Dissecting Tbh and Hangover function in ethanol tolerance in *Drosophila melanogaster*

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ABSTRACT

Development of ethanol tolerance is one behavior that is strongly associated with alcohol addiction in humans. *Drosophila melanogaster* has been established as a model to study the mechanistic bases of ethanol tolerance. Previously it was determined that at least two different mechanisms contribute to tolerance (Scholz *et al.*, 2005). One is acting at the level of a neuronal circuit to modulate brain function in response to ethanol and is mediated by octopamine (OA). OA is implicated in regulating organismal stress responses. The other one acting at cellular level is Hangover (Hang) dependent with Hang regulating cellular stress response likely to mediate neuroprotective mechanisms and to protect the CNS from ethanol-induced damage. In this thesis the two mechanisms were further investigated.

The key enzyme in OA synthesis is the tyramine-β-hydroxylase (Tbh) encoded by the *Tbh* gene. To get a better understanding of the molecular nature of known *Tbh* mutants the molecular organization of *Tbh* was investigated by PCR studies and Northern Blot analysis. At least eight transcripts were identified. In addition, three different antibody sera against Tbh were analyzed. Two of the antisera (Zhou *et al.,* 2008; Cibik, 2007) were confirmed to be Tbh specific. Using these antibody sera at least five Tbh isoforms were revealed. Tbh specificity of the third Tbh antiserum (Hampel, 2004) could not be confirmed completely but two additional putative Tbh isoforms were uncovered. Expression of four of the five identified Tbh isoforms was altered in Tbh^{nM18} mutants. However expression was still detectable. This indicates the mutant is not a null allele for all Tbh isoforms. Expression studies in larval CNS in combination with expression studies using head and body fractions in Western Blot analysis suggest that the identified protein isoforms are expressed in different sets of neurons and in different tissues and localized differently in the cells. To generate a complete loss of function of the *Tbh* gene, the new $Tbh^{R3\text{-}XP\text{-}del}$ mutant was generated by mutagenesis using FLP recombination. Tbh protein analysis revealed that the $Tbh^{R3\text{-}XP\text{-}del}$ mutant is also not a null allele for all Tbh isoforms. However, phenotypic analysis of the mutants further suggests that the altered isoforms (58kDa or/and 74kDa) specifically have a function in ethanol tolerance development. Further, using a heat inducible Tbh transgene it was shown that Tbh function most likely is required in the adult fly for tolerance development.

Hang is supposed to interact with RNA/DNA with *dunce* (*dnc*) being a potential target of Hang (Scholz and Klebes, unpublished data). $dnc^{\Delta 143}$ and $hang^{AE10}$ mutants share the same impairment in ethanol tolerance and in heat-ethanol cross tolerance (Scholz *et al.*, 2005; Franz, 2008). Here, it was detected that in the $dnc^{\Delta 143}$ mutant *dnc* transcripts

dnc^{RA} and *dnc*^{RL} are reduced and *hang* expression is increased. It could be shown that specifically *dnc*^{RA} is mediating ethanol tolerance. In contrast, in the *hang*^{AE10} mutant the *dnc* transcripts *dnc*^{RB} and *dnc*$^{RG/RN}$ are reduced. In the *dnc*^{1} mutant *dnc*^{RB} expression and ethanol tolerance is also reduced. Therefore a role for *dnc*^{RB} in ethanol tolerance is suggested. It is assumed that Hang is negatively regulated by Dnc^{PA} and Hang regulates *dnc*^{RB} expression. The results further suggest that there are two separate cAMP signaling pathways in which Dnc^{PA} and Dnc^{PB} operate to mediate normal ethanol tolerance. In the Dnc^{PA} dependent pathway Hang might be negatively regulated by Dnc^{PA}. This pathway is mediated only in a small set of neurons, in the PAM cluster of the mushroom body and in the F1 neurons of the fanshaped body. Interestingly, the same set of F1 neurons has been implicated in Homer dependent ethanol tolerance suggesting a common function for the neurons and/or putative interaction of Homer/*dnc*/ Hang. In the second Dnc dependent pathway specifically Dnc^{PB} might be required in a Hang dependent manner. Additional experiments show that Hang does not operate as a transcription factor for Dnc^{PB} isoforms indicating that this regulation is not on DNA but probably on RNA level. The Dnc^{PA} dependent pathway is disrupted in the *dnc*$^{\Delta 143}$ mutant whereas in *hang*^{AE10} mutants the other pathway is disrupted. In the *dnc*^{1} mutant most likely both Dnc dependent pathways regulating ethanol tolerance development are disrupted. This provides good tools to further investigate the two separate Dnc dependent pathways.

Taken together, Tbh isoforms and their relationship to the cellular stressor ethanol need to be further characterized to identify the ones required for ethanol tolerance. Furthermore, Hang might be activated by Dnc^{PA} and *dnc*^{RB} expression might be regulated by Hang in two separate pathways. This means that a third pathway regulating ethanol tolerance was found clarifying the high complexity and diversity underlying ethanol tolerance development.

ZUSAMMENFASSUNG

Toleranzentwicklung gegenüber Alkohol ist eine der Kriterien der Alkoholabhängigkeit bei Menschen. *Drosophila melanogaster* ist als Modellorganismus etabliert um zugrunde liegende Mechanismen der Alkoholtoleranzentwicklung zu untersuchen. In einer früheren Studie konnte gezeigt werden, dass wenigstens zwei verschiedene Mechanismen Toleranz vermitteln (Scholz *et al.*, 2005). Der eine Mechanismus moduliert die Gehirnfunktion in einem neuronalen Netzwerk in Antwort auf Ethanol und betrifft Oktopamin (OA). OA ist an der Regulierung von organismischem Stress beteiligt. Der andere Mechanismus wird vermittelt durch Hangover (Hang) und reguliert auf Zellebene die Antwort auf zellulären Stress. Hang schützt das zentrale Nervensystem wahrscheinlich vor von Alkohol versursachten Schäden. In dieser Arbeit wurden beiden Mechanismen weiter untersucht.

Das Schlüsselenzym in der OA Synthese ist die Tyramine-β-Hydroxylase (Tbh) welche vom *Tbh* Gen kodiert wird. PCR-Studien und Northern Blots wurden durchgeführt um die Organisation des *Tbh*-Gens besser zu beschreiben. Dabei wurden mindestens acht *Tbh* Transkripte identifiziert. Zusätzlich wurden drei verschiedene Tbh Antikörperseren auf ihre Tbh Spezifität hin untersucht. Für zwei der Antiseren (Zhou *et al.,* 2008; Cibik, 2007) konnte eine Tbh Spezifität bestätigt werden. Mithilfe dieser Antiseren wurden mindestens fünf verschieden Tbh Isoformen identifiziert. Tbh Spezifität für den dritten Antikörper (Hampel, 2004) konnte nicht vollständig bestätigt werden. Dieser Antikörper detektiert zwei zusätzliche mögliche Tbh Isoformen. Die Expression von vier der fünf bestätigten Isoformen war in der Tbh^{nM18} Mutante verändert. Tbh war jedoch noch detektierbar, was impliziert, dass die Mutante im Bezug auf alle Tbh Isoformen kein Nullallel ist. Expressionsstudien im larvalen Nervensystem in Kombination mit Expressionsstudien in Kopf und Körper von adulten Fliegen lassen darauf schließen, dass die verschiedenen Tbh Isoformen in unterschiedlichen neuronalen Untereinheiten und in unterschiedlichem Gewebe exprimiert werden. Desweiteren sind die verschiedenen Isoformen wahrscheinlich unterschiedlich in der Zelle lokalisiert. Die neue $Tbh^{R3\text{-}XP\text{-}del}$ Mutante wurde mithilfe von FLP-Rekombination hergestellt um einen vollständigen Tbh Funktionsverlust zu erzielen. Allerdings liegt in dieser Mutante Tbh weiter vor, was diese Mutante ebenfalls zu keiner Nullmutante für alle Tbh Isoformen macht. Die $Tbh^{R3\text{-}XP\text{-}del}$ Mutante zeigt reduzierte Ethanoltoleranz, was in Kombination mit der Tbh Expressionsstudie darauf schließen lässt, dass wahrscheinlich nur zwei bestimmte Isoformen (58kDa, 74kDa) Alkoholtoleranz vermitteln. Desweiteren konnte gezeigt

werden, dass die Tbh Funktion zur Vermittlung von Alkoholtoleranz womöglich erst in adulten Fliegen benötigt wird.

Es wird angenommen, dass Hang mit DNA/RNA interagiert, wobei *dunce* (*dnc*) ein mögliches Zielgen von Hang ist (Scholz und Klebes, unveröffentlichte Daten). $dnc^{\Delta 143}$ und $hang^{AE10}$ Mutanten weisen beide den gleichen Defekt in Alkoholtoleranzentwicklung und Hitze-Ethanol-Crosstoleranz auf (Scholz *et al.*, 2005; Franz, 2008). Es wurde hier gezeigt, dass in der $dnc^{\Delta 143}$ Mutante die *dnc* Transkripte dnc^{RA} und dnc^{RL} reduziert vorliegen und die *hang* Expression erhöht ist. Es konnte gezeigt werden, dass spezifisch dnc^{RA} die Ethanoltoleranz vermittelt. In der $hang^{AE10}$ Mutante hingegen sind die *dnc* Transkripte dnc^{RB} und $dnc^{RG/RN}$ reduziert. Im Vergleich mit der dnc^{1} Mutante, in der dnc^{RB} Expression und Ethanoltoleranz ebenfalls reduziert sind, zeigt sich, dass wahrscheinlich ebenfalls dnc^{RB} an der Entwicklung von Ethanoltoleranz beteiligt ist. Es wird vermutet, dass Hang negativ reguliert wird durch dnc^{RA} und Hang die Expression von dnc^{RB} reguliert. Desweitern lassen die Ergebnisse vermuten, dass es zwei separate cAMP Signalwege sind, in denen Dnc^{PA} und Dnc^{PB} agieren um Ethanoltoleranz zu vermitteln. Der Signalweg in dem Dnc^{PA} agiert wird Hang möglicherweise negativ reguliert von Dnc^{PA}. Es konnte gezeigt werden, dass dieser Signalweg in wenigen Neuronen vermittelt wird und zwar in den PAM Neuronen im Pilzkörper und in den F1 Neuronen im fächerförmigen Körper. Interessanterweise, wurde in einer früheren Studie gezeigt, dass die F1 Neurone ebenfalls Homer abhängige Ethanoltoleranz vermitteln. Dies lässt eine allgemeine Funktion dieser Neurone vermuten oder eine mögliche Interaktion von Homer/*dnc*/Hang. Im zweiten Dnc abhängigen Signalweg agiert wahrscheinlich Dnc^{PB} in Abhängigkeit von Hang. Weitere Ergebnisse zeigten, dass hierbei die Interaktion von Hang und dnc^{RB} nicht auf DNA Level ist, weil Hang kein Transkriptionsfaktor von dnc^{RB} ist. Hang reguliert dnc^{RB} wahrscheinlich auf RNA Ebene. In der $dnc^{\Delta 143}$ Mutante ist nur der Dnc^{PA} abhängige Signalweg gestört und in der $hang^{AE10}$ Mutante nur der Dnc^{PB} abhängige Signalweg. In der dnc^{1} Mutante hingegen sind vermutlich beide Signalwege unterbrochen. Die Mutanten bieten geeignete Tools um die beiden Signalwege weiter zu untersuchen.

Abschließend kann gesagt werden, dass die Tbh Isoformen und ihr Verhältnis zum zellulären Stressor Ethanol näher untersucht werden müssen um die Isoformen zu identifizieren, die für die Entwicklung von Ethanoltoleranz wichtig sind. Desweitern, im Bezug auf Ethanoltoleranz scheint Hang durch Dnc^{PA} aktiviert zu werden und dnc^{RB} Expression durch Hang reguliert zu werden und das wahrscheinlich in zwei separaten Signalwegen. Das bedeutet, dass ein dritter Signalweg, welcher Ethanoltoleranz vermittelt, gezeigt werden konnte. Dadurch wird die Komplexität und Diversität die der Entwicklung von Ethanoltoleranz zugrunde liegt deutlich.

1 INTRODUCTION

1.1 Ethanol tolerance: a criterion for alcoholism in humans

Alcoholism or alcohol use disorder (AUD) is a disease that affects people worldwide. According to information of the World Health Organization (WHO) more than 76 million people worldwide are addicted to alcohol. One in 25 deaths is caused by alcohol and worldwide yearly 2.5 million die due to diseases that are related with alcohol consumption. Alone in Germany, around 74.000 deaths yearly are caused by alcohol intake alone (26%) or by simultaneously consuming tobacco and alcohol (74%) (John and Hanke, 2002; Deutsche Hauptstelle für Suchtfragen e.V., Suchtbuch 2013). Ethanol tolerance is listed by the American Psychiatric Association in the Diagnostics and Statistical Manual DSM-IV as a criterion for alcoholism.

A distinction of different forms of ethanol tolerance is made between metabolic tolerance and functional tolerance (Tabakoff *et al.*, 1986). Metabolic tolerance affects metabolism and is mediated by factors regulating absorption, distribution, degradation or excretion of alcohol. The alcohol dehydrogenase (ADH) is one of these factors. ADH degrades ethanol to acetaldehyde (Holmes, 1994). Functional tolerance is defined as developed resistance to the effects of alcohol at the cellular level (Kalant *et al.*, 1971; Tabakoff *et al.*, 1986) with adaptive changes in the central nervous system (Fadda and Rossetti, 1998). Furthermore, ethanol tolerance is described as acute, rapid or chronic. Acute tolerance is acquired directly during a first exposure to ethanol. Rapid tolerance is induced and develops immediately after the first exposure to ethanol. It can be measured after a second exposure to ethanol. Chronic tolerance is evolved due to continuous or constantly repeated contact to ethanol eventually leading to addiction (Kalant *et al.*, 1971; Tabakoff *et al.*, 1986; Berger *et al.,* 2004). Ethanol tolerance can be a response to stress because ethanol causes oxidative stress. This means excessive generation of free radicals. Specific oxygen containing free radicals called ROS (reactive oxygen species) can damage or completely degrade essential molecules in the cells such as lipids, proteins and also DNA. It is shown that alcohol increases the generation of ROS (Sun *et al.*, 2001; Wu and Cederbaum, 2003; Albano, 2006). Alcohol tolerance might also be on the basis of reinforcing ethanol as

a positive stimulus indicating then a learned component (van Ree, 1979; Vogel-Sprott, 1997).

1.2 *Drosophila melanogaster*: a model to study ethanol tolerance

Behavior of *Drosophila melanogaster* after ethanol exposure is similar to that of humans. Excessive exposure to ethanol eventually leads to sedation along with losing postural control. In *Drosophila,* before flies are sedated an initial startle response can be observed followed by a period of hyperactivity (Singh and Heberlein, 2000; Scholz *et al.*, 2000; Wolf *et al.*, 2002; Wolf and Heberlein, 2003). Repeated exposures to ethanol lead to ethanol tolerance development. In *Drosophila,* ethanol tolerance is measured in different ways. Firstly, an increase of resistance towards ethanol can be detected by enhanced postural control after a second exposure to ethanol (Scholz *et al.*, 2000; Heberlein *et al.*, 2004; Scholz, 2005; Berger *et al.*, 2008). Another way to determine tolerance is to compare levels of sedation after first and second exposure to ethanol (Urizar *et al.*, 2007; Kong *et al.*, 2010). The recovery time after exposure to ethanol is also used to investigate ethanol tolerance in *Drosophila* (Berger *et al.*, 2004; Cowmeadow *et al.*, 2006; Krishnan *et al.*, 2012). Like in humans, continuous or constant repeated exposure to ethanol eventually leads to addiction also in *Drosophila* (Wolf and Heberlein, 2003; Devineni and Heberlein, 2010; Awofala, 2011; Robinson *et al.*, 2012). There are different pathways and mechanism shown to influence ethanol tolerance in *Drosophila*. Like in humans ADH affects alcohol induced behaviors in *Drosophila*. *Adh* mutants display impaired ethanol tolerance development (Ogueta et al., 2012) indicating tolerance regulation on a metabolic level. Further, ethanol tolerance in *Drosophila* can be regulated for example by neuronal signal transduction by the biogenic amine OA (Scholz *et al.*, 2000) but not by the biogenic amine dopamine (Bainton *et al.*, 2000). In addition, the neurotransmitter serotonin influences tolerance development. The serotonin transporter (SERT) transports back the neurotransmitter into the pre-synaptic neurons after serotonin was released into the synaptic cleft due to signal transmission. *dSERT* mutants fail to develop normal ethanol tolerance (Kaiser, 2009). Further, also for the phosphodiesterase Dnc, which regulates levels of the secondary messenger cAMP, a role in regulating ethanol tolerance was described

(Franz, 2008). In addition, it is also shown that a stress pathway defined by the Hang protein is required for ethanol tolerance development (Scholz *et al.*, 2005).

1.3 Octopamine mediates ethanol tolerance

1.3.1 Invertebrate ortholog of vertebrate norepinephrine

Firstly, octopamine (OA) was detected in the mollusc *Octopus vulgaris* (Ersparmer, 1948; Erspamer and Boretti, 1951). The OA of invertebrates is structural related to the vertebrate norepinephrine, also called noradrenaline. The only structural difference between these two molecules is that OA displays one hydroxyl group less in the phenol ring than norepinephrine (Fig. 1.3.1). OA and norepinephrine are both synthesized from tyrosine but via different pathways. OA is made out of tyrosine via tyramine whereas intermediate products of the norepinephrine pathway are DOPA and dopamine (Adamo 2008). In many invertebrate species, including insects, OA is detected (Axelrod and Saavedra, 1977; David and Coulon, 1985; Roeder, 1999). So far

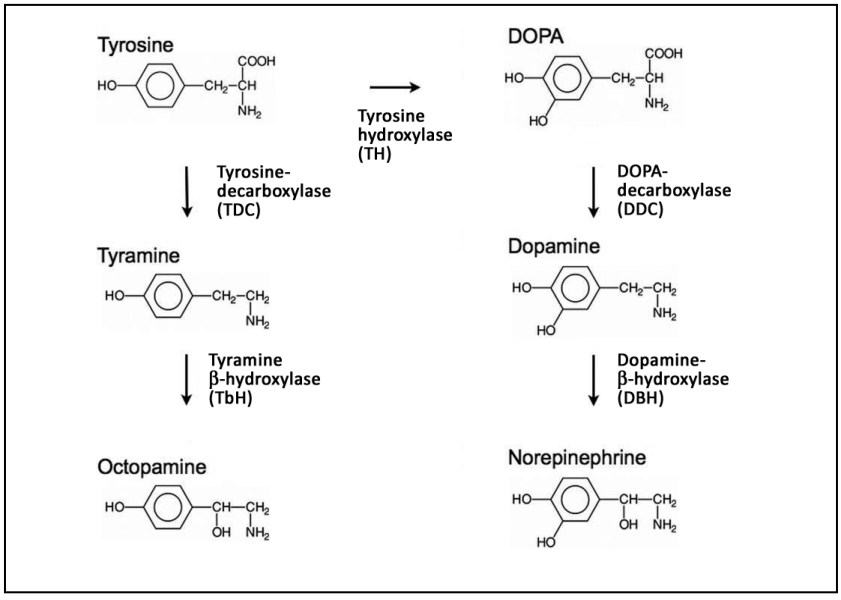


Fig. 1.3.1. Synthesis of OA and norepinephrine.
OA is synthesized in two steps. At first tyrosine is decarboxylated by the tyrosine-decarboxylase (TDC) to tyramine. Then tyramine is hydroxylated to OA by the tyramine-β-hydroxylase. Norepinephrine is synthesized of tyrosine as well but in three steps. Tyrosine is hydroxylated to DOPA by the tyrosine-hydroxylase. The DOPA-decarboxylase converts DOPA to dopamine. And in the final reaction dopamine is hydroxylated to norepinephrine by the dopamine-β-hydroxylase (modified after Barron *et al.*, 2010).

it is only known that insects use OA and not norepinephrine as a signaling molecule whereas molluscs use both (insects: Roeder, 1999; Schneider *et al.*, 2012; Scholz *et al.*, 2000; molluscs: Saavedra et al., 1974; Lacoste *et al.*, 2001-1; Lacoste *et al.*, 2001-2; Vehovszky *et al.*, 2005).

1.3.2 OA: A neurotransmitting, neuromodulating and neurohormonal function to mediate behavior in invertebrates

OA mediates different behaviors. It is shown for example that it initiates and mediates flight in moths and locusts (Claassen and Kammer, 1986; Sombati and Hoyle, 1984; Candy, 1978; Goosey and Candy, 1980). Furthermore OA influences aggression behavior in crickets and fruit flies (Stevenson *et al.*, 2005; Zhou *et al.*, 2008). In the honey bee OA also regulates foraging behavior, the division of labor and nestmate recognition (Page and Erber, 2002; Schulz *et al.*, 2003; Robinson *et al.*, 1999). It is also known that OA has an influence on learning and memory in fruit flies and honey bees (Schwärzel *et al.*, 2003; Menzel and Müller, 1996). In addition, ovulation of female fruit flies is regulated by OA as well (Monastirioti *et al.*, 1996; Monastirioti, 2003). By regulating these behaviors OA can operate as a neurotransmitter, neuromodulator and neurohormone (Orchard, 1982; Burrows, 1996; Farooqui, 2012). When released to the haemolymph of insects, one can say OA plays a neurohormonal role. For example, the fight-or-flight behavior in crickets was investigated in regard to concentration of OA in the haemolymph (Adamo *et al.*, 1995). However, neurotransmitters are endogenous molecules that are packaged into synaptic vesicles. After being released into the synaptic cleft they bind to specific receptors at the postsynaptic membrane. They transmit signals directly and do not modify the signal strengths. As a neurotransmitter OA for example regulates emission in the light organ of fireflies (Robertson and Carlson, 1976; Copland and Robertson, 1982). Neuromodulators can enhance or weaken incoming signal transmissions and therefore modify the output signal. A neuromodulatory role for OA is shown in different behaviors, like sting response and dance behavior in honey bees (Burrell and Smith, 1995; Barron *et al.*, 2007; Farroqui, 2007).

1.3.3 OA: stress and reward in invertebrates

OA is associated to be activated in response to stress. For example it is shown that OA levels are increased during flight-or-fight behaviors in locusts (Orchard *et al.*, 1993; Adamo *et al.*, 1995; Adamo and Baker, 2011). Also in honey bees it is shown that OA levels are changed in the brain due to cold stress (Chen *et al.*, 2008). Furthermore, in locusts OA is released into the haemolymph due to food deprivation (Davenport and Evans, 2008). In the american cockroach it is shown that the Tbh enzyme that synthesizes OA is upregulated due to mechanical stress which subsequently indicates an upregulation of OA (Châtel *et al.*, 2013). OA mediated regulation of stress either can lead to a change of the 'inner body/cell status' or a behavioral change. An example of the influence of OA changing in inner 'body/cell status' is the enhanced phagocytosis of hemocytes in cockroaches due to bacterial challenges (Baines and Downer, 1994). Also circulation of hemocytes in moths is upregulated by OA in response to bacterial stress (Kim and Kim, 2010). OA might also be involved in regulating cell volume during hypo-osmotic stress as shown in crustacean (Edwards and Pierce, 1986). An example of a behavioral change due to stress can be found in ethanol induced behavior in *Drosophila*. Ethanol causes oxidative stress. Flies lacking OA show reduced preference towards ethanol and are less tolerant (Scholz *et al.*, 2000; Schneider *et al.*, 2012) whereas heat-ethanol-cross tolerance is not impaired (Scholz *et al.*, 2005). However, influence of OA on alcohol induced behavior might also be on the basis of reinforcing ethanol induced rewards. OA is also considered to be the signal for the reward system in insects, including for example appetitive conditioning/olfactory memory and sugar reward (Hammer and Menzel, 1998; Menzel, 2001; Schwärzel *et al.*, 2003; Unoki *et al.*, 2005; Burke *et al.*, 2012; Schneider *et al.*, 2012; Perry and Barron, 2013).

1.3.4 Expression of OA in *Drosophila* larval CNS

Due to its neurotransmitting and neuromodulating role to mediate different behaviors in *Drosophila* OA occurs in the larval and in the adult CNS. But only in parts of the CNS OA is required for signal transmission and modulation. Therefore OA only appears in parts of the CNS. The OA immunoreactivity in the larval CNS was described by Monastirioti and colleagues (Monastirioti *et al.*, 1995) (Fig. 1.3.4). OA positive neurons are detected along the midline or close to it as single cells, pairs or

clusters. No OA positive cell bodies are found in the brain hemispheres. Here OA positive varicosities can be detected. In the ventral ganglion numerous OA immunoreactive varicosities are localized. Neuronal somata are detected in the ventral ganglion as well. In the subesophageal medial (SM) region about 10-14 cells are OA positive. In the thoracic region of the ventral ganglion three additional pairs of OA positive neurons flank the midline (PM, paramedical). Along the ventral midline in the abdominal ganglion OA reactive cells are present as well (AM, abdominal medial).

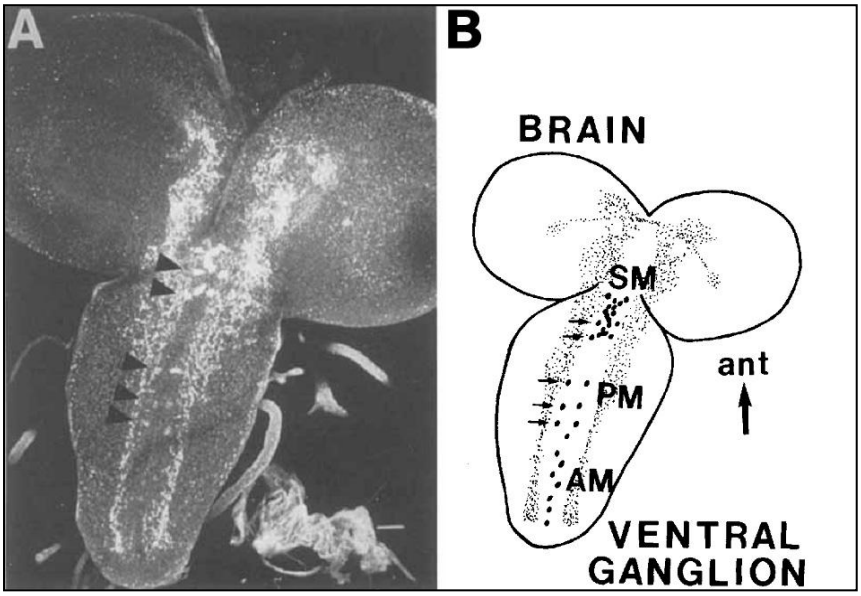


Fig. 1.3.4. OA immunoreactivity in the larval CNS.
A) OA distribution in the larval CNS. B) Schematic drawing of OA expression in the larval CNS. OA positive somata in the larval CNS are only detected in the ventral ganglion. The detected cells are located subesophageal medial (SM), paramedial (PM) and abdominal medial (AM). Furthermore plenty of OA immunoreactive varicosities can be seen in the two brain hemispheres and in the ventral ganglion (modified after Monastirioti *et al.*, 1995).

1.3.5 Tbh: The key enzyme for the OA synthesis

The enzyme tyramine-β-hydroxylase (Tbh) is the key enzyme in the OA synthesis. It converts tyramine to OA in the second step of the OA synthesis pathway (Fig. 1.3.1). The Tbh enzyme is a copper dependent hydroxylase. This class of enzymes is found in eukaryotes and it is shown that they play an important role in the biosynthesis of different neurotransmitters. Tbh is the insect homolog of the DBH which converts

dopamine to norepinephrine. The *Drosophila* Tbh protein and the mammalian DBH share 39% identity and 59% similarity (Monastirioti *et al.*, 1996). In vitro, DBH also can hydroxylate tyramine to OA (Goldstein and Contrera, 1961). Both the Tbh enzyme and the DBH enzyme bear two copper type II dependent monooxygenase domains which form the two copper centers Cu(II) that are essential for the postulated hydroxylation reaction (Gray *et al.*, 2006; Hess *et al.,* 2008). In the first step of the hydroxylation reaction the copper centers are being reduced by the co-substrate ascorbate. The second co-substrate O_2 then binds to one of the reduced copper centers forming a Cu(II)-superoxide intermediate. Afterwards an H atom is abstracted from the organic substrate tyramine producing an organic radical and Cu(II)-superoxide. The O-O bond within the Cu(II)-superoxide is split by an electron transfer from the second reduced unattached copper center. Water is released due to the electron transfer and the split of the O-O bond. The one oxygen leftover together with the copper is called Cu(II)-oxo species which eventually hydroxylates the organic radical at the tyramine molecule. OA is released and Tbh is ready for another turnover reaction of tyramine.

The Tbh enzyme in *Drosophila* is encoded by the *Tbh* gene consisting of eight exons. Currently two transcripts are annotated on flybase (http://flybase.org/reports/%20FBgn0010329.html; state: september 2013) that only differ in their 5'UTR region. The resulting proteins exhibit a size of 74 kDa. Several mutants for *Tbh* exist. The Tbh^{nM18} mutant was generated by P-element mutagenesis (Monastirioti *et al.*, 1996). The first 32 bp of the coding sequence within the second exon are deleted (Fig. 1.3.5.1). The mutant is rated as a null allele but it could be shown that *Tbh* transcript is still present in low concentration in the mutant (Ruppert, 2010). The gene is still transcribed and not fully disrupted. Nevertheless measurable levels of OA were not detected in the mutant (Monastirioti *et al.*, 1996). Consequently the level of tyramine is increased because tyramine cannot be hydroxylated. Tyramine may play its own role as a neurotransmitter in the nervous system (Kononenko *et al.*, 2009). Therefore within the Tbh^{nM18} mutant both OA and also tyramine transmitting function might be altered. The activity of TDC, the enzyme that synthesizes tyramine from tyrosine, is reduced in the mutants potentially to regulate the increased levels of tyramine (Gruntenko *et al.*, 2004). The Tbh^{nM18} mutant shows different behavioral phenotypes. For example rewarded olfactory memory and learning are strongly reduced whereas punishment learning is not

impaired (Schwärzel *et al.*, 2003; Sitaraman *et al.*, 2010; Yarali and Gerber, 2010). Larval locomotion is altered in the Tbh^{nM18} mutant. A linear locomotion pattern is lacking in mutant larvae due to fewer rhythmic bursts and less spiking activity (Fox *et al.*, 2006). Locomotion deficits can be partially rescued by feeding yohimbine, a tyramine receptor anatagonist which strengthens the assumption of tyramine operating as a neurotransmitter itself (Saraswati *et al.*, 2003). Besides locomotion Tbh^{nM18} flies also display problems in their flight performance (Brembs *et al.*, 2007). Also the jump muscle performance is reduced in this mutant (Harvey *et al.*, 2008). Other behavioral deficits of the mutant are impaired aggression and reduced sleep/increased waking activity (Hoyer *et al.*, 2008; Zhou *et al.*, 2008; Crocker and Sehgal, 2008). Furthermore female Tbh^{nM18} are sterile. They cannot lay eggs due to a non-functional egg laying mechanism (Monastirioti *et al.*, 1996; Monastirioti *et al.*, 2003).

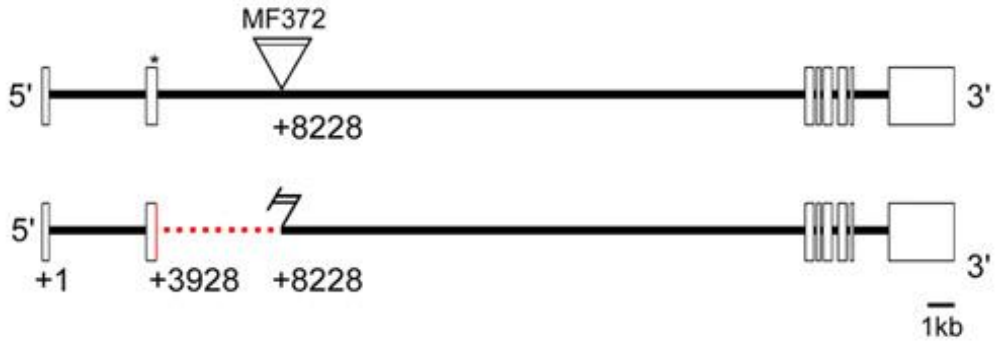


Fig. 1.3.5.1. Deletion mapping of the Tbh^{nM18} mutant.
The Tbh^{nM18} is generated by P-element mutagenesis with the MF372 transposon. The annotated genomic organization of the *Tbh* gene with its eight exons is shown. The given positions refer to the first base of the *Tbh* gene as +1. Only 32 bp of the coding sequence at the end of the second exon are deleted (dotted line; Ruppert, 2010).

The Tbh^{nM18} flies fail to develop normal ethanol tolerance (Scholz *et al.*, 2000; Fig. 1.3.5.2). Heat-ethanol cross-tolerance (Scholz *et al.,* 2005) and ethanol sensitivity (Scholz *et al.*, 2000; Fig. 1.3.5.2) of the mutant flies however are not impaired.

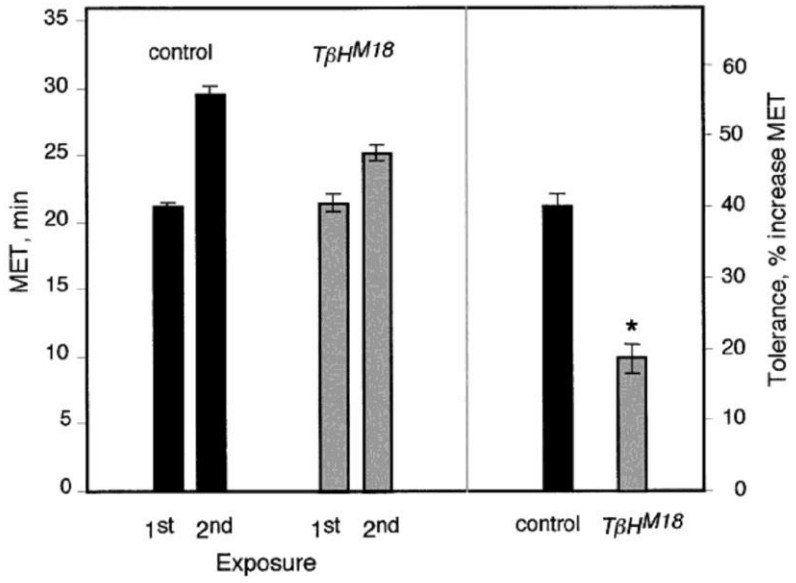


Fig. 1.3.5.2. The *Tbh^{nM18}* mutant displays reduced ethanol tolerance.
Control flies and *Tbh^{nM18}* mutant flies are tested in the inebriometer assay. The Mean Elution Times after a first (MET1) and second (MET2) exposure to ethanol are presented (left). The MET1 is associated with ethanol sensitivity. The percentage increase from MET1 to MET2 represents ethanol tolerance (right). Mutant *Tbh^{nM18}* flies show no change in ethanol sensitivity but fail to develop normal ethanol tolerance (Scholz *et al.*, 2000).

1.4 A Hangover dependent cellular stress component for ethanol tolerance development

1.4.1 The *hangover* gene

Firstly the *hangover* (*hang*) gene was described with its influence on a cellular stress pathway required for ethanol tolerance (Scholz *et al.*, 2005). The *hang^{AE10}* mutant was isolated in a screen of lines carrying different P-element insertions that were tested for their ability to develop ethanol tolerance (Scholz e*t al.*, 2005). In this mutant a P-element is inserted in the first exon of the gene into the coding sequence and therefore disrupts the reading frame (Fig. 1.4.1). Like *Tbh^{nM18}* mutants the *hang^{AE10}* mutants show reduced but still existent ethanol tolerance. Double *Tbh^{nM18}* and *hang^{AE10}* mutants show a complete loss of tolerance development which suggests that the development of tolerance relies on two parallel pathways, one affected by the

octopaminergic system and one regulated by Hang. Further, heat-ethanol-cross-tolerance is impaired in *hang*^{AE10} mutants but not in *Tbh*^{nM18} mutants suggesting that Hang defines a stress pathway required for ethanol tolerance development. Ethanol sensitivity of *hang*^{AE10} flies is not altered. Besides behavioral defects correlated with ethanol the *hang*^{AE10} flies however are not impaired in shock perception and in the perception of different odors and they do not display a defect in short term learning nd memory (Franz, 2008).

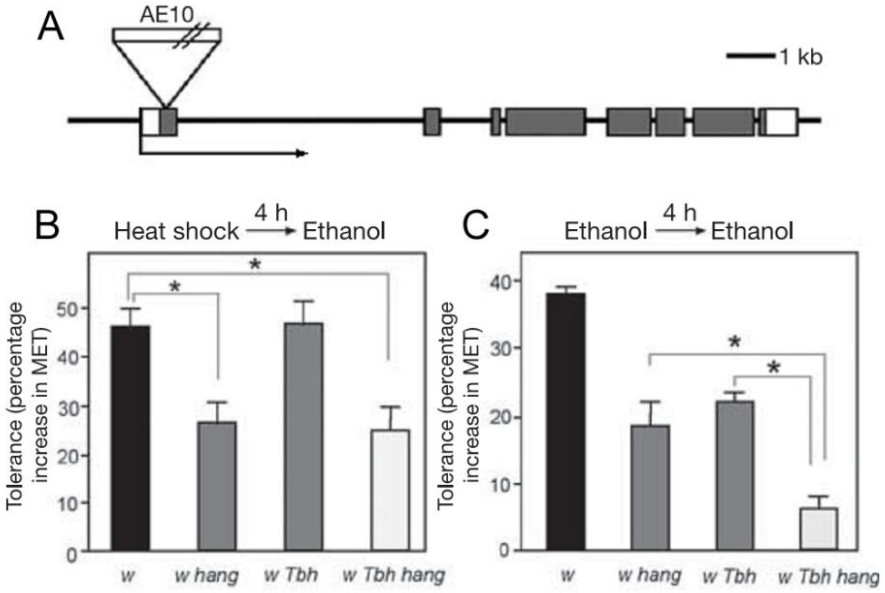


Fig. 1.4.1. The *hang*^{AE10} mutant is impaired in ethanol tolerance and in heat-ethanol-cross tolerance.
A) The insertion of the AE10 P-element in the genomic organization of the *hangover* gene is shown. The reading direction of the gene is presented with an arrow. Grey boxes represent coding sequences and white boxes non coding sequences. B) *hang*^{AE10} mutants but not *Tbh*^{nM18} mutants are impaired in heat-ethanol-cross tolerance. C) *hang*^{AE10}, *Tbh*^{nM18} double mutant show a lower level of ethanol tolerance than the two mutants themselves (Scholz *et al.*, 2005).

1.4.2 The Hangover protein

The *hang* gene encodes the Hangover protein (Hang) which is broadly expressed in the adult *Drosophila* brain (Scholz *et al.,* 2005). The Hang protein consists of 1901 amino acids and bears 15 zing finger domains of the C_2H_2 class (Scholz *et al.*, 2005; Fig. 1.3.2). The C_2H_2 domain consists of the amino acids cystidin (C) and histidin (H).

The C_2H_2 zinc fingers are associated with a nucleic acids binding motif and can be found for example in transcription factors and in RNA-binding proteins (Miller et al., 1985; Joho *et al.*, 1990; Jiang and Pan 2012). Zinc finger domains exhibit a specific secondary protein structure which is stabilized by a zinc ion that binds to the cystidin and histidin amino acids of the domain. Two of the 15 zinc finger motifs found in the Hang protein belong to the specific U1-like subclass that is particularly associated with RNA modifying proteins (Nelissen *et al.*, 1991). The high number of zinc finger domains of the Hang protein and two more specific domains among them suggests that Hang may bind to DNA and RNA. Furthermore the Hang protein also bears an EF-hand motif that is associated with Ca^{2+} binding proteins (Ikura *et al.*, 2002). Calcium can operate as a messenger substance to activate proteins. This suggests that Hang might be activated by calcium.

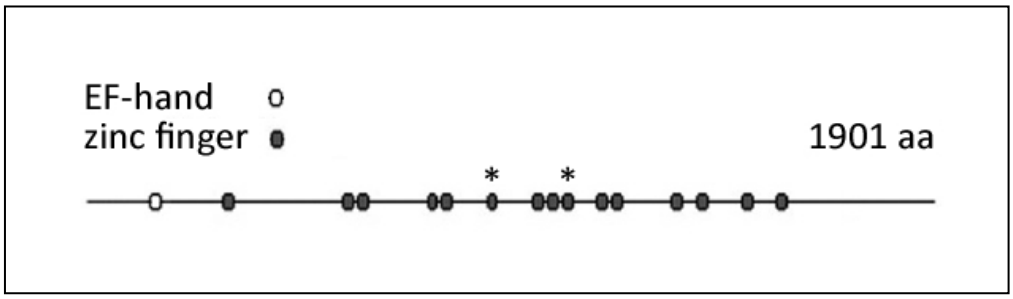


Fig. 1.3.2. The protein structure of the Hang protein.
The Hang protein with its protein domains is shown. The protein bears 15 zinc finger domains (filled circles) and one EF-hand motif (unfilled circles). Two of the 15 zinc finger motifs belong to the U1-like subclass (*) that is associated with RNA binding (modified after Franz, 2008).

1.4.3 *dunce* as a potential target of Hang

Due to its protein structure Hang probably can bind eiher DNA or RNA or both. To identify potential target genes of the Hang protein a cDNA microarray was performed (Scholz and Klebes, unpublished data). Therefore the *hang* mutant $hang^{AE10}$ was used to compare gene expressions in this mutant with the genes expressions in a wild type control. With this experiment it was shown that the *dnc* gene is a potential target of Hang because transcript levels of this gene are down regulated in the mutant (Fig. 1.3.3). *In vitro*, it can be shown that Hang binds to *dnc* (Franz, 2008).

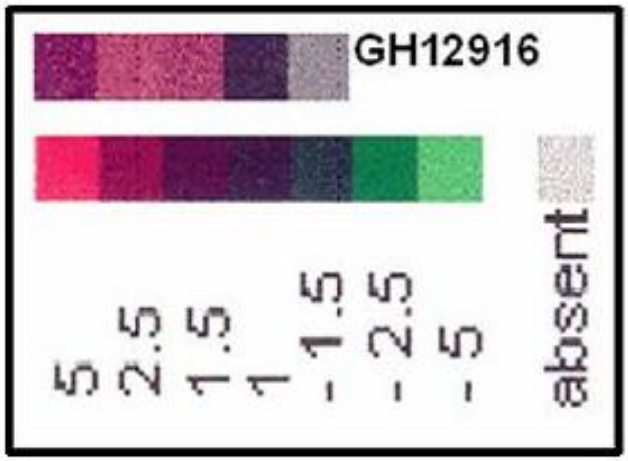


Fig. 1.3.3. *dunce* is a potential target of Hang.
A cDNA microarray where the transcriptome of wild type flies is compared to the transcriptome of *hang*^{AE10} mutants. Different EST clones are spotted on a microarray plate and are incubated afterwards with the cDNA of wild type control and mutant. Wild type cDNA is labeled with a red dye whereas the mutant cDNA is labeled with a green dye. Wild type and mutant cDNA compete for the binding sites of the EST clones. Red squares mean that the gene is higher expressed in the wild type and therefore down regulated in the mutant. A green square stands for an opposite gene regulation. A colour code is presented to show differences between wild type control and mutant. Only the transcript analysis of the *dunce* gene (GH12916) is shown here with more or less red squares. This indicates that the *dunce* gene is down regulated in the *hang*^{AE10} mutant (Scholz and Klebes, unpublished data).

1.4.4 Dunce is encoded by the *dnc* gene

The *dunce* gene encodes the phosphodiesterase 4b (PDE) homologue Dunce (Dnc). In *Drosophila* there are six PDE classes that either hydrolyses cAMP or cGMP or both (Day *et al.*, 2005). Dnc is a PDE that only hydrolyses cAMP (Davis and Kiger, 1981). The *dnc* gene is 163 kb long and very complex. New studies about the genomic organization of the *dnc* gene indicate that there are 18 coding exons. Eight transcripts were confirmed initiated from at least four different promotors and therefore with different transcription start sites (Gooi and Hendrich, unpublished data; Fig. 1.3.4). The transcripts are divided in four groups due to size and function of the associated proteins (modified after Qiu *et al.*, 1991). The function of group 1 including the longest transcript *dnc*^{RB} is not known yet, whereas the function of group 3 bearing transcript *dnc*^{RA} is shown to play a role in learning. Group 2 containing the transcripts *dnc*^{RJa} and *dnc*^{RJb} and group 4 with transcripts *dnc*^{RN}, *dnc*^{RG}, *dnc*^{RF} and

dnc^{RL} each share one half of the PDE functionality. Group 4 is also associated with female fertility. Within the *dnc* gene several other genes are located (Chen *et al.*, 1987; Furia *et al.*, 1990, www.flybase.org). Some of them are already characterized but seem not to be correlated with Dnc. In humans eleven different PDE families are existent. Dnc is assigned to the PDE4 family. The human homolog encoded by four different genes that are additionally alternatively spliced includes different isoforms that differ in their N terminal regions (Houslay and Adams, 2003). In *Drosophila* only the *dnc* gene is known to encode for different PDE4 isoforms. The human PDE4 proteins are divided in three categories: super-short, short and long isoforms are existent (Houslay, 2001). The long isoforms include two elements, namely the upstream conserved regions 1 and 2 (UCR1, UCR2). The short isoforms only exhibit UCR2 while the super-short isoforms not only lack UCR1 but also only have truncated version of UCR2. In the human PDEs the UCRs are shown to have a regulatory effect on the catalytic PDE function (MacKenzie *et al.*, 2000; Beard *et al.*, 2000). It is shown that the two UCR elements can form a module required for the activation of

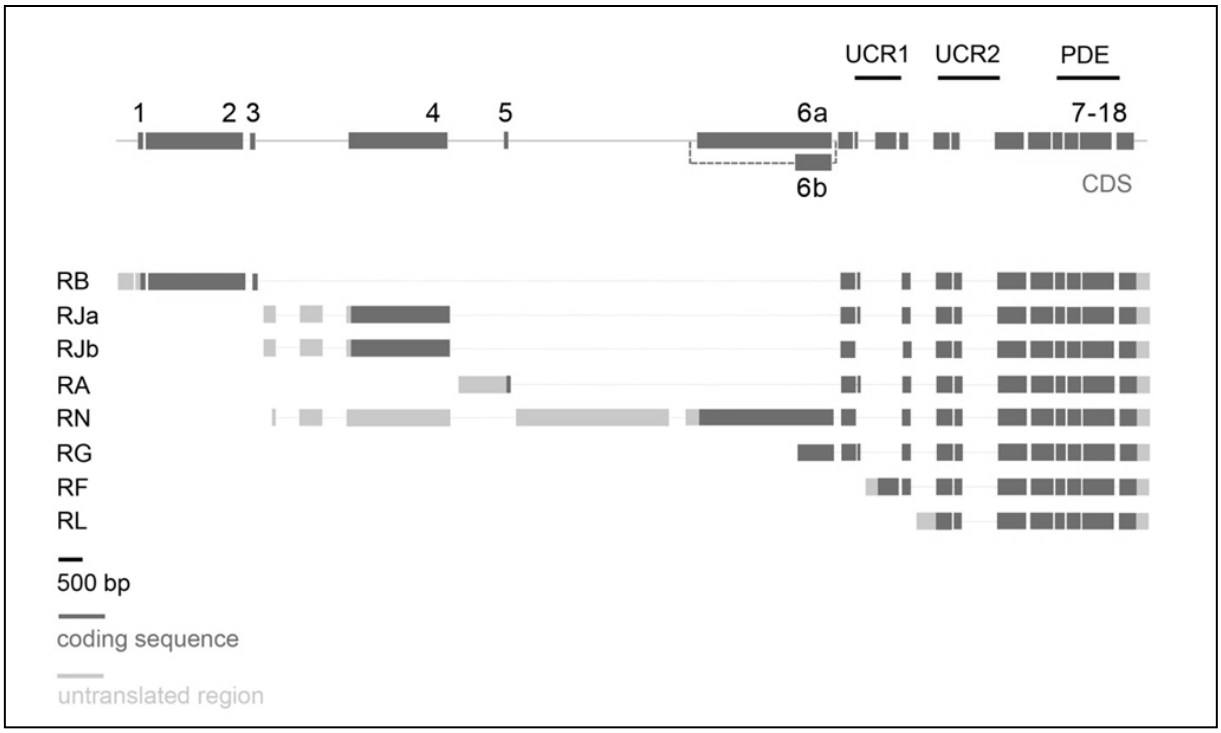


Fig. 1.3.4. The genomic organization of the *dunce* gene.
The *dunce* gene with its coding exons is shown (CDS). The eight transcripts with different transcription start sites are indicated with their coding sequence (dark grey) and untranslated regions (light grey). The introns are not in scale. The gene region that encodes the PDE activity in the C-terminal region of the protein and the UCR1/UCR2 regions are indicated as well (Scholz, unpublished).

PDE4 upon phosphorylation by the cAMP-dependent kinase (Beard *et al.,* 2000). The UCR2 element holds an autoinhibitory nature because removing this region leads to increased catalytic activity (Kovala *et al.*, 1997). The UCR1 element contains a PKA phosphorylation site being phosphorylated by PKA when cAMP levels are too high which leads to activation of PDE4 (MacKenzie et al., 2002). It is also postulated that PDE4s oligomerize to form a functional UCR1/UCR2 module (Richter and Conti, 2002; Conti and Beavo, 2007). The UCR regions of Dnc and the human PDE4 are almost 100% homolog to each other (Bolger *et al.*, 1993). The catalytic domain of phosphodiesterase activity is situated in the C-terminal section of the Dnc protein.

1.4.5 PDEs and their role in cAMP signaling

The function of PDEs is to regulate cAMP levels by hydrolyzing cAMP (cyclic adenosine monophosphate) to 5'AMP. cAMP is a secondary messenger molecule that serves for the intracellular transfer of an extracellular signal that cannot pass the cell membrane. cAMP is synthesized from ATP by adenylyl cyclases (AC) in response to the activation of membrane receptors belonging to the G-protein coupled receptors (GPCRs). The GPCRs are also known as seven transmembrane receptors because they pass through the membrane seven times. They operate through G-proteins and activate inside signal transduction. Functional selectivity to discriminate signals that use identical signaling pathways is achieved by different ligands and receptor subtypes. In a cAMP dependent signaling pathway cAMP levels are crucial because cAMP can activate protein kinase A (PKA) by phosphorylation. PKA is a tetrameric kinase consisting of two regulatory subunits (PKA-R) binding cAMP and two catalytic subunits (PKA-C) phosphorylating protein substrates. PDEs can interact with A-kinase-anchoring proteins (AKAPs) and AKAPs interact with PKAs to anchor the formed complexes in defined subcellular domains. In a regulatory loop, PKA activation by local present cAMP phosphorylates and activates PDE4 which in turn reduces cAMP (Conti and Beavo, 2007). The anchoring proteins in the AKAPs bind kinases to sites where they can be moved into the nucleus where they can phosphorylate physiologically relevant downstream targets such as transcription factors to activate them. It is shown that cAMP signaling plays a role in response to ethanol in *Drosophila*. As an acute response to ethanol the cAMP synthesis is activated while as a response to chronic ethanol exposure cAMP is reduced (Diamond

and Gordon, 1997; Bellen *et al.*, 1998). Also impairing other components of the cAMP dependent signaling pathway alters alcohol induced behaviors. *Amnesiac* encodes a neuropeptide that opertes as an AC increasing cAMP levels (Feany and Quinn, 1995) and *rutabaga* the Ca^{2+}-calmodulin sensitive AC (Livingstone *et al.*, 1984; Levin *et al.,* 1992). The major subunit of cAMP dependent protein kinase is encoded by the *DCO* gene (Lane and Kalderon, 1993). *Amnesiac*, *rutabaga* and *DCO* mutants show increased ethanol sensitivity towards ethanol (Moore *et al.*, 1998). The cAMP signaling pathway also is associated with responding to stress. Transcription factors of the FoxO class in vertebrates regulate the cellular responses to various stimuli such as energy deprivation (Greer *et al.*, 2007). In *Drosophila* it is shown that dFoxO (the *Drosophila* FoxO) regulates cAMP signaling by directly inducing the expression of an adenylate cyclase gene (Mattila *et al.*, 2009). Further, cAMP singling can activate the CREB transcription factor which is described to be involved in stress response in vertebrates (Nibuya *et al.*, 1996; Duman and Vaidya, 1998) and in *Drosophila* (Hendricks *et al.*, 2001; Honjo and Furukubo-Tokunaga, 2005).

1.4.6 Reduced ethanol tolerance of the $dnc^{\Delta 143}$ mutant

There are different available *dnc* mutants in *Drosophila* displaying reduced PDE activity (Davis and Kiger, 1981). Most of these mutants are sterile (Salz *et al.,* 1982; Lannutti and Schneider, 2001). The first *dnc* mutant isolated in a screen for defective olfactory learning and short term memory is the dnc^{1} mutant (Dudai *et al.*, 1976). The mutation of the dnc^{1} mutant is hypomorph in regard to the PDE activity. Other hypomorph *dnc* alleles are dnc^{2} and dnc^{CK}. The mutants dnc^{M11} and dnc^{M14} are considered to be amorphe alleles (Davis and Kiger, 1981). The defect of short term memory and olfactory learning is described also for other *dnc* mutants (Tully and Quinn, 1985; Roman and Davis, 2001; Franz, 2008). *Dnc* mutants are shown to have impaired other behaviors such as courtship (Greenspan and Ferveur, 2000; Gailey, 1984), proboscis extension response to sugar (Duerr and Quinn, 1982) and attention (van Swinderen, 2007). The *dnc* mutants dnc^{1} and dnc^{M11} were tested for ethanol sensitivity but no phenotype was detected (Moore *et al.*, 1998). Besides behavioral defects also neuranatomical and neurophysiological phenotypes are detected in *dnc* mutants (Shayan and Atwood, 2000; Davis, 1996). The detailed mutation mappings within the *dnc* gene of the different alleles are not known. But knowing the exact

mutation sites in the *dnc* gene would help to associate different transcripts with different behavior patterns. Therefore a new *dnc* mutant was generated by P-element mutagenesis in the Scholz lab by Anastasios Saratsis (Saratsis, 2006). This mutant is the $dnc^{\Delta 143}$ mutant. The deletion specifically affects the dnc^{RA} transcript group and thereby only the 5'UTR region and not the coding sequence (Fig. 1.3.1). The mutant flies show a defect in the cellular stress response due to defective heat-ethanol cross tolerance (Franz, 2008). The $dnc^{\Delta 143}$ flies display normal ethanol sensitivity but fail to develop normal ethanol tolerance (Franz, 2008). Besides the alteration of ethanol induced behaviors the mutant also shows another behavioral defect like other *dnc* mutants do namely a decreased function of short time memory (Franz, 2008). The flies are not impaired in shock perception and in the perception of different odors (Franz, 2008). The reduced ethanol tolerance of the $dnc^{\Delta 143}$ mutant can be restored to wild type level by induced expression of a Dnc protein fragment, containing the PDE-activity domain that exists in all *dnc* transcripts ($UAS\text{-}dnc^{All}$), in dnc^{RA}-GAL4 driven neurons shown by Mirjam Franz (Fig. 1.3.6.2 A; Franz, 2008). The dnc^{RA}-GAL4 line drives expression in a broad set of neurons throughout the brain amongst others in the mushroom body, the antennal lobes and in the central complex in the adult *Drosophila* brain (Fig. 1.3.6.2 B; Franz, 2008). The dnc^{RA}-GAL4 line (formerly characterized as dnc^{RMRA}-GAL4) was generated by Anastasios Saratsis (Saratsis, 2006). The promoter region that initiates the dnc^{RA} transcript was used to create the dnc^{RA}-GAL4 line (Fig. 1.3.6.1).

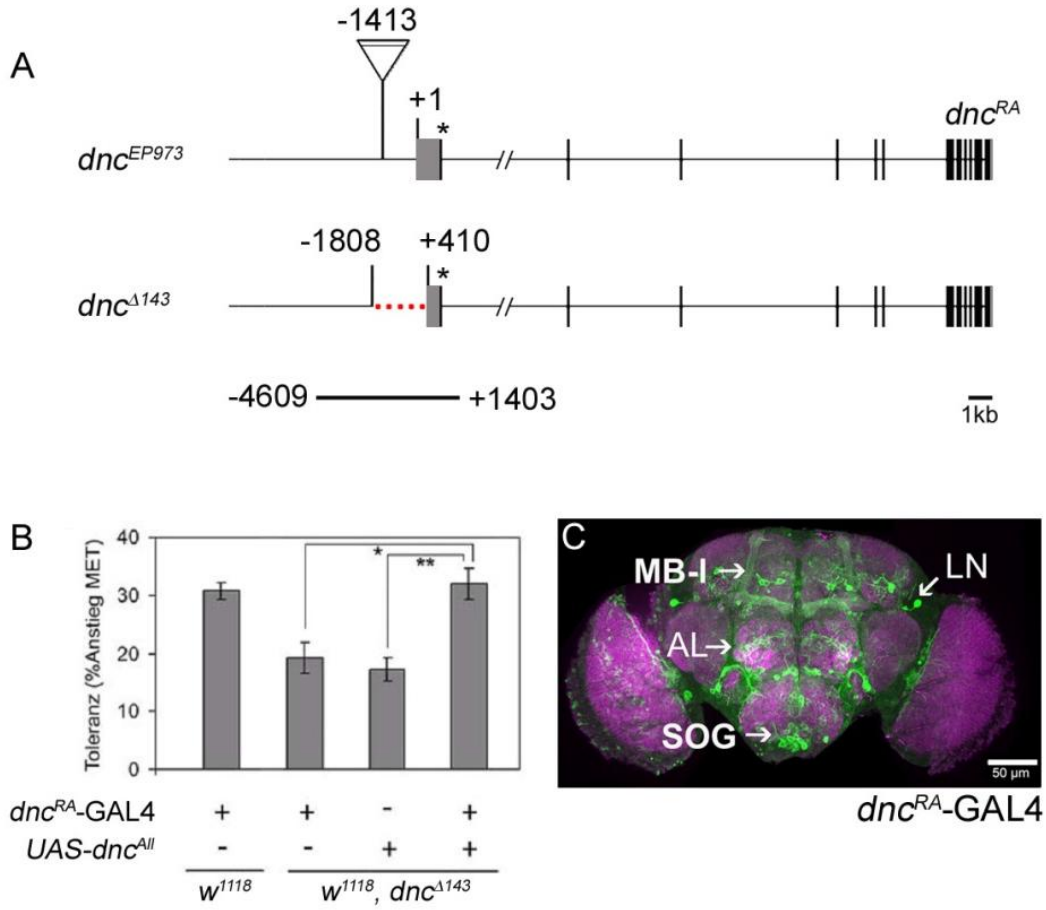


Fig. 1.3.6. Induced *dunce* expression in *dnc*^{RA}-GAL4 driven neurons restores reduced ethanol tolerance of the *dnc*$^{\Delta 143}$ mutant.
A) The *dunce* mutant *dnc*$^{\Delta 143}$ was generated by P-element mutagenesis (Saratsis, 2006). The *dnc*^{RA} region of the *dunce* gene is shown. The P-element jumpout specifically deleted sequence of the *dnc*^{RA} transcript (red dots). All positions refer to the first base pair of the gene as +1. The DNA fragment from -4609 to +1403 was used to generate the *dnc*^{RA}-GAL4 line. B) The reduced ethanol tolerance of the *dnc*$^{\Delta 143}$ is restored by *dunce* expression in *dnc*^{RA}-GAL4 driven neurons. C) The *dnc*^{RA}-GAL4 drives expression broadly througout in the adult *Drosophila* brain (MB: mushroom body, AL: antennal lobes, SOG: subesophageal ganglion, LN: lateral neurons) The scale bar represents 50 µm (modified after Franz, 2008).

1.5 The aim

The aim was to investigate the function of two stress related pathways for ethanol tolerance development in *Drosophila melanogaster*. Two processes are involved in ethanol tolerance (Scholz *et al.*, 2005). One acts on the cellular level likely to mediate neuroprotective mechanisms and the second one on the level of the organisms. To better understand how these two mechanisms function to form ethanol tolerance the function of genes implicated in these processes need to be further investigated. The biogenic amine octopamine (OA) and the Hangover (Hang) protein were the field of interest to examine the mechanisms and pathways underlying ethanol tolerance development.

OA is implicated in regulating organismal stress responses and ethanol tolerance. The key enzyme in OA synthesis is Tbh encoded by the *Tbh* gene. To understand how organismal stress mediates ethanol tolerance it is important to identify when and where Tbh function is required using Tbh^{nM18} mutants. However, previous studies implicated that Tbh^{nM18} mutants are not a null allele for Tbh function. Therefore the mutants need to be further investigated on molecular level and in turn the genomic organization of *Tbh*. In addition, in 2007 Stefanie Hampel already identified an alternatively spliced *Tbh* transcript. This raises the possibility of isoforms with different functions. Therefore it was questioned whether there are additional Tbh isoforms beside the one annotated Tbh protein. First the molecular organization of *Tbh* has to be revaluated. How many Tbh transcripts and isoforms are existent? To uncover putative transcripts the *Tbh* gene was investigated by RT-PCR and Northern Blot analysis. To analyze whether putative *Tbh* splice variants lead to functional proteins different antisera against Tbh were used for Western Blot analyses and neuroanatomical studies. This was done firstly to identify general presence in the fly and secondly to analyze putative expression in tyraminergic/octopaminergic neurons in the larval CNS. To further dissect additional Tbh function a new Tbh mutant was generated and molecular genetically and phenotypically analyzed. Therefore, qRT-PCRs, Western Blots and behavioral experiments to analyze ethanol tolerance were done. To analyze when Tbh function is required Tbh was induced firstly in adulthood by a heat shock inducible *Tbh* transgene to restore reduced ethanol tolerance. Further, to answer the question where Tbh function is required Tbh was expressed in

different sets of neurons in the in Tbh^{nM18} mutants to restore reduced ethanol tolerance.

Hang is shown to regulate cellular stress response required for ethanol tolerance development (Scholz *et al.*, 2005). Due to its protein structure Hang is supposed to interact with RNA/DNA. The *dunce* (*dnc*) gene is a potential target of Hang (Scholz and Klebes, unpublished data). *dnc* and *hang* mutants share the same impairment in ethanol tolerance development. To investigate the Hang/*dnc* interaction the *dnc* gene needs to be further investigated because of the eight Dnc isoforms. The question was whether Hang interacts with specific Dnc isoforms and therefore whether distinct Dnc isoforms are involved in ethanol tolerance development. In addition, it was questioned whether Hang interacts with *dnc* on DNA level as a transcription factor. Firstly, it was investigated which Dnc isoforms mediate ethanol tolerance and where Dnc is required. Therefore *dnc* transcript levels in several *dnc* mutants were analyzed by qRT-PCR. In addition, single *dnc* transcripts were overexpressed in the fly to test ethanol tolerance and they were expressed in the $dnc^{\Delta 143}$ mutant to restore reduced ethanol tolerance. To identify the neurons that mediate ethanol tolerance in a Dnc dependent manner *dnc* was expressed in different sets of neurons in the $dnc^{\Delta 143}$ mutant. To further investigate the Hang/*dnc* interaction, it was investigated whether *dnc* transcript levels are altered in the $hang^{AE10}$ mutant and whether *hang* expression is altered in *dnc* mutants. To identify whether the pathways regulating ethanol tolerance that are impaired in $dnc^{\Delta 143}$ and $hang^{AE10}$ mutants are the same a complementation test was done testing transheterozygous mutants for ethanol tolerance development. Furthermore, to test whether Hang mediates ethanol tolerance in the same set of neurons as Dnc, *hang* was expressed in the $hang^{AE10}$ mutant in the same neurons that require Dnc to restore reduced ethanol tolerance in the $dnc^{\Delta 143}$ mutant. To examine whether Hang operates as a transcription factor, GFP expression initiated by different *dnc* promoters was analyzed in wild type conditions and in the $hang^{AE10}$ mutant when Hang is not present.

2 MATERIAL & METHODS

2.1 Living material

2.1.1 Bacteria

E. coli XL1-blue	Agilent Technologies
E. coli BL21 (DE3)	Agilent Technologies

2.1.2 Flies

Genotype	**Chr.**	**Origin**
Canton-S (referred to as *CS*)		Lindsley & Zimm
w^{1118}	X	Lindsley & Zimm
w^{1118}, Tbh^{nM18}/FM7	X	Monastirioti *et al.*, 1996
w^{1118}, Tbh^{nM18}/FM7;; *hs-Tbh*	X, III	Monastirioti *et al.*, 2003
w^{1118};; *MKRS, hsFLP/TM6B,*Tb^{1}	III	Parks *et al.*, 2004
w^{1118}/*Bin*	X	Parks *et al.*, 2004
XP^{d01344}	X	The Exelixis Collection at Harvard Medical School
w^{1118}, XP^{d01344}	X	Manuela Ruppert (outcrossed)
XP^{d10000}/*FM7*	X	The Exelixis Collection at Harvard Medical School
w^{1118}, XP^{d10000}/*FM7*	X	Manuela Ruppert (outcrossed)

w^{1118}, *Tbh*$^{R3\text{-}XPdel}$/*FM7*	X	Manuela Ruppert, 2013
w^{1118}, NP938	X	Kyoto Stock Center
w^{1118}, NP208	X	Kyoto Stock Center
w^{1118}; *Appl*-GAL4	II	Torroja *et al*, 1999
w^{1118}, *Tbh*^{nM18}, *UAS-Tbh*/*FM7* (recombinant 31)	X	Henrike Scholz
w^{1118}, *Tbh*^{nM18}, *elav*-GAL4/*FM7*	X	Henrike Scholz
w^{1118}, *elav*-GAL4	X	Bloomington #458
w^{1118}; UAS-Tbh	II	Scholz
w^{1118}; *TDC2*-GAL4	II	Cole *et al.*, 2005
UAS-mCD8::GFP; UAS-mCD8::GFP UAS-mCD8::GFP	X, II, III	Sebastian Busch
w^{1118}; *dSert*^{1}	II	Andrea Kaiser, 2009
w^{1118}; *dSert*^{10}	II	Andrea Kaiser, 2009
w^{1118}; *dSert*^{16}	II	Andrea Kaiser, 2009
w^{1118}; *dSert*^{18}	II	Andrea Kaiser, 2009
w^{1118}, *dnc*$^{\Delta 143}$	X	Anastasios Saratsis, 2006
dnc^{1}	X	Dudai *et al.*, 1976
dnc^{M11}	X	Mohler, 1977
w^{1118}; MB247-GAL4	II	Zars *et al.*, 2000
w^{1118}; *TH*-GAL4	II	Friggi-Grelin *et al.*, 2003
w^{1118}; 78y-GAL4	II	Renn *et al.*, 1999
w^{1118};; NP6510-GAL4	III	Liu *et al.*, 2006
w^{1118}; Tub-GAL80^{ts}; NP6510-GAL4	III	Manuela Ruppert

w^{1118}, $dnc^{\Delta 143}$, UAS- dnc^{All} (recombinant 13, formerly known as w^{1118}, $dnc^{\Delta 143}$, UAS-dnc)	X	Henrike Scholz
w^{1118};; dnc^{RA}-GAL4 (formerly known as w^{1118};; dnc^{RMRA}-GAL4)	III	Anastasios Saratsis, 2006
w^{1118}, $hang^{AE10}$, UAS-dnc^{All} (formerly known as w^{1118}, $hang^{AE10}$, UAS-dnc)	X	Henrike Scholz
w^{1118}, $hang^{AE10}$	X	Henrike Scholz
w^{1118}, $hang^{AE10}$, UAS-hang	X	Isabell Schwenkert
w^{1118}, $hang^{AE10}$, D52-GAL4	X	Henrike Scholz
w^{1118}, $hang^{AE10}$, NP7145-GAL4	X	Henrike Scholz
w^{1118}, $hang^{AE10}$;; dnc^{RA}-GAL4	X, III	Henrike Scholz
w^{1118}, $dnc^{\Delta 143}$;; dnc^{RA}-GAL4	X, III	Manuela Ruppert
w^{1118}; UAS-dnc^{RL}_7.1 (referred to as w^{1118}; UAS-dnc^{RL})	II	Li Ming Gooi
w^{1118};; UAS-dnc^{RA}_6 (referred to as w^{1118};; UAS-dnc^{RA})	III	Li Ming Gooi
w^{1118}; UAS-dnc^{RG}_5 (referred to as w^{1118};; UAS-dnc^{RG})	III	Li Ming Gooi
w^{1118}; UAS-dnc^{RL}_7.1; UAS-dnc^{RA}_6 (referred to as w^{1118}; UAS-dnc^{RL}; UAS-dnc^{RA})	II, III	Manuela Ruppert

For all experiments the flies were cultivated on a standard *Drosophila* fly food, where 20 liters of food contain 160g agar, 300g dry yeast, 1200g polenta and 1600ml sugar beet molasses. As a preservative propionic acid and nipagin were added. Flies that were used for experiments were raised on constant conditions at 25°C and 65% humidity under a 12h/12h day-night rhythm.

2.2 DNA material

All primers were ordered and generated at Sigma-Aldrich. All positions refer to the first base of the first exon of the associated gene as +1. The following list shows the primers used.

Primer	**Sequence**	**Location (gene)**	**Position**
RplP0-sense	CAGCGTGGAAGGCTCAGTA	*RplP0*	+441
RplP0-anti	CAGGCTGGTACGGATGTTCT	*RplP0*	+617
actin5C-sense	TTAGCTCAGCCTCGCCACTT	*actin5C*	+1168
actin5C-anti	GCAGCAACTTCTTCGTCACA	*actin5C*	+1841
SuTpl-sense	TCCCAGAGCCACCGTTACAC	*Su(Tpl)*	+14774
SuTpl-anti	CTGGTTGCAGGCGTTTAGCGT	*Su(Tpl)*	+14874
L-Sert-RT	GTTGCCTCAGCATCTGGAAG	*dSERT*	+1544
R-Sert-RT	CAGCCGATAATCGTGTTGTA	*dSERT*	+2943
CG3419-L2	CAACTGGGCTACGTGCATC	*CG3419*	+175
CG3419-R1	GGAGTTGTTGAACTCCCAGGT	*CG3419*	+317
EST-F Primer	ACGCGCTTTCCACTTGTTCG	*Tbh*	+1
Unnamed sense	ATTCCGCTGCAGCTGAGCAG	*Tbh*	+3790

Unnamed anti	GGACTGACACTCACGGAGACA	*Tbh*	+4490
Sonde-v-L	CCAAGCTAACGGGACAAAAG	*Tbh*	+94
Sonde-v-R	GAGCAGCATCACTGGCATAA	*Tbh*	+3980
Frag2-L-EcoRI	CAAGAATTCGCAGCTGAGCAGTC AGGAT	*Tbh*	+3798
Frag2-R-XhoI	CAACTCGAGCATCCAGCTCAGCTT AATCTCC	*Tbh*	+26841
Frag3-L-EcoRI	GCGATGGTCGACTGGTACAAGCA	*Tbh*	+26860
Frag3-R-XhoI	GCGCTCGAGAACATCCATCTTGA AGACCTCACA	*Tbh*	+27073
Frag5-L-EcoRI	AAAGTCGACATGAAGCGCACGGA GCAT	*Tbh*	+28694
Frag5-R-XhoI	AAACTCGAGATAGATGCACTCCCC CAGCA	*Tbh*	+30220
Tbh-RT-L	ATCCGTACGTTCGACTGGAG	*Tbh*	+27771
Tbh-RT-R	TCGACATCTTGATGCGAAAG	*Tbh*	+28088
All_L	AGCATGCAGTGCAACAGGT	*Tbh*	+30057
All_R2	GGATTGTAGTTGGGGCACAG	*Tbh*	+30162
Mut_2.Intron_R3	AGCCGGATGACATTATCTGC	*Tbh*	+9285
Tbh-d01344-L1	TGGCACACACTTACGGGTTA	*Tbh*	-788
Tbh-d10000-L1	GTGCAAAGTGCTCACGCTTA	*Tbh*	+8515

RT-RMRA-sense	ACAACAACAACAGCCACCAG	*dunce*	+114855
RT-RMRA-anti	CGAAGGAGATTTGCTTCCAC	*dunce*	+114986
RT-RORB-sense	TCCGGAGGATTGTAATCTGG	*dunce*	+68954
RT-RORB-anti	GACGTCGTTGATCAGGGTCT	*dunce*	+69151
RT-RJRC-sense	CAGCAAATCCAACAGCTTCA	*dunce*	+103839
RT-RJRC-anti	CTGCTCGCTGCTTGTGATAA	*dunce*	+104068
RT-RGRN-sense	ACGAGGACGATGAGGATCAG	*dunce*	+137791
RT-RGRN-anti	GCGATCGCTGGTCATTAGAT	*dunce*	+138013
RT-RL-sense	AATTGCCTACCATGCTCCC	*dunce*	+157488
RT-RL-anti	GCCTGGATCTTGATGGATT	*dunce*	+157694
dunceAll-sense	GGACTGGTGCCTCGACCAGCT	*dunce*	+157823
dunceAll-anti	CGCAGCGATGGCAAGTCGAACT	*dunce*	+160815
Hang F1	GAACGGTCGGCGCGACAAAA	*hangover*	+666
Hang R1	CCGATCCTGCGGTGTAACCTGA	*hangover*	+6226
Mut_d01344_ white_L1	TTAGCTGCACATCGTCGAAC	within the XP element (GenBank #AY515149)	
Start-XPR	GGGAAACGCCTGGTATCTTT	within the XP element (GenBank #AY515149)	

The vectors used for cloning and the DNA-, RNA- and protein ladders used for gel electrophoresis are listed in the following.

Vectors	**Size**	**Company**
pCR®II-Topo	3973 bp	Life technologies
pET-28b	5368 bp	Novagen

DNA-, RNA- and protein ladders	**Company**
1 kb Plus DNA Ladder	Life Technologies
PageRuler prestained Protein Ladder	Fermentas
Amnion® Millenium™ RNA Marker	Life Technologies

2.3 Enzymes, chemicals and kits

All restriction enzymes were obtained from New England BioLabs. Standard chemicals were purchased from Sigma-Aldrich, Roche or Merck. Standard enzymes like DNase or RNase are either obtained from New England BioLabs as well or from Roche, Fermentas, Life Technologies or Novagen. The following tables show the source of specific custom-built enzymes and chemicals and the kits that were used.

Specific enzymes, chemicals	**Company**
SuperScript II Reverse Transcriptase	Life Technologies
polymerase mastermix MESA BLUE qPCR for SYBR® Assay	Eurogentec
Phusion® High-Fidelity DNA Polymerase	New England BioLabs
PerfectHyb™ Plus Hybridization Buffer	Sigma -Aldrich
CDP-*Star*®	Roche
7.5X gel loading buffer VIII	AppliChem

Kit	**Company**
NucleoSpin® Plasmid	Machery-Nagel
NucleoBond®	Machery-Nagel
QIAquick Gel Extraction Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
Ni-NTA Spin Columns	Qiagen
ECL Detection Reagents	GE Healthcare
DIG RNA Labeling Kit (SP6/T7)	Roche
DIG Wash and Block Buffer Set	Roche
TOPO®TA Cloning®Kit	Life Technologies

2.4 Antibodies

Antibody for Northern Blot Analysis

Epitope	Host	Dilution	Source
α Digoxigenin-AP	mouse	1:10000	Roche

Antibodies for Western Blot Analysis

Primary antibodies	Host	Dilution	Source
α Tbh Zhou	rabbit	1:5000	Zhou *et al*, 2008
α Tbh Cibik (2nd bleed)	rabbit	1:5000	Cibik, 2007
α Tbh Hampel	guinea pig	1:15000	Hampel, 2004
α 6x-His	mouse	1:2000	ThermoScientific
α β-actin	mouse	1:10000	abcam
α GFP	mouse	1:2000	Roche

Secondary antibodies	Dilution	Source
α mouse - Peroxidase	1:80000	Sigma-Aldrich
α rabbit - HRP	1:3000	GE Healthcare
α guinea pig - Peroxidase	1:3000	GE Healthcare

Antibodies for Immunostaining

Primary antibodies	Host	Dilution	Source
α Tbh Zhou	rabbit	1:500	Zhou *et al*, 2008
α Tbh Cibik (1st bleed)	rabbit	1:500	Cibik, 2007
α Tbh Hampel	guinea pig	1:1000	Hampel, 2004
α GFP	mouse	1:100	Life technologies
α GFP	chicken	1:1000	Life technologies
α nc82	mouse	1:50	Hofbauer, Würzburg
α TH	rabbit	1:200	Neckameyer

Secondary antibodies	Dilution	Source
α rabbit - Cy3	1:1000	Jackson Immuno Research
α guinea pig - Texas Red	1:100	Dianova
α mouse - Alexa488	1:200	Life technologies
α chicken - Alexa488	1:1000	Life technologies
α mouse - Cy3	1:200	Dianova

2.5 Buffer and Solution compositions

For DNA extraction

Homogenizing buffer	100mM NaCl 100mM Tris (pH 8.0) 50mM EDTA 0.5% SDS

For DNA gel electrophoresis

50x TAE buffer (pH 8.5)	40mM Tris base 20mM acetic acid 1mM EDTA
10x DNA Loading dye	40g Sucrose 0.2g Orange G 60g Glycerol bring to 100ml with ddH_2O

For bacterial cultures and plasmid transformation

LB medium (pH 7.0)	10g tryptone 5g yeast extract 10g NaCl bring to 1000ml with ddH_2O
LB medium +antibiotic (pH 7.0)	10g tryptone 5g yeast extract 10g NaCl bring to 1ltr with ddH_2O 100µg/ml antibiotic after autoclaving when lukewarm

LB plates (pH 7.0)	10g tryptone 5g yeast extract 10g NaCl 15g agar bring to 1000ml with ddH_2O
LB plates +antibiotic (pH 7.0)	10g tryptone 5g yeast extract 10g NaCl 15g agar bring to 1000ml with ddH_2O 100µg/ml antibiotic after autoclaving when lukewarm
SOB medium	0.5% yeast extract 2% tryptone 10mM NaCl 2.5mM KCl 10mM $MgCl_2$ 10mM $MgSO_4$
SOC medium	960µl SOB medium 40µl 1M glucose

For Northern Blot Analysis

ddH_2O^{DEPC}	1ltr ddH_2O 1ml DEPC
10x BPTE	3g PIPES (100mM) 6g Bis-Tris (300mM) 2ml 0.5M EDTA (10mM) 90ml ddH_2O
Glyoxal mix	6ml DMSO 2ml deionized glyoxal 1.2ml 10x BPTE

	0.6ml 80% glycerol 0.2ml ethidium bromide (10mg/ml)
20x SSC (pH 7-8)	88.23g Tris-Sodium-Citrate$X2H_2O$ 175.3g NaCl bring to 1000ml with ddH_2O
2x SSC/0.1%SDS	100ml 20x SSC 0.1g SDS bring to 1000ml with ddH_2O
0.1x SSC/0.1%SDS	5ml 20x SSC 0.1g SDS bring to 1000ml with ddH_2O

For purification of 6xHis-tagged protein

Lysis buffer (NPI-10) (pH 8.0)	50mM NaH_2PO_4 300mM NaCl 10mM imidazole
Wash buffer (NPI-20) (pH 8.0)	50mM NaH_2PO_4 300mM NaCl 20mM imidazole
Elution buffer (NPI-500) (pH 8.0)	50mM NaH_2PO_4 300mM NaCl 500mM imidazole

For protein extraction

RIPA buffer w/o inhibitors	150mM NaCl 50mM Tris (pH 8.0) 5mM EDTA 1mM EGTA

	1.0% NP-40 0.5% Na-Deoxycholate 0.1% SDS
Protease inhibitors	One cOmplete, Mini, EDTA-free tablet (Roche) dissolved in 700µl ddH_2O
RIPA buffer w/ inhibitors	875µl RIPA w/o inhibitors 125µl protease inhibitors
4x SDS gel loading buffer (pH 6.8)	250mM Tris 8.0% SDS 40% Glycerol 0.4% Bromphenol blue 5% β-Mercaptoethanol

For SDS-PAGE and Western Blot Analysis

10x Tris Glycine buffer	1.92M Glycine 0.25M Tris
1x SDS running buffer	100ml 10x Tris Glycine buffer 1g SDS 900ml ddH_2O
1x transfer buffer	100ml 10x Tris Glycine buffer 200ml Methanol 700ml ddH_2O
10x TBST	50mM Tris 150mM NaCl 0.2% Tween20 after autoclaving
Blocking solution	5% milk powder in 1x TBST

Mild stripping buffer (pH 2.2)	15g Glycine 1g SDS 10ml Tween20 bring to 1000ml with ddH_2O
Homemade chemiluminescence	10ml Solution1 + 10ml Solution2 Solution1: 10ml 100mM Tris (pH 8.5) + 44µl 90mM paracoumaric acid + 100µl 250mM luminol (paracoumaric acid and luminol dissolved in DMSO) Solution2: 10ml 100mM Tris (pH 8.5) + 7µl 30%-H_2O_2
Stripping buffer (pH 2.2)	15g Glycine 1g SDS 10ml Tween20 bring to 1ltr with ddH_2O

Acrylamid gel	Resolving gel		Stacking gel
	10%	20%	5%
ddH_2O	9.9ml	1.6ml	6.8ml
30% Acrylamide/ Bisacrylamide	8.3ml	16.6ml	1.7ml
1.5M Tris/HCl, pH 8,8	6.3ml	6.3ml	-
1.0M Tris/HCl, pH 6,8	-	-	1.3ml
10% SDS	250µl	250µl	100µl

10% APS	250µl	250µl	100µl
TEMED	20µl	25µl	10µl

For Immunostaining

Drosophila Ringer (pH 7.2)	46mM NaCl 182mM KCl 3mM $CaCl_2$ 10mM Tris
Phosphate buffered saline (PBS) (pH 7.4)	137mM NaCl 2.7mM KCl 2mM KH_2PO_4 10mM Na_2HPO_4
PBT (for adult CNS)	1x PBS 0.3% Triton X-100
PBT (for larval CNS)	1x PBS 0.5% Triton X-100
Blocking solution A	1x PBT 5% FCS
Blocking solution B	1x PBT 2.5% BSA 5% NGS

2.6 Methods on DNA level

2.6.1 Isolation of genomic DNA

To isolate genomic DNA of *Drosophila* an appropriate amount of flies or fly heads was collected in a 1.5ml tube on ice. A volume of 500µl homogenizing buffer was added to the tube. Afterwards the flies/fly heads were homogenized by mechanical

force with a micro pestle. Following this the tube was incubated for 30 minutes at 70°C. Once this step was completed 70µl 8M KAc were added. After briefly mixing the tube by hand, the tube was incubated for 15 minutes on ice before centrifugation at maximum rpm at 4°C. The supernatant contained the DNA and purified by phenol/chloroform extraction.

2.6.2 Phenol/chloroform purification of DNA

To purify a DNA containing solution an equal amount of phenol was added. Following incubation for five minutes at room temperature with shaking, the tube was spun down for two minutes. Afterwards the upper aqueous phase was transferred to a new tube and mixed with 0.5 volumes phenol and 0.5 volumes chloroform. The tube was mixed and centrifuged for five minutes before removing the upper aqueous phase to a new tube. To precipitate the DNA 2.5 volumes absolute ethanol were added. After a brief incubation the tube was spun down. The DNA pellet was washed with 70% ethanol, dried for five minutes and resuspended in an appropriate amount of ddH_2O.

2.6.3 Polymerase chain reaction (PCR)

To amplify copies of specific DNA fragments polymerase chain reaction is performed. The reaction consists of three phases. The first step is the denaturation phase where DNA is denaturated at 95°C. The second stage is the annealing phase where the primers hybridize to complementary DNA sequences. The temperature used for annealing is dependent on the GC-content of the primers. In the last step called the elongation the DNA polymerase synthesizes the complement DNA sequence in between the primers. For PCR the following ingredients were mixed:

- cDNA/gDNA
- Primer forward
- Primer reverse
- Deoxynucleotide triphosphates (dNTPs)
- DNA-Polymerase specific buffer
- DNA-Polymerase

When cDNA is used the PCR is called RT-PCR due to reverse transcription (RT). For standard PCR *Taq* DNA Polymerase from New England Biolabs according to the manufacturer's instructions was used. For amplification of PCR fragments needed for downstream applications such as cloning Phusion® High-Fidelity DNA Polymerase also from New England Biolabs with its specific protocol was used.

2.6.4 Quantitative Real Time PCR (qRT-PCR)

Performing qRT-PCR means using a technique based on polymerase chain reaction to compare cDNA quantities of a target gene in an experimental group with the cDNA levels of the target gene in a control group normalized to the expression of a reference gene in both samples. With this technique the real time progress of PCR using fluorescent labeled oligonucleotides as reporter molecules is monitored. The fluorescence emitted by the reporter molecules increases as the PCR product is being duplicated with each cycle of amplification.

cDNA of the control group and of the experimental group was synthesized and was adjusted to a concentration of 100ng/µl. A 25µl qRT-PCR reaction mixture consisted of:

- SYBR mastermix 12.5µl
- cDNA (100ng) 1µl
- primer sense (10µM) 1µl
- primer anti (10µM) 1µl
- ddH_2O 9.5µl

The SYBR mastermix contains SYBR® Green I Blue Dye, Taq DNA polymerase, dNTPs and optimized PCR buffer. For each sample to be measured, the reaction was carried out in triplicate. The PCR program performed for the qRT-PCR is indicated below:

Holding stage:	initial denaturation	95°C	5min	
Cycling stage:	denaturation	95°C	15s	40x
	hybridization	57°C	30s	
	elongation	72°C	30s	
Melting curve stage:		95°C	15s	
		60°C	60s	
	melting curve	60-95°C	15s	70x

The cycle number at a given threshold level of log-based fluorescence was defined as the Ct value. Ct values were received for the target gene and the reference gene both for the control group and the experimental group. To analyze the raw data in the form of Ct values the following calculation after Pfaffl was used (Pfaffl, 2001).

$$\frac{(E_{target})^{\Delta Ct\ target(control-experiment)}}{(E_{reference})^{\Delta Ct\ reference(control-experiment)}} = ratio$$

A suitable reference primer pair belonging to a gene not to be regulated in the experimental group was determined for every experimental group using the Microsoft Office Excel Add-In NormFinder (Andersen *et al.*, 2004). For the qRT-PCR experiments shown in this thesis PCR polymerase mastermix MESA BLUE qPCR for SYBR® Assay from Eurogentec was used and the experiments were carried out with the iCycler iQ5 Multicolor Real-Time PCR Detection System and its corresponding iQ5 Optical System Software from BioRad.

2.6.5 PCR purification

To purify PCR samples which means removing primers, dNTPs, enzymes and salts the QIAquick PCR Purification Kit was used according to protocol.

2.6.6 DNA Gel Electrophoresis

With gel electrophoresis DNA fragments were separated according to size. Agarose gels were prepared in TAE buffer and ethidium bromide was added (5µl/100ml TAE). Ethidium intercalates with DNA which leads to a strong fluorescence of ethidium bromide under UV light.

2.6.7 Gel extraction

To extract DNA from an agarose gel the desired band was cut out under UV light. Cutting out the band was done quickly because UV light can cause damages to the

DNA. Afterwards the extraction proceeded according to the protocol of the QIAquick Gel Extraction Kit.

2.6.8 DNA Sequencing

To sequence DNA fragments or plasmids sequencing service from GATC Biotech was used. There sequencing was performed using the Sanger method.

2.6.9 Restriction Digest

Restriction enzymes cut DNA specifically at their associated recognition sequence. A 20µl digest consisted of the following components. It can contain one single or two different restriction enzymes.

- DNA template 0.5-2µg
- restriction buffer (10x) 2µl
- restriction enzyme A and/or B 0.5µl each
- bring to 20µl with ddH_2O

The mixture was flicked, briefly centrifuged and incubated at 37°C for two hours. If the used restriction enzymes can be heat inactivated the reaction was stopped by incubation at 65°C for 20 minutes.

2.7 DNA cloning

2.7.1 Cloning strategy for a *Tbh* hybridization probe

To generate the *Tbh* hybridization probe for northern blotting a 525 bp long *Tbh* fragment consistent of parts of the first and the second exon was amplified with the primers Sonde-v-L and Sonde-v-R by RT-PCR. The PCR fragment then was brought into the pCR®II-TOPO® vector. The generated plasmid contained the *Tbh* fragment in 3' to 5' direction. The vector map is shown in the supplement. The DIG-labeled RNA antisense hybridization probe was generated by *in vitro* transcription using the DIG RNA Labeling Kit (Sp6/T7) from Roche.

2.7.2 Cloning strategy to generate pET28b plasmids bearing different coding sequences of *Tbh*

To express different Tbh protein fragments in *E. coli* BL21 cells *Tbh* specific coding sequences were cloned into the expression vector pET28b. Therefore three different *Tbh* fragments were amplified by linker RT-PCR with primers shown in the following table. Thereby an EcoRI restriction site was added at 5' end of the fragments and a XhoI restriction site at 3'.

***Tbh* fragment**	**primers**
F2	Frag2-L-EcoRI and Frag2-R-XhoI
F3	Frag3-L-EcoRI and Frag3-R-XhoI
F5	Frag5-L-EcoRI and Frag5-R-XhoI

The fragments were then cloned into the pET28b vector due to adequate restriction sites considering a 6xHis tag at the 3' end of the fragments. A detailed vector map is shown in the supplement.

2.7.3 Direct cloning of PCR fragments into the pCR®II-TOPO® Vector

After PCR purification PCR products can be cloned directly into the pCR®II-TOPO® Vector using the TOPO®TA Cloning® Kit according to the kits' protocol. After overnight ligation the plasmid was transformed into *E. coli* XL1-blue heat competent cells. Cells are plated on LB plates containing ampicillin and IPTG which allows blue/white screening of positive colonies.

2.7.4 Ligation of DNA fragments into vector plasmids

Specific DNA fragments can be integrated into a vector plasmid by ligation. Therefore DNA fragments were cut with the same restriction enzymes than the vector. With a ligation reaction the DNA fragment and the vector were fused due to the compatibility of the corresponding overhangs. To verify a successful ligation a part of the ligation was analyzed by gel electrophoresis. A 10µl ligation reaction consisted of the following components:

- Vector DNA, cut 50-100ng
- Insert DNA, cut x
- T4 ligase buffer (10x) 1µl
- T4 ligase 1µl
- bring to 10µl with ddH_2O

50 to 100ng of vector DNA were used for ligation. The right amount of insert DNA was calculated as follows.

$$\frac{bp(Insert) \times ng(Vector)}{bp(Vector)} \times 3 = ng(Insert)$$

The reaction was incubated overnight at 16°C. To prevent vector self-ligation the vector DNA was incubated for 30 minutes at 37°C with alkaline phosphatase (CIP, New England BioLabs) before ligation. CIP removes 5' phosphates from the DNA. For ligation at least one of the two compatible overhangs needs to have the 5' phosphate otherwise ligation is not happening.

2.7.5 Transformation of ligated plasmid DNA into *E. coli* XL1-blue heat competent cells.

The competent cells (50µl aliquots) were thawed on ice. An amount of 10-100ng of the ligation was added to the cells. The tube was flicked briefly and then incubated on ice for 30 minutes. Directly afterwards the cells received a heat shock for 45 seconds

at 42°C. Another incubation on ice for two minutes follows before 500µl of pre-warmed SOC medium followed. The tube was incubated at 37°C for one hour with shaking at 225-250rpm. After the incubation 50-250µl of the transformation were plated on LB agar plates and incubated at 37°C overnight. To avoid growing of empty cells, the agar plates contained specific antibiotics for which resistance was encoded by the vector.

2.7.6 Transformation of plasmid DNA into *E. coli* BL21 (DE3) heat and chemi-competent cells.

The competent cells (100µl aliquots) were thawed on ice. 1.7µl of β-Mercaptoethanol (1.42M) were pipetted to the cells. The tube was flicked briefly and then incubated on ice for ten minutes. Then an amount of 50-500ng of the plasmid DNA was added to the cells. The tube again was flicked briefly and then incubated on ice for 30 minutes. Right afterwards the cells were heat shocked for 45 seconds at 42°C. After the heat shock the procedural method was the same than described in the protocol for transformation into *E. coli* XL1-blue heat competent cells (2.7.5). Transforming plasmid DNA of an expression vector (e.g. pET28a-c) expression of the encoded protein cloned into the vector can be induced using the T7-lac-expression system.

2.7.7 Isolation of plasmid DNA from bacterial cells

The isolation of plasmid DNA from bacterial cells is carried out either with the NucleoSpin® Plasmid miniprep kit or with the NucleoBond® midiprep kit according to the manufacturer's instructions.

2.7.8 Protein induction/expression in *E. coli* BL21 (DE3) cells

Specific bacterial strains can be used to express proteins in vitro. To do so, the coding sequence of the gene of interest was cloned into the pET28b expression vector and transformed into *E. coli* BL21 (DE3) cells. A single bacterial colony was used to inoculate a 100ml culture. The culture was incubated at 37°C with shaking. As soon as the culture reached an OD_{600} between 0.5 and 0.6 IPTG (1mM end concentration) was added to the culture. IPTG induced the protein expression according to the T7-

lac-expression system. The culture was then incubated again at 37°C with shaking for four hours. Samples of 1ml were taken after the four hours after IPTG induction. The samples were centrifuged and the pellet was resuspended in SDS gel loading buffer for analysis by SDS-PAGE.

2.8 Methods on RNA level

2.8.1 Isolation of total RNA

To isolate total RNA from *Drosophila* an appropriate amount of flies or fly heads was collected in a 1.5ml tube on ice. A volume of 300µl of trizol was added to the flies. The tissue was then homogenized with a micro pestle. Another 700µl of trizol were added afterwards to achieve a total volume of 1ml. The homogenate was incubated at room temperature for five minutes. 200µl of chloroform were pipetted into the tube before it was mixed thoroughly by hand for 15 seconds. The tube was then incubated at room temperature for three minutes. Afterwards the tube was centrifuged for 15 minutes at 4°C and 12000xg. After the spinning step the aqueous phase was transferred into a new tube. A volume of 500µl isopropanol was added. The solution was mixed and incubated at room temperature for ten minutes. A centrifugation step at 4°C and 12000xg for ten minutes follows. The pellet was washed in two washing steps with 1ml 70% ethanol each and centrifugation at 7500xg and 4°C for five minutes. The pellet was dried after washing for five minutes. Then the dry pellet was dissolved in 50µl ddH_2O for ten minutes at 65°C. To digest the genomic DNA in the sample 1µl of DNase (RNase free) was added and the tube was incubated at 37°C for 30 minutes. The DNA digest reaction was stopped by incubation at 95°C for ten minutes.

2.8.2 Reverse transcriptase (cDNA synthesis)

Using reverse transcriptase (SuperScript II) isolated RNA is transcribed into cDNA. Therefore the following ingredients were pipetted together:

- total RNA 100ng-5µg
- 50µM Oligo(dT) primer 1µl
- 10mM dNTPs 1µl
- ddH_2O bring to 12µl

The mixture was incubated for five minutes at 65°C. The tube was quickly chilled on ice afterwards. During the incubation a mastermix including the following ingredient was prepared.

- 5x First-Strand buffer 4µl
- 0.1M DTT 2µl
- RNaseOUT 1µl

7µl of the mastermix were added into the pre-incubated tube before the tube was incubated at 42°C for two minutes. At last 1µl (200Units) of SuperScript II was pipetted to the mix and the reaction was incubated at 42°C for one hour. The reaction was inactivated by heating the tube for 15 minutes at 70°C. Finally the RNA was digested by adding 1µl of RNAse (DNase free) and subsequent incubation at 37°C for 30 minutes.

2.8.3 RNA sample labeling with Digoxigenin (*in vitro* transcription)

To detect hybridization of a RNA probe to a RNA target sequence, the probe was labeled with a non isotopic marker, in this case digoxigenin (DIG). Labeling of a RNA hybridization probe with digoxigenin was done with the DIG RNA Labeling Kit (SP6/T7) according to the manufacturer's instructions.

2.8.4 Northern Blot Analysis

To study gene expression by detection of RNA in one or more samples Northern blot analysis was performed. The first step was to isolate RNA. The isolated RNA was then denaturated by glyoxal as follows:

- RNA 10µg
- glyoxal mix 10µl

The reaction was incubated for 60 minutes at 55°C. Meanwhile a 1.2% agarose gel in 1x BPTE was prepared. After incubation the samples were collected by centrifugation and an appropriate amount of 7.5X RNA loading buffer was added before the samples were loaded onto the gel. The gel ran at 60V - 80V until the dye front left the gel. After electrophoresis the gel was rinsed in ddH_2O^{DEPC} twice for ten minutes. In the meantime the following was prepared:

- 5cm of dry paper towels
- 3 pieces of 3 MM Whatman blotting paper moistened in 20x SSC
- nitrocellulose membrane rinsed in ddH_2O^{DEPC}, moistened in 20x SSC for 5 minutes
- 1 piece of dry 3 MM Whatman blotting paper
- buffer bridge moistened in 20x SSC

After the gel electrophoresis was done the blotting stack was assembled. An empty gel tray was placed upside down into a dish. The gel tray was covered with the buffer bridge. Two pieces of moistened 3 MM Whatman paper were placed on top of the bridge. The gel was then transferred onto the bridge with its bottom side facing upwards. The gel was then covered with the membrane. One piece of moistened 3 MM Whatman paper was put on the top of the membrane on the stack. In the end the stack was covered with one piece of dry 3 MM Whatman paper and 5cm of dry paper towels before a heavy glass plate on top of everything weighted the stack down. The transfer ran for 16 hours. After transfer the stack was dismantled and the membrane was placed onto a piece of 3 MM Whatman paper soaked in 20x SSC before the damp membrane was cross linked by UV irritation. The cross linked membrane was rinsed briefly in ddH_2O^{DEPEC} and washed in 20mM TrisHCl for 20 minutes at 60°C. Afterwards the membrane was rinsed again briefly in ddH_2O^{DEPEC}. Hybridization of the membrane followed. The hybridization started with placing the membrane into a hybridization bottle and covering it with 10ml PerfectHyb™ Plus Hybridization Buffer. The membrane was pre-incubated in a hybridization oven at 55°C hybridization temperature with rotation for one hour. Meanwhile 350ng DIG-labeled RNA probe were added to 500µl PerfectHyb™ Plus Hybridization Buffer. The mixture was incubated at 95°C for five minutes, transferred to ice immediately

afterwards and brought to a volume of 3.5ml with PerfectHyb™ Plus Hybridization Buffer. After pre-incubation of the membrane the PerfectHyb™ Plus Hybridization Buffer was removed and the diluted RNA digoxigenin-labeled probe was added. The membrane was then incubated at 55°C hybridization temperature for six hours to overnight. Afterwards the membrane was washed twice with 2x SSC/0.1%SDS for five minutes at room temperature followed by two longer washing steps with 0.1x SSC/0.1%SDS each 15 minutes at 55°C. The digoxigenin detection followed using the DIG Wash and Block Buffer Set and CDP-*Star*® both from Roche according to the manufacturer's instructions. Enzymatic dephosphorylation of CDP- *Star*® by alkaline phosphatase results in light emission at 466nm which was detected on an X-ray film.

2.9 Methods on protein level

2.9.1 Protein extraction

To extract proteins from *Drosophila* tissue an appropriate amount of flies or fly heads were collected on ice in a 1.5ml tube. The flies/fly heads were snap frozen in liquid nitrogen or at -80°C. A volume of 100µl to 200µl RIPA w/ buffer was added to the frozen flies/fly heads before the tissue was homogenized with a pestle. The tube was incubated on ice for 30 minutes and was centrifuged afterwards at maximum speed for 20 minutes at 4°C. The supernatant containing the protein was transferred to a new tube. An appropriate amount of 4x SDS gel loading buffer was pipetted to the protein solution before the solution was boiled for five minutes at 95°C. Finally the tube was centrifuged shortly afterwards and kept on ice until loading onto the acrylamide gel.

2.9.2 Protein purification of 6xHis-tagged protein

A bacterial pellet from 5ml cell culture was resuspended in 630µl of lysis buffer NPI-10. 70µl lysozyme 10mg/ml lysozyme and 15 units of benzonase were added to the resuspended cells. The lysate was incubated at room temperature for 30 minutes. After this the lysate was centrifuged at 12.000xg for 30 minutes at 4°C. The Ni-NTA spin column was equilibrated with 600µl buffer NPI-10 and centrifuged for five minutes at 900xg. Up to 600µl of the cleared lysate containing the 6xHis-tagged

protein as loaded onto the pre-equilibrated Ni-NTA spin column and the column was centrifuged afterwards at 200xg for five minutes so that the histidin from the 6xHis-tag can bind to the Ni^{2+} ions in the column. The column was washed twice with 600µl buffer NPI-20 and centrifuged for two minutes at 900xg. The 6xHis-tagged protein was then eluted with 100µl of the elution buffer NPI-500 and a centrifugation step at 900xg for two minutes.

2.9.3 SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)

To separate proteins according to their size SDS-PAGE was performed. It consists of two layers, the stacking gel and the resolving gel. The stacking gel has a lower concentration of acrylamide than the resolving gel. The separation of the proteins is dependent on the amount of acrylamide in the gel and hence on the degree of cross-linking. Higher percentages are needed to resolve smaller proteins whereas proteins with high molecular weight can resolve better in lower percentage gels. Here standard 10% acrylamide gels were prepared if not described differently. In this thesis the gels were poured and run with the vertical electrophoresis cell equipment for SDS-PAGE from BioRad. Once gels have been prepared the vertical electrophoresis apparatus was assembled. Both the upper and the lower buffer tank were filled with 1x SDS running buffer. The protein samples (10-50µg/well) and the protein ladder (10µl) were loaded onto the gel. The gel was running at 80-120V. After electrophoresis the gels were used for western blot analysis.

2.9.4 Western Blot Analysis

Western blot analysis was used to detect specific proteins in specific given samples. In this thesis proteins resolved by the SDS-PAGE were transferred to a nitrocellulose membrane performing a wet transfer using the Mini Trans-Blot Cell from BioRad. Afterwards the membrane was stained with antibodies specific to target proteins. After SDS-PAGE the gel was carefully disassembled, the stacking gel was then cut off and the resolving gel was placed in transfer buffer. Four pieces of 3 MM Whatman blotting paper and two sponges were soaked in transfer buffer as well until use. The nitrocellulose membrane was incubated in methanol for five minutes before it was

transferred to transfer buffer. When gel, blotting paper, sponges, and membrane were soaked in transfer buffer for at least ten minutes the western blot transfer cassette was assembled in the following order:

- wet sponge (black side)
- 2 pieces of wet 3 MM Whatman blotting paper
- gel
- membrane
- 2 pieces of wet 3 MM Whatman blotting paper
- wet sponge (red/white side)

The blotting cell was filled completely with transfer buffer before the power supply was connected to the cell. Transfer ran for two hours at 200mA. After transfer the apparatus was disassembled and the membrane was transferred to TBST for a short washing step. Afterwards the membrane was incubated in blocking solution (5% milk in TBST) for one hour at room temperature. The first antibody diluted in blocking solution was applied for overnight incubation at 4°C. After overnight incubation the membrane was washed three times for 15 minutes with TBST before the secondary antibody diluted in blocking solution was applied for two to three hours at room temperature. Another three washing steps followed the incubation of the secondary antibody before chemiluminescence detection was performed.

2.9.5 Chemiluminescence Detection

Using chemiluminescence detection proteins were detected on a nitrocellulose membrane with the help of peroxidase tagged antibodies. Therefore two chemiluminescene detection reagents were brought together in a 1:1 ratio before application to the membrane. Using antibodies generated in mouse or guinea pig the Enhanced Chemiluminescene (ECL) Detection Reagents from GE Healthcare were used according to the manufacturer's instructions. Using antibodies generated in rabbit homemade chemiluminescene reagents 1 and 2 at a ratio of 1:1 were applied to the membrane for ten minutes with shaking. The peroxidase tagged to the secondary antibody catalyzes the oxidation of luminol present in one of the detection reagents.

The oxidation reaction emits energy in form of light at 428nm detectable by an X-ray film.

2.9.6 Mild stripping of Western Blot membranes for reprobing

To remove primary and secondary antibodies from Western Blot membrane for reprobing the membrane is washed with mild stripping buffer for 15 minutes twice. Afterwards the membrane is washed twice for 10 minutes with PBS and twice for five minutes with TBST. The membrane is then ready for blocking.

2.9.7 Protein expression analysis using pixel intensity

To compare the quantity of protein expression from different genotypes pixel intensity (PI) of the protein bands on western blots were put in relation. Therefore western blots developed with an X-ray film were scanned to obtain a digital image. With the computer program ImageJ (version 1.42q) the pixel intensities of the protein bands for the target protein and a loading control from both the test genotype and a control genotype were determined. The pixel intensities of the target protein were then normalized to the pixel intensities of the loading control. Afterwards the normalized values of test genotype and control genotype were put in relation. To test for statistical differences the student's t-test was done.

$$\frac{\mathrm{PI}_{\text{loading control}}(\text{test genotype})}{\mathrm{PI}_{\text{target protein}}(\text{test genotype})} \Big/ \frac{\mathrm{PI}_{\text{loading control}}(\text{control genotype})}{\mathrm{PI}_{\text{target protein}}(\text{control genotype})} = \text{ratio}$$

2.10 Immunostaining for *Drosophila* CNS

2.10.1 Immunostaining of larval CNS

Third instar larvae were collected and incubated in ice cold 70% ethanol for two minutes before their CNS was dissected in *Drosophila* ringer. The dissected brains were transferred to PBS. The brains were then fixed in 3.7% formaldehyde diluted in PBS for 30 minutes. The tissue was rinsed three times in 0.3% PBT subsequent to the

fixation and before the brains were washed three time for 15 minutes with 0.3% PBT. After washing blocking solution A was applied to the brains for one hour at room temperature with shaking. Incubation with primary antibody diluted in blocking solution A for overnight at 4°C follows. Three rinsing steps and three washing steps for 15 minutes with 0.3% PBT were carried out afterwards before the secondary antibody was applied to the brains. The secondary antibody was diluted in blocking solution A and was incubated for two to three hours at room temperature with shaking. The moment the secondary antibody was added all steps were carried out with aluminum foil wrapped around the tube to keep exposure to light to a minimum. Another washing procedure including three rinsing steps and three washing steps with 0.3% PBT followed the incubation of the secondary antibody. The tissue was incubated in 50% glycerol in PBS for 30 minutes after the last washing step. The brains were then mounted on slides and were analyzed by confocal fluorescence microscopy.

Using the α Tbh Cibik antibody the procedure described above was adjusted. The fixation was 2.5 hours at 4°C. Instead of blocking solution A blocking solution B was used both for blocking and for dilution of the antibodies. Incubation with the first antibody was for 48h at 4°C and for the secondary antibody overnight at 4°C.

2.10.2 Immunostaining of adult CNS

Immunostaining of adult CNS tissue was performed according to the protocol described in 2.10.1. However, instead of 0.3% PBT for washing and preparing blocking solutions a 0.5% solution was used.

2.10.3 Analysis of immunostained preparations

Immunostainings of larval and adult CNS of *Drosophila* were scanned with the confocal laser scanning microscope LSM 510 META from Zeiss. The preparations were scanned by doing optical sections of 1µm thickness. The stack of pictures was then converted into a Z-projection using the program ImageJ (version 1.42q).

2.11 *Tbh* mutagenesis using FLP recombination

To generate a new *Tbh* mutant a mutagenesis using flippase (FLP) recombination to delete a part of the *Tbh* gene due to flanking XP-elements was performed. The mutagenesis is based on the publication of Parks and colleagues in 2004. XP-elements consist of two FRT sites that flank an UAS sequence, an additional UAS sequence and a *white* gene (Fig. 2.11.1 A). If two XP-elements are present in transheterozygous in the fly flippase, when induced, recombines the two XP-elements due to their FRT sites by deleting the sequence in between (Fig. 2.11.1 B). A residual element tagging the deletion site including one *white* gene and one UAS sequence remains in the genomic DNA resulting in w+ deficiencies. Flippase can be induced by a heat shock that is given to the larvae when a *hsFLP* transgene is present in the fly.

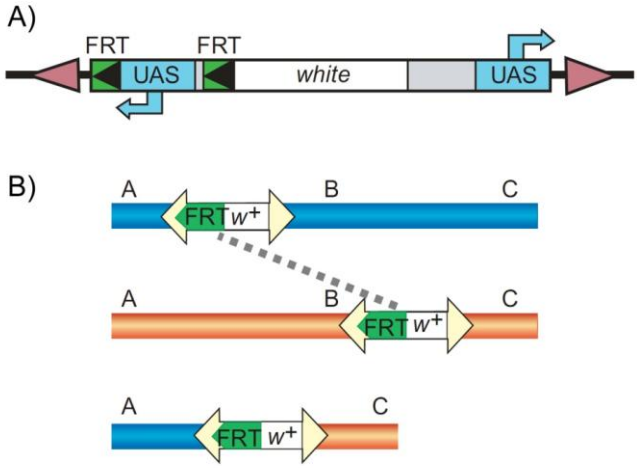


Fig. 2.11.1. Schematics of an XP-element and for deletion generation.
A) XP-element sequence includes two UAS- sites, a copy of the *white* gene and two FRT sites. The same orientation (arrow head) of FRT sites of two XP-elements are crucial for successful FRT-FLP deletion. B) Starting pairs of chromosomes with inserted XP-elements and FRT sites are shown in orange and blue. FRT recombination performed by flippase is indicated by the dashed line. Recombination leads to deletion of sequence B, the sequence between the XP-insertions (modified after Parks *et al.*, 2004).

The XP fly lines that were used to generate a deletion of the first and the second exon and a part of the second intron of the *Tbh* gene (X chromosome) were generated from investigators at Exelixis (The Exelixis Collection at Harvard Medical School). The XP-element for the left breaking point of the expected deletion is located in the upstream

region of the *Tbh* gene at position -555 referring to the first base pair of the *Tbh* gene. This line is the XP^{d01344} line. The location of the right XP-element is located in the second intron of the *Tbh* gene at position +8694 (Fig. 2.11.2). This line is the XP^{d10000} line. Homozygous XP^{d10000} are female sterile and are balanced with *FM7*.

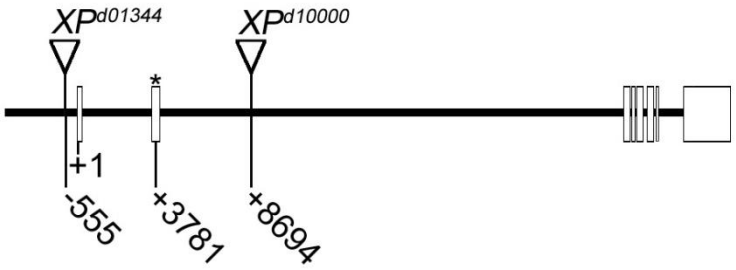


Fig 2.11.2. Schematic drawing of the positions of the XP-lines used for mutagenesis.
The *Tbh* gene located on the X chromosome is shown. The white boxes indicate the eight exons of the gene. The annotated start codon is indicated with an asterisk at position +3781. The left XP-element is located at position -555 and the right XP-element at position +8694 referring to the first base pair of the gene.

In the first crossing male flies carrying the *hs-FLP* transgene on the second chromosome were crossed with homozygous XP^{d01344} female virgins. 30 crossing with each 35 virgins and 15 males were set up. To identify the *hs- FLP* transgene this transgene was combined with the *MKRS* marker. Males of the F1 generation that carry both the XP-element and the *hs-FLP* transgene were crossed then to female virgins of the second XP-element line XP^{d10000}. XP^{d10000} flies were balanced over *FM7* due to female sterility when homozygous. Here 45 crossings with each 35 virgins and 15 males were set up. After three days the adult flies were removed from the vials and the larvae were then heat shocked for one hour each day for four days. During heat shock flippase was induced and recombination of the XP-elements occured in the oocytes of females being transheterozygous for both XP-elements and carrying the *hs-FLP* transgene. Progeny was raised to adulthood.

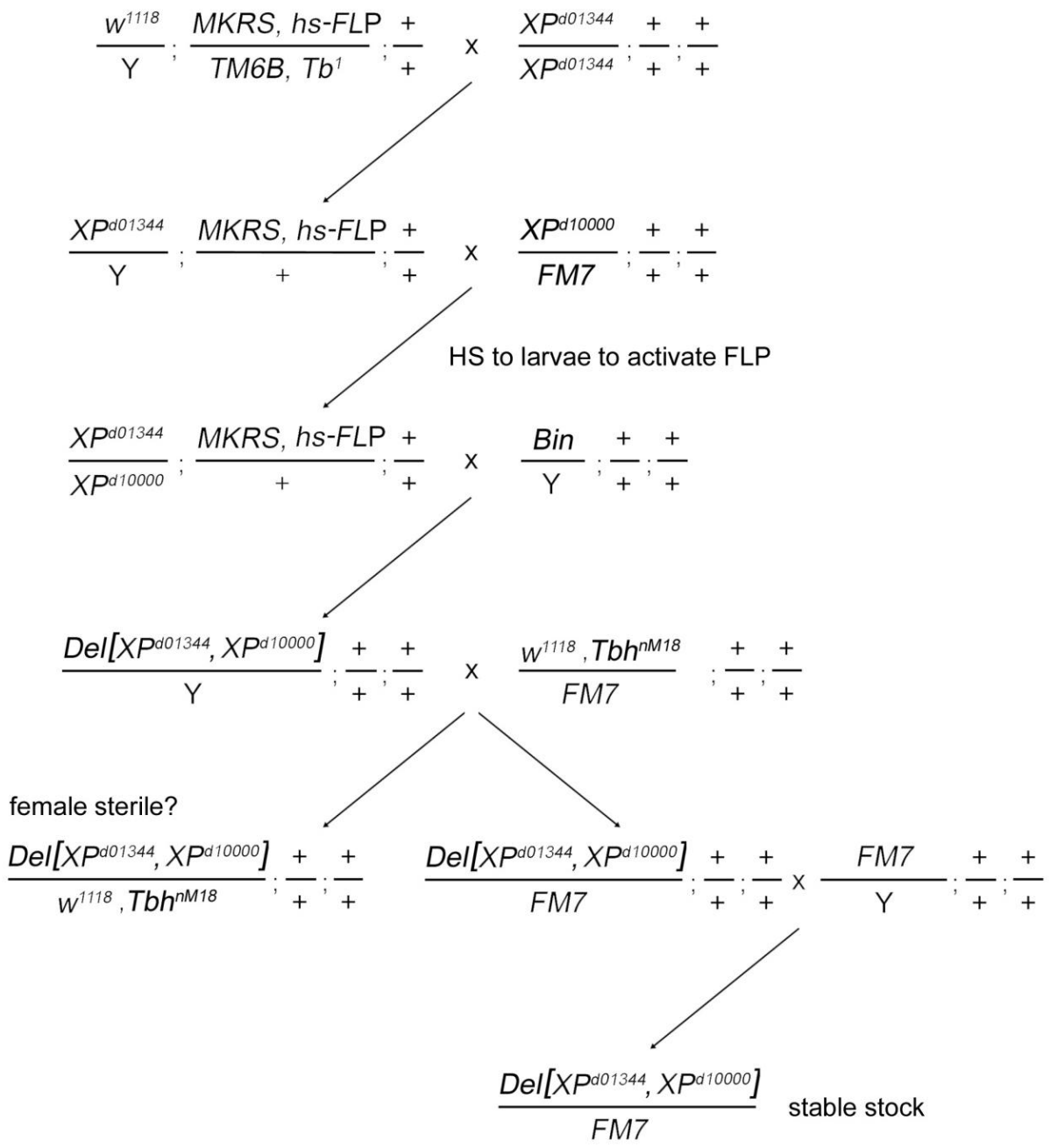


Fig. 2.11.3. Crossing scheme for *Tbh* mutagenesis to generate FLP-FRT-based deletions.
Crossings generate two FRT-bearing XP-element lines in trans in the presence of heat shock–driven FLP recombinase (*hs-FLP*). Activation of FLP recombinase results in the generation of *Tbh* deletions. Potential fly lines carrying the deletion are screened for female sterility. Potential lines carrying the deletion are established with the *FM7* balancer.

Virgin females were collected and crossed to males containing the balancer Bin. 30 crossings with each 35 virgins and 15 males were set up. Only some flies of the next generation carried the expected deletion. Therefore 350 single crossings were set up of single individual offspring males with $Tbh^{nM18}/FM7$ virgins.

Offspring females were screened then for female sterility because a *Tbh* deletion is shown to cause female sterility (Monastirioti *et al.*, 1996). The tested female flies carried one copy of the Tbh^{nM18} mutation and in addition either one of the original XP-elements or the expected deletion. Of the identified sterile stocks, virgins being heterozygous for the putative *Tbh* deletion and the *FM7* balancer were crossed to *FM7* balanced males to generate a stable stock of the putative deletion $Del[XP^{d01344}, XP^{d10000}]$ (Fig. 2.11.3). The putative lines carrying either the expected deletion or only the an initial XP-element insertion were tested by PCR. Furthermore the verified new *Tbh* mutant and the two XP-elements lines were crossed to w^{1118} for five generations to obtain the lines in the w^{1118} background. The w^{1118} background was needed for behavioral experiments so that w^{1118} can be used as an adequate control for behavioral experiments.

2.12 Measuring body balance towards ethanol exposure of *Drosophila* using the inebriometer

Body balance towards ethanol exposure is associated with ethanol sensitivity or tolerance of *Drosophila* and can be measured using the inebriometer. The inebriometer consists of a glass column with a series of platforms inside on which the flies can rest. The column is connected to an evaporator producing an ethanol/water vapor mixture which is introduced into the column from the top (Fig. 1.12). The ethanol/water vapor mixture is adjusted to the ratio 2.5/2.25. An outer glass tube around the inner column filled with water cools the system to a constant temperate of 20°C. 120 male flies 3 to 5 days are inserted into the inner column at the top of the apparatus. During one hour the flies are losing their body balance due to intoxication and they are falling down the column and leave the column through a laser barrier. Flies passing the laser barrier are recorded electronically by the computer program DFM28 every three minutes. The recording program calculates the Mean Elution Time (MET) giving a time value representing the average peak where most of the flies

fell out of the column. The first MET (MET1) represents alcohol sensitivity. The following formula shows the calculation of the MET.

$$\frac{\sum[\text{number of eluted flies at time point (every 3min)} \times \text{time point}]}{\text{total number of eluted flies}} = \text{MET}$$

Flies are collected after the first MET and are allowed to recover for three hours. After recovery they are inserted into the column again. The second MET (MET2) and the first MET (MET1) are used for calculation of tolerance giving the percentage increase of sensitivity.

$$\frac{(\text{MET2} - \text{MET1})}{\text{MET1}} \times 100 = \text{tolerance}$$

The data was analyzed for its significance using ANOVA and the post-hoc Tukey-test within the STATISTICA program software.

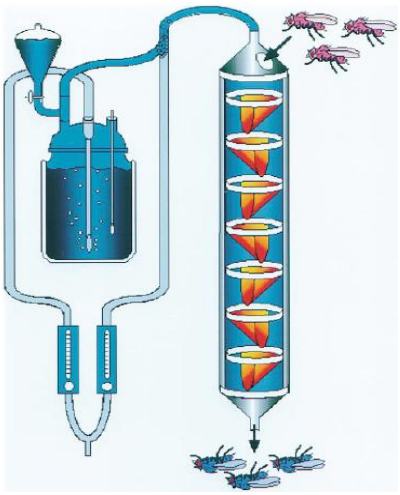


Figure 1.12. Inebriometer to measure alcohol sensitivity and tolerance of *Drosophila*.
Schematic drawing of an inebriometer connected to an evaporator. Ethanol vapor is brought into the column via the evaporator. Flies are inserted into the top of the columns and fall out the column at the bottom after they lost their body balance due to alcohol intoxication. (Bellen, 1998)

3 RESULTS

3.1 The *Tbh* gene encodes at least five transcripts resulting in different isoforms

The Tbh^{nM18} mutant, having a deletion in the *Tbh* gene, is impaired in developing normal ethanol tolerance (Scholz *et al.*, 2000). The *Tbh* gene encodes the key enzyme for the octopamine (OA) synthesis. On flybase the *Tbh* gene is annotated with two transcript (http://flybase.org/reports/FBgn0010329.html; state: september 2013) that only differ in their 5'UTR region. However RT-PCR studies showed that an additional alternative exon exists (Hampel, 2007). This suggests that *Tbh* is further alternatively spliced. To address how many other putative Tbh proteins exist the genomic organization of the *Tbh* gene needs to be investigated in more detail. In addition the functionality in relation to ethanol tolerance needs to be investigated.

3.1.1 The *Tbh* gene is alternatively spliced

The *Tbh* gene consists of eigth exons and according to flybase encodes two transcripts, *Tbh-RB* and *Tbh-RC* (http://flybase.org/reports/FBgn0010329.html; state: september 2013). Transcript *Tbh-RC* was added just recently and differs from *Tbh-RB* only in a shorter 5'UTR lacking the first exon. The resulting proteins are the same. Only the transcript of origin - *Tbh-RB* - will be considered in the following experiments. It was already shown that the first exon of the *Tbh* gene is further alternatively spliced (Hampel, 2007, transcript II in figure 3.1.1.1). To identify additional *Tbh* transcripts two approaches were carried out. At first RT-PCR studies followed by sequencing were performed using exon specific primers. Total RNA of whole wild type flies was used for cDNA synthesis. Three additional *Tbh* transcripts besides the annotated transcript *Tbh-RB* (transcript I) and the transcript identified by Hampel (transcripts II) were uncovered (Fig. 3.1.1.1). All five transcripts are summarized in figure 3.1.1 including their sequence sizes and the resulting putative protein sizes. Sequences of the alternatively spliced transcripts are shown in detail in the supplement. Notable aspects of the additional transcripts are firstly that transcripts I and II only differ in their 5'UTR and not in the resulting protein. Secondly alternatively splicing of transcript II leads to an alternative stop codon

compared to the other transcripts and alternatively splicing of transcript V leads to an alternative start codon.

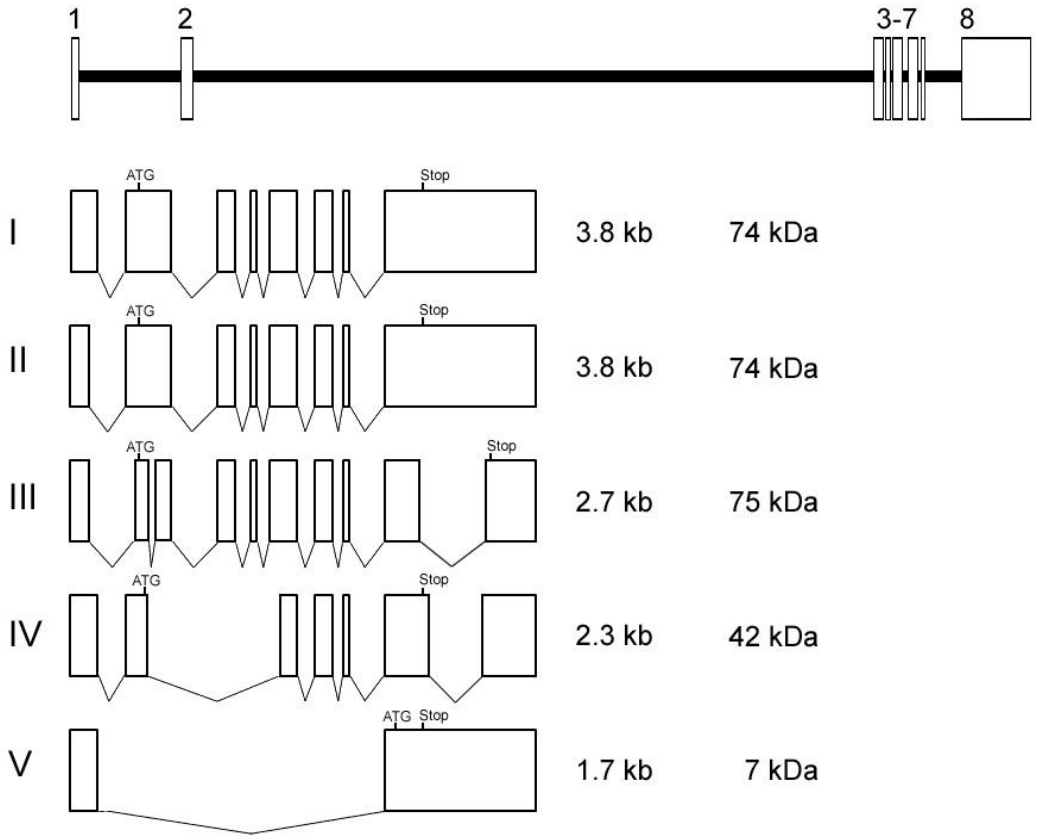
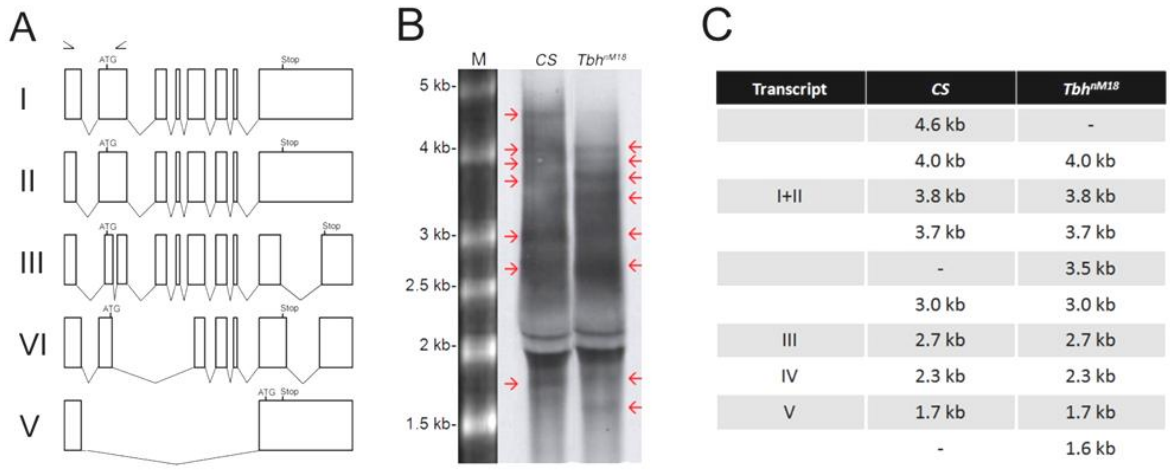


Fig. 3.1.1.1. PCR studies identify four additional *Tbh* transcripts.
The genomic organization of the *Tbh* gene with its eight exons is shown. The annotated *Tbh* transcript *Tbh-RB* (I) and four alternatively spliced transcripts (II-V) are presented with their sequence size, transcription start sites (ATG), transcription stop sites (Stop) and resulting protein sizes.

In addition to transcript analysis with RT-PCR, the sequences of available EST clones were used for comparison. One EST (GenBank ID: EY198604) indicates a larger *Tbh* 5'UTR as annotated. Additional 89 base pairs belonging to the *Tbh* 5'UTR region were confirmed performing RT-PCR with transcript specific primers (EST-F) that bind upstream to the *Tbh* gene. The additional base pairs start at the cytological position 7889641 on the X-chromosome. This position is referred to as position +1 for further position descriptions in following experiments.

To further support that additional *Tbh* splice variants exist, Northern Blot analysis was performed with *CS* cDNA from whole mount adult flies. For comparison RNA from Tbh^{nM18} mutants was used. The analysis uncovered eight *Tbh* transcripts in wild type (Fig. 3.1.1.2) using a hybridization probe that recognizes 525 base pairs of the first and the second exon (Fig. 3.1.1.2 A). The sequence was selected because it did not match any other sequence in the *Drosophila* genome. Eight *Tbh* transcripts in a range of 4.6 to 1.7 kb were identified using *CS* RNA (Fig. 3.1.1.2 B). The detected transcripts are listed in figure 3.1.1.2 C. According to their sequence size four of the detected transcripts match the five transcripts described in figure 3.1.1.2 C. The 4.6 kb and 4.0 kb sized transcripts are larger than the annotated transcript indicating additionally transcribed gene sequence than annotated. The transcript profile of the *Tbh* mutant Tbh^{nM18} differed from the transcripts of *CS* (3.1.1.2 B). Seven of the nine detected transcripts overlapped. The 4.6 kb transcript was lacking in the *Tbh* mutant. In contrast, in the *Tbh* mutant two additional transcripts of 3.5 kb and 1.6 kb were detected. Therefore the deletion in the Tbh^{nM18} mutant most likely deletes two transcripts, the missing 4.6 kb transcript and a second one, because two new



C

Transcript	CS	Tbh^{nM18}
	4.6 kb	-
	4.0 kb	4.0 kb
I+II	3.8 kb	3.8 kb
	3.7 kb	3.7 kb
	-	3.5 kb
	3.0 kb	3.0 kb
III	2.7 kb	2.7 kb
IV	2.3 kb	2.3 kb
V	1.7 kb	1.7 kb
	-	1.6 kb

Fig. 3.1.1.2. Northern Blot analysis reveals that the *Tbh* gene encodes for at least eight transcripts.
A) Primers used for generating the hybridization probe are indicated with arrowheads within the alternatively spliced *Tbh* transcripts showing that the hybridization probe consists of parts of the first and second exon. B) Northern Blot analysis identified eight *Tbh* transcripts in wild type *CS* RNA of adult whole flies indicated with an arrow. In Tbh^{nM18} mutants a different set of transcripts was detected lacking the 4.6 kb transcript and showing two additional transcripts of 3.5 kb and 1.6 kb. C) Detected *Tbh* transcripts from *CS* and Tbh^{nM18} in B are listed. It is indicated which transcripts match the alternatively spliced *Tbh* transcripts from figure 3.1.1.1.

transcripts with 3.5 kb and 1.6 kb could be detected in the mutant. It also can be said that the absent or truncated transcripts contain the sequence that is deleted in the *Tbh*^{nM18} mutant.

To summarize, the *Tbh* gene codes for at least eight transcripts whereof five are known by their sequence. To identify the alternative splicing of the other transcripts further RT-PCR studies followed by sequencing need to be performed.

3.1.2 The *Tbh* gene encodes more than one Tbh isoform

To confirm that all *Tbh* splice variants can form putative functional proteins, Western Blot analysis was performed using antibodies that were raised against different epitopes of the annotated Tbh protein. There are three available Tbh antibodies (Tab. 3.1.2). The antigen that was used to generate the antibody serum generated by Zhou and colleagues in 2008 consisted of the whole annotated Tbh protein persisting of 670 amino acids. Two other Tbh antibodies were generated in the Scholz group, one by Stefanie Hampel in 2004 and one by Osman Cibik in 2007. The antigen used by Hampel to generate the antibody serum in guinea pig consists of the first 191 amino acids of the annotated Tbh protein whereas the antigen that was used from Cibik to produce a Tbh specific antibody serum includes the amino acids 112 to 562.

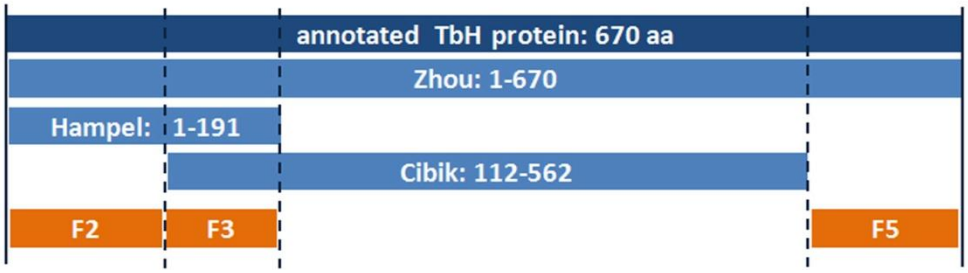
To use the different antibody sera as a tool to identify additional Tbh isoforms first it is neccesary to conform that the used Tbh antibody sera recognize Tbh epitopes. Therefore different Tbh protein fragments were used for Western Blot analysis to define the specificity of the antibodies. Firstly the antigens of the Tbh Cibik antiserum and the Tbh Hampel antiserum were used. The Tbh Cibik antigen is around 50 kDa and the Tbh Hampel antigen around 20 kDa. The 6-His tagged Tbh peptides were both expressed in *E. coli* BL21 cells and purified. The expression of the Tbh fusion peptide of Cibik and the Tbh fusion peptide of Hampel were first verified with an anti 6xHis antibody that detected both proteins in the lysate and in the purified protein fractions (Fig. 3.1.2.1 A). Detected peptides only in the purification fraction were analyzed for the three Tbh antisera due to unspecific binding in the lysate. The Tbh antibody serum from Zhou detected both Tbh peptides verifying the polyclonal character of this antibody serum (Fig. 3.1.2.1 B). The Tbh antibody serum from Cibik detected the Cibik Tbh antigen and but not the Hampel antigen (Fig. 3.1.2.1 C). The

antiserum from Hampel did not recognize the Hampel antigen. It was not clear whether the Cibik antigen was detected by the Hampel antiserum because of other unspecific detection at the same size (Fig. 3.1.2.1 C, D). However all three used Tbh antibody sera detected a protein at around 60 kDA in the lysate fraction of the Tbh Hampel antigen. The detected protein matches to a trimer of the Tbh Hampel antigen already detected and described by Hampel in 2004. The trimer most likely was not detected by the 6xHis antibody due to protein folding making the 6His tag not accessible for detection and purification. That might also be the reason why the trimer was not detected in the purified protein fraction as well.

A

Acceptor	Antibody	Designer	Amino acids
rabbit	a TbH Zhou	Zhou *et al*, 2008	aa 1-670
rabbit	a TbH Cibik	Cibik 2007	aa 112-562
guinea pig	a TbH Hampel	Hampel 2004	aa 1-191

B



Tab. 3.1.2. The three available Tbh antibody sera were generated against different Tbh antigens.
A) The available Tbh antibodies are listed showing the amino acids of the annotated *Drosophila* Tbh protein that were used to generate the antibodies. The animal that was used for antibody generation is listed as well as the origin of the antibodies. B) The annotated Tbh protein (dark blue) and the antigens used for generating the Tbh antibody sera (light blue) are shown in same scale to each other. The orange bars indicate Tbh peptides that were generated to test specificity of the antibodies.

In summary, the Tbh antisera of Zhou and Cibik detect Tbh specific epitopes. It is not sure whether the Hampel antibody serum is Tbh specific. It is possible that this antiserum detected a trimer of the used antigen but not the monomer. To investigate the specificity of the antibody sera in more detail additional Tbh peptides were generated for Western Blot analysis. The generated Tbh peptides F2, F3 and F5 are shown in table 3.1.2 B. Tbh peptide F2 contained amino acids 1 to 111 and peptide F3 amino acids 112 to 191. The third generated peptide F5 consisted of the amino acids 563 to 670. All three Tbh peptides were 6xHis tagged. The fusion proteins F2 and F5 including the 6xHis tag each had a size of 15 kDa, whereas the fusion protein F3 displayed a size of 13 kDa. The proteins were expressed in *E. coli* BL21 cells and purified afterwards. Their presences were verified with an anti 6xHis antibody which detected all three proteins in the lysate and in the purified protein fraction (Fig. 3.1.2.2 A). Detected peptides only in the purification fraction were analyzed with the three Tbh antisera due to unspecific binding in the lysate. The Tbh antibody from Zhou labeled the peptides F2 and F5 but not F3 (Fig. 3.1.2.2 B). The Tbh antibodies of Hampel and Cibik recognized none of the Tbh fragments (Fig. 3.1.2.2 C. D).

To summarize, the Tbh Zhou antibody is indeed polyclonal but does not recognize epitopes within amino acids 112 to 191. The Tbh antibody from Cibik seems to detect Tbh epitopes only within the annotated Tbh protein from amino acid 192 to 562 because other protein regions were not detectable. The Tbh antibody of Hampel did not detect any of the generated smaller Tbh peptides but a possible trimer of the Tbh Hampel antigen. This indicates that the antiserum may only detect polymeric Tbh protein structures. To identify the specificity of the Tbh antibodies of Hampel and Cibik in more detail additional Tbh protein fragments could be generated and tested. Also the purification conditions of the 6xHis tagged proteins should be improved to purify higher amounts of protein making it easier to detect the proteins.

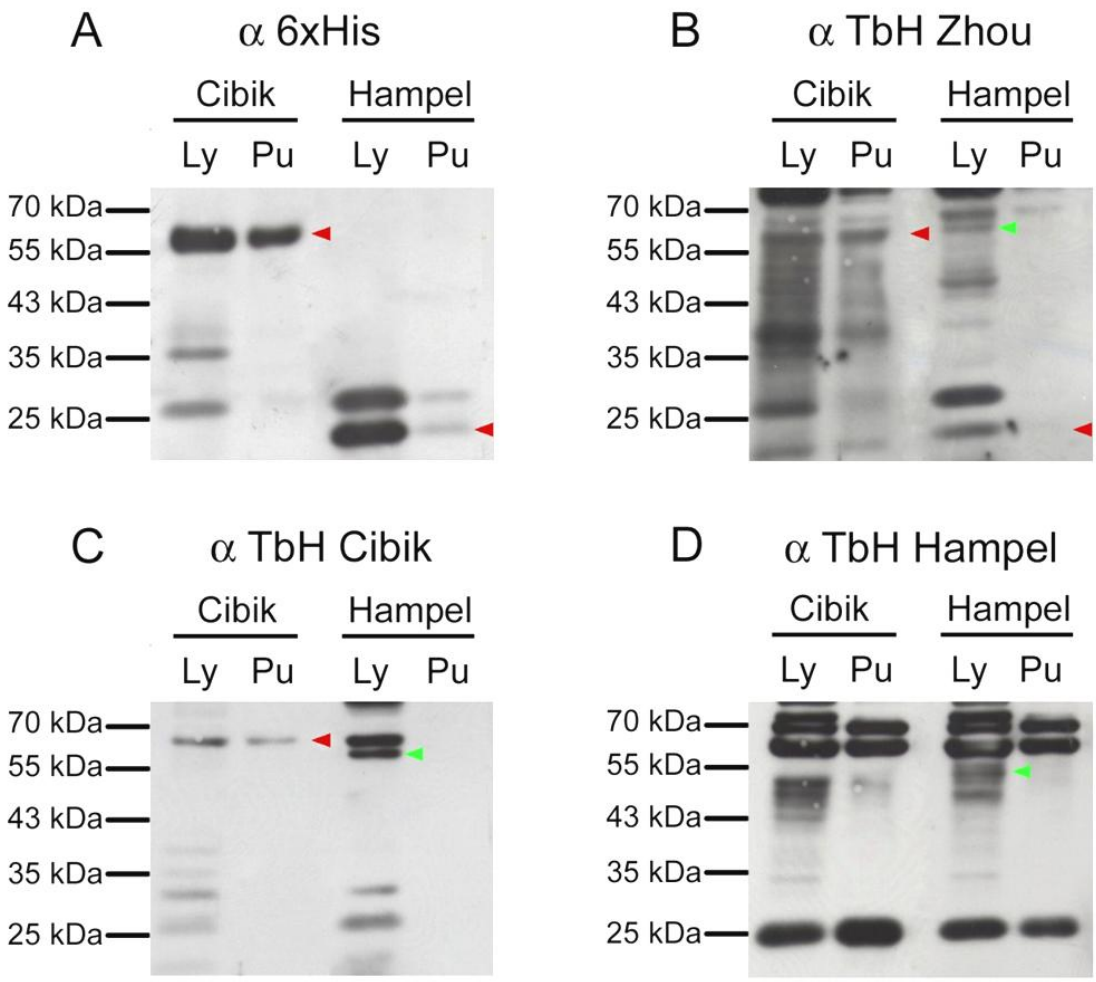


Fig. 3.1.2.1. The three available Tbh antibody sera specifically recognize Tbh peptides.
Western Blot using the Tbh Cibik antigen and the Tbh Hampel antigen is shown. By stripping the membrane after every detection, the same blot was be used for all four antibody detections. Lysate (Ly) and purified proteins from the first eluate (Pu) were loaded. A) Both antigens were verified purified protein fraction. B) The Tbh Zhou antiserum detected both the Tbh Cibik antigen and the Tbh Hampel antigen in the purified fraction (red arrows). Further, a possible trimer of the Tbh Hampel antigen was detected (green arrow). C) The antiserum of Cibik detected the Cibik antigen (red arrow) and the possible trimer of the Tbh Hampel (green arrow). D) The Tbh antiserum from Hampel only detected a possible trimer of the Tbh Hampel antigen (green arrow).

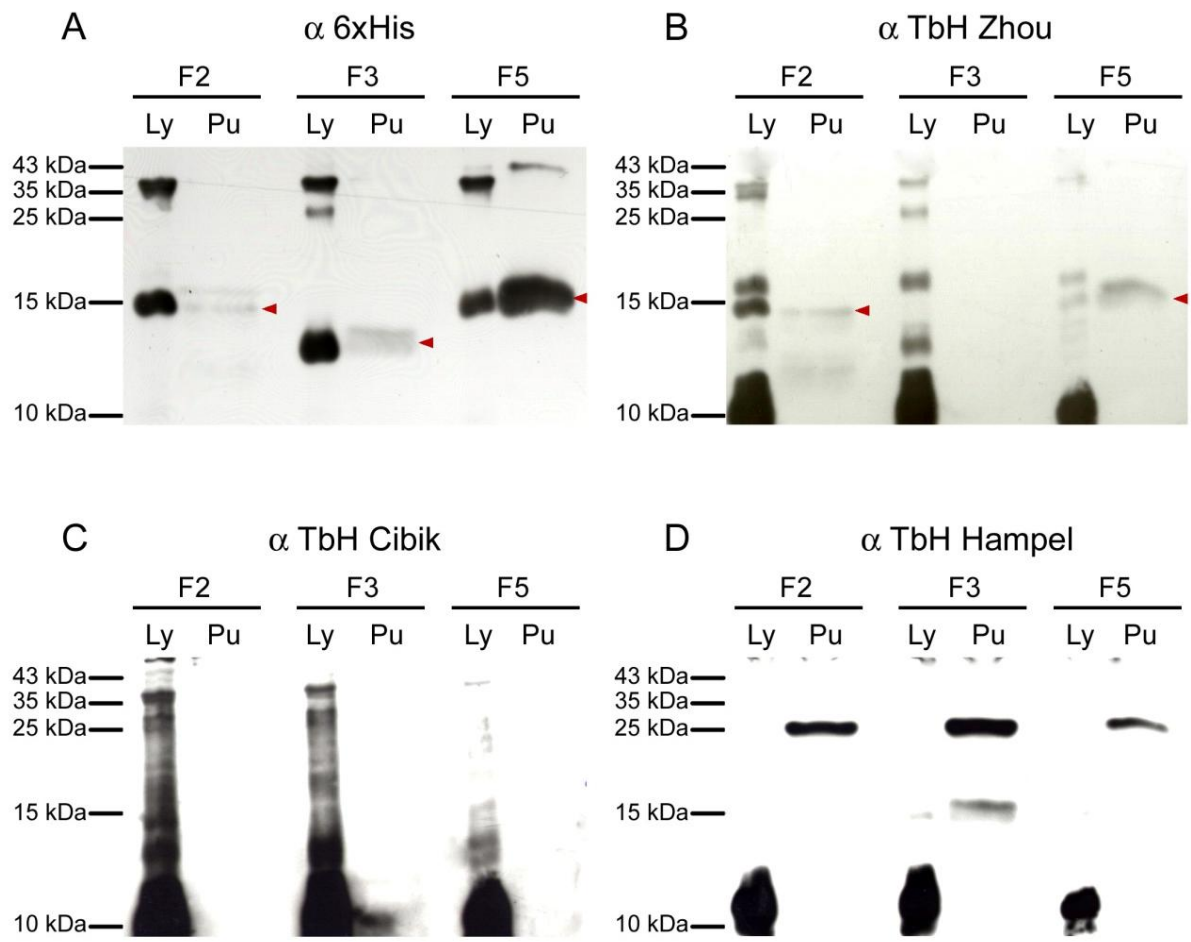


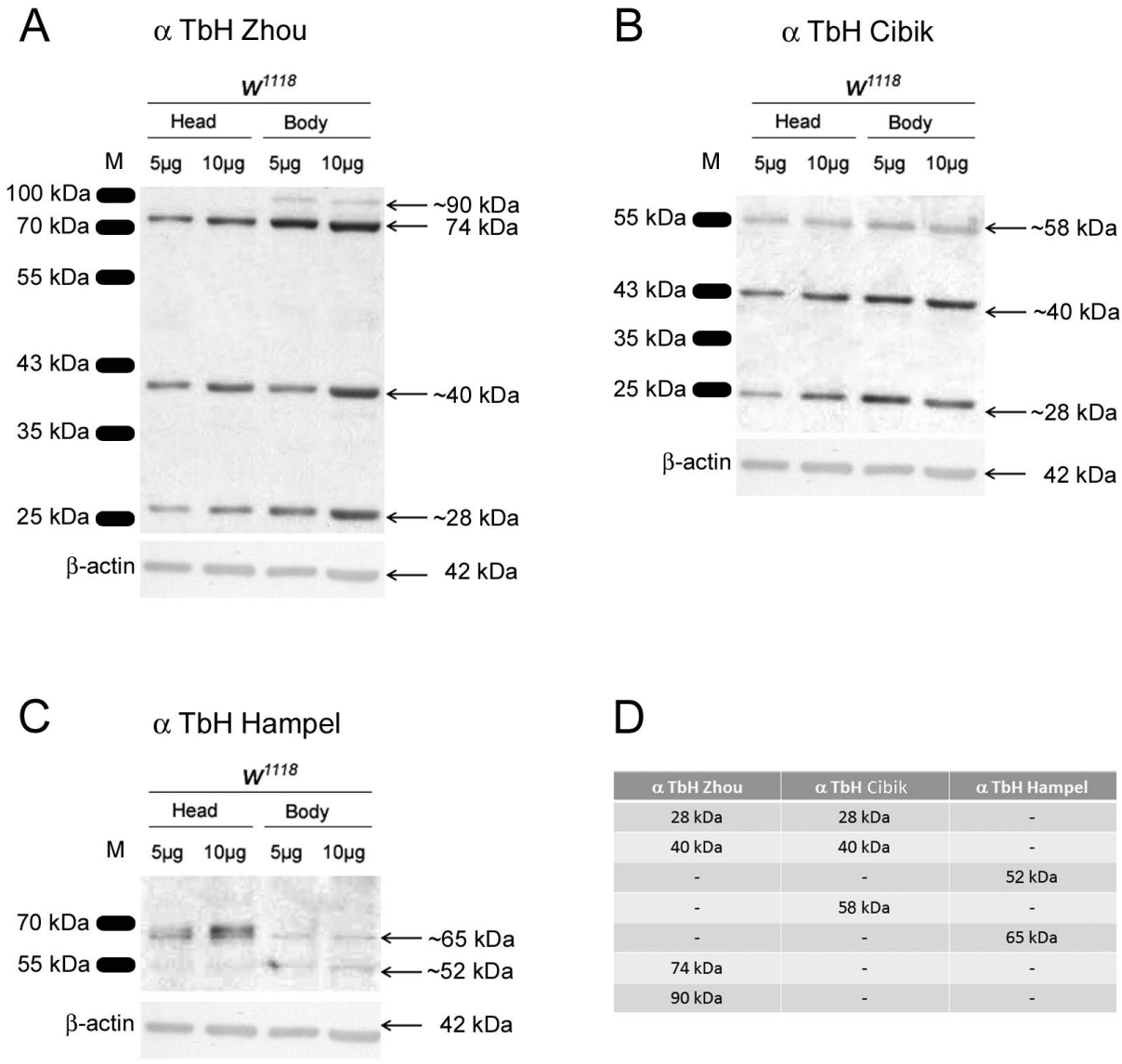
Fig. 3.1.2.2. The Tbh Zhou and Cibik antiserum detect specifically defined Tbh epitopes.
A 20% acrylamide gel was used for Western Blotting to separate the small Tbh peptides. Lysate (Ly) and purified proteins from the first eluate (Pu) of the generated Tbh peptide F2 (aa 1-111), F3 (aa 112-191) and F5 (aa 563-670) were loaded. One Western Blot was reused for four different antibody sera. Red arrow heads indicate Tbh specific bands. A) The existence of the three His tagged Tbh fusion proteins in the lysate and in the purified protein fraction was verified by a His antibody detection. B) The Tbh antibody serum of Zhou detected the fragments F2 and F5. C) The Tbh antibody from Cibik labeled none of the specific peptides. D) Also the Tbh antibody from Hampel did not detect any of the specific Tbh fusion proteins.

To conclude, the three available Tbh antibodies recognize different Tbh epitopes. The Zhou antiserum is polyclonal for the whole annotated Tbh protein. The Cibik antiserum only detects epitopes within amino acids 192-562 of the annotated Tbh protein and not within the first amino 191 acids of the annotated protein and the last 108 amino acids. The Hampel antiserum might only detect dimeric, trimeric or polymeric Tbh protein structures because none of the monomeric antigens were detected but a putative trimer of the Hampel antigen. In figure 3.1.2.3 the epitope detections of the antibody sera are schematically summarized.

Fig. 3.1.2.3. The Tbh antisera recognize different Tbh epitopes.
The full length of the annotated Tbh protein is shown in dark blue. The antigens to generate the Zhou, Cibik and Hampel antisera are indicated in relation to the annotated protein. The azure blue part represents the epitopes that were detected with the corresponding antiserum whereas the light blue region indicates the epitopes that were not detectable. To compare the isoforms with putative transcript sequence the annotated Tbh transcript is shown with its coding exons in relation to the annotated Tbh protein.

The partial specificity towards Tbh allows using the antibody sera to analyze the expression of Tbh isoforms in w^{1118} flies. Male flies were separated into body and head. More than one Tbh isoform was identified by using the three different Tbh antibody sera summarized in figure 3.1.2.4 D. The Tbh antibody serum of Zhou labeled four different protein bands (28 kDa, 40 kDa, 74 kDa, 90 kDa), one of them has the similar size than the annotated Tbh protein (74 kDa). Another one seems to be body specific (90 kDa; Fig. 3.1.2.4 A). The Tbh antiserum of Cibik detected three proteins (28 kDa, 40 kDa, 58 kDa) but definitely not the annotated Tbh protein (74 kDa; Fig. 3.1.2.4 B). Two of them were the same also detected by the Tbh antiserum

of Zhou (28 kDa and 40 kDa). The Tbh Hampel antibody labeled completely other proteins than the other two antisera. Two proteins seem to be recognized at the size around 65 kDA because one was missing in the body indicating a head specific Tbh protein. Another protein was detected at around 52 kDa (Fig. 3.1.2.4 C).



α TbH Zhou	α TbH Cibik	α TbH Hampel
28 kDa	28 kDa	-
40 kDa	40 kDa	-
-	-	52 kDa
-	58 kDa	-
-	-	65 kDa
74 kDa	-	-
90 kDa	-	-

Fig. 3.1.2.4. More than one Tbh isoform exists in male flies.
Western Blots were performed with the wild type proteome of male w^{1118} flies separated into body and head. As a loading control β-actin was chosen. A) The Tbh Zhou antibody detected four Tbh isoforms including the annotated Tbh protein of 74 kDa. B) The Tbh antibody from Cibik labeled three Tbh isoforms. C) The Tbh antibody from Hampel detected two Tbh isoforms among one seems to be a double band that is only detected in the body fraction indicating for a head specific Tbh isoform at around 65 kDa. D) The table lists all Tbh isoforms detected by the three antibodies indicating that the isoforms of 40 kDa and 28 kDa were both detected by anti Tbh Zhou and anti Tbh Cibik.

To summarize, the three Tbh antibody sera label different putative Tbh isoforms. For a first conclusion, the detected isoforms are compared to the described *Tbh* transcripts (Fig. 3.1.2.3 D). The annotated Tbh protein (74 kDa) resulting from transcript I is detectable only with the Zhou antiserum. Also the isoforms resulting from II and III could be included within this protein band because of a similar size. The isoform resulting from transcript IV matches the identified protein of 40 kDa detected by the Zhou and Cibik antisera. Tbh isoform V resulting from transcript V was not detected. This could be due to the expected small size of 7 kDa. The western blot condition chosen for the above mentioned experiment did not allow detecting proteins under the size of 10 kDa. Therefore it is still possible that the smaller Tbh isoform exists. A western blot with appropriate conditions could be done to investigate the small isoform. In addition, the proteins associated with transcript I/II and III were not recognized separately because they only differ in one kDa. 2D-gelectrophoresis might resolve this problem, because it allows separating proteins not only due to size but also due to the isoelectric point. Furthermore in the western blot analysis only male flies were used, there might be additional Tbh isoforms in female. This is likely because *Tbh* mutants have an egg laying phenotype (Monastirioti *et al.*, 1996) assuming a female specific Tbh isoform.

For a further conclusion, the putative isoforms are compared with the epitope specificity of the antisera to find out about their putative protein sequence and the associated transcripts (Fig. 3.1.2.4). The antiserum from Zhou is polyclonal for the whole annotated Tbh protein but did not detect epitopes within amino acids 112 to 191. So this region might not be accessible for antibodies. This is consistent with the other antibody sera. The peptide sequence is included in the Hampel antigen and the Cibik antigen neither can be detected with the Hampel antisera nor with the Cibik antisera. Further the Zhou antisera specifically labels epitopes within the first 111 amino acids and within the last 108 amino acids. So isoforms only detected with this antiserum (isoform of 74 kDa and 90 kDa) must contain the second or the eights exon. The antiserum from Cibik only detects Tbh epitopes from amino acid 192 to 562 the identified proteins definitely contain parts of this protein region. Transferring this fact on transcript level, the associated transcripts of the isoforms 28 kDa, 40 kDa and 58 kDa must contain fragments from the third to the eights exon. This is consistent with the identified 40 kDa isoform that reflects transcript IV (see 3.1.1.2). For the Hampel antibody serum no specific Tbh epitopes could be detected.

The results suggest that this antiserum might detect polymeric Tbh structures. Therefore the Tbh proteins (52 kDa and 65 kDa) detected by the Hampel antiserum might reflect dimers, trimers or in general polymers. The 65 kDa protein could be a dimer of the 28 kDa isoform detected by anti Zhou and Cibik. The antisera from Zhou and Cibik might not detect the dimer because of putative change in protein confirmation caused by dimerization. Also both detected proteins (52 kDa and 65 kDa) of the Hampel antibody might be polymers of the 7 kDa protein that is resulting from identified transcript V. To further investigate the putative isoforms *Tbh* mutants should be analyzed for Tbh expression. Changes in Tbh isoform expression then can be correlated to the mutation in the *Tbh* gene of the mutant to find out about the protein sequences.

3.1.3 Different Tbh isoforms are altered in the Tbh^{nM18} mutant

The best known mutation for the *Tbh* gene is the Tbh^{nM18} mutation being described as a *Tbh* null allele (Monastirioti *et al.*, 1996; Koon *et al.,* 2011) because Tbh immunoreactivity with specific Tbh antisera generated in the respective labs was shown to be missing in the mutant. To further investigate the specificity of the two Tbh antibodies generated in the Scholz lab and to further verify the identified Tbh isoforms, the Tbh^{nM18} mutant was used to examine putative Tbh isoform expression.

Western Blots were performed using proteins separated in head and body from males from the *Tbh* mutant and the wild type control w^{1118}. Tbh isoforms were detected with the Tbh antisera from Zhou, Cibik and Hampel (Fig. 3.1.3 A-C). To clarify putative differences in the expression of the isoforms in the mutant, pixel intensities of the protein bands were measured and then normalized to the loading control (β-actin) before they were compared to the wild type control. In the body fraction the detected proteins at 40 kDa, 58 kDa, 65 kDa and 74 kDa were reduced to around 60% (P=0,01; P=0,01; P=0,03; P=0,00) in the mutant. By trend the 52 kDa band was also reduced to around 60% in the body fraction but not significantly (P=0,13). In the head expression of the protein at size 65 kDa was three-fold increased (P=0,02) in the *Tbh* mutant (Fig. 3.1.3 D). By trend, a non significant two-fold increase of the 52 kDa and 90 kDa bands was seen in the head as well (P=0,26; P=0,19). Further, the 28kDa band was reduced to around 65% in the head (P=0,00). In addition to the

altered expression of isoforms, the antiserum of Zhou also detected an additional shifted protein at around 38 kDa indicating a truncated isoform (Fig. 3.1.3 A).

To summarize, expression of the Tbh isoforms at 28kDa, 40kDa, 58kDa, 65kDa and 74kDa was altered due to the *Tbhn*^{M18} mutation. Only proteins at 52kDa and 90kDa were not altered significantly. Furthermore, Tbh protein is not missing completely indication that this mutant is not a null allele. If the detected protein bands include more than one isoform, then a reduced intensity of this band could indicate that several isoforms completely lack. This could be the case for the 74 kDa band because three isoforms at 74/75 kDa were already identified. This is also consistent with expected changes due to the deletion of the *Tbh*^{nM18} mutant. The deletion affects the transcription start site and should disrupt annotated transcription. The normal protein should lack completely but a truncated protein due to an alternative start codon in the third exon could result. This would be consistent both with the truncated protein at 38 kDa specifically detected by the Zhou antiserum and the reduced band at 74 kDa. A 2D-gelectrophoresis might help to separate isoforms at the same size to investigate which isoforms really lack. Up and down regulations of isoforms could be a result of a disruption of regulatory elements. This would suggest that there are transcript variants that do not include the deleted sequence of the *Tbh* mutant within their coding sequence and therefore use alternative start codons downstream of the annotated start codon. This could then mean an additional promoter that initiates other transcripts than the described transcripts I to V. In addition, the fact that the putative Tbh isoform at 65kDa detected only by the Hampel Tbh antiserum is increased in the head and decreased in the body of the *Tbh* mutant indicates that this Tbh antibody really detects Tbh epitopes. But still this cannot be said for sure and the antibody serum needs to be further investigated to prove Tbh specificity

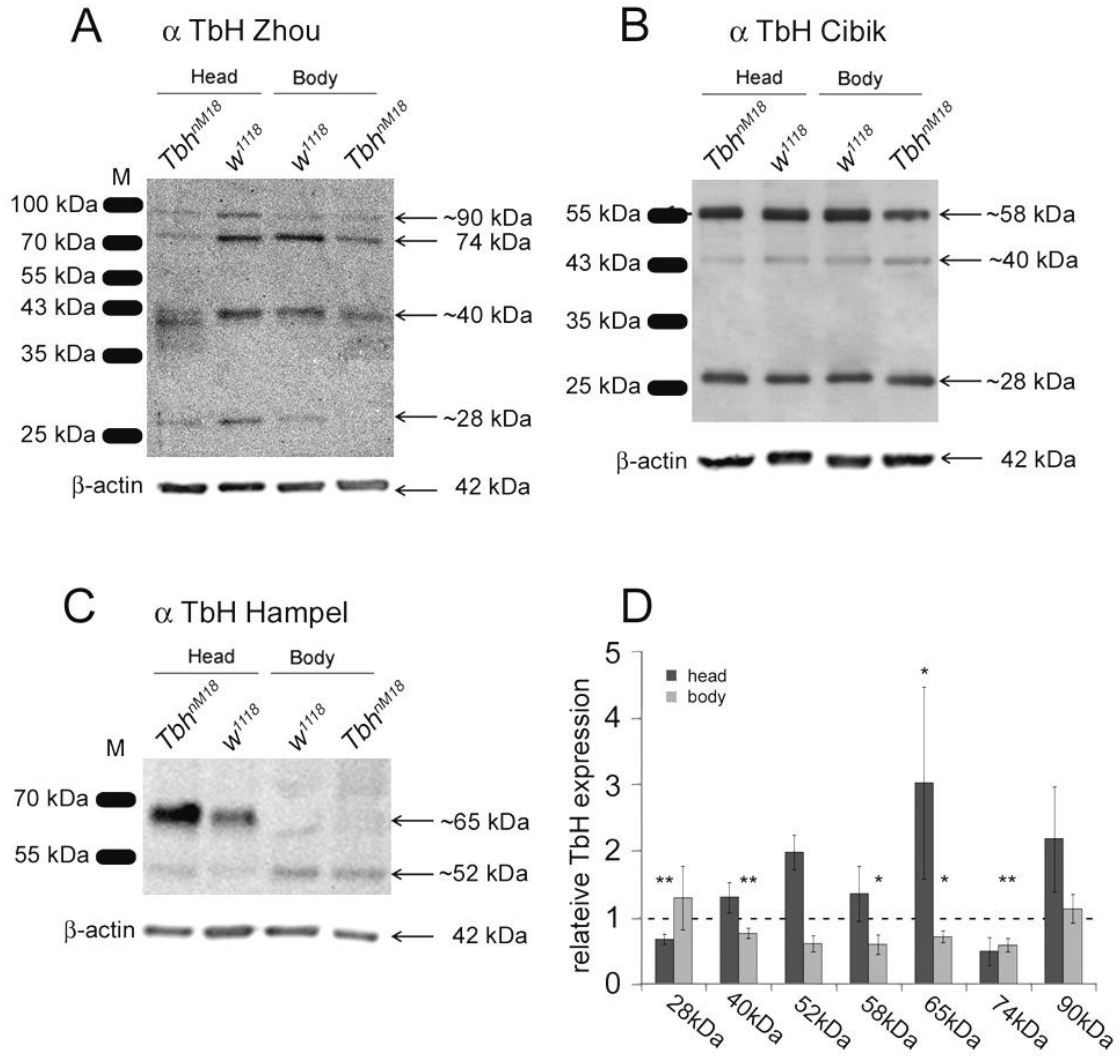


Fig. 3.1.3. The expression of putative Tbh isoforms is altered partially in the *Tbh^{nM18}* mutant

Proteins of male wild type *w^{1118}* flies and *Tbh^{nM18}* mutant flies were separated in head in body. Tbh isoforms were labeled with the three Tbh antisera from Zhou (A), Cibik (B) and Hampel (C). β-actin served as the loading control. Expression of the Tbh isoforms was partially altered. Furthermore the Zhou Tbh antiserum detected a shifted band at around 38 kDa. D) Tbh expression in the *Tbh* mutant was quantified by measuring pixel intensities of the bands. Intensities were normalized to loading control and set in relation to wild type control. Thereby the wild type expression was defined at 1, indicated by the dashed line. Proteins at 40kDa, 58kDa and 74kDa (associated with annotated protein) were significantly reduced in the body. In the head proteins at 65kDa were increased and at 28kDa were decreased (Head: 28kDa: 0,67 ± 0,08; 40kDa: 1,29 ± 0,23; 52kDa: 1,97 ± 0,26; 58kDa 1,35 ± 0,42; 65kDa: 3,02 ± 1,45; 74kDa:0,48 ± 0,21; 90kDa: 2,17 ± 0,79; Body: 28kDa: 1,29 ± 0,48; 40kDa: 0,76 ± 0,07; 52kDa: 0,60 ± 0,13; 58kDa:0,58 ± 0,15; 65kDa: 0,70 ± 0,09; 74kDa: 0,58 ± 0,10; 90kDa: 1,12 ±0,21). The error bars indicate SEM. Exemplary one blot of each antibody detection is presented in A-C but three to four blots were used for expression analysis in D.

3.1.4 The Tbh antibody serum of Cibik recognizes distinct cells in the larval CNS that do not colocalize with GAL4 expression of the *TDC2*-GAL4 driver

The different putative Tbh isoforms also might be expressed in cells required for OA synthesis (Monastirioti *et al.*, 1996). The *TDC2*-GAL4 driver line drives expression in a subset of tyraminergic/octopaminergic neurons (Busch *et al.,* 2009; Selcho *et al.*, 2012; Schneider *et al.*, 2012). Previously it has been shown that the expression of the epitopes recognized by the Zhou antibody serum is in parts overlapping with the *TDC2*-GAL4 expression pattern in the adult brain (Schneider *et al.,* 2012). To

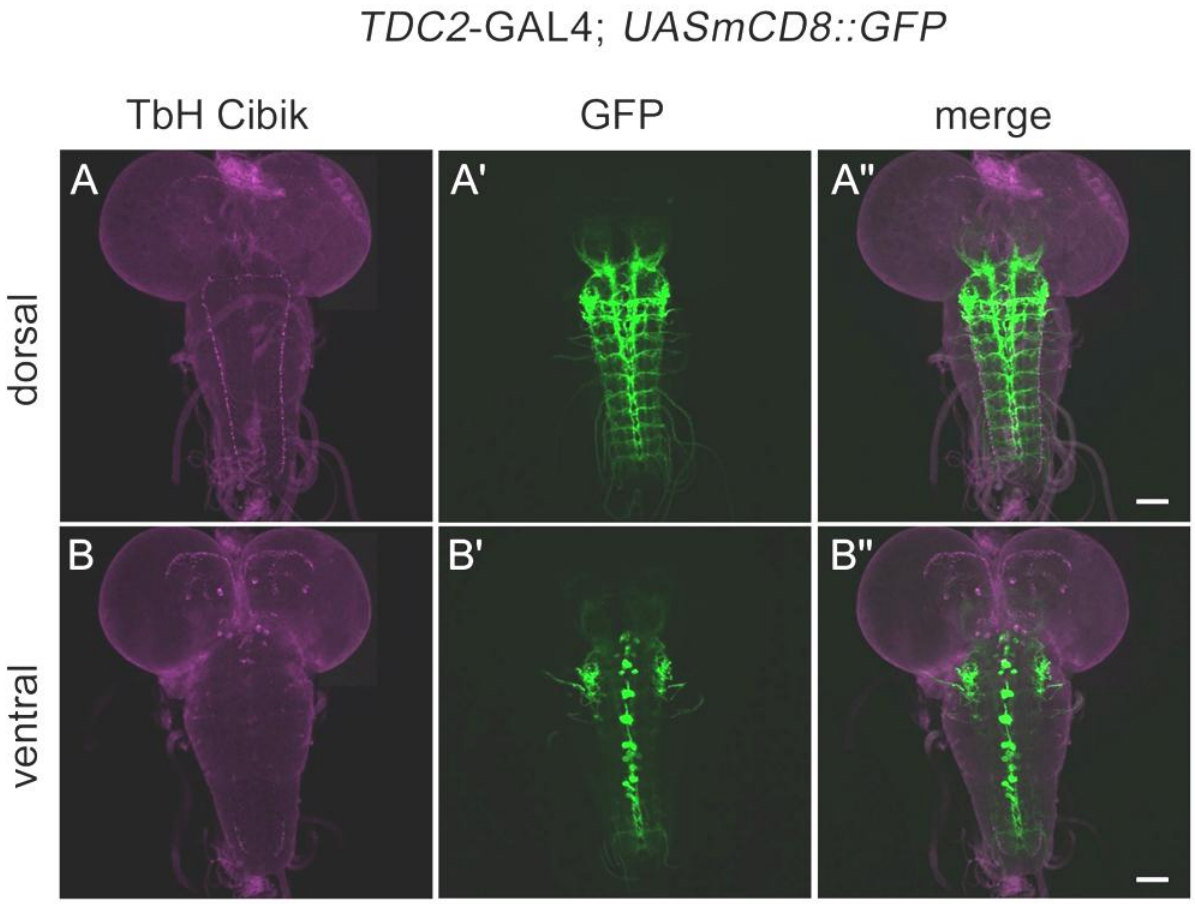


Fig. 3.1.4.1. The expression pattern of the *TDC2*-GAL4 driver line does not overlap with the expression pattern of a novel TBH antigen recognized by the Tbh Cibik antibody serum.
Larval brains of flies expressing GFP under the control of the *TDC2*-GAL4 driver line are shown with α Tbh Cibik (magenta) and α GFP (green) immunoreactivity. The merged image is presented and shows no detectable colocalizations of the Tbh Cibik expression pattern and the GFP expression. The dorsal and the ventral view of the staining are shown. The scale bars represent 50µm.

investigate whether the antigens recognized by the Cibik and Hampel antibody serum might also be expressed in the same set of cells, the expression pattern of the GAL4 line and the antigens were compared by immunohistochemistry. The GAL4 expression was visualized by a *UAS-mCD8::GFP* transgene in the larval brain and matched the expected *TDC2*-GAL4 associated expression (Honjo and Furukubo-Tokunaga, 2009; Vöme and Wegener, 2008; Fig. 3.1.4.1). The GAL4 expression was colabeled with either the Hampel or Cibik antibody serum. Using the Tbh antibody serum of Hampel no staining was achieved. Therefore no colocalization study could be performed. Using the Tbh antibody serum of Cibik cell bodies were labeled in the two hemispheres as well as in the ventral ganglion (Fig. 3.1.4.1). Besides the immunoreactivity of cell bodies also varicosities were labeled both in the hemispheres and in the ventral ganglion. But colocalization was not seen in the merged image on this level of resolution. Therefore the regions of interest were analyzed in higher magnification (Fig. 3.1.4.2). In the two hemispheres in the superior protocerebrum region immunoreactivity was only seen with the Tbh Cibik antiserum in the form of detected somata and varicosities but no GFP immunoreactivity was visible (Fig. 3.1.4.2 A). In the subesophageal region Tbh Cibik positive cell bodies were detected along the midline as paired or unpaired neurons. GFP positive cells were labeled as well in this region. But the Tbh Cibik positive cells were localized in between the detected GFP positive cells and therefore there was no colocalization (Fig. 3.1.4.2 B, C). In the ventral ganglion Tbh immunoreactive varicosities were observed surrounding the GFP signal but not colocalizing (Fig. 3.1.4.2 D).

In summary, even though the expression of epitopes detected by the Tbh Cibik antiserum in parts looks similar to the driven expression in the *TDC2*-GAL4 line no overlap was identified. Therefore not the same epitope is recognized by the Tbh antiserum of Cibik compared to the Tbh antiserum of Zhou. In comparison to the OA staining in the larval brain Tbh Cibik immunoreactive varicosities in the larval CNS looks very similar to OA expression (Monastirioti *et al.,* 1995). Therefore to confirm the functionality of the Tbh isoforms detected by the Tbh Cibik antibody colocalization studies could be performed directly colabling Tbh Cibik and OA. Also other GAL4 lines such as NP7088-GAL4 (Busch *et al.,* 2009) or Tbh-GAL4 lines (Hampel, 2007) expressing in different subsets of octopaminergic neurons could be used for colocalization studies.

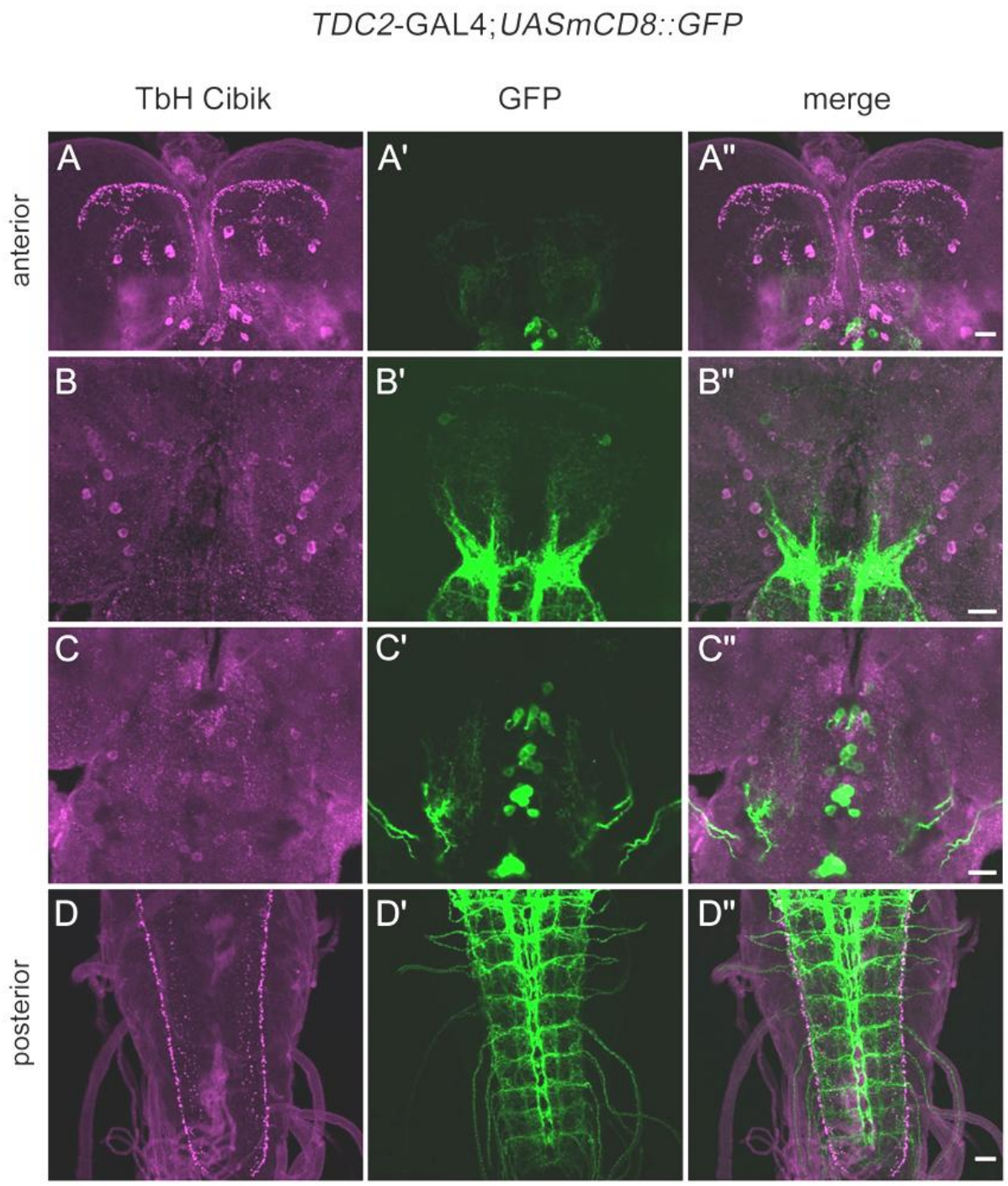


Fig. 3.1.4.2 Tbh Cibik positive cells do not colocalize with *TDC2*-GAL4 labeled neurons.
Larval CNS of flies expressing GFP under the control of the *TDC2*-GAL4 driver line are stained the Tbh antibody of Cibik (magenta) and GFP (green). 20x magnifications of regions of interest are presented. No colocalization between the GFP signal and the Tbh Cibik expression was detected. The Tbh antibody detected cell somata as well as varicosities in the two hemispheres and in the ventral ganglion. The scale bars represent 20 µm.

3.1.5 The Tbh isoforms are similar in their functional domains

The annotated Tbh protein is involved in the OA synthesis since Tbh mutants lack detectable amounts of OA (Monastirioti *et al.*, 1996). To identify whether the additional Tbh isoforms also share features with the known Tbh protein, amino acid sequences of the five Isoforms resulting from the five described *Tbh* transcripts were compared with Tbh of other related insects (Fig. 3.1.1.2). The Tbh proteins from the american cockroach *Periplaneta americana* (GenBank ID: JQ316453), the honey bee *Apis mellifera* (GenBank ID: NP_001071292) and the red flour beetle *Tribolium castaneum* (GenBank ID: XP_974169) were used. The alignment was done with the online alignment tool T-Coffee (http://tcoffee.vital-it.ch/apps/tcoffee/do:regular). Furthermore functional domains within the five Tbh isoforms were analyzed using the online tool Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motifscan).

The alignment of the five isoforms in *Drosophila* with the other three Tbh proteins of different insect species indicate high similarities between all of them (Fig. 3.1.4 A). Furthermore it can be shown that the *Drosophila melanogaster* isoforms I, II, III and the Tbh proteins of the other three insect species contain a DOMON domain, a copper type II dependent monooxygenase domain and a C-terminal copper type II dependent monooxygenase domain. *Drosophila* Tbh isoform IV lacks the DOMON domain. Further, this isoform contains a N-terminal copper type II dependent monooxygenase domain instead of the centric copper type II dependent monooxygenase domain. Isoform V of *Drosophila* Tbh bears no specific protein domains (Fig. 3.1.4 B).

Fig. 3.1.4. Protein alignment of Tbh protein sequences from different insect species including the five *Drosophila* isoforms.
Drosophila melanogaster isoforms (DmI-V) were compared to Tbh proteins from *Periplaneta americana* (Pa), *Apis mellifera* (Am) and *Tribolium castaneum* (Tc). A) Protein alignment was done using the online tool T-Coffe. Color coding is shown with red color indicating similarities between the sequences and blue indicating no similarities. B) Protein domains of the different Tbh proteins are shown in orange within the whole protein (white). DOMON domains, copper type II dependent monooxygenase domains (Monoox), C-terminal Monoox (Monoox-C) and N-terminal Monoox (Mono-N) are identified.

To summarize, it can be shown that the described *Drosophila* isoforms I to IV contain each two copper type II monooxygenase domains which are essential for proper functionality to hydroxylate tyramine to OA (Grey *et al.*, 2006; Hess *et al.,* 2008). Therefore it can be supposed that the Tbh isoforms I-V may also synthesize OA. However the function of Tbh isoform V is unclear because this isoform does not bear any functional domains.

3.2 The newly generated *Tbh*$^{R3\text{-}XPdel}$ line is impaired in ethanol tolerance development

The *Tbh*^{nM18} mutant is said to be a null allele (Monastirioti *et al.*, 1996). But former studies showed that *Tbh* gene expression in this mutant is still existent (Ruppert, 2010). The down regulation already leads to abnormal development of ethanol tolerance of this mutant (Scholz *et al.*, 2000). Additional *Tbh* alleles with differing lesions are required to study Tbh function more in detail. A null allele of the *Tbh* gene could give a deeper insight look of Tbh function with regard to alcohol induced behaviors. Therefore putative *Tbh* alleles were investigated and a new *Tbh* mutant was generated.

3.2.1 The NP938 P-element insertion is a putative new *Tbh* allele

To further investigate function of the Tbh isoforms and to uncover expression of the isoforms in the *Drosophila* CNS new *Tbh* alleles need to be identified. Tbh is associated with the development of ethanol tolerance (Scholz *et al.*, 2000). Therefore putative fly lines were tested for their ethanol tolerance behavior. The two GAL4 lines *NP938* and *NP208* were tested because they carry the P-element close to the 5'UTR region of the *Tbh* gene. The insertion of the transgenic element of the *NP938*-GAL4 (cytological position 7889235) line is closer to the first exon of the *Tbh* gene (cytological position 7889641) than the insertion of the transgenic element of the *NP208*-GAL4 (cytological position 7889150) line. For the *NP938*-GAL4 line it is already shown that it drives expression in both octopaminergic neurons and in Tbh Cibik positive cells (Hampel, 2007).

Flies were tested in the inebriometer with w^{1118} as a positive control. The positive control showed normal ethanol sensitivity (MET1: 23,33±1,15) and developed normal

ethanol tolerance (%Tolerance: 18,14±3,26; Fig. 3.2.1). Flies of the *NP208*-GAL4 line neither showed a change in sensitivity nor in tolerance compared to the control (MET1: 24,99±2,22; %Tolerance: 18,55±5,32; Fig. 3.2.1). However flies of the *NP938*-GAL4 line indeed displayed no impaired ethanol sensitivity compared to the w^{1118} control (MET1: 21,87 ±1,44) but ethanol tolerance development was increased by 100% (Fig. 3.2.1; *P*=0,02). To conclude the insertion of the P-element insertion of the *NP938*-GAL4 line influences alcohol tolerance and might be an additional *Tbh* allele. However, further tests need to be performed to investigate *Tbh* expression and the expression of the neighboring gene to validate whether alteration of the *Tbh* gene or of the neighboring gene causes the phenotype.

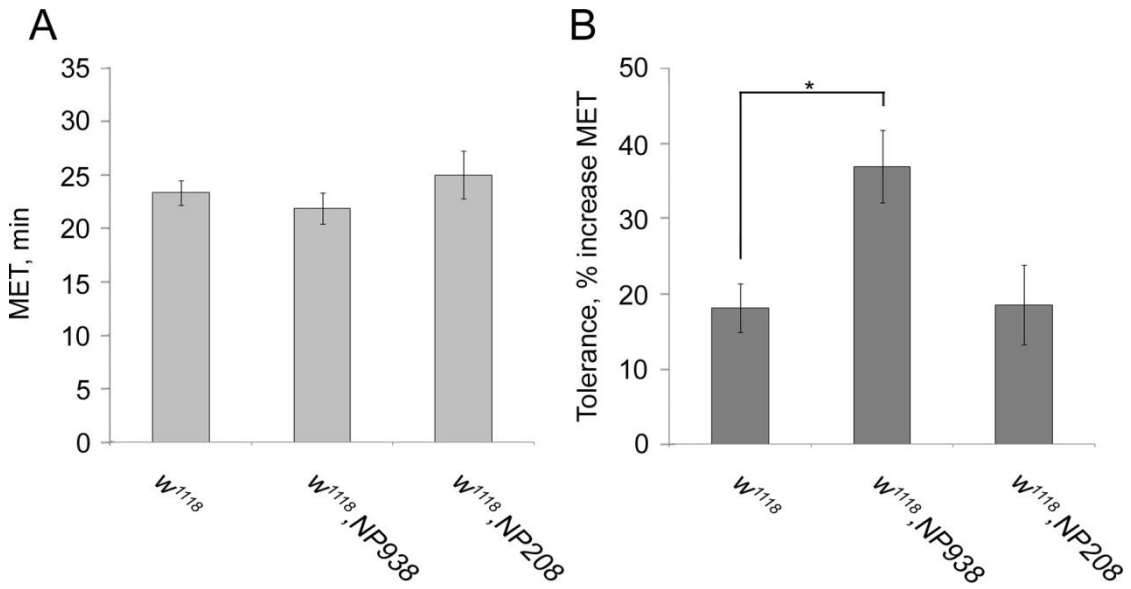
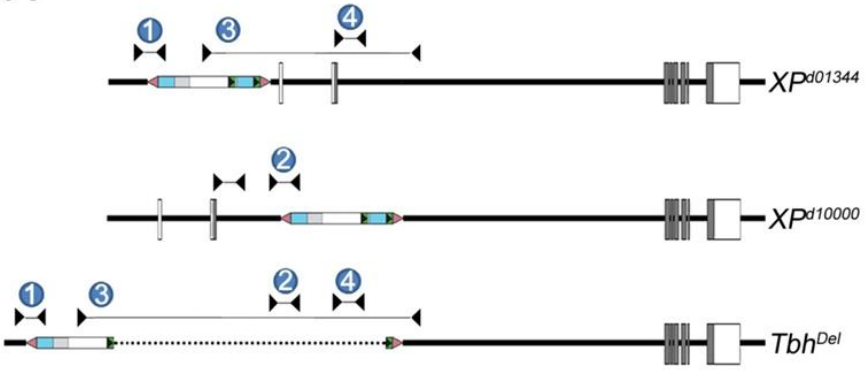


Fig. 3.2.1. The *NP938*-GAL4 line shows increased ethanol tolerance.
Ethanol sensitivity indicated by MET1 (A) and ethanol tolerance indicated by percentage increase of MET1 to MET2 (B) were investigated in the inebriometer assay for the GAL4 driver lines *NP938* and *NP208*. w^{1118} flies were used as the wild type control. A) No significant differences of the first MET were detected between the two GAL4 lines *NP983* and *NP208* and the wild type control w^{1118} (w^{1118}: 23,33 ± 1,15; w^{1118}, *NP938*: 21,87 ± 1,44; w^{1118}, *NP208:* 24,99 ± 2,22). B) Flies of the *NP938* line developed significant higher tolerance than the wild type control and the *NP208* line (w^{1118}: 18,14 ± 3,26; w^{1118}, *NP938*: 36,94 ± 4,82; w^{1118}, *NP208:* 18,55 ± 5,32). The error bars indicate SEM. $P^{*} \leq 0{,}05$, n=8

3.2.2 The generated *Tbh*$^{R3\text{-}XPdel}$ line carries a large deletion in the *Tbh* gene

Even though the *Tbh*^{nM18} mutant is said to be a null allele (Monastirioti *et al.*, 1996) *Tbh* gene expression in this mutant is detectable (Ruppert, 2010). To have a better tool to further investigating Tbh function the aim was to isolate a *Tbh* null mutant. Therefore the new *Tbh* mutant was generated performing a mutagenesis using a FLP recombination system (Parks *et al.*, 2004). This method bases on the use of two neighboring P-elements that are recombined to delete the genomic DNA sequence in between and to generate a new P-element that consist of parts of the two original P-elements. With this method the first two exons of the annotated Tbh transcript containing the transcription start site should be deleted. More precisely the expected deletion was from upstream of the gene at position -555 to the second intron at position +8694 and was caused by the upstream P-element insertion line *XP*^{d01344} and the downstream P-element insertion line *XP*^{d10000} (see 2.11; material and methods). In the last step of the performed mutagenesis flies were obtained that either carry one of the initial XP-element insertions or carry the expected deletion. 350 single crosses were set up to generate a stock of the putative new mutant. To find the right line among them it was screened for female sterility because it its known that a deletion in the *Tbh* gene can cause female sterility (Monastirioti *et al.,* 1996). It was not possible to screen for a specific eye color. Firstly, this was because both XP-element lines showed the same eye color. Further, due to a remaining *white* gene in the truncated version of the XP-element the eye color did not differ as well. A truncated XP-element would be present in flies carrying the expected deletion. A little more than 50 % of the tested fly lines were female sterile. This was expected because the *XP*^{d10000} insertion line was female sterile already and the distribution of the two XP-lines should be 50 % each. The female sterile lines were then tested by PCR. To confirm the expected deletion in of the putative fly lines and by knowing that both P-element insertions were inserted from 3' to 5', four different PCRs were done using genomic DNA isolated from whole male flies (Fig. 3.2.2.1).

A



B

	1	2	3	4
XP^{d01344}	460bp	-	-	720bp
XP^{d10000}		410bp	3100bp	720bp
Tbh^{Del}	460bp	-	2300bp	-

C

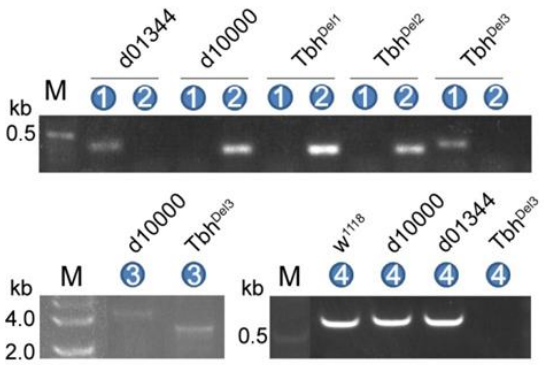


Fig. 3.2.2.1. The *Tbh*^{Del3} carries the desired deletion in the *Tbh* gene.
A) The genomic organization of the *Tbh* gene is shown including the insertions of the upstream (XP^{d01344}) and downstream (XP^{d10000}) XP-elements from 3' to 5'. The expected deletion is indicated as well as the primers used for the four PCRs. The first PCR and second PCR show whether the 3' region of the upstream P-element (1st PCR) or of the downstream P-element (2nd PCR) is present. The expected deletion can be excluded for flies only having 3' of the upstream P-element. When only the 3' region of the downstream P-element was present the third PCR was tested. The third PCR tests whether the present P-element is complete or truncated. The primer pair of the fourth PCR amplifies a fragment within the expected deletion and should be not be successful with flies carrying the deletion. B) The expected fragments for the four PCRs are listed for each possible genotype. C) According to PCR 1 and 2 the *Tbh*^{Del3} line contained only the 3' region of the upstream XP-element and not of the downstream XP-element. Further PCR 3 showed that the transgenic element in the *Tbh*^{Del3} was truncated. Also the expected band of PCR 4 lacked in this line. This confirmed the expected deletion in the *Tbh*^{Del3} line.

In the first PCR a specific primer pair (Tbh-d01344-L1 and Start-XPR) was used to identify whether the 3' region of the upstream P-element (XP^{d01344}) was present. A 460 bp fragment could only be amplified when the 3' region of the upstream P-element was present. The presence of the 3' region of the downstream XP-element (XP^{d10000}) was tested with a second PCR (primers: Tbh-d10000-L1, Start-XPR). If the 3' region of this element was present a fragment of around 410 bp was expected. The third PCR was done to investigate whether a truncated version of the P-element was present or the whole initial P-element (primers: Mut_d01344_ white_L1, Mut_2.Intron_R3). Only fly lines bearing just the 3' region of the upstream P-element were tested here because a successful deletion could be excluded for fly lines bearing only the 3' region of the downstream P-element. When the original downstream P-element is completely present a band of around 3100 bp could be amplified. When the P-element is truncated a smaller band at about 2300 bp would be expected. The primers for the fourth PCR (Unnamed sense and Unnamed anti) were set within the expected deletion. Therefore a band of around 720 bp only appears with flies not having the desired deletion (Fig. 3.2.2.1 A, B). Exemplary, first and second PCR of three potential *Tbh* mutants are shown. For the putative mutant fly lines Tbh^{Del1} and Tbh^{Del2} fragments only were amplified for the second PCR indicating that the 3' region of the downstream P-element was present. This shows that the expected deletion was definitely not present. For the putative mutant fly line Tbh^{Del3} a fragment only with the first PCR was amplified. This meant this line contained the 3' region of the upstream P-element. In the third PCR this line also showed a truncated smaller P-element. In the fourth PCR no fragment was amplified using the Tbh^{Del3} line (Fig. 3.2.2.1 C). The Tbh^{Del3} line was confirmed containing the expected deletion by PCR analysis (Fig. 3.2.2.2). The Tbh^{Del3} line was one out of the first ten tested putative *Tbh* fly lines which were preselected by female sterility. Including the preselection by female sterility it can be said that one fly line out of 20 carried the expected deletion. To conclude, far too many flies were set up to generate the deletion because the mutagenesis efficiency was about 5 %.

From here, the new *Tbh* mutant is referred to as the $Tbh^{R3\text{-}XPdel}$ line named after a combination of producer (R for Ruppert), number of potential fly line tested by PCR (3 for Tbh^{Del3}) and the type of deletion (XPdel for deletion by XP-element recombination). A final map of the genomic organization of the new *Tbh* mutant $Tbh^{R3\text{-}XPdel}$ is shown in figure 3.2.2.2.

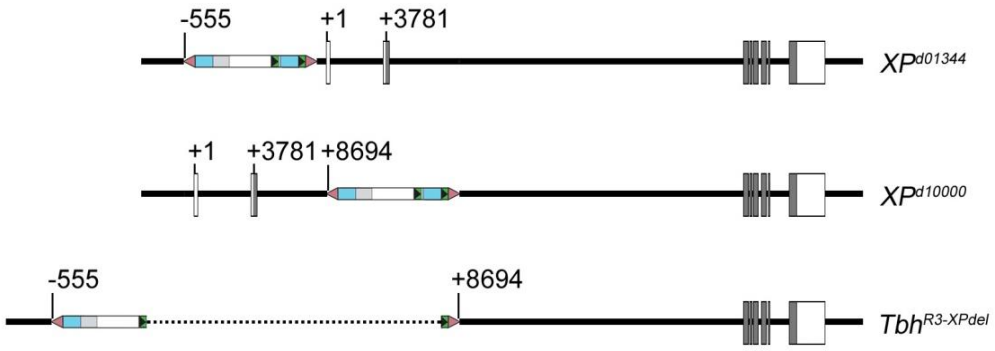


Fig. 3.2.2.2. Deletion mapping of the new *Tbh* mutant $Tbh^{R3\text{-}XPdel}$.
The two XP-element lines XP^{d10000} and XP^{d01344} used for generating the new *Tbh* mutant are shown with the exact insertion of the XP-elements in the *Tbh* gene. The positions refer to the first base pair of the *Tbh* gene. The deletion is caused due to recombination of the two XP-elements. The sequence in between the two XP-element lines is deleted and a residual truncated transgenic element evolves.

3.2.3 Specific *Tbh* transcripts are reduced in the new $Tbh^{R3\text{-}XPdel}$ mutant

In the Tbh^{nM18} mutant *Tbh* transcript expression is strongly down regulated (Ruppert, 2010). The deletion of this mutant includes less sequence than the deletion of the new $Tbh^{R3\text{-}XPdel}$ mutant. Therefore it is suggested that the deletion of the new mutant should affect *Tbh* transcript expression as well. To test whether the deletion affects *Tbh* transcript expression qRT-PCR was performed using *RplPo* as the loading control compared to normal *Tbh* expression in w^{1118}. As an additional control the XP-element lines used for mutagenesis were tested as well (Fig. 3.2.3).

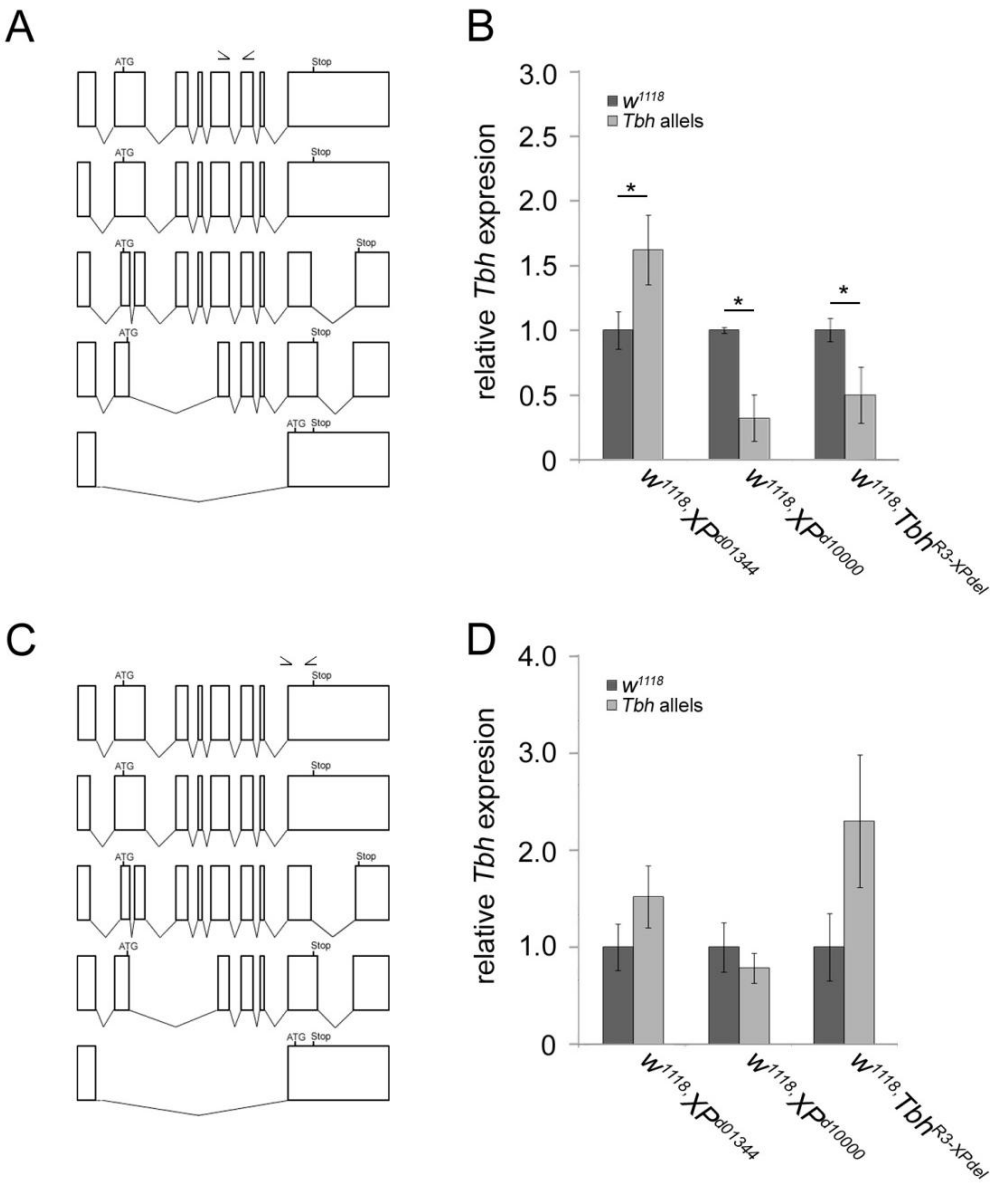


Fig. 3.2.3. The new *Tbh* mutant *Tbh*$^{R3\text{-}XPdel}$ displays *Tbh* transcript specific down regulation.

qRT-PCRs were performed on cDNA synthesized from whole male flies using *RplPo* as a loading control. *Tbh* expression in the w^{1118} control is normalized to a value of +1. *Tbh* expression is compared to the normalized *Tbh* expression of the control. A) *Tbh* primers used for the first qRT-PCR are indicated within the *Tbh* transcripts and are specific for *Tbh* transcripts I to IV. B) With the first qRT-PCR *Tbh* transcript specific up regulation in the left XP-element line and a down regulation in the right XP-element line was detected. Specific *Tbh* transcripts were down regulated in the new *Tbh* mutant as well (XP^{d01344}: 1,62 ± 0,14; XP^{d10000}: 0,32 ± 0,18; $Tbh^{R3\text{-}XPdel}$: 0,50 ± 0,21). C) Primers used for the second qRT-PCR are indicated with arrowheads including all shown transcripts. D) In the second qRT-PCR no change in *Tbh* expression was detected in none of the genotypes (XP^{d01344}: 1,52 ± 0,24; XP^{d10000}: 0,78 ± 0,15; $Tbh^{R3\text{-}XPdel}$: 2,30 ± 0,68). The error bars indicate SD. $P^{*} \leq 0{,}05$

Firstly qRT-PCR with a primer pair specific for *Tbh* transcripts I to IV except transcript V (Tbh-RT-L, Tbh-RT-R) was performed to quantify specific *Tbh* transcript expression (Fig. 3.2.3 A). The downstream XP-element line XP^{d10000} displayed a *Tbh* transcript specific down regulation to around 30% (*P*=0,02) whereas the upstream XP-element line XP^{d01344} displayed a *Tbh* transcript specific upregulation to around 160% (*P*=0,02). In the new *Tbh* mutant the expression of transcripts I to IV was significantly reduced to around 50% but still detectable (*P*=0,05; Fig. 3.2.3 B). To investigate the expression of all described *Tbh* transcripts a second qRT-PCR was performed using primers (All_L, All_R2) amplifying a sequence present in all transcript variants (Fig. 3.2.3 C). With this no significant differences in *Tbh* expression was detected with neither the new *Tbh* mutant (*P*=0,96) nor the XP-element lines (P=0,26; P=0,10; Fig. 3.2.3 D).

To conclude, the new *Tbh* mutant displays a *Tbh* transcript I to IV specific down regulation. However the right XP-element line that was used to generate this allele also shows a similar transcript specific down regulation. It cannot be said whether the generated deletion or the P-element insertion itself causes the change in *Tbh* transcript expression. To validate this, Tbh protein expression and behavioral phenotypes of both lines were investigated. Also notable is that both XP-element insertions already disrupt normal *Tbh* gene expression. In the case of the upstream XP-element insertion, inhibitory elements upstream of the *Tbh* gene seem to be affected due to upregulated gene expression. In case of the downstream XP-element insertion, regulatory elements within the second intron seem to be interrupted. Therefore both XP-element lines seem to be additional new *Tbh* alleles as well. To further investigate this, both fly lines should be characterized for Tbh protein expression and behavioral phenotypes.

3.2.4 Tbh isoform expression is differently altered in the XP^{d10000} line and in the new $Tbh^{R3\text{-}XPdel}$ mutant

It was shown before with the Tbh^{nM18} mutant that already a small deletion in the *Tbh* gene, affects both *Tbh* transcript expression and Tbh protein expression (Ruppert, 2010; Monastirioti *et al.*, 1996). To further investigate how the deletion in the *Tbh* gene of the newly generated *Tbh* mutant influences Tbh isoform expression western blots studies were performed. Also the XP-element line XP^{d10000} was tested to identify

the influence of the P-element insertion and to examine whether the deletion led to a stronger effect.

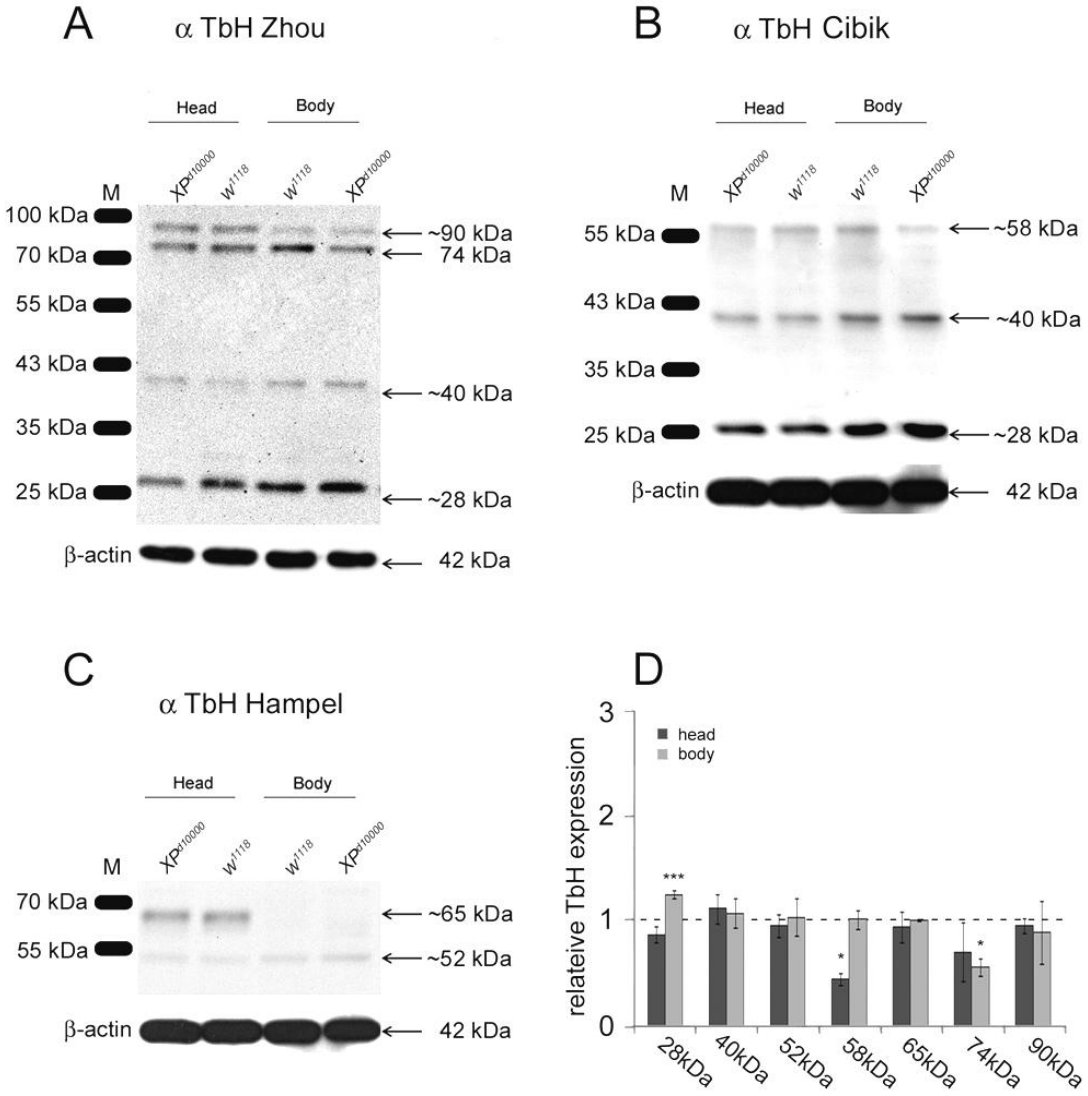


Fig. 3.3.4.1. In *XP^{d10000}* Tbh protein expression is partially altered.
Tbh isoforms were labeled with the three Tbh antisera from Zhou (A), Cibik (B) and Hampel (C). β-actin served as the loading control. Proteins of male *w^{1118}* flies and *XP^{d10000}* flies were separated in head in body. Tbh expression in the *XP^{d10000}* line was set in relation to the expression in the control measuring pixel intensities of the bands. Control expression was defined as +1, indicated by the dashed line. In the head only Tbh isoform at 58 kDa was reduced in the mutant. In the body protein at 28kDa was increased and protein at 74kDa was decreased (Head: 28kDa: 0,87 ± 0,07; 40kDa: 1,11 ± 0,14; 52kDa: 0,95 ± 0,11; 58kDa: 0,44 ± 0,06; 65kDa: 0,93 ± 0,15; 74kDa: 0,70 ± 0,28; 90kDa: 0,95 ± 0,07; Body: 28kDa: 1,25 ± 0,03; 40kDa: 1,07 ± 0,13; 52kDa: 1,03 ±0,18; 58kDa: 1,01 ± 0,09; 65kDa: 1,00 ± 0,00; 74kDa: 0,56 ± 0,08; 90kDa: 0,89 ± 0,29). The error bars indicate SEM. Exemplary one blot of each antibody detection is presented in A-C but three blots were analyzed in D.

Therefore the *Tbh*$^{R3\text{-}XPdel}$ mutant and the additional identified *Tbh* allele *XP*^{d10000} were analyzed for Tbh isoform expression with western blot analysis using proteins separated in head and body. Proteins of *w*^{1118} flies served as the wild type control. Tbh isoforms were detected with the Tbh antisera from Zhou, Cibik and Hampel. To clarify putative differences in the expression of the isoforms in the two genotypes, pixel intensities of the protein bands were measured and then normalized to the loading control (β-actin) before they were compared to the wild type control. For the *XP*^{d10000} line expression of Tbh isoform at 28kDa in the body was increases slightly to 120% (*P*=0,00). Further in the body also Tbh isoforms at 74kDa were decreased to around 60% (*P*=0,02). By trend the Tbh isoform at 74kDa also seemed to be decreased in the head but this was not significant (*P*=0,39). The expression of protein at 56 kDa isoform in the was detected to be reduced to around 45% (*P*=0,02). Other protein bands were not affected by the XP^{d10000} insertion (Fig. 3.2.4.1). In the newly generated *Tbh* mutant only significant difference in Tbh expression was detected in the body. Proteins at 28kDa (*P*=0,00), 52kDa (*P*=0,00), 58kDa (*P*=0,02) and 90kDa (*P*=0,00) were reduced to around 50-60%. By trend also proteins at 40kDa (*P*=0,16) and at 74kDa (*P*=0,09) were reduced. In the head no significant difference in Tbh expression was detected (Fig. 3.3.4.2).

In summary, Tbh isoforms are altered in the *XP*^{d10000} line as well as in the *Tbh*$^{R3\text{-}XPdel}$ mutant but Tbh is still detectable in both alleles indicating that they are not null alleles. In the new *Tbh* mutant more isforms were affected thatn in the XP-line. Therefore the deletion in the mutant causes other Tbh expression impairments than the P-element insertion itself. The less strong effect detected in the *XP*^{d10000} line leads to the conclusion that the *Tbh* transcript specific down regulation caused by this XP-element insertion either can be compensated on protein level or additional isoforms affected by the insertion are not detected by the used antibodies. If only regulatory elements are affected by the XP^{d10000} insertion then this would indicate an additional *Tbh* promoter. Compared to the *Tbh*^{nM18} mutant in the new *Tbh* mutant no protein shift was detected indicating that the sequence with the alternative start codon is deleted.

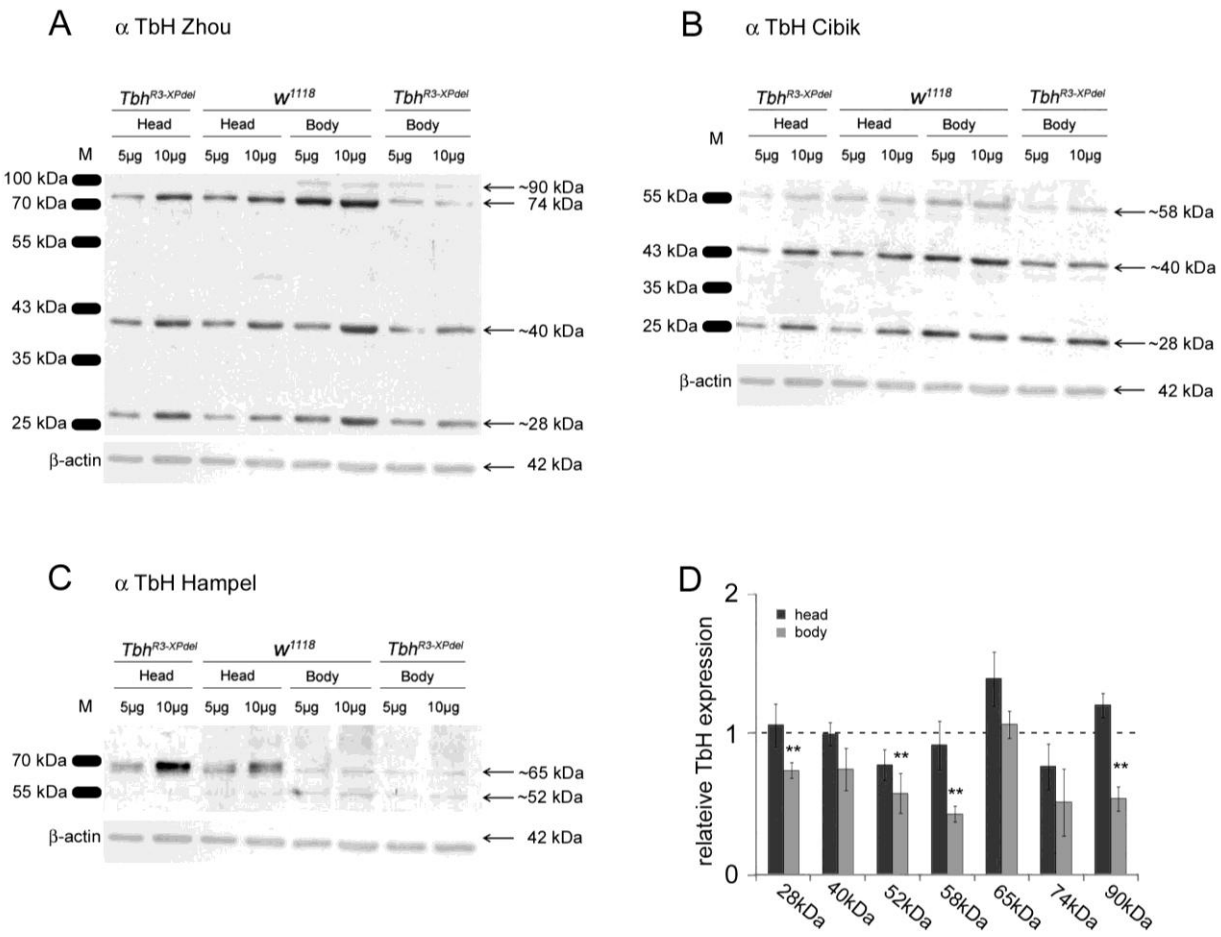


Fig. 3.3.4.2. Different Tbh isoforms are altered in the *Tbh$^{R3\text{-}DelXP}$* mutant. Proteins of male *w^{1118}* flies and *Tbh$^{R3\text{-}XPdel}$* mutant flies were separated in head in body. Tbh isoforms were detected with the Tbh antisera from Zhou (A), Cibik (B) and Hampel (C). β-actin was used as the loading control. Significantly different expression was only detected in the body for proteins at 28kDa, 52kDa, 58kDa and 90kDa. (Head: 28kDa: 1,06 ± 0,15; 40kDa: 1,00 ± 0,08; 52kDa: 0,78 ± 0,11; 58kDa: 0,92 ± 0,17; 65kDa: 1,39 ± 0,19; 74kDa: 0,77 ± 0,16; 90kDa: 1,20 ± 0,09; Body: 28kDa: 0,74 ± 0,06; 40kDa: 0,75 ± 0,15; 52kDa: 0,58 ± 0,14; 58kDa: 0,43 ± 0,06; 65kDa: 1,07 ± 0,10; 74kDa: 0,51 ± 0,24; 90kDa: 0,54± 0,09). The error bars indicate SEM. Exemplary one blot of each antibody detection is presented in A-C but three blots were used for expression analysis in D.

3.2.5 The *Tbh*$^{R3\text{-}XPdel}$ mutant develops reduced ethanol tolerance

The *Tbh*^{nM18} mutant shows normal ethanol sensitivity and reduced ethanol tolerance (Scholz *et al.*, 2000; Scholz *et al.*, 2005). To test whether the new isolated *Tbh*$^{R3\text{-}XPdel}$ mutant is also impaired in developing normal ethanol tolerance the mutant is tested in the inebriometer. In addition, the two XP-elements that were used to generate the new allele were tested as well to investigate the influence of the P-element insertions.

Flies of the *w*^{1118} genotype were used as the controls and showed normal ethanol sensitivity (MET1: 22,77±0,72) and ethanol tolerance (%Tolerance: 25,02±2,51). The upstream XP-element line *XP*^{d01344} line displayed a significantly reduced resistance of two minutes towards ethanol (*P*=0,03) but a normal level of ethanol tolerance compared to the control (%Tolerance: 22,50±2,24). Ethanol sensitivity of flies carrying the downstream XP-element *XP*^{d10000} was not significantly impaired (MET1: 25,21±0,81). However these flies develop a 50% reduced ethanol tolerance (*P*=0,00). The new *Tbh* mutant showed normal ethanol sensitivity (MET1: 23,52±0,50) and developed 50% reduced ethanol tolerance (*P*=0,00; Fig. 3.2.5). To conclude, the newly generated *Tbh* mutant is impaired in developing normal ethanol tolerance but shows normal resistance towards ethanol. The downstream XP-element line shows the same phenotype. These two observations are consistent with less *Tbh* expression causing reduced ethanol tolerance described for the *Tbh*^{nM18} mutant (Scholz *et al.,* 2000). In addition flies of the upstream XP-element line *XP*^{d10000} are less resistant towards ethanol. They also might be impaired in developing normal ethanol tolerance because the development of ethanol tolerance is dose dependant (Scholz *et al.*, 2000). Being firstly exposed to less ethanol would result in a lower level of ethanol tolerance compared to flies exposed to more ethanol in the first run. For the upstream XP-element line *XP*^{d10000} a normal level of ethanol tolerance was detected. Therefore these flies might develop increased levels of ethanol tolerance. To validate this, these flies could be tested for ethanol tolerance after being exposed to a uniform amount of ethanol like the control. Overexpressed levels of *Tbh* transcripts were not investigated so far therefore it cannot be said if the observed *XP*^{d01344} phenotype is as one would expect.

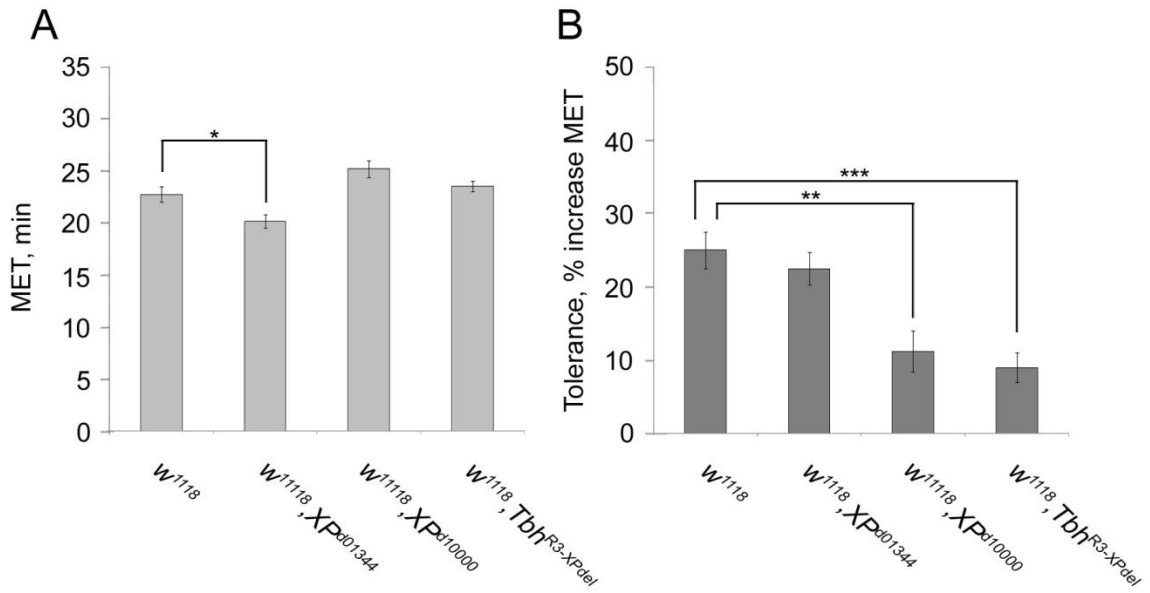


Fig. 3.3.5. The new *Tbh* mutant $Tbh^{R3\text{-}XPdel}$ displays normal ethanol
Ethanol sensitivity (A) and ethanol tolerance (B) were tested with the inebriometer for the new *Tbh* mutant $Tbh^{R3\text{-}XPdel}$ and the XP-element lines XP^{d10000} and XP^{d01344}. w^{1118} flies were used as the control. A) The XP^{d01344} flies showed reduced ethanol resistance. The new *Tbh* mutant and the XP^{d10000} line were not impaired in ethanol sensitivity (w^{1118}: 22,77 ± 0,72 28; w^{1118}, XP^{d01344}: 20,20 ± 0,64; w^{1118}, XP^{d10000}: 25,21 ± 0,81; w^{1118}, $Tbh^{R3\text{-}XPdel}$: 23,52 ± 0,50. B) The new *Tbh* mutant $Tbh^{R3\text{-}XPdel}$ and the XP^{d10000} line developed reduced ethanol tolerance. The ethanol tolerance of the XP^{d01344} line was not impaired.(w^{1118}: 25,02 ± 2,51; w^{1118}, XP^{d01344}: 22,50 ± 2,24; w^{1118}, XP^{d10000}: 11,20 ± 2,79; w^{1118}, $Tbh^{R3\text{-}XPdel}$: 9,01 ± 2,00). The error bars indicate SEM. $P^{*} \leq 0{,}05$, $p^{**} \leq 0{,}01$, $p^{***} \leq 0{,}001$, n=14-23

3.3 Tbh function is required in the adult fly to form normal ethanol tolerance

Flies of the *Tbh* mutant Tbh^{nM18} fail to develop normal ethanol tolerance but are wild type with regard to ethanol sensitivity (Scholz *et al.*, 2000). The question was where and when Tbh is required to from normal ethanol tolerance. Two different approaches were done. Firstly the aim was to figure out where in the fly Tbh is required and more precisely which neurons in *Drosophila* mediate ethanol tolerance. Therefore wild type *Tbh* was induced into different sets of neurons in the Tbh^{nM18} mutant to subsequently test ethanol tolerance. Secondly it was investigated whether Tbh function is required during embryonic and larval development or whether it is sufficient to have normal Tbh function during adulthood to develop wild type ethanol

tolerance. Therefore *Tbh* was restored in the Tbh^{nM18} mutant only in the adult stage of *Drosophila* with a heat shock driven *UAS-Tbh* transgene.

3.3.1 Reduced ethanol tolerance of Tbh^{nM18} mutant flies cannot be restored by induced pan-neuronal *Tbh* expression

It was already shown that the reduced ethanol tolerance of Tbh^{nM18} mutants cannot be restored by restoring Tbh in different subsets of octopaminergic neurons (Ruppert, 2010). Here Tbh was restored in the Tbh^{nM18} mutant in a pan-neuronal way including broader sets of octopaminergic neurons to test changes in ethanol tolerance. The pan-neuronal driver lines *Appl*-GAL4 (Torroja *et al.*, 1999) and *elav*-GAL4 (Yannoni and White, 1999) were used. Male flies were tested in the inebriometer for ethanol sensitivity and tolerance. In both experiments the positive control was w^{1118}.

Firstly, expression of *UAS-Tbh* in an *Appl*-GAL4 dependent manner in the *Tbh* mutant did not alter ethanol sensitivity (Fig. 3.4.1 A) and also did not restore normal ethanol tolerance (Fig. 3.4.1 B). The positive control w^{1118} showed normal ethanol sensitivity and tolerance. The MET1 of the experimental group (w^{1118}, Tbh^{nM18}, *UAS-Tbh*; *Appl*-GAL4) was not different from the mutant controls (P=0,76; w^{1118}, Tbh^{nM18}; *Appl*-GAL4 and P=0,31; w^{1118}, Tbh^{nM18}, *UAS-Tbh*). The data for ethanol tolerance showed that the experimental flies still showed reduced ethanol tolerance compared to the positive control (P=0,00) and not different to the mutant controls (P=0,99; P=0,78; Fig. 3.3.1 B). In addition, both the insertions of the *Appl*-GAL4 transgene and *the UAS-Tbh* transgene slightly affect ethanol sensitivity because the MET1 values of the flies carrying the *Appl*-GAL4 and or the UAS transgene were significantly higher than the wild type control w^{1118} (P=0,01; P=0,00; P=0,00; Fig. 3.3.1 A). Secondly, expression of *UAS-Tbh* in *elav*-GAL4 driven neurons did not influence ethanol sensitivity (Fig. 3.4.1 C) and did not restore normal ethanol tolerance in the Tbh^{nM18} mutant (Fig. 3.4.1 D). The positive control w^{1118} showed normal ethanol sensitivity and tolerance. The MET1 values of the mutant control carrying the *elav*-GAL4 transgene (w^{1118}, Tbh^{nM18}, *elav*-GAL4) was not different to the experimental group (w^{1118}, Tbh^{nM18}, *UAS-Tbh, elav*-GAL4; P=0,35). Further, the level of tolerance of the experimental group did not differ significantly from the two mutant control groups (P=0,25; w^{1118}, Tbh^{nM18}, *elav*-GAL4 and P=1,00; w^{1118},

Tbh^{nM18}, *UAS-Tbh*). They failed to develop normal ethanol tolerance. In addition, an influence of the *elav*-GAL4 insertion on ethanol sensitivity was detected as well as the effect of the *UAS-Tbh* transgene (like in 3.3.1 A) on ethanol sensitivity. The influence of the *elav*-GAL4 insertion was stronger than the insertion of the *UAS-Tbh* transgene. The MET1 values of the two mutant controls and the experimental group were significantly higher than the *w*^{1118} control (*P*= 0,00 ; *P*= 0,04; *P*=0,00) whereby the MET1 values of the flies carrying the *Appl*-GAL4 transgene were higher than the MET1 of flies only carrying the *UAS-Tbh* transgene (*P*= 0,00; *P*= 0,00).

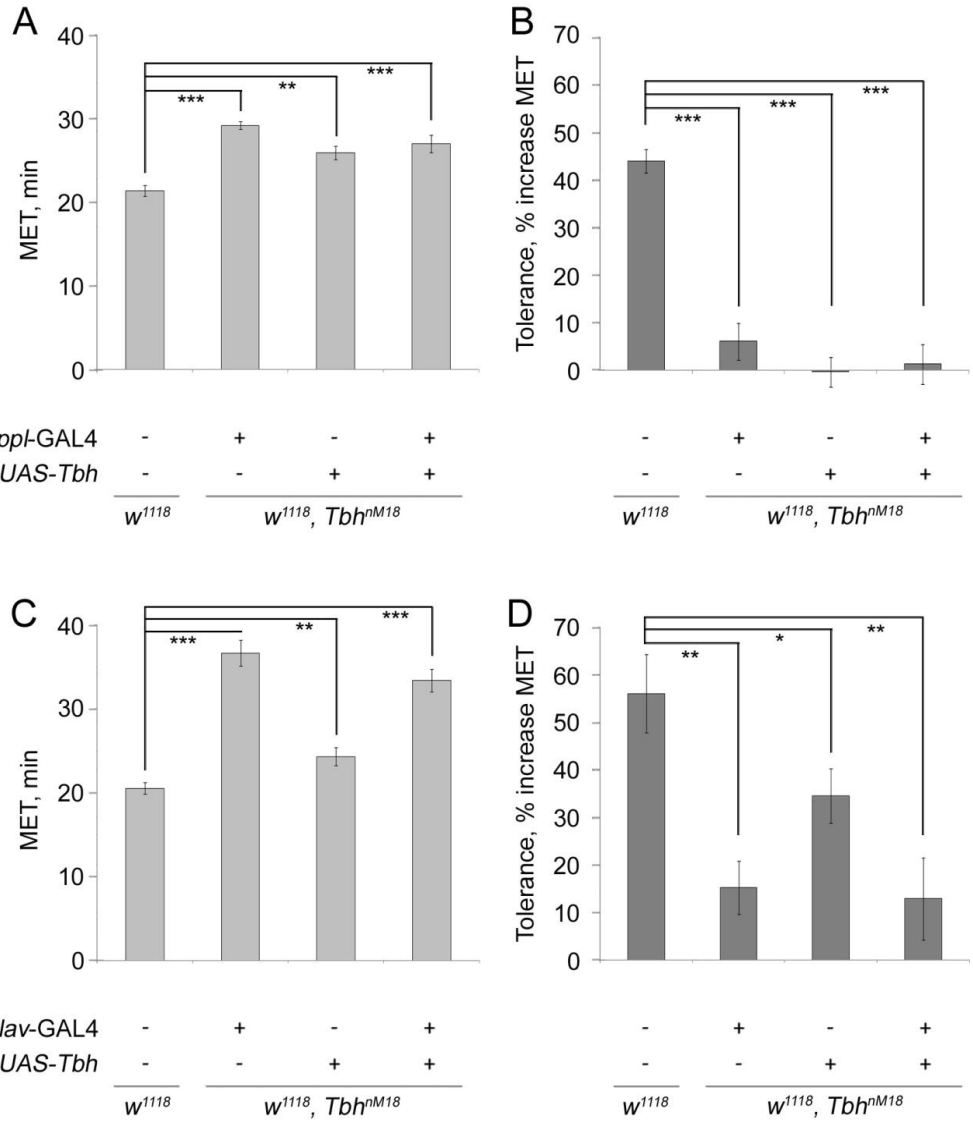


Fig. 3.3.1 Pan-neuronal Tbh expression in the *Tbh^{nM18}* mutant does not restore ethanol tolerance.
The inebriometer assay was used to test ethanol sensitivity (A, C) and ethanol tolerance (B, D). *Appl*-GAL4 was used (A, B) and *elav*-GAL4 (C, D). A) The MET1 of the experimental flies (*w^{1118}*, *Tbh^{nM18}*, *UAS-Tbh*; *Appl*-GAL4) and the mutant controls (*w^{1118}*, *Tbh^{nM18}*; *Appl*-GAL4 and *w^{1118}*, *Tbh^{nM18}*, *UAS-Tbh*.) were not different from each other but different from the *w^{1118}* control (*w^{1118}*: 21,38 ± 0,67; *w^{1118}*, *Tbh^{nM18}*; *Appl*-GAL4: 29,17 ± 0,45; *w^{1118}*, *Tbh^{nM18}*, *UAS-Tbh*: 25,90 ± 0,83; *w^{1118}*, *Tbh^{nM18}*, *UAS-Tbh*; *Appl*-GAL4: 27,02 ± 1,04). B) The level tolerance of the experimental was not different to the reduced level of the mutant controls. Therefore ethanol tolerance was not restored in the *Tbh* mutant (*w^{1118}*: 43,96 ± 2,43; *w^{1118}*, *Tbh^{nM18}*; *Appl*-GAL4: 6,11 ± 3,87; *w^{1118}*, *Tbh^{nM18}*, *UAS-Tbh*: 0,30 ± 3,14; *w^{1118}*, *Tbh^{nM18}*, *UAS-Tbh*; *Appl*-GAL4: 1,34 ± 4,18). n=4-10 C) MET1 values of the mutant controls (*w^{1118}*, *Tbh^{nM18}*, *elav*-GAL4 and *w^{1118}*, *Tbh^{nM18}*, *UAS-Tbh*) and the experimental flies (*w^{1118}*, *Tbh^{nM18}*, *UAS-Tbh*, *elav*-GAL4) were higher than the wild type control *w^{1118}*. (*w^{1118}*: 20,54 ± 0,72; *w^{1118}*, *Tbh^{nM18}*, *elav*-GAL4: 36,65 ± 1,52; *w^{1118}*, *Tbh^{nM18}*, *UAS-Tbh*: 24,33 ± 1,06; *w^{1118}*, *Tbh^{nM18}*, *UAS-Tbh*, *elav*-GAL4: 33,39 ± 1,33). D) Flies of the experimental group failed to develop normal ethanol tolerance as well as the mutant controls indicating that reduced ethanol tolerance was not restored in the *Tbh* mutant (*w^{1118}*: 56,05 ± 8,24; *w^{1118}*, *Tbh^{nM18}*, *elav*-GAL4: 34,54 ± 5,70; *w^{1118}*, *Tbh^{nM18}*, *UAS-Tbh*: 15,21 ± 5,60; *w^{1118}*, *Tbh^{nM18}*, *UAS-Tbh*, *elav*-GAL4: 12,90 ± 8,60). n=5-7. The error bars indicate SEM. *P**≤0,05, *P***≤0,01, *P****≤0,001

To conclude, neither expression of *Tbh* in an *Appl*-GAL4 and *elav*-GAL4 dependent manner restores the reduced ethanol tolerance of the *Tbh^{nM18}* mutant and does not restore altered ethanol sensitivity due to GAL4 transgene insertion. Therefore Tbh function is not required in the neurons driven by the two GAL4 lines to form normal ethanol tolerance. The two driver lines are said to express pan-neuronally (Torroja *et al.*, 1999; Yannoni and White, 1999) but it cannot be excluded that some neurons lack expression. So maybe the required neurons simply are excluded within the two used GAL4 lines. Therefore more specific GAL4 lines with specific octopaminergic or tyraminergic neurons should be used. Another option is that Tbh expression is required in a more specific set of neurons and expression in too many neurons already leads to a phenotype.

3.3.2 Ubiquitous Tbh expression in the adult stage of the *Tbh^{nM18}* mutant restores reduced ethanol tolerance

Tbh expression in the *Tbh^{nM18}* mutant in different sets of neurons failed to restore reduced ethanol tolerance (see 3.4.1; Ruppert, 2010). Previously it had been shown that heat induced Tbh expression in the adult fly restores positive associated learning and memory of the *Tbh^{nM18}* mutant (Schwärzel *et al.*, 2003) and female sterility (Monastirioti *et al.*, 2003). To determine whether Tbh function is required in the adult stage for ethanol tolerance and to ubiquitously expressing *Tbh*, Tbh function was restored in the *Tbh* mutant using a heat inducible transgene (*hs-Tbh*).

Tbh is involved in regulation of cellular stress which can be caused by heat (Scholz *et al.*, 2005). Therefore to determine whether heat induced Tbh levels are specific for the *hs-Tbh* transgene but not due to heat exposure, first the kinetics of the *Tbh* expression of the transgene was investigated performing qRT-PCR. qRT-PCR was done with *RplPo* as the loading control and using *Tbh* primers amplifying *Tbh* from the fifth to the sixth exon (Fig. 3.3.2 A). Using these primers it was shown before that *Tbh* is down regulated in the *Tbh^{nM18}* mutant to a level almost zero (Ruppert, 2010). Thereby *Tbh* levels of the *Tbh^{nM18}* mutant were used as a control for the heat shock. Heat shock was given for 30 minutes at 38°C four hours before RNA isolation. *w^{1118}* was used as the positive control. *Tbh* expression in *w^{1118}*, *Tbh^{nM18}*;; *hs-Tbh* flies without heat shock was as expected due to the *Tbh*n^{M18} mutation close to zero (rel.*Tbh*-expression: 0,05±0,03). In this flies heat shock increased *Tbh* levels to around 70-fold higher than the control (rel. *Tbh*-expression: 68,2±16,0). An elevation of *Tbh* expression was detected also in the *Tbh^{nM18}* mutant with heat shock (rel. *Tbh*-expression: 0,52±0,16) to half the *Tbh* expression in the control. However, *Tbh* increase was around 35-fold stronger with the *hs-Tbh* transgene (Fig. 3.3.2 B).

To conclude, *Tbh* expression is increased due to heat stress and with the *hs-Tbh* transgene *Tbh* can be induced. Induced *Tbh* expression in the *Tbh^{nM18}* mutant after heat shock is detected but due to the *Tbh^{nM18}* deletion it is not known whether the induced Tbh is functional. Further with the *hs-Tbh* transgene specifically the annotated *Tbh* transcript is expressed. Heat itself could induce expression of other Tbh isoforms as well. To test whether heat stress itself already restores reduced ethanol tolerance in the *Tbh* mutant, ethanol sensitivity and ethanol tolerance were tested in the inebriometer for the *w^{1118}* control and *Tbh^{nM18}* mutant flies both with

and without heat shock. Heat shock was given four hours before the experiment equal to the qRT-PCR. The data suggested that a heat shock itself cannot restore reduced ethanol tolerance of the Tbh^{nM18} mutant (Fig. 3.3.2 C, D). The ethanol sensitivity of w^{1118} showed normal sensitivity. With heat shock the MET1 of the control was increased due to the heat stress which is consistent with previous studies (Scholz *et al.*, 2005). The *Tbh* mutant flies without heat shock already showed an increased resistance towards ethanol indicated by a eight minutes higher MET1 than the w^{1118} control (P=0,00). This does not correspond with the literature that says that Tbh^{nM18} flies are not impaired in ethanol sensitivity (Scholz *et al.*, 2000). The MET1 of the mutant with heat shock was not further increased (P=0,55) which would have been expected due to literature (Scholz *et al.*, 2005). This was likely because of the high MET1 of the mutant without heat shock (Fig. 3.3.2 C). The data for ethanol tolerance showed that the wild type control without heat shock developed normal ethanol tolerance as well as the wild type control with heat shock. The *Tbh* mutant with and without heat shock showed reduced ethanol tolerance compared to the control without heat shock (P=0,01; P=0,01) even though the flies were exposed to more ethanol in the first run of the experiment (Fig. 3.3.2 D). Excluding the contradictions that the mutant showed abnormal resistance towards ethanol, the heat shock itself most likely is not sufficient to restore normal ethanol tolerance in the Tbh^{nM18} mutant. It is possible that with heat shock either the wrong isoforms or nonfunctional proteins due to the mutation were induced. But because of the existing contradictions concerning ethanol sensitivity of the *Tbh* mutant this experiment should be repeated to clarify the result.

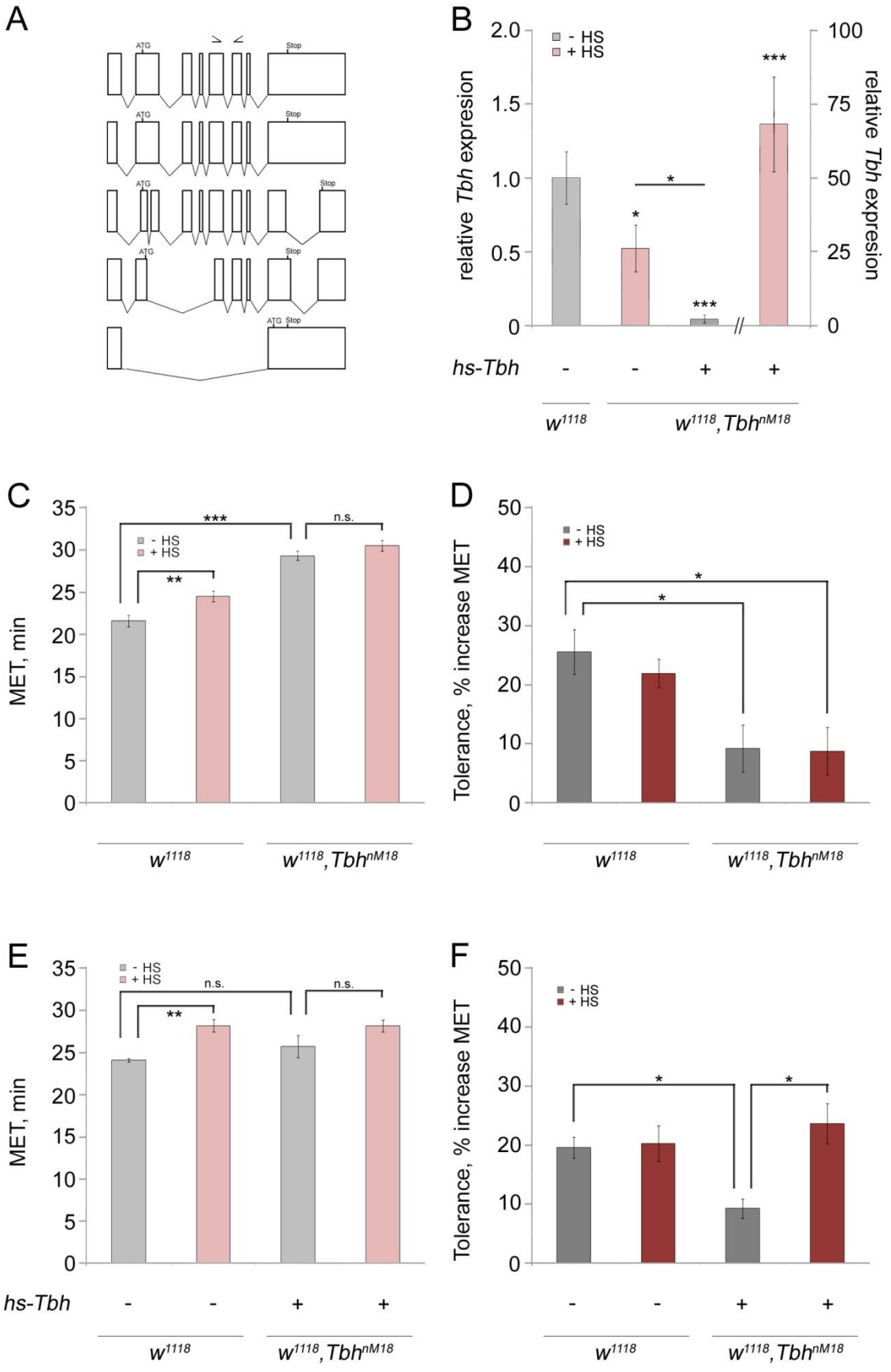

A
ATG
Stop
B
relative Tbh expresion
relative Tbh expresion
- HS
+ HS
hs-Tbh
w1118
w1118, TbhnM18
C
MET, min
n.s.
D
Tolerance, % increase MET
E
F

Fig. 3.3.2 Ubiquitous *Tbh* expression in the adult *Tbh^{nM18}* mutants restores wild type ethanol tolerance.
A) qRT-PCR was performed using RplP0 as the loading control. *Tbh* expression in the *w^{1118}* control was normalized to +1. *Tbh* primers used for the qRT-PCR are indicated with arrowheads in a scheme for the alternatively spliced *Tbh* transcripts. B) *Tbh^{nM18}* flies with heat shock showed higher *Tbh* levels (0,52 ± 0,16) than the mutant control *w^{1118}, Tbh^{nM18};; hs-Tbh* (0,05 ± 0,03) without heat shock. This level was still lower than *Tbh* expression in *w^{1118}*. Heat shock in *w^{1118}, Tbh^{nM18};; hs-Tbh* flies increased *Tbh* expression to a value of 68,2 ± 16,0. Therefore the *hs-Tbh* transgene is functional. Significances are relative to the *w^{1118}* control if not other indicated. C) The MET1 of the wild type control *w^{1118}* was increased by heat shock. The *Tbh^{nM18}* mutants showed abnormal increased MET1 that was not further increased by heat shock (*w^{1118}* -HS: 21,58 ± 0,67; *w^{1118}* +HS: 24,48 ± 0,66; *w^{1118}, Tbh^{nM18}* -HS: 29,32 ± 0,55; *w^{1118}, Tbh^{nM18}* +HS: 30,50 ± 0,64). D) Ethanol tolerances of *w^{1118}* with and without heat shock were wild type. The *Tbh^{nM18}* showed reduced tolerance with and without heat shock indicating that reduced tolerance was not restored (*w^{1118}* -HS: 25,53 ± 3,83; *w^{1118}* +HS: 21,90 ± 2,41; *w^{1118}, Tbh^{nM18}* -HS: 9,16 ± 3,96; *w^{1118}, Tbh^{nM18}* +HS: 8,72 ± 4,06). n=13. E) The MET1 of the wild type control *w^{1118}* was increased by heat shock again. *w^{1118}, Tbh^{nM18};; hs-Tbh* mutants showed wild type MET1 that was not increased by heat shock indicating an effect of *Tbh* expression on ethanol sensitivity (*w^{1118}* -HS: 24,06 ± 0,21; *w^{1118}* +HS: 28,15 ± 0,75; *w^{1118}, Tbh^{nM18};; hs-Tbh* -HS: 25,70 ± 1,317; *w^{1118}, Tbh^{nM18};; hs-Tbh* +HS: 28,14 ± 0,74). F) *w^{1118}* flies with and without heat shock developed wild type ethanol tolerance. The *w^{1118}, Tbh^{nM18};; hs-Tbh* mutants without heat shock showed reduced tolerance without whereas with developed normal ethanol tolerance. This indicates that the reduced tolerance was restored (*w^{1118}* -HS: 19,56 ± 1,78; *w^{1118}* +HS: 20,22 ± 3,03; *w^{1118}, Tbh^{nM18};; hs-Tbh* -HS: 9,22 ± 1,62; *w^{1118}, Tbh^{nM18};; hs-Tbh* +HS: 23,62 ± 3,40). n=5-7. The error bars indicate SEM. *P**≤0,05, *P***≤0,01, *P****≤0,001

Because most likely an effect of heat shock can be ruled out to influence ethanol tolerance in the *Tbh^{nM18}* mutant, Tbh then was expressed ubiquitously in the mutant using the *hs-Tbh* transgene. With this it was investigated whether Tbh is only required during adulthood to form normal ethanol tolerance. Therefore control *w^{1118}* flies and experimental *w^{1118}*, *Tbh^{nM18};; hs-Tbh* flies both were tested with and without heat shock in the inebriometer. Heat shock was given as described before. The MET1 values of the control and the experimental flies without heat shock were not different (*P*=0,53) . The heat shock increased MET1 in the control (*P*=0,01) as described in the literature (Scholz *et al.*, 2005). By trend this effect was seen in the experimental group as well but not significant (*P*=0,20; Fig. 3.3.2 E). The data for ethanol tolerance revealed that heat shock induced Tbh expression restored reduced ethanol tolerance

of the *Tbh* mutant. The wild type control with and without heat shock developed the same level of tolerance (*P*=1,00). The experimental flies without heat shock as expected showed reduced ethanol tolerance (P=0,04) whereas with heat shock the tolerance level was not different to the w^{1118} controls (*P*=0,68; Fig. 3.3.2 F).

Concluding, *Tbh* induction in the adult fly most likely restores wild type behavior of the *Tbh* mutant which means *Tbh* is only required in the adulthood and not during embryonic or larval development to form normal ethanol tolerance. But to make a definite conclusion the shown behavioral experiments need to be repeated. Firstly because of the contradictions concerning ethanol sensitivity of the *Tbh* mutant in the first behavioral experiment and secondly because the given heat shock in both shown behavioral experiments influenced ethanol sensitivity. Tolerance development is dose dependent. To exclude effects of dose dependency the heat shock should be given earlier than four hours before experiment to eliminate the effect of the heat shock itself on sensitivity.

3.4 A small set of neurons mediate ethanol tolerance in regard to *dunce* function

The $dnc^{\Delta 143}$ mutant fails to develop normal ethanol tolerance (Franz, 2008). The mutant phenotype is restored by induced expression of a Dnc peptide, containing the PDE-activity domain that exists in all *dnc* transcripts (*UAS-*dnc^{All}) in dnc^{RA}-GAL4 driven neurons (Franz, 2008). The dnc^{RA}-GAL4 line drives expression in a broad set of neurons throughout the brain amongst others in the mushroom body and in the central complex. To narrow down the neurons that mediate ethanol tolerance with regard to *dnc* other GAL4 lines were tested to restore the $dnc^{\Delta 143}$ mutant phenotype.

3.4.1 The $dnc^{\Delta 143}$ mutant phenotype in terms of ethanol tolerance can be rescued by induced *dunce* expression in NP6510-GAL4 driven neurons

The reduced ethanol tolerance of the $dnc^{\Delta 143}$ can be restored to a wild type level by dnc^{All} expression in a dnc^{RA}-GAL4 dependent manner. To narrow down the neurons required to form normal ethanol tolerance dnc^{All} was expressed in the *dnc* mutant in

neurons driven by the 78y-GAL4, the MB247-GAL4, the TH-GAL4 and the NP6510-GAL4 line.

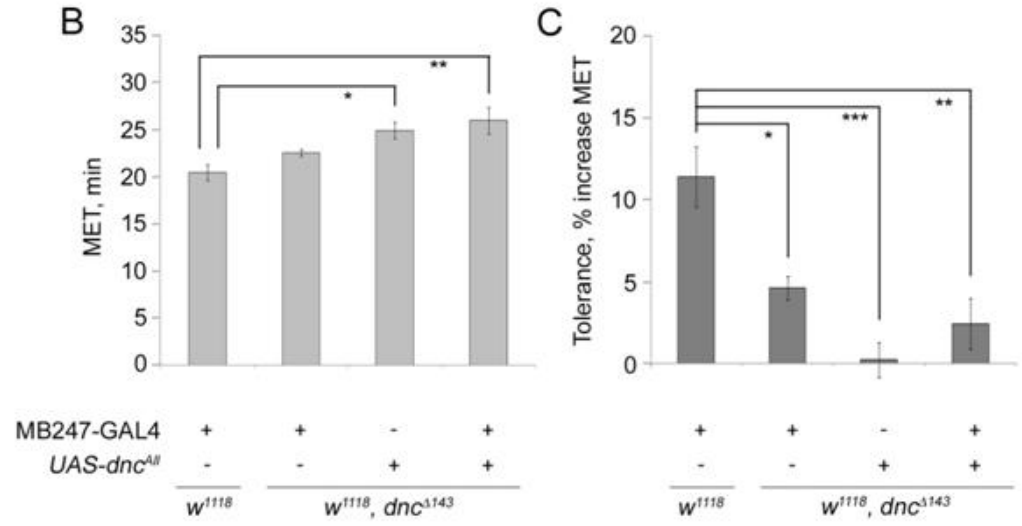
Firstly, the 78y-GAL4 line was used. The 78y-GAL4 line drives expression in small field neurons connecting ellipsoid body with protocerebral bridge in the central complex (Renn *et al.*, 1999). Male flies expressing dnc^{All} in the *dnc* mutant (w^{1118}, $dnc^{\Delta 143}$, *UAS-*dnc^{All}*;;* 78y-GAL4) were lethal. Therefore it was not possible to perform behavioral experiment (Fig. 3.4.1 A). Secondly, the MB247-GAL4 line was used (Fig. 3.4.1 B, C). MB247-GAL4 drives expression in the Kenyon cells of the mushroom body (Zars *et al.*, 2000). Flies carrying only the GAL4 transgene (w^{1118}; MB247-GAL4) were used as a positive control. The positive control showed normal ethanol sensitivity (MET1: 20,46±0,86). Flies carrying the *UAS-*dnc^{All} transgene displayed a higher resistance towards ethanol indicated by around four minutes higher MET1 values (P=0,021 and P=0,005; Fig. 3.4.1 B). This means that the *UAS-*dnc^{All} insertion influences ethanol sensitivity which is consistent with previous studies (Franz, 2008). Assessing the tolerance data it is shown that the positive control developed ethanol tolerance (%Tolerance: 11,41±1,85). The mutant controls (w^{1118}, $dnc^{\Delta 143}$; MB247-GAL4 and w^{1118}, $dnc^{\Delta 143}$, *UAS-*dnc^{All}) and the experimental group (w^{1118}, $dnc^{\Delta 143}$, *UAS-*dnc^{All}; MB247-GAL4) failed to develop ethanol tolerance on the same level (P=0,014, P=0,000, P=0,002; Fig. 3.4.1 C). Therefore flies carrying the *UAS-*dnc^{All} transgene showed a reduced level of ethanol tolerance even they were exposed to more ethanol in the first run of the experiment. Further, the reduced ethanol tolerance of the *dnc* mutant was not restored to normal by dnc^{All} expression in a MB247-GAL4 dependent manner. Thirdly, the NP6510-GAL4 was used (Fig. 3.4.1 D, E). NP6510-GAL4 drives expression only in a few neurons amongst others in the mushroom body (Aso *et al.*, 2010; Aso *et al.*, 2012) and in the fanshaped body (Liu *et al.*, 2006; Li *et al.*, 2009; Young and Armstrong, 2010). Flies of the w^{1118};; NP6510-GAL4 genotype were used as a positive control and showed normal ethanol sensitivity (MET1: 24,09±0,54). The MET1 of the mutant control w^{1118}, $dnc^{\Delta 143}$, *UAS-*dnc^{All} was increased significantly of around four minutes reflecting the known influence of the *UAS* insertion (P=0,001). The MET1 of the experimental flies (w^{1118}, $dnc^{\Delta 143}$, *UAS-*dnc^{All}) was not different to that (P=0,39; Fig. 3.4.1 D). Therefore expression of *dnc* did not alter ethanol sensitivity. The positive control developed normal ethanol tolerance (%Tolerance: 34,80±2,95). The mutant controls (w^{1118}, $dnc^{\Delta 143}$, *UAS-*dnc^{All} and w^{1118}, $dnc^{\Delta 143}$;;

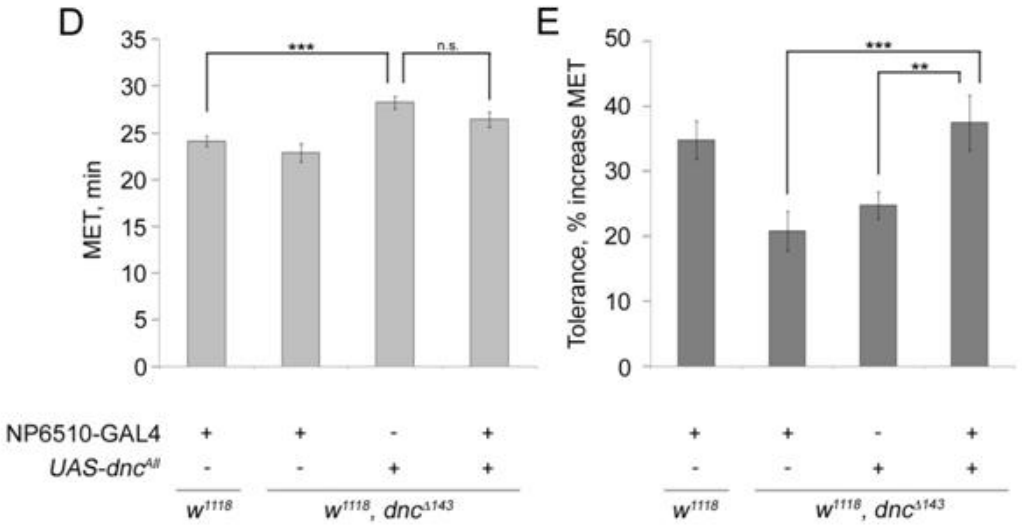
NP6510-Gal4) failed to develop normal ethanol tolerance (*P*=0,004, *P*=0,03). The level of tolerance of the experimental flies was not different to the positive control (*P*=0,85) but significantly different to the mutant controls (*P*=0,001, *P*=0,007). That means that *dnc* expression in neurons driven by the NP6510-GAL4 line was sufficient to restore reduced ethanol tolerance of the $dnc^{\Delta 143}$ mutant. The expression pattern of the NP6510-GAL4 was confirmed by immunohistochemistry (Fig. 3.4.1 F). GAL4 expression was visualized by a *UAS-mCD8::GFP* transgene in the adult brain and was detected in the PAM cluster of the mushroom body and in F1 neurons of the fanshaped body (Fig. 3.4.1 F). The brains were costained with nc82 to visualize brain compartments. GAL4 expression matched the described NP6510-GAL4 expression in the mushroom body (Aso *et al.*, 2010; Aso *et al.*, 2012) and in the fanshaped body (Liu *et al.*, 2006; Li *et al.*, 2009; Young and Armstrong, 2010).

To summarize, the neurons driven by the NP6510-GAL4 line are sufficient to mediate normal ethanol tolerance with regard to *dnc* whereas the neurons driven by MB247-GAL4 were not sufficient. Comparing the expression pattern of NP6510-GAL4 with dnc^{RA}-GAL4 it is seen that both lines drive expression in the PAM neurons of the mushroom body and in F1 neurons of the fanshaped body of the central complex. The mushroom body neurons driven by the NP6510-GAL4 line are dopaminergic (Aso *et al.*, 2010; Aso *et al.*, 2012). To further narrow down the required neurons to either fanshaped body neurons or mushroom body neurons the *TH*-GAL4 driver line was used to express dnc^{All} in the *dnc* mutant. The *TH*-GAL4 line drives expression in dopaminergic neurons throughout the adult *Drosophila* brain including the PAM neurons (Friggi-Grelin *et al.*, 2003). According to what is known *TH*-GAL4 does not express in the central complex. When reduced ethanol tolerance is not restored using *TH*-GAL4 the PAM neurons can be ruled out. However, experimental flies expressing dnc^{All} in a *TH*-GAL4 dependant manner in the $dnc^{\Delta 143}$ mutant were lethal (Fig. 3.4.1 A). Therefore the behavioral experiment could not be performed. Another way to rule out the PAM neurons is to test whether the PAM neurons driven by the NP6510-GAL4 line are the same than the neurons that are driven by the dnc^{RA}-GAL4 line. This could be tested by colabling dnc^{RA}-GAL4 expression with dopamine expression to see whether the driven PAM neurons are dopaminergic. If they are not, then the PAM neurons could be ruled out.

A

GAL4-line	Experimental Genotype	Male lethality
78y-GAL4	w^{1118}, $dnc^{\Delta 143}$, $UAS\text{-}dnc^{All}$;; 78y-GAL4	+
TH-GAL4	w^{1118}, $dnc^{\Delta 143}$, $UAS\text{-}dnc^{All}$; TH-GAL4	+





F

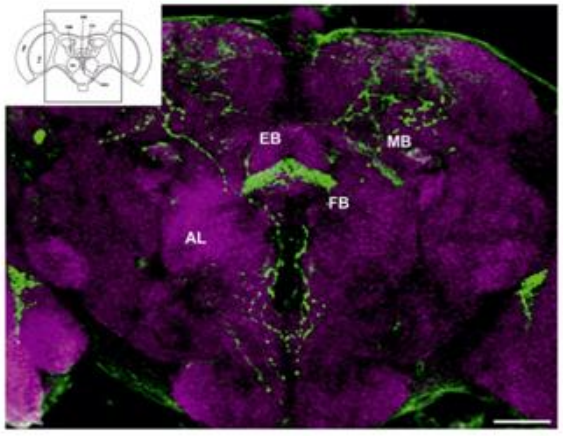


Fig. 3.4.1. Induced *dnc^{All}* expression in NP6510-GAL4 driven neurons rescues the mutant *dnc^{Δ143}* phenotype.
Dnc^{All} was expressed in the mutant *dnc^{Δ143}* in neurons driven by 78y-GAL4 (A), TH-GAL4 (A), MB247-GAL4 (B, C) and NP6510-GAL4 (D, E). Ethanol sensitivity and ethanol tolerance were tested. The positive controls (GAL4 insertion alone) showed normal sensitivity and tolerance. A) Flies expressing *dnc^{All}* in the *dnc^{Δ143}* mutant in a 78y-GAL4 and TH-GAL4 dependent manner were lethal. B) Flies carrying the *UAS-dnc^{All}* transgene were more resistant toward ethanol reflecting the influence on sensitivity of the *UAS* insertion. Expression of *dnc^{All}* did not change this (*w^{1118}*; MB247-GAL4: 20,46 ± 0,86, *w^{1118}*, *dnc^{Δ143}*; MB247-GAL4: 22,53 ± 0,37, *w^{1118}*, *dnc^{Δ143}*, *UAS-dnc^{ALL}*: 24,93 ± 0,89, *w^{1118}*, *dnc^{Δ143}*, *UAS-dnc^{ALL}*; MB247-GAL4: 25,98 ± 1,41). C) The mutant controls (*w^{1118}*, *dnc^{Δ143}*; MB247-GAL4 and *w^{1118}*, *dnc^{Δ143}*, *UAS-dnc^{ALL}*) and the experimental flies (*w^{1118}*, *dnc^{Δ143}*, *UAS-dnc^{ALL}*; MB247-GAL4) displayed significantly reduced tolerance. Therefore the reduced ethanol tolerance of *dnc^{Δ143}* was not restored (*w^{1118}*; MB247-GAL4: 11,41 ± 1,85, *w^{1118}*, *dnc^{Δ143}*; MB247-GAL4: 4,61 ± 0,71, *w^{1118}*, *dnc^{Δ143}*, *UAS-dnc^{ALL}*: 0,22 ± 1,08, *w^{1118}*, *dnc^{Δ143}*, *UAS-dnc^{ALL}*; MB247-GAL4: 2,42 ± 1,55). n=5 D) The MET1 of the mutant control *w^{1118}*, *dnc^{Δ143}*; NP6510-GAL4 was significantly higher than the positive control reflecting the influence of the *UAS* transgene. The MET1 of the experimental group (*w^{1118}*, *dnc^{Δ143}*, *UAS-dnc^{All}*;; NP6510-GAL4) was not different to that, indicating that *dunce* expression did not influence sensitivity (*w^{1118}*;; NP6510-GAL4: 24,09 ± 0,54; *w^{1118}*, *dnc^{Δ143}*;; NP6510-GAL4: 22,85 ± 0,97; *w^{1118}*, *dnc^{Δ143}*, *UAS-dnc^{All}*: 28,24 ± 0,68; *w^{1118}*, *dnc^{Δ143}*, *UAS-dnc^{All}*;; NP6510-GAL4: 26,42 ± 0,83). E) The mutant controls (*w^{1118}*, *dnc^{Δ143}*;; NP6510-GAL4 and *w^{1118}*, *dnc^{Δ143}*, *UAS-dnc^{All}*) showed reduced ethanol tolerance. Experimental flies (*w^{1118}*, *dnc^{Δ143}*, *UAS-dnc^{All}*;; NP6510-GAL4) developed a level of ethanol tolerance not different to the positive control but different to the mutant controls. The reduced ethanol tolerance of the *dnc^{Δ143}* mutant phenotype was restored to a normal level by *dnc^{All}* expression in NP6510-GAL4 driven neurons. (*w^{1118}*;; NP6510-GAL4: 34,80 ± 2,95; *w^{1118}*, *dnc^{Δ143}*;; NP6510-GAL4: 20,85 ± 3,05; *w^{1118}*, *dnc^{Δ143}*, *UAS-dnc^{All}*: 24,76 ± 2,06; *w^{1118}*, *dnc^{Δ143}*, *UAS-dnc^{All}*;; NP6510-GAL4: 37,45 ± 4,21). n=13-18. The error bars indicate SEM. *P**≤0,05, *P***≤0,01, *P****≤0,001 F) Expression of the NP6510-GAL4 line in adult brain is shown. GAL4 expression was visualized by a *UAS-mCD8::GFP* transgene (green). Brains were costained with nc82 (magenta) to label the neuropil of the different brain compartments. GFP expression was detected in the mushroom body and in the fanshaped body. The scale bar represents 50µm.

3.4.2 The *dnc^{RA}*-GAL4 line expresses in dopaminergic PAM neurons

Expression of *dnc* in a *dnc^{RA}*-GAL4 and in a NP6510-GAL4 dependent manner restores reduced expression in the *dnc$^{\Delta 143}$* mutant. NP6510-GAL4 expresses in dopaminergic PAM neurons in the mushroom body (Aso *et al.*, 2010; Aso *et al.*, 2012) and in F1 neurons of the fanshaped body (Liu *et al.*, 2006; Li *et al.*, 2009; Young and Armstrong, 2010). The expression of *dnc^{RA}*-GAL4 is broader whereat PAM neurons and fanshaped body neurons are also addressed (Franz, 2008). To test whether the driven PAM neurons are the same within both GAL4 lines it was tested whether the PAM neurons driven by *dnc^{RA}*-GAL4 are dopaminergic. Therefore adult brains of flies expressing a *UAS-mCD8::GFP* transgene under the control of the *dnc^{RA}*-GAL4 line were colabeled with GFP and TH (Fig. 3.4.2). GFP signal reflects GAL4 expression. TH labels dopaminergic neurons because TH is the rate limiting enzyme in the dopamine synthesis. An overview of the staining is shown in figure 3.4.2 A and B. To identify putative colocalizations in the mushroom body PAM neurons, the region of interest was scanned in a higher magnification (Fig. 3.4.2 C). Neurons of the PAM cluster were detected by TH and by GFP and some cells colocalized (Fig. 3.4.2 C”).

To conclude, the *dnc^{RA}*-GAL4 line drives expression in dopaminergic PAM neurons in the mushroom body. This means PAM neurons within *dnc^{RA}*-GAL4 and NP6510 might overlap. Therefore these neurons cannot be ruled out to play a role in mediating ethanol tolerance in regard to *dnc*. To further narrow down the required neurons, *dnc^{RA}*-GAL4 expression could be suppressed in dopaminergic neurons in the *dnc$^{\Delta 143}$* mutant by using *TH*-GAL80. Ethanol tolerance could be tested then.

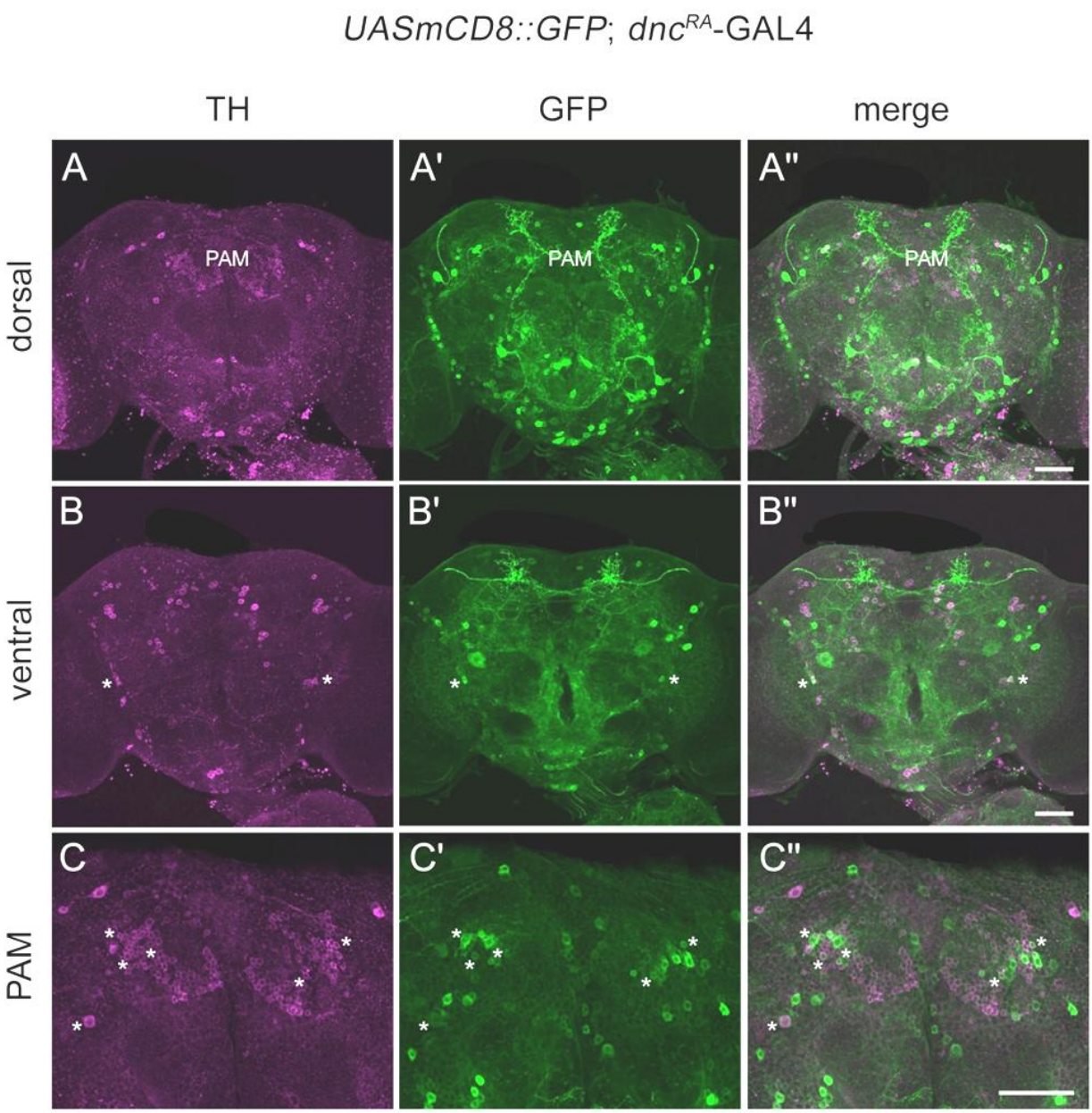


Fig. 3.4.2. PAM neurons driven by the *dnc*^{RA}-GAL4 line are partially dopaminergic.
Adult fly brains of the genotype *UAS-mCD8::GFP; dnc*^{RA}-GAL4 were costained with α TH (magenta) and α GFP (green). An overview of an exemplary brain from dorsal (A, A', A") to ventral (B, B', B") is shown. A higher magnification of the PAM neurons is shown in C to C". Cells that both express GFP and TH are displayed with an asterisk. GFP was expressed in some cells of the PAM cluster that partially colocalized with TH expression indicating that the *dnc*^{RA}-GAL4 line drives expression in dopaminergic PAM neurons. The scale bar indicates 50µm.

3.5 Ethanol tolerance in regard to *dunce* is specifically regulated by the dnc^{RA} transcript

Mutant $dnc^{\Delta 143}$ flies are impaired in developing normal ethanol tolerance but show wild type ethanol sensitivity (Franz, 2008). The *dnc* gene encodes at least eight transcripts. Therefore the question is whether there is a transcript specific regulation of ethanol tolerance or whether all transcripts are required to form normal ethanol tolerance. To test this *dnc* transcript levels in different *dnc* mutants were analyzed and compared to behavioral phenotypes regarding ethanol tolerance. Furthermore different *dnc* transcripts were specifically expressed in $dnc^{\Delta 143}$ flies to restore wild type ethanol tolerance to test the influence of the single transcripts.

3.5.1 Ethanol tolerance is impaired for the dnc^{1} allele but not for dnc^{M11}

The $dnc^{\Delta 143}$ mutant shows reduced ethanol tolerance indicating that *dnc* is involved to form normal ethanol tolerance. The *dnc* gene encodes at least eight transcripts (Fig. 3.5.1). Therefore the question is whether all *dnc* transcripts or only specific transcripts are required. That's why additional *dnc* alleles (dnc^{1} and dnc^{M11}) were tested in the inebriometer to investigate ethanol sensitivity and tolerance behavior. Furthermore the transcript levels of the different *dnc* transcript groups were analyzed in these mutants to correlate it with behavioral phenotypes.

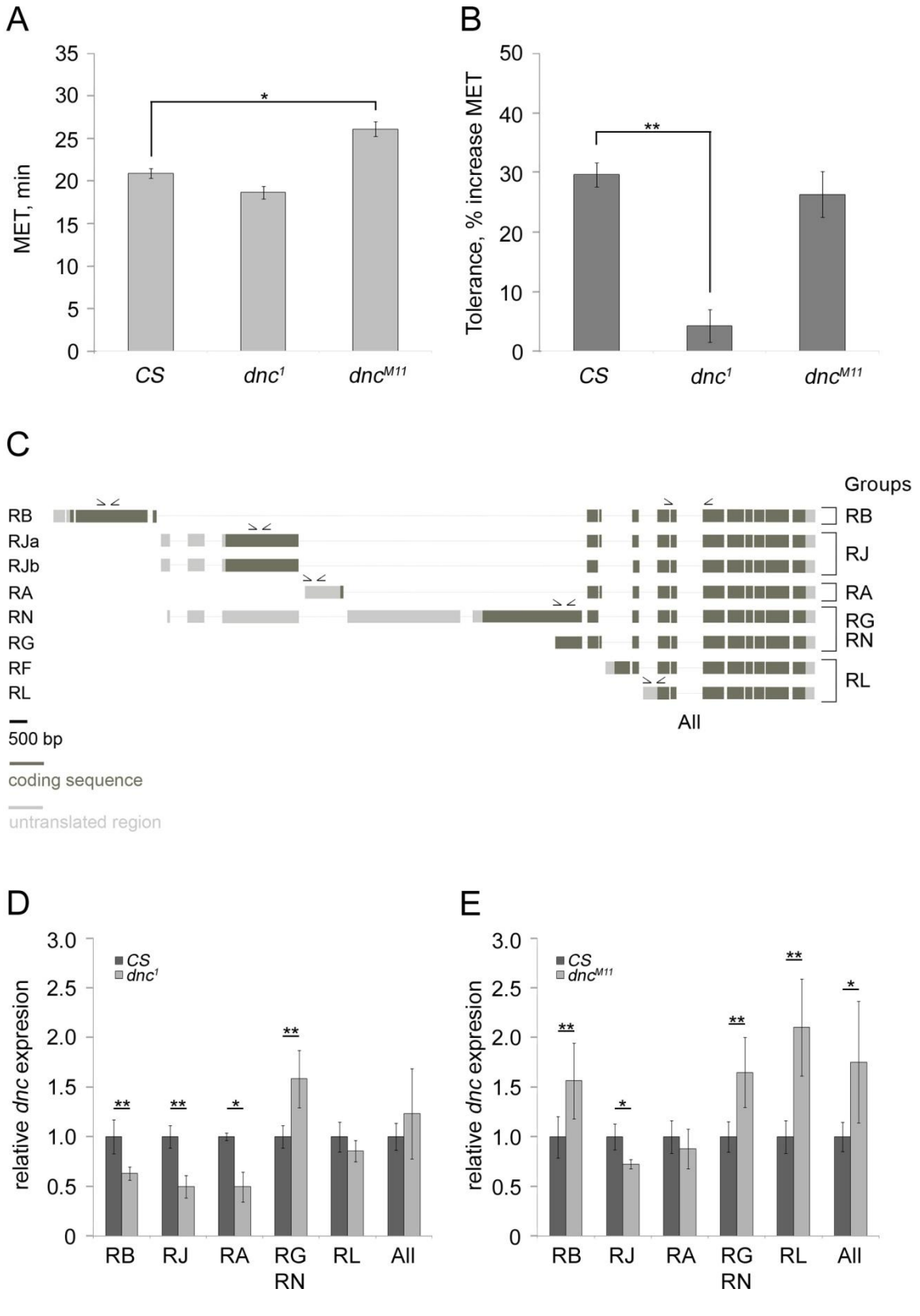
For the behavioral study *CS* flies were tested as the wild type control and showed normal ethanol sensitivity (MET1: 20,90±0,58) and ethanol tolerance (%Tolerance: 29,65±1,99). The dnc^{1} mutant showed normal ethanol sensitivity (MET1: 18,67±0,77) but ethanol tolerance was decreased to around 15%. However the dnc^{M11} mutant displayed a five minutes decreased resistance towards ethanol and developed a wild type level of tolerance (Fig. 3.5.1 A, B).

To conclude the two *dnc* mutants show contrariwise phenotypes. One is impaired in sensitivity and one in tolerance development. However a phenotype regarding ethanol tolerance for dnc^{M11} cannot be excluded completely because these flies were exposed to more ethanol in the first run of the experiment. Ethanol tolerance is dose dependent and therefore a higher level of tolerance would have been expected. To

investigate this, flies could be exposed to the same amount of ethanol as a first exposure using a modified inebriometer setup and then be tested for tolerance.

The two *dnc* alleles contain different mutations in the *dnc* gene that are not further characterized. To analyze the influence of the mutations on *dnc* expression qRT-PCR was performed using *actin* as the loading control. *Dnc* primers were used specific for five different transcript groups reflecting the functional groups defined by Qiu *et al.* (1991). Primer pairs including all *dnc* transcripts were used as well. *Dnc* transcript levels of the two mutants were compared to wild type *dnc* expression in the *CS* control. In the dnc^{1} mutant no change in expression regarding all *dnc* transcripts together was detected (*P*=0,38). But more specific a down regulation of transcripts of group RB (*P*=0,01), RJ (*P*=0,01) and RA (*P*=0,02) to around 50-60% was detected. Further, an up regulation of transcripts of group RG/RN to 160% was displayed (*P*=0,004). Transcript group RL was the only one not significantly altered in the dnc^{1} mutant (*P*=0,45; Fig. 3.5.1 D). In the dnc^{M11} mutant an up regulation of all *dnc* transcripts together to around 175% was detected (*P*=0,03). In addition an up regulation of transcript group RL (*P*=0,002), RB (*P*=0,003) and RG/RN (*P*=0,01) was detected to a level of 170-200 %. The transcript level of group RJ showed a down regulation of 28% (*P*=0,02). Only transcript group RA was not significantly altered in the dnc^{M11} mutant (*P*=0,27; Fig. 3.5.1 E).

In conclusion, it is shown that in the dnc^{1} and dnc^{M11} mutants *dnc* expression is affected differently. Comparing these results with the identified ethanol sensitivity and tolerance phenotypes, it is suggested that specifically *dnc* transcript group RA might play an important role in mediating ethanol tolerance. This is because group RA is not altered in dnc^{M11} but in dnc^{1} and only dnc^{1} displays a reduced ethanol tolerance. To further verify this, other *dnc* mutants, like the dnc^{A143} mutant, could be tested for *dnc* transcript expression to further compare that with behavioral phenotypes. In addition, specific transcripts could be expressed in the mutants to restore reduced ethanol tolerance or specific transcripts could be expressed in wild type to investigate behavioral changes.


A
MET, min
35
30
25
20
15
10
5
0
*
CS
dnc1
dncM11
B
Tolerance, % increase MET
50
40
30
20
10
0
**
CS
dnc1
dncM11
C
Groups
RB
RJa
RJb
RA
RN
RG
RF
RL
RB
RJ
RA
RG
RN
RL
All
500 bp
coding sequence
untranslated region
D
relative dnc expresion
3.0
2.5
2.0
1.5
1.0
0.5
0
CS
dnc1
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3.0
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RB
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RL
All

Fig. 3.5.1. *dnc*^{1} and *dnc*^{M11} mutants display different phenotypes with regard to ethanol sensitivity and tolerance and different alterations in *dunce* transcript expression.
Ethanol sensitivity indicated by MET1 (A) and ethanol tolerance indicated by percentage increase of MET1 to MET2 (B) were tested for *dnc*^{1} and *dnc*^{M11} in the inebriometer with *CS* as the wild type control. A) *dnc*^{1} showed normal ethanol sensitivity. *dnc*^{M11} was more resistant towards ethanol (*CS*: 20,90 ± 0,58, *dnc*^{1}: 18,67 ± 0,77, *dnc*^{M11}*:* 26,14 ± 0,89). B) *dnc*^{1} flies failed to develop normal ethanol tolerance whereas *dnc*^{M11} showed a normal level of tolerance (*CS*: 29,65 ± 1,99, *dnc*^{1}: 4,26 ± 2,72, *dnc*^{M11}: 26,35 ± 3,87). The error bars indicate SEM. n=18-20 C) The *dunce* transcript group specific primers used for qRT-PCR are indicated in a scheme of the eight transcripts. qRT-PCR with *actin* as the loading control was performed on cDNA synthesized from heads of male flies. *Dunce* expression in *CS* was normalized to a value of +1. D) In the *dnc*^{1} mutant *dunce* transcript groups RB, RJ and RA were down regulated whereas group RG/RN was upregulated. *Dunce* group RL was not altered. The level of all *dunce* transcripts together was not changed as well (*dnc*^{1}: RB: 0,63 ± 0,07, RJ: 0,50 ± 0,11, RA: 0,50 ± 0,15, RG/RN: 1,58 ± 0,28, RL: 0,86 ± 0,10, All: 1,23 ± 0,45). E) In the *dnc*^{M11} mutant the transcript level of all *dunce* transcripts together was upregulated representing the up regulations of the transcript groups RB, RG/RN and RL. Only transcript group RJ was reduced. No alteration of *dunce* transcript group RA was detected (*dnc*^{M11}: RB: 1,57 ± 0,38, RJ: 0,72 ± 0,05, RA: 0,88 ± 0,20, RG/RN: 1,65 ± 0,35, RL: 2,11 ± 0,49, All: 1,75 ± 0,61). The error bars of the qRT-PCR data represent SD. $P^{*} \leq 0{,}05$, $P^{**} \leq 0{,}01$

3.5.2 The *dnc*$^{\Delta 143}$ mutant shows a *dnc*^{RA} specific down regulation

Similar to the *dnc*^{1} mutant the *dnc*$^{\Delta 143}$ mutant shows normal ethanol sensitivity but develops reduced ethanol tolerance (Franz, 2008). In contrast to the *dnc*^{1} mutant, the exact deletion in the *dnc* gene is known for the *dnc*$^{\Delta 143}$ mutant. Specifically 5'UTR sequence of the *dnc*^{RA} transcript and no coding sequence are deleted. Therefore only expression of transcript group RA should be affected. To verify the transcript specific down regulation in the mutant qRT-PCR was performed with *actin* as the loading control. The used *dnc* primers were the same as described in 3.5.1 and specific for the five transcript groups. As expected the expression of transcript group RA was down regulated to a level almost zero (P= 0,04). Also expression of group RL was decreased to around 60% (P= 0,03; Fig. 3.5.2). *Dnc* transcript groups RB, RJ and RG/RN were not affected (P=0,93, P=0,89, P=0,43) which reflects that no modification of all transcripts levels together was detected as well (P= 99,98).

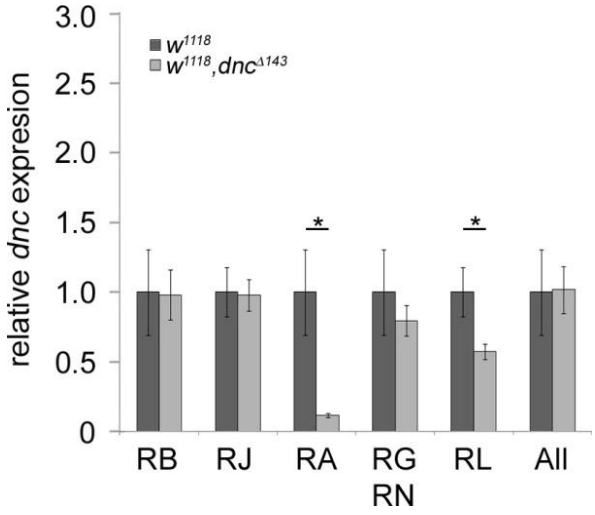


Fig. 3.5.2. The *dunce* mutant $dnc^{\Delta 143}$ displays a dnc^{RA} transcript specific down regulation.
qRT-PCR was performed on cDNA synthesized from heads of male flies with *actin* as the loading control. *Dunce* expression in the w^{1118} control was normalized to a value of +1. *Dunce* expression of the five functional *dunce* groups in the $dnc^{\Delta 143}$ mutant were compared to the normalized *dunce* expression of the w^{1118} control. *Dunce* group RA was highly down regulated in the *dunce* mutant. Group RL was down regulated as well. All other *dunce* groups were not altered as well as the level of all *dunce* transcripts together (w^{1118}, $dnc^{\Delta 143}$: RB: 0,98 ± 0,18,RJ 0,98 ± 0,11, RA: 0,12 ± 0,02, RG/RN: 0,79 ± 0,11, RL: 0,57 ± 0,05, All: 1,02 ± 0,17). The error bars indicate SD. $P^{*} \leq 0{,}05$

In summary, the transcript specific mutation in the $dnc^{\Delta 143}$ is confirmed. Deleting parts of the 5'UTR of transcript group RA leads to down regulation of dnc^{RA} to almost zero. This and the fact that the mutant is impaired in ethanol tolerance supports the theory that specifically dnc^{RA} is involved in mediating ethanol tolerance. However unexpectedly also group RL is down regulated in the $dnc^{\Delta 143}$ mutant. This indicates that the *dnc* group RL is also affected by the $dnc^{\Delta 143}$ specific deletion. Transcript dnc^{RA} might interact with dnc^{RL}.

3.5.3 Overexpression of dnc^{RA}, dnc^{RL} and dnc^{RG} does not affect ethanol tolerance

Over expression of dnc^{All} does not alter ethanol tolerance development (Franz, 2008). To test whether transcript specific overexpression can influence development of

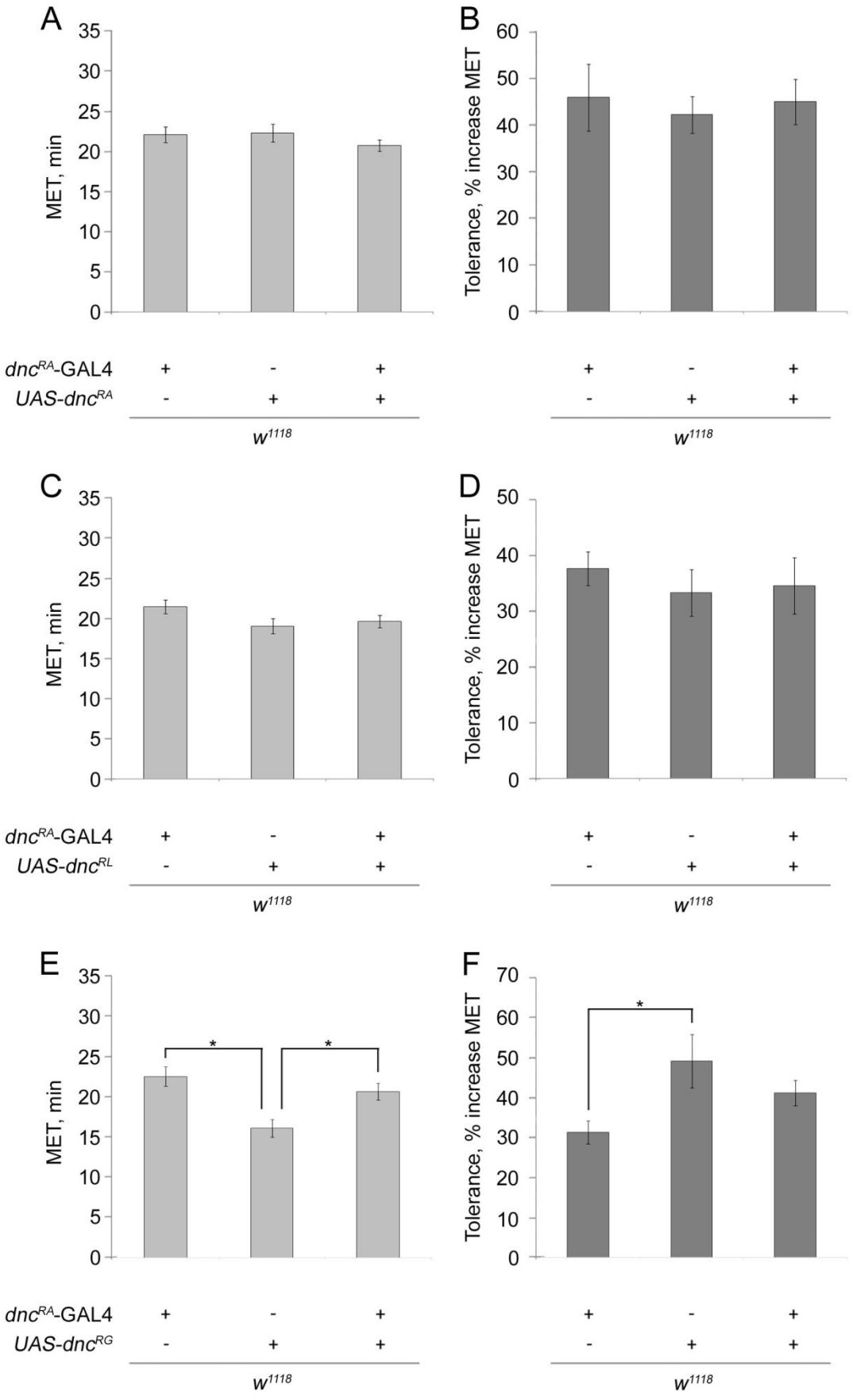
ethanol tolerance, *dnc*^{RA}, *dnc*^{RL} and *dnc*^{RG} were overexpressed in w^{1118} flies using the *dnc*^{RA}-GAL4 driver. Flies carrying only the *dnc*^{RA}-GAL4 transgene (w^{1118};; *dnc*^{RA}-GAL4) served as the positive control.

Firstly, *dnc*^{RA} was overexpressed (Fig 3.5.3 A, B). The positive control showed normal ethanol sensitivity (MET1: 22,09±0,97). The *UAS* control (w^{1118};; *UAS-dnc*^{RA}) showed normal ethanol sensitivity as well (%Tolerance: 22,28±1,10). The insertion of the transgene did not influence ethanol sensitivity. The MET1 of the flies overexpressing *dnc*^{RA} (w^{1118};; *UAS-dnc*^{RA}/*dnc*^{RA}-GAL4) did not differ from the positive control (*P*=1,00) indicating that overexpression of *dnc*^{RA} did not alter ethanol sensitivity (Fig. 3.5.3 A). The data for ethanol tolerance showed a normal level of tolerance for the positive control (%Tolerance: 45, 90±7,15). The tolerance level of the *UAS* control did not differ from the positive control (*P*=0,90). This indicates that the *UAS* insertion has no effect on tolerance development as well. The tolerance level of the experimental flies was not altered (%Tolerance: 44,95±4,84; Fig. 3.5.3 B). Therefore overexpressing *dnc*^{RA} does not influence development of tolerance. Secondly, *dnc*^{RL} was overexpressed (Fig. 3.6.3 C, D). The positive control showed normal ethanol sensitivity (MET1: 21,47±0,86). The MET1 values the *UAS* control (w^{1118}; *UAS-dnc*^{RL}) and the experimental group (w^{1118}; *UAS-dnc*^{RL}; *dnc*^{RA}-GAL4) were not different from the positive control (*P*=0,13, *P*=0,29; Fig. 3.5.3 C). Therefore the *UAS-dnc*^{RL} insertion has no effect on ethanol sensitivity and overexpressing *dnc*^{RL} does not alter ethanol sensitivity. The the level of tolerance development was normal for the positive control (%Tolerance: 37,71±3,03). Ethanol tolerance of the *UAS* control and the experimental group were not different from the positive control (*P*=0,75, *P*=0,86; Fig. 3.5.3 D). This indicates that the insertion of the *UAS* transgene did not influence tolerance development and that overexpressing *dnc*^{RL} does not alter tolerance development. Thirdly, *dnc*^{RG} was overexpressed (Fig. 3.5.3 C, D). The sensitivity of the positive control (MET1: 25,39±1,33) as well as the sensitivity of the experimental flies (w^{1118};; *UAS-dnc*^{RG}/ *dnc*^{RA}-GAL4; MET1: 23,27±1,16) were normal whereas the sensitivity of the *UAS* control was significantly increased by around five minutes compared to the positive control (*P*=0,002) and the experimental flies (*P*=0,02; Fig. 3.5.3 C). This indicates that the *UAS-dnc*^{RG} insertion influences ethanol sensitivity but overexpression of *dnc*^{RG} did not. The data for tolerance development showed normal tolerance development for the the positive control (%Tolerance: 31,35±2,91). The *UAS* control developed an increased level of tolerance, which was

different to the positive control (P=0,02) and not to the experimental group (P=0,38; Fig. 3.5.3 D). This means that the insertion of the *UAS-dnc^{RG}* transgene influences not only ethanol sensitivity but also ethanol tolerance development. Overexpression of *dnc^{RG}* did not alter impaired tolerance development.

To summarize, overexpression of *dnc^{RA}*, *dnc^{RL}* and *dnc^{RG}* did not alter ethanol tolerance development which means these transcripts alone may not mediate tolerance behavior in the *dnc^{RA}*-GAL4 driven neurons. Further, overexpression of *dnc^{RG}* influenced ethanol sensitivity indicating that this transgene is involved in regulating ethanol sensitivity. To test the influence of the other *dnc* transcripts, additional *UAS*-transgenes need to be generated. Then also overexpression of the other *dnc* transcripts could be analyzed. It is possible that not one *dnc* transcript alone mediates tolerance behavior. Therefore different transcripts could be overexpressed simultaneously.

Fig. 3.5.3. The reduced ethanol tolerance of the *dnc^{Δ143}* mutant can be partially rescued by induced expression of *dnc^{RA}*.
Ethanol sensitivity indicated by MET1 and ethanol tolerance indicated by percentage increase of MET1 to MET2 were investigated in the inebriometer for flies overexpression *dnc^{RA}* (A, B), *dnc^{RL}* (C, D) and *dnc^{RG}* (E, F). Flies carrying only the *dnc^{RA}*-GAL4 transgene were used as a positive control. A) MET1 values of positive control, *UAS* control (*w^{1118}*;; *UAS-dnc^{RA}*) and of the flies overexpressing *dnc^{RA}* were not different from each other (*w^{1118}*;; *dnc^{RA}*-GAL4: 22,09 ± 0,97; *w^{1118}*;; *UAS-dnc^{RA}*: 22,28 ± 1,10; *w^{1118}*;; *UAS-dnc^{RA}*/ *dnc^{RA}*-GAL4: 20,74 ± 0,71). B) Levels of ethanol tolerance of positive control, *UAS* control (*w^{1118}*;; *UAS-dnc^{RA}*) and of the flies overexpressing *dnc^{RA}* did not differ (*w^{1118}*;; *dnc^{RA}*-GAL4: 45,90 ± 7,15; *w^{1118}*;; *UAS-dnc^{RA}*: 42,17 ± 3,98; *w^{1118}*;; *UAS-dnc^{RA}*/ *dnc^{RA}*-GAL4: 44,95 ± 4,84). The overexpression of *dnc^{RA}* does not influence ethanol sensitivity and tolerance. n=12-14 C) Control flies, *UAS* control flies and flies overexpressing *dnc^{RL}* showed normal ethanol sensitivity (*w^{1118}*;; *dnc^{RA}*-GAL4: 21,47 ± 0,86; *w^{1118}*; *UAS-dnc^{RL}*: 19,05 ± 0,93; *w^{1118}*; *UAS-dnc^{RL}*; *dnc^{RA}*-GAL4: 19,64 ± 0,78). D) Ethanol tolerance of the flies overexpressing *dnc^{RL}* was not different from the positive control and the *UAS* control (*w^{1118}*;; *dnc^{RA}*-GAL4: 37,71 ± 3,03; *w^{1118}*; *UAS-dnc^{RL}*: 33,37 ± 4,21; *w^{1118}*; *UAS-dnc^{RL}*; *dnc^{RA}*-GAL4: 34,59 ± 5,06). Overexpressing *dnc^{RL}* has no effect on ethanol sensitivity and tolerance. n=12-13 E) MET1 of the *USA-dnc^{RG}* insertion line was higher than the MET1 of the positive control and of the flies overexpressing *dnc^{RG}*. Overexpression of *dnc^{RG}* restores the altered sensitivity caused by *UAS-dnc^{RG}* transgene insertion (*w^{1118}*;; *dnc^{RA}*-GAL4: 25,39 ± 1,33; *w^{1118}*;; *UAS-dnc^{RG}*: 18,15 ± 1,24; *w^{1118}*;; *UAS-dnc^{RG}*/ *dnc^{RA}*-GAL4: 23,27 ± 1,16). F) Level of ethanol tolerance of the *UAS-dnc^{RG}* line was significantly higher than the positive control. The insertion also affects development of ethanol tolerance. The tolerance level of flies overexpressing *dnc^{RG}* was not different to the *UAS* control (*w^{1118}*;; *dnc^{RA}*-GAL4: 31,35 ± 2,91; *w^{1118}*;; *UAS-dnc^{RG}*: 49,14 ± 6,72; *w^{1118}*;; *UAS-dnc^{RG}*/ *dnc^{RA}*-GAL4: 41,18 ± 3,17). Overexpressing *dnc^{RG}* effects ethanol sensitivity but not tolerance development. n=12-14 Error bars indicate SEM. P^{**}≤0,01.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3.5.4 Specific *dnc^{RA}* expression in the mutant *dnc$^{\Delta 143}$* partially restores reduced ethanol tolerance

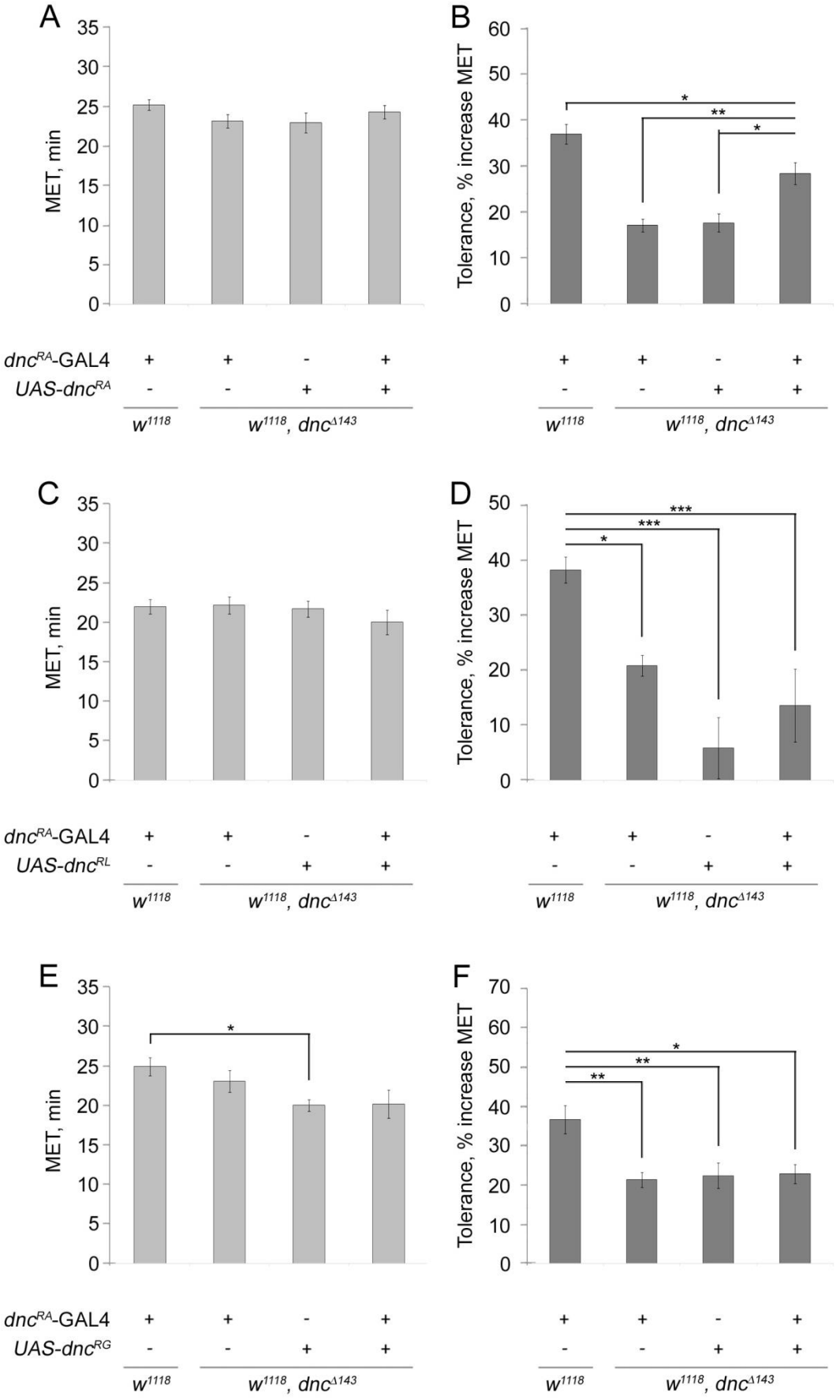
Reduced ethanol tolerance of the *dnc$^{\Delta 143}$* mutant is restored to normal by induced expression of *dnc^{All}* (Franz, 2008). Expression is sufficient in *dnc^{RA}*-GAL4 driven neurons where GAL4 is under the control of the *dnc^{RA}* specific promoter. Further, the *dnc$^{\Delta 143}$* mutant displays a strong transcript *dnc^{RA}* specific down regulation (see 3.5.2). Therefore the question was whether the *dnc^{RA}* transcript specifically is regulating ethanol tolerance. To investigate this, *dnc^{RA}*, *dnc^{RL}* and *dnc^{RG}* were expressed in the *dnc$^{\Delta 143}$* mutant in *dnc^{RA}*-GAL4 driven neurons to test ethanol tolerance in the inebriometer.

Firstly, because of strong down regulation in the mutant, *dnc^{RA}* was expressed by using a *UAS-dnc^{RA}* transgene. For positive control the GAL4 line alone (*w^{1118};; dnc^{RA}*-GAL4) was used. This control showed normal ethanol sensitivity and tolerance (Fig. 3.5.4.1 A, B). The MET1 values the two mutant controls (*w^{1118}, dnc$^{\Delta 143}$;; UAS-dnc^{RA}* and *w^{1118}, dnc$^{\Delta 143}$;; dnc^{RA}*-GAL4) and the experimental group (*w^{1118}, dnc$^{\Delta 143}$;; UAS-dnc^{RA}/dnc^{RA}*-GAL4) did not differ from the positive control (*P*=0,24, *P*=0,37, *P*=0,84; Fig. 3.5.4.1 A). Therefore expressing *dnc^{RA}* in the *dnc* mutant did not affect ethanol sensitivity. Ethanol tolerance data showed that the mutant controls displayed the expected reduced ethanol tolerance (*P*=0,00). The level of tolerance of the experimental group was higher than the level of the mutant controls (*P*=0,01, *P*=0,04) but also lower than the positive control (*P*=0,02; Fig. 3.5.4.1 B). This means that expressing *dnc^{RA}* in the *dnc* mutant did not restore reduced tolerance completely but at least improved reduced ethanol tolerance.

Secondly, *dnc^{RL}* was expressed in the *dnc$^{\Delta 143}$* mutant by using a *UAS-dnc^{RL}* transgene. This transcript is down regulated in the mutant as well (Fig. see 3.5.2). The wild type control (*w^{1118};; dnc^{RA}*-GAL4) showed normal ethanol sensitivity and tolerance (3.5.4.1 C, D). The MET1 values of the mutant controls (*w^{1118}, dnc$^{\Delta 143}$; UAS-dnc^{RL}* and *w^{1118}, dnc$^{\Delta 143}$; dnc^{RA}*-GAL4) and the experimental flies (*w^{1118}, dnc$^{\Delta 143}$;; UAS-dnc^{RL}*; *dnc^{RA}*-GAL4) were not different from the wild type control (*P*=0,98, *P*=1,00, *P*=0,61; Fig. 3.5.4.1 C). This means expressing *dnc^{RL}* in the mutant did not influence ethanol sensitivity. Assessing ethanol tolerance data it was shown that the mutant controls and the experimental group showed the same level of ethanol reduction compared to

the positive control (P=0,05, P=0,00, P=0,00; Fig. 3.5.4.1 D). In conclusion, the reduced ethanol tolerance of the $dnc^{\Delta 143}$ mutant cannot be restored by single transcript expression of dnc^{RL}.

Fig. 3.5.4.1. The reduced ethanol tolerance of the $dnc^{\Delta 143}$ mutant can be partially rescued by induced expression of dnc^{RA}.
Ethanol sensitivity indicated by MET1 and ethanol tolerance indicated by percentage increase of MET1 to MET2 were investigated in the inebriometer for flies expressing dnc^{RA} (A, B), dnc^{RL} (C, D) or dnc^{RG} (E, F) in the $dnc^{\Delta 143}$ mutant. Flies carrying only the dnc^{RA}-GAL4 transgene were used as a positive control. A) The MET1 values of the positive control, the two mutant controls (w^{1118}, $dnc^{\Delta 143}$;; dnc^{RA}-GAL4 and w^{1118}, $dnc^{\Delta 143}$;; *UAS-*dnc^{RA}) and the mutant flies expressing dnc^{RG} were all normal (w^{1118};; dnc^{RA}-GAL4: 25,13 ± 0,68; w^{1118}, $dnc^{\Delta 143}$;; dnc^{RA}-GAL4: 23,07 ± 0,83; w^{1118}, $dnc^{\Delta 143}$;; *UAS-*dnc^{RA}: 22,90 ± 1,25; w^{1118}, $dnc^{\Delta 143}$;; *UAS-*dnc^{RA}/ dnc^{RA}-GAL4: 24,21 ± 0,83). D) The ethanol tolerances of the mutant controls (w^{1118}, $dnc^{\Delta 143}$;; dnc^{RA}-GAL4 and w^{1118}, $dnc^{\Delta 143}$;; *UAS-*dnc^{RA}) were significantly different to the positive control w^{1118};; dnc^{RA}-GAL4 and also to the experimental group (w^{1118}, $dnc^{\Delta 143}$;; *UAS-*dnc^{RA}/ dnc^{RA}-GAL4). Flies of the experimental group developed not the same level than the positive control. Induced dnc^{RA} expression restored reduced ethanol tolerance of the $dnc^{\Delta 143}$ mutant only partially (w^{1118};; dnc^{RA}-GAL4: 36,88 ± 2,11; w^{1118}, $dnc^{\Delta 143}$;; dnc^{RA}-GAL4: 17,08 ± 1,35; w^{1118}, $dnc^{\Delta 143}$;; *UAS-*dnc^{RA}: 17,64 ± 1,95; w^{1118}, $dnc^{\Delta 143}$;; *UAS-*dnc^{RA}/ dnc^{RA}-GAL4: 28,36 ± 2,34). n=11-39 C) The MET1 values of the positive control, the two mutant controls (w^{1118}, $dnc^{\Delta 143}$;; dnc^{RA}-GAL4 and w^{1118}, $dnc^{\Delta 143}$; *UAS-*dnc^{RL}) and the mutant flies expressing dnc^{RL} did not differ from each other (w^{1118};; dnc^{RA}-GAL4: 22,05 ± 0,89; w^{1118}, $dnc^{\Delta 143}$;; dnc^{RA}-GAL4: 22,25 ± 1,09; w^{1118}, $dnc^{\Delta 143}$; *UAS-*dnc^{RL}: 21,77 ± 0,99; w^{1118}, $dnc^{\Delta 143}$; *UAS-*dnc^{RL}; dnc^{RA}-GAL4: 20,10 ± 1,55). D) The level of ethanol tolerances of the mutant controls (w^{1118}, $dnc^{\Delta 143}$;; dnc^{RA}-GAL4 and w^{1118}, $dnc^{\Delta 143}$; *UAS-*dnc^{RL}) and the experimental group (w^{1118}, $dnc^{\Delta 143}$; *UAS-*dnc^{RL}; dnc^{RA}-GAL4) were significantly different to the positive control not different from each other. Induced expression of dnc^{RL} did not restore reduced ethanol tolerance of the $dnc^{\Delta 143}$ mutant (w^{1118};; dnc^{RA}-GAL4: 38,19 ± 2,37; w^{1118}, $dnc^{\Delta 143}$;; dnc^{RA}-GAL4: 20,81 ± 1,85; w^{1118}, $dnc^{\Delta 143}$; *UAS-*dnc^{RL}: 5,88 ± 5,51; w^{1118}, $dnc^{\Delta 143}$; *UAS-*dnc^{RL}; dnc^{RA}-GAL4: 13,59 ± 6,57).n=10-22 E) Comparing all MET1 values, only MET1 of the positive control was different to the mutant control carrying the *UAS-*dnc^{RG} transgene. This reflected the influence of the *UAS* insertion on ethanol tolerance shown in 3.6.3 (w^{1118};; dnc^{RA}-GAL4: 25,03 ± 1,14、w^{1118}, $dnc^{\Delta 143}$;; dnc^{RA}-GAL4: 23,14 ± 1,37; w^{1118}, $dnc^{\Delta 143}$;; *UAS-*dnc^{RG}: 20,09 ± 0,75; w^{1118}, $dnc^{\Delta 143}$;; *UAS-*dnc^{RG}/ dnc^{RA}-GAL4: 20,22 ± 1,79). D) The ethanol tolerances of the mutant controls (w^{1118}, $dnc^{\Delta 143}$;; dnc^{RA}-GAL4 and w^{1118}, $dnc^{\Delta 143}$;; *UAS-*dnc^{RG}) were significantly different to the positive control and not different from each other. Induced expression of dnc^{RG} did not restore the $dnc^{\Delta 143}$ mutant phenotype (w^{1118};; dnc^{RA}-GAL4: 36,55 ± 3,58、w^{1118}, $dnc^{\Delta 143}$;; dnc^{RA}-GAL4: 21,28 ± 1,93; w^{1118}, $dnc^{\Delta 143}$;; *UAS-*dnc^{RG}: 22,32 ± 3,25; w^{1118}, $dnc^{\Delta 143}$;; *UAS-*dnc^{RG}/ dnc^{RA}-GAL4: 22,76 ± 2,45). n=6-14. The error bars indicate the SEM. P^{*}≤0,05, P^{**}≤0,01 P^{***}≤0,001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Tolerance, % increase MET
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dncRA-GAL4 + + - +
UAS-dncRA - - + +
w1118
w1118, dncΔ143
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UAS-dncRL - - + +
w1118
w1118, dncΔ143
E
MET, min
*
dncRA-GAL4 + + - +
UAS-dncRG - - + +
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w1118, dncΔ143
F
Tolerance, % increase MET
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w1118
w1118, dncΔ143

Thirdly, the *dnc*^{RG} transcript, a transcript that is not altered in the *dnc*$^{\Delta 143}$ mutant was expressed in the mutant as well. Thereby the wild type control (*w*^{1118}*;; dnc*^{RA}-GAL4) displayed normal ethanol sensitivity and developed normal ethanol tolerance. The data for ethanol sensitivity showed that flies carrying the *UAS-dnc*^{RG} transgene in the mutant background (*w*^{1118}*, dnc*$^{\Delta 143}$;; *UAS-dnc*^{RG}) displayed a lower MET1 than the wild type control (*P*=0,01). This is consistent with the influence of the *UAS-dnc*^{RG} insertion already shown in 3.5.3. The MET1 of the experimental flies (*w*^{1118}*, dnc*$^{\Delta 143}$;; *UAS-dnc*^{RG}*/ dnc*^{RA}-GAL4) was not different to both mutant controls (*P*=0,46, *w*^{1118}*, dnc*$^{\Delta 143}$;; *dnc*^{RA}-GAL4; *P*=1,00, *w*^{1118}*, dnc*$^{\Delta 143}$;; *UAS-dnc*^{RG}) and close to be significantly different to the positive control (*P*=0,08; Fig. 3.5.4.1 E). The data for ethanol tolerance showed that the mutant controls and the experimental group developed reduced ethanol tolerance (*P*=0,00, *P*=0,01, *P*=0,05) not different from each other (*P*=0,99, *P*=1,00; Fig. 3.5.4.1 F). So the reduced ethanol tolerance of the *dnc*$^{\Delta 143}$ mutant cannot be improved by *dnc*^{RG} expression.

To conclude, only expression of *dnc*^{RA} and not of *dnc*^{RL} or *dnc*^{RG} improved reduced ethanol tolerance of the *dnc*$^{\Delta 143}$ mutant. But expression of *dnc*^{RA} was not sufficient to restore tolerance in the mutant completely. Either the expression level of *dnc*^{RA} is not suitable or additional transcripts are require. To test this more copies of *UAS-dnc*^{RA} transgenes could be used to increase *dnc*^{RA} expression or other transcripts could be expressed together with *dnc*^{RA} in the mutant or. Transcripts *dnc*^{RA} and *dnc*^{RL} are both down regulated in the *dnc*$^{\Delta 143}$ mutant (see 3.5.2) that's why both transcripts were expressed in the *dnc*$^{\Delta 143}$ mutant simultaneously (Fig. 3.5.4.2). The positive control (*w*^{1118}*;; dnc*^{RA}-GAL4) displayed normal ethanol sensitivity and developed normal ethanol tolerance. Data for ethanol sensitivity indicated that flies carrying both the *UAS-dnc*^{RA} transgene and the *UAS-dnc*^{RL} transgene in the *dnc*$^{\Delta 143}$ mutant (*w*^{1118}*, dnc*$^{\Delta 143}$*; UAS-dnc*^{RL}*; UAS-dnc*^{RA}) displayed higher resistance towards ethanol than the positive control (*P*=0,01). This means only the insertions of both constructs together influenced ethanol sensitivity because insertions of *UAS-dnc*^{RA} and *UAS-dnc*^{RL} alone did not it (Fig. 3.5.4 A, C). However the MET1 of the experimental flies also carrying both insertions (*w*^{1118}*, dnc*$^{\Delta 143}$*; UAS-dnc*^{RL}*; UAS-dnc*^{RA}*/ dnc*^{RA}-GAL4) was not different to the positive control (*P*=0,25) and not to the mutant control carrying both insertions (*P*=0,97). This indicates that simultaneous expression of *dnc*^{RA} and *dnc*^{RL} might influence sensitivity in the mutant (Fig. 3.5.4.2 A). For

ethanol tolerance the mutant controls and the experimental group displayed reduced ethanol tolerance compared to the positive control (P=0,00) and not different from each other (Fig. 3.5.4.2 B).

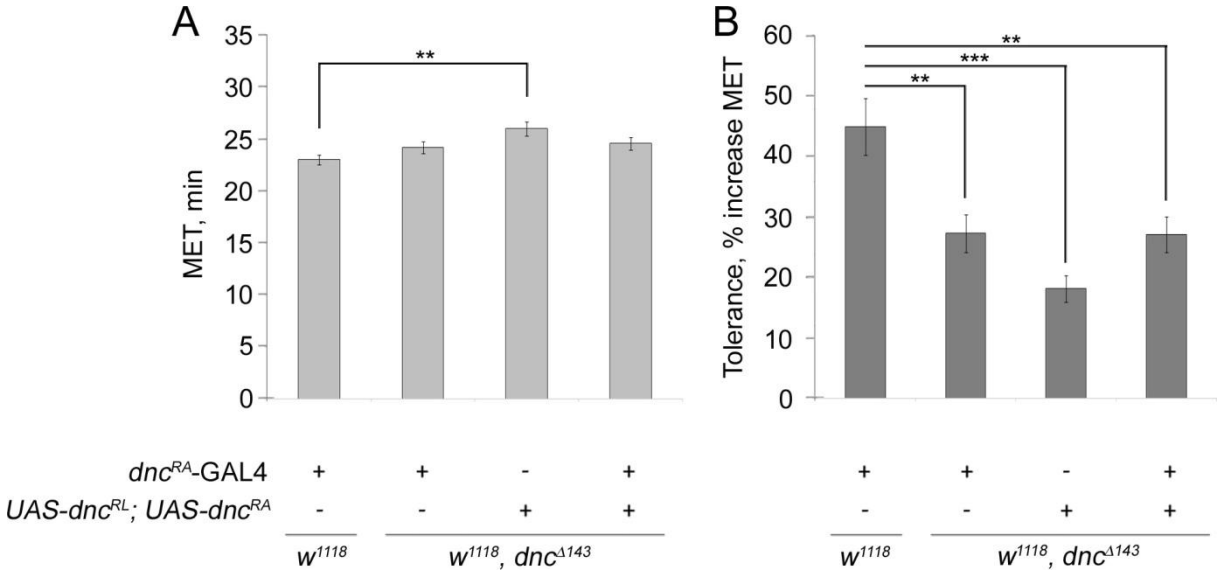


Fig. 3.5.4.2. Simultaneous expression of *dnc^{RA}* and *dnc^{RL}* does not restore wild type ethanol tolerance in the *dnc^{Δ143}* mutant.
Ethanol sensitivity (A) and ethanol tolerance (B) were tested in the inebriometer for flies expressing *dnc^{RA}* and *dnc^{RL}* in the *dnc^{Δ143}* mutant. Flies carrying only the *dnc^{RA}*-GAL4 transgene were used as a positive control. A) Insertion of both *dnc^{RA}* and *dnc^{RL}* UAS transgenes influenced ethanol sensitivity indicated by an increased MET1 of *w^{1118}, dnc^{Δ143}; UAS-dnc^{RL}; UAS-dnc^{RA}* flies. Simultaneous expression of *dnc^{RA}* and *dnc^{RL}* in the *dnc* mutant did not influence ethanol sensitivity (*w^{1118};; dnc^{RA}*-GAL4: 22,98 ± 0,49; *w^{1118}, dnc^{Δ143};; dnc^{RA}*-GAL4: 24,17 ± 0,59; *w^{1118}, dnc^{Δ143}; UAS-dnc^{RL}; UAS-dnc^{RA}*: 25,95 ± 0,65; *w^{1118}, dnc^{Δ143}; UAS-dnc^{RL}; UAS-dnc^{RA}/dnc^{RA}*-GAL4: 24,55 ± 0,60). B) Ethanol tolerances of the mutant controls (*w^{1118}, dnc^{Δ143};; dnc^{RA}*-GAL4 and *w^{1118}*, *dnc^{Δ143}*; *UAS-dnc^{RL}*; *UAS-dnc^{RA}*) and the experimental group (*w^{1118}*, *dnc^{Δ143}*; *UAS-dnc^{RL}*; *UAS-dnc^{RA}/dnc^{RA}*-GAL4) were significantly different to the positive control and not different from each other. Simultaneous expression of *dnc^{RA}* and *dnc^{RA}* did not restore the *dnc^{Δ143}* mutant phenotype (*w^{1118}*;; *dnc^{RA}* -GAL4: 44,86 ± 4,65; *w^{1118}*, *dnc^{Δ143}*;; *dnc^{RA}*-GAL4: 27,25 ± 3,12; *w^{1118}*, *dnc^{Δ143}*; *UAS-dnc^{RL}*; *UAS-dnc^{RA}*: 18,14 ± 2,21; *w^{1118}*, *dnc^{Δ143}*; *UAS-dnc^{RL}*; *UAS-dnc^{RA}*/ *dnc^{RA}* -GAL4: 27,05 ± 2,92). The error bars indicate SEM. n=10, P^{**}≤0,01, P^{***}≤0,001.

Therefore simultaneous expression of dnc^{RA} and dnc^{RL} did not restore reduced ethanol tolerance of the $dnc^{\Delta 143}$ mutant. Furthermore partial restoring by dnc^{RA} expression of the $dnc^{\Delta 143}$ mutant phenotype was not detected. Additional expression of dnc^{RL} might repress the improvement of the tolerance development.

In summary, dnc^{RA} expression in the *dnc* mutant only restored ethanol tolerance partially whereas dnc^{RL} and dnc^{RG} expression did not influence tolerance behavior. This indicates that specifically dnc^{RA} is involved in regulating ethanol tolerance. To further verify this, dnc^{RA} could be expressed in the dnc^{1} mutant to test ethanol tolerance because this mutant also displayed a reduced ethanol tolerance and dnc^{RA} transcript reduction.

3.6 A *dnc* transcript specific interaction with Hangover

Due to the protein structure of Hang it is likely that Hang can bind DNA and/or RNA. In a cDNA Microarray (Scholz and Klebes, unpublished data) to identify potential target genes of Hang the *dnc* gene was detected as a potential target. Furthermore the *hang* mutant $hang^{AE10}$ and the *dnc* mutant $dnc^{\Delta 143}$ share the same behavioral phenotype regarding ethanol tolerance and heat-ethanol cross tolerance (Franz, 2008; Scholz *et al.*, 2005). Both mutants fail to develop normal ethanol tolerance. The interaction of *dnc* and *hang* was investigated more in detail in the following by qRT-PCR, behavioral experiments and western blot analysis.

3.6.1 Gene expression is altered contrarily in the $dnc^{\Delta 143}$ and the $hang^{AE10}$ mutant

Dnc was identified as a potential target gene of Hang by using the $hang^{AE10}$ mutant in a cDNA microarray (Scholz and Klebes, unpublished data). To further investigate the interaction of *hang* and *dnc*, gene expression of *dnc* in the $hang^{AE10}$ mutant and of *hang* in $dnc^{\Delta 143}$ was tested by qRT-PCR.

Firstly, *dnc* transcript levels in the $hang^{AE10}$ mutant were analyzed (Fig. 3.6.1 A). *Su(Tpl)*, a gene that was not altered in the cDNA microarray (Scholz and Klebes, unpublished data) was used as the loading control. The used *dnc* primer pairs were the ones described in 3.5.1. A down regulation of all *dnc* transcripts together to

around 65% was detected in the *hang*^{AE10} mutant (*P*=0,01) reflecting the more specific down regulations of transcript groups RB (*P*=0,01) and RG/RN (*P*=0,01) was seen. The other *dnc* transcript groups RJ (*P*=0,21), RA (*P*=0,10) and RL (*P*=0,33) were not affected (Fig. 3.6.1 A). Secondly, *hang* expression was analyzed in the *dnc*$^{\Delta 143}$ mutant. The loading control was *actin*. *Hang* expression in the *dnc*$^{\Delta 143}$ mutant was increased to almost 600 % (*P*=0,00; Fig. 3.6.1 B).

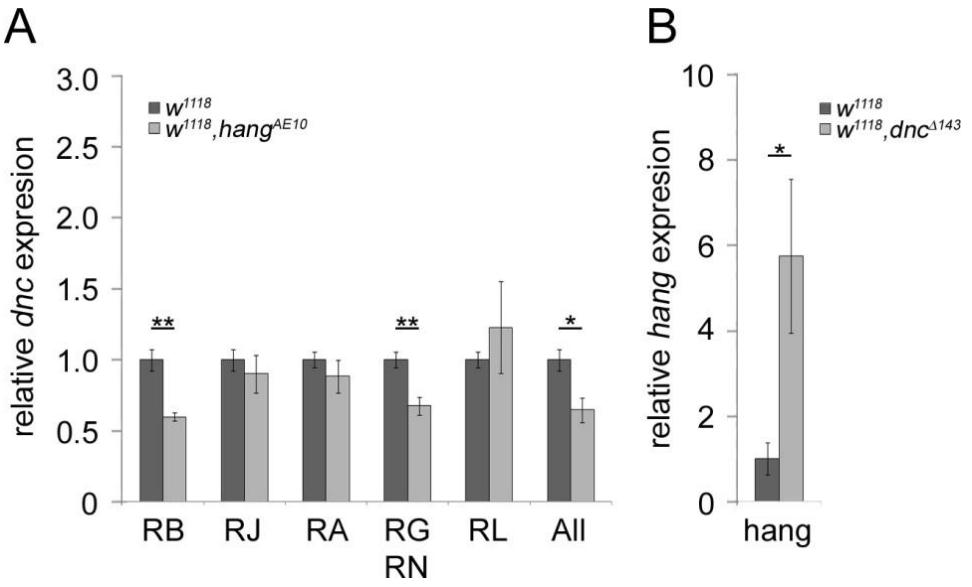


Fig. 3.6.1. Specific *dunce* transcripts are down regulated in the *hang*^{AE10} mutant whereas *hangover* expression is upregulated in the *dnc*$^{\Delta 143}$ mutant.
qRT-PCR data is shown performed on cDNA synthesized from heads of male flies. *Dunce/hangover* expression in the mutants was compared to *dunce/hangover* expression in *w*^{1118}. Loading control for the *dnc*$^{\Delta 143}$ mutant was *actin* and for the *hang*^{AE10} mutant *Su(Tpl)* A) Transcript levels of all *dunce* groups together were down regulated in the *hang*^{AE10} mutant reflecting the down regulated groups RB and RG/RN (*w*^{1118}, *hang*^{AE10}: RB: 0,60 ± 0,03; RJ: 0,90 ± 0,13; RA: 0,88 ± 0,11; RG,RN: 0,68 ± 0,06, RL: 1,23 ± 0,32, All: 0,65 ± 0,09). B) A) *Hangover* expression in the *dnc*$^{\Delta 143}$ mutant was increased (*w*^{1118}, *dnc*$^{\Delta 143}$: 5,75 ± 1,81). The error bars indicate SD. *P**≤0,05, *P***≤0,01.

To conclude, *dnc* expression is affected by the *hang*^{AE10} mutation and *hang* expression is affected by the *dnc*$^{\Delta 143}$ deletion. It is indicated that *hang* expression is activated by Dnc and that *dnc*^{RB} *and dnc*$^{RG/RN}$ expression is regulated by Hangover. However *dnc*^{RA} is not regulated by Hangover. Therefore the reduced ethanol tolerance of the *hang*^{AE10} mutant is not caused by *dnc*^{RA} but also *dnc*^{RB} and/or *dnc*$^{RG/RN}$ affect ethanol tolerance development. To test this, these transcripts should

be expressed in the *hang*^{AE10} mutant to test ethanol tolerance. To test whether *dnc*^{RA} and *dnc*^{RB} and/or *dnc*$^{RG/RN}$ operate in the pathway to regulate ethanol tolerance a complementation test of the *dnc*$^{\Delta 143}$ and *hang*^{AE10} mutant should be performed. .

3.6.2 Complementation test of *dnc*$^{\Delta 143}$ and *hang*^{AE10}

The *hang*^{AE10} and the *dnc*$^{\Delta 143}$ mutant are both impaired in developing reduced ethanol (Scholz *et al.*, 2005; Franz. 2008). But different dnc transcripts are reduced in the mutants – *dnc*^{RA} in the *dnc*$^{\Delta 143}$ mutant (see 3.5.2) and *dnc*^{RB} and *dnc*$^{RG/RN}$ in the *hang*^{AE10} mutant (see 3.6.1). To test whether the mutations of *hang*^{AE10} and the *dnc*$^{\Delta 143}$ mutants affect the same signaling pathway a complementation test was performed. Heterozygous *dnc*$^{\Delta 143}$ and *hang*^{AE10} flies and transheterozygous *dnc*$^{\Delta 143}$, *hang*^{AE10} flies were tested in the inebriometer. Because both mutations are located on the X chromosome females were tested. To exclude variation in tolerance development caused by different body mass, the female flies were fed for two days with autoclaved yeast before the experiment. *w*^{1118} served as a positive control for the assay.

Heterozygous *hang*^{AE10} and the *dnc*$^{\Delta 143}$ females showed normal ethanol sensitivity not different from each other (P=0,30). The MET1 of the transheterozygous *dnc*$^{\Delta 143}$, *hang*^{AE10} flies was not different to both MET1 values of the heterozygous females (P=0,14, P=97; Fig. 3.6.2 A). The heterozygous *hang*^{AE10} and the *dnc*$^{\Delta 143}$ flies developed a normal level of ethanol tolerance (%Tolerance: 57,71±4,46 and 60,69±3,24). The level of ethanol tolerance of the transheterozygous *dnc*$^{\Delta 143}$, *hang*^{AE10} was not different to the heterozygous mutants (P=0,79, P=0,69; Fig. 3.6.2 B). That means the transheterozygous *dnc*$^{\Delta 143}$, *hang*^{AE10} flies were not impaired in developing normal ethanol tolerance indicating that *hang*^{AE10} and *dnc*$^{\Delta 143}$ failed to complement each other. This experiment was performed by Mirjam Franz (2008) before. The number of n was too low to make a clear conclusion. That's why the experiment was repeated. Mirjam Franz showed a trend of an increased ethanol tolerance for transheterozygous *dnc*$^{\Delta 143}$, *hang*^{AE10} flies. This was not confirmed here. Here the tested flies showed normal tolerance development indicating that *dnc*^{RA} and *dnc*^{RB} and/or *dnc*$^{RG/RN}$ act in different signaling pathways to mediate ethanol tolerance. The *dnc*^{1} mutant, where *dnc*^{RB} transcripts are altered, could be used for

complementation tests with *hang*^{AE10} to confirm the interaction of Hang and specifically *dnc*^{RB} with regard to etanol tolerance development.

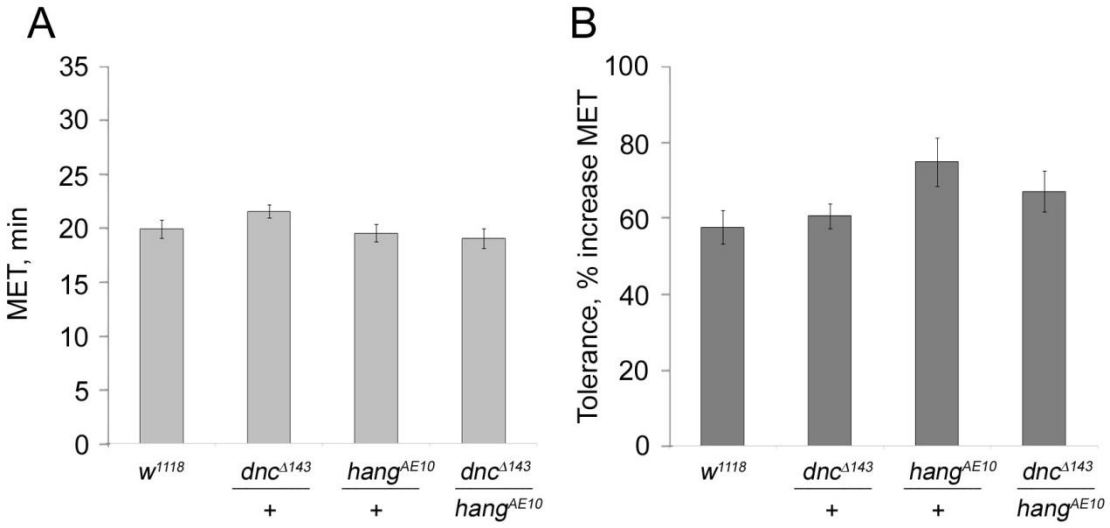


Fig. 3.6.3. Transheterozygous *dnc*^{Δ143}, *hang*^{AE10} flies are not impaired in ethanol tolerance development.
A) Heterozygous *dnc*^{Δ143} and heterozygous *hang*^{AE10} mutants showed normal ethanol sensitivity. Transheterozygous *dnc*^{Δ143}, *hang*^{AE10} flies showed were not impaired in ethanol sensitivity (w^{1118}: 19,97 ± 0,83; *dnc*^{Δ143}/+: 21,62 ± 0,58; *hang*^{AE10}/+: 19,60 ± 0,81; *dnc*^{Δ143}/*hang*^{AE10}: 19,10 ± 0,93). B) Ethanol tolerance development of heterozygous *dnc*^{Δ143} and *hang*^{AE10} flies was normal. The transheterozygous flies were not impaired in developing normal ethanol tolerance (w^{1118}: 57,71 ± 4,46; *dnc*^{Δ143}/+: 60,69 ± 3,24; *hang*^{AE10}/+: 74,91 ± 6,30; *dnc*^{Δ143}/*hang*^{AE10}: 67,18 ± 5,40) The *dnc*^{Δ143} and *hang*^{AE10} mutant did not complement each other. The error bars indicate SEM. n=8

3.6.3 Expression of Hang in the *hang*^{AE10} mutant in NP6510-GAL4 driven neurons might improve reduced ethanol tolerance

The reduced ethanol tolerance of the *dnc*^{Δ143} mutant can be restored by *dnc* expression in *dnc*^{RA}-GAL4 and NP6510-GAL4 driven neurons (Franz, 2008; see 3.4.1). The same phenotype of the *hang*^{AE10} mutant can be restored by *hang* expression also in *dnc*^{RA}-GAL4 driven neurons indicating that both genes regulate ethanol tolerance in the same set of neurons. This could be coincidence because the

expression pattern of the *dnc*^{RA}-GAL4 driver line is very broad. To test whether both genes really operate in the same set of neurons, *hang* was expressed in *hang*^{AE10} in a NP5610-GAL4 dependent manner.

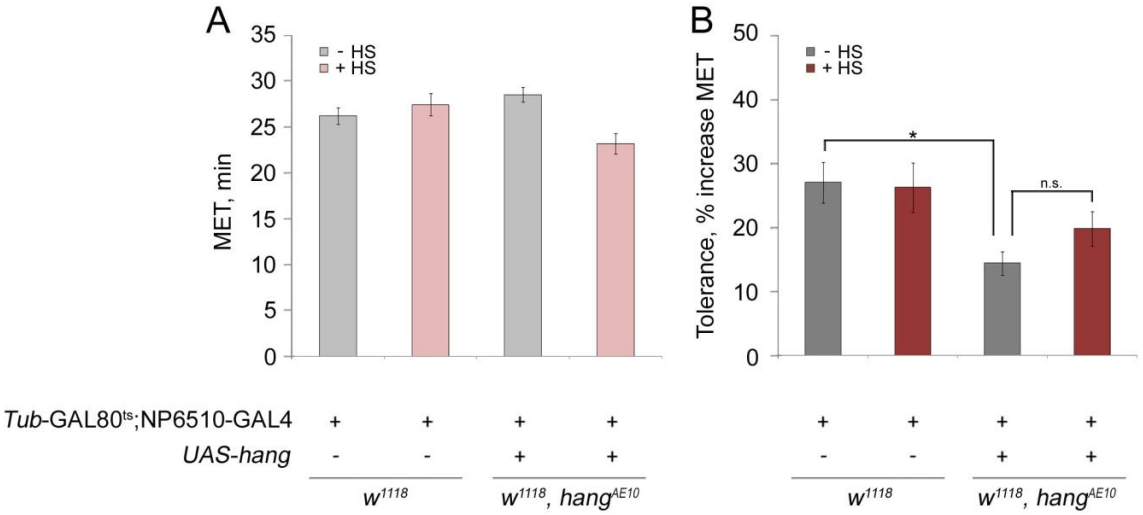


Fig. 3.6.3. *Hangover* expression in NP6510-GAL4 driven neurons might improve reduced ethanol tolerance of the *hang*^{AE10} mutant.
Ethanol sensitivity (A) and ethanol tolerance (B) were tested for flies expressing *hangover* in a *NP6510*-GAL4 dependent manner in the *hang*^{AE10} mutant. Grey colored bars indicate flies were tested without heat shock and red colored bars indicate flies tested with heat shock. Heat shock was set to activate *hangover* expression. A) Control flies (*w*^{1118};; *Tub*-GAL80^{ts}; NP6510-GAL4) and experimental flies (*w*^{1118}, *hang*^{AE10}, *UAS-hang*; *Tub*-GAL80^{ts}; NP6510-GAL4) with and without heat shock showed normal ethanol sensitivity. But by trend, the experimental flies were less resistant towards ethanol (*P*=0,19) (*w*^{1118};; *Tub*-GAL80^{ts}; NP6510-GAL4: -HS: 26,24 ± 0,94; +HS: 27,49 ± 1,23; *w*^{1118}, *hang*^{AE10}, *UAS-hang*; *Tub*-GAL80^{ts}; NP6510-GAL4: -HS: 28,56 ± 0,80; +HS: 23,21 ± 1,12). B) Control flies with and without heat developed normal ethanol tolerance. Flies of the experimental group without heat shock fail to develop normal ethanol tolerance reflecting the *hang*^{AE10} mutation. The ethanol tolerance of the experimental flies with heat hock was not different to the experimental flies without heat shock. By trend an improvement was seen (*P*=0,52) (*w*^{1118};; *Tub*-GAL80^{ts}; NP6510-GAL4: -HS: 27,06 ± 3,19; +HS: 26,27 ± 3,82; *w*^{1118}, *hang*^{AE10}, *UAS-hang*; *Tub*-GAL80^{ts}; NP6510-GAL4: -HS: 14,41 ± 1,88; +HS: 19,82 ± 2,71). The error bars indicate standard deviation. n=14-21, *P**≤0,05.

Flies expressing *hang* in NP6510-GAL4 driven neurons in the $hang^{AE10}$ mutant were lethal. That's why *hang* expression was firstly activated in the adulthood by a heat shock using *Tub*-$GAL80^{ts}$. GAL80 inactivates GAL4 by binding. A thermo sensitive version of GAL80 ($GAL80^{ts}$) can be inactivated by a heat shock. Inactivation of $GAL80^{ts}$ leads to activate GAL4 which induces *UAS* expression. Control flies (w^{1118};; *Tub*-$GAL80^{ts}$; NP6510-GAL4) and experimental flies (w^{1118}, $hang^{AE10}$, *UAS-hang*; *Tub*-$GAL80^{ts}$; NP6510-GAL4) were raised on 18°C to suppress *hang* expression until adulthood. One copy each was heat shocked (33°C, 30 min) 16 hours before tested in the inebriometer to activate *hang* expression. Another copy was tested without heat shock. Sensitivity of control flies with and without heat shock were normal and not significantly different from each other (MET1: 26,24±0,94; and 27,49±1,23; Fig. 3.6.3 A). This indicates that the heat shock of 33°C itself did not have an influence on ethanol sensitivity. Ethanol sensitivities of the experimental flies with and without heat shock were not different to the controls and not significantly different from each other (Fig. 3.6.3 A). But by trend sensitivity of experimental flies with heat shock was decreased, but not significantly (MET1: 28,56±0,80 vs 23,21±1,12; P= 0,19). This could indicate an effect of the *hang* expression. On tolerance level the controls showed normal tolerance development with and without heat shock (%Tolerance: 27,06±3,19 and 26,27±3,82; Fig. 3.6.3 B). The experimental flies without heat shock showed 50% reduced ethanol tolerance reflecting the $hang^{AE10}$ mutation (P=0,02). Tolerance level of the same flies with heat shock was not different to that, but by trend a nonsignificant 35% improvement was seen (%Tolerance: 14,41±1,88 vs. 19,82±2,71P=0,52; Fig. 3.6.3 B). Furthermore, including that the experimental flies with heat shock might have been exposed to less ethanol in the first run of the experiment and due to dose dependency of tolerance development, an improved level of tolerance in the mutant could be existent. Considering these two aspects, *hang* expression in the $hang^{AE10}$ mutant in NP6510-GAL4 driven neurons might improve reduced ethanol tolerance. The experiment needs to be repeated using a modified version of the inebriometer to expose flies to the same amount of ethanol in the first run to exclude the effect of dose dependency.

3.6.4 Hangover is not a transcription factor for the *dnc* transcript groups RB, RJ and RA

The Hang protein carries 16 zing finger domains of the C_2H_2 class which are associated with a nucleic acids binding motif (Scholz *et al.*, 2005). It was shown that *in vitro* Hang can bind RNA (Franz, 2008). To test whether Hang also binds DNA and operates as a transcription factor for *dnc* an *UAS-mCD8::GFP* transgene was expressed under the control of different *dnc* promoters in wild type control and in the $hang^{AE10}$ mutant. GFP expression was detected by western blot analysis and pixel intensities of the detected proteins were quantified with ImageJ (Fig. 3.6.4). In the $hang^{AE10}$ mutant Hang protein is not detectable (Scholz *et al.*, 2005). If Hang operates as a transcription factor GFP should be reduced or lacking in the $hang^{AE10}$ mutant (Fig. 3.6.4 A). Three GAL4 lines specific for three *dnc* promoters were used. The NP7145-GAL4 transgene is inserted in the promoter region of the transcript group RB (http://flybase.org/reports/FBti0037253.html). The D52-GAL4 line is specific for transcript group RJ (Ronald L. Davis). The dnc^{RA}-GAL4 was generated including the dnc^{RA} promoter region (Saratsis, 2006). GFP expression was not altered in the $hang^{AE10}$ mutant compared to the control using NP7145-GAL4 (P=0,31), D52-GAL4 (P=0,49) and dnc^{RA}-GAL4 (P=0,32; Fig. 3.6.4 B, C) indicating that the lack of Hang did not change expression of *dnc* transcript groups RB, RJ and RA. To conclude, Hang does not initiate the expression of *dnc* transcript groups RB, RJ and RA. To investigate whether Hang operates for the residual groups RG/RN and RL other GAL4 driver lines could be used that represent RG/RN and RL specific expression.

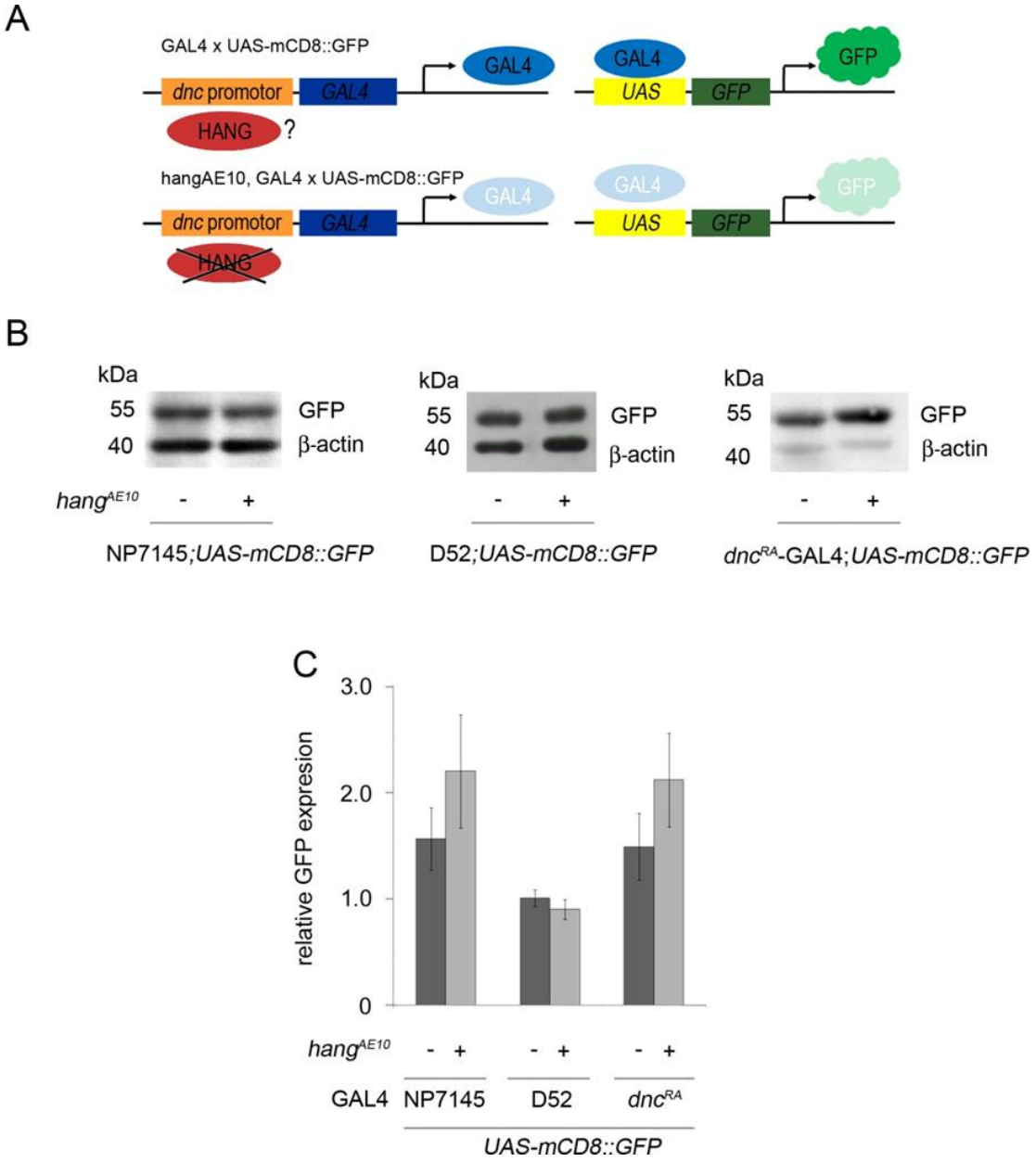


Fig. 3.6.4. Hangover does not initiate *dnc*^{RB}, *dnc*^{RJ} and *dnc*^{RA} expression.
A) GFP was expressed using *UAS-mCD8::GFP* under the control of specific *dunce* promoters (NP7145-GAL4, D52-GAL4, *dnc*^{RA}-GAL4). If Hangover operates as a transcription factor for *dunce,* in the *hang*^{AE10} mutant where Hangover is lacking GFP expression should be altered. B) One exemplary Western Blot is shown for each GAL4. Loading control was β-actin. NP7145-GAL4 (RB specific), D52-GAL4 (RJ specific) and *dnc*^{RA}-GAL4 (RA specific) did not display an obvious change in GFP expression in *hang*^{AE10} compared to GFP expression in the control. C) GFP pixel intensities from the Western Blots were compared of *hang*^{AE10} and control. No significant differences were detected indicating that Hangover is not a transcription factor for *dunce* transcript groups RB, RJ and RA.

3.7 The deletions in the *dSERT* mutants only affect *dSERT* expression and not the expression of the neighboring gene *CG3419*

Independent of investigating octopamine and Hangover mediated pathways that regulate ethanol tolerance, additional experiments were performed to characterize *dSERT* mutants on molecular level. The *dSERT* mutants $dSERT^{1}$, $dSERT^{10}$, $dSERT^{16}$ and $dSERT^{18}$ were generated by P-element mutagenesis from Andrea Kaiser (2009). The $dSERT^{1}$ mutant is described as a revertant because no deletion is detectable. The $dSERT^{10}$, $dSERT^{16}$ and $dSERT^{18}$ mutants carry deletions within the first intron of the *dSERT* gene (Fig. 3.7 A). The $dSERT^{16}$ mutant carries the largest deletion of 1178bp and the $dSERT^{18}$ mutant the shortest deletion of 838bp. The deletion of the $dSERT^{10}$ is 1121bp long. The deletions are close to the neighboring CG3419 gene. To test whether the mutations within the *dSERT* gene affect only *dSERT* expression or also expression of the neighboring gene qRT-PCR was performed. *RplPo* was used as the loading control. For the first qRT-PCR the *dSERT* primer pair was located in the third and fourth exon of the *dSERT* gene (Fig. 3.7 A). In the $dSERT^{1}$ mutant *dSERT* expression is not altered. In the $dSERT^{10}$ and $dSERT^{16}$ mutant *dSERT* transcript levels were reduced to almost zero ($dSERT^{10}$: 0,01±0,02; $dSERT^{16}$: 0,001±0,003). In the $dSERT^{18}$ mutant *dSERT* expression was highly upregulated to around 190% (Fig. 3.7 B). For the second qRT-PCR the *CG3419* primers were set in the first and second exon of the *CG3419* gene including both annotated transcripts (Fig. 3.7 A). The transcript levels of the *CG3419* gene were not altered in the $dSERT^{1}$ (P=0,39), $dSERT^{10}$ (P=0,85), $dSERT^{16}$ (P=0,58) and $dSERT^{18}$ (P=0,36) mutants (Fig. 3.7 C).

To summarize, the deletions in the *dSERT* mutants $dSERT^{10}$, $dSERT^{16}$ and $dSERT^{18}$ affect only expression of the *dSERT* gene and not expression of the neighboring *CG3419* gene. Consequently, behavioral phenotypes of the mutants like impaired ethanol sensitivity and ethanol tolerance development can be exclusively associated with serotonin transporter function. Because in the $dSERT^{1}$ mutant *dSERT* expression is not altered these flies can be used as a control for the $dSERT^{10}$, $dSERT^{16}$ and $dSERT^{1}$ mutants.

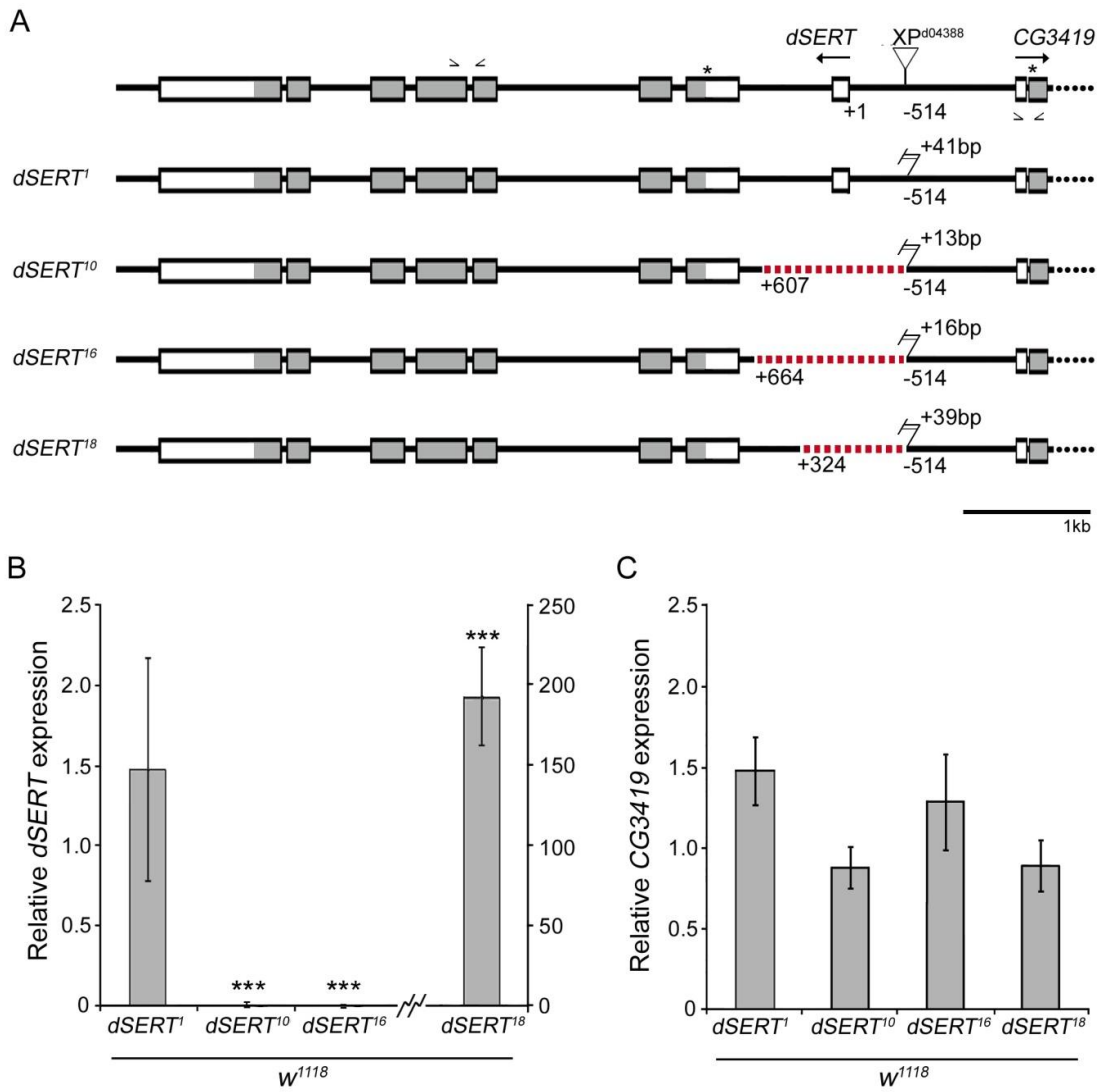


Fig. 3.7: Alteration of transcript levels of the *dSERT* gene and its neighboring gene in the *dSERT* mutants.

A) The genomic organization of the *dSERT* gene and 5' region of the neighboring gene *CG3419* is shown. Deletions and additional base pairs of the *dSERT*^{1}, *dSERT*^{10}, *dSERT*^{16} and *dSERT*^{18} flies are indicated with red dotted lines. The start codons are presented with an asterisk. White boxes indicate non coding exons whereas grey boxes indicate coding exons. *dSERT* primers used for qRT-PCR are indicated with arrowheads. qRT-PCR was performed on on cDNA synthesized from whole male flies with *RplPo* as the loading control. Gene expression of *dSERT* (B) and *CG3419* (C) were put in relation to expression in *w*^{1118} which was normalized to +1. B) *dSERT* transcript levels were down regulated in *dSERT*^{10} and *dSERT*^{16}. In *dSERT*^{18} a strong increase of *dSERT* transcript was detected. *dSERT*^{1} showed normal expression (*dSERT*^{1}: 1,48 ± 0,69; *dSERT*^{10}: 0,01 ± 0,02; *dSERT*^{16}: 0,001 ± 0,003, *dSERT*^{18}: 193,42 ± 30,64). C) *CG3419* transcript levels were not altered in all *dSERT* mutants (*dSERT*^{1}: 1,50 ± 0,21; *dSERT*^{10}: 0,90 ± 0,13; *dSERT*^{16}: 1,30 ± 0,30; *dSERT*^{18}: 0,91 ± 0,16). Error bars represent SD. *P***≤0,01, *P****≤0,001.

4 DISCUSSION

4.1 The *Tbh* gene encodes more than one Tbh isoform with putative different functions

PCR studies and Northern Blot analysis reveal that the *Tbh* gene encodes at least eight transcripts. Performing Western Blots using two different Tbh specific antisera (Cibik 2007, Zhou *et al.,* 2008) five different Tbh isoforms (90kDa, 74kDa, 58kDa, 40kDa, 28kDa) could be confirmed. The specificity for these antisera was confirmed because the antiserum detects the epitope that was used for generating the antiserum and expression of the isoforms is altered in different *Tbh* mutants (see 3.1.2; 3.1.3; 3.1.3). In addition there is evidence that more than one 74kDA protein exist for Tbh. Furthermore, by using a third Tbh antiserum (Hampel, 2004) that recognizes putatively polymeric Tbh structures two putative dimeric/polymeric Tbh structures (52kDa, 65kDa) were identified. However the specificity of this antibody serum needs to be further determined. The existence of multiple different splice variants in *Drosophila* is consistent with finding in the american cockroach *Periplaneta americana* where five isoforms are described (Châtel *et al.,* 2013).

In this study evidence from expression studies in larval CNS and in combination with expression studies using head and body fractions in Western Blot analysis suggest that the identified protein isoforms are expressed in different sets of neurons and in different tissues. Firstly, it was shown in a previous study using the antibody serum of Zhou (Zhou *et al.,* 2008) that Tbh is expressed in neurons of the VUM cluster driven by the *TDC2*-GAL4 line (Schneider *et al.*, 2012). In the ventral nerve cord of *Drosophila* larvae octopaminergic/tyraminergic VUM neurons were also described (Selcho *et al.*, 2012). The isoforms detected by the Tbh antiserum of Cibik (28kDa, 40kDa, 58kDa) are not expressed in the subset of octopaminergic cells addressed by the *TDC2*-GAL4 line suggesting that this serum might not detect Tbh or isoforms in other neuronal subsets. However the *TDC2*-GAL4 line does not drive GAL4 expression in all Tbh Zhou positive neurons (Schneider *et al.*, 2012). Further, it was shown that Tbh isoforms labeled by the Tbh Cibik antiserum are expressed in neurons that are addressed by *Tbh* promoter specific GAL4 lines (Hampel, 2007) indicating expression in other neuronal subsets. Secondly, Tbh is expressed in

different tissues. Tbh expression of putative isoforms detected by the Tbh Hampel antiserum was not detectable in the larval CNS of *Drosophila* performing immunohistochemistry, but in the body and head of adult flies performing Western Blots. It is possible that the Tbh Hampel antibody serum is not working with immunohistochemistry or Tbh isoforms are not present in the larval CNS but in other tissues. To test this, the antiserum should be used to immunostain of other tissues. The ovaries could be a possible target because Tbh mutants are female sterile and Tbh function is shown to be required for functional ovulation (Monastirioti *et al.*, 2003).

In addition to expression in different Tbh positive neurons the Tbh antigens might be localized in different cellular compartments. Tbh isoforms might be located differently in the cell. Tbh immunoreactivity of Tbh Cibik detection was found in cell bodies but also a high number of Tbh positive varicosities were found (see 3.1.4) whereas Tbh Zhou labels more projections and somata (Zhou *et al.,* 2008; Schneider *et al.*, 2012). Furthermore, Tbh antibody serum of the Budnik lab also labels varicosities in particular type II boutons (Koon *et al.*, 2011).

Different expression of the isoforms in the cell, in neuronal subsets and in tissues could indicate for diverse functionality. The expression pattern of Tbh Cibik labeled isoforms is similar to the described OA expression pattern in the larvae (Monastirioti *et al.*, 1995). However, this needs to be further investigated. Tbh Cibik expression should be colabeled with OA expression. Coexpression could indicate a role for OA synthesis for these Tbh isoforms. However, for example the 4.6 *Tbh*-GAL4 line under the control of a *Tbh* promoter fragment expresses in eight Tbh Cibik positive cells caudally localized in the brain which does not colocalize with OA expression (Hampel, 2007). This might indicate a function not associated with OA synthesis for specific isoforms in these neurons. A possible alternative pathway for Tbh in *Drosophila* could be the synthesis of norepinephrine because the domain architecture of the annotated two *Drosophila* Tbh isoforms is organized similar to DBH (dopamine-β-hydroxylase), the enzyme that hydroxylases dopamine to norepinephrine (Aravind, 2001; Kapoor *et al.*, 2011). Norepinephrine is the vertebrate ortholog of OA. So far it is only known that insects use OA and not norepinephrine as a signaling molecule whereas molluscs use both (insects: Roeder, 1999; Schneider *et al.*, 2012; Scholz *et al.*, 2012; molluscs: Saavedra et al., 1974;

Lacoste *et al.*, 2001-1; Lacoste *et al.*, 2001-2; Vehovszky *et al.*, 2005). However, norepinephrine was already detected in some butterfly species like the silkworm *Bombyx mori* (Naokuni *et al.*, 1991) and the cabbage armyworm *Mamestra brassicae* (Takeda *et al.*, 1993) but not in other lepidopterans including *Manduca sexta* (Geng *et al.,* 1993; Sparks and Geng, 1993). This suggests that norepinephrine indeed might have its function in insects. Further, *in vitro,* DBH can hydroxylate tyramine to OA (Goldstein and Contrera, 1961). This raises the possibility that Tbh might also be able to hydroxylate dopamine to norepinephrine. However, norepinephrine was not detected in *Drosophila* yet but it would be interesting for future experiments to look for norepinephrine in *Drosophila*.

As said before the structural architecture of Tbh is similar to DBH including a DOMON domain and two copper type II dependent monooxygenase domains. The here identified additional *Drosophila* isoforms II and III also contain these three domains indicating that they are functional for the hydroxylation reaction. Isoform IV lacks the DOMON domain which could indicate an alternative function. Initially, the DOMON domain was found in secreted and membrane proteins and was suggested to mediate extracellular adhesive interactions (Aravind, 2001). But computational analysis displayed high diversity of this domain involved in heme and sugar recognition (Iyer *et al.*, 2007). Further, analysis of DBH supposes that the DOMON domain potentially promotes tetramerization of the enzyme's subunits. The tetramers are composed of two disulfide-linked dimers whereby the dimers are formed out of the two copper type II dependent monooxygenase domains within the protein resulting in a tetrameric dimer. Dimerization of the monooxygenase domains within the protein is associated with enzymatic function (Saxena *et al.*, 1985; Robertson *et al.*, 1994; Gray *et al.*, 2006; Hess *et al.,* 2008). Lacking only the DOMON domain would result in a dimeric monomer but still with enzymatic function. It was shown for DBH that both dimers and tetramers show enzymatic activity. Thereby it was suggested that the different forms may originate in different tissue sources (Frigon and Stone, 1978). This could be consistent with DBH occurring both in soluble and membrane-bound forms (Winkler *et al.*, 1986). Lacking the DOMON domain therefore might be crucial for soluble or membrane-bound enzyme activity to synthesize OA.

In addition to the functionality of the isoforms, at least some should be stress dependent and inducible due to stress because OA is activated due to stress (Orchard *et al.*, 1993; Adamo *et al.*, 1995; Adamo and Baker, 2011; Chen *et al.*, 2008; Davenport and Evans, 2008; Châtel *et al.*, 2013). Further it is also shown in the american cockroach that Tbh is expressed due to stress (Châtel *et al.,* 2013). Future experiments should focus on isoform detection in samples with and without stress. This could reveal which isoforms exactly are stress dependent. Furthermore, *Drosophila* wild type flies and also Tbh^{nM18} mutants are more resistant towards ethanol when exposed to heat stress four hours before shown by heat-ethanol cross tolerance (Scholz *et al.*, 2005). Here (see 3.3.2) a heat shock was given to the Tbh^{nM18} mutants eight hours before measuring tolerance development. It was not clear whether Tbh^{nM18} mutants really do not restore reduced ethanol tolerance due to the heat shock because of an abnormal control. But if not then this could mean that there might be a Tbh isoform that is induced immediately after stress to regulate ethanol tolerance development but is already degraded after eight hours. So there might be isoforms that are induced to acute stress that only remain for a short time and isoforms that longer active.

Comparing Tbh isoform expression with behavioral phenotypes it is suggested that Tbh isoforms of a size of 74kDa and 58kDa could function in ovulation and ethanol tolerance development. This is suggested because the three *Tbh* alleles Tbh^{nM18}, $Tbh^{R3\text{-}XPdel}$ and XP^{d10000} are female sterile and they are impaired in ethanol tolerance development and the 58kDA and 74kDA expression is reduced.

4.2 The Tbh^{nM18} mutant and the newly generated *Tbh* mutant are not null alleles for all Tbh isoforms.

The newly generated *Tbh* mutant $Tbh^{R3\text{-}XPdel}$ and the Tbh^{nM18} mutant are not null alleles for all Tbh isoforms since in both *Tbh* mutants Tbh protein is still detectable with the used Tbh antibody sera (see 3.1.3; 3.2.4). However, so far the Tbh^{nM18} has been described in the literature as complete *Tbh* null allele (Monastirioti *et al.*, 1996; Koon *et al.*, 2011) because Tbh expression in the larval CNS and on Western Blots was shown to be missing using antibody sera of the respective labs. The inconsistency could be due to the diverse antibody sera. The antibody used in the publication of Monastirioti (Monastirioti *et al.*, 1996) apparently detected only the annotated Tbh

protein at 74kDa. Further, with this antibody it was claimed that no Tbh immunoreactivity in the *Tbh*^{nM18} was detected. However, there was still Tbh positive immunoreactivy that has been described as being nonspecific for Tbh (Monastirioti *et al.*, 1996). In the publication of Koon (Koon *et al.*, 2011) larval CNS were stained with the respective antibody serum of this lab. Here the Tbh expression was missing in the synaptic varicosities, however other putative expression domains in the ventral nerve cord were not shown.

It has been published that OA is not detectable in the *Tbh*^{nM18} mutant (Monastirioti *et al.*, 1996). This could indicate that mainly Tbh isoforms regulating OA synthesis are affected by the *Tbh*^{nM18} mutation but isoforms with other functions are still present and functional. Therefore, described phenotypes like reduced ethanol tolerance (Scholz *et al.*, 2000), impairment in rewarded olfactory memory and learning (Schwärzel *et al.*, 2003; Sitaraman *et al.*, 2010; Yarali and Gerber, 2010) or locomotion defects (Fox *et al.*, 2006) of the *Tbh*^{nM18} mutant cannot be associated with complete loss of Tbh function but might be associated with specific loss of OA function.

With regard to the *Tbh* gene organization it can be said that the genomic organization most likely is more diverse. There might be alternative promoters and exons. Both the *Tbh*^{nM18} mutant and the new *Tbh*$^{R3\text{-}XPdel}$ mutant carry a deletion in the annotated transcript but protein is still detectable. So the deletion might include or disrupt an alternative promoter region or regulatory elements and therefore Tbh isoforms are only altered in the level of expression and do not lack completely. This should be further investigated to examine Tbh function in more detail.

4.3 Tbh function is required in adulthood to form normal ethanol tolerance

Tbh function most likely is required in adulthood and not during embryonic and larval development to form normal ethanol tolerance because reduced ethanol tolerance of the *Tbh*^{nM18} is restored to normal by induced expression of Tbh firstly in the adult fly using a heat shock *Tbh* transgene (see 3.3.2). However, an effect of the heat shock itself on tolerance development could not be ruled out completely because an effect of the heat shock on ethanol sensitivity was seen and because tolerance

development is dose dependent (Scholz *et al.*, 2000). But it would be consistent with Tbh being activated due to stress. It has been shown previously that ethanol is able to cause oxidative stress (Sun *et al.*, 2001; Wu and Cederbaum, 2003; Albano, 2006). Therefore ethanol tolerance mediated by Tbh function would be a response to an acute stress situation and not an internal defined pathway evolved during development. In addition, Tbh function also is only required in adulthood to regulate egg lying in female flies (Monastirioti *et al.*, 2003) and to form sugar memory (Schwärzel *et al.*, 2003). In these studies ovulation and sugar memory in Tbh^{nM18} was restored using the same heat shock *Tbh* transgene (Monastirioti *et al.*, 2003) used in this study. To completely verify that Tbh function is required during the adult stage the heat shock effect on sensitivity and tolerance needs to be fully excluded. Future experiments will be to induce Tbh using the heat shock inducible transgene earlier than four hours before behavioral experiments. Kinetics for the heat shock Tbh transgene were shown in a previous study where Tbh was still present 16 hours after the heat shock (Ruppert, 2010).

The experiments conducted in this study could not reveal the Tbh positive neurons required for ethanol tolerance. Pan-neuronal expressed Tbh in Tbh^{nM18} did not restore reduced ethanol tolerance (see 3.3.1). It is possible, even the used GAL4 lines are described to express pan-neuronal that the specific Tbh requiring neurons were not addressed. In previous studies it was also shown that Tbh expression by different *Tbh* promoter specific GAL4 lines (1.3 *Tbh*-GAL4, 6.2 *Tbh*-GAL4, 6.6 *Tbh*-GAL4) and by the *TDC2*-GAL4 and NP7088-GAL4 (expression in subsets of octopaminergic neurons) is not sufficient to restore ethanol tolerance in the Tbh^{nM18} mutant (Ruppert, 2010). However, Tbh expression in the Tbh^{nM18} mutant using the 4.6 *Tbh*-GAL4 restores ethanol tolerance to normal levels (Fuchs, 2012). This GAL4 line is under the control of a *Tbh* promoter fragment (Hampel, 2007). It was shown that expression of this driver colocalizes with Tbh (Cibik specific antiserum) in eight cells described as caudally localized in the brain (Hampel, 2007). This reveals that Tbh function is sufficient in these eight neurons to mediate normal ethanol tolerance. Future experiments will focus on better describing these neurons to identify exact localization in the *Drosophila* brain.

Olfactory ethanol preference and ethanol tolerance might not be linked, since VUM neurons in the SOG are required for ethanol preference but not for ethanol tolerance

(Schneider *et al.,* 2012). This means that Tbh functional diversity is achieved by Tbh being expressed in different neurons.

4.4 A small set of neurons mediate Dnc dependent ethanol tolerance

The expression of *dnc* using the NP6510-GAL4 driver in $dnc^{\Delta 134}$ mutants restores ethanol tolerance. The expression of the NP6510-GAL4 in the PAM cluster of the MB (Aso *et al.*, 2010; Aso *et al.*, 2012) and the F1 neurons of FB (Liu *et al.*, 2006; Li *et al.*, 2009; Young and Armstrong, 2010) is very well described and was confirmed here. In a previous study it is shown that expression of *dnc* in a dnc^{RA}-GAL4 dependent manner also restores ethanol tolerance (Franz, 2008). The dnc^{RA}-GAL4 line drives transgene expression in a very broad set of neurons throughout the *Drosophila* brain, however, also in PAM neurons and neurons of the FB (Franz, 2008). Both MB and FB are associated with mediating different behaviors. For the MB a role in regulating olfactory learning and memory is described (McGuire *et al.*, 2001; Akala *et al.*, 2006; van Swinderen, 2009). Specifically dopaminergic PAM neurons in the MB are identified to induce aversive and reward odor memory (Aso *et al.*, 2012; Liu *et al.*, 2012). Previously it has been shown with structural mutants in the mushroom body that this brain structure is not involved in mediating ethanol tolerance (Scholz *et al.,* 2000). In addition, Pam neurons addressed by the NP6510-GAL4 line innervating the MB have been described to be dopaminergic (Aso *et al.*, 2010; Aso *et al.*, 2012). Interfering dopaminergic signaling by inhibition of neurotransmission using the *Th*-GAL4 driver line does not interfere with ethanol tolerance (Ritze, 2007). The fanshaped body is one the four substructures of the central complex (Renn *et al.*, 1999). In the central complex memory traces for other learning tasks, such as visual pattern memory in tethered flight, seem to reside (Liu *et al.*, 2006; Pan *et al.*, 2009). In addition, the CC is associated with regulating locomotion (Strauss and Heisenberg, 1993; Strauss, 2002; Popov *et al.*, 2004). The CC has been implicated in ethanol tolerance (Scholz *et al.,* 2000; Urizar *et al.*, 2007; Scholz, 2009). Development of ethanol tolerance can be described as an experience dependent change of behavior because flies are tested twice in the same assay with ethanol as a stimulus. This indicates a learning component in ethanol tolerance development. Tolerance development also affects locomotion. This is reflected by a loss of postural control

when exposed to ethanol. Therefore it might be more likely that in context with learning and locomotion defects it might be the F1 neurons in the CC neurons that mediate tolerance development. To verify this, the additional GAL4 expression of the NP6510-GAL4 and dnc^{RA}-GAL4 expression in dopaminergic MB neurons should be restricted to F1 neurons only and used to restore *dnc* expression in $dnc^{\Delta 143}$ mutants. This could be done using *Th*-GAL80. When reduced ethanol tolerance can be restored then, MB neurons can be ruled out and the F1 neurons would be confirmed to mediate normal ethanol tolerance development with regard to *dnc*. Further, GAL4 lines that only express in the PAM neurons or in the F1 neurons could be used. The R58E02-GAL4 line for example strongly labels the PAM neurons with little expression elsewhere (Liu *et al.*, 2012) and could be used to rule out the PAM neurons. The c42-GAL4 line drives expression mainly in the ellipsoid body and in the F1 neurons of the fanshaped body (Urizar *et al.*, 2007; Pan *et al.*, 2009) and could be used to verify the F1 neurons. In addition, interestingly the set of neurons addressed by the c42-GAL4 line have been implicated in Homer dependent ethanol tolerance (Urizar *et al.*, 2007) suggesting a common function for the neurons and/or further a putative interaction of Homer/*dnc*/Hang. Homer proteins interact with different synaptic receptors (Urizar *et al.*, 2007). It could be interesting to investigate the putative interaction of Homer/*dnc*/Hang in the future.

4.5 Dnc isoform specific interference with ethanol tolerance

It can be shown here that specific the Dnc^{PA} isoform is required for ethanol tolerance development. In dnc^{1} and dnc^{M11} a broad set of *dnc* transcripts are altered. However, dnc^{1} develops reduced tolerance whereas dnc^{M11} does not. In the dnc^{M11} mutant with normal tolerance dnc^{RA} expression is not altered. But in the dnc^{1} and $dnc^{\Delta 143}$ alleles dnc^{RA} expression is decreased (see 3.5.1 and 3.5.2) suggesting that dnc^{RA} is required for ethanol tolerance. In addition, only expression of dnc^{RA} in the $dnc^{\Delta 143}$ mutant and not expression of dnc^{RG} and dnc^{RL} improves reduced ethanol tolerance (see3.5.4). However, dnc^{RA} overexpression does not influence ethanol tolerance development indicating that excess levels of Dnc^{PA} do not influence tolerance. This would be consistent with overexpressing dnc^{All} which does not affect tolerance development either (Franz, 2008). A threshold level of Dnc^{PA} might be required to form normal ethanol tolerance. Reduced levels would result in impaired ethanol tolerance

development, consistent with the $dnc^{\Delta 143}$ and dnc^{1} mutant (Franz, 2008, see 3.5.1 and 3.5.2). Increased levels could be nonrelevant because the surplus proteins are not activated by other PDE interacting proteins that might be limited in the cells. The expression of dnc^{RA} only improves ethanol tolerance in $dnc^{\Delta 143}$, but does not fully restore ethanol tolerance to control levels. Dnc^{PA} might have a second function of regulating learning and memory because the $dnc^{\Delta 143}$ is also impaired in olfactory learning and memory. But Dnc^{PA} dependent tolerance development might also carry a learning and memory component. The isoforms Dnc^{PB}, Dnc^{PG}, Dnc^{PN}, Dnc^{PJ}, Dnc^{PF} might be involved in regulating other behavioral aspects like ethanol sensitivity (see 3.5.1), learning and memory (Tully and Quinn, 1985; Roman and Davis, 2001; Franz, 2008) or courtship (Greenspan and Ferveur, 2000; Gailey, 1984) because these behaviors are impaired in distinct *dnc* mutants and not in all. In addition, dnc^{RB} might also be involved in regulating ethanol tolerance but in a seperate pathway, because in the $hang^{AE10}$ and dnc^{1} mutants where ethanol tolerance is reduced as well, this transcript is reduced wheras dnc^{RA} is not altered (see 4.6 for detailed explanation).

It is supposed that the Dnc isoforms can function differently due to structural differences. It is reported for several PDE isoforms that PDEs form dimers due to GAF-A domains (Zoraghi *et al.*, 2005). It is supposed that dimerization or oligomerization is required to achieve catalytic PDE function. But false or disrupted dimerization can change affinity of the catalytic PDE domain (Richter and Conti, 2004). Indeed, PDE4s do not carry GAF-A domains but the highly conserved UCR1 and UCR2 domains which likely have a similar function (Conti and Beavo, 2007). Short splicing variants are said to be monomeric because they lack UCR1. Monomeric isoforms cannot be activated by PKA (Conti and Beavo, 2007) and therefore might display a different function. Dnc^{PL} is such a short splicing variant lacking UCR1. Therefore a PDE catalytic function of Dnc^{PL} might be nonexistent but a function of regulating active Dnc isoform levels by dimerization is possible. The results here assume that Dnc^{PL} might operate to inactivate Dnc^{PA} in a positive feedback regulation dependent manner where a specific ratio of Dnc^{PA} and Dnc^{PL} is required to mediate normal ethanol tolerance. This would mean when Dnc^{PA} is absent Dnc^{PL} expression is decreased because it is not required. This is consistent with the shown data because dnc^{RL} also is down regulated in the $dnc^{\Delta 143}$ mutant where dnc^{RA} is missing almost

completely (see 3.5.2). However, the 50% reduction of *dnc*^{RA} expression in the *dnc*^{1} mutant (see 3.5.1) might not be strong enough to also affect *dnc*^{RL} because *dnc*^{RL} is not altered in this mutant (see 3.5.1). Furthermore, when *dnc*^{RL} expression is overexpressed then *dnc*^{RA} expression might be initiated due to positive feedback. This would be consistent with the *dnc*^{RL} overexpression in wild type background does not affect tolerance behavior (see 3.5.3). In wild type *dnc*^{RA} expression can be induced to regulate the ratio of *dnc*^{RA} and *dnc*^{RL}. However, in the *dnc*$^{\Delta 143}$ mutant where the gene region of *dnc*^{RA} is mutated *dnc*^{RA} expression to regulate the ratio of *dnc*^{RA} and *dnc*^{RL} is not possible. Consistently, simultaneous expression of Dnc^{PA} and Dnc^{PL} in the *dnc*$^{\Delta 143}$ mutant (see 3.5.4) does not improve reduced ethanol tolerance whereas single expression of Dnc^{PA} does improve reduced tolerance (see 3.5.1). To test mutual regulation of *dnc*^{RA} and *dnc*^{RL}, *dnc* transcript levels of flies overexpressing *dnc*^{RL} or *dnc*^{RA} in wild type performing qRT-PCR could be done. In addition, Dnc^{PL} possibly also regulates other Dnc isoforms in separate pathways because in the *dnc*^{M11} mutant where Dnc^{RB} and Dnc^{RG} expression is increased Dnc^{PL} is increased as well. The other Dnc isoforms (Dnc^{PA}, Dnc^{PB}, Dnc^{PG}, Dnc^{PN}, Dnc^{PJ}, Dnc^{PF}) all carry the UCR1 and UCR2 domain indicating dimerization and therefore a functional activation of the PDE catalytic domain.

There are two possible mechanisms to achieve functional diversity of the Dnc isoforms. Firstly, some isoforms might be located differently within the cell and therefore only function in a specific sub-cellular compartment which is consistent with the vertebrate PDE4d with at least 4 different isoforms that are expressed in different sub-cellular domains (Chandrasekaran *et al.,* 2008). Isoforms Dnc^{PG} and Dnc^{PN} carry a NLS (nuclear localization site) motif which indicates distinct localization in the cell nucleus of these isoforms. To verify this and to test where the other isoforms are located on the cellular level GFP tagged *UAS* transgenes for all transcripts will be generated. Therefore expression of the transcripts can be visualized by GFP detection. Another possible mechanism to achieve functional diversity is that Dnc isoforms are expressed in different neuronal subsets. The expression of the *dnc*^{RA}-GAL4 line reflects the expression of Dnc^{PA} due to the *dnc*^{RA} specific promoter element. Expression is rather broad and throughout the whole brain. To test this different GAL4 lines with different *dnc* promoter elements could be generated or endogenous expression could be determined by protein expression

analysis using specific Dnc antibodies against different Dnc isoforms. Having all the described *dnc* transcript group specific *UAS* and GAL4 transgenes will help to further investigate isoform specific regulation of different behavioral aspects because all isoforms then can either be expressed in *dnc* mutants or overexpressed in wild type but both in the appropriate neuronal subset.

4.6 Hangover interacts with specific *dnc* transcripts

Hang interacts with different Dnc isoforms to form normal ethanol tolerance. Firstly, Hang might be regulated by Dnc^{PA} because Hang expression is increased in the $dnc^{\Delta 143}$ mutant in which dnc^{RA} expression is reduced (see 3.6.1). For Dnc^{PA} is shown that this isoform specifically mediates ethanol tolerance (see 4.5). However, dnc^{RA} transcript levels are not altered in the $hang^{AE10}$ mutant. But $hang^{AE10}$ mutants like the $dnc^{\Delta 143}$ mutants are impaired in developing ethanol tolerance. In the $hang^{AE10}$ mutants $dnc^{RG/RN}$ and dnc^{RB} expression is reduced suggesting that one of this tanscripts is also involved in mediating ethanol tolerance. There are two indications why specifically the Hang/dnc^{RB} interaction might be involved in ethanol tolerance development. Firstly, in the dnc^{1} mutant which shows reduced ethanol tolerance dnc^{RB} transcripts are reduced whereas $dnc^{RG/RN}$ expression is not altered. Secondly, in the dnc^{M11} mutant where dnc^{RB} and $dnc^{RG/RN}$ expression is increased, no change in ethanol tolerance is detected (see 3.5.1). This is consistent with overexpression of Dnc not affecting ethanol tolerance (Franz, 2008). The regulation of $dnc^{RG/RN}$ by Hang might concern other behavioral aspects than ethanol tolerance development. Concluding, Hang might be activated by Dnc^{PA} and dnc^{RB} expression is regulated by Hang. It is suggested that there are two separate cAMP signaling pathways in which Dnc^{PA} and Dnc^{PB} operate to mediate normal ethanol tolerance. This is supposed because indeed $dnc^{\Delta 143}$ and $hang^{AE10}$ mutants both show reduced ethanol tolerance but the kinetics in tolerance development is different (Franz, 2008). Long-term tolerance development after 16 hours is only impaired in $hang^{AE10}$ but not in $dnc^{\Delta 143}$. Furthermore, $dnc^{\Delta 143}$ and $hang^{AE10}$ mutants do not complement each other (see 3.6.2). In addition, dnc^{RA} is involved in regulating ethanol tolerance development (see 4.6) but dnc^{RA} is not altered in the $hang^{AE10}$ mutant (see 3.6.1). Concluding, in $hang^{AE10}$ and in $dnc^{\Delta 143}$ two seperate pathways are disrupted both resulting in

reduced ethanol tolerance. The first pathway is Dnc^{PA} dependent (see 4.6 for detailed explanation) possibly including a learned component. A learned component is suggested because *dnc*$^{\Delta 143}$ flies display defects in olfactory learning and memory (Franz, 2008) whereas *hang*^{AE10} mutants are not impaired in olfactory learning and memory (Franz, 2008). In the other pathway specifically Dnc^{PB} might be involved. This pathway is disrupted in the *hang*^{AE10} mutant. In addition, the second described pathway might carry a long-term component for tolerance development because long-term tolerance development after 16 hours is only impaired in *hang*^{AE10} but not in *dnc*$^{\Delta 143}$. In the *dnc*^{1} mutant most likely both pathways are disrupted because these mutants show no tolerance development at all and transcript levels of *dnc*^{RB} and *dnc*^{RA} are altered. For future experiments the *dnc*^{1} mutant should be used for a complementation test together with *hang*^{AE10}. In addition, the *dnc*^{1} mutant should be used to specifically express *dnc*^{RB} to restore reduced ethanol tolerance. These experiments then could further confirm that Hang and *dnc*^{RB} operate in the same pathway.

The two pathways might mediate ethanol tolerance in different sets of neurons. It is already shown that *dnc*^{All} expression in a *dnc*^{RA}-GAL4 dependent manner does not restore reduced tolerance in the *hang*^{AE10} mutant (Franz, 2008). Expression of more specific transcripts (*dnc*^{RB}) in suitable neurons driven by more specific promoter lines (*dnc*^{RB}-GAL4) might be required. Due to the proposed two separate pathways the neurons that mediate ethanol tolerance in the Dnc^{PA} dependent pathway might not be the same than for the Hang/*dnc*^{RB} dependent pathway. This would be consistent with the assumption that different *dnc* transcripts are expressed in different neuronal subsets to achieve functional diversity. Indeed it is shown that reduced tolerance of the *hang*^{AE10} mutant can be restored by Hang expression in Dnc^{PA} associated neurons but the expression of the used *dnc*^{RA}-GAL4 line is also very broad (Franz, 2008). So this could have been coincidence and neurons with *dnc*^{RB} specific expression might be included. For future experiments it is planned to express *dnc*^{All} and *dnc*^{RB} in the *hang*^{AE10} mutant in *dnc*^{RB} specific neurons to try to restore reduced ethanol tolerance.

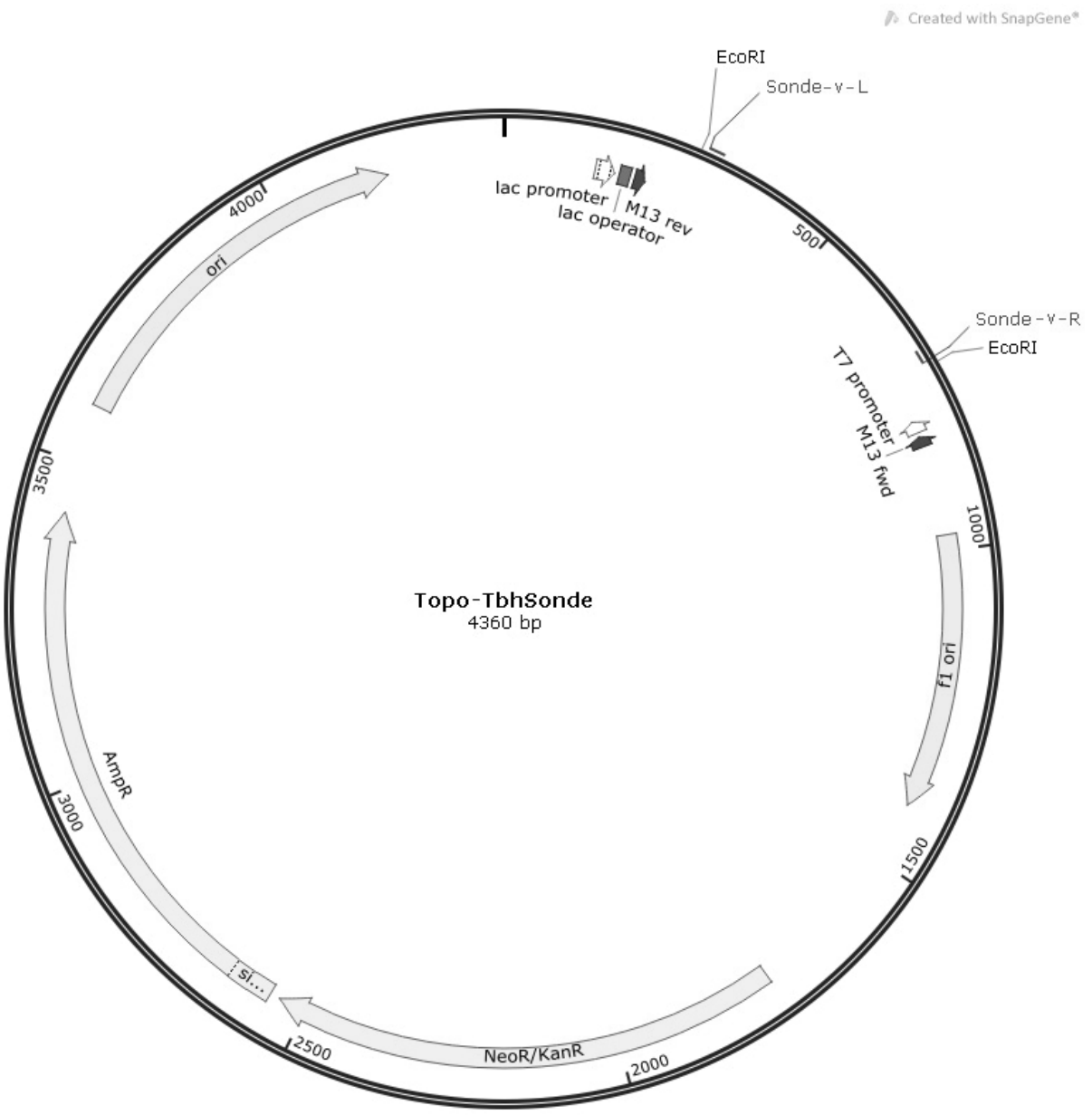
The proposed interaction of Hang and *dnc*$^{RG/RN}$ and/or *dnc*^{RB} most likely is not on DNA level because it can be shown here that Hang is not a transcription factor for *dnc* transcript groups RB, RJ and RA. In addition, the other groups RG/RN and RL should be tested to completely rule out the role of Hang as a transcription factor for

dnc. It is more likely that Hang in response to cellular stress modifies the transcripts $dnc^{RG/RN}$ and dnc^{RB} directly. This is consistent with previous findings that Hang in *Drosophila* can bind *dnc* in vitro (Franz, 2008). Furthermore, making a linkage to higher organisms it is shown that cellular stress can alter RNA processing in higher organisms (Kedersha and Anderson, 2007) and more specific that the human Dnc homolog PDE4 is altered in response to cellular stress (Hill *et al.*, 2006; Brown *et al.*, 2007; Erdogan *et al.*, 2008). Along with the Hang related protein ZNF699 in humans which is associated with alcohol dependence and which is significantly reduced in alcoholics (Riley *et al.*, 2006), cellular stress response to ethanol may be conserved between insects and higher organisms.

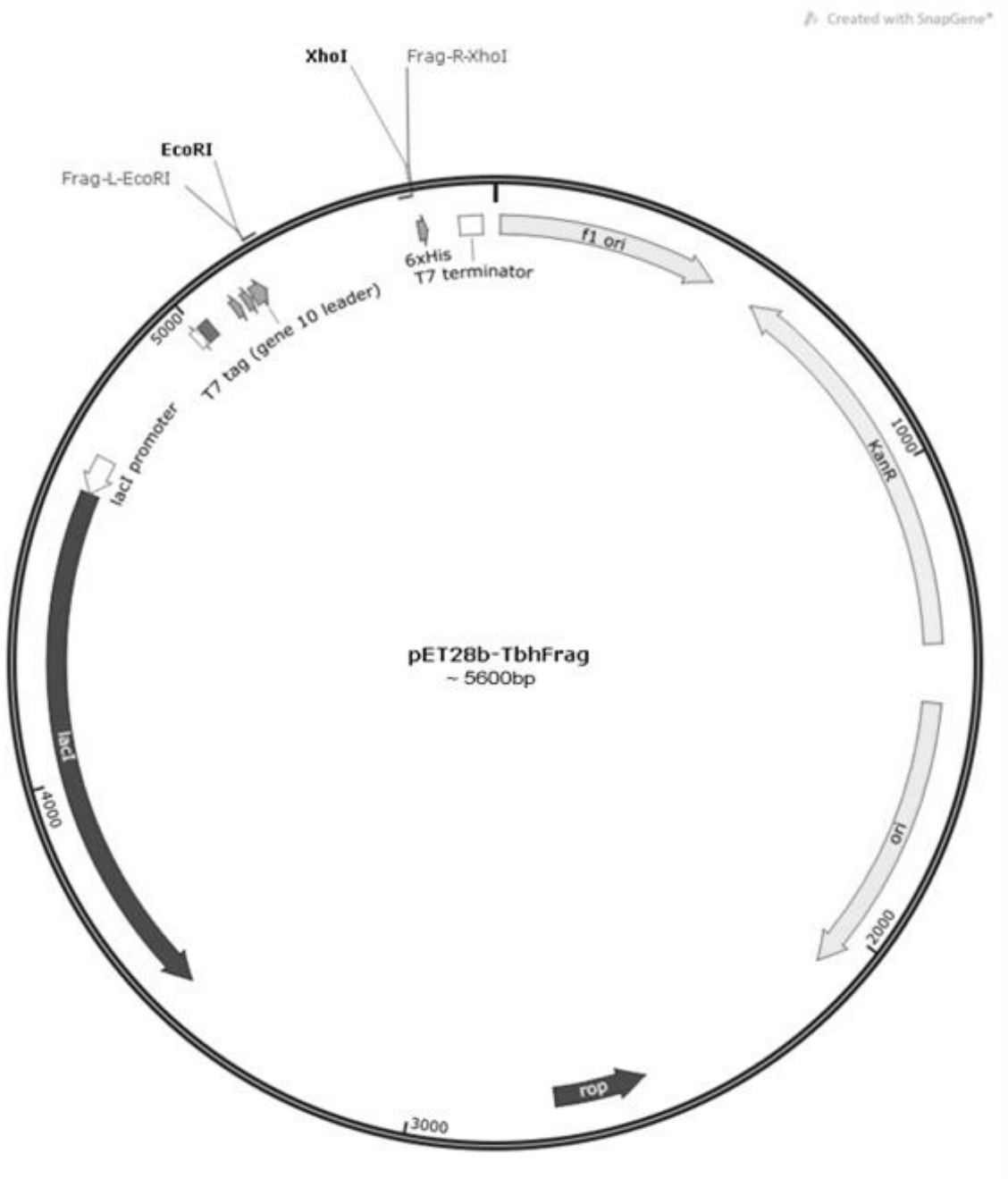
5 SUPPLEMENT

Vector maps

- Topo-TbhSonde
  (pCR®II®-Topo vector with *Tbh* fragment for hybridization probe for Northern Blot)



- pET28b-Frag2, pET28b-Frag3, pET28b-Frag5
  (pET28b vector with *Tbh* fragments for Tbh peptide expression in *E. coli* BL21 (DE3) cells)



Tbh transcript sequences

Legend: ATG/STOP Exon1 Exon2 Exon3 Exon4 Exon5 Exon6 Exon7 Exon8

- Transcript III

ACGCGCTTTCCACTTGTTCGTGCTATTCGTTACGCGATTTCTCTGACGAAAGCGTAGAAGCGCGCCAAAAAAAGC
GCGCGCAAAAACAAAAAGCCAAGCTAACGGGACAAAAGTCGCGCGCGACTCTGATTAGCGATAAACGATATCCGA
GAACAATAATTCCGCCACCGATCTGCCGGCCGTGCAATCTCAAATCTCAAA**ATG**CTTAAAATTCCGCTGCAGCTG
AGCAGTCAGGATGGCATTTGGCCAGCCCGATTCGCCAGGCGACTCCATCACCACCACCAACTGGCTTATCATCAT
CACAAGCAGCAGCAGCAGCAGCAGCAGGCGAAACAGAAACAAAAGCAAAATGGAGTGCAGCAAGGACGTTCGCCG
ACATTTATGCCAGTGATGCTGCTCCTCCTAATGGCCACACTGCTCACGCGCCCGCTGAGCGCCTTCTCCAACCGC
TTATCCGACACAAAGCTGCACGAGATCTACCTGGACGACAAGGAGATTAAGCTGAGCTGGATGGTCGACTGGTAC
AAGCAGGAGGTGCTCTTCCACTTGCAGAATGCTTTCAACGAACAGCACCGCTGGTTCTATCTGGGTTTCTCCAAG
CGCGGCGGCCTGGCGGATGCGGATATTTGCTTTTTTGAGAATCAGAATGGATTCTTCAATGCGGTAACCGATACG
TACACCAGTCCGGATGGACAGTGGGTGAGACGGGACTACCAGCAGGACTGTGAGGTCTTCAAGATGGATGAGTTC
ACGTTGGCGTTTAGGCGCAAGTTTGACACCTGCGACCCTTTGGATTTGCGACTCCATGAGGGCACAATGTACGTG
GTTTGGGCCCGTGGTGAAACGGAACTGGCCCTGGAGGATCACCAGTTCGCTCTGCCCAATGTGACGGCACCGCAC
GAGGCGGGTGTTAAGATGCTACAGCTACTACGGGCCGACAAGATACTTATACCCGAAACCGAGTTGGATCACATG
GAGATCACACTGCAGGAGGCGCCAATTCCCAGTCAGGAGACCACGTACTGGTGTCACGTTCAGCGACTGGAGGGC
AATCTCCGGCGTCGCCATCATATCGTTCAGTTCGAGCCGCTCATCCGAACGCCGGGCATCGTGCATCACATGGAA
GTGTTTCACTGCGAGGCCGGTGAGCACGAGGAGATTCCCCTGTACAACGGCGACTGTGAACAGTTGCCGCCACGG
GCCAAGATCTGCTCAAAAGTGATGGTCCTGTGGGCCATGGGCGCGGGCACCTTTACCTATCCTCCGGAAGCCGGT
CTACCAATCGGCGGACCCGGCTTCAATCCGTACGTTCGACTGGAGGTACATTTCAATAATCCGGAGAAGCAGTCG
GGCTTGGTGGACAACTCCGGCTTTCGCATCAAGATGTCGAAGACACTGCGTCAGTATGACGCCGCCGTTATGGAA
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GCGGACTGCACACGAGCCGCTCTGCCGGCGACGGGCATCATCATCTTTGGCTCTCAGCTGCATACGCATCTGCGT
GGCGTTCGCGTCCTAACCCGGCACTTTCGCGGCGAACAGGAGCTGCGCGAGGTGAACCGCGATGACTACTACTCG
AATCACTTCCAGGAGATGCGCACCCTGCACTACAAGCCGCGTGTCCTGCCCGGCGACGCTTTGGTAACCACTTGT
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- Transcript IV

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- Transcript V

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6 LIST OF ABBREVIATIONS

5HT	5-Hydroxytryptamin (Serotonin)
AC	Adenylyl cyclase
ADH	Alcohol dehydrogenase
AKAP	A-kinase-anchoring protein
AUD	Alcohol use disorder
cAMP	Cyclic adenosine monophosphate
CDS	Coding sequence
CNS	Central nervous system
DBH	Dopamine-β-hydroxylase
DDC	DOPA-decarboxylase
Dnc	Dunce
dSERT	Drosophila Serotonin Transporter
FLP	Flippase
FRT	Flippase recognition target
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
Hang	Hangover
HS	Heat shock
MET	Mean elution time
OA	Octopamine
PDE	Phosphodiesterase
PKA	Protein kinase A
PKA-C	Catalytic subunit of Protein kinase A
PKA-R	Regulatory subunit of Protein Kinase A

SD	Standard deviation
SEM	Standard error of the mean
SERT	Serotonin Transporter
Tbh	Tyramine-β-hydroxylase
TDC	Tyrosine-decarboxylase
UAS	Upstream activating sequence
UCR	Upstream conserved region
UTR	Untranslated region

Neuroanatomical abbreviation:

AL	Antennal lobes
AM	Abdominal medial
FB	Fanshaped body
LN	Lateral neurons
MB	Mushroom body
PM	Paramedical
SM	Subesophageal medial
SOG	Subesophageal ganglion
CC	Central Complex

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TEILPUBLIKATIONEN

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Schneider A., **Ruppert M**., Hendrich O., Giang T., Ogueta M., Hampel S., Vollbach M.,, Büschges A. and Scholz H. 2012. „Neuronal Basis of Innate Olfactory Attraction to Ethanol in *Drosophila*“. *PLoS ONE* 7(12).

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ERKLÄRUNG

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzen Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen –, die anderen Werken im Worlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kennlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegt hat, dass sie – abgesehen von oben angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverafhrens nicht vornehmen werde.

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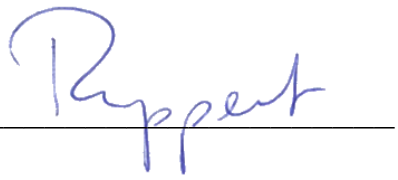

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