universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Functional analysis of the courtship neuronal circuit of *Drosophila melanogaster*

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag. rer.nat)

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Wien, am	20. September 2009

Contribution and acknowledgements

I would like to thank following people who supported me throughout my thesis:

Barry Dickson allocated me the opportunity to work on this exciting project. As my supervisor he guided me all along my diploma project .

In particular I would like to thank Jai Yu providing me with Gal4 Lines and TNTfruFLP stocks, Tanxiao Liu for providing me with Kir2.1fruFLP fly Stocks for silencing experiments.

Laszlo Tirian for introduction on the song recording system and Alex Keene for helpful feedback concerning the song analysis with LifeSong X.

Anne von Philipsborn for initial supervision and critical view throughout my work.

I am grateful to all other members of the Dickson lab for constructive and critical feedback on my work.

Finally I would like to thank Piotre Danzinger who was always there for me in this time.

Dedicated to my family.

Contents

Abbreviations

CNS	central nervous system
CPG	central pattern generator
CPP	cycles per pulse
FLP	FLP recombinase
FRT	FLP recombinase recognition target site
IPF	intrapulse frequency
IPI	interpulse interval
Kir2.1	potassium inwardly rectifier
PPT	pulses per train
PTL	pulse train length
SOG	suboesophagial ganglion
syb/VAMP	neuronal synaptobrevin
TxTe/TNT	tetanus toxin light chain from bacteria
VNC	ventral nerve cord

Synopsis

Males of most fruitfly species (*Drosophilidae)* perform a courtship ritual , an innate behavior, which is characterized by a sequence of stereotyped steps. The genetic basis of this behavior is determined by one gene: *fruitless*.

This gene is exclusively expressed in certain neurons of the nervous system of *Drosophila*.

Fruitless expressing neurons are located in the brain as well as in the ventral nerve cord of the fly and it is suggested that those neurons are assembled to form a neuronal circuit, at least in some parts of the nervous system, that encode for the different steps of that innate behavior.

The aim of this work is to examine the role of *fruitless* neurons in the control/ accomplishment of a certain motor pattern that is performed by male flies during the courtship ritual: the courtship song.

The courtship song of male *Drosophila* is generated by vibrating one wing whiles the male orient towards the female. The fact that only male flies sing and mutations in the *fruitless* gene affect courtship song traits as well as its production indicate that certain *fruitless* populations may play a role in courtship song pattern generation or may have a modulatory function relating the motor program. The courtship song of *Drosophila melanogatser* is characterized by certain song traits. With the help of effector-genes and the FLP-in technique sub populations of the „*fruitless* circuit" in male *Drosophila* were silenced and the courtship song of those flies was analyzed. During the course of this work it was examined that silencing of some *fruitless* clusters indeed affect certain song traits and I tried to classify those changes. The results support the hypothesis that some fruitless neurons are required for generating a proper courtship song.

Synopsis

Die Männchen der meisten Fruchtfliegenarten (*Drosophiliden*) zeichnen sich durch ein angeborenes Balzverhalten aus, das aus stereotypen Verhaltensmustern besteht. Die molekulargenetische Grundlage für dieses Verhalten wird durch ein einzelnes Gen determiniert, *fruitless*.

Dieses Gen wird ausschließlich in bestimmten Neuronen im Nervensystem von *Drosophila* expremiert. Diese *fruitless* Neurone kommen sowohl im Gehirn als auch im Ventralganglion dieser Insekten vor und man nimmt an, dass diese Neurone ein Netzwerk bilden, welches für die unterschiedlichen Schritte des Balzverhaltens codiert. Diese Arbeit hat sich zum Ziel gesetzt die Kontrolle und Ausführung eines bestimmten motorischen Signals während des Balzverhaltens der männlichen Fliegen zu untersuchen und zu verstehen: der Balzgesang. Der Gesang männlicher *Drosophiliden* wird durch Flügelvibrationen erzeugt, wobei das Männchen sich dem Weibchen zuwendet. Die Tatsache, dass nur Männchen singen können und *fruitless* Mutationen Effekte auf die Generierung des Gesangs haben, legt die Vermutung nahe, dass bestimmte neuronale Subpopulationen, die den *fruitless* Faktor beinhalten, an der Mustergenerierung des Gesangs teilhaben oder zumindest eine modulatorische Funktion im Zusammenhang mit dem motorischen Programm des Singens besitzen. Der Gesang der männlichen *Drosophiliden* zeichnet sich durch ganz bestimmte Charakteristika aus. Mithilfe von Effektorgenen und der FLP-in Technik wurden bestimmte neuronale Subgruppen des *fruitless* Netzwerkes still gelegt und der Gesang dieser Fliegen wurde analysiert. Im Zuge der Experimente hat sich herausgestellt, dass die Stilllegung von bestimmten Subpopulationen das Gesangmuster beeinflusst. Des Weiteren wurde versucht diese Veränderungen im Gesang zu klassifizieren. Diese Ergebnisse unterstützen die Hypothese, dass einige *fruitless* Neurone notwendig sind um einen Wildtyp-ähnlichen Liebesgesang zu erzeugen.

Introduction

Overview

The nervous system of the fly

The nervous system of insects consists of a brain and a ventral nerve cord (VNC). The brain is dorsally located in the head and the VNC runs through the thorax ventrally (Fig.1). Neurons of the central nervous system of the fly constitutes to a complicated meshwork of different neuronal circuits with certain functions (Power 1943, Strausfeld 1976).

The brain of *Drosophila* consists of three main parts, the central brain and the optical lobes on either side of the central brain (Ito 2008). The optical lobes function as lower-order sensory centers for visual information processing whereas the central brain function as lower-order center for sensation of other information (such as taste, olfaction etc.). The central brain consits of centers for association, integration and higher-order motor control (Power ME, Ito 2008).

The ventral nerve cord (VNC), the equivalent of the spinal cord in vertebrates, consists of three pairs of thoracic ganglia that innervate muscles for locomotion, the legs and wings whereas the abdominal ganglion innervates posterior body parts, for example reproductive organs. Anatomically the VNC can be further subdivided from anterior to posterior in different regions : the prothoracic segment, the anterior dorsal complex, the mesothoracal and metathoracal segments and the abdominal segment (Yu 2009).

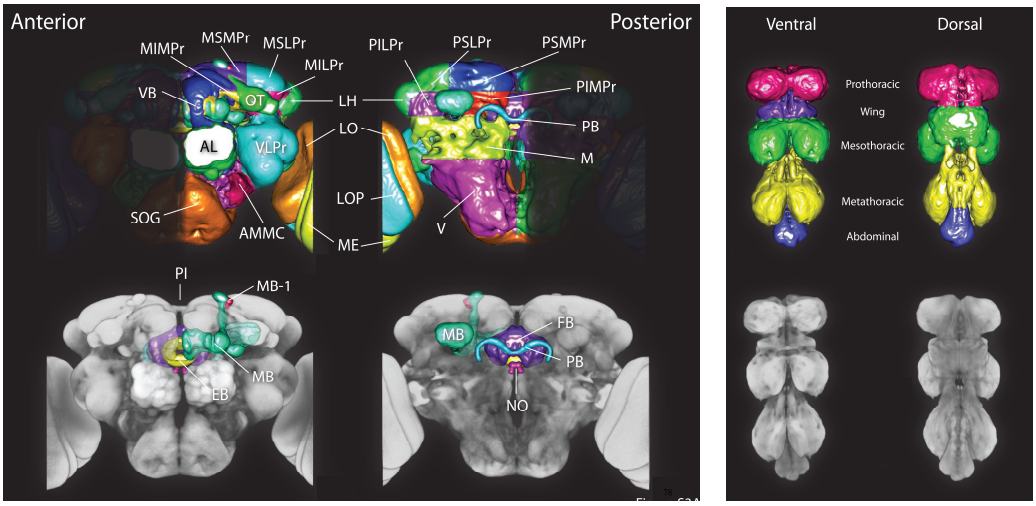

Figure 1. **The central nervous system of adult *Drosophila melanogaster*.**

The brain (picture on the left) as well as the VNC (picture on the right) can anatomically and functionally be divided in different regions: AL-antennal lobe, AMMC-antennal mechanosensory and motor center, EB-elipsoid body, FB-fan shaped body, M-medial posterior, SOG-suboesophagial region, MB-mushroom body, PB-protocerebral bridge,, OT-olfactory tubule, VB-ventral body, VLPr-ventrolateral protocerebrum, V-posterior ventral (pictures adopted from Yu, 2009).

It is thought that the adult nervous system of *Drosophila* consists of about 100.000 neurons: Approximative, 30.000 neurons are part of the central brain, 15.000 neurons are part of each optical lobe and another 15.000 neurons are part of the ventral nerve cord. In addition there are about 3.500 neurons that connect the brain and the VNC through the so called cervical connective via ascending and descending neurons.

Concerning the size of the whole CNS of the fly (Fig.1), cell bodies of neurons can be rather big (2-5 µm) and dendritic fields may span up to 50 µm. In contrast to vertebrates, in invertebrates cell bodies are located on the cortical rind of the nervous system and all neuronal processes project in the inside the CNS to form the synaptic neuropil (compartments of axon tracts, dendritic arborizations and glial sheaths).

The classification of neurons depends on how one defines a cell type:
Neurons can be classified by their anatomy (morphology, connectivity etc.), their function (e.g. which neurotransmitter(s) the neuron uses), by their origin of lineage or even by their expression profiles (Luo 2008).

For a functional analysis of the nervous system a cell type can be seen as the smallest unit that can be monitored and manipulated.
This thesis deals with a neuronal circuit in the CNS of *Drosophila melanogaster* which is defined by the expression of a certain gene that encodes a transcription factor. In this case the classification of neurons occurs by the presents of this factor in neurons of the CNS of *Drosophila*. The neurons that contain this certain factor are required and sufficient for courtship behavior in male flies and the factor is called *fruitless*/fru^{M} (Demir and Dickson, 2005).

Behavior and the nervous system

Behavior, as the ability of living organisms to respond to their surroundings, always fascinated scientists. How an animal respond to its environment is a consequence of evolutionary changes through its natural history (Greenspan, 2008).

One presumption in neurobiology is that the nervous system and behavior are in a way the same thing. In this view all aspects of behavior can be seen as a consequence of the anatomy of the nervous system, its structure and function. Meaning, understanding the nervous system´s function lead to a better understanding of behavior. Moreover, understanding the structure and function of the nervous system may lead to a better understanding of behavior.

Functionally the nervous system can be seen as input-output device for information sensation, processing and reaction. This device himself is in turn made up of interconnected input-output devices until you reach the smallest unit of the system: the neuron, which is also able to process information in the way of an input-output device.

To investigate the principals that govern how neuronal circuits govern behaviours one need a robust system to work with. This is the case for courtship behaviour in *Drosophila melanogaster*.

The courtship behaviour is innate. Thus it is robust, developmentally fixed, uniform and stereotyped, sex and species-specific. First-time performances of innate behaviours are completely functional. Male flies are able to perform this behaviour without prior exposure to other flies, no prior experience is needed to perform it (Hall, 1994). Additionally one can reverts to years of investigation of courtship behavior in male *Drosophila* aswell as the fact that the fly as model system has several advantages not only because it is genetically defined but also because of the

availability of genetic techniques to trace subsets of neurons to dissect neuronal circuits (Luo 2008, Simpson 2009).

Courtship behavior in *Drosophila*

The courtship behavior of male flies consists of series of consecutive steps that can easily be observed and quantified.

The male orients towards the female, follows her and taps her with his forelegs. Shortly thereafter he extends one wing and vibrates it to produce a courtship-specific sound. Later, the male contacts the females genitalia and extends his mouthparts. Finally, the male curls his abdomen until copulation occurs (Fig. 2).

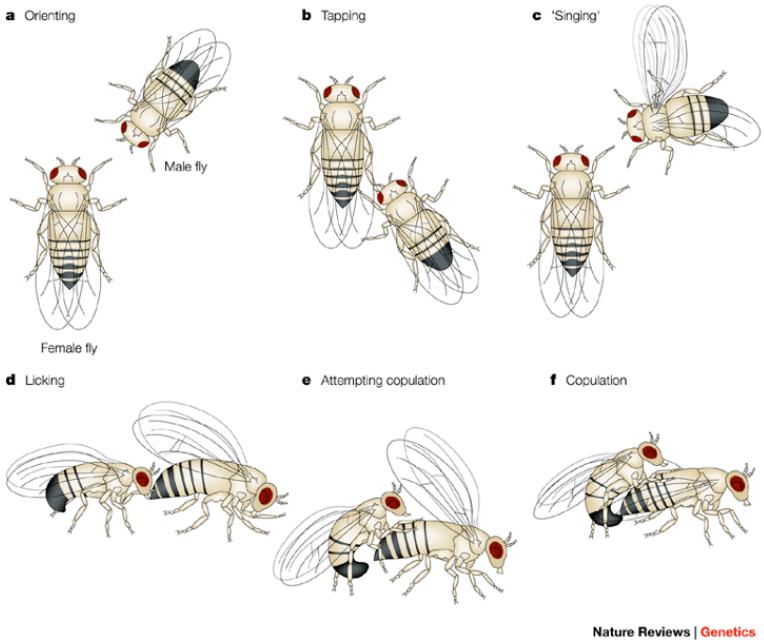

Figure 2. **The *Drosophila* male courtship ritual** (adopted from Sokolowski 2001).

***Fruitless* and the courtship circuit**

It is thought that sex-specific differences in courtship behavior is determined by structural and/or functional variations of the underlying neuronal circuits. The expression of a key regulatory gene in this organism determines this reproductive behavior in many aspects (Demir and Dickson 2005). This gene is called *fruitless*.

Fruitless (fru) is expressed in about 2% of neurons in the nervous system of the fly. The gene encodes a putative transcription factor of the BTB-zinc finger family (Ito 1996, Ryner 1996). The genetic locus encodes for a whole set of transcription factors (Ito 1996, Ryner 1996) leading to the expression of different isoforms.

One isoform, Fru^M , exists only in male flies due to alternative splicing of one transcript and expression of this isoform. Male *Drosophila* expressing a mutated form of Fru^M cannot perform the courtship ritual anymore and in female *Drosophila* ectopic expression of this *fruitless* isoform lead to the performance of male-specific behavior in those flies . Thus, this regulatory gene is not only necessary but also sufficient for the performance of the male courtship ritual (Demir and Dickson 2005).

Fruitless expressing neurons are well defined by their anatomy, not only because targeted insertions of Gal4 into the *fruitless* locus (fruGal4) lead to the identification of their localization in the nervous system (Manoli 2005, Stockinger 2005), but also due to the work of Jai Yu, a member of Barry Dickson´s research group, who described the detailed anatomy of *fruitless* neurons in males and females of *Drosophila melanogaster*. Figure 3 illustrates the map of the „*fruitless* circuit“ (Yu, 2009).

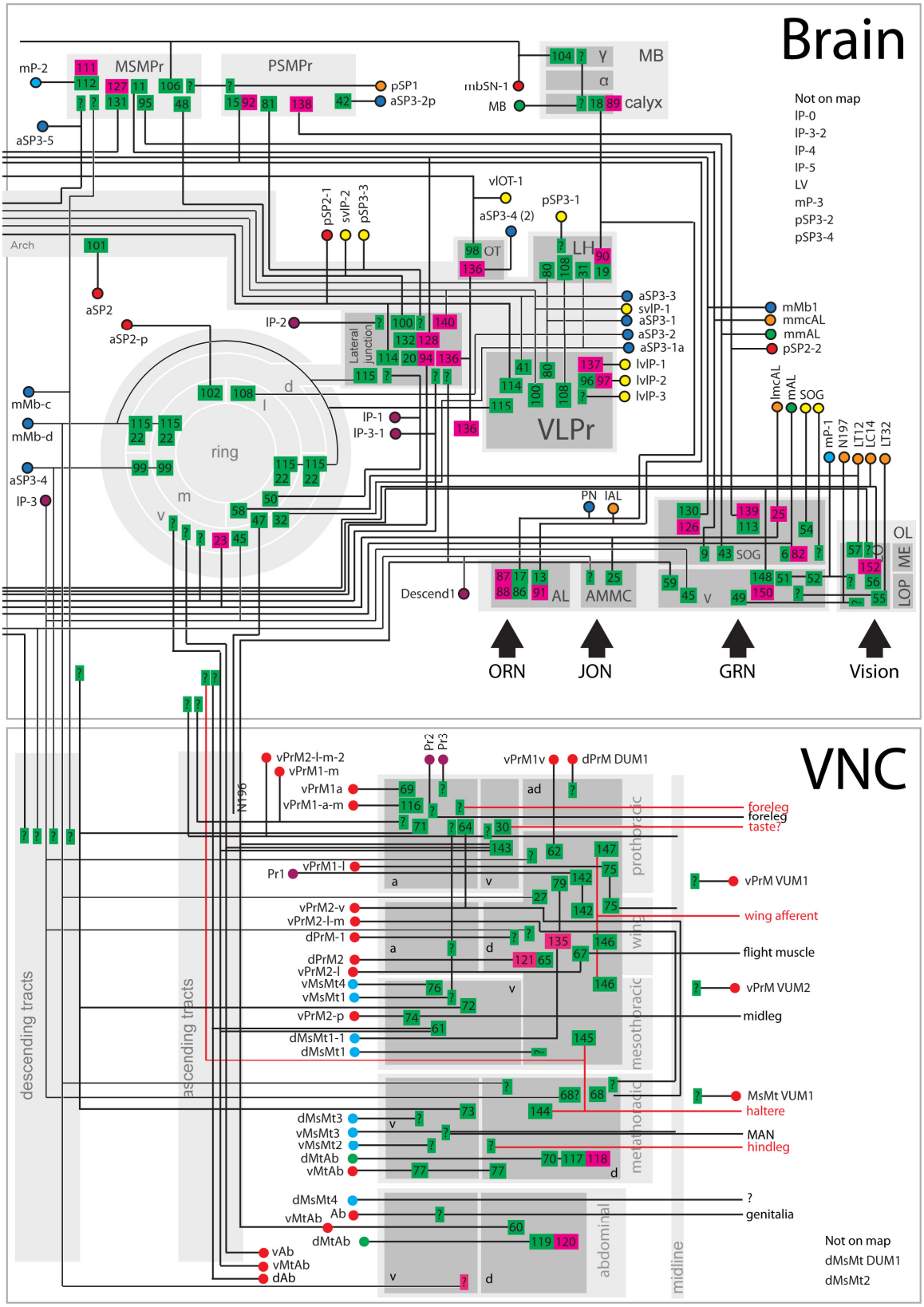

Figure 3. **The „fruitless circuit“** (adopted from Yu 2009).

The Courtship Song of *Drosophila melanogaster*

During courtship both partners, male and female, exchange information via chemicals such as pheromones, through tactile or visual cues and also via acoustic communication (Ewing 1983). Acoustic signals are exclusively produced by male flies using wing vibration and the female fly receives the sound with a transmitter organ on the third antennal segment, the aristae (Burnet 1971, Eberl 1997).

The courtship sound in males is produced by extending one wing to an angle of about 90 degree to the side of the body and vibration of this wing whiles they orient towards the female. The courtship song consists of two different motor patterns. The pulse song and the sine (or hum) song (von Schilcher 1976, Tauber 2003).

It is thought that the pulse song has the function to identify the species as well as for intraspecific mate choice (Talyn 2004).

Characteristically for the sine song is its continuous oscillation with a frequency of 140-170 Hz (von Schilcher 1975). The emitted sound pulses have a higher frequency (150-300Hz) and have certain intervals, the so called Interpulse Interval (IPI) that is a species specific (Ewing & Bennnet-Clark 1968). In *Drosophila melanogaster* the IPI is about 35ms. The number of bursts in each pulse can vary (2-50 pulses per train) and each pulse is made up of 1-3 cycles that last between 5-10 ms (Fig. 4).

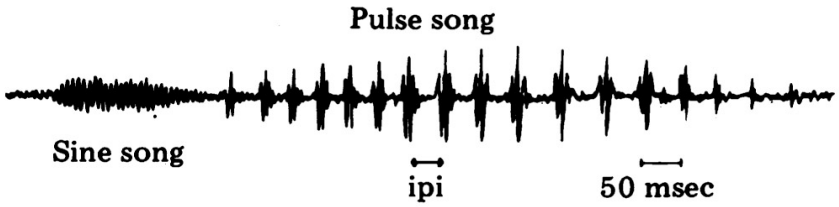

Figure 4. **Oscillogram of the courtship song of *Drosophila melanogaster* males** (adopted from Kyriacou and Hall 1980).

Fruitless **and song production**

The neuronal basis for courtship song production was first described using sex-mosaic flies which were partially male and female (von Schilcher and Hall 1979). It is still not clear which neurons contribute to the motor pattern generation of the love song of the fly. Nevertheless, several studies show that *fruitless* neurons are involved not only because of the fact that fru^M mutant male flies do not sing (Rideout 2007).

The mesothoracic segment of the ventral nerve cord contains fru^M expressing neurons that are sexual dimorphic and it is suggested that this region can be the putative song generator in male flies (Hall 1979, von Schilcher and Hall 1979, Lee 2000). A specific region containing *fruitless* neurons span the prothoracic region, the mesothoracic region and the dorsal wing region in the ventral nerve cord. In this region the so-called anterior dorsal mesothoracic nerve (ADMN)is located that innervates the wing and flight muscles. This nerve extends posterior from this complex and is a *fruitless* neuron.

In addition one descending *fruitless* neuron projects into this complex. This region is called the anterior dorsal complex (Yu 2009).

The *fruitless* neurons in this region is also present in female flies and a former study could demonstrate that activation of those neurons in an artificial way induces wing extensions in female flies and also a courtship like sound, although this artificial love song was abnormal to some extend (Clyne and Miesenböck 2008).

However an alternative hypothesis is that *fruitless* neurons are not part of the central pattern generator for song but may play a role in modulation an/or control of the pattern generator itself.

To examine the function of *fruitless* neurons in courtship song it is necessary to dissect the circuit.

Genetic dissection of the „*fruitless* circuit“

For circuit analysis it is necessary to have genetic access to certain populations of neurons, at least to subsets of neurons or neuronal clusters.

The experimental basis of this project is made by a detailed anatomical description of the „fruitless circuit“ (Yu 2009, Fig. 3). In course of Yu´s work the FLP-in technique was used to genetically label subsets of *fruitless* expressing neurons with mCD8.GFP or tauLacZ (Lee and Luo 1996, Callahan and Thomas 1994) to reconstruct a wiring diagram of the „fruitless circuit“. Although it is not demonstrated yet that *fruitless* neurons are interconnected, except between the first and second order olfactory neurons (Datta 2008), one can suggest a potential connectivity at least in some cases based on overlapping arborization between different neuronal classes (Jefferis 2007)

An existing collection of Drosophila Lines , so-called Gal4 drivers, with overlapping expression in subsets of *fruitless* neurons were chosen to further investigate the requirement of these neurons in song production by introducing neuronal silencers in these neurons using the FLP-in technique.

The Gal4 Lines were chosen based on their expression of the driver in certain *fruitless* neurons (the more restricted the more accurate the analysis) and by anatomical criteria (e.g. *fruitless* neurons in the mesothoracic ganglion).

The GAL4/UAS FLP-in System

Gal4 encodes a yeast transcription factor protein (881 amino acids) that specifically recognizes a yeast upstream activating DNA element (UAS element) to activate transcription (Giniger 1985). This UAS element consists of four related 17bp sites and can be introduced in the genome of the fly to drive expression of GAL4 because both, the protein and the DNA sequence, are not present in *Drosophila*. The introduction of this powerful genetic system was first demonstrated by Andrea Brand and Norbert Perrimon at Harvard Medical School (Brand and Perrimon 1993).

If one bring a reporter gene under the control of an UAS element, the reporter will be expressed in those cells where the Gal4 protein is present. There are a lot of Gal4-lines available, so called Driver-lines, and this is also true for UAS-Reporters (Duffy 2002).

To genetically trace only subsets of the fruM neurons the FLP-in technique was used. The FLP recombinase is a recombinase from *Saccharomyces cereviciae* that recognizes FRT target sites to promote site-specific DNA recombination between those sites (Golic 1989).

A modified UAS Responder line was used to achieve that a certain reporter/effector gene will be present in subsets of fru^{M} neurons:

The responder carries in its genome an UAS recognition site and the reporter/ effector gene. The reporter/effector gene is separated by a transcriptional termination site, a „stop-cassette“, flanked by two FRT sites and is thus inactive as long no FLP recombinase excsises the „sop-cassette“. The FLP-recombinase gene is present in the same Line on another chromosome and is under the control of the fruitless promoter (fru^{FLP}) thus the enzyme will only be present in fru^{M} cells and therefore the reporter/effector.

This method lead to an effective, restricted expression of the reporter/effector in overlapping cell populations between the UAS Resopnder Line and the GAL4 Driver Line. Limitations are only due to the random activity of the FLP recombinase. Sometimes, especially when the overlapping cell subset is small, it can be the case that the reporter/effector is not expressed. This is also true for the strength of the Gal4 Line used. The used Gal4 driver lines have the driver gene introduced on different loci and with different copy number, thus expressing the transcription factor not with the same level (Abmad and Golic 1996).

A diagram to better illuminate the UAS/Gal4 FLP-in technique is shown in Figure 5.

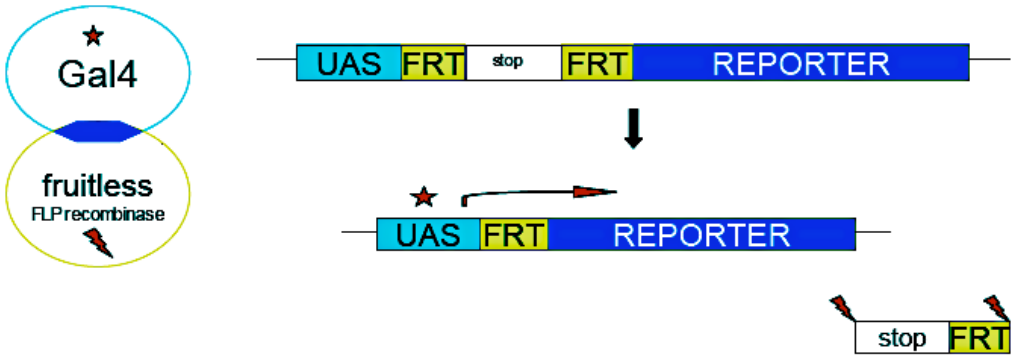

Figure 5. **The UAS-Gal4 FLP-in technique**

To trace only sub populations of fruM neurons in the nervous system of *Drosophila melanogaster* a modified UAS/Gal4 technique was used. The Reporter/Effector gene is separated by a transcriptional termination sequence flanked by two FRT-sites. A FLP-recombinase that is able to introduce a site-specific DNA recombination between FRT sites is under the control of a *fruitless* (fru^{M}) promoter thus the expression of the Reporter/Effector occurs only in *fruitless* neurons. The diagram on the left illustrates the restricted expression in the overlap of the Gal4 driver and the UAS responder.

Functional analysis of the *„fruitless* circuit“

Once it is possible to target genetically neurons there are many ways to control neuronal activity in vivo using „effector genes“ (Simpson 2008).

For silencing certain neurons or neuronal clusters two different neuronal inhibitors were used: Tetanus Toxin, Toxic light chain from bacteria, and Kir2.1, a vertebrate inwardly rectifying potassium channel.

Tetanus toxin light chain (TxTe) is a protease that cleaves neuronal Synaptobrevin (n-Syb/VAMP) and blocks thus vesicle fusion in chemical synapses (Martinet 2002, Sweeny 1995). n-Syb is an essential factor for neurotransmitter release as it regulates fast synaptic vesicle fusion in a Calcium dependent manner (Kidokoro 2003). The disadvantage of this effector is that is has primarily no effect on electrical synapses (Phelan and Starich 2001). In addition TxTe-action may differ according to the cell-type in which it is expressed (Kaneko 2000).

Kir2.1 is a potassium channel that is open in rest and thus increases the excitability of the neuron by hyperpolarization . Thus, the effector is able to block action potential generation and has not only an influence on synaptic transmission but also on electrical transmission among neurons (Bainess 2001, Paradis 2001, Wu 2008, Nitaback 2002).

Summary

The function of neural circuits are defined by mechanisms by which assemblies of neurons generate perception, neuronal states and behavior .

To get an insight how neuronal circuits are organized and how this organization lead to a certain behavior a robust model system is needed. This is the case for courtship behavior in Drosophila, an innate behavior which consists of stereotyped sequences .

In this work attention was paid especially on the courtship pulse song production taking advantage of the relatively easy way to quantify this male-specific behavior, since the pulse song of *Drosophila* males consists of certain traits: the Interval between pulses (Inter pulse Interval, IPI), the number of cycles per pulse (CPP) or the pulse number in each song train (Pulses per Train, PPT).

With the help of „effector genes“ that act as neuronal silencers the aim of this diploma project is to get a better insight in the deeper mechanistic function of *fruitless* neurons for courtship song generation in male *Drosophila melanogaster*.

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Materials and Methods

Fly stocks and Genetics

UAS>stop> Effector; fruFLP flies

The FLP in constructs were initially cloned into UAS-reporter plasmids. The used FRT sites were wild type sequences flanked by two transcriptional termination sequences (SV40 3´UTR and tubulin 3´UTR as described in Stockinger 2005). The UAS>stop>TxTe and the UAS>stop>TxTe(inactive) are insertions on chromosome II. The UAS>stop>Kir2.1 construct is an insertion on an attP landing site (19a1) on the second chromosome.

Fly stocks for behavioral experiments are in an w+/Canton-S background and are double-balanced with Curly O (CyO) on the second chromosome and with TM3 Serrate (TM3Ser) on the third chromosome.

Canton S

A Canton S strain(laboratory stock) was used to collect male flies as wilde type control for courtship song recordings. The flies were held in bottles with cornmeal-agar-molasses-yeast medium.

yhh Canton S

Virgins for song recordings were collected from a heatshock *hid* stock carrying the hshid gene on the Y chromosome (yhh CS). *Hid* (*head involution defective*) is a proapoptotic gene. If it is expressed from a heat shock promotor it induces ectopical cell death after a heat shock of embryos at 37°C. The heat shock hid gene is present on the Y chromosome thus a heat shock of 37°C for one hour kills all male flies (Grether, 1995). Virgins were collected after hatching (> 6 hrs) and aged for 3-5 days before song recordings.

All flies were raised in controlled temperature and light conditions: 25°C with 12hr light:12hr dark condition (8am to 8pm).

Silencing of neuronal Fru^M clusters (Gal4 Screen)

Silencing of *fruitless* neurons was achieved using a set of Gal4 Driver lines and UAS-Responder Lines as described above. Therefore 10-15 virgins from the UAS>stop>Effector Line were crossed with 5-7 males from each Gal4 Driver line.

The flies were cultivated on power food in small plastic bottles within a 12hr day: 12hr night cycle from eight am to eight pm at 25°C and a humidity of 60%.

After ten days newly hatched F1 males were collected and aged 4-6 days in 96 well plates with power food (cormeal-agar-molasses-yeast medium), again with a temperature of 25°C and with 12 hr light: 12 hr dark condition starting at 08:00.

For each Gal4 driver at least 8 males were used for recordings. Some Gal4 driver lines showed a higher variation in their song traits after silencing their fruitless neurons. In these cases up to 32 male flies were used for recordings.

50 Gal4 driver lines were chosen for the screen based on two criteria: First, Gal4 lines with a small *fruitless* overlap and secondly, lines with a *fruitless* overlap in the mesothoracic ganglion were selected for screening of pulse song phenotypes (Supplementary Table 1 and Table 2). The screen was initially started with TeTx as silencer and after this first screen silencing was done with Kir2.1 because it turned out that Kir2.1 acts in a more specific way compared to TeTx (based on observations from other lab members, data not shown).

Courtship Song recording and analysis

Courtship recordings were performed in an insulated anechoic box where the recording chambers were placed (8 chambers) together with the microphones.

Recordings were performed at a temperature of 22°C since some song traits are temperature dependent (Ritchie 2001).

Aged flies were placed in one chamber (diameter of 1mm, closed with a nylon mesh) and the courtship sound was recorded with small microphones placed on the side of the courtship chamber. Single pair recordings were performed with G.R.A.S (Vibration and sound) microphones Coax Preamplifier Type AG0002 (12,7 mm diameter, 104 mm lenght) coupled to an ICP preamplifier with constant voltage. Courtship songs were recorded for 5 min with a LabView 8.2 recorder (National Instruments). The frequency cut off for the recording of raw data was 80-500 Hz . Raw data files were stored and converted with a MatLab script to .wav files.

For Song analysis LifeSongX version 0.75 software was used . This software is Mac OS X-based and was developed by John Rieffel and Adriana Vilella at Brandeis University for courtship behaviour and song (http://lifesong.bio.brandeis.edu) (Lin 2005, Vilella 2005).

Since LifeSong X cannot read .wav files, Matlab output files (.wav files) were converted with Amadeus Pro Software for Macintosh into Quick Time Movie files (.mov files). The courtship song pulses were selected per hand to eliminate noise. Following preferences for pulse song were used: The IPI was detected using a minimum of 15ms and a maximum of 100ms. With this setting pulses with a higher intrapulse interval of 100ms were not included in a certain song trait. The minimum pulse train length for detection of song trains was 3 and pulses were detected with a pulse S/N cut off of 4. For each fly a song report was generated and exported to Excel for further statistical analysis.

Following spectral characteristics of pulse song components were analyzed: The intrapulse interval, the number of pulses within a song train, the wing cycles per pulse and the Intrapulse (or carrier) frequency (Wheeler 1988).

The **interpulse interval (IPI)**, a species specific pulse song trait in *Drosophila*, is the time between the beginning of one pulse and the end of the following pulse. The IPI was calculated by determining the duration of the pulse song trains divided by the number of all pulses within the train minus 1.

The number of **pulses per train (PPT)** were counted for each recording and averaged for each genotype.

The **cycles per pulse (CCP)** are the half of the number of peaks plus the number of troughs minus one.

Intrapulse frequency (or carrier frequency) **(IPF)** is the high energy maximum after the Fourier transformation of a certain pulse.

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Results and Discussion

Sound production in *Drosophila* and some genetics

The love song produced by male flies during courtship is genetically programmed and has a rhythmic repetitive structure of pulse sounds that play a critical role in mate recognition. In this study I tried to investigate the function of *fruitless* neurons in this behavior in a proximate, mechanistic way.

Since the first analysis of *Drosophila* courtship sound (Shorey 1962) several song mutants have been identified that affect different sound parameters.

The first one, *cacophony*, produces polycyclic pulses . The gene encodes an alpha-1 calcium channel subunit with several different isoforms which are generated by alternative splicing (von Schilcher 1976, Wheeler 1989).

An allele of *no-on-transientA*, a RNA binding protein involved in alternative splicing, called *dissonance*, is a gene required for proper pulse song production. Mutations in the 3´end of the open reading frame lead to pulses that appear normal at the beginning of the sound but become polycyclic at the end of the trains (Kulkarni et al., 1988, Rhendal and Hall, 1996). Another pulse trait, the interpulse interval, is also altered by this gene (Wheeler et al. 1989).

There are several other genes that have an influence on the cycles per pulse in a song. For example *croaker* and *cysteine-string-protein,* that are not well characterized at the molecular level . *slowpoke*, a calcium-activated potassium channel, controls the cycles per pulse and the intrapulse frequency (Yamamoto et al. 1997, Peixoto and Hall 1998).

But how does *fruitless* affect the song production?

Former studies reported that some alleles of *fruitless* eliminate the pulse song and other alleles affect the length of the mean IPI (Vilella et al. 1997, Wheeler et al. 1988).

Sine song seems to be not altered by *fruitless* and that was the main reason that only pulse songs were analyzed in this study. The other reason is that the sine song is hard to detect, since the amplitude of this second song pattern is very low. Furthermore, electrophysiological studies show that the sine song derives from the flight circuitry (a reduced-power flight motor pattern) and that the underlying neuronal circuit for pulse song seems to be different (Ewing 1979b, c).

Fru^{M} neurons are required for courtship song

Since it is known that Fru^{M} mutant male flies are not able to generate a proper pulse song (Ryner 1996, Vilella 1996, Vilella 1997), it was first tested if silencing of *fruitless* neurons in a Gal4 Line with a broad overlap affects the pulse song production.

One Gal4 Line, called G314, was therefore tested. The *fruitless* overlap of this line contains 60 cell clusters (out of 92) , 41 clusters in the brain and 19 clusters in the VNC (Yu 2009). The *fruitless* neurons of G314 are given in Figure 6.

Silencing of those 60 fru neuronal clusters with TeTx (n=32) or with Kir2.1 (n=32) indeed affects the ability of pulse song generation in male *Drosophila*.

This effect was not that specific compared to the results obtained when Kir2.1 was used as effector gene. About 30% (9 males out of 32) of G314∩fruFLP::TeTx males produced pulse song trains with a high pulse frequency and slightly higher wing cycles per pulse compared to wild-type *Drosophila* and controls (heterozygous flies carrying the Effector TeTx but not Gal4, supplementary Figure 1.). This effect could not be due to the fact that TeTx only acts on chemical synapses whereas Kir2.1 affects the whole neuron by altering the excitability (Sweeny 1995, Paradis 2001) because only some individuals generated a song. This result is more likely due to the stochastic action of the FLP-recombinase.

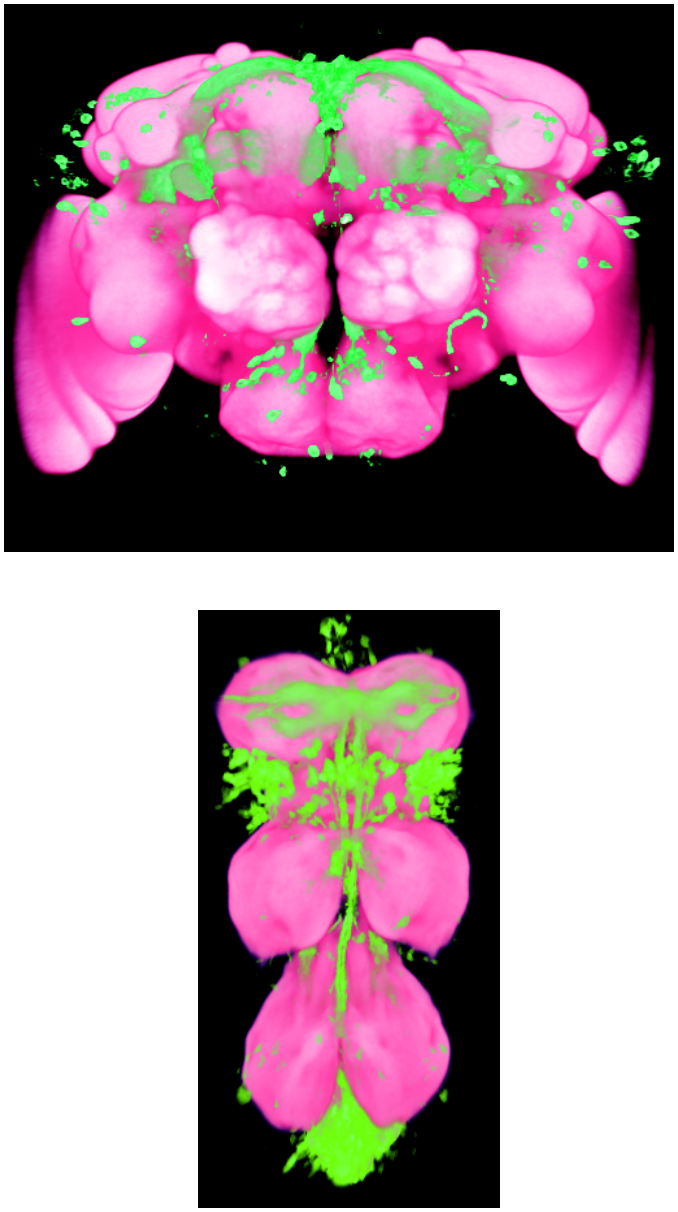

Figure 6. **The *fruitless* overlap of G314 Gal4 in male *Drosophila*.**
Fruitless neurons in the central nervous sytsem of G314Gal4 are labeled with mCD8GFP (shown in green) using the FLP-in technique (A) brain, B) VNC, the neuropil is shown in magenta). This line was used to examine if the requirement of *fruitless* neurons for proper pulse song generation in Drosophila males could be reproduced with the FLP-in system using two different neuronal silencers (TeTx and Kir2.1). The picture was taken from BrainGazer and is an computational average of 5 male flies.

Silencing of *fruitless* neuronal subsets lead to different song phenotypes

Interestingly, several song defects could be investigated. The following summarizes the obtained results and categorizes the different phenotypes concerning the pulse song pattern to identify the neuronal fruitless clusters required for a certain trait.

The Inter Pulse Interval (IPI)

The inter pulse interval, as the amount of time between two pulses in a song train, is species specific and the most important parameter for mate recognition in the melanogaster subgroup (Ewing and Bennet-Clark 1968).

The mean IPI is around 35 ms in *Drosophila melanogaster* (Cowling and Burnet 1981, Wheeler 1988). The mean IPI of Canton-S flies (laboratory stock) was caclulated (n=30) using LifeSongX software and a value of 35ms was calculated for those recordings (Supplementary figure). Thus, Canton-S was used as wilde type-control in this study.

Previous studies reported that mutations in the *fruitless* gene cause courtship pulse song abnormalities affecting the Inter pulse Interval length. Various *fruitless* mutants tended to have higher mean IPIs (>40ms) compared to wilde type *Drosophila* (Vilella 1997).

Silencing of *fruitless* subpopulations (37 Gal4 lines) with TeTx did not affect this song trait significantly in those lines that were tested (data not shown). However, using Kir2.1 as effector gene lead to a slightly, but significant shorter mean IPI in other Gal4 lines (not tested with TeTx) as shown in Table 1 (Fig.6; F-test, $p<0{,}001$).

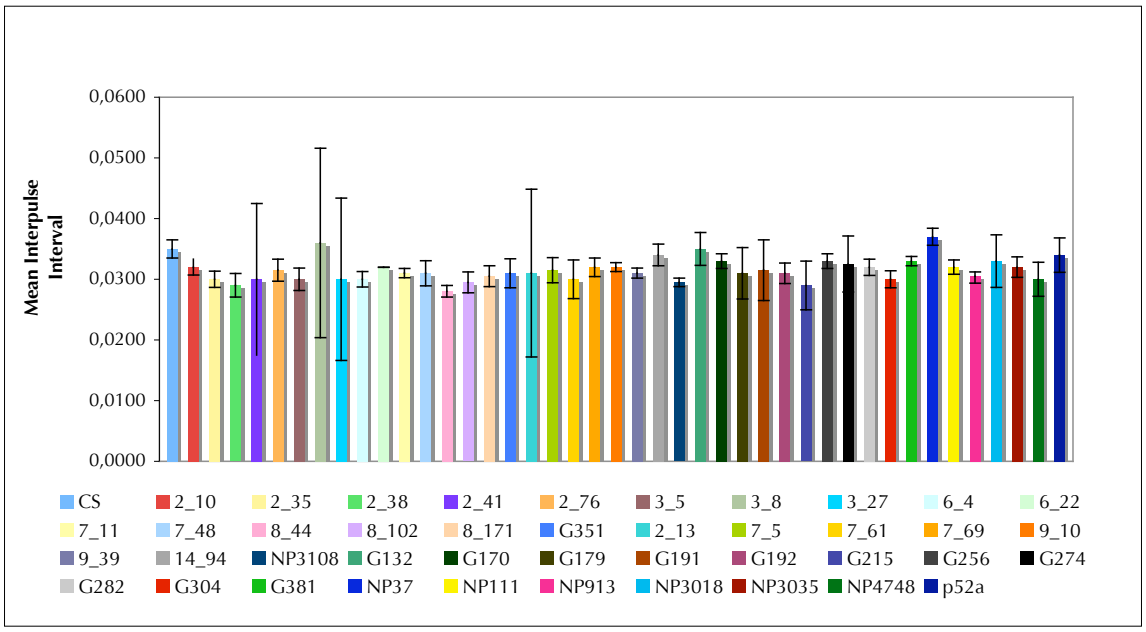

Figure 6. **Mean IPIs of various Gal4 Lines after silencing of their *fruitless* neuronal sub populations using Kir2.1.**
The numbers in the legend show the Gal4 line used for silencing their *fruitless* neuronal sub populations (Gal4∩fruFLP::Kir2.1) using the FLP-in technique and Kir2.1 as neuronal silencer (see Material and Methods). CS: Canton-S was used a s wild-type control. For Gal4nfruFLP::Kir2.1 n>8 flies and the mean IPI of all recordings (22°C) was calculated. The bars indicate the S.E.M.

The actual IPI length oscillates in a sinusoidal pattern with a period of 55-60s and a minimum length and maximum length of 28 to 40 ms, respectively. It is known that the *period* locus controls this oscillation and that they play an important role in female receptivity. Although it is not known to which extend this song trait is relevant in natural populations, it lead to statistically significant higher responses in song playback experiments (Kyriacou and Hall 1980, Alt 1998, Kyriacou et al. 1990, Wheeler et al. 1991). In this work it was not observed if the silenced Gal4 lines had different IPI period length´s or not because it was not possible with LifeSong X software to analyze this. However, one cannot exclude that the oscillation of Inter pulse Intervals was affected after silencing certain *fruitless* neurons with or without affecting the overall mean IPI.

Gal4∩fruFLP::Kir2.1	n	number of pulses	mean IPI (+/- SD)	number of *fruitless* neurons (brain/VNC)
Canton-S	30	3968	35,00 (1,53)	
Canton-S∩fruFLP::Kir2.1	16	1897	35,00 (1.76)	
2-38	9	865	29,00 (1,94)	26/15
3-5	9	1398	30,00 (1,84)	15/18
6-4	8	1145	30,00 (1,28)	23/15
6-22	12	1163	32,00 (0,09)	2/4
8-44	16	4278	28,00 (0,95)	19/14
8-102	11	2789	29,50 (1,72)	34/13
8-171	10	1645	30,50 (1,71)	2/2
G215	8	1354	29,00 (4,00)	7/3
G304	8	982	30,00 (1,41)	27/16
NP913	8	2487	30,50 (0,71)	27/14
NP3108	9	1156	29,50 (0,71)	16/11

Table 1. **Silencing of certain *fruitless* neuronal subset lead to a decrease of the mean IPI** (F-Test, $p<0,001$). The mean IPI lenght was compared to wilde type (Canton -S) and to heterozygous flies carrying the fruFLP and the effector gene in their genome. Numbers indicate the name of the Gal4 line, n= number of flies, the mean IPI is given together with the standard error of the mean (+/- SD).

The neuromuscular events generating the wing movement for pulse production is well characterized. The hypothesis is as follow: The indirect, myogenic flight muscles (dorsal longitudinal depressor and dorsal ventral elevators) produce the power for wing vibration, as in flight. The so called axillary wing muscles depress the wing movement by activating the depressors thus producing one up-and-down movement of the wing. One muscle, the sternobasalar muscle uncouples this periodicity producing pulse sound. Sine song is produced by the same muscles as for flight except that the power is reduced (Ewing 1979b, c). There is evidence that one *fruitless* neuron, vPrM2-l (ventrally located motor neuron) innervates one of the

sternobasalar muscles (Yu, 2009). Additionally it might be the case that this neuron therefore regulates the interpulse interval. The Gal4 collection that was used for the screening in this work contains only a few lines that lable this motor neuron and the expression strength of the driver and thus the effector in those lines is not that strong compared to other Gal4 drivers.

As shown in Table1, eleven lines had an statistically significant altered mean IPI lenght and only two of them have an overlap with vPrM2-l. Hence, the involvement of this motorneuron in regulating the interpulse interval within the pulse song is still speculation. The most prominent neuron that overlap between them is another ,in the mesothorax ventrally located, neuron: vMsMt1. Seven out of eleven lines express the silencer in this neuron and it might be the case that vMsMt is an interneuron that is part of the pattern generator for pulse song. To further asses the function of vPrM2-l or vMsMt1 more restricted lines would be needed that express Gal4 strongly enough. In addition a more detailed functional charcterization would be needed in which precise measurements of wing vibration (e.g with high speed video analysis or vibrometry) are taken. Furthermore, activating these neurons and electrophysiology could help to understand their function.

Pulses Per Train (PPT)

The pulse number per train in wilde type *Drosophila* of the *melanogaster* group consists of about seven pulses per train on average. Silencing *fruitless* neurons in eight Gal4 lines lead to a up to 3-fold increase in pulse number per train (p>0,001, T-test). In addition some lines showed an significant decrease in pulse number and those lines were considered to be non singers (see section „Non Singers“). Tables 2A and 2B list those lines with a significant increase in pulse number.

This song trait is potentially interesting because very high pulse numbers could be an indication for affected sensory feedback loops that involve higher integration centers.

mmcAL is the only neuron which all of the „high pulse number“-Gal4 lines have in common. The superior medial protocerebrum is connected via this neuron to the SOG. The lateral protocerebral complex is the region most likely involved in higher order sensory integration. All sensory modalities are received into this brain region and the taste information is relayed into the SOG via the superior medial protocerebrum (Yu 2009).

Except 8-171Gal4 and 2-29Gal4 all lines involved in this kind of phenotype label a lot of neurons, indicating that there are more neurons involved in producing wilde type like pulse train numbers. Again, to further asses the integration of sensory information a closer dissection of the neuronal circuit is needed.

Gal4∩fruFLP::TeTx	n	number of pulses	mean PPT (+/- SD)	number of *fruitless* neurons (brain/VNC)
Canton-S (CS)	30	3074	7,19 (2,36)	
CS∩fruFLP::TeTx	12	1990	6,34 (1,44)	
12-5	9	1324	21,55 (7,48)	22/15
8-102	11	2789	20,93 (3,45)	34/13
2-29	4	956	19,39 (2,64)	28/11
6-22	3	629	19,12 (4,85)	19/14

Gal4∩fruFLP::Kir2.1	n	number of pulses	mean PPT (+/- SD)	number of *fruitless* neurons (brain/VNC)
Canton-S (CS)	30	3074	7,19 (2,36)	
CS∩fruFLP::Kir2.1	12	1990	6,34 (1,44)	
p52a	3	419	17,50 (2,12)	2/4
G132	4	574	16,00 (2,36)	32/20
6-4	8	1673	14,50 (5,03)	23/15
8-171	10	1645	13,00 (3,30)	2/2

Table 2A and 2B: **Mean PPT of lines silenced with Kir2.1.**
The mean pulse number per train is given with the standard error of the mean (+/- SD), n=number of flies recorded.

Non Singers

Except G314Gal4∩fruFLP::Kir2.1 Gal4 lines are indicated to be non singers are listed in Table3A and B. These lines produced either no pulses at all or only 2-3 pulses . Trains with less than 3-4 pulses are per definition no songs (Tauber 2003).

Except G332, there was one other non singer (G94) after silencing with TeTx. Using Kir2.1 to silence *fruitless* neurons in this line (eight out of ten males were analyzed) did not significantly affect the song itself or any song trait analysed compared to wild type flies. This may be due to the strength of this Gal4 line or the more general effect of Kir2.1 compared to TeTx.

These Gal4 lines have relative broadly overlaps with the *fruitless* circuit (see Table 3) and therefore it is hard to interpret these data.

Gal4∩fruFLP::TeTx	**n**	**% of non singing flies**	**number of *fruitless* neurons (brain/VNC)**
Canton-S (CS)	48	<15%	
CS∩fruFLP::TeTx	16	<15%	
G332	24	>95%	15/6
G94	24	>90%	19/0

Table 3A. **Non singing males with silenced fruitless neuronal sub populations.**
The upper table (3A) summarizes results obtained with TeTx, non singers are given as percentage of all recorded males of a line.

Gal4∩fruFLP::Kir2.1	n	% of non singing flies	number of *fruitless* neurons (brain/VNC)
Canton-S (CS)	48	<15%	
CS∩fruFLP::Kir2.1	24	<10%	
11-58	24	>95%	20/0
20-5	24	100%	27/18
G30	24	>90%	24/14
G122	16	>95%	26/13
G332	16	100%	15/6
G336	16	>95%	36/19
G364	16	>95%	35/18

Table 3B. **Non singing males with silenced *fruitless* neuronal sub populations.**
The upper table (3A) summarizes results obtained with TeTx, non singers are given as percentage of all recorded males of a line.

Cycles per Pulse

Each pulse in a song train is made up of 1 to 3 cycles lasting between 5 and 10 ms in *Drosophila melanogaster* (Kulkarni and Hall 1987). To investigate if the mean cycles per pulse are affected at least 500 pulses from >4 different male flies were used for analysis (Supplementary Figure 3). Some Gal4 lines showed a dramatic different song pattern concerning the cycles in each pulse (p>0,01, T-test, Fig. 7). Lines with a higher mean CPP than 1,8 were considered to be „polycyclic“. Table 3 summarizes Gal4 Lines with this phenotype.

Gal4∩fruFLP::TeTx	**n**	**number of pulses**	**mean CPP (+/- SD)**	**number of *fruitless* neurons (brain/VNC)**
Canton-S (CS)	30	3074	1,56 (0,23)	
CS∩fruFLP::TeTx	12	1990	1,59 (0,27)	
8-102	6	1174	2,48 (0,02)	34/13
8-171	6	1360	2,35 (0,33)	2/2
G351	8	4272	2,34 (0,11)	7/8
8-44	5	2782	2,28 (0,59)	19/14
2-29	4	956	2,12 (0,49)	28/11
2-76	5	1133	2,10 (0,09)	25/12
3-8	5	2756	2,09 (0,07)	22/12

Table 3A. **Mean CPP (*fruitless* neuronal subsets silenced with TeTx).**
Mean CPP (+/- standard error of the mean) of male song pulses of Gal4 Lines silenced with Tetanus toxin light chain (TexT) using the FLP-in technique to trace *fruitless* neuronal subsets are listed above. n= number of male flies recorded. Wild type males (Canton S strain) and heterozygous males containing fruFLP as well as the effector gene TeTx in their genome were used as reference.

Gal4∩fruFLP::Kir2.1	n	number of pulses	mean CPP (+/- SD)	number of *fruitless* neurons (brain/VNC)
Canton-S (CS)	30	3074	1,56 (0,23)	
CS∩fruFLP::Kir2.1	16	1498	1,52 (0,21)	
8-102	6	1358	1,72 (0,15)	34/13
8-171	4	514	1,80 (0,19)	2/2
G351	8	1678	2,05 (0,36)	7/8
8-44	8	1651	2,00 (0,23)	19/14
2-29	6	1043	1,96 (0,16)	28/11
NP3108	3	765	2,37 (0,29)	7/7
G274	6	501	2,17 (0,29)	2/3
NP111	4	570	2,15 (0,20)	12/4
7-5	6	1564	2,14 (0,27)	16/11
3-8	4	478	2,10 (0,32)	22/12
9-10	4	544	2,06 (0,03)	0/9
2-41	3	453	2,05 (0,18)	11/18
2-13	6	1256	2,05 (0,89)	3/3
NP3018	4	465	1,99 (0,43)	15/0
p52a	3	491	1,85 (0,03)	2/4
G170	6	2044	1,89 (0,14)	16/5

Table 3B. **Mean CPP (*fruitless* neuronal subsets silenced with Kir2.1).**
Mean CPP (+/- standard error of the mean) of male song pulses of Gal4 Lines silenced with Kir2.1 using the FLP-in technique to trace *fruitless* neuronal subsets are listed above. n= number of male flies recorded. Wildtype males (Canton S strain) and heterozygous males containing fruFLP as well as the effector gene TeTx in their genome were used as reference.

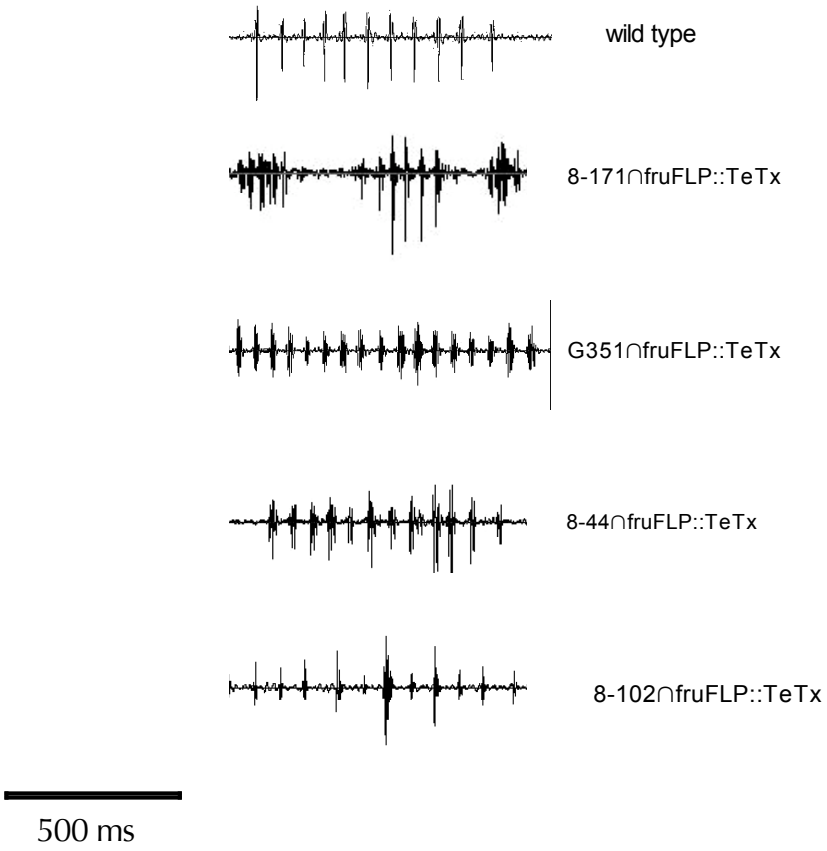

Figure 7. **Song defects in male flies using TeTx to silence *fruitless* neurons.**

Seven Gal4 lines had a polycyclic phenotype using TeTx and were re-tested with Kir2.1. This phenotype was reproducible using the other effector gene, although it had not such a dramatic effect on the pulse cycle number. In sum sixteen lines showed this polycyclic phenotype.

What do these lines all have in common?

As indicated in Table 3 all of them express the driver in various subsets of *fruitless* neurons and some of these neurons overlap among them. In addition some have a broader overlap than others. 12 out of 16 „polycyclic“ lines overlap in 2 *fruitless* neurons, one in the VNC and one in the brain (Yu, 2009). The neuron in the VNC is ventrally located and is called vPrM1-l.

The neuron in the brain is called mmcAL. In addition 3 other neurons could be found after comparing those Gal4 lines: aSP-2 (ten lines out of sixteen express the effector gene in this neuron), vMsMt1 and vMtAb (nine out of sixteen lines express the effector in those neurons).

Based on the *fruitless* map (Yu 2009) the following conclusions can be made:

vPrM1-l is a neuron located in the prothoracic segment and contributes to the anterior dorsal complex. This complex contains neurons that are most likely part of the song generator in male flies. Early studies using electrophysiology and other studies suggest that the song generator in flies evolutionary derived from the flight circuit (Ewing 1979b, Crossley 1990). Meaning that the central pattern generator for song produces a phasic output and that also sensory feedback is involved in which proprioceptive wing stretch receptors dampen wing oscillations in that way that only single cycle pulses are produced (Wolf 1991). There are some mechanosensory mutants that are not able to produce this sensory feedback thereby generating songs which are often polycyclic (Tauber and Eberl 2001). From that view one possibility would be that vPrM1-l is involved in maintaining this feedback.

The other prominent neuron found in this study is mmcAL. This neuron connects the Pars Intercerebralis (PI) with the anterior Subesophageal Ganglion (SOG) and may also be involved in this mechanosensory feedback in a pathway that connects to the brain without involvement of higher order brain centers (Yu 2009). aSP-2 is the second *fruitless* neuron in the brain that is most likely required for producing a normal courtship song pattern concerning pulse cycles. It is an interneuron with ipsilateral projections to the lateral protocerebral complex-arch, a neurite trackt that connects the protocerebral complex of both brain hemispheres. Two other ventrally located neurons in the VNC may be involved in producing proper courtship sound pulses: vMsMt1 and vMtAb. vMsMt1 is a projection neuron located in the mesothoracic ganglion, the region of the VNC that is mostly considered as the region containing the central pattern generator for song (von Schilcher 1979).

Intrapulse Frequency (IPF)

The intrapulse frequency of a certain pulse is the high energy peak of a certain pulse after Fourier transformation of that pulse. The IPF is sometimes called Carrier frequency (Wheeler 1988). Neurons that are required for a certain Intrapulse frequency may be involved in modulating the pattern generator for song.

In *Drosophila melanogaster* the mean IPF lies between 230 and 280 Hz. In this study songs with significantly higher and significantly lower intrapulse frequencies were observed (p>0,001, T-test) as indicated in Table 4A and 4B.

Gal4∩fruFLP::TeTx	**n**	**number of pulses**	**mean IPF (+/- SD)**	**number of *fruitless* neurons (brain/VNC)**
Canton-S (CS)	30	3074	250 (18)	
CS∩fruFLP::TeTx	12	1990	279 (21)	
8-102	6	1174	191 (17)	34/13
8-171	6	1360	348 (24)	2/2

Table 4A. **Mean IPFs of courtship songs with silenced *fruitless* neuronal subsets using Tetanus Toxin Light chain (TeTx) as effector.**

It is almost impossible to trace that behavior down to a certain *fruitless* cluster since the lines shown in Table 4A and 4B do not have a common overlap with the circuit.

But the fact that both, high frequency and low frequency pulse songs could be observed indicate that parts of the circuit are likely to activate or inhibit the song output. Nine lines showed a phenotype with Kir2.1 whereas only two lines had an IPF-phenotype when TeTx was used. Although all of these lines were both tested with TeTx and Kir2.1. This indicates that there may be electrical couplings between neurons that are crucial for producing a certain carrier frequency.

Gal4∩fruFLP::Kir2.1	n	number of pulses	mean IPF (+/- SD)	number of *fruitless* neurons (brain/VNC)
Canton-S (CS)	30	3074	250 (18)	
CS∩fruFLP::Kir2.1	16	1498	237 (21)	
3-8	4	478	198 (93)	22/12
G132	6	1785	200 (19)	23/20
8-171	6	1360	204 (25)	2/2
8-102	6	1174	225 (20)	34/13
2-38	6	565	212 (12)	26/15
NP37	4	519	214 (11)	6/0
G351	8	1678	315 (53)	7/8
G274	6	501	327 (84)	2/3
NP3108	4	465	371 (20)	7/7

Table 4B. **Mean IPFs of courtship songs with silenced *fruitless* neuronal subsets using Tetanus toxin Light chain (TeTx) as effetcor.**

Summary and Outlook

The Information flow in the nervous system of *Drosophila*, as in all other animals, is gernerated by electrically pattern changes along neurons. The underlying mechanism is the depolarization of neurons which are connected to one another other via chemical or electrical couplings (synapses). Neuronal circuits have the probability to store and process information by synaptic connectivity and strength (neuronal plasticity). A common feature of neuronal circuits in all organisms are the involvement of feedback mechanisms that are able to produce certain patterns and states (Lu 2009, Spencer and Ghausi 2003).

Although some behaviors have been mapped to neuronal circuits (e.g. in Drosophila: learning, circadian rhythms, sleep, aggression, responses to drugs and courtship) it is still unclear how the information processing occurs that underlies this behavior. For instance, fundamental roles of sensory feedback mechanisms that influence motor patterns are not well understood as well as the integration of sensory modalities that are then transformed to influence motor outputs. The possibility to manipulate neurons for a functional study is a powerful tool to dissect neuronal circuits and also to reprogram these circuits that may lead to predictable changes in behavior in well defined circuits. Computational models can then be used to fit certain behaviors after changing well defined circuit properties or to replay different activities artificially.

The results in this work educes that the selective activation of some thoracic f*ruitless* neurons indeed seem to be involved in the love song production of *Drosophila melanogaster*. It is likely that the f*ruitless* circuit is either part of the central pattern generator for pulse song or modulates it. At least the production of proper pulse cycle numbers. Furthermore, *fruitless* neurons in higher brain centers seem to be involved. For instance, the PPT phenotype shows that some of these neurons are required for this song trait.

During the course of this work it became more and more clear that there are various technical limitations. For instance, the availability of driver lines. If one use the network theory as working hypothesis, one cans imagine that deleting certain parts of a network may affect the outcome differently. Thus, it is of importance to have enough lines that overlap among each other to get an idea how the network is organized. To overcome this problem in the future more specific driver lines are needed that express the reporter/effector in only a small subset of neurons. Currently, thousands of enhancer trap lines are being generated to create a more specific and more reproducible tool for functional studies of the nervous system in *Drosophila melanogaster* (Rubin et al. 2008, Dickson et al. unpublished).

On the other hand it turned out that the analysis of certain song traits was almost impossible with the available recording system. The sine song could not be analyzed, simply because of the high signal-to-noise ration of the recorder. Because of technical problems the analysis was done manually. In future it would be more suitable to have a more precise and noise resistant, automated system . Then one can program an algorithm that is able to detect the pulse and the sine song and their properties in a certain song pattern, for instance with Matlab. In addition, correlations among certain song traits and statistics could easily be done.

To ask if certain *fruitless* neurons are not only required, but also sufficient for pulse song generation in males, one should activate certain *fruitless* neuronal subsets e.g. with TrpA1 or other effector genes that are able to activate neurons (Miesenböck et al. 2008, Hamada 2008).

The combination of electrical silencing and activation together with their electrophysiological characterization and immunohistochemistry may give a deeper insight into the function and involvement of *fruitless* neurons in the song circuitry.

In this view this study represents only a part of the experimental approaches that have to be done to dissect the neuronal circuit underlying the song generation in male flies and the specific role of *fruitless* neurons within this circuit.

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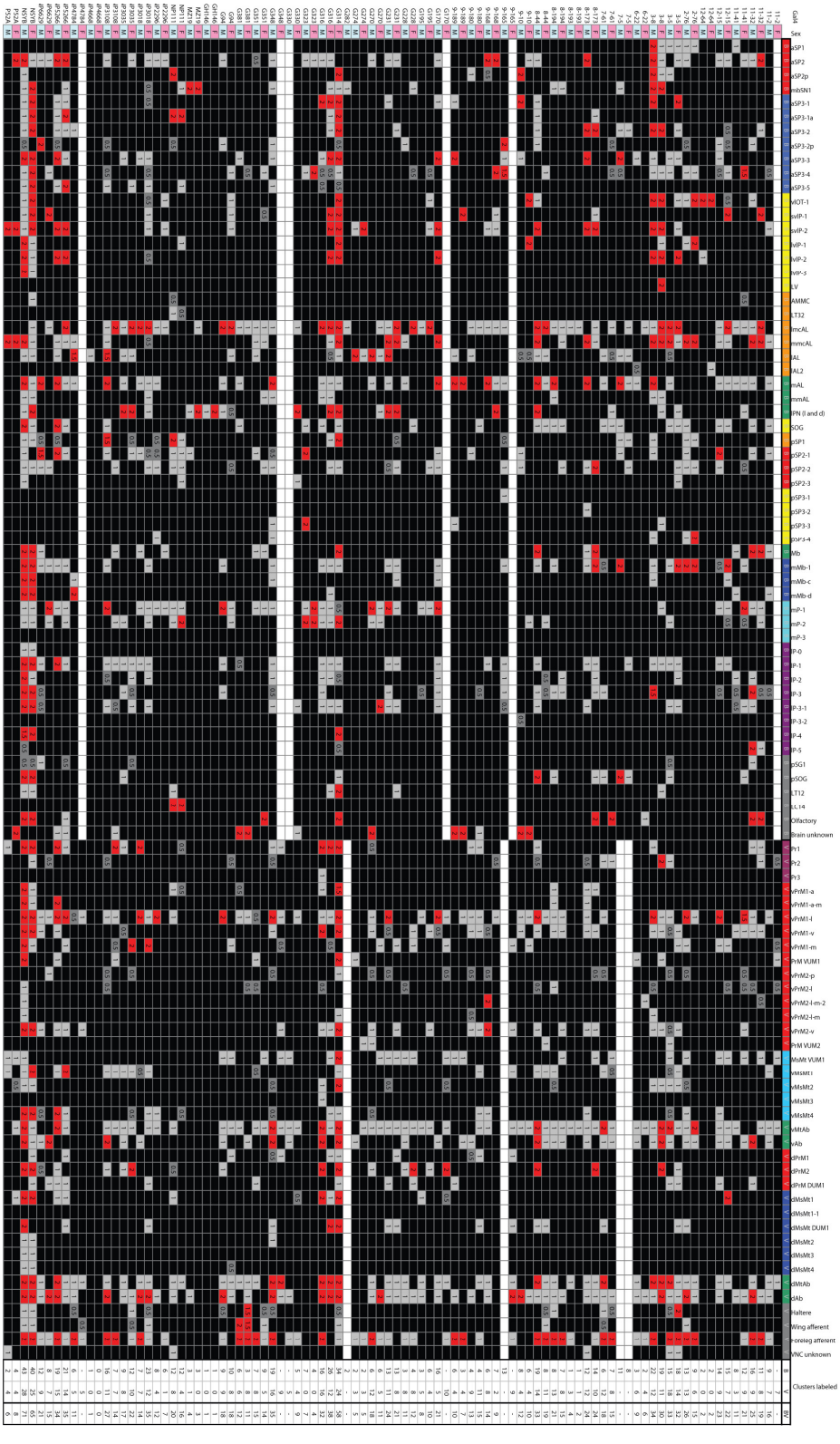
Appendix

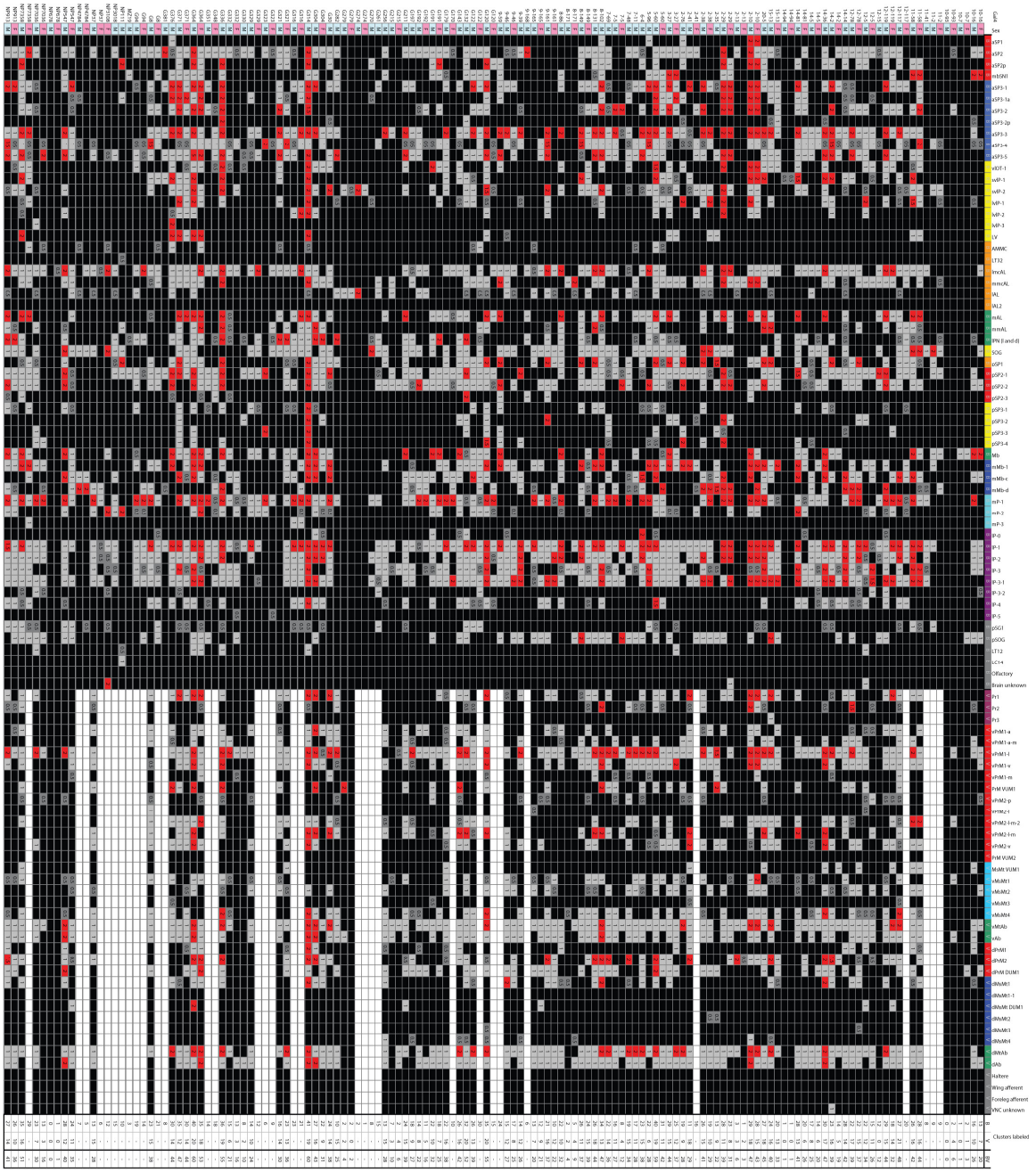
Gal4 Driver	Brain fruitless subset	Brain fruitless subset	tested using the Kir2.1 effector	tested using the TeTx effector	pulse song phenotype
2-10	29	18	+	-	
2-13	3	3	+	-	CPP
2-29	28	11	+	+	PPT, CPP
2-38	26	15	+	-	IPI, IPF
2-41	11	18	+	-	CPP
2-76	25	12	+	-	CPP
3-5	15	18	+	-	IPI
3-8	22	12	+	+	CPP, IPF
3-27	29	15	+	-	
5-60	40	19	+	-	NS?
6-4	23	15	+	-	IPI, PPT
6-22	19	14	+	-	IPI, PPT
7-5	16	11	+	-	CPP
7-11	17	11	+	-	
7-48	22	17	+	-	
7-61	7	8	+	-	
7-69	22	14	+	-	
8-44	19	14	+	+	IPI, CPP
8-86	22	10	+	-	
8-102	34	13	+	+	IPI, PPT, CPP, IPF
8-171	2	2	+	+	IPI, PPT, CPP, IPF
8-193	1	1	-	-	
9-10	0	9	+	-	CPP
9-39	14	12	+	+	
9-165	13	-	-	-	
9-168	6	8	-	-	
10-7	1	0	-	-	
11-2	9	7	-	-	
11-58	20	0	-	-	NS
12-5	22	15	+	-	PPT
12-15	12	-	-	-	
12-78	28	4	+	-	
12-119	18	14	+	+	

Gal4 Driver	Brain fruitless subset	Brain fruitless subset	tested using the Kir2.1 effector	tested using the TeTx effector	pulse song phenotype
14-14	28	14	-	-	
14-36	30	17	+	-	
20-5	27	18	+	-	NS
G30	24	14	+	-	NS
G94	19	-	+	-	NS
G132	32	20	+	-	PPT, IPF
G122	26	13	+	-	NS
G170	16	5	+	+	CPP
G195	3	5	+	-	
G215	7	3	+	-	IPI
G256	13	15	+	-	IPI
G274	2	3	+	-	CPP, IPF
G304	27	16	+	-	IPI
G314	41	19	+	+	NS
G316	34	24	+	+	NS?,
G321	16	14	+	-	NS?
G323	7	-	+	-	
G332	15	6	+	-	NS
G336	36	19	+	-	NS
G348	18	-	+	-	
G351	7	8	+	-	CPP, IPF
G364	35	18	+	-	NS
NP37	6	-	+	-	IPF
NP111	12	4	+	+	CPP
NP913	27	14	+	-	IPI
NP3018	16	4	+	-	CPP
NP3035	9	8	+	+	
NP3108	7	7	+	+	IPI, CPP, IPF
NP4748	6	5	+	-	
p52a	2	4	+	+	CPP
ppk	?	?	+	+	
nSyb	43	28	+	+	NS?

Supplementary Table1. ***Fruitless*** **sub populations of driver lines used in the screen**

The table shows the fruitless overlap of each analyzed driver in male flies (brain vs. VNC)the effector that was used and the pulse song phenotype (NS= non singer, NS? = only 8 flies tested, IPI= interpulse interval, PPT= pulsen number per train, CPP= cycles per pulse, IPF= intrapulse frequency). Data are adopted from Yu 2009.

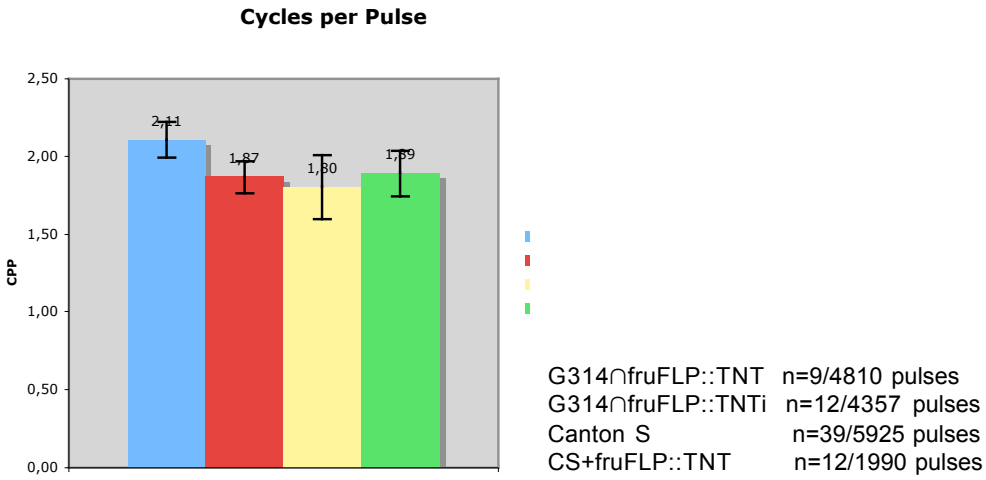




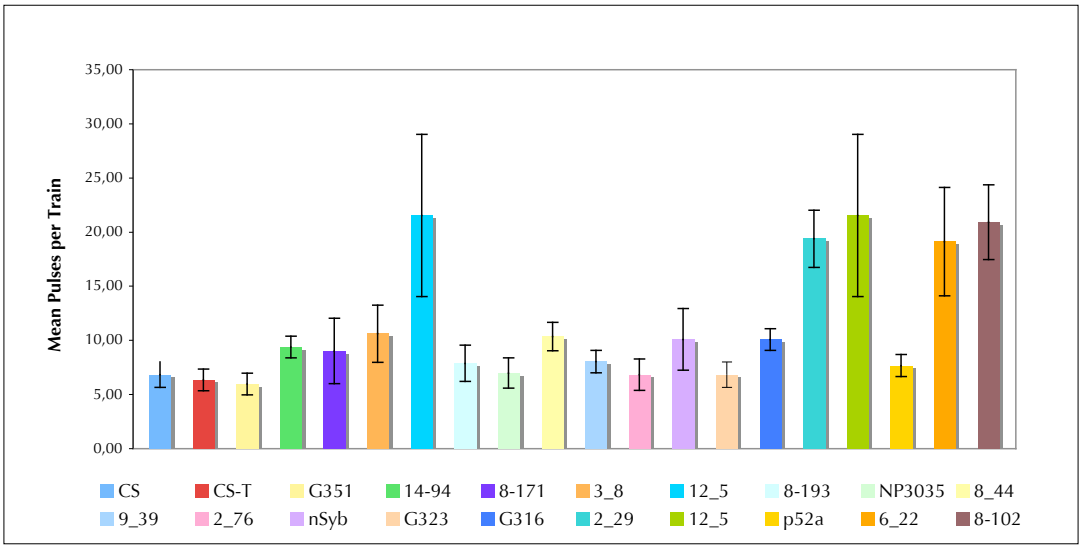
Supplementary Table 2A (page 59) and 2B (this page).

Gal4 lines labeling fru^{M} clusters.

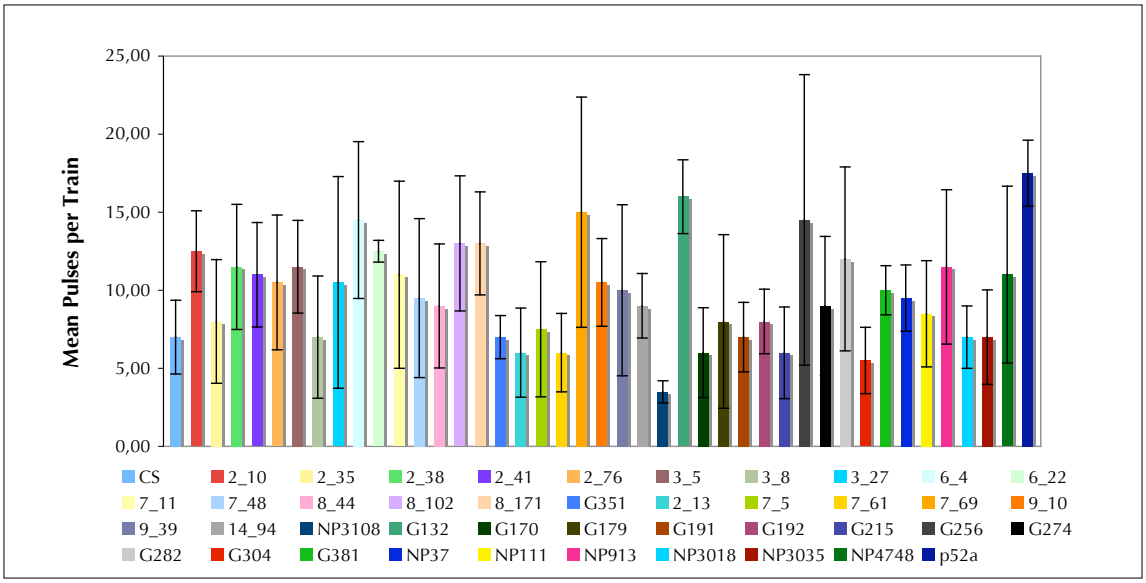
The Table includes selected Gal4 lines that were chosen for the screening in this study. Figure 2A shows Gal4∩FruFLP::mCD8GFP and Figure 2B shows Gal4∩FruFLP::tauLacZ. (M=males, F= females, B=brain, V= VNC). The strength of expression of the reporter genes is given in numbers (0=no labeling to 2=strong labeling). Tables adopted from Yu 2009.



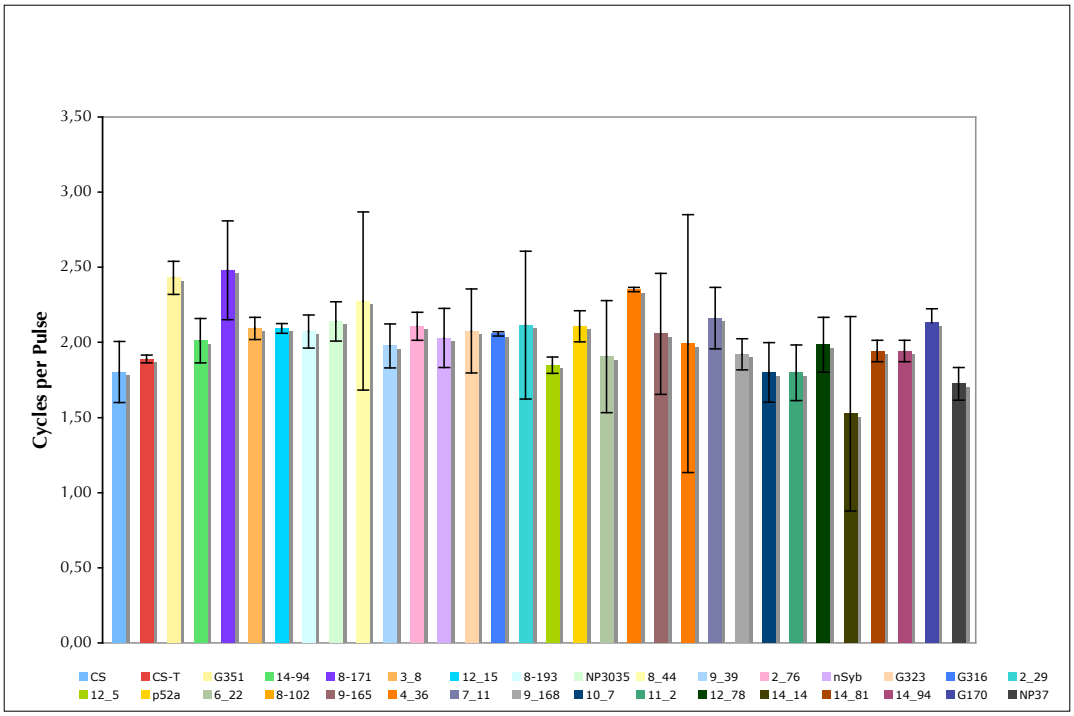
Supplementary Figure 1.**Mean CPP of G314nfruFLP::TeTx.**
Bars indicate +/-S.E.M



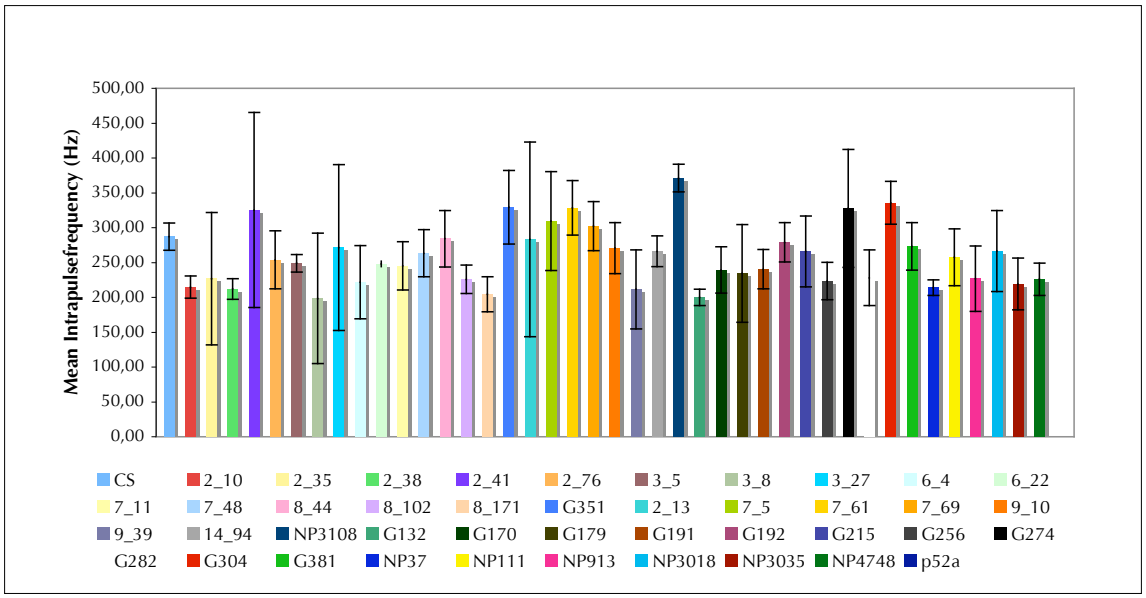
Supplementary Figure 2A. **Mean PPT of lines silenced with fruFLP::TeTx.**
Bars indicate +/- S.E.M.



Supplementary Picture 2B: **Mean PPT of lines silenced with fruFLP::Kir2.1.**
Bars indicate +/- S.E.M



Supplementary Figure 3. **Mean CPP of pulse songs from lines with silenced *fruitless* neuronal subsets**.
Bars indicate +/- S.E.M.



Supplementary Figure 4. **Mean IPF of Gal4 lines with silenced *fruitless* neuronal subsets**.
Bars indicate +/- S.E.M.

Curriculum vitae

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