Genome-wide analysis of innate electric shock- and odour-avoidance, punishment- and relief- learning

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Zusammenfassung

Entscheidend für das Überleben eines Tieres ist es, die Zeitspanne zu verringern, in der es einem schädlichen Reiz ausgesetzt ist. In den evolutionär konservierten Vermeidungsstrategien spiegelt sich dies wider. In meiner Doktorarbeit habe ich einige dieser Strategien auf der genetischen Ebene am etablierten Modelorganismus *Drosophila melanogaster* (Fruchtfliege) untersucht. *Drosophila* ist bekannt dafür, eine Brücke zwischen Genen und Verhalten zu schlagen. Als schädlichen Reiz verwendete ich Elektroschock, welcher heute allgemein üblich in vielen verschiedenen experimentellen Systemen ebenfalls angewendet wird. In Fruchtfliegen löst diese Methode sehr unterschiedliche Verhaltenseffekte aus. Erstens, das angeborene Fluchtverhalten, das in einem T-Labyrinth nachgewiesen wird. Zweitens können Fruchtfliegen konditioniert werden, einen Duft, der vor dem Elektroschock präsentiert wird, später zu meiden. Dies wird als Bestrafungslernen bezeichnet. Drittens entwickeln die Fruchtfliegen eine Präferenz für einen Duft, wenn sie vorher so konditioniert wurden, dass derselbe Duft erst nach dem Elektroschock kommt (Erleichterungslernen). Diese drei Strategien (Elektroschockvermeidung, Bestrafungslernen und Erleichterungslernen), die dazu dienen die Zeitdauer der Aussetzung an einen schädlichen Reiz zu verringern, wurden von mir in vergleichbarem Kontext untersucht.

Um deren genetische Effektoren zu vergleichen, benutzte ich eine Methode, die die natürliche mit der genetischen/transkriptomischen Variation kombiniert. Ich charakterisierte 38 Fliegeninzuchtstämme in Elektroschockvermeidung, Bestrafungslernen, Erleichterungslernen und zusätzlich in der Vermeidungsreaktion auf zwei verschiedene Düfte (3-Octanol und Benzaldehyd), die auch für deren Lernverhalten verwendet wurden. Diese 38 Stämme wurden einer natürlichen Fliegen-Population entnommen, dass heisst, alle Fliegen eines Stammes sind direkte Nachkommen eines befruchteten Fliegenweibchens. Unter diesen wurde für mehr als 20 Generationen Inzucht betrieben, so dass nun alle Fliegen eines Stammes das gleiche Genom besitzen, das sich wiederum vom Genom der anderen Stämme unterscheidet. Nun wurden jeweils genomweite Expression und Nukleotidpoly-

morphismen (SNPs) und Verhaltensdaten kombiniert, um Assoziationen zwischen diesen zu erhalten. Das Ergebnis daraus ist eine Liste an Kandidatengenen für jede der beschriebenen Verhaltensweisen.

Zunächst fand ich keine Korrelation zwischen den gemessenen Verhalten, was darauf hinweist, dass nicht alle genetischen Effektoren gleich sind. Dies ist tatsächlich der Fall beim Vergleich der Kandidatengene, der zwar eine signifikante, aber keine komplette Übereinstimmung ergab. Daher kann ich sagen, dass meine Vorgehensweise sowohl Ähnlichkeiten als auch Unterschiede zwischen den genetischen Effektoren dieser Verhaltensweisen aufgedeckt hat. Weitere Analysen zeigen, dass fast alle Kandidatengenlisten zum Beispiel eine signifikante Anreicherung an bekannten Fortbewegungs- und Lerngenen enthalten, aber auch spezifische Anreicherung zum Beispiel von bekannten Nozizeptionsgenen innerhalb der Elektroschockvermeidungskandidatengene. Diese Anreicherungen sprechen für die Qualität der Kandidatengenlisten.

In Bezug auf die Elektroschockvermeidungs-Expression assoziierten Gene wurde erstens ein Geninteraktionsnetzwerk auf der Basis von bekannten Protein-Protein Interaktionen errechnet. Dieses Netzwerk war angereichert mit Genen, die eine Rolle in der Entwicklung sensorischer Borsten spielen, den Härchen auf der Oberfläche der Fliege, die zum Beispiel wichtig sind für die Mechanorezeption. Zweitens habe ich Mutanten für 19 Elektroschockkandidatengene getestet und konnte die Rolle von 10 dieser Gene in der Elektroschockvermeidung validieren. Des Weiteren untersuchte ich diese Kandidatengene unter der Hypothese, dass Elektroschock molekulare Mechanismen benutzt, die sich für natürliche Reize entwickelt haben. Als Beispiele hierfür fand ich das Gen für den Hitzekanal trpA1 und ein stressbezogenes Gen npfr1, deren Rolle in der Elektroschockvermeidung ich in Mutanten validierte. Obwohl Elektroschock allgemein üblich als schädlicher Reiz benutzt wird, ist noch nichts bekannt über dessen periphere Wahrnehmung oder zentrale sowie motorische Schaltkreise. Meine Ergebnisse stellen hoffentlich eine nützliche Quelle dar, um diese Wissenslücken zu füllen. In Bezug auf die anderen Verhaltensweisen müssen diese Nachuntersuchungen noch durchgeführt werden. Im Speziellen wird die Kandidatengenliste für das Erleichterungslernen sehr nützlich sein, weil im Vergleich zu Bestrafungslernen noch so gut wie nichts bekannt ist. In Zukunft können Orthologe dieser Gene zu Säugetiergenen interessante Forschungsziele für die jeweiligen Verhaltensweisen sein, welche auch einen Einfluss auf psychiatrische Erkrankungen haben können, wie z.B. dem post-traumatischen Stress Syndrom (PTSD) oder weiterführenden Lernstörungen.

Schliesslich, inspiriert durch die Unterschiede an Kandidatengenen zwischen

Bestrafungs- und Erleichterungslernen und das Fehlen einer Korrelation zwischen diesen in Bezug auf die Inzuchtstämme, habe ich die Gedächtniseigenschaften der beiden Lernarten in meine Untersuchungen einbezogen. Ich konnte bestätigen, dass ein Teil des Bestrafungsgedächtnisses resistent ist gegenüber einer Kälteanaesthesie (ARM). Ferner konnte ich zeigen, dass das Erleichterungsgedächtnis nicht Kälteresistent ist. Da ARM von anderen molekularen Faktoren abhängt als das anaesthesiesensible Bestrafungsgedächtnis, ist es möglich, dass das Erleichterungsgedächtnis insgesamt von unterschiedlichen molekularen Mechanismen abhängt im Vergleich zum Bestrafungsgedächtnis. Dies stellt eine interessante Hypothese für zukünftigen Forschungsarbeiten dar.

Summary

Minimizing exposure to noxious events is critical for an animal's survival; this is reflected by the existence of evolutionarily conserved behavioural strategies for doing so. In this thesis, I compared a number of these at the level of their genetic effectors, using a well-established model, fruit fly *Drosophila melanogaster*, which is famous for its use in bridging between genes and behaviour. As a noxious stimulus, I employed electric shock, which is commonly used in a variety of experimental systems. In flies, electric shock exerts a multitude of behavioural effects: First, flies innately avoid electric shock, which can be quantified by a *mass* T-maze assay. Second, when flies are trained such that an odour is presented slightly preceding an electric shock, they avoid this odour later in a *mass T-maze* test, as a signal for punishment. Third, if flies are trained with a reversed order of event, i.e., the odour is presented upon the offset of electric shock, flies later on approach this odour as a signal for relief. I took these three kinds of behaviour, i.e., innate shock avoidance, punishment learning and relief learning as three different strategies for minimizing exposure to a noxious stimulus.

To compare the critical genetic effectors, I used a strategy that combines natural variation and genomic / transcriptomic data. I characterized 38 inbred fly strains in innate shock avoidance, punishment learning, relief learning and in addition innate avoidance of the two kinds of odour (3-octanol and benzaldehyde) used in the learning assays. These 38 strains had been derived from a natural population; i.e. all flies within a strain are the offspring of one mated female, inbred for over 20 generations and thus bear identical genomes whereas flies from different strains genomically differ. With respect to these 38 strains, I thus combined my behavioural data with the available genome-wide expression level- and single nucleotide polymorphism (SNP)-data testing for associations. This gave me lists of candidate genes for each kind of behaviour I looked at.

First, I found no obvious correlation between the different kinds of behaviour, suggesting that not all genetic effectors are common. Indeed, a comparison of candidate genes

across behaviours revealed much less than complete overlap, which however was well above chance-level. Thus my approach seems to have successfully revealed both the commons and the distinctions between the genetic effectors of these kinds of behaviour. Second, a closer look into these candidate gene lists revealed significant enrichment of, e.g., known locomotion and learning genes for most candidate lists but specific enrichment for nociception genes among the innate shock avoidance-candidates. These enrichments speak for the 'quality' of the candidate gene lists. With respect to innate shock avoidance, I extended my analyses by constructing a gene interaction network that integrates independent empirical protein-protein interaction data with my association results. This network turned out to be enriched for genes involved in sensory bristle development, i.e. sensory hairs on the body surface, which are critical for mechanosensation. Furthermore, with respect to 19 candidate genes I tested for a causal role in shock avoidance using P-element insertion mutants, revealing 10 cases where the mutant had significantly different scores than a corresponding control. Finally, I looked at my candidate gene list with the hypothesis that electric shock may use the molecular mechanisms evolved for detecting other more natural noxious stimuli. As examples, I found heat receptor gene *trpA1* and a stress related gene *npfr1* to be associated with shock avoidance and validated their roles using specific mutants. Although electric shock is a commonly used experimental noxious stimulus, yet nothing is known about its peripheral sensation or the central or motor circuits governing shock avoidance. My results hopefully provide a useful resource for starting to fill in these gaps.With respect to the other kinds of behaviour I looked at, these additional steps are yet to be taken. Especially, the candidate gene list for relief learning should be extremely useful, as we know close to nothing about genetic or neuronal mechanisms of relief learning, despite our deep knowledge about punishment learning. In the future, mammalian orthologues of these genes may be interesting targets for research in the respective kinds of behaviour and may be even in related psychiatric conditions such as post-traumatic stress syndrome (PTSD) or learning disorders.

Finally, prompted by the difference in terms of candidate genes and lack of correlation between punishment- and relief-learning between inbred strains, I tested for a difference in memory properties. I could confirm that part of the punishment memory is resistant to cold anesthesia (ARM). Further I showed that relief memory is completely abolished upon cold anesthesia. As ARM is relying on different molecular mechanisms than the anesthesia sensitive memory, it might be that relief memory consists of different molecular mechanisms than punishment memory which would be interesting to dissect in the future.

Publication and Contribution

Part of this work has been published:

S. Diegelmann, S. Preuschoff, M. Appel, T. Niewalda, B. Gerber, A. Yarali. Memory decay and susceptibility to amnesia dissociate punishment- from relief-learning. *Biol. Lett.*, 9(4):20121171, 2013.

S.Diegelmann, S. Preuschoff and M. Appel contributed equally to this study.

In this thesis, only the amnesia experiment was presented, which was carried out and analysed by M. Appel.

Another part of this work is in preparation to submission:

M. Appel, C.-J. Scholz, T. Müller, M. Dittrich, T. Schauer, M. Bockstaller, T. Oguz, D. Galili, C. Margulies, H. Tanimoto, A. Yarali. Genome-wide Identification of Genes Underlying Electric Shock Avoidance in Drosophila melanogaster.*in preparation*

For this work and the remaining part of the thesis the contributions were as follows:

Under supervision of A. Yarali, M. Appel carried out the major part of all behavioural experiments with inbred strains and P-element insertion mutants and did RTQ-PCR experiments. Furthermore, statistical analysis of behaviours, choice of candidate genes, enrichment analysis, manually retrieving known learning gene list, analysis with 'Homophila' and analyses of overlapping genes for all behaviours were mainly done by M. Appel. S. Kocabey, S. Gdegdak, S. Savage and A. Yarali helped with punishment- and relief learning experiments of inbred strains for approximately one to two months each. M. Bockstaller and T.Oguz tested 3-4 P-element insertion strains, respectively. A. Yarali tested *dTrpA1* mutants in electric shock avoidance and locomotion, manually retrieved known locomotion and nociception gene lists. C.-J. Scholz did the bioinformatic analysis of the microarray and SNP data and the linear regression. T. Müller and M. Dittrich conducted the gene-network analysis for electric shock avoidance.

Chapter 1

Introduction

This thesis mainly focuses on the genetic contribution to innate versus learned avoidance behaviour in the fruit fly *Drosophila melanogaster*. These behaviours are at the one hand avoidance of electric shock and the odours benzaldehyde (BA) and 3-octanol (OCT) as innate responses and punishment- and relief- learning on the other hand. In the following paragraphs I first give an overview of the motivation and goals of this thesis and then in the following sections go into detail of all relevant topics. There, I mainly describe investigated behaviours along with the important context and also the genome-wide approach used to discover candidate genes for them. In the last part, I compare punishment and relief memories in terms of their memory phases, i.e. long-term memory and anaesthesia resistant memory.

1.1 Motivation and goals of the thesis

Finding candidate genes associated with electric shock avoidance

Perceiving and avoiding dangerous stimuli such as noxious temperature, harsh touch and irritating chemicals is critical for the survival of animals including humans (e.g.[300, 122, 231]. Therefore, learning to minimize the exposure to these stimuli in the future will be of great benefit. *Drosophila melanogaster* has been used successfully as a model organism to find molecular factors influencing behaviour and some findings were even translatable to mammalians ([114, 207, 369, 61, 162], especially heat avoidance [231]). Apart from chemical, thermal and mechanical stimuli, electric shock induces very strong defensive and aversive behaviour in a variety of animals; in humans, it also elicits subjective pain and fear, e.g., [149, 93, 194, 335, 90, 222, 264]. Electric shock has become very important in lab-

oratories world-wide to investigate different phenomena and diseases in various organisms ranging from humans to *Drosophila*, such as learning and memory (e.g. [331, 72, 367]) and post traumatic stress disorder (PTSD) (e.g. [293, 314]). Despite this we do not know the molecular sensors and pathways specific to electric shock. Does it exploit those evolved for other noxious stimuli? Which genes influence variation in avoidance of electric shock and which of those are conserved across species? I am using a genome-wide approach to obtain first candidate genes leading to further hypothesis driven experiments in the purpose to fill this gap of knowledge.

Finding candidate genes associated to two different odours

Also, perception of odours and odour-guided behaviour is beneficial for example to distinguish good from bad food, to distinguish territories and to find mating partners. These factors have an influence on the survival of the organism (e.g., dogs:[28], rats:[69], moths:[130], flies: [13]). Animals, including humans, can distinguish many different odours because they possess many different receptors in distinct neurons [107, 35, 341]. In *Drosophila*, activation of one or a certain combination of receptors by an odour elicits approach or avoidance behaviour [74, 286]. However, although many receptors and other molecules are known to play a role, odour detection processes are still not fully understood. Even less is known about genetic factors influencing odour avoidance specifically. Which molecular pathways and thus variation in which genes influences differences in detection and avoidance of odours? Some attempts have already been made on the genome-wide level to identify key players and environmental impact, especially on benzaldehyde (BA) avoidance [9, 94, 347, 273, 346, 12, 267, 312, 313, 311]. Using a genome-wide approach to identify candidate genes for BA and OCT avoidance, gives me the unique opportunity to on the one hand compare my study to previous ones using BA avoidance and on the other hand detect candidates that might be specific to each odour and candidates more general to odour avoidance. Thus, this study serves as a source for future hypothesis and further detailed investigation.

Finding candidate genes associated with punishment- and relief-learning

Learning which events predict upcoming dangerous stimuli or signal relief from them is very useful for an organism to adjust it's behaviour in the future. Cues associated with painful and relieving events have been studied in a variety of species, including humans and might thus underlie common principles [302, 319, 365, 8, 7]. It is thought that these

processes underlie phenomena such as addiction to extreme sports but also diseases like PTSD [302, 7]. *Drosophila melanogaster* is used as a model organism to study punishment and relief learning [319, 365, 364, 363, 78]. If, for example, *Drosophila* is trained such that an odour precedes electric shock, it will avoid the odour later on as a signal for punishment (punishment learning) and if, under otherwise same conditions, the odour is presented shortly after electric shock is gone, it approaches the odour later on as a signal for relief (relief learning)[319].

Punishment learning is very well studied in *Drosophila* as over hundred genes are known to affect it (for a review see [127, 72] and Table S21 for a list of learning genes). However, most of these genes have very different functions suggesting many more factors, yet unknown, to play a role. Furthermore, it is not known whether innate responses to odourants and shock are mediated by the same molecular pathways as learned responses to theses stimuli. In the future, this study can thus be of advantage to those tearing apart these molecular circuits as it analyses in parallel innate and learned behaviour in the same inbred strains. Using a genome-wide approach for punishment learning, candidate genes can be suggested that could help finding more parts of the molecular puzzle.

In comparison to punishment learning, relief learning was largely ignored in the past. To date, two genes are known to affect relief learning, *white*[364] and *synapsin* (personal communication with Ayse Yarali/Bertram Gerber/Soeren Diegelmann). The fact that one gene, *white*, affects both kinds of learning in opposite directions, i.e. making punishment learning better and relief learning worse [364], raised the question of a genetically alterable balance between them. Also, do they rely on the same molecular mechanisms? In this case, my genome-wide approach which suggests candidate genes for relief learning will be of enormous help to start unraveling molecular mechanisms underlying this trait and later compare these results with those for punishment learning.

On top of this, genetic dissection of punishment learning has revealed different phases of memory, i.e. short-term memory (STM), middle-term memory (MTM), long-term memory (LTM) and anesthesia-resistant memory (ARM). These have different genetic requirements and also differ depending on the training assay(e.g. [328, 329, 211]). Given these differences in punishment learning, it is interesting to ask: Does relief learning consist of the same phases? If translatable to human research, these results could advance knowledge and treatments about diseases related to a traumatic experience [78].

Genome-wide approach to find associated genes for electric shock avoidance, odour avoidance, punishment- and relief-learning

Individual animals, including humans, differ in their phenotype, especially, when it comes to quantitative complex traits [126]. Electric shock avoidance, odour avoidance, punishment and relief learning are quantitative complex traits as they are influenced by many factors such as genetic, sex and environmental differences which can also interact with each other [126]. Here I focus on the question: How does genetic variation influence differences in the aforementioned traits in *Drosophila melanogaster*? Apart from it's short generation time, easy handling and genetic manipualtion [14], genetic variation can be 'frozen' in *Drosophila* through inbreeding [207]. Therefore, the Drosophila Genetic Reference Panel (DGRP)generated 38 inbred fly strains, in which all flies in one strain have the same genome but differ from flies in other strains. Ayroles and collegues assessed the transcriptome and single nucleotide polymorphisms (SNPs) of these strains [21, 208]. Bringing together genetic and behavioural measurements of complex traits in these strains, associations between behaviour and expression level or SNP of a gene can be seen, respectively. This method was already applied successfully to identify candidate genes for traits such as sleep, aggression, starvation resistance and chill coma recovery (e.g.[125, 88, 21, 208]). Genome-wide association studies (GWAS) are a powerful tool to detect small effects of genes which contribute to natural variation in a complex trait. However, candidate genes have to be further investigated through looking for enrichment of functional categories, doing a gene network analysis and validation through reverse genetics [291, 80, 21, 139] to tell apart 'random' and 'real' lists. While association enrichment analysis is reported for all five behaviours and their candidate genes, validation using reverse genetics and network analysis is provided in this thesis on the example of electric shock avoidance.

To sum up, my major focus lies on the question which genes influence variation in electric shock avoidance, odour avoidance, punishment- and relief- learning. Therefore I use a Genome-wide approach with the advantage of detecting even small influences of genes on behaviour in a naturally derived population. I tested 38 inbred fly strains [21], in electric shock avoidance, odour avoidance, punishment- and relief- learning to obtain individual scores for the different strains. Bringing these together in an association analysis with known gene expression levels and SNPs of the strains genomes [21, 208] results in a candidate gene list for every behaviour. These lists enable me to compare across behaviours as well as looking for enrichment in specific functions. Also, as this study is the first comparing innate and learned behaviour on the genome-wide level, these lists will be

a valuable resource for future hypothesis driven research. Additionally, I independently validated the electric shock avoidance associated genes using reverse genetics and I present a gene-interaction network for this trait. Finally, I compared punishment- and relief-learning in terms of memory phases, specifically resistance to cold anesthesia.

1.2 Nociception and electric shock avoidance

Nociception is referring to an organisms ability to sense potentially harmful stimuli [300]. This sensation mostly leads to a reflexive avoidance behaviour which is critical for the survival of the animal [300]. Hence, this has led to a strong conservation of this trait across species. Avoidance responses to noxious chemical stimuli for example, could already be observed in the single cell organism *Paramecium* [111], which swims away from polluted areas. Numerous other examples can be found in vertebrates as well as in invertebrates such as leeches, nematodes and the fruit fly [300], [326], [299]. In *Drosophila* nociception is studied extensively using noxious heat, touch and chemicals (reviewed by [144]). This led to the discovery of many receptors and molecular pathways needed for these behaviours. Many of these receptors are transient receptor potential channels which are distributed across the surface, but also inside the fly body [99]. Some of the discovered molecules playing a role in fly nociception were found to have similar functions in mammals (e.g.[231]).

First articles about nocious effects of electric shock appeared in 1898 [242], attempting to investigate why people and animals are dying from large electric shocks and urging electricity factories to develop guidelines to avoid these tragedies. Later, more elaborate studies on animals emerged that distinguish tickle, pain, injury and death induced by electric shock (for a review see [97]). Electric shock could be easily applied and strictly timed as a stimulus. Suitably, it has become a traditional reinforcement in aversive associative learning research across species, including humans, e.g., [335, 251, 47, 331, 187, 198, 182, 95, 188, 210, 10, 8, 7]. Until now, it is thought that electric shock circumvents sensory receptors [123, 263]. Yet, surprisingly little is known about the molecular pathways recruited by shock.

However, mutations in three genes are mentioned in literature to specifically affect electric shock avoidance or habituation to it in *Drosophila*. These are *turnip* (electric shock avoidance, [219]), *mbm* (*mushroom body miniature*, electric shock habituation, [3]) and one recombinant line of the *Su-var(3)6*^{01} mutant (*Protein Phosphatase 1*, electric shock avoidance, [19]). All of them also affect olfactory punishment learning. It is further known,

through which neurons the negative reinforcement signal of electric shock is conveyed to the learning centre of the fly brain, the mushroom body (MB, see Fig.1.1, [127]). If there is an electric shock, dopaminergic neurons carry the signal to the MB [282, 18], Dopamine binds to its G-protein coupled receptors in the target neurons and a cAMP signal cascade is elicited where genes such as PKA, dunce and rutabaga play a role [195], [85], [86] (see Learning and Memory section for details and Fig.1.5 from [301]). Apart from this, it is not clear whether innate electric shock avoidance uses the same or another pathway and molecular messengers as learning nor how many genes affect variation in this trait.

1.3 Olfaction and odour avoidance

Humans and animals alike, are alarmed by the smell of fire or attracted by the smell of food. Finding food, distinguishing territories or finding mates are supported by odour guided behaviours and are beneficial for survival across species (e.g., dogs:[28], rats:[69], moths:[130], flies: [13]). Evolutionary conserved or functionally similar molecules can be found that are involved in olfaction [131], [307], [4], [34]. For example odour receptor genes were first detected in rodents [44], later, functionally similar molecules were found in worms [287] and afterwards in flies [63], [340], [35]. In contrast, another kind of odour receptors, ionotropic receptors (IRs) are more ancient and can also be found in bacteria and plants [35, 2]. The olfactory system is relatively well studied in the model organism *Drosophila melanogaster*. Nevertheless, this behaviour involves many complex processes and the influence of most genes and molecular mechanisms underlying differences in innate approach and avoidance towards specific odours still need to be elucidated. In general, given the strong relatedness between *Drosophila* and other insects, knowledge about basic steps of this behaviour can be of common use for example to produce better insect repellents.

What do we know about the factors influencing olfactory behaviour, especially odour avoidance in *Drosophila*? *Drosophila* mainly perceives odours through sensillae on the surface of the antennae and maxillary palps [48, 42, 43]. Olfactory receptor neurons (ORNs) transmit the odour signal from the sensillae to the antennal lobe (AL), where ORNs expressing the same receptor project to the same ball shaped anatomical structure, the glomeruli ([236], see also Fig.1.1 and Fig. 1.2). Interneurons in the AL may play a role in processing the incoming information (reviewed in [212]). From the AL, projection neurons (PNs) project to the mushroom bodies (MB) (Fig. 1.1), which are thought to be the centre for learning and memory in the fly brain [127]. Also via the PNs information is transmitted

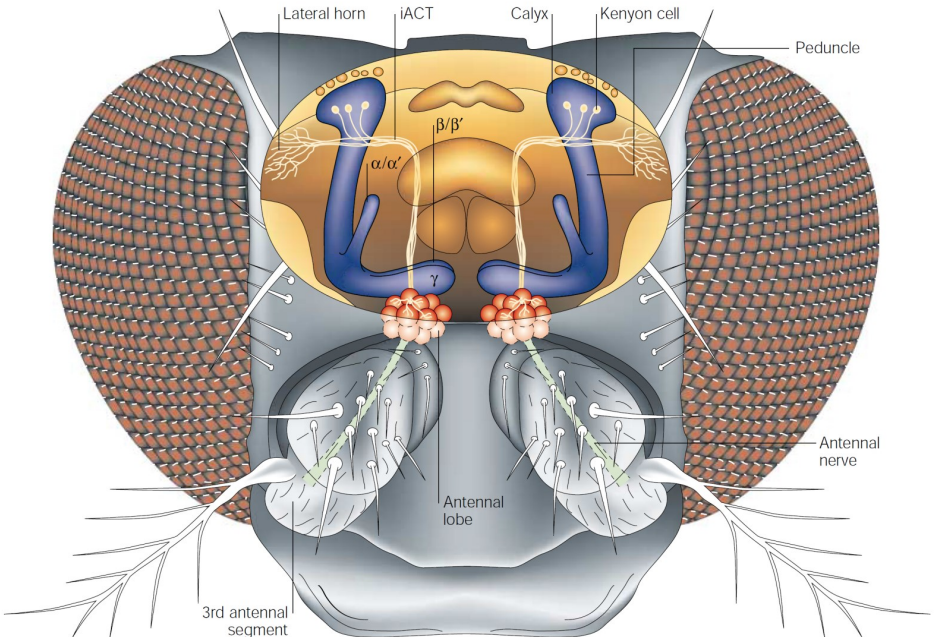


Figure 1.1: *Drosophila* head with olfactory pathway. Figure taken from [127]. Olfactory information is carried from the third antennal segment and maxillary palps (not shown) to the antennal lobe, where olfactory receptor neurons are sorted according to their odour specificity in about 40 glomeruli. This information is further processed in the dorsolateral protocerebrum (lateral horn) and the calyx of the mushroom body. The inner antennocerebral tract (iACT) connects individual glomeruli to both areas. α/α', β/β' and γ mark three different mushroom body systems.

to the lateral horn (LH) ([259, 212], see Fig. 1.1). There is evidence that olfactory stimuli inducing avoidance or attraction, respectively, are represented in different regions of the LH [220, 308, 309]. But attraction can be also influenced on the level of the MBs [348]. Further processing steps are not known in detail. Thus, molecules inducing morphological changes to these neuropils or altering transmission can possibly change odour avoidance.

The differently formed sensillae of antennae and maxillary palps contain haemolymph enriched with odourant binding proteins (OBPs) (reviewed in [271]). They circulate in the haemolymph and are very concentrated around the dendrites of olfactory (and gustatory) receptor neurons(see Fig.1.2). OBPs bind volatile molecules and shuttle them to their recognition sites (Fig.1.2, [102, 128, 113, 271]). A genome-wide screen identified 51 different OBPs [128]. Some of them, if knocked down, changed the fly's behaviour towards certain odours, i.e. approach or avoidance [312]. Thus, despite of being shuttles for incoming odourants to the respective receptor, OBPs very likely play a role in receptor specificity,

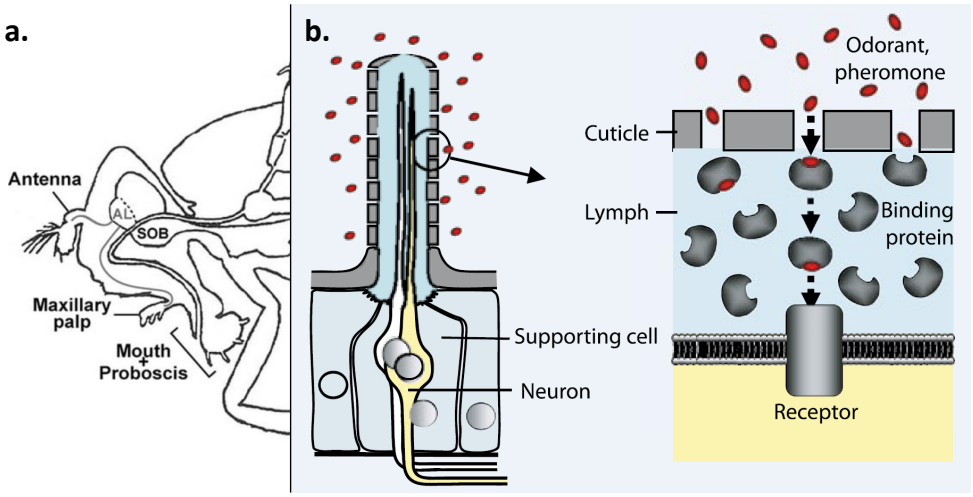


Figure 1.2: Odour sensation in the fruit fly. Adapted from [271] and [283] Odour sensation in the fruit fly. a. shows the maxilary palps and antennae of the fly, where olfactory receptor neurons (ORNs) can be found which have theire endings in small hairs called sensillae that project to the Antennal lobe (AL). b. Processes that happen in a sensillum during odour application. The odour (red dots) passes through holes in the cuticle into the hemolymph (blue) and is bound by odourant binding proteins (OBPs, grey half circles) which then serve as shuttles to the receptor that sits in the membrane (black line) of an ORN.

counting as factors affecting odour avoidance.

A good overview of olfactory receptors, cellular structures and molecules that play a role in further processing and following neuronal processes in adult flies is provided by [341, 101, 35, 212, 271]. In total $\sim$ 120 receptors are discovered in *Drosophila* to date that bind odours. These are devided into $\sim$ 60 odourant receptors (ORs) and $\sim$ 60 ionotropic receptors (IRs) [340, 35] with binding specificities ranging from one molecule to specific chemical structures [178]. ORNs are equipped with one specific receptor [35] that forms a heterodimer with the ubiquitous OR83B for ORs or IR8a or IR25a for IRs [185], [234], [34, 2]. Although ORs and IRs are mostly expressed in distinct sensillae forming heterodimers with their own kind, co-expression occurs, suggesting the formation of heterodimers between ORs and IRs [2]. Some receptors are restricted to e.g. the antennae, others not (for a review see [341] and [35]). The whole system guarantees for a good navigation in odour space as a range of different odours can be readily detected and distinguished (see a review by [107]). Small changes, i.e. SNPs in receptor genes can already lead to a shift in odour avoidance [267]. Hence, variations in odourant receptor genes can influence variation in odour avoidance, but which receptor changes the avoidance

to which odour specifically is not investigated to an exhaustive extent. In the AL, specific second messenger pathway molecules such as IP3-receptor, PLC, RY-receptor have been found to play a role in odour signalling as well as GABA and Ach receptors (for an overview see [228]). Again, there are still many gaps in our knowledge about the molecules in these pathways and how they effect the flies' response to odours.

Furthermore, odour avoidance has been found to change upon different activation pattern of ORNs and thus glomeruli [286] and in addition release of the neuropeptide tachykinin (DTK) by the interneurons of the AL [142]. Other than that, mutational screens and GWAS with already mentioned DGRP strains, using mainly BA avoidance, have revealed many genes, networks of genes and environmental factors influencing odour avoidance [347, 273, 12, 267, 346, 311]. Together, these findings imply many more genes to be involved in this simple odour avoidance response than previously thought.

1.4 Learning and memory

Learning is the ability of an organism to change its behaviour according to previous experience [323] and this information can then be retrieved as a memory for some time. After many years of research, still a lot of questions remain to be answered in detail about all kinds of memory. Notably, here, I focus on one form of learning, called classical conditioning, introduced by Ivan Pavlov [251] who trained dogs to associate a tone (conditioning stimulus, CS) with food reward (unconditioned stimulus, US) such that they later salivated upon hearing the tone only (conditioned response, CR). Electric shock can also be used as US to condition animals, including humans as it induces strong negative reactions (see nociception and electric shock avoidance above). Moreover, according to the opponent process theory every painful event has some relieving end to it (see Fig. 1.4 a. and b.,[303]). In general, the opponent process theory assumes that if the organism is in a neutral state, a novel unconditioned stimulus evokes a State A in an organism such as pain. After reaching the peak of state A, the organism shows adaptation to the stimulus and after offset of the stimulus the organism falls into state B which is opposite to state A, here relief. Both states can be associated with different CS [302]. The opponent process theory explains psychological phenomena such as extreme sports, self-administered aversive stimuli or addiction and stimuli associated with them. Both processes and forms of learning can be found across different species [302, 319, 365, 8, 7].

Strikingly, the fruit fly *Drosophila melanogaster* is able to learn to associate electric

shock as punishment or sugar as reward with other sensory stimuli such as visual or olfactory cues [85, 331, 282] [306]. Moreover, relief of electric shock can also be learned [319, 365]. One central theme of my study is olfactory punishment and relief learning. Briefly, for punishment learning, flies are presented with an odour A (CS) shortly before the onset of an electric shock as punishment (US). An odour B is presented alone in an unpaired way. After this training, flies are tested in a T-maze with odour A on one and odour B on the other side. Flies subsequently avoid odour A. Another group is trained reciprocally with odour B as paired CS and avoids it later in the test. This can be quantified calculating a learning index (see Material and methods and Fig. 1.3 [108]). Importantly, for relief learning, the odour is presented after the shock and flies later on approach this odour ([319], see Fig. 1.4 c. and d.). Although the effect is not as high as punishment or reward learning, relief learning has been independently validated across different experimenters and using different odours [365] and thus is a stable learning form.

Which genes have been found already to affect learning in the fruit fly? For punishment learning, already more than 100 mutants could be discovered that had a learning defect, e.g. *dunce, rutabaga* and *PKA* (e.g. [195], [85], [86]), only to name a few. Through one mutation *mushroom body miniature*, it was soon even possible to find a region in the fly brain where learning takes place, the mushroom body (reviewed in [127, 45], see also Fig. 1.1). These results, supported by experiments in Aplysia (e.g. [49]), led to a model of what might happen in neurons during learning, i.e. in the MB of the fly (see also Fig.1.5, [301, 127, 45]). Briefly, olfactory information is transmitted to the MB via the PNs coming from the AL (Fig. 1.1). They increase the Calcium level in Kenyon cells of the MB which also increases cyclic adenosine monophosphate (cAMP) levels. Electric shock is signaled from unknown regions through dopaminergic neurons to the MB. Also modulatory dorsally paired medial neurons (DPM) which make synapses on the same Kenyon cells (Fig.1.5) influence conditioning. Simultaneous activity of the US and CS pathways stimulates adenylate cyclase (Ac), encoded by *rutabaga*, which then increases levels of the second messenger cAMP. This increases excitability of the synapse such that if next time only the CS is given, i.e. the odour, the neuron fires. Thus we see the conditioned response. The model can be further refined/tuned through inclusion of about 100 molecules discovered for punishment learning in the MB and other brain regions (for details, see a learning gene list in Table S21). However, learning is a complex process and there are several questions that remain unsolved for punishment learning. For example, what are the molecules playing a role at the output of the MB? Which molecular processes

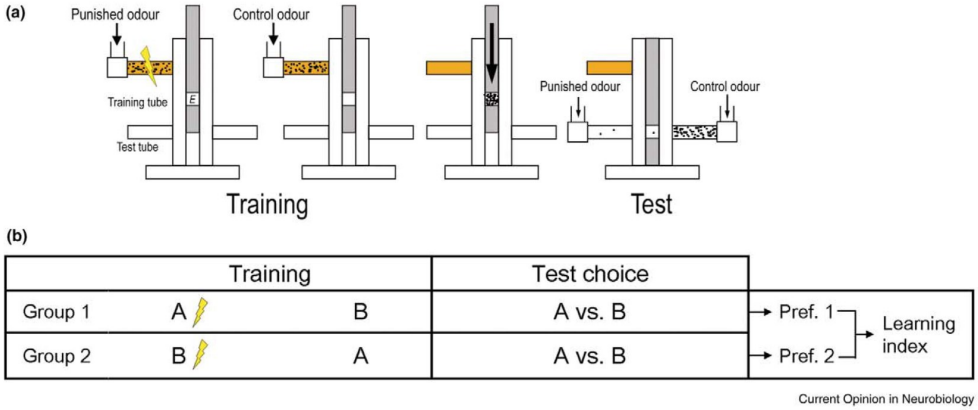


Figure 1.3: Conditioning device and reciprocal olfactory learning. Figure taken from [108]. The reciprocal design for *Drosophila* olfactory discrimination learning. (a) Training and test tubes are aspirated to produce a constant flow. A group of flies (black dots) are put into the training tube covered by a copper grid (orange) for electrification. During training, flies receive one odour together with an electric shock, and the control odour without shock. Subsequently, flies are transferred into the elevator compartment (E) and shuttled to a choice point where they can distribute between the previously punished and the control odour. Typically, flies avoid the previously punished odour. (b) To measure associative learning, a reciprocal experimental design is essential: two groups of flies receive either odour A with shock and B without or odour B with shock and A without. For both groups, the preference between odour A and B is measured after training. The learning index is then calculated by taking the mean preference of the two reciprocally trained groups, and thus purely represents associative memory, excluding any non-associative effects (e.g. sensitization by shock, habituation to the odours). Note that the learning index is unaffected by any overall bias for either of the two odours.

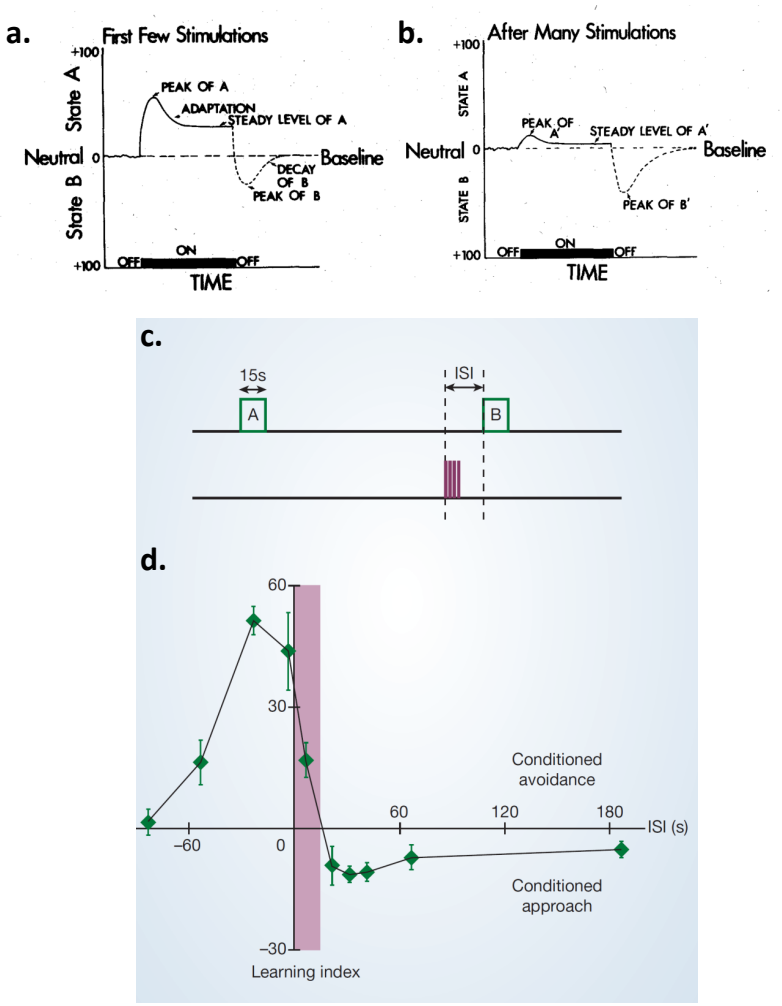


Figure 1.4: The opponent process theory and relief learning, pictures taken from [302] and [319]. a. The standard pattern of the opponent process theory is depicted. It assumes that if the organism is in a neutral state, a novel unconditioned stimulus evokes a State A in an organism such as pain. After reaching the peak of state A, the organism shows adaptation to the stiumulus. After offset of the stimulus (black bar on the bottom) the organism falls into state B which is opposite to state A, i.e. relief. b. Reaction of the organism to the same unconditioned stimulus after repeated stimulation. Here, only a small onset peak and a bigger offset peak is shown. c. Training paradigm for relief learning in the fruit fly. First an odour A (green box) is given for 15 s and after a significant amount of time, 4 electric shocks are given (purple lines) followed by odour B. Importantly, odour B is given while the fruit fly is in state B as shown above. The inter-stimulus interval (ISI) is the time from onset of electric shock to onset of odour stimulus. d. ISI is varied and for each ISI a learning index is calculated to whether the flies had shown conditioned approach (negative values) or avoidance (positive values) towards odour B. Indeed flies can learn to associate an odour with both states as described in a. and b.

shape learning in neurons? Which genes contribute to individual variation in learning?

Importantly, this model includes only findings for olfactory punishment learning which are partly also true for reward learning. Relief learning, however, remains in the dark so far. There are only two genes known to affect relief learning, *white* and *synapsin*. Mutants of one gene,*white*, showed opposing effects in punishment and relief learning, making relief learning worse and punishment learning better [364]. The other gene, *synapsin*, reduces both kinds of learning (personal communication with (Bertram Gerber/Soeren Diegelmann/Ayse Yarali). Although the same stimuli are used, punishment and relief learning seem to differ according to the molecular mechanisms underlying them. A genome-wide approach suggesting many candidate genes will thus be the beginning of the dissection of relief learning.

Moreover, the punishment learning genes are found to affect different phases of memory (e.g., [328, 330, 211], see Fig. 1.6). Immediately after the training, short term memory (STM) and middle term memory (MTM) are build up. STM consists of two components, one is sensitive to anesthesia, such as cold shock, and thus called anesthesia-sensitive memory (ASM) which has a fast decay. An anesthesia-resistant part of memory (ARM) builds up and is already visible 5 min after training [171], i.e. if they are tested after cold shock flies still retain some of the memory even up to 24 h [329]. Long-term memory (LTM) also lasts 24 h but is formed when flies are trained several times with an inter-training interval of 15 min (e.g. [329]). These intervals allow activation of protein synthesis which stabilizes the memory. Specific mutants, for example *rutabaga* and *dunce* [85, 84] have an effect on STM and amnesiac on MTM [328], whereas *radish* and *bruchpilot* [98, 171] can be found to exclusively affect ARM and others like *nalyot* and *dCreb2-b* [366, 75], affect LTM. Indeed some effort has been made to further distinguish LTM and ARM. For example, if the chemical cyclo hexamide (CXM), a protein synthesis blocker, is fed before training, LTM is disrupted but ARM is not affected. In contrast, if ethacrynic acid (EA), a deactivator of ATPase, is fed, ARM is disrupted and LTM is still intact [76]. Based on these experiments, the current model is, that after spaced training, LTM is build and after massed training, ARM is build(see Fig. 1.6, [146, 73]).

For relief learning, no memory phases have been reported so far. There is, however, evidence for relief learning in *Drosophila* staying on the same level for at least 2 hours [365]. This suggests different decay rates compared to punishment learning described above. But the existence of ARM or other memory components is unclear and thus further investigated in this thesis (see also [78].

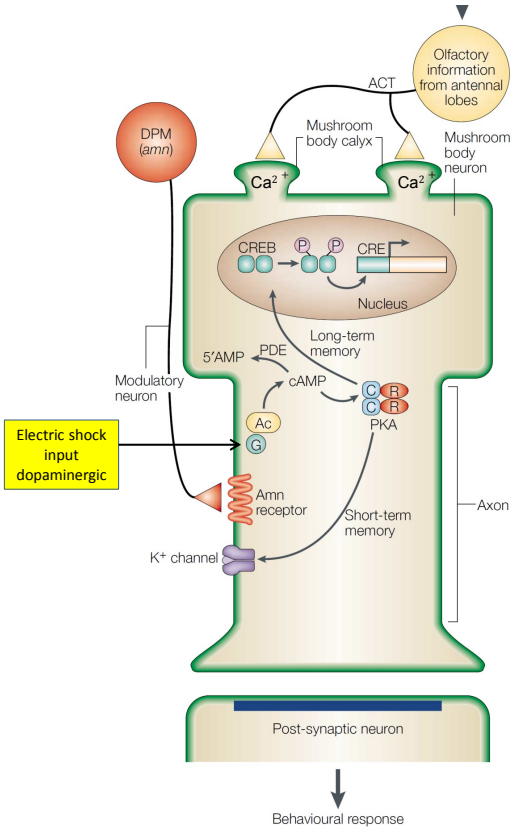


Figure 1.5: A model for olfactory aversive learning in *Drosophila* in the mushroom body. Figure taken and adapted from [301]. A mushroom body neuron gets olfactory information from: first, the antennal lobes through ACT (antennoglomerular tract) interneurons that synapse with the calyx of a mushroom body neuron and increase the Calcium level upon odour detection; and second, from DPM (dorsally paired medial neurons) and Dopaminergic neurons from different brain regions, which release the modulatory amnesic (Amn) neuropeptide and Dopamine after the delivery of an electric shock to the fly. The simultaneous activity of odour and shock pathways causes the stimulation of adenylate cyclase (Ac)–encoded by rutabaga(rut)– which is principally expressed in the axons and axon terminals of mushroom body neurons. The stimulated Ac then activates a G-protein-coupled receptor (G), which causes elevated cAMP levels. The increase in cAMP gives rise to either a short-lived change in the excitability of the mushroom body neuron (short-term memory) or a long-lasting change (long-term memory). The dunce-encoded cAMP phosphodiesterase (PDE) and the catalytic (C) and regulatory (R) subunits of protein kinase A (PKA) are among several genes that are preferentially expressed in mushroom body neurons. When PKA is activated for a short period of time, it is thought that downstream changes in the K^+ channels of the axon affect output from the post-synaptic neuron. Post-translational modifications and changes in gene expression thought to be involved in long-term memory occur partly through the phosphorylation of the transcriptional activator CREB by PKA, which then, in turn, binds to cAMP-responsive elements (CRE) that are located in the upstream regions of cAMP-inducible genes. P, phosphorylation.

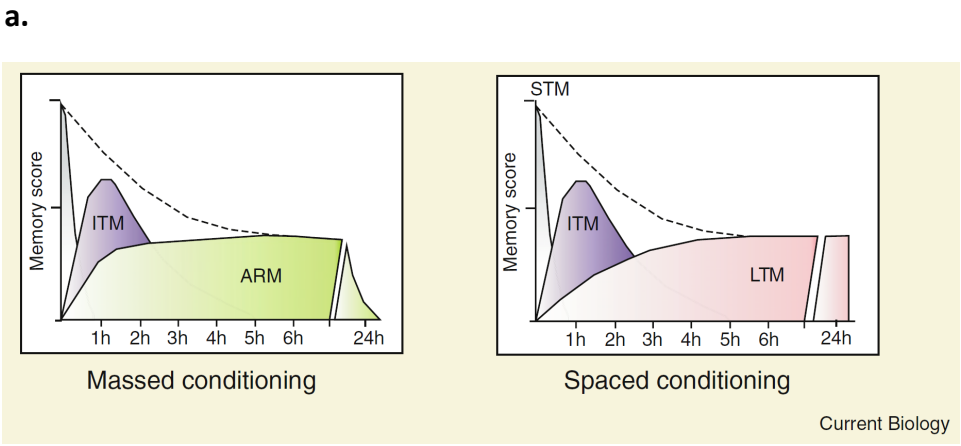


Figure 1.6: Memory phases in *Drosophila* taken and adapted from [146, 73]. One model of memoryphases in *Drosophila* for olfactory punishment memory in spaced (15 min inter-trial-interval and 5-10 trials) and massed conditioning (5-10 trials directly following each other) training paradigms. The earliest memory with the highest score is short-term memory (STM, white). While STM declines middle- or intermediate-term memory (ITM, purple) is build up. In addition in massed training (left), an anaestesia-resistant memory (ARM, green) is build up and while ITM declines, ARM persists over 24 h. After spaced training (right), protein synthesis dependent long-term-memory (LTM, pink) is build up, also persisting over 24 h.

Furthermore, findings in rats and humans support the similarity between relief and reward processing in the brain. Lesions in the amygdala, the centre of fear conditioning in the rat abolished punishment learning, but left relief learning intact whereas lesions in the ventral striatum, the reward region, destroyed relief learning and left punishment learning intact [7]. Corresponding brain imaging analysis in humans showed respective activation in amygdala for punishment learning and in the striatum for relief learning [7]. In flies, subsets of octopaminergic neurons projecting to the MB are required for reward learning but not for punishment and relief learning whereas certain subsets of dopaminergic neurons were important for punishment, but not relief learning [282, 363]. This suggests that relief learning might take place in other neurons of the fly brain than punishment learning and might be mediated by different receptors. However, the dopamine receptor dDA1 plays a role in punishment and reward learning [167]. On top of this, punishment and reward seem to be signaled by different clusters of dopaminergic neurons in the brain [18, 199], leaving the possibility open for relief being similar to reward learning. Therefore, it is interesting to find hints in a large candidate list about where to start searching for molecular mechanisms for relief learning.

1.5 *Drosophila* as a model organism in Genome-wide association studies

Drosophila melanogaster is serving as a model organism to uncover basic molecular processes for many different behaviours and diseases (reviewed by [29]). There are many reasons for this. One is that flies are very cheap and easy to handle. Their generation time, meaning the development from egg over larval stages to adult is 10 days at room temperature [14]. It also has a high fecundity. One female lays 50-80 eggs per day [241] which allows a scientist to perform experiments with high sample sizes and thus enables good statistical analysis. Another reason is the rich availability of genetic tools making it easy to manipulate *Drosophila*. Vast amounts of mutants are available, generated using UV and X-rays or feeding of specific chemicals that alter the DNA [14]. Additionally, targeted P-element insertions can disrupt genes, induce deletions, overexpress or block genes even in a tissue specific manner (reviewed by [141]). To date, the P-element disruption project covers already 2/3 of protein coding genes in *Drosophila* [31, 322, 30]. This development was enhanced by mapping and sequencing of the genome [225, 5]. Currently it is assumend, that the genome of *Drosophila melanogaster* contains about 16000 genes

[129]. Importantly, the fly is able to perform very complex behaviours such as learning and memory, has a smaller brain than mammals but a large genetic homology as about 75% are orthologs to the human genome [59, 37]. This paves the way for translational research. All in all, this makes *Drosophila* one of the best genetically tractable model organisms.

The *Drosophila* Genetic Reference Panel (DGRP) is providing inbred fly strains and their genomic and transcriptomic information to study genome-wide association of SNPs (quantitative trait loci,QTLs) and expression levels (expression quantitative trait loci, eQTLs) to different quantitative complex traits in *Drosophila melanogaster*. Quantitative complex traits are influenced by multiple factors which can be genetical or environmental and thus vary in their quantity among individuals [126]. Also electric shock avoidance, odour avoidance, punishment and relief learning, traits investigated in this study, are quantitative complex traits. Genome-wide association studies (GWAS) are one tool to gain inside into the genes influencing variation in these traits. *Drosophila* harbours a very high natural genetic variation which is maximized between inbred fly strains, such that one obtains a homozygous fly strain with minimized genetic variation which genetically differs from other homozygous fly strains (see more in the discussion). In contrast to associations using polymorphic markers with limited discovery of gene-phenotype associations [292, 278] used earlier, it is now possible to sequence the whole genome and use expression microarray technologies providing a vast amount of information about the genetic differences between 38 inbred fly strains generated by Ayroles et al. [21, 208]. Now, that the genotype is known, the phenotype of interest, in my case electric shock avoidance, odour avoidance, punishment and relief learning, needs to be measured for each of the 38 strains. Bringing together either expression level and behavioural scores or SNPs and behavioural scores in an association analysis reveals candidate genes for each trait (see Fig. 1.7). Through usage of a high number of individuals, the chances increase to detect even small contributions of genes [203]. As it provides an unlimited number of individuals of the same genotype to test for each trait and is highly genetically accessible, *Drosophila* is a powerful model organism to use in GWAS.

Using inbred strains from the DGRP, many phenotypes have been studied such as starvation resistance, chill coma recovery, aggression, alcohol sensitivity and sleep, validating this approach (e.g.[125, 88, 226, 21, 12, 62, 157, 139, 156, 208, 311]). Through these studies, many candidate genes could be suggested and some were validated using reverse genetics and supported by gene networks. The power of GWAS lies in assessing naturally occurring variation in the whole genome/transcriptome of each genotype in a population

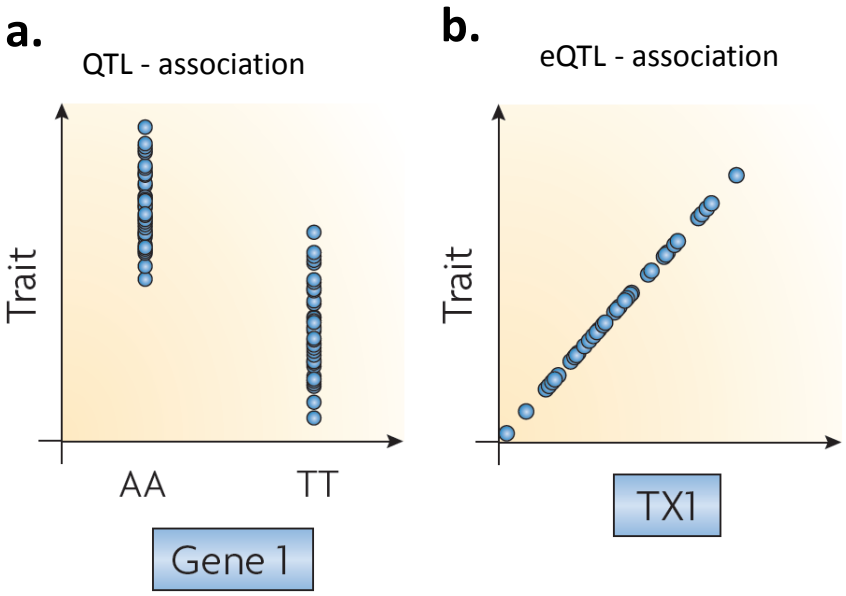


Figure 1.7: Quantitative trait loci (QTL) and expression quantitative trait loci (eQTL) mapping, adapted from [209]. a. The measured scores of a trait, i.e. a certain behaviour, in a population, i.e. inbred strains, is plotted depending on their genotype in Gene 1. If there is a difference between the two groups, there is an association with this QTL. b. Measured scores of a trait in a population are plotted against respective expression levels of a gene (TX1). If there is a significant correlation between these two, it is called an eQTL association.

to then tell which part of this variation contributes to the observed phenotype [92], leaving an estimated error coming from non-genetical effects. Not only, because of statistics but also because of genetic mechanisms it is possible to detect false positives (for more details see advantages and caveats of Genome-wide association). Therefore it is appropriate to check the candidate genes of a GWAS in a subsequent mutant analysis. As genes are either interacting on the expression and the protein level, more and more algorithms were developed during the last few years, to calculate networks that are associated with a trait (e.g. co-expression network: [21], protein-protein interaction network: [80]).

Summing up, in my study I assessed the complex traits electric shock avoidance, odour avoidance, punishment and relief learning in 38 inbred fly strains generated by [21] and then associated each with expression and sequence QTLs recorded by [21] and [208]. Especially relief learning and electric shock avoidance have never been investigated on a genome-wide scale. Also comparisons between these traits have not been done on that scale. Additionally, a newly developed network, based on protein-protein interactions was inferred for the eQTLs associated with electric shock avoidance [80]. In case of electric shock avoidance, I also validated the candidate gene list testing P-element insertion mutants.

Chapter 2

Material and Methods

2.1 Flies

Flies were kept in mass culture on standard cornmeal-molasses food [118] at 60-70% relative humidity and 25°C temperature under a 12:12 h light:dark cycle. 1- 3 days-old adults were collected into fresh food bottles and kept under the mentioned culture conditions but at 18°C temperature, for 1-3 days, so that they were 2- 4 days old at the experimental day. For the association analyses, I used 38 inbred fruit fly *Drosophila melanogaster* strains from the *Drosophila* Genetic Reference Panel collection (strain numbers are given in Fig. 3.3, 3.11, 3.10, 3.14; all available from the Bloomington Stock Center). These had been generated by full-sib inbreeding of iso–female strains from Raleigh, North Carolina, USA for more than 20 generations (for details see [21, 208]). For independent testing of candidate genes as well as for targeting *dNpfR1*, I used appropriate P-element insertion mutants of the Berkeley *Drosophila* Genome Project [31, 322, 30] (see Fig.2.1 for P-elements used) along with the corresponding co-isogenic controls (for details, please see Table S24). For targeting Transient receptor potential A1 (dTrpA1, FBgn0035934), the mutants $dTrpA1^{ins}$ (generated by Hamada et al. [122] via homologous recombination; kind gift from P. Garrity) and *dTrpA1[1]* (generated by Kwon et al. [181] via homologous recombination; Bloomington Stock Center) were used. Both of these had originally been on a w^{1118} background with copies of the mini white gene. Thus, for *dTrpA1[1]*, both w^{1118} and wild type Canton S (CS) were used as controls. As the 1st chromosome of the $dTrpA1^{ins}$ had been exchanged with that of CS, for this mutant CS was used as the only control.

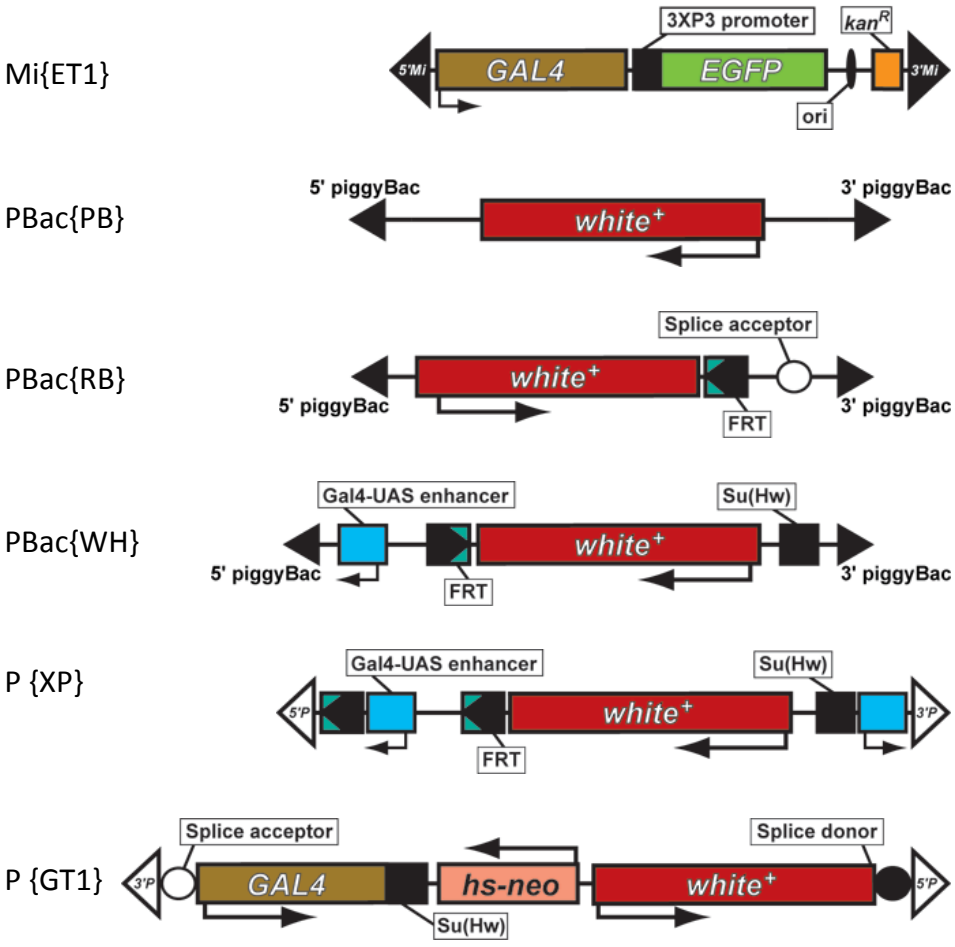


Figure 2.1: Figure adapted from [31], [30, 322]. Schematic drawings of P-element constructs, no scale used. Different P-elements contain various genes; Marker genes, such as *white* and *GFP* to recognize insertion as well as different promoters to initiate genomic transcription (3XP3, Su(Hw)); hs-neo and Gal4 transcription factor, Gal4/UAS enhancer and FRT site can be used in combination with other tools not described here; Antibiotic resistance gene with bacterial transcription origin (kan^R, ori) for cloning, Splice acceptors and donors and inverted repeats at the 5' and 3' ends, specific to the kind of P-element and needed for transposition, respectively.

2.2 Behavioural assays

All experiments took place at 21- 23 °C temperature and 60- 70% relative humidity under bright fluorescent light or red light in case of cold-anesthesia resistance of punishment and relief learning. They were performed using a set-up comparable to that used in [282]. The airflow to suck the odours or in some cases (see different behaviour sections) just air from outside through the arms of the set-up to the pump was produced by a vacuum pump (ME1, Vacuubrand, Aresing, Germany) and was adjusted to 4.5 l/min at the level of the pump for every experiment. For innate behaviours flies were tested in groups of $\sim$ 50, for learning 100-150 flies were used. The experimental setup [331] had four positions for processing four groups in parallel. The testing of each genotype at each position was balanced. This was critical because the position in the setup seemed to affect most behavioural scores (Kruskal-Wallis test on the data in Fig. 3.3, 3.11, 3.10 and 3.14, respectively, comparing scores between the four positions in the setup, after pooling across inbred strains: shock: H= 16.30, d.f.= 3, $P = 0.001$, N= 212, 218, 202, 220, BA: H= 39.36, d.f.= 3, $P < 0.0001$, N= 79, 83, 76, 80, OCT: H= 12.04, d.f.= 3, $P = 0.0072$, N= 79, 83, 76, 80, punishment: H=24.27, d.f.= 3, $P < 0.0001$, N= 93, 87, 90, 87, relief: H= 7.04, d.f.= 3, $P = 0.0707$, N= 235, 218, 239, 225). Please also note, that although male, female and unisex scores are calculated based on the same experiment, scores calculated with < 5 flies were excluded from further analysis. At 0:00 min of each assay, flies were gently introduced into a tube of 9 cm length and 1.5 cm inner diameter, coated inside with a copper wire coil, perforated at one end and this shock tube was then attached to the experimental setup. Further steps were individually designed for each behavioural assay.

2.2.1 Electric shock avoidance

At 1:00 min, with rigorous shaking, flies were transferred to the Mid-compartment (cylindirical: 1.5 cm-diameter, 1 cm-length). At 4:00 min, they were gently moved to the choice point of a T-maze with two shock tubes as arms. At 6:00 min, one of the arms was electrified with 4 pulses of $\sim$ 100 V, DC; each pulse lasted 1.2 s and had an onset-to-onset interval of 5 s to the next shock. In Fig. 3.8, the number and intensity of shock was varied. 10 s after the last shock-pulse, the arms of the T-maze were sealed and flies of each gender in each arm were counted to calculate a 'unisex', a 'female' or a 'male' score as follows:

$$shock\ avoidance\ score = \frac{(\#\ shock - \#\ no\ shock) \cdot 100}{\#\ total} \tag{2.1}$$

, where # denotes the respective number of flies. The resulting values ranged between -100 and 100, more negative values meaning stronger avoidance of shock. The side of the electrified maze arm with respect to the setup was switched in alternating experiments to cancel out possible bias.

2.2.2 Locomotion

The locomotion assay was designed such that it was directly comparable to the present shock avoidance assay in that it employed the same setup and had the same temporal flow. At 1:00 min, flies were transferred to the Mid-compartment and at 4:00 min they got access to the T-maze with two shock tubes as two arms. At 6:00 min, instead of delivering shock pulses, rigorous shaking of the setup was applied such that all flies fell to the end of one arm of the maze (i.e. 'startarm'). Then, the set-up back was gently put to its horizontal position and the flies could disperse back towards the 'Opposite arm' for 25 s, in the absence of shock, before sealing the maze and counting flies in each arm and in the mid-compartment. The ratio of flies that travelled more than a tube-length (locomotion score) was quantified as:

$$locomotion\ score = 1 - \frac{\#\ startarm}{(\#\ startarm + \#\ opposite\ arm + \#\ Midcompartment)} \quad (2.2)$$

, where # denotes the respective number of flies. The resulting values ranged from 0 to 1 and larger values meant that more flies made it out of the Start arm.

2.2.3 Odour avoidance

Aiming for a better comparison across behaviours, I designed this assay such that the experimental flow and odour concentration is the same as for the test situation in the learning experiments (3.1). At 1:00 min, with rigorous shaking, flies were transferred to the 'Mid-compartment' (cylindrical: 1.5 cm-diameter, 1 cm-length). At 2:00 min, flies were moved to the choice point of a T-maze that consisted of two tubes of 78 mm length and 14 mm diameter covered with a 'mosquito net' at the end. Odour cups were attached at each side, one was empty and the other contained either a 14 mm diameter cup of 250 μl pure 3-octanol (VWR, 8.21859 , Darmstadt, Germany) or a 5 mm diameter cup of 50 μl pure benzaldehyde (VWR, 20863.291, Darmstadt, Germany). The air was sucked through the

middle of the T-maze so that the odour could not flow to the opposite compartment but flies were easily able to cross. After 2 min of choice, at 6:00 min experimental time, the T-maze was closed and flies on each side were counted. Then a benzaldehyde or 3-octanol avoidance score was calculated, respectively as for shock avoidance:

$$odour\ avoidance\ score = \frac{(\#\ odour - \#\ no\ odour) \cdot 100}{\#\ total} \tag{2.3}$$

, where # denotes the respective number of flies. The resulting values ranged between -100 and 100, more negative values meaning stronger avoidance of the respective odour. For better comparability, 3-octanol was always presented in the back and benzaldehyde always in the front arm of the T-maze as was the case for the learning experiments described in the next section.

2.2.4 Punishment learning

For this assay I used the same odour cups with pure benzaldehyde and 3-octanol as odours as described in the odour avoidance assay. At 1:00 min, an odour A was presented for 15 s as a control stimulus. At 4:15 odour B was presented for 15 s as the odour to be learned followed by 4 electrical shock pulses of $\sim$ 100 V, DC at 4:30; each pulse lasted 1.2 s and had an onset-to-onset interval of 5 s to the next shock. Thus, the inter stimulus interval (ISI, from shock onset to odour onset) was -15 s. To lower the disturbance of the flies right after the trial, flies were taken out of the set-up at 9:00 min (see Fig. 3.1) and rested in food vials for 14 min. During this time, the reciprocal group was inserted to the tubes. This group received odour B as control odour and odour A as odour to be learned. Please note that the procedure for relief learning consists of 6 such training trials for each reciprocal group. For the test, flies were treated as in the odour avoidance assay except that they had the choice between 3-octanol and benzaldehyde in the T-maze. After 2 min, the T-maze was closed and flies on each side counted for each reciprocal group to calculate a learning index (LI). First, the formula for the odour avoidance score was used to calculate the avoidance/approach of the learned odour for each reciprocal group, that is, 'odour' is replaced by 'odour to be learned' and 'no odour' by 'control odour' in the formula. Then, the LI was calculated combining both reciprocally trained groups (A and B) as follows:

$$LI = \frac{(\#\ odour\ avoidance\ score\ A - \#\ odour\ avoidance\ score\ B) \cdot 100}{2} \tag{2.4}$$

The resulting values ranged between -100 and 100, more negative values meaning stronger conditioned avoidance and thus stronger punishment learning.

2.2.5 Relief learning

Throughout this thesis, punishment and relief learning were made comparable and thus, the same treatment was used for both, except that flies received the odour to be learned at 5:10 min for 15 s (ISI = +40, Fig. 3.1). A Learning index was calculated as described for punishment learning, with more positive values meaning stronger conditioned approach and thus stronger relief learning.

2.2.6 Resistance to cold-anesthesia

In Fig. 3.20, the experiment used a 2x2 experimental design. This means that either flies were trained for punishment or relief learning as described above and after one hour in food vials, they were put into empty glas vials and either did or did not receive a cold-shock for 2 min on an ice bath of 0°. Afterwards, flies were placed back into food vials for 1 h. Their memory was then tested 2 h after the training as described above (see Fig.3.1 and 3.20).

2.3 Statistical analysis of behavioural scores

Behavioural scores were analyzed using Statistica version 11.0 (StatSoft, Hamburg, Germany) and R version 2.15.1 [1] on a PC. I used non-parametric statistics: Kruskal-Wallis tests for global comparisons, Mann-Whitney U-tests for pair-wise comparisons, and one-sample sign tests for comparing single groups to zero. In Fig.2.1, as 19 Mann-Whitney U-tests were performed in parallel, Benjamini Hochberg False Discovery Rates (FDR) were quantified [32]. E.g., a significance threshold of $FDR < 0.05$ indicated that up to 5% of the cases that were taken to be positive were expectedly false positives. In Fig. 3.8, due to a lower number of parallel comparisons, we used the more stringent Bonferroni correction to control for multiple testing, which restricted the experiment-wide error rate to 5% by dividing the critical P-value 0.05 by the number of tests.

[1] www.r-project.org

2.4 Gene expression level - behaviour association analysis

Also, R version 2.15.1 was used for these analyses. Raw Affymetrix GeneChip *Drosophila* Genome 2.0 expression microarray data [21] for the 38 inbred strains were downloaded from [2] (accession number EMEXPE-MEXP-1594) using the R Affy package [105]. The raw data covered 18 952 probe-sets and included four expression arrays per strain, two for each gender. For the strain 399 the data from one female sample was excluded from analysis, because the distribution of expression levels across the probe-sets rather resembled the typical male distribution. For all remaining data, perfect match probe intensity values were pre-processed with variance stabilization normalization (VSN) and summarized with the median polish method to obtain probe-set expression levels using the command vsnrma with the default parameter settings [140]. After such pre-processing, for each probe-set, a fixed-effects ANOVA (Expression $\sim$ Sex + Strain + Sex::Strain) was performed, followed by calculation of Benjamini Hochberg False Discovery Rates (FDR) [32]. Those probe-sets with $FDR < 0.001$ for either the Strain or the Sex::Strain terms were included in the next step of analysis. In order to get brain specific transcripts for punishment and relief learning, probe-sets of transcripts that are not expressed in the brain, i.e. callrate zero according to FlyAtlas [3], were excluded from further analysis at this point. Expression levels of these probe-sets were averaged for each strain to obtain unisex expression levels. These were then tested for effects on the unisex behavioural scores (Fig. 3.3, 3.11, 3.10, 3.14), using the following linear model:

$$behavioural\ score \sim \beta 0 + \beta 1 \cdot mean\ expression\ level + \beta 2 \cdot position\ in\ experimental\ setup \tag{2.5}$$

$\beta 0$ was the intercept and $\beta 1$ the estimate for the effect of the mean expression level. The position in the setup (please see above for a description of the setup) was a categorical variable and $\beta 2$ was the estimate for its effect. The significance of the $\beta 1$ was tested with a two-tailed t-test (d.f.= 356), followed by calculation of Benjamini Hochberg FDRs [32]. Please note that in this analysis all behavioural scores from all strains are taken into account. That is, for a given probe-set and a given behaviour, the model in (2.5) looks for a linear relationship between 38 mean expression values and 38 x N behavioural

[2] www.ebi.ac.uk/arrayexpress
[3] www.flyatlas.org

scores, where N is the sample size per strain for the respective behavioural assay. This procedure has a disadvantage: Namely, the N behavioural scores obtained from one strain co-vary with each other, whereas they vary independently from the scores obtained from another strain. Thus, the current analysis mixes co-varying and independent data with each other, which may lead to confound. Thus, at the time this thesis was written, two alternative analyses commenced. (I) To the right hand-side of the model (in 2.5.) 'strain' as a random variable was added to account for the co-variance between scores obtained from the same strain. (II) The model in (2.5.) was adapted to look for linear relationships between 38 expression values and 38 median behavioural scores ignoring the position in the set-up. A comparison between the three analysis strategies will hopefully increase the confidence in the results. For now, I take the results from the model as depicted in 2.5.. The probe-sets with an $FDR < 0.01$ were considered to be associated with investigated behaviours, except for relief learning. Due to the low statistical power (small effect and high variation in behaviour) to detect associations the threshold was set to $FDR < 0.1$ for relief learning, bearing in mind the higher rate of false positives in the list. From the prob-sets fulfilling these statistical thresholds, we excluded those with _x_ or _s_ qualifiers in their Affymetrix IDs, as according to the Affymetrix terminology these contained one or more probes that hybridize with products of different genes. The remaining probe-sets were annotated using FlyBase IDs given by Affymetrix [4] (for *Drosophila_2* arrays, the annotation file was downloaded in February 2012). These FlyBase IDs were then inserted to the Batch processing tool or 'Jump to Gene' of FlyBase [5] to obtain the newest ID. FB.IDs of other species and secondary IDs were removed. If there were no or several FB.IDs leading to different genes, but a 'Transcript ID' or if not a 'gene symbol' given by Affymetrix, this was used to retrieve the newest FB.ID through FlyBase as described above. Now, if FlyBase found either no gene or two different genes matching to this Transcript ID, there is no FB.ID entry in the tables as well as for genes with withdrawn gene models. Tables S1-5 list the final associated probe-sets for each behaviour along with the relevant statistics (i.e., Mean $\beta 1$, SEM of $\beta 1$, t, P and FDR) and corresponding gene FlyBase IDs. Those genes for which at least one corresponding probe-set fulfilled the statistical criterion for association were considered to be candidates (Table S1-5). Tables S11-S15 include only those candidate genes that have a FlyBase ID.

[4] `http://www.affymetrix.com/analysis/netaffx`
[5] www.flybase.org

2.5 Single nucleotide polymorphism (SNP) - behaviour association analysis

The Ilumina and 454 SNP calls of the respective strains [208] were downloaded from [6] . We pre-selected bi-allelic, homo-/ hemizygous single nucleotide polymorphisms (SNPs) with minor allele frequency (MAF) > 0.1 and call-rate > 0.7. For each such SNP, we tested for an effect on the behavioural scores using the following linear model:

$$behavioural\ score \sim \beta 0 + \beta 1 \cdot allele + \beta 2 \cdot position\ in\ experimental\ setup \tag{2.6}$$

$\beta 0$ was the intercept. The minor and major alleles took the values 2 and 0, respectively. $\beta 1$ was the estimate for the effect of the allele. Position at the setup and $\beta 2$ were as explained above for the expression - behaviour analysis. $\beta 1$ was subjected to a two-tailed t-test. With respect to autosomal SNPs, this was done using the 'unisex' behavioural scores (Fig. 3.3, 3.11, 3.10, 3.14). Please note, that all behavioural scores are used here as described above for the genome-wide expression analysis and the same analysis variations I and II are running in parallel while I am writing this thesis. For the current analysis, the cases with $P < 0.05/1387514 = 3.6 \cdot 10^{-8}$, corresponding to a Bonferroni correction over all tested SNPs, were considered as significant associations. For relief learning, which had a lower power to detect associations, a threshold of $P < 10^{-5}$ was used. With respect to the sex-chromosome SNPs, this analysis was done separately for each sex, using the sex-specific behavioural scores (Fig. 3.3, 3.11, 3.10, 3.14) and those SNPs that had $P < 3.6 \cdot 10^{-8}$ (for relief learning $P < 10^{-5}$) in at least one sex were considered as associated with the respective behaviour. Considering the hemizygous state of the males, the male $\beta 1$ values were multiplied by two. All SNPs were annotated according to *Drosophila melanogaster* reference genome version 5.35. In the attached tables, all SNPs that were annotated to inter-genic regions or to multiple genes were excluded, because during given time it cannot be determined which gene they affect and further analysis in this thesis does not build on them. Gene symbols were used to retrieve the newest FlyBase ID which was downloaded using the batch processing tool of FlyBase [7]. If this tool did not give a hit, a manual search was performed on the same webpage. Also if a symbol lead to many possible genes, the exact location of the SNP was looked up and the exact, matching gene ID taken. In case of

[6] http://dgrp.gnets.ncsu.edu/data/
[7] www.flybase.org

many matching IDs there is no ID entry. S6-10 list the autosomal (a) and sex-chromosomal (b) SNPs, respectively, along with the relevant statistics (i.e., Mean $\beta 1$, SEM of $\beta 1$, t, P), type of SNP as well as gene FlyBase IDs. A comprehensive list of all candidate genes can be found in S11-15. For these, SNPs without a FlyBase ID in S5-10 were excluded. For calculating the significance of the proportion of untranslated region (UTR) SNPs in the overlap with expression associated SNPs, Statistica version 11.0 (StatSoft, Hamburg, Germany) was taken to perform Pearson Chi-square tests.

2.6 Functional enrichment among candidate genes

The online database PUBMED [8] was scanned for publications reporting the effects of genes on fruit fly locomotion as well as nociceptive and learning behaviour (Table S21). In addition, I downloaded from http://superfly.ucsd.edu/homophila/ a list of fruit fly orthologs to human genes implicated in congenital pain insensitivity [59] ($BLASTE - value < 10^{-20}$; Table S21). I marked these kinds of genes in the list of candidates (Tables S11-15). In terms of bristle-related function, two gene lists were used: The first one had emerged from two mutagenesis screens [205, 240] and consisted of 287 genes for which, the respective P-element insertions significantly affected the number of bristles (ANOVA; $P < 0.05$). The second list was based on an RNAi-screen [227] and included 1 847 genes whose knockdown in the notum affected either bristle-number or -morphology to different extents (no statistics were reported in the original publication). Fold enrichment of a certain kind of gene, e.g., locomotion-genes, among the candidate genes was followed by a P-value calculation according to a hypergeometric test [68, 260]. The formula for enrichment (also called representation factor) was as follows:

$$fold\ enrichment\ of\ locomotion\ genes = \frac{\frac{\#\ locomotion\ genes\ in\ candidate\ list}{(\#\ candidate\ genes}}{\frac{\#\ locomotion\ genes)}{\#\ all\ fly\ genes}} \tag{2.7}$$

, where # candidate genes contained all SNP and expression associated candidate genes of the respective behaviour, unless stated otherwise; whereas # All fly genes was taken as 16 000 [129]. The exact formula for the P-value can be found on [9] [260]. I uploaded the list of candidate genes for every behaviour (Table S11-15) to the online bioinformatics tool

[8] www.ncbi.nlm.nih.gov
[9] http://nemates.org/MA/progs/overlap_stats.html

DAVID 6.7 (http://david.abcc.ncifcrf.gov/home.jsp) [68], [291] and selected the *Drosophila melanogaster* genome as background. Enrichments and P-values are calculated as described above by DAVID. Tables S16-20 show the GO Biological Function, Molecular Function and Cellular Compartment terms with a significant enrichment (Multiple correction method: Benjamini Hochberg $FDR < 0.05$).

2.6.1 Overlapping candidate genes

For comparing candidate gene lists across behaviours, the newest FlyBase IDs were retrieved using FlyBase Batch processing tool as described above. Significance of overlaps between candidate gene lists was calculated using the online calculator of 'Nematode bioinformatics' of the Lund lab at University of Kentucky, especially developed for this task [260] [10] using calculations described above. 16 000 was taken as number of genes in the genome [129], because most candidate genes were SNP based and thus associated compared to the whole genome. It has to be noted though, that a small proportion ($< 10\%$) of punishment and relief learning candidates, namely those associated with expression, are restricted to brain expressed transcripts which might confound the results.

2.7 Gene network analysis

This analysis followed up on the gene expression level shock avoidance associations. On the one hand, for each of the 10 121 probe-sets that had a highly variable expression level across strains (see above), gene FlyBase IDs were obtained, using the R Bioconductor package drosophila2.db [11] and the Batch Processing Tool of FlyBase [12]. On the other hand, the protein - protein interaction data were downloaded from FlyBase[13]. The intersection of these two datasets gave a network of 3 578 genes and 32 888 interactions for subsequent analyses. For each of these genes, the P-value for the association between the expression level and shock avoidance (see above) was converted to a network score using the routines implemented in the BioNet package [27], based on a Beta uniform mixture model and a log likelihood ratio [80]. These scores were then adjusted to an FDR of 0.001. Using an integer linear programming formulation the optimally scoring sub-module was calculated exactly

[10] http://nemates.org/MA/progs/overlap_stats.html
[11] www.bioconductor.org
[12] www.flybase.org
[13] ftp://ftp.flybase.net/releases/FB2012_06/precomputed_files/genes/physical_interactions_fb_2012_06.tsv.gz

[80], resulting in a smaller, shock avoidance-associated network. This network was then plotted using the routines in the BioNet framework [27]. Table S23 lists all network genes along with annotation as well as relevant statistics of their association to shock avoidance (i.e., Mean β1, SEM of β1, t, P, FDR).

2.8 RNA extraction, reverse transcription and real-time quantitative PCR

Flies aged 1- 3 days after adult-hatching were collected into food vials and stayed at 18 °C overnight, so that on the day of sample preparation they were 2- 4 days old. Flies were transferred into pre-frozen empty vials, which were then quickly put into liquid nitrogen. Frozen flies were sorted on a Petri dish on dry ice into groups of 10 males or 10 females. Each such group was placed back into a falcon tube and put back into liquid nitrogen to later be stored at -80 °C until RNA extraction. For RNA extraction, I used the Trizol Reagent following the manufacturers protocol (Sigma Aldrich, Taufkirchen, Germany; product number T9424-200ML). The resulting RNA content was measured by spectrophotometry on a Nanodrop2000 (PeqLab Biotechnology GmbH, Erlangen, Germany) and each RNA sample was accordingly diluted with RNAse free water to a final concentration of 1 g/ l. 10 l of each such sample was then used for reverse transcription with Superscript III and random primers (Life technologies, Invitrogen, Darmstadt, Germany; product numbers 18080-044 and 48190-011), following the manufacturers protocol. The resulting cDNA samples were stored at -20 °C until real time quantitative PCR (RTQPCR), which was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) following the manufacturers protocol, thus using 8l of cDNA sample, 2l of a primer-mix (5 pmol/ l per primer) and 10l FastSybrGreen Master Mix (Applied Biosystems, Darmstadt, Germany; product number 4385614) to reach a total of 20 l reaction volume. For each gene of interest and the housekeeping gene FBgn0002626 (*rp49*), we designed 20 b forward and reverse oligonucleotide primers, such that the amplified product size ranged 70- 150 bp (see Table 2.8). For primer-design, the software Primer3 version 4.0 (http://frodo.wi.mit.edu/, [270]) together with the *Drosophila* mis-priming library was used. The oligonucleotides were synthesized by Metabion (Martinsried, Germany). I analyzed with RTQPCR 2-12 independent cDNA samples for each genotype and sex (Table S24). Each sample that was included in a given RTqPCR run was loaded twice, once for amplifying from the gene of

interest and once for amplifying from the housekeeping gene *rp49* [14]. For each case, a CT value was calculated as the number of RTQPCR cycles to reach a particular amount of amplified product, characterized by a fluorescence intensity threshold of ~ 0.05, which was in the linear phase of the cycle number fluorescence intensity curves. Then, for each sample, a Delta CT was calculated by subtracting the CT value of the *rp49*-amplification from the CT value of the gene of interest-amplification. These Delta CT values were then plotted comparatively between mutants and controls for each sex (Fig. 3.7). To assess the fold changes in Table S24, I calculated for each genotype and sex the Median Delta CT values. If, e.g, the Median Delta CT of a mutant was 4 units larger than that of the control, this implied a $24 = 16$- fold decrease in the level of the respective mRNA in the mutant (Table S24).

Targeted gene	**Forward primer 5' to 3'**	**Reverse primer 5' to 3'**
FBgn0014417	TCCAAAAGGGATTCTTCACG	GATCCCTTCAACGAACTGGA
FBgn0032192	ATCGTTCCAATGACCAGGAC	ACTGCAACCTGTGGAGGAAC
FBgn0031689	GGAACCTCAATCGTTCAAGC	CGCCAAACCCAAAGAATAGA
FBgn0032733	CCAAACTCCGTAACCTGCAT	TGTCGCACTTGTCACAGTCA
FBgn0038873	TAGCCATCGATACGCAACTG	AACGACTGCTGCACCTTTCT
FBgn0034711	TTTCCACTACCAACGCATGA	ACCTTTTCACCGGAGTGATG
FBgn0040344	GTTCCCACACTCACCTTCGT	TGCAGTCAGGGAGGTACACA
FBgn0033130	GCCGCTGTATCCGAAAGATA	GCGCAGTAGAGTGCATCAGA
FBgn0031925	GTGGACTTCTCGCAGAAAGG	GAATAGGTCCACGCACGTTT
FBgn0000216	AGAACTCCCAGAACGAGCAA	TTAGAGACCGAATCCGTTGG
FBgn0038067	ACAGTGCTCACACGAGATGG	TGGTCCAGATTGCTGACAAA
FBgn0017551	TGGACGTACAACCAGCACAT	TCCTGACCTCGTCAAGATCC
FBgn0028919	AGCCCACAACTGGGACATAG	TAAGTGCCGTCCACTCACTG
FBgn0031935	GAGAACGGAAGGGACATGAA	TGCGACAATAGCTGAGGAAGT
FBgn0037862	CTGCACAGGCATGGCTACTA	TATGTGACCATGCGTTCGTT
FBgn0039620	CTTTGGCCAGCTTGATGATT	CATGGAGAACGTCAACATCG
FBgn0034728	AATTGAATCGCTGTCCATCC	ACCGGCGAGGAAAACTTTAT
FBgn0041702	CTGGAACCATCGAGCCTTAG	GAGTTGGTGGATGAGGAGGA
FBgn0038251	GGACGCCTAAATTGTGAGGA	GAGGCGTCGTTTGCAGATA
FBgn0002626	CGGATCGATATGCTAAGCTGT	GCGCTTGTTCGATCCGTA

Table 2.1: RTQPCR primer for tested electric shock avoidance candidate genes and the housekeeping control gene. The flybase identification number of the targeted gene is given in the first column, the DNA sequences of the forward and reverse primers are given from 5' to 3' end in the 2nd and 3rd column, respectively.

[14] http://www.roche-applied-science.com/sis/rtpcr/upl/index

Chapter 3

Results

3.1 Genome-wide analysis

I tested 38 inbred fly strains [21] in electric shock avoidance, avoidance of the odours BA and OCT and for punishment and relief learning (for detailed paradigms, see Fig. 3.1) to later on perform a Genome-wide association analysis between behavioural score and expression level and SNPs, respectively. Therefore these strains underwent a set of analysis steps (see Fig.3.2). First, I tested them in each behaviour to get behavioural scores for each strain. I found significant variation among strains for all behaviours tested, which is a requirement for further analysis (for details see subsections for each behaviour). Next, whole genome expression-levels for 18 900 probe-sets per strain and 1 387 514 SNPs [21, 208] were downloaded for each strain and pre-processed (to gain better readability, more details of the whole procedure can be found in Material and Methods). Briefly, to obtain the probe-set expression-levels that vary significantly between strains on the microarray, an ANOVA was performed and corrected for multiple testing using a False discovery rate (FDR) of 0.001 (allowing 1 in 1000 to be wrong). There were 10 121 probe-sets varying. After that, linear regression, with an FDR of 0.01, was used to look for significant associations between behavioural scores and expression-levels. Notably, for most existing transcripts, there was at least one probe-set on the microarray, but for one gene there could be a varying number of probe-sets. Association analysis was performed on the probe-set level but candidate gene analysis on the gene level. Linear regression was also used to look for significant SNP – behaviour associations. It should be noticed, that there was a different number of SNPs per gene, also in terms of that SNP being intronic, missense, synonymous, in an untranslated region (UTR), etc.. The analysis was performed on SNP level. To restrict the

number of candidate genes, a very stringent Bonferroni correction was used. The candidate gene lists were obtained by taking out inter-genic SNPs and further analysis was done on the gene level.

3.1.1 Electric shock avoidance

I tested 38 wild-derived inbred fruit fly strains in electric shock avoidance uncovering significant natural variation (Fig. 3.3, Kruskal-Wallis test: $H = 158.57, d.f. = 37$ and $P < 0.0001$), even if male and female avoidance scores are analyzed separately (Fig. 3.3 Kruskal-Wallis test: females $H = 145.0985, d.f. = 37$ and $P < 0.0001$, males $H = 107.597, d.f. = 37$ and $P < 0.0001$). This enabled testing for associations to gene expression levels and SNPs.

Now, an association analysis was performed, as described above, revealing 444 probe-sets and 3 183 SNPs that were significantly associated with electric shock avoidance (Table S1 and S6). These corresponded to a total of 1 515 candidate genes, 347 associated with expression and 1 104 with SNPs and 64 with both (for a full list, see Table S11, Fig. 3.4). The 64 overlapping genes contained significantly more SNPs in UTRs, suggesting a regulatory function for expression (Pearson Chi-square: $chi^2 = 14.86, df = 1, P = 0.0001$, with 27 UTR SNPs out of 173 overlapping SNPs and 256 UTR SNPs out of 3183 associated SNPs).

Functional enrichment among candidate genes

For an overview of the common functions of associated genes, the candidate gene list was probed for enrichment in Gene Ontology (GO) terms [15, 68]. Meaningfully, terms related to, e.g. neurotransmitter receptor activity, plasma membrane bound molecules, central nervous system- and muscle-development as well as locomotion were enriched (Hypergeometric test with multiple correction: $FDR < 0.05$; see Table S16 for a complete list). These represent many steps necessary to display electric shock avoidance.

As locomotion is an obvious pre-requisite for avoiding shock, a literature-based list of 140 fly locomotion-genes was manually compiled additionally (Table S21 and Material and Methods) and probed for an overlap to the shock avoidance candidates. Among the candidate genes, 33 had previously been implicated in locomotion (marked in Table S11), corresponding to a 2.5-fold enrichment as compared to the rest of the genome (Fig. 3.4, Hypergeometric test: $representation\ factor = 2.5, P < 0.0001$; for a list of all fly locomotion-affecting genes, see Table S21). This makes sense as locomotion is surely critical

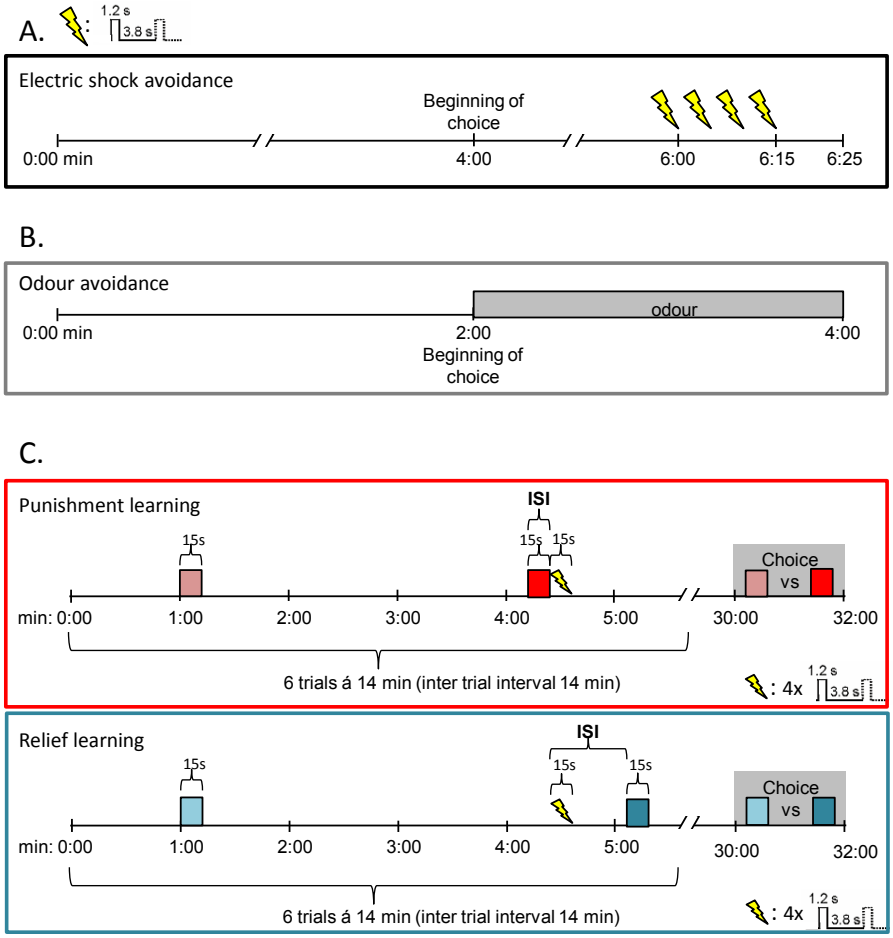


Figure 3.1: Behavioural Paradigms shown as a time line sketch. A. Electric shock avoidance treatment. Flies were put into the set-up at 0.00 minutes and had the choice between two tubes covered with copper wire at 4:00 min. After 2 min, one tube was electrified with 4 pulses, each lasting 1.2 s with a break of 3.8 sec. 10 s after the last shock, the door between the tubes was closed. B. Odour avoidance treatment. Flies entered the set-up as described in A. At 2:00 min, flies had the choice between a scented and an unscented (odour depicted as a grey bar) arm in a T-maze for two minutes. C. Punishment and relief learning treatment. Time line for punishment in red and relief in blue. Small quadrants represent the odourant stimulus and a lightning represents 4 electric shock pulses, each lasting 1.2 s with an inter shock interval of 5 s as shown in the lower right corner. Quadrants (odour A and odour B) in bright colours are for the control odour and in dark for the odour to be learned. Note, that for relief learning, everything stays the same as in punishment learning except the second odour is moved behind the electric shock. A.-C. Calculation of behavioural scores for each treatment is described in Material and Methods

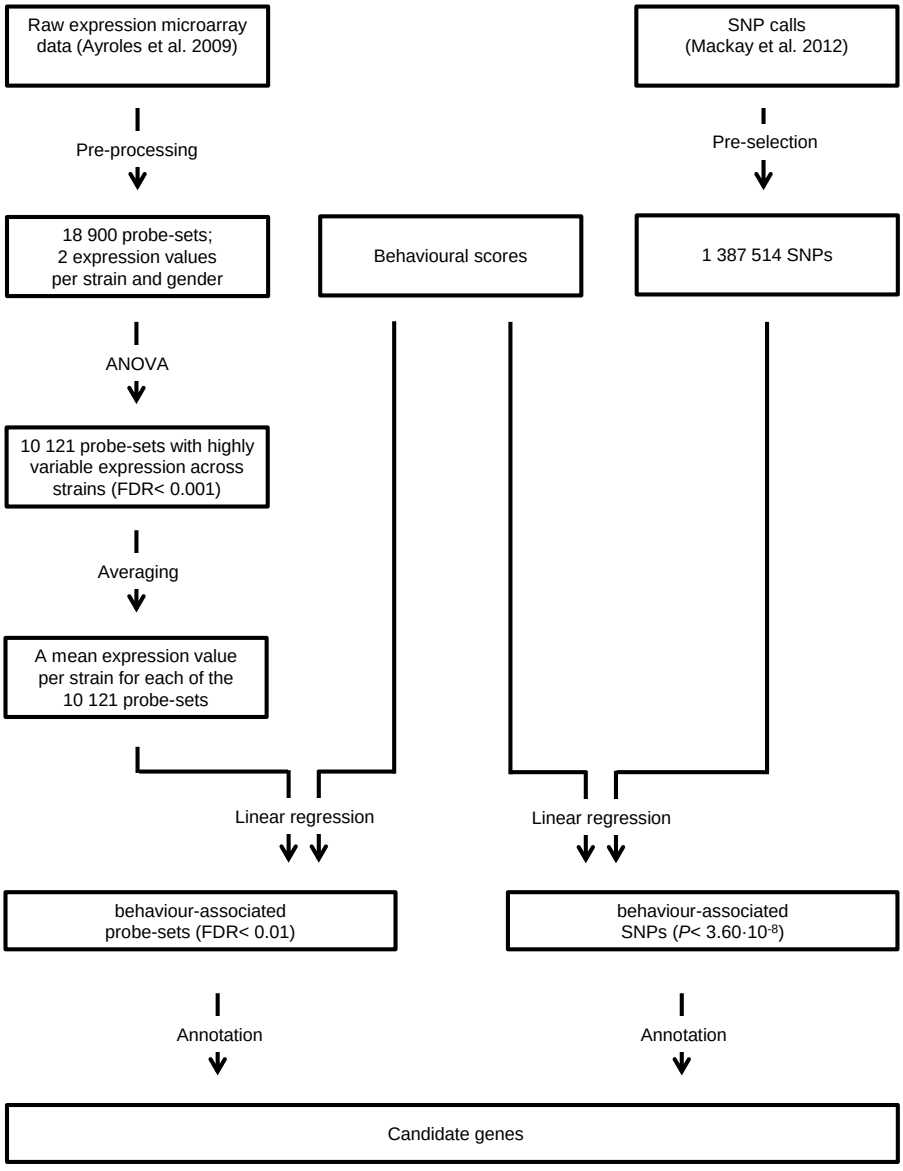
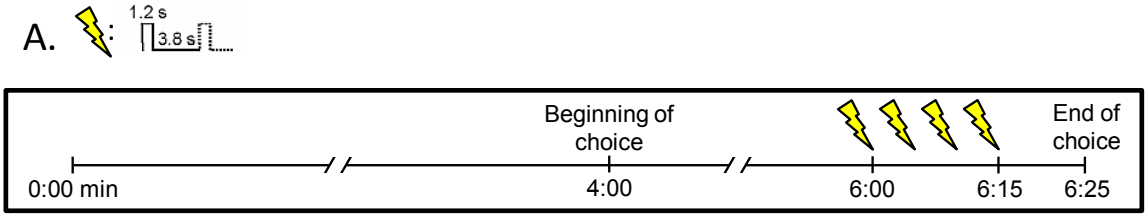


Figure 3.2: Different steps in the Genome-wide analysis. In the middle, behavioural tests with inbred strains are indicated which are later used for analysis shown on the left and right side, respectively. On the left, the steps and numerical results of the gene expression level - behaviour association analysis are sketched. On the right, the single nucleotide polymorphism (SNP) - behavioural association analysis is represented. For pre-processing, variance stabilization normalization (vsn) was used as described in Material and Methods. As a multiple correction method, FDR = False discovery rate was used on the left and Bonferroni on the right.

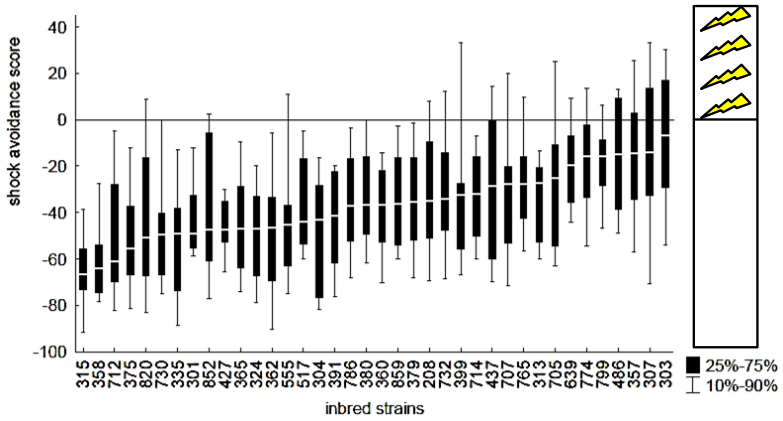
for avoiding shock. Furthermore, a manually compiled list of 50 nociception-genes (Table S21) was compared to the candidate gene list. 9 of the candidate genes had been reported to affect fruit fly nociceptive behaviour (marked in Table S11), corresponding to a 1.9- fold enrichment (Fig. 3.4, Hypergeometric test: $representation\ factor = 1.9, P = 0.043$; see Table S21 for a list of all fly nociceptive behaviour-related genes). Thus, shock likely recruits molecular networks of other noxious modalities, pointing to the value of the present candidate list in discovering novel nociception mechanisms. Also as *Drosophila* learning literature is dominated by electric shock reinforcement it is not surprising, that shock candidate genes were 1.9-fold enriched compared to a manually compiled list of 135 learning genes retrieved from literature, with 24 overlapping genes (3.4, Hypergeometric test: $representation\ factor = 1.9, P = 0.002$). To assess the translational potential of such discoveries, I analyzed shock avoidance candidates for orthology to human genes *SCN9A* and *NTRK1*, respective associates of the two main heritable pain insensitivity diseases. 16 of the candidate genes were orthologous to *NTRK1* (BLAST $E - value < 10 - 20$; marked in Table S11; no orthologs to *SCN9A* with this threshold were found among candidates), corresponding to an encouraging 4.6-fold enrichment as compared to the rest of the genome (Fig. 3.4, Hypergeometric test: $representation\ factor = 4.2, P < 0.0001$; Table S21 lists the 40 fly orthologs of *SCN9A* and *NTRK1* with the statistical threshold mentioned above).

Figure 3.3 *(following page)*: Natural variation in Electric shock avoidance A. Shows the time for treatments flies underwent in the electric shock avoidance assay. B.-D. The 38 tested inbred strains had significantly different shock avoidance scores for unisex (B.), male (C.) and female (D.) (Kruskal-Wallis test for each $P < 0.0001$). Box plots show the median as the midline, 25 and 75 % as the box boundaries and 10 and 90 % as the whiskers. Sample sizes were from left to right for B: N = 32, 16, 22, 24, 24, 16, 16, 24, 26, 28, 16, 24, 28, 34, 16, 32, 24, 22, 18, 18, 22, 32, 16, 16, 24, 16, 28, 18, 22, 16, 30, 16, 20, 24, 16, 20, 32, 24; for C: N = 23, 16, 16, 19, 21, 16, 18, 15, 14, 24, 34, 13, 25, 14, 26, 28, 17, 28, 23, 32, 23, 24, 20, 16, 15, 21, 32, 22, 24, 18, 29, 22, 16, 27, 32, 24, 21, 15; and for D: N = 16, 15, 23, 14, 24, 16, 25, 26, 18, 18, 22, 19, 32, 14, 16, 34, 16, 18, 24, 32, 24, 32, 24, 21, 16, 24, 16, 30, 16, 22, 14, 28, 28, 23, 24, 31, 20, 22.

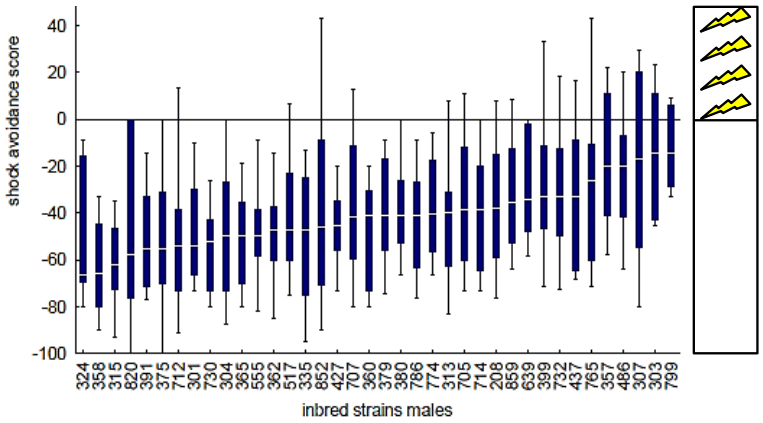
A.


1.2 s
3.8 s
Beginning of choice
End of choice
0:00 min
4:00
6:00
6:15
6:25

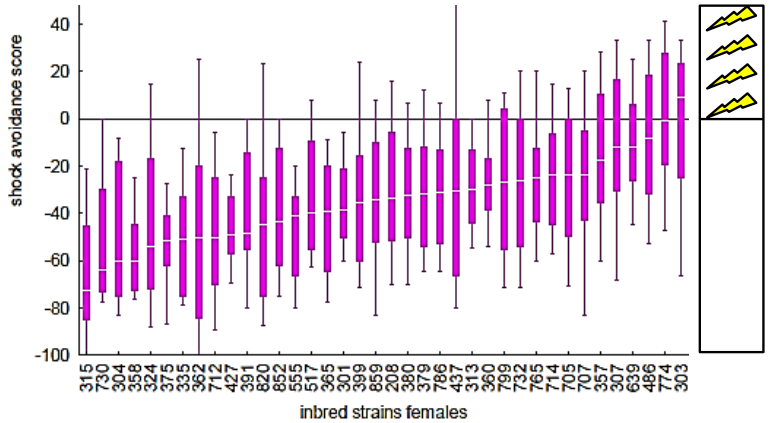
B.


shock avoidance score
40
20
0
-20
-40
-60
-80
-100
315 358 712 375 820 730 335 301 852 427 365 324 362 555 517 304 391 786 380 360 859 379 208 732 399 714 437 707 765 313 705 639 774 799 486 357 307 303
inbred strains
25%-75%
10%-90%

C.


shock avoidance score
40
20
0
-20
-40
-60
-80
-100
324 358 315 820 391 375 712 301 730 304 365 555 362 517 335 852 427 707 360 379 380 786 774 313 705 714 208 859 639 399 732 437 765 357 486 307 303 799
inbred strains males

D.


shock avoidance score
40
20
0
-20
-40
-60
-80
-100
315 730 304 358 324 375 335 362 712 427 391 820 852 555 517 365 301 399 859 208 380 379 786 437 313 360 799 732 765 714 705 707 357 307 639 486 774 303
inbred strains females

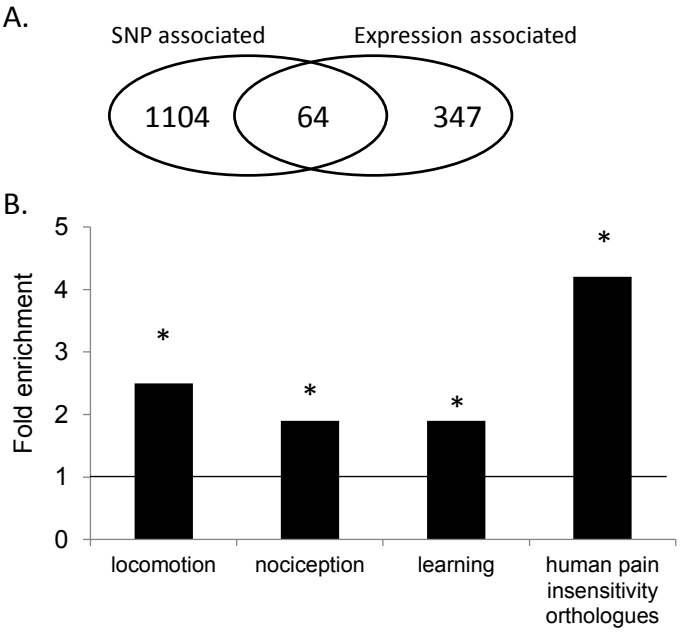


Figure 3.4: Candidate genes associated with electric shock avoidance. A. Number of candidate genes associated with SNPs and electric shock avoidance on the left and expression and electric shock avoidance on the right and overlapping candidates in the middle. B. Enrichment among electric shock avoidance candidates compared to locomotion, nociception, learning gene lists manually compiled through literature search and orthologs to human pain insensitivity disease genes as described in Material and Methods. * indicates $P < 0.05$ in a Hypergeometric test and the black line the expected amount of overlap between compared gene lists.

Gene-interaction network of candidate genes

In order to explore the functional interactions between the candidate genes, a large, experimental evidence-based fruit fly protein-protein interaction network[1] was used. 'Pruning' this network according to the gene expression level-shock avoidance associations (please see the Material and Methods for details) revealed a smaller shock avoidance-relevant network of 52 genes and 63 interactions (Fig. 3.5 and Table S23). As an emergent property, the genes that were positively associated with shock avoidance (i.e. the higher the expression level, the better the shock avoidance scores; Fig. 3.5, shades of green) and those that were negatively associated with shock avoidance (Fig. 3.5, shades of red) clustered to some extent separately. For 30 of the network genes, FlyBase listed phenotypic effects of genetic interference[2]. Although most of these had pleiotropic roles, including those essential for survival, a common theme seemed to be the effect on the number and morphology of bristles (14 genes, encircled blue in Fig. 3.5; see Table S11 for references). Bristles are external sense organs distributed throughout the flys body surface, inviting the speculation that shock sensation may be mediated by sensory neurons harboured therein. Furthermore, the bristle-related genes were not dispersed throughout the network, but were mostly inter-connected; thus, particular genes with several bristle-related interaction partners (e.g. FBgn0025716) may now be attractive for probing for a role in bristle-function.

Independent, unbiased validation of candidate genes

Next, for an initial independent validation, I picked 19 candidate genes, which were associated with shock avoidance in terms of expression level (Table S24). In this selection, I disregarded the genes known functions; thus, the main restricting criterion for choice was the availability of appropriate homozygous-viable P-element insertion mutants with co-isogenic controls [31], [322], [30] (please see Table S24 for details of the used strains). For 10 candidate genes, I found a significant difference in scores between the respective P-element insertion mutant and its control (Fig. 3.6, first two rows: Mann-Whitney U-tests: $FDR < 0.05$; for a full statistical report, please see Table S24). For the genes *Hexim, rad50, CG15107* and *CG3711*, also the respective mRNAs were dramatically down-regulated in the mutant as revealed by real time quantitative PCR (see Fig. 3.7 FBgn0038251, FBgn0034728, FBgn0041702 and FBgn0040344; see also Table S24). This agreed well with the respective behavioural impairment, because these four genes were associated with shock avoidance

[1] www.flybase.org
[2] www.flybase.org

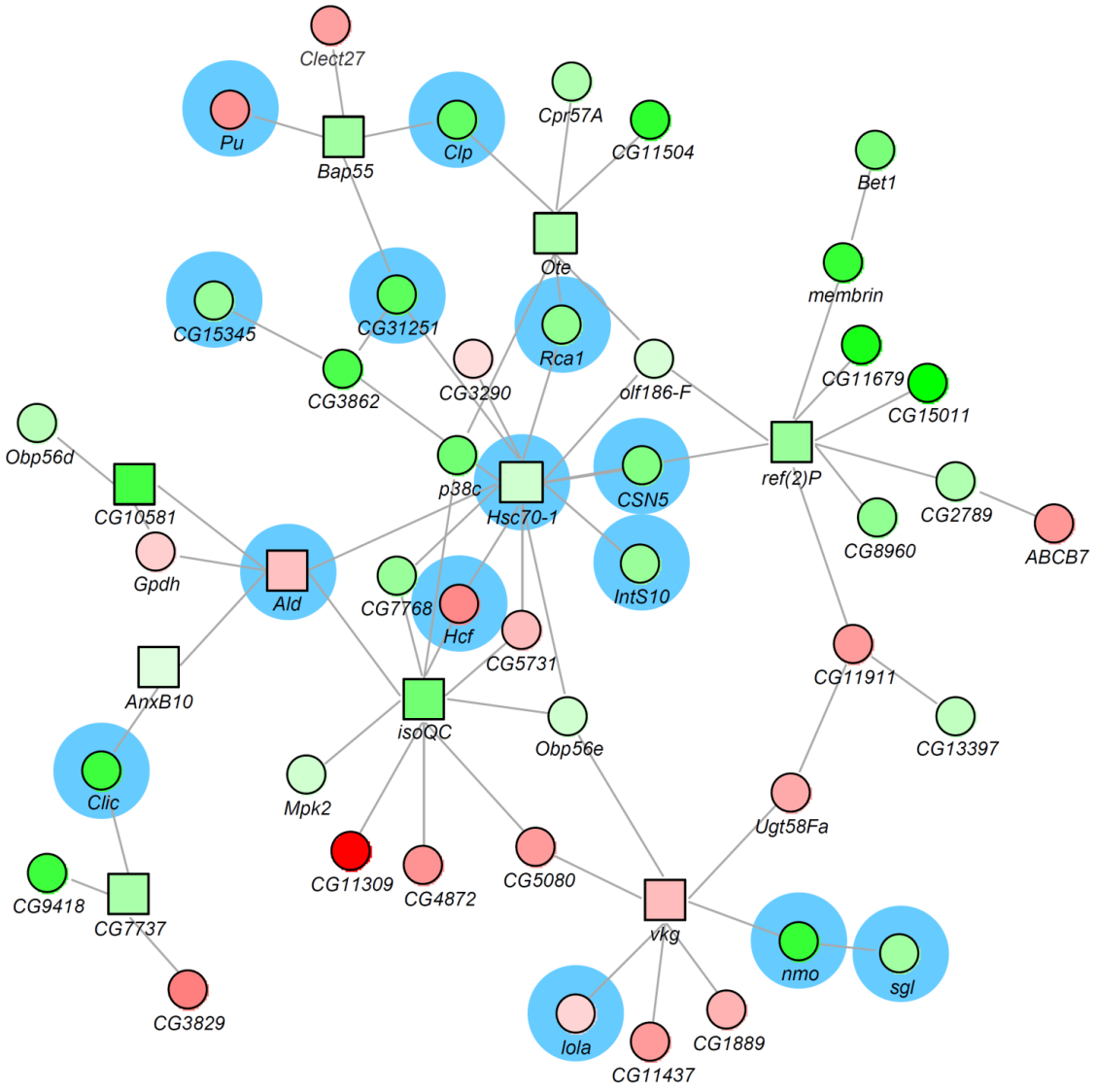


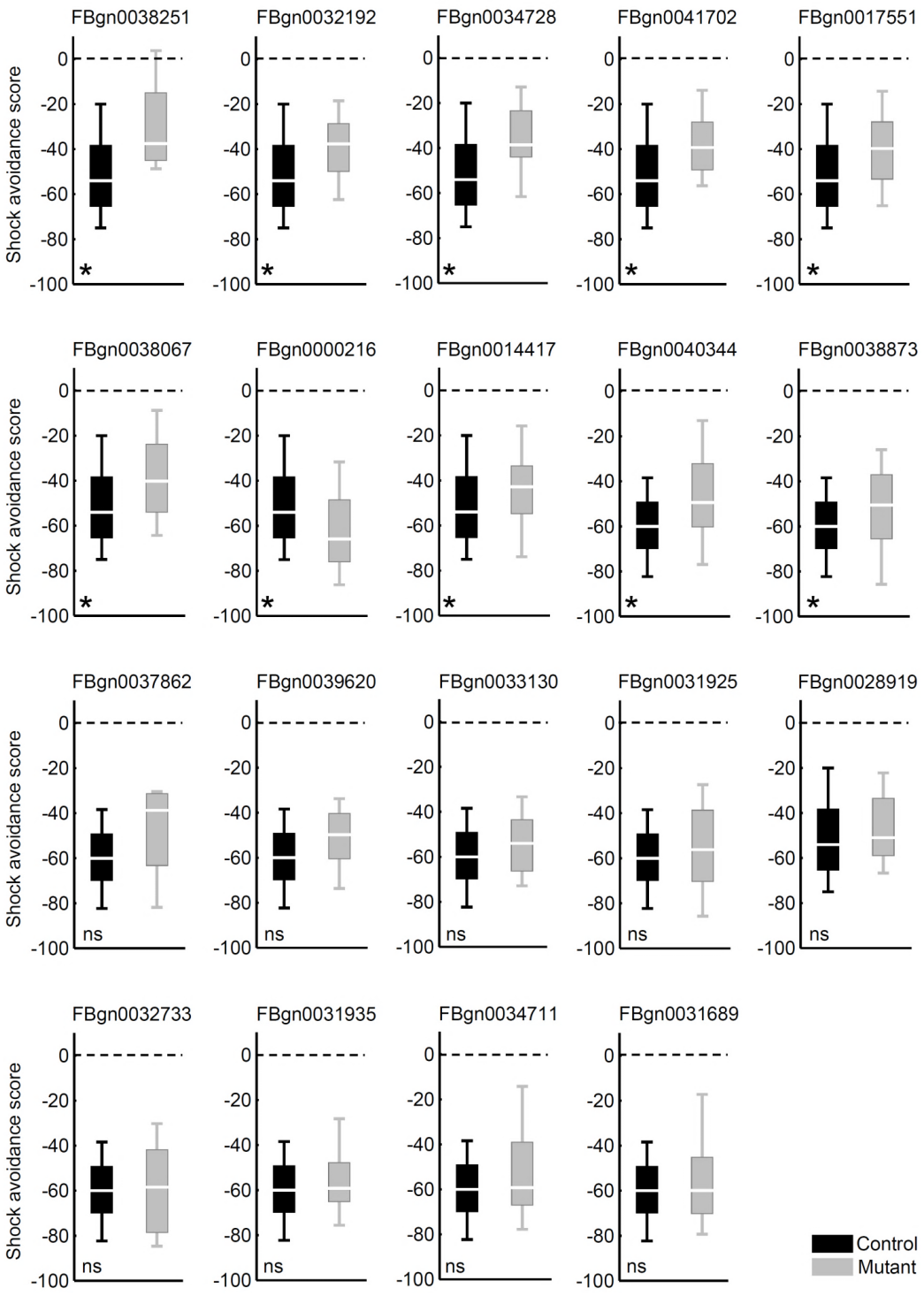
Figure 3.5: A shock avoidance associated gene interaction network. Each of the 52 nodes in the network represents a gene associated with shock avoidance in terms of expression level. Each edge indicates a pair-wise physical interaction between the proteins encoded by the respective genes, based on independent empirical evidence. Shades of green mean that the higher the respective genes expression level was, the stronger was the shock avoidance; shades of red mean vice versa (i.e. the higher the expression level, the weaker shock avoidance). The darker the shading, the larger the respective estimated effect of expression level on shock avoidance. Circles represent genes with statistically very strong association to shock avoidance (network-score $FDR < 0.001$). Those genes with less strong associations (network-score $FDR > 0.001$; represented by squares) were included for being critical to form connections between the other more strongly associated ones. Those genes implicated in bristle-function are haloed blue.

such that the higher their expression levels were, the stronger were the behavioural scores. For the remaining 6 genes I found no evidence for a change in the respective mRNA level due to the P-element insertion (Fig. 3.7, Table S24), thus one cannot compare the direction of these genes associations to shock avoidance with the direction of the respective mutant-effects on shock avoidance. For the remaining 9 candidate genes, the respective P-element insertions had no formally significant effect on the shock avoidance scores (Fig. 4, last two rows: Mann-Whitney U-tests: $FDR > 0.05$; also see Table S24), although, for the *CG4706* and *CG1443* the mutants tended to avoid shock less strongly than the controls (FBgn0037862 and FBgn0039620, Mann-Whitney U-tests: $FDR = 0.06$ and 0.1 respectively; also see Table S24). But there was no effect on the mRNA level whereas for 3 of the others, there was (see Fig. 3.7 and Table S24).

Biased validation of candidate genes

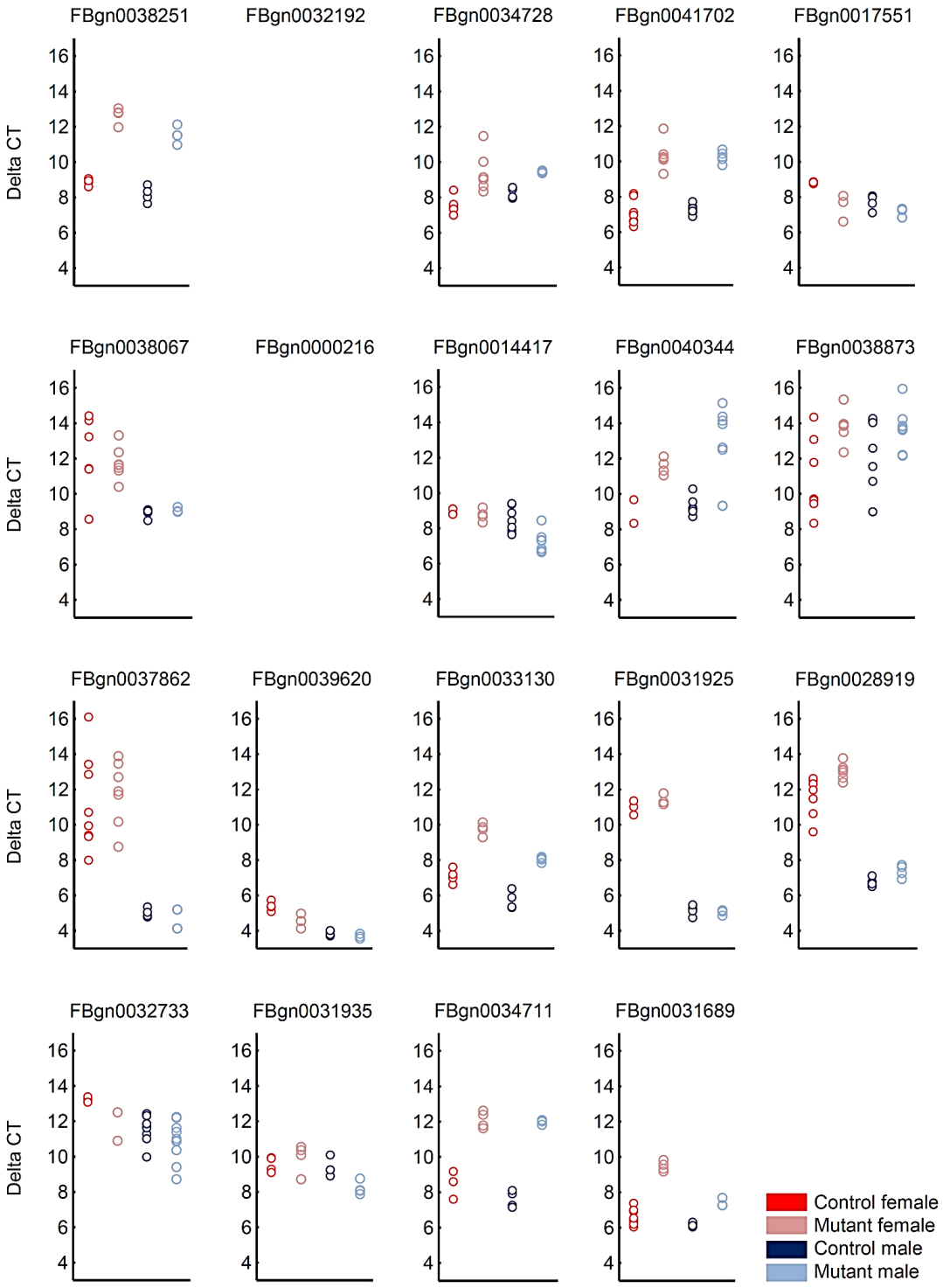
All analyses reported so far were unbiased for known function and thus somewhat exploratory. Therefore, the candidate gene list was exploited also with a hypothesis-driven approach. The enrichment of genes implicated in nociceptive behaviour (see above) suggested that shock may share molecular targets with other nociceptive modalities. To further explore in this direction, the candidate list was extended to 4 794 genes by loosening our statistical significance thresholds to $FDR < 0.10$ for the gene expression level-associations and to $P < 10 - 5$ for SNP-associations. This extended list contained 25 genes implicated in nociceptive behaviour (listed in Table S22), corresponding to a $\sim$ 2-fold enrichment (Pearson Chi-square test: $\chi^2 = 9.60, P = 0.002$). Among these genes was the well-studied, multimodal noci-sensor *Transient receptor potential A1* (*dTrpA1*) [338, 268, 269, 122, 181, 160, 165, 179, 233, 159, 372, 318, 343]. In order to independently

Figure 3.6 *(following page)*: Each panel shows, for a selected candidate gene, the shock avoidance scores of an appropriate P-element insertion mutant vs. those of a co-isogenic control. In 10 out of 19 cases (top two rows) I found a significant difference in shock avoidance between the genotypes. Box plots show the median as the midline, 25 and 75 % as the box boundaries and 10 and 90 % as the whiskers. Sample sizes were from left to right for black boxes: 90-102, for grey boxes: N = 16, 50, 20, 16, 50, 54, 70, 46, 48, 56, 16, 16, 32, 34, 64, 34, 18, 54, 34. For each panel, a Mann-Whitney U-test was used to compare the control- and the mutant-scores. The respective False Discovery Rates (FDRs) were then calculated over all 19 tests. *: $FDR < 0.05$, ns: $FDR > 0.05$. Please see Table S24 for details.


FBgn0038251
FBgn0032192
FBgn0034728
FBgn0041702
FBgn0017551
FBgn0038067
FBgn0000216
FBgn0014417
FBgn0040344
FBgn0038873
FBgn0037862
FBgn0039620
FBgn0033130
FBgn0031925
FBgn0028919
FBgn0032733
FBgn0031935
FBgn0034711
FBgn0031689
Shock avoidance score
0
-20
-40
-60
-80
-100
*
ns
Control
Mutant

test for a role of *dTrpA1* in shock avoidance, two independent loss-of-function mutants were used. Both of these avoided shock significantly less than the controls (Fig. 3.8 Mann-Whitney U-tests: $dTrpA1^{ins}$ vs. CS: $U = 530.00$, $P < 0.05/3$; *dTrpA1[1]* vs. CS: $U = 319.50$, $P < 0.05/3$; *dTrpA1[1]* vs. w^{1118}: $U = 148.50$, $P < 0.05/3$; One sample sign tests for $dTrpA1^{ins}$ and *dTrpA1[1]*: $P < 0.05/4$). Importantly, neither mutant was significantly impaired in a locomotion assay that used the same experimental setup (Fig. 3.8, Mann-Whitney U-tests: $dTrpA1^{ins}$ vs. CS: $U = 35.00, P = 0.04$; *dTrpA1[1]* vs. CS: $U = 66.00, P = 0.75$; but see the tendency in $dTrpA1^{ins}$), suggesting that the impairment in shock avoidance was not secondary to a locomotion defect. To better assess the relative contribution of dTRPA1 to shock avoidance, the parameters of shock were varied (Fig. 5C). *dTrpA1* mutants seemed to lack around one third of the full avoidance when 4 or 12 pulses of 100 V were used (Fig. 5C: Mann-Whitney U-tests: 4 pulses of 100V: $dTrpA1^{ins}$ vs. CS: U= 367.00, $P < 0.05/8$; *dTrpA1[1]* vs. CS: $U = 293.50, P < 0.05/8$; 12 pulses of 100V: $dTrpA1^{ins}$ vs. CS: $U = 40.00, P < 0.05/8$; *dTrpA1[1]* vs. CS: $U = 33.00, P < 0.05/8$). Given that these mutants were both strong loss-of-function cases, the remaining shock avoidance was likely due to dTRPA1-independent mechanisms. Interestingly, both dTrpA1 mutants avoided a single pulse of 100 V or 4 pulses of 50 V comparably to the control (Fig.5: Mann Whitney U-tests: 1 pulse of 100V: $dTrpA1^{ins}$

Figure 3.7 *(following page)*: This figure presents, with the same order as in Fig. 3.6, the effects of the P-element insertions on the respective mRNA levels, as measured by RTQPCR. For each case, the difference in expression from the control gene *rp49*, represented as Delta CT value, is compared between the mutant and the control for each sex in a scatter plot. E.g. as in the case of FBgn0038251 females, if the control had an average Delta CT of 9, while the mutant had 13, this indicates that the mutant mRNA level was $2(9 - 13) = 1/16th$ of the control. Thus, the mRNA levels of FBgn0038251, FBgn0034728, FBgn0041702 and FBgn0040344 were clearly decreased in the respective mutants, accompanying the impairment in shock avoidance (Fig. 3.6). For FBgn0017551, FBgn0038067, FBgn0014417 and FBgn0038873, the mutants defective shock avoidance (Fig. 3.6) was not paralleled by an effect on the mRNA levels. As for FBgn0032192 and FBgn0000216, for which I found an effect of the P-element on shock avoidance (Fig. 3.6), the RTQPCR measurements turned out unfeasible due to low expression levels (modENCODE Temporal Expression Data [www.flybase.org]). For FBgn0033130, FBgn0034711 and FBgn0031689, RTQPCR revealed a clear decrease of the respective mRNA level in the mutant, although shock avoidance was comparable to control (Fig. 3.6). For the remaining cases, the P-element insertion seemed to affect neither the respective mRNA levels, nor the shock avoidance scores (Fig. 3.6). For further details, please see Table 1, Table S24 and Material and Methods.


FBgn0038251
FBgn0032192
FBgn0034728
FBgn0041702
FBgn0017551
FBgn0038067
FBgn0000216
FBgn0014417
FBgn0040344
FBgn0038873
FBgn0037862
FBgn0039620
FBgn0033130
FBgn0031925
FBgn0028919
FBgn0032733
FBgn0031935
FBgn0034711
FBgn0031689
Delta CT
16
14
12
10
8
6
4
Control female
Mutant female
Control male
Mutant male

vs. CS: $U = 116.50, P = 0.68$; *dTrpA1[1]* vs. CS: U= 127.50, P= 0.98; 4 pulses of 50V: $dTrpA1^{ins}$ vs. CS: $U = 117.50, P = 0.71$; *dTrpA1[1]* vs. CS: $U = 108.00, P = 0.46$). Thus, these shock levels were perhaps too moderate to recruit dTrpA1. Given the strong disruption of the *dTrpA1* gene in the used mutants, the intact avoidance was likely independent of *dTrpA1*. To summarize, *dTrpA1* significantly contributes to shock avoidance, but other, independent mechanisms also exist.

In addition to *dTrpA1*, I found *Neuropeptide F* in the candidate gene list (see Table S11). Neuropeptide F and it's receptor Neuropeptide F receptor 1 *dNpfR1* are implicated in several stress responses in fly, also in heat nociception (reviewed in [230]). Using electric shock avoidance as a stress response, I tested the available P-element insertion line for *Neuropeptide F receptor 1* which has already been successfully used by [176]. I found significantly weaker shock avoidance scores in the *dNpfR1* P-element insertion strain compared to its co-isogenic control (Fig. 3.9, Mann Whitney U-test: $U = 145, P = 0.0081$). Moreover, the *dNpfR1* P-element insertion strain showed no deficit in locomotion in comparison to the control (Mann-Whitney U-test: $U = 114, P = 0.6156$). This result for the first time suggests that a similar mechanism to that for natural stressful stimuli is activated through electric shock.

3.1.2 Odour avoidance

Testing 38 wild-derived inbred fruit fly strains in avoidance to the odourants benzaldehyde (BA) and 3-octanol (OCT), respectively (see Fig. 3.10 and 3.11), I found that odour avoidance scores of these strains differ significantly from each other (Fig. 3.10 and 3.11: Kruskal-Wallis test: OCT $H = 100.1089, d.f. = 37$, $P < 0.0001$, BA $H = 104.6034, d.f. = 37$, $P < 0.0001$). Also, if male and female avoidance scores are analyzed separately, strains differ significantly from each other (Fig. 3.11 and 3.10, Kruskal-Wallis test: females OCT $H = 82.3614, d.f. = 37$, $P < 0.0001$ BA $H = 94.9500, d.f. = 37$, $P < 0.0001$, males OCT $H = 86.9572, d.f. = 37$, $P < 0.0001$, BA $H = 104.6034, d.f. = 37$, $P < 0.0001$).

Afterwards, an association analysis was performed, as described above, revealing 85 probe-sets significantly associated with BA avoidance and 109 with OCT avoidance (for details, please see above and Materials and Methods, see Table S2 and S3). Additionally, 4 268 SNPs were found to be significantly associated with BA avoidance and 569 with OCT avoidance (see Table S7a, S7b and S8). Subsequent analysis steps pool these into a total number of 1 009 candidate genes for BA avoidance and 349 for OCT avoidance; 69 genes were solely associated with expression and BA and 98 with OCT avoidance; 931 genes were

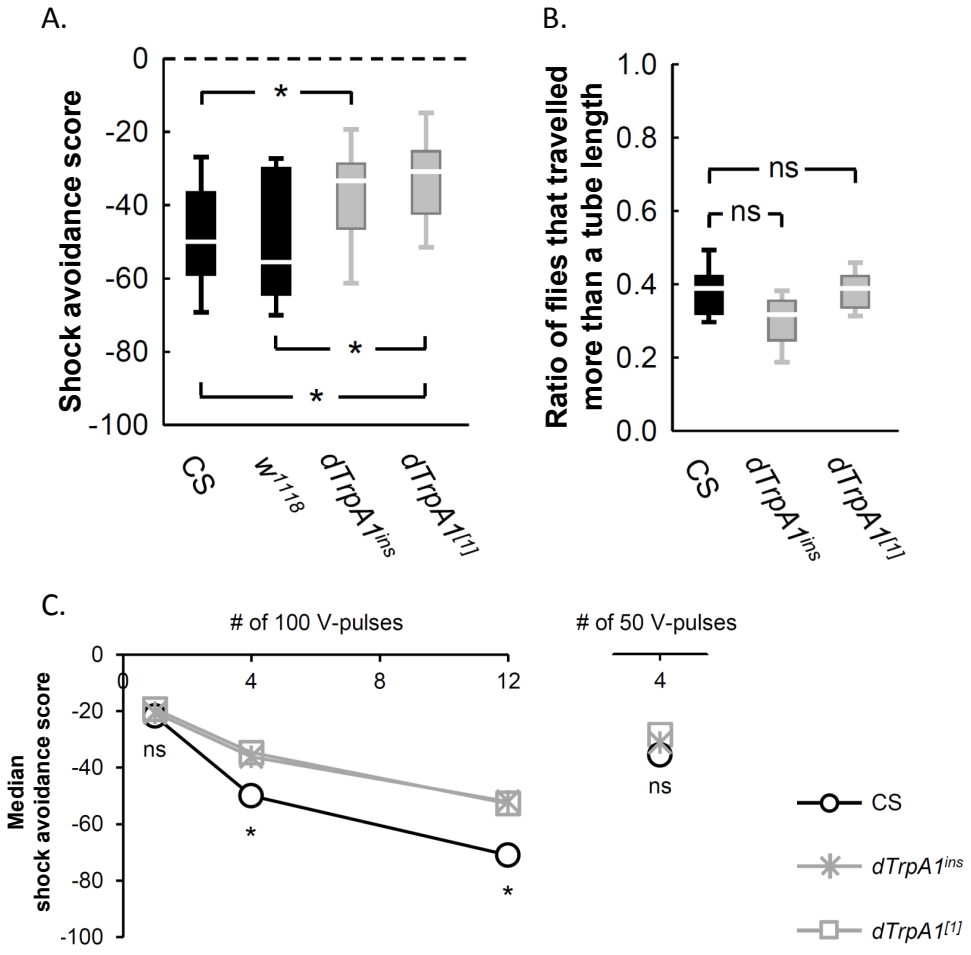


Figure 3.8: A. Two different *dTrpA1* loss-of-function mutants, $dTrpA1^{ins}$ and *dTrpA1[1]* had significantly weaker shock avoidance scores than the controls CS and w^{1118}. Box plots as explained in Fig. 3.3 *: $P < 0.05/3$ in Mann Whitney U-tests. Sample sizes were from left to right N= 56, 24, 32, 24. B. Both $dTrpA1^{ins}$ and *dTrpA1[1]* performed comparably to the CS control in a locomotion assay, quantifying the ratio of flies travelling one shock-tube length within 25 s in the absence of shock. Box plots show the median as the midline, 25 and 75 % as the box boundaries and 10 and 90 % as the whiskers. ns: $P > 0.05/2$ in Mann-Whitney U-tests. Sample size was N= 12, each. C. When 1 pulse of 100 V or 4 pulses of 50 V were used, both $dTrpA1^{ins}$ and *dTrpA1[1]* mutants showed comparable avoidance to the CS control. Using 4 or 12 pulses of 100 V on the other hand revealed the partial shock avoidance impairment. Median shock avoidance scores are plotted. *: $P < 0.05/8$, ns: $P > 0.05/8$ and in Mann-Whitney U-tests. Sample sizes were from left to right for CS: N= 16, 40, 16, 16, for $dTrpA1^{ins}$: N= 16, 30, 16, 16 and for *dTrpA1[1]*: N= 16, 16, 16, 16.

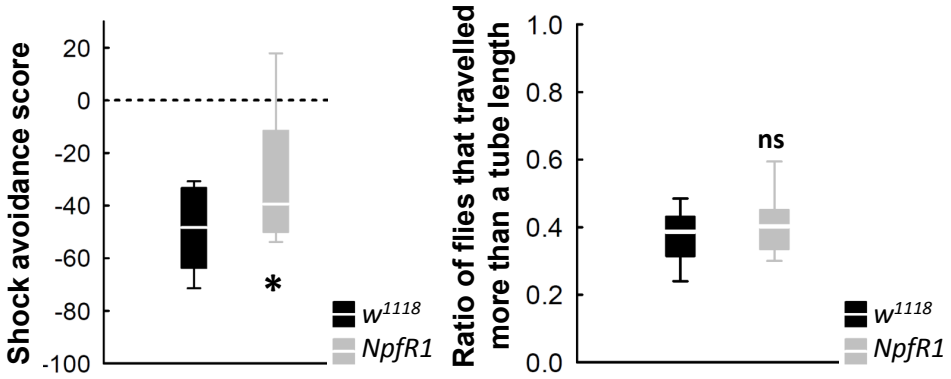


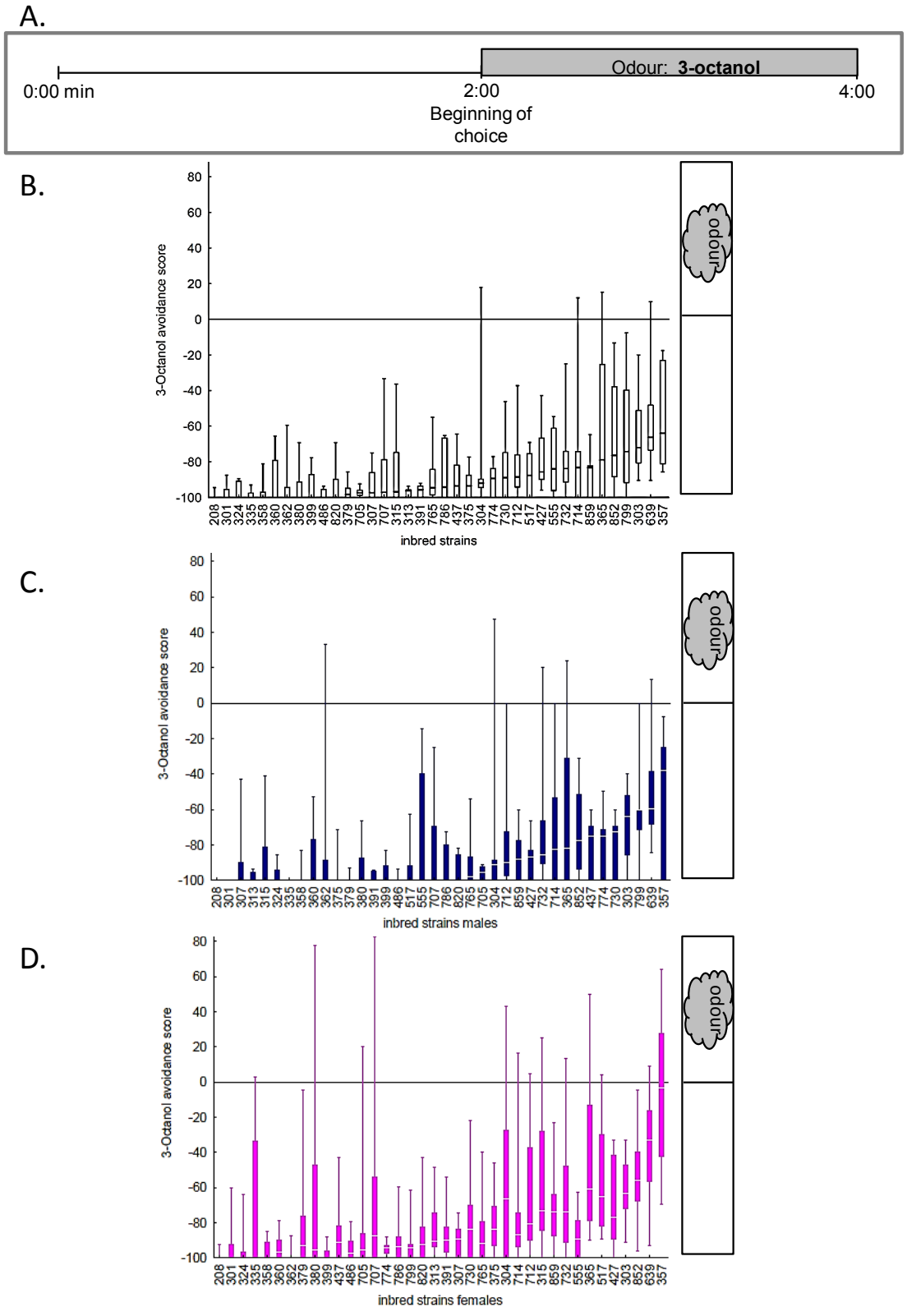
Figure 3.9: *dNpfR1* affects electric shock avoidance. On the left, a P-element insertion upstream of *dNpfR1*, shown in grey, had significantly weaker shock avoidance scores than the co-isogenic control w^{1118} shown in black. Box plots show the median as the midline, 25 and 75 % as the box boundaries and 10 and 90 % as the whiskers. *: $P < 0.05$ in Mann Whitney U-tests. Sample sizes were from left to right N = 22, 24. On the right, the P-element insertion mutant *dNpfR1* performed similarly to the co-isogenic control w^{1118} in a locomotion assay, quantifying the ratio of flies travelling one shock-tube length within 25 s in the absence of shock. Box plots as explained above ns: $P > 0.05$ in Mann-Whitney U-tests. Sample size was N = 16 each.

solely associated with SNPs and BA and 246 with OCT avoidance. 9 genes were in the overlap between expression and SNP associations for BA avoidance and 5 in the overlap for OCT avoidance (see Table S12 and S13 for complete candidate gene lists, Fig. 3.12 and 3.13). Furthermore, there was a significant overlap of 50 candidate genes between BA and OCT avoidance (Hypergeometric test: $representation\ factor = 2.3, P < 0.0001$).

Enrichment analysis

Gene-ontology enrichment analysis for BA avoidance with DAVID ([68], $FDR < 0.05$) resulted in enrichment of the Biological process categories muscle organ development, signal transducer activity, neurogenesis and adult behaviour, the Cell cycle category plasma

Figure 3.10 *(following page)*: Natural variation in 3-octanol avoidance. A. 3-octanol avoidance treatment as described in Fig. 3.1. B.-D. The 38 tested inbred strains had significant variation in avoidance to the pure odour 3-octanol (OCT) for unisex (B.), male (C.) and female (D.) (Kruskal-Wallis test for each $P < 0.0001$). Box plots show the median as the midline, 25 and 75 % as the box boundaries and 10 and 90 % as the whiskers. Sample sizes were for B. $N = 8 - 10$, C. $N = 5 - 10$ D. $N = 3 - 10$


A.
Odour: 3-octanol
0:00 min
2:00
Beginning of choice
4:00
B.
3-Octanol avoidance score
inbred strains
odour
C.
3-Octanol avoidance score
inbred strains males
odour
D.
3-Octanol avoidance score
inbred strains females
odour

membrane and the Molecular function categories neurotransmitter binding and neurotransmitter receptor activity. These categories make sense because they represent different steps from odour recognition towards odour avoidance. Interestingly, among the receptors found, were two odorant receptors (Or2a and Or9a) and two Gustatory receptors (Gr57a, Gr5a). There were also 3 odorant binding proteins (obp18a, obp19c, obp8a) and 4 ionotropic receptors (Ir92a, Ir94f, Ir11a, Ir7b). The OBPs found, were previously reported to affect BA avoidance [312]. Or2a is activated mildly by a certain BA concentration during Calcium imaging [3].

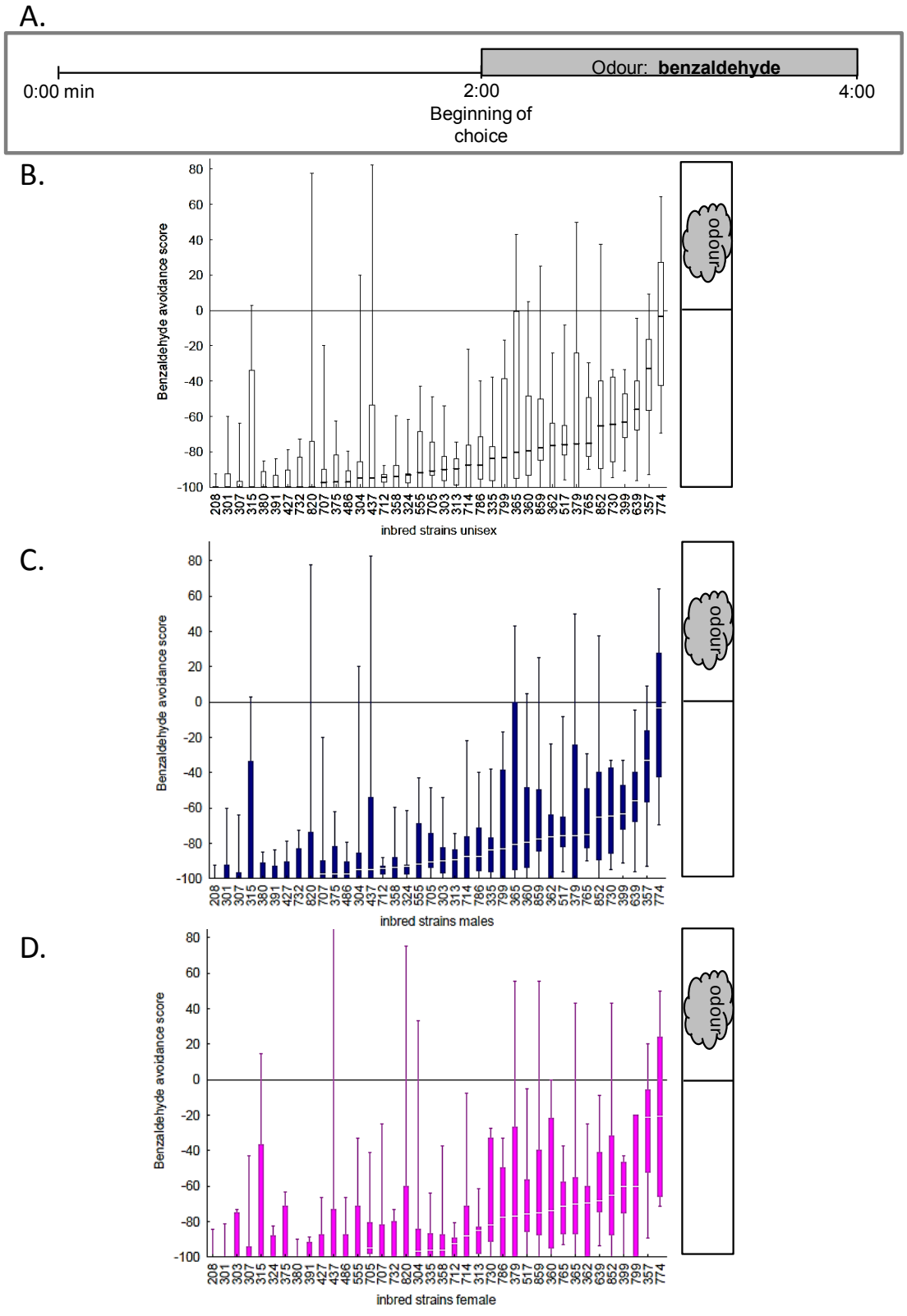
For OCT avoidance, there were only 3 categories significantly enriched and all of them had to do with cell differentiation. In general, there were two gustatory and two ionotropic receptors associated, Gr57a, Gr58a, Ir41a and Ir67c, none of them being reported for 3-octanol so far.

In addition, I compared both of my candidate gene lists to another for BA avoidance that used a larger DGRP collection and solely associated SNPs with BA avoidance [313] (multiple comparison threshold of this study $P < 10^{-5}$). It has to be kept in mind, that this other list used a different assay with another concentration and time for choice and showed no SNPs associated significantly with Bonferroni correction. 24 candidates were overlapping between the two lists, resulting in a significant overlap ($representation\ factor = 2.2, P = 0.0003$). Among the 24 overlapping candidate genes are three known learning genes, *dunce*, *white* and *Fas2* (see Table S12). In addition, *frizzled* is in the overlap. It plays an important role in axon guidance, especially in sensory organs and the MB, where odour information is transmitted [374].

Furthermore, candidate genes for BA and OCT avoidance were compared to the literature based lists for locomotion, nociception and learning, as reported in electric shock avoidance. Both lists were significantly enriched for locomotion and learning genes (Fig. 3.12 and 3.13, Hypergeometric test: locomotion-BA: 2.8 fold, $representation\ factor = 2.8, P < 0.0001$; locomotion-OCT: 3.3 fold, $representation\ factor = 3.3, P = 0.001$;

[3] http://neuro.uni-konstanz.de/DoOR/default.html

Figure 3.11 *(following page)*: Natural variation in 3-octanol avoidance. A. 3-octanol avoidance treatment as described in Fig. 3.1. B.-D. The 38 tested inbred strains had significant variation in avoidance to the pure odour benzaldehyde (BA) for unisex (B.), male (C.) and female (D.) (Kruskal-Wallis test for each $P < 0.0001$). Box plots show the median as the midline, 25 and 75 % as the box boundaries and 10 and 90 % as the whiskers. Sample sizes were for A. $N = 8 - 10$, B. $N = 5 - 10$, C. $N = 4 - 10$


A.
Odour: benzaldehyde
0:00 min
2:00
Beginning of choice
4:00
B.
Benzaldehyde avoidance score
odour
inbred strains unisex
C.
Benzaldehyde avoidance score
odour
inbred strains males
D.
Benzaldehyde avoidance score
odour
inbred strains female

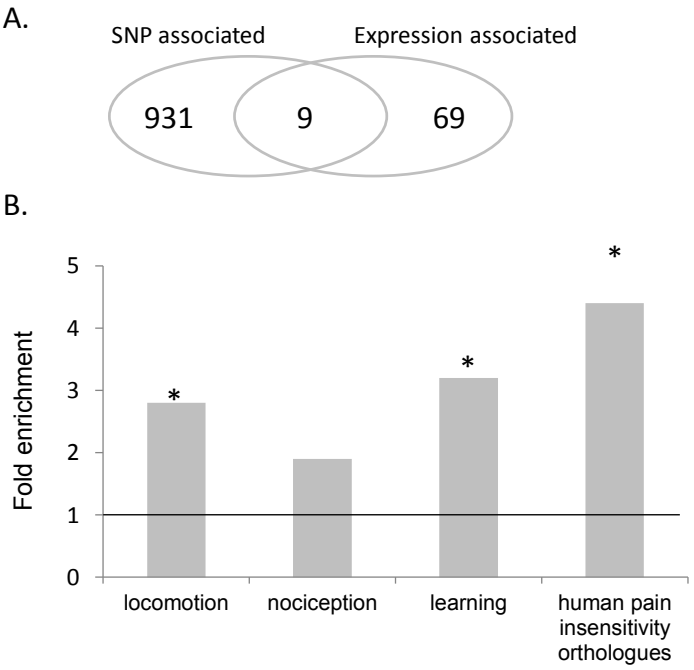


Figure 3.12: Candidate genes associated with benzaldehyde avoidance. A. Number of candidate genes associated with SNPs and benzaldehyde avoidance on the left and expression and benzaldehyde avoidance on the right and overlapping candidates in the middle. B. Enrichment among benzaldehyde avoidance candidates compared to locomotion, nociception, learning gene lists manually compiled through literature search and orthologs to human pain insensitivity disease genes as described in Material and Methods. * indicates $P < 0.05$ in a Hypergeometric test and the black line the expected amount of overlap between compared gene lists.

learning-BA: 3.2 fold, $representation\ factor = 3.2, P < 0.0001$; learning-OCT: 3.1 fold, $representation\ factor = 3.1, P = 0.003$), arguably because locomotion is necessary and odour avoidance plays an important role in learning and olfactory learning is over-represented in *Drosophila* learning literature. None of the lists was enriched in nociception genes, which speaks for relative specificity of the lists. Interestingly, BA avoidance candidates were enriched for the human pain insensitivity disease orthologues described in electric shock avoidance above (Fig. 3.13, Hypergeometric test: 4.4 fold, $representation\ factor = 4.4, P < 0.0001$, OCT avoidance candidates show a tendency for enrichment: 3.4 fold, $representation\ factor = 3.4, P = 0.056$).

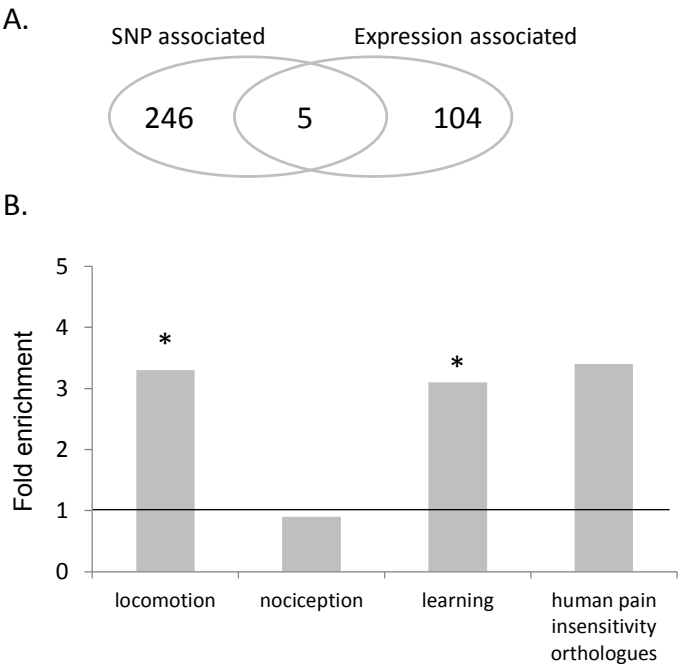


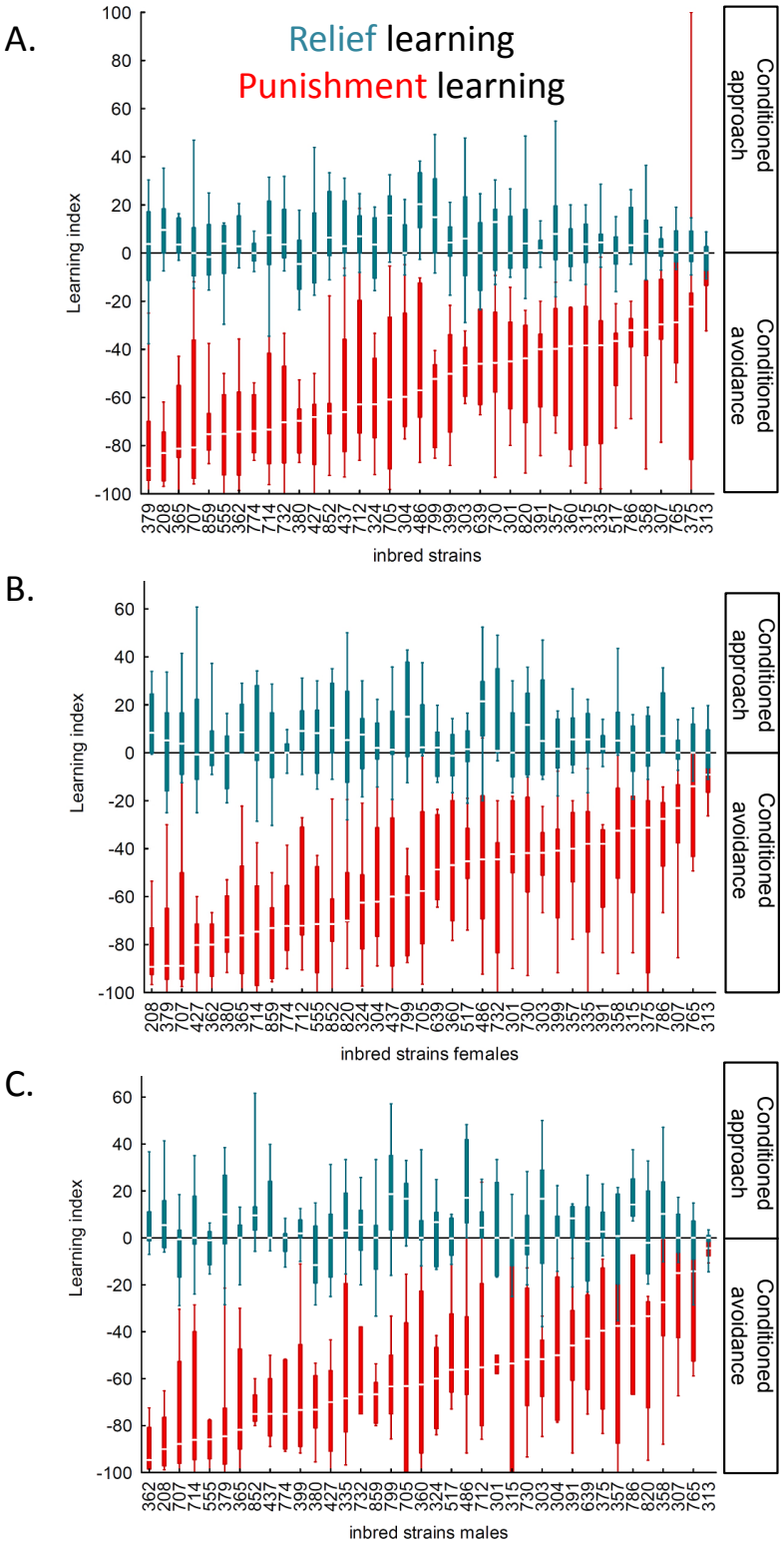
Figure 3.13: Candidate genes associated with 3-octanol avoidance. A. Number of candidate genes associated with SNPs and 3-octanol avoidance on the left, expression and 3-octanol avoidance on the right and overlapping candidates in the middle. B. Enrichment among 3-octanol avoidance candidates compared to locomotion, nociception, learning gene lists manually compiled through literature search and orthologs to human pain insensitivity disease genes as described in Material and Methods. * indicates $P < 0.05$ in a Hypergeometric test and the black line the expected amount of overlap between compared gene lists.

3.1.3 Punishment and relief learning

Using the assays for punishment and relief learning described above, I tested 38 wild-derived inbred fruit fly strains. This revealed significant natural variation among strains for punishment learning and relief learning alike (Fig. 3.14 Kruskal-Wallis test: punishment $H = 109.1823, d.f. = 37,\ P < 0.0001$, relief $H = 65.1856, d.f. = 37,\ P = 0.0029$). This is not always the case if male and female LIs are analyzed separately, i.e. for relief learning in females no significant natural variation could be observed (Fig.3.14, Kruskal-Wallis test: punishment females $H = 110.7393, d.f. = 37,\ P < 0.0001$, males $H = 101.2393, d.f. = 37,\ P < 0.0001$ and relief females $H = 49.1103, d.f. = 37,\ P = 0.0879$, males $H = 80.665, d.f. = 37,\ P < 0.0001$, Fig. 3.14).

Association analysis was performed as described in the beginning of this chapter and presented here for unisex behavioural scores (see also Material and Methods for details). As the detection power for associations to relief learning was low and to allow a more comprehensive enrichment analysis, the multiple correction rate was loosened for expression and SNP relief learning associations ($FDR = 0.1, P-value = 10^{-5}$, respectively), keeping in mind, that this allows also more false positives. To summarize, association analysis uncovered 79 probe-sets significantly associated with punishment learning and 12 with relief learning (Table S4 and S5). Additionally 1 190 SNPs were found to be significantly associated with punishment learning and 330 with relief learning (see Table S9a, S9b and S10). Subsequent analysis steps pool these into a total number of 588 candidate genes

Figure 3.14 *(following page)*: The 38 tested inbred strains showed significant variation in punishment and relief learning for unisex (A.), male (B.) and female (C.), except for C. relief females (Kruskal-Wallis test for A. punishment $P < 0.0001$, relief $P < 0.0029$, for B. punishment $P < 0.0001$, relief $P < 0.0001$, for C. $P < 0.0879$). Box plots show the median as the midline, 25 and 75 % as the box boundaries and 10 and 90 % as the whiskers. Sample sizes were from left to right for A. punishment N = 8, 9, 9, 23, 7, 9, 20, 11, 8, 8, 8, 8, 9, 8, 9, 9, 8, 9, 10, 8, 8, 7, 8, 8, 8, 7, 17, 8, 11, 8, 8, 9, 7, 8, 9, 8, 9, 11; relief N = 24, 25, 24, 25, 24, 24, 26, 23, 25, 24, 25, 24, 23, 24, 24, 24, 25, 25, 24, 24, 24, 24, 24, 25, 24, 24, 24, 23, 24, 24, 25, 24, 23, 26, 24, 25, 21; for B: punishment N = 8, 9, 15, 6, 6, 8, 7, 4, 4, 11, 5, 8, 7, 6, 3, 5, 5, 8, 8, 7, 8, 9, 8, 2, 6, 6, 7, 7, 8, 8, 4, 7, 3, 5, 8, 8, 7, 11; relief N = 17, 22, 19, 14, 13, 15, 13, 10, 13, 18, 14, 22, 19, 19, 18, 17, 18, 22, 9, 15, 16, 16, 21, 7, 16, 14, 18, 17, 11, 19, 10, 12, 12, 16, 16, 24, 21, 17; for C: punishment N = 9, 7, 19, 7, 13, 8, 8, 5, 7, 10, 8, 9, 10, 5, 9, 9, 7, 6, 8, 8, 11, 9, 9, 5, 6, 8, 6, 8, 7, 8, 14, 8, 6, 7, 4, 9, 8, 11; relief N = 23, 19, 23, 18, 16, 24, 20, 19, 24, 24, 21, 21, 21, 19, 21, 14, 20, 23, 22, 21, 14, 20, 19, 14, 18, 23, 16, 21, 17, 21, 17, 20, 19, 17, 18, 24, 20, 19.


A.
Relief learning
Punishment learning
Learning index
Conditioned approach
Conditioned avoidance
inbred strains
B.
Learning index
Conditioned approach
Conditioned avoidance
inbred strains females
C.
Learning index
Conditioned approach
Conditioned avoidance
inbred strains males

for punishment learning and 199 for relief learning; 71 genes were solely associated with expression and punishment and 11 with relief learning; 509 genes were solely associated with SNPs and punishment and 187 with relief learning. 4 genes were in the overlap between expression and SNP associations for punishment and 1 in the overlap for relief learning (Fig. 3.15).

Enrichment analysis

In terms of Gene-ontology (GO) categories, punishment learning candidate genes are enriched for example in the categories axon guidance (Biological Process) and the signal transducer and receptor activity (Molecular Function). Among the receptor genes were odorant, ionotropic and gustatory receptors, which makes sense, as we look at olfactory learning. Interestingly, they differed from those found for BA and OCT avoidance alone. In addition, *Serotonin receptor 2* was in the list of enriched receptor genes. It was recently discovered to play a role in olfactory STM and LTM [152].

Additionally, I compared the candidate genes for punishment learning with a manually compiled learning gene list retrieved from literature, focusing mainly on olfactory learning genes. I found significant enrichment of learning genes among the candidate genes for punishment learning (see Table S14 and S21, Pearson chi-square: 2.6 fold, $representation\ factor = 2.6, P = 0.001$. Fig. 3.15). This refers to 13 learning genes. On top of this, 7 were mainly those for long term aversive olfactory memory (*murashka*, *visgun*, *dFoxo*, *approximated*[6], *foraging* [216], *tequila* [77]). As the training protocol for olfactory LTM is similar to the one used in this study, this comes very close to an independent validation of candidate genes. Additionally, 3 of the remaining 6 genes were found to be involved in visual- (*tolloid*, *Strn-Mlck* [150]) and courtship-rejection- (*fruitless* [352]) LTM, respectively. This means 9 of 13 genes associated with punishment learning are involved in long-term memory. But also 2 genes so far tested for olfactory aversive STM and acquisition were found in the enriched list (*PHM*[143], *gilgamesh*[317]). Unexpectedly, the *Octopamine receptor in mushroom bodies (OAMB)* gene was also found among them, which has a known role in appetitive learning, but not short term aversive learning [168]. All those genes are suggested as strong candidates for punishment learning.

Furthermore, comparisons to the manually compiled locomotion and nociception gene lists described above (see Table S14 and S21 and Fig. 3.15), revealed enrichment for locomotion genes (Pearson chi-square: 3.1 fold, $representation\ factor = 3.1, P < 0.0001$), but not for nociception genes among punishment learning candidates. This speaks for

the specificity of the list. Nevertheless, to test for the translatable potential of the list to mammalian research and as punishment learning has to deal with the 'painful' side of the unconditioned stimulus, I probed for human insensitivity to pain disease gene orthologues as described for electric shock avoidance. The punishment learning candidates are enriched for these orthologues (Hypergeometric test: 4.1 fold, $representation\ factor = 4.1, P = 0.003$) confirming translatability to mammalian research once more.

The relief learning list contained 199 candidate genes in total (see Table S15) which is smaller than the candidate lists for other behaviours. A GO-category enrichment analysis showed very few and broad categories such as organ and system development probably due to the small list [68] and higher FDR.

No enrichment was found for locomotion, nociception or human pain insensitivity homologues suggesting relative specificity of this list. However, compared to the manually compiled list of known learning genes, I found a significant enrichment among relief learning candidates (Fig. 3.15, Hypergeometric test: 3.6 fold, $representation\ factor = 3.6, P = 0.007$). These refers to 6 genes. One is *fruitless* which plays a role in courship-rejection long-term memory [352]. Another one is *gilgamesh* known to play a role in STM and MTM [317]. *Approximated* and *Gp210* are involved in LTM [6, 83]. Furthermore, there is *tau* which plays a role in STM and also has a human homologue implicated in Alzheimer's disease [173]. The last gene is *DopR*, which is involved in olfactory aversive and appetitive STM [167]. The first 3 also overlap with punishment learning candidates and the latter 3 were only found in the relief list. This strongly suggests common molecular pathways between relief learning and other forms of learning in *Drosophila* and thus suggests very strong candidates to test for relief learning.

3.1.4 Correlations among behaviours

To get a broader picture of the comparability of these behaviours and future association results, I investigated how these related behaviours are correlated within the 38 inbred strains using median behavioural scores of each strain for the Spearman correlation (see Fig.3.16, 3.17 and 3.18. Surprisingly, there was no significant correlation between punishment and relief learning ($rs = -0.0616, P = 0.7055$, Fig. 3.16). Also, I found no correlation between electric shock avoidance and the two kinds of learning (punishment: $rs = -0.0795, P = 0.6350$, Fig. 3.16 relief: $rs = -0.0140, P = 0.9294$, Fig. 3.17), nor odour avoidance and electric shock avoidance (BA: $rs = 0.2391, P = 0.1481$, OCT: $rs = 0.2273, P = 0.1699$, Fig. 3.18). Neither did I find a correlation between odour

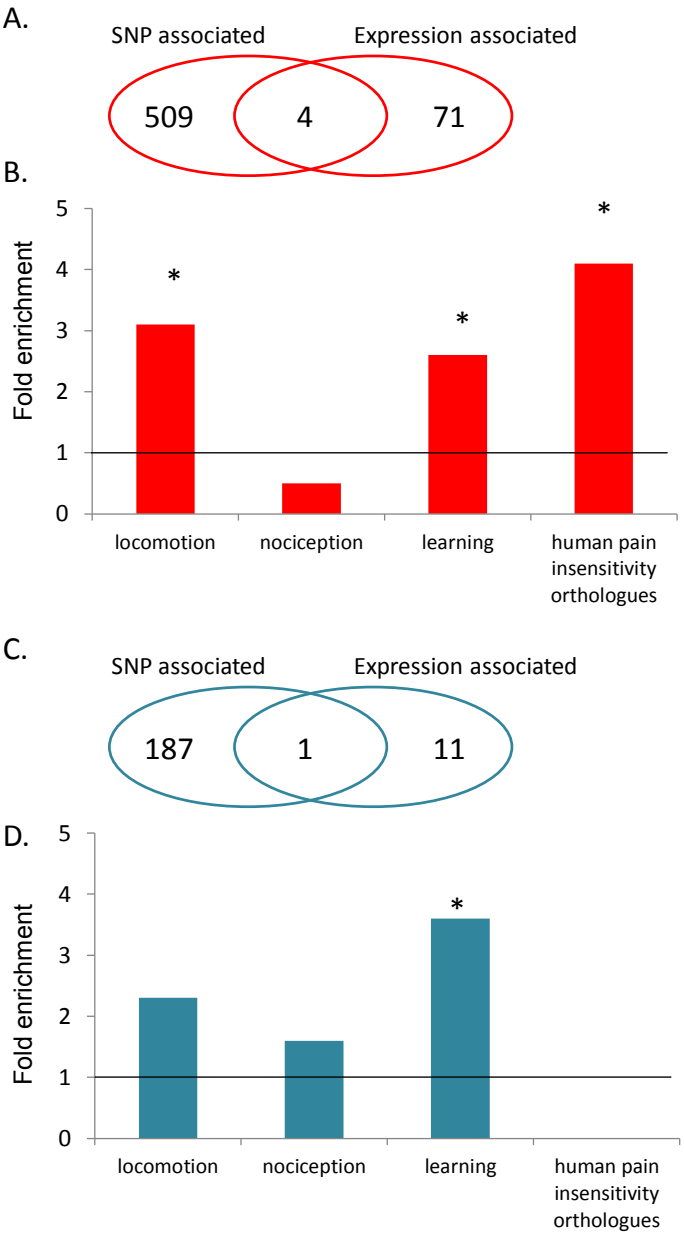


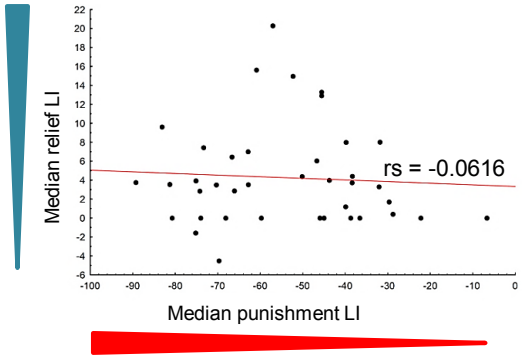
Figure 3.15: Candidate genes associated with punishment (red) and relief (blue) learning. A. and C. Number of candidate genes associated with SNPs and punishment and relief learning on the left and expression and punishment and relief learning on the right and overlapping candidates in the middle. B. and D. Enrichment among punishment and relief learning candidates compared to locomotion, nociception, learning gene lists manually compiled through literature search and orthologs to human pain insensitivity disease genes as described in Material and Methods. * indicates $P < 0.05$ in a Hypergeometric test and a black line indicates the expected overlap.

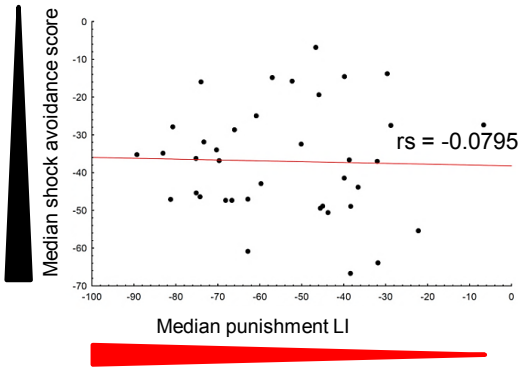
avoidance and the two kinds of learning (OCT: punishment: $rs = -0.1368, P = 0.4128$, relief: $rs = -0.0036, P = 0.9830$, BA: punishment: $rs = -0.0133, P = 0.9367$, relief: $rs = 0.0847, P = 0.6131$, Fig. 3.16, 3.17). But 3-Octanol avoidance was significantly correlated with Benzaldehyde avoidance ($rs = 0.3495, P = 0.0315$, Fig. 3.18), meaning that flies that do not avoid one odour are very likely to have problems in avoiding the other odour. Little correlation among behavioural results suggest a higher chance of finding specific genes for each behaviour.

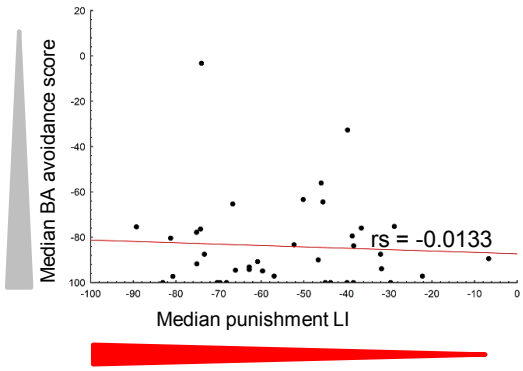
3.1.5 Overlapping candidate genes between learning and innate behaviour

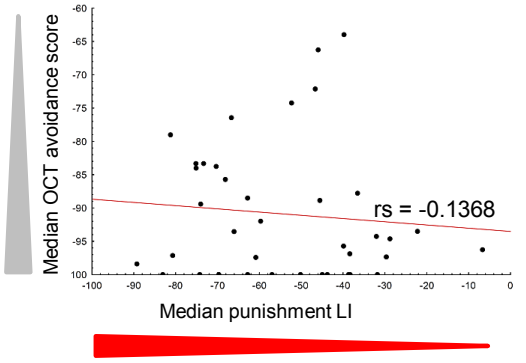
In order to gain a more complete picture of the specificity and pleiotropy of candidate genes, I report here the number of associated genes that overlap between behaviours as given in Fig. 3.19. On top of the the reported significant overlap between BA and OCT avoidance candidates, there were in total 48 significantly overlapping candidate genes between punishment and relief learning ($representation\ factor = 6.6, P < 0.0001$, for exact numbers of all overlapping genes, see Fig. 3.19). In addition to overlapping learning associations, 195 genes overlapped between punishment learning and electric shock avoidance ($representation\ factor = 2.8, P < 0.0001$), 78 genes with BA avoidance ($representation\ factor = 1.8, P < 0.0001$) and 50 genes with OCT avoidance ($representation\ factor = 3.2, P < 0.0001$), all three being significant. This leaves us with 317 candidates specific for punishment learning. For relief learning, 86 genes overlapped with electric shock avoidance ($representation\ factor = 4.4, P < 0.0001$), 35 with BA avoidance ($representation\ factor = 2.7, P < 0.0001$) and 24 with OCT avoidance ($representation\ factor = 5.4, P < 0.0001$), again all overlaps were significant. Thus, 60 genes are specifically associated with relief learning (see Fig. 3.19 for overlaps and Table S11-S15 for details). 235 candidate genes overlapped between BA avoidance and electric

Figure 3.16 *(following page)*: Correlations between punishment learning and other behaviours measured in 38 inbred strains. The 38 tested inbred strains showed no significant correlation of punishment learning (red) to relief (blue), OCT avoidance (grey), BA avoidance (grey) or electric shock avoidance (black). For each behaviour combination, a scatterplot of median behavioural scores for each inbread strain was plotted and a linear correlation line is fitted through the data points. Colored bars at the axis indicate in which direction learning or avoidance gets better through increased thicknes of the bar.


Median relief LI
rs = -0.0616
Median punishment LI


Median shock avoidance score
rs = -0.0795
Median punishment LI


Median BA avoidance score
rs = -0.0133
Median punishment LI

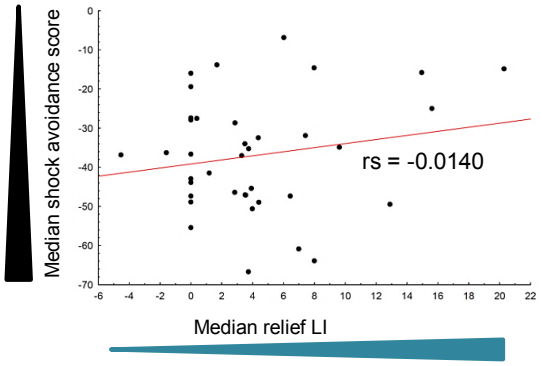

Median OCT avoidance score
rs = -0.1368
Median punishment LI

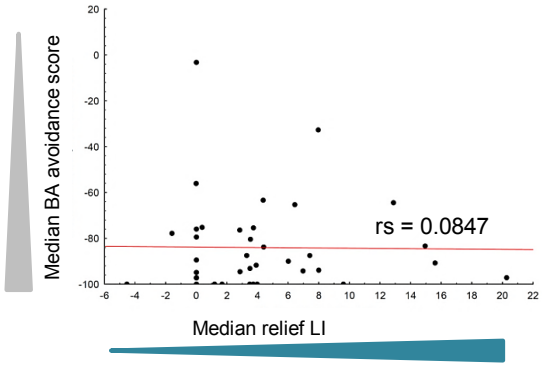
shock avoidance ($representation\ factor = 2.2, P < 0.0001$) and 115 genes between OCT and electric shock avoidance ($representation\ factor = 3.0, P < 0.0001$). Thus, despite little correlation among behaviours of inbred strains, all candidate gene lists are overlapping significantly suggesting a high redundancy of pathways influencing these behaviours. To add up, this leaves us with 1 013, 711 and 180 genes solely associated with shock avoidance, BA and OCT avoidance, respectively (Fig. 3.19). Three genes overlap with all behaviours, namely *fruitless*, *Protein tyrosine phosphatase 99A* and *defective proventriculus*. According to FlyBase, these are involved in courtship learning, motorneuron axonguidance and leg joint morphogenesis .

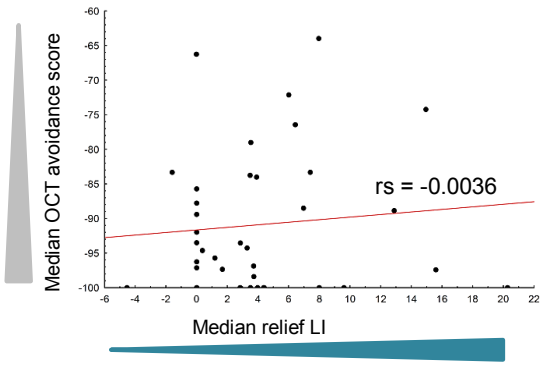
3.2 Comparison between relief and punishment memory phases

In terms of absolute learning scores, punishment memory is obviously much stronger than relief memory as reported previously [319], [365] when using the treatment depicted in Fig. 3.1. This is true even for 2 h memory, although relief memory scores stay the same but punishment memory has already decayed as reported in [78]. Despite decaying slower than punishment memory, relief seems to be more sensitive to cold-anesthesia (see Fig. 3.20, [78]). Implementing a cold shock 60 min after training, relief memory is eliminated compared to its control and not significantly different from zero (MWU test: $P < 0.05, U = 41, N = 14, 14$, OSS test: $P < 0.05/2$ for control, $P = 0.79$ for cold amnesia group) whereas punishment memory is reduced by a half compared to its control (MWU test: $P < 0.05, U = 54, N = 14, 14$, OSS test: $P < 0.05/2$ for for each group). These results are already published in a paper accepted in the refereed journal 'Biology Letters' [78].

Figure 3.17 *(following page)*: Correlations between relief learning and other behaviours measured in 38 inbred strains. The 38 tested inbred strains showed no significant correlationof relief learning (blue) to OCT avoidance (grey), BA avoidance (grey) or electric shock avoidance (black). For each behaviour combination, a scatterplot of median behavioural scores for each inbread strain was plotted and a linear correlation line is fitted through the data points. Colored bars at the axis indicate in which direction learning or avoidance gets better through increased thicknes of the bar.


Median shock avoidance score
rs = -0.0140
Median relief LI


Median BA avoidance score
rs = 0.0847
Median relief LI


Median OCT avoidance score
rs = -0.0036
Median relief LI

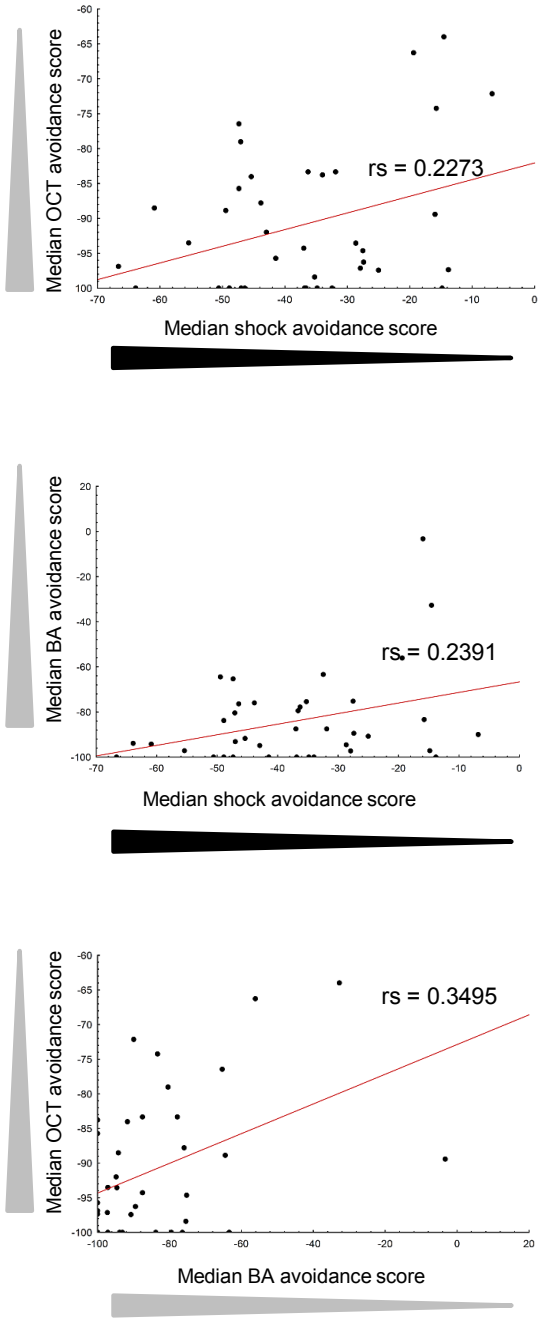


Figure 3.18: Correlations between avoidance behaviours measured in 38 inbred strains. The 38 tested inbred strains showed tendential correlation between 3-Octanol and Benzaldehyde avoidance (grey) but no correlation between odour and electric shock avoidance (black). For each behaviour combination, a scatterplot of median behavioural scores for each inbread strain was plotted and a linear correlation line is fitted through the data points. Colored bars at the axis indicate in which direction learning or avoidance gets better through increased thicknes of the bar.

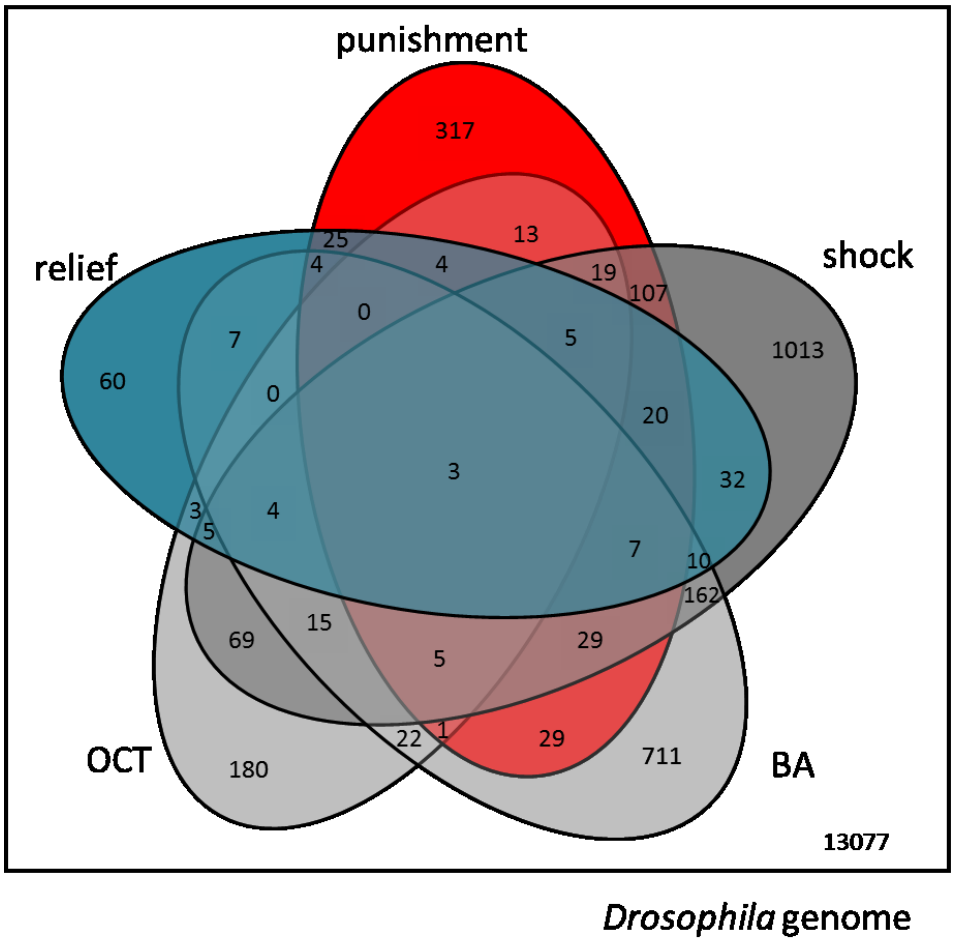


Figure 3.19: Number of overlapping candidate genes between behaviours tested. Venn diagrams containing numbers of candidate genes for punishment learning (red circles), relief learning (blue circles), electric shock avoidance (black circles) and BA and OCT avoidance (grey circles). Numbers of overlapping genes are indicated in the overlap between the respective circles.

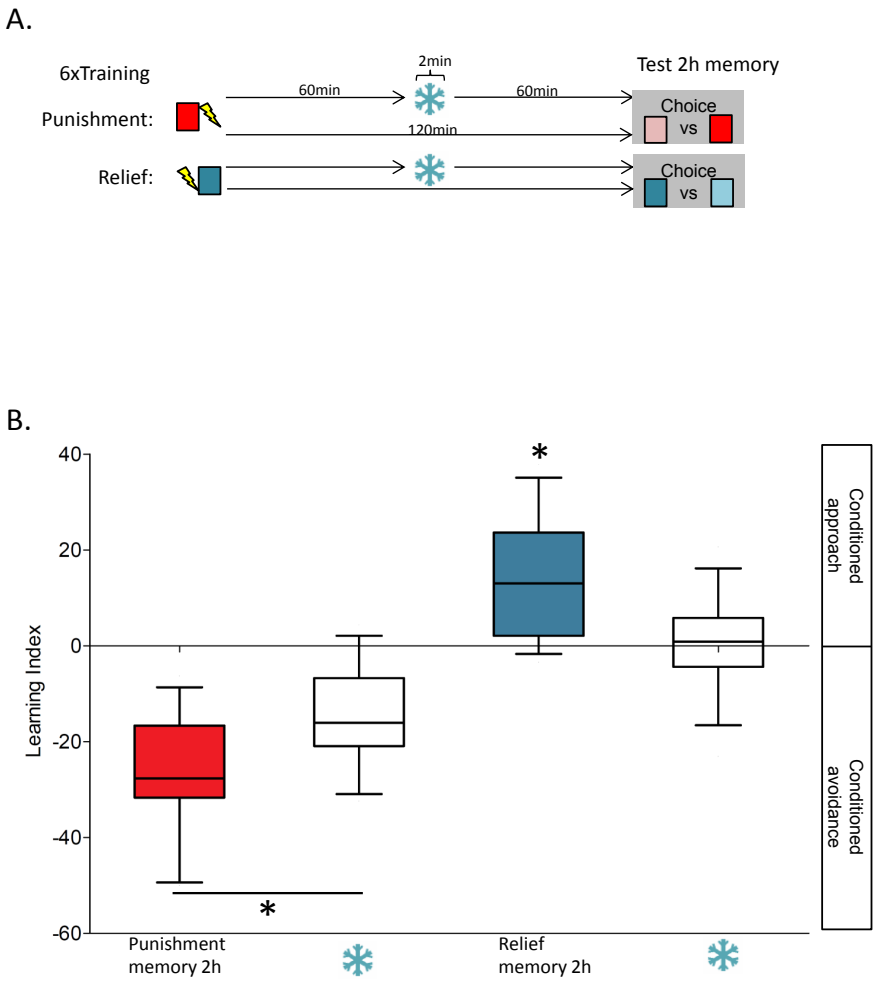


Figure 3.20: Punishment and relief memory resistance to cold amnesia adapted from [78] A. After subjecting flies to a training for punishment and relief learning as described in Fig. 3.1, they were either given a cold shock on an ice bath for 2 min or left on room temperature at 60 min after training offset. Memory was tested 120 min after training offset. B. Box plots containing the median learning index (LI) as the midline, 25 and 75 % as the box boundaries and 10 and 90 % as the whiskers are presented for punishment and relief learning in red and blue, respectively. Grouped with either kind of learning, white indicates LIs where a cold shock was given. Stars are for significant difference to zero with $P < 0.05/2$ and $N = 14$ for all groups.

Chapter 4

Discussion

4.1 Summary of results

Measured behavioural scores in 38 inbred fly strains vary significantly between strains, are not correlated among behaviours and are associated with expression level and SNPs of different and overlapping candidate genes enriched in specific categories, also showing translational potential. Significant variation between inbred strains in electric shock avoidance, BA avoidance, OCT avoidance, punishment and relief learning can be caused to a certain extent by genetic factors. The fact that behaviours are not correlated with each other, except BA and OCT avoidance, speaks for different genetic regulation of the traits. Some genetic factors could be revealed using genome-wide association analysis to expression levels and SNPs. Indeed many distinct candidate genes were discovered. But lists also overlapped significantly suggesting either pleiotropic functions or common behavioural steps underlying these behaviours. The latter is supported by enrichment found in most lists for locomotion genes and in all for learning genes. But enrichment in nociception genes for electric shock avoidance and clustering of LTM genes in punishment learning speaks for the specificity of the candidate gene lists. Furthermore, these lists might contribute to translational research as shock avoidance and punishment learning candidates are enriched in fly-human orthologues for pain insensitivity disease. Hence, this study gives a deeper insight into genes influencing variation in electric shock avoidance, odour avoidance, punishment and relief learning, suggesting candidates enriched in plausible functional categories for further investigation.

Additionally, candidates for electric shock avoidance could be validated in an unbiased and biased way. That is, an unbiased gene network, based on protein-protein interactions

revealed a major role of bristle related genes in electric shock avoidance. Furthermore 10/19 genes could be independently validated using P-element insertion mutants. Interestingly, three of these are also implicated in bristle development confirming the network results. Based on the enrichment in nociceptive genes among shock candidates, a role of *trpA1* in electric shock avoidance was shown for the first time. Another gene, *npfr1*, which is suggested to modulate stress responses, also affects the response to electric shock. Thus, bristle development, a natural noci-sensor and a putative stress regulator are prominent candidates to investigate further in electric shock avoidance.

In addition to different genes that influence variation in punishment and relief memory, these memories have distinct properties in terms of resistance to a cold shock and long term memory [78]. Punishment memory decays quickly but survives 24 hours, but relief memory scores remain at the same level for up to 4 hours and the memory is gone at 24 hours. Also, whereas punishment memory partly survives the cold shock, relief memory is erased completely. This suggests different processes underlying relief and punishment learning.

4.2 Advantages and caveats of Genome-wide association studies in *Drosophila*

The power of using *Drosophila* in genome-wide association studies is that its large genetic variation can be 'frozen' through inbreeding [207, 92]. Flies within one strain become largely homozygous during inbreeding of small sub-populations due to genetic drift [258]. But which genotype becomes homozygous is different in each inbred strain. Thus genetic variation within a strain is minimized and variation between strains is maximized. Additionally, multiple measurements of one individual line can be obtained, which allows assessment of the noise created by environmental influences. This enables better detection of QTLs which depends on the effect size. The effect size is determined by the difference in the trait phenotype between strains divided by the standard deviation of the trait within a strain [209, 92]. Hence, in homozygous strains, the probability increases to detect small effects of genes on the variation in the trait.

Furthermore, the DGRP is a source of inbred fly strains provided to the scientific community together with their genetic information [21, 208]. Inbred fly strains were generated

and whole genome expression microarrays and sequencing performed on each of them. This data can now be used by every laboratory around the world to test the strains for different traits and look for genetic associations. Therefore, comparisons between different traits affected by the same genotype are facilitated and pleiotropic or common effects can be detected as demonstrated within this study.

In addition, using the DGRP, sex specific effects can be separated through testing of males and females of the same genotype [21, 208]. Male and female phenotypes can be associated to the corresponding genetic information and potentially antagonistic genes can be detected. However, a problem arises if doing so due to unequal effect sizes of X-chromosomal genes in males and females and incomplete dosage compensation, i.e. inactivation of transcription of one X-chromosome in females (reviewed by[248]). Not only is it impossible to tell, which X-chromosome was inactivated by looking at the sequencing data of the DGRP, but also which genes were inactivated due to incomplete dosage compensation. Focusing on expression data could bring more insight here. Nonetheless, if handled with care, it might be an interesting outlook for this study to look at sex specific effects of gene expression or SNPs on the behavioural scores as many genes seem to be dimorphic [21, 213].

However, it has to be kept in mind that only genes that vary in expression or sequence in this fly population can be detected [207]. Genes that were lethal, not varying in expression nor had they important SNPs or shared the SNP with other strains are ignored by this kind of analysis. In other words, this analysis is population specific and if one wants to assess the contribution of all possible genes to a phenotype, it would be necessary to use many different populations in the future and do a mutagenesis screen in addition. This problem is addressed in [139] where totally different SNPs were associated with the same trait by creating a new population from the DGRP strains. Nevertheless, the associated SNPs converged on the same gene network. Thus, a GWAS is no attempt to find all possible genes affecting a trait; it rather detects genes whose natural variation is involved in a gradual change in phenotype.

Furthermore, candidate gene lists are bound to include false candidates for several reasons. The first is statistical error due to multiple statistical comparisons by associating every single gene to the same trait. Therefore, multiple correction methods have been developed such as FDR [32] or Bonferroni correction [132] allowing for a limited number of false positives. The other reasons are of biological nature and include inter-correlations between gene expression levels and inter-dependency of alleles at various polymorphic loci

(i.e. linkage disequilibrium) [21, 208]. Inter-correlations between gene expression levels can lead, for example, to an association of transcripts that are up-or down-regulated through the same transcription factor, but only one of these transcripts truly has an effect on the trait [21]. Linkage disequilibrium (LD) leads to detection of two or more alleles in the candidate list, because they are segregating together, but only one is contributing to the phenotype [126]. In the DGRP, LD is very low [208]. Therefore it is advantageous to further validate the lists of associated genes using reverse genetics.

Another caveat is epistasis which applies to both, GWAS and mutagenesis screens. Epistastatic genes mask the effects of other genes on the phenotype and thus these occluded genetic effects cannot be detected in the GWAS or mutant (reviewed by e.g., [254, 126]). In the past, very few such studies have been reported, but recently, evidence for it's occurrence has increased. Moreover, using DGRP and another population, called 'fly-land', in a GWAS with the same trait, associations did not overlap [139]. However, the gene-networks of both candidate lists overlapped extensively. Evidence for epistasis was also shown for P-element insertion mutants used for validation of these screens. Depending on the genetic background they either affected the trait or not [313]. These studies already include suggestions to a solution for this problem in form of creating a gene network or, using different inbred populations and mutants in different genetic backgrounds to gain as much information as possible.

To functionally interpret large candidate gene lists, enrichment analysis is commonly used as a tool, also in *Drosophila* [68, 332]. However, it is still in a developmental stage as many functions of genes are still unknown or predicted based on protein domain similarity [332]. Also the number of genes in the candidate list influences the power of obtaining significant enrichment categories [68]. Nevertheless it is currently the only tool to gain an overview of general and specific functions of candidate genes and grasp their 'non-randomness'.

In addition the knowledge about the detailed interplay between QTL and eQTL on a genomic level is still very sparse [280],[89]. For example, associated SNPs do not always have an effect on the expression level of nearby genes. They could effect protein function or translation. Also SNPs can change regulation of expression of genes further away or in a tissue specific manner. All of these differences are impossible to detect with the current whole body genome-wide-analysis presented here with *Drosophila*. In this sense, a caveat of the current study is, that intergenic regions were not analysed further. Future detailed bioinformatic and genetic analysis could reveal more links between QTLs and

eQTLs through inclusion of the intergenic SNPs. Furthermore, with the newly available cis-eQTL map in *Drosophila* [213] it might be much easier to do this. This is an exciting outlook to gain more insight into QTL and eQTL interactions.

Using the DGRP GWAS, several human homologues have been detected influencing related traits in *Drosophila* emphasizing the contribution to translational research. For example GWAS in alcohol sensitivity or sleep and waking activity [124, 226] found human homologues implicated in traits such as alcohol consumption, daytime sleepiness and sleep duration. Due to the small sample size and power of human GWAS, it is difficult to detect genes with small effects on disease phenotypes which leads to an overestimation of genes with large effects while ignoring the genetic architecture behind it [207]. Thus, taking into account advantages discussed above, *Drosophila* is a very useful and powerful model to fill this gap as many genes are conserved across species and fulfill similar functions.

All in all, *Drosophila melanogaster* is a useful model for genome-wide association studies due to the higher power in detecting small effects of QTLs and eQTLs using inbred strains. But it has to be kept in mind that only genes can be associated that vary in expression or sequence to a certain frequency in the investigated population and association of X-chromosomal SNPs seems to be difficult because of unknown dosage compensation effects [248]. Through foundation of the DGRP traits can be tested and compared within genotypes also revealing pleiotropic genetic effects. However, it is advantageous to use enrichment and gene-network analysis and reverse genetics to at least partly circumvent caveats such as LD, statistical error and epistasis. Furthermore, candidates can be suggested for translational research.

4.3 New insights into electric shock avoidance

Taken together SNP and expression associations to this trait revealed a list of $\sim$ 1 500 'candidate shock avoidance genes'. These likely encode for proteins with developmental or acute functions in various steps from peripheral sensation of shock down to the muscle contractions for avoidance. Thus, this list can serve as a source for further investigation dissecting this behaviour.

However, because of caveats discussed above, the causal nature of the gene - shock avoidance relationships suggested by this analysis must be scrutinized independently using reverse genetic methods. Other GWAS using the same inbred strains but testing different traits have validated their candidate gene lists to different extents using P-element insertion

lines (e.g., [21, 88, 125, 226, 350]). I attempted this with 19 candidate genes and found a highly significant effect of a respective P-element insertion mutation on shock avoidance in 10 cases. This confirms the hypothesis that many genes affect one trait.

In all reported GWA studies the P-element insertions were in or near the candidate genes suggesting different severity on the gene function, mostly affecting mRNA levels if measured. In this study, RTQ-PCR results confirm a reduction in transcipt level in only some of those lines that showed a phenotype and vice versa. This could have many reasons, e.g. these were either true false positives in the list or the amount of transcript was probably not reduced enough to affect behavior or not high enough to detect differences. Significant behavioral effects without changed transcript level could be due to truncated, but still transcribed genes, which are not functional at the protein level. Also, detected levels of transcript are too low to detect a significant difference in the adult fly, but the transcript could play a role earlier in development. Furthermore, alternative splice forms and genetic background effects can occlude effects of the disrupted gene. The latter was demonstrated in a study where the same P-element insertions had a different effect depending on the genetic background of an inbred strain [313]. Together, P-element mutants can reveal many, but not all causal effects, inviting further scrutiny using independent genetic methods, including rescue experiments.

In the future, detailed analyses can follow as to which stages of shock processing are affected by these genes and where in the fly body these effects manifest. Only four of these 'validated' genes have so far been investigated in any appreciable detail. Among these, *bearded* may be particularly interesting due to its well-established role in the development of bristles [193], which are external sensory organs distributed across the fly's body surface. Interestingly, bristle-related roles have been also attributed to two other 'validated' genes, *Regulator of cyclin A1* and *rad50* [227, 60].

Furthermore, gene network analysis seems to show consistent results even across population specific association of a trait [139] adding a high value to meta-analysis. Integrating the results of the electric shock association analyses with the existing protein-protein interaction data resulted in a shock avoidance-relevant network which included many genes with effects on bristle number and morphology (Table S23). This confirmed also enrichment analysis results. However, most of the above mentioned genes also have bristle-unrelated functions (according to FlyBase) which may potentially be the intermediate to their effects on shock avoidance. Nevertheless, it is tempting to speculate that bristle-function may be an 'endophenotype' for shock avoidance and that shock sensation may be at least partially

mediated through bristle-associated sensory neurons. Thus, these functionally-unbiased approaches to the candidate gene list provided an interesting cellular hypothesis about shock-sensation.

In addition, the candidate list was enriched in genes implicated in nociceptive behaviour (Table S11), which suggested a molecular hypothesis for shock-sensation: Shock may recruit sensors and downstream signaling cascades of other noxious modalities. Indeed, upon loss-of-function of the famous 'noci-sensor Transient receptor potential A1 (dTrpA1), shock avoidance was impaired (Fig.3.8). Various isoforms of the TRPA1 channel in fruit flies are key to sensing noxious heat [338, 268, 122, 181, 269, 23, 233, 159, 372, 318, 343], harmful chemicals [160, 165, 179, 159, 372] and harsh touch [372]. The channel-nature of TRPA1 as well as the lack of locomotor impairment upon loss of its function (Fig. 3.8) renders it an attractive candidate for a molecular shock-sensor. It may be that one or several of TRPA1 isoforms are directly activated by shock (for voltage-sensitivity of the fruit fly and mammalian TRPA1, see [159, 343], [272]; also see [339] for other mammalian TRP channels). Alternatively, shock may trigger thermal and/ or mechanical changes in the fly-tissue, resulting in TRPA1-opening. Of course, shock or other effects secondary to it may also bear upon TRPA1 indirectly, through signaling cascades. Indeed, TRPA1-activation by mild heat as well as noxious chemicals seems to rely on Phospholipase C signaling [181, 165, 179]. Interestingly, several genes of this pathway came up in the association analyses (Table S22: CDP *diglyceride synthetase*, *lazaro*, *retinal degeneration A*). The remaining shock avoidance ability upon loss of dTrpA1 function (Fig. 3.8) points to other, independent mechanisms. These may be implemented by other nociception-related channels which I found to be associated with shock (Table S22). Importantly, previously unknown 'noci-sensors' are likely also present among our list of candidate genes. An exciting further outlook would be to map out the cellular site and role of TRPA1 and other sensors in shock sensation, as e.g., the various behavioural effects of TRPA1 seem to be implemented in distinct types of neurons [122, 181, 165, 179, 233, 159, 372, 318].

Furthermore, it has been shown that NPY family peptides are implicated in stress and pain responses in a variety of species [26, 325, 324, 355, 358, 230]. The *Neuropeptide F receptor 1, NPFR1* gene and it's G-protein coupled receptor NPFR1 is the distant *Drosophila* homologue to the vertebrate NPY receptor, is expressed in interneurons and turned out to play a role in several behaviours, such as aggression, ethanol sensitivity, noxious food avoidance, food-associated memory and heat nociception (see a review by [230]). For heat nociception, it seems to suppress the activity of another noci-sensor

in *Drosophila* called TrpA encoded by the *painless* gene[358]. The fact that I found a role for *NPFR1* in electric shock avoidance further supports the hypothesis that shock explores molecular pathways evolved for natural aversive stimuli and their stress response systems. In addition, studies on aggression showed that NPF reduces while Serotonin (5-HT) enhances aggression [79]. As the Serotonin transporter gene was highly associated with electric shock avoidance, this could be another possible pathway over which shock avoidance is influenced. Thus it would be exciting to narrow down the possible molecular pathway through which *NPFR1* acts to change electric shock avoidance by making use of the sophisticated genetic tools developed in *Drosophila*.

Furthermore, orthologous genes to those found in a heat nociception screen using *Drosophila* as a model [231], were positively tested for pain perception in mice. Enrichment in the shock avoidance candidate gene list for human-fly orthologues for congenital insensitivity to pain suggests possible candidates to mammalian models for pain/nociception (as for example in [231]). As the high number of related disease genes between fly and human suggests (75% based on the online tool 'Homophila' with $E < 10^{-20}$, Bier et al. 2005, Chien et al. 2002), this data can be useful to predict phenotypes in mammalian paradigms in the future.

To conclude the present list of candidate shock avoidance genes and the suggested interaction network provide useful hypotheses about shock sensation and thus can indeed give a head-start to studying particularly the molecular but also the cellular mechanisms of shock avoidance in the fruit fly. I hope that what we learn from these studies would then to some extent be translatable to nociceptive behaviour in evolutionarily higher animals, including man.

4.4 Interesting candidate genes for avoidance to two different odours

The avoidance of benzaldehyde was associated with 994 candidate genes and the avoidance to 3-octanol revealed 351 candidate genes also uncovering 41 genes significantly overlapping between both behaviours. This already suggests that the proteins encoded by those candidates cover various steps involved in the avoidance of an odour, such as smelling, giving a value to the odour and locomotion. General odour avoidance candidate genes are suggested from the overlap whereas more specific candidates to avoidance of specific odours could possibly be found in the non-overlapping part. This study offers the opportunity to

differentiate odour specific versus general odour avoidance genes, providing the basis of studying them more in detail in the future.

Specificity of several, but yet not all OBPs to certain odourants has been demonstrated in the past [312]. Thus they are not solely seen as shuttling molecules. Among the three odourant binding proteins detected for BA avoidance, one (*obp18a*) was independently tested in an RNAi knockdown screen for response to several odours [312] and showed a significant change in response to benzaldehyde. Moreover, this change took place in males and females underlining the focus on unisex association in this study. Furthermore, two other OBPs were associated with variation in olfactory response to BA in another genome-wide study [12]. Thus, these studies confirm the specificity of the BA avoidance candidate gene list.

Two classes of odorant receptors have been found in *Drosophila* which are currently thought to have distinct odour class specificities. According to electrophysiological data recorded from four sensilla each expressing a set of different IRs [295], IRs seem to be specific to amines and acids. As for both, OCT and BA avoidance candidate genes, IRs were detected, this contradicts the current opinion. Therefore it is interesting to further investigate these candidates. It has to be noted that most deteced IRs have not been tested for specific odorants yet. Responses of glomeruli specific to certain ORs detected for BA avoidance have been tested using Calcium imaging and a summary can be seen at the DoOR website [1]. One of the two detected ORs showed a response to BA in the respective glomeruli. However, as very different response patterns can be generated depending on the concentration of the odour, it can be that the pure concentration of BA used in my study elicits response also in the other ORs associated. In this context it would be interesting to test the response of the associated receptors also for odour concentrations used in this study.

Comparison to another BA avoidance GWAS [313] can be useful, but no complete overlap can be expected, because of several reasons. First, the assay differs as I used a different odour concentration and time frame, second a different number of inbred strains were used and third strains probably were reared on different food. Odour concentration is a very important factor as flies change their avoidance to approach behaviour when the concentration of some odours is decreased [348]. Different brain centers mediate these responses and also different neuron patterns are activated upon different concentrations [348, 74]. Furthermore, the shared part of immediate avoidance genes might be less as flies had more

[1] http://neuro.uni-konstanz.de/DoOR/default.html

time to avoid the odour. The usage of more inbred strains unavoidably leads to more multiple comparisons, as there is more variation in the genome to be explored. Therefore, many candidate genes might fall below the correction threshold. Moreover, whether or not larvae are reared on different food has a large impact (about 50%) on the variability of odour guided behaviour [273]. For example, flies reared on tomato versus standard versus alcohol food differed in their olfactory response to BA. This suggests strong dependence of the detected genetic effects of BA avoidance on environmental factors, population size of the DGRP and kind of assay used. Despite all these differences, candidates from another GWAS study using a different BA avoidance assay [313] overlapped significantly with my candidate genes for BA avoidance speaking for the usefulness of the list and encouraging further comparisons between GWAS.

Furthermore, enrichment in GO-categories such as nervous system development and receptor activity for OCT and BA avoidance are encouraging. Further enrichment in locomotion and learning genes makes sense, as flies have to move in order to avoid the odour and these odours are used for olfactory learning (e.g. [329, 6, 171]). These categories can be taken to specify and choose interesting candidates for further investigation. Additionally, these candidates can be used to specifically study for example avoidance behaviour itself, especially in tissues already found to play a role here such as glomeruli [286].

Benzaldehyde is a very special odour. For example, when the antennae and maxillary palps were removed, flies still avoided BA [163]. Among the receptors detected in the candidate gene list were also Gustatory receptors. In support, in electrophysiological studies response to BA in the maxillary palps, a taste organ in the fly, was abolished in loss of function mutants of the gene *chemosensory jump 6* [20], suggesting taste organs for sensing BA in the fly. Indeed, different kinds of sensory receptors from IRs to GRs were associated. Interestingly, GRs were also associated for OCT avoidance, making them a strong candidate for further investigations.

Additionally, Guo et al.[117] used BA as a punishment in a visual conditioning paradigm which implies that this odour might use similar mechanisms as other punishment stimuli such as electric shock or heat. Indeed, when heat is used as a punishment in the same paradigm, but flies were pre-exposed to BA, heat-visual associations were abolished [200] through a yet unknown mechanism. In addition, I present an enrichment in BA avoidance candidates for fly-human homologues implicated in insensitivity to pain disease. This suggests that this odour is possibly processed similar to noxious stimuli in the brain. But further experiments are needed to verify this hypothesis.

There is further evidence that responses to some odours are male and female specific [96, 74, 313]. For example, genetically blocking input to specific antennal glomeruli changed avoidance of BA and proprionic acid at certain concentrations [74]. Also genetic feminization of glomeruli changed sexual behaviour of males [96]. Thus an exciting outlook would be to look at sex specific effects of genes among the candidates for BA and OCT avoidance.

In summary, odour specific genes could be confirmed in comparison to other odour response screens and additional candidate genes were suggested for OCT and BA avoidance. Among them were different receptor types, that are either already shown to react to these odours and thus validating the list of candidate genes further or that bring up new hypothesis and thus are strong candidates to be investigated in the future. Furthermore, BA could be confirmed as a special odour in that the candidate genes were enriched in fly-human homologues to insensitivity to pain, suggesting noxious pathways used by this odour.

4.5 Common and diverging molecular pathways of punishment and relief learning, odour and shock avoidance

A striking variability observed in punishment and relief learning among the naturally derived inbred fly strains. They all resemble laboratory 'wildtype' strains, ranging from strains that do not learn both punishment and relief over strains that are only good in one kind of learning to strains that are very good in both (Fig.3.14). Of course, in nature, these effects would be reduced due to heterozygozity of the flies. Nonetheless, the genome of natural populations harbours this kind of variation. This indeed raises the question of where to put the border between 'normal' and 'pathological' behaviour as currently discussed for humans [115].

Moreover, in a common sense olfactory learning is expected to depend on the amount of innate avoidance to the unconditioned and response to the conditioned stimulus in naive animals. This might be because avoidance tests are commonly thought to reflect the fly's perception of these stimuli which is not necessarily true. At least in this population of inbred strains either kind of learning does not correlate with innate avoidance of electric shock or the two odours used. However, significant overlaps could be found between candidate gene lists for every behaviour. But there are also many genes that do not overlap

with any other tested behaviour here. Thus, these genes' functions might represent common and specific steps for each behaviour. Supporting this assumption, enrichment in known locomotion and learning genes among candidates can be found for every behaviour, whereas for example nociception genes are only enriched for electric shock avoidance. This suggests separate and shared molecular mechanisms for innate and learned behaviours.

In animal models for anxiety disorders (e.g. PTSD: [293]; [314]; and memory (e.g. [70]) electric shock is mostly used either as a reinforcer or as a traumatic event. Here, for both punishment learning and shock avoidance candidates, enrichment was found in human pain insensitivity homologues. It thus is no far-fetched speculation that this study could pave the way for translational studies in mammals.

About a hundred genes are already known for olfactory punishment learning and only a couple for relief learning (see introduction and Table S21 for a detailed list with references). The presented genome-wide association analysis revealed in total 713 candidate genes for punishment learning and 195 for relief learning to be tested further. It has to be noted here, that, due to the lower power of detecting effects with relief learning which is caused by small differences between strains compared to high variability within strains. Therefore, I loosened the multiple correction for relief learning for further analysis. Thus the chances are higher to have false positives in the list. Nevertheless, these candidates represent a rich source for and will facilitate further hypothesis driven experiments and genetic dissection of learning.

Memory consists of many phases, at least for olfactory punishment learning. LTM is only obtained via a spaced training paradigm and lasts for 24 hours. Some of the known learning genes affect specifically LTM [329, 146]. In my study I used a slightly different spaced training paradigm testing flies already after 30 min. Interestingly, punishment learning candidates are enriched for known learning genes, showing a specific cluster consisting of 7 olfactory LTM genes, 2 visual LTM gene [150] and 1 courtship rejection LTM gene [352] out of 13 in total. This nicely 'validates' the candidate gene list for punishment learning and adds the assumption that these genes already play a role immediately after the training and not only 24 h afterwards. Although for many of them, no exact molecular function is currently known, there are hints towards certain molecular mechanisms being involved, from neurotransmitters to second messengers, for example for the Serotonin receptor gene *5HT2*. In mammals, the Serotonin receptor 2 (5HT2) family plays a role in higher order behaviours including learning and memory [351, 237]. 5HT2 in flies has been detected within the protocerebrum and ellipsoid body (EB) [237]. So far, 5-HT2 receptor

function has been implicated in circadian rhythms, visual processing, and aggression and lately also short- and long-term memory [238, 237, 151]. By comparison to mammalian 5HT2 function it has been suggested that 5HT2 neurons are facilitating the integration of sensory information coming to the mushroom bodies, the centre for learning and memory. As the *Serotonin transporter* gene can be found in the list of shock avoidance one could speculate of a possible link here. *dFoxo* affects long-term memory [6] in a more general way. It especially influences long-term neurogenesis in the mushroom body of adult flies, thus sustaining neuronal plasticity [294] which could in turn improve learning. In addition, the *foraging* gene, which encodes for the second messenger PKG, tells us a lot about how natural variation can selectively influence different parts of memory. One Allele of this gene leads to a defect in short-term memory whereas another leads to a defect in long-term memory [216]. As these examples show, although all of the known genes affect LTM, they fulfill many different functions and more genes functioning in the respective pathways or genes connecting these pathways might be found among the other candidates in the list.

Known learning genes are sometimes only tested for STM and knowledge about other memory phases or other kinds of memory is missing. Two known genes found in the punishment learning list affect STM and aquisition, respectively. *PHM*, which is the key enzyme in neuropeptide biosythesis, influences short-term memory and could be found mainly in the mushroom body and Antennal lobes [143]. On the level of projection neurons which bring olfactory information to the mushroom body, *PQBP* seems to downregulate the NMDA receptor subunit 1 [315] and thus olfactory learning might be modulated this way leading to aquisition problems. Surprisingly, the gene *Octopamine receptor in mushroom bodies, OAMB* showed up among the candidates for punishment learning. It is known to play a role in appetitive but not aversive olfactory short term memory in *Drosophila* using one trial training [168]. Hence, it is tempting to test for a role of these genes and those in the same pathway in the presented punishment learning paradigm.

So far, only one gene, *synapsin*, has been shown to have the same effect on punishment and relief learning (see introduction). Here, *fruitless*, which was shown to play a role in long-term memory of courtship rejection in males [352] can be found in both punishment and relief candidate lists with different number of SNPs. There is supporting information for fruitless possessing a role in adult neuronal plasticity (reviewed in [361]). As courtship is a relatively complex behaviour involving many behavioural steps and interaction between males and females, including olfactory cues it might be possible that it plays a role in aversive and relieving olfactory memories. Thus, it will be interesting to investigate further

mechanisms here. Also *tau*, *gilgamesh* and *approximated*, genes that play a role in aversive olfacory short-, middle- and long-term memory, respectively, are found in both lists. *Gish* encodes for the constitutively active casein kinase I (CKI) affecting the olfactory memory trace independent of the *rut* memory trace and in *alpha/beta* mushroom body neurons at early times (min) after conditioning [317]. Hence this independent memory trace might be common for punishment and relief memory. It has to be noted, that conditions similar to relief learning were applied as a control during the reported study using 12 shocks and 45 s inter stimulus interval. Although only one trial was applied and no specific statistical test was performed, *gish* mutants showed a slight decrease in learning [317]. This raises hope to find a similar function for *gish* in relief memory, but needs to be validated further with 6 training trials. *Tau* serves as a *Drosophila* model for Alzheimer's disease and is thought to regulate neurodegeneration in humans through microtubule function, but in the fruit fly it reduces aversive olfactory short-term memory without degenerating neurons [215]. Thus this refers to a more general mechanism that could affect both kinds of learning in either the same or different tissues. The current molecular function of *approximated* in long-term memory is unknown. All these genes are very strong candidates and can be used to detect common molecular mechanisms between both kinds of learning.

Interestingly, the *Dopamine receptor, dDA1* which plays a role in both, appetitive and aversive olfactory learning [167], was found only among the significant relief associations. One reason of lack of detection of this receptor for punishment learning might lie in the fact that it is needed in specific neuronal tissues(e.g. [17]) where it might not necessarily vary in inbred strains. Further tests need to be done to first validate the role of *dDA1* in relief learning and later to find out if relief and reward learning share the same tissue wherein *dDA1* acts. As for humans and rats, the reward centre in the brain seems to play a role in relief learning, whereas the centre for fear conditioning leaves relief learning unaffected [7], this might be also the case in flies. Thus, dDA1 is a very strong candidate to test for relief especially by using existing genetic tools for tissue specificity.

Taken together, common and distinct genes affect all tested behaviours, suggesting partly overlapping but also distinct molecular pathways between innate and learned behaviour. Especially candidate gene lists of punishment and relief learning give new insights suggesting similar molecules to play a role in both learning forms as well as new links to other forms of learning such as appetitive and courtship learning. In addition, there might be diverging molecular mechanisms that only play a role in one kind of memory phase or learning. Importantly, as this study looks at the whole body, it is necessary to also test for

tissue specificity of these genes involving more sophisticated tools and experiments future projects. There it can be also advantageous to start with already known learning genes where there are tools and a hint towards the brain region. My study narrows down the list of candidates to start with and at the same time suggests previously unknown candidates. This offers a basis for new hypothesis based approaches. Finally, creating gene networks for all tested behaviours would be enlightening in that one might tell apart converging and specific parts of networks.

4.6 Distinct properties of punishment and relief memory

The finding that punishment and relief memories are influenced by distinct genes such as specific LTM genes that are mainly in the candidate list for punishment but not for relief learning, resembles the result of distinct memory properties, i.e. no LTM and ARM could be found for relief learning [78]. Regarding cold-anesthesia, both punishment and reward memories have been shown to be only partially susceptible within the first 2 hours following training [78, 320, 281, 177, 329, 146, 98, 170, 171]. That is, cold-anesthesia typically spares a so-called amnesia-resistant component of reward and of punishment memory. Indeed I confirmed that punishment memory 1 hour after training is composed of an anesthesia-sensitive component and an anesthesia-resistant component ([78], Fig.3.20). Critically however, cold-anesthesia abolishes relief memory completely ([78], Fig.3.20). Given that for punishment memory, anesthesia-sensitive versus resistant components seem to have partially different genetic requirements (i.e. anesthesia-resistance: *radish*, *bruchpilot*, and anesthesia-sensitive: *synapsin*, *rutabaga*, *amnesiac*, see [146, 98, 170, 171]), it will be interesting to look for roles of these genes in relief learning.

Current literature supports the theory that ARM and LTM are mutually exclusive in *Drosophila* [146, 256]. That is, LTM is produced using a spaced training protocol (described in the introduction) and also is protein synthesis dependent [329]. ARM is built up during massed training and is protein synthesis independent [76, 50]. Supporting this, mutants of *ala*, specific for LTM, had decreased learning scores with increased number of spaced training trials [249, 146]. Also activity of a certain pair of oscillatory dopaminergic neurons can influence if LTM or ARM is built depending on the starvation status of the fly [256]. However, whereas mutually exclusiveness can be supported for 24 hours memory using spaced and massed training, it is still not clear how many memory phases co-exist after 2

hours. Interestingly, in this study a slightly different spaced training paradigm to previous studies was used and an anesthesia-resistant punishment memory part remains after 2 h. Further experiments need to be carried out to clarify whether this remaining memory is protein synthesis dependent or not.

Given observable similarities of punishment and relief learning between fly and mammals, including humans [8, 7] my findings could contribute to uncovering common mechanisms. This means, apart from punishment memories being stronger than relief memories in fly and human, anesthesia can erase relief memories in flies and maybe also in humans. This can have following implications on treatment of punishment and relief memories towards the same traumatic event in psychiatric disorders such as PTSD: while trying to erase punishment memory, one may unwittingly also erase relief memory. Dependent on the relative strength of these memories and the relative effectiveness of treatment, the net effect of such manipulation may make the overall-mnemonic effect of the traumatic episode even more adverse [78].

4.7 Conclusion

First, genome-wide analysis of variation among inbred strains in relief and punishment learning and their underlying innate responses to electric shock and odour avoidance together with their association to gene-expression levels and SNPs revealed many common and distinct genes influencing these traits. This suggests many pleiotropic and overlapping gene functions among these traits as well as specific ones only found for one trait. Second, enrichment analysis supports the comprehensiveness of these gene lists, as for example nocicptive genes are enriched in the electric shock avoidance candidate gene list or clustering of LTM genes can be found in the punishment learning list. Third, the gene-network for electric shock avoidance was also enriched for bristle function. In total, the function of 12 candidate genes was independently validated using mutants, among them, bristle genes, a heat receptor and a receptor implicated in stress responses suggesting promising results also for the remaining candidate lists and a good starting point for deeper analysis of mechanisms involved in electric shock avoidance. Furthermore, human homologues for insensitivity to pain found in electric shock avoidance and punishment learning raises hope for the translatability of this study to mammalian research. Fourth, the finding, that relief memory has no amnesia-resistant component and no LTM is a large step forward in dissecting this so far ignored kind of memory. Further investigation of these different

mechanisms underlying opposing memories might considerably advance our understanding of psychiatric diseases.

Appendix S

Supplementary tables

For Supplementary tables see attached CD.

Table S.1: Gene expression level associations for electric shock avoidance. For each probe-set that had a strongly strain-variable expression level, a linear regression was performed between the mean expression level and the unisex shock avoidance scores. $\beta 1$ is the respective estimate for the effect of the expression level on shock avoidance. Negative $\beta 1$ values indicate that the higher the expression level was the stronger was the shock avoidance; positive $\beta 1$ values reflect vice versa. The t and P values refer to the results of a two-tailed t-test comparing $\beta 1$ to zero. Based on the P values of all 10 121 strain-variable probe-sets, Benjamini Hochberg False Discovery Rates (FDR) were calculated. I list probe-sets with $FDR < 0.01$, excluding those that correspond to multiple genes. FlyBase gene IDs are based on R Bioconductor package drosophila2.db and FlyBase as described in Material and Methods.

Table S.2: Gene expression level associations for BA avoidance. Procedure is as described in S1 but for BA avoidance scores.

Table S.3: Gene expression level associations for OCT avoidance. Procedure is as described in S1 but for OCT avoidance scores.

Table S.4: Gene expression level associations for punishment learning. Procedure is as described in S1 but for punishment learning.

Table S.5: Gene expression level associations for relief learning. Procedure is as described in S1 but for relief learning.

Table S.6: Single nucleotide polymorphism (SNP) associations for electric shock avoidance. For each autosomal SNP with a favorable minor allele frequency and call rate, a linear regression was performed between the allele type and the unisex shock avoidance scores. $\beta 1$ is the respective estimate for the effect of allele type on shock avoidance. The t and P values refer to the results of a two-tailed t-test comparing $\beta 1$ to zero. I list SNPs with $P < 0.05/1387514 = 3.6010 - 8$ corresponding to a Bonferroni correction over all 1 387 514 tested SNPs. Annotations are based on *Drosophila melanogaster* reference genome version 5.35 and FlyBase as described in Material and Methods. We excluded those SNPs that were mapped to inter-genic regions or to multiple genes. Sex-chromosome single nucleotide polymorphism (SNP) associations are treated the same except the analyses were done for each sex separately , using the sex-specific shock avoidance scores and double effect size for male associations as described in Material and methods. SNPs with $P < 3.6010 - 8$ for the SNP with the lowest P-value for each gene are listed. Other types of SNPs are separately listed as well.

Table S.7: Single nucleotide polymorphism (SNP) associations for BA avoidance. Procedure is as described in S6 but for BA avoidance.

Table S.8: Single nucleotide polymorphism (SNP) associations for OCT avoidance. Procedure is as described in S6 but for OCT avoidance.

Table S.9: Single nucleotide polymorphism (SNP) associations for punishment learning. Procedure is as described in S6 but for punishment learning.

Table S.10: Single nucleotide polymorphism (SNP) associations for relief learning. Procedure is as described in S6 but for relief learning.

Table S.11: Candidate genes for electric shock avoidance revealed by expression level- and / or SNP-associations (from Tables S1-10). Genes with ambiguous FlyBase IDs are excluded as described in Material and Methods. Independent validation in electric shock avoidance refers to the results presented in Fig. 3.6 and Table S24. For details of implication in locomotion or nociceptive behaviour as well as orthology to human pain insensitivity disease genes, please refer to Table S21. For details of bristle-implication, see in the results.

Table S.12: Candidate genes for BA avoidance. Procedure is as described in S11 but for BA avoidance.

Table S.13: Candidate genes for OCT avoidance. Procedure is as described in S11 but for OCT avoidance.

Table S.14: Candidate genes for punishment learning. Procedure is as described in S11 but for punishment learning.

Table S.15: Candidate genes for relief learning. Procedure is as described in S11 but for relief learning.

Table S.16: Enriched Gene Ontology (GO) terms for electric shock avoidance. The candidate gene list in Table S11 was analyzed against the background of fruit fly genome for enrichment in GO terms for Biological Process, Cellular Compartment and Molecular Function using the online tool DAVID 6.7. P values and Benjamini Hochberg False Discovery Rates (FDR) refer to the results of Fishers exact tests.

Table S.17: Enriched Gene Ontology (GO) terms for BA avoidance. Procedure is as described in S16 but for BA avoidance.

Table S.18: Enriched Gene Ontology (GO) terms for OCT avoidance. Procedure is as described in S16 but for OCT avoidance.

Table S.19: Enriched Gene Ontology (GO) terms for punishment learning. Procedure is as described in S16 but for punishment learning.

Table S.20: Enriched Gene Ontology (GO) terms for relief learning. Procedure is as described in S16 but for relief learning.

Table S.21: Locomotion-, nociceptive, learning behaviour-genes and orthologs of human pain insensitivity disease genes. Lists of locomotion- and nociceptive behaviour-genes are manually compiled based on literature. Orthology to human congenital pain insensitivity genes is based on http://superfly.ucsd.edu/homophila/ and refers to a BLAST $E-value < 10^{-20}$.

Table S.22: Candidate shock avoidance genes implicated in nociception - extended list. 25 fruit fly genes implicated in behavioral responses to noxious heat, cold, touch or chemicals are listed, which are associated with shock avoidance in terms of expression level and/ or single nucleotide polymorphisms with a lower statistical threshold for significance than used in the previous analyses ($FDR < 0.1$ and $P < 10 - 5$, respectively)

Table S.23: Network genes. By superimposing the results of the gene expression level shock avoidance analyses on the existing protein-protein interaction network, a shock avoidance-relevant network of 52 genes was obtained. These genes are listed along with the statistics of their association to shock avoidance, as well as their known effects on bristles.

Table S.24: Independent validation of electric shock avoidance candidate genes. For 19 candidate genes from Table S4, I compared appropriate P-element insertion mutants to controls in terms of shock avoidance and the level of the respective mRNAs (by real-time quantitative PCR- RTQPCR). Here, the results of these analyses are documented in detail listing FlyBase IDs of targeted genes, genotype and FlyBase ID of P-element insertion strains, insertion site, control genotypes, statistics (i.e. U- and P-value and sample size N) and finally RTQPCR results as described in Fig.3.7.

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