask if SPAbG and pMP8 are connected this method can be used to see if these neurons are forming synaptic connections.

As both primary antibodies for mCherry and GASP1-10 were obtained from rabbits, we stained 2 individual brains for either SPAbG or pMP8 and looked for overlap with the reconstituted GFP signal (Figure 3.4). The upper panel shows stainings for SPAbG, the lower for pMP8. The split Gal4 driver for SPAbG is very weak, therefore only a punctuated structure of an arbor projecting down from the dorsal part of the brain can be seen. pMP8 arborizations are clearly visible in the lower panel. Both images of SPAbG and pMP8 show no clear signal for the reconstituted GFP. Together with the double labeling finding this experiment suggests that there are no connections between these two neurons. Although this result is not a final proof for SPAbG and pMP8 being not connected, it lowers the chances of pMP8 being involved in the direct signal processing coming from SPAbG.

HBS-GFP11,UAS-GFP1-10/VT50405ADZ ; VT8469Hap1,HBS-mCherry/VT45154DBD
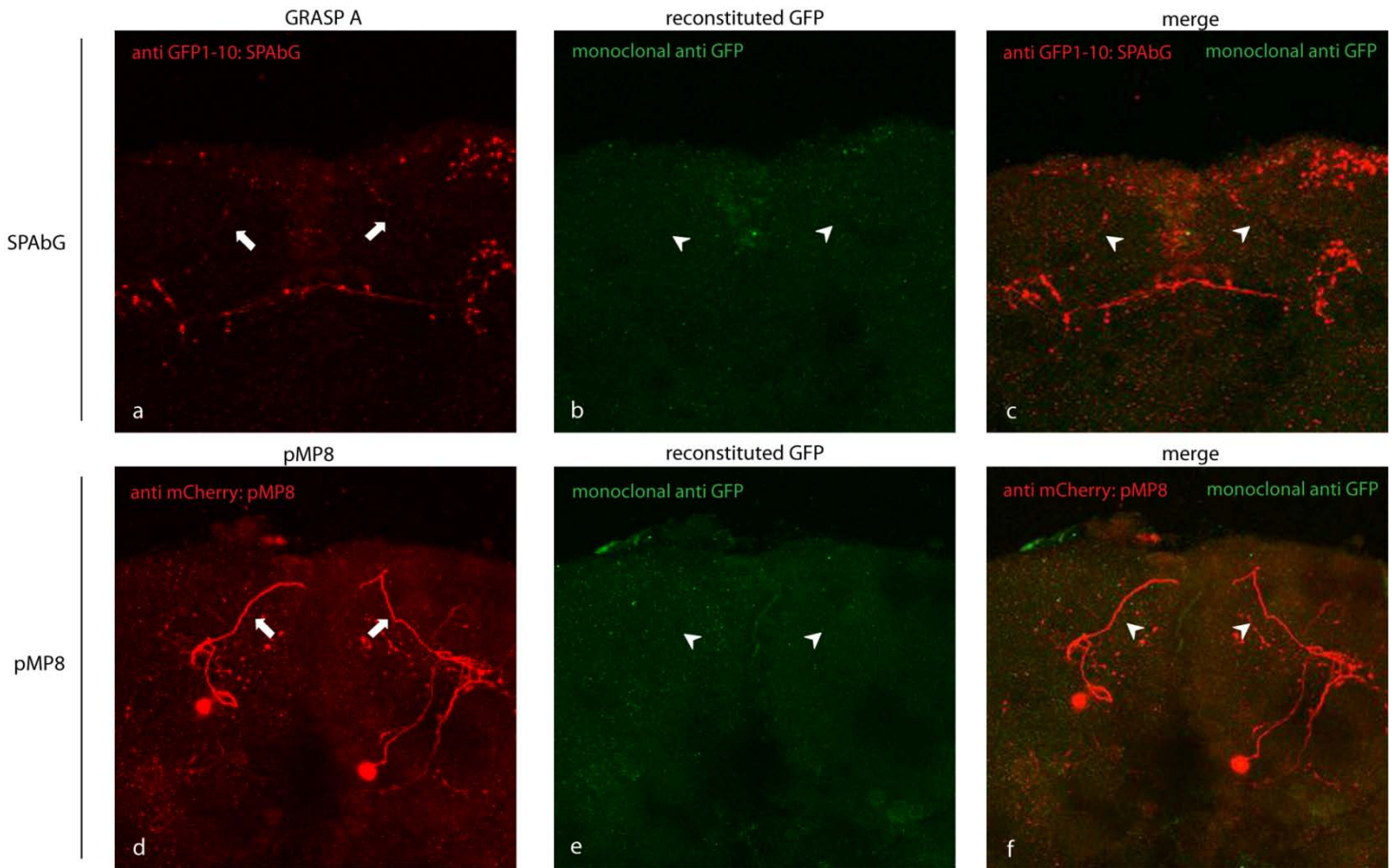
Figure 3.4 GRASP experiment of pMP8 and SPAbG

(a) SPAbG projections visualized with antibody against GRASP1-10 (white arrows).

(b) No reconstituted GFP signal can be detected in the area of interest (white arrowheads).

(c) Merged image of (right) and (middle).

(d) pMP8 neurons visualized with antibody against mCherry.

(e) No reconstituted GFP signal can be detected in the area of interest (white arrowheads).

(f) Merged image of (right) and (middle).

Discussion

It is important to note that silencing experiments in a receptivity assay for the VT-Hap1 labeling pMP8 were carried out in parallel and after our experiments described here. Support for our results comes from these silencing experiments. Silencing pMP8 neurons in female flies did not show any phenotype in a receptivity assay (Yang Wu and Barry Dickson, unpublished). In contrast when SPAbG neurons are silenced, females become less receptive (Kai Feng, Mark Palfreyman, Martin Haesemeyer and Barry Dickson, unpublished). If SPAbG directly signals to pMP8, silencing of pMP8 should result in a similar reduction of receptivity.

Our results show that pMP8 and SPAbG mostly run next to each other (Figure 3.3) and it is unlikely that they form direct synaptic connections (Figure 3.4). A potential argument against the GRASP experiment result is a missing GFP11 construct in our transgenic fly and therefore we do not see any reconstituted GFP signal. Transgenic flies were created by recombining GFP1-10 and GFP11 under different transcription factor binding sites on the second chromosome. This recombination was necessary to use the split GAL4 hemi-drivers that label specifically SPAbG in our experiments. When recombining the two GFP constructs PCR verified both half's to be present in the fly's genome. Nevertheless to further show that this is the case a positive control for our GRASP experiment would be beneficial. This positive control should fulfill two criteria. First we would need a VT line with a very spares expression pattern that labels exclusively one neuronal cell population and second this neurons should be known to form synaptic connection with pMP8. So far we have not identified any neuron that would fulfill these two criteria and therefore not carried out this control experiment.

Conclusion from our silencing and our experiments leave the question about the actual function of pMP8 open. Upon neuronal activation of pMP8 female flies show a following phenotype, a stereotypic behavior seen during male courtship. When looking at videos of two female flies placed together in one behavioral arena and pMP8 activation at the same time, flies often orient head to head and start extending their forelegs towards the opposite fly. This behavior can also be observed in female aggression assays (Nilsen et al., 2004). Whether or not this behavior can be indeed seen as aggression has to be verified in a new set of experiments that specifically address an aggression paradigm. This approach is one way to identify a behavioral function of pMP8 and might allow better characterization of its role within the female's nervous system.

Experimental procedures

Fly stocks

Flies were reared at 25°C and 60% humidity in a 12/12 hour light circle on standard cornmeal yeast agar medium. VT8469Hap1p65, HBS-mCherry, HBS-Gal4, HBS-GFP11, UAS-GFP1-10, UAS-GFP were created in our lab. The split GAL4 line used was made by replacing GAL4 with pZpGAL4DBD (for VT45154 in attp2 landing site) or p65ADZ (for VT50405 in attp40 landing site). UAS-mCD8GFP flies were used for double labeling (Lee and Luo, 1999).

For recombination on the second chromosome of HBS-GFP11 (19a landing site) with UAS-GFP1-10 (260b landing site) flies containing transgene were crossed to each other. Virgins containing both transgenes were then crossed to w⁻ double balancer males. From this cross single, *cyo* males were isolated and crossed to w⁻ double balancer females. Newly hatched c*yo* males were isolated from this cross and used for single fly genomic DNA preparation to afterwards verify the recombination by PCR. Single fly genomic DNA preparation was done by adding 200 μg/ml proteinkinase K to the squishing buffer (SB) and mashing each fly in 50 μl of it. The mixture was then incubated for 30 minutes at 25°C before it was heated up to 95°C to inactivate proteinkinase K. 1 μl of this digestion was used for PCR.

Squishing buffer (SB)	PCR mix	PCR program
10 mM Tris-Cl pH 8.2	35 μl dH$_2$O	1. 94°C 2:00 min
1 mM EDTA	5 μl Tag buffer	2. 94°C 0:30 min
25 mM NaCl	1 μl dNTP (10 nm)	3. 62°C 0:30 min
200 μg/ml proteinkinase K	3 μl MgCl (25 nm)	4. 72°C 0:30 min
	2 μl each primer (10 yM)	5. from step 2, 34 times
	1 μl Tag polymerase	6. 72°C 10:00
	1 μl DNA	7. 4°C forever

Primer sequences

260b LS19 (forward): **TAGGTACGGCATCTGCGTTGAGTCG**

260b LS25 (reverse): **GCCGCTGAGAAAGCCGTTAGATGAG**

19a LS20 (forward): **TGTTTGTGTACTCCCACTGGTATAGCCTTCTT**

19a LS21 (reverse): **CGTGTGCAAGTGTGCGTGTTTGTTT**

Immunohistochemistry and image analysis

Flies were aged for 5-7 days after eclosion at 25°C, 60% humidity on powerfood before dissection of their brain and VNC. Flies were anesthetized with CO_2 before transferred into PBS buffer for dissection. The tissue was fixed in 4% paraformaldehyd in PBST for 20 minutes and then washed with PBST for 20 – 25 minutes before put into blocking solution (10% normal goat serum in PBST) for at least 2 hours. Brains and VNCs were incubated in primary and secondary antibody solution for 48-72 hours with an intermediate washing step between the two solutions. Another overnight washing step at 4°C in PBST, followed by at least 4 hours of washing at room temperature was performed before mounting tissues on cover slides. Antibodies used were:

      mouse monoclonal anti reconstituted GFP (1:100, Sigma G6539; (Gordon and Scott, 2009))

      rabbit anti GFP (1:6000, Torri Pines)

      rabbit anti mCherry (1:1000, Living Colors)

      mouse monoclonal NC82 (1:20, Hybridoma Bank)

      secondary Alexa 488, 567, 647 (all 1:1000, Invitrogen).

Confocal stacks were obtained with Zeiss LSM700 with a Multi Immersion NeoFluor 25x/0.8 objective on a multislide holder. ImageJ (NIH) was used to analyze stacks and z-projections.

A detailed, stepwise description of the staining protocol and mounting procedure can be found in the supplement.

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Conclusions and outlook

We could identify VT lines labeling neuronal clusters that are potentially involved in following behavior. Nevertheless all hits label more than one neuronal subpopulation which makes it difficult to conclude which neuron is the putative output neuron. A first strategy to identify a neuron that is common in several hits is double labeling of 2 expression patterns with two different reporters like GFP (Boda et al., 2001; Lee and Luo, 1999) and mCherry (Shaner et al., 2004) driven by different expression systems and look for overlapping stained cell bodies and arborizations within the fly's nervous system. Nevertheless this will not enable us to specifically target such neuron in future experiments. Therefore refined genetic approaches have to be taken to reduce broad expression patterns and label a smaller fraction of the neurons seen in the stainings. One way would be to subtract overlapping expression patterns of a VT-GAl4 line with a VT-LexA line, by driving GAL80 with LexAop (Lee and Luo, 1999). This should lead to a sparser labeling and thereby increase specificity of our results. A second way to reduce broad expression patterns is to use an intergenetic split-GAL4 strategy, called the split Gal4 system. This method makes use of the separated DNA binding and activation domain of GAL4 that can be combined with a leucine zipper (Luan et al., 2006). When driving expression of these two halves with different enhancer tiles, a reporter signal will only be seen in overlapping expression patterns of these two enhancer tiles. With this method it is possible to reproducibly target a very small fraction of neurons in the fly's brain. We already created a matrix with our hits found in the epistasis screen, in which we first combine all GAL4 hits with either pZpGAL4DBD (DNA binding domain, DBD) or p65ADZ (activation domain, AD). These combinations will be tested with the same setup as we quantified our positive VT lines. Any reduction in following behavior will be analyzed in detail and stainings of the corresponding expression pattern will hopefully allow us to identify an output neuron for pMP8 that is responsible for following and common to both sexes. It will be interesting to see if this split-GAL4 approach has the power to refine the resolution for functional dissection of a neuronal circuit that is responsible for following behavior in Drosophila.

Future identification of such a neuron will automatically raise the question of how relevant such a finding is. Drawing a complete neuronal circuit diagram for any animal behavior and being able to explain how brains work has been a long lasting wish for scientists (Finger, 1994). Identification of circuit components is the first basic step to draw a circuit. But in order to understand how a network functions is it necessary to understand how each component transfers signals and transmits information to its

interconnected neighbors. This will allow to gain insight into how the whole network processes information to guide an animal's behavioral decision. Moreover this information flow can be changed by memory processes and neuronal modulation (Kandel, 2012; Marder, 2012). The ultimate goal is to elucidate how a human brain works and functions, and what makes *Homo sapiens* unique in its cognitive abilities. To shine light onto these hidden mechanisms it is necessary to come back to model organisms and start discovering their neuronal basis for behavior. Conclusions from such studies combined with future methods will maybe allow scientists of the next generation to come a step closer to the mystery of how brains work.

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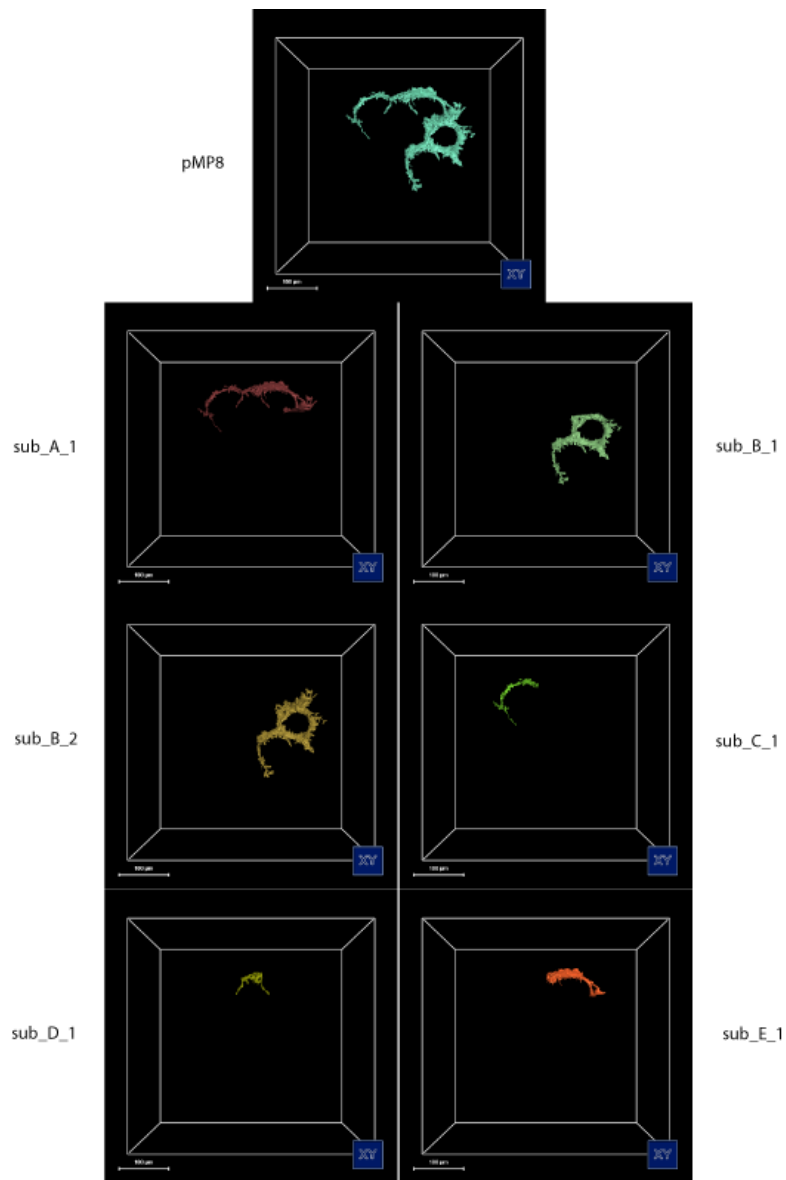
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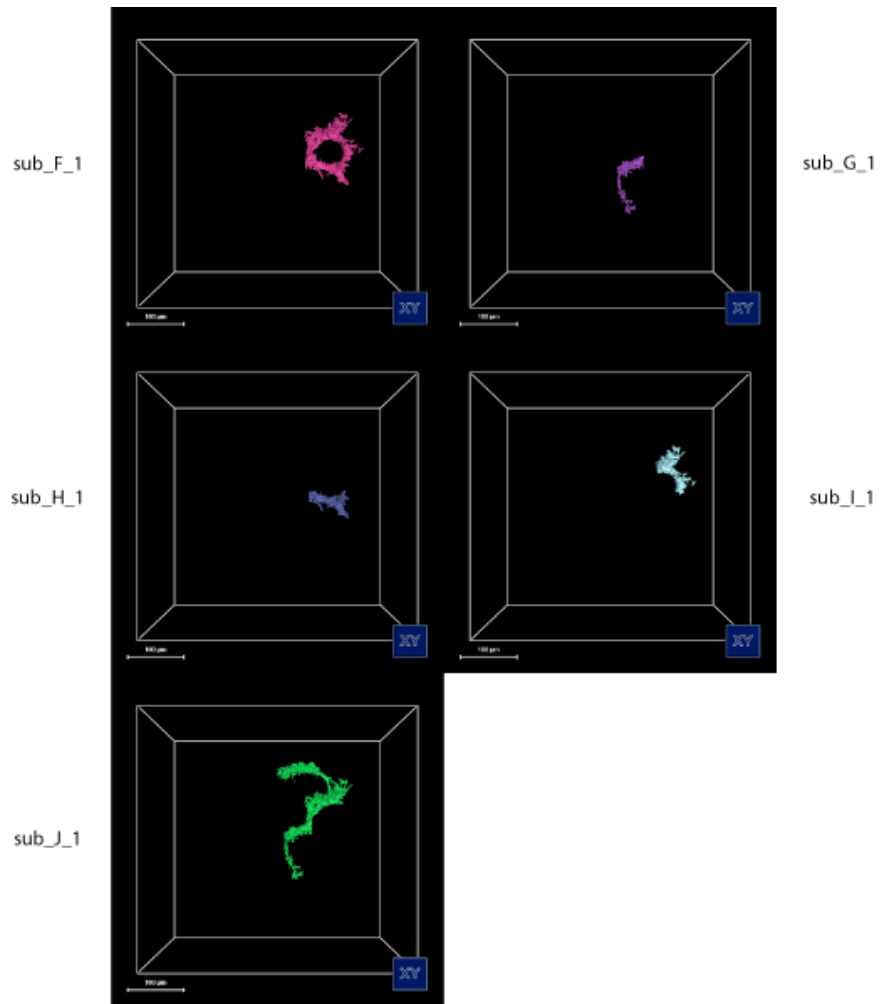
Supplement

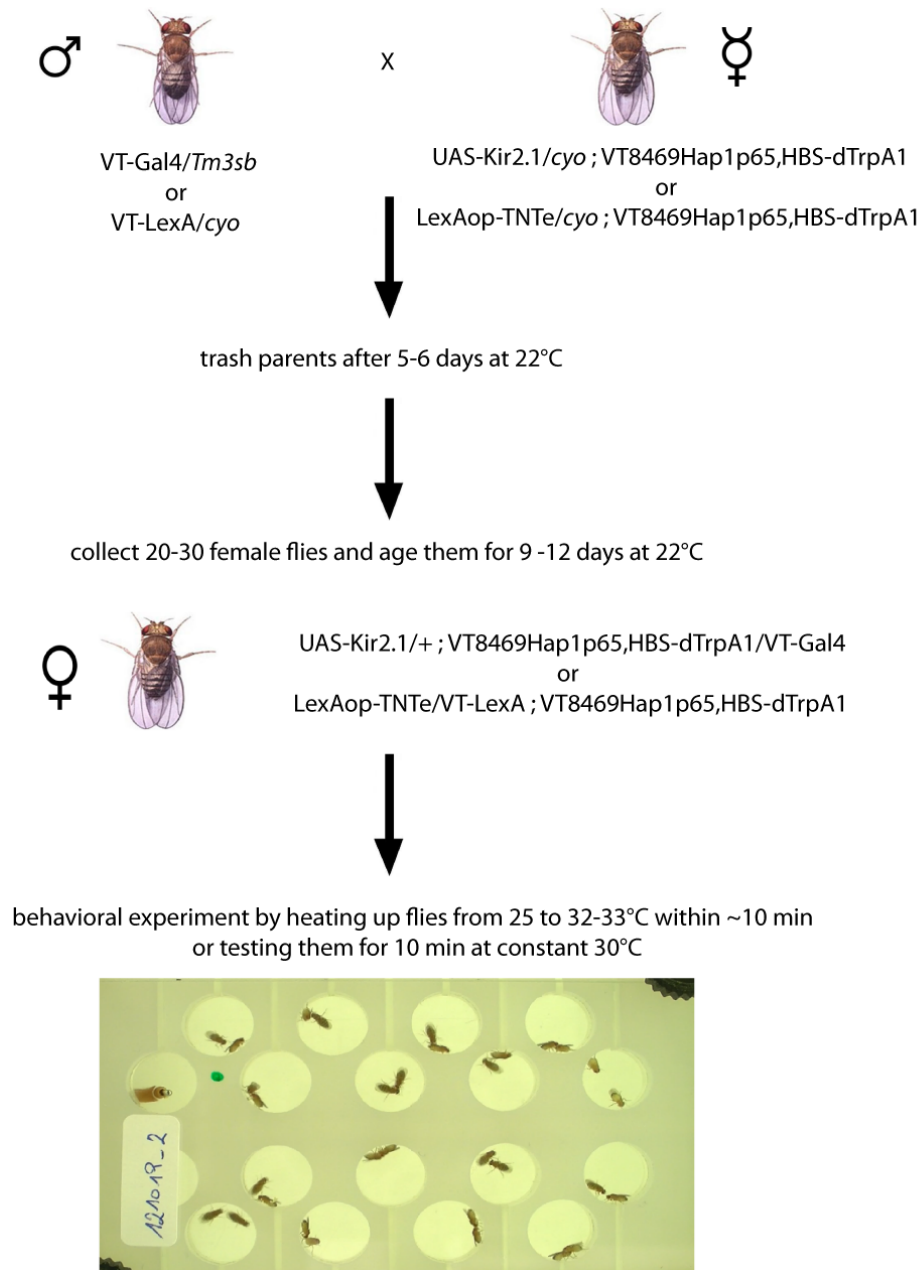## Subdivisions

Figure 1.1. Subdivision of pMP8.

Subdivision were used in the Braingaizer query for the search of overlapping neurons in the male nervous system with pMP8.

Figure 1.2. Subdivison of pMP8.

Subdivision were used in the Braingaizer query for the search of overlapping neurons in the male nervous system with pMP8.

<u>Workflow behavior</u>

Figure 2. Workflow diagram for behavior experiments.

Candidate VT lines were crossed with pMP8 activation stock that harbored a neuronal silencer on the second chromosome. Progeny was collected soon after eclosion and aged between 9 to 12 days. Flies were either tested on a temperature gradient or at constant temperature for quantification.

Locomotion (following without silencing)
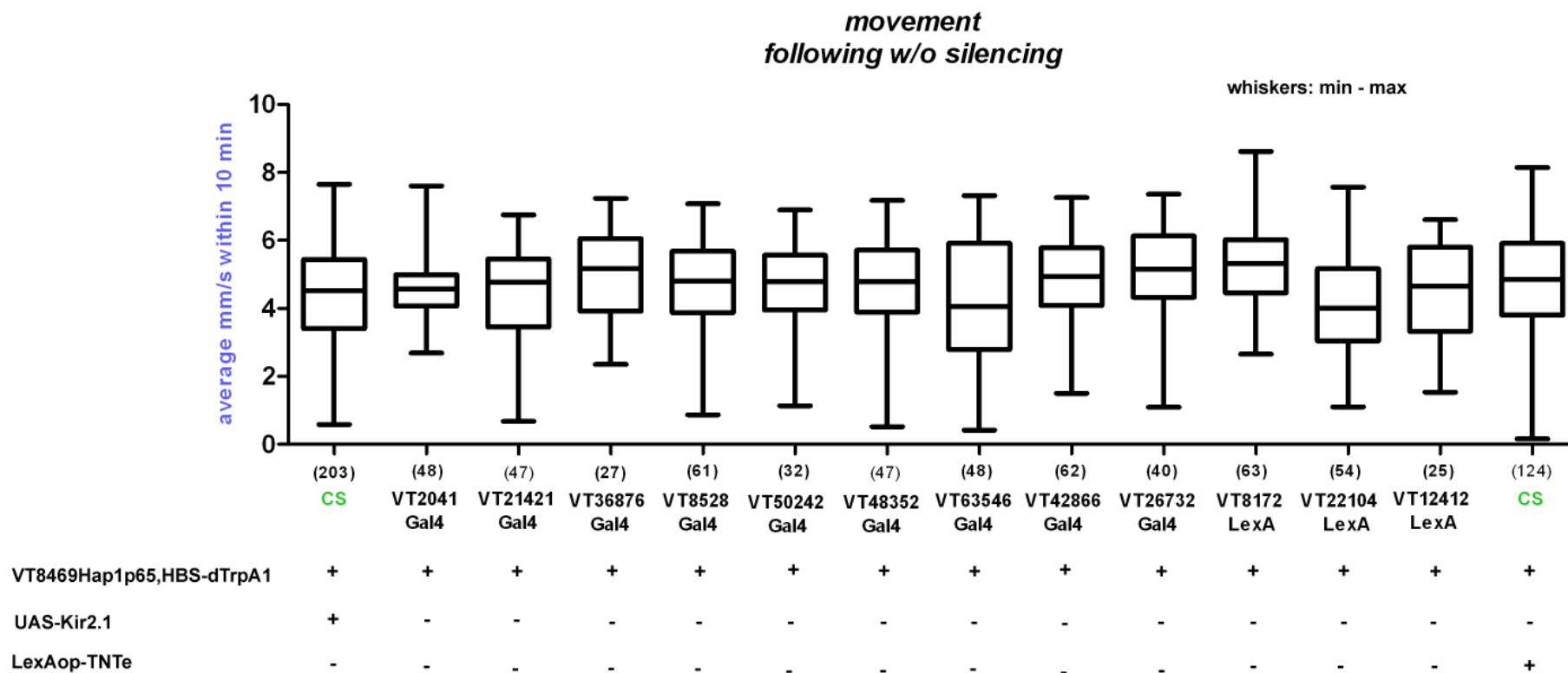
Figure 3. Locomotion is normal when neuronal silencers are not expressed.

Locomotion is not affected when VT lines positive for the absence of the following phenotype do not drive expression of a neuronal silencer.

Staining protocol

Staining procedure was developed by Jai Yu (Yu et al., 2010).

1. Dissect in **PBS** and store brains in PBS on ice. Dissect as carefully as possible, only high quality brains will register. Dissect in batches of 30 minutes.

2. Incubate in 300 μl **Formaldehyde solution** for **20 - 30 minutes** at room temperature to fix tissue.

Temperature (C°)	19-21	22	23	24	25
Time (min)	~30	~25	22	21	20

   a. **DO NOT FIX AT TEMPERATURES ABOVE 25ºC** otherwise there will be high background

3. Wash in 500 μl **PBST**, 3 X 10-15 minutes. **DO NOT WASH AT TEMPERATURES ABOVE 25ºC** otherwise there will be high background.

4. Block in 300 μl **Blocking solution** for at least 2 hours at room temperature (usually about 4 hours).

5. Incubate in 300 μl **Primary antibody solution** for approx. 48 hours at 4ºC.

   a. Only re-use primary solution once.

6. Wash in 500 μl **PBST**, 2-3 X 10-15 minutes at room temperature. Wash overnight at 4ºC.

7. Incubate in 300 μl **Secondary antibody solution** (spin down for 5 minutes before use) for approx. 48 to 72 hours at 4ºC. I have tried 96 hours.

   a. 72 hours is preferable.

8. Wash in 500 μl **PBST**, 2-3 X 10-15 minutes at room temperature. Wash overnight at 4ºC.

9. Wash again for a few hours (every hour, >4 hours). Wash for 5-10 minutes in 500 μl PBS before mounting.

10. Mount on slide with small drop of **Anti-fade solution** for microscopy according to registration protocol.

Formaldehyde solution

PBS

Paraformaldehyd 4%

10 μl 10% Triton X

Blocking solution

normal goat serum (NGS) 10%

PBST

Primary antibody solution

Antibody (see Experimental procedures)

Normal goat serum (NGS) 5%

PBST

PBST

PBS

0.3% Triton X

Secondary antibody solution

Antibody (see Experimental procedures)

Normal goat serum (NGS) 5%

PBST

Anti-fade-solution

Vectashield H-1000

References

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Curriculum Vitae

David Mahringer

Address: Obere Donaustrasse 31, 1020 Vienna
Birth: 23.10.1987
Permanent mail: d.mahringer@gmail.com

Education

2010   Master studies Molecular Biology at the University of Vienna focusing on Neuroscience

2010   Bachelor degree in Biology at the University of Vienna

2007   Bachelor studies Biology at the University of Vienna focusing on Molecular Biology

2007   Civilian service at the Austrian Red Cross Linz-Stadt

2007   Paramedic Training

2006   Graduation Khevenhüllergymnasium Linz

Experience

2012   Research Institute for Molecular Pathology, IMP Vienna
Master thesis, research group Barry Dickson
Assignment: **"**Identification of sexually shared components of the Drosophila courtship circuit"
Address: Dr. Bohrgasse 7, 1030 Vienna, Austria

2011   Centre for Brain Research, Medical University of Vienna
Institute lab rotations
Assignment: Introduction to behavioural assays, molecular/physiological techniques and imaging methods in neuroscience
Address: Spitalgasse 4, 1090 Vienna, Austria

2011   voestalpine AG Linz
Assignment: Shift work division LogServ
Address: voestalpine Straße 1, 4020 Linz, Austria

2011   Tutor at the University of Vienna, Max F. Perutz Laboratories
Assignment: Laboratory course in Molecular Biology (Dr. Lilli Winter)
Address: Dr. Bohrgasse 3, 1030 Vienna, Austria

2011   Research Institute for Molecular Pathology, IMP Vienna
Internship, research group Barry Dickson
Assignment: Genetic screen to identify neural components of the male Drosophila melanogaster courtship ritual
Address: Dr. Bohrgasse 7, 1030 Vienna, Austria

2010   Tutor at the University of Vienna, Max F. Perutz Laboratories
Assignment: Laboratory course in Molecular Biology (Prof. Andreas Hartig)
Address: Dr. Bohrgasse 3, 1030 Vienna, Austria

2010   Laboratory for Tumor Cytogenetics and Molecular Biology, Hospital of the Barmherzigen Schwestern Linz
            Internship, research group Gerald Webersinke
            Assignment: Preparation and analysis of human hematopoietic cells for tumour characterization
            Address: Seilerstätte 4, 4010 Linz, Austria

2010   Center for Brain Research, Medical University of Vienna
            Bachelor thesis, research group Michael Kiebler
            Assignment: Establishing antibodies for 2 neuronal ribonuclear proteins
            Address: Spitalgasse 4, 1090 Vienna, Austria

2009   Austrian Red Cross Linz-Stadt
            Assignment: Emergency medical service
            Address: Körnerstraße 28, 4020 Linz, Austria

2008   voestalpine AG Linz
            Assignment: Shift work division LogServ
            Address: voestalpine-Straße 1, A-4020 Linz, Austria

2007   voestalpine AG Linz
            Assignment: Shift work division LogServ
            Address: voestalpine-Straße 1, A-4020 Linz, Austria

2006   voestalpine AG Linz
            Assignment: Facility management
            Address: voestalpine-Straße 1, A-4020 Linz, Austria

2006   Chamber of Labour Linz
            Assignment: Facility management
            Address: Volksgartenstraße 40, 4020 Linz, Austria

2004   voestalpine AG Linz
            Assignment: Shift work division construction
            Address: voestalpine-Straße 1, A-4020 Linz, Austria

Academic scholarships

2010   merit scholarship awarded from the University of Vienna

Language and Computer skills

- ❖ Fluent in German (mother tongue) and English
- ❖ Basic knowledge in Spanish and Latin
- ❖ European Computer Driving Licence (Word, Excel, PowerPoint, Outlook)
- ❖ Adobe Photoshop and Illustrator
- ❖ Basic knowledge in Perl programming language

Personal interests

- ❖ Diving instructor assistant („First Austrian Professional Diving Instructor Association")
- ❖ Hiking / Camping
- ❖ Analogue and digital photography