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Expression and Function of Fruitless Isoforms in *Drosophila melanogaster*

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Synopsis

Male sexual behaviour is hardwired in the *Drosophila* nervous system due to the expression of male-specific *fruitless (fru)* products. Those appear as alternative splice variants, termed the Fru^{M} isoforms Fru^{MA}, Fru^{MB} and Fru^{MC}. Each Fru^{M} isoform carries a distinct zinc-finger domain that likely allows it to function as a transcriptional regulator. Accordingly, we hypothesised that distinct isoforms have specific functions in sub-behaviours of the courtship ritual which might also be reflected in distinct expression patterns and molecular functions. For that purpose we generated mutants affecting the zinc-finger domains of Fru^{MA}, Fru^{MB} and Fru^{MC} as well as isoform specific antibodies and myc tagged alleles. Here, we show that all of the *fru* isoform mutants display distinct differences in overall courtship performance as well as in sub-behaviours. This holds true if aspects of the behaviour are analysed quantitatively as well as qualitatively. The strongest impairment is seen in fru^{C} mutants, which do not generate courtship song and fail to copulate. In search for the basis of those differences we first investigated the expression pattern of each of the *fru* isoforms and found that there is surprisingly little difference between their expression patterns, i.e. each isoform is expressed in the majority of the *fru* neurons, yet we also observe that some distinct expression. Different behavioural functions therefore are likely to be based in both the distinct molecular actions and the distinct expression pattern of the Fru^{M} isoforms. Furthermore we addressed the cellular phenotype of the *fru* isoform mutants on the anatomical level of the sexually dimorphic *fru* circuit. The most severe phenotype is seen again in the fru^{C} mutant where a majority of neurons display a female like anatomy. We hence postulate that sexually dimorphic circuit anatomy is highly relevant for the sex-specific courtship behaviour. Finally, we attempt to map the neuronal substrates in which Fru^{MC} is required for generation of song. Initial results suggest the known song neurons P1 and vPR6 as well as some novel neurons.

Zusammenfassung

Das Paarungsverhalten männlicher Fruchtfliegen der Art *D. melanogaster* ist angeboren und basiert damit auf, in der Entwicklung fixierten, neuronal Netzwerken. Der genetische Faktor, der sowohl ursächlich als auch instruktiv für das männliche Nervensystem und Verhalten ist, ist *fruitless* (*fru*). Bei Fru^{M} handelt es sich um einen potentiellen Transkriptionsfaktor der spezifisch in Männchen in drei Spleißvarianten exprimiert wird. Diese Varianten Fru^{MA}, Fru^{MB} und Fru^{MC} unterscheiden sich deutlich in ihren Zink-Finger Domänen. Diese Zink-Finger machen ihre Bindungseigenschaften als Transkriptionsfaktor aus. Daraus ergibt sich die folgende Hypothese: Verschiedene Fru^{M} Varianten haben unterschiedliche Funktionen für das Paarungsverhalten. Die Ursachen hierfür können sowohl in ihrem Expressionsmuster als auch in ihrer molekularen Funktionsweise liegen. Um diese Hypothese zu Untersuchen haben wir einerseits Mutanten erzeugt, die speziell die Zink-Finger Domänen der Isoformen betreffen und darüber hinaus Antikörper, die diese Domänen erkennen. Wir beobachten, dass sich die fru^{A}, fru^{B} und fru^{C} Mutanten erheblich in ihrem Paarungsverhalten unterscheiden, sowohl quantitativ als auch qualitativ. Die stärkste Beeinträchtigung zeigen Mutanten der Fru^{MC} Isoform, die den wichtigen Paarunsgesang nicht generieren können und damit auch nicht erfolgreich sind bei ihren Kopulationsversuchen. Interessanterweise sind die drei Isoformen dennoch in einem großen Teil aller *fru* Neurone präsent. Dennoch gibt es eine Reihe von Neuronentypen die nur eine einzige Isoform exprimieren. Die unterschiedlichen Funktionen werden daher vermutlich zum einen von der Spezifität der Zink-Finger Domäne und zum anderen von der Spezifität der Expressionsmuster hervorgerufen. Um die zellulären Ursachen für die verschiedenen Verhaltensphänotypen zu Untersuchen haben wir die neuronale Netzwerkmorphologie genauer analysiert. Es ist bekannt, dass die *fru* Neurone sex-spezifisch beziehungsweise sexuell dimorph sind. Wir können zeigen, dass

diese Dimorphismen der Neurone abhängig von Fru^{M} sind und zwar hauptsächlich von der Fru^{MC} Isoform. Wir postulieren daher, dass die neuronale Morphologie maßgebend für die Maskulinisierung des *fru* Netzwerkes und damit des Paarungsverhaltens ist. Schließlich versuchen wir die Neurone zu identifizieren, in denen Fru^{MC} notwendig ist um den Paarunsgesang hervorzurufen. Erste Ergebnisse deuten an, dass es sich dabei um die P1 und vPR6 Neurone, die schon bekannt sind für ihre Rolle im Paarunsgesang, und einige bis jetzt unbekannte Neurone handelt.

1 Introduction

1.1 Motivation

Behaviour is a unifying feature of all living animals and is essential for their survival and reproduction. It comprises all observable actions that are elicited in response to stimuli. Only recently at the beginning of the 20th century the first systematic steps have been taken to understand the organisation and elicitation of animal behaviours. But it was the technical progress in the fields of genetics and neurophysiology that allowed the analysis of the underlying mechanisms at a level beyond the observation of organism behaviour. Subsequently it was recognized that genes instruct the development and physiology of the nervous system, the organ controlling behaviour. At present, it is a major goal of neuroscience to investigate in molecular detail how nervous systems are built and function in order to generate behaviour.

1.2 Genes, Neurons and Behaviour

Three men were instrumental to the field of ethology in the first half of the 20th century with their studies on the organisation and elicitation of individual and social animal behaviours: Max von Frisch, Nikolaas Tinbergen and Konrad Lorenz. Behaviours can have proximate as well as ultimate causes. Proximate, or direct, causes relate to the immediate external or internal stimuli that trigger behaviour. Ultimate, or indirect, causes concern the evolutionary relevant aspects of behaviour. These are the causes of behaviour but what is the basis? In other words, to which extent are behaviours determined by genes and to which extent by environment?

One of the first studies that pointed out the heritability of certain human abilities was carried out by Francis Galton in the 19th century. The field of behavioural genetics has gained recognition in the middle of the 20ths century and had a highly controversial history, specifically concerning human behaviour. It is often associated with the "nature versus nurture" debate. A logic approach to assessing questions in this field is to keep one variable, either the genetic information or the environment, constant while varying the other. This has naturally limitations in studies of human behavioural genetics and the research in this field has long been limited to twin or adoption studies.

Modern genetic analysis does however allow correlation studies by linking certain behavioural traits to their genetic basis. The combination of genome with phenotype studies on an individual or population level highlighted the heritability of human traits. In this context it has been valuable to study natural variation of genes in cases where there are obvious phenotypic effects like human diseases. Those studies pinpointed to cases where single genes are critical factors in certain human behavioural traits, like Huntingtons disease. On the other hand it became apparent that most complex behavioural traits and diseases, like diabetis, in humans are multigenic. Those multigenic traits are often under high influence of environmental factors.

The genetic impact on simple or complex behaviours can be examined much more stringently in model organisms like mice and fruit flies. Tremendous insights in how genes govern behaviour came from the work of Seymour Benzer with the fruit fly *Drosophila melanogaster (D. melanogaster)* in the second half of the 20th century. His lab discovered one of the first genes that had a unique role in behaviour (Konopka and Benzer, 1971). This gene was called *period (per)* since flies carrying mutations in this gene displayed arrhythmic circadian behaviour. In fact it appeared that it is the complex interactions of several oscillating circadian genes, among them *per,* in combination that lead to circadian behaviour (Allada, 2003). Circadian behaviour in *D. melanogaster* is one example of a behavioural phenotype being an emergent property

of the action of gene networks. In contrast, evidence for a single gene shaping a behavioural phenotype is also rare in *D. melanogaster.* The most prominent representative is the gene *fruitless* (*fru*) that was shown to be instructive for male courtship behaviour and is subject of investigation in this thesis. But even in fruit flies behavioural phenotypes are not only under genetic constrains but also plastic and modifiable by environmental influences or experience.

The picture that emerges from human as well as animal studies is the following: All behaviour is shaped by the interplay of genes and environment. Behaviour is thus intimately related to genes. Genes are in turn under evolutionary selection from the environment. The complex relationship between genes and behaviour can be best described as: all animal behaviours are gene-dependent, but no behaviour is gene determined.

The question therefore is not so much whether there are behaviours that are instructed by genes but rather how (Baker et al., 2001). What is the link between genes and behaviour? Genes were found to unfold their roles in behaviour through their action in cells. Specifically, genes instruct the development and physiology of the nervous system and its functional units, the neurons. On the structural level genes guide circuit building while on the physiological level they guide the activity patterns of the circuit. Thereby, genes can set both rigid innate behaviours as well as provide the nervous system with flexibility to adapt and learn from environment cues.

The quest for understanding the mechanisms by which a nervous system senses, interprets and processes information in order to generate appropriate behavioural outputs is known as neuroethology. With the development of sophisticated experimental techniques for addressing a neuron`s physiology from the 1970s on the knowledge in this field grew. As a consequence several concepts on how neurons are organised and function in order to generate behaviours have been established. The

major goal of understanding the molecular and cellular basis of a behaviour in detail, from the genetic make-up of neurons via their assembly in a neuronal circuit and its functional mechanisms, has however not been accomplished yet.

In order to achieve this aim the fruit fly *D.melanogaster* serves as an ideal model to understand behaviour from genes to organism. With its 10^5 neurons the nervous system of *D.melanogaster* is relatively simple compared to mammals. Furthermore, the small fly possesses a rich repertoire of behaviours from simpler locomotion associated behaviours like sleep, flight and gap crossing to sensory behaviours like gravitaxis, olfaction and nociception to more complex behaviours like courtship, aggression and learning. The most important advantage *D.melanogaster* offers is the extensive array of genetic tools that allows the dissection of the function of genes and neuronal circuits relevant for behaviour (Simpson, 2009).

1.3 Organisation and Function of the Nervous System in *D.melanogaster*

In adult fruit flies sensory neurons in legs, mouthparts, antennae, wings, eyes, halters and genitalia transmit chemical, visual, thermal and mechanical information about the insects` environment from the periphery to the central nervous system. The brain and the ventral nerve cord are the two primary structures of the central nervous system and are composed of fused ganglia (Figure 1 A) (Cranston and Gullan, 2004). The neuronal cell bodies are located on the surface of the brain and ventral nerve cord, called the rind, while the neurites project inside to form the neuropil which contains neural fibers and synaptic arborisations. Glial cells can be found both in the rind and in the neuropil.

The brain can be divided into three parts: the optic lobes, the protocerebrum and the suboesophageal ganglion. The latter two are also referred to the central brain. The

optic lobes are the primary neuropils for processing of visual information from the eyes. The suboesophageal ganglion is innervated by nerves from sensory neurons of the mouthparts and is the primary center for processing of gustatory input. The protocerebrum includes the sensory neuropils for the antennal lobes that convey olfactory information as well as the antennal mechanosensory and motor center for auditory information (North and Greenspan, 2007). The ventral nerve cord is composed of fused thoracic and abdominal ganglia. It is innervated by nerves form the sensory neurons of the wings, legs and abdomen. Higher neuronal integration centers are believed to be located in the central brain and include the mushroom body and the central complex, which are functionally well described, as well as the lateral horn, the lateral complex and superior, inferior, ventromedial and ventrolateral neuropils, which are functionally poorly described (Zars, 2000; Strauss, 2002).

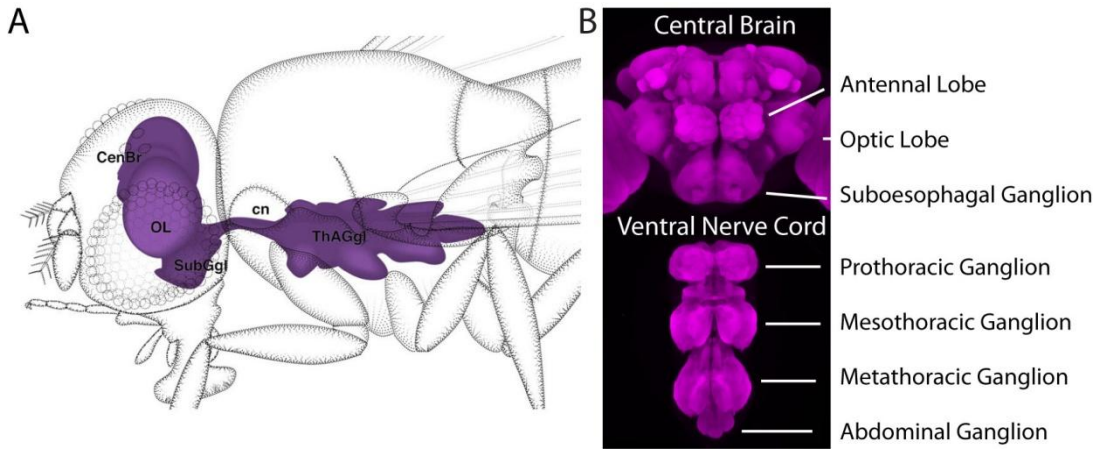


Figure 1. Organisation of the central nervous system in *Drosophila*

(A) Schematic of the adult fly. The head contains the brain consisting of the central brain (CenBr), the optic lobes (OL) and the suboesophageal ganglion (SubGgl). The thoracic ganglion (ThAGgl) lies in the thorax and is connected with the brain via the cervical nerve (cn). (Hartenstein, 1993) (B) A standard central brain and ventral nerve cord with their important ganglia are displayed. Staining is performed against *nc82* (Yu et al., 2010).

The ventral nerve cord is composed of four major neuropils the prothoracic, the mesothoracic, the metathoracic and the abdominal ganglion (Figure 1 B). Motor neurons are mainly situated in the ventral nerve cord from where they innervate the leg, wing and abdominal muscles. Furthermore, motor neurons are also found in the suboesophageal ganglion where they send axonal projections to the mouthparts. Central pattern generators are supposedly located in the ventral nerve cord but at present remain barely understood.

There are thought to be about 100 000 neurons in the central nervous system of *D.melanogaster*. Of these 100 000 neurons, one third is located in the two optic lobs, one third in the central brain and the other third in the ventral nerve cord. The brain and the ventral nerve cord are connected by approximately 3600 axons of ascending or descending neurons that pass through the cervical connective. Fruit flies use the canonical neurotransmitters acetylcholine, glutamate, GABA, histamine, dopamine, serotonin, tyramine and octopamine. In contrast to vertebrates, where the major excitatory neurotransmitter in the central nervous system is glutamate, in *D.melanogaster* it is acetylcholine (Simpson, 2009).

1.4 Genetic Toolbox of *D. melanogaster*

In *D.melanogaster* highly sophisticated genetic manipulations are possible and thereby permit functional analysis on the molecular as well as cellular level (Venken and Bellen, 2007). The following section aims to provide an overview over the history of genetics in *D.melanogaster* and the techniques used in this study in order to elucidate gene and cell function. The roots of *D.melanogaster* genetics can be found with the classic experiments from Herman Muller in the 1920s when he showed for the first time that radiation leads to lethal mutations in fruit flies. This classic technique of x-ray

mutagenesis was later complemented by the use of alkylating agents like ethane methyl sulfonate (Fishbein et al., 1970).

An important step towards targeted transgenesis in *D. melanogaster* was the discovery of transposons called P-elements in wild type strains of *D.melanogaster* in the 1970s. Since P-elements were not present in the laboratory strains they were used to not only disrupt genes but also for the introduction of genetic material into the fruit fly`s genome (Rubin and Spradling, 1982; Karess and Rubin, 1984). One of the attempts to eliminate position effects, that were acting on P-elements, lead to the development of *in vivo* gene targeting through homologous recombination. This is achieved either via 'ends-in' or insertional gene targeting (Rong and Golic, 2000) or 'ends-out' or replacement gene targeting (Gong and Golic, 2003). Another method to control for position effects is to insert the transgene at a known site. This technique uses the bacteriophage ϕC31 integrase (Groth et al., 2004). This integrase catalyzes the stable recombination of an *attB* containing plasmid with an *attP* containing landing site that has been introduced into the *D.melanogaster* genome via P-element. Importantly, ϕC31 integrase mediated transgenesis allows the integration of larger fragments into the genome than P-element mediated transgenesis (Venken et al., 2006).

One avenue that has proved very productive for understanding neuronal function has come from the ability to genetically manipulate neuronal subpopulations. These manipulations depend on effective transgenic techniques and over the past decade truly came into the state of the art in *D.melanogaster*. One of the key advancements has been to take exogenous binary expression systems from other organisms. This allows one an unprecedented level of combinatorial control since any genetic element can be expressed under any control element without the need to generate a new transgenic fly each time. There are three binary expression systems in *D.melanogaster.* All of them exploit the specific binding of an exogenous transcription factor to its defined binding site: The yeast GAL4/UAS system, the bacteria LexA/LexAO system and

the Q system from fungi (Brand and Perrimon, 1993; Lai and Lee, 2006; Potter et al., 2010). Out of these systems the GALl4/UAS system is the most widely used (Duffy, 2002). These systems contain two parts: 1) the driver consisting of the exogenous transcription factor expressed under the control a native *D.melanogaster* promoter or enhancer and 2) the reporter consisting of the desired genetic element under the control a native *D.melanogaster* minimal promoter engineered to contain the respective binding site for the exogenous transcription factor.

A crucial aspect is the appropriate spatio-temporal expression of the driver element, most importantly when analyzing neuronal function. There is little understanding of how the composition and assembly of regulatory genetic elements reflect the resulting expression pattern. Drivers that have been generated by P-element insertion as enhancer traps usually recapitulate the expression of the most proximate gene (Brand and Perrimon, 1993). Due to the influence of multiple enhancers on this enhancer trap line its expression is rather broad (Hayashi et al., 2003). But recently it was demonstrated that the expression of a genetic element can be directed to distinct small subsets of cells by inserting it in proximity of a 3-kb enhancer fragment (Pfeiffer et al., 2008). In combination, numerous techniques exist for the spatial and temporal restriction of transgene expression (Luan and White, 2007; McGuire et al, 2003; Basler and Struhl, 1993).

Finally, there are two types of genetic reporter elements whose expression elucidates gene function. On the one hand classic tissue specific rescue experiments and on the other hand tissue specific knockdown via RNA interference can be performed (Kennerdell and Carthew, 2000; Dietzl et al., 2007; Haley et al., 2008). For cell physiology a variety of genetic elements can be used in reporter constructs to label, eliminate, silence and activate neurons or just simply monitor their activity (Simpson, 2009).

1.5 Courtship Behaviour in *D. melanogaster*

The courtship behaviour of the male fruit fly is an ideal model to investigate the genetic and neuronal basis of behaviour. *D.melanogaster* courtship is both a robust and a complex behaviour. Most importantly, this stereotypic sequence is innate and therefore likely to be hardwired in the nervous system throughout development. *D.melanogaster* courtship was first described almost a century ago by Sturtevant and has since been extensively investigated (Spieth, 1974; Siegel et al., 1984; Hall, 1994; Greenspan and Ferveur, 2000).

The behaviour consists of a series of actions enabling the exchange of visual, auditory, olfactory, gustatory and tactile information between the sexes (Figure 2 A). The male, once he has encountered a female, orients towards and pursues her in order to touch her abdomen and hind legs. At this point the male is able to assess the pheromonal profile of the female and hence the identity of sex and species (Shorey and Bartell, 1970; Ferveur et al., 1997, Bray and Amrein, 2003). Next the male circles around the female and extends and vibrates a wing to generate the courtship song, a species specific component of courtship behaviour (Figure 2 B) (Shorey, 1962). The song consists of two components: 1) a continuous oscillation termed `sine song` and 2) a train of pulses the `pulse song`. The time between two pulses, the inter pulse interval, is species dependent. For *D.melanogaster* it is typically 35 ms. Each pulse consists of 1-3 cycles and the number of pulses in one train can vary between 2 and 50 (Kulkarni and Hall, 1987). The final courtship steps comprise the male touching the female`s abdomen with its proboscis, bending its abdomen for attempted copulation and eventually successful copulation.

What are the relative roles or contributions of the different steps concerning copulations success? Initial visual and pheromonal inputs are multimodal and therefore slightly more redundant. Loss of either vision or olfaction or both in combination does

not abolish the entire behaviour but decreases the courtship level (Gailey et al., 1986). One of the more important aspects of successful courtship performance for the male is the correct performance of courtship song, specifically the appropriate length and cycling of the inter pulse interval of the pulse song. The sine song has a more minor role and is thought to prime females prior to mating. Males that are not able to produce courtship song hardly succeed in copulation (Bennet-Clark et al., 1976). The occurrence of polycyclic pulse song does, however, not decrease mating success (Kulkarni and Hall, 1987). While touching the abdomen once more conveys pheromonal information, the crucial action is the successful grabbing of the female with the help of the sex combs on the male`s foreleg and the bending of the abdomen (Ng and Kopp, 2008; Finley et al., 1997).

The female part of the mating behaviour is, though from the observers' point of view rather straightforward, as sophisticated as the males` part. If she did not mate within the last 6 days she is receptive and slows down upon evaluation of the male pheromonal profile and courtship song to allow copulation (Manning, 1962; Bennet-Clark et al., 1976; Kurtovic et al., 2007; Stowers and Logan, 2008). If a female has recently mated she is unreceptive and will not accept the courting male. Rather, she actively displays rejection behaviours comprising ovipositor extrusion, kicking and wing flicking (Manning 1967). Furthermore the female carries traces of the male pheromone cis-vaccenyl acetate (cVA) a remnant of recent mating (Kurtovic et al., 2007). Subsequently the male will have learned to interpret those cues and be conditioned to suppress courtship towards mated females (Siegel and Hall, 1979). This demonstrates that though male courtship behaviour is per se innate it is also modifiable by experience. This is known as courtship conditioning.

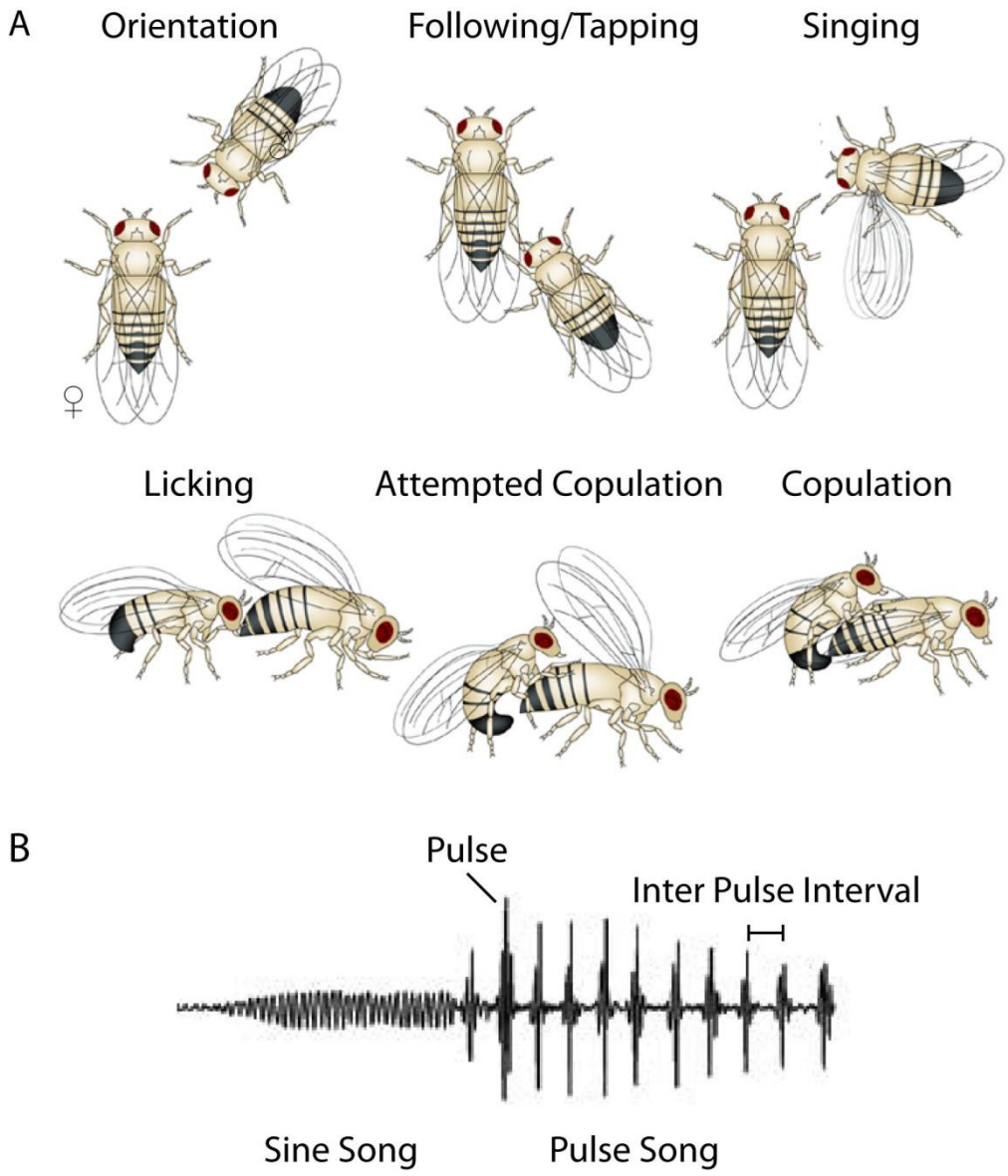


Figure 2. Courtship ritual and song of *D. melanogaster*

(A) The courtship steps that are sequentially performed by the male fruit fly. The early steps are orienting towards the female, following and tapping her. Subsequently, he generates the courtship song. Once the female slows down, the male initiates pre-copulation steps like licking and attempted copulation. (Adapted from Sokolowski, 2001) (B) The courtship song consists of two components, the sine song and the pulse song. The inter pulse interval is a critical feature of the pulse song and is defined by the time between two pulses in a song train.

The exact sequence and composition of the sub-behaviours of the entire courtship ritual varies between different *Drosophila* species (Markow and O'Grady, 2005). Some Hawaiian species for instance lack sine song or generate unusual song that is neither pulse nor sine song (Hoy et al., 1988).

Premating behaviours are however not the only sex-specific behaviours in *D.melanogaster*. The circadian rhythm, foraging and aggression behaviour have been shown to differ between males and females (Huber et al., 2004; Nilson et al., 2004; Ribeiro and Dickson, 2010). Furthermore, the male courtship activity or male sex drive has been shown to be one of the behaviours that are under control of the circadian clock (Fujii et al., 2007; Fujii and Amrein, 2010).

1.6 Genetic Substrates of Courtship Behaviour

The year 1963 marks the birth of investigating the genetic basis of courtship behaviour. K.S. Gill reported in an abstract on an x-ray induced recessive mutation, located on the third chromosome that renders male fruit flies sterile while females remain fertile. Additionally, mutant males grouped together in the absence of females display the formation of lines or circles of courting flies. Since the mutant males did not display abnormalities in the reproductive system the sterility was assumed to have behavioural origins (Gill, 1963). The gene carrying this mutation was later on termed *fruitless (fru)* and is part of the sex-determining hierarchy in *D.melanogaster* (Figure 3) (Baker, 1989; Steinmann-Zwicky, 1992; Ryner and Swain, 1995).

In brief, in fruit flies sex is determined cell-autonomously by the ratio of X chromosomes(X) to autosomes (A). In males (XY) the X/A ratio is 0.5 and causes the male sexual fate while in females (XX) the ratio is 1. The female X/A ratio enables sufficient accumulation of X chromosomal linked transcription factors that *trans*

activate the expression of the master control gene *sex lethal* (*sxl*) (Cline, 1993). Subsequently, the Sxl protein induces the expression of *transformer (tra)* by acting as a splice factor (Inoue et al., 1990). *Tra* in conjunction with non-sex-specific *transformer-2* (*tra-2*) regulates splicing of *doublesex* (*dsx*) and *fru* pre mRNA (Nagoshi et al., 1988; Hoshijima et al., 1991 ; Ryner et al., 1996; Heinrichs et al., 1997).

The male and female specific Doublesex proteins Dsx^M and Dsx^F, or the male specific Fru protein Fru^M, respectively are putative transcription factors and represent a terminal branch point of the sex determination pathway. Dsx proteins are master regulators of somatic sexual development outside the nervous system and determine the sex of the gonads, genitalia and external sexual characteristics (Keisman et al., 2001; Camara et al., 2008).

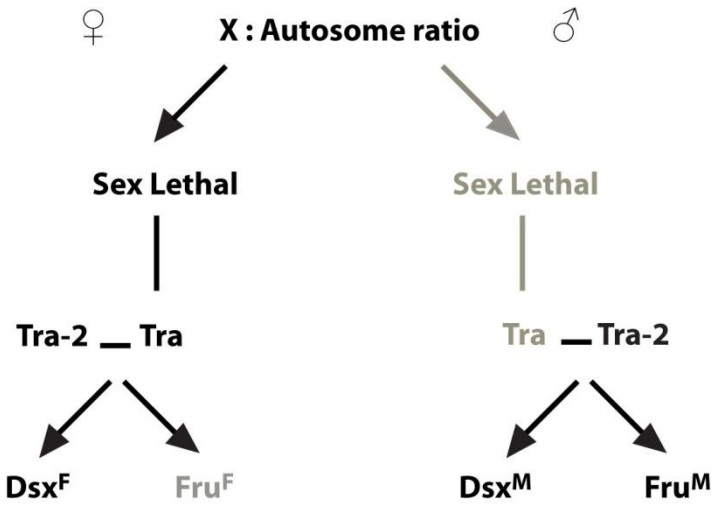


Figure 3. The sex-determining hierarchy in *Drosophila*

The ratio of X chromosomes to autosomes determines the sex of a cell in Drosophila. Upon expression of the gene *sxl* a cascade of splicing events leads to two downstream effectors that are different between the sexes. In females Dsx^F but no product of *fru* is present, while in males Dsx^M and Fru^M are generated.

Dsx is expressed in a number of tissues with dimorphic features such as foreleg, cuticle, oenocytes, fat body, muscles, digestive system and reproductive organs (Ferveur et al., 1997; Rideout et al., 2010; Robinett et al., 2010).

How about the second branch that is established by *fru*? Since the first reference fifteen years passed until scientist again focused their attention to the original fru^1 mutant phenotype and thoroughly quantified it (Hall, 1978). The next two decades brought up new *fru* mutants generated by P-element insertion (Gailey et al., 1991; Ito et al., 1996; Villella et al., 1997). The specific phenotypes of those mutants strengthened the idea that *fru* is required for all aspects of male courtship behaviour (Baker et al., 2001).

The elucidation of the molecular structure of *fru* revealed that it is a large, highly complex locus consisting of four promoters (P1-P4) with alternative splicing at the 5` and 3` prime ends of the primary transcripts (Figure 4 A and B) (Ito et al., 1996; Ryner et al., 1996; Goodwin et al., 2000; Usui-Aoki et al., 2000). Transcripts of the most distal P1 promoter are spliced sex-specifically under the control of Tra and Tra-2 proteins. They contain the S-exon that harbours *tra* binding sites. In females those sites are used and generate a non functional transcript carrying an early stop codon that is therefore not translated into protein. In males the absence of Tra causes the usage of default splice sites so that the S exon is fully included into the transcript which gives rise to the Fru^M protein with a unique N-terminal 101 amino acid extension (Figure 4 C). This extension contains no known functional domains.

The original fru^1 as well as the subsequently recovered *fru* alleles that cause a courtship phenotype were all associated with the P1 transcripts that are sex-specific (Goodwin et al., 2000; Anand et al., 2001). The specific impairment of courtship could further be correlated with the genetic composition of the *fru* locus. fru^1 mutants, for example, court females but fail to copulate, additionally they vigorously court males.

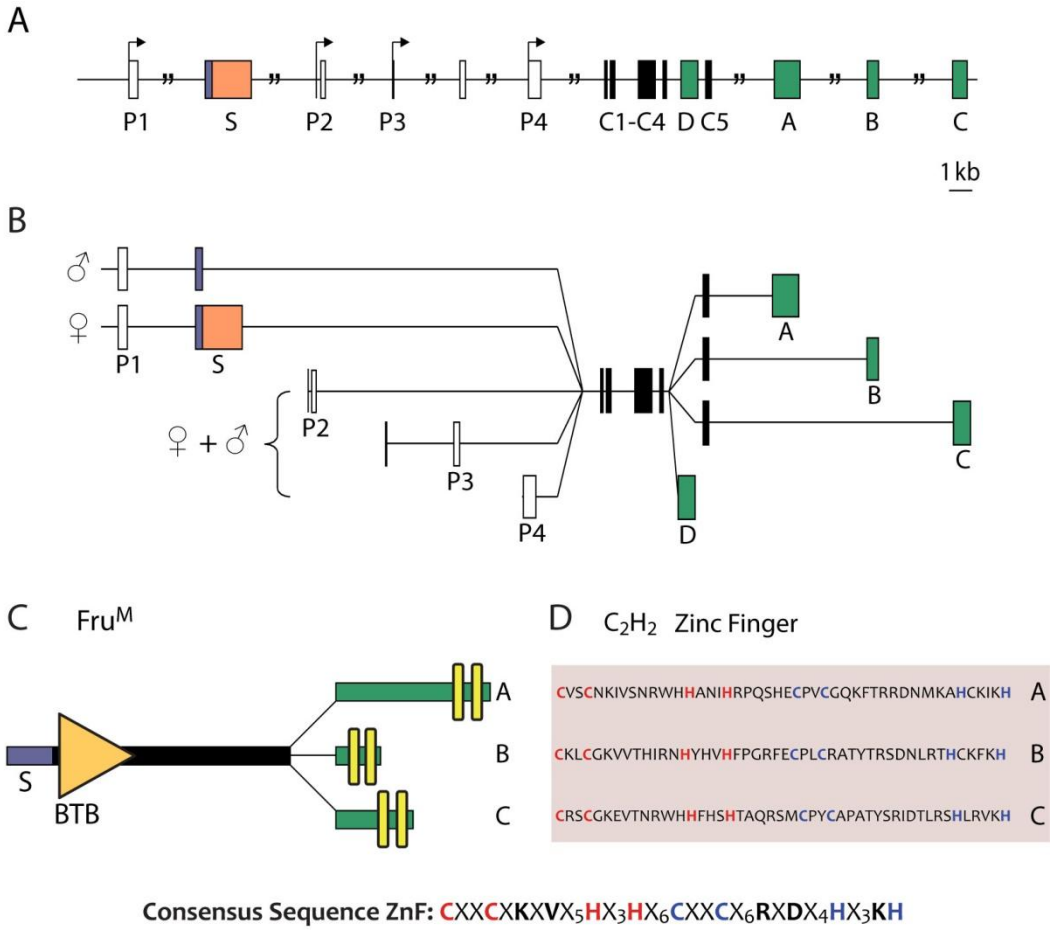


Figure 4. Structure of the *fru* gene, transcripts and proteins

(A) The genomic locus of *fru* spans about 140 kb and is very complex. It contains four promoters (P1-P4), four exons common to all transcripts (C1-C4) and four alternative 3' exons. (B) All theoretically possible transcripts are displayed. Transcripts from the P1 promoter are spliced sex-specifically at the S exon. (C) Three types of proteins arise only in males from the P1 promoter due to alternative splicing at the 3'end. They carry a BTB and zinc-finger domain. (D) The zinc-finger domains of the A, B and C exon consist of two C_2H_2- zinc fingers. Relevant cysteine and histidine amino acid residues are marked in colour. The consensus sequence of all three zinc fingers is presented.

This mutation is an inversion associated with P1 promoter and mutants lack sex-specific transcripts in certain subsets of the central nervous system. Conversely, the *fru*$^{3-4}$ mutants display a dramatic decrease of courtship towards females and lack courtship song. These *fru* alleles are P-element insertions in the intronic regions downstream of P1 and do not affect the expression pattern but the structure of the sex-specific transcripts (Goodwin et al., 2000). Together, this data provided first insights into how different expression patterns or transcript structures can lead to distinct *fru* dependent phenotypes.

The downstream promoters P2-P4 give rise to transcripts that are not sex-specifically expressed. The transcripts from P2-P4 are collectively called Fru^{Com} and play vital roles in development of both sexes (Ryner et al., 1996; Anand et al., 2001). Mutants lacking transcripts from the P3 promoter often fail to eclose, while mutants lacking products from all promoters die at an early pupal stage.

One known domain can be identified within all *fru* transcripts. These domains are: the bric-a-brac, the tramtrack and the broad complex domains. The BTB domain is a common motif in *Drosophila* transcription factors allowing homo- as well as heteromultimerisation of proteins. Apart of the generation of sex-specific and non-sex-specific isoforms by the use of alternative promoters and splicing at the 5' end of the *fru* gene alternative splicing at the 3` end generates further variation. Each of the four alternative exons at the C-terminus carries a zinc finger (ZnF) DNA binding domain, the A, B, C exons carry C_2H_2-ZnF while the D exon carries a TTF-ZnF. The BTB as well as the ZnF domains strongly imply that Fru proteins are transcription factors (Figure 4 D).

Due to the unique behavioural phenotype of *fru* P1 mutants the expression of Fru^{M} was hypothesized to be neuronal. Indeed, studying the expression of Fru^{M} protein confirmed its absolute restriction to the nervous system both in adulthood and development (Lee et al., 2000). Fru^{M} expression starts in a few neurons of the third

instar larval brain and ventral nerve cord. It extends to about 1600 neurons in the pupa nervous system where it reaches an intensity maximum at mid pupa stage. In the adult nervous system the number of labeled cells remains at approximately 1600 but the expression intensity decreases. These cell counts are excluding the *fru* positive neurons in the mushroom body as well as optic lobe which can be estimated with 200-500 (Stockinger et al., 2005). Fru^{M} is however not expressed at any life stage in females. Importantly co-labeling with the pan-neuronal marker *elav* confirmed that Fru^{M} is expressed in neurons but not glia (Lee et al., 2000). Furthermore it is localised in the nucleus supporting its putative function as a transcription factor.

Fru^{M} is clearly in the right general place to generate courtship behaviour, but is this also true for Fru^{COM} proteins? Given the early lethality of *fru* null mutants, it is perhaps not surprising that Fru^{COM} proteins are expressed at all stages of development in both neuronal and non-neuronal tissues (Lee et al., 2000; Song et al., 2002). Non-neuronal tissues include embryonal muscle, epidermal and tracheal tissues; larval imaginal discs, muscles and gonads; pupal appendages, epidermal tissue and flight muscles as well as adult flight muscles and follicle cells. Within the nervous systems, Fru^{COM} can be found in numerous neurons and glia of the embryonic and larval nervous system. Interestingly, its expression is shut down at early stages of pupation when expression from the P1 promoter is initiated. Fru^{COM} proteins are only transiently expressed again in a time window encompassing late pupae to young adults in neurons and glia. It should also be noted that this expression pattern does not overlap with the Fru^{M} pattern. In terms of the non-sex-specific promoters, all three promoters (P2-P4) display partially overlapping expression patterns (Dornan et al., 2005). P2 transcripts are found in whole pupae and adult heads. P3 transcripts are present in the entire development from embryos to adults but excluding adult heads. P4 transcripts are present over the entire development from embryo to adult in all tissues.

Genes that can activate an entire program for morphogenesis have been discovered first in *D.melanogaster* and are considered to be master control genes (Yamamoto, 2007). Could these master control genes also exist for behavioural patterns? Based on the sex-specific neuronal expression pattern of Fru^M, the sex-specific courtship phenotype of the *fru* P1 mutants and its position in the sex determination pathway the following hypothesis was raised: In analogy to Dsx^M and Dsx^F being the master regulators of sexual morphology, could Fru^M be a master regulator or switch gene for sexual behaviour in *D.melanogaster*? This question was elegantly tackled by on the one hand enforcing endogenous Fru^M expression in females due to the genetic elimination of *tra* binding sites and on the other hand feminizing *fru* neurons using *tra* RNAi or *fru* transgenes (Demir and Dickson, 2005; Manoli et al., 2005). These experiments consistently show that females expressing Fru^M display almost all aspects of male courtship behaviour towards other females. These results suggest that in fruit flies there is a dichotomy, or separation, of body and mind sexual development with *dsx* and *fru* being the respective master regulators (Shirangi and McKeown, 2007). But does this strict separation really hold true?

Interfering with *fru* function in males does not completely eliminate all male courtship behaviour but only the one that is directed towards females. Furthermore inducing Fru^M in females only generates the early steps of courtship behaviour but no attempted copulation. These observations suggest other factors are at play. One candidate is Dsx^M on the evidence that it appears to have behavioural roles as well as those in body morphology. *dsx* mutants show deficits in some aspects of courtship behaviour like courtship song and Dsx^M is sufficient to more fully generate the later steps of male courtship behaviour in Fru^M expressing females (Villella and Hall, 1996; Rideout et al., 2007). One has to be careful with interpreting these contributions of Dsx^M into the light of regulation of male courtship behaviour since some aspects of behaviour, like copulation, are just naturally constrained by morphology (Demir and Dickson, 2005).

Interestingly, of the about 900 *dsx* positive neurons in the male central nervous system the vast majority is also *fru* positive (Cachero et al., 2010). This suggests that even though the two factors together might be shaping courtship behaviour that they unfold their actions in the same neuronal substrates of this behaviour.

Taken together these experiments underline the crucial role of *fru* in acting as a master regulator for the male courtship behaviour. Per definition the underlying circuitry of this innate behaviour is hardwired in the brain and *fru* is the determination factor that renders the nervous system male. This opens a unique opportunity to study the development and physiology of the neuronal substrates of male courtship behaviour.

1.7 Neuronal Substrates of Courtship Behaviour

Having established *fru* as a master regulator for courtship behaviour, a decisive question to be tackled was whether the *fru* positive neurons are indeed the neuronal substrates of this behaviour. This was answered by making use of the GAL4/UAS system. The GAL4 transcription factor can be expressed under the endogenous *fru* promoter by placing it near the S-exon (Stockinger et al., 2005) or replacing the S-exon (Manoli et al., 2005). The expression of exogenous elements under the P1 promoter defines the set of *fru* neurons that is, dependent on the genetic element, largely overlapping with the Fru^M antibody staining (Stockinger et al., 2005; Manoli et al., 2005; Yu et al., 2010). In combination with a UAS-transgene that disrupts synaptic transmission, all *fru* neurons were silenced and thereby shown to be exclusively dedicated to male courtship but not to other behaviours such as locomotion or phototaxis. With the converse experiment sufficiency for some courtship behaviours, most prominently for song, could be shown by acutely exciting *fru* neurons (Clyne and Miesenböck, 2008; von Philipsborn et al., 2011; Kohatsu et al., 2011).

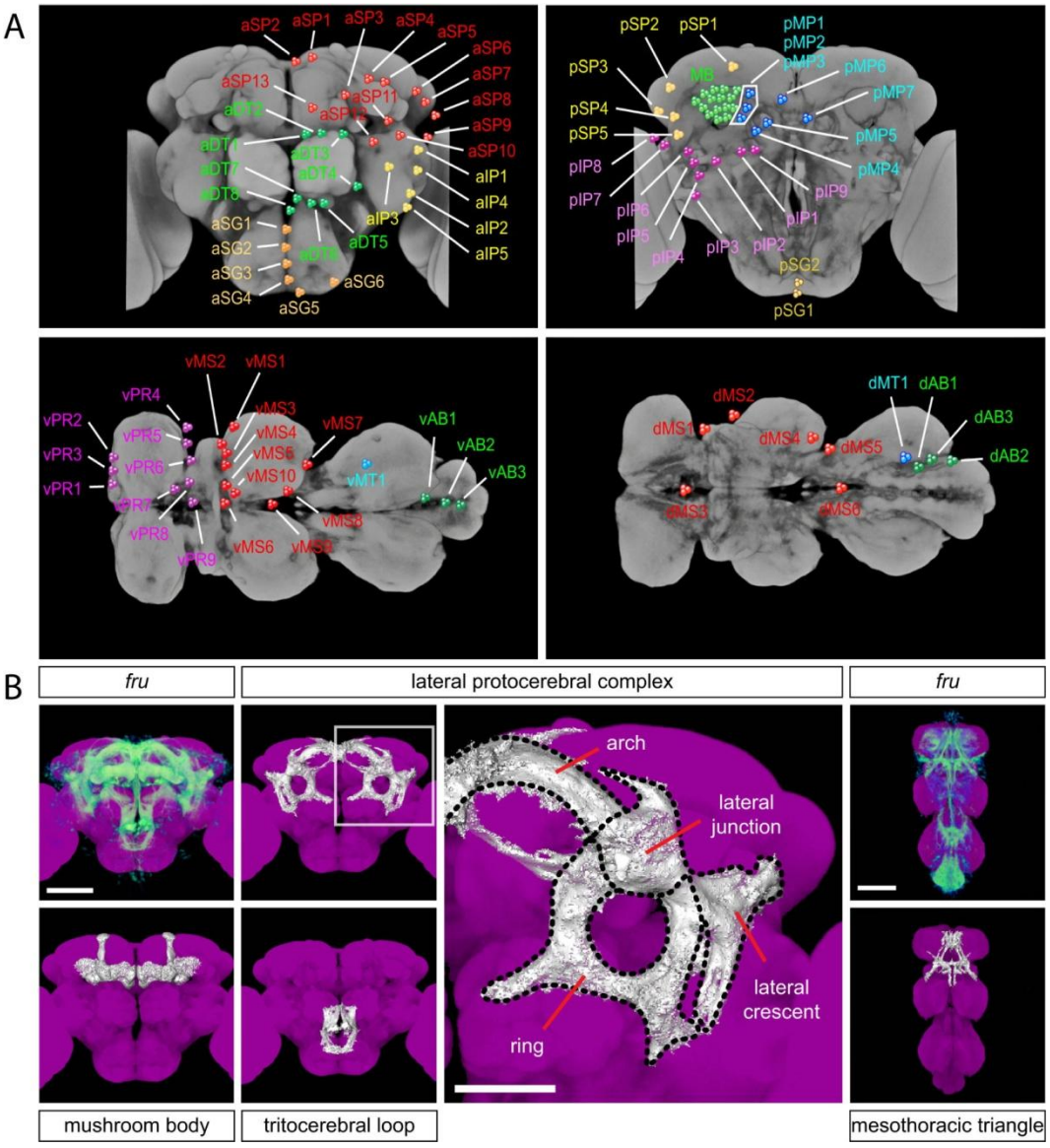


Figure 5. Atlas of the *fru* neurons and relevant neuropil regions of the *fru* circuit

(A) Upper panel displays atlas of the anterior and posterior brain and lower panel of the ventral and dorsal ventral nerve cord. Neurons are colour coded according to classes. About 100 distinct types of neuron can be identified based on anatomy. (B) *fru* neurons send projections into a few core regions of the nervous system namely the mushroom body, tritocerebral loop and lateral protocerebral complex in the brain and the mesothoracic triangle in the ventral nerve cord. (Adapted from Yu et al., 2010)

Similarly, silencing of *dsx* neurons also impairs courtship behaviour (Rideout et al., 2010). This is perhaps not surprising as most *dsx* neurons are also *fru* positive.

Fru^M expressing neurons encompass only about 2 % of all the neurons in the nervous system. Despite such a small subset of neurons, Fru^M expressing neurons represent all levels of information flow from sensory input relevant for courtship behaviour in the antenna, proboscis, maxillary pulps, and forelegs via distinctive classes of clustered neurons in the central nervous system to motor neurons in the mesothoracic and abdominal ganglion innervating the direct flight muscles and reproductive organs respectively (Stockinger et al., 2005; Manoli et al., 2005; Billeter et al., 2006a, Yu et al., 2010).

The neuronal substrates governing this circuit are organised into a network. In a putative circuit the individual elements need to be connected with each other not only anatomically but also functionally. Evidence for anatomical connections arises from experiments focusing on the overlapping arborisation patterns of *fru* neurons which according to Peters` rule implies connectivity (Stockinger et al., 2005; Koganezawa et al., 2011; Cachero et al., 2010; Yu et al., 2010; Ruta et al., 2010). Yet the only proof of functional connectivity comprises the cVA processing sub-circuit consisting out of four neuronal levels of information transfer and processing (Datta et al., 2008; Ruta et al., 2010).

The essential question of how this circuit functions only became fully addressable when its functional units have been mapped (Yu et al., 2010; Cachero et al., 2010). This led to a number of insights concerning the general structure of the *fru* circuit. First, focusing on the central brain and ventral nerve cord and based on their unique morphology or lineage each study identifies about 100 distinct classes of neurons (Figure 5 A). Second, the central brain holds putative integration or decision making neurons that are potentially connected to the ventral nerve cord by descending potential command

neurons. Third, the circuit could comprise from sensory input to motor output a minimum of six synaptic connections. Fourth, many neurons send projections into a few core regions namely the lateral protocerebral complex, the dorsal medial protocerebrum, the mushroom body and the tritocerebral loop in the central brain and the mesothoracic triangle and abdominal ganglion in the ventral nerve cord (Figure 5 B). Fifth, the circuit is largely sexual dimorphic both in the presence of and the morphology of the neurons.

Up to the present there are only few neurons or even sub-circuits that have been functionally identified. The main reason for this was the lack of GAL4 lines specifically labeling one type of neuron. This limitation could however be addressed with growing success within recent years (see section 1.4.).

One pheromone input and processing sub-circuit is analysed in great detail. Fru^{M} is expressed in olfactory receptor neurons in the antenna, those neurons project to sexually dimorphic glomeruli in the antennal lobe where they connect to *fru* positive first order olfactory projection neurons. Silencing those olfactory receptor neurons leads to an impairment of male courtship behaviour (Stockinger et al., 2005). One class of those receptor neurons that innervate the DA1 glomerulus expresses the odorant receptor 67d that responds to the volatile pheromone cVA. This pheromone elicits sex-specific behaviours (Ejima et al., 2007; Kurtovic et al., 2007; Billeter et al., 2009; Wang and Anderson 2010). It is generated by the male fly and transferred to the female during copulation. In males it promotes male-male aggression, suppresses male courtship towards males and mated females while in females it conveys receptivity to courting males. The second order DA1 projection neuron (aDT3) sends sexually dimorphic arborisations to the lateral horn region where it connects to male specific DC1 (aSP5). This neuron projects to the lateral triangle in the lateral protocerebral complex where it connects to male specific DN1 fourth order neuron which is descending and arborises in the mesothoracic triangle. All those neurons have been

shown to be functionally connected, e.g. the most downstream component responds electrically to *in vivo* cVA application (Ruta et al., 2010). While the cVA pathway is the best studied sensory pathway in sexual behaviour, there is at least one more less well studied putative pheromonal pathway.

Gustatory receptor 32a neurons in the forelegs are required for appropriate unilateral wing extension directed to the female during singing. Furthermore, the axon terminals of those gustatory receptor neurons co-localize with the dendritic arborisations of the mAL (aDT2) neurons in the central brain that convey information to the lateral junction of the lateral protocerebral complex. Males that are deprived of this input at either of the neuronal levels extend both wings simultaneously (Koganezawa et al., 2010).

Several types of *fru* neurons in the central brain have been identified to be crucial for the appropriate execution of courtship associated behaviours. Fru^M expression in mushroom body neurons is required for courtship conditioning (Manoli et al., 2005). Fru^M co-localizes with octopaminergic neurons in the suboesophageal ganglion that have been suggested to modulate male specific aggression patterns (Certel et al., 2007).

Neurons of the circadian rhythm circuitry overlap with *fru* neurons. Disturbing the cycling of the clock genes in those neurons renders the male sex drive arrhythmic (Fujii et al., 2007; Fujii and Amrein, 2010). For the sequential execution of the individual components of courtship behaviour the mcAL median bundle neurons (aDT6) which receive sensory input from various modalities have been suggested to require Fru^M (Manoli and Baker, 2004).

Recently central components of the song circuit have been mapped in detail beginning with the putative integration or decision making P1 (pMP4) neurons that arborise mainly in the lateral protocerebral complex and could pass on information to the potential P2b (pIP10) command neurons for the song motor program (Kohatsu et al.,

2011; von Philipsborn et al., 2011). The P2b neurons are descending and arborise in the mesothoracic triangle where they overlap with three types of ventral nerve cord neurons that determine song structure dPR1, vPR6 and vMS11. Functional connectivity between those types of neurons has not been shown but any of them, except vMS11, is sufficient to generate courtship song when thermogenetically activated (von Philipsborn et al., 2011). In a more precise analysis the P1 and P2b neurons have been shown to be sufficient for more than just singing but to trigger the initial steps of courtship including following and tapping. P1 seems to further be a major player for integrating sensory information as revealed by imaging P1 activity (Kohatsu et al., 2011).

Finally, there is little known about which *fru* neurons are involved in the various motor outputs. Fru^{M} is required in some abdominal motor neurons for the proper formation of the Muscle of Lawrence that is suggested to play a role in copulation (Gailey et al., 1991; Taylor and Knittel, 1995; Lee et al., 2001; Billeter et al., 2006). Furthermore a subset of serotonergic neurons innervating the reproductive organs is proposed to regulate ejaculation during copulation (Lee et al., 2001). For the generation of courtship song motor neurons innervating the direct flight muscles are required (Ewing, 1979) and at least one such neuron (vMS2) has been identified anatomically in the *fru* circuit (Yu et al., 2010).

There is growing evidence that *fru* neurons are also relevant for female mating behaviour. Specifically, silencing of all *fru* neurons with fru^{GAL4} or indeed only a subset of neurons near the uterus leads to a switch in mating behaviour, i.e. virgin females reject males and lay their eggs (Kvistiani and Dickson, 2006; Häsemeyer et al., 2009; Yang et al., 2009).

Taken together, *fru* neurons do not only constitute the neuronal substrate for courtship behaviour but individual types of neurons, even sub-circuits, underlying distinct behavioural modules have been identified.

1.8 Anatomy of the *fru* circuit

After having determined *fru* as a master regulator for male courtship behaviour one remaining crucial question is how does *fru* masculinise the nervous system. As a putative transcription factors it could act either directly or indirectly to express genes needed for development or functionality. The *fru* positive neurons comprise only 2 % of all neurons in the nervous system hence their mere presence could have been the difference between males and females. This issue could only be addressed once a GAL4 enhancer trap line recapitulating *fru* expression was generated (Stockinger et al., 2005; Manoli et al., 2005). Surprisingly, on a gross anatomical level the *fru* neurons are not only present in females but also seem to innervate similar regions. This notion was revised within the past five years and it is now clear that many subtle morphological differences do exist between male and female brains. This refinement was accomplished thanks to the ability to label ever smaller subsets of the *fru* circuit (Kimura et al., 2005; Stockinger et al., 2005; Billeter et al., 2006; Rideout et al., 2007; Kimura et al., 2008; Datta et al., 2008; Yu et al., 2010, Cachero et al., 2010, Rideout et al., 2010; Ruta et al., 2010).

The nature of these differences can be two fold. Neurons can be sex-specific, i.e. the neuron is either never born or it dies in one sex. Apart from simple presence and absence, the neurons can also be sexually dimorphic, i.e. the neuron is present in both sexes but the morphology of its projections is different. One intriguing finding is that both *dsx* and *fru* are relevant for shaping neuronal anatomy. In the large number of

structurally different neurons that are *dsx* and *fru* double positive both factors act in concert. The general picture seems to be that both *dsx* and *fru* are instructing the existence of a neuron while *fru* alone is determining its morphology.

One example for this mode of action is the P1 (pMP4) neuron which is sex-specific and occurs exclusively in males. It was shown to be sufficient when present in females to trigger the early steps of courtship behaviour. In females the P1 neurons are born but die due to the action of Dsx^F. In males the presence of P1 is not dependent on Fru^M, however, Fru^M is required for the correct positioning of the terminals of P1 neurites (Kimura et al., 2008). There are additional neuronal populations that are sex-specific and Fru^M and Dsx^M positive. For those both factors determine the cell number (Billeter et al., 2006a; Rideout et al., 2007; Rideout et al., 2010).

The first sexually dimorphic neuron discovered was the mAL (aDT2) class of *fru* positive *dsx* negative neurons that is located medially above the antennal lobe and plays a role in defining the laterality of wing display during song. In females a fraction of these neurons dies in development due to the absence of Fru^M and those neurons that do not die show a *fru* and *hunchback (hb)* dependent altered arborisation pattern in the suboesophagal ganglion (Kimura et al., 2005; Goto et al., 2011).

Interestingly, the various components of the pheromonal cVA circuit are also sexually dimorphic (Datta et al., 2008; Ruta et al., 2010). The DA1 glomerulus that houses the arborisations of the olfactory receptor neurons and the corresponding projection neurons (aDT3) is larger in males than in females. The DA1 projection neurons themselves have dimorphic axonal projections into the lateral horn. These dimorphisms are *fru* dependent. The third order DC1 (aSP5) neurons display sexual dimorphic arborisations in the ventral lateral horn while the fourth order DN1 neurons are male specific (Stockinger et al., 2005; Datta et al., 2008; Ruta et al., 2010).

Gustatory receptor neurons from the foreleg have been shown to display midline crossing in the ventral nerve cord only in males. In females midline crossing is suppressed by Dsx^F and in males it is promoted by Fru^M via the Robo paralogues (Mellert et al., 2010). One example for male specific neurons in the optic lobe are the *fru* neurons in the medulla (Kimura et al., 2005). Taken as a whole, recent evidence elucidates roughly 30 novel dimorphisms in the nervous system suggesting that there are more dimorphic neurons than was initially appreciated and that the degree of dimorphism increases in putative higher order integration neurons relative to those in the periphery (Figure 6) (Yu et al., 2010 and Cachero et al., 2010).

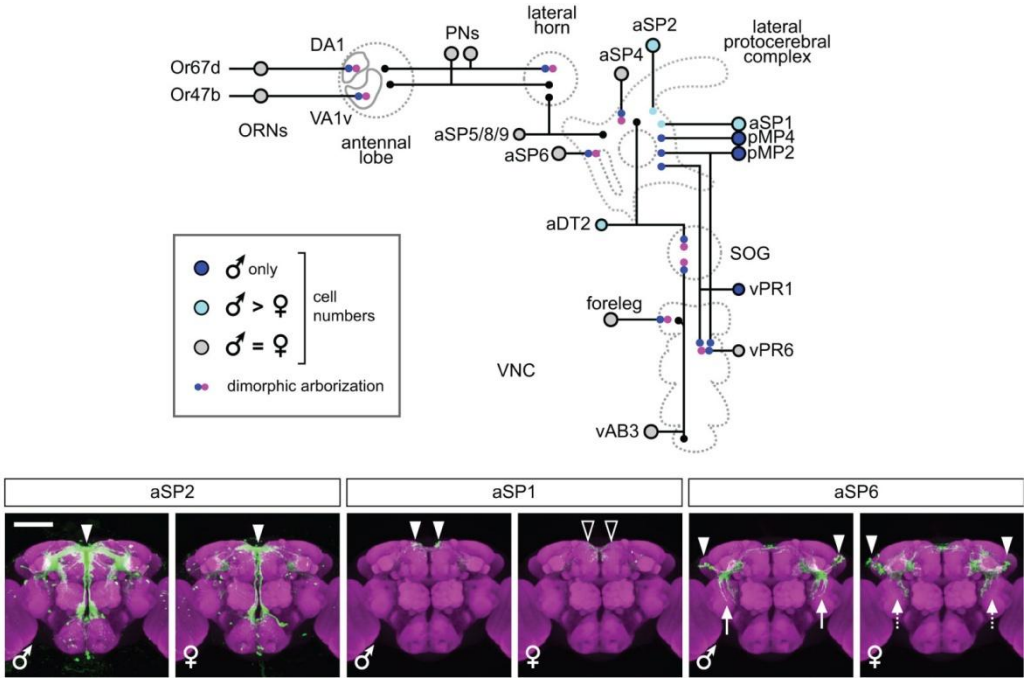


Figure 6. Sexually dimorphic *fru* neurons

(A) The schematic displays core regions and ganglia of the *fru* circuit that are innervated by sex-specific or sexually dimorphic neurons. Sex-specific neurons like aSP2 have colour coded cell bodies while sexually dimorphic neurons have coulour coded arborisations. (B) Three types of neurons in the brain that are either sex-specific (aSP2 and aSP1) or sexually dimorphic (aSP6). (Adapted from Yu et al., 2010)

At present, the P1 neurons are the only example demonstrating the functional importance of the sex-specific features of *fru* neurons (Kimura et al., 2008). This raises the question on how relevant these sexual differences are. However, the existence and Fru^M/Dsx^M dependence of numerous additional sexually dimorphic neurons implies further functional relevance for those neuronal classes.

These structural features demonstrate that *fru* acts developmentally to masculinise the neurons anatomically. But does is also act post-developmentally? Many adult neurons are born during the larval stages and first pupal stage (Hartenstein, 1993). Fru^M expression likewise starts in third instar larva, peaks in mid pupal stages and stays constant at lower level in adulthood. This suggests a potential requirement not only throughout development but also in adulthood. This question was addressed only indirectly in *tra* mutant females that conditionally express functional Tra protein (Belote and Baker, 1987; Arthur et al., 1998). These investigations showed that male behaviour is programmed in a critical time window during pupation but that there is also plasticity for male determination in adults as well.

Conceptually we have learned that *fru* plays an instructive role in the masculinisation of the neuronal substrates relevant for male courtship behaviour. At the same time absence of Fru^M is likely to feminize those neurons. Importantly, shaping the morphology of neurons is a property that strengthens the suggestion of *fru* being a master regulator of courtship behaviour.

1.9 Existence of Different Fru^M Variants

fru is a large and complex gene, which spans about 140 kb. There are not only four different promoters but alternative splicing occurs at the 5` and 3` end. Alternative choice of promoters and splicing in general lead to an increase in the number of

proteins that can be derived from one gene, these variants are called isoforms. They can be different, similar or antagonistic in their molecular function both as a result of being different proteins but also as a result of their putatively distinct expression patterns. Together these mechanisms can lead to different cellular and behavioural phenotypes. Alternative splicing is a key mechanism that allows a limited number of genes to fulfill a variety of sophisticated functions in eukaryotes. One of the most dramatic examples of this is found in the *D.melanogaster* gene d*own syndrome cell adhesion molecule (Dscam).* In theory alternative splicing of *Dscam* could give rise to about 40 000 different isoforms. The key factors for alternative splicing by the spliceosome machinery are exonic/intronic splicing enhancers or silencers. These are specific sites in the exon or intron that are recognized by certain proteins. The presence or absence of these proteins can instruct what splice site is used in what cell and at what time.

Alternative splicing is a common theme for the entire *fru* locus in *D.melanogaster*, indeed for the entire sex determination pathway. The 3` A, B and C exons are incorporated into the transcripts from the four different promoters. P1 transcripts in the central nervous system contain all three exons (Billeter et al., 2006).The same is true for P3 or P4 transcripts in embryos (Song et al., 2002) while only P2 transcripts containing the A exon are detected in adult heads (Billeter et al., 2006*)*. Although the D exon is annotated to belong to an open reading frame there are no transcripts reported at any life stage (Billeter et al., 2006). The A, B and C exons differ in encoding alternative zinc finger domains. As zinc finger domains are known to bind DNA, it was hypothesized that these transcripts may provide distinct cellular and behavioural functions. Different functions have already been suggested as only *UAS-fru*^{A} and *UAS-fru*^{C} but not *UAS-fru*^{B} transgenes provided a rescue of axonal path finding defects in *fru* mutants embryos (Song et al., 2002). In addition an EMS screen isolated a *fru* allele affecting only the *fru*^{C} isoform. As these *fru*$^{\Delta C}$ mutants display a reduction of fertility

and severe impairment of several aspects of courtship behaviour, it seems unlikely the isoforms are purely redundant (Billeter et al., 2006). Finally, it is clear that different cells have different requirements for the isoforms. For example, Fru^{C} alone is required and sufficient for the development of the male specific Muscle of Lawrence while conversely a combination of all three isoforms is necessary for the development of serotonergic neurons in the abdominal ganglion (Usui-Aoki et al., 2000; Billeter et al., 2006).

In addition to these functional differences, there may additionally be differences in expression patterns. This was shown to be the case for the non-sex-specific transcripts in embryos where the isoforms are expressed in a tissue specific pattern outside of the central nervous system but in a highly overlapping manner within. For the sex-specific isoforms expression pattern differences may also exist. For instance, Fru^{M} isoform expression patterns seem to be distinctively regulated since all Fru^{M} expressing abdominal neurons in pupae are also fru^{MC} but only in part fru^{MA} positive (Billeter et al., 2006). Whether these last examples of difference in expression pattern relate to functional requirements is currently unclear. Together, these studies give a first glimpse on how fru^{M} isoforms differ in expression and function.

At the same time these findings challenge the notion of *fru* being a single master regulator for courtship behaviour.

The sequence of the important *fru* domains and the concept of alternative and sex-splicing are conserved throughout insect evolution and suggest that *fru* is an ancient gene with conserved functions as the prototypic gene of male sexual behaviour among many holometabolous insects (Gailey et al., 2005; Bertossa et al., 2009). Taking the genomic make up of the *fru* locus in other species into consideration the complexity grows with an extension to 6 promoters and zinc fingers carrying exons from A to G (Figure 7).

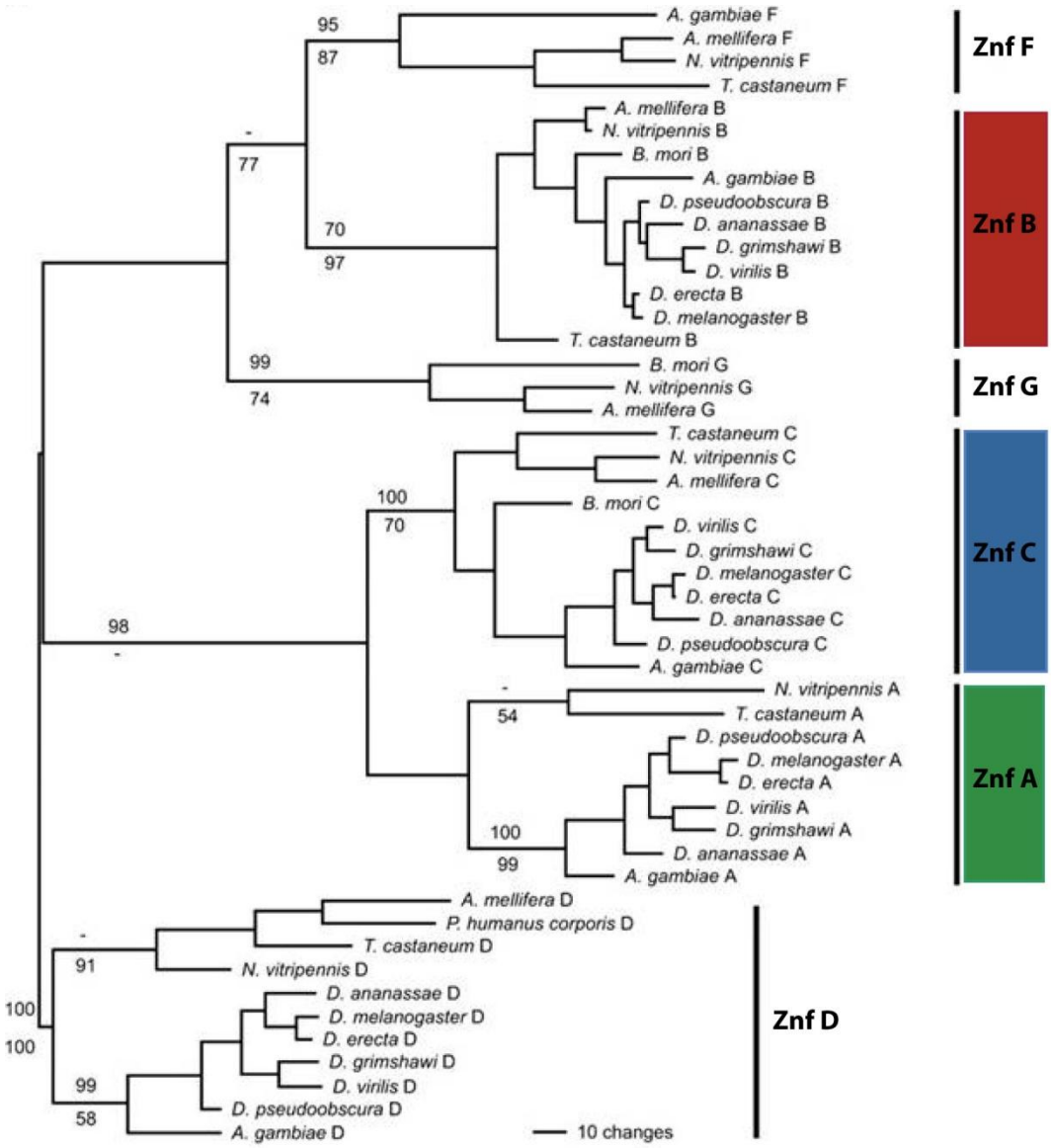


Figure 7. *fru* zinc finger phylogenetic tree

Maximum parsimony tree of nucleotide sequences of *fru* zinc fingers from various insect taxa. Numbers above and below branches are baysian posterior probabilities or nonparametric bootstrap proportions, respectively. ZnF D is used as outgroup. In *D. melanogaster* a common ancestor duplicated early and gave rise to two zinc fingers. One is the ancestor of ZnF B and ZnF F, the latter got lost, and the other one of ZnF A and ZnF C. (Adapted from Bertossa et al., 2009)

A phylogenetic analysis revealed that *fru* zinc fingers in general are most closely related within the *Drosophilids*, followed by another Dipteran species *Anopheles gambiae*. Despite lower sequence conservation, the *fru* locus and the alternative zinc fingers are clearly present in the evolutionarily more distant non-dipteran insects like the butterfly *Bombyx mori*, the beetle *Tribolium castaneum* and bees and wasps like *Apis mellifera* and *Nasonia vitripennis*. Therefore virtually all *fru* zinc fingers were likely present in a common ancestor of all holometabolous insects. The *fru* C_2H_2 zinc fingers show remarkable conservation among each other and have the same consensus sequence (Figure 4 C). Phylogenetic tree analysis indicates that the ancestral *fru* locus contained two C_2H_2 zinc finger types. One duplicated to give rise to G and the ancestor of B and F. The other one duplicated to give origin to A and C. These duplications occurred very early in insect evolution and some exons subsequently got lost in some species. Taken together fru^A and fru^C are more closely related to each other than to fru^B (Bertossa et al., 2009). Furthermore it was shown that the fru^A zinc finger displays the highest amount of amino acid substitutions outside of Drosophilids and is absent in *A. mellifera* and *B. mori* (Gailey et al., 2006; Bertossa et al., 2009). This suggest that it is evolutionary less constrained which could lead either to acquisition of new functions or even disappearance (Bertossa et al., 2009).

1.10 **Aim of Thesis**

D. melanogaster male courtship behaviour is an ideal model to study how genes determine the neuronal circuitry underlying behaviour. The putative transcription factor *fru* is a master regulator for courtship behaviour and masculinises the neuronal circuit governing this behaviour. This circuit consists of about 100 different types of neurons and for about a dozen of these subtypes a function is known. The current aim for the field is to understand how each type of neuron functions in concert with its

partners to generate all the different aspects of male mating behaviour. On top of that the ultimate goal is to elucidate *fru* dependent molecular mechanisms that specify the cellular phenotypes of individual neurons.

In order to address the above-mentioned questions one crucial step has to be taken: The analysis of the expression and function of the different Fru^{M} isoforms. There is some evidence for differences in the expression pattern and function of the isoforms coming from alternative splicing of the 3` end (Billeter et al., 2006). In this work I want to extend this initial study and answer the following questions: 1) Do the Fru^{M} isoforms have distinct or overlapping functions? 2) Are they expressed in distinct or overlapping neurons? Using mutants in each isoform we want to analyse their functions at a cellular as well as behavioural level. We will further address the global expression pattern of all three Fru^{MA}, Fru^{MB} and Fru^{MC} isoforms in the entire CNS throughout development at cellular resolution. Finally, we ask whether isoform specific phenotypes can be mapped to certain types of neuron.

2 Results

2.1 Behavioural Phenotypes of *fru* Isoform Mutants

Here, we aim to elucidate the contributions of each Fru^M isoform to the different modules of courtship behaviour. The global function of a protein can be analysed best by gaining mutants in the corresponding gene. We therefore introduced loss-of-function mutations into the 3` zinc finger containing exons of the *fru* gene either by chemical mutagenesis or gene targeting (Figure 8 A and B). The chemical mutagenesis screen was based on the fact that Fru^M is not only required for normal male mating behaviour, but also suppresses female mating and egg-laying behaviours. Accordingly, females carrying one copy of the $fru^{\Delta tra}$ allele are sterile due to expression of Fru^M (Demir and Dickson, 2005). This provided a convenient assay to screen for intragenic revertant alleles, in which fertility is restored due to an additional loss-of-function mutation in the dominant $fru^{\Delta tra}$ allele.

In such a chemical mutagenesis screen, 9 fertile revertants of the $fru^{\Delta tra}$ allele were isolated. Five of these revertants were found by sequence to be in the alternatively spliced zinc fingers (Figure 9 B and C). Two revertants have mutations in the fru^B zinc finger domain while three revertants have mutations in the fru^C zinc finger domain. They are referred to as fru^{B1}, fru^{B2}, fru^{C1}, fru^{C2} and fru^{C3} (Figure 8 A and C) Sequencing of the remaining exons in these five revertants did not uncover any additional mutations. Three lines of evidence support the fru^B and fru^C mutants being recessive loss-of-function, and likely null, alleles. First, the revertant females regained fertility. Since the fru^B mutants are less fertile than the fru^C mutants, Fru^{MB} might not be as important as Fru^{MC} for the sterility phenotype. Second, the mutations are missense and nonsense mutations affecting the crucial amino acids cysteine and histidine of the C_2H_2 zinc finger DNA binding domain (Figure 8 C and 9 C).

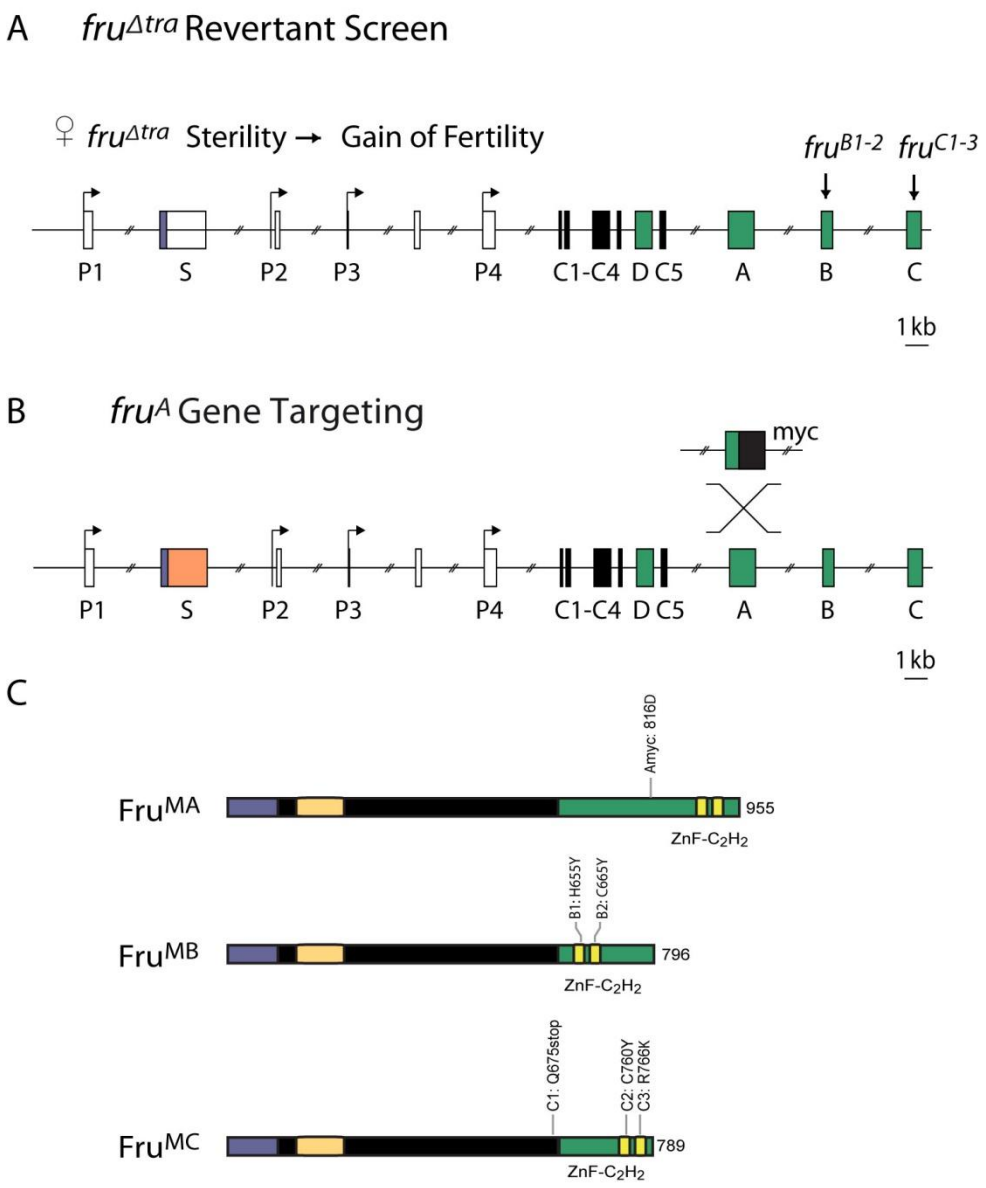


Figure 8. Generation of *fru* isoform mutants

(A) and (B) Two different strategies for generating *fru* isoform mutant alleles have been exploited. A $fru^{\Delta tra}$ revertant screen recovered two fru^{B} and three fru^{C} mutant alleles while the $fru^{\Delta A}$ mutant allele was generated by gene targeting. (C) Mutant male isoform proteins are displayed and the exact position of the mutation is indicated. Zinc fingers are marked in yellow, isoform specific exon in green, common exon in black, BTB domain in skin colour and male specifc N-terminus in magenta.

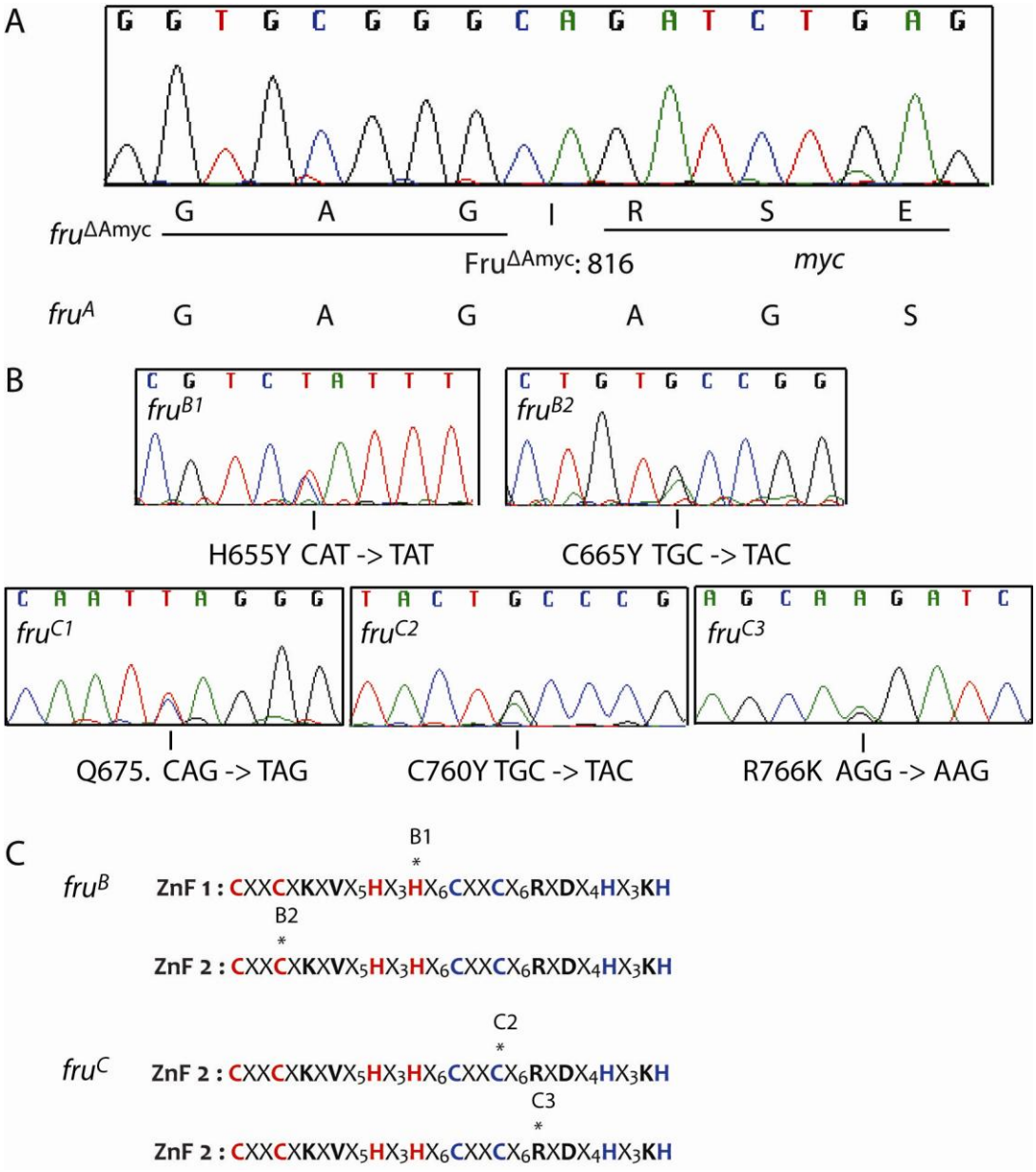


Figure 9. Validation of *fru* isoform specific alleles

(A) Sequence of fru^{A} exon in $fru^{\Delta Amyc}$ flies shows replacement of endogenous sequence from amino acid 816 on with 4 c-myc epitope tags. (B) Sequence reads on fru^{B} *and* fru^{C} *mutant* heterozygous flies show double peak at indicated mutated bases. Sequencing was performed in heterozygous flies since homozygous fru^{B} and fru^{C} mutants are lethal. (C) fru^{B} and fru^{C} missense mutations are indicated and affect conserved cysteine and histidine residues of the zinc fingers.

Third, neither the fru^{B} nor the fru^{C} mutants are homozygous viable. This suggests both types of mutants contribute to the vital defects seen in fru^{Com} mutants.

As no fru^{A} mutant allele was recovered we generated a targeted mutation by ends-in homologous recombination that is designed to be a loss-of-function due to the absence of the entire zinc finger binding domain and is referred to as $fru^{\Delta A}$ (Figure 8 B). The absence of the zinc finger in the $fru^{\Delta A}$ allele was validated by PCR and Sequencing (Figure 9 A). These mutants are homozygous viable which suggests that Fru^{ComA} contributes little to the vitality phenotype associated with the fru^{Com} mutants.

Collectively these mutants allowed a comparative analysis of the diverse behavioural phenotypes resulting from functional loss of a single Fru^{M} isoform.

In relation to courtship behaviour, three scenarios are possible concerning the phenotypic relevance of each isoform: 1) only one isoform is relevant, 2) the isoforms are completely redundant or 3) each isoform contributes a unique role. In order to exclusively address the functional loss of the transcripts from the sex-specific P1 promoter, we tested the *fru* isoform mutants over a fru^{F} allele. This allele forces expression of the female transcript, which does not generate a protein product, from the P1 promoter. Since fru^{F} is effectively a null in the sex-specific functions of *fru*, we were able to uniquely assess the sex-specific roles for the isoform mutants while retaining the Fru^{Com} isoforms. To gain a general impression of the mutants, I first tested them in a standard courtship assay. All mutants showed a phenotype in this assay. Interestingly, the phenotype of different isoform mutants was not equivalent. When tested for the success in copulating with a female in a standard assay and compared to the 80 % success rate of controls $fru^{\Delta Amyc}$ males flies have a mild reduction to about 50 %, fru^{B1} and fru^{B2} males a dramatic reduction to about 15 % and fru^{C1}, fru^{C2},fru^{C3} the most severe reduction to almost 0 % (Figure 10 A). Since the phenotypes for the copulation success of the different fru^{B} and fru^{C} mutants were highly similar all

following experiments were performed only with *fru*^{B2} and *fru*^{C1} flies. To gain further insight into the specific defect that lead to the decrease in success rate in the copulation assay, I tested individual steps within the courtship ritual. First, I examined the latency of the wing extension. Wing extension provides an indirect measure of the male response to female chemosensory cues.

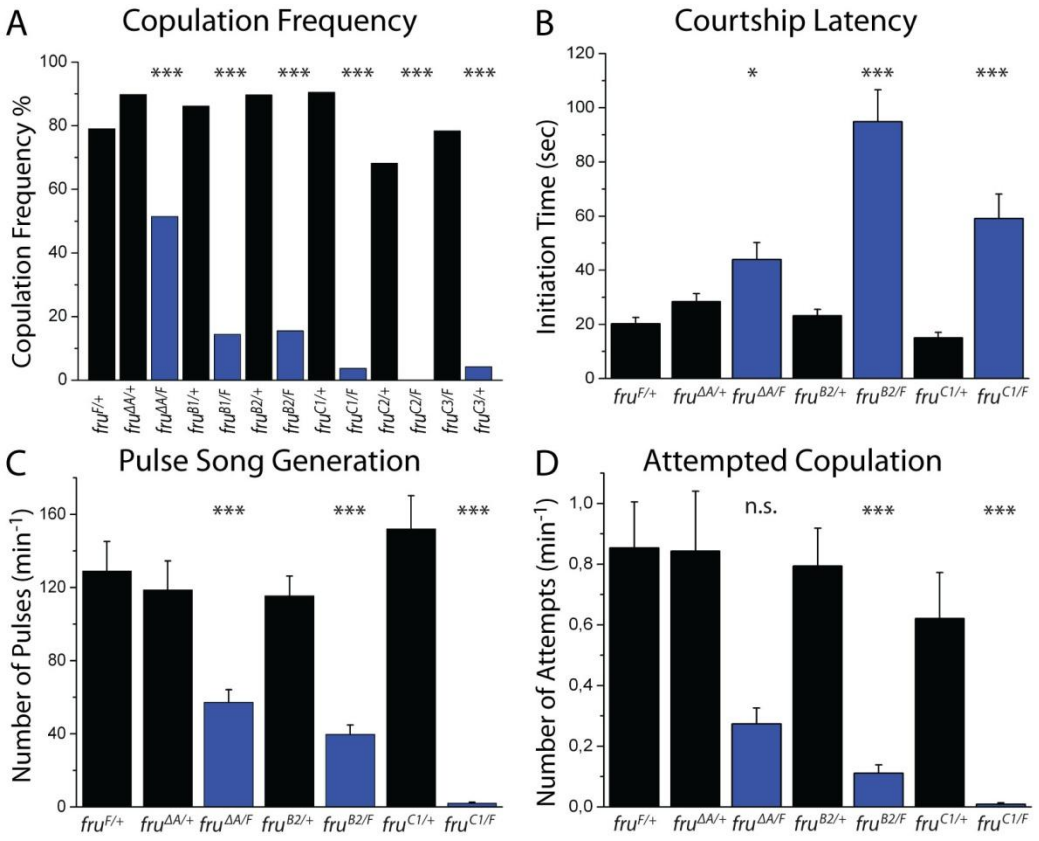


Figure 10. *fru* isoform mutants display distinct impairments of courtship behaviour

fru Isoform mutants are crossed with *fru*^{F} and analysed in standard courtship assays. Statistical tests are performed by comparing mutants in blue with their matched controls in black. (A) Copulation frequency, n = 97-109, Fisher`s exact test, p<0.001 *** (B) Courtship initiation, n = 59, s.e., Mann-Whitney, p<0.001 ***, p<0.05 *. (C) Pulse song quantification, n= 30, s.e., Mann-Whitney, p<0.001 ***. (D) Attempted copulation, n = 49, s.e., Mann-Whitney, p<0.001 ***, n.s. not significant.

Again, all mutants showed a phenotype. The $fru^{\Delta Amyc}$ mutant had the weakest defect with a 1.5 times increase; however, unlike the copulation assay the fru^{B2} mutant exhibited the most dramatic detriment with a five times increase and the fru^{C1} mutant an intermediate effect with a three times increase of initiation time (Figure 10 B). Similar results were seen when analysing the latency of orientation and following (data not shown).

In *D. melanogaster* the courtship song provides a crucial component in determining male sexual success (Bennet-Clark et al., 1976). The major component of courtship song is a train of pulses, generated by vibrating the wing. All mutants had defects in courtship song. Importantly, there is a complete absence of pulse song in fru^{C1} mutants while $fru^{\Delta Amyc}$ and fru^{B2} mutants generate about 60 % and 40% of the pulses seen in controls (Figure 10 C). Interestingly, the reduced song that remains in fru^{B2} and $fru^{\Delta Amyc}$ mutants is aberrant in two major parameters. The species specific inter pulse interval is slightly increased from about 33 ms in controls to 39 ms in both mutants (Figure 11 A and C). Furthermore, the $fru^{\Delta Amyc}$ but not fru^{B2}males display twice as many cycles per pulse in their song. They produce about 2.2 ± 0.5 cycles per pulse compared to the 1.2 ± 0.3 cycles per pulses of control flies (Figure 11 B and C). In agreement with all the above experiments, I also find that the number of attempted copulation is highly reduced in $fru^{\Delta Amyc}$ (0.3 ± 0.4 per min) as well as fru^{B2} mutants (0.1 ± 0.2 per min) compared to controls (0.9 ± 1.1 per min). Notably, copulation attempts are completely absent in fru^{C1} mutants (0.0 ± 0.0 per min) (Figure 10 D).

Having assayed individual courtship defects, I additionally performed competitive mating assays in which a wild type female is asked to choose between two competing males. All mutant males lost when competing with a wild type male. While $fru^{\Delta Amyc}$ mutant males lost in 75 % the other mutants lost in almost 100 % of the cases. By contrast, both $fru^{\Delta Amyc}$ and fru^{B2} mutants won when competing with fru^{F} males, which

eliminate all sex-specific transcripts. Any difference between *fru*^{F} and *fru*^{C1} males could not be seen as there was no copulation observed within 30 minutes (Figure 12).

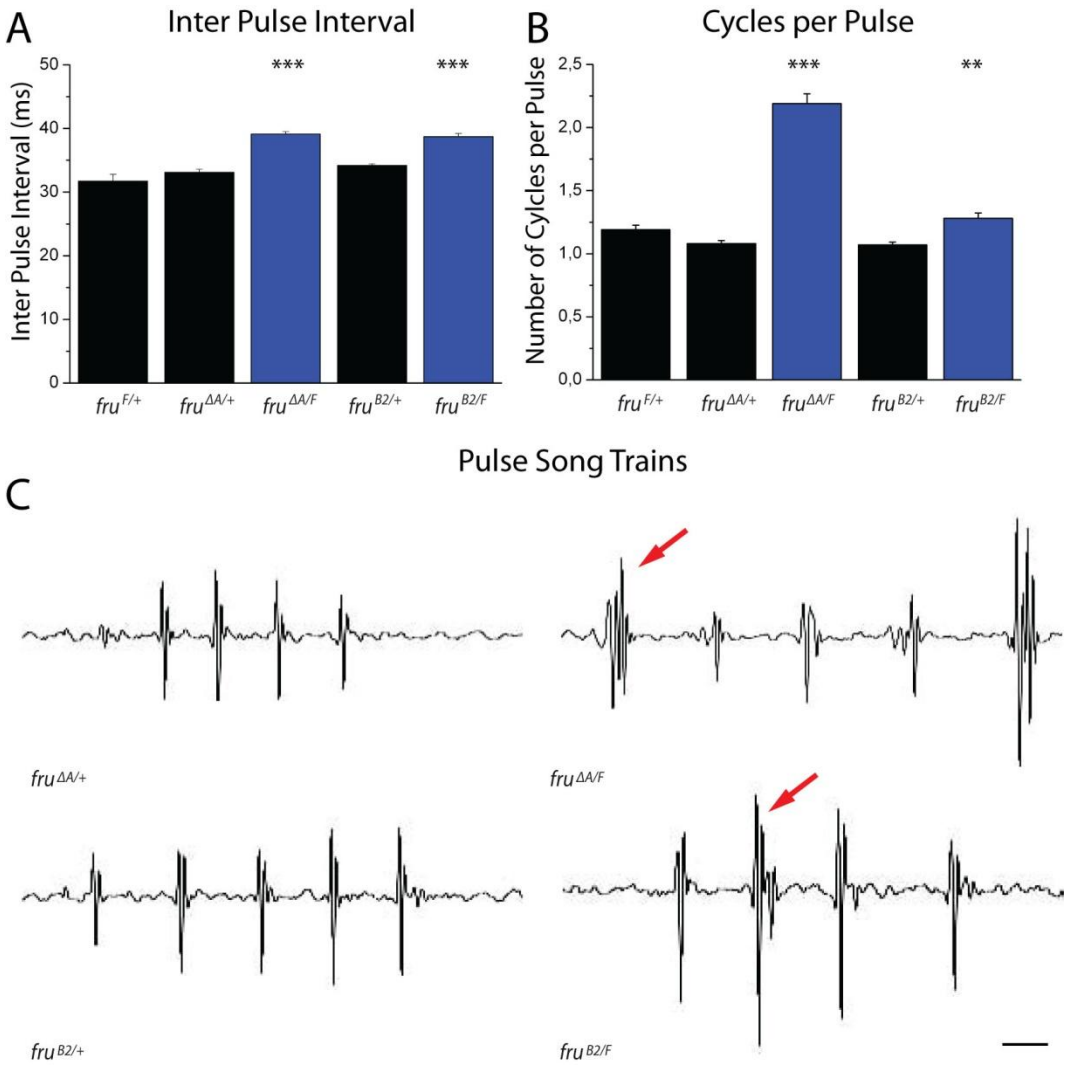


Figure 11. *fru* isoform mutants sing aberrant song

(A) Inter pulse interval is slightly elevated in *fru*$^{\Delta A}$ and *fru*^{B2} mutants, n= 25, s.e., Mann-Whitney, p<0.001 ***. (B) *fru*$^{\Delta A}$ mutants sing polycyclic song, 120 pulses from 20 flies, s.e., Mann-Whitney, p<0.001 ***, p<0.01 **. (C) Example traces of pulse song trains, left panel controls, right panel mutants, red arrow points to polycyclic pulses, scale bar 20 ms.

When the isoform mutants were directly competing against each other, *fru*$^{\Delta Amyc}$ males always won over both *fru*^{B2} and *fru*^{C1} males; and *fru*^{B2} males always won over *fru*^{C1} males (Figure 12). Collectively, these results show that all isoform mutants have a phenotype and that the behavioural phenotype of each isoform is quantitatively as well as qualitatively different. *fru*$^{\Delta Amyc}$ males have the smallest decrement in courtship performance. *fru*^{B2} males perform courtship at an intermediate level and show the largest defect in latency of courtship initiation. *fru*^{C1} males have the most severe impairment and most notably display no pulse song. In sum, these data lead us to favour the third hypothesis: that each isoform has a function in courtship behaviour. Different *fru*^{M} isoforms mutants have however partially overlapping impairment of courtship behaviour suggesting a certain redundancy.

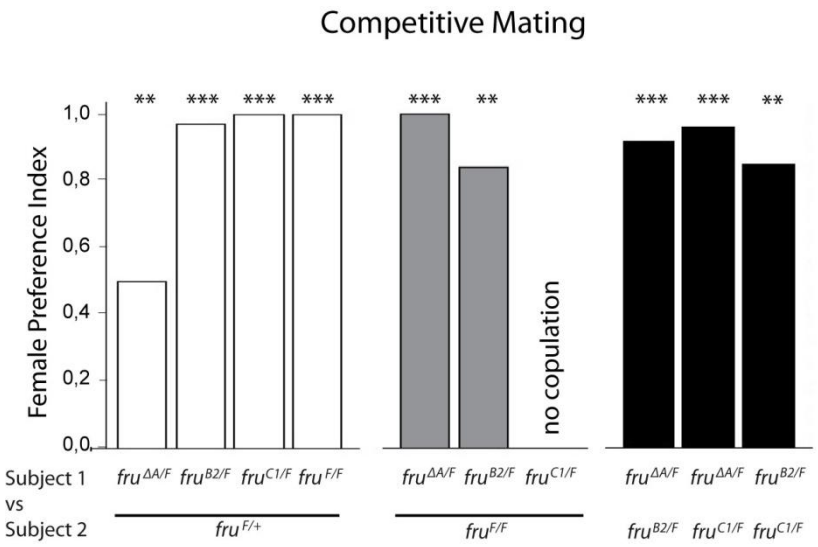


Figure 12. *fru* isoform mutants display different success in competitive mating assays

Competitive mating assay, n= 26-40, t= 30 min, Female Preference Index is calculated from [(Sum Copulations Object2-Sum Copulations Object 1)/Sum Copulations], Fisher`s exact test, p<0.001 ***, p<0.01 **.

2.2 **Expression of Fru Isoforms in the Central Nervous System**

Next we analysed the expression of the *fru* isoforms in the central nervous system. We figured that the pattern of expression could give insights into the neuronal basis of the observed distinct roles in courtship behaviour. We therefore took two parallel approaches: 1) we generated myc tagged versions of the proteins and 2) we generated isoform specific antibodies.

The tagged *fru* isoform alleles (*fru*^{Amyc}, *fru*^{Bmyc}, *fru*^{Cmyc},and *fru*^{Dmyc}) were validated for the insertion of the myc tag using exon specific primers (Figure 13 A). In a second PCR we confirmed the loss of the duplication that arose from ends-in targeting strategy (Data not shown). In the second approach, we attempted to generate polyclonal antibodies to each isoform. While we did not succeed in generating a functional Fru^{B} antibody, we were successful in generating antibodies to Fru^{A} and Fru^{C}.

The specificity and functionality of the Fru isoform specific antibodies was tested by overexpressing each isoform in third instar larva. Specifically, I used the peptidergic driver *CCAP-GAL4* to express the cDNAs for the respective isoforms. The Fru^{A} antibody only stains *CCAP* neurons in strains overexpressing the Fru^{MA} cDNA. The Fru^{C} antibody stains both the *CCAP* neurons overexpressing the Fru^{MC} cDNA as well as additional neurons (Figure 13 B). These additional neurons are likely to be those expressing the sex-non-specific Fru^{ComC}, which is expressed at this stage of development (Lee et al., 2000). Co-staining of adult brains of *fru*^{Amyc} strains for Fru^{A} and the myc tag, or alternatively *fru*^{Cmyc} strains for Fru^{C} and the myc tag, revealed perfect overlap and underlines the specificity of Fru^{A} and Fru^{C} antibodies (Data not shown).

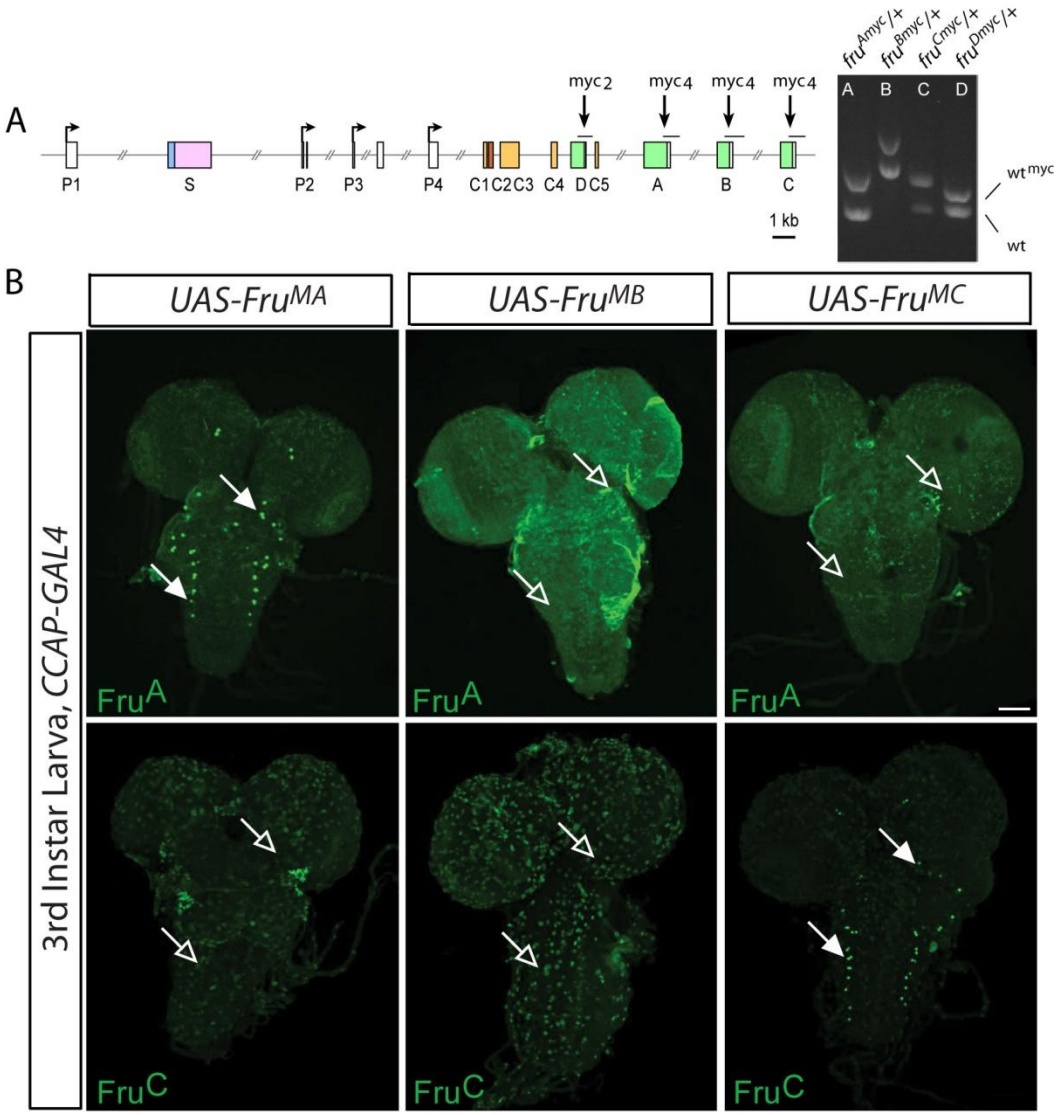


Figure 13. Validation of tools for Fru isoform expression analysis

(A) Schematic of the four *fru* alleles harbouring 2-4 myc tags (fru^{Amyc}, fru^{Bmyc}, fru^{Cmyc},and fru^{Dmyc}). The tagged isoforms were generated by end-in homologous recombination and validated by PCR for the insertion of the myc tag in heterozygous background. (B) Anti-Fru^{A} and Fru^{C} antibodies exclusively detect the appropriate isoform. From left to right Fru^{MA}, fru^{MB} and fru^{MC} cDNA transgenes are overexpressed in peptidergic neurons with *CCAP-GAL4* in third instar larvae. Solid arrows indicate specific labeling of the appropriate isoform in *CCAP* neurons. Empty arrows indicate absence of unspecific labeling in the other isoforms. Scale bar 50 µm.

The way the *fru* isoforms are expressed could give valuable hints about their cellular specificity, i.e. when and in which neurons they are required in order to masculinise the *fru* circuit and thereby determining aspects of the courtship behaviour. There are three possible outcomes of the expression study. First, each isoform is present in all *fru* neurons or second, the isoforms are expressed in exclusive subsets and third, their expression overlaps with each other to a certain extent. I first stained for the myc tag in the background of a strain expressing lamin-GFP under the control of the *fru* driver, fru^{GAL4}. fru^{GAL4} reproduces the expression pattern of the sex-specific transcripts derived from the *fru* P1 promoter. By contrast, the myc tagged isoforms should show expression from all *fru* promoters, both the sex-specific P1 promoter as well that the sex-non-specific P2-4 promoters.

fru^{GAL4} thus allowed me to compare the expression of each isoform in relation to the entirety of the sex-specific expression pattern (Figure 14 A). At a gross level, I observe little evidence for cell specific splicing, as the three isoforms Fru^{Amyc}, Fru^{Bmyc} and Fru^{Cmyc} are present in a major subset of *fru* neurons in males. By contrast, I do not detect expression of Fru^{Dmyc} in adult males or females. This observation is consistent with the reported absence of fru^{D}-mRNA in adult head tissues (Figure 15) (Billeter et al., 2006). In fru^{Bmyc} strains, expression from the sex-non-specific promoter, Fru^{ComB}, was seen in young adults .Specifically, I see staining for fru^{Bmyc} in both females and males (Figure 14 A and B). The cells expressing this sex-non-specific transcript don't overlap with the sex-specific transcripts, as defined by the fru^{GAL4} expression pattern in females (Figure 14 B-insert). These cells are labeled both in the cortex and the neuropil indicating that they are likely to represent both neurons and glia. The expression of these non-sex-specific, Fru^{ComB}, transcripts begins at the end of pupation, peaks in freshly eclosed adults, and is absent in 8 d old adults (Figure 16). This transient expression in neurons and glia is in line with the Fru^{Com} expression pattern previously observed (Lee et al., 2000).

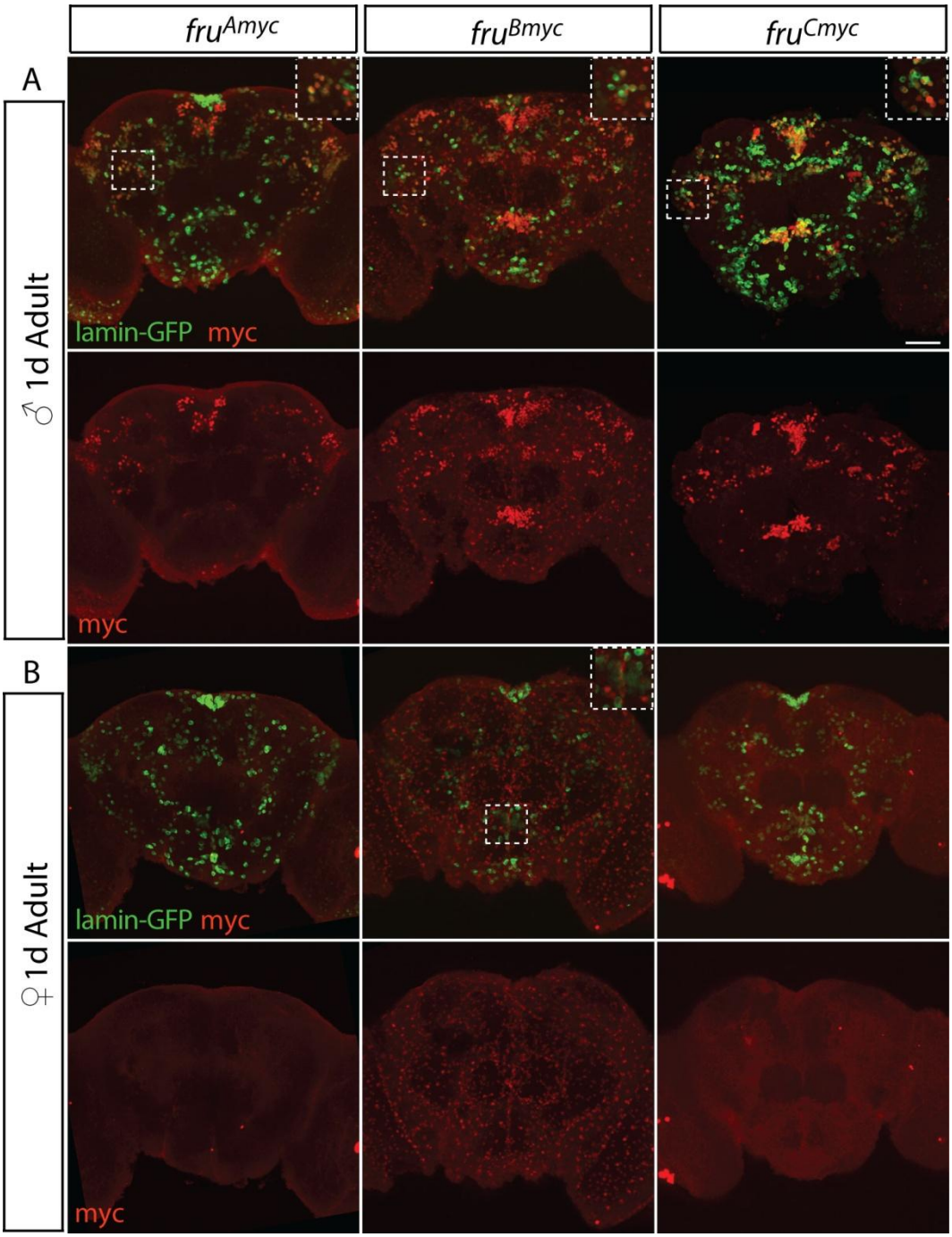


Figure 14. Fru isoforms are expressed in a subset of *fru*^{GAL4} neurons

Staining for myc tagged isoforms in 1 day old males (A) or females (B). The brains display expression of lamin-GFP driven by Fru^{GAL4}, along with anti-myc staining for the respective myc

tagged isoforms. The upper panels display double labeling with GFP in green and myc staining in red. The lower panels show the myc staining alone. From left to right the tagged isoform being expressed is: fru^{Amyc}, fru^{Bmyc}, fru^{Cmyc}. Insets show enlargement of marked regions and presents (A) or absence (B) of overlap between Fru^{Bmyc} and Fru^{GAL4}.

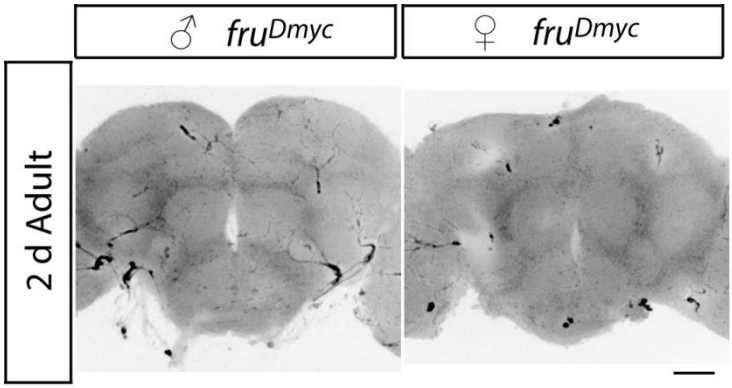


Figure 15. Fru^{Dmyc} is not expressed in the adult brain

Brains of adult male and female fru^{Dmyc} flies labeled for the myc tag show no expression. Scale bar 50 µm.

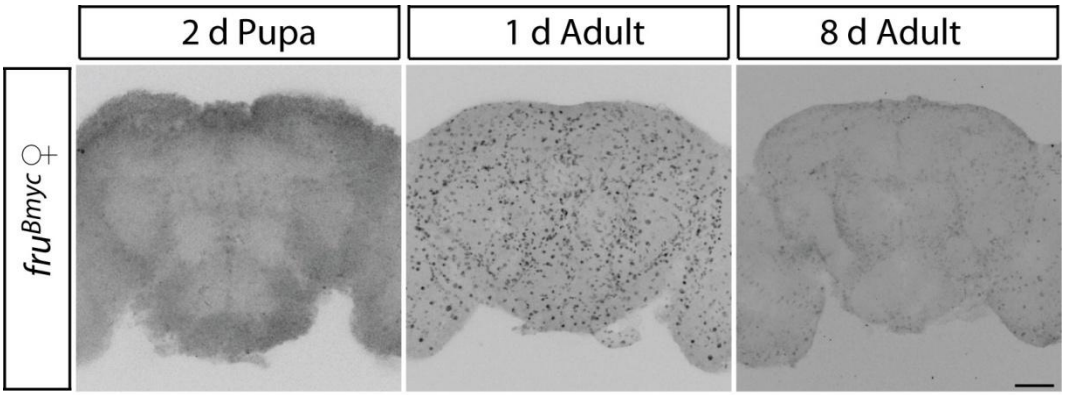


Figure 16. Fru^{ComB} is expressed transiently during development

Staged brains of fru^{Bmyc} females labeled for the myc tag (in red). From left to right: 2 d pupa, 1 d adult and 8 d adult. The sex-non-specific, Fru^{ComB}, is expressed transiently between late pupation and young adult stages. Scale bar 50 µm.

My initial stainings of the myc tagged isoforms suggested a highly overlapping, but not identical expression pattern. To further assess the degree of overlap of the Fru^{M} isoforms, brains and ventral nerve cords of adult fru^{Bmyc} flies were stained in parallel with antibodies against the myc- tag, Fru^{A} and Fru^{C} (Figure 17 A). Stainings were done in 8 day adults, to assess the contribution of the sex-specific isoforms to adult function and to circumvent detection of Fru^{ComB} expression. Despite the high degree of overlap, there are nonetheless pronounced differences between the expression patterns of the three different Fru^{M} isoforms. Fru^{A} is for example expressed in some neurons that are Fru^{Bmyc} and Fru^{C} negative, namely the mushroom body, the optic lobe and the mesothoracic ganglion neurons.

The fru^{M} mRNA peaks at the mid pupal stage where it likely functions in masculinising the nervous system; therefore, we assessed the *fru* isoform expression in 48 h pupal brain and ventral nerve cord. Again, the three isoforms are expressed in a highly overlapping manner, similar to the expression in the adult central nervous system (Figure 17 B). We further quantified the expression by counting the neurons labeled by each isoform. The relative numbers of cells expressing each isoform does not change through development (Table 1). Fru^{M} labels approximately 1600 neurons in the adult central nervous system and roughly the same number in 48 h pupa (Stockinger et al., 2005; Yu et al., 2010; Manoli et al., 2005). These cell counts do not contain the *fru* positive mushroom body and optic lobe neurons because they are too small and dense to be reliably counted. The entire expression of Fru^{A}, Fru^{Bmyc} and Fru^{C} in the central brain (excluding the mushroom body neurons) and the ventral nerve cord reconstitutes this cell number with 1573 and 1607 labeled neurons in pupa and adult central nervous system, respectively. Each individual isoform is expressed in about 1000 neurons i.e. 2/3 of all *fru* neurons. All three Fru^{M} isoforms overlap to a major degree, i.e. in 604 of the 1607 neurons (38 %). In terms of specificity, there are a higher proportion of exclusively Fru^{A} positive neurons than Fru^{Bmyc} or Fru^{C}.

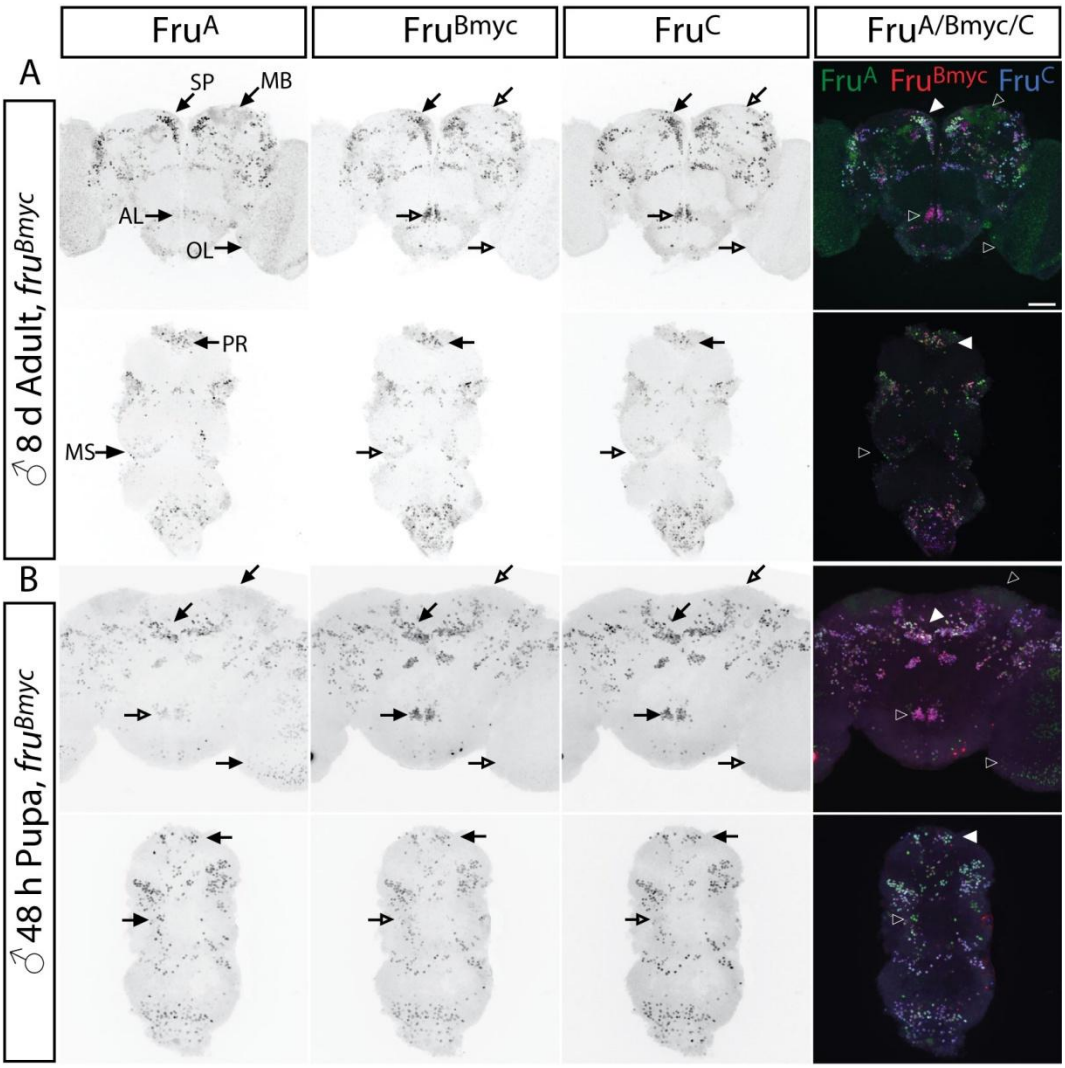


Figure 17. Fru^{M} isoforms are expressed in highly overlapping expression patterns

Brains and ventral nerve cords of male 8 d adult (A) and 48 h pupal (B) flies triple labeled for Fru^{A}, Fru^{Bmyc} and Fru^{C} in gray and colour coded overlay. Solid arrows indicate regions of triple labeling like medial superior protocerebrum (SP) and ventral prothoracic ganglion (PR). Empty arrows indicate areas with exclusive Fru^{A} expression (in green) like mushroom body (MB), optic lobes (OL) and mesothoracic ganglion (MS) or areas with exclusive Fru^{Bmyc} and Fru^{C} double labeling like ventral antennal lobe (AL).

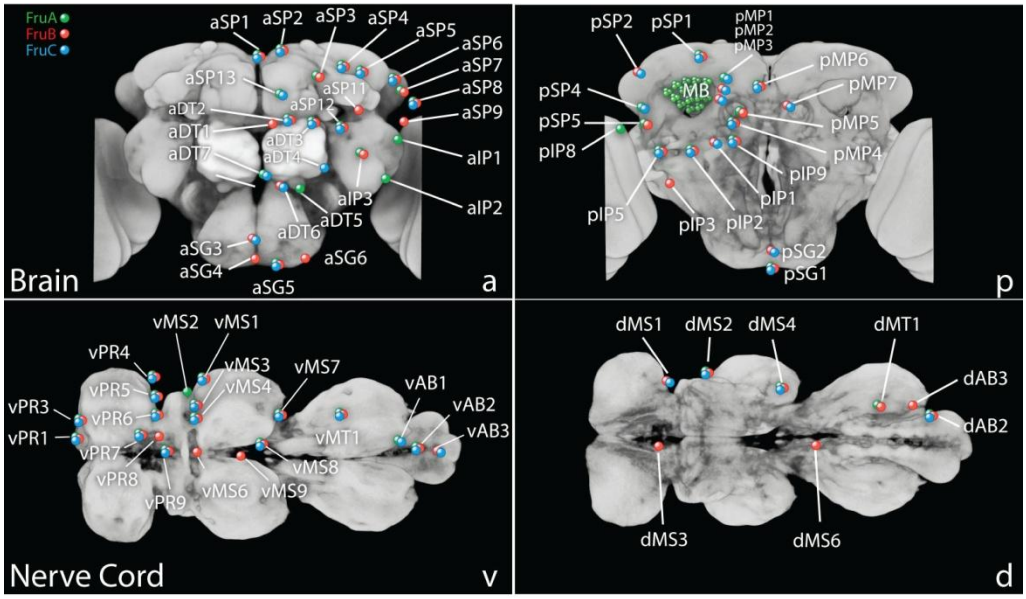
	Absolute Counts			Percentages %		
	Pupa Cells	**Adult Cells**	**Neuronal Classes**	**Pupa**	**Adult**	**Neuronal Classes**
Sum all	1573 ± 89	1607 ± 123	70	100	100	100
A all	1131 ± 40	1062 ± 109	49	72	66	70
B all	1246 ± 104	1132 ± 108	56	79	70	80
C all	1055 ± 73	1003 ± 69	44	67	62	63
A∩B	124 ± 43	76 ± 23	6	8	5	9
B∩C	235 ± 53	276 ± 17	6	15	17	9
A∩C	44 ± 13	28 ± 12	5	3	2	7
A∩B∩C	728 ± 76	604 ± 69	32	46	38	46
A\(B∪C)	235 ± 47	354 ± 78	6	15	22	9
B\(A∪C)	159 ± 54	175 ± 44	12	10	11	17
C\(A∪B)	49 ± 12	94 ± 21	1	3	6	1

Table 1. Cell counts and neuronal class analysis display Fru^M isoform expression patterns

Absolute counts contain absolute cell or neuron counts. Percentages contain absolute counts for every category as a percentage value of the sum of the entire set of positive cells or neurons. For absolute cell counts mean and s.d. are displayed, n=5. Data for cell counts was obtained using Imaris software and for neurons extracted from the Isoform Map in Figure 12. Cell counts do not include mushroom body and optic lobe neurons that are Fru^A positive.

This becomes most evident when also taking the mushroom body and optic lobe neurons into consideration that are mainly Fru^{A} positive. Furthermore, the overlap between Fru^{B} and Fru^{C} is larger than for the other combinations. Consistent with the visual impression, the cell number and overlap is highly similar between 48 h pupal and adult central nervous systems, indicating that *fru* isoform expression is stable over development. The similarity of the cell counts between pupal and adult central nervous system becomes further apparent from the Venn diagrams (Figure 19).

The *fru* neurons have been subdivided into distinct classes (Yu et al., 2010; Lee et al., 2000; Billeter and Goodwin 2004).



(adapted from Yu et al, 2010)

Figure 18. Fru^{M} isoform expression within defined neuronal classes

A schematic of the anterior (left) and posterior (right) brain as well as the ventral (left) and dorsal (right) ventral nerve cord is shown. Neuron clusters are labeled and colour coded according Fru isoforms expression: green for Fru^{A}, red for Fru^{B} and blue for Fru^{C}. Fru isoform maps are reconstructed from the data contained in the appendix and mapped onto the classes defined in Yu, 2010.

We therefore sought to map the Fru^M isoform expression onto these distinct classes of neurons. The most recent publication suggests 100 distinct classes (Yu et al., 2010). We mapped the isoform expression in 70 out of the 100 classes (Figure 18 and Appendix). The remaining classes were not mapped due to either the neurons having their cell body in the periphery or to the inability to unambiguously identify the cluster. Briefly, the technique involved using GAL4 drivers to identify clusters combined with antibody staining to identify the isoform within the defined cells (Appendix).

We find that Fru^A is expressed in 49, Fru^B in 56, and Fru^C in 44 classes of neurons (Figure 18 and Appendix). In 32 types of neurons all three isoforms are expressed, which represents 46 % of the unambiguously identified clusters (Table 1). In 6 types of neurons I find Fru^A exclusively expressed -aDT5, MB, aIP1, aIP2, pIP8 and vMS2. In 12 types of neurons I find Fru^B exclusively expressed -aDT1, aSG4, aSG6, aSP9, aSP11, pIP3, vPR8, vMS6, vMS9, dMS3, dMS6 and dAB3. In only 1 type of neuron is Fru^C exclusively expressed -aDT4 (Figure 18).

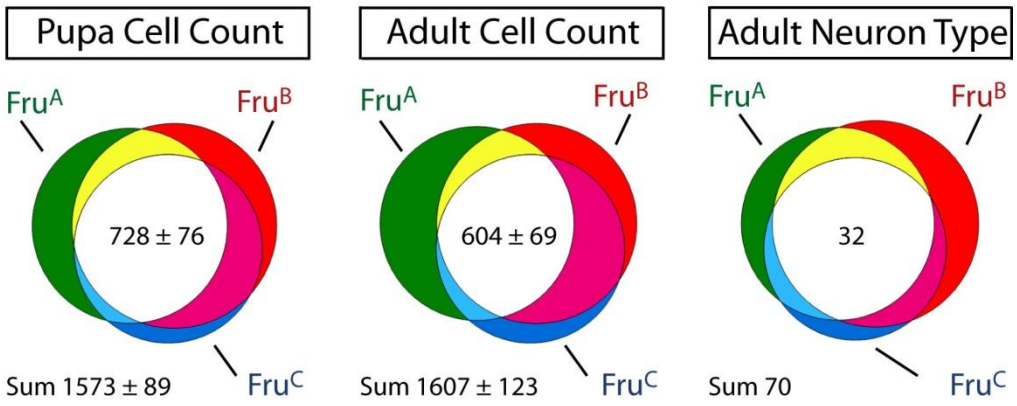


Figure 19. Venn Diagrams highlight overlapping and distinct isoform expression

Cell counts for the expression of each isoform from pupal (left) and adult (middle) central nervous system based on data in Table 2, n = 5. Neuronal class counts (right) are extracted from the Fru^M isoform expression map in Figure 18 and Table 2. Further details are found in the Appendix and Figure 12.

Further details of the expression analysis are provided in the appendix. This cellular expression study corresponds to the quantitative analysis of the Fru^M isoform expression (Figure 19).

In summary, we find considerable overlap between the isoforms around 40% of the cells expressing all three and 60% at least two isoforms. Nonetheless, some differences are apparent with around 37% of the cells expressing only a single isoform. Together, this analysis helps to guide further understanding of the different functional roles that we identified for the Fru^M isoforms.

2.3 Cellular Phenotypes of *fru* Isoform Mutants

Having established that the Fru^M isoforms play partially different roles in courtship behaviour and are expressed in a largely overlapping however somewhat distinct manner, I now turned to a detailed examination of the cellular substrate for courtship behaviour, the *fru* circuit. While there is little known about the sex-specific physiology of the *fru* circuit, it is now apparent that anatomical differences exist (Kimura et al., 2005; Datta et al., 2008; Yu et al., 2010, Cachero et al., 2010; Ruta et al., 2011). The differences range from changes in cell number to changes in cell morphology.

Tools exist to label most of the neurons showing anatomical differences between the sexes –specifically GAL4 enhancer trap lines that are restricted within the *fru* circuit (Yu et al., 2010). I therefore used these GAL4 enhancer trap lines to examine the requirement for each isoform in the described anatomical differences (Yu et al.,2010). I placed the *fru* mutants over the fru^{FLP} allele, which replaces the expression of the sex-specific *fru* transcripts with expression of the flipase. On one hand it is a null allele for the transcripts from the P1 promoter and on the other hand allows restriction of GAL4 expression to *fru* neurons (Yu et al., 2010).

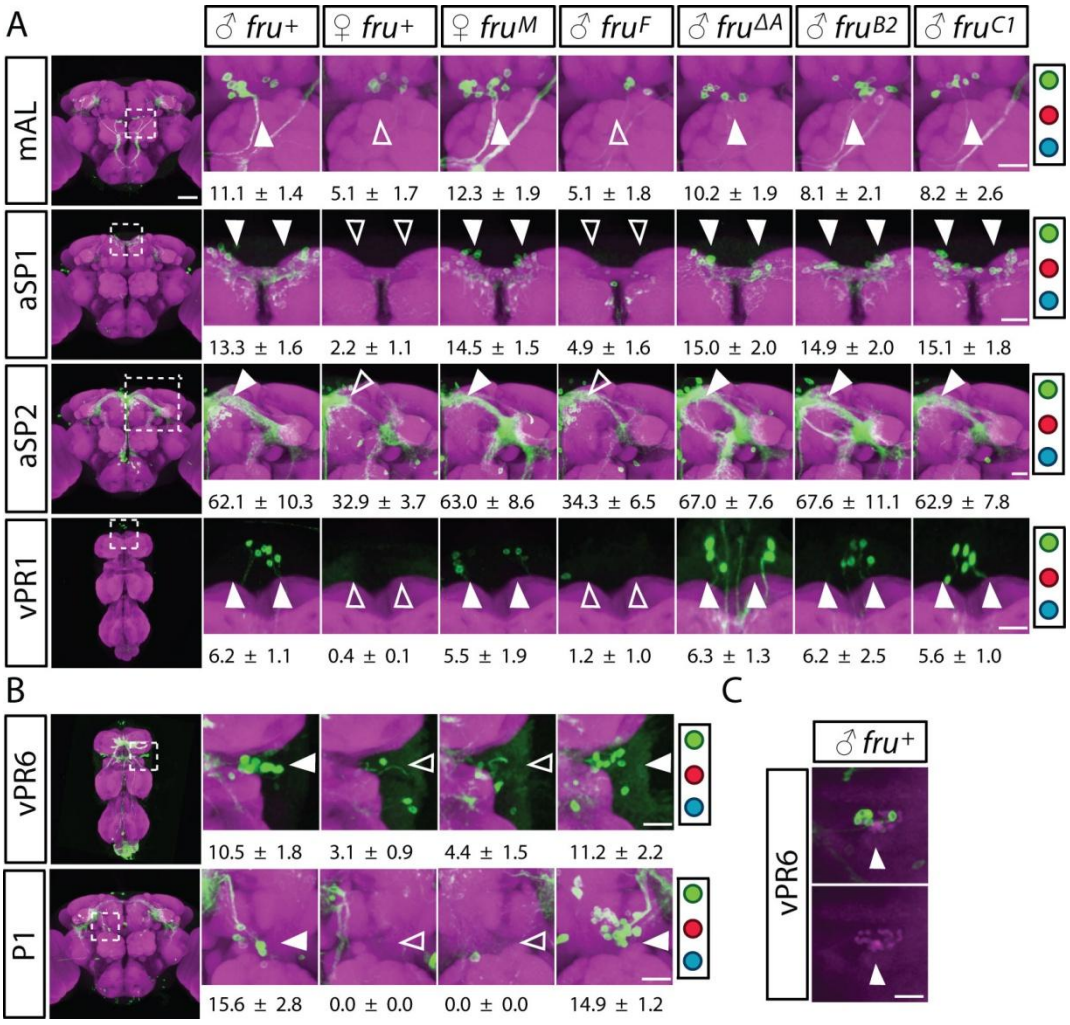


Figure 20. Fru^{M} specifies cell number in some neurons but the isoforms act redundantly

Rows show sex-specific neurons in the following genetic backgrounds *fru*$^{+}$, *fru*^{M}, *fru*^{F}, *fru*$^{\Delta Amyc}$, *fru*^{B2}, *fru*^{C1}. All isoform mutants are tested over *fru*^{FLP}. (A) Fru^{M} dependent cell number dimorphisms. For mAL, aSP1, aSP2 and vPR1 cell number dimorphisms Fru^{M} is necessary and sufficient but no single isoform is required. (B) Fru^{M} independent cell number dimorphisms. Fru^{M} is neither necessary nor sufficient to determine the cell number within the P1 and vPR6 neurons. Neurons labeled with mCD8-GFP in green, neuropil is labeled with nc82 in magenta, scale bar overview picture 50 µm, blow-up 20 µm, cell counts n= 10, mean ± s.d. (C) Staining of vPR6 neurons (green) shows that this cluster is positive for Dsx^{M} (magenta). Scale bar 25 µm. Traffic light displays Fru^{M} isoform expression on labeled neurons (Compare to Appendix).

Thus, I was able to uniquely look for contributions of the sex-specific role of the individual isoforms. Fru^M necessity was addressed in fru^F males, which lack Fru^M, whereas Fru^M sufficiency was addressed in fru^M females, which gain endogenous Fru^M.

In four out of six neuronal classes examined, I find that the cell number is dependent on Fru^M (Figure 20 A). In these neuronal classes, Fru^M is both necessary and sufficient, since cell counts in fru^F males equal those in females and expression of Fru^M in female gives the same count as a wild type male. Unlike those four neuronal classes aSP1, aSP2, mAL(aDT2) and vPR1 , the cell numbers in the vPR6 and the P1 (pMP4) clusters are not dependent on Fru^M (Figure 20 B). The presence of the mAL (aDT2) and P1 (pMP4) classes have previously been shown to depend on Fru^M and Dsx^M respectively, thus nicely validating our approach (Kimura et al., 2005; Kimura et al., 2008). The vPR6 class of neurons is located in the mesothoracic ganglion and is Fru^M/Dsx^M double positive (Figure 20 C). It has been shown that the cell number of a *dsx* positive set of neurons (TN1) in this area is Dsx^M dependent (Rideout et al., 2010). Therefore I suggest that TN1 and vPR6 are likely to be the same class of neurons. Having established that the cell numbers in the aSP1, aSP2, mAL (aDT2) and vPR1 clusters depends on Fru^M. I now sought to determine which isoform controlled the cell number. Surprisingly, each of the *fru* isoform mutants displays a wild type male cell number in all four clusters examined (Figure 20 A). Thus, in terms of the cell number, the three isoforms seem to function redundantly.

The analysis of the dimorphic arborisations was carried out by registering and averaging the stained samples (see methods). In six out of six clusters examined, I find that the sex-specific arborisation is dependent on Fru^M (Figure 21). In five out of six cases, aDT2, vAB3, pMP2, aSP4 and aSP6, I could show that Fru^M is both necessary and sufficient. Unlike these five clusters, Fru^M is not sufficient in females for shaping, the sexual dimorphism in the midline crossing of foreleg neurons (LAN). The necessity, but

lack of sufficiency for Fru^M in the LAN dimorphism is in agreement with previous observations (Mellert et al., 2010).

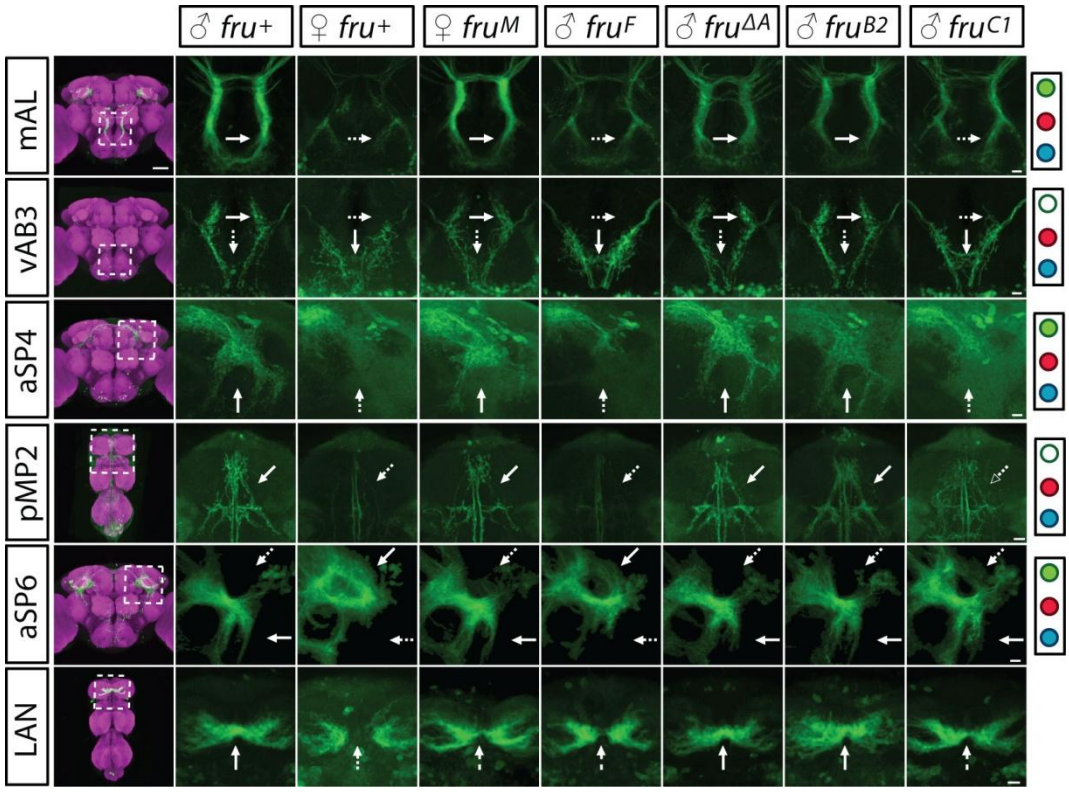


Figure 21. Fru^M and specifically Fru^{MC} determine sexually dimorphic arborisations in neurons

Fru^M dependent anatomical dimorphisms in selected *fru* neurons are displayed. Arrows indicate sex-specific arborisations (normal arrow presence of arbor, doted arrow reduction or lack of arbor). Each dimorphism was analysed in the following genetic backgrounds fru^+, fru^M, fru^F, $fru^{\Delta Amyc}$, fru^{B2}, fru^{C1}. Isoform mutants are tested over fru^{FLP}. In mAL, vAB3, aSP4, pMP2, aSP6 Fru^M is necessary and sufficient for the arborisation pattern. In all cases fru^{C1} is the major determinant. First panel shows the location of the cluster analysed, scale bar 50 µm. Panel 2-8 show blow-ups of averaged projection, scale bar 20 µm. The data for averaging come from 7-10 samples per genotype. Neuronal arborisations are labeled with mCD8-GFP in green, neuropil is labeled with nc82 in magenta. Traffic light displays Fru^M isoform expression on labeled neurons (Compare to Appendix).

Having established that the dimorphisms in all six clusters are depended on Fru^{M}, I now sought to determine which isoform was responsible for the dimorphisms. In sharp contrast to the cell number counts, I find phenotypes for some of the isoform mutants, suggesting that the isoforms do not function redundantly in determining sex-specific morphology (Figure 21). In all six neuron types, $fru^{\Delta Amyc}$ males never show a phenotype. In the mAL (aDT2) neurons, fru^{B2} males show a slight phenotype. In all types of neurons, fru^{C1} males display the strongest phenotype. I observe a complete transformation into the female pattern in the mAL (aDT2), vAB3 and aSP4 clusters and a strong transformation in the aSP6, pMP2 and LAN clusters.

The differences in phenotypes between the isoforms cannot be attributed to Fru^{M} isoform expression patterns since all but 2 dimorphic classes express the full set of isoforms. In the two classes that only express two isoforms, the phenotype can be attributed to Fru^{C} since Fru^{A} is absent in those neurons and fru^{B} mutants do not display a phenotype. In sum, I can conclude that most anatomical dimorphisms I examined are dependent on Fru^{M}. Within the Fru^{M} dependent dimorphisms, the Fru^{M} isoforms act largely redundantly to specify cell number, but largely non-redundantly to determine cell morphology. In all cases of sex-specific morphological differences, Fru^{C} appears to be the important determinant, as neither Fru^{A} nor Fru^{B} mutants have major alterations in arborisations.

2.4 Cellular Basis of Behavioural Phenotypes

At both, a gross behavioural level and a detailed anatomical level the most important isoform appears to be Fru^{MC} since fru^{C} mutants display dramatically reduced courtship, including lack of song, and feminised arborisation patterns in sexually dimorphic neurons. The *fru* circuit can be subdivided into distinct modules. For example, pMP4

(P1), pIP10, dPR1, vPR6, and vMS11 all contribute to distinct aspects of the courtship song (Kohatsu et al, 2011; von Philipsborn et al., 2011, Kimura et al., 2009) –and might therefore constitute a courtship song module. We conducted a small-scale screen to determine the importance of Fru^{MC} within known neurons of the courtship song module. Moreover we sought to uncover novel neuronal components in which Fru^{C} is required for song. The ultimate goal of this candidate song screen is to identify neurons in which Fru^{MC} is required to generate song and to elucidate the Fru^{MC} dependent cellular phenotypes underlying this behavioural phenotype.

In *D.melanogaster*, it is possible to knock-down gene function in a cell type specific manner by using RNAi constructs expressed under tissue or cell specific drivers (Kennerdell and Carthew, 2000; Dietzl et al., 2007). We therefore generated a fru^{C} specific short micro-RNAi (*fru^{C}-ShmiR or fru^{C}-IR*) construct according to procedures described previously (Haley et al., 2008). The specificity of this *fru^{C}-IR* was validated in behavioural assays and with immunohistochemistry. Flies expressing the *fru^{C}-IR* under the pan-neuronal driver *elav-GAL4* do neither generate pulse song nor succeed in copulation (Figure 22 A and B). Importantly, they also do not appear to have any general defects that would suggest off-target effects. Immunohistochemistry additionally demonstrated that the Fru^{C} isoform is selectively targeted. Specifically in the flies expressing *fru^{C}-IR* under the pan-neuronal driver elav-GAL4, I find no staining for Fru^{C}, while staining for Fru^{A} nor Fru^{B} remains unaffected at this level of analysis (Figure 22 C).

In an initial step, I used a small set of candidate GAL4 lines in order to selectively knock down Fru^{C}. (Yu et al., 2010; von Philipsborn et al., 2010). It is also important to note one major caveat of this approach: the *fru^{C}-IR* construct does not selectively target the sex-specific transcripts of *fru*. Unlike the previous behavioural analysis, there is no straightforward way of solving this caveat.

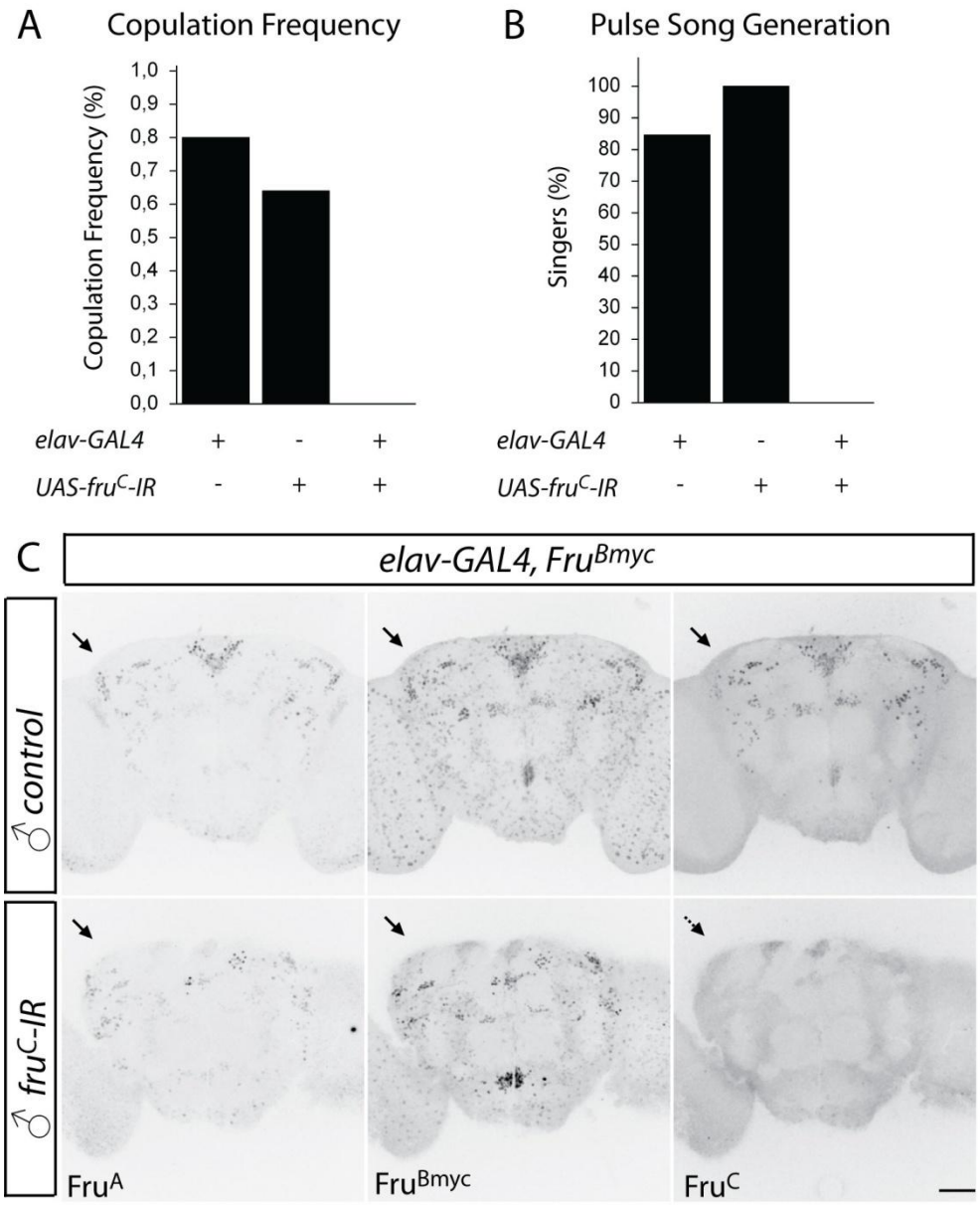


Figure 22. Validation of *fru^{C}-IR* specificity

Flies expressing *fru^{C}-IR* under e*lav-GAL4* display (A) absence of copulation success and (B) pulse song, n=5-16. (C) Accessing expression of all three Fru^{M} isoforms in *fru^{Bmyc}* strains expressing *fru^{C}-IR* under e*lav-GAL4* reveals specific loss of Fru^{C} but neither Fru^{A} nor Fru^{Bmyc} expression. Scale bar 50 µm.

Nonetheless, as Fru^{ComC} acts on various aspects of neuronal and general development we exclude flies having a high lethality rate or obvious behavioural impairments. The test set of GAL4s was composed of 4 enhancer trap lines and 19 molecularly defined enhancer-GAL4 transgenes. The lines were chosen from larger collections (Yu et al., 2010; von Philipsborn et al., 2010) according to two criteria. First, the driver lines needed to label a restricted set of maximally 10 types of *fru* neurons (Figure 23 A). Second, the driver lines were primarily chosen to contain previously defined neurons required for singing, namely P1 and vPR6. All of the identified neurons within the song module are known to be sex-specific (von Philipsborn et al., 2011). We also included driver lines that are expressed in sexually dimorphic neurons into our test set.

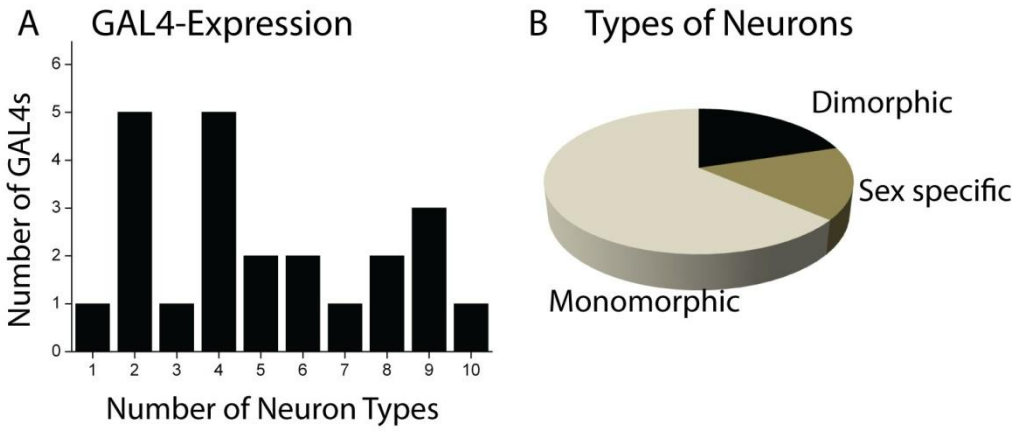


Figure 23. Properties of GAL4 lines tested in the courtship song assay

The test set was composed out of 23 GAL4 lines. (A) Distribution of GAL4s according to the amount of neurons labeled in the adult central nervous system. Each line labels less than 10 classes of neurons. (B) GAL4 lines were selected to contain sex-specific or sexually dimorphic neurons. The test set contains 40 % of such neurons, 30% more than a random set would do.

First we figured they are likely to be involved in the generation of song. Second, a subsequent analysis of the molecular functions of Fru^C is straightforward due to the observable morphology of the neurons. Nonetheless, the lines chosen do include some monomorphic classes of neurons but much less than a random set would do (Figure 23 B). From the 23 tested GAL4 lines 17 gave a phenotype that was statistically significant (Figure 24).

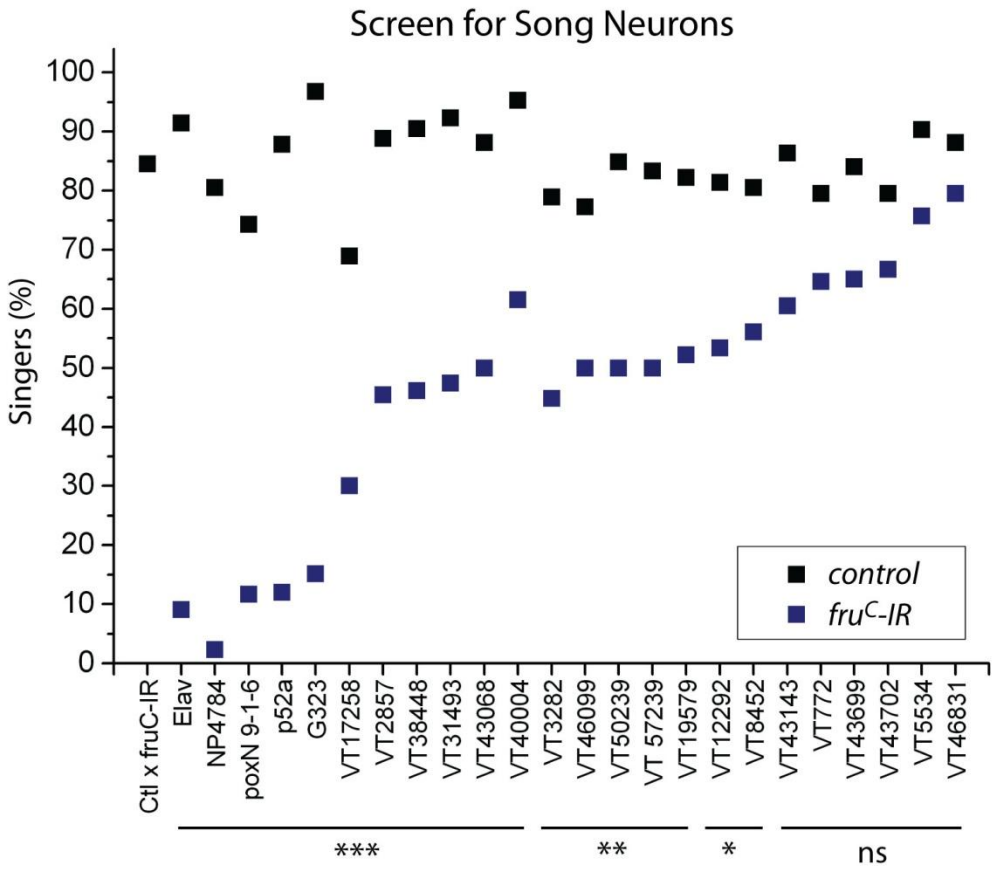


Figure 24. The majority of GAL4 lines cause a song phenotype in a small-scale screen

17 out of 23 GAL4 lines were found to cause a significant reduction of the percentage of flies generating courtship song when strains were also carrying *fru^C-IR* (blue) compared to controls (black). n= 25-50, Fisher`s exact test, $p<0.001$ ***, $p<0.01$ **, $p<0.05$ *.

The phenotypes for 8 of the 17 lines can be explained by reduction of Fru^{MC} function in the known clusters of the song neurons P1 and vPR6.Two lines containing P1 neurons are positive (G323 and VT43068) and 6 out of 8 lines containing vPR6 neurons are positive (NP4784, VT17258, VT3282, VT46099, VT57239 and VT19579). Therefore, it is likely that at least within these clusters, Fru^{MC} is the major isoform.

GAL4	Significance	Neurons	
pox9-1-6	***		vAB3
p52a	***	vPR1 vMS9	aDT6 aSP12 vMS8
VT2857	***	aSP4	dAB6
VT38448	***	vMS9	vPR7 vMT1 dMS5 pIP6_2 PMP1_1
VT31493	***	aSP4 vPR1 aDT2	aDT5 aSP8 aSP13 mbSN2 Brain_u
VT40004	***		aSP2_3 pIP9 Brain_u VNC_u
VT50259	**	aDT2	aSP5 pIP5 pIP7 pMP6 vMS4 VNC_u
VT12292	*		aSG MB vAB2 Brain_u
VT8452	*	aSP4	aSP2_3

Table 2. **List of positive GAL4 lines and the putative novel song neurons they label**

The listed GAL4s show a significant absence of song which cannot be explained by a known song neuron. First column refers to the GAL4 line and the second column to the level of significance of the phenotype. The third column displays all the neurons that are labeled by the GAL4 sorted for occurrence in the entire set of positive GAL4s. The left side contains vPR1, aSP4, aDT2 and vMS9 that are present multiple times in different GAL4 lines. The right side contains neurons that are labeled only in that particularly GAL4 line.

Additionally, the tested set contained 9 GAL4 lines that have not been implicated in song yet and still gave a phenotype. Within these lines the following 4 clusters were present multiple times and strongly enriched: aSP4, vPR1, mAL, vMS9 (Table 2). The positive set of neurons contained 30 more classes, which were less or not enriched. One of them vAB3 is worth mentioning since it is represented by one GAL4 pox-9-1-6 that exclusively labels this neuron. These clusters make attractive candidates to be novel neuronal components of the courtship song module.

In summary, I could give strong correlative evidence that two of the known song neurons P1 and vPR6 are likely to require Fru^{MC}. Finally, I was able to tentatively suggest 5 neuron types, which had not previously been identified as being involved in singing.

3 Discussion

3.1 *fru* Isoform Mutants Display Differences in Courtship

In my thesis research I aimed to elucidate the cellular expression pattern as well as the functions of the Fru^M isoforms to extend our understanding of the genetic and cellular basis of male courtship behaviour in the fruit fly. The molecular functions of proteins can be manifold. The *fru* isoforms are putative transcription factors that are important for male courtship behaviour. Due to the broad range of phenotypes that have been seen in *fru* mutants, I postulate that the targets of Fru^M may include both genes involved in neuronal development and neuronal physiology.

The function of a gene is classically inferred by analyzing the phenotype that results from manipulating the activity of the gene, its mRNA, or its protein product. A classic approach is the generation of loss-of-function mutations and the analysis of resulting phenotypes. Therefore, we generated flies carrying specific mutations in 3 of alternative isoforms of Fru^M (FruA, B, C). Fru^{MD} was not expressed and therefore was not considered further.

I first rigorously analysed the courtship behaviour in the mutants. The mutants of each isoform are impaired to different degrees. The fru^C mutants display the strongest phenotype since they have increased courtship initiation time, do not generate courtship song, do not attempt copulation, and almost never succeed in copulation. The fru^B mutants show an intermediate phenotype with a much longer delay in courtship initiation, a defective but present courtship song, a low level of attempted copulation, and some copulation success. Finally, fru^A mutants perform all courtship behaviours much better than fru^B or fru^C mutants and succeed in copulation in 50 % of the cases. This suggested that the protein variants might have partially overlapping but also distinct functions. The qualitative analysis of courtship song supports distinct

functions, since only fru^{A} but not fru^{B} mutants display an abnormal polycyclic pulse song. The fru^{B} and fru^{C} mutants show severe decrements in copulation success. Still the mutants differ in their respective decrements in the other pre mating steps. The behavioural phenotype of an already existing $fru^{\Delta C}$ null mutant is very similar to the one we observe in our fru^{C1-3} mutants (Billeter et al., 2006). This mutant, like ours, displays an almost complete absence of copulations success and an increase of courtship initiation time.

Up until recently, courtship has been described using a single parameter, the courtship index, which takes into account only the time until mating is initiated. (Gailey et al., 1986). In reality, courtship is a highly complex behaviour. Courtship involves a stereotyped series of steps, yet to what extent the steps are interdependent is a matter of some debate. For instance, is accumulation of sensory information necessary to proceed from one step to the next? In a related question, are the steps even sequential –can a step be bypassed? While I cannot answer these questions in my current work, it is important to realize that a defect in copulation attempts may arise indirectly from a defect in any number of the courtship steps that lead up to this final step. It will be important in future to directly test the mutants at each step in as controlled a fashion as possible. This may not be possible for all experiments, but one could for instance play wild- type song to a courting fru^{C} mutant male to ask whether there is a rescue of copulation success. A full rescue of copulation success in such an experiment would imply a specific role for FruC in the generation of courtship song.

In summary, we find that the *fru* isoform mutants display quantitative as well as qualitative differences in the performance of courtship behaviour. Can the basis for these differences be found in the expression pattern of the isoforms?

3.2 Fru^M Isoforms are Expressed in a Largely Overlapping Manner

I set out to identify the expression patterns of the Fru^M isoforms in order gain further understanding of unique phenotype seen in the respective mutants. One has to keep in mind however, that the role a gene plays for a certain behavioural phenotypes is determined by both the expression pattern and the molecular functions of its protein. We find that the Fru^M isoforms do not show obvious temporal specificity; specifically, all three isoforms are broadly expressed within the *fru* circuit throughout pupal and adult stages. While all three isoforms are widely expressed, I do find distinct neurons that express unique combinations or at times single isoforms. Each isoform is expressed at least in 2/3 of all *fru* neurons. All three isoforms overlap in about 40-70 % and are present uniquely in approximately 38 % of the neurons.

This expression pattern sheds some light on how the observed behavioural phenotypes of the mutants indeed arose. All isoforms are expressed in the majority of *fru* neurons and required for courtship behaviour however to a different extent. This coud be interpreted in one of two ways. First, it is possible that only a subset of cells have a major role in courtship behaviour. Thus, the cells in which Fru^{MC} and fru^{MB} are expressed might provide a guide to the cells that have the greatest role in behavioural phenotype. Alternatively, it is possible that the expression pattern is not as important as the genes expressed from the respective isoforms. Namely, the Fru^{MC} isoform might have a unique subset of downsteam target genes that have a major role in the development or function of the *fru* circuit, with the other isoforms playing a less important role.

Although at first glance it appears counterintuitive that the fru^A mutants have the smallest impairment of courtship despite being expressed in the largest subset of neurons, a large number of neurons also express the B and C isoforms. Thus it is

possible that it is not the expression pattern but the different molecular function each isoform exerts that is important. Therefore the weak phenotype of *fru*^{A} mutants suggests that its molecular functions are redundant.

I have more specifically looked at the expression with respect to the specific *fru* clusters identified previously (Yu et al., 2010). These results are in well accordance with the cell counts. As more types of *fru* neurons become accessible and functionally analysed, it will be useful to additionally elucidate their isoform expression profile.

In summary, I have shown that the expression patterns of the three Fru^{M} isoforms are highly overlapping in the adult central nervous system. This goes in line with and greatly extends what was previously known about Fru^{MA} and Fru^{MC} expression in the abdominal ganglion of two day old male pupae (Billeter et al., 2006).

3.3 Molecular Mechanisms of Fru^{M} Isoform Function

Fru^{M} is required in a large and heterogeneous subset of neurons for the complex performance of male courtship behaviour. Therefore, it likely plays various roles for development of the underlying circuit and its physiology. One might conceptually explain these widespread roles from the existence of the different isoforms and their partially independent expression patterns. The presence of the Fru^{M} isoforms in overlapping and distinct subsets of neurons provides the general framework under which I know try to understand the molecular functions of the distinct isoforms. In this section the different mechanisms will be discussed and how my data supports one or the other concept.

The Fru^{M} isoforms are expressed in largely overlapping manner. Either all three isoforms or at least two are present in a large fraction (70%) of all *fru* neurons. In those

neurons the isoforms could interact molecularly in two different ways. First, several isoforms could co-operate on determining a certain cellular feature either by heteromultimerisation or individual action and hence have similar molecular functions. Second, the isoforms are required to determine different cellular features of the type of neuron they are expressed in and therefore have distinct molecular functions. In order to distinguish between these possibilities we studied the cellular anatomy in *fru* mutants.

Importantly, sexually dimorphic neurons, addressed in this study, express almost always all three isoforms. A different cellular phenotype must therefore arise from the distinct molecular functions of the Fru^M isoforms. Our data suggests that both above mentioned scenarios are likely for Fru^M isoform function. First, we found that a set of 5 male specific neurons does not display a reduced female specific cell number in the different mutants. The absence of a phenotype in each isoform mutant favors to the conclusion that all isoforms have similar functions in regulation of cell fate and therefore are redundant in function. In this scenario the isoforms could either act directly or indirectly on different genetic substrates, of which each could be partially required to determine cell fate. Additionally, it is possible that all isoforms act in concert on one genetic target and can substitute each other. One could distinguish between these two hypotheses by identifying genetic targets or more specifically the genomic binding sites of each Fru^M isoform.

Conceptually it is an interesting question on how Fru^M establishes sex-specific neurons. Two hypotheses can be distinguished. Firstly, Fru^M acts in neuroblasts and establishes a sex-specific lineage that gives rise to neurons only in one sex. Secondly, neurons are born in both sexes and upon Fru^M expression specifically die or survive in one sex but not the other. The latter scenario has been shown to be the case for the sex-specific mAL neurons (Kimura et al., 2005; Kimura et al., 2008).

How about the sexually dimorphic neurons? The major determinant for them seems to be Fru^{MC} since only the fru^{C} mutants displayed a transformation into the female pattern of neuronal arborisation. The transformation was however partially incomplete in three out of six types of neuron. Since these sexually dimorphic neurons express two or three isoforms the Fru^{C} specific phenotype can only be attributed to the different molecular functions. Fru^{C} most likely functions in sexually dimorphic neurons directly or indirectly on axon guidance while Fru^{A} and Fru^{B} do not exert this function.

My experiments suggested that the isoforms function redundantly in specifying cell number, but non-redundantly in setting up arborisations. This observation is at odds with at least one previous set of experiments. $fru^{\Delta C}$ mutants display a dramatic reduction in male specific abdominal serotonergic neurons. In addition, fru^{A}, fru^{B} and fru^{C} transgenes are not redundant in their ability to rescue these neurons –specifically the number of neurons present in the rescue experiments is not the same from isoform to isoform (Billeter et al., 2006). While my studies do not directly address those neurons, the reduction of cell number in male fru^{C} mutants does contradict my general finding that the isoforms are redundant when it comes to cell number. One explanation is that with six types of neurons we have analysed a too small set and would observe the same changes of cell number in those neurons. Another possibility is that upon manipulation of the Fru^{M} proteins not the cell number but the expression of serotonin, that was used a marker, changed in the previous study.

My finding of non-redundant action of the Fru^{M} isoforms concerning neuron morphology is supported by a previous analysis of the midline crossing of forleg afferents in the ventral nerve cord (Mellert et al., 2010).

3.4 Correlation of Fru^M Isoform Expression and *fru* Mutant Phenotypes

A general purpose of our behavioural and expression studies was to deepen our understanding of the neuronal basis of certain behavioural modules. Our cellular analysis shows that some types of neurons are only expressing one isoform. This offers the opportunity to tentatively link the expression of a specific isoform in a certain type of neuron to a specific behavioural phenotype of the associated mutant. It has to be kept in mind, however that the seemingly specific phenotypes can also be attributed to the distinct molecular functions of the isoforms in other than the suggested neurons.

One example is the time until courtship initiation in fru^B mutants that is much higher than in the other two isoform mutants. Intriguingly, Fru^B is the only isoform that is expressed in neurons of the suboesophageal ganglion with local arborisations. This ganglion has been implicated as a primary center for taste/pheromone processing (Singh and Nayak, 1985). We therefore hypothesise that Fru^B is required in those neurons for appropriate processing of pheromone signal.

Are there specific functions for Fru^{MA}? Interestingly, neurons in the mushroom body, neurons in the optic lobe and several neurons in the lateral protocerebrum express only Fru^{MA}. It has been shown that Fru^M is required in the mushroom body neurons for courtship conditioning, which is the learned component of courtship (Manoli et al., 2005). Additionally, the male courtship drive displays a circadian rhythm that requires the clock genes to be functioning in *fru* neurons in the lateral protocerebrum (Fujii and Amrein, 2010). The large ventral lateral neurons (lLNv) can be unambiguously identified (in our nomenclature they are known as pIP8) –they uniquely express Fru^{MA}. These findings suggest that Fru^{MA} might play a modulatory role on the courtship circuit.

Although *fru*^{C} mutants display the most severe impairment of courtship behaviour it is not possible to directly deduce neurons underlying this from the expression pattern. The Fru^{C} isoform is expressed largely overlapping with the other two isoforms.

Our data allows the formulation of hypotheses concerning the neuronal basis of some aspects of behaviour. Those hypotheses can be carefully tested now by manipulating both physiology of the suggested neurons as well as the expression of the respective Fru^{M} isoform in those neurons by driving specific reporter constructs with restricted GAL4 lines. Those fly strains can then be tested for the recurrence of the isoform specific mutant phenotype.

3.5 Mapping Neurons in which Fru^{MC} is Required for Song

In this work we have elucidated the expression pattern and functional relevance of the different *fru* isoforms. This is meant to build the fundament for teasing apart the courtship circuit including molecular functions of Fru^{M}. One of the crucial findings of our behavioural studies was the fact that the isoform mutants have quantitatively and qualitatively different pulse songs. This opens a unique opportunity to further analyse the genetic and neuronal basis of courtship song.

The absence of courtship song in *fru*^{C} mutants is especially interesting since only *fru*^{C} is required for the male specific arborisation patterns of sexually dimorphic neurons. These neurons project in the three major regions of the *fru* circuit: the lateral protocerebral complex, the tritocerebral loop and the mesothoracic triangle. Those regions have been implied to be relevant areas for the generation of courtship song which is specifically lacking only in fru^{C} mutants (von Phillipsborn et al., 2011).

Several neuronal clusters are known to have a role in generating courtship song, yet we do not currently know the isoform requirement within these song centers (Kimura et al., 2008; Kohatsu et al., 2011, von Philipsborn et al., 2011). My experiments have now determined the requirement for Fru^{MC} within two of those clusters: P1 and vPR6. Additionally, I found the sex- specific vPR1 and mAL (aDT2), sexually dimorphic aSP4 and vAB3 and monomorphic vMS9 neurons to be potential components of the song circuit. This data is however preliminary and needs further validation.

P1 is an interneuron in the brain that is suggested to integrate sensory information and compute the command for song generation while vPR6 is an interneuron in the ventral nerve cord that might be part of the central pattern generator for song (Kimura et al., 2008, Yu et al., 2010; Kohatsu et al., 2011; von Philipsborn et al., 2011). Interestingly, both aSP4 and vAB3 neurons could act up-stream of the putative integration-center P1. The aSP4 neurons are dopaminergic interneurons, which are generally known for the modulation of arousal and choice behaviours, with ipsilateral and contralateral arborisation in the ring where P1 neurons project too (Riemensperger et al., 2011; Yu et al., 2010). The ascending vAB3 neurons instead are located in the ventral abdominal ganglion where they have post-synaptic processes and arborise in the region of the forleg afferent crossing indicating that they receive pheromonal input from the genitalia and forlegs. They have presynaptic processes in the suboesophageal ganglion, where they very likely connect to mAL neurons, and processes in the lateral horn (Yu et al., 2010). The mAL neurons have been shown to be relevant for song posture while the lateral horn is known to be a relay center for pheromonal information from the antennal lobes (Koganezawa et al., 2008; Datta et al., 2008). Finally, the vPR1 and vMS9 neurons are interneurons in the ventral nerve cord and could constitute a part of the central pattern generator for song. (Yu et al., 2010).

These yet unknown types of neurons are therefore highly promising candidates as components of the courtship song circuit. The next steps for validation comprise the

exact identification of those types of neurons in the GAL4s that display a phenotype in song generation either by using multiple other GAL4s that label mutual exclusive additional neurons or by methods of restriction for one GAL4 line like heat-shock inducible Flipase or split GAL4 (Luan and White, 2007). Since the *fru^{C}-IR* we used in this small-scale screen could act on all *fru^{C}* transcripts one crucial aspect is to unambiguously associate the song phenotype specifically to the repression of only Fru^{MC} but not Fru^{ComC}. The latter possibility can be excluded by introducing a ternary temperature sensitive factor into the test strains and restricting *fru^{C}-IR* expression to the pupal and adult stages where only Fru^{MC} is expressed.

The Fru^{M} isoforms can be further used as tools for understanding the generation of courtship song molecularly and cellularly. The usage of *fru^{A}* and *fru^{B}*-RNAi could reveal how aberrant song in those mutants specifically arises and in which neurons these isoforms are required. Additionally, thermogenetics could be used to activate relevant neurons for song in the isoform mutant backgrounds in order to determine where in the course of information processing and execution which isoform functions (von Philipsborn et al., 2011).

The more distant goal is, however, to identify whether the underlying molecular function of Fru^{MC} as a determinant for neuron morphology is indeed the cause of the behavioural phenotype and what the molecular targets are that are orchestrating this transformation. A first step towards this is to analyse the dimorphic morphology of the song neurons in those strains that specifically lack Fru^{MC} in those neurons and display a song phenotype. The gene *roundabout* (*robo)* is an axon guidance molecule that has been shown to act downstream of Fru^{M} in shaping the male anatomy of the forleg afferent neurons (Mellert et al., 2010). However, no phenotype has been observed yet in strains lacking of Fru^{M} or Robo in forleg neurons. In order to test other candidate genes that might act downstream of *fru* an RNAi approach seems to be most straightforward. Finally, the sufficiency of Fru^{MC} both for the cellular dimorphism and

the song generation could be established by expression of a fru^{MC} transgene in the relevant neurons in either fru^{C} mutant males or even females. Taken together, these results could reveal how a gene functions on a neurons anatomy and thereby influences specific behaviours. In vPR6 and P1 neurons, that already have been shown to be involved in courtship song, Fru^{MC} most likely masculinises the physiology since Dsx^{M} is required for the existence of those neurons (Rideout et al., 2010). Candidate downstream factors that have been shown to be involved in song generation are *dissonance*, *cacophony*, *croaker*, *period*, *paralytic* and *slowpoke* (Tauber and Eberl, 2003). These genes do not need to be direct targets of Fru^{MC} but could also indirectly be involved in the song production. One way to test this is to ask whether their expression pattern in song neurons is sex-specific and dependent on Fru^{MC}.

3.6 Implications for the Evolution of *fru*

The evolution of multiple isoforms of a gene is a common theme in the evolution of higher organisms with the requirement for more complex and versatile gene functions. This is obviously also the case for *fru*. It was already known that its` sex-specific and non-sex-specific isoforms have distinct functions for development and behaviour. Furthermore there was initial evidence for the zinc finger Fru^{M} isoforms to possess different functions as well. In this work I have added substantial support to this notion. Theoretical and empirical evidence suggests that duplicated gene domains are maintained because ancestral functions are shared among duplicates or at least one of them develops novel functions (Force et al., 1999). My data on the redundant as well as non-redundant molecular functions of the Fru^{M} isoforms goes along line with this. The most conserved zinc finger domains are fru^{B} and fru^{C} therefore it is likely that they convey ancient and conserved functions for courtship behaviour (Gailey et al., 2006;

Bertossa et al., 2009). The fru^{A} zinc finger is least conserved and even absent in *A. melifera* and *B. mori* which might account for the redundant molecular and behavioural functions we observe and the distinct courtship associated functions we suggest for Fru^{MA}.

Since the 101 amino acid N-terminal male specific extension of Fru^{M} does not contain any known motifs it is under debate whether it possesses any functional relevance. One hypothesis is that there is little difference concerning the molecular function of the Fru^{M} and Fru^{Com} zinc finger isoforms. Instead the specific role of Fru^{M} isoforms for male courtship behaviour is established purely by its sex-specific expression in distinct neuronal subsets. One line of evidence arises from experiments that showed that fru^{FomC} cDNA was sufficient to induce the Muscle of Lawrence in female fruit flies (Usui-Aoki et al., 2000). Along this line, one would expect that both types have similar molecular functions. It was shown that Fru^{ComA} and Fru^{ComC} have a role in axonal path finding in embryos (Song et al., 2002). In this study I could provide evidence that Fru^{MC} acts either directly or indirectly on neuronal arborisation patterns as well.

If the alternative zinc fingers possess similar functions in sex-specific and non-sex-specific Fru proteins one can hypothesise that they have evolved prior to the sex-specific splice site. It was shown that most *fru* zinc fingers where present before the radiation of holometabolous insect species. However, sex-specific splicing of *fru* transcripts is also present in the dipteran species *A.gambiae* as well as the parasitic wasp *N.vitripennis* and therefore has also ancient origin (Bertossa et al., 2009). In order to fully resolve when and how *fru* has acquired its sex-specific role in comparison to its non-sex-specific functions the structure of the *fru* locus and all its transcripts need to be analysed in more insect species.

The notion that *fru* zinc finger isoforms have similar functions in sex-specific and non-sex-specific Fru proteins could however explain the high degree of their conservation

since their evolution is under much stronger functional constrains. A zinc-finger could not evolve without affecting the function of the respective Fru^{M} and Fru^{Com} isoform. In this context it is most likely that evolution does not act primarily on the structure of the zinc finger domains but rather the expression patterns of the different Fru^{M} isoforms in order to modify courtship behaviour.

4 Conclusion and Outlook

Here we have shown that the master regulator of male courtship behaviour Fru^{M} performs its functions via three isoforms Fru^{MA}, Fru^{MB} and Fru^{MC}. These isoforms display shared and distinct expression patterns and impairments of courtship behaviour. Furthermore, their molecular functions are partially redundant and partially non-redundant. Alternative splicing of Fru^{M} has therefore proven an important element for simplifying complex expression pattern and function. Considering this, can one still speak of a single master regulator for courtship behaviour? Perhaps one can! Fru^{M} is a putative transcription factor indicating that it most likely diversifies its actions already at the subsequent genetic level. Many master regulators of morphological development are also transcription factors. Compared to the many possible downstream factors of fru^{M} the subfunctionalisation into three isoforms seems negligible.

A master regulator of behaviour is thought to instruct the morphology and physiology of the neuronal circuit underlying this behaviour. We have shown that Fru^{M}, specifically Fru^{MC}, is necessary and sufficient for the majority of structural dimorphisms of the *fru* circuit thereby highlighting its role as a master regulator for courtship behaviour. Additionally, this led us to suggest that it is the feminized neuronal anatomy in the fru^{C} mutants that causes the most severe courtship impairments, specifically in song generation, observed in those mutants.

The ultimate goal however was to identify neuronal substrates of behavioural modules and the molecular function Fru^{M} exerts in those neurons. Therefore we performed a candidate approach to map the sex-specific or sexually dimorphic neurons in which Fru^{MC} is required for proper generation of pulse song. We identified a number of known and novel song neurons. The further validation and characterization of those candidate neurons can lead to valuable insights into how the gene *fru* renders the

anatomy and physiology of a neuronal circuit male like in order to generate crucial aspects of courtship behaviour.

5 Methods

5.1 Generation of *fru* Isoform specific Mutants and RNAi

fru$^{\Delta tra}$ Reversion Screen

The *fru*$^{\Delta tra}$ allele was marked by recombining it with a miniwhite insertion on the third chromosome (*fru*$^{\Delta tra}$ *w*$^{+}$). Females carrying the *fru*$^{\Delta tra}$ *w*$^{+}$ chromosome have the same reduced fertility as the original *fru*$^{\Delta tra}$ females. *Fru*$^{\Delta tra}$ *w*$^{+}$/TM3 males were EMS treated according to standard methods. The mutagenised males were crossed to Ly, hs-hid/TM3 virgins. Males were removed after 3 days and females transferred to fresh medium every 2 days. The progeny were heat shocked as late third instar larvae in order to diminish all non-*fru*$^{\Delta tra}$ *w*$^{+}$ progeny. The eclosing males and females (appr. 85000) were tested in small groups (appr. 10 females and males) for reversion of female fertility. Vials with more than 10 pupae were kept and the progeny crossed inter-se after the presence of the w^{+} insertion had been confirmed. Stocks were established from single males and tested for the presence of the *fru*$^{\Delta tra}$ insertion. Established stocks were crossed to Df(3R)Exel6179, a deficiency removing *fru* and a few neighbouring genes completely, and tested for lethality in order to map the mutation to the *fru* locus. The alleles that were lethal with Df(3R)Exel6179 were crossed to *fru*$^{4-40}$ an allele removing the male specific P1 promoter and tested for male fertility. All protein coding exons were sequenced from lethal and male sterile/reduced fertility stocks. The five alleles containing a mutation in either the B or C DNA binding domains were backcrossed to an isogenic background (*w*^{1118}; iso2; iso3) for at least five generations for systematic characterization of lethality and fertility. The *fru*^{C} mutants were further backcrossed to an isogenic w+ background prior to detailed behavioural analysis. The already reduced fertility of the *fru*^{B1} and *fru*^{B2} mutant revertants decreased further during isogenisation and especially in the *w*$^{+}$ background. The

mutants were therefore maintained as balanced heterozygotes, specifically w^+; iso2; fru^B/Tm3,Sb males were generated by crossing w1118/Y; iso2; fru^B/TM3 *Sb* males to w^+; iso2; *Dr* (iso3 background)/TM3 *Sb* virgins and used for behavioural assays.

Gene Targeting fru^A Isoform

Since we did not recover a fru^A specific mutant in this screen a $fru^{\Delta Amyc}$ allele was generated by ends-in homologous recombination, essentially as described (Gong and Golic, 2003). The donor construct contained a total of ~ 7 kb of homology to the genomic sequence immediately upstream of the A exon, with an I-SceI site approximately in the middle of the homology region. The myc tag and stop codon was placed after the codon for G816 in the A exon (FBpp0083063; REFSEQ NP_732347), thereby deleting codons for the final 139 residues of Fru^A. A further ~ 1.5 kb homology following the endogenous stop codon, an I-CreI site, and the $white^+$ marker was added. Donor constructs were prepared through standard cloning procedures, with genomic fragments amplified by PCR from wild type *Drosophila*. Ends-in targeting results in a duplication, which was resolved by using *hsI-CreI* to introduce a double-stranded break at the I-CreI site and selecting progeny for the loss of the $white^+$ marker. The $fru^{\Delta Amyc}$ flies were validated for the resolved duplication and replacement of 139 residues in the A exon with 4 c-myc tags via PCR and Sequencing. The recombinant flies were backcrossed for 5 generations to w+; iso2; iso3 background prior to behavioural and lethality tests.

Generation fru^C-RNAi

The strategy for targeting the fru^C transcripts was based on microRNA interference (Haley et al., 2008). The targeting construct was designed using an online hairpin design tool. The targeting hairpin had the following sequence:

ctagcagtCTGGCCATAAATCGCATCAGAtagttatattcaagcataTGTGATGCGAATTATGGCCAGgcg

The targeting construct was cloned in the pNE expression vector and inserted into the fly genome. The presence of the fru^C targeting insertion in the fly genome had no apparent effect on health or viability of the transgenic animals. The specificity of the mRNAi knock-down was tested by looking at the behavioural consequence of expressing the fru^C targeting construct under the pan-neuronal e*lav-GAL4* driver. Additionally, the same flies were tested by antibody staining and were found to specifically lack Fru^C and not Fru^A and Fru^B expression.

5.2 Generation of Tools for Fru Isoform Expression Analysis

To examine the expression patterns of the various *fru* isoforms, we designed tools for fru^A, fru^B, fru^C and fru^D. We generated four new *fru* alleles by ends-in homologous recombination, adding c-myc epitope tags to the carboxy terminus of either the Fru^A, Fru^B, Fru^C or Fru^D isoform. We refer to these four alleles as fru^{Amyc}, fru^{Bmyc}, fru^{Cmyc} and fru^{Dmyc}. The four novel *fru* alleles were generated in a similar manner as the $fru^{\Delta Amyc}$ allele, exept that the first homology region was followed by 4 in-frame c-myc epitope tags for fru^A, fru^B and fru^C and 2 c-myc tags for fru^D (amino acid sequence EQKLISEEDLGS) without the deletion of endogenous sequences (Gong and Golic, 2003). The final recombinant lines were verified by genomic PCR and DNA sequencing across the targeted region, and confirmed to be wild type for *fru* function in courtship assays.

Fru^A,Fru^B and Fru^C antisera were obtained from rabbit immunised with a GST fusion protein containing the entire isoform specific zinc finger exon. The sera were purified against their antigen on column and the eluted antibodies dialysed in PBS containing 50 % glycerol. Functionality and specificity were tested by staining with the antibodies in strains overexpressing *fru* isoform specific transgenes in third instar larva *CCAP* neurons. The Fru^A and Fru^C antibodies demonstrated specific staining in this assay. Fru^B

antibodies did not give specific staining and where not subsequently used. Additionally, an anti-guinea-pig Fru^C antibody was generated to carry out Fru^A, Fru^B and Fru^C triple labeling. The reference sequences for the amino acid are for Fru^A: FBpp0083063; REFSEQ NP_732347, for Fru^B: FBpp0083065; REFSEQ:NP_732345 and for Fru^C: FBpp0083061; REFSEQ:NP_732344.

5.3 **Fly Stocks**

For analysis of overlap of Fru^M isoforms with fru^{GAL4}, the myc tagged *fru* alleles were crossed to w^-*; UAS-lamin-GFP;* fru^{GAL4} flies (Stockinger et al., 2005). In the triple isoform staining w^-; $fru^{B\text{-}myc}$ were used together with the Fru^A and Fru^C specific antibodies. The validation of the Fru^M isoform antibody specificity was carried out by crossing w^-*, CCAP-GAL4* with w^-*;UAS-*Fru^{MA}, w^-*;UAS-*Fru^{MB} and w^-*;UAS-*Fru^{MC} flies (Kim et al., 2006; Song et al., 2002). The UAS-FruMA,B, and C all express the respective cDNA for the isoform. Experiments for analysis of cellular phenotypes of the *fru* isoform mutants were done crossing *w-; UAS>>mCD8GFP;* fru^X with *w-; Y-GAL4;* fru^{FLP} (Yu et al., 2010). X stands for the *fru* alleles and Y for the GAL4 enhancer trap lines. In order to avoid confusion with the novel $fru^{C1\text{-}C3}$ alleles the existing fru^C control allele was termed fru^+ in this study. This *fru* allele is a control for the targeting procedure and only contains a footprint, an FRT site, which is also present in the other *fru* alleles (Demir and Dickson, 2005). Behavioural experiments on *fru* isoform mutant phenotypes were performed by crossing the novel *fru* alleles w^+*;* $fru^{\Delta A}$, w^+*;* $fru^{B1\text{-}B2}$ *and* w^+*;* $fru^{C1\text{-}C3}$ *to* w^+*;* fru^F (Demir and Dickson, 2005). Experiments knocking down the Fru^C isoform using RNAi were conducted crossing w^-*; UAS-*Fru^C*-ShmiR* flies with GAL4 lines from either an enhancer trap collection (Yu et al., 2010) or from an enhancer tile collection (C.M., S.S.B., A. Stark, and B.J.D., unpublished). The latter strategy is described in Pfeiffer et al, 2008.

w^+, iso2, iso3 flies used for backcrossing and behaviour were generated from a *w1118, iso2, iso3* stock (Ryder et al., 2004).

5.4 Immunohistochemistry and Image Analysis

Unless indicated, staining of adult central nervous system was carried out by collecting freshly eclosed flies and aging them for 2 to 4 days before dissection. For pupal staining, white prepupae were separated by sex and aged for 48 h in food vials. Wandering larvae were used for staining of third instar larval central nervous system. The central nervous system was dissected, fixed for 20 min in 4 % paraformaldehyde, washed four times for 15 min and blocked in 10 % normal goat serum for 4 hours at room temperature. Primary and secondary antibody incubations were carried out in 5 % normal goat serum at 4 °C and each incubation was followed by four steps of washing within one hour at room temperature and one subsequent wash over night at 4 °C. Prior to mounting in Vectashield (vector labs) the samples were washed every hour for at least 4 hours. Antibodies were used at the following concentrations rabbit anti-Fru^A (1:4000), guinea-pig anti-Fru^C (1:8000), rat anti-myc (1:8000, Abcam), mouse anti-nc82 (1:20, Hybridoma Bank), rat anti-Dsx^M (1:1000, Hempel and Oliver, 2007), chicken anti-GFP (1:10 000, abcam) and secondary Alex-Fluor 488, 568 and 647 antibodies (1:1000, Invitrogen). The basis for all required solutions was PBS containing 0.3 % TritonX and 0.1 % NaN_2.

Confocal stacks were taken on a Zeiss LSM510 with a Multi Immersion Plan NeoFluar 25x/0.8 objective. ImageJ was used for image processing, which included rotation, noise reduction, superimposition and contrast/brightness adjustments. Cell number analysis of Fru^M isoform expression in the central nervous system was carried out with Imaris (Bitplane) using a cell diameter of 3.5 µm for spot detection and correcting

manually for wrongly detected spots. For analysis of sexual dimorphisms image registration and processing was performed as described previously (Yu et al, 2010). Non-rigid image registration was done without prior processing except inversion or rotation. Registration was verified manually in ImageJ by superimposing the template with the neuropil (NC82 stained) channel of the sample. Subsequently registered images were averaged using Amira (Visage Imaging) software. Amira was also used to segment the arborisation of the averaged images from the aSP6 neurons displayed in Figure 19 in order to clearly reveal their arborisations.

5.5 **Behavioural Analysis**

Flies were raised on defined medium (Backhaus et al., 1984) at 25 °C in a 12hr:12hr light:dark cycle and collected as virgins after eclosure. Females were kept in groups up to 20 and males were housed individually. All behavioural experiments were conducted with 5-7 day old males and 4-6 day old females of the genotype: *w^+; iso2; iso3* as objects. Copulation frequency and attempted copulation assays were performed in single pair assays in chambers of 1 cm diameter for 10 min under constant light. Courtship initiation latency was assessed in a chamber with 10 cm diameter. Competitive courtship assays were carried out similarly but with an observation period of 30 min. In the competitive courtship assay, the genotype of the competing males was distinguished by applying a terra cotta mark to the thorax at least 24 h prior to testing. The mark itself did not affect courtship performance. All the behavioural assays were videotaped and analysed manually. In RNAi experiments, the assays were performed in the dark with an assay time of 10 min. Courtship Song was recorded from single males female pairs housed in a soundproof chamber measuring 1 cm in diameter. Recordings were taken for 3 min or until copulation. Pulse song was analysed

with LifeSong (Bernstein et al., 1992). Settings in Lifesong were: signal/noise ratio: 5, IPI detection 15-65 ms, minimum train length: 3 pulses. In the *fru*^{C} RNAi screen, flies were scored as “singers” and “non singers” depending on whether the number of pulses per minute was above or below 3. When data was obtained describing a population property, like for copulation frequency, competitive mating and the amount of “singers” in the GAL4 candidate song screen, the statistical significance of the differences was assessed using Fisher`s Exact Test. In all the other cases where a specific parameter was determined for every individual fly, such as time until courtship initiation, the mean and standard error were calculated. This data was subjected to the Mann-Whitney Test since it was not normally distributed.

6 Bibliography

Allada, R. (2003). Circadian clocks: a tale of two feedback loops. *Cell , 112*, 284-286.

Anand, A., Villella, A., Ryner, L. C., Carlo, T., Goodwin, S. F., Song, H. J., et al. (2001). Molecular genetic dissection of the sex-specific and vital functions of the Drosophila melanogaster sex determination gene fruitless. *Genetics , 158*, 1569-1595.

Arthur, B. I., Jallon, J. M., Caflisch, B., Choffat, Y., & Nöthiger, R. (1998). Sexual behaviour in Drosophila is irreversibly programmed during a critical period. *Curr Biol , 8*, 1187-1190.

Backhaus, B., Sulkowski, F., & Schlote, F. (1984). A semi-synthetic, general-purpose medium for Drosophila melanogaster. *Dros Inf Serv , 60*, 210-212.

Baker, B. S. (1989). Sex in flies: the splice of life. *Nature , 340*, 521-524.

Baker, B. S., Taylor, B. J., & Hall, J. C. (2001). Are complex behaviors specified by dedicated regulatory genes? Reasoning from Drosophila. *Cell , 105*, 13-24.

Belote, J. M., & Baker, B. S. (1987). Sexual behavior: its genetic control during development and adulthood in Drosophila melanogaster. *Proc Natl Acad Sci U S A , 84*, 8026-8030.

Bennet-Clark, H. C., Dow, M., Ewing, A. W., Manning, A., & Schilcher, F. v. (1976). Letter: Courtship stimuli in Drosophila melanogaster. *Behav Genet , 6*, 93-95.

Bernstein, A., Neumann, E. K., & Hall, J. (1992). Temporal analysis of tone pulses within the courtship song of two sibling Drosophila species, their interspecific hybrid, and behavioral mutants of D. melanogaster. *Journal of Insect Behavior , 5*, 15-36.

Bertossa, R. C., Zande, L., & Beukeboom, L. W. (2009). The Fruitless gene in Nasonia displays complex sex-specific splicing and contains new zinc finger domains. *Mol Biol Evol , 26*, 1557-1569.

Billeter, J.-C., & Goodwin, S. F. (2004). Characterization of Drosophila fruitless-gal4 transgenes reveals expression in male-specific fruitless neurons and innervation of male reproductive structures. *J Comp Neurol , 475*, 270-287.

Billeter, J.-C., Atallah, J., Krupp, J. J., Millar, J. G., & Levine, J. D. (2009). Specialized cells tag sexual and species identity in Drosophila melanogaster. *Nature , 461*, 987-991.

Billeter, J.-C., Rideout, E. J., Dornan, A. J., & Goodwin, S. F. (2006). Control of male sexual behavior in Drosophila by the sex determination pathway. *Curr Biol , 16*, R766--R776.

Billeter, J.-C., Villella, A., Allendorfer, J. B., Dornan, A. J., Richardson, M., Gailey, D. A., et al. (2006). Isoform-specific control of male neuronal differentiation and behavior in Drosophila by the fruitless gene. *Curr Biol , 16*, 1063-1076.

Brand, A. H., & Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development , 118*, 401-415.

Bray, S., & Amrein, H. (2003). A putative Drosophila pheromone receptor expressed in male-specific taste neurons is required for efficient courtship. *Neuron , 39*, 1019-1029.

Cachero, S., Ostrovsky, A. D., Yu, J. Y., Dickson, B. J., & Jefferis, G. S. (2010). Sexual dimorphism in the fly brain. *Curr Biol , 20*, 1589-1601.

Camara, N., Whitworth, C., & Doren, M. V. (2008). The creation of sexual dimorphism in the Drosophila soma. *Curr Top Dev Biol , 83*, 65-107.

Certel, S., Savella, M. G., Schlegel, D. C., & Kravitz, E. A. (2007). Modulation of Drosophila male behavioral choice. *Proc Natl Acad Sci U S A , 104*, 4706-4711.

Cline, T. W. (1993). The Drosophila sex determination signal: how do flies count to two? *Trends Genet , 9*, 385-390.

Clyne, J. D., & Miesenböck, G. (2008). Sex-specific control and tuning of the pattern generator for courtship song in Drosophila. *Cell , 133*, 354-363.

Cranston, P., & Gullan, P. (2004). *The Insects: An Outline of Entomology.* Wiley-Blackwell.

Datta, S. R., Vasconcelos, M. L., Ruta, V., Luo, S., Wong, A., Demir, E., et al. (2008). The Drosophila pheromone cVA activates a sexually dimorphic neural circuit. *Nature , 452*, 473-477.

Demir, E., & Dickson, B. J. (2005). fruitless splicing specifies male courtship behavior in Drosophila. *Cell , 121*, 785-794.

Dietzl, G., Chen, D., Schnorrer, F., Su, K.-C., Barinova, Y., Fellner, M., et al. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. *Nature , 448*, 151-156.

Dornan, A. J., Gailey, D. A., & Goodwin, S. F. (2005). GAL4 enhancer trap targeting of the Drosophila sex determination gene fruitless. *Genesis , 42*, 236-246.

Duffy, J. B. (2002). GAL4 system in Drosophila: a fly geneticist's Swiss army knife. *Genesis , 34*, 1-15.

Ejima, A., Smith, B. P., Lucas, C., Naters, W., Miller, C. J., Carlson, J. R., et al. (2007). Generalization of courtship learning in Drosophila is mediated by cis-vaccenyl acetate. *Curr Biol , 17*, 599-605.

Ewing, A. (1979). The neuromuscular basis of courtship song in Drosophila: The role of direct and auxillary wing muscles. *J Comp Physiol. , 130*, 87-93.

Ferveur, J. F., Savarit, F., O'Kane, C. J., Sureau, G., Greenspan, R. J., & Jallon, J. M. (1997). Genetic feminization of pheromones and its behavioral consequences in Drosophila males. *Science , 276*, 1555-1558.

Finley, K. D., Taylor, B. J., Milstein, M., & McKeown, M. (1997). dissatisfaction, a gene involved in sex-specific behavior and neural development of Drosophila melanogaster. *Proc Natl Acad Sci U S A , 94*, 913-918.

Fishbein, L., Flamm, W. G., & Falk, H. L. (1970). *Chemical mutagens. Environmental effects on biological systems.* Academic Press. New York.

Force, A., Lynch, M., Pickett, F. B., Amores, A., Yan, Y. L., & Postlethwait, J. (1999). Preservation of duplicate genes by complementary, degenerative mutations. *Genetics , 151*, 1531-1545.

Fujii, S., & Amrein, H. (2010). Ventral lateral and DN1 clock neurons mediate distinct properties of male sex drive rhythm in Drosophila. *Proc Natl Acad Sci U S A , 107*, 10590-10595.

Gailey, D. A., & Hall, J. C. (1989). Behavior and cytogenetics of fruitless in Drosophila melanogaster: different courtship defects caused by separate, closely linked lesions. *Genetics , 121*, 773-785.

Gailey, D. A., Billeter, J.-C., Liu, J. H., Bauzon, F., Allendorfer, J. B., & Goodwin, S. F. (2006). Functional conservation of the fruitless male sex-determination gene across 250 Myr of insect evolution. *Mol Biol Evol , 23*, 633-643.

Gailey, D. A., Lacaillade, R. C., & Hall, J. C. (1986). Chemosensory elements of courtship in normal and mutant, olfaction-deficient Drosophila melanogaster. *Behav Genet , 16*, 375-405.

Gailey, D. A., Taylor, B. J., & Hall, J. C. (1991). Elements of the fruitless locus regulate development of the muscle of Lawrence, a male-specific structure in the abdomen of Drosophila melanogaster adults. *Development , 113*, 879-890.

Gill, K. (1963). A mutation causing abnormal courtship and mating behavior in males of Drosophia melanogaster. *American Zoologist , 3*, 507.

Gong, W. J., & Golic, K. G. (2003). Ends-out, or replacement, gene targeting in Drosophila. *Proc Natl Acad Sci U S A , 100*, 2556-2561.

Goodwin, S. F., Taylor, B. J., Villella, A., Foss, M., Ryner, L. C., Baker, B. S., et al. (2000). Aberrant splicing and altered spatial expression patterns in fruitless mutants of Drosophila melanogaster. *Genetics , 154*, 725-745.

Goto, J., Mikawa, Y., Koganezawa, M., Ito, H., & D.Yamamoto. (2011). Sexually dimorphic shaping of interneuron dendrites involves the hunchback transcription factor. *J Neurosci , 31*, 5454-5459.

Greenspan, R. J., & Ferveur, J. F. (2000). Courtship in Drosophila. *Annu Rev Genet , 34*, 205-232.

Groth, A. C., Fish, M., Nusse, R., & Calos, M. P. (2004). Construction of transgenic Drosophila by using the site-specific integrase from phage phiC31. *Genetics , 166*, 1775-1782.

Haley, B., Hendrix, D., Trang, V., & Levine, M. (2008). A simplified miRNA-based gene silencing method for Drosophila melanogaster. *Dev Biol , 321*, 482-490.

Hall, J. C. (1978). Courtship among males due to a male-sterile mutation in Drosophila melanogaster. *Behav Genet , 8*, 125-141.

Hall, J. C. (1994). The mating of a fly. *Science , 264*, 1702-1714.

Hartenstein, V. (1993). *Atlas of Drosophila development.* Cold Spring Harbor Laboratory Press.

Häsemeyer, M., Yapici, N., Heberlein, U., & Dickson, B. (2009). Sensory neurons in the Drosophila genital tract regulate female reproductive behavior. *Neuron , 61*, 511-518.

Hayashi, S., Ito, K., Sado, Y., Taniguchi, M., Akimoto, A., Takeuchi, H., et al. (2002). GETDB, a database compiling expression patterns and molecular locations of a collection of Gal4 enhancer traps. *Genesis , 34*, 58-61.

Heinrichs, V., Ryner, L., & Baker, B. (1997). Regulation of Sex-Specific Selection of fruitless 59 Splice Sites by transformer and transformer-2. *Molecular and Cellular Biology , 18*, 450-458.

Hoshijima, K., Inoue, K., Higuchi, I., Sakamoto, H., & Shimura, Y. (1991). Control of doublesex alternative splicing by transformer and transformer-2 in Drosophila. *Science , 252*, 833-836.

Hoy, R. R., Hoikkala, A., & Kaneshiro, K. (1988). Hawaiian courtship songs: evolutionary innovation in communication signals of Drosophila. *Science , 240*, 217-219.

Huber, R., Hill, S. L., Holladay, C., Biesiadecki, M., Tononi, G., & Cirelli, C. (2004). Sleep homeostasis in Drosophila melanogaster. *Sleep , 27*, 628-639.

Inoue, K., Hoshijima, K., Sakamoto, H., & Shimura, Y. (1990). Binding of the Drosophila sex-lethal gene product to the alternative splice site of transformer primary transcript. *Nature , 344*, 461-463.

Ito, H., Fujitani, K., Usui, K., Shimizu-Nishikawa, K., Tanaka, S., & Yamamoto, D. (1996). Sexual orientation in Drosophila is altered by the satori mutation in the sex-determination gene fruitless that encodes a zinc finger protein with a BTB domain. *Proc Natl Acad Sci U S A , 93*, 9687-9692.

Karess, R. E., & Rubin, G. M. (1984). Analysis of P transposable element functions in Drosophila. *Cell , 38*, 135-146.

Keisman, E. L., Christiansen, A. E., & Baker, B. S. (2001). The sex determination gene doublesex regulates the A/P organizer to direct sex-specific patterns of growth in the Drosophila genital imaginal disc. *Dev Cell , 1*, 215-225.

Kennerdell, J. R., & Carthew, R. W. (2000). Heritable gene silencing in Drosophila using double-stranded RNA. *Nat Biotechnol , 18*, 896-898.

Kim, Y.-J., Zitnan, D., Galizia, C. G., Cho, K.-H., & Adams, M. E. (2006). A command chemical triggers an innate behavior by sequential activation of multiple peptidergic ensembles. *Curr Biol , 16*, 1395-1407.

Kimura, K.-I., Hachiya, T., Koganezawa, M., Tazawa, T., & Yamamoto, D. (2008). Fruitless and doublesex coordinate to generate male-specific neurons that can initiate courtship. *Neuron , 59*, 759-769.

Kimura, K.-I., Ote, M., Tazawa, T., & Yamamoto, D. (2005). Fruitless specifies sexually dimorphic neural circuitry in the Drosophila brain. *Nature , 438*, 229-233.

Koganezawa, M., Haba, D., Matsuo, T., & Yamamoto, D. (2010). The shaping of male courtship posture by lateralized gustatory inputs to male-specific interneurons. *Curr Biol , 20*, 1-8.

Kohatsu, S., Koganezawa, M., & Yamamoto, D. (2011). Female contact activates male-specific interneurons that trigger stereotypic courtship behavior in Drosophila. *Neuron , 69*, 498-508.

Konopka, R., & Benzer, S. (1971). Clock mutants of Drosophila melanogaster. *Proc Natl Acad Sci U S A , 68*, 2112-6.

Kulkarni, S. J., & Hall, J. C. (1987). Behavioral and cytogenetic analysis of the cacophony courtship song mutant and interacting genetic variants in Drosophila melanogaster. *Genetics , 115*, 461-475.

Kurtovic, A., Widmer, A., & Dickson, B. (2007). A single class of olfactory neurons mediates behavioural responses to a Drosophila sex pheromone. *Nature , 446*, 542-546.

Kvitsiani, D., & Dickson, B. J. (2006). Shared neural circuitry for female and male sexual behaviours in Drosophila. *Curr Biol , 16*, R355--R356.

Kyriacou, C. P., & Hall, J. C. (1980). Circadian rhythm mutations in Drosophila melanogaster affect short-term fluctuations in the male's courtship song. *Proc Natl Acad Sci U S A , 77*, 6729-6733.

Lai, S.-L., & Lee, T. (2006). Genetic mosaic with dual binary transcriptional systems in Drosophila. *Nat Neurosci , 9*, 703-709.

Lee, G., Foss, M., Goodwin, S. F., Carlo, T., Taylor, B. J., & Hall, J. C. (2000). Spatial, temporal, and sexually dimorphic expression patterns of the fruitless gene in the Drosophila central nervous system. *J Neurobiol , 43*, 404-426.

Lee, G., Villella, A., Taylor, B. J., & Hall, J. C. (2001). New reproductive anomalies in fruitless-mutant Drosophila males: extreme lengthening of mating durations and infertility correlated with defective serotonergic innervation of reproductive organs. *J Neurobiol , 47*, 121-149.

Luan, H., & White, B. H. (2007). Combinatorial methods for refined neuronal gene targeting. *Curr Opin Neurobiol , 17*, 572-580.

Manning, A. (1962). A Sperm Factor Affecting the Receptivity of Drosophila Melanogaster Females. *Nature , 194*, 252-253.

Manning, A. (1967). The control of sexual receptivity in female Drosophila. *Anim Behav , 15*, 239-250.

Manoli, D. S., & Baker, B. S. (2004). Median bundle neurons coordinate behaviours during Drosophila male courtship. *Nature , 430*, 564-569.

Manoli, D. S., Foss, M., Villella, A., Taylor, B. J., Hall, J. C., & Baker, B. S. (2005). Male-specific fruitless specifies the neural substrates of Drosophila courtship behaviour. *Nature , 436*, 395-400.

Markow, T. A., & O'Grady, P. M. (2005). Evolutionary genetics of reproductive behavior in Drosophila: connecting the dots. *Annu Rev Genet , 39*, 263-291.

McGuire, S., Le, P. T., K., A. O., & Davis, R. L. (2003). Spatiotemporal rescue of memory dysfunction in Drosophila. *Science , 302*, 1765-1768.

Mellert, D., Knapp, J.-M., Manoli, D., Meissner, G. W., & Baker, B. S. (2010). Midline crossing by gustatory receptor neuron axons is regulated by fruitless, doublesex and the Roundabout receptors. *Development , 137*, 323-332.

Nagoshi, R. N., McKeown, M., Burtis, K. C., Belote, J. M., & Baker, B. S. (1988). The control of alternative splicing at genes regulating sexual differentiation in D. melanogaster. *Cell , 53*, 229-236.

Ng, C. S., & Kopp, A. (2008). Sex combs are important for male mating success in Drosophila melanogaster. *Behav Genet , 38*, 195-201.

Nilsen, S. P., Chan, Y.-B., Huber, R., & Kravitz, E. A. (2004). Gender-selective patterns of aggressive behavior in Drosophila melanogaster. *Proc Natl Acad Sci U S A , 101*, 12342-12347.

North, G., & Greenspan, R. (2007). *Invertebrate Neurobiology.* Cold Spring Harbor Laboratory Press.

Pfeiffer, B. D., Jenett, A., Hammonds, A. S., Ngo, T.-T. B., Misra, S., Murphy, C., et al. (2008). Tools for neuroanatomy and neurogenetics in Drosophila. *Proc Natl Acad Sci U S A , 105*, 9715-9720.

Philipsborn, A. C., Liu, T., Yu, J. Y., Masser, C., Bidaye, S. S., & Dickson, B. J. (2011). Neuronal control of Drosophila courtship song. *Neuron , 69*, 509-522.

Potter, C., Tasic, B., Russler, E. V., Liang, L., & Luo, L. (2010). The Q system: a repressible binary system for transgene expression, lineage tracing, and mosaic analysis. *Cell , 141*, 536-548.

Ribeiro, C., & Dickson, B. (2010). Sex peptide receptor and neuronal TOR/S6K signaling modulate nutrient balancing in Drosophila. *Curr Biol , 20*, 1000-1005.

Rideout, E., Billeter, J.-C., & Goodwin, S. F. (2007). The sex-determination genes fruitless and doublesex specify a neural substrate required for courtship song. *Curr Biol , 17*, 1473-1478.

Rideout, E., Dornan, A. J., Neville, M. C., Eadie, S., & Goodwin, S. F. (2010). Control of sexual differentiation and behavior by the doublesex gene in Drosophila melanogaster. *Nat Neurosci , 13*, 458-466.

Riemensperger, T., Isabel, G., Coulom, H., Neuser, K., Seugnet, L., Kume, K., et al. (2011). Behavioral consequences of dopamine deficiency in the Drosophila central nervous system. *Proc Natl Acad Sci U S A , 108*, 834-839.

Robinett, C. C., Vaughan, A. G., Knapp, J.-M., & Baker, B. S. (2010). Sex and the single cell. II. There is a time and place for sex. *PLoS Biol , 8*, e1000365.

Rong, Y. S., & Golic, K. G. (2000). Gene targeting by homologous recombination in Drosophila. *Science , 288*, 2013-2018.

Rubin, G. M., & Spradling, A. C. (1982). Genetic transformation of Drosophila with transposable element vectors. *Science , 218*, 348-353.

Ruta, V., Datta, S. R., Vasconcelos, M. L., Freeland, J., Looger, L. L., & Axel, R. (2010). A dimorphic pheromone circuit in Drosophila from sensory input to descending output. *Nature , 468*, 686-690.

Ryder, E., Blows, F., Ashburner, M., Bautista-Llacer, R., Coulson, D., Drummond, J., et al. (2004). The DrosDel collection: a set of P-element insertions for generating custom chromosomal aberrations in Drosophila melanogaster. *Genetics , 167*, 797-813.

Ryner, L. C., & Swain, A. (1995). Sex in the '90s. *Cell , 81*, 483-493.

Ryner, L. C., Goodwin, S. F., Castrillon, D. H., Anand, A., Villella, A., Baker, B. S., et al. (1996). Control of male sexual behavior and sexual orientation in Drosophila by the fruitless gene. *Cell , 87*, 1079-1089.

Shirangi, T. R., & McKeown, M. (2007). Sex in flies: what 'body--mind' dichotomy? *Dev Biol , 306*, 10-19.

Shorey, H. H., & Bartell, R. J. (1970). Role of a volatile female sex pheromone in stimulating male courtship behaviour in Drosophila melanogaster. *Anim Behav , 18*, 159-164.

Shorey, H. (1962). Nature of the Sound Produced by Drosophila melanogaster during Courtship. *Science. , 137*, 677-8.

Siegel, R. W., & Hall, J. C. (1979). Conditioned responses in courtship behavior of normal and mutant Drosophila. *Proc Natl Acad Sci U S A , 76*, 3430-3434.

Siegel, R. W., Hall, J. C., Gailey, D. A., & Kyriacou, C. P. (1984). Genetic elements of courtship in Drosophila: mosaics and learning mutants. *Behav Genet , 14*, 383-410.

Simpson, J. (2009). Mapping and manipulating neural circuits in the fly brain. *Adv Genet , 65*, 79-143.

Singh, R., & Nayak, S. (1985). Fine structure and primary sensory projections of sensilla on the maxillary palp of Drosophila melanogaster meigen. *Int. J. Insect Morphol. Embryol , 14*, 291–306.

Sokolowski, M. B. (2001). Drosophila: genetics meets behaviour. *Nat Rev Genet , 2*, 879-890.

Song, H.-J., Billeter, J.-C., Reynaud, E., Carlo, T., Spana, E., Perrimon, N., et al. (2002). The fruitless gene is required for the proper formation of axonal tracts in the embryonic central nervous system of Drosophila. *Genetics , 162*, 1703-1724.

Spieth, H. T. (1974). Courtship behavior in Drosophila. *Annu Rev Entomol , 19*, 385-405.

Steinmann-Zwicky, M. (1992). How do germ cells choose their sex? Drosophila as a paradigm. *Bioessays , 14*, 513-518.

Stockinger, P., Kvitsiani, D., Rotkopf, S., Tirián, L., & Dickson, B. (2005). Neural circuitry that governs Drosophila male courtship behavior. *Cell , 121*, 795-807.

Stowers, L., & Logan, D. (2008). LUSH shapes up for a starring role in olfaction. *Cell , 133*, 1137-1139.

Strauss, R. (2002). The central complex and the genetic dissection of locomotor behaviour. *Curr Opin Neurobiol , 12*, 633-638.

Struhl, G., & Basler, K. (1993). Organizing activity of wingless protein in Drosophila. *Cell , 72*, 527-540.

Tauber, E., & Eberl, D. F. (2003). Acoustic communication in Drosophila. *Behav Processes , 64*, 197-210.

Taylor, B. J., & Knittel, L. M. (1995). Sex-specific differentiation of a male-specific abdominal muscle, the Muscle of Lawrence, is abnormal in hydroxyurea-treated and in fruitless male flies. *Development , 121*, 3079-3088.

Usui-Aoki, K., Ito, H., Ui-Tei, K., Takahashi, K., Lukacsovich, T., Awano, W., et al. (2000). Formation of the male-specific muscle in female Drosophila by ectopic fruitless expression. *Nat Cell Biol , 2*, 500-506.

Venken, K. J., & Bellen, H. J. (2007). Transgenesis upgrades for Drosophila melanogaster. *Development , 134*, 3571-3584.

Venken, K. J., Hoskins, R. A., & Bellen, H. J. (2006). P[acman]: a BAC transgenic platform for targeted insertion of large DNA fragments in D. melanogaster. *Science , 314*, 1747-1751.

Villella, A., & Hall, J. C. (1996). Courtship anomalies caused by doublesex mutations in Drosophila melanogaster. *Genetics , 143*, 331-344.

Villella, A., Gailey, D. A., Berwald, B., Ohshima, S., Barnes, P. T., & Hall, J. C. (1997). Extended reproductive roles of the fruitless gene in Drosophila melanogaster revealed by behavioral analysis of new fru mutants. *Genetics , 147*, 1107-1130.

Wang, L., & Anderson, D. J. (2010). Identification of an aggression-promoting pheromone and its receptor neurons in Drosophila. *Nature , 463*, 227-231.

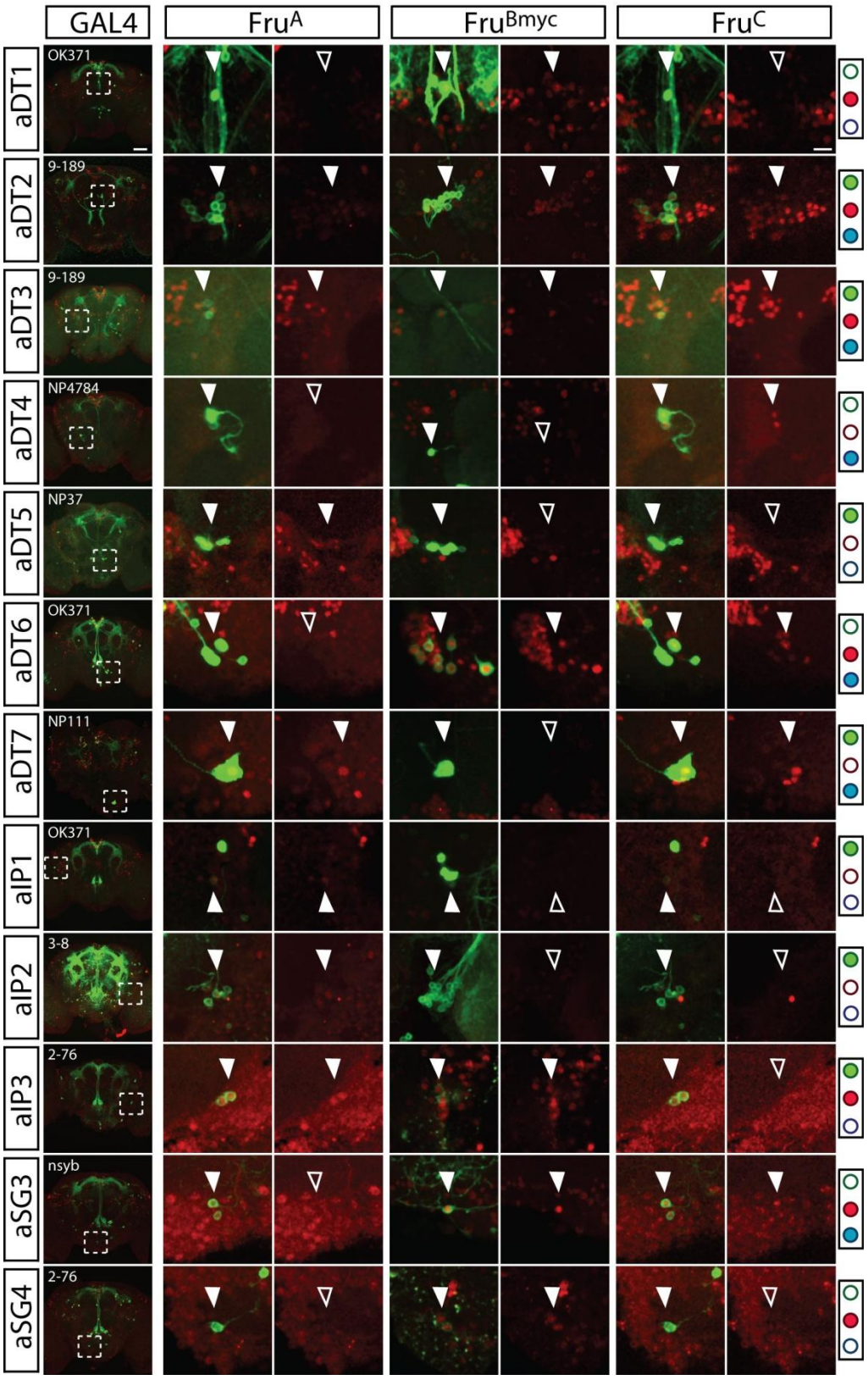
Yamamoto, D. (2007). The neural and genetic substrates of sexual behavior in Drosophila. *Adv Genet , 59*, 39-66.

Yang, C.-H., Rumpf, S., Xiang, Y., Gordon, M. D., W.Song, Jan, L. Y., et al. (2009). Control of the postmating behavioral switch in Drosophila females by internal sensory neurons. *Neuron , 61*, 519-526.

Yu, J., Kanai, M. I., Demir, E., X., G. S., & Dickson, B. J. (2010). Cellular organization of the neural circuit that drives Drosophila courtship behavior. *Curr Biol , 20*, 1602-1614.

Zars, T. (2000). Behavioral functions of the insect mushroom bodies. *Curr Opin Neurobiol , 10*, 790-795.

Appendix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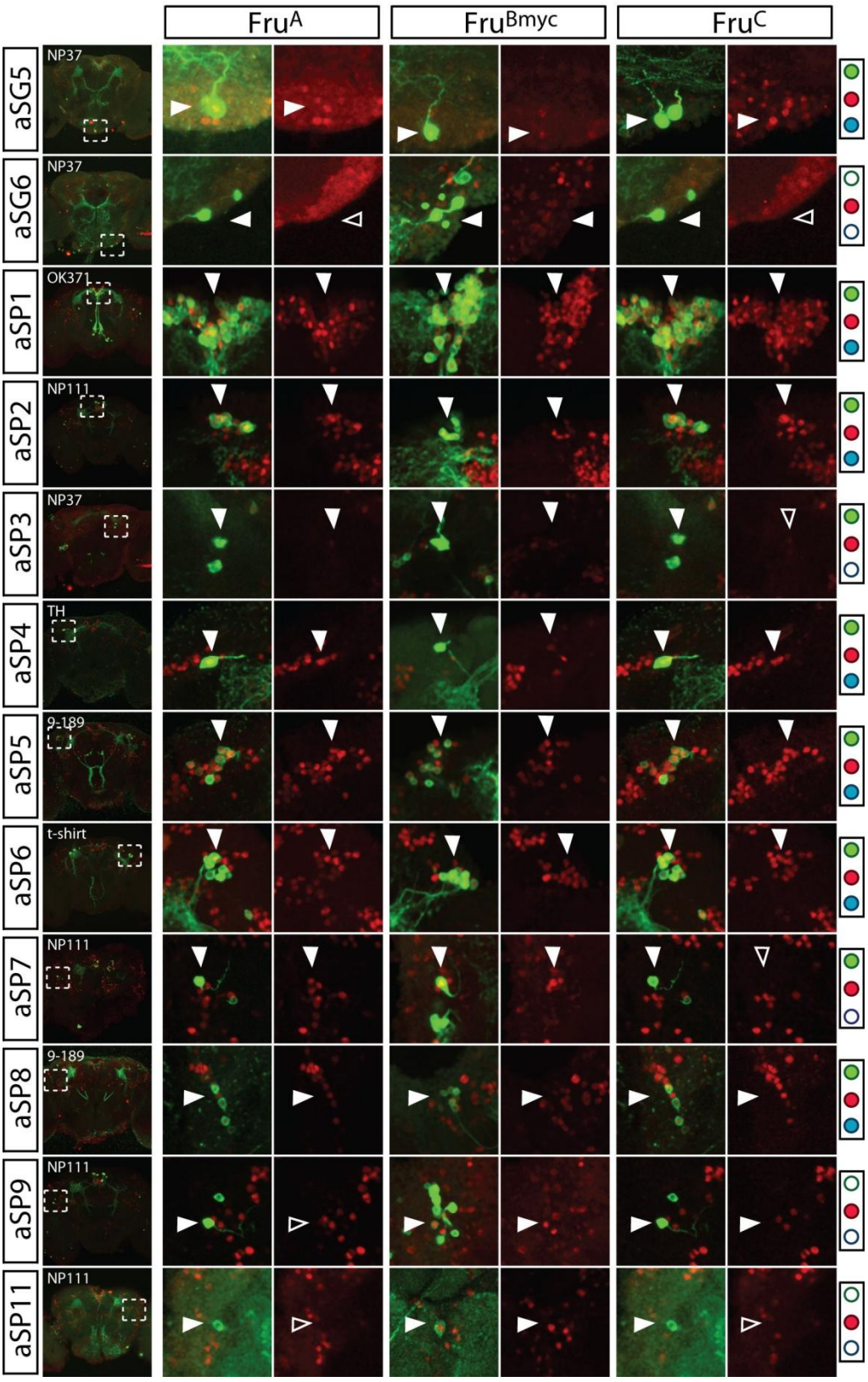
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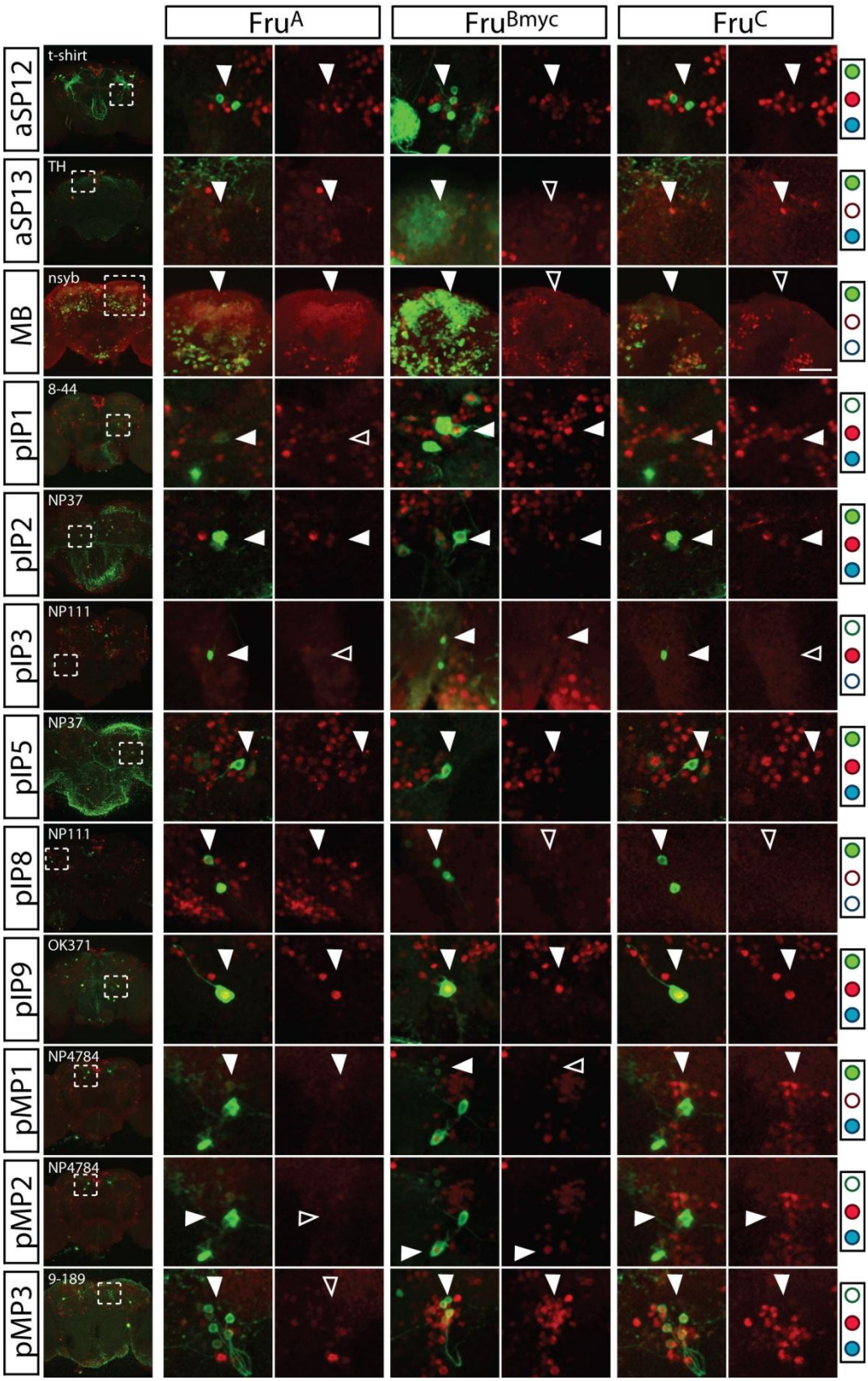
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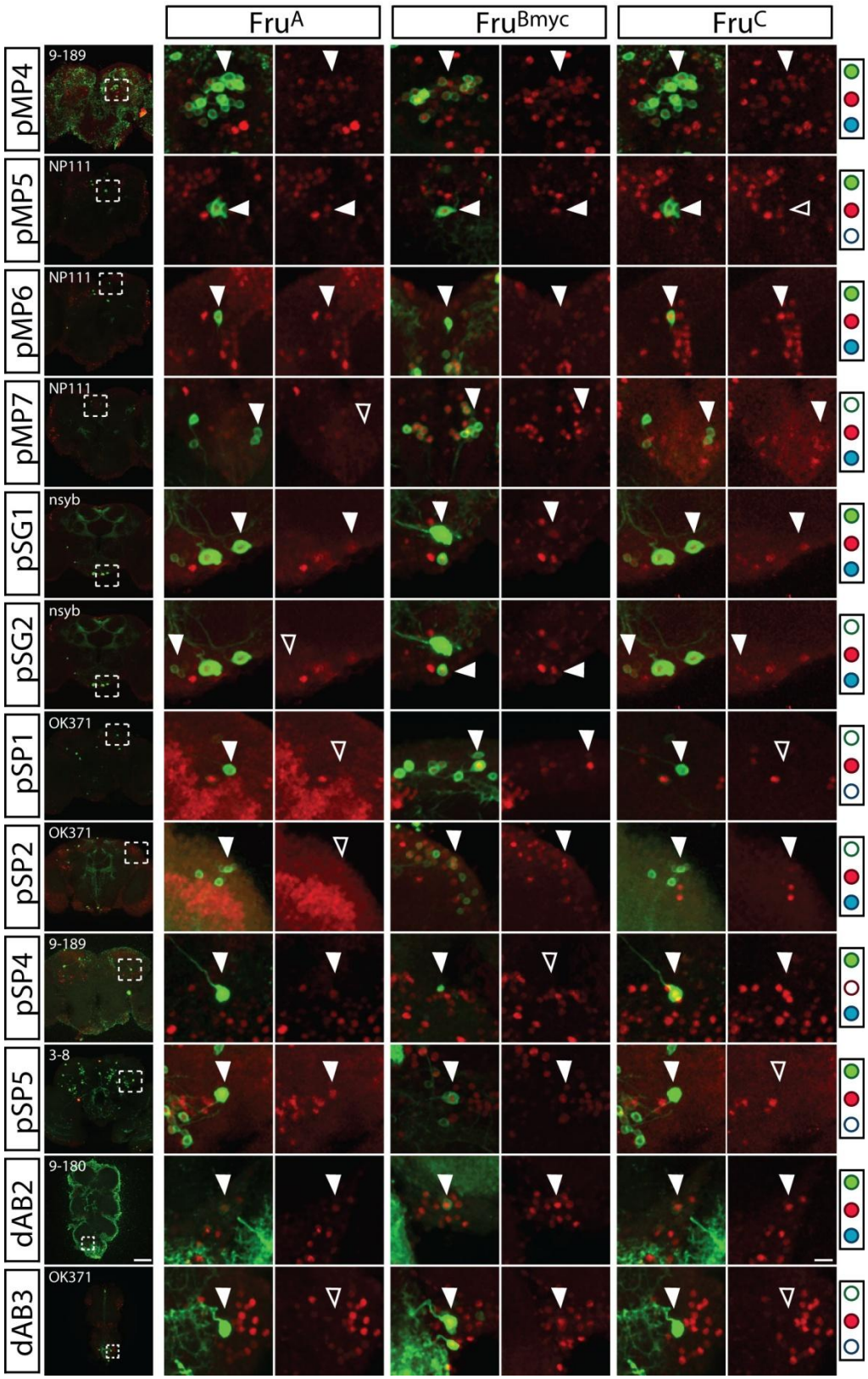
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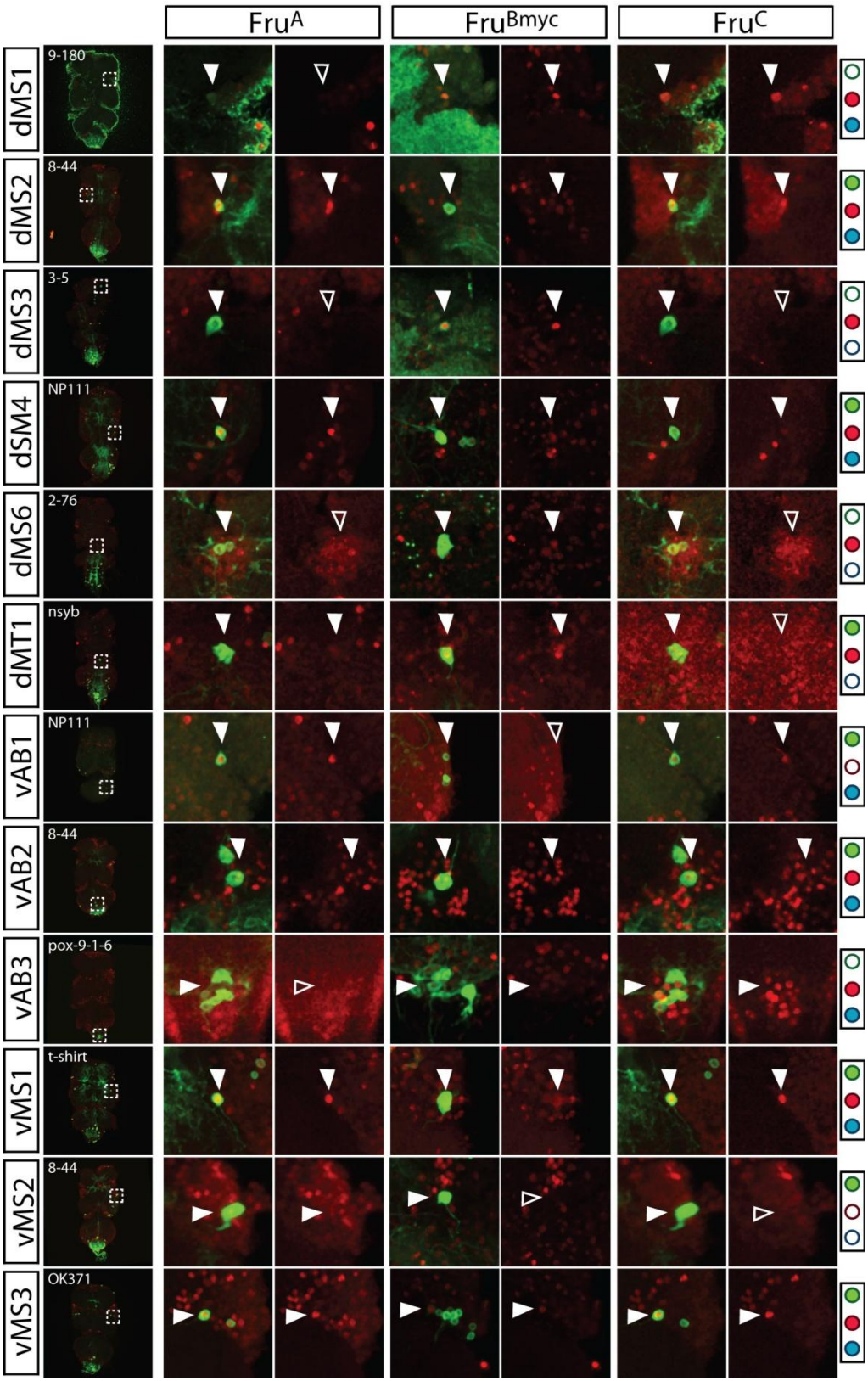
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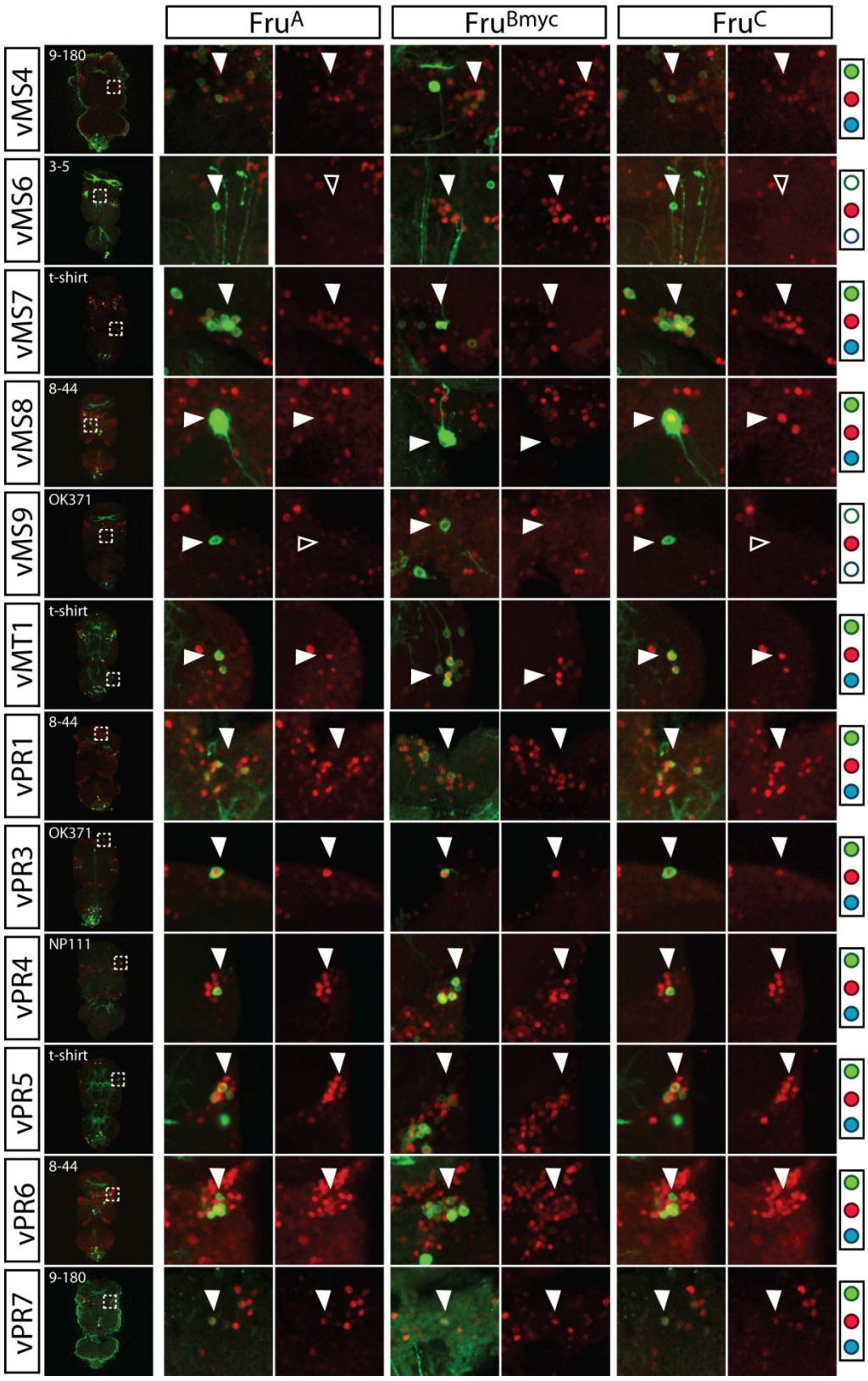
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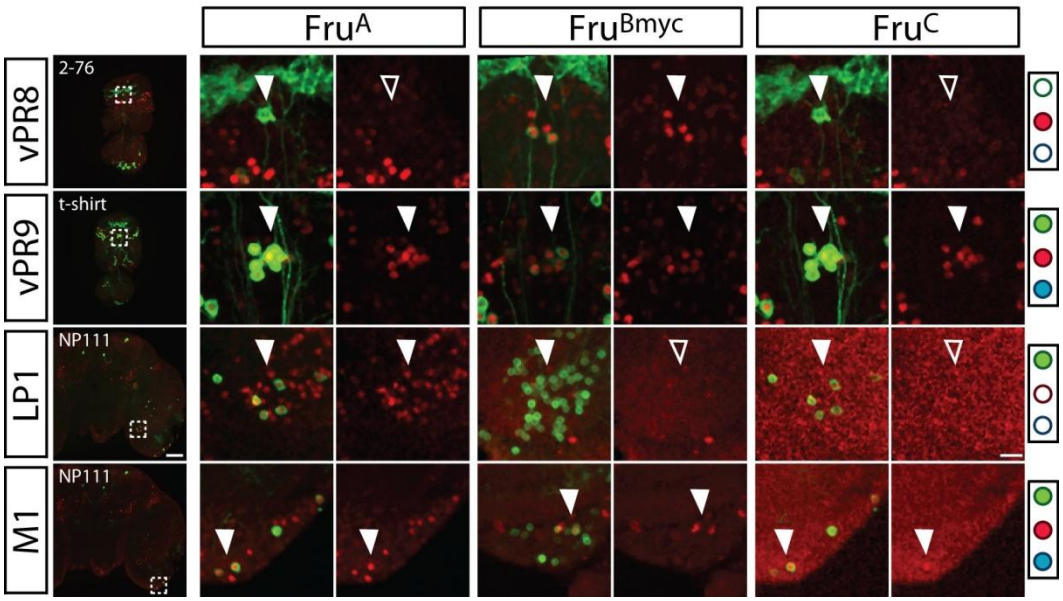

From the 100 distinct *fru* neurons 72 are addressed here. 70 Classes are localized in the central brain and ventral nerve cord and two classes in the optic lobes. The neuron name is displayed at the left site and the isoform stained for at the top. Every row represents one type of neuron. The first panel shows the GAL4 lines used, its expression with mCD8-GFP in green and the Fru^{A} staining in red. The boxed region is shown in panel 2-7 with the GAL4::mCD8-GFP staining in green and the isoform stainings in red. Fru^{A} and Fru^{C} have been detected in the same samples while Fru^{Bmyc} was detected in a separate sample. Solid arrowheads mark the presence of staining and empty arrowheads the absence of staining. Finally, at the right site a traffic light summarizes the expression with a full circle standing for presence and empty circle for absence of labeling and Fru^{A} being green, Fru^{Bmyc} being red and Fru^{C} being blue. Scale bar first panel 50 µm. Scale bar other panels 10 µm.

Curriculum Vitae

Personal Details

Date of Birth	November 20th 1982
Place of Birth	Halle/Saale, Germany
Nationality	German
Marital Status	Unmarried

Education

06/2007- present	PhD studies in Molecular Biology, Institute of Molecular Pathology, Vienna, Austria
05/2007	Diploma degree in Biochemistry, predicate 1.4 (upper 1st class)
2002-2007	Studies in Biochemistry, University of Leipzig, Germany
06/2002	A-Levels, predicate 1.1 (upper 1st class)
1993-2002	High School– "Wilhelm von Humboldt", Halle/Saale, Germany

Research Experience

PhD Thesis

06/2007-present	"Expression and Function of Fruitless Isoforms in *Drosophila melanogaster*", Dr. B. Dickson, Research Institute of Molecular Pathology, Vienna, Austria

Diploma Thesis

10/2006-03/2007	"Proteomic Analysis of Cholinergic SN56.B5.G4 Cells after Beta-Amyloid (1-42) Exposure", Prof. R. Schliebs, Medical Faculty, University of Leipzig, Germany

Undergraduate Research Education

10/2005-03/2006	"Quantification of Intergenic Transcripts by RT-PCR", Dr. P. Khaitovich, Department of Evolutionary Genetics, Max-Planck-Institute of Evolutionary Anthropology, Leipzig, Germany
08-09/2005	"Expression of mGluR 2/3 in Epileptogenic Hippocampus of Rats", Prof. G. Sperk, Prof. F. Ferraguti, Institute of Pharmacology, Medical University of Innsbruck, Austria

Internships

03/2004	"Enzyme Mediated Peptide Synthesis", Dr. F. Bordusa, Department of Organic Synthesis, Max-Planck-Research Unit for Enzymology of Protein Folding, Halle/Saale, Germany
09-10/2003	"Isolation of a Putative Promoter of the Codeinon-Reductase", Prof. T. Kutchan, Department of Natural Product Biotechno-logy, Leipnitz Institute of Plant Biochemistry, Halle/Saale

Additional Skills

03-11/2008	Co-Organization of the Annual "Vienna Biocenter PhD Student Symposium" on Synthetic Biology, Vienna, Austria
06-07/2008	Participation in "Neurobiology of *Drosophila*" course, Cold Spring Harbor, USA
10/2005-03/2006	Student assistant for undergraduate courses in "Enzymology and Molecular Biology", University of Leipzig, Germany
2002-2007	Member of Students` Council, University of Leipzig, Germany
Language	German (native), English (fluent), French, Italian, Latin (basic)
IT Knowledge	LaTeX, Origin, Graph Pad, MS Office, Adobe Illustrator